

# Identification of EP4 as a Potential Target for the Treatment of Castration-Resistant Prostate Cancer Using a Novel Xenograft Model

Naoki Terada<sup>1</sup>, Yosuke Shimizu<sup>1</sup>, Tomomi Kamba<sup>1</sup>, Takahiro Inoue<sup>1</sup>, Atsushi Maeno<sup>1</sup>, Takashi Kobayashi<sup>1</sup>, Eijiro Nakamura<sup>1,6</sup>, Toshiyuki Kamoto<sup>1,7</sup>, Toshiya Kanaji<sup>8</sup>, Takayuki Maruyama<sup>8</sup>, Yoshiaki Mikami<sup>2</sup>, Yoshinobu Toda<sup>3</sup>, Toshiyuki Matsuoka<sup>4</sup>, Yasushi Okuno<sup>5</sup>, Gozoh Tsujimoto<sup>5</sup>, Shuh Narumiya<sup>4</sup>, and Osamu Ogawa<sup>1</sup>

## Abstract

More effective therapeutic approaches for castration-resistant prostate cancer (CRPC) are urgently needed, thus reinforcing the need to understand how prostate tumors progress to castration resistance. We have established a novel mouse xenograft model of prostate cancer, KUCaP-2, which expresses the wild-type androgen receptor (AR) and which produces the prostate-specific antigen (PSA). In this model, tumors regress soon after castration, but then reproducibly restore their ability to proliferate after 1 to 2 months without AR mutation, mimicking the clinical behavior of CRPC. In the present study, we used this model to identify novel therapeutic targets for CRPC. Evaluating tumor tissues at various stages by gene expression profiling, we discovered that the prostaglandin E receptor EP4 subtype (EP4) was significantly upregulated during progression to castration resistance. Immunohistochemical results of human prostate cancer tissues confirmed that EP4 expression was higher in CRPC compared with hormone-naïve prostate cancer. Ectopic overexpression of EP4 in LNCaP cells (LNCaP-EP4 cells) drove proliferation and PSA production in the absence of androgen supplementation *in vitro* and *in vivo*. Androgen-independent proliferation of LNCaP-EP4 cells was suppressed when AR expression was attenuated by RNA interference. Treatment of LNCaP-EP4 cells with a specific EP4 antagonist, ONO-AE3-208, decreased intracellular cyclic AMP levels, suppressed PSA production *in vitro*, and inhibited castration-resistant growth of LNCaP-EP4 or KUCaP-2 tumors *in vivo*. Our findings reveal that EP4 overexpression, via AR activation, supports an important mechanism for castration-resistant progression of prostate cancer. Furthermore, they prompt further evaluation of EP4 antagonists as a novel therapeutic modality to treat CRPC. *Cancer Res*; 70(4); 1606–15. ©2010 AACR.

## Introduction

Prostate cancer is one of the most frequently diagnosed cancers in the Western world (1). Because prostate cancer development is initially dependent on androgens, medical or surgical castration is the mainstay therapy for patients with advanced prostate cancer. However, most patients ultimately relapse after a period of initial response to this therapy, progressing to castration-resistant prostate cancer

(CRPC). Effective therapeutic approaches for CRPC are extremely limited. Treatment with docetaxel was established as a new standard of care for CRPC patients (2). However, it is not curative, and optimal timing of administration remains controversial. Consequently, it is highly desirable to explore new therapeutic strategies based on detailed molecular mechanisms for the development of castration resistance in prostate cancer.

The generation of suitable *in vivo* models is critical to better understand the processes associated with the development and progression of prostate cancer. We have previously reported a novel prostate cancer xenograft model named KUCaP-1 (previously referred to as KUCaP; ref. 3). KUCaP-1 tumors harbor the W741C mutant androgen receptor (AR), regress soon after castration in mice, and do not regrow with long-term follow-up (4). We have now established another novel xenograft model named KUCaP-2 using locally recurrent CRPC specimens derived from a different patient. The KUCaP-2 tumors harbor wild-type AR, regress soon after castration, and restore their ability to proliferate after 1 to 2 months without AR mutation. As the sequential changes of the xenograft resemble the clinical behavior of prostate cancer, this model may provide an excellent system to

**Authors' Affiliations:** <sup>1</sup>Department of Urology, Kyoto University Graduate School of Medicine; <sup>2</sup>Department of Diagnostic Pathology, Kyoto University Hospital; <sup>3</sup>Anatomical Center, Kyoto University Graduate School of Medicine; <sup>4</sup>Department of Pharmacology, Faculty of Medicine, Kyoto University; <sup>5</sup>Department of Genomic Drug Discovery Science, Kyoto University Graduate School of Pharmaceutical Sciences, Kyoto, Japan; <sup>6</sup>Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts; <sup>7</sup>Department of Urology, Miyazaki University, Miyazaki, Japan; and <sup>8</sup>Development Research Laboratories, Research Headquarters, Ono Pharmaceutical Co., Ltd., Osaka, Japan

**Corresponding Author:** Osamu Ogawa, Department of Urology, Kyoto University Graduate School of Medicine, 54, Shogoinkawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. Phone: 81-75-751-3325; Fax: 81-75-761-3441; E-mail: ogawao@kuhp.kyoto-u.ac.jp.

doi: 10.1158/0008-5472.CAN-09-2984

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study the mechanisms associated with castration-resistant progression of prostate cancer and to evaluate new treatment modalities for CRPC.

In KUCaP-2, prostaglandin E receptor EP4 subtype (EP4) expression significantly increased with the development of castration resistance. We explored the function of EP4 in prostate cancer cells as a potential target for the treatment of CRPC.

## Materials and Methods

**Generation of xenograft model.** Clinical materials were used after informed consent was obtained, according to protocols approved by the institutional review board at Kyoto University Hospital. All experiments involving laboratory animals were done in accordance with the Guideline for Animal Experiments of Kyoto University. Local recurrent tumors after radical prostatectomy were resected trans-urethrally, minced into 20 to 30 mm<sup>3</sup> tumor bits, and transplanted s.c. into 5-wk-old male nude mice (Charles River Japan) with 50 µL of Matrigel (Becton Dickinson) injected around the implant. The KUCaP-2 xenograft was established ~10 mo after the first inoculation. The xenograft tumors were extracted and transplanted to several mice without Matrigel. Ninety percent of the tumor was serially transplantable.

**Sequence analysis.** Genomic DNA from the xenograft tissue was extracted and all of the exons of the *AR* gene were sequenced as previously reported (3).

