



# Human castration resistant prostate cancer rather prefer to decreased $5\alpha$ -reductase activity

Takeo Kosaka, Akira Miyajima, Hirohiko Nagata, Takahiro Maeda, Eiji Kikuchi & Mototsugu Oya

Department of Urology, Keio University School of Medicine, Shinjuku-ku, Tokyo 160-8582, Japan.

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Correspondence and  
requests for materials  
should be addressed to  
A.M. (akiram@a8.  
keio.jp)

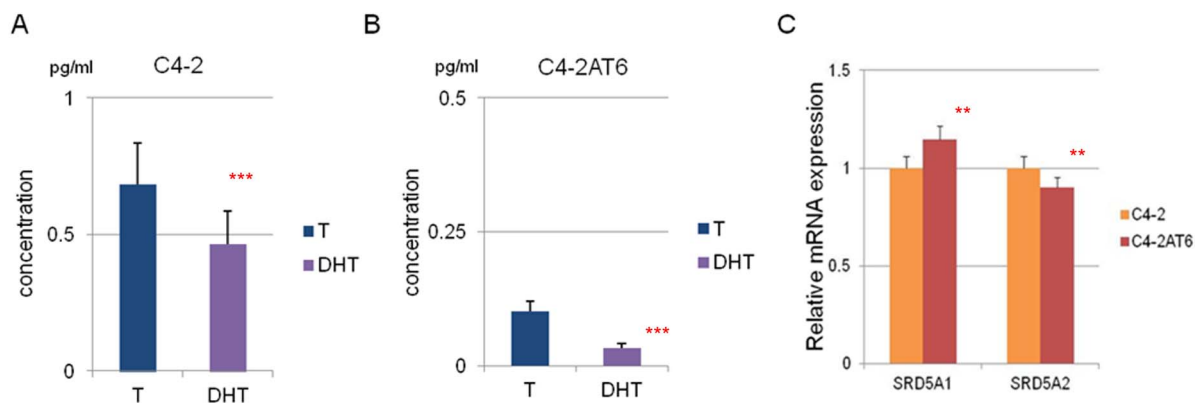
Physiologically relevant steroid  $5\alpha$ -reductase (SRD5A) activity that is essential for dihydrotestosterone (DHT) biosynthesis in human castration-resistant prostate cancer (CRPC) has not been fully characterized yet. In this study to ascertain the potential SRD5A activity, we cultured two human CRPC cell lines, C4-2 and C4-2AT6, with the steroid precursor:  $^{13}\text{C}$ -[2,3,4]-androstenedione (13C-Adione), and analyzed the sequential biosynthesis of  $^{13}\text{C}$ -[2,3,4]-testosterone (13C-T) and  $^{13}\text{C}$ -[2,3,4]-DHT (13C-DHT) by liquid chromatography/mass spectrometry (LC/MS/MS). The 13C-DHT/13C-T concentration ratio detected by LC/MS/MS in C4-2AT6 cells appeared to reflect the SRD5A activity. The ratio in C4-2AT6 was significantly lower than that in C4-2. An increased concentration of DHT did not have a positive effect on cell proliferation, rather it exhibited inhibitory effects.  $5\alpha$ -reductase inhibitors did not have any inhibitory effect at clinically achievable concentrations. These results indicate that CRPC cells may have an unknown regulation system to protect themselves from an androgenic suppressive effect mediated by SRD5A activity.

Prostate cancer (PCa) is one of the most commonly diagnosed malignant tumors in men and the second leading cause of cancer-related deaths in the United States<sup>1</sup>. Androgen ablation is the gold standard treatment for advanced PCa. One of the most troublesome aspects of PCa is that androgen-dependent PCa inevitably progresses to highly aggressive and life-threatening castration-resistant prostate cancer (CRPC) after androgen ablation therapy<sup>2</sup>. Recurrent tumors frequently express androgen receptor (AR) target genes<sup>3</sup>, such as prostate-specific antigen (PSA), and about 30% of patients with progressive disease respond to additional hormonal manipulations<sup>4,5</sup>. These findings suggest that many recurrent prostate cancers are neither hormone refractory nor androgen independent, but maintain a clinically relevant reliance on the AR signaling axis.

More recently, intratumoral conversion of adrenal androgens and *de novo* steroid synthesis have been proposed as potential causes of PCa progression<sup>6,7</sup>. The reported high intratumoral testosterone and dihydrotestosterone (DHT) concentrations in CRPC patients with castrated serum androgen levels also suggested that CRPC maintains a clinically relevant reliance on the AR signaling axis. Androgen receptor activation by androgens converted from adrenal androgens or synthesized intratumorally via the *de novo* route has been proposed as one of the mechanisms of castration resistance<sup>7-11</sup>. However, DHT production by PCa has not been fully characterized yet<sup>12,13</sup>. Although  $5\alpha$ -reductase (5AR), which is essential for DHT biosynthesis, has been detected at the mRNA level in CRPC metastases<sup>9-11,14</sup>, physiologically relevant 5AR activity has not been fully demonstrated in human CRPC yet. Recent advances have shed light on the relationship between androgens and the development or the progression of PCa<sup>15-18</sup>. The use of  $5\alpha$ -reductase inhibitors (5ARI) to prevent progression of Pca, continues to be widely discussed<sup>17-19</sup>. Does progression to CRPC depend on DHT produced by  $5\alpha$ -reductase? Is it effective to treat CRPC using 5ARIs? The effects of finasteride or dutasteride on metastatic prostate cancer or progression to CRPC have not yet been evaluated.

