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Prostate cancer stromal cells and LNCaP cells coordinately activate the androgen receptor through synthesis of T and DHT from DHEA.

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Abstract

One of the mechanisms through which advanced prostate cancer (PCa) usually relapses after androgen deprivation therapy (ADT) is the adaptation to residual androgens in PCa tissue. It has been observed that androgen biosynthesis in PCa tissue plays an important role in this adaptation. In the present study, we investigated how stromal cells affect adrenal androgen DHEA metabolism in androgen-sensitive PCa LNCaP cells. DHEA alone had little effect on PSA promoter activity and the proliferation of LNCaP cells. However, the addition of prostate stromal cells (PrSC) or PCa-derived stromal cells (PCaSC) increased DHEA-induced PSA promoter activity via androgen receptor activation in the LNCaP cells. Moreover, PCaSC stimulated the proliferation of LNCaP cells under physiological concentrations of DHEA. Biosynthesis of testosterone or DHT from DHEA in stromal cells and LNCaP cells was involved in this stimulation of LNCaP cell proliferation. Androgen biosynthesis from DHEA depended upon the activity of various steroidogenic enzymes present in stromal cells. Finally, the dual 5α -reductase inhibitor dutasteride appears to function not only as 5α -reductase inhibitor but also as 3β -hydroxysteroid dehydrogenase inhibitor in LNCaP cells. Taken together, this coculture assay system provides new insights of coordinate androgen biosynthesis under the microenvironment of PCa cells before and after ADT, and offers a model system for the identification of important steroidogenic enzymes involved in

PCa progression and for the development of the corresponding inhibitors of androgen biosynthesis.

For Review Only

Introduction

Prostate cancer (PCa) is the most common malignancy and the second leading cause of cancer-related death of men in the United States (1). Since advanced PCa is initially dependent upon androgens, androgen-deprivation therapy (ADT) is the first choice for advanced PCa. Unfortunately, after an initial response to ADT, PCa eventually loses responsiveness to the androgen blockade and progresses into what is termed an androgen non-responsive phenotype.

Multiple molecular mechanisms that could account for the development of resistance to ADT have been proposed (2) that typically invoke the androgen receptor (AR) as a key mediator in the progression of PCa (3,4). Moreover, alterations of AR itself, which are either absent or at low frequency in the original androgen-dependent state, result in an androgen-hypersensitive situation where stimulation of PCa growth occurs at castrate levels of androgens (4). One of the AR alterations that occurs is AR mutation that results in promiscuous ligand specificity (5). Therefore, in addition to its normal ligands, testosterone (T) and dihydrotestosterone (DHT), both androstenediol, a precursor of T, and estradiol can activate the AR and stimulate the proliferation of LNCaP cells which have a mutated AR (6,7). T and the more active androgen DHT are important factors in PCa progression. These hormone are still present in PCa tissue after ADT. Specifically, when PCa patients are treated with ADT, serum T and DHT decreases to less than

one-tenth of pretreatment levels (8). However, T and DHT in PCa tissue are still present at 20 to 40% of pretreatment values (7-12). These remaining androgens that are still present post-therapy may continue to promote AR activation and account for the observation that combination therapy with a LH-RH agonist, to block androgen production, and an antiandrogen, to block ligand binding to the AR, is more effective for PCa treatment than either therapy alone (8,13,14).

T and DHT in PCa tissue after medical or surgical castration are synthesized locally in the prostate from dehydroepiandrosterone (DHEA) of adrenal origin (7-12). The metabolism from DHEA to DHT in peripheral target tissues depends upon the level of expression of various steroidogenic enzymes in the specific cell types of these tissues (15). Adrenal DHEA is converted to T by 17β -hydroxysteroid dehydrogenase (17β -HSD) and 3β -HSD. T is then converted to DHT by 5α -steroid reductase (SRD5A) in the prostate (15-18). Currently, two types of 3β -HSD, fifteen types of 17β -HSDs and 3 types of SRD5A have been identified and localized in various peripheral tissues, including the prostate, with specific expression patterns in each tissue (18,19). For example, 3β -HSD and type 5 17β -HSD were localized in basal cells of alveoli, stromal cells and endothelial cells of blood vessels of the prostate (20). Fung et al. have observed increased expression of AKR1C3 (type 5 17β -HSD) in PCa tissue (21) while Stanbrough et al. confirmed that ADT-resistant PCa and bone marrow metastases expressed increased levels of multiple

genes responsible for androgen metabolism (HSD3B2, AKR1C3, SRD5A1, AKR1C2, AKR1C1 and UGT2B15) (22). These studies provide support for the concept that PCa tissues can perform local biosynthesis of T and DHT resulting in activation of the AR (23).

It remains unclear; however, in which cell types T and DHT are converted from DHEA to other androgens in PCa tissue, although the products from DHEA and the relevant steroidogenic enzymes are definitively present in the prostate. In this study, we explored the hypothesis that PCa stromal cells contribute to the biosynthesis of T and DHT in PCa. We demonstrated that T and DHT synthesized from DHEA in stromal cells activated AR in PCa epithelial cells in a paracrine fashion and thus contribute to the development of ADT resistance in PCa.

Materials and Methods

Isolation of Stromal Cells from Prostate Carcinoma Tissue. All studies were approved by the Institutional Review Board. We obtained informed consent for experimental use of all specimens obtained from prostate needle biopsy or surgical procedure. The characteristics of PCa patients is described in Table 1. Stromal cells were isolated using a modification of a previously described method (24). Briefly, small pieces of PCa tissue were minced with scissors and washed twice with

phosphate-buffered saline. The fragments were then digested in 0.25% trypsin-EDTA (Invitrogen, Carlsbad, CA) for 30 min at 37°C. After digestion, the dispersed stromal cells were cultured in RPMI supplemented with 1% penicillin/streptomycin and 10% FCS (Sigma, St. Louis, MO) (RPMI-10% FCS) on 6 cm dishes. Bone-derived stromal cells 1 (BDSC-1) were obtained from the 11th rib of a 48-year-old man during left adrenalectomy for pheochromocytoma. Bone-derived stromal cells 2 (BDSC-2) were obtained from the 11th rib of a 58-year-old man during left nephroureterectomy for localized ureteral cancer. These bone samples were cut into bone chips and further processed with a bone grinder (25). Bone chips were then cultured in RPMI-10% FCS like prostate-derived stromal cells.

Cell Culture and Cell Proliferation Assay. LNCaP cells were cultured in Dulbecco's modified Eagle medium including phenol red-5% FCS (DMEM-5% FCS). Normal prostate-derived stromal cells, PrSC, commercially available (Cambrex, East Rutherford, NJ) were cultured using SCGM BulletKit (Cambrex). Twenty-four h after 3×10^4 LNCaP cells were seeded on 12-well plates, aliquots of 3×10^4 stromal cells were plated onto Cell Culture Inserts (1.0 μ m pore size 12-well format; Becton Dickinson, Franklin Lakes, NJ) with DMEM including phenol red-5% charcoal-stripped FCS (CCS; Hyclone, Logan, UT) in 12-well plates for 24 h. Cells were then treated with DHEA, (Sigma, St. Louis, MO), and cultured for 4 days. As a

positive control, LNCaP cells were treated with 0.1 nM DHT for 4 days for 24 h without stromal cells. Medium and reagents were replaced every 2 days. At the end of the culture period, cells were trypsinized and counted in triplicate using a hemocytometer. All coculture studies were performed in DMEM-5% CCS. These experiments were performed at least twice to obtain reproducible data.

Coculture, Transfection, and Luciferase Assay. To evaluate AR transcriptional activity, 24 h after plating 5×10^4 cells on 12-well plates in DMEM including phenol red-5% CCS, LNCaP cells were transfected using Lipofectamine transfection reaction (Invitrogen, Carlsbad, CA) using 0.4 μ g of luciferase reporter plasmid, pGLPSAp-5.8, driven by a 5.8 kb PSA promoter including androgen-response elements (26). Twelve h after transfection, LNCaP cells were cocultured with 5×10^4 stromal cells for 12 h, followed by the addition of indicated concentration of DHEA for 24 h. Cells were then harvested 24 h after addition of reagents and lysed in luciferase lysis buffer (Promega WI). As a positive control, transfected LNCaP cells were treated with 0.1 nM DHT for 24 h without stromal cells. To block AR and SRD5A activity, we used 1 μ M bicalutamide and 5 μ M dutasteride (Nacalai tesque Kyoto, Japan). To knockdown AR expression in LNCaP cells by RNA interference, 5×10^4 LNCaP cells were transfected with 20 nM Non-target (NT) siRNA, AR siRNA-1, or AR siRNA-2 (Invitrogen) using

RNAiMAX (Invitrogen) for 12h, LNCaP cells were transfected with pGL3PSAp-5.8 for 12 h. Then after changing medium, transfected LNCaP cells were cocultured with or without PrSC. Consequently, cells were treated with or without 100 nM DHEA (+) for 24 h. As a positive control, cells were treated with 0.1 nM DHT for 24 h. For knockdown of AR expression in PrSC by RNA interference, we transfected 3×10^5 PrSC on a 6 cm dish with 50 nM Stealth RNAi Negative Control Duplex or AR Stealth RNAi (5'-CAUAGUGACACCCAGAAGCUUCAUC-3') (Invitrogen) for 48 h with Lipofectamine RNAiMAX. Transfected cells were counted and 5×10^4 siRNA-transfected PrSC were cocultured with 5×10^4 LNCaP cells transfected with pGL3PSAp-5.8 12 h before coculture. Total RNA was extracted from the aliquot of PrSC transfected with AR siRNA. Knockdown of AR expression was confirmed by RT-PCR analysis. These experiments were performed at least twice to provide reproducible data.

