

Generation 2.5 Antisense Oligonucleotides Targeting the Androgen Receptor and Its Splice Variants Suppress Enzalutamide-Resistant Prostate Cancer Cell Growth

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Abstract

Purpose: Enzalutamide (ENZ) is a potent androgen receptor (AR) antagonist with activity in castration-resistant prostate cancer (CRPC); however, progression to ENZ-resistant (ENZ-R) CRPC frequently occurs with rising serum PSA levels, implicating AR full-length (AR_{FL}) or variants (AR-Vs) in disease progression.

Experimental Design: To define functional roles of AR_{FL} and AR-Vs in ENZ-R CRPC, we designed 3 antisense oligonucleotides (ASO) targeting exon-1, intron-1, and exon-8 in AR pre-mRNA to knockdown AR_{FL} alone or with AR-Vs, and examined their effects in three CRPC cell lines and patient-derived xenografts.

Results: ENZ-R-LNCaP cells express high levels of both AR_{FL} and AR-V7 compared with CRPC-LNCaP; in particular, AR_{FL} levels were approximately 12-fold higher than AR-V7. Both AR_{FL} and AR-V7 are highly expressed in the nuclear fractions of ENZ-R-

LNCaP cells even in the absence of exogenous androgens. In ENZ-R-LNCaP cells, knockdown of AR_{FL} alone, or AR_{FL} plus AR-Vs, similarly induced apoptosis, suppressed cell growth and AR-regulated gene expression, and delayed tumor growth *in vivo*. In 22Rv1 cells that are inherently ENZ-resistant, knockdown of both AR_{FL} and AR-Vs more potently suppressed cell growth, AR transcriptional activity, and AR-regulated gene expression than knockdown of AR_{FL} alone. Exon-1 AR-ASO also inhibited tumor growth of LTL-313BR patient-derived CRPC xenografts.

Conclusions: These data identify the AR as an important driver of ENZ resistance, and while the contributions of AR_{FL} and AR-Vs can vary across cell systems, AR_{FL} is the key driver in the ENZ-R LNCaP model. AR targeting strategies against both AR_{FL} and AR-Vs is a rational approach for AR-dependent CRPC. *Clin Cancer Res*; 21(7); 1675–87. ©2015 AACR.

Introduction

First-line treatment for metastatic prostate cancer is androgen deprivation therapy (ADT), which reduces serum testosterone levels and androgen receptor (AR) activity. Despite high initial response rates, remissions are temporary with emergence of castration-resistant prostate cancer (CRPC), largely driven by AR reactivation despite low levels of serum testosterone. Serum prostate-specific antigen (PSA) is an AR-regulated protein that

serves as a useful marker of response and prognosis to ADT (1); indeed, rising PSA is the earliest sign of CRPC (2, 3). AR reactivation in CRPC involves AR gene amplification, mutations or splice variants (AR-Vs), as well as intratumoral steroidogenesis, increased coactivator expression, and activation of signal transduction pathways that sensitize AR to low levels of androgens (4–7). These mechanisms work in concert to drive CRPC progression, and highlight that targeting the AR remains a critical component of novel CRPC therapies (8, 9).

More potent AR pathway inhibitors like abiraterone (10, 11) and enzalutamide (ENZ; refs. 12, 13) suppress ligand levels and binding to AR, respectively, inhibiting AR nuclear translocation and transcriptional activity. While these AR pathway inhibitors significantly prolong survival in CRPC, cancers often recur with rising serum PSA levels indicative of persistent AR activity. Therefore, loss of suppression of AR activity despite potent AR pathway inhibition remains a major problem in CRPC and additional novel agents with activity in abiraterone- or ENZ-resistant tumors is critical to improve control of CRPC.

Antisense oligonucleotides (ASO) offer one approach to selectively target genes and their splice variants. While ASOs are primarily used to inhibit "undruggable" targets (14, 15), they may also be of use against drug-resistant targets like nuclear AR in ENZ-resistance (ENZ-R). While AR extinction approaches using

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Translational Relevance

Suppression of androgen receptor (AR) signaling with potent inhibitors like enzalutamide (ENZ) remains a therapeutic goal for castration-resistant prostate cancer (CRPC); however, ENZ-resistant (ENZ-R) CRPC frequently occurs. We characterized functional consequences of changes in AR full-length (AR_{FL}) and variants (AR-Vs) levels associated with ENZ treatment response and resistance using AR-ASOs selectively targeting AR_{FL} alone or AR_{FL} + AR-Vs in several ENZ-R models. This study indicates the AR is an important driver of ENZ resistance and that while both AR_{FL} and AR-V7 levels are induced, AR_{FL} is the key mediator of ENZ resistance in the LNCaP model. However, the role of AR_{FL} and AR-Vs in progression to ENZ-R CRPC appears cell-line and context-dependent, and therefore ASO strategies targeting AR_{FL} and AR-Vs is a rational third-line approach for AR pathway inhibitor-resistant CRPC.

ASOs (16) or shRNA (17) can reduce AR levels and inhibit tumor growth in CRPC models, they have not been studied in the context of ENZ-R disease and their knockdown effects on full-length AR (AR_{FL}) and AR-Vs are undefined. This is particularly important as AR-Vs are emerging as a mechanism driving ligand-independent and ENZ-R AR transcription (18–20), which could be targeted with appropriately designed ASO.

In this study, we characterized changes in AR_{FL} and AR-Vs levels associated with ENZ treatment response and resistance, and compared effects of AR-ASOs targeting either AR_{FL} or AR_{FL} and AR-Vs. ENZ exposure induces expression of both AR_{FL} and AR-V7 in CRPC, and high levels of AR_{FL} and AR-V7 are associated with ENZ-R CRPC. However, AR_{FL} mRNA levels are 12 times higher than AR-V7. Despite differential effects on AR-Vs knockdown, all AR-ASO potently silenced AR_{FL} levels and similarly induced apoptosis and cell growth inhibition in ENZ-R LNCaP cells. In contrast, knockdown of both AR_{FL} plus AR-Vs more potently suppressed 22Rv1 cell growth, AR transcriptional activity, and AR-regulated gene expression, compared with knockdown of AR_{FL} alone. Overall, these results highlight AR_{FL} as an important driver of ENZ-R CRPC and that while both AR_{FL} and AR-V7 levels are induced during the evolution to ENZ-R, their biologic effects appear cell line and context dependent.

Materials and Methods

Cell lines

LNCaP cells were provided by Dr. Leland W.K. Chung (1992, MD Anderson Cancer Center, Houston, TX) and authenticated with short tandem repeat (STR) profile analysis at Genetics Resources Core Facility (GRCF) at John Hopkins (Baltimore, MD) in January 2013. PC-3 and 22Rv1 cells were obtained from ATCC and authenticated at GRCF in January 2013 and at IDEXX BioResearch in September 2014, respectively. M12 cells stably expressing AR-V567es cDNA (M12 AR-V567es) were provided by Dr. Stephen R. Plymate (University of Washington School of Medicine, Seattle, WA; refs. 5, 21) and no further authentication was done by our laboratory. LNCaP and 22Rv1 cells and PC-3 cells were maintained in RPMI 1640 and DMEM (Invitrogen Life

Technologies, Inc.), respectively, with 5% FBS. M12 AR-V567es stable cells were cultured as described previously (5, 21).

Generation of castration- and ENZ-R LNCaP xenografts and cell lines

CRPC and ENZ-R LNCaP xenografts and cell lines were generated as previously described (22–24). The ENZ-R cell lines MR1F, MR57A, MR49F, and MR49C were maintained in RPMI1640 with 10 μ mol/L ENZ (Haoyuan Chemexpress Co.) + 5% FBS; CRPC LNCaP VehD and VehA cells were derived from CRPC LNCaP vehicle-treated tumors maintained in RPMI1640 with 5% charcoal-stripped serum (CSS). The authentication of MR49F, MR49C, and VehD was performed at GRCF in January of 2013; the other lines were not authenticated.

