Androgen receptor is a tumor suppressor and proliferator in prostate cancer

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Targeting androgens/androgen receptor (AR) functions via androgen deprivation therapy (ADT) remains the standard treatment for prostate cancer. However, most tumors eventually recur despite ADT. Here we demonstrate that the prostate AR may function as both a suppressor and a proliferator to suppress or promote prostate cancer metastasis. Results from orthotopically recombining stromal WPMY1 cells with epithelial PC3 prostate cancer cells in mice demonstrated that restoring AR in epithelial PC3 cells or knockdown of AR in stromal WPMY1 cells suppressed prostate cancer metastasis. Knockdown of the AR in epithelial CWR22rv1 prostate cancer cells also resulted in increased cell invasion in vitro and in vivo. Restoring AR in PC3 cells (PC3-AR9) results in decreased invasion in bone lesion assays and in vivo mouse models. Mice lacking the prostate epithelial AR have increased apoptosis in epithelial luminal cells and increased proliferation in epithelial basal cells. The consequences of these two contrasting results led to the expansion of CK5/CK8-positive intermediate cells, and mice developed larger and more invasive metastatic tumors in lymph nodes and died earlier than wild-type littermates. Mechanistic dissection suggested that androgens/AR might directly or indirectly modulate metastasis-related genes and suppression of TGFβ1 signals results in the partial inhibition of AR-mediated metastasis. Collectively, our understanding of these opposing roles of prostatic AR may revolutionize the way we combat prostate cancer, and allow the development of new and better therapies by targeting only the proliferative role of AR.

Androgen deprivation therapy | testosterone | TGFβ1 | metastasis

Early studies suggested that the prostatic epithelial androgen receptor (AR), when activated by androgens, increased cellular proliferation (1, 2). Clinical studies also pointed out that androgen deprivation therapy (ADT) with suppression of androgens/AR functions, is an effective treatment for most prostate cancer patients (3, 4). However, most prostate tumors regress over 12–18 months of continuous ADT (1–4). The detailed mechanisms of why suppression of androgens/AR ultimately fails and cancers recur as a more aggressive type and metastasize remain unclear.

The conventional concept of the AR role in prostate cancer is to promote cancer progression, and positive AR staining can be found in many prostate tumors even at the later stages. In addition to androgens, other factors could also affect AR activity, such as (a) AR mutations or amplification, (b) changes in AR and AR coregulators interactions, or (c) growth factors/kinases signal pathways that activate AR activity at the castration level of androgen (1–4). However, why patients receiving ADT tended to have an earlier development of more aggressive types of cancer and whether AR has a differential role in different prostatic cells and/or in different prostate cancer stages remain unclear.

Here, we report the generation of a mouse cancer model lacking the AR only in its prostatic epithelium (pes-ARKO-TRAMP), which develops prostate cancer spontaneously with an intact immune system. Notably, through AR gain- and loss-of-function in epithelial–stromal cell coculture and coimplantation experiments, we demonstrated that the AR could function in epithelial basal intermediate cells as a tumor suppressor to suppress prostate cancer metastasis, in epithelial luminal cells as a surviving factor, and in stromal cells as a proliferator to stimulate cancer progression. These contrasting data challenge the currently used ADT that systematically suppresses androgen actions, and thus reduces both proliferative and suppressor functions of AR. Our results suggest the need for better therapies that only target the proliferative function of AR.

Results

AR is a Proliferator in Stroma to Promote Prostate Cancer Progression and a Suppressor in Epithelium to Suppress Prostate Cancer Cell Invasion. PC3-v cells vs. PC3-AR9 cells. To dissect how the AR influences prostate cancer metastasis, we stably transfected an AR cDNA whose expression is regulated by a natural AR promoter (5) into AR-negative prostate cancer PC3 cells (designated PC3-AR9). Unlike other cell models in which the AR is overexpressed with strong viral promoters (6), leading to an unnatural build-up of AR, these PC3-AR9 cells express a normal quantity of functional AR and are activated by dihydrotestosterone (DHT) (Fig. 1A and B). Using the Boyden chamber invasion assay, we found PC3-AR9 cells to be significantly less invasive than the parental PC3 cells stably transfected with the vector only (designated PC3-v) (Fig. 1C).

