Human castration resistant prostate cancer rather prefer to decreased 5α-reductase activity

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Physiologically relevant steroid 5α-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: 13C-[2,3,4]-androstenedione (13C-Adione), and analyzed the sequential biosynthesis of 13C-[2,3,4]-testosterone (13C-T) and 13C-[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. 5α-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. 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. Recurrent tumors frequently express androgen receptor (AR) target genes, such as prostate-specific antigen (PSA), and about 30% of patients with progressive disease respond to additional hormonal manipulations. 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. 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. However, DHT production by PCa has not been fully characterized yet. Although 5α-reductase (5AR), which is essential for DHT biosynthesis, has been detected at the mRNA level in CRPC metastases, 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. The use of 5α-reductase inhibitors (5ARI) to prevent progression of PCa, continues to be widely discussed. Does progression to CRPC depend on DHT produced by 5α-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. 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 SRD5A activity in human CRPC cell lines C4-2 and C4-2AT6. To ascertain the potential of SRD5A activity, we developed a co-culture system using the steroid precursor C13-[2,3,4]-progesterone with C4-2 and C4-2AT6 cells. We...
analyzed the sequential biosynthesis of androgens from each C13-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 ± 0.12 and 0.46 ± 0.17 pg/mL, respectively. In C4-2AT6 cells, the concentration of T and DHT was 0.101 ± 0.01 and 0.033 ± 0.002 pg/mL, respectively. These results indicated that in C4-2 and C4-2AT6 cells 5α-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 5α-reductase essential for DHT biosynthesis. Compared with C4-2 cells, quantitative PCR (qPCR) for C4-2AT6 cells showed 1.3 ± 0.2 fold increases of the expression of SRD5A1(Fig. 1C, p < 0.01). qPCR for

**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

**Figure 2** | (A) Simplified schematic representation of the co-culture system with 13C steroid precursors. (B) Typical selected ion recordings of the 13C-T and 13C-DHT extracted from cultured medium. (C) 13C-T and 13C-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.
C4-2AT6 cells showed reduced SRD5A2 mRNA expression in C4-2AT6 cells compared to C4-2 cells (Fig. 1C, p < 0.01).

Comparison of 5α-reductase enzyme activities using 13C-[2,3,4]-androstenedione (13C-Adione). Although 5α-reductase includes type-1 and type-2 5α-reductase, the actual enzymatic activity in CRPC has not been elucidated yet. To determine the activity of SRD5As, we developed a co-culture system with the C13 steroid precursor 13C-[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 13C-[2,3,4]-testosterone (13C-T), as well as the concentration of 13C-[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 ± 6703 and 799 ± 321 pg/mL; thus the DHT/T ratio was 0.157 ± 0.026 (Fig. 2C). In contrast, in C4-2AT6 cells, the concentrations of 13C-T and 13C-DHT were 8707 ± 2283 pg/mL and 151 ± 48.5 pg/mL, respectively, and the DHT/T ratio was 0.017 ± 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 in C4-2 (p < 0.01). These results indicated that both C4-2 and C4-2AT6 cells expressed direct 5α-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 5α-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. 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).
Is it effective to treat CRPC using 5α-reductase inhibitors? To determine whether inhibition of 5αR 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 cells28–30, 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 over 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 C13 steroid precursors to obtain direct evidence of 5α-reductase activity in CRPC cells. 13C-T and 13C-DHT were detected by LC/MS/MS. The activity of 5α-reductase changed in CRPC cells under androgen ablation. This is the first report showing direct evidence of changes in 5αR 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 expression12,13,19. 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 resistance7–11. 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 PCa31–33. Although 5AR, which is essential for DHT biosynthesis, was detected at the mRNA level in CRPC metastases9–11,14, 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 C13 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
C4-2 cells were routinely maintained in RPMI-1640 (Invitrogen, Carlsbad, CA). These results may provide the grounds for debate about 5ARI in PCa. ther finasteride nor dutasteride showed a positive effect on CRPC cells.

