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EBP1, an ErbB3-binding protein, is decreased in prostate cancer and implicated in hormone resistance

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Abstract

Aberrant activation of the androgen receptor (AR) by the ErbB2/ErbB3 heterodimer contributes to the development of hormone resistance in prostate cancer. EBP1, an ErbB3-binding protein, acts as an AR corepressor. As EBP1 is decreased in preclinical models of hormone-refractory prostate cancer, we studied the expression of EBP1 in human prostate cancer. We found that the expression of the *EBP1* gene was significantly decreased in prostate cancer tissues compared with benign prostate at both mRNA and protein levels. Restoration of EBP1 expression in the hormone-refractory LNCaP C81 cell line led to an amelioration of the androgen-independent phenotype based on established biological criteria and a reduction in the expression of a cohort of AR target genes. The ability of the ErbB3 ligand heregulin (HRG) to stimulate growth and AKT phosphorylation of hormone-refractory prostate cancer cells was abolished. Abrogation of EBP1 expression by short hairpin RNA in hormone-dependent LNCaP cells, which undergo apoptosis in response to HRG, resulted in HRG-stimulated cell growth. Restoration of EBP1 expression decreased the tumorigenicity of C81 xenografts in female mice, whereas elimination of EBP1 expression enhanced the ability of LNCaP cells to grow in female mice. Our data support a role for EBP1 in the

development of hormone-refractory prostate cancer via inhibition of both AR- and HRG-stimulated growth and present a novel strategy for treating androgen-refractory prostate cancer. [Mol Cancer Ther 2008;7(10):3176–86]

Introduction

Prostate cancer begins as an androgen-dependent tumor that undergoes clinical regression in response to pharmacologic and surgical strategies that reduce testosterone concentration. Despite this treatment, the cancer eventually regrows as a uniformly fatal hormone-refractory tumor. The androgen receptor (AR) is central to the initiation and growth of prostate cancer and to the therapeutic response to hormones. AR continues to be expressed even in hormone-refractory tumors and aberrant AR signaling is an important mechanism of progression to androgen independence (1). Increases in AR protein levels are associated with hormone-refractory disease clinically (2, 3) and in xenograft models of prostate cancer (4). Thus, new approaches to silence AR signaling are important for the therapy of hormone-refractory prostate cancer.

ErbB family members and their ligands have been implicated in the development of androgen independence due to their stimulatory effects on AR function (5). It was initially shown that androgen-independent sublines of human prostate cancer xenografts expressed higher levels of ErbB2 than androgen-dependent sublines (6). Ectopic expression of ErbB2 sensitized cells to extremely low levels of androgen (7). Inhibition of ErbB2/3 signaling resulted in suppression of AR signaling and cell growth. ErbB2/3 kinase activity stabilized AR protein levels via inhibition of ubiquitin-mediated degradation and increased recruitment of AR to AR-responsive promoters (5). Inhibition of ErbB2 kinase activity also impaired AR recruitment to the prostate-specific antigen (PSA) promoter (8) and triggered apoptosis in androgen-independent C4-2 cells (9). ErbB3 has also recently emerged as an important factor in prostate cancer progression. Overexpression of ErbB3 was linked to a less favorable prognosis in prostate cancer (10). A secreted form of the ErbB3 receptor, MDA-BF-1, was associated with prostate cancer metastases (11). A high frequency of nuclear ErbB3 expression was associated with hormone-refractory prostate cancer (12) and nuclear ErbB3 expression was increased in prostate cancer cells found in lymph node and bone metastases (13). The ErbB3/4 ligand heregulin (HRG), present in normal human adult prostate and in benign prostatic hyperplasia, may function as a paracrine physiologic differentiation factor (14, 15). HRG had opposing effects on growth of hormone-dependent versus hormone-refractory cells lines. HRG inhibited growth and induced differentiation of hormone-sensitive ErbB1-, ErbB2-, and ErbB3-expressing LNCaP prostate

cancer cells (14, 15) and induced the expression of the tumor suppressor p53 and the cyclin-dependent kinase inhibitor p21 (16). Tal-Or et al. (17) similarly showed that neuregulin (HRG) activated ErbB2/ErbB3 heterodimers and induced apoptosis of LNCaP cells. In contrast, HRG enhanced growth of CWR-R1 and 22Rv1 hormone-refractory cells and activated AR (18, 19). Such effects were more pronounced in medium with castrate levels of androgens, pointing to the importance of ErbB signaling pathways in androgen-deprived patients.

Our laboratory has cloned EBP1, a protein that binds both the ErbB3 receptor and the AR. EBP1, the product of the *PA2G4* gene, is the human homologue of the mouse cell cycle-regulated, DNA-binding protein p38-2G4 (20). EBP1 has a predicted amphipathic helical domain for protein/protein or protein/DNA interactions and an LXXLL motif flanked by basic regions present in many nuclear receptor binding proteins (21). EBP1 physically associated with AR and suppressed AR signaling (22). Overexpression of the *EBP1* gene in androgen-dependent LNCaP cells down-regulated expression of AR and its target genes. EBP1 decreased transcription of AR-activated genes in part by binding to an AR response element in AR target genes and recruiting the corepressors Sin3A and HDAC2 to chromatin (23). EBP1 bound AR targets in the presence of bicalutamide, providing evidence of EBP1 function in prostate cancer therapy. The ability of EBP1 to bind AR-regulated promoters and inhibit AR promoter activity was enhanced by HRG (24). The ectopic expression of EBP1 resulted in growth inhibition both *in vivo* and *in vitro* (23). As EBP1 expression was reduced in two models of hormone-refractory prostate cancer (25), we postulated that lack of EBP1 function might contribute to growth of cells in an androgen-deprived environment.

In this study, we determined the expression of EBP1 in clinical prostate cancer and the effects of EBP1 on the hormone-refractory phenotype in prostate cancer cells. We found that expression of EBP1 was down-regulated in clinical prostate cancer at both mRNA and protein levels. In addition, increased expression of EBP1 in hormone-refractory cells led to an amelioration of the androgen-independent phenotype. These studies suggest the therapeutic potential of EBP1 in hormone-refractory prostate cancer.

Materials and Methods

Cell Culture and Reagents

The LNCaP-C81 cell line (C81), a hormone-refractory variant of LNCaP cells, was a kind gift of Dr. Ming-Fong Lin (University of Nebraska; ref. 26). All other cell lines were obtained from the American Type Culture Collection and routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum. HRGβ1 was obtained from R&D Systems.

Creation of Stably Transfected Cell Lines

Subconfluent C81 cells in 100 mm tissue culture dishes were transfected with 10 μg CMV-10 or CMV10-*EBP1* expression plasmids using Fugene-6 according to the

manufacturer's protocol. Cells were selected in G418 (800 μg/mL) for 5 weeks and resistant colonies were expanded. Both individual clones and mass transfectants were obtained.

To generate EBP1-silenced cell lines, LNCaP cells were seeded into 96-well plates and transduced with lentiviral particles (multiplicity of infection = 25) corresponding to different short hairpin RNA (shRNA) constructs targeted to the *PA2G4 (EBP1)* gene (Mission shRNA; Sigma) according to the manufacturer's protocol. Cell lines were selected in 2 μg/mL puromycin and surviving colonies of cells were expanded as mass cultures. Five individual shRNA lentiviral particle constructs were tested. Only one construct (13C) corresponding to nucleotides 302 to 322 of *PA2G4* (Genbank NM_006191.1) inhibited EBP1 expression. Another construct, 16A, did not inhibit EBP1 expression and served as a control.

