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Laboratory-Prostate cancer

Cell-based evidence regarding the role of FSH in prostate cancer

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Abstract

Introduction: Conversion of androgen-responsive prostate cancer (CaP) to castration-resistant CaP is associated with an acceleration of the disease that often requires treatment modalities other than androgen deprivation therapy only. Recently, follicle-stimulating hormone (FSH) has been shown to play a role in CaP growth, and clinical data showed that high serum concentration of FSH in chemically castrated CaP patients was associated with a shorter time of progression to castration-resistant CaP. In this study, we sought to investigate if FSH could have direct effects on CaP cells, possibly through the androgen receptor and androgen receptor regulated genes, such as prostate-specific antigen (PSA).

Materials and methods: The human CaP cell lines PC-3, LNCaP and C4-2, and nonmalignant PNT1A cells, were utilized to investigate the effects of FSH. qPCR, Western blotting analysis, and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymetoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium assays were performed in order to analyze the FSH effects.

Results: The FSH receptor was present in all cell lines except PNT1A. FSH significantly increased PSA mRNA ($P < 0.01$) and protein ($P < 0.03$) levels in C4-2 cells in a dose-dependent manner. In LNCaP cells, FSH also increased PSA protein level, although to a lesser extent than in C4-2 cells, and the expression was reduced by the antiandrogen enzalutamide. In PC-3 cells, FSH was shown to increase their proliferation ($P < 0.03$) and β-catenin expression.

Conclusion: These findings demonstrate that FSH may have a direct effect in CaP in an androgen-depleted environment. However, further research is needed to understand the significance of direct FSH action in the maintenance of CaP growth at the different phases of transition from androgen dependence to androgen independence.

Keywords: Follicle-stimulating hormone receptor; Prostate-specific antigen; Signaling pathway; Castration-resistant prostate cancer

1. Introduction

Androgen deprivation therapy (ADT) is the cornerstone in the treatment of metastatic prostate cancer (CaP). Initially, tumors regress and prostate-specific antigen (PSA) level declines following ADT in the majority of patients. However, despite tumor regression, patients eventually invariably develop castration-resistant CaP (CRCaP) [1]. The mechanisms suggested to relate to this state of disease include androgen receptor (AR) mutation, AR hypersensitivity, and aberrant activation of AR signaling pathways by ligands other than androgens [2]. Accordingly, the cancer remains AR-driven in most patients, a concept that has been proven by the clinical success of abiraterone and enzalutamide, which have antiandrogenic effects [3–6].

ADT is implemented today through chemical castration using gonadotropin-releasing hormone (GnRH) agonists or antagonists. Clinical trials have shown that the GnRH agonist-induced suppression of luteinizing hormone results in rapid decrease in the levels of testosterone and PSA, whereas a rebound in the follicle-stimulating hormone (FSH) level is observed after initial suppression [7,8]. In contrast, with GnRH antagonists, sustained suppression of both gonadotropins is achieved [7,9]. Despite this potential

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advantage of GnRH antagonists, treatment with agonist is still more frequent.

The best evidence so far regarding the importance of keeping FSH suppressed has been obtained from clinical and preclinical data showing that increased serum level of FSH correlates with shorter time to CRCaP development, and that FSH receptor (FSHR) expression is found in CaP tissues at a higher level than in benign prostatic hyperplasia and normal prostate tissue [10–15].

FSHR belongs to the G-protein-coupled receptor gene family [16]. The physiological responses to FSH are mediated by different signaling pathways, starting with activation of cyclic adenosine monophosphate (cAMP) and transduced to mitogen-activated protein kinase, extracellular signal-regulated kinase, and the downstream effector cAMP response element-binding protein [17]. Furthermore, FSH has been shown to activate the phosphoinositide 3-kinase and Akt (PI3K/Akt) pathway [18–20]. Activation of this pathway is more likely pertinent to pathological conditions [21].

Extragonadal expression of the FSHR has been found in the prostate, breast, and blood vessels in a number of malignant tumors [12,13,15,22,23], suggesting the involvement of FSHR in the development and progression of multiple cancer types. Our knowledge about the role of FSH in CaP development is yet in its infancy and the mechanisms underlying the conversion of androgen responsive to androgen unresponsive state of CaP is poorly understood. In the current study, the effects of FSH on gene and protein regulation of human CaP cell lines, representing different stages of the tumor progression from androgen dependence to independence, was investigated by monitoring the expression of the AR target genes PSA and the prostatic tumor suppressor gene NKX3.1 as well as cell proliferation.