Quantitative Analysis of androgens in the medium by LC-ESI-MS/MS. Before harvesting cells, we collected the cultured medium and froze it at -30°C until LC-ESI-MS/MS was performed as described previously (7). For LC-ESI-MS/MS, 0.5 ml of the cultured medium was diluted with 0.5 ml of distilled water and then was 5 ng of androstenediol- $^2\text{H}_4$ (A-d₄), 5 ng [16,16,17- $^2\text{H}_3$]-T (100 pg), [17,16,16- $^2\text{H}_3$]-DHT (100 pg), [2,2,4 α ,6- $^2\text{H}_4$]-DHEA (200 pg), [2,2,4,6,6,16,16- $^2\text{H}_7$]- androstenedione

(100 pg), and [2,2,4,6-²H₄]-Adiol (100 pg) as internal standards were added to the individual samples.

LC-ESI-MS/MS was performed using an API-4000 triple stage quadrupole mass spectrometer equipped with an ESI ion source (Applied Biosystems, Foster City, CA) and an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) with HTC PAL auto-sampler (CTC Analytical, Zwingen, Switzerland).

The assay was validated to ensure that the result was within the 20% range of accuracy and precision. We confirmed that endogenous DHEA, Adiol, Adione, T, and DHT in DMEM-5% CCS were below detectable methods.

RNA Extraction and RT-PCR. Twenty-four h after plating 5×10^4 LNCaP cells or stromal cells were treated with or without 10^{-8} M DHT for 24 h at which time total RNA was purified with RNeasy mini kit (QIAGEN, Maryland, USA). Complementary DNA (cDNA) was made by reverse-transcription (RT) of 200 ng each total RNA using ThermoScript RT-PCR system (Invitrogen). Each cDNA sample was amplified with ExTaq (TAKARA, Japan). The sense and antisense primers used and RT-PCR conditions are shown in Table 2. The amplified PCR products were visualized using electrophoresis on a 1.5% agarose gel. For quantification of mRNA expression, real-time PCR was performed according to the manufacturer's instructions and using the LightCycler TaqMan Master Solution, PCR primers and

Universal probe (Roche Applied Science, Switzerland). The gene expression in each sample was quantified as the yield of the target gene relative to that of the glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) gene.

Statistical analysis. Student's *t*-test, for bivariate comparisons, or ANOVA with Fischer's least significant difference post-hoc test, for multivariate comparisons, were used to determine the statistical significance of differences of proliferation and luciferase assays. *. $P < 0.05$ was considered statistically significant.

Results

Effect of DHT and DHEA on LNCaP cells in the presence of stromal cells

In order to investigate AR activity, we transfected LNCaP cells with a luciferase expression plasmid driven by the prostate specific antigen (PSA) promoter, pGL3PSAp-5.8, and performed luciferase assay because this method is simple, effective, and highly sensitive compared to ELISA for PSA protein and quantification of mRNA by RT-PCR (7). When LNCaP cells transfected with pGL3PSAp-5.8 were treated with DHEA, PSA promoter activity was induced in a dose-dependent manner as previously

described (Fig. 1A) (7). However, high concentrations of DHEA were needed to induce PSA promoter activity. On the other hand, coculture of LNCaP cells with normal prostate-derived PrSC increased DHEA-induced PSA promoter activity in direct relation to the number of added PrSC (Fig. 1A). At the addition of 40,000 PrSC increased 100 nM DHEA-induced PSA promoter activity three-fold.

To study the effect of DHEA and of PrSC on LNCaP proliferation, LNCaP cells were cocultured with PrSC in chambers separated by a membrane and treated with DHEA for 4 days (Fig. 1B). LNCaP cell proliferation in the absence of PrSC peaked at 0.1 nM DHT as previously described (7). LNCaP cell proliferation in the absence of PrSC was stimulated by DHEA in a dose-dependent manner, as previously described (7). LNCaP cell proliferation was stimulated by coculture with PrSC in the absence of DHEA, which suggested that some factors secreted from PrSC stimulated the proliferation of LNCaP cells. The addition of 10-30 nM DHEA to the coculture of LNCaP cells and PrSC induced greater proliferation of LNCaP cells compared to the addition of PrSC alone. The induction of proliferation produced by DHEA was the same that could be attained by 0.1 nM DHT, which stimulates maximal proliferation as previously described (7). At 100 nM DHEA, the LNCaP cell proliferation began to decrease. This finding reflects the previously reported observation that DHT induces a biphasic response on LNCaP cell growth (7).

To determine if the effect of DHEA on PSA promoter activity in the presence of PrSC is

mediated through the AR present in LNCaP cells, PrSC, or both we first used the antiandrogen bicalutamide to block AR. Bicalutamide (1 μ M) blocked DHEA-induced PSA promoter activity in the LNCaP and PrSC co-culture indicating that the DHEA-induced PSA promoter activity required the AR (Fig. 1C). However, this experiment does not indicate if the impact on LNCaP AR or PrSC AR or both is what mediates the effect. Accordingly, we next blocked AR expression in LNCaP cells to determine if the LNCaP AR was required for DHEA-mediated induction of the PSA promoter. We cotransfected LNCaP cells with AR siRNA expression plasmids and pGLPSAp-5.8 and treated cells with DHEA in the absence and presence of PrSC. Transfection of LNCaP cells with AR siRNA resulted in greater than 90% decrease of AR expression (Fig. 1D). Knockdown of AR expression in LNCaP cells diminished DHEA-induced PSA promoter activation in both the absence and presence of PrSC (Fig. 1D). This observation suggests that LNCaP AR expression is required for DHEA-mediated PSA activation both in LNCaP cells alone and the increase of DHEA-mediated activation of the PSA promoter in the presence of PrSC (as observed in Fig. 1A). To determine if DHEA also mediates its effects on LNCaP cells through a requirement for AR in the PrSC, we knocked-down AR expression in PrSC using AR siRNA which resulted in decreasing AR expression >90% (Fig. 1E). Coculture of PrSC transfected with AR shRNA and LNCaP had no impact on DHEA-induced PSA promoter compared to coculture of PrSC transfected with

non-targeting control shRNA. These results indicate that DHEA action was increased in the presence of PrSC by a mechanism other than via AR in PrSC cells, such as paracrine factors produced in PrSC from DHEA metabolites. (Fig. 1E right).

Effect of PCa-derived stromal cells on LNCaP cells

To further investigate if the ability of PrSC to promote androgen activity in the LNCaP cells was prostate-specific, we examined stromal cells from different tissues. Coculture of LNCaP cells with human bone-marrow derived stromal cells (HMSC) and lung-derived stromal cells (HLFa) enhanced DHEA-induced PSA promoter activity, although these effects were somewhat lower than with PrSC (Fig. 2A).

We next investigated if PCa-derived stromal cells (PCaSC) could regulate DHEA stimulatory effects on PSA promoter in LNCaP cells. We performed primary cultures of several PCaSCs from PCa tissue obtained by prostate needle biopsy for diagnosis (pathology and stage of patients are described in Table 1). All PCaSCs expressed vimentin and keratinocyte growth factor (KGF), thus confirming their stromal origin (Fig. 2B). All of PCaSCs increased DHEA-induced PSA promoter activity. PCaSC-1, 2, 5, 8 and 9 stimulated greater DHEA-induced PSA promoter activity than the PrSC (Fig. 3C). Since PCa

often metastasizes to bones, we also cocultured LNCaP cells with bone-derived stromal cells (BDSC-1 and -2). To confirm that bone origin of the BDSC, we examine for expression of osteoblast factors. RT-PCR confirmed that core binding factor $\alpha 1$ (cbfa1), osteopontin (OPN), and osteocalcin (OCN) were expressed in both BDSC-1 and 2 indicating that they were of bone origin (Fig. 2B). Both BDSCs increased PSA promoter activity induced by DHEA at a level 5.4-7.6-fold higher than observed with PrSC (Fig. 2C).

We next investigated in further detail how coculture with PCaSCs affected the proliferation of LNCaP cells in the presence of DHEA. When LNCaP cells were cultured without stromal cells, more than 10 nM DHEA was necessary to stimulate cell proliferation (Fig. 1B). Furthermore, when LNCaP cells were cocultured with PCaSC-5, 8, or 9 in the absence of DHEA, the proliferation of LNCaP cells was barely stimulated (compare Fig. 1B with Fig. 2D). In contrast, the addition of DHEA to the coculture of PCaSC-5, 8, or 9 with LNCaP cells stimulated LNCaP cell proliferation similar to the induction of the PSA promoter activity as described in Fig. 2C (Fig. 2D). This effect was seen even at low DHEA concentration (1 nM) in the LNCaP cells cocultured with PCaSC-8 and 9.

