


## Research Paper

# Nuclear receptor ERR $\alpha$ contributes to castration-resistant growth of prostate cancer via its regulation of intratumoral androgen biosynthesis

Zhenyu Xu<sup>1,2\*</sup>, Taiyang Ma<sup>2\*</sup>, Jianfu Zhou<sup>2,3</sup>, Weijie Gao<sup>2</sup>, Youjia Li<sup>2</sup>, Shan Yu<sup>2</sup>, Yuliang Wang<sup>2</sup> and Franky Leung Chan<sup>2</sup>

1. Precision Medicine Centre, Yijishan Affiliated Hospital of Wannan Medical College, Wuhu, Anhui Province, PR China
2. School of Biomedical Sciences, Faculty of Medicine, the Chinese University of Hong Kong, Hong Kong, China
3. Department of Urology, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, China

\*Zhenyu Xu and Taiyang Ma contributed equally to this article.

 Corresponding authors: Yuliang Wang and Franky Leung Chan, School of Biomedical Sciences, the Chinese University of Hong Kong, Shatin, Hong Kong, China; Phone: +86 (852) 3943 6841; Fax: +86 (852) 2603 5031; E-mails: yuliang.wang816@gmail.com; franky-chan@cuhk.edu.hk

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## Abstract

Enhanced intratumoral androgen biosynthesis and persistent androgen receptor (AR) signaling are key factors responsible for the relapse growth of castration-resistant prostate cancer (CRPC). Residual intraprostatic androgens can be produced by *de novo* synthesis of androgens from cholesterol or conversion from adrenal androgens by steroidogenic enzymes expressed in prostate cancer cells via different steroidogenic pathways. However, the dysregulation of androgen biosynthetic enzymes in CRPC still remains poorly understood. This study aims to elucidate the role of the nuclear receptor, estrogen-related receptor alpha (ERR $\alpha$ , ESRRA), in the promotion of androgen biosynthesis in CRPC growth.

**Methods:** ERR $\alpha$  expression in CRPC patients was analyzed using Gene Expression Omnibus (GEO) datasets and validated in established CRPC xenograft model. The roles of ERR $\alpha$  in the promotion of castration-resistant growth were elucidated by overexpression and knockdown studies and the intratumoral androgen levels were measured by UPLC-MS/MS. The effect of suppression of ERR $\alpha$  activity in the potentiation of sensitivity to androgen-deprivation was determined using an ERR $\alpha$  inverse agonist.

**Results:** ERR $\alpha$  exhibited an increased expression in metastatic CRPC and CRPC xenograft model, could act to promote castration-resistant growth via direct transactivation of two key androgen synthesis enzymes CYP11A1 and AKR1C3, and hence enhance intraprostatic production of dihydrotestosterone (DHT) and activation of AR signaling in prostate cancer cells. Notably, inhibition of ERR $\alpha$  activity by an inverse agonist XCT790 could reduce the DHT production and suppress AR signaling in prostate cancer cells.

**Conclusion:** Our study reveals a new role of ERR $\alpha$  in the intratumoral androgen biosynthesis in CRPC via its transcriptional control of steroidogenic enzymes, and also provides a novel insight that targeting ERR $\alpha$  could be a potential androgen-deprivation strategy for the management of CRPC.

Key words: ERR $\alpha$ , nuclear receptor, AKR1C3, CYP11A1, intratumoral steroidogenesis, castration resistance, prostate cancer

## Introduction

Androgen receptor (AR) signaling is the major pathway in prostate cancer and thus androgen-deprivation therapy still remains the current principal

treatment option for locally advanced and metastatic prostate cancer. However, acquired resistance to therapy still invariably develops in patients and

progress to therapy-resistance. Failure to androgen-deprivation or hormone therapy developed in advanced metastatic prostate cancer patients is the primary cause of mortality among patients and is still a significant clinical problem [1]. Among multiple adaptive mechanisms in response to hormone therapy, persistent AR signaling is believed as one of the major factors contributing to recurrence of therapy- or castration-resistant prostate cancer (CRPC) [2, 3]. Besides AR gene-related mechanisms (such as AR gene amplification and its overexpression, mutation, and splice variation), intratumoral androgen biosynthesis is considered as another key factor responsible for the persistent elevated tissue levels of androgens causing the reactivation of AR signaling in CRPC tissue despite low levels of circulating androgens upon the systemic androgen-deprivation therapy or castration [4]. Based on this understanding, targeting the persistent AR signaling axis is still the current mainstay therapeutic option for the management of CRPC [5, 6]. In fact, xenograft models of CRPC and CRPC tissues show increased expressions of multiple key steroidogenic enzymes involved in the biosynthesis of testosterone (T) and dihydrotestosterone (DHT) using several pathways, including the classical (canonical or front-door) pathway (by conversion of adrenal androgens dehydroepiandrosterone/DHEA→androstenediol→T→DHT), alternative “backdoor” pathway (progesterone→androstenediol→DHT or DHEA→5 $\alpha$ -Adione→DHT) or direct *de novo* synthesis from cholesterol [7-12]. Based on this, it forms the pharmacological basis of inhibition of systemic or intratumoral biosynthesis of androgens by targeting the androgen synthesis enzymes (such as the CYP17A1 inhibitor abiraterone acetate) as a novel hormone or androgen-deprivation therapy for the management of metastatic CRPC [13, 14].

Estrogen-related receptor alpha (ERR $\alpha$ , NR3B1, *ESRRA*) is a constitutive active ligand-independent orphan nuclear receptor belonging to the nuclear receptor superfamily. ERR $\alpha$  shares significant structural similarities with the ligand-dependent estrogen receptors (ER $\alpha$  and ER $\beta$ ) but does not bind estrogens [15]. Studies in past decades characterize that ERR $\alpha$ , working with its co-regulators peroxisome proliferator-activated receptor  $\gamma$  coactivator-1  $\alpha$  and  $\beta$  (PGC-1 $\alpha$  and PGC-1 $\beta$ ), performs a central role in regulation of cellular energy metabolism and mitochondrial functions, and functions in many biological processes [16]. Increased expression of ERR $\alpha$  and dysregulated function of ERR $\alpha$ -PGC-1 axis are demonstrated in different cancers and also associated in their unfavorable clinical outcomes [16, 17]. We and others demonstrate that ERR $\alpha$  exhibits an

up-regulation pattern in advanced prostate cancer [18, 19], and is implicated as a negative prognostic marker for prostate cancer [20]. Our previous study shows that ERR $\alpha$  can function to promote the hypoxic growth adaptation of prostate cancer cells via its direct interaction with HIF-1 $\alpha$  and enhancement of HIF-1 signaling [21]. Study in an intra-osseous prostate cancer xenograft model shows that overexpression of ERR $\alpha$  can promote the *in vivo* growth of prostate cancer cells in bone via stimulation of bone cells and modulation of extracellular matrix in stroma [22]. Our recent study shows that increased co-expression of ERR $\alpha$  and an oncogenic transcription factor ERG expressed by TMPRSS2:ERG fusion gene is closely associated with the Gleason scores and metastasis status in prostate cancer, and both ERR $\alpha$  and ERG can synergistically regulate each other at transcriptional level and form a reciprocal regulatory loop to promote the advanced growth of prostate cancer [18].

It is believed that up-regulation of steroidogenic enzymes involved in androgen biosynthesis in prostate cancer tissues is a main driving force for the CRPC. However, the mechanism or factors involved in the regulation of androgen biosynthetic enzymes still remains not well understood. The main goal of the present study was to determine the role of ERR $\alpha$  in the growth regulation of CRPC. Here, we showed that ERR $\alpha$ , which exhibited an up-regulation pattern in metastatic CRPC and also xenograft model of CRPC, could function to promote the resistance to androgen-deprivation in prostate cancer cells and also enhance the intratumoral androgen biosynthesis via its transcriptional regulation of some key androgen biosynthetic enzyme genes, including CYP11A1 and AKR1C3. Our results also implicate that ERR $\alpha$  could be a potential therapeutic target as its inhibition could suppress the activation of AR signaling in prostate cancer cells.

## Methods

### Cell lines and cell culture

Three human prostate cancer cell lines (LNCaP, VCaP and DU145; ATCC, Manassas, VA), one embryonic kidney cell line HEK293 and its SV40 large T-antigen-transformed subline HEK293FT (Invitrogen), and the viral packaging mouse cell line PA317 (ATCC) were used in this study. LNCaP cells were maintained in RPMI1640 supplemented with 4.5 g/liter glucose, 1 mM sodium pyruvate, 10 mM HEPES and 10% FBS; VCaP and PA317 cells in DMEM with 10% FBS; DU145 cells in MEM with 1 mM sodium pyruvate and 10% FBS; HEK293 and HEK293FT cells in DMEM with 10% FBS; with cells

cultured with or without 50 U/ml penicillin-streptomycin mixture. The cells and their sublines were harvested when grown to 70-80% confluence for various analyses.

### Gene expression data analysis

The survival analysis of ERR $\alpha$  expression was performed in CRPC patients using an exome sequencing (RNA-seq) dataset (GSE35988; Grasso et al., 2012) from the Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>) [23]. The expression profiles of ERR $\alpha$  and two key steroidogenic enzymes (CYP11A1 and AKR1C3) was analyzed in CRPC patients with bone metastasis using a gene expression microarray dataset (GSE32269; Cai et al., 2013) [24].

### Plasmid construction

(a) Expression plasmids, pBABE-FLAG-ERR $\alpha$ , pcDNA3.1-FLAG-ERR $\alpha$ , pcDNA3.1-FLAG-ERR $\alpha$  truncated mutants ( $\Delta$ DBD,  $\Delta$ LBD-AF2 and  $\Delta$ AF2), pcDNA3-PGC-1 $\alpha$ /PGC-1 $\alpha$ 2 $\times$ 9 mutant, were constructed as described previously [21]. Lentiviral plasmids pLKO.1-shERR $\alpha$  containing shRNA cassettes targeting ERR $\alpha$  were obtained from Dharmacon Inc. (b) Reporter gene plasmids. Luciferase reporter plasmids (CYP11A1 and AKR1C3) were constructed by cloning the respective gene promoter fragments, PCR-amplified from genomic DNA extracted from VCaP cells, into pGL3 basic vector. Sequences of the primers used for promoter cloning are listed in the Table S1. All plasmid constructs were confirmed by DNA sequencing before use.

### Viral transduction

For retrovirus production, pBABE-FLAG-ERR $\alpha$  or empty vector was transfected into PA317 packaging cells; for lentivirus production, pLKO.1-shERR $\alpha$  or pLKO.1-shScramble was transfected into 293FT cells as described previously [25, 26]. LNCaP and DU145 cells were infected with pBABE-FLAG-ERR $\alpha$  or pLKO.1-shERR $\alpha$ , followed by antibiotic selection, for generation of stable ERR $\alpha$ -overexpressed or -knockdowned clones. All ERR $\alpha$ -transduced or shERR $\alpha$ -infected clones were validated by immunoblotting before use.