Microarray Analysis and Bioinformatics

RNA was prepared using the Trizol reagent as described previously (22). Microarray processing and data analysis was done at Genome Explorations. U133A oligonucleotide arrays (Affymetrix) containing ~33,000 full-length annotated genes together with additional probe sets designed to represent expressed sequence tags were used. Only genes with a minimum expression level of 500 were included in this analysis. Genes whose expression varied more than 2.9-fold with a $P < 0.05$ were considered to be significantly different between the two cell lines. Oncomine analysis was done by using Oncomine3.0.[6]

Real-time Quantitative Reverse Transcription-PCR

Both cell culture samples and a commercial panel of cDNAs from normal prostate and prostate tumors (TissueScan; Origene) were examined. The method of Nakanishi et al. (27) was used as described previously. Real-time quantitative reverse transcription-PCR was done on the LightCycler (Roche) platform. The following forward and reverse primers were selected using Primer Express software and synthesized by the Core Laboratory of University of Maryland School of Medicine: EBP1 sense 5′-GCACGCCAATAGAAGG-3′ and antisense 5′-GTAAA-CGGCATGGCATC-3′, kallikrein-2 sense 5′-CATCCAGTC-TCGGATTG-3′ and antisense 5′-CTCATATTGTAGAG-CGGGT-3′, testis-specific protein, Y-encoded (TSPY) sense 5′-CAGGGCTTCTCATTCCACTC-3′ and antisense 5′-CC-ATCATATTCAACTCAACAACTGG-3′ (28), AGR2 sense 5′-ATTGGCAGAGCAGTTTCTCC-3′ and antisense 5′-GA-GCTGTATCTGCAGGTTCGT-3′ (29), and β-actin sense 5′-GCTATCCAGGCTGTGCTATC-3′ and antisense 5′-TGT-CACGCACGATTTCC-3′. A SYBR Green PCR kit was used as per the manufacturer's instructions (Applied Biosystems) and the analyses were done in duplicate or triplicate. Target mRNA values were normalized using β-actin mRNA as an internal control. The relative quantitation of gene expression was done using the comparative $\Delta\Delta C_t$ (threshold method) using β-actin as an internal control.

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[6] www.oncomine.org

Tissue Microarray and Immunohistochemical Analysis

An intermediate density tissue microarray of prostate cancer and benign prostate tissues was received from the Cooperative Prostate Cancer Tissue Resource (National Cancer Institute). For the benign group, 15 normal prostate tissues were taken from prostate cancer patients and 27 prostate tissues from normals were used. For the malignant group, 153 tumors from patients with both localized and metastatic prostate cancer (Gleason grades 4-10) were evaluated. The Vectastain Elite ABC kit purchased from Vector Laboratories was used for immunohistochemical analysis. Immunohistochemical analysis was done using an EBP1 antibody (Upstate 1:200; pH 7.4) as described previously (30). Immunohistochemical staining was assessed independently by two observers, and a consensus of grading was reached. Immunostaining was evaluated manually and intensity was scored on the following scale: 0 = negative, 1 = weak, 2 = moderate, and 3 = strong. The nonparametric Wilcoxon rank-sum test was conducted to determine significant differences between the benign prostate epithelium and the malignant tissue.

Measurement of PSA Levels

Serum PSA levels were determined using a PSA ELISA kit from DSL as described previously (25).

Western Blot Analysis

Western blot analysis was done as described previously (31). The EBP1 antibody was from Upstate, the polyclonal antibody to actin was from Sigma, and the AKT and phosphorylated AKT antibodies were from Cell Signaling. The AGR2 antibody was described previously (32).

Cell Growth Assays

Cell growth measurement in complete medium was done as described using a hemocytometer (33). For soft-agar growth assays, increasing concentrations of cells
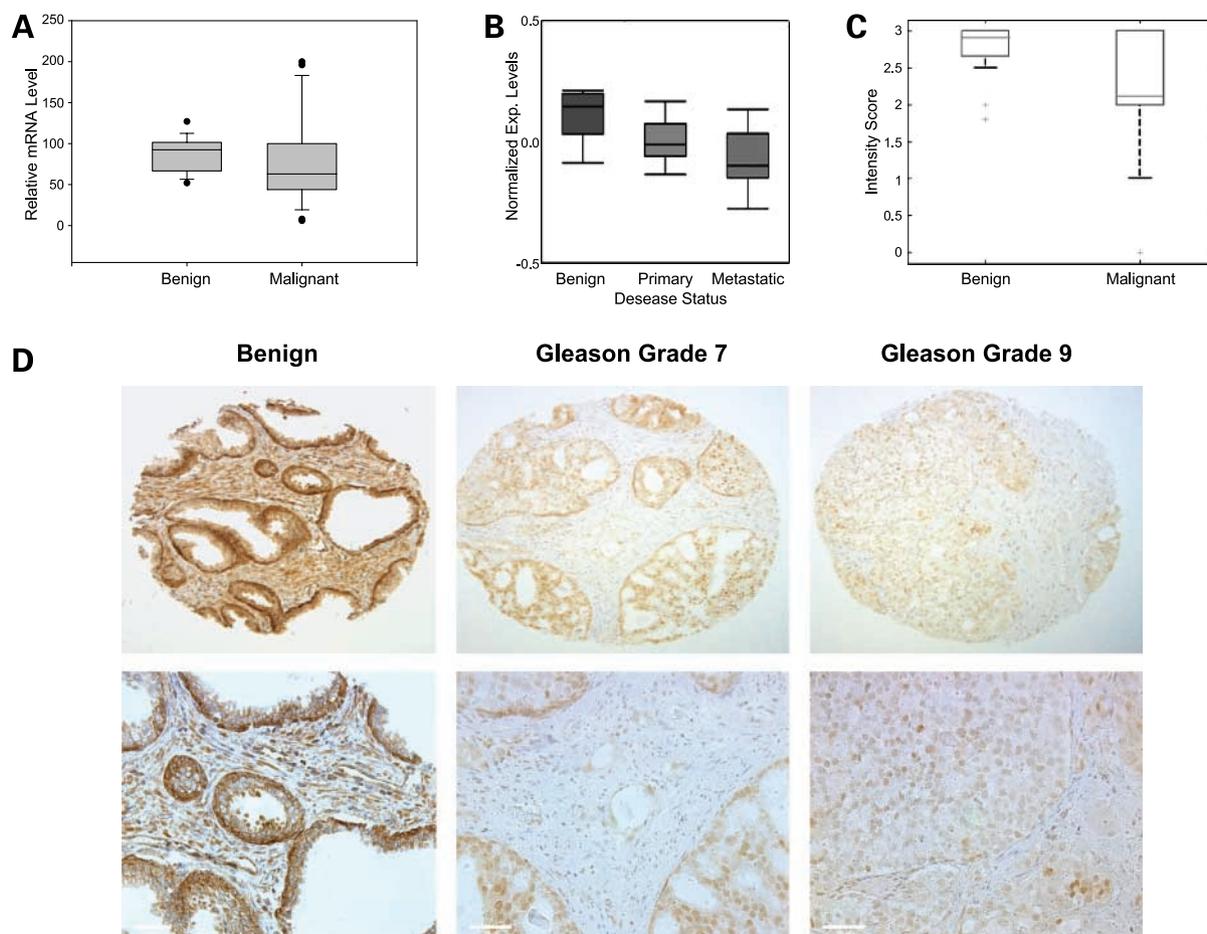