Molecular profiling

Transcriptome sequencing was performed using Illumina GA-II (Michael Smith Genome Sciences Centre, Vancouver, British Columbia, Canada) in four ENZ-R LNCaP and two CRPC LNCaP xenografts using established protocols (25). We used TopHat (26) to map RNA-Seq reads to the genome (hg19). Determination of AR expression (including splice variants) and an estimate of the ratio of AR-V7 to AR_{FL} are shown in Supplementary Materials and Methods.

Genome copy number profiling was performed on Agilent SurePrint G3 Human CGH Microarray Kit, 4 $\times$ 180 K in 4 ENZ-R LNCaP and 2 CRPC LNCaP xenografts tumors as described previously (27). Array CGH copy number data are available at GEO accession number GSE55345. To test for the F876L mutation, we performed PCR and Sanger sequencing across exon 8 of the AR gene in genomic DNA from the MR49F cell line, using standard techniques.

Antisense, siRNA, and plasmid transfection

Antisense AR (AR-ASO) used in this study contain constrained-ethyl (cEt) chemistry (Gen 2.5) and were identified by screening over 1400 ASOs against the full-length AR genomic sequence. The AR-ASO targeting exon-1, intron-1, exon-8, and scrambled (SCRB) control sequences were 5'-GCGACTACTACAACTT-3', 5'-CAACCATTAAATCAAC-3', 5'-GCCACGGGAAGTTTAG-3' and 5'-CAGCGCTGACAACAGTTTCAT-3', respectively. SCRB oligonucleotides with the same chemistry were supplied by ISIS Pharmaceuticals. Prostate cells were treated with oligonucleotides using protocols described previously (28, 29). For siRNA transfection, cells were treated with AR Exon 2b (30), AR Exon CE3 (31), or Scramble siRNA using Oligofectamin as described previously (28). AR-V7 plasmid (a generous gift from Dr. Stephen R. Plymate, University of Washington School of Medicine, Seattle, WA) was transfected using Lipofectin following the manufacturer's protocol.

Western blot analysis

Total proteins were extracted using radioimmunoprecipitation assay buffer and submitted to Western blot analysis as previously described (32). Primary antibodies are shown in Supplementary Materials and Methods. NuCLEAR Extraction Kit (Sigma-Aldrich) was used according to manufacturer's protocol.

Quantitative reverse transcription-PCR

Total RNA was extracted using TRIzol (Invitrogen Life Technologies, Inc.) as previously reported (29). Primers (Supplementary

Figure 1.

AR_{FL} and AR-V7 are highly expressed in ENZ-R cells and xenografts. A, castration resistant (CR) and ENZ-R LNCaP cells were cultured in medium with 5% CSS and AR_{FL}, AR-V7, and PSA protein and AR_{FL} and AR-V7 mRNA expression levels were measured by Western blotting and quantitative real-time PCR. AR-V7 protein was detected with anti-AR-V7 monoclonal antibody (AG10008), which only detects AR-V7. B, mRNA was extracted from 6 CR LNCaP and 6 ENZ-R LNCaP xenograft tumors and AR_{FL} and AR-V7 mRNA were analyzed by quantitative real-time PCR. C, transcriptome sequencing (4 ENZ-R LNCaP and 2 CRPC LNCaP xenografts) detected many AR-Vs in ENZ-R-LNCaP cells, but only AR-V7 was overtly expressed. AR_{FL} and AR-V7 mRNA expression (C, left) and mean ratio (C, right) were examined in ENZ-R LNCaP xenograft tumors. Please note that the F876L mutation was not detectable in the ENZ-R LNCaP xenograft tumors. D, LNCaP and MR49F cells were cultured for 3 days in media supplemented with CSS and ENZ followed by 1 nmol/L R1881. Cells were harvested and fractionated into nuclear and cytoplasmic extracts, protein extracts were analyzed by Western blotting for AR_{FL} and AR-V7. Lamin B1 and α -tubulin are shown as markers for nuclear and cytoplasm, respectively. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

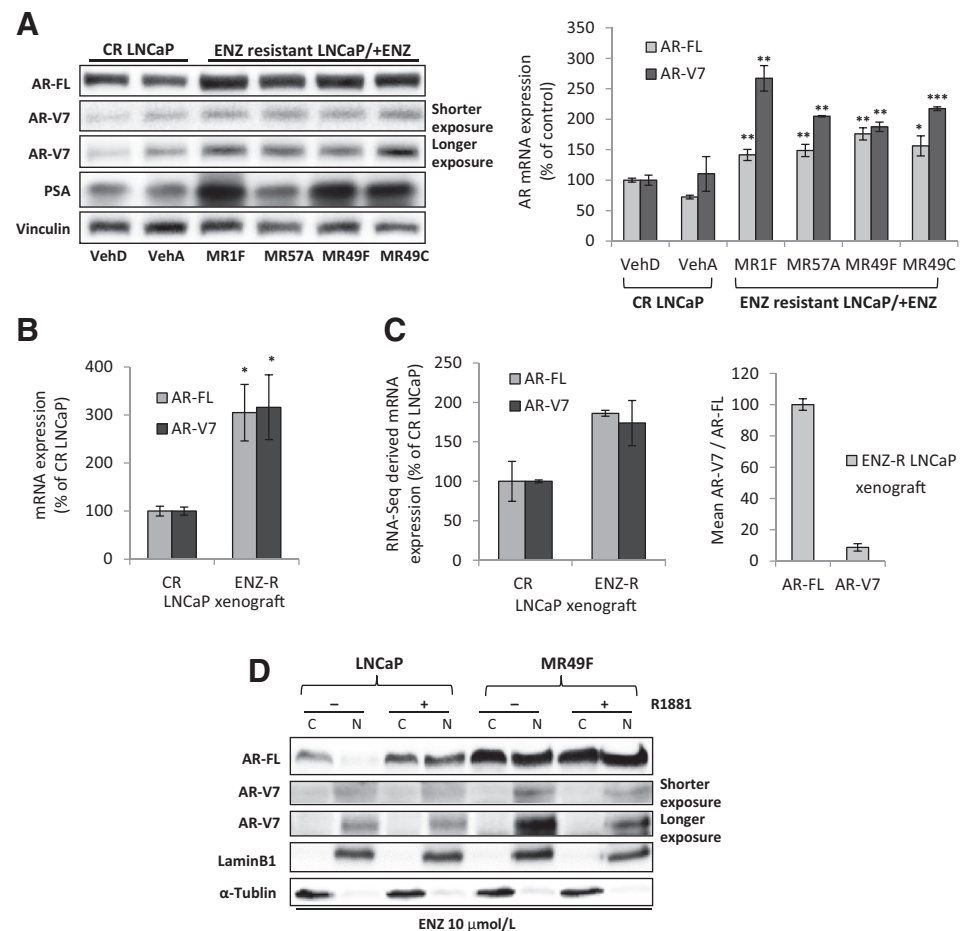


Table S1) were normalized to β -actin levels as an internal standard, and the comparative cycle threshold (C_t) method was used to calculate relative quantification of target mRNAs. Each assay was conducted in triplicate.

AR transcriptional activity

Cells were seeded at a density of 2.5×10^5 in 6-well plates and treated with AR-ASO or SCRB in CSS media. The next day, cells were transfected with ARR-3 luciferase reporter along with *Renilla* plasmid as described previously (29). All experiments were carried out in triplicate.

Cell proliferation and cell-cycle assays

Cells were cultured in CSS media, transfected with AR-ASO or SCRB, and cell growth measured by crystal violet assay as previously described (33). Cell-cycle distribution was analyzed by flow cytometry (Beckman Coulter Epics Elite, Beckman, Inc.) as described previously (29). Each assay was done in triplicate three times.