WPMY1-v cells vs. WPMY1-ARsi cells. The above results, which contradict the classical concept that the prostatic AR functions as a proliferator for prostate cancer progression, prompted us to coculture epithelial PC3-v cells with human prostatic stromal WPMY1 (7) cells to verify the stromal AR roles in prostate cancer cell invasion. Early reports demonstrated that the functional AR expressed in WPMY1 cells promoted androgen-dependent gene expression (8). We knocked down endogenous AR expression in WPMY1 cells with stably transfected AR-siRNA (designated WPMY1-ARsi) and cocultured these cells with PC3-v cells on different layers of the Boyden chamber (Fig. 1D) for the cell invasion assay. The result suggested that knockdown of the stromal AR in WPMY1-ARsi cells resulted in suppression of epithelial PC3-v cell invasion (Fig. 1E and F). In contrast, from the Boyden chamber coculture of either PC3-v or PC3-AR9 cells with WPMY1 cells transfected with the vector (WPMY1-v), our results indicated that addition of AR in PC3-AR9 prostate cancer epithelial cells results in suppression of epithelial PC3-v cell invasion (Fig. 1E and F).


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data are consistent with the results of Fig. 1C and suggest that the epithelial AR may function as a suppressor of cell invasion and the stromal AR may function as a stimulating factor in prostate cancer cell invasion.

CWR22rv1-AR+/+ cells vs. CWR22rv1-AR++/− cells. We then used a homologous gene recombination strategy to knockdown the AR in human CWR22rv1 cells isolated from a prostate tumor growing despite ADT (9) (CWR22rv1-AR+/-). CWR22rv1-AR+/- cells expressed much less AR than the parental CWR22rv1 or AR-/- cells compared to parental CWR22rv1-AR+++/− cells (Fig. 1G). Results of the invasion assay indicated that knockdown of the AR in CWR22rv1 (CWR22rv1-AR+/-) cells increases their invasive ability as compared to parental CWR22rv1-AR++,−/− cells (Fig. 1G). In a different approach using AR-siRNA (10) to knockdown AR in CWR22rv1-AR+/- (CWR22rv1-AR+++/−ARsi) cells, we found similar results, with enhanced invasive ability as compared to parental CWR22rv1-AR+++/− cells transfected with scramble RNA; adding functional AR in CWR22rv1-AR+++/−ARsi cells resulted in decreased cell invasion as compared to CWR22rv1-AR+++/− cells (Fig. 1H).

Together, based on four different approaches (knock-in of AR, knockdown of AR with genetic recombination, knockdown of AR with siRNA, and epithelium–stroma coculture system) with different prostate cancer cells, results shown in Fig. 1 all demonstrated that epithelial AR could function as a suppressor, and the stromal AR may function as a promoter for prostate cancer cell invasion.

Addition of Functional AR in PC3 Cells (PC3-AR9) Results in Decreased Invasion in Bone Lesion Assay and in Vivo Mouse Models. As PC3 cells were isolated from a bone metastasis, we examined the influence of the AR on PC3-AR9 cell invasion by measuring osteoclastogenes is in a bone-wafer absorption assay (11). We cocultured PC3-v or PC3-AR9 cells with bone cells from newborn rat bone marrow, onto bone wafer and quantified osteoclast formation. Compared to PC3-v cells, the PC3-AR9 cells on bone wafer had decreased numbers of osteolytic lesions (pitted areas) (Fig. 2A). To evaluate invasion in vivo, we injected cells into the tibia of nude mice (12). PC3-v tumors grew more aggressively and more invasively (Fig. 2B and C) than PC3-AR9 tumors as determined by x-ray analysis. Collectively, these data from knock-in of the functional human AR suggest that loss of the AR signaling in prostate epithelial cells promotes invasion both in vitro and in vivo.

We also tested the roles of AR in a metastatic assay in an in vivo mouse prostate cancer model. PC3-v or PC3-AR9 cells were orthotopically inoculated into the anterior prostate of nude mice. Consistent with the above findings, mice inoculated with PC3-v cells...
developed bigger metastatic tumors in pelvic lymph nodes (PLN) than mice inoculated with PC3-AR9 cells (Fig. 2D). Similar results also occurred when we replaced PC3/PC3-AR9 cells with CWR22rv1-AR$^{-/-}$/CWR22rv1-AR$^{+/+}$ cells, in which knockdown of AR in CWR22rv1-AR$^{-/-}$ cells led to the development of bigger metastatic tumors in lymph nodes (data not shown).