Figure 1. EBP1 expression is decreased with prostate cancer progression. **A,** quantitative reverse transcription-PCR was done for EBP1 mRNA expression on a panel of benign (7 normal and 11 benign prostatic hyperplasia), prostate cancer stage I and II (21), and prostate cancer stage III and IV (9) tissues obtained from Origene. The relative levels of all test mRNAs were normalized to β-actin. **B,** a public data set was obtained by Yu et al. and processed by Oncomine 3.0 for EBP1 expression. The average gene expression level at three different stages (benign $n = 23$, primary $n = 64$, and metastatic $n = 25$) of the disease is presented. **C,** box plots of immunohistochemical staining intensity of normal prostate tissue ($n = 42$) or tissue from patients with prostate cancer ($n = 153$). **D,** representative staining of (*left*) normal prostate epithelium, (*middle*) grade 7 Gleason showing nuclear staining of prostate cancer cells, and (*right*) Gleason grade 9 at both low (*top*) and high (*bottom*) magnification.

(as indicated) were plated in 35 mm Petri dishes in 0.3% agar in complete medium and colonies were counted after 10 days of incubation (33). For studies assessing the effect of DHT, HRG, or drugs on cell growth, cells (5×10^3) were plated in 96-well plates in complete medium. After a 24-h attachment period, the medium was replaced with steroid-free medium [phenol red-free RPMI 1640 and 5% charcoal-stripped serum (Sigma)]. After 48 h of steroid depletion, cells were re-fed with fresh steroid-reduced medium with or without the indicated concentrations of DHT, bicalutamide (Casodex; 10 μmol/L), HRG, or the chemotherapeutic drugs at the concentrations indicated. Relative cell growth was determined using a Promega Proliferation Reagent (Promega) as per manufacturer's instructions with absorbance being read at 490 nm using a Dynex plate reader.

Luciferase Reporter Assays

Cells (5×10^4) were plated in 12-well plates in complete medium. When cells reached 50% to 60% confluence, they were transfected using the Fugene-6 reagent (Roche) according to the manufacturer's instructions. Cells were transfected with 0.5 μg MMTV-luciferase reporter plasmid and 5 ng TK-*Renilla* plasmid (Promega) as an internal control. Complete medium was replaced 24 h after transfection with phenol red-free RPMI 1640 with charcoal-stripped serum with or without R1881 (10^{-8} mol/L). Luciferase activity was determined using the Promega dual-luciferase assay kit as described by the manufacturer.

In vivo Studies in Athymic Mice

Female nude athymic NCr-*nu/nu* mice, ages 4 to 6 weeks, were purchased from the National Cancer Institute. Animals were housed in a pathogen-free environment under controlled conditions of light and humidity and received food and water *ad libitum*. Cells were suspended in Matrigel (10 mg/mL; Collaborative Research) at 1.5×10^7/mL. Each mouse received s.c. injections at one site on each flank with 300 μL cell suspension. Tumors were measured with calipers. Tumor volumes were calculated by the formula: width^2 × length / 2. Mean ± SD tumor volume was calculated and plotted against time. The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Maryland.

Statistical Analysis

Results of growth assays were analyzed using a two-sided Student's *t* test. Significance was established at $P < 0.05$. The proportion of developed tumors in control and *EBP1* transfectants was compared using Fisher's exact test. All hypothesis tests were two-sided. The different groups were compared at the 0.05 level of significance.

Results

Reduced Expression of the *EBP1* Gene in Prostate Cancer Patients

As we have found reduced expression of EBP1 in preclinical models of prostate cancer (25), we first screened a commercially prepared cDNA panel consisting of non-

paired benign prostate and prostate tumor samples using quantitative reverse transcription-PCR. *EBP1* expression was significantly decreased ($P < 0.03$) in prostate cancers compared with normal tissue (Fig. 1A). We then determined the expression of the *EBP1 (PA2G4)* gene in patients using public gene expression data bases. Oncomine analysis of a data set posted by Yu et al. (34) revealed that *PA2G4* was significant decreased in localized prostate tumors ($n = 64$) relative to normal prostate samples ($n = 23$; $P < 0.00001$) and further down-regulated in metastases ($n = 25$; $P < 0.00001$; Fig. 1B). The studies of Vanaja et al. (35), Singh et al. (36), and LaTulippe et al. (37) also indicate that *EBP1* expression is similarly decreased ($P < 0.05$, $P < 0.1$, and $P < 0.01$, respectively; data not shown). To examine the expression of EBP1 protein in human prostate tumor tissues, immunohistochemical analysis was done on a tissue array containing benign ($n = 42$) and prostate cancer ($n = 153$) specimens. EBP1 staining intensity was decreased in the cytoplasm of the prostate tumor samples compared with the benign samples (Fig. 1C, 2.73 versus 2.11, $P < 0.002$). EBP1 staining was predominantly detected in the cytoplasm of both benign epithelial and prostate tumor cells (Fig. 1D). Nuclear and cytoplasmic staining was observed in 30% of prostate cancer specimens (Fig. 1D, *middle*) but not in benign tissue. This resembles the nuclear and cytoplasmic distribution of EBP1 observed in cell culture (refs. 31, 38, 39; Fig. 1D).

Restoration of EBP1 Expression in Hormone-Refractory C81 Cells Ameliorates the Hormone-Refractory Phenotype

The C81 LNCaP subline has been made androgen independent by continuous long-term passage in complete medium (26). To analyze whether the increased expression of EBP1 could ameliorate the hormone-refractory phenotype, we established FLAG-*EBP1* or empty vector stably transfected C81 cell lines. EBP1 expression was increased approximately 3-fold (Fig. 2A). We then examined *EBP1* transfectants based on established biological criteria for androgen independence: growth rate, response to hormones, and chemotherapy. The growth rate of the C81-*EBP1* transfectants in complete medium was significantly decreased ($P < 0.05$) compared with that of the vector control (Fig. 2B, *left*) and resembled that of low-passage LNCaP cells. Ectopic expression of *EBP1* in the C81 cell line also decreased colony growth in soft agar approximately 50% at the highest cell concentration tested (Fig. 2B, *right*) and was similar to that of low-passage LNCaP cells.