We have previously reported a useful model of human CRPC<sup>20-25</sup>. Briefly, we cultured the PTEN-null, androgen receptor (AR) positive, PSA producing CRPC cell line C4-2 for more than 6 months under androgen ablation conditions and named it C4-2AT6. These cells harbor the following characteristics: aggressive angiogenic properties, and elevated phosphorylated Akt expression. These two cell lines may reproduce the aspect of clinical CRPC progression and offer an excellent model system with which to study their complicated biology.

In this study, we sought to determine whether there was physiologically relevant SRD5As activity in human CRPC cell lines C4-2 and C4-2AT6. To ascertain the potential of SRD5As activity, we developed a co-culture system using the steroid precursor  $\text{C}^{13}$ -[2,3,4]-progesterone with C4-2 and C4-2AT6 cells. We



**Figure 1 | Detection of T and DHT in CRPC cells by LC/MS/MS analysis.** (A) T and DHT concentrations in the supernatant of C4-2 incubated for 6 hrs \*\*\*  $p < 0.001$ . (B) T and DHT concentrations in the supernatant of C4-2AT6 incubated for 6 hrs \*\*\*  $p < 0.001$ . (C) The mRNA expression of SRD5A1 and SRD5A2 in C4-2 and C4-2AT6 cells. \*\*  $p < 0.01$

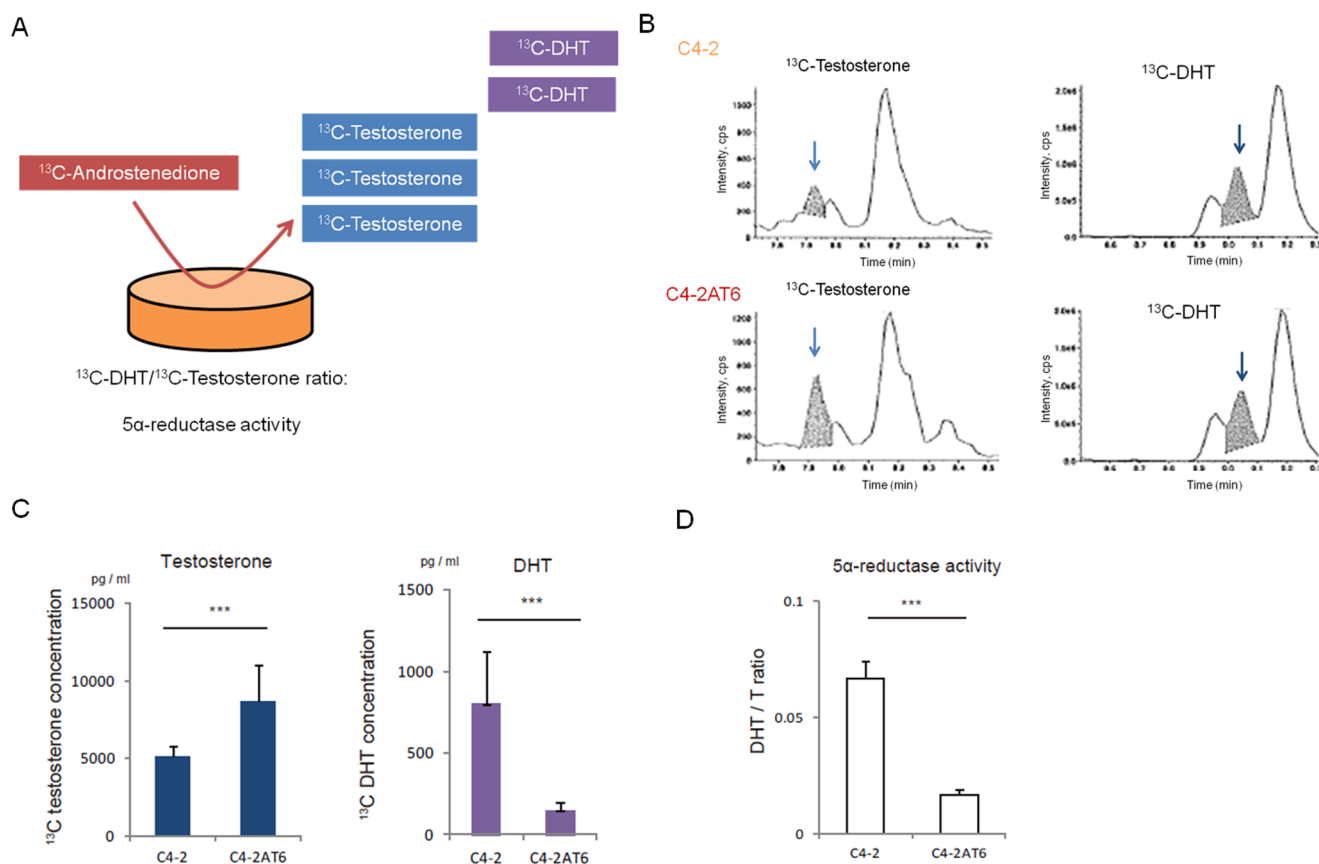
analyzed the sequential biosynthesis of androgens from each  $C^{13}$ -precursor and found direct evidence of reduced biosynthesis of DHT in CRPC.