### In vitro cell growth analysis

Cells were seeded at density of  $2 \times 10^3$  cells/well in 96-well plates and cultured in normal medium with FBS or charcoal-stripped (CS) FBS medium for 1-7 days with fresh medium replaced every 3 days. Viable cells grown for every other days were determined by MTT assay as described previously [25]. Briefly, cells

were incubated with 100 $\mu$ l/well methylthiazolyl-diphenyl-tetrazolium bromide (MTT, 0.5 mg/ml) in phenol red-free RPMI1640 medium for 4 h at 37°C, followed by incubation with 100  $\mu$ l/well SDS-HCl solution (10% SDS, 0.16% 6 M/10 mM HCl and 5% isobutanol) overnight in a CO<sub>2</sub>-free incubator at 37°C to dissolve the formed formazan dyes. Absorbance A<sub>570</sub> was measured in a microplate spectrophotometer. XCT790 and steroids were purchased from Sigma-Aldrich.

### CRPC xenograft tumors

CRPC xenograft model VCaP-CRPC was established based on the castration-resistant growth of tumors formed by subcutaneous inoculation of VCaP cells in castrated SCID mice as described previously [27]. Tumor needle biopsies were acquired at same days when castration was performed and 8<sup>th</sup>-week post-castration (castration-relapse or CRPC) for mRNA and protein analysis.

### In vivo tumorigenicity assay

ERR $\alpha$  and vector-transduced clones ( $3 \times 10^6$  cells suspended in 100  $\mu$ l 1:1 growth medium-Matrigel mixture) were s.c. injected into the flank of intact male SCID mice and allowed to grow for 7-8 weeks for *in vivo* tumorigenicity evaluation as described previously [28]. At about 9<sup>th</sup> week, mice were orchietomized for castration-resistance evaluation of clones following procedures as described previously [27]. For *in vivo* C29 (Jiangsu Aikon Biopharmaceutical R&D Co. Ltd., China) treatment study, once the castration-relapsed VCaP/LNCaP-pBABE tumors regrew to the precastration sizes, tumor bearing mice were randomly assigned to daily intraperitoneal injection of vehicle (PBS containing 5.2% (v/v) of polyethylene glycol and 5.2% (v/v) of Tween 80) or C29 (10 mg/kg/day in vehicle) for 3 weeks [29]. At the end of experiments, tumors were harvested and snap-frozen in liquid nitrogen for gene expression analyses, and androgen extraction and measurement.

### PCR and immunoblot analyses

#### Quantitative real-time qRT-PCR analysis.

SYBR Green-based qRT-PCR was performed following procedures as described previously [25]. Relative mRNA levels were determined by the comparative 2<sup>- $\Delta\Delta$ CT</sup> method and normalized to  $\beta$ -actin. The specificity of primers was validated by the melting-curve detection. PCR cycle times or CT values  $\geq 35$  were considered to be very low levels or levels below detection. Information on primer sequences is listed in the Table S2.

## Immunoblot analysis

An enhanced chemiluminescence method was used for immunosignals detection following procedures as described previously [30]. Primary antibodies used are as follows: ERR $\alpha$  (ab76228, abcam), AKR1C3 (ab84327, abcam), CYP11A1 (D8F4F, Cell Signaling Technology) and  $\beta$ -actin (13E5, Cell Signaling Technology). Results were confirmed by at least three independent experiments.

## Molecular biology analyses

### Chromatin immunoprecipitation (ChIP) -PCR assay

ChIP-PCR assay of CYP11A1 and AKR1C3 gene promoters was performed in VCaP cells following procedures as described previously [28] and using a commercial kit (SimpleChIP Enzymatic Chromatin IP kit, Cell Signaling Technology). Briefly, cross-linked and sonicated DNA samples obtained from VCaP cells were immunoprecipitated with anti-ERR $\alpha$  or control anti-IgG antibody followed by PCR analysis using primers specific for human *CYP11A1* and *AKR1C3* promoters listed in the Table S3.

### Luciferase reporter assay

Dual luciferase reporter assay was performed in HEK293 cells co-transfected with reporter plasmids (pGL3-AKR1C3 or pGL3-CYP11A1), expression plasmids [pcDNA3-ERR $\alpha$  or its truncations, pcDNA3-PGC-1 $\alpha$  (2 $\times$ 9)] and *Renilla* control reporter pRL-CMV using Lipofectamine 2000. Reporter activity was determined following procedures as described previously [25]. All assays were repeated at least three times independently and results were presented as mean  $\pm$  SD.

## Androgen measurement

Androgen levels in xenograft tumors were measured by ultra-high performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UPLC-MS/MS) following procedures as described previously [27]. Briefly, androgens were extracted from cultured cells and snap-frozen tumor samples homogenized in PBS. To evaluate the AKR1C3 activity for its conversion of 5 $\alpha$ -androstanedione (5 $\alpha$ -Adione) to dihydrotestosterone (DHT), cultured cells were first serum starved in phenol red-free medium with 1% charcoal-stripped serum for 48 h and then treated with androgen metabolite 5 $\alpha$ -Adione (100 nmol/L) or 0.1 % ethanol (vehicle control) before androgen extraction. Cultured cells were collected, washed with PBS and lysed with cell lysis buffer. For frozen tumor samples, samples were weighed individually and homogenized in PBS. To serve as an internal controls,

50 pg of 3-deuteride-testosterone (T-3d) and 3-deuteride-dihydrotestosterone (DHT-3d) were added to the cell lysates or tumor homogenates. Each sample was extracted with 8 mL of diethyl ether, followed by freezing the aqueous phase in a dry-ice-ethanol bath. The residue was re-suspended in 0.5 mL of water and extracted with methylene chloride. Standards for T and DHT were prepared in parallel. The consequent oximes were analyzed by UPLC-MS/MS using an Agilent 1290 LC and Agilent 6460 Triple Quadrupole LC/MS system. Ions monitored were 289.4 > 109.2 and 292.4 > 109.2 for T and T-d3; and 291.2 > 255.4 and 294.2 > 258.4 for DHT and DHT-d3, respectively. The lower limits of quantification (LLOQ defined as a signal/noise  $\geq$  5) for T and DHT were 5 pg/ml and 10 pg/ml, respectively.

## Immunohistochemistry

Peroxidase immunohistochemistry (IHC) of ERR $\alpha$  and AKR1C3 was performed in a same construct of prostatic tissue microarray (TMA) slides containing benign prostatic hyperplasia (BPH,  $n = 3$ ), and prostate cancer ( $n = 93$ ) tissues using same antibodies as used in immunoblotting and following procedures as described previously [19]. The ERR $\alpha$  and AKR1C3 immunosignals in stained sections were evaluated by a semiquantitative immunoreactivity scoring (IRS) method as described previously [31].

## Statistical analysis

All experiments were performed at least three times. Results are expressed as mean  $\pm$  SD and analyzed by Student's *t*-test using the Graphpad 6.0 software with *P* values < 0.05 considered statistically significant.

## Results

### CRPC patients with high ERR $\alpha$ expression show poor clinical outcome

Our previous studies show that ERR $\alpha$  performs oncogenic roles in the advanced growth of prostate cancer via its promotion of hypoxia growth adaptation [21] and also transcriptional regulation of TMPRSS2:ERG fusion gene [18]. In order to elucidate further its clinical significance in the advanced progression of prostate cancer, we performed immunohistochemistry of ERR $\alpha$  in a prostatic tissue microarray slide containing benign prostatic hyperplasia (BPH,  $n = 3$ ), and prostate cancer ( $n = 93$ ) tissues of different Gleason scores (GS), and results showed that few cells showing positively-stained nuclei were detected in BPH and low-grade prostate cancer lesions, whereas significant increase of malignant cells with intense nuclear immunoreactivity was noticed in