**Tissue sampling and DNA microarray analysis.** The mice bearing KUCaP-2 tumors were castrated and the sequential changes in tumor volume were analyzed as previously reported (3). Serum samples were obtained at sacrifice to measure prostate-specific antigen (PSA) values. Xenograft tissues of KUCaP-2 were collected during various stages and total RNA was isolated and purified using the RNeasy Mini Kit (Qiagen). Changes in gene expression were analyzed using DNA microarray analysis with an Affymetrix Human Genome U133 Plus2.0.

**Real-time PCR.** cDNA was synthesized from total RNA using a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech). Real-time PCR was performed using SYBR green PCR Master Mix (Applied Biosystems) and monitored using GeneAmp 5700 (Applied Biosystems) in triplicate. The thermal cycling conditions were 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The values were normalized to the levels of amplified glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequences of primers were as follows: EP4, 5'-GGAAATGACCAGGCCAAGAC-3' (sense) and 5'-CAACCCTGGACCTCACACCTA-3' (antisense); PSA, 5'-GGAAATGACCAAGGCCAAGAC-3' (sense) and 5'-CAACCCTGGACCTCACACCTA-3' (antisense); AR, 5'-CTTCACCAATGTCAACTCCA-3' (sense) and 5'-TCATTCGGACACACTGGCTG-3' (antisense); and GAPDH, 5'-GAATATAATCCCAAGCGGTTTG-3' (sense) and 5'-ACTTCACATCACAGCTCCCC-3' (antisense).

**Antibodies and reagents.** Anti-AR (C-19; sc-815) and anti-PSA (C-19; sc7638) antibodies were obtained from Santa Cruz Biotechnology. Anti-β-actin antibody (AC-15; ab6276) was purchased from Abcam. Anti-EP4 antibody (COOH terminus:

101775) for Western blotting was obtained from Cayman Chemical and anti-EP4 antibody (N terminus: LS-A3898) for immunohistochemistry was obtained from MBL International. The EP4-specific antagonist ONO-AE3-208 was provided by Ono Pharmaceutical Co. (5). 5α-Dihydrotestosterone was purchased from Sigma. Forskolin, an activator of adenylyl cyclase, and dibutyryl cyclic AMP (dbcAMP), a cAMP analogue, were purchased from Nacalai Tesque. H-89, a cAMP-dependent protein kinase (PKA) inhibitor, was obtained from Biomol International. An expression vector, pcDNA3.1-EP4, was constructed by inserting the cDNA of human EP4, digested from a cloning vector, pBluescript-EP4 (6), into *HindIII-BamHI* sites of pcDNA3.1(-). Vectors were transfected into the cells using Lipofectamine 2000 reagent (Invitrogen) and transfectants were selected by geneticin (Nacalai Tesque).

**Western blotting and immunohistochemistry.** Western blotting was performed with each primary antibody (AR, 1:400; PSA, 1:400; EP4 1:700; β-actin, 1:5,000) as previously reported (7). Immunohistochemistry was performed by standard indirect immunoperoxidase procedures using each primary antibody (AR, 1:100; PSA, 1:100; EP4 1:400), and the reaction was enhanced by microwave only in EP4 immunohistochemistry. Hormone-naïve prostate cancer (HNPC) tissues were derived from radical prostatectomy specimens of localized prostate cancer patients as tissue microarrays constructed as previously reported (8, 9). CRPC tissue samples were local tumors obtained from patients undergoing transurethral resection or autopsy. The expression intensity was graded as none, weak, moderate, and strong by a clinical pathologist (Y.M.) who was blind to the clinicopathologic data. The grading was determined based on the intensity of staining for at least 20% of the cancer cells.

**Cell culture.** The prostate cancer cell lines LNCaP, DU145, and PC3 were obtained from the American Type Culture Collection, passaged for fewer than 6 mo after resuscitation. The cells were routinely cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum. For androgen-depleted conditions, cells were cultured in phenol red-free RPMI 1640 (Invitrogen) supplemented with 10% charcoal-stripped fetal bovine serum (CSFBS; Hyclone). To analyze the cell proliferation *in vitro*,  $1.0 \times 10^5$  cells per well were seeded into six-well plates and grown for indicated days, and then cell numbers were counted in triplicate by a hemocytometer. For the assessment of *in vivo* tumor growth,  $0.5 \times 10^7$  to  $1.0 \times 10^7$  cells were inoculated with 100 µL Matrigel in the flank region of 5-wk-old male nude mice, and tumor volumes were measured once weekly.

**RNA interference.** AR knockdown was performed using stealth RNAi [stAR(1);HSS100620 and stAR(2);HSS100619] compared with control nonspecific stealth RNAi (stCtr;12935-400) purchased from Invitrogen. Cells were seeded at  $5.0 \times 10^5$  per well in six-well plates and incubated for 24 h. Each 160 pmol of stealth RNAi was transfected using Lipofectamine 2000 reagent.

**Luciferase assay.** Cells were seeded at  $1.5 \times 10^5$  per well in 24-well plates and were transiently cotransfected with 250 ng

of pcDNA3.1-EP4, 250 ng of pGL3-PSAp-Luc, and 5 ng of pTK-RL using Lipofectamine 2000 reagent. After 24 h of incubation, the medium was changed to create androgen-depleted conditions and the cells were incubated again for 24 h. The luciferase activity of the cell lysate was measured using the Dual-Luciferase Reporter Assay System (Promega) with a luminometer (MicroLumat Plus LB96V, Berthold Technologies) in triplicate.

**cAMP assay.** Cells were seeded at  $1.0 \times 10^5$  per well in 96-well plates and incubated for 24 h. Cells were washed once with PBS and cultured for 1 h in androgen-depleted conditions. The intracellular cAMP concentrations were assayed using the cAMP-EIA kit (RPN225; Amersham-Pharmacia Biotech) in duplicate. ONO-AE3-208 was added 10 min before the assay.

**Statistical analysis.** The data were expressed as mean  $\pm$  SD and their statistically significant differences were determined by one-way ANOVA. Age, serum PSA levels, Gleason sums, and tumor volumes were compared by the Mann-Whitney *U* test, and EP4 staining levels were compared by the  $\chi^2$  test. Statistical analyses were all performed using SPSS software.

## Results

**KUCaP-2 is an androgen-dependent prostate cancer xenograft harboring wild-type AR, producing PSA, and developing castration resistance without AR mutation.** Tumor tissues used for the establishment of KUCaP-2 were histologically diagnosed as prostate cancer based on positive AR and PSA immunohistochemistry staining (Fig. 1A). Western blotting analysis revealed that KUCaP-2 cells expressed AR and PSA (Fig. 1B). In mice, the KUCaP-2 tumor regressed soon after castration and reproducibly regrew after 1 to 2 months. Sequence analysis of *AR* in KUCaP-2 tumors before and after castration showed no *AR* mutation.

