

# Androgen Receptor and Invasion in Prostate Cancer

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## Abstract

**Activation of androgen receptor (AR) stimulates the growth of not only androgen-dependent but also of androgen-refractory prostate cancer. However, neither the role of AR in invasion/metastasis nor the relationship between invasiveness and androgen-refractory status has been established. In this study, we used the androgen-dependent prostate cancer cell line MDA PCa 2b, derived from a human bone metastasis, to generate an invasive subline (MDA-I) using a Matrigel chamber. MDA-I cells expressed higher levels of AR and prostate-specific antigen than their less invasive parental cells. Blocking AR function or removal of androgen suppressed the invasion of MDA-I cells, whereas stimulating AR increased invasion. In addition, forced AR overexpression increased the invasiveness of MDA PCa 2b cells. Next, we showed that an androgen-refractory subline (MDA-hr) of MDA PCa 2b cells also expressed higher levels of AR and were more invasive than their parental androgen-dependent cells. Blocking AR function suppressed the invasiveness of MDA-hr cells. Gelatin zymography indicated that matrix metalloproteinase 2 (MMP-2) and MMP-9 activities were regulated by AR signaling and closely correlated with the invasiveness of the androgen-dependent and androgen-refractory prostate cancer cells. These data suggest that AR promotes the invasiveness of both androgen-dependent and androgen-refractory prostate cancer and that a more invasive phenotype might develop through AR activation during cancer progression. These findings potentially support the use of adjuvant hormonal therapy and the future development of more potent androgen blockade therapy.** [Cancer Res 2008;68(4):1128–35]

## Introduction

Prostate cancer is the most commonly diagnosed cancer among men; >30,000 men die of prostate cancer each year in the United States (1). Recently, prostate-specific antigen (PSA) screening has become more widespread, and the number of patients diagnosed with early stage prostate cancer has increased. Although the majority of diagnosed prostate cancers remain localized and rarely threaten life expectancy, about one third of prostate cancers invade surrounding tissue, metastasize to distant organs, such as bone, and consequently cause death. Survival of a patient with prostate cancer is directly related to the spread of the tumor. Median survival for patients with localized prostate cancer is >5 years,

compared with 1 to 3 years for patients with metastatic prostate cancer. Therefore, an understanding of the mechanisms responsible for the regulation of invasion and metastasis is urgently required in prostate cancer.

Because prostate cancer growth is initially androgen-dependent, medical or surgical castration has been the standard treatment for metastatic prostate cancer. However, the effect of hormonal therapy is temporary, and most tumors become “androgen refractory” (resistant to androgen-ablation therapy) within a few years. This presents a major obstacle in the treatment of metastatic prostate cancer. Many patients with androgen-refractory prostate cancer experience painful bone metastases; up to 84% of patients have been reported to have bone metastases at autopsy (2).

Androgen stimulates the growth of androgen-dependent prostate cancer through the activation of androgen receptor (AR). It has recently been shown that AR plays a critical role even in the growth of androgen-refractory cancers (3–8). However, the role of AR in invasion and metastasis of prostate cancer has not been established. A relationship between invasive properties and androgen-refractory status has also not been reported. Androgen-independent PC3 and DU145 cells are much more invasive than androgen-dependent LNCaP cells (9). However, because PC3 and DU145 cells do not express AR and PSA, an androgen-dependent marker for prostate cancer, it is unclear whether or not androgen-refractory prostate cancer cells expressing endogenous AR are indeed more invasive than their isogenic androgen-dependent cells.

MDA PCa 2b is an androgen-dependent prostate cancer cell line derived from an androgen-refractory bone metastasis (10) that expresses AR and PSA similar to clinical prostate cancer (11). In the current study, we examined the role of AR in the invasive properties of both androgen-dependent and androgen-refractory prostate cancer using cell lines derived from MDA PCa 2b cells, because (a) bone is the most common site for prostate cancer metastasis, (b) like most clinical prostate cancers, all sublines expressed endogenous AR and PSA, and (c) we could compare directly the invasive properties of androgen-dependent cells with those of isogenic androgen-refractory cells. To model androgen-dependent prostate cancer, we used a novel invasive subline, MDA-I, which was created from MDA PCa 2b cells using a Matrigel chamber assay. For androgen-refractory prostate cancer, we used the MDA-hr subline, which was previously generated from the MDA PCa 2b cells after long-term growth suppression by androgen-depleted culture to mimic the clinical progression of prostate cancer during an androgen ablation therapy (7). The androgen-dependent, AR-positive LNCaP prostate cancer cell line was also used to confirm results seen in the MDA PCa 2b cells.

## Materials and Methods

**Cell culture.** The androgen-dependent human metastatic prostate cancer cell line MDA PCa 2b was obtained from the American Type Culture Collection (ATCC) and maintained in this growth medium: Ham's

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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F-12K (Invitrogen Corporation), 25 ng/mL cholera toxin, 10 ng/mL epidermal growth factor, 5  $\mu$ mol/L phosphoethanolamine, 100 pg/mL hydrocortisone, 45 nmol/L selenious acid, 5  $\mu$ g/mL insulin, and 20% fetal bovine serum (FBS; Omega Scientific, Inc.), at 37°C in 5% CO<sub>2</sub>. The androgen-independent MDA-hr subline established from MDA PCa 2b was maintained in DCC medium, which is the same growth medium but with 20% dextran charcoal-stripped FBS (DCC-FBS; Omega Scientific, Inc.) instead of regular FBS. 293T cells were obtained from ATCC and were cultured in Iscove's modified DMEM (Mediatech, Inc.) with 10% FBS. LNCaP cells (ATCC) were maintained in RPMI with 10% FBS. All culture media were supplemented with 5 units/mL penicillin and 5  $\mu$ g/mL streptomycin.

