Dominant-Negative Androgen Receptor Inhibition of Intracrine Androgen-Dependent Growth of Castration-Recurrent Prostate Cancer

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Abstract

Background: Prostate cancer (CaP) is the second leading cause of cancer death in American men. Androgen deprivation therapy is initially effective in CaP treatment, but CaP recurs despite castrate levels of circulating androgen. Continued expression of the androgen receptor (AR) and its ligands has been linked to castration-recurrent CaP growth.

Principal Finding: In this report, the ligand-dependent dominant-negative ARΔ142–337 (ARΔTR) was expressed in castration-recurrent CWR-R1 cell and tumor models to elucidate the role of AR signaling. Expression of ARΔTR decreased CWR-R1 tumor growth in the presence and absence of exogenous testosterone (T) and improved survival in the presence of exogenous T. There was evidence for negative selection of ARΔTR transgene in T-treated mice. Mass spectrometry revealed castration-recurrent CaP dihydrotestosterone (DHT) levels sufficient to activate AR and ARΔTR. In the absence of exogenous testosterone, CWR-R1-ARΔTR and control cells exhibited altered androgen profiles that implicated epithelial CaP cells as a source of intratumoral AR ligands.

Conclusion: The study provides in vivo evidence that activation of AR signaling by intratumoral AR ligands is required for castration-recurrent CaP growth and that epithelial CaP cells produce sufficient active androgens for CaP recurrence during androgen deprivation therapy. Targeting intracrine T and DHT synthesis should provide a mechanism to inhibit AR and growth of castration-recurrent CaP.

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Introduction

Prostate cancer (CaP) is the most common non-skin cancer diagnosed in American men. Despite earlier detection and improved treatment, more than 33,000 deaths are anticipated in 2011 [1]. For decades, androgen deprivation therapy has been the preferred treatment for locally advanced or metastatic CaP. Androgen deprivation therapy is effective initially but remissions are temporary. CaP that recurs responds poorly to most treatments and almost all men succumb to the disease. A molecular role for the androgen receptor (AR) in the transition to castration-recurrent CaP is supported by the continuous expression of AR [2–4] and androgen-regulated genes [5].

Many mechanisms contribute to AR transactivation despite castrate levels of circulating testicular androgens [reviewed by Feldman [6]]. However, other studies including our own have demonstrated that castration-recurrent CaP maintains tissue levels of dihydrotestosterone (DHT) sufficient to activate AR [4,7–9]. Persistent tissue testosterone (T) and DHT during androgen deprivation therapy may derive from adrenal androgens, such as dehydroepiandrosterone (DHEA) and androstenedione [7], from androstenediol through the backdoor pathway of DHT synthesis [10,11] and/or de novo production from cholesterol [12]. In castration-recurrent CaP, AR induces the androgen-dependent expression of prostate-specific antigen and the transmembrane protease, serine 2-γ-ets erythroblastosis virus E26 oncogene
AR\textsuperscript{ΔTR} inhibits Castration-Recurrent CaP

The effect of intracrine androgen synthesis and AR\textsuperscript{ΔTR} inhibition of endogenous AR and castration-recurrent CaP growth was determined in CWR-R1 cells derived from the castration-recurrent CWR22 human CaP xenograft. CWR-R1 cells transduced with lentivirus expressing AR\textsuperscript{ΔTR} or LacZ under control of the CMV promoter exhibited high expression (Fig. 1). The efficacy of AR\textsuperscript{ΔTR} inhibition of AR transcriptional activity was determined using MMTV-Luc transfected into lentivirus-transduced CWR-R1 cells in the absence and presence of 0.1 nM DHT because the prostate-specific antigen-luciferase reporter gene is only weakly activated by endogenous AR [10,21,28]. Addition of 0.1 nM DHT LacZ-transduced CWR-R1 cells increased luciferase activity by 3-fold (Fig. 2). Under the same conditions, luciferase activity in AR\textsuperscript{ΔTR}-transduced CWR-R1 cells was low with or without 0.1 nM DHT, an androgen concentration that maximally stimulates androgen-regulated genes in CWR-R1 cells [29]. The results demonstrate a dominant negative effect of AR\textsuperscript{ΔTR} on endogenous AR transcriptional activity of MMTV-luc in the absence and presence of DHT.

