Elevated E2F1 Inhibits Transcription of the Androgen Receptor in Metastatic Hormone-Resistant Prostate Cancer

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Abstract

Activation of E2F transcription factors, through disruption of the retinoblastoma (Rb) tumor-suppressor gene, is a key event in the development of many human cancers. Previously, we showed that homozygous deletion of Rb in a prostate tissue recombination model exhibits increased E2F activity, activation of E2F-target genes, and increased susceptibility to hormonal carcinogenesis. In this study, we examined the expression of E2F1 in 667 prostate tissue cores and compared it with the expression of the androgen receptor (AR), a marker of prostate epithelial differentiation, using tissue microarray analysis. We show that E2F1 expression is low in benign and localized prostate cancer, modestly elevated in metastatic lymph nodes from hormone-naive patients, and significantly elevated in metastatic tissues from hormone-resistant prostate cancer patients (P = 0.0006). In contrast, strong AR expression was detected in benign prostate (83%), localized prostate cancer (100%), and lymph node metastasis (80%), but decreased to 40% in metastatic hormone-resistant prostate cancer (P = 0.004). Semiquantitative reverse transcription-PCR analysis showed elevated E2F1 mRNA levels and increased levels of the E2F-target genes dihydrofolate reductase and proliferating cell nuclear antigen in metastatic hormone-independent prostate cancer cases compared with benign tissues. To identify a role of E2F1 in hormone-independent prostate cancer, we examined whether E2F1 can regulate AR expression. We show that exogenous expression of E2F1 significantly inhibited AR mRNA and AR protein levels in prostate epithelial cells. E2F1 also inhibited an AR promoter-luciferase construct that was dependent on the transactivation domain of E2F1. Furthermore, using chromatin immunoprecipitation assays, we show that E2F1 and the pocket protein family members (p107 and p130) bind to the AR promoter in vivo. Taken together, these results show that elevated E2F1, through its ability to repress AR transcription, may contribute to the progression of hormone-independent prostate cancer.

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Introduction

Prostate cancer is the number one diagnosed cancer in males in the United States other than skin cancer and is the second leading cause of cancer-related deaths (1). Locally defined disease is often successfully treated with surgery and/or radiotherapy; however, disease recurs in an estimated 15% to 30% of patients (2). The androgen receptor (AR) is the mediator of the physiologic effects of androgen. It regulates the growth of normal and malignant prostate epithelial cells. Upon ligand binding, AR translocates to the nucleus, binds to DNA recognition sequences, and activates transcription of target genes, including genes involved in cell proliferation, apoptosis, and differentiation (reviewed in ref. 3). Androgen ablation therapy is highly successful for the treatment of hormone-sensitive prostate cancer; however, hormone resistance significantly limits its benefits. Hormonal ablation therapy will control metastatic disease for 18 to 24 months (4), but once metastatic prostate cancer ceases to respond to hormonal therapy, median survival decreases significantly (5). Although the focus of rigorous study, the molecular mechanisms underlying the progression of prostate cancer to hormone-independent disease remain unclear.

The E2F family of transcription factors consists of at least eight characterized family members (E2Fs 1–8), which can form heterodimers with differentiating protein family members (DP1, DP2), giving rise to functional E2F activity (6, 7). E2F controls the progression through the cell cycle by regulating the transcription of genes that are essential for DNA synthesis and cell cycle progression (8). Several E2Fs, including E2F1, behave as oncogenes, and are able to induce quiescent cells to enter the cell cycle, override various growth-arrest signals, and transform primary cells (9–11). Elevated E2F1 can induce hyperproliferation, hyperplasia, and p53-dependent apoptosis in E2F1 transgenic mice (12). E2Fs also act like tumor suppressors by activating apoptotic pathways (13), suggesting that E2Fs affect multiple downstream targets depending on cell type and environmental context.

Aberrant expression of E2F1 has been documented in human cancers, including amplification of the E2F1 gene in erythroleukemia cell lines (14), and overexpression of E2F1 protein in breast cancer cell lines (15) and head and neck carcinoma cell lines (16). Elevated E2F1 expression has been reported in invasive ductal breast carcinomas (15) and non–small cell lung carcinomas (17), where high levels of E2F1 were associated with advanced disease and poor prognosis. E2F3 was found to be elevated in localized prostate cancer and is associated with decreased overall survival (18). Recently, Rhodes et al. (19) reported that genes with E2F binding sites are significantly overexpressed in a wide variety of human cancers, further supporting the view that activation of the E2F pathway is a prevalent event in certain human cancers.

We have previously shown that disruption of the retinoblastoma (Rb)-E2F complex by homozygous deletion of Rb in a prostate tissue recombination model exhibited increased E2F activity and activation of E2F-target genes in vitro and predisposed prostate epithelium to hormonal carcinogenesis in vivo (20–22). We hypothesized that enhanced E2F1 expression may be a common event in prostate human cancer and may contribute to prostate
cancer development and progression. In this study, we used tissue microarray (TMA) analysis to examine protein expression of E2F1 and AR during prostate cancer progression. A TMA containing 667 tissue cores designed to examine prostate cancer progression to hormone-independent disease was used to assess the relationship between E2F1 protein expression and prostate cancer progression. We observed that E2F1 was significantly elevated in hormone-resistant prostate cancer. Interestingly, we also observed that overall AR levels were significantly down-regulated in hormone-resistant prostate cancer. To define a role for elevated E2F1 in hormone-resistant prostate cancer, we investigated whether E2F1 could regulate the expression of the AR and found that E2F1 acts as a transcriptional repressor of the AR gene. This is the first report investigating E2F1 expression during prostate cancer progression using TMA and the first study to show that E2F1 may be a critical component of AR transcriptional regulation.