## Results

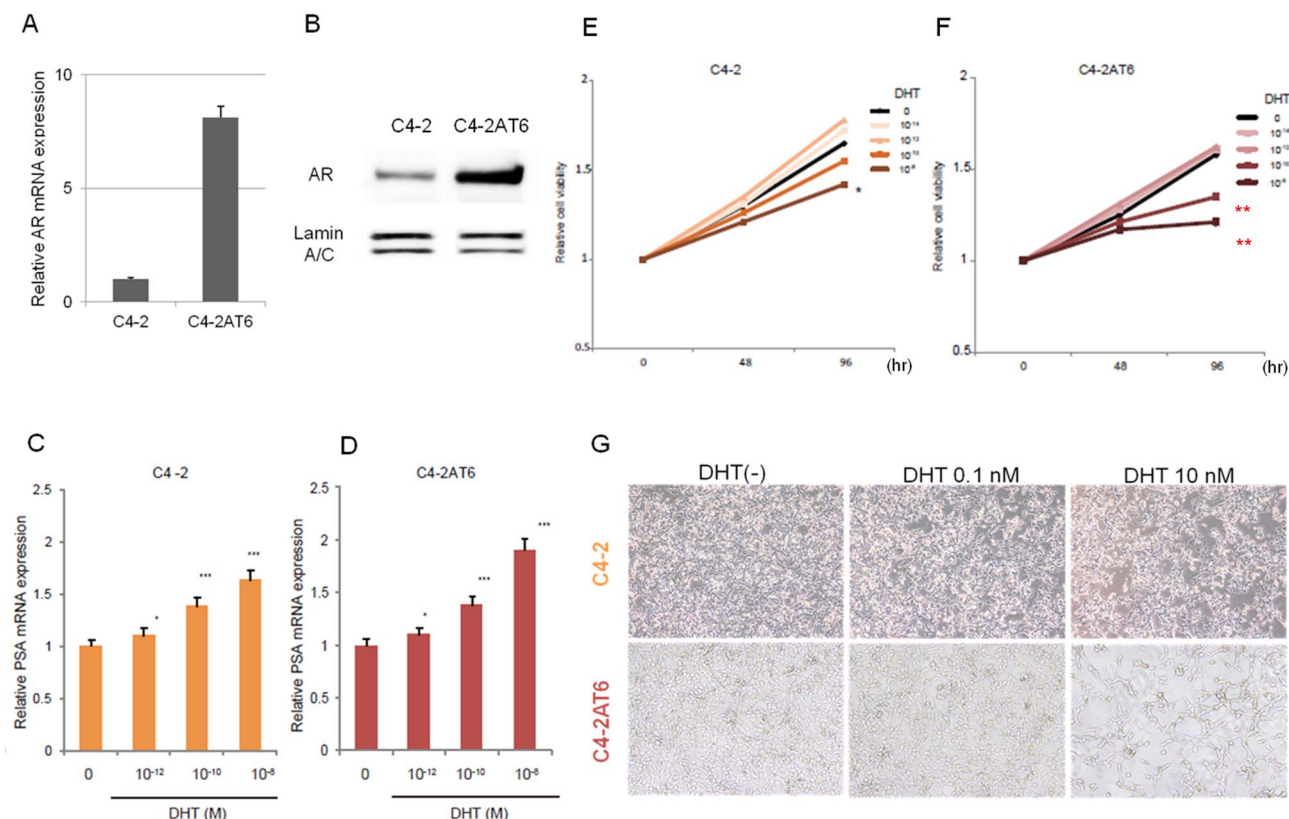
**Concentration of androgens in the supernatant of C4-2 and C4-2AT6 cells.** To determine whether prostate cancer cells have the ability to *de novo* synthesize androgen (Fig. 1A), we investigated the concentrations of testosterone (T) and dihydrotestosterone (DHT) in the supernatant of C4-2 and C4-2AT6 cells for 6 hr by LC/MS/MS analysis (Fig. 1B). In C4-2 cells, the concentration of T

and DHT was  $0.68 \pm 0.12$  and  $0.46 \pm 0.17$  pg/mL, respectively. In C4-2AT6 cells, the concentration of T and DHT was  $0.101 \pm 0.01$  and  $0.033 \pm 0.002$  pg/mL, respectively. These results indicated that in C4-2 and C4-2AT6 cells  $5\alpha$ -reductase was active.