Inhibition of endogenous AR transcriptional activity by AR\textsuperscript{ΔTR} in the absence of added DHT raised the possibility that endogenous AR ligands induced AR\textsuperscript{ΔTR} dimerization [30] and heterodimerization between AR\textsuperscript{ΔTR} and full-length endogenous AR [31]. Liquid chromatography tandem mass spectrometry analysis demonstrated 3.38±0.26 fmol T/million cells (n = 4) and 1.84±0.16 fmol DHT/million cells (n = 4) (Fig. 3). Since AR dimerization and DNA binding are androgen-dependent [30], the results suggested that AR\textsuperscript{ΔTR} inhibitory activity can serve as a surrogate indicator for intracellular active androgens.

The effect of AR\textsuperscript{ΔTR} on androgen-dependent Nkx3.1 gene expression [32] was assessed further to characterize the inhibitory activity of AR\textsuperscript{ΔTR} on AR signaling. Addition of DHT decreased Nkx3.1 protein levels in AR\textsuperscript{ΔTR}-transduced CWR-R1 cells

\begin{figure}[h]
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\caption{Overexpression of AR\textsuperscript{ΔTR} or LacZ transgene in lentiviral vector transduced CWR-R1 cells. Western blot analysis of CWR-R1 protein lysates (20 μg) demonstrated that endogenous AR (M, 110 kDa, lanes 1–2) is detected in both AR\textsuperscript{ΔTR} and LacZ transduced CWR-R1 cells using AR goat polyclonal antibody. LacZ (M, 60.5 kDa, lane 1) expression is only observed in LacZ-transduced CWR-R1 cells using LacZ rabbit polyclonal antibody and AR\textsuperscript{ΔTR} (M, 84 kDa, lane 2) expression is detected using AR goat polyclonal antibody in AR\textsuperscript{ΔTR}-transduced CWR-R1 cells. Endogenous β-actin was detected using β-actin AC-15 mouse monoclonal antibody (M, 43 kDa, lanes 1 and 2) and served as the loading control for both AR\textsuperscript{ΔTR} and LacZ-transduced CWR-R1 cells.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.png}
\caption{Endogenous AR transcriptional activity in LacZ and AR\textsuperscript{ΔTR} lentiviral vector transduced CWR-R1 cells. CWR-R1 cells were transiently transfected with the MMTV-luciferase reporter and assayed for luciferase activity in the presence and absence of DHT. In the presence of 0.1 nM DHT, CWR-R1 cells expressing the LacZ transgene demonstrated an increase in MMTV-luciferase reporter activity compared to control cells without DHT. Expression of AR\textsuperscript{ΔTR} reduced MMTV-luciferase reporter activity in the absence or presence of 0.1 nM DHT compared to LacZ-transduced control cells. Columns represent mean luciferase activity in relative light units from 3 independent experiments; bars ± standard deviation.}
\end{figure}
but increased Nkx3.1 protein in LacZ-transduced CWR-R1 control cells. Addition of DHT increased CWR-R1-LacZ cell proliferation, but there was no increase in ARΔTR-transduced CWR-R1 cell proliferation. In the absence of added DHT, both LacZ- and ARΔTR-transduced CWR-R1 cells exhibited minimal proliferation.

**ARΔTR inhibits CWR-R1 tumor growth**

LacZ or ARΔTR-transduced cells were injected into nude mice to generate CWR-R1 tumors to assess the impact of ARΔTR on tumor growth and endogenous AR signaling. Tumor growth rates were altered by ARΔTR expression. A rigorous mixed modeling statistical analysis that considered individual tumor volume trajectories demonstrated significant differences among the 4 groups. The growth rate of LacZ controls differed with exogenous T (p = 0.04) (Fig. 6A and 6B), which indicated
different controls were required for the two ARΔTR experimental groups. ARΔTR reduced tumor growth rates compared to controls, an effect that was similar without (p = 0.01) or with exogenous T (p = 0.0004) (Fig. 6C and 6D). Results were similar when tumor growth was assessed using two additional parameters. LacZ and LacZ+T controls differed by slope (p = 0.01) and doubling time (p = 0.02), which confirmed the need for different controls for each ARΔTR experimental group. T supplements to optimize ARΔTR function decreased tumor growth compared to controls by slope (p = 0.004) and doubling time (p = 0.0007). Without T supplements, tumor growth slope (p = 0.07) and doubling time (p = 0.37) were similar. Inspection of the individual tumor trajectories and actual tumor volumes suggested that the effect of ARΔTR was a combination of slowing the rate of growth and delaying the onset of tumor growth, and that this effect occurred more often when exogenous T is provided to enhance the effect of ARΔTR (Fig. 6E).