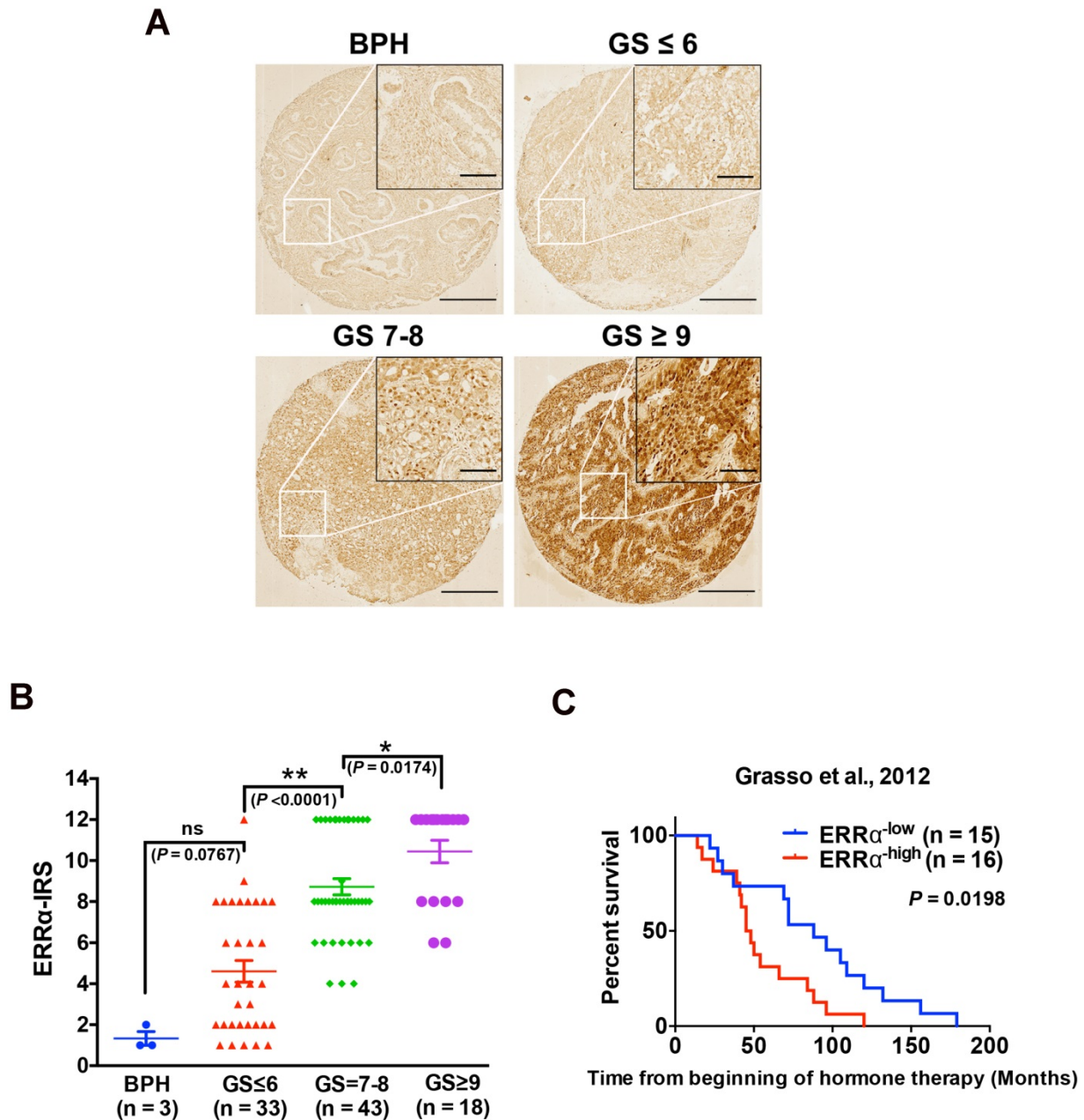


high-grade prostate cancer lesions. (Figure 1A). IRS analysis further confirmed that higher GS lesions ( $GS \geq 7$ ) exhibited significantly higher  $ERR\alpha$  immunoreactivity scores as compared to lower GS lesions ( $GS \leq 6$ ) and BPH tissues (Figure 1B). In addition, we also analyzed the expression profile of  $ERR\alpha$  in clinical samples of CRPC (including 31 CRPC patients totally) using a RNA-seq dataset from one CRPC cohort (Grasso et al., 2012) [23], which revealed that CRPC patients with higher expression of  $ERR\alpha$  were positively correlated with shorter overall survival

(Figure 1C). Together, these results showed increased expression of  $ERR\alpha$  in advanced prostate cancers is associated with their poor clinical and pathological outcomes.

**Castration-relapse prostate cancer xenograft tumors expresses higher levels of  $ERR\alpha$  and some key steroidogenic enzymes**

To provide insights into whether  $ERR\alpha$  would play a role in the castration-relapse growth in prostate cancer, we next examined the expression patterns of



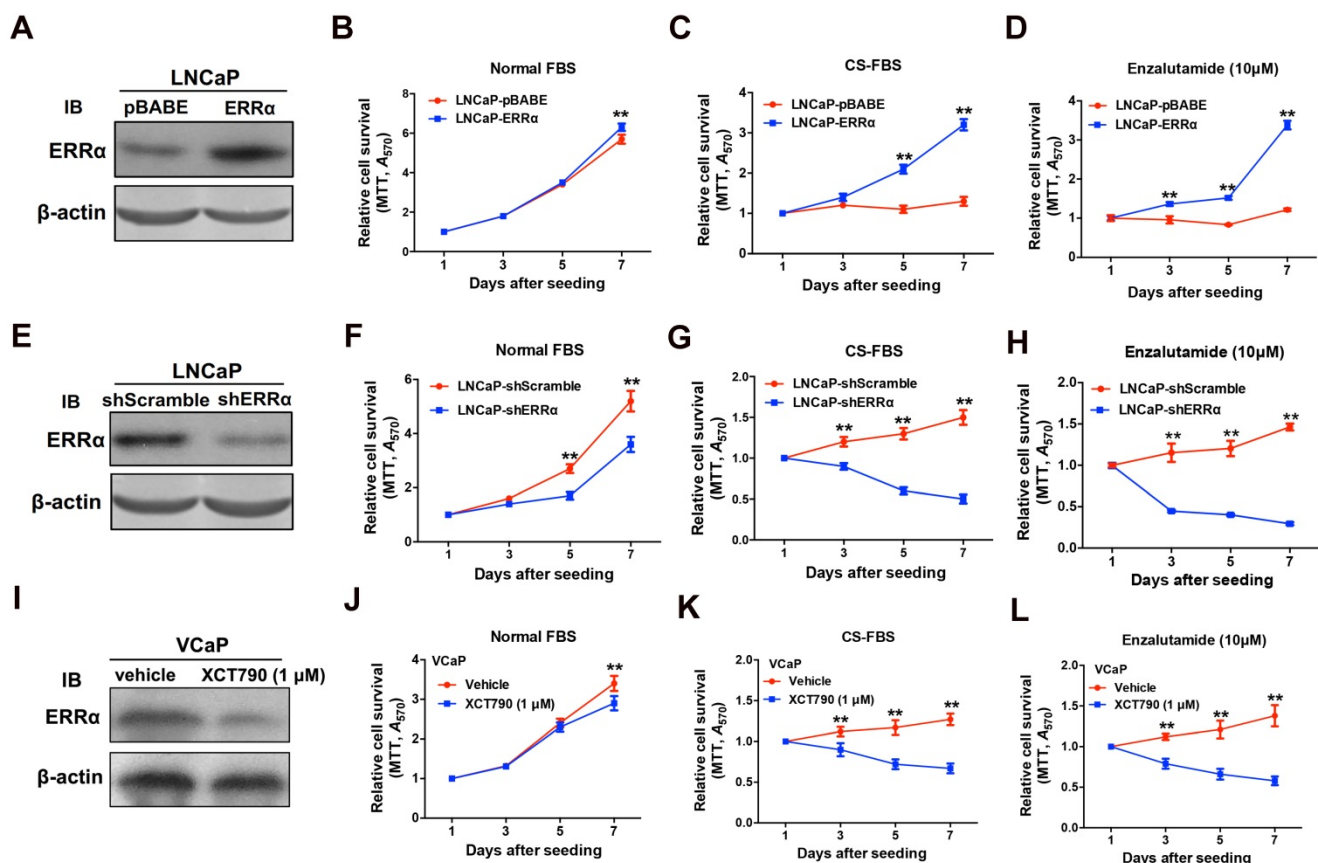
**Figure 1.** Up-regulation of  $ERR\alpha$  is associated with CRPC. **(A)**  $ERR\alpha$  immunohistochemistry. Representative micrographs of  $ERR\alpha$ -immunostained tissue microarray spots of BPH and malignant lesions. A significant increase in malignant cells with positive and intense nuclear  $ERR\alpha$  immunosignals was detected in adenocarcinoma lesions with higher Gleason scores. Magnification,  $\times 40$ ; scale bars, 250  $\mu m$ . Insets show the enclosed areas at higher magnification. Magnification,  $\times 200$ ; scale bars, 50  $\mu m$ . **(B)**  $ERR\alpha$  immunoreactivity score analysis ( $ERR\alpha$ -IRS) performed on BPH and neoplastic prostatic tissues. Adenocarcinomas with higher Gleason scores ( $GS \geq 7$ ) showed significantly higher  $ERR\alpha$  expression than BPH tissues. **(C)** Kaplan-Meier analysis of Grasso et al. 2012 study cohort (GSE35988) of CRPC revealed that higher  $ERR\alpha$  mRNA expression was positively correlated with shortened overall survival of prostate cancer patients relapsed from hormone therapy.

ERR $\alpha$  and also several major steroidogenic enzymes in an established castration-relapse xenograft tumor model VCaP-CRPC, in which AR-axis signaling was reactivated in castration-relapse VCaP xenograft tumors as compared to that in pre-castration [18, 27]. Results of qRT-PCR and immunoblot analyses showed that the castration-relapse VCaP-CRPC xenograft tumors expressed higher levels of ERR $\alpha$  and also several key steroidogenic enzymes involved in androgen biosynthesis (Figures S1A-C). Fradet *et al.* previously analyzed a gene expression microarray dataset of CRPC with bone metastasis (GSE32269), showing that tumor tissues from metastatic CRPC displayed higher expression of ERR $\alpha$  than that in primary localized prostate cancer [22, 24]. Analysis of the same dataset revealed that bone-metastatic CRPC tumor samples exhibited higher expression levels of two key steroidogenic enzymes, AKR1C3 and CYP11A1, than that in primary prostate cancer (Figures S1D-E). These results suggest that ERR $\alpha$  might play a role in intratumoral androgen biosyn-

thesis in castration-relapse growth of prostate cancer.

### ERR $\alpha$ overexpression confers *in vitro* resistance to androgen-deprivation and antiandrogen in AR-positive prostate cancer cells

Since ERR $\alpha$  exhibited an up-regulation expression pattern in clinical CRPC tissues and CRPC xenograft tumors, we hypothesize that ERR $\alpha$  might play a supportive role in CRPC growth. To evaluate the functional impact of ERR $\alpha$  in prostate cancer growth, we generated both stable ERR $\alpha$ -overexpression and -knockdown transductants in two prostate cancer cell lines (including AR-positive: LNCaP; AR-negative: DU145) for *in vitro* and *in vivo* growth phenotype analyses (Figure 2 and Figure S2). *In vitro* growth analyses showed that when being cultured in normal serum condition, the LNCaP-ERR $\alpha$  transduced clones proliferated at similar rate as the empty vector LNCaP-pBABE clones until Day-7. However, the LNCaP-ERR $\alpha$  cells exhibited



**Figure 2.** ERR $\alpha$  enhances *in vitro* growth resistance to androgen-deprivation and antiandrogen in prostate cancer cells. **(A-D)** ERR $\alpha$ -overexpression analysis. **(A)** Immunoblot validation of LNCaP-ERR $\alpha$  transduced clones. **(B-D)** *In vitro* growth responses of LNCaP-ERR $\alpha$  cells under androgen-deprivation culture condition (CS-FBS) and Enzalutamide treatment assayed by MTT. **(B)** Both LNCaP-pBABE and LNCaP-ERR $\alpha$  transduced cells grew at comparable rates in culture with normal FBS until Day-7. **(C and D)** However, when being cultured with CS-FBS or Enzalutamide, LNCaP-ERR $\alpha$  cells grew at normal rates, in sharp contrast to LNCaP-pBABE cells that did not grow. **(E-H)** ERR $\alpha$ -knockdown analysis. **(E)** Immunoblot validation of shERR $\alpha$ -transduced clones. **(F-H)** *In vitro* growth responses of LNCaP-shERR $\alpha$  cells toward cultures with CS-FBS and Enzalutamide as assayed by MTT. The LNCaP-shERR $\alpha$  cells grew at slower rate than the LNCaP-shScramble cells and did not grow in culture conditions with CS-FBS and Enzalutamide. **(I-J)** XCT790 treatment of VCaP cells. **(I)** Immunoblot analysis showed that XCT790 treatment could significantly reduce or abolish the protein level of ERR $\alpha$  in VCaP cells. **(J-L)** XCT790 treatment could significantly suppress the *in vitro* growth of VCaP cells cultured with CS-FBS or Enzalutamide. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus vector control LNCaP-pBABE, LNCaP-shScramble cells or vehicle treatment.