**SRD5A1 and SRD5A2 mRNA expression in human castration-resistant prostate cancer cell lines.** We quantified and compared transcripts of SRD5A1 and SRD5A2. SRD5A is a  $5\alpha$ -reductase essential for DHT biosynthesis. Compared with C4-2 cells, quantitative PCR (qPCR) for C4-2AT6 cells showed  $1.3 \pm 0.2$  fold increases of the expression of SRD5A1 (Fig. 1C,  $p < 0.01$ ). qPCR for



**Figure 2 | (A)** Simplified schematic representation of the co-culture system with  $^{13}C$  steroid precursors. **(B)** Typical selected ion recordings of the  $^{13}C$ -T and  $^{13}C$ -DHT extracted from cultured medium. **(C)**  $^{13}C$ -T and  $^{13}C$ -DHT concentrations in the supernatant of C4-2 and C4-2AT6 cells. \*\*\*  $p < 0.001$ . **(D)** DHT/T ratio appeared to reflect 5AR activity in cancer cells. C4-2AT6 cells exhibited significantly reduced 5AR activity, compared to C4-2.



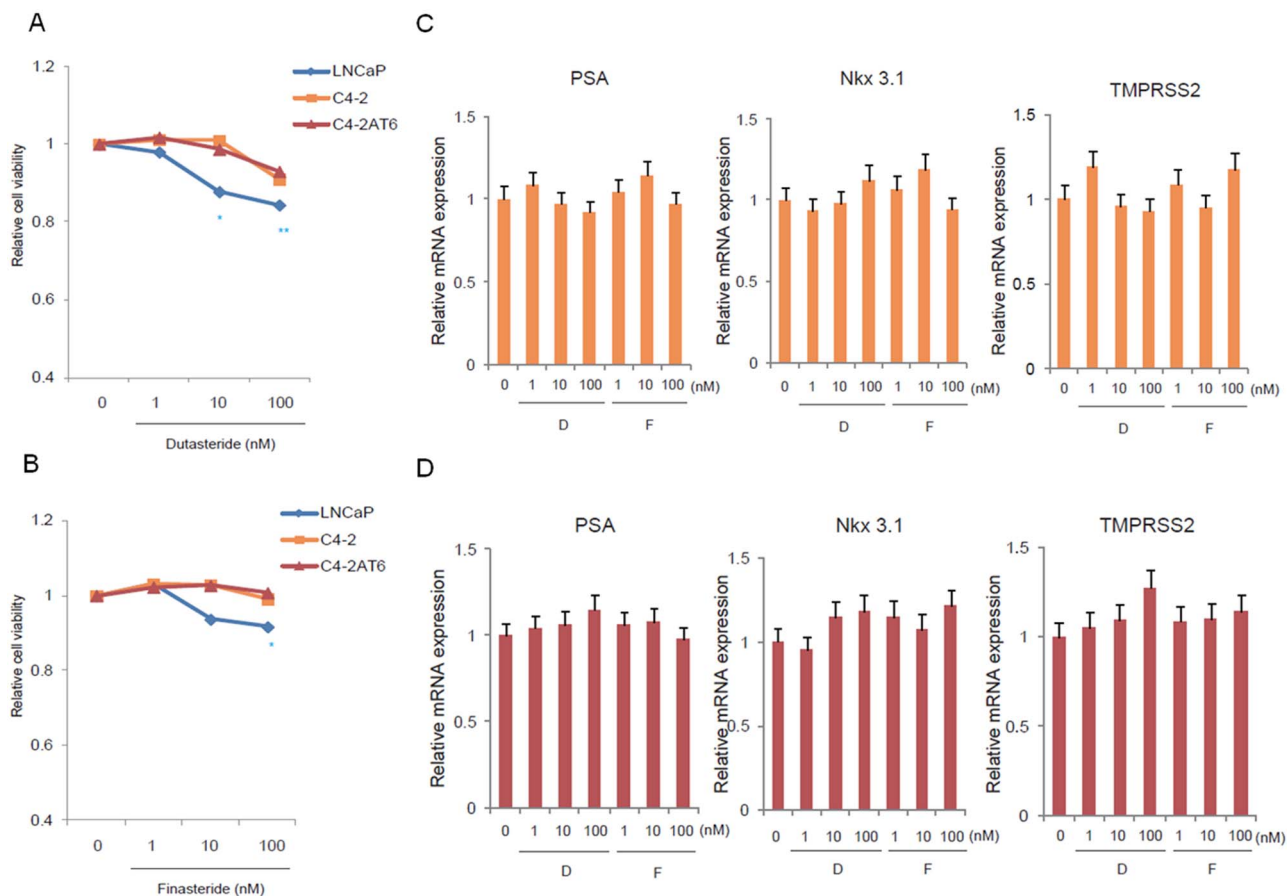
**Figure 3 | At high concentrations DHT exhibited inhibitory effects on C4-2AT6 cells.** (A) mRNA expression of AR in C4-2 and C4-2AT6 cells. \*\*\*  $p < 0.001$ . (B) Western blot analysis of AR expression in the nucleus in C4-2 and C4-2AT6 cells. (C) mRNA expression of PSA in C4-2 cells increased in a dose-dependent manner. (D) mRNA expression of PSA in C4-2AT6 cells increased in a dose-dependent manner. \* $p < 0.05$ , \*\*\*  $p < 0.001$ . (E) WST cell viability assay exhibited no significant proliferative response to DHT at  $10^{-14}$  M to  $10^{-12}$  M DHT concentration in C4-2 cells. DHT treatment at a concentration of  $10^{-8}$  M resulted in significant decreased cell viability. \* $p < 0.05$ . (F) When treated with  $10^{-10}$  M DHT or  $10^{-8}$  M DHT, C4-2AT6 cells showed significant decreased cell viability compared with that at lower DHT concentrations. \* $p < 0.01$ . (G) Response to DHT in C4-2 and C4-2AT6 cells detected by phase contrast field.