Negative selection of ARΔTR in CWR-R1 tumors

The similar time to euthanasia based on tumor size between CWR-R1-ARΔTR and LacZ mice in the absence of androgen suggested that ARΔTR cells select against ARΔTR expression. To

Figure 6. Individual mouse CWR-R1 tumor volume (cm³) trajectories and predicted mean tumor volumes from each cohort. Tumors were measured using digital calipers and volumes calculated from day 14 to day 160 after CWR-R1 cell inoculation. Tumor volumes, LacZ (A, n = 22), LacZ+T (B, n = 21), ARΔTR (C, n = 21) or ARΔTR+T (D, n = 21), are shown from day 21 when growth began through day 96 beyond which only 1 mouse in each group remained. Mixed modeling statistical analysis showed a statistically significant difference among the 4 groups. ARΔTR-transduced CWR-R1 tumor growth rates were reduced compared to LacZ controls in the absence (p = 0.01, panel A vs. C) or presence (p = 0.0004, panel C vs. D) of T. (E) Predicted mean CWR-R1 tumor volumes (cm³) were plotted against time of first tumor harvest for the LacZ+T (open squares), LacZ (open diamonds), ARΔTR+T (solid circles) and ARΔTR (open circles) groups. ARΔTR-expressing CWR-R1 tumors (circles) exhibit decreased growth rates compared to LacZ-transduced CWR-R1 control cells.

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Figure 7. Kaplan-Meier plots from each cohort. Day of euthanasia was based on 1.5 cm³ tumor size for mice in the LacZ+T (dashed line, n = 21), LacZ (dotted line, n = 22), ARΔTR (solid line, n = 21) or ARΔTR+T (broken line, n = 21) groups. The median survival time differed in groups that received exogenous T (broken line vs. dashed line, p = 0.008) but not in groups without T (solid line vs. dotted line, p = 0.34).

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test this, the AraTR and LacZ vector copy number and protein expression were determined in CWR-R1 tumors. AraTR or LacZ vector copy numbers and protein expression were determined in transduced CWR-R1 cells prior to inoculation and at the time of tumor harvest. AraTR or LacZ vector copy number per cell measured in CWR-R1-transduced cells prior to inoculation based on PCR amplification of the Woodchuck hepatitis virus post-transcriptional regulator element was 5.46 and 4.53, respectively. Mean (± standard deviation) vector copy number per cell for AraTR and LacZ-transduced CWR-R1 tumors at the time of harvest was 0.49±0.58 for AraTR+T and 2.59±1.68 for AraTR (p=0.001), and 3.10±1.77 for LacZ+T and 3.49±1.45 for LacZ (p=0.4) (Fig. 8).

To characterize further the effect of AraTR on vector copy number, ratios of vector copy number per cell in CWR-R1 cells were calculated at the time of tumor harvest and at the time prior to CWR-R1 cell inoculation for AraTR and LacZ tumors in the presence and absence of T. Statistical analysis of median vector copy number per tumor cell ratio for AraTR and LacZ expressing vectors showed that the AraTR expression vector cassette was selected against (p=0.006, Fig. 9A). A similar statistical result was obtained in the median vector copy number per tumor cell ratio for AraTR and LacZ expressing vectors in the presence of T (p<0.0001, Fig. 9B). Overall, AraTR-transduced CWR-R1 cells in the presence or absence of exogenous T selected against the AraTR vector compared to LacZ vector controls.

The altered median AraTR and LacZ vector copy number per tumor cell ratios suggested decreased AraTR protein expression compared to LacZ controls. Harvested CWR-R1 tumors with sufficient amounts of tissue (79 tumors) for analysis of endogenous AR and expressed AraTR or LacZ protein were analyzed on immunoblots. Expression of endogenous AR and AraTR protein was demonstrated in pTK909-AraTR transduced CWR-R1 cells prior to subcutaneous inoculation. AraTR-transduced CWR-R1 tumors in the presence of T (Fig. 10A), expressed endogenous AR in all 22 CWR-R1-AraTR+T tumors, although AR protein was low to undetectable in 3 samples (samples 21–23). In contrast, AraTR protein was negligible or absent in all CWR-R1-AraTR tumors in the presence of T supplements.