Xenograft tissues of KUCaP-2 were transplanted into 12 mice and collected during androgen-dependent growth (AD), castration-induced regression nadir (ND), and castration-resistant regrowth (CR) stages ( $n = 4$ , each; Fig. 1C). The tumor volumes were  $3,012 \pm 467$ ,  $562 \pm 208$ , and  $1,962 \pm 560$  mm<sup>3</sup>, and the median PSA values of the mice were 166.0, 4.0, and 50.9 ng/mL for the AD, ND, and CR stages, respectively. There was no histologic difference among KUCaP-2 tumors of each stage (Fig. 1D, a–c). The nuclear expression levels decreased from the AD stage to the ND stage, indicating that the depletion of circulating androgen suppressed nuclear expression of AR in KUCaP-2 tumors at the ND stage. Nuclear expression recovered at the CR stage to levels similar to those at the AD stage (Fig. 1D, d–f).

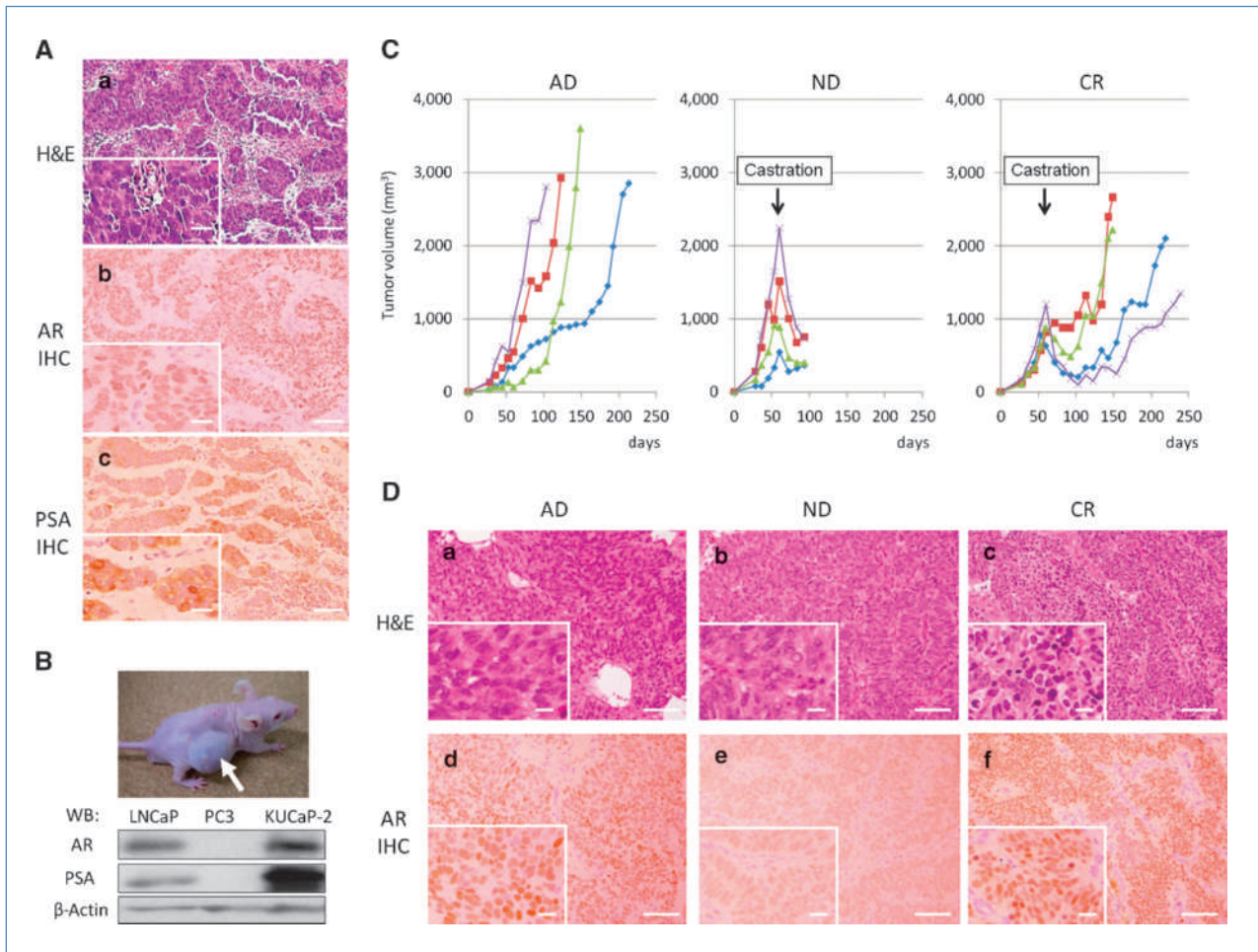
**EP4 expression was upregulated with the progression of castration resistance in KUCaP-2 tumors.** To elucidate the mechanisms responsible for the development of castration resistance, we evaluated the gene expression profiles of tumors at each stage using DNA microarray analyses. In total, for 2,476 genes, there was a significant difference ( $P < 0.05$ ) in expression between at least two stages. The *k*-means clustering ( $k = 10$ ) of these genes was performed to select candidate

genes (Fig. 2A). Previous reports on DNA microarray analysis in several different xenograft models showed that *AR* was the only gene consistently upregulated during castration-resistant progression (10). In our study, *AR* expression slightly increased from the AD stage to the ND stage (ratio = 2.7,  $P = 0.006$ ), with no difference between the ND and CR stages (ratio = 1.1,  $P = 0.280$ ). The *PSA* expression of tumors slightly and not significantly decreased from the AD stage to the ND stage (ratio = 0.6,  $P = 0.138$ ) and recovered at the CR stage. To find genes associated with castration resistance, we explored genes in the cluster whose expression levels were low in both the AD and ND stages but high in the CR stage. Among 111 genes in this cluster, the CR/ND ratio of *EP4* expression was the highest (ratio = 15.7,  $P = 0.029$ ; Table 1). These results were validated by real-time PCR analysis (Fig. 2B). Moreover, *EP4* expression was higher in the androgen-independent cell lines (DU145, PC3) compared with an androgen-dependent cell line (LNCaP; data not shown), consistent to other reports (11–13).