We also inoculated different combinations of epithelial cancer cells (PC3-$v$ and PC3-AR9) and stromal cells (WPMY1-$v$ and WPMY1-ARsi) into the anterior prostate of nude mice. These in vivo results are consistent with the in vitro data shown in Fig. 1, and demonstrate that mice inoculated with recombinants, with either restoration of the epithelial AR (PC3-AR9 cells) or knockdown of the stromal AR (WPMY1-ARsi cells), developed smaller metastatic tumors in lymph nodes (Fig. 2E).

Together, using either knockdown or knock-in of the AR in various human prostate cells, our in vitro cell invasion results and in vivo mouse tumor data consistently demonstrated that the epithelial AR may suppress, and the stromal AR may promote, prostate cancer metastasis. Generation of pes-ARKO-TRAMP Mice Lacking AR in Epithelial Luminal and Basal Cells. All of the above data were generated from human prostate cancer cells that were already transformed. We were interested in using a mouse model that underwent carcinogenesis
spontaneously with an intact immune system. We first mated female floxAR (C57BL/6) mice with TRAMP (FVB) mice (13), to generate floxAR-TRAMP (C57BL/6-FVB) mice. We then crossed these mice with Pb-Cre (C57BL/6) male mice (14) to generate pes-ARKO-TRAMP (C57BL/6-FVB) mice that lack the AR only in the prostatic epithelium (15). This pes-ARKO-TRAMP mouse was further bred with ROSA26R-β-Gal mouse (16), and from their offspring we found probasin-cre expressed in all of the epithelial luminal cells and some epithelial basal cells [supporting information (SI) Fig. S1 A and B, n = 3]. We confirmed that AR was knocked down in epithelial cells, but not in stromal cells, of pes-ARKO-TRAMP mice using (a) laser capture microdissection (LCM)-separating epithelial and stromal cells and (b) prostate immuno-histochemical staining with the antibody specific to the AR C-terminal region. (Fig. S1 C-E). Other urogenital organs from both wild-type AR-TRAMP (WT-TRAMP) and pes-ARKO-TRAMP (pes-ARKO-TRAMP) mice developed normally (Fig. S1F).

**Increased Apoptosis in Epithelial Luminal Cells and Increased Proliferation in Epithelial Basal Cells of pes-ARKO-TRAMP Mice.** Recent studies suggested that prostate cell growth develops from AR negative (AR−) to AR positive (AR+) cells via prostate stem cells (AR+), to basal cells (AR−), to basal-intermediate cells (mix of AR− and AR+), to luminal cells (AR+) (17). Using double staining of the luminal cell marker CK8 red-fluorescence (18) and the apoptotic marker TUNEL green-fluorescence detection (Fig. 3), we found a significantly higher level of apoptosis in CK8-positive luminal cells in 16 wk-old pes-ARKO-TRAMP mice compared to those of WT-TRAMP littermates. In contrast, using double staining of basal cell marker CK5 red-fluorescence (18) and a proliferation marker (Ki67 staining or BrdU incorporation as green-fluorescence) (Fig. 3), we found a significantly higher proliferation rate in CK5-positive basal cells in 16-wk-old pes-ARKO-TRAMP mice compared to WT-TRAMP mice (Upper). The quantitative data are shown (Lower, n = 3). (D) Increased CD44-positive cells in primary tumors of 24-wk-old pes-ARKO-TRAMP compared to WT-TRAMP littermates (n = 3).