**Invasion assay.** The *in vitro* invasive properties of prostate cancer cells were assessed using commercially available Matrigel invasion chambers (BD Biosciences). Cells were seeded in the upper compartment of a 24-well invasion chamber at  $1 \times 10^5$  cells per well in reduced growth medium with 1% FBS instead of 20% FBS for MDA cells. For invasion assay with LNCaP cells, RPMI 1640 with 1% FBS was used instead. The lower compartment of the chamber was filled with regular growth medium for each cell line. Both compartments were separated by an 8- $\mu$ m pore-size membrane filter, coated with Matrigel matrix. After 24 or 48 h of incubation at 37°C in 5% CO<sub>2</sub>, the cells at the upper surface of the filters were wiped away with a cotton swab, and the filters were fixed in 2% paraformaldehyde for 10 min followed by staining with 0.1% crystal violet for 2 min. The filters were removed from the invasion chamber using a scalpel blade and mounted onto glass microscope slides. Invasion was quantified by counting the number of cells on the filter under a microscope and expressed as percentage of control group, with control values setting at 100%. The invasive properties of MDA-hr cells were assessed with the same method as MDA PCa 2b cells, using DCC-FBS instead of regular FBS in the medium. In the experiments studying the effects of R1881 (Perkin-Elmer Life Science, Inc.), bicalutamide (purchased as Casodex tablets and extracted in our company), LY294002 (Sigma), and metalloproteinase 2 (MMP-2)/MMP-9 inhibitor II (Calbiochem) on invasion, these reagents were added to the medium in both upper and lower compartments. In the experiments studying the effects of androgen depletion or R1881 addition, the cells were preincubated in the DCC medium with or without R1881, respectively, overnight before the invasion assay was conducted. In each experiment, we seeded the same number of cells as used in the invasion assay in 24-well plates for cell growth at the same time and confirmed that the growth rate was not different between control and the treatment groups during the period of the invasion assay.

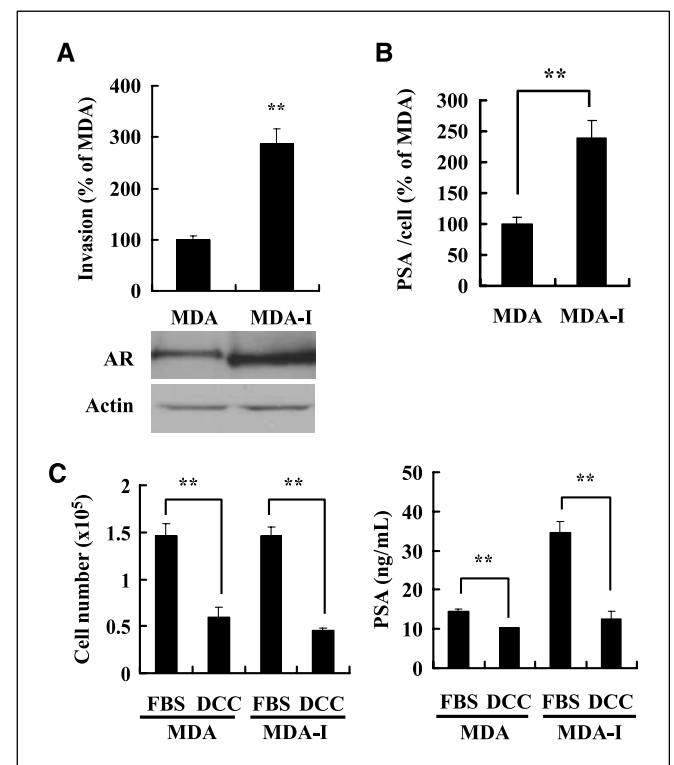
**Western blot analysis.** Western blot analysis was performed as described previously (7). Primary antibodies used were as follows: AR (Santa Cruz Biotechnology, Inc.), actin (Sigma), phosphorylated Akt (pAkt; Cell Signaling Technology, Inc.), Akt (Cell Signaling Technology, Inc.), phosphorylated epidermal growth factor receptor (pEGFR; Biosource International); EGFR (Santa Cruz Biotechnology, Inc.), phosphorylated Her2 (pHer2; Santa Cruz Biotechnology, Inc.), Her2 (Santa Cruz Biotechnology, Inc.), phosphorylated Erk1/2 (Santa Cruz Biotechnology, Inc.), and Erk1 (Santa Cruz Biotechnology, Inc.).

**Gelatin zymography.** MMP-2 and MMP-9 activities in the conditioned medium were determined by gelatin zymography. MDA PCa 2b cells and MDA-hr cells were incubated in their growth medium minus serum for 2 days, and then, the medium was collected and concentrated by centrifugation using Centricon filter with a  $M_w$  3,000 cutoff (Millipore Corporation). Proteins (15  $\mu$ g) from the concentrated media were separated by SDS-PAGE on a 10% gel containing 1.0 mg/mL gelatin (Sigma) under nonreducing conditions. Then, the gel was washed to remove SDS in 2.5% Triton X-100, incubated in a developing buffer [50 mmol/L Tris-HCl (pH 7.4), 200 mmol/L NaCl, 5 mmol/L CaCl<sub>2</sub>, and 0.02% Brij 35] for 20 h at 37°C. After being stained with Coomassie blue solution (0.5% Coomassie blue, 50% methanol, and 10% acetic acid), the gel was destained in 50% methanol:10% acetic acid. MMP-2 and MMP-9 activities were visualized as two clear bands against a blue background.

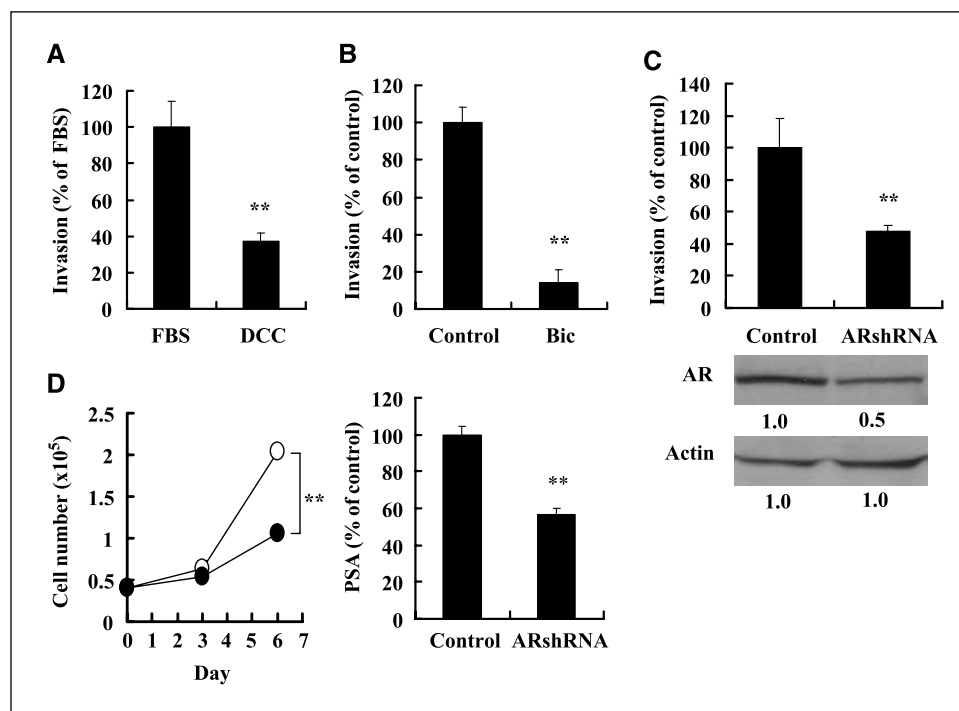
**AR knockdown and overexpression.** AR knockdown and overexpression were achieved by infecting prostate cancer cells with lentivirus expressing AR-specific short hairpin RNA (shRNA) and AR gene, respectively. The