C4-2AT6 cells showed reduced SRD5A2 mRNA expression in C4-2AT6 cells compared to C4-2 cells (Fig. 1C,  $p < 0.01$ ).

**Comparison of 5 $\alpha$ -reductase enzyme activities using  $^{13}\text{C}$ -[2,3,4]-androstenedione (13C- Adione).** Although 5 $\alpha$ -reductase includes type-1 and type-2 5 $\alpha$ -reductase<sup>26</sup>, the actual enzymatic activity in CRPC has not been elucidated yet<sup>27</sup>. To determine the activity of SRD5As, we developed a co-culture system with the  $\text{C}^{13}$  steroid precursor  $^{13}\text{C}$ -[2,3,4]-androstenedione(13C-Adione). We cultured C4-2 cells with 13C-Adione for 6 hr and examined the conversion ratio of 13C-Adione to  $^{13}\text{C}$ -[2,3,4]-testosterone (13C-T), as well as the concentration of  $^{13}\text{C}$ -[2,3,4]-dihydrotestosterone (13C-DHT) in the cultured supernatant (Fig. 2A). To compare the activity of SRD5A in CRPC cells, we estimated the concentration ratio of DHT/T (DHT/T ratio), which appeared to reflect 5AR activity in cancer cells.

13C-T and 13C-DHT were detectable by LC/MS/MS (Fig. 2B). In C4-2 cells, the concentrations of 13C-T and 13C-DHT were  $5104 \pm 703$  and  $799 \pm 321$  pg/mL; thus the DHT/T ratio was  $0.157 \pm 0.026$  (Fig. 2C). In contrast, in C4-2AT6 cells, the concentrations of 13C-T and 13C-DHT were  $8707 \pm 2283$  pg/mL and  $151 \pm 48.5$  pg/mL, respectively, and the DHT/T ratio was  $0.017 \pm 0.002$  (Fig. 2C). The concentration of 13C-T was significantly higher in C4-2 than in C4-2AT6 ( $p < 0.01$ ), and the concentration of 13C-DHT was significantly higher in C4-2AT6 than that in C4-2 (Fig. 2D,  $p < 0.01$ ). In C4-2AT6 cells, the DHT/T ratio was significantly higher than that in C4-2 ( $p < 0.01$ ). These results indicated that both C4-2 and C4-2AT6 cells expressed direct 5 $\alpha$ -reductase activity, and the activity was significantly higher in C4-2AT6 cells than in C4-2 cells.

**Response to DHT in C4-2 and C4-2AT6 cells.** The reduced DHT/T ratio and 5 $\alpha$ -reductase activity may reflect the decreased dependence of C4-2AT6 cells proliferation on DHT, compared with C4-2 cells. To investigate the response to DHT in C4-2 and C4-2AT6 cells, we determined AR expression at the mRNA (Fig. 3A) and protein levels (Fig. 3B). C4-2AT6 cells showed an 8.1 fold increase of AR mRNA expression, accompanied by AR protein expression in the nucleus. Next, we investigated the expression of AR target gene: PSA was determined by qPCR at different concentrations of DHT at 12 h (Fig. 3C, D). The mRNA expression of PSA in C4-2 and C4-2AT6 cells increased in a dose-dependent manner. To determine whether C4-2 or C4-2AT6 cells show a proliferative response to DHT, we investigated and compared the viability of cancer cells treated with DHT at various concentrations for 96 h (Fig. 3E, F, G). C4-2 cells showed no significantly response to DHT when treated for 96 h at  $10^{-14}$  M to  $10^{-12}$  M DHT (Fig. 3E, G,  $p < 0.05$ ). When treated with higher concentrations of  $10^{-8}$  M DHT, C4-2 cells showed significantly decreased cell viability compared with that at lower DHT concentrations (Fig. 3E, G). In C4-2AT6 cells, treatment with  $10^{-8}$  M DHT showed significantly decreased cell viability compared with that at lower concentrations of DHT (Fig. 3F, G). When treated with  $10^{-10}$  M DHT, C4-2AT6 cells showed significant decreased cell viability compared with that at lower DHT concentrations (Fig. 3F, G,  $p < 0.05$ ). Increasing the concentration of DHT did not have a positive effect on cell proliferation, rather the inhibitory effects on C4-2AT6 cells were more marked than those on C4-2 cells treated with the same DHT concentration (Fig. 3G).