2. Materials and methods

2.1. *Cell culturing*

The human CaP cell lines PC-3, LNCaP, and C4-2 were purchased from the American Type Culture Collection (Manassas, VA) and the immortalized noncancer cells PNT1A from Sigma Aldrich (St. Louis, MO). The authenticity of the cell lines was confirmed by Eurofins Genomics (Ebersberg, Germany) before use. The C4-2 cells are derived from LNCaP cells implanted into a male athymic nude mouse. The host was castrated at 8 weeks and a single tumor specimen was excised 4 weeks after castration. Specimen was then implanted into a castrated mouse to produce a second generation cell line, C4-2 [24]. This subline was found to be androgen independent and capable of growing in castrated hosts. Cells were routinely grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin-neomycin (Life Technologies, Paisley, UK), and 2 mM L-glutamine (Life Technologies).

2.2. *Cell treatments*

For protein detection, cells were grown in phenol red-free RPMI medium containing 2% charcoal stripped-serum for 24 hours prior to treatment with FSH (Gonal-F, Merck Serono, Darmstadt, Germany) or 5α-dihydrotestosterone (DHT, Steraloids Inc., Newport, RI) for further 24 hours at concentrations of 50, 100, 200, and 400 IU/l or 10 nM, respectively. For AR induction, PC-3 cells were treated with FSH at concentrations of 400, 800, and 1,600 IU/l. In the mRNA detection, assay cells were treated with 50 and 100 IU/l FSH or 10 nM DHT for 6 or 24 hours. For combination treatment, cells were exposed to 10 μM enzalutamide (Astellas Pharma, London, UK) for 1 hour before the treatment with the aforementioned substances for further 24 hours. Forskolin (Sigma Aldrich, St. Louis, MO) is an agent known to induce cAMP by direct activation of adenylyl cyclase, and was therefore used as an FSH control. In controls, cells were left untreated. For the detection of phosphorylated Akt (p-Akt), cells were pretreated with 10 μM of the PI3K inhibitor, LY294002 (Sigma Aldrich) 1 hour before treatment with FSH at concentrations of 50, 100, and 200 IU/l for 24 hours.

2.3. *Western blot analysis*

For Western blot analysis, total protein was extracted using radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 20 mM EDTA, 1% NP40, 0.1% SDS, 1 mM Na3VO4, 1 mM NaF, and 1 mM phenylmethylsulfonylfluoride) (Invitrogen Carlsbad, CA) supplemented with the protease inhibitor cocktail Complete Mini (Roche, Mannheim, Germany). Total protein concentration was measured using bicinchoninic acid protein assay (Pierce, Rockford, IL). Protein samples (20–30 μg) were loaded on 4% to 12 % SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred onto a nitrocellulose or PVDF membrane (Bio-Rad, Hercules, CA) and subjected to electrophoretic analysis and blotting. Membranes were probed overnight at 4°C with the relevant primary antibodies anti-AR (PG-21), anti-FSHR, anti-β-catenin, and anti-p-Akt from Cell Signaling Technology (Beverly, MA), anti-PSA (Dako, Glostrup, Denmark) and anti-β-actin (Sigma Aldrich), washed and incubated in secondary antibodies horseradish peroxidase (HRP)-conjugated antimouse Immunglobulin G (IgG) and antirabbit IgG (GE Healthcare, Stockholm, Sweden) for 1 hour at room temperature. Bands were visualized using enhanced chemiluminescence (Pierce, Rockford, IL) and images were acquired using the Bio-Rad Western workflow (Bio-Rad, Hercules, CA). Densitometric quantification of immunoblots was performed by the ImageJ Image Analysis Software (NIH, Baltimore, MD) and represented as fold change relative to control, normalized relative to β-actin bands.