Androgen-independent cells in culture can be either refractory to hormone stimulation or sensitized to low doses of hormone. In comparison with the parental LNCaP cells, C81 cells are no longer growth stimulated by DHT (26). We found that the growth of *EBP1* transfectants was reduced to one-third of that of the vector controls in the absence of androgens (data not shown). The vector control C81 cells were unresponsive to androgen (Fig. 2C, *left*) as reported previously (26). In contrast, *EBP1*-transfected C81 cells responded to androgen in a similar manner as the original low-passage LNCaP androgen-sensitive cells

(Fig. 2C, left; ref. 26). To determine if the androgen-stimulated growth of the *EBP1*-transfected cells was mediated via the AR, we examined the effect of the antiandrogen bicalutamide (Casodex) on DHT-stimulated growth. The DHT-stimulated growth of the *EBP1*-transfected cells was suppressed by 10 μmol/L bicalutamide (Fig. 2C, right), suggesting that the increased growth in the presence of androgens was mediated via the AR.
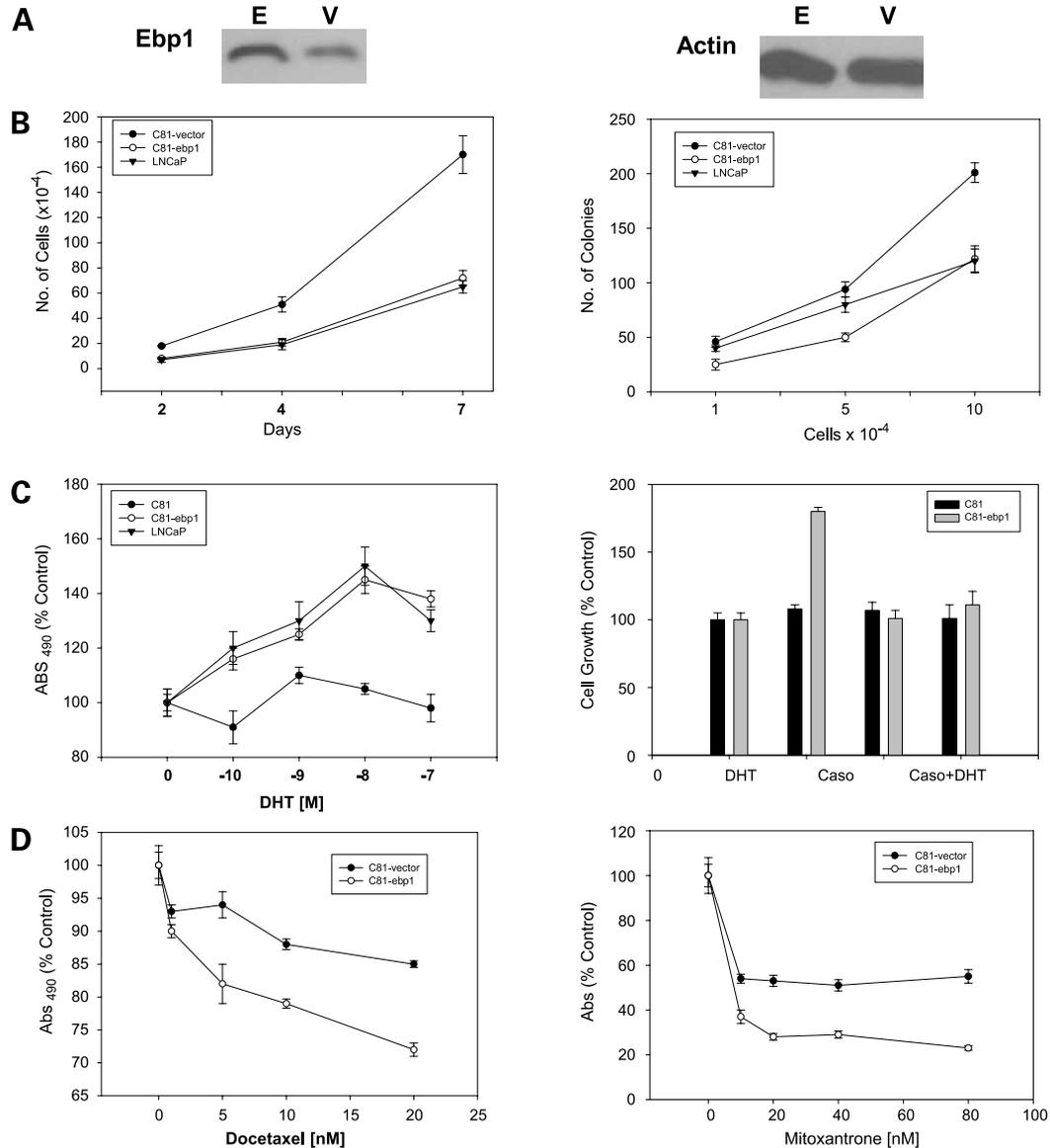

Figure 2. EBP1 restoration ameliorates the hormone-refractory phenotype. **A,** Western blot analysis of C81 cells transfected with a vector control (*V*) or *EBP1* (*E*) expression plasmid. **B,** *left,* growth in monolayer culture. Equal numbers (5 × 10⁴) of vector or *EBP1* stably transfected cells were plated at day 0 in complete medium and viable cell numbers were determined at the indicated times. *Points,* mean of three wells; *bars,* SE. Representative of three experiments (*right*). Colony growth in soft agar. C81 cells stably transfected with either pcDNA or *EBP1* were plated in soft agar at the cell densities indicated and colony numbers were assessed at day 10. *Point,* mean of three dishes; *bars,* SE. Representative of three experiments. **C,** *left,* response of *EBP1*- and vector-transfected C81 cells to DHT. *EBP1* and vector control C81-transfected cells and early-passage LNCaP cells were plated in complete medium at 5 × 10³ per well in 96-well plates for 1 d. The next day, cells were switched to steroid-reduced medium for 2 d as described in Materials and Methods. DHT was then added at the indicated concentrations. Cells were re-fed medium every 3 d. Total cell numbers were determined 7 d later by MTT assays. *Points,* mean of six wells; *bars,* SE. Representative of two experiments. *Right,* effect of bicalutamide on DHT-stimulated growth. Cells were plated as described in Materials and Methods. After 2 d in steroid-reduced medium, cells were fed with fresh steroid-reduced medium with or without 10 μmol/L bicalutamide (Casodex) in the presence or absence of 10 nmol/L DHT. Total cell numbers were assessed 7 d later by MTT assay. Cells were re-fed medium every 3 d. *Points,* mean of six wells; *bars,* SE. Similar results were found in two independent experiments. **D,** EBP1 expression sensitizes C81 cells to chemotherapeutic drugs. C81 vector control or *EBP1* transfectants were plated in 96-well plates in complete medium and allowed to attach overnight. The cells were then placed in steroid-reduced medium for 2 d and then re-fed with steroid-reduced medium in the presence of the indicated concentrations of docetaxel (*left*) or mitoxantrone (*right*). Total cell numbers were determined by MTT assays 5 d later. *Points,* mean of six wells; *bars,* SE. Representative of two experiments.
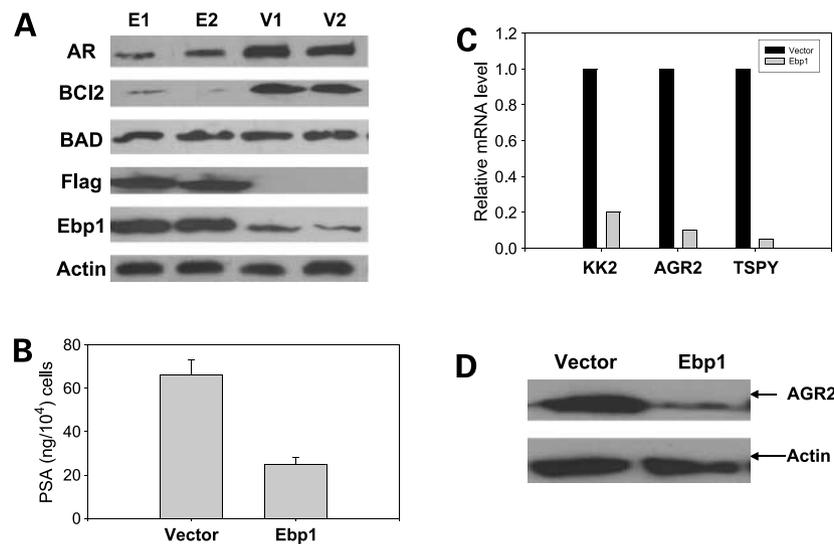
Figure 3. Expression of genes associated with androgen independence is decreased in C81 EBP1 transfectants. **A**, lysates of logarithmically growing clones of C81 *EBP1* transfectants (*E1* and *E2*) or vector controls (*V1* and *V2*) were resolved by SDS-PAGE and immunoblotted for AR, BCL-2, BAD, FLAG, EBP1, and actin as indicated. Representative of three experiments. **B**, secreted PSA as measured by ELISA. *EBP1* and vector control cells were plated at 1×10^6 per 100 mm dish in complete medium. Forty-eight hours later, conditioned medium was collected and frozen. PSA activity was assessed by ELISA. Levels of PSA were adjusted to total cell number. Representative of two experiments. **C**, validation of mRNA levels in EBP1 transfectants. Quantitative reverse transcription-PCR was done for the *kallikrein-2, AGR2*, and *TSPY* genes in *EBP1* and vector transfectants. The relative levels of all test mRNAs were normalized to *β-actin*. Representative of three experiments using different sets of cells. **D**, expression of AGR2 protein in vector and EBP1 C81 transfectants. Lysates of vector control or *EBP1*-transfected cells grown in complete medium were resolved by SDS-PAGE and analyzed by Western blotting with antibody to AGR2.