In vivo studies

Athymic mice were castrated and inoculated subcutaneously with 2×10^6 ENZ-R MR49F cells; mice were treated with ENZ 10 mg/kg/each *per os* daily for maintenance of ENZ resistance. Once tumors reached 200 mm³, mice were randomly assigned to 12.5 mg/kg exon-1 or exon-8 AR-ASO or SCRB intraperitoneally once daily for 5 days and then 3 times per week thereafter. Each

experimental group consisted of 11 mice. Tumor volume and serum PSA were measured as previously described (29). When tumor volume reached 10% or more of body weight, mice were sacrificed and tumors were harvested for evaluation of protein expression by Western blot analyses, mRNA expression by real-time monitoring of PCR, and immunohistochemistry. All animal procedures were carried out according to the guidelines of the Canadian Council on Animal Care and appropriate institutional certification.

Patient-derived LTL-313BR CRPC xenograft tumors (www.livingtumorlab.com) were grafted under the renal capsules as previously described (34). Once tumors reached palpable, mice were randomly assigned to 40 mg/kg exon-1 AR-ASO or SCRB. Each experimental group consisted of 9 mice. PSA was measured on days 17 and 24. Tumors were harvested on day 24 and tumor volume and tumor weights were measured as previously described (34).

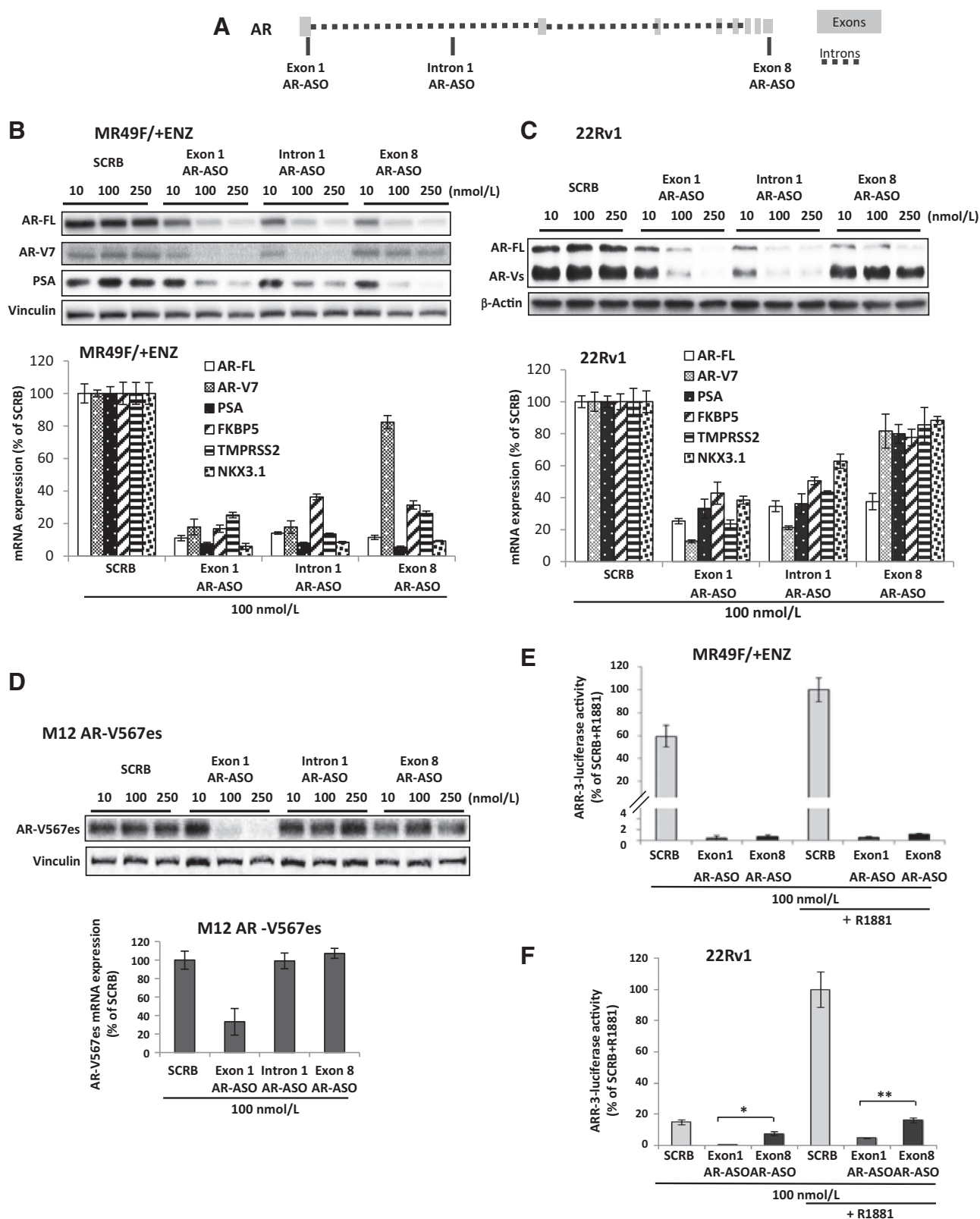
Immunohistochemistry

Immunohistochemistry was performed as previously described previously (29).

Statistical analysis

All *in vitro* data were assessed using the Student *t* test and one-way ANOVA test. Tumor volume and serum PSA levels of mice were compared using the Kruskal-Wallis and Wilcoxon rank

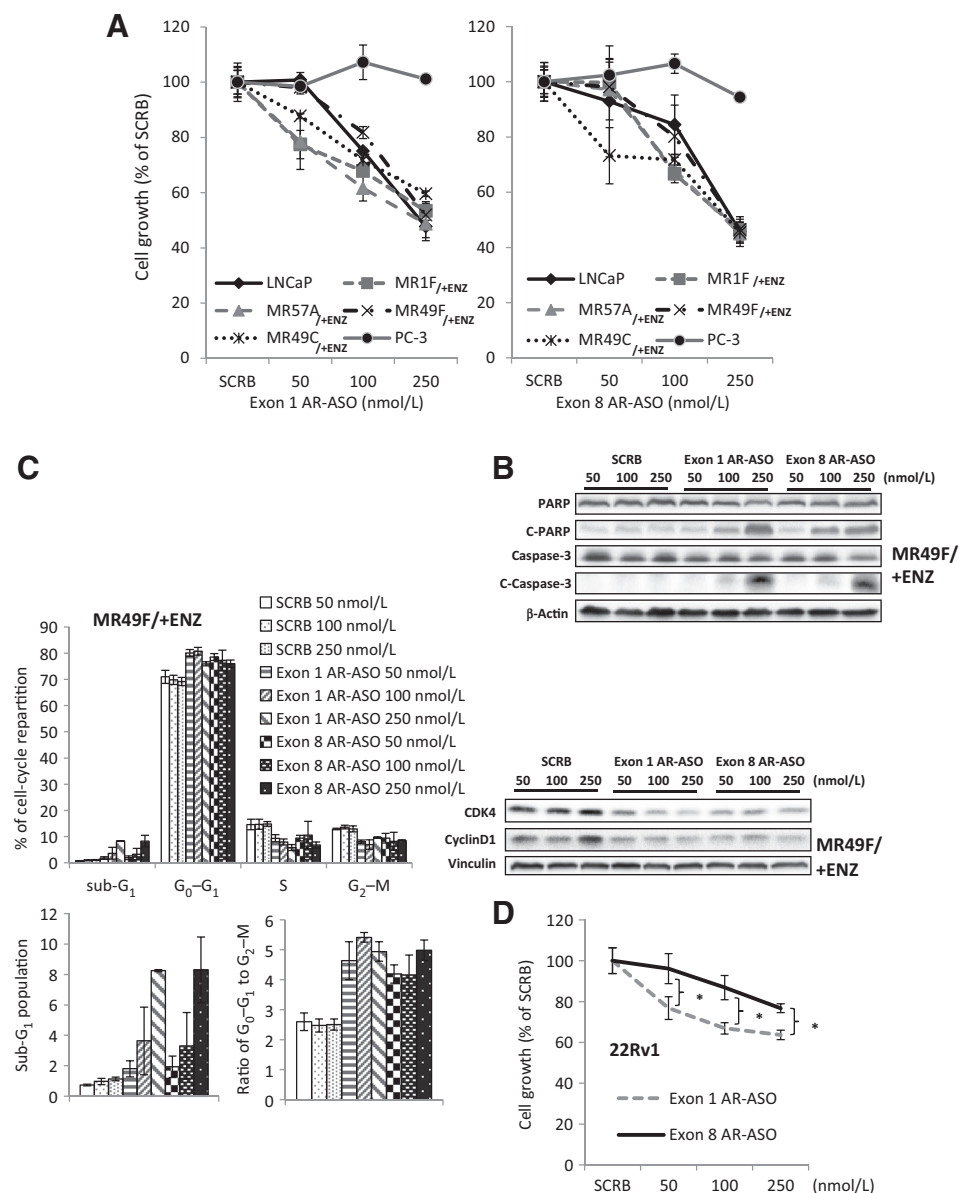
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**Figure 2.**