significant/higher resistance to cultures with CS-FBS (mimicking androgen-deprivation condition) and antiandrogen (Enzalutamide) as compared to the LNCaP-pBABE cells that did not grow under these treatments (Figures 2A-D). Conversely, knockdown of  $ERR\alpha$  in LNCaP-sh $ERR\alpha$  cells could substantially enhance the growth inhibition induced by androgen deprivation and antiandrogen as compared to LNCaP-shScramble cells (Figure 2E-H). Furthermore, treatment of another AR-positive VCaP cells with two  $ERR\alpha$  inverse agonists XCT790 and Compound 29 (C29) could significantly sensitize the growth inhibition as exerted by CS-FBS and Enzalutamide (Figures 2I-L and Figures S3A-D) [32, 33]. Interestingly, the suppressed growth of LNCaP and VCaP cells cultured with CS-FBS could be restored upon supplement with R1881 (a synthetic androgen agonist), and the change in proliferation rate (LNCaP- $ERR\alpha$  vs. LNCaP-pBABE and VCaP upon treatment with XCT790 vs. vehicle) in CS-FBS induced by enhancing or suppressing  $ERR\alpha$  activity could be abolished by R1881 (Figures S3E-H). In addition, no significant change in growth proliferation was observed in AR-negative and  $ERR\alpha$ -knockdown DU145-sh $ERR\alpha$  cells when they were cultured with CS-FBS or Enzalutamide until Day-7 (Figures S3I-L). Together, these results suggest that  $ERR\alpha$  overexpression could enhance *in vitro* resistance to androgen deprivation and antiandrogen in AR-positive but not AR-negative prostate cancer cells.

### **$ERR\alpha$ overexpression promotes *in vivo* castration-resistant tumorigenicity and enhances intratumoral androgen levels**

To further validate the enhanced resistance to androgen deprivation exerted by  $ERR\alpha$  overexpression in prostate cancer cells, we then evaluated the *in vivo* tumorigenicity of LNCaP- $ERR\alpha$  transduced cells in castrated host mice. Results showed that the xenograft tumors formed by LNCaP- $ERR\alpha$  clones showed no response or resistance to castration and continued to grow aggressively, in sharp contrast to tumors formed by empty vector clones that stopped to grow and became shrunk in castrated hosts (Figures 3A-C). To further investigate the cause driving the *in vivo* castration-resistant growth capacity acquired in LNCaP- $ERR\alpha$  clones, we analyzed the intratumoral androgen levels (testosterone and DHT) in LNCaP-derived tumors grew in castrated hosts by UPLC-MS/MS. Results showed that the LNCaP- $ERR\alpha$ -derived tumors grew in castrated hosts contained significant higher levels of androgens, as compared to their vector counterparts (Figure 3D). These results strongly suggest that the castration-resistant LNCaP- $ERR\alpha$ -derived tumors exhibited higher capacity of

androgen production. Based on these results, we hypothesize that  $ERR\alpha$  overexpression could increase *in situ* steroidogenesis or androgen biosynthesis in prostate cancer cells, leading to increased androgen levels in LNCaP- $ERR\alpha$ -derived tumors under castration condition, which promotes, at least in part, *in vivo* castration-resistant tumorigenicity.

### **$ERR\alpha$ overexpression enhances expressions of key steroidogenic enzymes in prostate cancer cells**

To verify our hypothesis, we next examined the expression profiles of key steroidogenic enzymes involved in androgen biosynthesis in AR-positive prostate cancer cells with either stable  $ERR\alpha$ -overexpression or -knockdown. qRT-PCR analysis of  $ERR\alpha$ - or sh $ERR\alpha$ -transduced prostate cancer clones showed that the LNCaP- $ERR\alpha$  transduced clones expressed significant higher mRNA levels of three steroidogenic enzymes (CYP11A1, AKR1C3 and CYP17A1), whereas the LNCaP-sh $ERR\alpha$  transduced clones exhibited significant reduced levels of three steroidogenic enzymes (CYP11A1, AKR1C3 and HSD17B3) (Figures 4A and B). qRT-PCR analysis showed that suppression of  $ERR\alpha$  activity by XCT790 or C29 could significantly reduce the transcript levels of CYP11A1 and AKR1C3 in another AR-positive VCaP cells (Figure 4C and Figure S4A). Immunoblot analysis also validated that overexpression of  $ERR\alpha$  could enhance AKR1C3 and CYP11A1 expression in LNCaP- $ERR\alpha$  cells whereas  $ERR\alpha$ -knockdown and XCT790 treatment could reduce their expression in LNCaP and VCaP cells, respectively (Figure 4D). The regulation of AKR1C3 and CYP11A1 expression by  $ERR\alpha$  was also observed in AR-positive LNCaP and VCaP cells under androgen-deprivation culture condition (CS-FBS) and in AR-negative DU145 cells (Figures S4B-D). Furthermore, we performed AKR1C3 IHC on the same construct of prostatic tissue TMA slides used for  $ERR\alpha$  IHC (Figure S4E). IRS analysis revealed that higher GS lesions (GS  $\geq$  7) exhibited significantly higher AKR1C3 IRS scores as compared to lower GS lesions (GS  $\leq$  6) and BPH tissues (Figure S4F), suggesting that  $ERR\alpha$  and AKR1C3 manifested a positive correlation in their immunoreactivity scores (Figure 4E). Finally, the expression of scavenger receptor B1 (SR-B1) encoded by *SCARB1* gene, which is reported to facilitate the precursor cholesterol uptake as needed to drive steroidogenic and non-steroidogenic biogenic pathways in prostate cancer [34], showed no change in either LNCaP- $ERR\alpha$  or LNCaP-sh $ERR\alpha$  cells, suggesting that SR-B1-mediated precursor cholesterol uptake might not be affected by  $ERR\alpha$  (Figure S4G). These results showed that  $ERR\alpha$  overexpression exhibited a

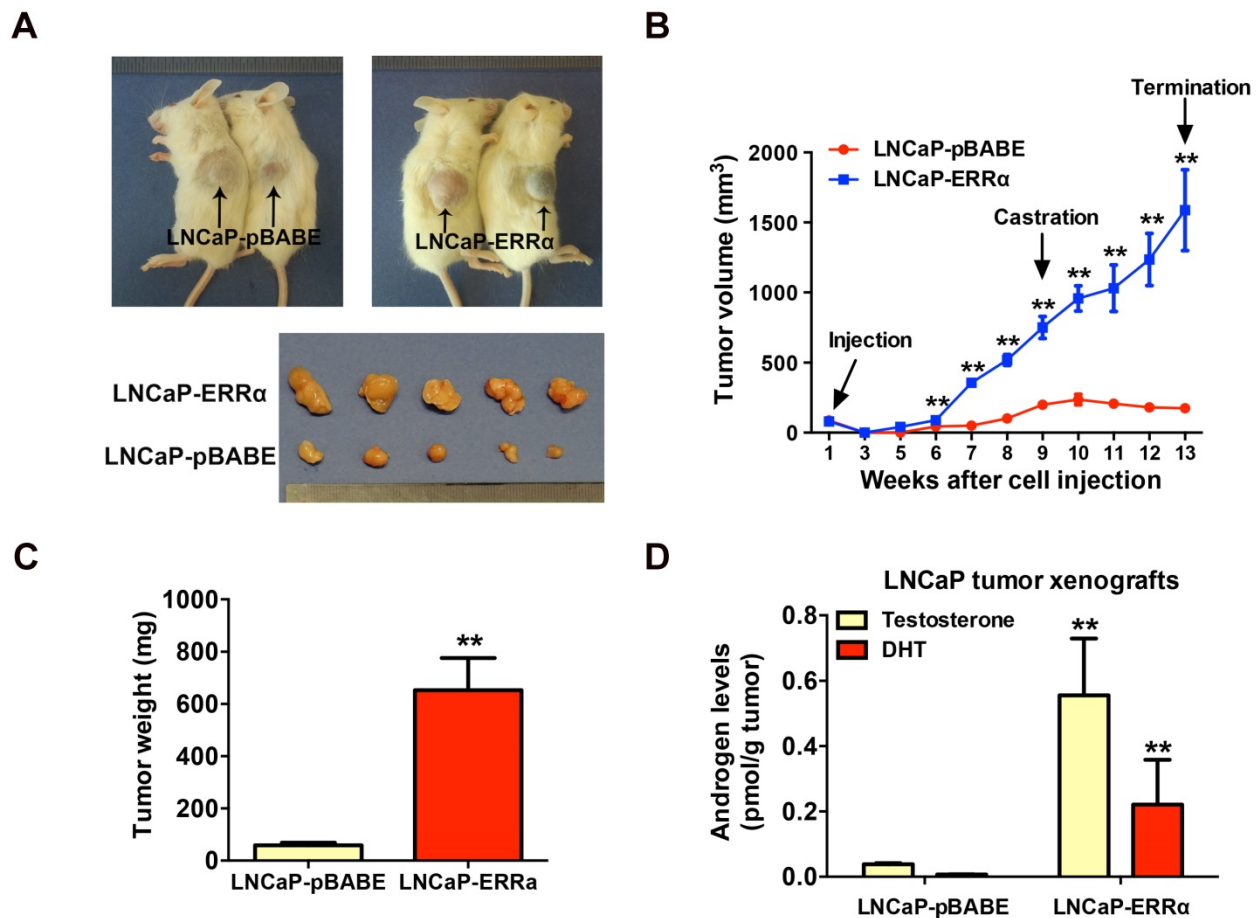


positive correlation with the up-regulation of two steroidogenic rate-limiting enzyme genes, CYP11A1 and AKR1C3, involved in androgen biosynthesis in prostate cancer cells, suggesting that ERR $\alpha$  could function to promote the castration-resistant growth via the up-regulation of steroidogenic enzymes involved in androgen biosynthesis.