pCSCG (control), pCSUACG (U6-shRNA-AR-CMV-GFP), and pCSCA plasmids (CMV-AR) were generously provided by Dr. Charles Sawyers (8). Lentiviral vector production and transduction were performed according to the method modified from that previously described (12). Briefly, in lentiviral vector production, 293T cells were plated on a 10-cm dish in 10 mL of medium and, on the following day at 70% confluency, were transfected with 2  $\mu$ g of VSV-G packaging plasmid, 5  $\mu$ g of p $\Delta$ Vpr packaging plasmid, and 5  $\mu$ g of pCSCG or pCSUACG or pCSCA vector by calcium phosphate precipitation. The transfections proceeded for 16 h, with medium replacement after 16 h and virus collection 48 h later. Viral supernatants were filtered through 0.45- $\mu$ m pore size filters and stored at -80°C until used for transduction. In lentiviral vector transduction, cells ( $8 \times 10^5$ ) at six-well plates were transfected with 2 mL of the viral supernatants for 2 h in the presence of 8  $\mu$ g/mL Polybrene. After 2 h, virus supernatants were removed, and 2 mL of medium was added to the cells. The cells were used for invasion and growth assays 1 week after the transduction.

**Fractionation of highly invasive subpopulation.** Highly invasive subpopulations of MDA PCa 2b cells were fractionated based on their *in vitro* invasive ability in a Matrigel chamber. Cells that invaded through the membrane filter coated with Matrigel matrix in the same manner as the invasion assay described above were trypsinized, collected, and then cultured. The fractionated cells showed a highly invasive phenotype and were designated as MDA-I cells.



**Figure 1.** Invasive capability, PSA secretion, and growth of MDA-I cells. **A**, invasion assay and Western blot of AR in MDA PCa 2b and MDA-I cells. Cells were seeded at  $1 \times 10^5$  per well as described in Materials and Methods. After 48 h, the cells that had invaded the filter were counted, and invasion was expressed as percentage of control group (MDA). **B**, PSA measurement. Cells were plated at  $1 \times 10^5$  per well. After 48 h, the culture medium was collected for ELISA. PSA levels were expressed as percentage of control group (MDA). All data are shown as mean  $\pm$  SE ( $n = 4$ ). \*\*,  $P < 0.01$  versus the MDA group by unpaired Student's  $t$  test. **C**, MDA PCa 2b and MDA-I cells were plated at  $4 \times 10^4$  per well in either regular medium or DCC medium in 24-well plates. After 6 d, cells were counted (*left*) and the culture media were assayed for PSA levels (*right*). Columns, mean ( $n = 3$ ); bars, SE. \*\*,  $P < 0.01$  by unpaired Student's  $t$  test.



**Figure 2.** Inhibition of invasion in MDA-I cells by androgen removal (A), AR antagonist bicalutamide (B), and AR knockdown using shRNA (C). D, inhibition of growth (left) and PSA secretion (right) by AR knockdown using shRNA. For invasion assays, cells were seeded at  $1 \times 10^5$  per well as described in Materials and Methods, in regular FBS (control) or DCC media (A), regular medium containing DMSO (control) or 100  $\mu\text{mol/L}$  of bicalutamide (Bic; B). C, invasion assay and Western blot of MDA-I cells infected with control vector or the AR shRNA lentiviral vector; protein bands were quantified as indicated below each gel picture, with control values set to 1. Invasive cells were counted after 48 h, and invasion was expressed as percentage of control group (control, 100%). Columns, mean SE ( $n = 4$ ); bars, SE. \*\*,  $P < 0.01$  versus control by unpaired Student's  $t$  test. D, MDA-I cells infected with either control vector (○) or AR shRNA lentiviral vector (●) were plated at  $4 \times 10^4$  cells per well in regular growth medium in 24-well plates. Cells were counted at 3 and 6 d (left), and the culture medium was assayed for PSA levels on day 6 (right). Columns, mean ( $n = 3$ ); bars, SE. \*\*,  $P < 0.01$  by unpaired Student's  $t$  test on day 6.

**Cell proliferation and PSA secretion assays.** In the experiment studying the androgen dependence of MDA-I cells, the cells were plated in 24-well plates at  $4 \times 10^4$  cells per well in the regular growth medium and the DCC medium. After incubation at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  for 6 days, the culture medium was removed for the measurement of PSA by ELISA (American Qualex), and the cells were trypsinized and counted with a hemocytometer. In the experiment studying the effect of shRNA against AR, cells were plated in 24-well plates at  $4 \times 10^4$  cells per well in their growth media. Cell growth and PSA secretion were examined as described above.

**Statistical analysis.** Data are shown as mean  $\pm$  SE. In the invasion and cell growth assays, differences between means of the control and treated groups were analyzed by Dunnett's test or unpaired Student's  $t$  test with Holm's correction for repeated tests. A value of  $P \leq 0.05$  was considered significant.

## Results

**Elevated AR expression in invasive androgen-dependent prostate cancer cells.** To study prostate cancer invasion in the androgen-responsive setting, we used the androgen-dependent prostate cancer cell line MDA-PCa 2b (shortened name: MDA) to generate a more invasive subline. Using the invasion chamber assay, MDA cells were fractionated into invasive (MDA-I) and noninvasive populations, and maintained separately. MDA-I cells showed significantly higher invasiveness than their parental cells (Fig. 1A, top). Furthermore, MDA-I cells showed increased AR expression compared with the parental MDA (Fig. 1A, bottom). PSA secretion in MDA-I was also elevated (Fig. 1B), suggesting an association of AR and AR activity with increased invasiveness.