Effects of AR-ASO on AR_{FL}, AR-Vs, AR-regulated gene expression levels and AR transcriptional activity in ENZ-R LNCaP and 22Rv1 cells. A, three AR-ASO sequences were designed to target exon-1, intron-1, and exon-8 in AR. B, MR49F cells were treated for 48 hours in media with ENZ supplemented with CSS with the indicated AR-ASO. (Continued on the following page.)

Figure 3.

Exon-1 and -8 AR-ASO similarly inhibit cell growth and induce apoptosis in ENZ-R LNCaP cells. A, AR-positive LNCaP, four ENZ-R derived LNCaP cell lines, and AR-negative PC3 cell lines were cultured in CSS media for 96 hours with the indicated AR-ASO. Cell growth was determined by crystal violet assay and compared with SCRB. B, MR49F cells were treated in CSS media with exon-1 and -8 AR-ASO for 96 hours and PARP and caspase-3 expression levels were measured by Western blotting. C, MR49F cells were treated in CSS media with exon-1 and -8 AR-ASO for 96 hours and the proportion of cells in sub-G₁, G₀-G₁, S, and G₂-M was determined by propidium iodide staining (left). C, right, MR49F cells were treated with exon-1 and -8 AR-ASO for 96 hours and CDK4 and Cyclin D1 expression levels were measured by Western blotting. D, 22Rv1 cells were cultured in CSS media for 96 hours with the indicated AR-ASO and cell growth was determined by crystal violet assay and compared with SCRB. *, $P < 0.05$.



sum test (JMP version 8). Levels of statistical significance were set at $P < 0.05$.

Results

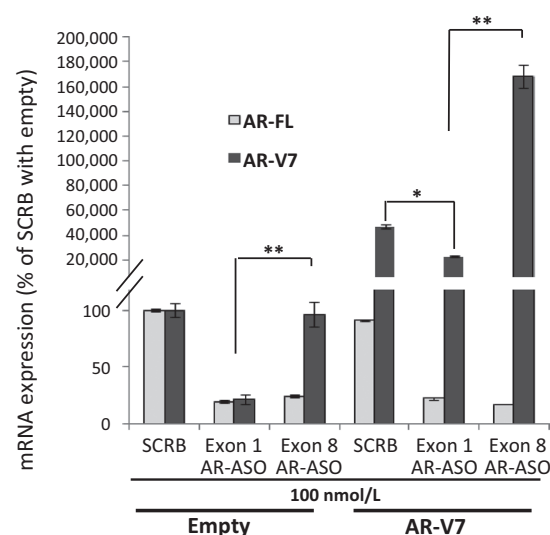
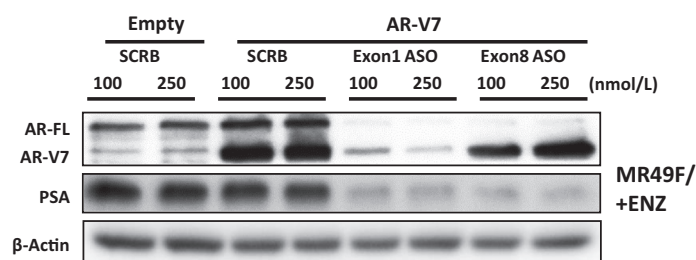
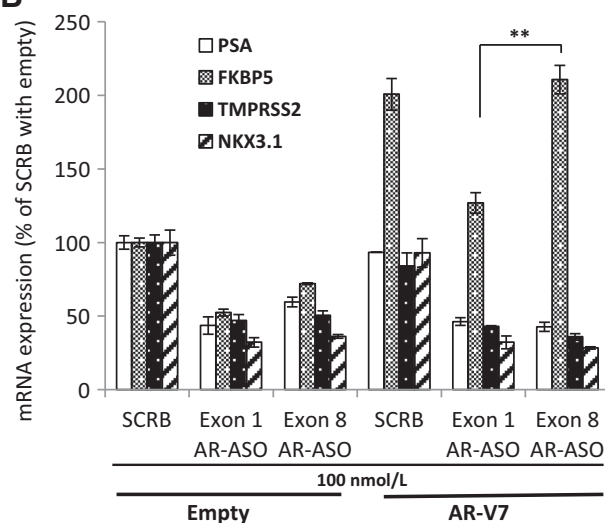
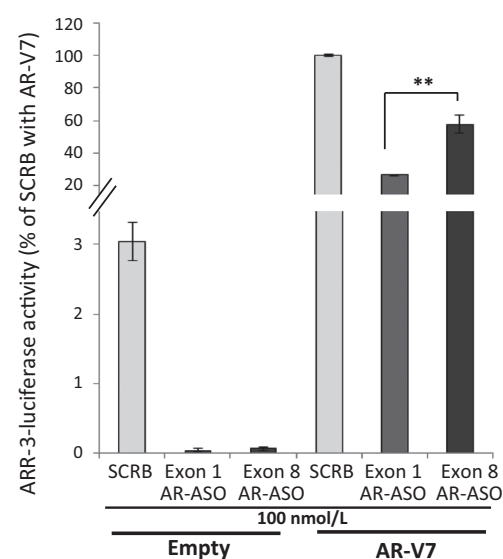
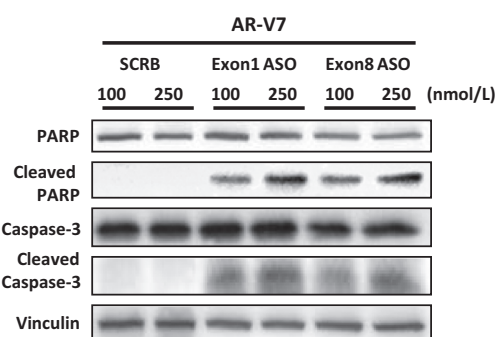
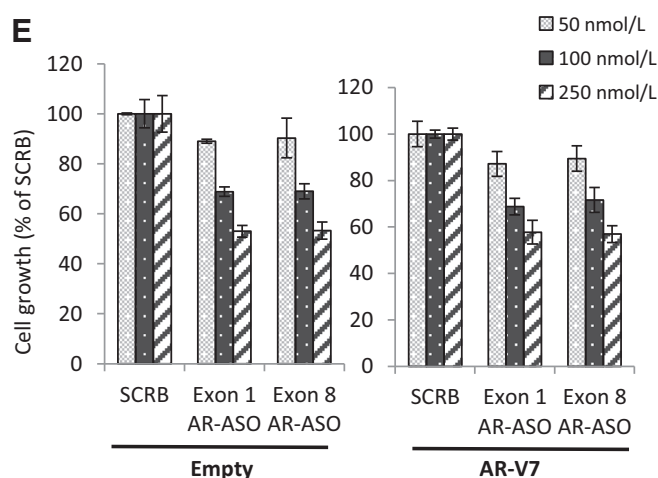
AR_{FL} and AR-V7 are highly expressed in ENZ-R cell lines and xenograft tissues

To characterize the role of AR signaling in ENZ resistance, the effects of ENZ treatment on AR_{FL}, AR-V expression, and PSA levels