Androgen concentration in medium after coculture with stromal cells

We hypothesized that the change of AR activity in the LNCaP cells induced by stromal cells in the presence of DHT and DHEA was influenced by the change of metabolism of androgens caused by stromal cells. To test this hypothesis, we examined the change in the concentrations of various androgens in the culture medium following coculture with stromal cells. After transfection with pGLPSAp-5.8, LNCaP cells cocultured with PrSC or PCaSC-8 were treated with 100 nM DHEA. When LNCaP cells were cocultured with PrSC or PCaSC-8 in the presence of DHEA, PSA promoter activity was activated 2.5 and 8.0 times more than when LNCaP cells were cultured alone, respectively (Fig. 3A).

In the absence of DHEA, we could not detect T and DHT in LNCaP and stromal cells using LC-MS/MS (data not shown). When LNCaP cells were cultured alone, the addition of 100 nM DHEA resulted in media concentrations of 75.3 pM T and 12.4 pM DHT in 24 h (Fig. 3B upper graphs). The addition of PrSC increased the concentration of T and DHT in the medium to 104.6 pM and 23.6 pM 24 h after addition of 100 nM DHEA, respectively (Fig. 3B upper graphs). Moreover, coculture of LNCaP cells with PCaSC-8 increased T and DHT to 522.6 pM and 128.9 pM 24 h after addition of DHEA, respectively (Fig. 3B upper graphs). The degree of increase of T and DHT by coculture corresponded with DHEA-induced PSA promoter activity in the presence or absence of stromal cells as observed in the previous experiments.

To examine how DHEA was metabolized to T in this culture system, we measured the concentration of intermediate metabolites, androstenedione (Adione) and androstenediol (Adiol), in the medium (Fig. 3C lower graphs). LNCaP monoculture contained 6,456 pM Adione at 24 h from addition of 100 nM DHEA; whereas, in the presence of stromal cells it was approximately 9,500 pM. This finding indicates that there was more 3 β -hydroxysteroid dehydrogenases (3 β -HSDs) activity in the coculture as opposed to the LNCaP monoculture (Fig. 3C lower graphs). We also found that the concentration of Adiol in medium from the LNCaP and PrSC coculture was reduced compared with LNCaP monoculture. In contrast, the concentration of Adiol synthesized in the PCaSC-8 and LNCaP coculture in the presence of DHEA was increased to 4,374 pM at 24 h. These data indicate that DHEA is converted into T mainly via Adione in the presence of PrSC and via both Adione and Adiol in the presence of PCaSC-8.

Expression of androgen biosynthesis enzymes

Several enzymes are involved in the conversion pathway of DHEA to T and DHT. Accordingly, we quantified mRNA expression level of AR and androgen biosynthesis enzymes in LNCaP, PrSC and BDSC (Fig. 4). Real-time PCR analysis revealed that AR expression level of all stromal cells was less than 10 times that of the levels in LNCaP cells. Type 1 and 2 3 β -hydroxysteroid dehydrogenase (HSD),

which catalyzes the transformation of DHEA into Adione and Adiol to T, was expressed in LNCaP cells and barely detectable in all stromal cells. Stromal cells expressed types 2 and 4 17β -HSD at relatively high level. The level of expression of type 2 and 4 17β -HSD, which catalyzes the transformation of T into Adione, was different among stromal cells. Type 3 17β -HSD, which is mainly expressed in the testes and catalyzes the transformation of Adione into T (27), was expressed in LNCaP cells and all stromal cells at a very low level. The expression level of type 5 17β -HSD (aldo-keto reductase, AKR1C3), which is believed to catalyze the transformation of DHEA and Adione into Adiol and T, respectively (28), is expressed at different levels among stromal cells although LNCaP cells show a low level of expression. The expression level of type 5 17β -HSD of PrSC and PCaSC-8 was not correlated with DHEA-induced PSA promoter activity in the presence of PrSC or PCaSC-8.

T is converted to DHT by 5α -steroid reductase (SRD5A) in the prostate. Until now, 3 types of SRD5A were identified (15-19). Type 1 SRD5A was ubiquitously expressed in all cells compared with type 2 SRD5A. Type 3 SRD5A, which has been recently identified (19), was expressed in LNCaP cells at a relatively high level and well expressed in all stromal cells compared with Type 1 and 2 SRD5A. AKR1C2 which catalyses the transformation from DHT into the inactive form androstane- 3α , 17β -diol (androstenediol) was expressed in all cells at a relatively low level, especially in LNCaP cells.

Type 5 17 β -HSD does not contribute to testosterone biosynthesis in PrSC

Dufort et al. (28) reported that Type 5 17 β -HSD may be involved in DHEA metabolism in stromal cells. This is supported by our findings that several stromal cells, including PCaSC-8, synthesized large amounts of Adiol from DHEA (Fig. 3C). However, since expression levels of type 5 17 β -HSD mRNA in stromal cells were not correlated with PSA activity as described in Fig. 2C, we focused on the activity (as opposed to expression level) of type 5 17 β -HSD. In order to inhibit type 5 17 β -HSD activity, we used naproxen which is a nonsteroidal anti-inflammatory drug and an AKR1C3 inhibitor (29). Naproxen did not inhibit PCaSC-8-induced DHEA activity in the coculture of LNCaP and PCaSC-8 cells (Fig. 5B). To further confirm these data, we cocultured LNCaP cells with PCaSC-8 in which type 5 17 β -HSD was knocked down using shRNA. Knockdown of type 5 17 β -HSD expression in PCa-SC-8 did not repress DHEA-induced PSA promoter activity (Fig. 5C). These data suggest that type 5 17 β -HSD does not significantly contribute to the conversion of Adione into T in PCaSC.

Effect of a dual 5 α -reductase inhibitor on DHT biosynthesis in stromal cells

Since SRD5A type 1 and 3 are expressed in all stromal cells and DHT is synthesized from DHEA in stromal cells, we investigated if the dual 5 α -reductase inhibitor, dutasteride, could block

DHEA-induced PSA promoter activity in the presence of stromal cells. Coculture of LNCaP cells with PCaSC-8 or 9 increased DHEA-induced PSA promoter activity to 10-fold or 30-fold, respectively (Fig. 6A). Five μM dutasteride significantly inhibited PSA promoter activity induced by 100 nM DHEA as well as 0.1 nM T, regardless of the presence of PCaSC-8 or 9. Simultaneously, we investigated how T was metabolized in LNCaP monoculture by dutasteride and how DHEA were metabolized to other androgens by PCaSC-8 and 9 in the presence of dutasteride. When LNCaP cells were treated with T in the presence of 5 μM dutasteride there was minimal production of DHT and the metabolism of T was reduced in the medium after 24 h (Fig. 6B). Dutasteride inhibited conversion of DHEA to DHT in the LNCaP and PCaSC-8 or 9 coculture (Fig. 6C). Dutasteride also inhibited T biosynthesis from DHEA in LNCaP cells by approximately 80% at 24 h. This inhibition of T biosynthesis was also observed in coculture with PCaSC-8 or 9 by approximately 75% and 66%, respectively (Fig. 6C). We also measured the concentration of Adione and Adiol 24 h after DHEA and dutasteride treatment (Fig. 6D). The concentration of Adione was decreased by dutasteride in accordance with the decrease in the concentration of T. In contrast, the concentration of Adiol was not changed by dutasteride regardless of PCaSCs.

Comparison of androgen concentrations on coculture with those on PCaSC-8 and 9 monoculture

The androgen concentrations measured in Fig. 6C and 6D were totals of androgens metabolized by LNCaP cells and PCaSC-8 or 9. Therefore, we measured the androgen concentrations in medium of monoculture cells treated with DHEA for 24 h (Fig. 6E and 6F). The sum of T and DHT concentrations from the monocultures of the LNCaP cells and the PCaSCs was less than T and DHT concentrations in coculture without dutasteride (compare Fig. 6E with 6C). This suggests that the presence of the two cell types in the coculture stimulates T and DHT synthesis in a synergistic fashion. On the other hand, LNCaP cells produced much higher levels of Adione in monoculture than either PCaSC line (Fig. 6F). The total Adione in the coculture is approximates that of the LNCaP monoculture (compare Fig. 6D with 6F) suggesting that in coculture the total Adione may be primarily due to production by 3β -HSD in LNCaP cells, although this has not been definitively demonstrated. The Adiol levels in coculture with PCaSC-8 or 9 were more than the sum of Adiol produced from DHEA by the monocultures (Compare Fig. 6D with 6F). This suggests that the presence of the two cell types in the coculture stimulates Adiol synthesis in a synergistic fashion.