Materials and Methods

TMA construction. The tissue donation program and rapid autopsy program was approved by the Institutional Review Board of the University of Michigan. Benign and localized prostate cancer tissues were constructed from transurethral resection of the prostate and prostatectomy samples taken from 28 patients with prostate cancer seen at the University of Michigan from 1994. Regional lymph node metastases from hormone-naïve patients were derived from radical prostatectomy series at the University of Ulm (Ulm, Germany) from 1997 to 2000. In this time interval, 49 patients had lymph node metastases, and, in nine cases, the representative paraffin block of the lymph node provided enough metastatic tissue suitable for TMA construction. Metastatic tissues from hormone-resistant prostate cancer patients were acquired from the rapid autopsy procurement program at the University of Michigan. Formalin-fixed, paraffin-embedded tissue blocks with tumor samples from each case were identified. One pathologist (M.A.R.) reviewed the H&E-stained slides and circled areas of prostate cancer, which were then used as template for the TMA. To take tumor heterogeneity into account, four TMA cores (0.6-mm diameter) were sampled from each donor block using a validated sampling method. The TMA was constructed using a manual tissue arrayer (Beecher Instruments, Silver Spring, MD) as previously described (23).

Evaluation of E2F1 and AR protein expression by immunohistochemistry. Standard biotin-avidin complex immunohistochemistry was done using an E2F1 antibody (PharMingen, Franklin Lakes, NJ) and an AR antibody (Santa Cruz Biotechnology, Santa Cruz, CA). All cores on the TMA were assigned at diagnosis and evaluated using TMA Profile, an internet-based image evaluation tool that uses zoomable TMA images generated by the BLISS Imaging System (Bacus Lab, Lombard, IL). Tissue cores were examined by one pathologist (K.J.W.) blinded to each patient’s identity and clinical features. Based on previous work, E2F1 staining intensities were examined by one pathologist (K.J.W.) blinded to each patient’s identity and the BLISS Imaging System (Bacus Lab, Lombard, IL). Tissue cores were sampled from each donor block using a validated sampling method. The tumor heterogeneity into account, four TMA cores (0.6-mm diameter) were sampled from each donor block using a validated sampling method. The TMA was constructed using a manual tissue arrayer (Beecher Instruments, Silver Spring, MD) as previously described (23).

Statistical analysis. A comparison of expression levels between different cohorts was done using the Mann-Whitney test of the Wilcoxon signed-ranks test for dependent samples. For metastatic hormone-resistant samples, the protein expression was investigated for associations with clinical and pathologic variables using the t test and the Mantel-Haenszel \( \chi^2 \) test. Protein expression scores are presented in a graphical format using error bars with 95% confidence intervals. \( P \) values \(<0.05\) were considered statistically significant.

Cell lines. LNCaP and 293 cells were purchased from the American Type Culture Collection (Manassas, VA). All cells were maintained in RPMI 1640 supplemented with 5% fetal bovine serum (FBS), 0.1% penicillin/streptomycin, and 0.1% l-glutamine.

Semiquantitative reverse transcription-PCR analysis. Total RNA from cell lines was isolated using Qiagen RNA Easy kit using the manufacturer’s protocol (Qiagen, Valencia, CA). Frozen human prostate tissue samples used for semiquantitative reverse transcription-PCR (RT-PCR) analysis were evaluated by a pathologist to confirm pathologic disease. Two to three 10-μm sections were used to isolate total RNA using Qiagen RNA Easy kit using the manufacturer’s protocol. RNA was quantitated spectrophotometrically and 2 μg of RNA were used to make cDNA using Thermoscript RT-PCR Reaction System (Invitrogen, Carlsbad, CA) per manufacturer’s protocol. PCR reaction was done using the following primers: human E2F1, 5’-CATTAGCTATGGCAAGAAG-3’ and 5’-GATGCCACCTAACAGGTCTCTCA-3’; hypoxanthine phosphoribosyltransferase, 5’-CAGTACAGGCCCCAAATGTT-3’ and 5’-TTAAGCGGATGGTC-CA-3’; proliferating cell nuclear antigen (PCNA), 5’-GACCTCCGGCCAC-CATGTTCG-3’ and 5’-GCTTAAGATCCTTCTTAATCCTC-3’; dihydrofolate reductase (DHFR), 5’-GTTGTTGTTGCTGAATGCTAC-3’ and 5’-CCTTTTCTCTCTCTGACATCAG-3’; and actin, 5’-CCTGGAGCTTGACAGAAGAAG-3’ and 5’-GAACGCAAATGCTATGCTC-3’. PCR was done using a Thermocycler with an annealing temperature of 59°C. Ten-microliter aliquots were removed at cycles 25, 28, 31, and 34. Samples were run in triplicate and reaction products were electrophoresed in a 0.8% agarose gel as previously described (20). Densitometric analysis was done using Image J Software downloaded from NIH (Bethesda, MD).