SCIENTIFIC REPORTS

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% CO2. C4-2AT6 cells were established from C4-2 as previously reported6. Briefly, C4-2 cells were grown in RPMI-1640 containing 10% charcoal stripped fetal bovine serum at 37°C in a humidified 5% CO2 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. 13C-[2,3,4]-androstenedione (13C-Andro) was purchased from Hayashi Pure Chemical Ind., Ltd. (Osaka, Japan) and CDN Isotope (Quebec, Canada). Bond Elut C18 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 x 3 mm ID, 3μm μm), and CAPCELL PAK SCX UG80 pre-column (35 mm x 2 mm ID, 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-400 triple stage quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) connected to Agilent 1100 (Agilent Technologies, FTC-PAL (CTC Analytics), and an ES-I ion source was employed. A 13C-DHEA (1000 pg) as the internal standard (IS) and diethyl ether (4 mL). The cell lysates or 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 1x deionized water solution to stop the reaction, the resulting mixture was loaded onto the Bond Elut C18 cartridge column that had been pre-conditioned with methanol and water. The cartridge was washed with purified water, and then with 30% acetonitrile solution. The purified extracts were eluted with 80% acetonitrile solution. After the solvent was evaporated to dryness using a centrifugation evaporator at 35–55°C, the residue was dissolved in 40% acetonitrile solution, and a 20 μL aliquot of the solution was subjected to LC-ESI-MS/MS analysis. Subsequently, the desired substances were eluted with 80% acetonitrile solution.

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 (Hs00395685_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.

References:
1. Jemal, A. et al. Cancer statistics, 2009. CA Cancer J Clin 59, 225–249 (2009).
2. Scher, H. I. & Sawyers, C. L. Biology of progressive, castration-resistance prostate cancer: directed therapies targeting the androgen-receptor signaling axis. J Clin Oncol 23, 8253–8261 (2005).

Methods

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3. Chen, Y., Sawyers, C. L. & Scher, H. I. Targeting the androgen receptor pathway in prostate cancer. *Curr Opin Pharmacol* 8, 440–448 (2008).

4. Lam, J. S., Leppert, J. T., Vemulapalli, S. N., Shvarts, O. & Belldegrun, A. S. Secondary hormonal therapy for advanced prostate cancer. *J Urol* 175, 27–34 (2006).

5. Ryan, C. J. & Small, E. J. Role of secondary hormonal therapy in the management of recurrent prostate cancer. *Urology* 62 Suppl 1, 87–94 (2003).

6. Montgomery, R. B. et al. Maintenance of intratumoral androgens in metastatic prostate cancer: a mechanism for castration-resistant tumor growth. *Cancer Res* 68, (2008).

7. Stanbrough, M. et al. Increased expression of genes converting adrenal androgens to testosterone in androgen-independent prostate cancer. *Cancer Res* 66, 2815–2825 (2006).

8. Mostaghel, E. A., Montgomery, B. & Nelson, P. S. Castration-resistant prostate cancer: targeting androgen metabolic pathways in recurrent disease. *Urol Oncol* 27, 251–257 (2009).

9. Thomas, L. N. et al. Type 1 and type 2 1α,25-dihydroxyvitamin D3 receptor expression in the development and progression of prostate cancer. *Eur Urol* 53, 244–252 (2008).

10. Thomas, L. N. et al. Levels ofts-1 and type 2 are increased in localized high grade compared to low grade prostate cancer. *J Urol* 179, 147–151 (2008).

11. Thomas, L. N. et al. Differential alterations in 1α,25-dihydroxyvitamin D3 receptor type 1 and type 2 levels during development and progression of prostate cancer. *Prostate* 63, 231–239 (2005).

12. Locke, J. A. et al. Steroidogenesis inhibitors alter but do not eliminate androgen synthesis mechanisms during progression to castration-resistance in LNCaP prostate xenografts. *J Steroid Biochem Mol Biol* 115, 126–136 (2009).

13. Locke, J. A. et al. Androgen levels increase by intratumoral de novo steroidogenesis during progression of castration-resistant prostate cancer. *Cancer Res* 68, 6407–6415 (2008).

14. Rainey, W. E. & Nakamura, Y. Regulation of the adrenal androgen biosynthesis. *J Steroid Biochem Mol Biol* 108, 281–286 (2008).

15. Morgentaler, A. & Traish, A. M. Shifting the paradigm of testosterone and prostate cancer: the saturation model and the limits of androgen-dependent growth. *Eur Urol* 55, 310–320 (2009).

16. Morgentaler, A. Testosterone and prostate cancer: an historical perspective on a modern myth. *Eur Urol* 50, 935–939 (2006).

17. Theoret, M. R. et al. The risks and benefits of 1α,25-dihydroxyvitamin D3 receptor inhibitors for prostate-cancer prevention. *N Engl J Med* 365, 97–99 (2011).

18. Walsh, P. C. Chemoprevention of prostate cancer. *N Engl J Med* 362, 1237–1238 (2010).

19. Parker, C. What (if anything) to do about low-risk prostate cancer. *Lancet* 379, (2012).

20. Shirotate, S. et al. Regulation of monocyte chemotactic protein-1 through angiotensin II type 1 receptor in prostate cancer. *Am J Pathol* 180, 1008–1016 (2012).

21. Kosaka, T. et al. Long-term androgen ablation and docetaxel up-regulate phosphorylated Akt in castration resistant prostate cancer. *J Urol* 185, 2376–2381 (2011).