We also examined the effect of dutasteride on the androgen levels in coculture versus monocultures. Five μ M dutasteride inhibited DHT synthesis from T in LNCaP, PCaSC-8, and PCaSC-9

monocultures and also Adione synthesis from DHEA in LNCaP monoculture. However, dutasteride did not inhibit Adione synthesis in PCaSC-8 and 9 (Fig. 6F). This result indicates that reduction of T concentration observed in coculture in the presence of dutasteride is due to inhibition of Adione synthesis by dutasteride in LNCaP cells but not PCaSCs.

Discussion

The present study shows that PrSC can increase AR activity induced by the adrenal precursor DHEA in androgen-sensitive PCa cells and stimulate the proliferation of PCa cells. Furthermore, PCaSC and BDSC induce greater androgen activity than PrSC or lung-derived stromal cells. The physiological concentration of serum DHEA is between 3 and 15 nM although that of DHEA-sulfate (DHEA-S) ranges between 1 and 10 μ M. This concentration of DHEA did not stimulate the proliferation during monoculture of LNCaP cells. We also confirmed that 10 μ M DHEA-S had almost no ability to activate AR even in the presence of stromal cells (data not shown), thus suggesting that the LNCaP and stromal cells studied have little steroid sulfatase activity.

Our results show that physiological concentrations of DHEA stimulate the proliferation of LNCaP cells in the presence of stromal cells, especially cancer-derived stromal cells. The present data strongly suggest that biosynthesis of T and DHT in stromal cells mediates paracrine stimulation of PCa cells. This activity could readily contribute to androgen deprivation-refractory PCa after ADT. Recently, Arnold et al. described a similar study using coculture of LAPC4 androgen-sensitive PCa cells with stromal cells (30). According to their study, PCa-derived stromal cells also increased PSA mRNA expression induced by DHEA. However, they did not identify that the proliferation of LAPC4 cells was stimulated by DHEA in the presence of PCa-derived stromal cells in contrast to the proliferation of LNCaP cells in our study. The difference between our study and their study may be due to a difference in sensitivity for androgens between LNCaP and LAPC4.

Several mechanisms explaining why advanced PCa relapses during ADT have been considered (2,31). AR amplification, action of cytokines on the AR, induction of AR coactivators, or STAT-3 activation (32-34) can all cause an androgen-hypersensitive state in PCa. It has also been shown that coculture of stromal cells with PCa-derived epithelial cells enhanced AR activity via recruitment of coregulators (35). Pathways which are involved in the development of treatment-resistant PCa are also related to the microenvironment surrounding cancer cells. In fact, PCa-stroma interactions play an

important key factor for prostate development and carcinogenesis (36). Increased expression of chemokines, cytokines, and growth factors from stromal cells drive PCa cells to a more invasive and malignant state (37-39). Accordingly, alterations in the stromal microenvironment as well as PCa cells themselves cooperate to promote malignant transformation of PCa.

It is very likely that residual androgens in PCa tissue are involved in the progression of PCa that has become androgen-hypersensitive. Our data indicate that the adrenal androgen precursor DHEA acts as precursor for synthesis of residual androgens in PCa tissues. Recently, many physicians have adopted combined androgen blockade using an LH-RH agonist and an antiandrogen as initial treatment for advanced PCa. However, metastatic PCa relapses and serum PSA values increase a few years later. Ketoconazole, which inhibits the synthesis of adrenal DHEA, is often used for HRPc in West European countries, and have a response rate of about 60% but the response is of short duration. Furthermore, in phase II studies using the CYP17A inhibitor abiraterone, which blocks DHEA synthesis, PSA declines of >50% in 12/21 (57%) patients have been observed (40) although cessation of the antiandrogen, by itself, could have caused a significant effect on PSA. These results suggest that adrenal DHEA contributes to >50% of relapses of advanced metastatic PCa.

Even after ADT, DHT remains in the prostate cancer tissue at 20–40% of untreated prostate tissue

although serum DHT levels decrease to <10% after ADT (7, 8, 11, 41). Mohler et al. demonstrated that recurrent PCa tissue retains T at around 50% of benign prostatic tissue (42). Recently, Montgomery has demonstrated that T levels within metastases from anorchid men were significantly higher than levels within primary PCa from untreated eugonadal men (43). These reports indicate that T and DHT accumulate in PCa tissue and metastatic lesions after ADT. These data as well as the presence of steroidogenic enzymes in PCa tissue strongly suggest that T is synthesized from adrenal DHEA not only in the testes but also in PCa tissue and metastatic lesions, by 'intracrine synthesis' (8, 15).

The mechanisms through which T and DHT are synthesized in PCa tissue and how they contribute to PCa progression are unclear. Our data indicate that stromal cells promote androgen production in PCa either directly or through stimulation of PCa epithelial cell-mediated production of androgens. The PSA promoter activity induced by DHEA was only moderate in LNCaP monocultures. However, coculture with stromal cells, especially, PCaSCs, strongly activated PSA promoter activity induced by DHEA. Although our results are consistent with Arnold et al's observation that T synthesized from DHEA in PCa-derived stromal cells contributed to PSA induction in LAPC4 (30), we extended these studies further to elucidate which pathways were involved in T synthesis. Specifically, we identified that the increased level of DHEA-induced AR activity by stromal cells was dependent on the synthesized level of T and

DHT achieved by coculture. (Fig. 3 and Fig. 6). Furthermore, the ability the predominant pathways (via Adiol and/or via Adione) of T biosynthesis from DHEA were dependent on stromal cells. These results suggest that enzymatic activities which catalyze conversion of DHEA to T or DHT via Adiol or Adione differ among stromal cells and coordinately with cancer cells.

When we examined the expression of androgen biosynthesis enzymes, expression pattern was different among each of stromal cells. Furthermore, the expression pattern of type 5 17β -HSD in stromal cells, whose expression has been reported to be increased in PCa (21), did not correlate with increase of DHEA-induced PSA promoter activity by stromal cells. This discrepancy may be due to several reasons: 1) expression level of androgen biosynthesis enzymes mRNA does not always reflect its enzymatic activity; 2) total activity of all 17β -HSDs may be important for the global conversion of DHEA to Adiol or Adione to T; and 3) recently, type 15 17β -HSD has been proposed to convert Adione to T and thus may impact the overall response (18).

DHT is usually synthesized from T by SRD5A1 and 2 in the prostate. Although SRD5A2 is predominantly expressed in the normal prostate, recent evidence shows that SRD5A1 is highly expressed in PCa and HRPC; whereas, SRD5A2 is not highly expressed (44). Consistent with this finding, we also observed that SRD5A1 was highly expressed in LNCaP cells and all stromal cells. Uemura et al.

discovered SRD5A3 in HRPC (19). SRD5A3 is predominantly expressed in HRPC but not in androgen-sensitive PCa. We observed that both LNCaP cells and PCaSC from PCa patients expressed SRD5A3 mRNA and that dutasteride almost completely blocked PSA promoter activity induced by T and DHEA in the presence or absence of stromal cells. Dutasteride may inhibit SRD5A3 activity as well as SRD5A1 and SRD5A2 activity. Interestingly, when LNCaP cells were treated with DHEA regardless of the presence or absence of stromal cells, dutasteride inhibited not only DHT biosynthesis but also T and Adione biosynthesis from DHEA in LNCaP cells. However, inhibition of Adione biosynthesis from DHEA by dutasteride was not observed in PCaSC-8 and 9 (Fig. 6F). It is not clear why dutasteride inhibited Adione synthesis only in LNCaP cells. Recently, Lazier et al. demonstrated that dutasteride not only inhibits the conversion from T to DHT but also acted as an antiandrogen (45). We also confirmed that dutasteride inhibited PSA promoter activity induced by DHT in LNCaP cells (data not shown). Taken together, dutasteride may have a variety of inhibitory functions on androgen biosynthesis and AR action.

On the basis of our data and previous reports, we propose an interactive model for the interaction between PCa cells and the surrounding stromal cells (Fig. 7). The proliferation of PCa is highly dependent upon T and DHT secreted from the testes, the T of testicular origin adds to the T made locally from DHEA (15). Although ADT induces apoptosis of many PCa epithelial cells, stromal cells surrounding PCa cells

can still survive. Then oncogenes, cytokines, and growth factors may affect survival and progression, especially when only partial androgen blockade or monotherapy is used. On the other hand, PCaSC can synthesize T and DHT from DHEA coordinately with cancer cells, and secrete them to the surrounding area in a paracrine fashion. Surviving PCa cells receive an androgenic supply from stromal cells, and progression of the cancer continues. However, we cannot eliminate that some growth factors or cytokines induced by DHEA in either stromal or epithelial cells affect LNCaP proliferation independent of AR.

In conclusion, we have demonstrated that the concentration of T and DHT in PCa tissue is higher than in serum after ADT. This results from biosynthesis of T from DHEA in PCa-derived tissues. It is possible that both stromal and epithelial cells may contribute to the conversion of DHEA to T. Our coculture assay system is extremely useful for measuring total DHEA activity which results in AR activation in PCa and stromal cells. Such data could support the development of new drugs to inhibit biosynthesis of androgens. Moreover, using this assay system, there may be a possibility of predicting relapse and the time of relapse at diagnosis of advanced PCa, depending upon the level of steroidogenic enzyme expression. In the present study, we used stromal cells from high-grade PCa tissue (Gleason 7, 8, and 9). It would be interesting if we could culture stromal cells from low grade PCa tissue and perform the same study; however, these were not readily available for our studies. Additional studies are needed to

assess which enzymes are the most involved in the biosynthesis of androgens in PCa tissue. The present data also indicate that monotherapy of PCa with medical or surgical castration alone or an antiandrogen alone is an insufficient treatment for PCa at any stage of the disease, due to the major importance of the local biosynthesis of androgens from DHEA.