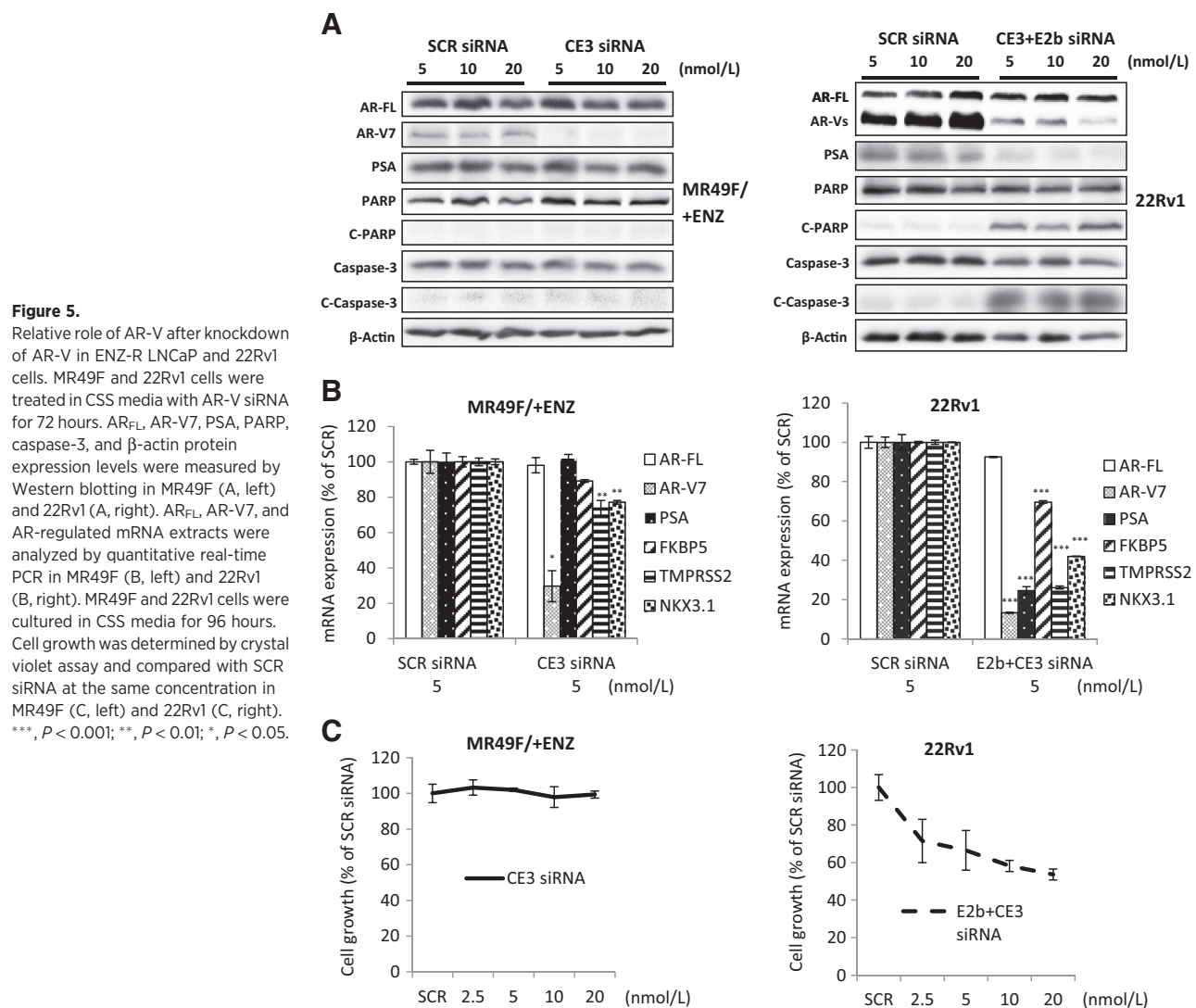
were assessed in several ENZ-R cell lines. AR_{FL} and AR-V7 protein (Fig. 1A, left) and mRNA (Fig. 1A, right) levels were increased in ENZ-R LNCaP-derived cell lines compared with CRPC LNCaP-derived (VehD and VehA) cells; moreover, short-term ENZ treatment induced AR_{FL} and AR-V7 mRNA levels in parental LNCaP cells (Supplementary Fig. S1). AR-V7 protein levels, however, were much lower than AR_{FL} protein levels and most apparent on Western blot analysis with prolonged exposure (Fig. 1A, left).

(Continued.) AR_{FL}, AR-V7, PSA and vinculin protein were analyzed by Western blotting. AR_{FL}, AR-V7, and AR regulated target mRNA expression were analyzed by quantitative real-time PCR. AR-V7 protein was detected with anti-AR-V7 monoclonal antibody. C, 22Rv1 cells were treated for 48 hours in CSS media with 3 types of AR-ASO and AR_{FL}, AR-Vs, and β-actin protein were analyzed by Western blotting and AR_{FL}, AR-V7, and AR regulated target mRNA expression were analyzed by quantitative real-time PCR. AR proteins were detected with AR N-20 antibody, which detects both AR_{FL} and AR-Vs. D, M12 cells stably expressing AR-V567es were treated for 48 hours with the AR-ASO and protein and mRNA extracts were analyzed by Western blotting and real-time PCR for AR-V567es. AR-V567es protein (80 kDa) was detected with AR N-20 antibody. E, MR49F cells were treated with 100 nmol/L AR-ASO in CSS media with ENZ. F, 22Rv1 cells were treated with 100 nmol/L AR-ASO in CSS media. The next day, cells were transiently transfected with 1 μg of AR-3-luciferase, followed by 1 nmol/L R1881 treatment for 12 hours and luciferase activity was determined. **, $P < 0.01$; *, $P < 0.05$.

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A**B****C****D****E****Figure 4.**

Relative role of AR_{FL} versus AR-V in AR activity after transient transfection of AR-V7 in MR49F LNCaP cells. MR49F cells were transiently transfected with empty vector or AR-V7 cDNA and then treated in CSS media with exon-1 and -8 AR-ASO for 72 hours. A, AR_{FL}, AR-V7, PSA, and β-actin protein expression levels were measured by Western blotting (left). (Continued on the following page.)