**Increased Basal-Intermediate Cells in pes-ARKO-TRAMP Mice.** Loss of epithelial AR in pes-ARKO-TRAMP mice resulted in increased

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**Fig. 3.** Loss of epithelial AR in pes-ARKO-TRAMP leads to higher proliferation in basal cells and higher apoptosis in luminal cells with increased basal intermediate cells. (A) Using the TUNEL assay, the apoptosis signals in the luminal epithelial cells from 16-wk-old pes-ARKO-TRAMP prostatic epithelium were higher than those from WT-TRAMP (arrowheads). CK8 immunostaining (red) was used to identify the luminal epithelial cells (Upper). Quantitative results showed the differences were 18% vs. 2% (Lower). (B) The growth rates of prostate epithelium were demonstrated by Ki67 immunostaining (Top) and BrdU incorporation (Middle) in 16-wk-old WT-TRAMP vs. pes-ARKO-TRAMP. The mice were i.p. injected with BrdU (10 μg/g body weight) every 6 h, and killed 24 h later. Paraffin-fixed tissue sections were stained by the BrdU detecting kit (Zymed Laboratories). The results from double immunofluorescent staining of BrdU (green) and CK5 (red) indicated that the higher proliferative prostate cells belong to the CK5 positive-basal cells in 16 wk-old pes-ARKO-TRAMP (Bottom with overlapped image, yellow color). The quantitative data were shown (n = 3). (C) Loss of the AR in the epithelium of pes-ARKO-TRAMP led to the expansion of the basal intermediate cell populations (yellow), with increased CK5/CK8-positive signals in prostates of 16-wk-old pes-ARKO-TRAMP compared to WT-TRAMP mice (Upper). The quantitative data are shown (Lower, n = 3). (D) Increased CD44-positive cells in primary tumors of 24-wk-old pes-ARKO-TRAMP compared to WT-TRAMP littermates (n = 3).

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**Supporting Information:**
- Fig. S1: Additional experimental details and results.
- Table S1: Summary of experimental conditions and results.
- Table S2: Statistical analysis of experimental data.
apoptosis in epithelial luminal cells and increased proliferation in epithelial basal cells. The consequences of these two contrasting results lead to the expansion of CK5/CK8-positive basal-intermediate cells in pes-ARKO-TRAMP mice (Fig. 3C, n = 6), which is further confirmed by the increase of CD44-positive cells in the primary tumors of 24-wk-old pes-ARKO-TRAMP mice compared to Wt-TRAMP mice. Our observation (Fig. 3D, n = 6) is consistent with another report that CD44+ prostate cancer cells are highly tumorigenic and metastatic (22). These results suggest that knockout of AR in epithelium resulted in the cell population changes, with expansion of epithelial basal-intermediate–like cells and decrease of epithelial luminal cells in pes-ARKO-TRAMP prostates. This is in agreement with a recent report showing that after ADT in prostate cancer patients, the percentage of CK5-positive basal–intermediate cells increased significantly (from 29% to 75%) (18).

**pes-ARKO-TRAMP Mice Develop More Aggressive and Invasive Metastatic Tumors.** Increased apoptosis in epithelial luminal cells and increased proliferation in epithelial basal cells in pes-ARKO-TRAMP mice also led to increased size of metastatic tumors in PLN in 24-wk-old pes-ARKO-TRAMP compared to Wt-TRAMP mice (P < 0.05; 3.0 vs. 1.7 mg, n = 7 for each genotype) (Fig. 4A and B). In addition, more prostate cancer metastatic foci were observed within the livers of pes-ARKO-TRAMP mice (n = 6) than those of Wt-TRAMP mice (n = 6) (Fig. 4C). Western blotting analysis confirmed loss of the AR in primary and PLN metastatic tumors in pes-ARKO-TRAMP mice (Fig. 4D).

Using invasion assay (23) of primary cultured PLN tumor cells isolated from both genotypes of TRAMP mice, we found that the primary culture PLN tumor cells from pes-ARKO-TRAMP mice (n = 5) were more invasive than those from Wt-TRAMP mice (n = 5) (Fig. 4E). Importantly, restoring a functional AR by infecting retrovirus-AR reduced the invasiveness of PLN tumor cells from pes-ARKO-TRAMP mice (Fig. 4E). These results (Fig. 4 A–E) showed that loss of the prostatic epithelial AR leads to the development of more invasive and metastatic prostate cancers and that gain of AR function reverses these characteristics. Consequently, increased tumor invasiveness and metastases lead to lower survival rates in pes-ARKO-TRAMP mice (n = 20) compared with Wt-TRAMP littermates (n = 30) (Fig. 4F).