**EP4 expression was higher in clinical CRPC than in HNPC.** EP4 was mainly expressed in cellular membranes or in the cytoplasm of KUCaP-2 tumor cells, with more expression at the CR stage compared with the AD stage (Fig. 2C, a,b). Using KUCaP-2 samples from CR and AD stages as positive and negative controls, respectively, staining intensity of EP4 in clinical materials from 27 HNPC and 31 CRPC patients was graded (Fig. 2C, c–f). The characteristics of these patients were shown in Table 2. All the CRPC patients had PSA relapse. The serum PSA level and the Gleason sum were higher in CRPC than in HNPC. The EP4 expression level was significantly higher in CRPC than in HNPC ( $P = 0.0001$ ).

**EP4 overexpression induced castration-resistant progression of LNCaP cells through AR activation.** We examined whether EP4 overexpression induced castration-resistant progression using LNCaP. LNCaP cells were stably transfected with pcDNA3.1-EP4, and two monoclonal EP4-overexpressing LNCaP clones were established and named LNCaP-EP4(A) and LNCaP-EP4(B). The EP4 signal activates adenylate cyclase, which results in acceleration of the production of cAMP (14). The intracellular cAMP concentrations, PSA expression levels, and cell proliferation ratio of LNCaP-EP4 without androgen were higher compared with those in vector alone-transfected LNCaP (LNCaP-mock) cells, indicating that overexpressed EP4 protein activated adenylate cyclase and induced PSA expression and cell proliferation without androgen (Fig. 3A).

To examine whether AR activation is associated with androgen-independent PSA expression and cell proliferation in LNCaP-EP4, AR expression was attenuated using a stealth RNAi system. PSA expression without androgen was suppressed more significantly by the attenuation of AR in LNCaP-EP4 cells than in LNCaP-mock cells. Further, the suppression of androgen-independent cell proliferation was statistically significant in LNCaP-EP4 cells, but not in LNCaP-mock cells, indicating that AR activation was associated with androgen independence of LNCaP-EP4 cells (Fig. 3B). We examined the effect of EP4 on AR activation using



**Figure 1.** KUCaP-2, a novel established prostate cancer xenograft expressing AR and PSA, regresses after castration of mice and develops castration resistance with nuclear expression of AR. A, H&E staining (a), AR immunohistochemistry (IHC; b), and PSA immunohistochemistry (c) of tumor tissues used for the establishment of KUCaP-2. Scale bars, 50  $\mu$ m, inset 5  $\mu$ m. B, KUCaP-2 tumor (arrow, top) expressing AR and PSA detected with Western blotting (WB; LNCaP and PC3 presented as a positive and negative control; bottom). C, the sequential changes in xenograft tumor volume of KUCaP-2 before the tumor extraction at the AD, ND, and CR stages ( $n = 4$  each). D, H&E staining (a–c), AR immunohistochemistry (d–f) at AD, ND, and CR stages of tumors. Scale bars, 50  $\mu$ m, inset 5  $\mu$ m.

a luciferase reporter assay in LNCaP cells. EP4 signaling promoted AR activation without androgen to ~50% of the level achieved with androgen stimulation and did not induce additional AR activation in the presence of androgen. Then, we examined whether the increase in cAMP levels and the acceleration of PKA activity was associated with AR activation. PSA expression in LNCaP cells without androgen was induced by both forskolin and dbcAMP but was inhibited by H-89 (Fig. 3C). These results indicate that the signal activation of EP4-cAMP-PKA-AR axis is associated with the castration resistance of LNCaP cells.

The castration of mice decelerated xenograft tumor growth in LNCaP-mock cells but not in LNCaP-EP4 cells (Fig. 3D). The serum PSA values of castrated mice bearing LNCaP-EP4 xenografts were significantly higher than those of mice bearing LNCaP-mock xenografts (median PSA at sacrifice: 4.0 and 32.5 ng/mL in LNCaP-mock and LNCaP-EP4 cells, respectively,  $P < 0.05$ ). These results show that EP4

overexpression induces castration-resistant progression of LNCaP cells *in vivo*.

**EP4 antagonist suppressed castration-resistant progression of LNCaP-EP4 and KUCaP-2 tumors.** ONO-AE3-208 is an EP4-specific antagonist (5). The Ki values of ONO-AE3-208 for the prostanoid receptors are 1.3, 30, 790, and 2,400 nmol/L for EP4, EP3, FP, and TP, respectively, and >10,000 nmol/L for the other prostanoid receptors (15). To examine the EP4 antagonistic effect of ONO-AE3-208 on LNCaP-EP4 cells, intracellular cAMP concentrations were examined under a variety of ONO-AE3-208 concentrations in androgen-depleted conditions, indicating that 10 to 100 nmol/L of ONO-AE3-208 is sufficient to antagonize overexpressed EP4. This concentration of ONO-AE3-208 reached the Ki of EP3 and could also antagonize EP3. The EP3 signal inhibits adenylate cyclase, and thus the antagonism of EP3 increases intracellular cAMP concentrations (15). However, the suppression level of cAMP was proportional to the ONO-AE3-208 concentrations, suggesting