2.4. Real-time quantitative PCR analysis

Total RNA was isolated from the cells using RNeasy Mini Kit (Qiagen, West Sussex, UK) following the manufacturer's protocol. Each cDNA was synthesized by reverse transcription from 500 ng of total RNA using the RevertAid First-Strand Synthesis System and Oligo(dT)$_{18}$ primers (Life Technologies, Thermo Fisher Inc.). Real-time polymerase chain reaction (PCR) was performed with SYBR Green QPCR master mix (Life science, Thermo Fisher Inc) in AriaMx detection system (Agilent, Technologies, Willoughby, Australia) with an initiation step at 95°C for 10 minutes followed by 40 cycles at 95°C for 30 seconds, 56°C for 1 minute, and 72°C for 30 seconds. The following primers for each gene were used: AR (F 5′-CCTGGCTTCCGCAACTTACAC-3′ and R 5′-GGACTTGTGCATGCGGTACTCA-3′); PSA (F 5′-AGGCCTTCCCTGTACACCAA-3′ and R 5′-GTCTTGGCCTGGTCATTTCC-3′); NKX3.1 (F 5′-GTACCTGTCGGCCCCTGAACG-3′ and R 5′-GCTGTTATACACGGAGACCAGG-3′); FSHR (F 5′-GATGTTTTCCACGGAGCCTC-3′ and R 5′-ATCTCTGACCCCTAGCCTGA-3′); cMyc (F 5′-GGCGGGCACTTTGCACTGGA-3′ and R 5′-TCGCGGGAGGCTGCTGGTTT-3′); β-actin (F 5′-CGTGGGGCGCCCCAG-3′ and R 5′-TTGGCCTTGGGGTTCAGGGGG-3′). The mRNA amount was determined by using the ΔΔCt method. Samples were analyzed in triplicates and the data compared with the expression of mRNA in nontreated control which was set as reference value.

2.5. Proliferation assay

Proliferation of PC-3 cells was determined by a viability assay examined by trypan blue exclusion and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymetoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium assay (MTS) (Promega Biotech, Nacka, Sweden), according to the protocol from the manufacturer. Approximately 3×10^3 cells/well were cultured in 96-well plates for 24 hours in 5% serum complemented serum before being treated with FSH (100, 400 IU/l, or 10 nM/l DHT) for 24 and 48 hours. Cells were washed with phosphate buffered saline, and then 80 μl new medium containing 20 μl 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymetoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium was added to each well. Cells were incubated at 37°C for 2 hours and measured for optical density values at 490 nm on Milenia Kinetic Analyzer (Diagnostic Products Corporation, DPC, LA). Wells containing medium only served as a blank control. Three independent experiments were carried out.

2.6. Statistical analysis

Results were expressed as the mean ± standard deviation (SD). Statistical significance was determined with unpaired Student's t test. All statistical analyses were conducted using SPSS version 24 (SPSS Inc., Chicago, IL). Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Expression of FSHR in CaP cells

Expression of FSHR protein was demonstrated in all 3 cancer cell lines (Fig. 1, upper panels), but not in the PNT1A cells (Supplementary Figure 1). High to moderate expression of FSHR protein was found in the C4-2 and PC-3 cells, whereas in LNCaP cells the originally low protein expression was increased following FSH treatment (Fig. 1, upper panels). At mRNA level, FSHR expression was markedly increased in PC-3 and LNCaP cells treated with 100 IU/l FSH as compared with controls (Fig. 1, lower panels). C4-2 cells are androgen independent that have high basal expression of FSHR at protein and mRNA. Hormonal treatment therefore only changed it marginally.

3.2. FSH-induced PSA expression in androgen independent cells

FSH treatment significantly increased the protein level of PSA and AR in C4-2 cells ($P < 0.03$) at the concentration of 50 IU/l (Fig. 2A). The effect of FSH on PSA induction was less pronounced in LNCaP cells with a nonsignificant trend at concentration of 50 IU/l (Fig. 2B). The significant values were determined by densitometric analysis shown in lower panels. A significant induction of PSA mRNA was also detected in C4-2 cells in response to treatment with 100 IU/l FSH. The induction was observed after 6 hours ($P < 0.01$) and 24 hours ($P < 0.03$) of exposure (Fig. 2C). Moreover, in addition to PSA an increase of NKX3.1 gene expression was detected after 24 hours exposure to FSH at concentration of 50 IU/l, but the induction was narrowly short of statistical significance ($P > 0.05$). No significant induction of PSA mRNA was observed in LNCaP cells treated with FSH (Fig. 2D).