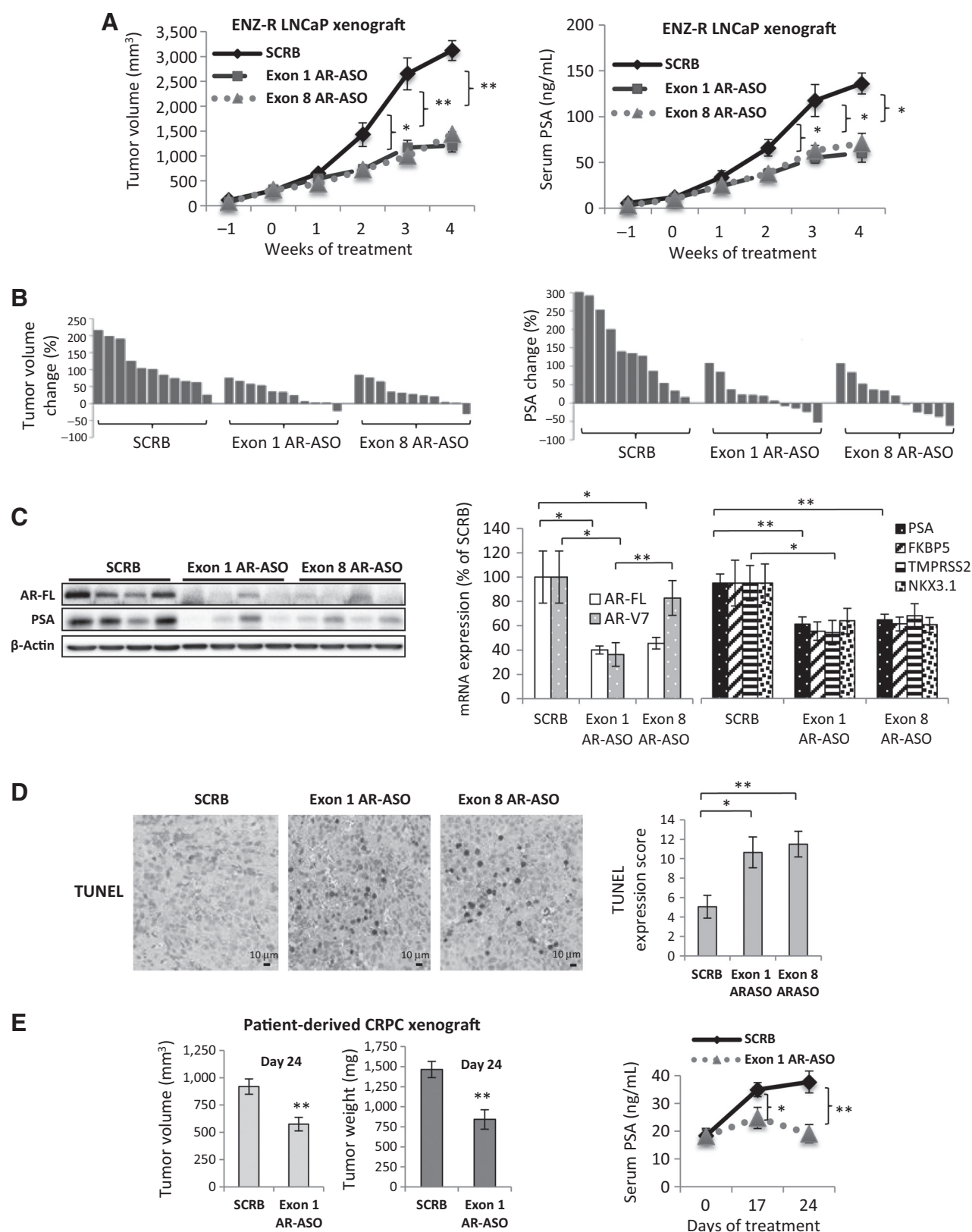
Next, levels of AR_{FL} and AR-V7 were assessed in several ENZ-R xenografts. Quantitative RT-PCR (Fig. 1B) and RNA seq (Fig. 1C, left) demonstrated higher AR_{FL} and AR-V7 mRNA levels in tumor tissues from ENZ-R LNCaP xenografts compared with CRPC LNCaP xenografts; however, AR_{FL} mRNA levels were approximately 12-fold higher than AR-V7, as assessed by RNA seq (Fig. 1C, right).

While transcriptome sequencing detected many AR-Vs in tumor tissues from ENZ-R LNCaP xenografts, only AR-V7 was predominantly expressed (35). Recent reports have linked a mutation in the ligand-binding domain of AR (F876L) to ENZ resistance (36–38). This mutation was not detectable by RNA-Seq in the ENZ-R LNCaP xenografts. However, PCR and Sanger sequencing across the AR ligand-binding domain in MR49F cells was positive for the

F876L mutation, as well as the parental T877A mutation. DNA copy number analysis revealed an absence of focal AR amplification (ref. 39; Supplementary Fig. S2A). Therefore, high AR mRNA expression appears independent of genetic alterations, potentially indicating epigenetic mechanisms of upregulation and epithelial plasticity. The integrative genome viewer (IGV) analysis on mRNA sequencing data across exon 8 of the AR has been performed in LNCaP-derived MR49F and VehD CRPC cells as well as LTL-313BR tumors. It shows that MR49F carries the F876L mutation at a frequency of 63% (blue) and there is no detection of mutation F876L in LTL-313BR and VehD CRPC. As a control, the native LNCaP T877A mutation (orange) is clearly present in 100% of reads (Supplementary Fig. S2B).

(Continued.) A, AR_{FL}, AR-V7 mRNA extracts were analyzed by quantitative real-time PCR (right). B, AR-regulated mRNA extracts were analyzed by quantitative real-time PCR. C, the next day cells were transiently transfected with 1 μg of ARR-3-luciferase, followed in media with ENZ supplemented with CSS and luciferase activity was determined. D, following transient transfection with empty vector or AR-V7 cDNA and treatment in CSS media with exon-1 and -8 AR-ASO for 96 hours, PARP, and caspase-3 expression levels were measured by Western blotting. E, cell growth was determined by crystal violet assay and compared with SCR at the same concentration. *, $P < 0.05$; **, $P < 0.01$.

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**Figure 6.**

AR-ASO suppress ENZ-R LNCaP and patient-derived CRPC tumor growth *in vivo*. When MR49F xenograft tumors reached 200 mm³, mice were randomly assigned to 12.5 mg/kg exon-1, exon-8 AR-ASO or SCRB. The mean tumor volume (A, left) and the serum PSA level (A, right) were compared among the 3 groups $\pm$ SEM ($n = 11$ per group). (Continued on the following page.)

Subcellular fractionation studies detected higher levels of both AR_{FL} and AR-V7 proteins in the nucleus in MR49F compared with parental LNCaP in response to ENZ treatment (Fig. 1D). Importantly, this high nuclear accumulation of AR_{FL} is seen even in the absence of exogenous androgens, suggesting that AR_{FL} is driving ENZ resistance. Immunohistochemical analysis also demonstrated higher levels of AR_{FL} in the nucleus of ENZ-R compared with castrate-resistant LNCaP tumors (Supplementary Fig. S3). Collectively, these data indicate that ENZ induces increased expression levels of AR_{FL} and AR-V7, and both are associated with development of ENZ resistance in the LNCaP model. However, although levels of AR-V7 are low compared with AR_{FL}, the latter is localized in both cytoplasm and nucleus of ENZ-R LNCaP cells, while AR-V7 is confined almost exclusively to the nuclear fraction.

AR-ASO knockdown of AR_{FL} and AR-Vs

To define the functional role of AR_{FL} and AR-Vs in ENZ resistance, 3 ASO sequences were designed to target exon-1, intron-1, or exon-8 in AR pre-mRNA (Fig. 2A) to selectively knockdown AR_{FL} alone, or knockdown AR_{FL} plus AR-Vs simultaneously. All three AR-ASO dose dependently decreased AR_{FL} in both parental LNCaP (Supplementary Fig. S4A) and ENZ-R LNCaP MR49F (Fig. 2B, top) and MR1F (Supplementary Fig. S4B). As AR-Vs are COOH-truncated, exon-1 and intron-1 AR-ASOs potently suppressed levels of AR_{FL} and AR-V7, whereas exon-8 AR-ASO only reduced levels of AR_{FL}. Quantitative real-time PCR analysis confirmed that both exon-1 and intron-1 AR-ASO, but not exon-8, reduced AR-V7 mRNA levels (Fig. 2B, bottom).

22Rv1 cells express high levels of endogenous AR-Vs, but exhibit relatively low levels of AR activity and PSA expression. While all 3 ASO potently reduced AR_{FL} levels, only exon-1 and intron-1 AR-ASO dose dependently reduced AR-Vs; in contrast and similar to LNCaP-derived ENZ-R cell lines, exon-8 AR-ASO did not alter AR-Vs protein levels (Fig. 2C, top). Quantitative real-time PCR indicated that AR-V7 mRNA was decreased by exon-1 and intron-1 AR-ASO but not by exon-8 AR-ASO (Fig. 2C, bottom).

M12 prostate cancer cells, which are engineered to stably express AR-V567es, were used to further assess differential effects of the 3 AR-ASO on silencing AR-V567es. As expected, only exon-1 AR-ASO decreased AR-V567es protein and mRNA levels (Fig. 2D). Intron-1 ASO did not silence AR-V567es because the construct lacks intron-1.