RNA isolation and Northern blot analysis. Total RNA was prepared using Qiagen RNA Easy kit per manufacturer’s protocol (Qiagen). Twenty micrograms of RNA were resolved by gel electrophoresis under denaturing conditions and RNA was transferred to a Duralon-UV membrane (Stratagene, La Jolla, CA) overnight by capillary action in 20× SSC buffer (3 mol/L NaCl and 0.3 mol/L Na citrate). RNA was cross-linked to the membrane by Stratagene UV cross-linking. A 1.6 kb human AR cDNA fragment was isolated from CMV3-HA3I (kindly provided by D. Robins, University of Michigan, Ann Arbor, MI) using a HindIII and Nhel restriction enzyme sites. The human and mouse AR cDNA fragments were gel purified using Qiagen Gel Purification kit, according to the manufacturer’s protocol, and subsequently labeled with [α-32P]dATP using the random oligonucleotide primer labeling kit (Stratagene) and purified on Stratagene Nucleotide Push Columns following the manufacturer’s protocol. The [α-32P]dATP-labeled probes were hybridized to a Duralon-UV membrane (Stratagene) at 65°C overnight in hybridization buffer [0.25 mol/L NaHPO4 (pH 7.2) and 7% SDS] while rotating. The membrane was subsequently washed twice for 45 minutes each in 20 mol/L NaHPO4 (pH 7.2) and 5% SDS followed by two additional washes for 45 minutes each in 20 mol/L NaHPO4 (pH 7.2) and 1% SDS. The membranes were exposed to X-ray film (Kodak, Rochester, NY) overnight and the RNA was visualized by autoradiography.

Western blot analysis. Cells were trypsinized, centrifuged, and washed once with PBS. Cell pellets were lysed in radioimmuno precipitation assay buffer [50 mmol/L Tris (pH 8.0), 120 mmol/L NaCl, 0.5% NP40, 1.0 mmol/L EGTA, 200 μg/mL phenylmethylsulfonyl fluoride (PMSF), 50 μg/mL aprotinin, 5 μg/mL leupeptin, and 200 μmol/L sodium orthovanadate] and protein concentrations were determined using Bradford Protein Assay Reagent (Bio-Rad, Hercules, CA), following the manufacturer’s protocol. For Western blot analysis, 50 μg of protein extract were subjected to gel electrophoresis on a Tris-glycine polyacrylamide gel (Invitrogen). The gel was transferred to Optitran nitrocellulose membrane (Schleicher & Schuell Biosciences, Inc., Keene, NH) by electrophoresis for 1 hour at 43 V. The membrane was blocked in 10% nonfat dry milk in TBST (10 mmol/L Tris, 250 mmol/L NaCl, 1% Tween 20) for 1 hour at room temperature and immunoblotted with primary antibodies for AR (Santa
Cruz Biotechnology), E2F1 (PharMingen), Rb (PharMingen), prostate-specific antigen (PSA; DAKO, Carpinteria, CA), cyclin E (Santa Cruz Biotechnology), PCNA (Santa Cruz Biotechnology), or β-actin (Santa Cruz Biotechnology). The membrane was incubated with a secondary antibody conjugated to horseradish peroxidase, and the bands were detected using enhanced chemiluminescence (Pierce, Rockford, IL) detection system following the manufacturer’s protocol.

Luciferase assay. LNCaP cells were plated at 2 × 10⁵ per six-well dish and incubated at 37°C overnight. Cells were cotransfected with 1 μg of the following promoter-luciferase reporter constructs: DHFR-Luc, E2F-Luc, CRE-Luc (kindly provided by G. Denis, Boston University, Boston, MA; ref. 24), and 2.0 kb human AR promoter-Luc (kindly provided by F.H. Sarkar, following promoter-luciferase reporter constructs: DHFR-Luc, E2F-Luc, and incubated at 37°C.

After 24 h, streptomycin, and 0.1% L-glutamine. For each immunoprecipitation, 1 μg of pSV-β-galactosidase (Promega) was added to each sample as an internal control. DNA was transfected using Tfx50 transfection reagent (Promega) at a ratio of 3:1 (DNA/Tfx50) following the manufacturer’s protocol. After 24 h, control DNA was transfected using a Monolight 2010 luminometer. Samples were assayed in triplicate and luciferase activity was normalized to the manufacturer’s protocol using Monolight 2010 luminometer.