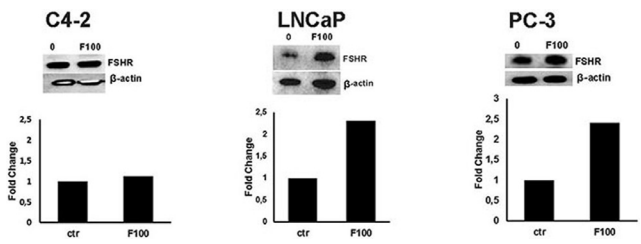

Fig. 1. FSHR expression in CaP cell lines. Upper panels are Western blot analyses of FSHR protein levels in C4-2, LNCaP, and PC-3 cells. Lower panels are qPCR analyses of FSHR transcript level in the aforementioned cells in the absence (ctr) or presence of 100 IU FSH (F100). FSHR = follicle-stimulating hormone receptor.

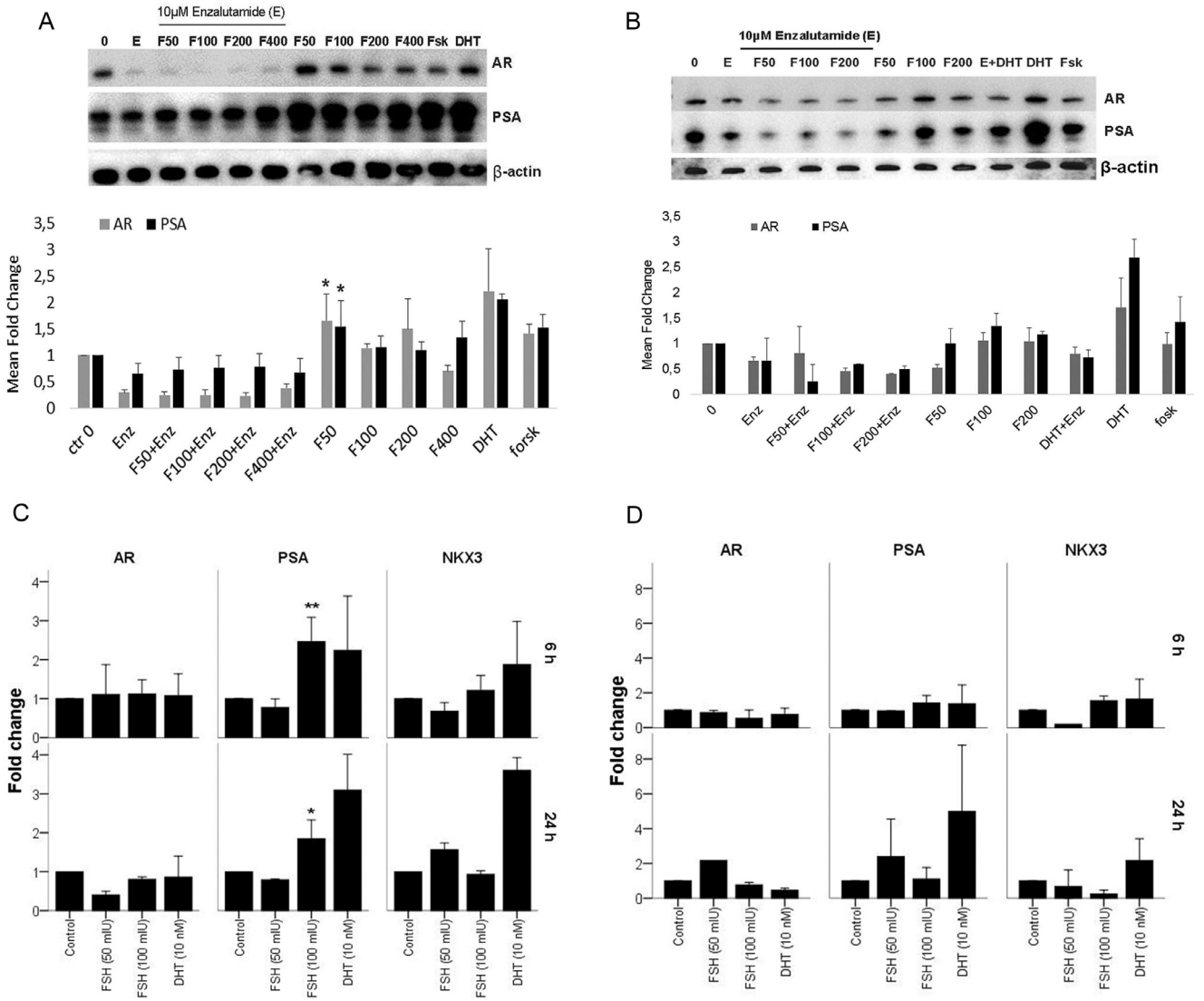

Fig. 2. PSA expression in CaP cell lines. (A and B) protein levels in C4-2 and LNCaP cells. Cells were treated with FSH (F) in the absence or presence of the AR blocker enzalutamide (E) for 24 hours. Lower panels are densitometric analyses of at least 3 Western blot analyses. (C and D) transcript levels of PSA and AR in C4-2 and LNCaP cells treated with FSH or DHT. Expression values were normalized to β-actin and determined by the $\Delta\Delta$Ct method. The y axis shows fold change in arbitrary unit. The values are mean $\pm$ SD of at least 3 independent experiments. $^*P < 0.05$; $^{**}P < 0.01$. CaP = prostate cancer; DHT = 5α-dihydrotestosterone; FSH = follicle-stimulating hormone.