Effects of AR-ASO on AR-regulated genes and AR transcriptional activity

Differential knockdown of AR-Vs by exon-1 versus exon-8 AR-ASO was used to evaluate their relative contribution to the overall AR-regulated transcriptome in ENZ-R LNCaP (MR49F) and 22Rv1 cells. Quantitative real-time PCR was used to measure expression levels of several AR-regulated genes, PSA, FKBP5, TMPRSS2, and NKX3.1. In MR49F cells, all three AR-ASO similarly decreased the

basal mRNA levels of these AR-regulated genes (Fig. 2B, bottom). Both exon-1 and exon-8 AR-ASO also potently suppressed R1881-stimulated AR transactivation in MR49F (Fig. 2E). As exon-8 AR-ASO does not reduce AR-V7 levels, these data suggest that most AR transcriptional activity in ENZ-R LNCaP cells is driven by AR_{FL}.

In contrast, in 22Rv1 cells, which express high levels of endogenous AR-Vs but relatively low levels of AR_{FL} activity and PSA expression, exon-1 and intron-1 AR-ASO more potently suppressed AR-dependent gene expression compared with exon-8 AR-ASO. In fact, near complete inhibition of AR_{FL} alone without inhibition of AR-V7 (using exon-8 ASO) had very little effect on the expression of the 4 AR-regulated genes (Fig. 2C, bottom). Similarly, AR reporter activity using an ARR-3 luciferase reporter assay in 22Rv1 cells indicates that both basal and R1881-stimulated AR transactivation were reduced to a greater extent by exon-1 AR-ASO compared with exon-8 AR-ASO (Fig. 2F).

Effects of AR-ASO on cell growth and apoptosis

Differential knockdown of AR_{FL} and AR-Vs by exon-1 versus exon-8 AR-ASO was used to evaluate relative biologic contributions of AR_{FL} versus AR-V7 in regulating ENZ-R LNCaP and 22Rv1 cell survival and growth. Cell growth assays were performed in castrate-sensitive LNCaP, 4 different ENZ-R LNCaP-derived cells, 22Rv1, and AR-negative PC3 cell lines (as controls). As expected, AR-ASO treatment did not affect PC-3 cell growth over the concentrations used. Despite differential effects on AR_{FL} and AR-V knockdown (Fig. 2B and Supplementary Fig. S4), all AR-ASO similarly inhibited castrate-sensitive and ENZ-R LNCaP cell growth (Fig. 3A). Exon-1 and exon-8 AR-ASO similarly induced apoptosis in ENZ-R cells as measured by caspase-3 and PARP cleavage (Fig. 3B). The fraction of ENZ-R LNCaP cells undergoing apoptosis (sub-G₁ fraction) was similarly increased by exon-1 and exon-8 AR-ASO, and was accompanied by cell-cycle arrest (Fig. 3C, left) and decreased levels of cell-cycle proteins, CDK4, and cyclin D1 (Fig. 3C, right). These data suggest that, as both AR-ASO similarly silenced AR_{FL} levels, inhibited cell growth, and induced apoptosis in LNCaP-derived ENZ-R despite differential effects on AR-V knockdown, most AR pathway-derived cytoprotection is driven by AR_{FL} in ENZ-R LNCaP model. In 22Rv1 cells, however, knockdown of both AR_{FL} and AR-Vs more potently suppressed cell growth compared with knockdown of AR_{FL} alone, suggesting a significant role for AR-Vs in this cell system (Fig. 3D).

As ENZ-R LNCaP cells express low levels of AR-V7 (~8%) compared with AR_{FL}, we transiently transfected AR-V7 in MR49F cells to further assess relative role of AR_{FL} versus AR-V in AR pathway activation in ENZ-R LNCaP cells. Transient transfection of AR-V7 increased levels of ligand-independent AR transactivation (Supplementary Fig. S5). Exon-1 ASO reduced both AR_{FL} and AR-V7 levels (Fig. 4A), and more potently suppressed FKBP5 mRNA expression (Fig. 4B) and AR transactivation (Fig. 4C)

(Continued.) Waterfall plots of greatest percent decline in tumor volume (B, left) and the serum PSA level (B, right) from baseline at any time. C, total proteins were extracted from 4 representative xenograft tumors from each group and AR_{FL} and PSA were analyzed by Western blotting (left). C, mRNA were extracted from 11 xenograft tumors from each group and AR_{FL}, AR-V7, and AR regulated mRNA were analyzed by quantitative real-time PCR (right). D, tumors (n = 11 per group) were collected and TUNEL was evaluated by immunohistochemistry. E, when patient-derived CRPC xenograft tumors became palpable through the abdominal wall, mice were randomly assigned to 40 mg/kg exon-1 or SCRB. The mean tumor volume and tumor weight (E, left) and the serum PSA level (E, right) were compared between the 2 groups ± SEM (n = 9 per group). **, P < 0.01; *, P < 0.05.

compared with exon-8 AR-ASO. However, exon-1 and 8 AR-ASO treatment in ENZ-R LNCaP AR-V7–overexpressing cells similarly decreased PSA protein and mRNA expression (Fig. 4A and B), induced apoptosis as measured by caspase-3 and PARP cleavage (Fig. 4D) and cell growth inhibition (Fig. 4E). These results confirm that the biologic consequences of AR activity in ENZ-R LNCaP cells are principally driven by AR_{FL} and suggest that some AR target genes such as FKBP5 may be preferentially regulated by AR-V7.

Effects of specific AR-V silencing in ENZ-R LNCaP and 22Rv1 cells

While ENZ-R LNCaP cells express low levels of AR-V7 compared with AR_{FL}, this variant always exists in nucleus and has constitutive ligand-independent activity. ENZ-R LNCaP cells expressed AR-V7 without DNA rearrangement while 22Rv1 cells have a rearrangement with 35 kb tandem duplication encompassing AR exon 3 and high expression of truncated AR-Vs AR 1/2/3/2b and AR 1/2/3/CE3 (AR-V7; refs. 20, 40). To further define the functional role of AR-Vs in ENZ-R LNCaP and 22Rv1 cells, we used a CE3 siRNA that specifically silenced AR-V7, but not AR_{FL} in ENZ-R LNCaP cells, and used CE3 and E2b siRNA to specifically silence AR-Vs in 22Rv1. In ENZ-R LNCaP cells, AR-V7 knockdown did not decrease PSA protein or mRNA levels, did not induce apoptosis as measured by caspase-3 and PARP cleavage (Fig. 5A, left and 5B left), and did not inhibit cell growth (Fig. 5C, left). In 22Rv1 cells, AR-V7 knockdown decreased PSA protein and mRNA levels, and induced apoptosis (Fig. 5A and B, right) and cell growth inhibition (Fig. 5C, right). These data indicate that the biologic consequences of AR-V are cell-type-specific, with 22Rv1 cells driven by AR-Vs (20), whereas ENZ-R LNCaP cells are driven predominantly by AR_{FL}.

AR-ASO inhibit ENZ-R LNCaP and patient-derived CRPC tumor growth *in vivo*

The *in vivo* activity of exon-1 and exon-8 AR-ASO were evaluated using MR49F LNCaP xenografts. At baseline, mean tumor volume and serum PSA levels were similar in both groups. Exon-1 and Exon-8 AR-ASO significantly reduced mean tumor volume from 2,653 mm³ to 1,168 and 994 mm³ by 3 weeks (**, $P < 0.01$ and **, $P < 0.01$ respectively), compared with SCRB (Fig. 6A, left); serum PSA levels were also significantly lower (*, $P < 0.05$; *, $P < 0.05$; Fig. 6A, right). There was no significant difference between the two AR-ASO treatment groups. Waterfall plots of best tumor volume and serum PSA decline per mouse at any time are shown in Fig. 6B.

AR_{FL} and PSA protein expression in tumors collected from representative MR49F xenografts ($n = 4$ per group) similarly decreased after treatment with exon-1 and exon-8 AR-ASO (Fig. 6C, left). Exon-1 ASO significantly decreased expression of both AR_{FL} and AR-V7 mRNA; in comparison, exon-8 ASO significantly decreased AR_{FL} without reducing AR-V7 mRNA levels in tumors collected from MR49F xenografts (Fig. 6C, right). PSA mRNA levels were significantly decreased after treatment with both exon-1 and exon-8 AR-ASO compared with SCRB; mRNA levels of AR-regulated genes were also reduced (Fig. 6C, right) by both AR-ASOs. Immunohistochemical analysis revealed that tumors treated with exon-1 or -8 AR-ASO had significantly higher apoptosis rates than SCRB controls (Fig. 6D, right) as shown by increased TUNEL staining (Fig. 6D left). Collectively, these data suggest that exon-1 AR-ASO inhibited expression levels of AR_{FL}

and AR-V7 with suppression of ENZ-R LNCaP tumor growth and the biologic consequences of AR activity in ENZ-R LNCaP cells are principally driven by AR_{FL}.