Chromatin immunoprecipitation assay. Human 293 cells were maintained in RPMI 1640 supplemented with 10% FBS, 0.1% penicillin/streptomycin, and 0.1% L-glutamine. For each immunoprecipitation, 1 × 10⁶ cells were used. Cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature on a shaking platform. Cross-linking was stopped by the addition of 0.125 mol/L glycine. Cells were subsequently washed twice with cold PBS, harvested using a cell scraper, and pelleted by centrifugation. Cells were resuspended in lysis buffer [5 mmol/L PIPES (pH 8.0), 85 mmol/L KCl, 0.5% NP40, 200 μg/mL PMSF, 50 μg/mL aprotinin, 5 μg/mL leupeptin] and incubated on ice for 10 minutes. Nuclei were pelleted by centrifugation, resuspended in nuclei lysis buffer [50 mmol/L Tris-HCl (pH 8.1), 10 mmol/L EDTA, 1% SDS, 200 μg/mL PMSF, 50 μg/mL aprotinin, 5 μg/mL leupeptin] and then incubated on ice for 10 minutes. Chromatin was sonicated to an average length of 600 bp with four rounds of 15-second pulse using a Misonix 300 Sonicator while keeping samples on ice. Samples were subsequently centrifuged at 14,000 rpm for 10 minutes at 4°C. Chromatin was precleared with protein A–agarose beads containing sonicated salmon sperm DNA and bovine serum albumin for 15 minutes at 4°C. Samples were centrifuged for 5 minutes at 4°C and diluted in IP dilution buffer [0.01% SDS, 1% Triton X-100, 1.2 mmol/L EDTA, 167 mmol/L Tris (pH 8.1), 167 mmol/L NaCl, plus protease inhibitors]. Samples were incubated overnight at 4°C with 5 μg of the respective antibody; E2F1 (Santa Cruz Biotechnology), E2F4 (Santa Cruz Biotechnology), Rb (PharMingen), p107 (Santa Cruz Biotechnology), p130 (Santa Cruz Biotechnology), and IgG (Santa Cruz Biotechnology). Immunocomplexes were collected by adding protein A–agarose beads, and pellets were washed twice with a low salt buffer [0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris (pH 8.1), 150 mmol/L NaCl], twice in a high salt buffer [0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris (pH 8.1), 500 mmol/L NaCl], twice with LiCl buffer [0.25 mol/L LiCl, 1% NP40, 1% deoxycholate, 1 mmol/L EDTA, 10 mmol/L Tris (pH 8.0)], and twice with TE. Antibody-protein-DNA complexes were eluted with IP elution buffer (1% SDS and 0.1 mol/L NaHCO₃). Formaldehyde cross-links were reversed by adding 0.3 mol/L NaCl and RNase A and by incubating samples at 65°C for 4 to 5 hours. Protein-DNA complexes were precipitated with ethanol overnight at −20°C and centrifuged at 14,000 rpm for 20 minutes at 4°C. Proteinase K was added for 1 to 2 hours at 45°C and DNA was purified using Qiagen DNA purification columns. PCR reaction was done using the following primers for AR promoter: 5’-CCCGAGTTTGCCAGAGGTAA-3’, 5’-TCTTGTACGTCGTCGTGAATC-3’, with an annealing temperature of 95°C for 36 cycles. PCR samples were electrophoresed on a 1.0% agarose gel in 0.5× TBE buffer and visualized under UV light.

Results

Clinical features of patient population. Two TMAs containing 667 tissue cores were evaluated for E2F1 and AR expression by immunohistochemistry. One array contained 188 tissue cores representing benign prostate, localized prostate cancer, metastatic lymph nodes from hormone-naïve prostate cancer patients, and metastatic tissues from men who died from hormone-resistant prostate cancer. The second array contained 479 tissue cores representing benign prostate and metastatic hormone-resistant prostate cancer from rapid autopsies of 30 men who died of hormone-resistant prostate cancer. Benign and localized prostate cancer tissues were constructed from transeptural resection of the prostate and prostatectomy samples from 28 patients, with a median age of 65 years and median PSA value of 5.8 ng/mL. Pathologic stage was T₁ in 26 patients and T₂ in two patients. Regional lymph node metastases were constructed from radical prostatectomy samples from nine patients, with a median age of 64 years and median PSA value of 43.1 ng/mL. All patients had a pathologic stage T₃, and eight patients had seminal vesicle involvement. Metastatic hormone-resistant prostate cancer tissues were acquired from rapid autopsies done on 30 men who died of advanced hormone-resistant prostate cancer. The median age at time of death was 71 years. Twenty-eight men were initially diagnosed with clinically localized prostate cancer, but developed widely disseminated disease after 5 to 10 years. Two men were initially diagnosed with metastatic prostate cancer. All patients were treated with androgen deprivation therapy.