### ERR $\alpha$ can directly transactivate steroidogenic enzyme genes AKR1C3 and CYP11A1 in prostate cancer cells

We next sought to determine whether the up-regulation of the two androgen biosynthetic enzyme genes AKR1C3 and CYP11A1 could be the result of direct targeting of ERR $\alpha$  in prostate cancer cells. Promoter sequence analysis predicted multiple potential ERR $\alpha$ -binding sites (ERREs) present in the promoter or regulatory regions of AKR1C3 (17 $\beta$ -hydroxysteroid dehydrogenase 5) and CYP11A1 (cholesterol desmolase) genes respectively (Figures

5A and B). ChIP-PCR assay validated that two DNA fragments of AKR1C3 gene (designated as P1 and P3 sites located at about -1,838 bp and -6,787 bp upstream) and one DNA fragment of CYP11A1 gene (designated as P7 site located at -4,308 bp upstream) could be PCR-amplified in the ERR $\alpha$ -immunoprecipitated DNA extracted from the VCaP cells (Figure 5C). Reporter gene assays performed in HEK293 cells confirmed that the AKR1C3 and CYP11A1 gene promoters-driven reporter constructs could be dose-dependently transactivated by the transfected intact ERR $\alpha$ , with further potentiation by co-transfection with an ERR $\alpha$ -specific co-regulator PGC-1 $\alpha$  (2 $\times$ 9) (Figures 5D-J). However, deletion of the identified ERREs in the AKR1C3 or CYP11A1 promoter inserts in reporters abolished their transactivation by ERR $\alpha$ , further suggesting that these ERREs were essential for the ERR $\alpha$ -mediated transactivation. Further truncation analysis of the functional domains of ERR $\alpha$  demonstrated that the



**Figure 3.** ERR $\alpha$  overexpression promotes *in vivo* castration-resistant growth capacity and enhances intratumoral androgen levels in LNCaP-ERR $\alpha$ -derived xenograft tumors. **(A)** Images show the representative castrated SCID mice bearing the xenograft tumors formed by LNCaP-ERR $\alpha$  and LNCaP-pBABE cells and the dissected xenograft tumors formed by the corresponding cells after 1-month growth in castrated host. **(B)** Growth curve shows the growth responses of LNCaP-ERR $\alpha$  and LNCaP-vector clones in intact host mice for initial 9 weeks followed by 4-week growth in same hosts after castration. LNCaP-ERR $\alpha$  clones formed larger tumors than vector clones in intact mice before castration and continued to grow aggressively in castrated hosts, as compared to tumors formed by LNCaP-pBABE clones that became atrophied after host castration. **(C)** Measurement of wet weights of xenograft tumors. LNCaP-ERR $\alpha$  clones formed tumors heavier than their vector counterpart in castrated hosts. **(D)** Measurement of androgens in xenograft tumors by LC-MS/MS. Significant higher levels of testosterone and DHT were detected in LNCaP-ERR $\alpha$ -derived tumors as compared to its vector counterpart. Data are represented as mean  $\pm$  SD (n = 5) and analyzed by Students' t-test. \*, P < 0.05; \*\*, P < 0.01 versus vector control LNCaP-pBABE cells.

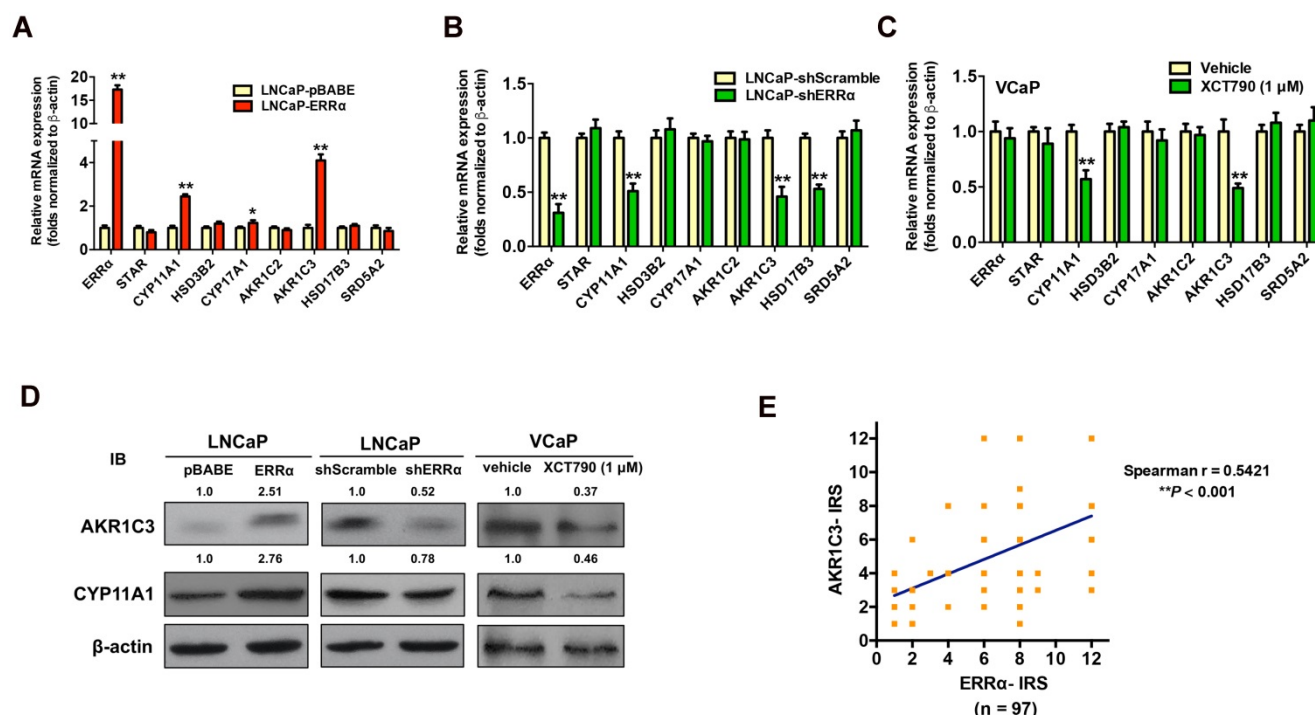


AKR1C3-Luc and CYP11A1-Luc reporters could only be transactivated by the intact ERR $\alpha$  but not its DBD/AF2-deleted mutants. Together, these results suggest that ERR $\alpha$  could directly transactivate the AKR1C3 and CYP11A1 genes through its direct binding to multiple binding motifs in their promoter or regulatory regions.

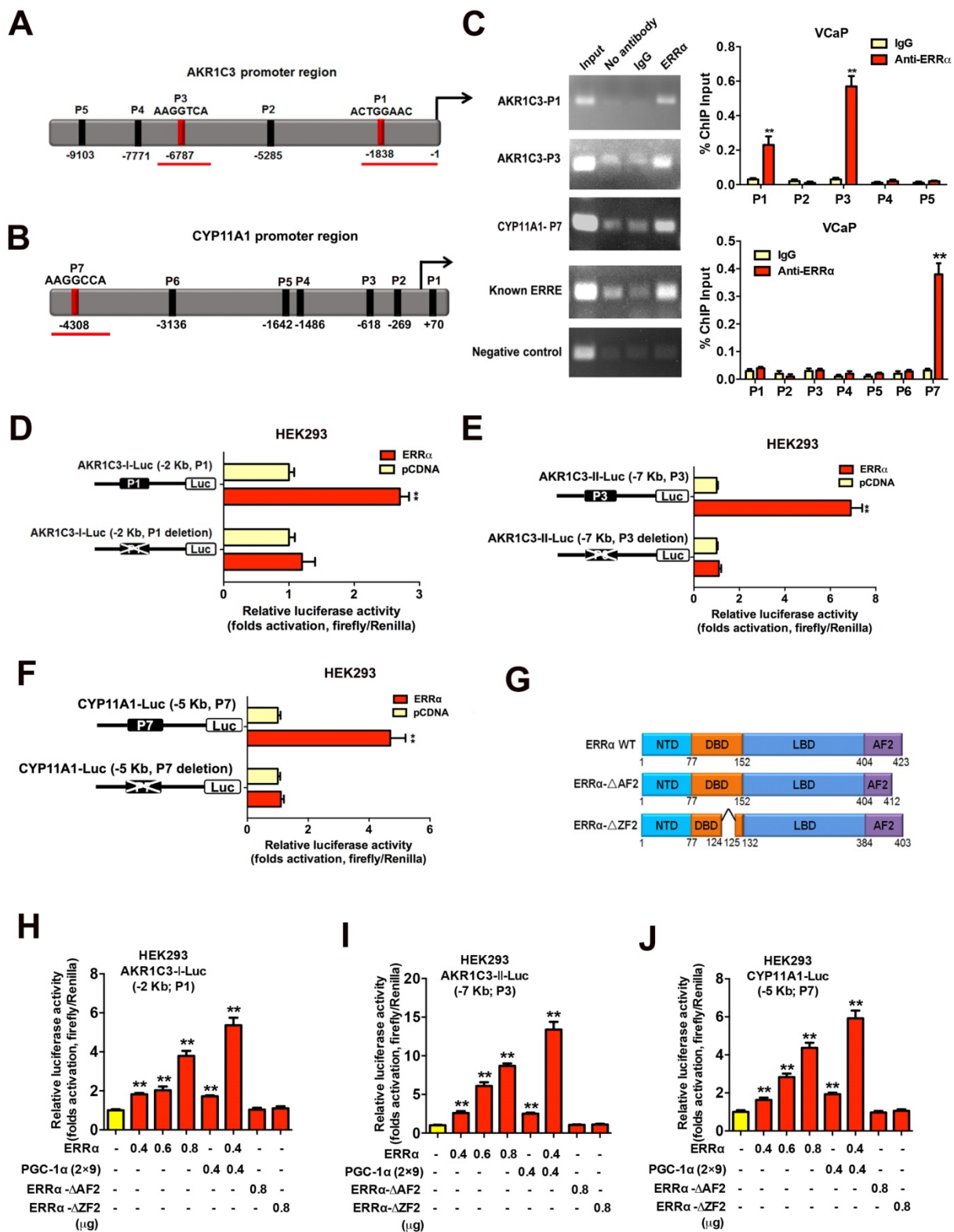
### ERR $\alpha$ -mediated AKR1C3 expression enhances the DHT production in prostate cancer cells

It is known that AKR1C3 is a key steroidogenic enzyme involved in the biosynthesis of DHT via its reduction of androgen intermediates to DHT bypassing testosterone biosynthesis, in an alternative androgen biosynthesis pathway known as the “backdoor” pathway (Figure 6A) [10, 35]. Powell *et al.* revealed that testosterone precursors androstenedione and androstenediol were converted to DHT at very low levels in VCaP cells, suggesting that the 5 $\alpha$ -Adione pathway (secondary backdoor pathway) has a more profound impact on DHT biosynthesis in prostate cancer cells [36]. Since the expression of CYP11A1 is rather low in prostate cancer cells and hardly detectable in clinical prostate cancer tissues, we next mainly investigated the significance of ERR $\alpha$ -enhanced AKR1C3 expression or activity in the