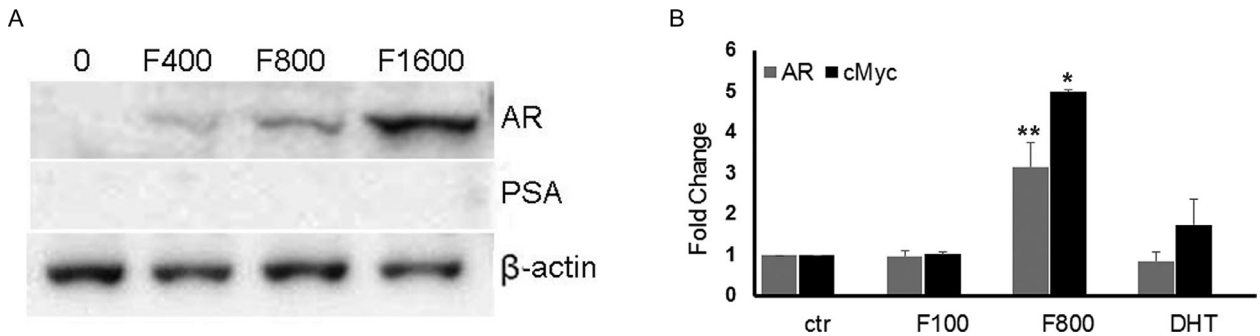

Fig. 3. AR and PSA expression in PC-3 cells. (A) Protein levels of PC-3 cells and (B), transcript levels of AR and c-Myc expression in PC-3 cells. Expression values were normalized to β-actin and determined by the $\Delta\Delta$Ct method. The y axis shows fold change in arbitrary unit. The values are mean $\pm$ SD of at least 3 independent experiments. $^*P < 0.05$; $^{**}P < 0.004$. F = follicle-stimulating hormone.

3.3. FSH-induced AR expression in PC-3 cells

In PC-3 cells, FSH was able to induce low level of AR protein at a very high concentration of 800 and 1,600 IU/l but not at lower concentrations (Fig. 3A). However, it failed to induce PSA protein in these cells. In contrast, qPCR showed a statistically significantly increased amount of AR ($P < 0.004$) upon treatment with 800 IU/l of FSH (Fig. 3B). FSH at high concentration also induced c-Myc ($P < 0.01$) in PC-3 cells.