Immunohistochemistry and quantitative analysis of E2F1 staining in prostate tissues. The staining intensity of E2F1 was evaluated in 61 benign, 56 localized prostate cancer, 36 regional lymph node metastasis, and 35 hormone-resistant prostate cancer cores. Nuclear E2F1 staining was observed in 27.1% of benign, 14.3% of localized prostate, 38.9% of hormone-naïve lymph nodes, and 54.8% of metastatic hormone-resistant prostate cancer (χ² P = 0.0006). The number of positive E2F1 nuclei was low in benign and localized prostate cancer (Fig. 1A) with mean expression values of 1.77% [SD, 3.18; 95% confidence interval (95% CI), 0.98–2.58] for benign and 1.07% [SD, 3.06; 95% CI, 0.217–1.93] for localized prostate cancer (Fig. 1B). The number of positive E2F1 nuclei increased in lymph nodes from hormone-naïve patients with a mean expression value of 3.61% [SD, 5.6; 95% CI, 1.79–5.43; Fig. 1A and B]. Metastatic hormone-resistant prostate cancer had the greatest number of E2F1-positive nuclei, with a mean expression value of 13.04% [SD, 8.3; 95% CI, 8.45–17.62]. There was wide variation in the number of nuclei exhibiting staining in hormone-resistant metastatic tissues. The data were, therefore, stratified into eight categories: negative, ≤5%, 6% to 10%, 11% to 20%, 21% to 40%, 41% to 60%, 61% to 80%, and 81% to 100%. The distributions of staining for benign, localized prostate cancer, hormone-naïve, and hormone-resistant metastatic prostate cancer are shown in Fig. 1C. These analyses show that high levels of nuclear E2F1 staining (≥20%) are restricted to hormone-resistant metastatic prostate cancer. Two patients with liver metastasis had over 60% of cells staining positive for nuclear E2F1.
Figure 1. E2F1 expression during prostate cancer progression. A, immunohistochemical staining of E2F1 in benign prostate (Benign), localized prostate cancer (Tumor), metastatic lymph nodes from hormone-naive patients (HN Mets), and metastatic hormone-resistant prostate cancer (HR Mets). Magnification, ×5 (a, c, e, g) and ×20 (b, d, f, h). B, E2F1 expression in benign prostate, localized prostate cancer, metastatic lymph nodes from hormone-naive patients, and metastatic hormone-resistant prostate cancer. Points, mean nuclear E2F1 protein expression; bars, 95% CI. N, number of cases indicated for each tissue type. C, distribution of E2F1 staining in benign prostate, localized prostate cancer, metastatic lymph nodes from hormone-naive patients, and metastatic hormone-resistant prostate cancer. Columns, percentage of samples in each category. D, stain intensity of nuclear E2F1 for benign prostate, localized prostate cancer, metastatic lymph nodes from hormone-naive patients, and metastatic hormone-resistant prostate cancer. Points, nuclear E2F1 intensity; bars, 95% CI.
The staining intensity of E2F1 was quantified on a scale of 0 to 3; 0 representing absence of stain, 1 representing weak stain, 2 representing moderate stain, and 3 representing intense stain. Mean expression values for nuclear E2F1 stain intensity according to diagnosis is shown in Fig. 1D. E2F1 immunoreactivity was low in benign prostate and localized prostate cancer with a mean stain intensity score of 0.215 (SD, 0.816; 95% CI, 0.052–0.378) and 0.214 (SD, 0.63; 95% CI, 0.05–0.38) for benign and localized prostate cancer, respectively. Metastatic lymph nodes from hormone-naive patients had the strongest nuclear E2F1 stain intensity, with 28% having intense nuclear E2F1 stain and a mean stain intensity score of 1.03 (SD, 1.07; 95% CI, 1.89–2.6; Fig. 1D). Metastatic tissues from hormone-resistant patients had a wide distribution of E2F1 staining intensity, with 29% with low stain intensity, 16% with moderate stain intensity, and 10% with intense staining. These results show that benign and localized prostate cancer have low E2F1 stain intensity and metastatic tissues from hormone-naive and hormone-resistant patients have the greatest percentage of cells with intense E2F1 stain, suggesting a role of E2F1 in prostate cancer progression.

**AR expression in prostate cancer progression.** A central issue to understanding hormone-resistant prostate cancer is to identify the role of AR during prostate cancer progression; we, therefore, assessed AR expression during prostate progression in the same TMA described above. Nuclear AR expression was detected in 83% of benign, 100% of localized prostate cancer, 80% of metastatic lymph nodes, and 40% of metastatic hormone-resistant prostate cancer (Fig. 2A). The number of positive AR nuclei was similar between benign, tumor, and hormone-naive lymph nodes with mean expression values of 45.5%, 52.21%, and 45.83%, respectively (Fig. 2B). Expression of AR was down-regulated in metastatic tissues from hormone-resistant patients with a mean expression value of 21.2% (Fig. 2B). AR stain intensity was greatest in benign and localized prostate cancer, decreased slightly in hormone-naive lymph nodes, and was lowest in metastatic hormone-resistant prostate cancer (Fig. 2C). Sixty percent of hormone-resistant tissues had <10% of tumor cells staining positive for AR.

**Figure 2.** AR expression during prostate cancer progression. A, AR expression detected by immunohistochemistry. Example of positive AR staining in benign prostate (a, e), localized prostate cancer (b, f), and metastatic lymph nodes from hormone-naive patients (c, g). Example of negative AR staining in a liver metastasis from a hormone-resistant patient (d, h). Magnification, ×5 (a–d) and ×20 (e–h). B, AR expression for benign prostate, localized prostate cancer, metastatic lymph nodes from hormone-naive patients, and metastatic hormone-resistant prostate cancer. Points, mean nuclear AR protein expression; bars, 95% CI. C, stain intensity of nuclear AR expression for benign prostate, localized prostate cancer, metastatic lymph nodes from hormone-naive patients, and metastatic hormone-resistant prostate cancer. Points, mean AR protein expression; bars, 95% CI.
E2F1 and AR expression in metastatic hormone-resistant prostate cancer. We wanted to expand our assessment of E2F1 expression in metastatic prostate tissues from hormone-resistant patients; therefore, we stained a second prostate TMA containing 479 metastatic tissue cores from 30 patients who had died from hormone-resistant prostate cancer. Metastatic tissues included bone, liver, lung, dura, bladder, lymph node, adrenal gland, pancreas, and soft tissues. Nuclear E2F1 stain was detected in 80% (382 of 479) of hormone-resistant cases and the mean expression value of E2F1 was 28% (range 0–90%). The distribution of nuclear E2F1 staining in hormone-resistant cases based on tissue type is shown in Fig. 3A. E2F1 staining was evaluated in the prostate gland from 13 hormone-resistant patients and showed increased nuclear E2F1 expression with a mean value of 32.5% (SD, 23.23, 95% CI, 26.36–39.24) compared with 4.4% (SD, 10.14, 95% CI, 1.2–10.1) for benign prostate. Figure 3B (a and b) shows low E2F1 staining in benign prostate tissue versus strong E2F1 staining in the prostate of a patient with hormone-resistant disease. In some cases, perinuclear E2F1 staining was observed, as shown in a metastatic bladder sample (Fig. 3B, c) and a bone metastasis (Fig. 3B, d). We observed wide heterogeneity of E2F1 expression between different organ sites and patients; however, there was no pattern to the differences in E2F1 expression between the different organ sites that could be distinguished.