Mitoxantrone or docetaxel is currently the standard palliative treatment in hormone-refractory prostate cancer patients (40). C81 cells were relatively refractory to docetaxel in androgen-deprived conditions as reported previously (41), but *EBP1*-transfected cells were significantly more sensitive to 5 to 20 nmol/L docetaxel under these same conditions (Fig. 2D, *left*). The sensitivity of both vector control and *EBP1* transfectants to mitoxantrone plateaued at 20 nmol/L. However, growth of *EBP1* transfectants was decreased approximately 80% as opposed to 40% for the vector controls at this concentration (Fig. 2D, *right*). In contrast, the sensitivity of the *EBP1* transfectants to both drugs was the same as vector controls when cells were grown in complete medium (data not shown).

Restoration of EBP1 Protein Levels Significantly Reduces Expression of Molecules Associated with the Androgen-Refractory Phenotype

To elucidate how restoration of *EBP1* gene expression in C81 cells mitigates the hormone-refractory phenotype, we examined the expression levels of proteins that play a key role in androgen independence. Western blot analysis of two individual C81 clones stably transfected with a FLAG-tagged *EBP1* or vector controls (Fig. 3A) showed that ectopic expression of *EBP1* markedly reduced the protein levels of AR, consistent with our previous report for LNCaP cells (25), and BCL-2, another molecule associated with the androgen-independent phenotype. In contrast, the level of the proapoptotic protein BAD remained the same as the vector controls.

We next conducted a microarray analysis of mass-transfected *EBP1* and vector-transfected C81 cell lines to determine the spectrum of differentially expressed genes contributing to the hormone-refractory phenotype (Supplementary Tables 1 and 2).[7] Six AR target genes potentially involved in androgen-independent growth were found to be down-regulated at least 3-fold in *EBP1*-overexpressing cells compared with controls (Table 1). These include *PSA (kallikrein 3), kallikrein 2, TMPRSS2*, and *prostate differentiation factor* as reported previously for LNCaP cells (25). In addition, the expression of the AR-regulated gene *AGR2* (32) and the candidate oncogene *TSPY* (28) was significantly down-regulated in the *EBP1* transfectants.

We next validated the expression of four representative genes associated with prostate cancer progression. Secreted PSA protein levels were decreased 60% in *EBP1* transfectants compared with C81 vector controls (Fig. 3B). Expression of the kallikrein 2 gene, a potential marker for prostate cancer progression (42), was down-regulated over 80% in *EBP1* transfectants compared with vector controls (Fig. 3C). *TSPY* mRNA was also down-regulated over 90%. *AGR2*, the human homologue of the *Xenopus anterior gradient 2* gene, is regulated by AR and its overexpression is associated with enhanced metastasis (43). Real-time reverse transcription-PCR indicated that *AGR2* mRNA

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[7] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

was down-regulated over 90% in *EBP1* transfectants compared with vector controls (Fig. 3C). Western blot analysis detected decreased AGR2 protein in C81-*EBP1* transfectants (Fig. 3D).

Restoration of EBP1 Expression Blocked HRG-Activated Phosphatidylinositol 3-Kinase/AKT Signaling

HRG-activated pathways contribute to ligand-independent activation of AR (44). We therefore determined if EBP1 could affect the responsiveness of C81 androgen-independent cells to HRG. Growth of *EPB1* transfectants in androgen-depleted medium was reduced by two-thirds compared with vector-transfected controls (Fig. 4A). HRG induced the growth of vector control C81 cells in androgen-free medium as expected (19). However, the ectopic expression of *EBP1* completely abrogated the ability of C81 cells to grow in response to HRG (Fig. 4A). In contrast, the response of C81 *EBP1* transfectants to epidermal growth factor was unchanged (Supplementary Fig. S1).[7]

As restoration of *EBP1* expression inhibited HRG-induced growth, we examined if *EBP1* could attenuate HRG signaling. We examined the AKT pathway as HRG-induced AKT phosphorylation and activation is important in growth of androgen-independent cells (45) and inhibition of AKT phosphorylation delays the transition to androgen independence (46). AKT was basally phosphorylated in C81 vector- and *EBP1*-transfected cells as expected (Fig. 4B) presumably due to loss of PTEN expression (47). Densitometry indicated that HRG induced a 4-fold increase in AKT phosphorylation at 5 min that gradually declined in vector control cells (Fig. 4C). In contrast, HRG failed to induce an increase in AKT phosphorylation in *EBP1* transfectants.