In the absence of supplemental T, AR protein persisted in 10 of 15 CWR-R1-AraTR tumors (tumor numbers 28, 29, 31, 32, 34, 35, 38, 39, 43 and 44), and endogenous AR was not detected in 5 tumors that also did not express AraTR (samples 24–27 and 33) (Fig. 10B). CWR-R1-AraTR tumors 32 and 35 without the T supplement expressed AR but not AraTR. AraTR was co-expressed in 8 CWR-R1 tumors (tumor numbers 28, 29, 31, 34, 36, 39, 43 and 44).

In contrast, LacZ protein was expressed in all tumors except tumor 72 in the absence and presence of T (Fig. 10C). Empty vector control CWR-R1 tumors with T (samples 92 and 93) and without T (samples 94 and 95) and 293T cells did not express LacZ or AraTR protein. The consistent expression of LacZ protein corresponded with vector copy number calculated in LacZ-transduced tumors.

The results suggest negative selection of AraTR is not limited to CWR-R1-AraTR tumors propagated in mice with supplemental T. In AraTR-transduced tumors without supplemental T, AraTR selection was less possibly due to low serum T and/or altered intracrine T biosynthesis.

**Intracrine synthesis of active androgens in castration-recurrent CaP**

The absence of CYP17A1 in mouse adrenals [33] provides a model to test whether castration-recurrent CaP produces intracrine androgens. Generation of the CWR-R1-AraTR tumors provided an opportunity to investigate an effect of AR signaling on intracrine active androgen biosynthesis. The similar CWR-R1-AraTR tumor growth, tumor doubling times and slopes with and without T, and measurements of DHT in CWR-R1 cells suggested that CWR-R1 tumors produced active androgens in the absence of circulating T.

To identify possible differences in androgen profiles between CWR-R1-AraTR and LacZ tumors, T, DHT, androstenedione and androsterone were measured using liquid chromatography tandem mass spectrometry. Tissue levels of 0.32 nM DHT (p=0.59) and 0.05 nM androsterone (p=0.23) measured at the time of tumor harvest were similar in AraTR and LacZ-transduced tumors (Table 2) and sufficient to activate endogenous AR and AraTR [see *in vitro* results; Fig. 2]. Furthermore, 3.25 nM T in LacZ tumors was similar to T levels in castration-recurrent CaP [4,9] that was sufficient to activate endogenous AR-H874Y [29]. However, in CWR-R1-AraTR tumors, T levels were ~4-fold less than LacZ tumors, which suggested alteration in T biosynthesis by the dominant negative AR. Decreased T biosynthesis in AraTR-transduced CWR-R1 tumors correlated with the accumulation of ~1 nM androstene-dione, an androgen precursor to T. In contrast, CWR-R1-LacZ tumors contained 1.05 nM androstenedione. Quantitation of 5α-reduced and unsaturated androgen precursors in AraTR and LacZ-transduced CWR-R1 tumors suggested intracrine androgen biosynthesis contributed to AR-dependent castration-recurrent CaP growth.

**Table 1. Log-Rank Test p-values in Order of Stepdown Pair-Wise Comparisons of Overall Survival Family-Wise Error Rate (FWR) Controlled p-values.**

| Comparison     | log-rank test p-value |
|----------------|-----------------------|
| LacZ+T        | LacZ                  | 0.03 |
| LacZ+T        | AraTR+T               | 0.008 |
| LacZ          | AraTR                 | 0.34 |
| AraTR         | AraTR+T               | 0.59 |
| Overall       |                        | 0.009 |

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Figure 8. AraTR and LacZ mean vector copy number/cell in harvested CWR-R1 tumors with and without T. Vector copy number per cell was determined using quantitative PCR of the Woodchuck hepatitis virus post-transcriptional regulator element. The mean vector copy number per cell is 0.49±0.58 for AraTR+T, 2.58±1.68 for AraTR, 3.10±1.77 for LacZ+T and 3.48±1.45 for LacZ tumors. A statistically significant decrease in vector copy number per cell was observed for AraTR+T vs AraTR tumors (p=0.001) but not LacZ+T vs. LacZ (p=0.4) tumors. doi:10.1371/journal.pone.0030192.g008