AR expression was evaluated in the same tissue samples (Fig. 3B, f–j). Nuclear AR expression varied across tumor samples with a median expression value of 63.3% (SD, 38.5; 95% CI, 58.78–67.81) and median stain intensity value of 2.15 (SD, 1.22; 95% CI, 2.04–2.26). One patient had a complete loss of AR in all metastatic sites examined, including lymph node, liver, pancreas, and soft tissue, as well as the prostate gland. Our results are similar to the study by Shah et al. (28), which reported a wide variation of AR and PSA expression in hormone-resistant prostate cancer from rapid autopsy patients.

Elevated E2F1 mRNA and E2F-target genes in metastatic hormone-resistant prostate cancer. Because E2F1 protein expression was significantly elevated in metastatic hormone-resistant prostate cancer compared with benign prostate tissue, we wondered whether E2F1 mRNA transcripts were also increased to the same degree as protein expression. We analyzed E2F1 transcripts from nine metastatic hormone-resistant prostate cancer patients and four benign cases by semiquantitative RT-PCR analysis. As shown in Fig. 4A, E2F1 transcripts were elevated in hormone-resistant prostate cancer when compared with benign prostate tissues. Densitometric analysis of mRNA transcripts revealed up to 47-fold activation of E2F1 mRNA expression (after normalizing to actin) in metastatic hormone-resistant prostate cancer compared with benign tissues (Fig. 4B). When we compared the band intensity of E2F1 mRNA transcripts with the expression value of nuclear E2F1 stain reported on the TMA for each individual case, we found a statistically significant correlation between E2F1 mRNA and E2F1 protein expression (Fig. 4C; Pearson correlation coefficient r = 0.87393, P < 0.01). These results showed that both E2F1 mRNA and protein expression are significantly elevated in metastatic hormone-resistant prostate cancer compared with benign tissues.

To investigate whether increased E2F1 expression resulted in increased E2F1 transcriptional activity, the same set of benign and hormone-resistant prostate cancer cases was evaluated for mRNA expression of two established E2F-target genes, DHFR and PCNA.
E2F1 down-regulates AR expression. Overall, our TMA results clearly show increased E2F1 protein expression and decreased AR expression in hormone-resistant prostate cancer compared with benign and localized prostate cancer. Based on these observations, we hypothesized that elevated levels of E2F1 may down-regulate AR expression. To test this hypothesis, we examined whether overexpression of E2F1 could inhibit endogenous AR mRNA levels in prostate epithelial cells. LNCaP cells were transiently transfected with increasing amounts of a plasmid-containing human E2F1 or empty pcDNA3 control vector. Total RNA was harvested and subjected to Northern blot analysis for the detection of AR and E2F1 mRNA. As shown in Fig. 5A, AR mRNA levels decrease with increasing amounts of E2F1 in LNCaP cells. The 28S and 18S rRNAs are shown as a loading control. Figure 5B shows decreased AR protein expression in E2F1-overexpressing cells. As a positive control to show increased E2F activity, we show increased protein expression of cyclin E and PCNA, two well-characterized E2F-target genes (Fig. 5B). We also show decreased protein expression of the AR-target gene, PSA, which is most likely due to decreased AR protein expression. These results suggest that E2F1 down-regulates AR expression in prostate epithelial cells.

E2F1 expression results in repression of both mouse and human AR promoters. To assess whether E2F1 can regulate AR promoter activity, we used a 2 kb human AR promoter construct containing −900 to +1,129 bp relative to the transcription start site cloned upstream of a luciferase reporter gene (29). The human AR promoter/luciferase construct (hAR-Luc) was cotransfected into human LNCaP cells with either empty pcDNA3 vector (control), wild-type E2F1, or a dominant-negative E2F1 construct, along with a cytomegalovirus (CMV) promoter-driven β-galactosidase reporter plasmid as an internal control (Fig. 6A). E2F1 repressed AR promoter activity by 62% in LNCaP cells when compared with cells transfected with empty pCDNA3 plasmid. The dominant-negative E2F1 did not significantly inhibit AR promoter activity. As a positive control, we show that E2F1 activates an E2F-inducible promoter, which contains four adjacent E2F consensus binding sites (E2F-Luc). To show promoter specificity, we show that E2F1 does not have a similar effect on a CRE-Luc promoter, which contains four adjacent cyclic AMP (cAMP) regulatory elements in front of a luciferase reporter construct. These results show that E2F1 inhibits AR promoter activity.

We next examined the effects of different E2F1 mutants on AR promoter activity in LNCaP cells. A mutation in the DNA binding domain (EcoI32) slightly relieved E2F-mediated inhibition, whereas deletion of the transactivation domain of E2F1 (E2F1_{1,280}) completely abrogated the inhibitory effect of E2F1 (Fig. 6B). As expected, the E2F1 mutants did not activate the E2F-Luc promoter activity, which requires both the E2F1 transactivation and DNA binding domains for activation. These results indicate that the transactivation domain of E2F1 is essential for E2F1-mediated repression of the AR promoter.