**Human Clinical Data from Prostate Cancer Patients.** Recent clinical data from 254 prostate cancer patients found that ADT treatment resulted in the promotion of metastatic prostate tumors (24). We also evaluated AR expression in primary (97 cases) and metastatic (28 cases) prostate tumors and observed a significant difference between AR expression in primary tumors (91.75%) and metastatic tumors (67.86%), (P < 0.01) (Fig. S2). These in vivo clinical data

![Image](https://www.pnas.org/cgi/doi/10.1073/pnas.0804700105) Niu et al.
are consistent with a recent study (25) that used tissue arrays from prostate cancer patients who received radical prostatectomy, in which it was concluded that AR expression was significantly decreased in metastatic prostate cancer as compared to primary prostate cancer or normal prostate (mean 1.30 vs. 3.49, P < 0.01). Together, these clinical findings all support the suppressor roles of AR in metastasis, as we demonstrated above in human prostate cancer cells and various mouse prostate tumor models.

**Molecular Mechanisms by Which AR Can Function as a Suppressor of Prostate Metastasis.** It is now well accepted that few, if any, solid cancers rely on a single pathway mecanism for tumor progression and metastasis. For example, breast cancer may use at least 6 different factors to regulate its progression (26). We also hypothesized that the AR may modulate prostate cancer metastasis via multiple pathways. Therefore, using DNA microarray, we evaluated the differential gene expression of CWR22rL-AR+/+ and CWR22L-AR−/− cells. Among these genes up-regulated or down-regulated by knockdown of AR, we focused on those that have been linked to prostate cancer metastasis and confirmed the results using Q-PCR. The results (Table 1) suggest that the relative mRNA expression of those metastasis/invasion-related genes correlate well with the metastatic status in PLN tumors from Wi-TRAMP and pes-ARKO-TRAMP mice as well as xenografted tumors from PC3-v vs. PC3-AR9 (Table 1). The above data support the concept that AR can function as a suppressor of prostate metastasis. Further mechanistic dissection. (Fig. S3 and Fig. S4) suggests that the AR might use multiple mechanisms, including modulation of TGFβ1 signals, to modulate those metastasis/invasion-related genes. Suppression of TGFβ1 signals could then partially reverse the AR-mediated suppression of metastasis.

**Impact to current clinical treatment and development of new therapy to battle prostate cancer.** Results from the above studies all point out that AR has multiple and distinct functions: as a proliferator in stromal cells and a survival factor in epithelial luminal cells to promote cancer progression, or a metastatic suppressor in epithelial basal cells to inhibit tumor metastasis. These results are in stark contradiction to the accepted role of the prostatic AR to function only as a proliferator. Therefore, the conclusions drawn from these data may influence current clinical prostate cancer therapy. Based on our findings, systemic targeting of androgens/AR signals via ADT may result in either suppression or promotion of prostate cancer, depending on which role of AR (proliferator vs. suppressor) is more dominant at a particular stage of prostate cancer progression (27). Indeed, clinicians also face similar case decisions in which some prostate cancer patients do respond well when treated with androgens instead of antiandrogens (Nicholas Vogelzang, personal communication).

Therefore, the ideal therapeutic approach(es) could be to target the proliferative function or suppressive function of AR separately, which could be achieved by development of specific vehicles that carry androgens only to those prostate cells with AR suppressive function, or carry antiandrogens only to those prostate cells with AR proliferative function. Alternatively, a drug might be developed to specifically target the AR suppressor with its unique associated coregulators, or the proliferative AR with its unique associated coregulators. Before such an ideal therapy can be developed, perhaps a combination of anti-androgens/AR signals with antime-tastasis signals, such as antagonists for Akt, Cox-2, or MMP-9 signals (28), could be applied to battle prostate cancer.

**Methods.** Please see SI Text for the following detailed methods: (i) Cell culture, plasmids, and reagents; (ii) construction of retroviral vector with human AR cDNA and AR-siRNA; (iii) generation of pes-ARKO-TRAMP mice; (iv) in vitro bone-wafer resorption and osteoclastogenesis assay; (v) BrdU incorporation assay; (vi) TUNEL assay; (vii) immunohistochemistry stainings of Ki67, CK5, CK8, and CD44 on mouse prostate tumors; (viii) immunofluorescence stainings of CK5 and CK8 on mouse prostate tumors; (ix) isolation of primary culture prostate PLN tumor cells for invasion assay; (x) laser capture microdissection to separate the prostate stromal and epithelial cells; and (xi) statistical analyses of results.

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