Intracrine synthesis of active androgens in castration-recurrent CaP

The absence of CYP17A1 in mouse adrenals [33] provides a model to test whether castration-recurrent CaP produces intracrine androgens. Generation of the CWR-R1-AraTR tumors provided an opportunity to investigate an effect of AR signaling on intracrine active androgen biosynthesis. The similar CWR-R1-AraTR tumor growth, tumor doubling times and slopes with and without T, and measurements of DHT in CWR-R1 cells suggested that CWR-R1 tumors produced active androgens in the absence of circulating T.
Discussion

Studies in this report were based on the premise that a dominant negative form of AR with a deletion of the NH₂-terminal transactivation domain requires androgen for dimerization [30] and inhibition of transcriptional activity of full-length AR [27]. We have shown that stable expression of dominant negative AR<sub>DTR</sub> inhibits endogenous AR-H874Y transcriptional activity and slows or delays CWR-R1 tumor growth in the absence and presence of supplemental T. Dominant negative AR<sub>DTR</sub> activity was also indicated by the decrease in Nkx3.1 protein and luciferase reporter activity in the presence of DHT.

Results from the study have revealed two additional important findings. First, there was essentially 100% negative selection against the dominant negative form of AR during castration-recurrent CWR-R1 tumor growth in the presence of supplemental T. This contrasted nearly 100% retention of the LacZ control gene. Both dominant negative AR<sub>DTR</sub> and LacZ were integrated into the genome using lentivirus expression and cell selection prior to cell inoculation and tumor growth. Second, approximately 50%...
exclusion of dominant negative AR occurred in CWR-R1-ARTR tumors propagated in the absence of supplemental T. These results, together with mass spectrometry measurements of T and DHT in cultured CWR-R1 cells, provide evidence for intracrine synthesis of active androgens needed for AR signaling in CaP. Selective loss of a dominant negative AR demonstrates the genetic versatility of castration-recurrent CaP cells to maximize AR signaling.

Intracrine synthesis of androgen in castration-recurrent CaP

Inhibition of luciferase activity in CWR-R1 cells by dominant negative ARTR in the absence of exogenous T suggested intracellular synthesis of T. This was supported by mass spectrometry measurements of T and DHT in CWR-R1 cells. Our findings are in agreement with previous evidence that androgen-starved LNCaP cells synthesize androgens from 14C-acetate labeled cholesterol [34]. CaP cells alter cholesterol metabolism and processing to support androgen biosynthesis [35]. Intracrine biosynthesis of androstenedione, DHEA and T was also demonstrated in CWR-R1 and PC-3 cells, and implicated CYP17A1 activity in AR positive and negative CaP cells [36]. In clinical specimens, cholesterol and androgen biosynthetic enzymes were up-regulated in castration-recurrent CaP [12,37,38].

In the CWR-R1-LacZ tumors, intratumoral T and DHT levels were sufficient to activate AR-H874Y and promote tumor growth. Intratumoral DHT levels in ARTR and LacZ-transduced tumors were similar. However, in ARTR-transduced CWR-R1 xenograft tumors, T levels were lower and androstenedione levels were greater than CWR-R1-LacZ tumors. Lower T levels in the CWR-R1-ARTR tumor suggested that dominant negative ARTR selected against cells expressing aldo-keto reductase 1C3, an enzyme that reduces androstenedione to T in castration-recurrent CaP, or increased the oxidation of T to androstenedione by NAD+ dependent 17β-hydroxysteroid dehydrogenase-15 [48] may explain why men with castration-recurrent CaP and high baseline androstenedione levels survived longer when treated with ketoconazole [49]. DHT levels remain similar even though the T:androstenedione ratios in LacZ and ARTR tumors were inversed. The low and similar DHT levels (~10−11) measured in LacZ and ARTR-transduced CWR-R1 tumors may be the optimum intratumoral concentration for CWR-R1 cell proliferation [42] and AR-H874Y coordinated DNA replication licensing [50].

**Negative selection to enhance AR activity**

The growth inhibitory effects of ARTR were complicated by the fact that the ARTR transgene was negatively selected against during CWR-R1 tumor growth most efficiently in the presence of supplemental T. Expression of the ARTR transgene was lost in all of the 22 ARTR+T tumors by the time of harvest, while the ARTR transgene was retained in ~50% of tumors in the absence of supplemental T. The mean tumor volume was larger than ARTR+T tumors, which may be associated with a partial switch from T to androstenedione biosynthesis by these tumors. Although the timing of negative selection against the ARTR transgene was not rigorously tested, preliminary studies suggested loss of the transgene occurred approximately 10 days after tumor cell inoculation on day 23.