We next examined the human AR promoter for potential E2F1 consensus binding sites using MatInspector computational analysis. We identified two potential E2F1 binding sites located at positions 617 to 630 and 961 to 977 relative to the transcription start site, located on the complimentary DNA strand. To investigate whether E2F1 regulates the AR promoter in vivo, we used the chromatin immunoprecipitation (ChIP) assay using primers designed to include the two potential E2F1 binding sites identified at positions 617 to 630 and 961 to 977 on the AR promoter. The ChIP assays were done in the human 293 cell line because these cells routinely work well in ChIP assays and have been used to easily show the binding of E2F1 to endogenous promoters. As shown in Fig. 6C, a strong PCR signal was seen when samples were incubated with an antibody to E2F1. No signal was detected with

DHFR and PCNA transcripts were high in metastatic hormone-resistant prostate cancer compared with benign tissues with up to 10-fold activation of DHFR and 30-fold activation of PCNA in metastatic prostate cancer (Fig. 4A and B). There was a statistically significant ($P < 0.001$) positive correlation between E2F1 and DHFR expression (Pearson correlation coefficient, $r = 0.852219$) and E2F1 and PCNA expression (Pearson correlation coefficient, $r = 0.76305$). These results suggest that increased E2F1 expression in hormone-resistant prostate cancer results in increased E2F1 transcriptional activity.

Figure 4. Molecular analysis of E2F1 mRNA and E2F-target genes in benign and metastatic hormone-resistant prostate cancer. A, results of semiquantitative RT-PCR analysis for evaluation of mRNA levels of E2F1, DHFR, PCNA, and actin in four benign prostate tissues (lanes 1–4) and nine metastatic hormone-resistant prostate cancer cases (lanes 5–13). Actin, loading control. B, band intensities for E2F1 (white columns), DHFR (gray columns), and PCNA (black columns) transcripts. Each sample was normalized to actin and represented as arbitrary units $\times 10^7$. C, semiquantitative RT-PCR analysis of E2F1 mRNA in benign and metastatic hormone-resistant prostate cancer (white columns). Levels are normalized to corresponding actin values for each case. Black columns, mean expression value of nuclear E2F1 protein for each tissue as determined by TMA. Note the different scales.
other antibodies, including IgG, which was used as a control for nonspecific precipitation of protein-DNA complexes or E2F4. The samples were also subjected to PCR reactions using primers to actin to ensure that the immunoprecipitations were not binding nonspecifically. Because E2F1 can transcriptionally repress promoters by recruiting Rb pocket protein family members, we decided to perform ChIP assays with antibodies to Rb and the pocket protein family members p107 and p130. Strong PCR bands were detected when anti-p107 and anti-p130 antibodies were used, but not when anti-Rb antibodies were used. These results suggest that E2F1, p107, and p130, but not Rb, bind to the AR promoter in vivo and may suggest that p107 and p130 cooperate with E2F1 to mediate repression of the AR.

**Discussion**

Activation of E2F transcription factors by disruption of the Rb pathway is thought to be a key event in human cancer; however, detailed assessment of E2F1 expression has not been previously examined in human prostate cancer. Using TMA technology, we have shown that E2F1 is low in benign and localized prostate cancer, modestly elevated in hormone-naive metastatic lymph nodes, and significantly elevated in metastatic tissues from men who died of hormone-resistant prostate cancer (P = 0.0006). On a larger TMA, E2F1 immunoreactivity was detected in 80% of metastatic tissues from 30 men who died of hormone-resistant prostate cancer with an overall mean expression value of 28% (range 0–90%) compared with benign tissues with a mean expression value of 4.4% (range 0–15%). Elevated E2F1 protein expression correlated with increased E2F1 mRNA and increased expression of E2F1-target genes DHFR and PCNA, suggesting that E2F1 expression is elevated in advanced prostate cancer.

E2F1 expression has been evaluated in other human cancers and high levels of E2F1 have been associated with advanced-stage disease and poor prognosis in breast cancer and non–small cell lung cancer patients (15, 17). Elevated E2F1 expression has been reported in lymph node metastasis of breast cancer patients and amplification of the E2F1 gene has been reported in metastatic tumors of human colorectal cancer (15, 30). These studies are in agreement with our study, which demonstrat low E2F1 expression in benign tissues and elevated levels in late-stage disease.

The oncogenic capacity of E2Fs has been attributed to their ability to transactivate genes involved in DNA synthesis and cell cycle progression. In this study, we have shown that human metastatic hormone-resistant prostate cancer tissues had increased levels of E2F-target genes DHFR and PCNA, which are essential components for DNA replication. DHFR is required for purine biosynthesis and is thus important for the production of nucleotides required for DNA synthesis and S-phase progression (29). PCNA is a nuclear cofactor for DNA polymerase δ and is widely used as a marker of cell proliferation. PCNA expression in prostate cancer has been correlated with disease progression and negatively correlated with survival (31, 32). High levels of E2F1 may provide tumor cells with a proliferative advantage by transcriptionally activating downstream targets involved in DNA synthesis and thus contribute to tumor progression. With the use of cDNA microarray technology, hundreds of genes have been identified as E2F targets, including genes involved in cell cycle regulation, mRNA splicing, chromatin modification, angiogenesis, apoptosis, invasion, and metastasis (33–35). We have recently shown that cyclooxygenase 2 and DNA methyltransferase 1 are downstream targets of E2F1 in prostate epithelial cells and may contribute to hormonal carcinogenesis (20, 21), suggesting that E2Fs play multiple roles in tumorigenesis.