**Figure 4** | Efficacy of 5ARIs assessed using PCA cell lines. The efficacy of 5ARIs in prostate cancer cells was assayed by incubation with dutasteride (A, C) or finasteride (B, D) at various concentrations for 96 h. In LNCaP cells, dutasteride and finasteride exhibited significant inhibitory actions within the clinically achievable 5ARI concentration of 10 nM. In C4-2 and C4-2AT6 cells, neither dutasteride nor finasteride inhibited cell viability at the same concentrations. \* $p < 0.05$ , \*\* $p < 0.01$ . (C, D) qPCR analysis of PSA, Nkx3.1, TMPRSS2 in C4-2 (C) and C4-2AT6 (D) after treated with 5ARIs. Dutasteride and finasteride did not show a significant effect on these AR target genes. (D) Dutasteride, (F) Finasteride.

**Is it effective to treat CRPC using 5 $\alpha$ -reductase inhibitors?** To determine whether inhibition of 5AR activity with 5-ARIs: dutasteride or finasteride, showed some efficacy in C4-2 or C4-2AT6 cells, we investigated cell viability in cells treated with various concentrations of 5-ARIs for 96 h (Fig. 4A, B). It has been reported that 5ARIs exhibit some inhibitory actions in LNCaP cells<sup>28–30</sup>, therefore, we used the LNCaP cell line as a control in our experiments to compare the effects of 5ARIs on prostate cancer cell lines. As previously shown, dutasteride or finasteride exhibited significant inhibitory actions in LNCaP cells within the clinically achievable 5ARI concentration of 10 nM. On the other hand, these two 5ARIs did not have inhibitory effects on C4-2 and C4-2AT6 cells at clinically achievable 5ARI concentrations of <100 nM. To investigate the expression of AR target gene in response to finasteride and dutasteride in C4-2 and C4-2AT6 cells, we determined the expression of PSA, Nkx3.1 and TMPRSS2 by qPCR at different concentrations (Fig. 4C, D). qPCR analysis revealed 5ARIs had no effect on these AR target genes in C4-2 and C4-2AT6 cells.

## Discussion

In this study, we developed a co-culture system with C<sup>13</sup> steroid precursors to obtain direct evidence of 5 $\alpha$ -reductase activity in CRPC cells. 13C-T and 13C-DHT were detected by LC/MS/MS. The activity of 5 $\alpha$ -reductase changed in CRPC cells under androgen ablation. This is the first report showing direct evidence of changes in 5AR enzyme activity in CRPC cells. Moreover, we showed that the

capacity of DHT to influence the proliferation of CRPC is limited, probably due to saturation effects at very low concentrations.

Studies in CRPC cancer tissue have measured intraprostatic testosterone or the active metabolite DHT in quantities sufficient to stimulate AR-mediated gene expression<sup>12,13,19</sup>. AR activation by androgens converted from adrenal androgens or synthesized intratumorally via the *de novo* route has been proposed as one of the mechanisms of castration resistance<sup>7–11</sup>. It has been reported that men with a Gleason score of >7 had lower intraprostatic dihydrotestosterone (DHT) than men with a Gleason score of <6, raising the possibility that a low-androgen environment predisposes men to development of high-grade PCa<sup>31–33</sup>. Although 5AR, which is essential for DHT biosynthesis, was detected at the mRNA level in CRPC metastases<sup>9–11,14</sup>, physiologically relevant 5AR activity in human CRPC has not yet been fully demonstrated. In this study, to ascertain potential 5AR activity, we co-cultured C4-2 and C4-2AT6 cells with the C<sup>13</sup> steroid precursor 13C-Adione. We analyzed the sequential biosynthesis of the androgens 13C-T and 13C-DHT, and obtained direct evidence of *de novo* sequential biosynthesis of androgens in CRPC, C4-2 and C4-2AT6 cells found to express 5AR activity. C4-2AT6 cells showed lower 5AR activities than C4-2 cells, although C4-2AT6 cells showed significantly higher SRD5A1 mRNA expression. These results indicated that 5AR activity changed under androgen ablation in CRPC cells and 5AR activity was not necessarily paralleled by SRD5As mRNA expression. To determine whether dutasteride and finasteride have the ability to inhibit the conversion into DHT in CRPC cells, we investigated the concentration of 13C-DHT after treatment with these 5ARIs. LC/MS/MS analysis was not





able to detect 13C-DHT in C4-2 and C4-2AT6 cells (data not shown). These results indicate 5ARIs were able to inhibit the conversion into 13C-DHT in C4-2 and C4-2AT6 cells, although the 5ARIs did not have an anti-proliferative effect.