3.4. Signaling pathway utilized by FSH

FSH enhanced the phosphorylation of Akt in all 3 cell lines to a varying degree, being most pronounced in the PC-3 cells (Fig. 4A). The PI3K/Akt inhibitor, LY294002 abrogated phosphorylation of Akt and reduced the level of PSA induced by FSH in both LNCaP and C4-2 cells (Fig. 4A). Moreover, FSH at concentration of 50 and 100 IU/l also increased the level of β-catenin in PC-3 and C4-2 cells, but not in LNCaP, as compared with controls (Fig. 4B). When C4-2 cells treated with the AR blocker, enzalutamide, together with FSH, they exhibited reduced Akt phosphorylation at concentrations of 50 and 100 IU/l, whereas, at higher concentrations, 200 IU/l, FSH seemed to restore phosphorylation of Akt (Fig. 4C).

3.5. Proliferation

Cell proliferation increased when exposed to high FSH concentration in PC-3 cells (Fig. 5A). At concentration of 100 IU/l, PC-3 proliferation was observed after 48 hours, whereas at concentration of 800 IU/l the proliferation started already after 24 hours and continued to the end of the 48 hours experiment ($P < 0.03$). This was in agreement with the cell viability determined by trypan blue exclusion assay (Fig. 5B). In contrast, DHT treatment did not contribute to PC-3 cell proliferation.

4. Discussion

The current study provides experimental evidence that FSH is able to induce PSA expression in CaP cell lines. Moreover, FSH can enhance the PI3K/Akt signaling pathway and induce cell proliferation. The high level of FSHR