22. Hasegawa, M. et al. Low-dose docetaxel enhances the sensitivity of 5-1 in a xenograft model of human castration resistant prostate cancer. *Int J Cancer* 130, 431–442 (2012).

23. Kosaka, T., Miyajima, A., Shirotate, S., Kikuchi, E. & Oya, M. Phosphorylated Akt up-regulates angiotensin II type-1 receptor expression in castration resistant prostate cancer. *Prostate* (2011).

24. Kosaka, T. et al. Ets-1 and hypoxia inducible factor-1α inhibition by angiotensin II type 1 receptor blockade in hormone refractory prostate cancer. *Prostate* 70, 162–169 (2010).

25. Kosaka, T. et al. Angiotensin II type 1 receptor antagonist as an angiogenic inhibitor in prostate cancer. *Prostate* 67, 41–49 (2007).

26. Dillard, P. R., Lin, M. F. & Khan, S. A. Androgen-independent prostate cancer cells acquire the complete steroidogenic potential of synthesizing testosterone from cholesterol. *Mol Cell Endocrinol* 295, 115–120 (2008).

27. Yamaoka, M., Hara, T. & Kusaka, M. Overcoming persistent dependency on androgen signaling after progression to castration-resistant prostate cancer. *Clin Cancer Res* 16, 4319–4324 (2010).

28. Bologna, M., Muzzi, P., Biordi, L., Festuccia, C. & Vicentini, C. Finasteride dose-dependently reduces the proliferation rate of the LNCaP human prostatic cancer cell line in vitro. *Urology* 45, 282–290 (1995).

29. Lazr, C. B., Thomas, L. N., Douglas, R. C., Vessey, J. P. & Rittmaster, R. S. Dutasteride, the dual 1α,25-dihydroxyvitamin D3 receptor inhibitor, inhibits androgen action and promotes cell death in the LNCaP prostate cancer cell line. *Prostate* 58, 130–144 (2004).

30. Xie, H., Dalrymple, S. L., Becker, R. E., Denmeade, S. R. & Isaac, J. T. Pharmacologic basis for the enhanced efficacy of dutasteride against prostate cancers. *Clin Cancer Res* 12, 4072–4079 (2006).

31. Nishiyama, T., Ikariashi, T., Hashimoto, Y., Wako, K. & Takahashi, K. The change in the dihydrotestosterone level in the prostate before and after androgen deprivation therapy in connection with prostate cancer aggressiveness using the Gleason score. *J Urol* 178, 1282–1288; discussion 1288–1289 (2007).

32. Nishiyama, T., Ikariashi, T., Hashimoto, Y., Suzuki, K. & Takahashi, K. Association between the dihydrotestosterone level in the prostate and prostate cancer aggressiveness using the Gleason score. *J Urol* 176, 1387–1391 (2006).

33. Nishiyama, T., Hashimoto, Y. & Takahashi, K. The influence of androgen deprivation therapy on dihydrotestosterone levels in the prostatic tissue of patients with prostate cancer. *Clin Cancer Res* 10, 7121–7126 (2004).

34. Thompson, I. M. et al. The influence of finasteride on the development of prostate cancer. *N Engl J Med* 349, 215–224 (2003).

35. Andriole, G. L. et al. Effect of dutasteride on the risk of prostate cancer. *N Engl J Med* 362, 1192–1202 (2010).

36. Zhao, H. Y. et al. Androgen-repressed phenotype in human prostate cancer. *Proc Natl Acad Sci U S A* 93, 15152–15157 (1996).

37. Cinar, B. et al. Androgen receptor mediates the reduced tumor growth, enhanced androgen responsiveness, and selected target gene transactivation in a human prostate cancer cell line. *Cancer Res* 61, 7310–7317 (2001).

38. Chu, C. P., Hitipakka, R. A., Fukuuchi, J., Kokoantis, J. M. & Liao, S. Androgen causes growth suppression and reversion of androgen-independent prostate cancer xenografts to an androgen-stimulated phenotype in athymic mice. *Cancer Res* 65, 2082–2084 (2005).

39. Chu, C. P. et al. Androgen suppresses proliferation of castration-resistant LNCaP-104-R prostate cancer cells through androgen receptor, Skp2, and c-Myc. *Cancer Sci* 102, 2022–2028 (2011).

40. Chu, C. P. et al. Inhibition of tumor growth and progression of LNCaP prostate cancer cells in athymic mice by androgen and liver X receptor agonist. *Cancer Res* 66, 6482–6486 (2006).

41. Ariai, S. et al. Effect of castration monotherapy on the levels of adrenal androgens in cancerous prostatic tissues. *Steroids* 76, 301–308 (2011).