Recent advances have shed light on the relationship between androgens and the development or the progression of PCA<sup>15–18</sup>. The use of 5AR inhibitors to prevent progression of PCA continues to be widely discussed. Discussion has been fueled by the findings of two large randomized, placebo-controlled trials: the Prostate Cancer Prevention Trial (PCPT) with finasteride<sup>34</sup> and the Reduction by Dutasteride of Prostate Cancer Events (REDUCE) trial<sup>35</sup>. The PCPT trial was the first large-scale study to investigate the role of finasteride in the context of prostate cancer development. Tumors found in patients treated with finasteride were of a higher grade than the tumors in those administered a placebo. Gleason scores between 7 and 10 were found in 6.4% of the tumors in the finasteride group, while in only 5.1% of those in the placebo group. The REDUCE trial showed an overall reduction in the number of tumors with a Gleason score of 5–6 in patients receiving dutasteride versus those given a placebo (19.9% compared to 25.1%, respectively). However, during 3 and 4 year periods, tumors with a Gleason score of 8–10 were more frequent in the dutasteride-treated group than in the placebo group. The FDA reanalyzed these two major trials and cited the fact that the absolute incidence of tumors with Gleason scores between 8 and 10 was increased by 0.7% with finasteride and by 0.5% with dutasteride. In December 2010, the US Food and Drug Administration's Oncologic Drugs Advisory Committee voted against recommending 5-ARI for the indication to reduce PCA risk, because the risk of more aggressive tumors outweighed their potential for chemoprevention<sup>17</sup>. These observations still cannot be fully explained. It may also be possible that finasteride or dutasteride has little or no effect on more aggressive tumors with high Gleason scores. Whether 5ARI increases the rates of high-grade disease remains a matter of debate. The decision by FDA not to approve the use of 5ARIs to prevent prostate cancer indicates that further basic and clinical investigations exploring the role of 5-ARI in the development and progression of PCA are warranted.

The reduced 5-AR activity we observed in C4-2AT6 cells raised a critical question: Does the viability of C4-2AT6 cells depend on DHT? Thus, we investigated the effects of DHT on human CRPC cell proliferation. C4-2 and C4-2AT6 cells exhibited reduced cell viability when treated with DHT. C4-2AT6 cells exhibit elevated and functional AR expression and produce PSA in response to DHT in a dose-dependent manner; however, C4-2AT6 cells showed significantly lower cell viability at the same concentration of DHT than C4-2 cells. The suppressive effect of DHT on PCA cells is not limited to C4-2 or C4-2AT6 cells. Some reports showed that CRPC could be treated with androgens due to the inhibitory action of excess androgens<sup>36–40</sup>. Accumulating evidence has suggested that AR has a finite ability to bind to T or DHT and that at higher concentrations T or DHT has no further effect on prostate growth when all ARs are bound to T or DHT<sup>36–40</sup>. It has been proposed this be termed the saturation point. Due to this saturation point, excess DHT may result in the suppression of androgenic-induced proliferation of C4-2AT6 cells. CRPC cells may have an unknown regulation system to protect themselves from the androgenic suppressive effect mediated by 5AR activity.

In this study, we clearly showed reduced DHT dependence, accompanied by reduced 5 $\alpha$ -reductase activity in CRPC cells. Moreover neither finasteride nor dutasteride showed a positive effect on CRPC cells. These results may provide the grounds for debate about 5ARI in PCA.

## Methods

**Cell lines and culture.** C4-2 cells were obtained from UroCor (Oklahoma City, OK). C4-2 cells were routinely maintained in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. C4-2AT6 cells were established from C4-2 as previously reported<sup>24</sup>. Briefly, C4-2 cells were grown in RPMI-1640 containing 10% charcoal stripped fetal bovine serum at

37°C in a humidified 5% CO<sub>2</sub> atmosphere. Cells were passaged upon attaining confluence during a 6 month period. We named this cell line C4-2AT6; that is, C4-2 cells subjected to androgen ablated treatment for 6 months.

**Chemicals.** <sup>13</sup>C-[2,3,4]-androstenedione (13C-Andro) was purchased from Hayashi Pure Chemical Inc., Ltd. (Osaka, Japan) and CDN Isotope (Quebec, Canada). Bond Elut C<sub>18</sub> cartridge was purchased from Varian Medical Systems KK (Tokyo), and 4-dimethylaminopyridine (DAP), 2-methyl-6-nitrobenzoic anhydride (MNBAn), and picolinic acid (PA) were from Tokyo Kasei Industry. Triethylamine (TEA) was from Wako Pure Chemical Industries (Osaka). Cadenza CD C-18 column (250 mm × 3 mm I.D., 3 μm) and CAPCELL PAK SCX UG80 pre-column (35 mm × 2 mm I.D., 5 μm) were from Intact (Kyoto, Japan) and Shiseido (Tokyo), respectively.