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Legends

Fig. 1 Effect of PrSC on PSA promoter activity and proliferation of LNCaP cells in the presence of DHEA.

(A) After 5×10^4 LNCaP cells were transfected with $0.4 \mu\text{g}$ pGLPSAp-5.8 for 12 h, the cells were cocultured in DMEM-5% CCS with 0, 2×10^4 , or 4×10^4 PrSC cells for 12 h. Consequently, both cells were treated with DHEA for 24 h and cells were lysed. As a positive control, transfected LNCaP cells were treated with 0.1 nM DHT for 24 h without PrSC (column of oblique lines). The absolute luciferase activities were measured. (B) LNCaP cell proliferation in the absence or presence of PrSC. Twenty-four h after 3×10^4 LNCaP cells were cultured on the lower chamber, PrSC cells were seeded on the upper chamber. Twelve h later, cells were treated with increasing concentrations of DHEA for 4 days and counted. As a positive control, LNCaP cells were treated with 0.1 nM DHT for 4 days for 24 h without PrSC (column of oblique lines). (C) LNCaP cells transfected with pGL3PSAp-5.8 were cocultured with or without PrSC for 12 h. Those cells were treated with 100 nM DHEA in the absence or presence of $1 \mu\text{M}$ bicalutamide for 24 h, and then luciferase activities were measured. Transfected LNCaP cells were also treated with 0.1 nM DHT with or without bicalutamide for 24 h as a positive control. (D) AR knockdown of AR in LNCaP cells. After AR of LNCaP cells were knocked-down using AR siRNA-1, or AR siRNA-2,

cells were transfected with pGL3PSAp-5.8 for 12 h. Transfected LNCaP cells were cocultured with or without PrSC. Consequently, cells were treated with or without 100 nM DHEA (+) for 24 h. As a positive control, cells were treated with 0.1 nM DHT for 24 h. (E) LNCaP cells transfected with pGL3PSAp-5.8 were cocultured with PrSC transfected with 50 nM NT siRNA or AR siRNA-1 for 48 h. The aliquot of transfected PrSC were used for RT-PCR analysis of AR knockdown. Those cells were treated with 100 nM DHEA for 24 h, and then luciferase activity was measured. These coculture experiments were performed at least twice with reproducible data. The data are presented as the mean \pm SD of triplicate measurements.

Fig. 2 Effect of stromal cells from different tissues and PCa tissues on DHEA and DHT-induced PSA promoter activity and proliferation. (A) Effect of stromal cells from different tissues on DHEA and DHT-induced PSA promoter activity. Stromal cells from different tissues, PrSC, HMSC, and HLFa., were cocultured with LNCaP cells transfected with pGL3PSAp-5.8 and treated with 100 nM DHEA for 24 h and luciferase activities were measured. (B) Confirmation of stromal cells from different PCa patients and the normal rib bone. Expression of vimentin and KGF was observed in prostate-derived stromal cells by RT-PCR. Expression of cbfa1 and osteopontin (OPN) was also observed in BDSC-1 and BDSC-2 by

RT-PCR. (C) Effect of stromal cells from different PCa patients and the normal rib bone on PSA promoter activity. Columns indicate the relative acceleration ratio compared with 100 nM DHEA alone in the absence of stromal cells, and the number above each column represents relative induction ratio compared with PrSC. (D) Effect of different PCaSCs on LNCaP cell proliferation. Twenty-four h after 3×10^4 LNCaP cells were cultured on the lower chamber, PCaSC-5, 8, or 9 cells were seeded on the upper chamber. Twelve h later, cells were treated with increasing concentrations of DHEA for 4 days and counted. Medium was changed every 2 days and DHEA was added to medium. These coculture experiments were performed at least twice with reproducible data. The data are presented as the mean \pm SD of triplicate measurements.

Fig. 3 Androgen biosynthesis from DHEA in PrSC and PCaSC-8. (A) LNCaP cells transfected with pGL3PSAp-5.8 were cocultured with PrSC or PCaSC-8, treated with 100 nM DHEA for 24 h, and measured luciferase activity as described in Fig. 1. As a positive control, transfected LNCaP cells were treated with 0.1 nM DHT for 24 h (red column). This coculture experiments were performed at least twice with reproducible data. (B) Concentration of T, DHT, Adione, and Adiol in the medium used for coculture with LNCaP and stromal cells after treating with 100 nM DHEA. Twelve h after starting culture of 5×10^4

LNCaP cells with or without 5×10^4 PrSC or PCaSC-8 cells, 100 nM DHEA was added to the medium. Then aliquots of medium were collected after 12 h and 24 h for measuring concentration of T, DHT, Adione, and Adiol by LC-ESI-MS/MS. A red line shows concentration of DHT in the medium used for monoculture of LNCaP cells after treating with 0.1 nM DHT as described in Fig. 3A. The lower limit of quantitation of T, DHT, DHEA, Adione, and Adiol were 5, 2.5, 5, 2.5, and 1 pg/assay, respectively. The data are presented as the mean \pm SD of triplicate measurements.

Fig. 4 Real-time RT-PCR analysis of AR and androgen biosynthesis enzymes mRNA in LNCaP cells and stromal cells. Expression of AR, various HSD17s, HSD3B-1 and 2, SRD5As, and AKR1C2 mRNA in various stromal cells was confirmed using real-time RT-PCR analysis. 1. LNCaP cells treated without androgen for 24 h in DMEM-5% CCS; 2. LNCaP cell treated with 10 nM DHT for 24 h; 3. PC-3 cells; 4. DU145 cells; 5. PrSC; 6. PCaSC-1; 7. PCaSC-2; 8. PCaSC-5; 9. PCaSC-6; 10. PCaSC-7; 11. PCaSC-8; 12. PCaSC-9; 13. BDSC-1; 14. BDSC-2.

Fig. 5 Effect of type 5 17β -HSD on DHEA-induced PSA promoter activity. (A) Effect of HSD17B-5 inhibitor, naproxen and HSD17B-5 shRNA on DHEA-induced PSA promoter activity. After 5×10^4

LNCaP cells were transfected with 0.4 μg pGLPSAp-5.8 for 12 h, the cells were cocultured with 0 or 5×10^4 PCaSC-8 cells for 12 h. Consequently both cells were treated with 100 nM DHEA in the absence or presence of 5 μM naproxen for 24 h, and then the absolute luciferase activities were measured. (B) knockdown of HSD17B-5 mRNA and PSA promoter activity. After PCaSC-8 was transfected with 50 nM HSD17B-5 siRNA for 48 h, RNA was purified and subjected to RT-PCR of HSD17B-5 mRNA. As a negative control, 100 nM non-targeting (NT) siRNA was also transfected. LNCaP cells transfected with pGLPSAp-5.8 were cocultured with siRNA-transfected PCaSC-8. Then LNCaP and PCaSC-8 cells were treated with 100 nM DHEA for 24 h and luciferase activities were measured. These coculture experiments were performed at least twice with reproducible data. The data are presented as the mean \pm SD of triplicate measurements. NS means not statistically significant difference.

Fig. 6 Effect of 5α -reductase inhibitor, dutasteride, on T or DHEA-induced PSA promoter activity and concentration of various androgens in the medium. (A) After 5×10^4 LNCaP cells were transfected with 0.4 μg pGLPSAp-5.8 for 12 h, the cells were cocultured with 5×10^4 PCaSC-8 or PCaSC-9 cells or without stromal cells for 12 h. Consequently both cells were treated with 100 nM DHEA with or without 5 μM dutasteride (Dut) for 24 h, and luciferase assay was performed. As a positive control, transfected

LNCaP cells were treated with 0.1 nM T for 24 h. These coculture experiments were performed at least twice with reproducible data. The data are presented as the mean \pm SD of triplicate measurements. (B) Concentration of T, DHT, Adione, and Adiol in the medium treated with 0.1 nM T with or without Dut. Twenty-four h after treating cocultured LNCaP cells in the presence of 0.1 nM T with or without Dut the medium was collected for LC-ESI-MS/MS as described in Fig. 3. (C) and (D) Concentration of T and DHT (C), Adione and Adiol (D) in the medium treated with 100 nM DHEA with or without dutasteride. Twenty-four h after treating cocultured LNCaP cells with PCaSC-8 or 9 in the presence of 100 nM DHEA with or without Dut, the medium was collected for LC-ESI-MS/MS. (E) and (F) Concentration of T and DHT (E) and Adione and Adiol (F) in the medium of LNCaP, PCaSC-8, and PCaSC-9 monoculture treated with DHEA with or without dutasteride. Twenty-four h after treating LNCaP cells, PCaSC-8, and PCaSC-9 in the presence of 100 nM DHEA with or without dutasteride, the medium was collected for LC-ESI-MS/MS. The data are presented as the mean \pm SD of triplicate measurements.