Negative selection of the ARTR transgene was supported by the decreased genome vector copy number in both ARTR and LacZ-transduced CWR-R1 tumors in the absence or presence of exogenous T. Loss of transgene expression has been shown to occur 10 to 15 days after lentiviral transduced embryonic carcinoma cells [51]. The delay in ARTR+T tumor growth supported selection against the transgene after 10 days in the CWR-R1 model. It could be argued that loss of the transgene resulted from vector escape from variegation and/or extinction events. On the other hand, random gene or chromosomal deletion and transgene silencing [51] were not supported, as there was nearly complete retention of the control LacZ transgene in the CWR-R1 tumors.

|                      | Androstenedione | Testosterone | Dihydrotestosterone | Androsterone |
|----------------------|-----------------|--------------|---------------------|--------------|
| LacZ                 | 1.05±0.18       | 3.25±0.37    | 0.28±0.17           | 0.07±0.02    |
| ARTR                 | 13.6±4.9        | 0.79±0.33    | 0.32±0.11           | 0.05±0.02    |

Table 2. Mean androgen levels (nM, ± SEM) measured in LacZ (n = 8) and dominant-negative ARTR (n = 11) CWR-R1 androgen-independent tumors without exogenous T pellets.
Recent studies have demonstrated that CWR-R1 cells, as well as the parental CWR22 CaP xenografts, contain replication competent retroviruses identical to the xenotropic murine leukemia related virus (XMRV) found in human CaP cells [52,53]. XMRV evolved by recombination between two endogenous retroviruses in nude mice carrying the CWR22 xenografts [52,53]. XMRV proviruses were not detected in samples obtained from early CWR22 tumor passages, which is consistent with newer reports that XMRV is not involved in the development of CaP in humans. The data presented herein does not exclude the unlikely possibility that XMRV infection contributes to castration-independence of CWR-R1 cells (derived from CWR22) [53]; Paprotka et al. found this scenario most unlikely. The experiments were well controlled and XMRV infection was present in experimental and control animals. Overall, the results presented here demonstrate inhibition of the CWR-R1 tumor growth by dominant negative inhibition of AR signaling, and indicate the central role of tumor-derived androgens in the development of castration-resistant CaP. The decrease and delay in castration-recurrent tumor growth, coupled with a 36% extension of survival in the presence of T, supported the importance of inhibition of AR expression/activity as a clinical target [54,55].

Materials and Methods

Generation of ARΔTR and LacZ transduced CWR-R1 cells

The coding region of pCMV-hARα142–337 with wild-type LBD and FXXLF motif binding motif [20] was subcloned into the BamHI site of SK+ plasmid (Stratagene, La Jolla, CA). The plasmid was digested with Xhol/SpeI and the fragment was subcloned into the Xhol/XbaI site of HIV-1 based vector pTK642 upstream of the IRES-GFP-BSD cassette. The resulting vector pTK989 expressed ARΔTR under control of the human cytomegalovirus promoter (Fig. 11). LacZ expression vector pTK1027 was generated by ligation of the SmaI/XhoI fragment that contains LacZ cDNA pTK1022 into pTK642 digested with Hpal/Xhol. All plasmid sequences were confirmed using direct sequencing.

HIV-1 based vectors were generated using a transient 3-plasmid transfection method. The standard calcium phosphate protocol was used as described [56]. Briefly, vector constructs (15 μg), vesicular stomatitis virus glycoprotein expression plasmid (5 μg) and ΔNRF packaging cassettes (10 μg) were transfected into 293T cells (ATCC #11268). Lentiviral particles were collected 60 h after transfection from filtered conditioned medium. Lentivirus stocks were aliquoted and stored at −80°C. Protein lysates (20 μg) prepared from CWR-R1 cells or pulverized CWR-R1 tumor specimens were separated using 4–8% acrylamide gradient gels containing SDS and electroblotted to nitrocellulose membranes (Nitrobind®, Osmonics, Inc., Minnetonka, MN). Antihuman AR goat polyclonal antibody (Abcam, Cambridge, MA, catalog #ab19066, 1:1000) targeted AR N-terminal amino acids 2–16. Rabbit polyclonal LacZ antibody (Millipore, Billerica, MA, catalog #AB1211) and rabbit polyclonal GADPH (Santa Cruz Biotechnology, Santa Cruz, CA, catalog # sc-25776) were used at 1:1000 dilution. Nkx3.1 goat polyclonal antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, catalog #sc-15021) and β-actin AC-13 mouse monoclonal antibody (Abcam, Inc, catalog #ab6276) were used at 1:100 and 1:5000 dilutions, respectively. Secondary rabbit-antigoat, goat-antirabbit or anti-mouse IgG antibodies conjugated to horseradish peroxidase were used at 1:10,000 dilution (Pierce, Rockford, IL). Specific signals were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