Recent data showed that increased AR expression is correlated with androgen-refractory tumor growth (8); therefore, we asked whether MDA-I cells still require androgen for their growth by culturing them in normal FBS medium versus androgen-depleted DCC-FBS medium. Compared with the parental cell lines, MDA-I cells showed similar growth inhibition upon androgen withdrawal (Fig. 1C, left), as well as a similar reduction in PSA secretion (Fig. 1C, right). In addition, MDA-I cells grew at a similar rate

to parental MDA cells in androgen-repleted medium, and there was no difference in attachment between MDA and MDA-I cells. Thus, this highly invasive subline remains androgen dependent despite elevated AR expression.

**AR mediates invasiveness of androgen-dependent prostate cancer cells.** Because AR expression is increased in MDA-I cells, we asked whether invasion is affected by or dependent on AR activity. Invasiveness was reduced by  $>50\%$  when MDA-I cells were cultured in androgen-depleted DCC medium (Fig. 2A), implying that reduction of AR activity through androgen removal significantly decreased invasive capacity. Indeed, treatment of MDA-I cells with bicalutamide, an AR antagonist, resulted in as much as 90% inhibition of invasion (Fig. 2B), confirming that invasion is mediated by AR activity. Furthermore, suppressing AR expression by shRNA knockdown via lentivirus vector in MDA-I cells also reduced invasiveness significantly by 50% (Fig. 2C). We confirmed that reduced AR level achieved by knockdown significantly decreased the growth rate and PSA secretion of MDA-I cells (Fig. 2D). Note that there was no difference in growth rate on day 3 between control and AR shRNA-treated groups, showing evidently that the knockdown effect on invasion determined on day 2 was independent of MDA-I growth suppression. Thus, removal of AR ligand, AR receptor blockade, and AR knockdown all greatly inhibit the invasiveness of MDA-I. These data suggest that AR is necessary for the invasive properties of androgen-dependent prostate cancer cells.

Next, we asked whether the presence of AR in this system is sufficient to promote invasion. Stimulation of AR activity with exogenous synthetic androgen R1881 in MDA-I cells promoted invasiveness in a dose-dependent manner, with almost 5-fold increase at the highest dose (Fig. 3A). A similar trend was observed when the parental cell line MDA was transduced with an AR-expressing lentiviral vector. Overexpression of AR significantly increased invasiveness in MDA-AR cells compared with control (Fig. 3B). Thus addition of ligand or forced expression of AR is sufficient to promote invasion of prostate cancer cells. Together

these results provide direct evidence for AR signaling in mediating invasion in androgen-dependent prostate cancer cells.

An effect of AR overexpression on cell growth was also observed. Interestingly, there was no significant difference in growth rate between MDA-AR cells and control cells cultured in regular FBS (Fig. 3C). In contrast, MDA-AR cells showed greater growth by day 6 when cultured in the absence of androgen (Fig. 3D), suggesting that high levels of AR expression could confer androgen-refractory status to MDA cells. Thus, increased expression of AR is sufficient to promote both androgen refractory and invasive behavior.

To determine if the involvement of AR in prostate cancer invasion was limited to the MDA-I cell line, we performed parallel studies in the androgen-dependent cell line LNCaP (Supplementary data). As with MDA-I, the dependence of invasion on AR activity was confirmed by AR blockade using bicalutamide, RNA interference, and growth in androgen-depleted medium (Supplementary Fig. S1A–C). All three treatments reduced the invasiveness of LNCaP cells between 60% and 90%, confirming that invasiveness is dependent on functional AR and presence of ligand. Furthermore, stimulation with synthetic androgen or overexpression of AR also increased invasiveness of LNCaP cells (Supplementary Fig. S1D, E). As with MDA cells transduced with AR, LNCaP-AR cells could grow in the absence of androgen, consistent with previous studies showing that AR overexpression confers resistance to androgen deprivation.

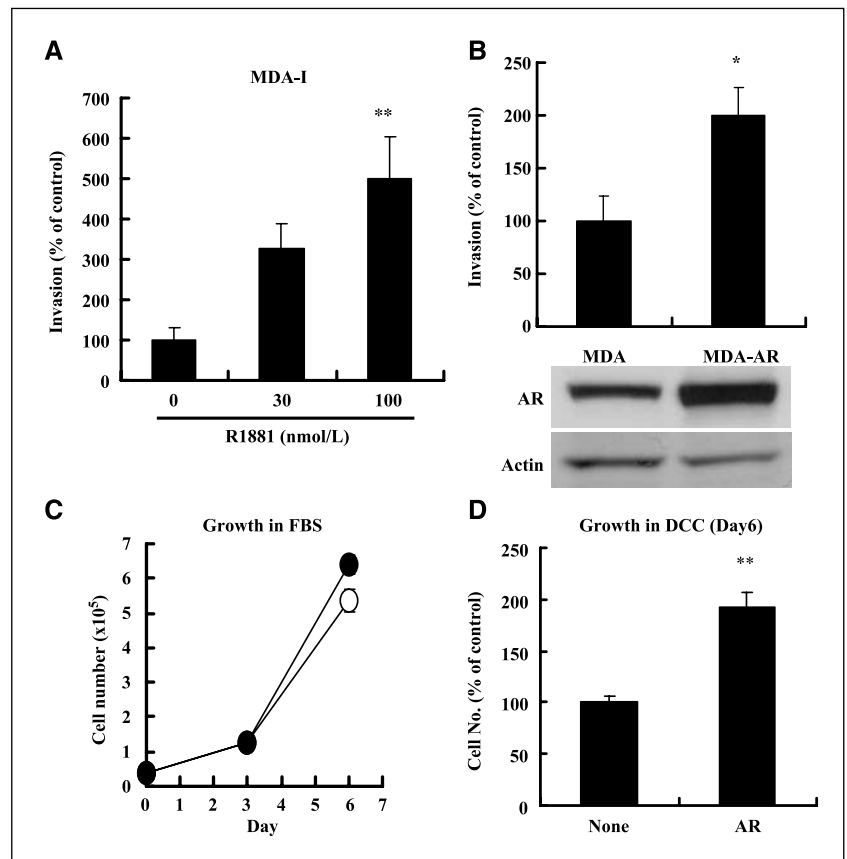
**Androgen-refractory prostate cancer cells are more invasive than androgen-dependent prostate cancer cells.** Because AR activity seems to mediate invasion in androgen-dependent cells, we wanted to see if this is also the case in androgen-refractory cancer