Fig. 7 Mechanism of how DHEA contributes to androgen therapy non-responsive PCa. Prior to androgen deprivation therapy (ADT), T from the testes plays a major role in the androgen activity in the prostate;

whereas, the adrenal androgen DHEA plays a minor role. After ADT, in addition to the growth factors and cytokines from PCa-derived stromal cells that stimulate the proliferation of PCa, residual PCa cells become androgen-hypersensitive through various mechanisms and simultaneously PCa-derived stromal cells synthesize T, DHT, and precursors from DHEA coordinately. These synthesized androgens induce AR activity and stimulate the progression of PCa in a paracrine fashion.

For Review Only

Stromal cells	PSA (ng/ml) on diagnosis	Gleason score	Stage on diagnosis
PCaSC-1	4799	5+5	T3a, N0, M1
PCaSC-2	263	4+4	T3b, N0, M0
PCaSC-5	76.9	4+3	T3a, N0, M1
PCaSC-6	46.4	4+4	T3a, N1, M0
PCaSC-7	648	4+3	T4, N1, M1
PCaSC-8	246	4+5	T4, N0, M1
PCaSC-9	180	5+4	T3b, N0, M0

Table 1 Characteristics of PCa patients on diagnosis

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	Forward (5'-3')	Reverse (5'-3')	Condition
GAPDH	GACCACAGTCCATGCCATCA	TCCACCACCCTGTTGCTGTA	94C for 15 sec, 56C for 30 sec, 72C for 30 sec
hAR	TCCAAATCACCCCCAGGAA	GACATCTGAAAGGGGCATG	94C for 15 sec, 56C for 30 sec, 72C for 30 sec
HSD17B-1	ACAATCATCTCCCACCTCCCAACGC	CATGGTGAGACTGTGGGAGAGGCT	94C for 15 sec, 65C for 30 sec, 72C for 30 sec
HSD17B-2	AACAATGCTGGGGTGTGGCTTTC	GGCGTGAATAGGCAAAGGGCTCT	94C for 15 sec, 65C for 30 sec, 72C for 30 sec
HSD17B-3	CCTGTTTCCTGGCCTCTCTACTCC	GGGACTGGTGAGGAAAAGTTGTGC	94C for 15 sec, 65C for 30 sec, 72C for 30 sec
HSD17B-4	GGCTGCTTTGTACCGCCTCAGTGGA	GTAGTGTGCCCTTCAGAGCTTGGCG	94C for 15 sec, 65C for 30 sec, 72C for 30 sec
HSD17B-5	GAGAAGTAAAGCTTTGGAGGTCACA	TAGCAATTTACTCCGGTTGAAATAC	94C for 15 sec, 65C for 30 sec, 72C for 30 sec
HSD17B-6	AGATGGAGAGCATCGCTGCAGCTAC	TGCACCGATGTCAGAGCATGTTCCA	94C for 15 sec, 65C for 30 sec, 72C for 30 sec
HSD17B-7	AAGGCAAGGAACCCCTACAGCTCTTC	CTCAAGAGATCCTCCCACCTCATCC	94C for 15 sec, 65C for 30 sec, 72C for 30 sec
HSD17B-8	CTGCTTTTCTCGCCACCATCTGTC	TGCGACCACATCTGCCACATCCTCA	94C for 15 sec, 65C for 30 sec, 72C for 30 sec
HSD17B-10	TGTGGCTGCCTTCGAGGGTCAGGTT	TGGGCTTCTCCCAAGCTGGAGAGT	94C for 15 sec, 65C for 30 sec, 72C for 30 sec
HSD17B-11	CATGTCTCGGTCCCCTTCTACTGG	GGAAACGCTCAGGAAGGATCCTTTC	94C for 15 sec, 65C for 30 sec, 72C for 30 sec
HSD3B-1&2	AGCAAAAAGCTTGCTGAGAAGGCTG	TTCTGCTTGGCTTCTCCAGCTGT	94C for 15 sec, 65C for 30 sec, 72C for 30 sec
SRD5A1	TGGAGAAATCATGGAGTGGTGTGGC	TCCTCCTCTATCCCCAAGTTCTCCA	94C for 15 sec, 65C for 30 sec, 72C for 30 sec
SRD5A2	ATACCCCTGATGGGTGGTACACAGAC	GCTTCAGTTGGGGATCAGGATAGGG	94C for 15 sec, 65C for 30 sec, 72C for 30 sec
SRD5A3	GCAGGAGTGGTCATTCAGTGAACC	CTGTACTTTGGGCTCCAGACTTCAC	94C for 15 sec, 65C for 30 sec, 72C for 30 sec
AKR1C2	TAAAAGTAAAGCTCTAGAGGCCGTC	CAGCAGTTTTCTCTGGTTGAAGTAA	94C for 15 sec, 65C for 30 sec, 72C for 30 sec
vimentin	TGCAGGCTCAGATTCAGGAACAGCA	AGGTGACGAGCCATTTCTCCTTCA	94C for 15 sec, 65C for 30 sec, 72C for 30 sec
KGF	GGAAATCAGGACAGTGGCAGTTGGA	CAAATCCTGCCAGAAGCCTGTAGGT	94C for 15 sec, 65C for 30 sec, 72C for 30 sec
cbfa1	CCCACGAATGCACTATCCAGCCACC	ATTCGAGGTGGTGGTGCATGGCGG A	94C for 15 sec, 65C for 30 sec, 72C for 30 sec
OPN	TATGATGGCCGAGGTGATAGTGTGG	TAATCTGGACTGCTTGTGGCTGTGG	94C for 15 sec, 65C for 30 sec, 72C for 30 sec

Table 2 Sequence of primers and reaction condition of PCR

Fig. 1 Mizokami et al.

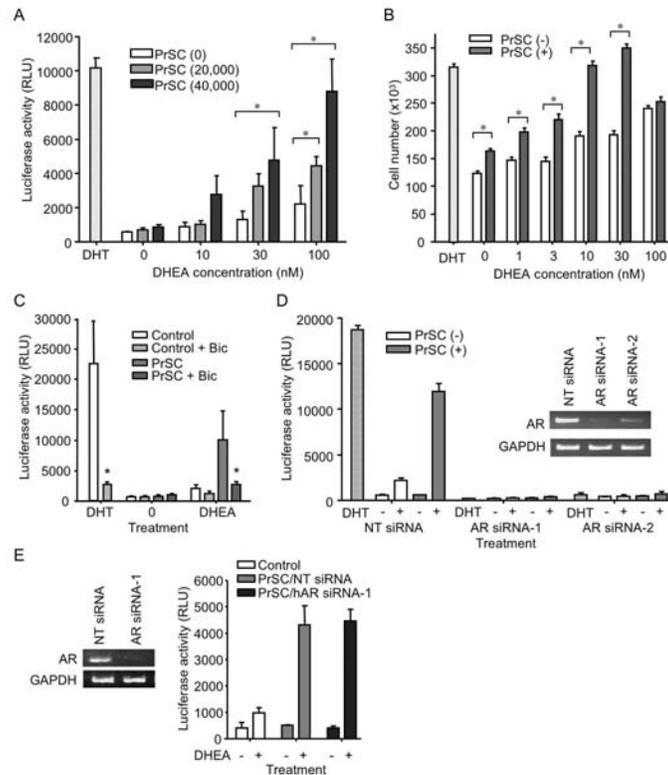


Fig. 1 Effect of PrSC on PSA promoter activity and proliferation of LNCaP cells in the presence of DHEA. (A) After 5×10^4 LNCaP cells were transfected with $0.4 \mu\text{g}$ pGLPSAp-5.8 for 12 h, the cells were cocultured in DMEM-5% CCS with 0, 2×10^4 , or 4×10^4 PrSC cells for 12 h. Consequently, both cells were treated with DHEA for 24 h and cells were lysed. As a positive control, transfected LNCaP cells were treated with 0.1 nM DHT for 24 h without PrSC (column of oblique lines). The absolute luciferase activities were measured. (B) LNCaP cell proliferation in the absence or presence of PrSC. Twenty-four h after 3×10^4 LNCaP cells were cultured on the lower chamber, PrSC cells were seeded on the upper chamber. Twelve h later, cells were treated with increasing concentrations of DHEA for 4 days and counted. As a positive control, LNCaP cells were treated with 0.1 nM DHT for 4 days for 24 h without PrSC (column of oblique lines). (C) LNCaP cells transfected with pGL3PSAp-5.8 were cocultured with or without PrSC for 12 h. Those cells were treated with 100 nM DHEA in the absence or presence of $1 \mu\text{M}$ bicalutamide for 24 h, and then luciferase activities were measured. Transfected LNCaP cells were also treated with 0.1 nM DHT with or without bicalutamide

for 24 h as a positive control. (D) AR knockdown of AR in LNCaP cells. After AR of LNCaP cells were knocked-down using AR siRNA-1, or AR siRNA-2, cells were transfected with pGL3PSAp-5.8 for 12 h. Transfected LNCaP cells were cocultured with or without PrSC. Consequently, cells were treated with or without 100 nM DHEA (+) for 24 h. As a positive control, cells were treated with 0.1 nM DHT for 24 h. (E) LNCaP cells transfected with pGL3PSAp-5.8 were cocultured with PrSC transfected with 50 nM NT siRNA or AR siRNA-1 for 48 h. The aliquot of transfected PrSC were used for RT-PCR analysis of AR knockdown. Those cells were treated with 100 nM DHEA for 24 h, and then luciferase activity was measured. These coculture experiments were performed at least twice with reproducible data.