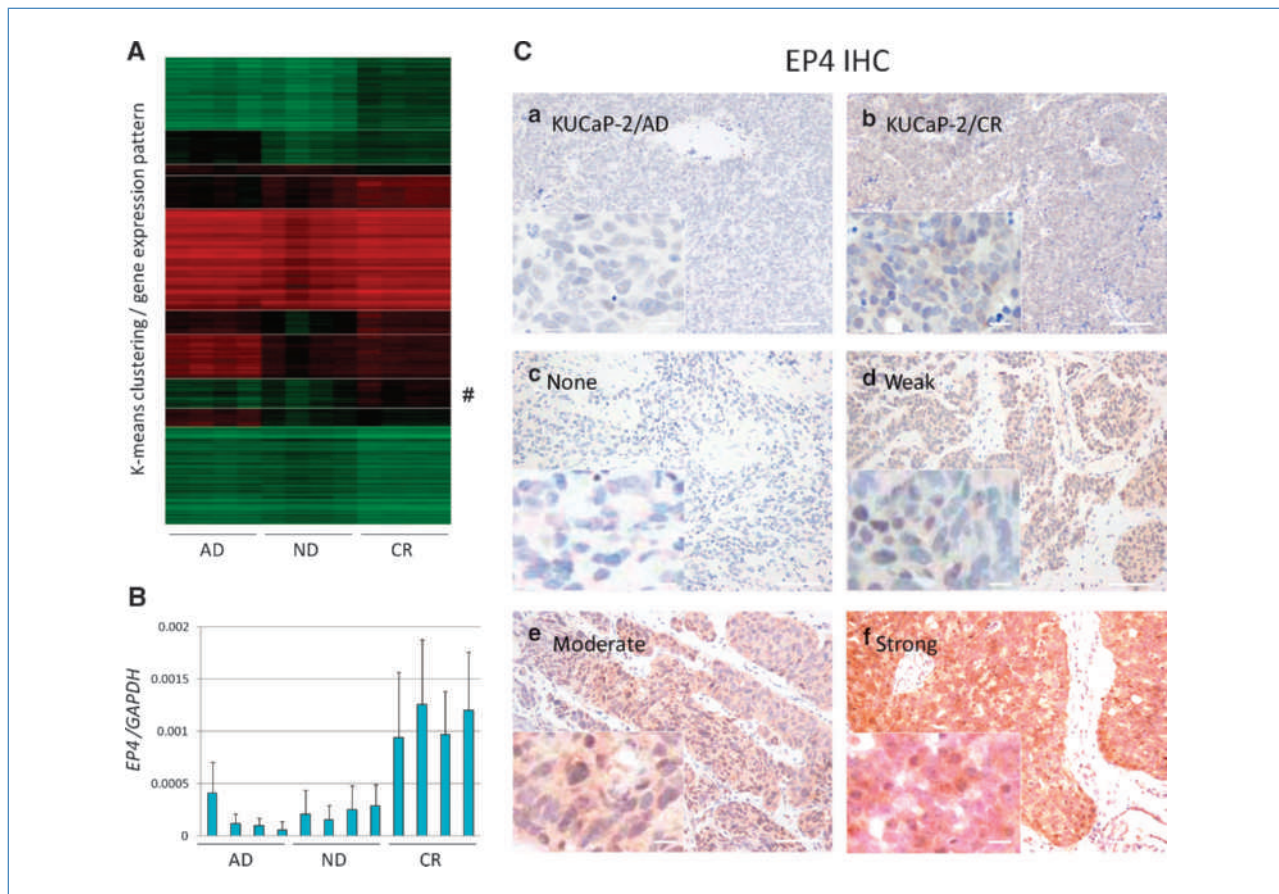
that antagonistic effect against EP3 might be slight. The PSA expression of LNCaP-EP4 cells without androgen was also suppressed by the same concentrations of ONO-AE3-208 (Fig. 4A).

We then examined the *in vivo* antitumor effect of ONO-AE3-208. I.p. injection of ONO-AE3-208 (10 mg/kg; once a day) suppressed the castration-resistant growth of LNCaP-EP4 xenograft tumors (Fig. 4B). The serum PSA values of LNCaP-EP4 xenograft mice were also significantly decreased (median PSA at sacrifice: 5.7 and 3.7 ng/mL in controls and AE3-208, respectively,  $P < 0.05$ ). The mean body weight of mice in the control and AE3-208 groups were almost the same, and no mice died during the treatment, indicating that ONO-AE3-208 was well tolerated at the concentrations used. The same dose of ONO-AE3-208 also suppressed the castration-resistant growth of KUCaP-2 tumors (Fig. 4C). The PSA production of KUCaP-2 tumors was significantly decreased (median PSA at sacrifice: 17.4 and 9.4 ng/mL in controls and AE3-208, respectively,  $P < 0.05$ ). There were no significant differences in EP4 expression between the tumors of the control and AE3-208 groups (data not shown), indicating that

ONO-AE3-208 antagonized EP4 without suppressing the receptor expression. In summary, EP4 antagonism with ONO-AE3-208 might be an effective and tolerable treatment modality for CRPC, in which EP4 overexpression induced castration-resistant progression (Fig. 4D).

### Discussion

As CRPC is a heterogeneous group of diseases (16), many experimental models are required to elucidate the mechanisms for castration resistance. However, limited tissue availability for molecular studies and few available human prostate cancer cell lines with both AR- and androgen-dependent states have restricted prostate cancer research. Xenografts are models in which human tissue is transplanted into an immunodeficient mouse. In this way, human prostate cancer can be propagated *in vivo* for long periods to allow the study of tumor progression under different experimental hormonal conditions and to support the testing of novel therapies. Before 1993, only one prostate cancer xenograft, LNCaP



**Figure 2.** EP4 expression was upregulated during the castration-resistant progression of KUCaP-2. A, the *k*-means clustering of the DNA microarray data for AD, ND, and CR stage tumors of KUCaP-2. The EP4 is in the cluster indicated with #. B, expression levels of EP4 validated by real-time PCR analysis. C, the EP4 immunohistochemistry of AD stage (a) and CR stage (b) tumors of KUCaP-2. The staining intensity of EP4 in clinical samples of prostate cancer patients graded as none (c), weak (d), moderate (e) and strong (f). Scale bars, 50  $\mu$ m, inset 5  $\mu$ m.

**Table 1.** Significantly upregulated genes at CR compared with AD and ND stages (10 highest CR/ND ratio)

Gene name (symbol)	CR/AD		CR/ND	
	Ratio	P	Ratio	P
Prostaglandin E receptor EP4 subtype (EP4)	15.7	0.01048	15.7	0.02903
Lumican (LUM)	23.6	0.00110	14.2	0.01095
Natriuretic peptide receptor C (NPRC)	2.3	0.19044	8.1	0.04921
N-methyl-D-aspartate 3A (GRIN3A)	9.1	0.00006	7.4	0.00029
Neurologin 1 (NLGN1)	7.9	0.01844	6.1	0.04429
Coiled-coil domain containing 68 (CCDC68)	7.4	0.00013	4.4	0.00460
Tissue factor pathway inhibitor (TFPI)	4.1	0.04105	4.2	0.04678
Secretoglobin, family 1D, member 2 (LIPB)	8.2	0.01950	3.8	0.06641
Nudix-type motif 11 (UNDT11)	4.0	0.00444	3.7	0.01913
Eukaryotic translation initiation factor 1A Y-linked (EIF1AY)	2.6	0.07360	3.3	0.04354

(17), had been reported to be androgen dependent. LNCaP tumors shrink slightly after castration, usually with less than a 10% reduction in volume, and regrow less than 5 weeks after castration. Thereafter, several androgen-dependent xenografts have also been established. The LAPC-4 (18), LuCaP-23 (19), and PC346P (20) xenograft models reportedly show a response to castration similar to that of LNCaP. The CWR22 (21) and LAPC-9 (22) models showed recurrent growth after androgen ablation after 3 to 6 months, which was similar to our established xenograft, KUCaP-2. Similar to these models that mimic the clinical behavior of prostate cancer, KUCaP-2 may provide an excellent system to study the mechanisms associated with the castration-resistant progression of prostate cancer and help us develop novel treatment modalities against CRPC.