**LC-ESI-MS/MS.** For the measurement of T and DHT in cultured medium, an API-4000 triple stage quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) connected to Agilent 1100 (Agilent Technologies), HTC-PAL (CTC Analytical), and an ESI ion source were employed, as previously shown<sup>41</sup>. The column was a Cadenza CD-C<sub>18</sub> column (150 mm × 3 mm I.D., particle size 3 μm) and used at 40°C. The mobile phase consisted of 0.1% formic acid (Solvent A) and methanol: acetonitrile (1 : 1, Solvent B). For gradient elution, A/B was used at 20/80 to 10/90 between 0 to 10 min, 0/100 between 10 and 12.5 min, and 0/100 to 20/80 between 12.5 and 15 min. The flow rate was 0.4 mL/min. The following ESI conditions were used: spray voltage, 5,000 V; collision gas, nitrogen, 45 psi; curtain gas, 11 psi; ion source temperature, 450°C; and ion polarity, positive. For the quantification of <sup>13</sup>C-[2,3,4]-testosterone (13C-T) and <sup>13</sup>C-[2,3,4]-dihydrotestosterone (13C-DHT) transitions were at m/z 290/112, and 334/111, respectively. Preparation of the derivatization reagent for picolinyl ester derivatives was prepared as follows: Ten milligrams of DAP, 20 mg of MNBAn, and 25 mg of PA were dissolved into 1 mL of tetrahydrofuran (THF), and then agitated. After 10 min, the reagent solution was used. Pretreatment of the cultured medium: The cultured medium was mixed with 13C-DHEA (1000 pg) as the internal standard (IS) and diethyl ether (4 mL). The organic layer was separated after the aqueous phase became frozen, and the organic layer was then evaporated to dryness at 40°C under N<sub>2</sub> gas. The residue was dissolved in 20% methanol-water and the solution was applied onto the Bond Elut C<sub>18</sub> cartridge column that had been pre-conditioned with methanol and purified water. The cartridge was washed with purified water, and then with 30% acetonitrile solution. Subsequently, the desired substances were eluted with 80% acetonitrile solution. The purified extracts were dissolved in the reagent mixture prepared as described above. TEA was added to this mixture, and the resulting mixture was allowed to stand at room temperature for 30 min. After dilution of the reaction mixture with 1% acetic acid solution to stop the reaction, the resulting mixture was loaded onto the Bond Elut C<sub>18</sub> cartridge that had been pre-conditioned with methanol and purified water. After the cartridge was washed with water and 40% acetonitrile solution, derivatives were eluted with 80% acetonitrile solution. After the solvent was evaporated to dryness using a centrifugation evaporator at 53–55°C, the residue was dissolved in 40% acetonitrile solution, and a 20 μL-aliquote of the solution was subjected to LC-ESI-MS/MS. A series of treated samples (200 μL) spiked with authentic 13C-T or 13C-DHT at concentrations of 1.0, 5.0, 10, 50, 100, or 1,000 pg, and with chlormadione (CHM, 1000 pg) and 13C-DHEA (1000 pg) as the internal standard were prepared. A calibration curve was obtained for 13C-T or 13C-DHT by assigning the concentration of 13C-T or 13C-DHT to x and the peak area ratio of the PA derivatives of 13C-T or 13C-DHT to that of the corresponding chlormadione to y. Subsequently, a 1/x weighting linear regression was performed to construct the calibration curve. To prepare QC samples, 13C-T or 13C-DHT standard solution at 20, 100, and 800 pg were similarly added to purified water with chlormadione. The calibration curves of individual compounds showed good linearity with correlation coefficients (r) of more than 0.998–0.999. Intra-day assay accuracy and precision were evaluated by measuring multiple replicates (n = 5) of QC samples. The accuracy and precision of 13C-T or 13C-DHT at mid level were 2–13%, respectively, while the inter-day accuracy and precision at mid level, were 88–108% and 2–15%, respectively.

**Real-time quantitative PCR.** Total RNA was isolated using RNeasy Mini kit (Qiagen, Hilden, Germany), and the quantity and quality were evaluated by spectrophotometry. Reverse transcription of RNA to cDNA was done using High Capacity cDNA Archive Kit (Applied Biosystems). The reaction mixture (1 μL) was then used as a template in a TaqMan Fast real-time quantitative PCR assay using Taqman Universal PCR Master Mix and the 7500 Fast Real-time PCR system (Applied Biosystems). The primers and TaqMan probe sets (TaqMan Gene Expression Assays) for SRD5A1(Hs00971643\_g1), SRD5A2(Hs00165843\_m1), PSA(Hs02576345\_m1), Nkx3.1(Hs00171834\_m1), TMPRSS2(Hs01120965\_m1) and human GAPDH endogenous control (Hs99999903\_m1) were purchased from Applied Biosystems (sequences not disclosed). The cycling conditions were 50°C for 10 minutes, 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute.

**Statistics.** Experiments were carried out in two or more replicates and statistical analysis was performed by Student's t test. P values < 0.05 were considered significant.

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## Author contributions

T.K. performed a significant amount of the experimental work. A.M. and M.O. supervised the project. T.K. and A.M. wrote the manuscript. H.N., T.M., and E.K. prepared the figures.

## Additional information

**Competing financial interests:** The authors declare no competing financial interests.

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