Abrogation of EBP1 Protein Expression by shRNA in LNCaP Cells Results in a Hormone-Refractory Phenotype

To further test the hypothesis that EBP1 is important in the reversal of the hormone-refractory phenotype, we created LNCaP cells in which EBP1 expression has been ablated by transduction with a lentivirus targeting the *EBP1* gene. EBP1 expression was knocked down in the C13
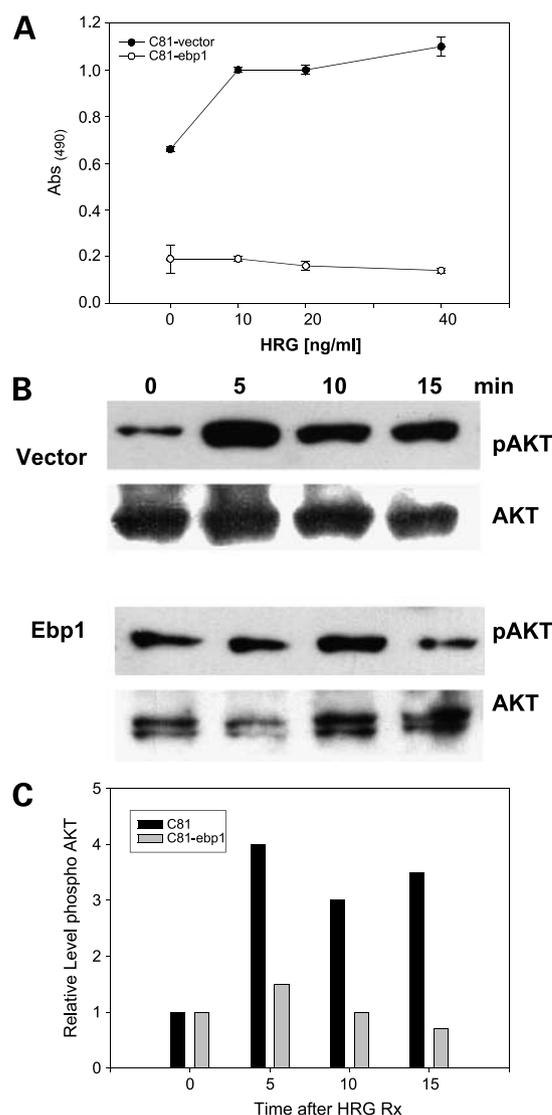
Table 1. Androgen-regulated genes decreased in *EBP1*-transfected C81 cells

Accession no.	Fold change		Gene name
NM_003308	TSPY	90	0.002
AF088867	AGR2	50	0.002
NM_003527	POV-1	4.8	0.001
U17040	PSA	30	0.001
BC005196	Prostatic kallikrein 2	2.9	0.004
AF003934	Prostate differentiation factor	7.0	0.0002
AF2700487	TMPRSS2	4.0	0.01

NOTE: A microarray analysis of *EBP1*-transfected and vector control cells was done as described in Materials and Methods. The expression of 60 genes (minimum of 2.9-fold change) was found to differ significantly between the two cell lines. Nine genes were induced and 51 were repressed. AR signaling-associated genes are presented.

Figure 4. EBP1 overexpression inhibits the response to HRG. **A,** *EBP1* and vector control transfected cells were plated in complete medium at 5×10^3 per well for 1 d. The next day, cells were switched to steroid-reduced medium and HRG was added at the indicated concentrations. Total cell numbers were determined 7 d later by MTT assay. *Points,* mean of six wells; *bars,* SE. Representative of three experiments. **B,** AKT activation in response to HRG C81-*EBP1* or vector control cells were serum starved overnight and then treated with HRG (20 ng/mL) for the indicated times (min). Cell lysates were immunoblotted with antibody to phosphorylated AKT or total AKT as indicated. **C,** densitometric evaluation of AKT phosphorylation. Representative of two independent experiments.

but not in the A16 cell line (Fig. 5A). HRG inhibited the growth of A16 cells as it does for LNCaP cells (17). In contrast, HRG stimulated the growth of C13 LNCaP cells in which EBP1 expression has been ablated, similar to its effects on androgen-independent cell lines (Fig. 5B, *left*). To examine the potential role of phosphatidylinositol 3-kinase/AKT signaling pathway in this phenomenon, we treated EBP1 knockdown cells with HRG and measured phosphorylated AKT. AKT was basally phosphorylated in

both LNCaP and EBP1 knockout cells. However, HRG induced a 2-fold increase in pAKT protein levels in LNCaP cells and a 3.5-fold increase in pAKT levels in EBP1-depleted LNCaP cells as determined by densitometry (Fig. 5B, *right*).

We further showed that the expression of two genes associated with hormone-refractory growth was altered in EBP1 knockout cells. Levels of secreted PSA in conditioned medium of log-phase cells were 12.5 ng/10[4] cells for A16 cells and 75 ng/10[4] cells for the sh*EBP1*-transduced C13 cells. Abrogation of the expression of the *EBP1* gene also led to an up-regulation of AGR2 (Fig. 5C, *left*). We next tested the transcriptional response of an AR-regulated

MMTV promoter to R1881. The response was significantly (*P* < 0.05) enhanced in EBP1 knockout cells (Fig. 5C, *middle*). Finally, we examined the response of A16 and C13 cells to docetaxel. The A16 control cells were sensitive to docetaxel with a 65% reduction in growth observed at 5 nmol/L of the drug, In contrast, C13 cells were relatively resistant to docetaxel with a maximum of 15% growth inhibition at 20 nmol/L (Fig. 5C, *right*).

Manipulation of EBP1 Expression Affects Growth in Animal Models

To corroborate if the effects observed *in vitro* are observed *in vivo*, we examined the consequence of expression of *EBP1* on the tumorigenicity of C81 cells in
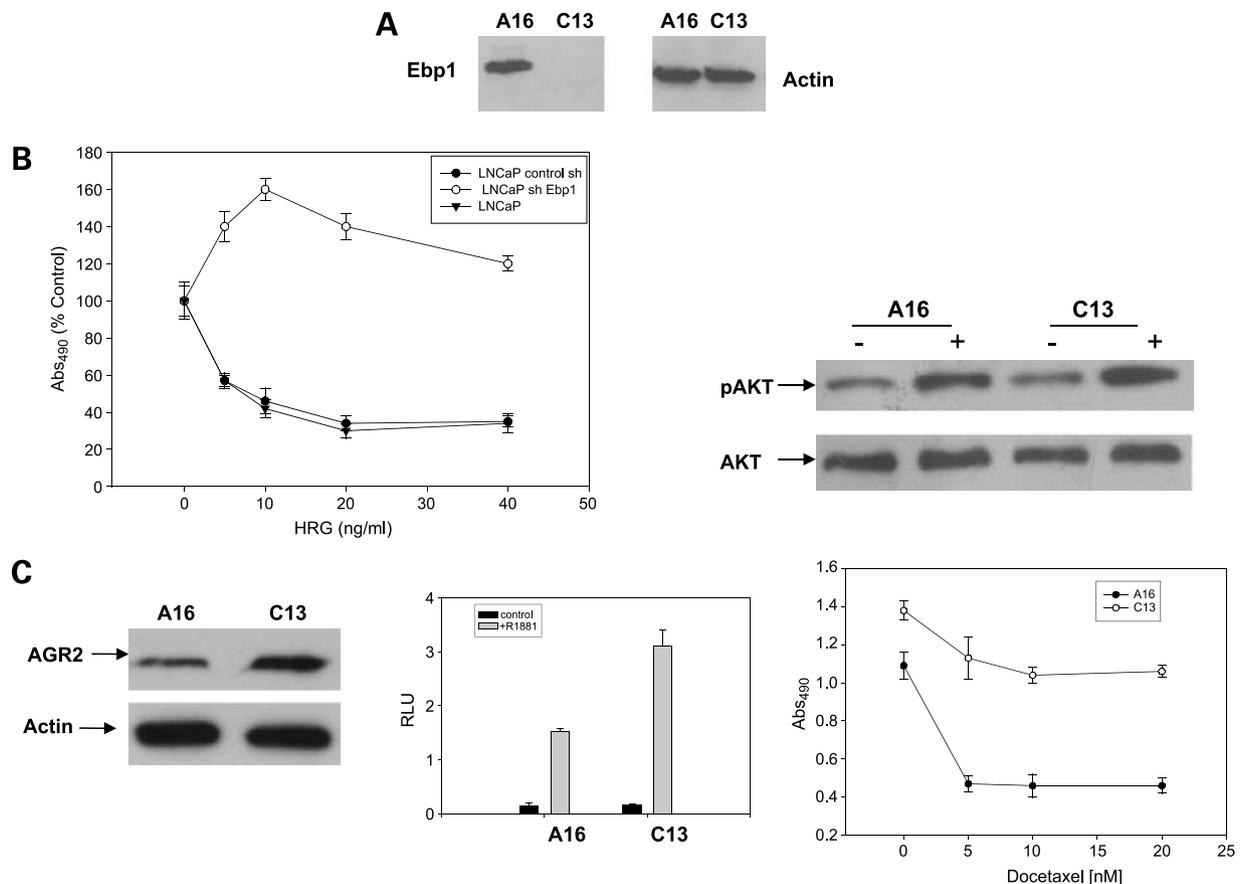