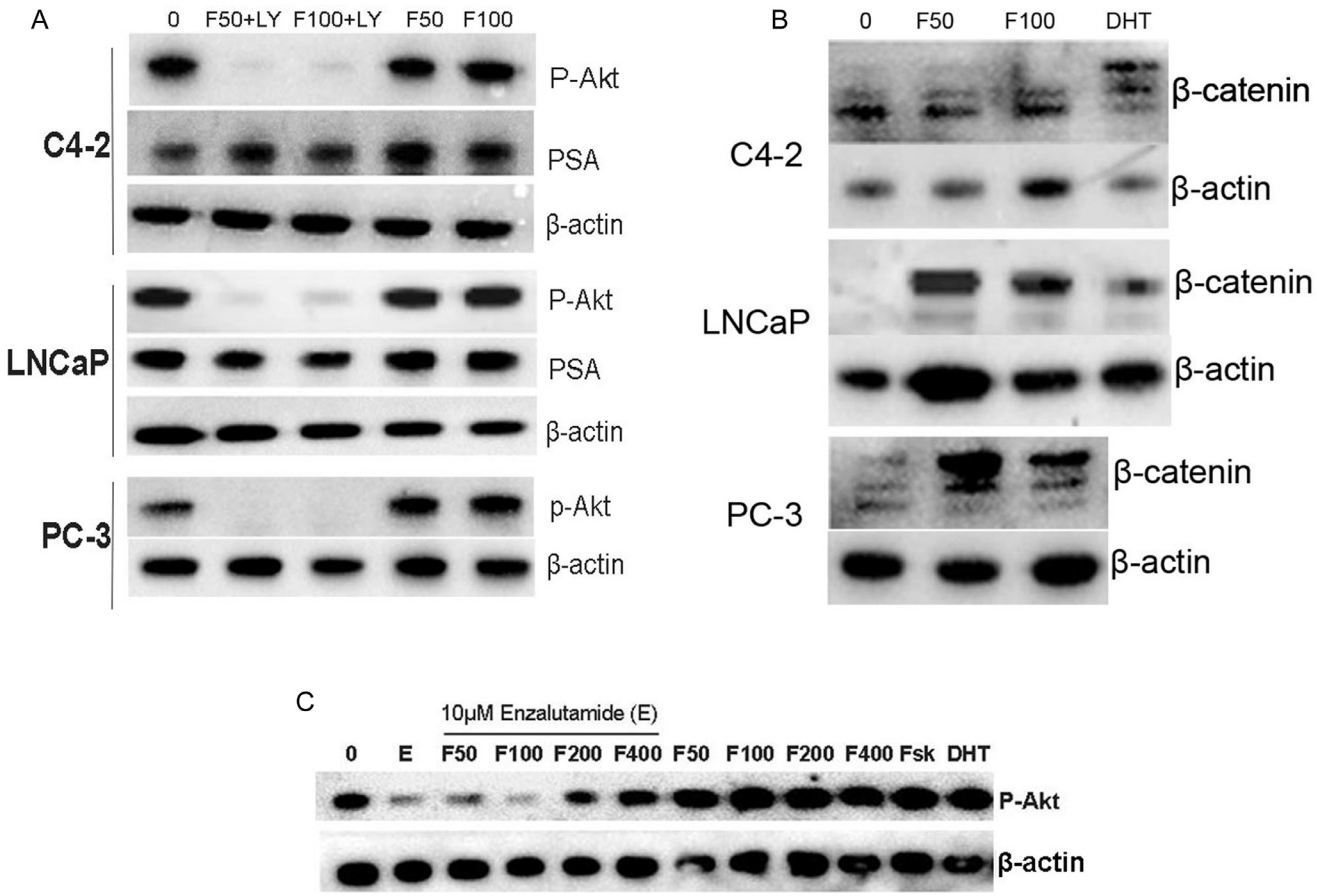

Fig. 4. FSH induced Akt phosphorylation and β-catenin expression in CaP cells. Western blot analyses of protein extracts show the phosphorylation of Akt (Ser^{473}) in (A) C4-2, LNCaP, and PC-3 cells after treatment with the FSH (F) in the presence and absence of PI3K/Akt inhibitor, LY294002 (LY); (B) expression of β-catenin in the C4-2, LNCaP, and PC-3 cells; (C) expression of phosphorylated Akt in C4-2 treated with FSH (F) in the absence or presence of enzalutamide (E). CaP = prostate cancer; FSH = follicle-stimulating hormone.

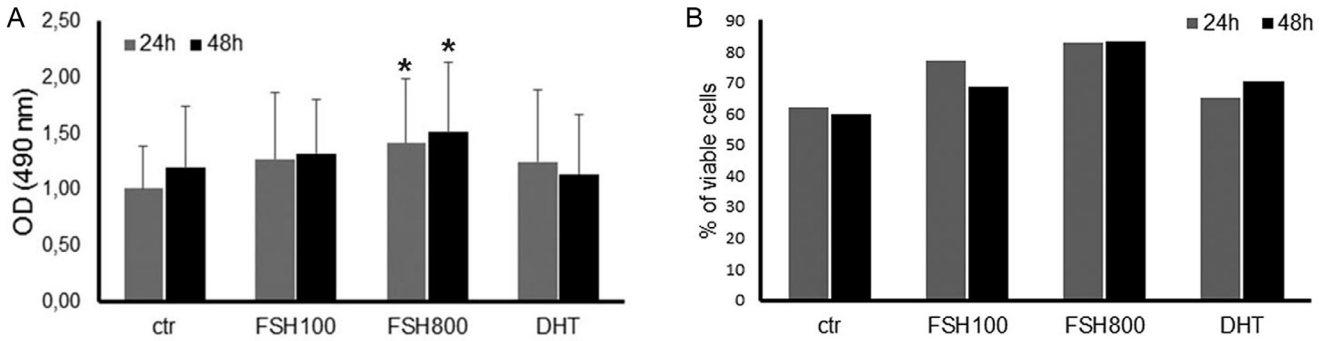

Fig. 5. (A) MTS assay showing the proliferation of PC-3 cells treated with FSH or DHT at concentrations and times indicated. (B) viability assay examined by trypan blue exclusion. The values in MTS are mean ± SD of 3 independent experiments.

* $P < 0.03$. DHT = 5α-dihydrotestosterone; FSH = follicle-stimulating hormone.

detected in C4-2 and PC-3 and low in LNCaP cells suggests that the FSH responsiveness may emerge during ADT and facilitate tumor cell growth and resistance to ADT. Conversely, the absence of FSHR in normal prostatic epithelial cells, PNT1A, shows that the FSHR is exclusively confined to cancer cells, and especially to androgen independent cells. These results were in line with the previously reported studies in humans, in whom FSHR expression was absent in most noncancerous cells, in contrast to malignant ones [11–13].

Rising serum PSA in patients after ADT indicates relapse and that malignant cell growth is restored in an AR-independent fashion [25]. The significant induction of PSA in the metastatic C4-2 cells is in accordance with the clinical scenario that patients undergoing ADT, and as a consequence of this having high serum levels of FSH, also had higher PSA levels [9,26]. In contrast, FSH did not show significant effect on PSA induction in LNCaP cells. The difference in response to FSH in these 2 cell lines is likely due to the fact that LNCaP cells are not under selection pressure of the androgen-depleted environment [27], whereas C4-2 cells are not depending on androgens to proliferate although they induce AR and PSA upon treatment with androgens.