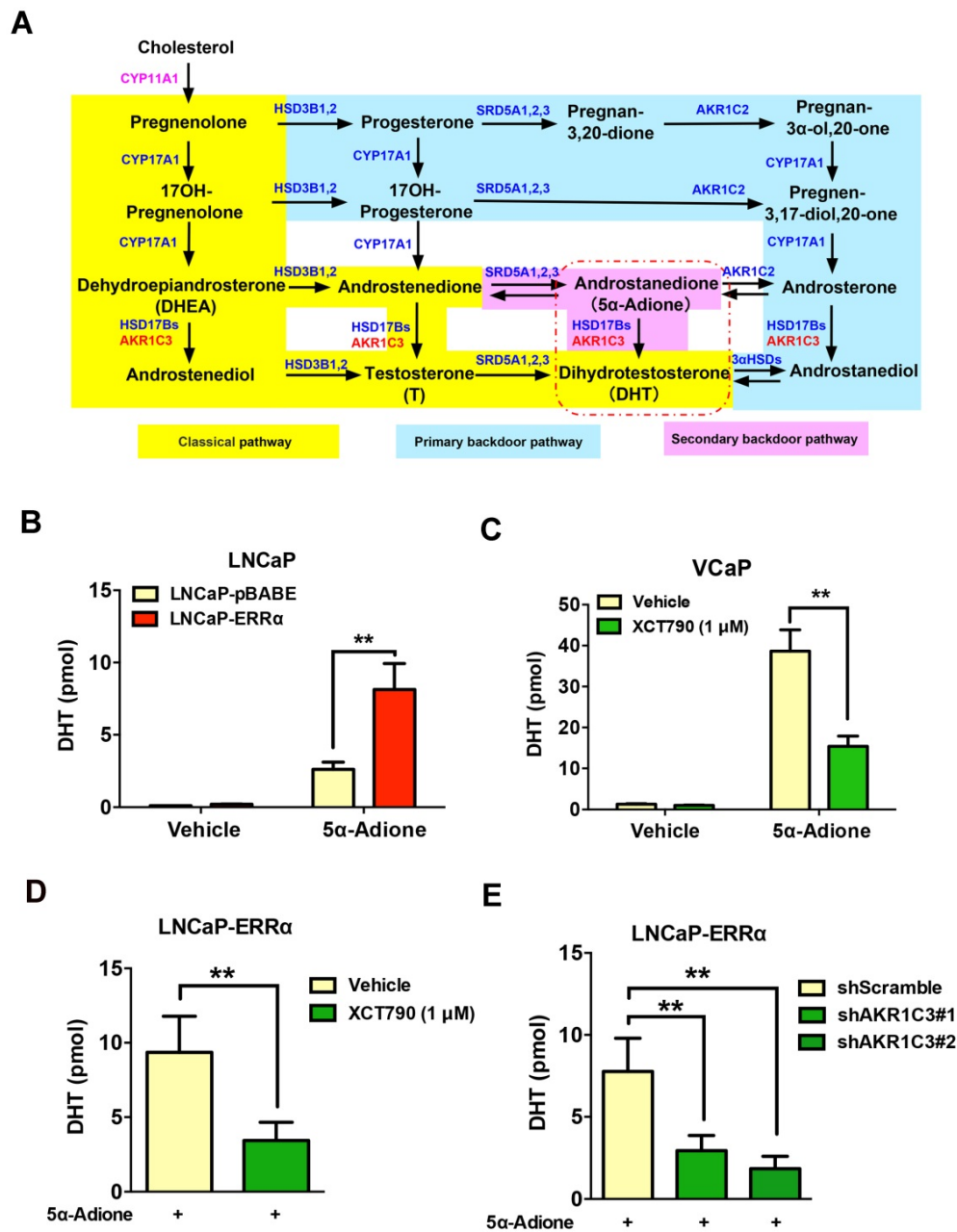
DHT biosynthesis in AR-positive prostate cancer cells. UPLC-MS/MS analysis showed that the LNCaP-ERR $\alpha$  transduced cells displayed higher capacity of DHT production upon supplement with precursor 5 $\alpha$ -androstenedione (5 $\alpha$ -Adione), whereas such 5 $\alpha$ -Adione-enhanced DHT production capacity was significantly attenuated by XCT790 treatment in VCaP and LNCaP cells (Figures 6B-C and Figures S5A-D). Importantly, the increased DHT biosynthesis induced by ERR $\alpha$  overexpression could be abolished by either XCT790 treatment or shRNA-mediated knockdown of AKR1C3 (Figures 6D-E and Figures S5E-F). Furthermore, XCT790 treatment and AKR1C3 silencing could re-sensitize LNCaP-ERR $\alpha$  cells to enzalutamide or culture condition with CS-FBS (Figures S5G-H), suggesting that AKR1C3, at least partially, was involved in the ERR $\alpha$  overexpression-mediated *in vitro* resistance to antiandrogen and androgen-deprivation condition. Overall, these results suggest that ERR $\alpha$  could function to promote the intratumoral DHT biosynthesis in prostate cancer or CRPC via its direct regulation of AKR1C3 expression and also pharmacological suppression of ERR $\alpha$  activity could reduce the DHT production in prostate cancer cells.



**Figure 4.** ERR $\alpha$ -overexpression enhances whereas its knockdown or suppression attenuates the expressions of some key steroidogenic enzymes in prostate cancer cells. **(A-C)** qRT-PCR analysis. **(A)** LNCaP-ERR $\alpha$  clones expressed higher transcript levels of CYP11A1 and AKR1C3 as compared to empty vector LNCaP-pBABE clones. **(B)** ERR $\alpha$ -knockdown in LNCaP-shERR $\alpha$  cells. ERR $\alpha$ -knockdown could significantly suppress the mRNA levels of CYP11A1 and AKR1C3 in LNCaP-shERR $\alpha$  clones as compared to their shScramble clones. **(C)** XCT790 treatment. XCT790 treatment could significantly suppress the mRNA levels of CYP11A1 and AKR1C3 in VCaP cells as compared to their vehicle treatment. **(D)** Immunoblot analysis of AKR1C3 and CYP11A1 in LNCaP-ERR $\alpha$  cells, LNCaP-shERR $\alpha$  cells and XCT790-treated VCaP cells. ERR $\alpha$ -overexpression enhanced AKR1C3 and CYP11A1 expression in LNCaP-ERR $\alpha$  cells whereas ERR $\alpha$ -knockdown and XCT790 treatment suppressed AKR1C3 and CYP11A1 expression in LNCaP and VCaP cells, respectively. **(E)** Linear regression analysis between ERR $\alpha$  and AKR1C3 immunoreactivity scores (IRS). Results showed ERR $\alpha$  and AKR1C3 manifested a positive correlation in their IRS.



**Figure 5.** Direct transactivation of AKR1C3 and CYP11A1 genes by ERR $\alpha$  in prostate cancer cells. **(A and B)** Schematic diagrams depict the putative ERR $\alpha$ -binding sites (ERREs), as predicted by the online program MatInspector (<https://www.genomatrix.de>), located in the **(A)** AKR1C3 (P1-P5) and **(B)** CYP11A1 (P1-P7) gene promoter/regulatory regions. **(C)** ChIP-PCR assay of AKR1C3 and CYP11A1 gene promoters performed in VCaP prostate cancer cells. Results validated that two ERRE sites (P1 and P3) located respectively at 1.8 kb and 6.8 kb upstream of the transcription start site of AKR1C3 promoter (upper graph), and one ERRE site (P7) located at 4.3 kb upstream of the CYP11A1 promoter (lower graph), were enriched of ERR $\alpha$ . \*\*,  $P < 0.01$  versus non-immune IgG-treated DNA. **(D-F)** Luciferase reporter assay of AKR1C3-Luc and CYP11A1-Luc reporters performed in ERR $\alpha$ -transfected HEK293 cells. The AKR1C3-Luc and CYP11A1-Luc reporter constructs, containing inserts of ERR $\alpha$  promoter/regulatory regions, could be significantly transactivated by the transfected ERR $\alpha$ . Deletion of identified ERREs in the reporters (P1 and P3 in AKR1C3-Luc; P7 in CYP11A1-Luc) abolished or significantly reduced the ERR $\alpha$ -induced transactivation. **(G)** Schematic diagram shows the functional domains of wild-type ERR $\alpha$  protein and two of its truncated mutants generated. **(H-J)** Luciferase reporter assays of AKR1C3-Luc and CYP11A1-Luc reporters performed in HEK293 cells. The AKR1C3-Luc and CYP11A1-Luc reporters could be dose-dependently transactivated by ERR $\alpha$  and further potentiated by co-transfection with co-regulator PGC-1 $\alpha$  (2 $\times$ 9), but not by ERR $\alpha$ - $\Delta$ AF2 and ERR $\alpha$ - $\Delta$ ZF2 truncated mutants. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus empty vector pcDNA3.1. Data are presented as mean  $\pm$  SD (n = 3) and analyzed by Students' *t*-test.



**Figure 6.** ERR $\alpha$ -overexpression can promote while its suppression can attenuate DHT production in prostate cancer cells. **(A)** Diagram shows the classical (canonical or front-door) and alternative (back-door) pathways of androgen biosynthesis. Androgens are synthesized from cholesterol via multiple enzymatic steps. CYP11A1 is responsible for converting cholesterol to pregnenolone by side chain cleavage of cholesterol. Pregnenolone is then converted to dehydroepiandrosterone (DHEA) and androstenedione by CYP17A1. The classical pathway for testosterone biosynthesis involves conversion of major adrenal DHEA and androstenediol to testosterone in the testis, followed by 5 $\alpha$ -reduction of testosterone to dihydrotestosterone (DHT) by 5 $\alpha$ -reductases (SRD5As). On the other hand, DHT biosynthesis via 5 $\alpha$ -reduction of upstream steroids bypassing testosterone can be achieved by two other back-door pathways. In the primary backdoor pathway, 17OH-progesterone is 5 $\alpha$ - and 3 $\alpha$ -reduced by SRD5As and AKR1C2 before the 17,20-lyase reaction of CYP17A1, yielding the androsterone and then to androstenediol by HSD17Bs and AKR1C3. In the secondary backdoor pathway, androstenedione is converted to 5 $\alpha$ -androstenedione (5 $\alpha$ -Adione) by SRD5As and then to DHT by HSD17Bs and AKR1C3. Through these backdoor pathways, DHT is synthesized without using testosterone as intermediate. **(B and C)** UPLC-MS/MS measurement of DHT in LNCaP-ERR $\alpha$  cells and XCT790-treated VCaP cells. Results showed that upon supplement with 5 $\alpha$ -Adione, LNCaP-ERR $\alpha$  cells contained significant higher level of DHT than the empty vector LNCaP-pBABE cells. On the other hand, suppression of ERR $\alpha$  activity by XCT790 could significantly reduce the DHT level in 5 $\alpha$ -Adione-supplemented VCaP cells. **(D and E)** UPLC-MS/MS measurement of DHT in LNCaP-ERR $\alpha$  cells upon XCT790 treatment and shRNA-mediated knockdown of AKR1C3. Results showed that ERR $\alpha$  overexpression-induced increase of DHT biosynthesis could be abolished by either XCT790 treatment or AKR1C3 knockdown. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus empty vector pBABE or vehicle.