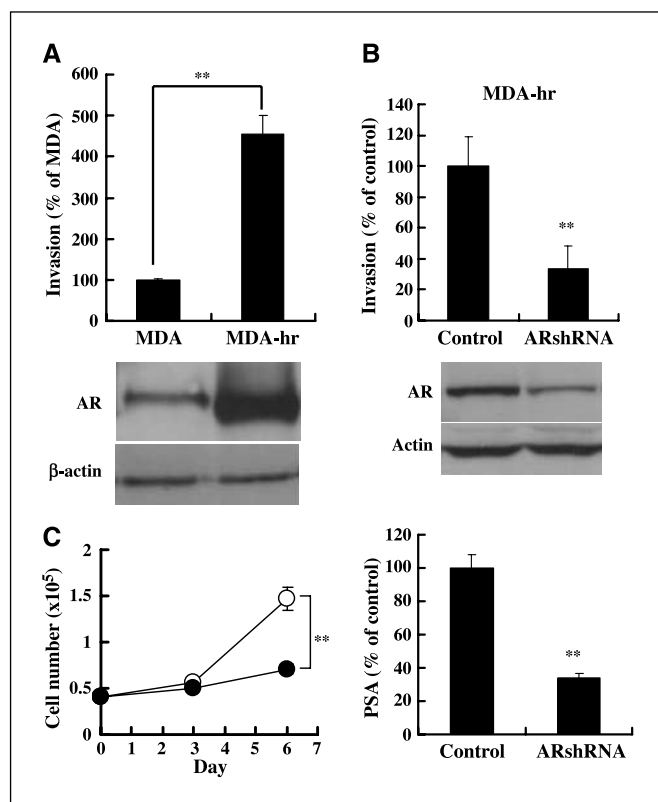
cells and whether there is a potential link between androgen resistance and invasiveness. The invasive properties of the parental MDA cell line and its androgen-refractory derivative, designated MDA-hr, were examined. The latter was established by serial passaging of the parental cell line in androgen-depleted medium, and its androgen-refractory status was confirmed by *in vivo* tumor growth in castrated mice (7). MDA-hr was 4-fold more invasive than parental MDA and also expressed higher levels of AR (Fig. 4A). This is consistent with the above finding that AR overexpression (both in MDA-Pca2b and LNCaP cells) is sufficient to confer androgen-independent status. These data suggest that progression to androgen-refractory cancer might be accompanied by increased invasiveness.

**AR, but not activated Akt, is necessary for the enhanced invasiveness of androgen-refractory prostate cancer cells.** As in the case of androgen-dependent MDA-I cells, AR also mediates invasion of MDA-hr cells. AR knockdown using shRNA suppressed invasiveness by >50% (Fig. 4B). Bicalutamide treatment had a similar effect (see below; Fig. 5C). AR knockdown significantly decreased the growth rate and PSA secretion of MDA-hr cells on day 6 after cells were seeded, but not on day 3 when the change in invasiveness was first noted (Fig. 4C). This emphasizes the specific effects of AR on invasion, independent of cell growth.

To further explore the signaling pathway of AR-mediated invasion in prostate cancer cells, we chose to focus on the phosphatidylinositol 3-kinase (PI3K)–Akt pathway. It has been reported by other groups that androgen depletion increases activation of Akt, which is required for progression to androgen-refractory status in LNCaP cells (13), whereas activation of Akt can

**Figure 3.** Stimulation of invasion by synthetic androgen R1881 in MDA-I cells (A) and AR overexpression in MDA cells (B) and stimulation of growth in AR overexpressing MDA-AR cells (C and D). For invasion assays, cells were seeded at  $1 \times 10^5$  per well as described in Materials and Methods. A, MDA-I cells were treated in DCC medium with DMSO (control) or 30 and 100 nmol/L of R1881 for 48 h, and invasive cells were quantified as percentage of control. Columns, mean ( $n = 4$ ); bars, SE. \*\*,  $P < 0.01$  versus the control group by Dunnett's test. B, invasion assay (top) and Western blot (bottom) of MDA cells infected with either control vector or the AR-expressing lentiviral vector. Columns, mean ( $n = 4$ ); bars, SE. \*,  $P < 0.05$  versus the control vector-infected group by unpaired Student's *t* test. C, MDA cells infected with either control vector (○) or the AR-expressing lentiviral vector (●) were plated at  $4 \times 10^4$  per well in 24-well plates in growth medium with regular FBS. Cells were counted at 3 and 6 d. Points, mean ( $n = 3$ ); bars, SE. There was no statistical significance between the two groups. D, the same experiment as in C was set up in DCC medium, and cells were counted on day 6. Columns, mean ( $n = 3$ ); bars, SE. \*\*,  $P < 0.01$  by unpaired Student's *t* test.





**Figure 4.** Increased invasiveness of hormone-refractory MDA-hr cells via AR activity compared with androgen-dependent MDA cells. Invasion assay (top) and Western blot of AR (bottom) in MDA cells (control, regular FBS) and MDA-hr cells (DCC-FBS; A) and MDA-hr cells (DCC-FBS) infected with the control vector or AR shRNA lentiviral vector (B). For invasion assays, cells were seeded at  $1 \times 10^5$  per well as described in Materials and Methods. Invasive cells were counted after 48 h, and invasion was expressed as percentage of control group (control, 100%). Columns, mean ( $n = 4$ ); bars, SE. \*\*,  $P < 0.01$  versus control by unpaired Student's *t* test. C, MDA-hr cells infected with either control vector (○) or AR shRNA lentiviral vector (●) were plated at  $4 \times 10^4$  per well in DCC medium in 24-well plates. Cells were counted at 3 and 6 d (left), and the culture medium was assayed for PSA levels on day 6 (right). Points, mean ( $n = 3$ ); bars, SE. \*\*,  $P < 0.01$  by unpaired Student's *t* test on day 6.

also induce an invasive phenotype accompanied by increased MMP-2 activity (14). As shown in Fig. 5A, MDA-hr cells not only had higher AR expression, but also exhibited higher levels of activated Akt (pAkt) compared with the parental cells. In addition, our signaling pathway analysis also showed higher levels of activated EGFR (pEGFR) and pHer2 in MDA-hr cells but no detectable change in Erk1/2 or its activated form. These findings suggest that the PI3K-Akt pathway is activated in this androgen-refractory cell line and raised the possibility that activated Akt might be involved in the enhanced invasiveness of MDA-hr cells. To address this question, MDA-hr cells were first treated with the PI3K inhibitor LY294002 at different doses to determine the amount required to block Akt phosphorylation (Fig. 5B). Next, MDA-hr cells were exposed to either the AR antagonist bicalutamide or LY294002 and their effects on invasiveness analyzed. Bicalutamide, but not LY294002, significantly suppressed invasiveness in MDA-hr cells by 50% (Fig. 5C). These results suggest that AR, but not activated Akt, is required for the invasive behavior of MDA-hr cells.