The data are presented as the mean \pm SD of triplicate measurements.

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Fig. 2 Mizokami et al.

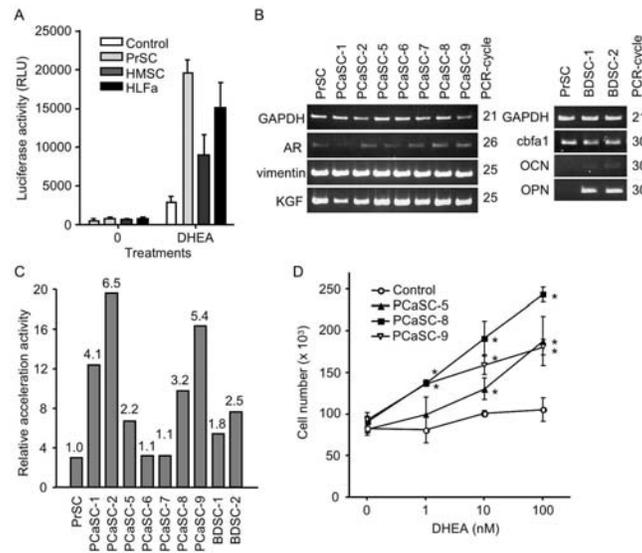


Fig. 2 Effect of stromal cells from different tissues and PCa tissues on DHEA and DHT-induced PSA promoter activity and proliferation. (A) Effect of stromal cells from different tissues on DHEA and DHT-induced PSA promoter activity. Stromal cells from different tissues, PrSC, HMSC, and HLFa., were cocultured with LNCaP cells transfected with pGL3PSAp-5.8 and treated with 100 nM DHEA for 24 h and luciferase activities were measured. (B) Confirmation of stromal cells from different PCa patients and the normal rib bone. Expression of vimentin and KGF was observed in prostate-derived stromal cells by RT-PCR. Expression of cbfa1 and osteopontin (OPN) was also observed in BDSC-1 and BDSC-2 by RT-PCR. (C) Effect of stromal cells from different PCa patients and the normal rib bone on PSA promoter activity. Columns indicate the relative acceleration ratio compared with 100 nM DHEA alone in the absence of stromal cells, and the number above each column represents relative induction ratio compared with PrSC. (D) Effect of different PCaSCs on LNCaP cell proliferation. Twenty-four h after 3×10^4 LNCaP cells were cultured on the lower chamber, PCaSC-5,

8, or 9 cells were seeded on the upper chamber. Twelve h later, cells were treated with increasing concentrations of DHEA for 4 days and counted. Medium was changed every 2 days and DHEA was added to medium. These coculture experiments were performed at least twice with reproducible data. The data are presented as the mean \pm SD of triplicate measurements.
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Fig. 3 Mizokami et al.

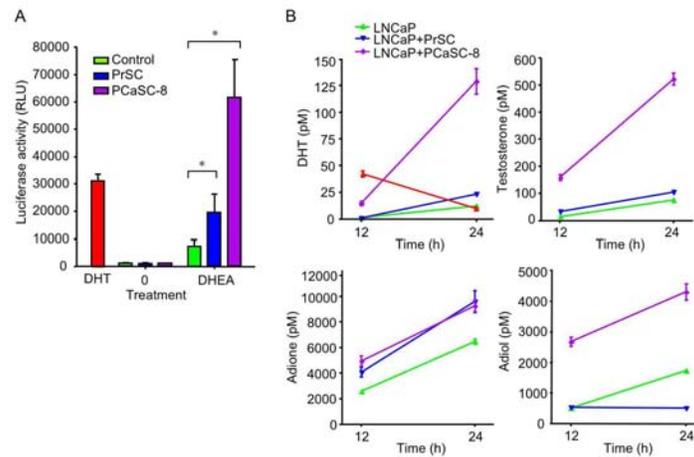


Fig. 3 Androgen biosynthesis from DHEA in PrSC and PCaSC-8. (A) LNCaP cells transfected with pGL3PSAp-5.8 were cocultured with PrSC or PCaSC-8, treated with 100 nM DHEA for 24 h, and measured luciferase activity as described in Fig. 1. As a positive control, transfected LNCaP cells were treated with 0.1 nM DHT for 24 h (red column). This coculture experiments were performed at least twice with reproducible data. (B) Concentration of T, DHT, Adione, and Adiol in the medium used for coculture with LNCaP and stromal cells after treating with 100 nM DHEA. Twelve h after starting culture of 5×10^4 LNCaP cells with or without 5×10^4 PrSC or PCaSC-8 cells, 100 nM DHEA was added to the medium. Then aliquots of medium were collected after 12 h and 24 h for measuring concentration of T, DHT, Adione, and Adiol by LC-ESI-MS/MS. A red line shows concentration of DHT in the medium used for monoculture of LNCaP cells after treating with 0.1 nM DHT as described in Fig. 3A. The lower limit of quantitation of T, DHT, DHEA, Adione, and Adiol were 5, 2.5, 5, 2.5, and 1 pg/assay, respectively. The data are presented as the mean \pm SD of triplicate

measurements.
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Fig. 4 Mizokami et al.

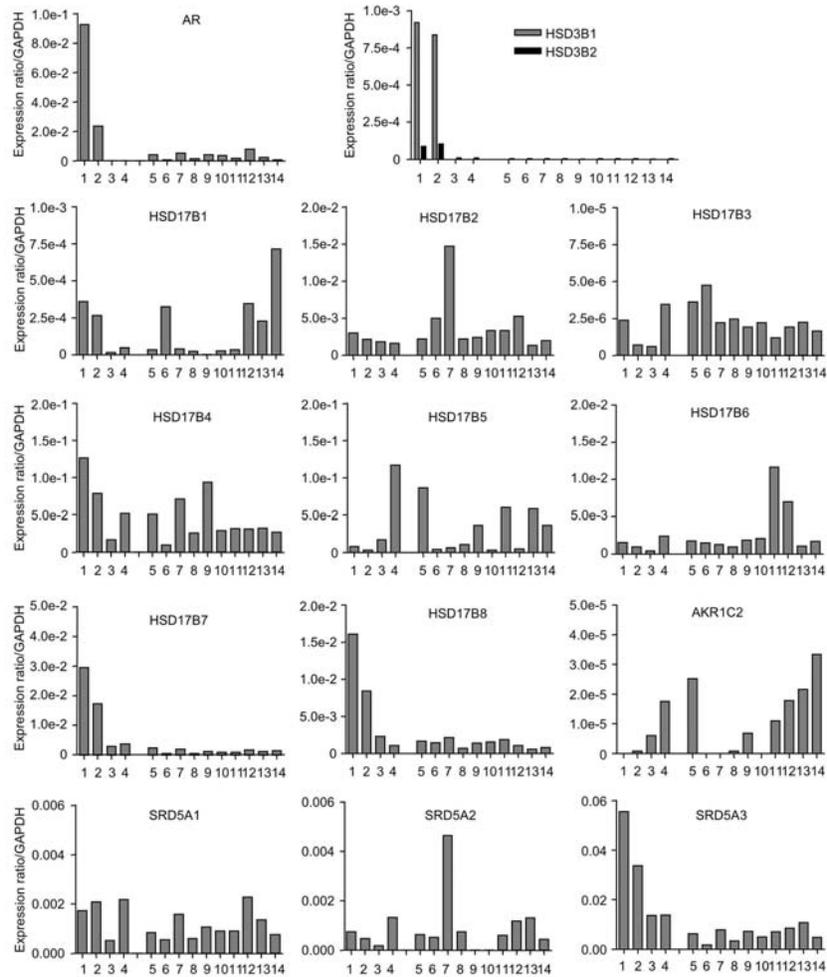


Fig. 4 Real-time RT-PCR analysis of AR and androgen biosynthesis enzymes mRNA in LNCaP cells and stromal cells. Expression of AR, various HSD17s, HSD3B-1 and 2, SRD5As, and AKR1C2 mRNA in various stromal cells was confirmed using real-time RT-PCR analysis. 1. LNCaP cells treated without androgen for 24 h in DMEM-5% CCS; 2. LNCaP cell treated with 10 nM DHT for 24 h; 3. PC-3 cells; 4. DU145 cells; 5. PrSC; 6. PCaSC-1; 7. PCaSC-2; 8. PCaSC-5; 9. PCaSC-6; 10. PCaSC-7; 11. PCaSC-8; 12. PCaSC-9; 13. BDSC-1; 14. BDSC-2.