### ERR $\alpha$ can function to activate AR signaling in prostate cancer cells

Finally, we investigated the functional significance of ERR $\alpha$ -mediated up-regulation of AKR1C3 in activation of AR signaling in prostate cancer cells. Immunoblot analysis of AR and its target

PSA in prostate cancer cells showed that supplement with an androgen precursor 5 $\alpha$ -Adione could enhance the levels of nuclear AR level (without change in total AR level) and PSA in both LNCaP-pBABE and LNCaP-ERR $\alpha$  transduced cells, with significant higher levels in LNCaP-ERR $\alpha$  cells (Figure 7A).

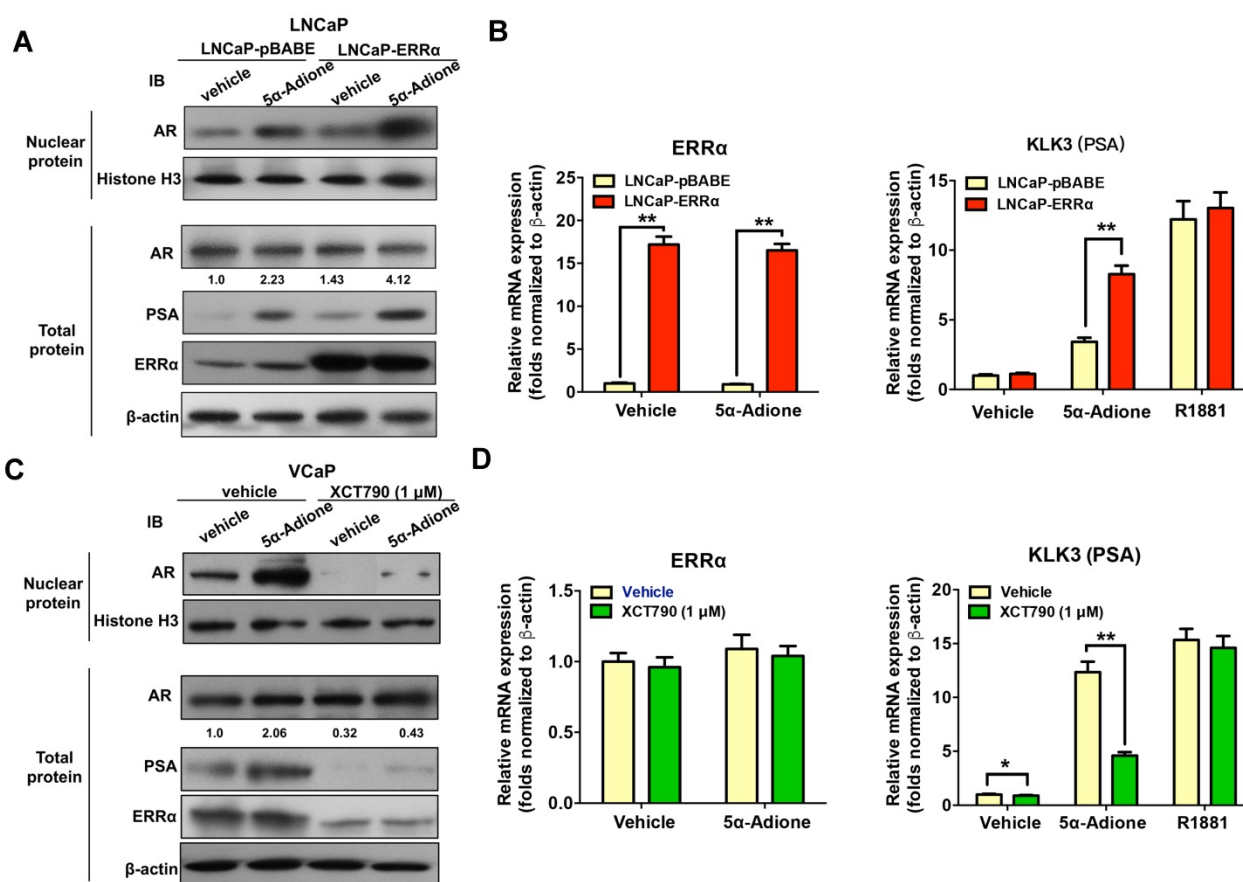


qRT-PCR analysis revealed that 5 $\alpha$ -Adione supplement could significantly increase the PSA mRNA levels in both LNCaP-ERR $\alpha$  and LNCaP-pBABE transduced cells, with much higher levels induced in LNCaP-ERR $\alpha$  cells (Figure 7B). We also confirmed that suppression of ERR $\alpha$  activity by XCT790 could significantly reduce or abolish the levels of AR (nuclear) and PSA in AR-positive VCaP cells supplemented with or without 5 $\alpha$ -Adione (Figures 7C and D), accompanied with the decreased ERR $\alpha$  protein expression upon XCT790 treatment. Similar reductions of levels of AR (nuclear) and PSA were also confirmed in stable ERR $\alpha$ -knockdown LNCaP-shERR $\alpha$  cells with or without 5 $\alpha$ -Adione (Figure S6). Together, these results demonstrate the significance of ERR $\alpha$ -mediated up-regulation of AKR1C3 in the activation of AR signaling in prostate cancer cells through the enzymatic role of AKR1C3 in the backdoor pathway of androgen biosynthesis.

Overall, these results suggest that ERR $\alpha$  could play a role in activation of AR signaling in prostate cancer cells via its regulation of AKR1C3 expression.

### Pharmacological targeting of ERR $\alpha$ can suppress castration-resistant growth of prostate cancer *in vivo*

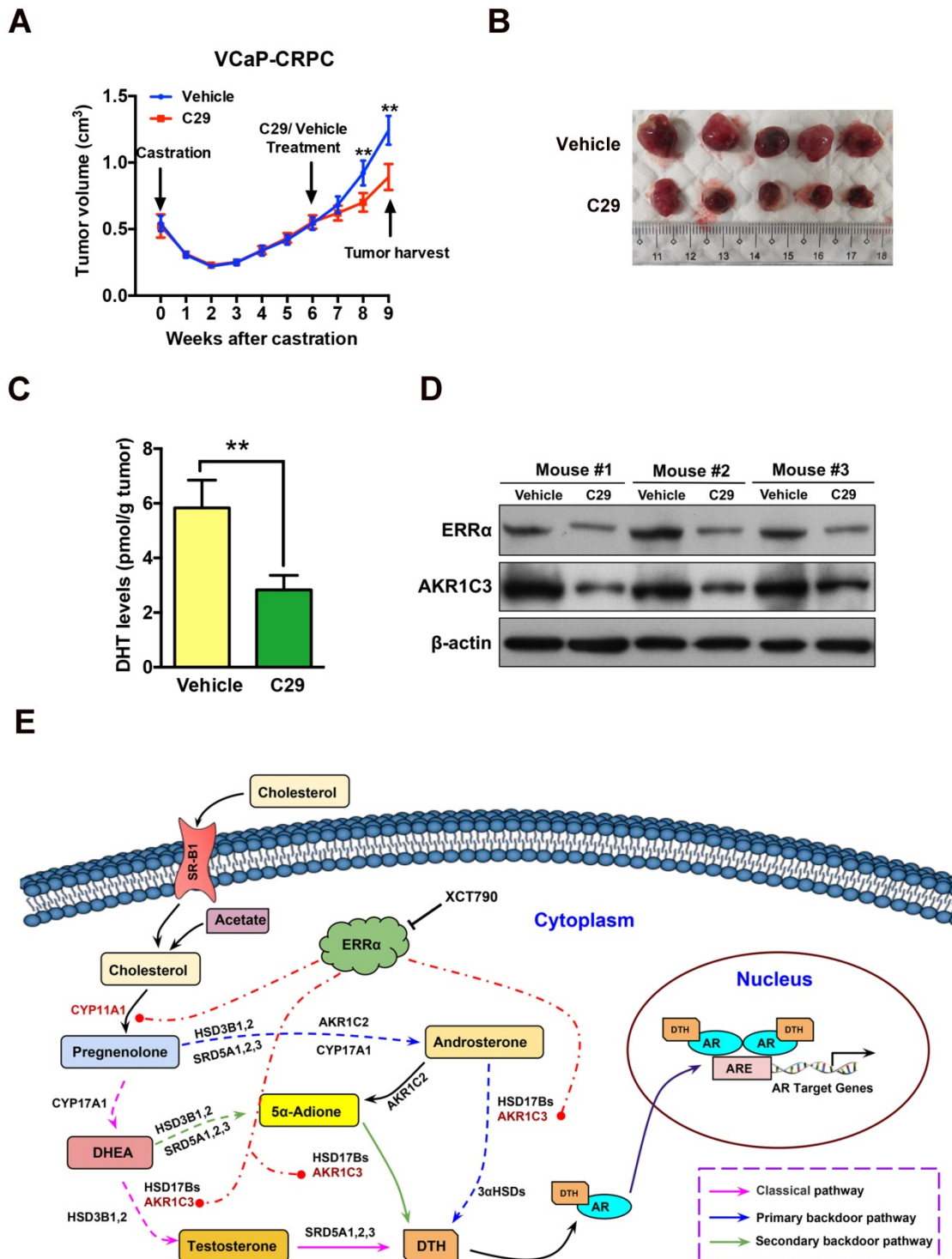
To evaluate the therapeutic significance and application value of targeting ERR $\alpha$  in CRPC, we assessed the anti-tumor effect of suppression of ERR $\alpha$  in the VCaP-CRPC model by intraperitoneal injection of C29 or vehicle. The results showed that C29 could significantly prevent the castration-relapsed tumor growth as compared to vehicle, accompanied by decrease of intratumoral DHT production and expression of ERR $\alpha$  and AKR1C3 (Figures 8A-D). A similar inhibitory effect as exerted by C29 treatment was also observed in castration-resistant LNCaP-pBABE and LNCaP-ERR $\alpha$  xenografts, in which the