Androgens and AR are critical components for prostate gland development, prostatic function, and are involved in prostate tumorigenesis. AR expression has been observed in primary prostate cancer and can be detected throughout progression in both hormone-sensitive and hormone-resistant cancers (36, 37); however, the importance of AR during late stages of prostate cancer is not entirely clear. Initial studies showed that AR mRNA is present in androgen-sensitive prostate cancer cell lines, but is absent or expressed at low levels in androgen-independent cell lines (38). Recent studies have shown heterogenous expression of AR throughout prostate cancer. Sadi and Barrack (39) showed that cancer cells in patients with advanced prostate cancer who responded poorly to therapy exhibited a significant heterogeneity in the staining intensity of AR. de Vere White et al. (40) showed a loss of AR expression in 33% of prostate cancer patients following combined androgen blockade therapy and Hobisch et al. (41) showed complete loss of AR in prostate cancer lymph node metastases. Kinoshita et al. (42) showed a significant loss of AR

**Figure 5.** E2F1 inhibits AR mRNA and protein expression. A, Northern blot analysis to detect AR and E2F1 gene expression in LNCaP cells transfected with 0, 5, 10, or 15 μg E2F1. Total RNA was harvested for Northern blot analysis using the indicated probes. Equivalent loading of RNA was confirmed by 28S and 18S rRNAs. B, Western blot analysis of whole-cell lysates harvested from LNCaP cells transfected with 0, 5, 10, and 15 μg E2F1 for the detection of AR, E2F1, cyclin E, PCNA, and PSA. PARP, poly(ADP)ribose polymerase: protein loading control.
expression in advanced prostate cancer and found that methylation of the AR promoter was tightly linked to metastatic hormone-resistant tumors. In our study, 60% of hormone-resistant prostate tumors had <10% of tumor cells staining positive for AR and a complete loss of AR expression was observed in several metastatic tumors. These results suggest that loss of AR expression during prostate cancer progression may be associated with the development of androgen independence in a subset of patients. It is important to note that these studies do not rule out the importance of AR amplification and mutated forms of AR, which have shown to confer androgen independence of prostate cancer cells (reviewed in refs. 43–45). A variety of AR mutations have been identified in prostate cancer cell lines and human tumors with the most common mutations occurring in the ligand binding domain. Mutations in the ligand binding domain alter the specificity of the AR; enhancing the binding of estrogens, progesterone, and antiandrogens; and thereby decreasing its dependency on androgens while stimulating cell growth (46, 47). In addition to AR mutations, a variety of growth factors, including insulin-like growth factor I, epidermal growth factor, and keratinocyte growth factor, can activate androgen-responsive genes via the AR, suggesting that androgen independence could arise from overexpression of growth factors in the local environment (48). Clearly, mutations of the AR and activation of AR via growth factors play a role in androgen-independent growth. In this study, we are suggesting that decreased expression of AR may also play a role in the selection of androgen-independent tumors. Mechanisms for AR downregulation in prostate cancer have been proposed, including promoter silencing by DNA methylation (49) and transcriptional repression by transcription factors nuclear factor-B and nuclear factor-κB (50, 51). This is the first study to show that E2F1 represses AR mRNA and protein expression and inhibits AR promoter activity. We have also shown that physiologic levels of E2F1 can bind to the AR promoter in vivo in human 293 cells. E2F1 primarily functions as a positive regulator; nevertheless, in addition to the present report, a negative regulatory role of E2F1 has also been described for a number of genes, including urokinase-type plasminogen activator (uPA; ref. 52), the antiapoptotic protein Mcl-1 (26), and human telomerase reverse transcriptase (htERT; ref. 53). Unlike its positive regulatory function, which is mediated by direct interaction of E2F1 to DNA binding sites, the mechanism(s) for E2F1-mediated negative regulation are still largely unknown. Crowe et al. (53) identified two putative E2F binding sites in the Mcl-1 promoter that were important for E2F1-mediated repression. The E2F1 mutant (E132), which lacks the DNA binding domain of E2F1, was inefficient at repressing Mcl-1 (26), and human telomerase reverse transcriptase (htERT; ref. 53). Unlike its positive regulatory function, which is mediated by direct interaction of E2F1 to DNA binding sites, the mechanism(s) for E2F1-mediated negative regulation are still largely unknown. Crowe et al. (53) identified two putative E2F binding sites in the Mcl-1 promoter that were important for E2F1-mediated repression. The E2F1 mutant (E132), which lacks the DNA binding domain of E2F1, was inefficient at repressing Mcl-1 promoter activity, suggesting that the DNA binding domain was essential for repression. In another study by Croxton et al. (26), direct repression of the Mcl-1 promoter by E2F1 also required the DNA binding domain, but not the transactivation domain. Koziczak et al. (52) showed that both the DNA and transactivation binding domains of E2F1 were necessary for the negative regulation of the uPA and plasminogen activator inhibitor 1 genes; however, E2F1 repressed promoter activity independently of the pocket protein Rb. Our data show that the carboxyl-terminal transactivation domain was essential for E2F1 suppression of the AR promoter (Fig. 5). Several proteins are known to bind to this region and regulate transcription, including cAMP-responsive element binding protein–binding protein (54), MDM2 (55), and TRRAP-Tip60 complex (56). The transactivation domain also interacts with the basal transcription factor III and the Rb pocket protein family members (57). Our ChIP analysis show that p107 and p130 are recruited to the AR promoter along with E2F1 and suggest that p107 and p130 may cooperate with E2F1 to mediate AR transcriptional repression. Future studies are being done to determine if p107 and p130 are required for E2F1-mediated repression of the AR.
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References


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