**Regulation of MMP-2 and MMP-9 activities by AR in androgen-dependent and androgen-refractory prostate cancer cells.** Given that MMP-2 and MMP-9 are known to regulate

invasiveness, we next asked whether they might play a role in AR-mediated invasion of prostate cancer cells. Using gelatin zymography, the activities of MMP-2 and MMP-9 in MDA-I and its parental line were measured. Consistent with the invasive pattern (Fig. 1A), MDA-I cells showed higher MMP-2 and MMP-9 gelatinolytic activities than parental cells (Fig. 6A, left), suggesting that increased invasiveness of MDA-I cells may be caused by change in MMP activity. Treatment with R1881 stimulated both MMP activities (Fig. 6A, middle) in MDA-I cells in a dose-dependent manner, whereas treatment with bicalutamide suppressed both MMP activities (Fig. 6A, right). These data parallel that of the invasion assays (Figs. 2B and 3A), suggesting that MMP-2 and MMP-9 activities play a role in the regulation of AR-mediated invasion in MDA-I cells. To confirm the role of MMPs further, MDA-I cells were treated with a specific inhibitor of MMP-2/MMP-9, and its effect on invasiveness examined. As expected, the inhibitor significantly suppressed invasion of MDA-I cells both at 25 and 40  $\mu\text{mol/L}$  (Fig. 6C, left), demonstrating the involvement of MMPs in promoting invasion.

Similar analyses were performed on the androgen-independent MDA-hr cells. Higher gelatinolytic activities were observed in MDA-hr cells compared with parental cells (Fig. 6B, left). Treatment with either bicalutamide or AR shRNA suppressed the gelatinolytic activities of MMP-2 and MMP-9 (Fig. 6B, middle and right). These data suggest that MMP-2 and MMP-9 activities are regulated by AR and are associated with the increased invasiveness of the hormone-refractory MDA-hr cells (Figs. 4B and 5C). Finally, treatment with the MMP-2/MMP-9 specific inhibitor significantly suppressed the invasion of MDA-hr cells (Fig. 6C, right), demonstrating that MMPs are causative in AR-mediated invasion of this cell line.

## Discussion

Accumulating evidence suggests that AR plays a critical role in the growth of both androgen-dependent and androgen-independent prostate cancer (3–6, 8). However, the role of AR in invasion and metastasis in prostate cancer is not clear (15). Recent studies showed that forced expression of AR in AR-negative PC3 cells and ARCaP cells decreased their invasive properties, and treatment with androgen further reduced invasion of these cells (16, 17). The AR-negative PC3 and DU145 cell lines are more invasive than the AR-positive LNCaP cell lines (9). These observations suggest that AR is inversely correlated with and may suppress invasion. In contrast, Liao and colleagues recently reported that testosterone induces MMP-2 expression in LNCaP cells, suggesting an association between androgen and genes that regulate invasion (18). In the current study, we evaluated the role of AR in invasion of both hormone-dependent and hormone-refractory prostate cancer cells upon observing that AR expression was elevated in invasive and hormone-refractory sublines of the hormone-dependent cell line MDA (7). Pharmacologic or genetic blockade of AR signaling suppressed the invasive properties of both cell lines, whereas androgen treatment increased invasiveness. Forced expression of AR in the noninvasive MDA parental line also conferred an invasive phenotype. These data clearly link AR expression to invasion in both androgen-dependent and androgen-refractory AR-positive prostate cancers.

One important finding of our study is that the hormone-refractory MDA-hr cell line is more invasive than its androgen-dependent parent, which suggests a link between invasion and androgen independence. In the case of MDA, this link is mediated

by increasing AR levels. Interestingly, we previously reported that N-cadherin expression is up-regulated in hormone-refractory variants of the LAPC-9 and LAPC-4 prostate cancer xenografts. It is also expressed in the AR-negative hormone-refractory PC3 cell lines. It is interesting to speculate that genes involved in cancer invasion and metastasis may be associated with or play a role in hormone-refractory prostate cancer progression.