CWR-R1 cell and tumor immunoblot analysis

AR, ARΔTR, LacZ and Nkx3.1 homeobox protein immunoblot analyses were performed as described [15] using CWR-R1 cells treated with 1 nM DHT or CWR-R1 tumor specimens stored at −80°C. Protein lysates (20 μg) prepared from CWR-R1 cells or pulverized CWR-R1 tumor specimens were separated using 4–8% acrylamide gradient gels containing SDS and electroblotted to nitrocellulose membranes (Nitrobind®, Osmonics, Inc., Minnetonka, MN). Antihuman AR goat polyclonal antibody (Abcam, Cambridge, MA, catalog #ab19066, 1:1000) targeted AR N-terminal amino acids 2–16. Rabbit polyclonal LacZ antibody (Millipore, Billerica, MA, catalog #AB1211) and rabbit polyclonal GADPH (Santa Cruz Biotechnology, Santa Cruz, CA, catalogue # sc-25776) were used at 1:1000 dilution. Nkx3.1 goat polyclonal antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, catalog #sc-15021) and β-actin AC-13 mouse monoclonal antibody (Abcam, Inc, catalog #ab6276) were used at 1:1000 and 1:5000 dilutions, respectively. Secondary rabbit-antigoat, goat-antirabbit or anti-mouse IgG antibodies conjugated to horseradish peroxidase were used at 1:10,000 dilution (Pierce, Rockford, IL). Specific signals were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

CWR-R1 tumor studies

Mice were maintained and experimental procedures performed in accordance with the National Institute of Health, the University of North Carolina School of Medicine Institutional Animal Care and Use Committee and the Institutional Biosafety Committee. Male athymic nude mice 4 to 5 weeks old were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN) and housed individually in the Division of Laboratory Medicine facility at...
the University of North Carolina School of Medicine. Each mouse was identified using a numbered ear tag. Two days after castration, mice were divided into two groups of 45 mice each. One T pellet (12.5 mg, 90-day release) was implanted subcutaneously in each of 22 mice from each group to normalize circulating levels to ~4 ng/mL T (Innovative Research, Sarasota, FL). Mice were inoculated subcutaneously on one flank with 1.25 x 10^6 CWR-R1 bcl-2-selective cells suspended in Matrigel (1:1 mixture, BD Biosciences, Bedford, MA). Matrigel was used to promote xenograft formation in the castrated microenvironment of the inoculation site [57]. Mice received cells transduced with lentivirus-ARTR or lentivirus-LacZ. The tumor growth suppressing or promoting activity of host innate immune system was accounted for by comparing ARTR to control lentivirus-LacZ groups. Tumor volume was measured at least 3 times per week using digital calipers and volume calculated using the equation L_x(L_y) x 0.523. Mice were euthanized and tumors excised when tumor weight reached 1.5 cm^3. Tumors were cut into 100 mg pieces with 1/3 frozen in liquid nitrogen and stored at −80°C, 1/3 immersed in RNAlater (Ambion, Austin, TX) for 24 h and stored at −80°C, and 1/3 fixed in 10% formalin-buffered saline for 24 h, washed in phosphate buffered saline and embedded in paraffin.