Figure 5. Suppression of EBP1 by shRNA promotes a hormone-refractory phenotype. **A,** Western blot analysis of EBP1. Lysates of LNCaP cells stably transduced with a control lentivirus (A16) or an *EBP1*-targeted shRNA (13C) were collected and resolved by SDS-PAGE. Western blots were probed for endogenous EBP1 or actin as indicated. **B,** inhibition of EBP1 expression abrogates the ability of HRG to arrest growth. *Left,* control and shRNA *EBP1*-targeted cells were plated in complete medium at 5×10^3 per well for 1 d. The next day, cells were switched to steroid-reduced medium and HRG was added at the indicated concentrations. Total cell numbers were determined 7 d later by MTT assay. *Points,* mean of six wells; *bars,* SE. Representative of three experiments. *Right,* AKT phosphorylation in EBP1 knockout cells. Control (A16) or EBP1 knockout cells (C13) were treated with HRG (2O ng/mL) for 15 min and cell lysates were assessed for the presence of phosphorylated and total AKT as indicated. **C,** *left,* AGR2 is overexpressed in C13 LNCaP cells that do not express *EBP1*. Lysates of logarithmically growing A16 and C13 cells were resolved by SDS-PAGE and analyzed by Western blotting using antibodies to AGR2 and actin as indicated. *Middle,* elimination of EBP1 expression enhances the activity of an AR-regulated promoter. A16 or C13 cells were transfected with a MMTV-luciferase reporter plasmid. Twenty-four hours after transfection, cells were switched to phenol red-free RPMI 1640 with 1% charcoal-stripped serum containing R1881 (10^{-8} mol/L) or vehicle control. Sixteen hours later, luciferase activity was measured. *Points,* mean of triplicate wells; *bars,* SE. Representative of three experiments. *Right,* elimination of EBP1 expression affects the response to docetaxel. C13 or A16 cells were plated in 96-well plates in complete medium and allowed to attach overnight. The cells were then placed in steroid-reduced medium for 2 d and then re-fed with steroid-reduced medium in the presence of the indicated concentrations of docetaxel. The viability of the cells was determined by MTT assays 5 d later.

female nude mice. *EBP1*- and vector-transfected cells were injected s.c. into female nude mice and tumor growth was monitored in two independent experiments (Fig. 6). The first measurable tumor derived from C81 vector transfectants was detected on day 8. In contrast, tumor development of *EBP1* transfectants was much slower, with the first tumor being observed on day 20. On day 28 post-transplantation, tumors were observed at only 1 of 26 of the *EBP1* inoculation sites as opposed to 24 of 26 of the sites for vector controls. At the end of both studies, tumors had developed at 25 of 26 of the sites injected with the vector control cells, whereas 10 of 26 of the sites inoculated with *EBP1* transfectants developed tumors (Fig. 6A; $P = 0.00001$, Fisher's exact test, two-sided). Average tumors volumes for the *EBP1* transfectants observed at the end of the first study (3 animals per group, 6 sites per group) were 104 ± 70 mm^3 compared with 998 ± 206 mm^3 (mean $\pm$ SE) for the vector controls ($P = 0.01$). In the second experiment (10 animals per group, 20 sites per group), the average tumor volume was 206 ± 54 for the *EBP1* transfectants and 561 ± 47 for the vector controls ($P = 0.0001$; Fig. 6B).

We also examined the ability of A16 and C13 cells (in which EBP1 expression had been decreased) to form tumors in female nude mice. At the concentrations used, we found that parental low-passage LNCaP cells formed tumors in the same manner as the A16 cells, with measurable growth being observed at all sites at day 50 (data not shown). However, growth of LNCaP controls and A16 cells leveled off at day 58. In contrast, C13 cells continued to grow. At the termination of the experiment at day 68 post-inoculation, the average tumor volume was 307 ± 64 mm^3 for the A16 controls and 542 ± 67 mm^3 for the C13 EBP1 knockout cells ($P = 0.04$; Fig. 6C). Wet weights of the tumors were also significantly different: A16 = 0.150 ± 0.03 versus C13 = 0.288 ± 0.05 g ($P = 0.03$).

Discussion

Emerging studies indicate the importance of ErbB receptor signaling in hormone-refractory prostate cancer (44). EBP1, an ErbB3-binding protein, also functions as an AR corepressor, suggesting its role in suppression of ErbB-mediated activation of AR. In these studies, we found that restoration of EBP1 expression ameliorated the hormone-refractory phenotype of C81 cells and that elimination of EBP1 expression resulted in a hormone-refractory phenotype in hormone-dependent LNCaP cells. These studies suggest the therapeutic potential of EBP1, an endogenous AR corepressor, for treatment of advanced prostate cancer.

Restoration of *EBP1* gene expression in hormone-refractory C81 cells led to a reversal of the androgen-independent phenotype based on a series of established criteria. These biological changes were likely due in part to the decreased expression of proteins contributing to the androgen-independent phenotype. AR protein levels were decreased in C81-*EBP1* transfectants, consistent with our previous finding obtained from low-passage hormone-dependent LNCaP cells (25). The decreased expression of these AR