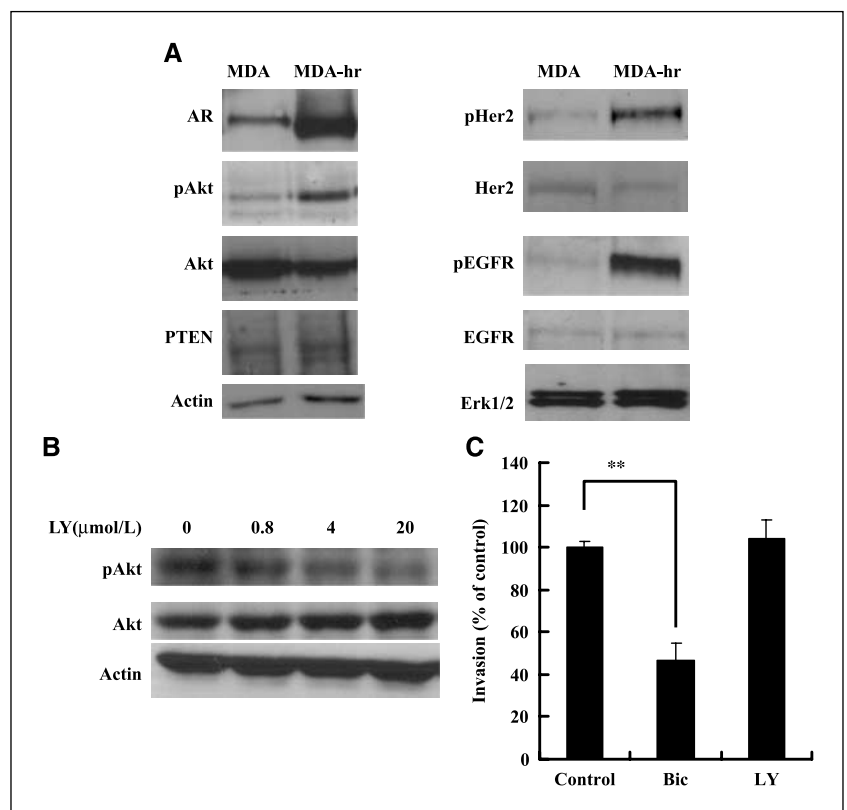
Our data suggest that the level of AR expression is critical in determining whether a prostate cancer cell becomes invasive versus invasive and hormone refractory. MDA-I cells are invasive but remain hormone dependent, whereas MDA-hr cells are both invasive and hormone refractory. MDA-I cells express an intermediate level of AR, whereas MDA-hr cells express the highest level of AR. Forced overexpression of AR resulted in AR levels similar to those observed in MDA-hr, leading to both an invasive and hormone-refractory phenotype. These findings suggest that intermediate levels of AR are sufficient to mediate invasion, but not hormone independence, and that the pathways governing invasion and hormone-refractory growth are distinct. At the clinical level, these data imply that partial blockade of AR might affect hormone-refractory progression while having no effect on invasion.

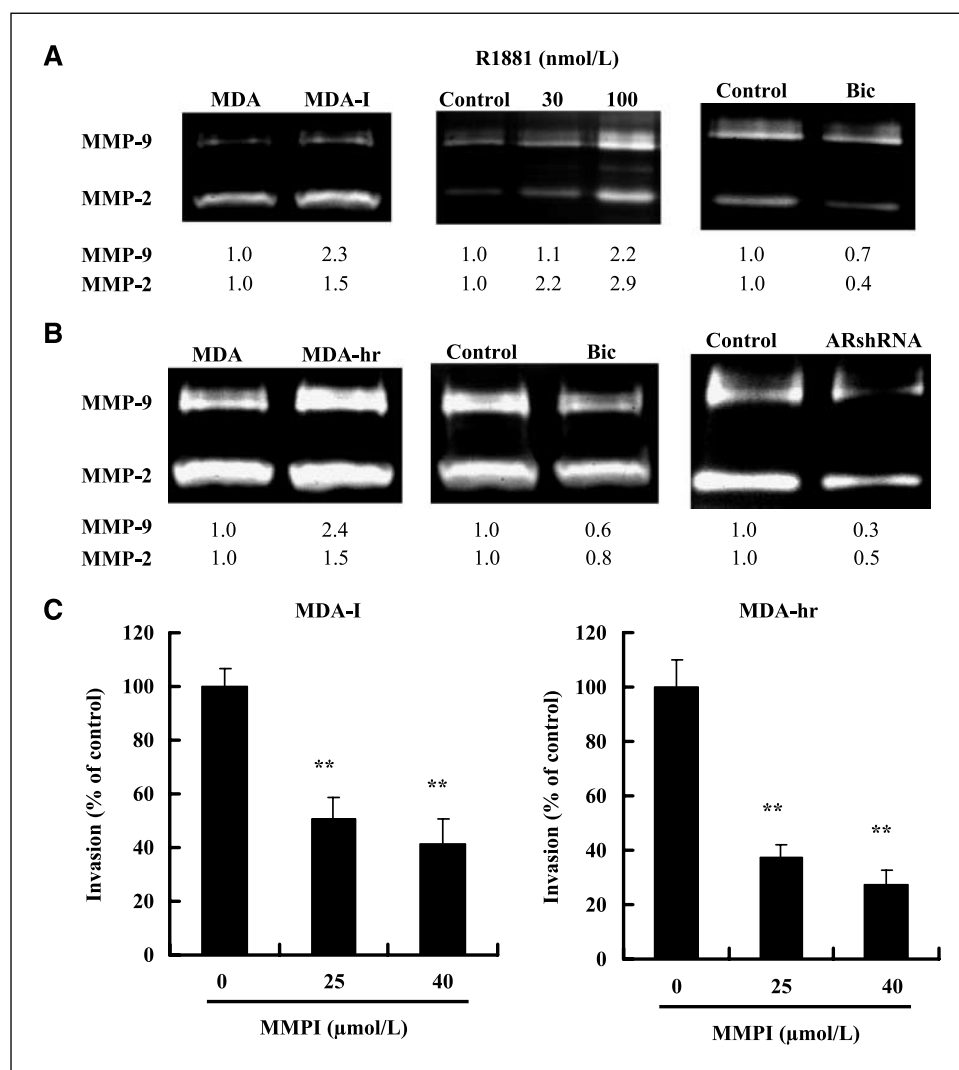
One of the key steps in the process of cancer invasion and metastasis is the degradation of the extracellular matrix. A wide range of proteinases, including MMPs, are involved in the process. Among different MMPs, MMP-2 and MMP-9, called gelatinases, disrupt the basement membrane by cleaving type IV collagen. Many studies have shown that increased levels of MMP-2 or MMP-9 in the serum or tissue samples of prostate cancer patients are correlated with advanced stage (19–26). Whereas MMP expression is regulated by growth factors and cytokines, as well as oncogenes (27–29), regulation of MMP-2 or MMP-9 activities by AR signaling

in prostate cancer cells is controversial. It has been reported that androgen decreases MMP-9 secretion in PC-3 cells stably expressing AR (19), suggesting that androgen suppresses MMP-9 activity. On the other hand, androgen has been shown to increase MMP-2 or MMP-9 activities in androgen-dependent LNCaP cells (18). In this study, we showed that androgen stimulates both MMP-2 and MMP-9 activities in androgen-dependent MDA-I cells. Furthermore, we showed that blocking AR signaling by an AR antagonist or AR knockdown decreases both MMP-2 and MMP-9 activity in MDA-I and androgen-refractory MDA-hr cells. Together with the findings that a MMP-2/MMP-9-specific inhibitor suppressed the invasion of MDA-I and MDA-hr cells, our data clearly show that both MMP-2 and MMP-9 activities are stimulated by AR signaling and are closely correlated with the invasiveness of androgen-dependent and androgen-refractory prostate cancer cells that express endogenous AR. The discrepancy between our results and those in PC3 may be attributed to the presence of AR in MDA cells compared with PC3 and the existence of an intact AR signaling pathway in the former.