**Figure 7.** ERR $\alpha$  functions to activate AR signaling in prostate cancer cells. Cultured cells were first serum-starved in phenol red-free medium with 1% CS-FBS for 48 hours and then treated with androgen metabolite 5 $\alpha$ -Adione (100 nmol/L) or vehicle control (0.1% ethanol) for 24 hours before mRNA or protein analyses. **(A)** Immunoblot analysis of AR and PSA in LNCaP-pBABE and LNCaP-ERR $\alpha$  transduced cells. Results showed that supplement with 5 $\alpha$ -Adione could enhance the nuclear AR (with no change in total AR level) and PSA expression levels in both LNCaP-pBABE and LNCaP-ERR $\alpha$  cells, with significant higher levels in LNCaP-ERR $\alpha$  cells. **(B)** qRT-PCR analysis of ERR $\alpha$  and KLK3 (PSA) expression in 5 $\alpha$ -Adione-supplemented LNCaP-pBABE and LNCaP-ERR $\alpha$  transduced cells. Supplement with 5 $\alpha$ -Adione induced no change in mRNA levels of ERR $\alpha$  in both LNCaP-pBABE and LNCaP-ERR $\alpha$  cells. However, 5 $\alpha$ -Adione supplement could significantly increase PSA mRNA levels in both LNCaP-pBABE and LNCaP-ERR $\alpha$  cells, with significant higher levels in LNCaP-ERR $\alpha$  cells. Supplement with R1881 induced increase of PSA mRNA levels in both LNCaP-pBABE and LNCaP-ERR $\alpha$  cells at same levels. **(C)** Immunoblot analysis of AR and PSA in VCaP cells treated with 5 $\alpha$ -Adione and XCT790. Results showed that suppression of ERR $\alpha$  activity by XCT790 could significantly reduce or abolish the protein levels of nuclear AR, PSA and ERR $\alpha$  in VCaP cells with or without 5 $\alpha$ -Adione supplement. **(D)** qRT-PCR analysis of ERR $\alpha$  and PSA expression in VCaP cells treated with 5 $\alpha$ -Adione and XCT790. Results showed that XCT790 treatment did not affect the mRNA levels of ERR $\alpha$ , but significantly suppressed the mRNA levels of KLK3 in VCaP cells supplemented with 5 $\alpha$ -Adione. Supplement with R1881 induced increase of PSA mRNA levels in VCaP cells upon treatment with XCT790 or vehicle at same levels. \*\*,  $P < 0.01$  versus empty vector pBABE transduced cells or vehicle.

C29-induced growth inhibition was more significant in LNCaP-ERR $\alpha$  xenografts as compared to that in LNCaP-pBABE xenografts (Figures S7A-D). These results suggest that ERR $\alpha$  is a potential therapeutic

target of CRPC and pharmacological suppression of ERR $\alpha$  could potentiate the sensitivity of prostate cancer cells to hormone or AR-axis targeting therapy.



**Figure 8.** ERR $\alpha$  inverse agonist C29 suppresses castration-resistant growth of prostate cancer *in vivo*. **(A)** Growth curve of VCaP-CRPC xenograft tumors upon C29 or vehicle treatment. Once the castration-relapsed VCaP-CRPC tumors regrow to sizes as that in pre-castration at 6-7 week post-castration, tumor-bearing mice were randomly assigned to intraperitoneal injections of C29 or vehicle for additional 3 weeks. Results showed that C29 could significantly retard the castration-relapsed growth of VCaP-CRPC tumors as compared to vehicle. **(B)** Images show the representative dissected VCaP-CRPC xenograft tumors upon 3-week treatment with C29 or vehicle in castrated host. **(C)** Measurement of DHT in VCaP-CRPC xenograft tumors by LC-MS/MS. Significant reduction of DHT levels was detected in tumors upon C29 treatment as compared to vehicle. **(D)** Immunoblot analysis. VCaP-CRPC tumors upon C29 treatment expressed lower protein levels of ERR $\alpha$  and AKR1C3 as compared to vehicle. \*\*,  $P < 0.01$  versus vehicle. **(E)** Schematic diagram depicts the hypothetical role of ERR $\alpha$  in the promotion of intratumoral androgen biosynthesis and reactivation of AR signaling in CRPC via its direct transactivation of some key steroidogenic enzyme genes.

## Discussion

Enhanced intratumoral androgen biosynthesis caused by up-regulation of androgen synthesis enzymes in CRPC tissues is regarded as one of the key factors responsible for the reactivation of AR signaling in CRPC and also its relapse growth. Based on this, selective inhibition of the key enzyme in early steroidogenesis, CYP17A1 by abiraterone acetate, is developed as a novel androgen-deprivation therapy and its clinical use can prolong the survival of patients with locally advanced or metastatic CRPC [37-39]. However, resistance to abiraterone acetate still inevitably occurs in CRPC patients [40]. A few studies on CRPC xenograft models show that resistance to abiraterone acetate may involve multiple mechanisms, including up-regulation of CYP17A resulting in enhanced *de novo* androgen biosynthesis [9, 41], induction of ligand-independent AR splice variants and progesterone-sensitive mutated AR [9, 41], and acquisition of mutated steroidogenic enzyme 3 $\beta$ HSD1 that can accelerate the conversion of adrenal steroid DHEA to DHT [42]. On the other hand, the mechanisms involved in the up-regulation of androgen synthesis enzymes in CRPC and also resistance to abiraterone still remain largely unknown. Here we show that the orphan nuclear receptor ERR $\alpha$  could be a key factor for the transcriptional regulation of some key androgen synthesis enzymes in prostate cancer cells and its overexpression would be responsible for the enhanced intratumoral androgen biosynthesis in CRPC.

In this study, we showed that ERR $\alpha$  displayed a higher expression pattern in advanced prostate cancer and a CRPC xenograft model VCaP-CRPC, and its up-regulation was positively correlated with poor clinical outcome. These results validate the previous reports by us and others that ERR $\alpha$  exhibits an up-regulation pattern in advanced prostate cancer and also its usefulness as a poor prognostic marker for prostate cancer [18-20, 22]. Furthermore, our present study showed that its overexpression could confer *in vitro* resistance to androgen-deprivation and antiandrogen, and also *in vivo* castration-resistant growth capacity of AR-positive cancer cells. Our results suggest that overexpression of ERR $\alpha$  could play a significant role in the development and progression of CRPC. However, the mechanisms responsible for the up-regulation of ERR $\alpha$  in prostate cancer are still unclear. Genomic amplification of *ESRRA* gene and down-regulation of an ERR $\alpha$ -targeting microRNA miR-125a are shown to be responsible for the up-regulation of ERR $\alpha$  in oral squamous cell carcinoma [43, 44]. Indeed, genotype with amplification at chromosome 11q13.1, where the

*ESRRA* gene is located, is detected in localized prostate cancer tumors by comparative genomic hybridization (CGH) analysis and such genotype is shown as a positive predictor of post-operative disease recurrence and metastasis [45]. Recently, we demonstrate that the transcription factor ERG, expressed by the TMPRSS2:ERG fusion gene, can directly transactivate the *ESRRA* gene in advanced prostate cancer [18]. These studies provide indirect evidence that genetic factors, such as gene amplification and transactivation by transcription factors, could be one of the mechanisms responsible for the increased expression of ERR $\alpha$  in advanced prostate cancer.

One key finding we showed in this study is that overexpression of ERR $\alpha$  could confer *in vitro* resistance to androgen-deprivation and *in vivo* castration-resistant growth capacity in AR-positive prostate cancer cells via a mechanism of direct transactivation of some key androgen synthetic enzyme genes, leading to enhanced intracellular *de novo* androgen production in prostate cancer cells. In a recent study, we also demonstrate that another nuclear receptor LRH-1 (NR5A2), which exhibits an increased expression in CRPC, can play a supportive role in intratumoral androgen biosynthesis in CRPC via a similar mechanism of direct transactivation of key androgen synthetic enzyme genes [27]. Interestingly, comparing their specific targets, it is noted that ERR $\alpha$  can target the CYP11A1 and AKR1C3 (involved in the *de novo* and backdoor pathways of DHT biosynthesis), whereas LRH-1 can target the STAR, CYP11A, CYP17A1 and HSD3B2 (involved in similar pathways except the secondary backdoor pathway), suggesting that the two nuclear receptors may perform differential roles in biosynthesis of DHT via their regulation of different steroidogenic enzymes involved in the intratumoral androgen biosynthesis in CRPC. These results also implicate that the prostate cancer cells in CRPC can shift to employ alternative pathways of DHT biosynthesis via their flexible or adaptive transcriptional control of androgen synthetic enzyme genes by different nuclear receptors including ERR $\alpha$  and LRH-1.

Although ERR $\alpha$  is regarded as an orphan nuclear receptor, its transactivation activity can be targeted or modulated by synthetic ligands, such as the inverse agonist XCT790. One noteworthy finding of this study is that ERR $\alpha$  could be druggable as the intracellular DHT production and AR signaling in prostate cancer cells could be significantly attenuated or suppressed by treatment with XCT790, likely via its direct repression on AKR1C3 enzyme and reduction of ERR $\alpha$  protein level. This result also implicates that



besides inhibition of activities of steroidogenic enzymes (e.g. CYP17A1 targeting abiraterone), targeting their upstream regulators (such as ERR $\alpha$ ) could be a potential therapeutic strategy for the suppression of intratumoral androgen biosynthesis in CRPC.

Recently, it is shown that the oncogenic transcription factor ERG, expressed by the TMPRSS2:ERG fusion gene, could upregulate the AKR1C3 expression in prostate cancer [36]. Our recent study also shows that ERR $\alpha$  and ERG can synergistically regulate each other at the transcriptional level and both form a reciprocal regulatory loop to promote the advanced growth of prostate cancer. Together, these findings suggest that besides CYP17A1, AKR1C3 and ERR $\alpha$  could also be the potential therapeutic targets in the management of CRPC.

## Conclusions

In summary, our present study shows that the nuclear receptor ERR $\alpha$  could perform a supportive role in the intratumoral androgen biosynthesis in prostate cancer via its direct transactivation of at least two key steroidogenic enzyme genes CYP11A1 and AKR1C3, and through this regulation it could help to promote the advanced growth of CRPC by activation of AR signaling (Figure 8E). Our study also provides a novel insight that targeting ERR $\alpha$  could be a potential androgen-deprivation strategy for the management of CRPC as pharmacological suppression of its activity could help to attenuate the intracellular production of DHT and AR signaling in prostate cancer cells.

## Abbreviations

5 $\alpha$ -Adione: 5 $\alpha$ -androstane-3-one; AR: androgen receptor; CGH: comparative genomic hybridization; ChIP: chromatin immunoprecipitation; CRPC: castration-resistant prostate cancer; CS: charcoal-stripped; DHEA: dehydroepiandrosterone; DHT: dihydrotestosterone; ERR $\alpha$ : estrogen-related receptor alpha; GEO: gene expression omnibus; qRT-PCR: quantitative real-time PCR; RNA Sequencing: RNA-Seq; T: testosterone.

## Supplementary Material

Supplementary figures and tables.

<http://www.thno.org/v10p4201s1.pdf>

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## Competing Interests

The authors have declared that no competing interest exists.

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