Next, the *in vivo* activity of exon-1 AR-ASO was evaluated in LTL-313BR patient-derived xenografts. At baseline, mean serum PSA levels were similar in both groups. Exon-1 AR-ASO significantly reduced mean tumor volume and tumor weight from 919 mm³ to 574 mm³ and from 1,463 mg to 841 mg by 24 days (**, $P < 0.01$; **, $P < 0.01$ respectively), compared with SCRB (Fig. 6E, left); serum PSA levels were also significantly lower (**, $P < 0.01$; Fig. 6E, right).

Discussion

The AR remains a central driver of CRPC progression following first-line ADT (17), resulting from AR gene amplification, promiscuous AR mutants, altered expression of coregulators, activation by oncogenic signaling pathways, and increased androgen biosynthesis (4–6, 41). ENZ is an AR antagonist that binds potently to its ligand-binding domain (LBD) to inhibit AR nuclear translocation and transactivation (12), and results in 37% reduction in risk of death in post-docetaxel CRPC (42). Despite this benefit, primary resistance to ENZ is observed in about 30% of patients, and acquired resistance develops in initial responders, often associated with reactivation of AR signaling and rising PSA levels (12, 13). One mechanism of persistent AR signaling in ENZ-resistant CRPC involves treatment-induced AR mutations in the LBD. We detected the F876L AR mutation, reported to be ENZ-specific, in the LBD of AR_{FL} in MR49F cells, which may contribute to ENZ resistance (36–38).

Another potential mechanism of persistent AR signaling in ENZ-resistant CRPC involves treatment-induced AR splicing to encode for transcriptionally active COOH-terminally truncated AR-V proteins containing the NH2-terminal and central DNA-binding domains that lack the LBD (30). AR-Vs facilitate ligand-independent transcriptional activity and cell growth in various model systems (4, 5, 30, 43). AR-V7 is one of the most studied AR-Vs and reported to be associated with recurrence following surgery (4) and poor survival (43) and accumulating evidence links AR-Vs with CRPC and resistance to AR pathway inhibitors like abiraterone (18) and ENZ (19, 20). AR-V overexpression has been reported to provide a growth advantage under castrate conditions (4, 5, 31).

While recent reports link AR-Vs to CRPC, the biologic significance of AR-Vs in AR-regulated cell survival and proliferation, independent of AR_{FL}, remains a controversial issue of clinical relevance as new AR antagonists are designed to also inhibit AR-V activity. For example, the activity of AR-Vs has been reported to remain dependent on endogenous AR_{FL} (44). Our study using ASO-directed knockdown also suggests that the biologic consequences of AR-V7 are cell line-dependent, and driven mainly by AR_{FL} in ENZ-R LNCaP cells. While we found AR-V7 expression is induced by ENZ and highly expressed in ENZ-R LNCaP cells, AR_{FL} levels also increase and remain more than 12-fold higher than AR-V7 levels in all ENZ-R LNCaP cells. While AR_{FL} silencing has been reported to increase AR-V7 levels (19), we did not detect increased AR-V7 expression in parental and ENZ-R LNCaP cells after treatment with exon-8 AR-ASO targeting only AR_{FL}. We did, however, see increased AR-V7 after AR_{FL} knockdown when AR-V7 was transiently transfected, which may reflect challenges with

detecting induction when initial AR-V7 levels are very low and AR_{FL} knockdown induces apoptosis.

To define the biologic consequences of AR_{FL} and AR-V7 induction in ENZ resistance, ASOs were designed to suppress both AR_{FL} and AR-Vs, or AR_{FL} alone. Despite differential effects on AR-V knockdown, all AR-ASO potently silenced AR_{FL} levels and similarly induced apoptosis and inhibited cell growth in LNCaP-derived ENZ-R cell lines. These data suggest that while ENZ induces both AR_{FL} and AR-V7 levels, the biologic consequences are mainly driven by AR_{FL} in the ENZ-R LNCaP model (44). On the other hand, in 22Rv1 cells endogenously expressing higher AR-Vs levels and lower AR_{FL} levels, exon-1 AR-ASO more potently suppressed endogenous AR_{FL} and AR-V7 expression levels, cell growth, AR transcriptional activity, and AR-regulated gene expression compared with exon-8 ASO. Cell line dependence may partially explain this disparate data regarding biologic relevance of AR_{FL} versus AR-Vs in CRPC cell survival and AR pathway inhibitor resistance. 22Rv1 cells contain an intragenic rearrangement of AR with tandem duplication encompassing AR exon 3 and high expression of AR-Vs (20, 39). Compared with LNCaP cells, AR signaling in 22Rv1 is regulated to a much greater extent by AR-Vs, possibly reflecting structural AR gene alterations in 22Rv1 than in LNCaP (20, 39). A recent study evaluated the roles of AR_{FL} and AR-V species in CRPC ENZ responsiveness in the context of rearrangement-driven changes in AR splicing (20). Unlike CRPC LNCaP-derived cells, 22Rv1 cells display robust growth under castrate conditions and ENZ treatment, despite inhibition of AR-regulated genes. Knockdown of AR-Vs, but not full-length AR, reduced the androgen-independent growth rate of 22Rv1 cells (20).

Although, AR targeting approaches with ASOs or siRNA have been reported previously (16, 45, 46), no study so far has reported on pharmacologic AR inhibition *in vivo* in the context of ENZ-resistant disease. ASOs are chemically modified stretches of single stranded DNA complementary to an mRNA region in a target gene that inhibit gene expression by forming RNA/DNA duplexes that are then degraded (15). Short tissue half-life of first-generation phosphorothioate ASO, along with *in vivo* delivery of siRNA, remain significant barriers to clinical development of ASO or siRNA targeting the AR. In this study, we used a panel of highly optimized, next-generation constrained-ethyl (cEt) modified ASOs (Gen 2.5), which demonstrated favorable physicochemical, biochemical, and pharmacokinetic properties *in vivo* (47, 48). The improved resistance against nuclease-mediated metabolism results in a significantly improved tissue half-life *in vivo*, resulting in a longer duration of action and a more intermittent dosing schedule. Moreover, Gen 2.5-modified ASOs display significantly superior efficacy in tissues that are less sensitive to earlier generation ASO chemistries, including tumor cells. In this study, we show for the first

time that systemic administration of an AR-ASO potently suppressed levels of both AR full-length and AR-Vs and inhibited AR activity *in vivo*. An exon-1 targeting AR-ASO inhibited *in vivo* growth of ENZ-R LNCaP tumors, with significant knockdown of AR and AR-V7 expression *in vivo*, providing preclinical proof as a promising next-generation anti-AR agent. No AR knockdown or antitumor activity was observed with multiple control oligonucleotides.

In summary, these results highlight the AR as an important driver of ENZ resistance and that while both AR_{FL} and AR-V7 levels are induced, AR_{FL} is an important mediator of ENZ resistance in the ENZ-R LNCaP model. However, the role of AR_{FL} and AR-V7 in progression to ENZ-R CRPC appears to be context-dependent, and therefore, ASO strategies that target AR_{FL} and AR-Vs is a rational third-line approach for AR pathway inhibitor-resistant CRPC.

Disclosure of Potential Conflicts of Interest

Y. Lorient is a consultant/advisory board member for Astellas. No potential conflicts of interest were disclosed by the other authors.

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Generation 2.5 Antisense Oligonucleotides Targeting the Androgen Receptor and Its Splice Variants Suppress Enzalutamide-Resistant Prostate Cancer Cell Growth

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