Recently, it has been reported that androgen depletion increases PI3K-Akt activation, which is required for the progression to an androgen-refractory state in LNCaP cells (13). In addition, it has been reported that Akt activation induces an invasive phenotype accompanied by increased MMP-2 and MMP-9 activity in COMMA-1D mouse mammary epithelial cells and in HT1080 human fibrosarcoma cells (14, 30). Consistent with the association of PI3K-Akt activation with androgen independence, we show in this study that pAkt expression levels are higher in MDA-hr cells than in parental MDA cells. However, we could not show the requirement of pAkt for invasion of these cells. One possibility is that the role of pAkt in invasion is context dependent. Consistent with this

**Figure 5.** A, increased expression of activated Akt (pAkt), pEGFR, pHer2 in MDA-hr cells, analyzed by Western blot. B, titration of LY294002 (LY) to determine the sufficient concentration for blocking pAkt; samples were analyzed by Western blot. C, invasion assay after treatment of MDA-hr cells with either bicalutamide or LY294002. MDA-hr cells were seeded at  $1 \times 10^5$  per well as described in Materials and Methods in DCC medium and treated with either DMSO (control), 100  $\mu\text{mol/L}$  of bicalutamide, or 4  $\mu\text{mol/L}$  of LY294002. Invasive cells were counted after 48 h, and invasion was expressed as percentage of control group (control, 100%). Columns, mean ( $n = 4$ ); bars, SE. \*\*,  $P < 0.01$  versus the control group by unpaired Student's *t* test with Holm's correction.





**Figure 6.** Regulation of MMP-2 and MMP-9 activities by AR signaling. Gelatin zymography was performed as described in the Materials and Methods. Proteins (15  $\mu$ g) from the concentrated conditioned media were used for gelatin zymography. The number below each picture indicates the relative density of the band when setting the control to 1. *A*, parental MDA and MDA-I cells were serum-starved for 2 d (*left*), MDA-I cells were treated with DMSO (control), 30, or 100 nmol/L of R1881 (*middle*), and MDA-I cells were treated with control serum or 100  $\mu$ mol/L of bicalutamide (*right*) for 2 d. *B*, MDA and MDA-hr cells were serum-starved for 2 d (*left*), MDA-hr cells in DCC medium were treated with DMSO (control) or 100  $\mu$ mol/L bicalutamide for 2 d (*middle*), and MDA-hr cells transduced with scrambled (control) or AR shRNA lentiviral vector were serum-starved for 2 d (*right*). *C*, MMP-2/MMP-9-specific MMP inhibitor (MMPI) inhibited invasion in MDA-I and MDA-hr cells. The invasion assay was performed as described in Fig. 5C except 25 and 40  $\mu$ mol/L of MMP inhibitor were used instead. *Columns*, mean ( $n = 4$ ); *bars*, SE. \*\*,  $P < 0.01$  versus control by Dunnett's test.

possibility, Majumder et al. showed that prostate-restricted Akt hyperactivity caused prostatic intraepithelial neoplasia but not invasive cancer in the MPAKT (murine prostate restricted Akt kinase transgenic) mouse model (31). Further studies are required to elucidate the role of Akt activation in prostate cancer invasion and progression to androgen independence.

A major finding in the prostate cancer field has been the presence of gene fusions and translocations in a majority of tumors. These genetic changes typically involve the linkage of an androgen responsive promoter to an oncogene, such as the androgen responsive promoter of TMPRSS2 to the oncogenes ERG, ETV1, and ETV4. Interestingly, since this manuscript was initially submitted, Tomlins et al. reported that both LNCaP and MDA Pca 2b cells carry genetic rearrangements that lead to overexpression of ETV1 (32). Even more recently, Cai et al. published data linking ETV1 to MMP activity and to invasiveness in LNCaP cells (33). It will be interesting to further explore the link between AR, ETV1, and invasiveness. Of equal importance is to determine if AR mediates invasiveness only in prostate cancers that have an ETV1-activating translocation and if ETV1 is the primary downstream target of AR activity causing invasiveness. A clear link between AR, ETV1, and invasiveness might improve our ability to identify early-stage prostate cancers at high risk to metastasize and kill their host.

Our study has a number of important clinical implications. For one, our data suggest that the level of AR (and PSA) expression might predict tumor stage and/or clinical progression to metastasis or hormone-refractory status in the clinic. AR overexpression in malignant epithelium with loss of AR in the adjacent periepitheial stroma was shown to be associated with higher clinical stage and poor clinical outcome (34). Higher levels of AR and AR-regulated genes have also clearly been shown in clinical specimens of metastatic prostate cancer, consistent with the hypothesis that AR regulates both metastasis and resistance to castration (3, 20). It was also recently reported that higher levels of AR expression are correlated with lymph node status, extracapsular extension, seminal vesicle invasion, Gleason score, and decreased biochemical recurrence-free survival in prostate cancer treated with radical prostatectomy (35). However, previous studies failed to show any prognostic significance of AR level after radical prostatectomy (36, 37). One possible reason for the disparity in published studies is that quantification of AR staining in clinical material is difficult. Likewise, AR overexpression in a small percentage of cancer cells may affect tumor progression, which could be missed when looking at global AR expression levels in tumors. Additional studies using newer methods of tissue quantification will be needed to determine the true prognostic implications of AR expression level in clinical material.

A second implication of our study is that AR blockade may prevent invasive and/or metastatic progression in men with high-risk tumors. Indeed, recent studies in the surgical and radiation literature have supported the use of concomitant or adjuvant hormonal therapy to retard cancer progression. Adjuvant hormonal therapy after surgery in men with small volume node-positive prostate cancer has been shown to increase survival compared with surgery and delayed hormonal blockade (38). Similarly, long-term hormonal therapy has been shown to improve survival of men with locally advanced and high-grade disease in men undergoing radiation therapy (39, 40). One caveat to our hypothesis is that early hormonal therapy might speed the

development of hormone-refractory disease, which might be more invasive than untreated disease. The tradeoff of early inhibition of invasion compared with earlier development of more invasive hormone-refractory disease needs to be examined further in animal models of prostate cancer (41, 42).

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