Quantitative PCR

Quantitative PCR of the Woodchuck hepatitis virus post-transcriptional regulatory element was performed to assess ARTR and LacZ transgene integration and retention using the 7500 Real Time PCR System and SDv1.1x Software (Applied Biosystems, Foster City, CA). DNA was isolated from RNAlater treated frozen tumor samples and prepared for real-time PCR as described [58]. In brief, tumor DNA were digested with restriction enzyme DpnI to degrade plasmid/vector DNA that remained after transfection. Genomic DNA (10 ng) was normalized using human β-globin gene (2 copies/diploid cell), DNA (1 ng) was calculated equal to 151.5 copies for 1 copy/diploid cell, or 303 copies for 2 copies/diploid cell. An equal β-globin equivalent was used to amplify viral DNA. DNA isolated from FP9 cells that contained a single copy of provirus per diploid cell was used as a standard [59]. The FP9 cell line was generated by the Flip-In System (Invitrogen) employing Flip-mediated recombination to introduce a single lentivector genomic into HEK293 genome containing a single FRT site. Primers for β-globin gene were forward primer 5'-CGAAGGATGCTATTGCTTAC-3' and reverse primer 5'-GCCCTACCAAGAAGCT-3'. Lentivirus specific primers to amplify the WRPE sequence were forward primer 5'-ACGTCCTTCTGCTAAGTCC-3' and reverse primer 5'-AAAGGGAGATCCGACCGACTGC-3'. The quantitative PCR reaction contained 1× Master mix (Promega, Madison, WI), 0.08× SYBR® Green I (Cambrex Bio Science, Rockland, ME), 300 nM forward primer, 300 nM reverse primer and template in a total volume of 15 μL. Quantitative-PCR conditions were 95°C for 5 min; 40 cycles at 95°C for 15 sec; annealing at 63°C for 30 sec; and extension at 72°C for 30 sec. A minus template incubation was used as control. An aliquot (10 μL) of each PCR reaction was subjected to gel electrophoresis. PCR products were visualized using ethidium bromide staining. Each experiment was performed 3 times.

Androgen measurements in CWR-R1 tumors

Tumor samples stored at −80°C were pulverized using liquid nitrogen. Tissue was transferred into high pressure liquid chromatography grade water (50 mg/mL) containing deuterated internal standard, 5α-androstan-17β-ol-3-one, 16,16,17-d3 (1.0 ng, CDN isolopes, Pointe-Claire, Quebec, Canada). Samples were homogenized and extracted 3 times with 1 mL chloroform:acetone (9:1). Organic extracts were combined and dried. Androgens were purified and concentrated using solid phase C18 extraction cartridges (Varian, Palo Alto, CA). Liquid chromatography tandem mass spectrometry was performed as described [9] using the AB SCIEX Triple Quad™ 3000 system (Applied Biosystems, Foster City, CA) with modification. Steroids were eluted using a linear gradient of 65% to 80% mobile phase A (0.4 mM ammonium formate) for 2.25 min and mobile phase B (0.4 mM ammonium formate in methanol) followed by isocratic elution at 95% mobile phase A for 13 min at flow rate 175 μL/min at 60°C. A Phenomenex Luna C18 column (3.0 μm, 150x2 mm) was used to separate T, DHT, androstenedione and androsterone. The parent–product positive ion pairs monitored (mass to charge ratio) were 289.2 to 97.0 for T, 291.2 to 255.2 for DHT, 294.2 to 258.2 for 5α-androstan-17β-ol-3-one, 16,16,17-d3 internal standard, 287.2 to 97 for androstenedione and 291.2 to 255.2 for androsterone.

Statistical Methods

Tumor volumes were measured serially and analyzed using random coefficient modeling beginning on day 14 after cell inoculation until the tumor volume exceeded 1.5 cm^3 when the mice were euthanized (the longest was day 204). The primary interest of this analysis was to investigate the pair-wise difference in growth rates of CWR-R1-ARTR, ARTR+T, LacZ and LacZ+T tumors. This approach was chosen a priori that used a step-down approach to control the family-wise error rate for statistical comparisons of interest. The nonparametric Wilcoxon rank-sum test (with Van der Waerden normal scores) was used for each of the pair-wise 2-group comparisons, when summary measures of slopes and doubling times were compared. The Kruskal-Wallis test (with Van der Waerden normal scores) was used when more than 2 groups were compared. These comparisons yielded essentially equivalent results to parametric random coefficient modeling. The Kaplan-Meier method was used to estimate the time to euthanasia function for each of the 4 groups. The log-rank test was used to test for differences among survival curves. Statistical analyses were performed using SAS statistical software, version 9.2 from the SAS Institute, Inc., Cary, NC.

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Author Contributions

Conceived and designed the experiments: TK EW JM. Performed the experiments: MT BZ BK XL KH DM. Analyzed the data: MT BZ BK XL KH DM JM EW TK. Contributed reagents/materials/analysis tools: TK BJ JM EW MT DM. Wrote the paper: MT BZ EW JM TK.

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