Most androgen-dependent xenografts were derived from patients with CRPC, as seen in KUCaP-2, because of the difficulty to obtain enough samples from patients with HNPC. It was suggested that prostate cancer contain a heterogeneous mixture of cells that vary in their dependence on androgen for growth and survival, and that treatment with androgen

ablation therapy provides selective pressure and alters the relative concentration of these cells, thereby leading to the outgrowth of CRPC (22). These tumors presumably contain a mixture of growth-arrested, androgen-responsive tumor cells in addition to androgen-independent cells at the time of implantation into mice. In the androgenic environment of the intact male mouse, the androgen-responsive cells would gain a growth advantage and eventually develop into androgen-dependent xenografts.

The castration-resistant KUCaP-2 tumors expressed AR in their nuclei and produced PSA, suggesting that AR was activated with significantly low circulating androgen and is associated with the castration-resistant progression. Recent findings suggest that AR is an important transcription factor that mediates survival and proliferation signaling not only in HNPC but also in CRPC (23, 24). The androgen-independent activation of AR is mediated by several pathways (25, 26). The acquisition of mutations in *AR* is likely to be an important pathway (3, 27). However, KUCaP-2 harbors wild-type AR and progresses to castration resistance without AR mutation. Another possible pathway is its hypersensitivity to low levels

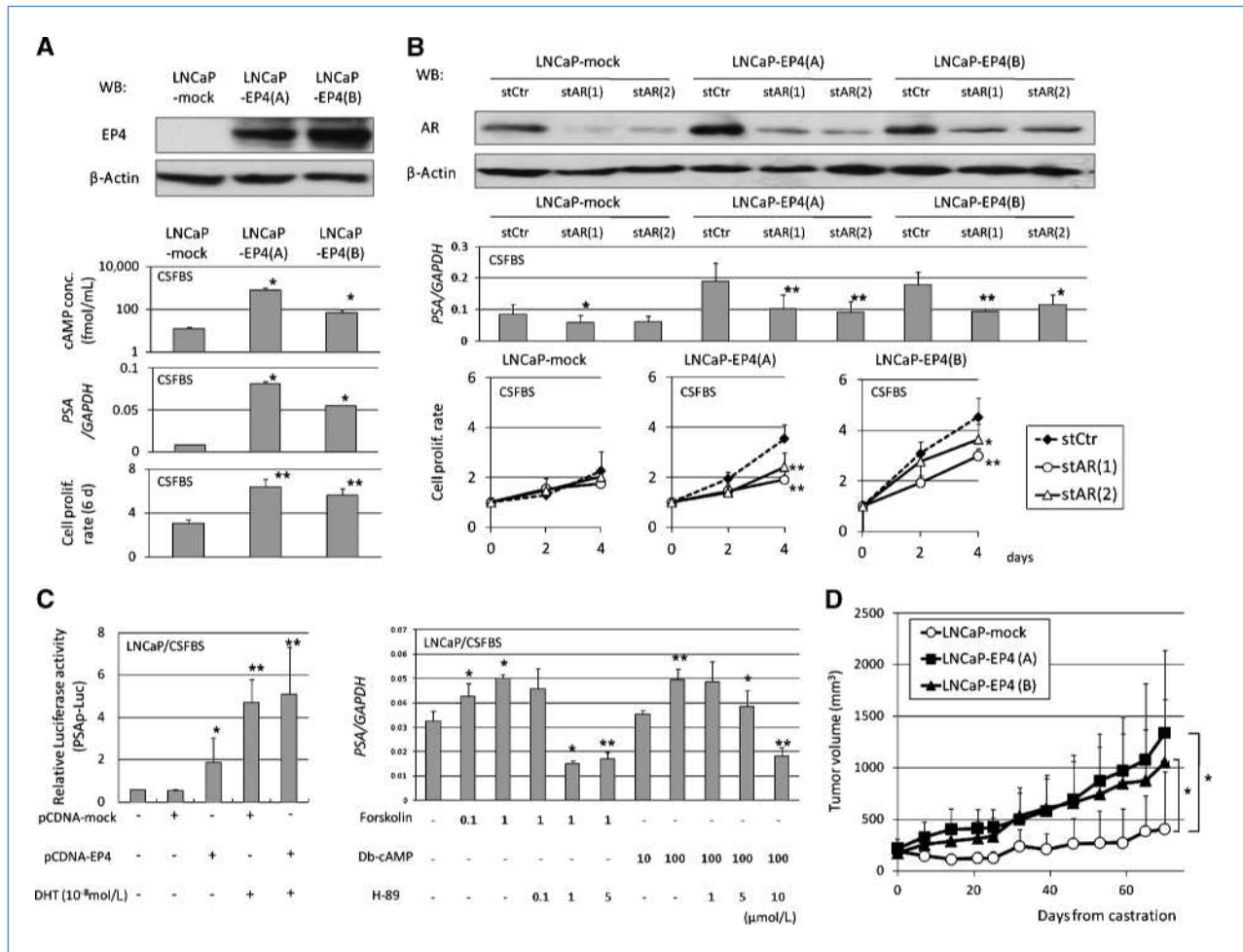
**Table 2.** Patient characteristics and EP4 staining grade in HNPC and CRPC

	HNPC	CRPC	P
Number	27	31	
Median serum PSA (ng/mL)	7.9 (3.8–31.3)	15.5 (0.5–949)	0.0066*
Median Gleason sum	7 (3–9)	9 (6–10)	0.0001*
EP4 staining grade <sup>†</sup>			0.0001 <sup>‡</sup>
None	10 (37.0%)	5 (16.1%)	
Weak	17 (63.0%)	9 (29.1%)	
Moderate	0 (0%)	10 (32.2%)	
Strong	0 (0%)	7 (22.6%)	

\*Mann-Whitney *U* test.

<sup>†</sup>The grading was determined on the intensity of staining for at least 20% of the cancer cells.

<sup>‡</sup> $\chi^2$  test between HNPC and CRPC.