target genes may have restored the physiologic response of C81 cells to DHT as we observed and resulted in the growth inhibition induced by Casodex. The increased dependence of C81 *EBP1*-transfected cells on androgens
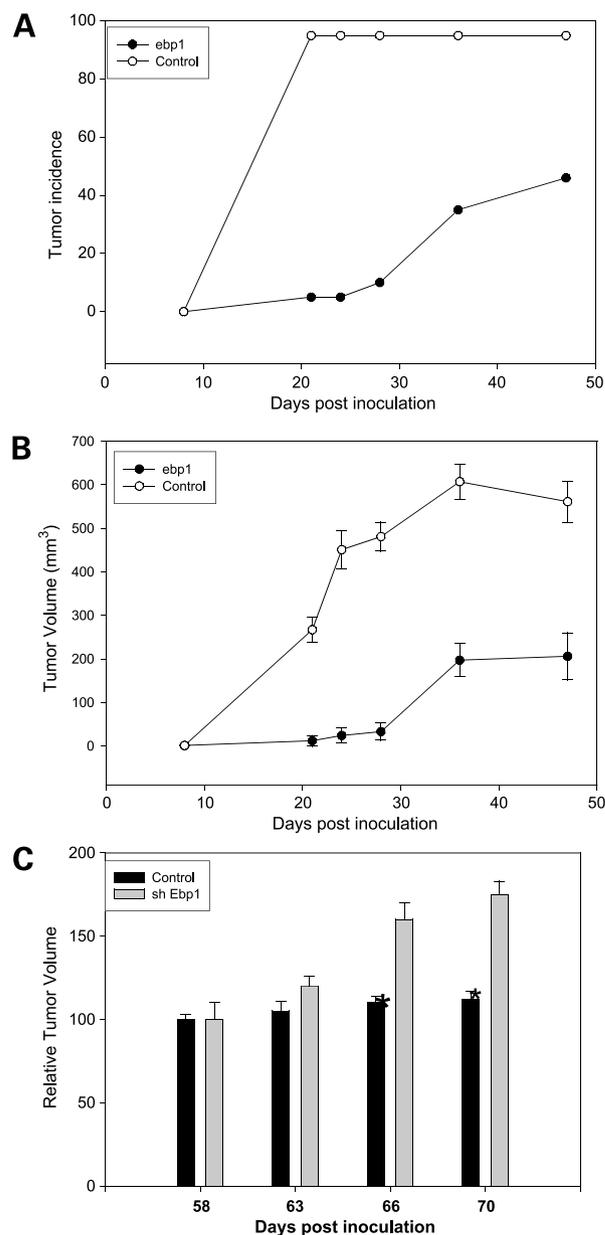

Figure 6. Manipulation of EBP1 expression affects growth of prostate cancer cells *in vivo*. **A** and **B**, ectopic expression of *EBP1* reduces the growth of androgen-independent cells in athymic female mice. C81 vector- or *EBP1*-transfected cells were injected s.c. into female athymic mice. **A**, tumor development with time ($n = 10$ animals, 20 sites per group). **B**, tumor volumes and growth rate. Tumor volumes were calculated as described in Materials and Methods. *Points*, mean (10 mice per group injected at 20 sites); *bars*, SE. **C**, elimination of EBP1 expression enhances growth of androgen-dependent LNCaP cells *in vivo*. A16 lentivirus control or C13 *EBP1* shRNA-transduced LNCaP cells were injected s.c. into female athymic mice. Tumor volumes were calculated as described in Materials and Methods. *Points*, mean (10 mice per group injected at 20 sites); *bars*, SE. *, $P = 0.04$.

was also evidenced by the fact that *EBP1*-transfected cells grew poorly in female mice.

Expression of the androgen-regulated genes *AGR2* and *TSPY* was decreased in C81-*EBP1* transfectants. Further, inhibition of EBP1 protein expression in LNCaP cells led to up-regulation of AGR2. *AGR2*, the human homologue of the *Xenopus anterior gradient 2* gene, is normally expressed in LNCaP cells only in response to androgen treatment and overexpressed in primary prostate adenocarcinoma (32). Elevated AGR2 expression is significantly associated with poor survival of prostate cancer patients (48). As there is no effective therapy for metastatic prostate cancer, the possible role of EBP1 as a modulator of *AGR2* gene expression warrants further investigation.

Mitoxantrone or docetaxel is currently the standard palliative treatment in hormone-refractory prostate cancer patients. BCL-2, which is overexpressed in human refractory prostate cancers (49), is a potent inhibitor of apoptosis induced by these drugs. Unexpectedly, the protein level of BCL-2 was decreased in *EBP1*-transfected C81 cells. The mechanism of the inhibition of BCL-2 expression in C81-*EBP1* transfectants is not known. Our microarray data showed no changes in *BCL-2* mRNA levels in *EBP1* transfectants compared with vector controls. EBP1 can directly bind *BCL-2* mRNA, suggesting that EBP1 may have effects on translation efficiency (50). Alternatively, EBP1 may alter the stability of the BCL-2 protein. We suggest that the increase in sensitivity to docetaxel and mitoxantrone observed in *EBP1*-transfected cells in the absence of androgens was due to the down-regulation of BCL-2. It is of interest that changes in sensitivity were only observed in androgen-deprived conditions. Studies of drug sensitivity of LNCaP C33 and C81 variants were similarly conducted in steroid-free medium (41). It is possible that activation of alternative pathways, such as ErbB signaling, enhances cell survival after drug treatment in the androgen-deprived environment and EBP1, by interfering with such pathways, inhibits growth.

A growing literature indicates that ErbB signaling in androgen-deprived conditions is vital to androgen-refractory growth and that hormone-naive and hormone-refractory cells respond differently to such signaling. The ErbB2/ErbB3 ligand HRG augments the growth of CWR-R1 hormone-refractory cells in androgen-deprived conditions (19) but inhibits growth of hormone-dependent cells (17). It has been postulated that changes in the profiles of ErbB receptors and downstream signaling pathways account for such differences (19). We found that ectopic expression of *EBP1*, a member of the ErbB3 signal transduction pathway, completely abrogated the ability of HRG to induce growth of C81 androgen-independent cells in androgen-deprived medium. Knockdown of EBP1 expression resulted in HRG-induced growth stimulation, rather than growth inhibition, in hormone-dependent LNCaP cells. We suggest that EBP1 may be one member of an ErbB signal transduction pathway that is important in regulating the response of prostate epithelial cells to HRG. Thus, HRG-induced signals that would ordinarily inhibit growth in hormone-

dependent cells because of EBP1 or similar proteins may increase growth when these proteins are nonfunctional. Of interest, the inhibition of HRG-induced growth appeared to be specific, as the response to epidermal growth factor was not affected.

Although androgen-independent survival of prostate cancer cells through the AKT pathway has been shown (45), the role that the inhibition of AKT activation observed may have played in the EBP1-induced growth suppression in response to HRG is not yet clear. We initially examined the AKT pathway due to its role as a mediator of HRG-induced cell survival. In addition, both phosphatidylinositol 3-kinase, an activator of AKT, and EBP1 bind the cytoplasmic domain of ErbB3. This raised the possibility that EBP1 might affect recruitment of phosphatidylinositol 3-kinase to ErbB3 after HRG stimulation. Although AKT signaling was suppressed in EBP1 transfectants, changes in other downstream signaling pathways may also have contributed to EBP1-induced growth inhibition. For example, ongoing studies in our laboratory indicate that EBP1 expression affects mitogen-activated protein kinase signaling in a complex pattern that is dependent on cell type, ligand concentration, and duration of exposure. Thus, at this time, there is uncertainty regarding the importance of AKT modulation versus other downstream pathways. In addition, we do not know if the biological effects of EBP1 are due to its direct effects on AR, modification of the effects of ErbB on AR, or modulation of ErbB effects that are unrelated to AR signaling.

In summary, we show that overexpression of EBP1, an AR corepressor that interacts with ErbB3, modulated the hormone-refractory phenotype both *in vitro* and *in vivo*. These studies support the role of EBP1 as an endogenous negative regulator of AR signaling and provide a rationale for the design of EBP1-based approaches for the treatment of hormone-refractory prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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