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Fig. 5 Mizokami et al.

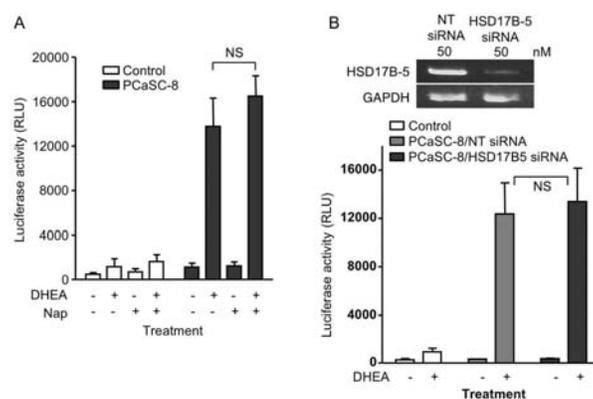


Fig. 5 Effect of type 5 17 β -HSD on DHEA-induced PSA promoter activity. (A) Effect of HSD17B-5 inhibitor, naproxen and HSD17B-5 shRNA on DHEA-induced PSA promoter activity. After 5×10^4 LNCaP cells were transfected with 0.4 μ g pGLPSAp-5.8 for 12 h, the cells were cocultured with 0 or 5×10^4 PCaSC-8 cells for 12 h. Consequently both cells were treated with 100 nM DHEA in the absence or presence of 5 μ M naproxen for 24 h, and then the absolute luciferase activities were measured. (B) knockdown of HSD17B-5 mRNA and PSA promoter activity. After PCaSC-8 was transfected with 50 nM HSD17B-5 siRNA for 48 h, RNA was purified and subjected to RT-PCR of HSD17B-5 mRNA. As a negative control, 100 nM non-targeting (NT) siRNA was also transfected. LNCaP cells transfected with pGLPSAp-5.8 were cocultured with siRNA-transfected PCaSC-8. Then LNCaP and PCaSC-8 cells were treated with 100 nM DHEA for 24 h and luciferase activities were measured. These coculture experiments were performed at least twice with reproducible data. The data are presented as the mean \pm SD of triplicate measurements. NS means not statistically

significant difference.
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Fig. 6 Mizokami et al.

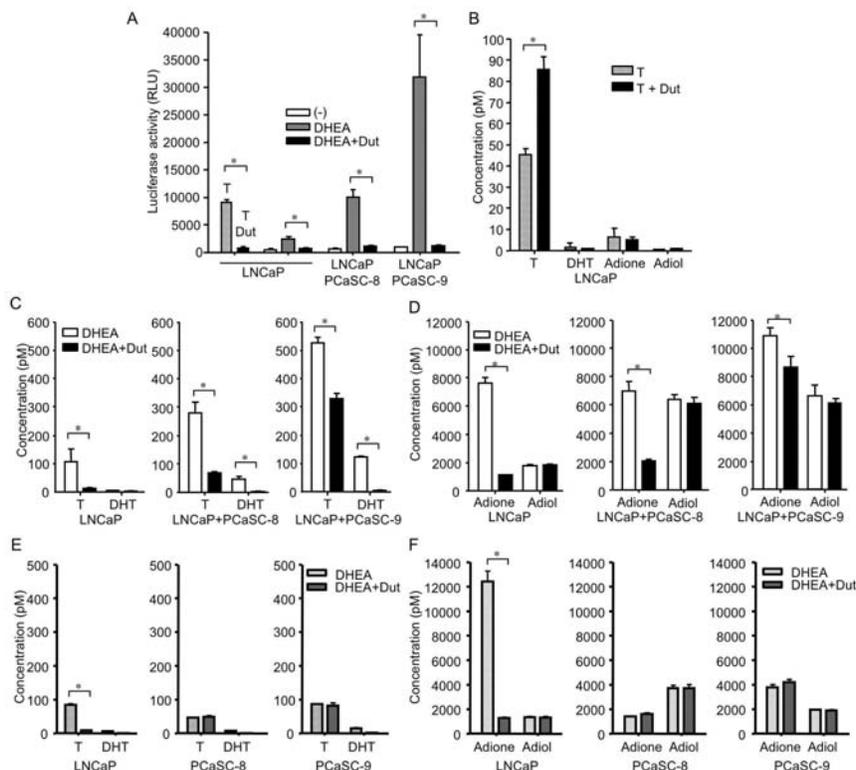


Fig. 6 Effect of 5 α -reductase inhibitor, dutasteride, on T or DHEA-induced PSA promoter activity and concentration of various androgens in the medium. (A) After 5×10^4 LNCaP cells were transfected with $0.4 \mu\text{g}$ pGLPSAp-5.8 for 12 h, the cells were cocultured with 5×10^4 PCaSC-8 or PCaSC-9 cells or without stromal cells for 12 h. Consequently both cells were treated with 100 nM DHEA with or without 5 μM dutasteride (Dut) for 24 h, and luciferase assay was performed. As a positive control, transfected LNCaP cells were treated with 0.1 nM T for 24 h. These coculture experiments were performed at least twice with reproducible data. The data are presented as the mean \pm SD of triplicate measurements. (B) Concentration of T, DHT, Adione, and Adiol in the medium treated with 0.1 nM T with or without Dut. Twenty-four h after treating cocultured LNCaP cells in the presence of 0.1 nM T with or without Dut the medium was collected for LC-ESI-MS/MS as described in Fig. 3. (C) and (D) Concentration of T and DHT (C), Adione and Adiol (D) in the medium treated with 100 nM DHEA with or without dutasteride. Twenty-four h after treating cocultured LNCaP cells with

PCaSC-8 or 9 in the presence of 100 nM DHEA with or without Dut, the medium was collected for LC-ESI-MS/MS. (E) and (F) Concentration of T and DHT (E) and Adione and Adiol (F) in the medium of LNCaP, PCaSC-8, and PCaSC-9 monoculture treated with DHEA with or without dutasteride. Twenty-four h after treating LNCaP cells, PCaSC-8, and PCaSC-9 in the presence of 100 nM DHEA with or without dutasteride, the medium was collected for LC-ESI-MS/MS. The data are presented as the mean \pm SD of triplicate measurements.
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Fig. 7 Mizokami et al.

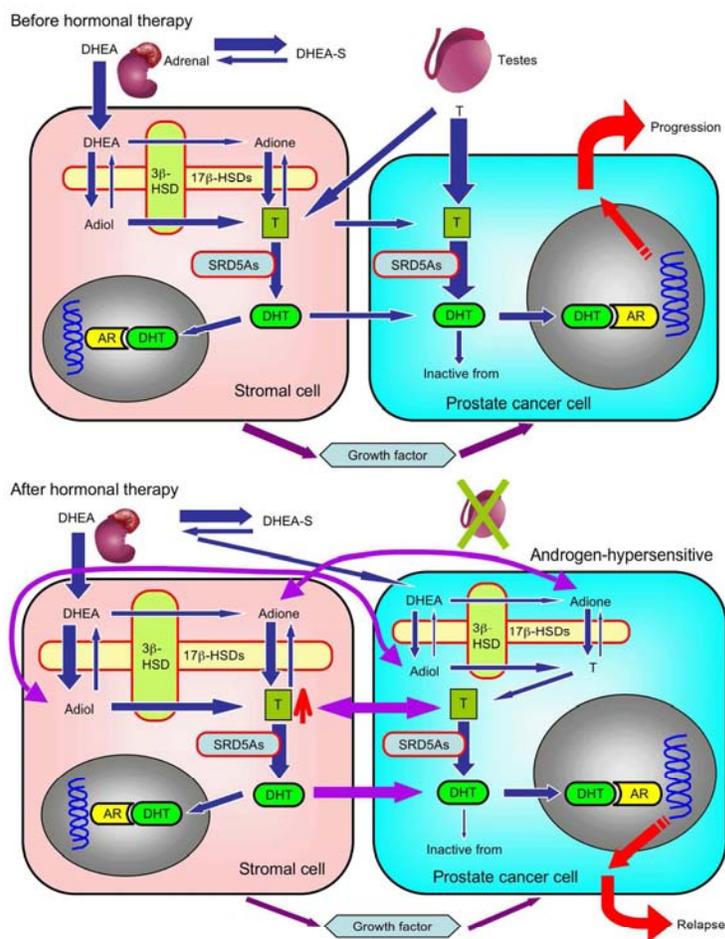


Fig. 7 Mechanism of how DHEA contributes to androgen therapy non-responsive PCa. Prior to androgen deprivation therapy (ADT), T from the testes plays a major role in the androgen activity in the prostate; whereas, the adrenal androgen DHEA plays a minor role. After ADT, in addition to the growth factors and cytokines from PCa-derived stromal cells that stimulate the proliferation of PCa, residual PCa cells become androgen-hypersensitive through various mechanisms and simultaneously PCa-derived stromal cells synthesize T, DHT, and precursors from DHEA coordinately. These synthesized androgens induce AR activity and stimulate the progression of PCa in a paracrine fashion.

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