**Figure 3.** Overexpression of EP4 with cAMP-PKA signal activation promoted the castration-resistant progression of LNCaP cells through AR activation. A, EP4 protein expression detected with Western blotting in LNCaP-EP4(A) and LNCaP-EP4(B) compared with LNCaP-mock (top), intracellular cAMP concentrations, PSA expression, and cell proliferation ratio versus day 0 in androgen-depleted conditions (CSFBS) for 6 d (bottom). \*,  $P < 0.05$ ; \*\*,  $P < 0.005$  versus LNCaP-mock. B, AR expression attenuated using a stealth RNAi system detected with Western blotting (top), PSA expression levels on day 4 (middle), and cell proliferation ratio on days 2 and 4 versus day 0 (bottom) in CSFBS. \*,  $P < 0.05$ ; \*\*,  $P < 0.005$  versus LNCaP-mock. C, relative luciferase activities of PSAP-Luc in LNCaP cells with transfection of pcDNA3.1-mock and pcDNA3.1-EP4 in CSFBS and 5 $\alpha$ -dihydrotestosterone (DHT) stimulation, respectively. \*,  $P < 0.05$ ; \*\*,  $P < 0.005$  versus control (left). PSA expression of LNCaP cells in CSFBS for 6 d under the administration of forskolin, dbcAMP, and H-89. \*,  $P < 0.05$ ; \*\*,  $P < 0.005$  versus preadministration (right). D, the sequential changes in xenograft tumor volume after the castration of mice ( $1 \times 10^7$  cells,  $n = 5$  each). \*,  $P < 0.05$ .

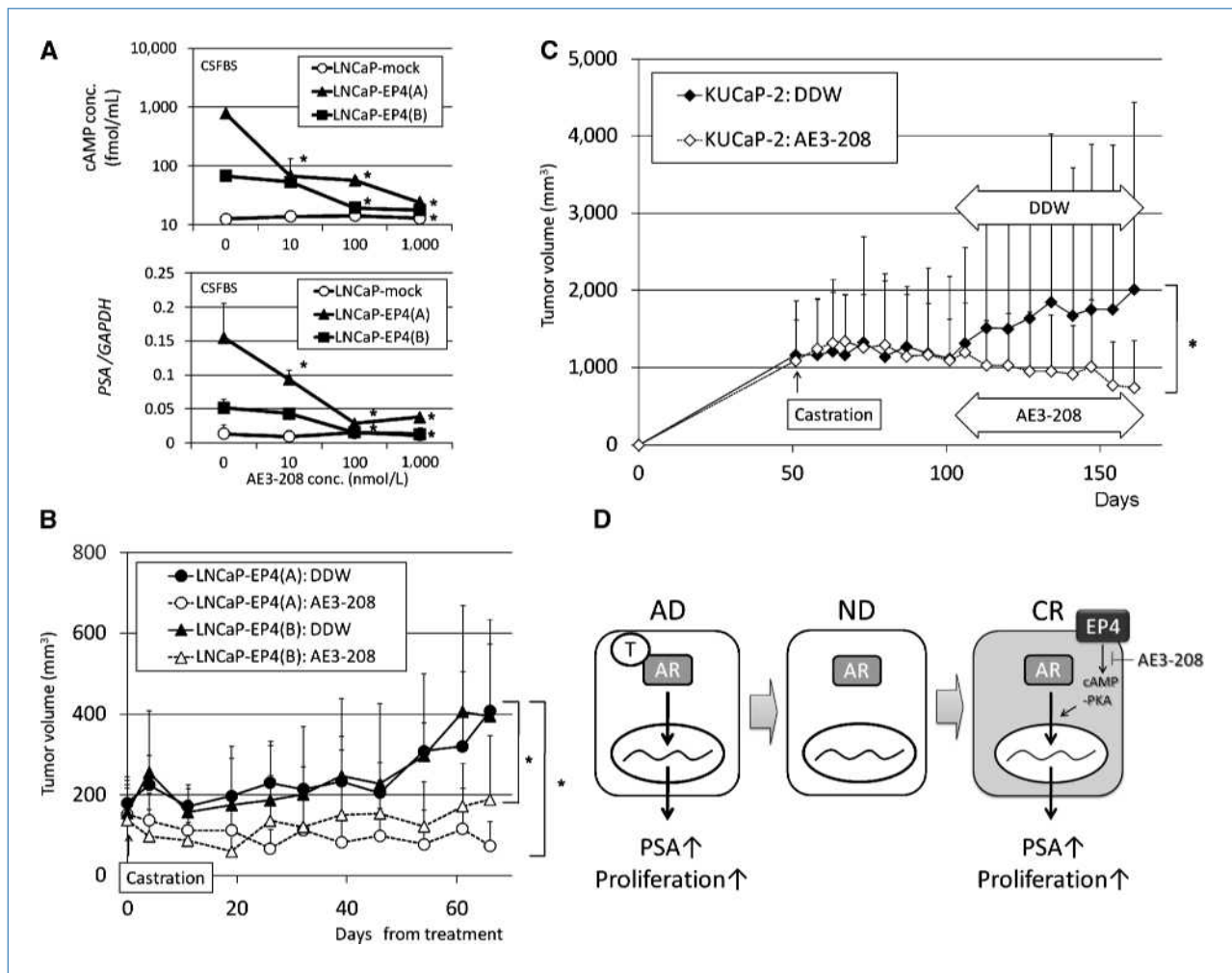
of androgens, induced by increased expression of the AR itself (10). In KUCaP-2, the *AR* mRNA expression slightly increased from the AD to ND and CR stages. However, the AR protein expression levels decreased at the ND stage and recovered at the CR stage to the same level as the AD stage both in the AR immunohistochemistry (Fig. 1D) and Western blotting (data not shown) analysis. One possible explanation for this discrepancy is that the AR protein might be degraded without androgen at ND stage and stabilized at the CR stage (28, 29). These results indicated that the upregulation of AR might merely be an adaptation of tumor cells to the condition of low androgen stimulation and not an essential indicator of castration-resistant progression. EP4 upregulation was observed during castration-resistant progression in KUCaP-2. EP4 expression was higher in clinical CRPC than

in HNPC. The xenograft of EP4-overexpressing LNCaP cells developed castration resistance through AR activation. These results revealed that EP4 upregulation might lead to AR activation, resulting in the castration-resistant progression of prostate cancer. It was reported that the activation of a membrane-localized G protein-coupled receptor induced nuclear partition and activation of AR through the accumulation of intracellular cAMP and PKA activation (30). As EP4 is a G protein-coupled receptor, our data showing that EP4-cAMP-PKA axis activates AR are consistent to the report.

EP4 is one of the prostaglandin E2 (PGE2) receptors. PGE2, the product of cyclooxygenase-2 (COX-2) conversion of plasma membrane phospholipids, is the most common prostanoid and is associated with inflammatory disease (14) and cancer (31, 32). It was suggested that inflammation plays a

role in prostate carcinogenesis (33, 34) and the regular consumption of non-steroidal anti-inflammatory drugs (NSAID) may reduce the risk of prostate cancer (35–37). Therefore, NSAIDs, including COX-2 inhibitors, have been tested in the treatment (38) and prevention (39) of prostate cancer. However, these approaches have met with limited success (40) and, sometimes, severe cardiovascular side effects (41), probably because COX-2 produces multiple products with pleiotropic effects in addition to PGE2. Therefore, targeting downstream signaling pathways of PGE2 may represent an attractive new strategy. There are four subtypes of PGE2 receptors, EP1 to EP4. The intracellular signaling differs among the receptor subtypes; EP1 is coupled to calcium mobilization, EP3 inhibits adenylate cyclase, and EP2 and EP4 stim-

ulate adenylate cyclase in various types of cells (42). The effects of PGE2 are dependent on the ligand concentration and the target cell receptor expression (32). Experimental studies have suggested that increased EP2 and EP4 expression is important during colorectal and prostate cancer progression (43, 44). In KUCaP-2, EP2 expression did not increase significantly during castration-resistant progression (data not shown), indicating that EP4 might be more strongly associated with castration resistance than EP2 in this model. To examine the association of PGE2 and cancer progression, the serum PGE2 concentrations of mice bearing KUCaP-2 were examined by PGE2 Express EIA kit (500141; Cayman Chemical). Unfortunately, reproducible results could not be obtained, probably because of the instability of PGE2.



**Figure 4.** Castration-resistant progression of LNCaP-EP4 and KUCaP-2 was suppressed by ONO-AE3-208 treatment. A, the cAMP concentration (top) and PSA expression (bottom) of LNCaP-mock, LNCaP-EP4(A), and LNCaP-EP4(B) under the *in vitro* administration of ONO-AE3-208 in CSFBS. \*,  $P < 0.05$  versus LNCaP-mock. B, the sequential changes in LNCaP-EP4(A) and LNCaP-EP4(B) xenograft tumors treated with i.p. injection of 200  $\mu$ L/d distilled water (DDW) and 10 mg/kg/d ONO-AE3-208 (AE3-208) started soon after the castration of mice and continued for 70 d ( $0.5 \times 10^7$  cells,  $n = 5$  each). \*,  $P < 0.05$ . C, the sequential changes in KUCaP-2 tumors treated with the same volume of DDW and AE3-208 solution started 50 d after castration and continued for 60 d ( $n = 5$  each). \*,  $P < 0.05$ . D, schematic representation of the relationship between EP4 overexpression and castration resistance. After castration, the upregulated EP4 induces activation of the AR without androgen and promotes castration-resistant cell proliferation and PSA production, which is suppressed by ONO-AE3-208 administration. T, androgen.



Therefore, it might be difficult to examine the serum PGE2 concentrations in clinical samples. The secreted PGE2 concentrations in LNCaP-EP4 cells were higher than in LNCaP-mock cells [ $20.3 \pm 15.4$ ,  $48.7 \pm 4.9$ , and  $44.7 \pm 11.5$  pg/mL in LNCaP-mock, LNCaP-EP4(A), and LNCaP-EP4(B), respectively]. However, the administration of PGE2 into LNCaP-EP4 could not induce cell proliferation and PSA production. To elucidate the association of PGE2 and cancer progression needs further examinations.

The cell proliferation of LNCaP-EP4 was significantly higher than that of LNCaP-mock under androgen-depleted medium but not under normal medium (data not shown), indicating that EP4 overexpression enhanced androgen-independent but not androgen-dependent proliferation of LNCaP cells *in vitro*. However, the *in vivo* tumor growth of LNCaP-EP4 was significantly higher than that of LNCaP-mock in intact mice ( $193 \pm 76$  and  $121 \pm 46$  mm<sup>3</sup> on day 30, respectively,  $P = 0.003$ ). Moreover, the xenograft tumor take-up rate of LNCaP-EP4 was higher than that of LNCaP-mock (~100% and 60%, respectively). It was reported that PGE2 regulated angiogenesis in PC3 cells through EP2 and EP4 (44). Therefore, it was suggested that EP4 overexpression might increase cell proliferation of LNCaP cells *in vivo* through angiogenesis. The PSA expression of LNCaP-EP4 was significantly higher than that of LNCaP-mock under androgen-depleted medium. Although EP4 expression was higher in LNCaP-EP4(B) than in LNCaP-EP4(A), the intracellular cAMP concentration and PSA expression levels were higher in LNCaP-EP4(A) than in LNCaP-EP4(B). To investigate the reasons for the discrepancy, we performed transient transfection analyses with several different amounts of pcDNA3.1-EP4. The PSA expression levels were correlated with EP4 expression levels in LNCaP cells transiently transfected with the EP4 expression vector, suggesting that the reasons for this discrepancy might be clonal variations of LNCaP-EP4 (data not shown). The expression of TMPRSS2, one of other AR-regulated genes (45), was also increased with EP4 overexpression and decreased by the attenuation of AR (data not shown). These results indicated that EP4 may increase PSA expression partly in an AR-dependent manner; however, we do not exclude possibilities that EP4 increases PSA and

TMPRSS2 expressions through an AR-independent manner. Analysis of these mechanisms needs further investigations.

It was suggested that the EP4-cAMP-PKA axis can activate the  $\beta$ -catenin/TCF signaling pathway, leading to cancer progression (46), and that the EP4-specific antagonist, ONO-AE3-208, inhibits the progression of EP4-expressing colorectal cancer (47, 48). The present study is the first report showing that ONO-AE3-208 reduces the castration-resistant progression of prostate cancer cells induced by EP4 overexpression. ONO-AE3-208 did not suppress the proliferation of KUCaP-2 in intact mice (data not shown), suggesting that EP4 antagonism might have no antitumor effect against HNPC. DU145 and PC3 are AR-negative prostate cancer cells with high EP4 expression. The *in vitro* administration of 100 nmol/L ONO-AE3-208 decreased intracellular cAMP concentrations of these cells. However, it did not suppress their cell proliferation *in vitro* and *in vivo* (data not shown). It was suggested that the EP4-cAMP-PKA axis might not be associated with their cell proliferation.

In conclusion, we found that EP4 overexpression is one of the mechanisms responsible for progression to CRPC using a novel xenograft model KUCaP-2. The administration of EP4 antagonist *in vivo* suppressed the castration-resistant progression of KUCaP-2, indicating that EP4 may be a potential target for the treatment of CRPC.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Acknowledgments

We thank Tomoko Kobayashi and Megumi Kishida for their valuable technical assistance.

#### Grant Support

Grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Received 8/11/09; revised 11/30/09; accepted 12/2/09; published OnlineFirst 2/9/10.

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