Unlike the gonads, where cAMP is the main second messenger of FSH action, the major signaling cascade activated by FSHR in CaP cells is PI3K/Akt. FSH has been reported to utilize the PI3K/Akt signaling pathway in the progression and migration of many cancers including breast cancer [15,20–22]. In the present study, we showed that FSH augmented levels of p-Akt and also increased β-catenin levels in both C4-2 and PC-3 cells. It is tempting to speculate that reduced androgen action is compensated through signal transduction pathways shared with FSH, as recently demonstrated in the testis tissue [28].

In the majority of studies investigating PC-3 cells, the AR is not detected. However, some studies have stated that the AR is only detectable after DHT treatment [29]. We found that very high concentration FSH was able to induce AR expression in PC-3 cells and the expression was correlated with significant cell proliferation. This finding may corroborate the role of AR, as in the presence of FSH, these cells did not require androgens to proliferate. This finding may also explain the absence of AR detection in some studies. We speculate that FSH can act as a substitute for androgens though activation of the mitogenic signals that may amplify the activity of the AR in a low-ligand environment, possibly mediated by targeting the transcriptional machinery or the AR itself, via genomic and nongenomic mechanisms. Further, we observed that enzalutamide abrogated FSH-stimulated induction of PSA and p-Akt in C4-2 cells and in parallel, a p-Akt inhibitor not only inhibited p-Akt, but also resulted in reduced PSA concentration. An important finding was that FSH also led to the increase of β-catenin, which is a downstream target of Akt and known to play a role in the proliferation and migration in CaP cells [30]. These results together suggest a cross-talk between AR and FSHR signaling pathways, likely also affecting downstream targets.

During ADT of CaP patients with GnRH agonists, FSH levels drop initially and then gradually rebound toward pretreatment levels [7,8]. This, and the evidence of the correlation between levels of FSH and CaP progression as well as the shorter time to the development of CRCaP [10,14] suggests that FSH/FSHR may play a pivotal role in this context. The potential mechanisms of how FSH influences the progression to CRCaP may include (1) activation of PI3K/Akt a signaling shared by AR and FSH, (2) increased β-catenin, which has been reported to be a relatively specific coactivator of the AR, (3) expression of the c-Myc gene, related to tumor growth regardless of the AR, and (4) the FSH signaling pathway promoting the interaction between transcription factors and AR, and thereby by increasing the transactivation of the AR to initiate transcription of genes normally exclusively regulated by androgen as reflected by PSA expression (Fig. 6).

More studies are warranted in order to elucidate the significance of FSH action in the context of the progression of CaP.

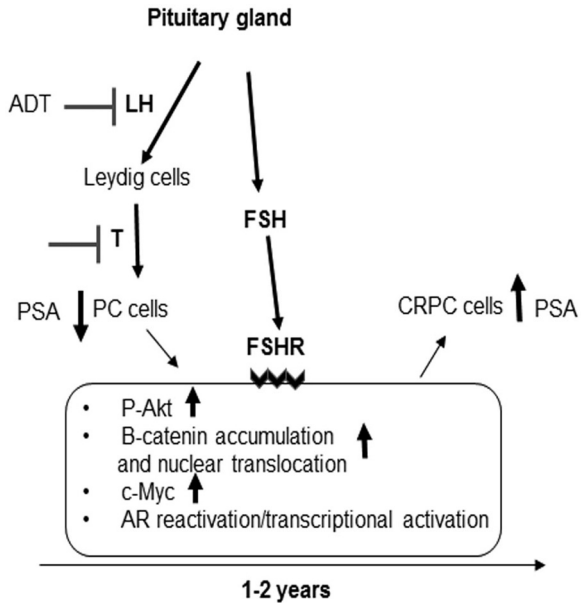

Fig. 6. The potential mechanisms of how FSH influences the progression of androgen dependent prostate cancer (PC) to castration resistant PC (CRPC). ADT = Androgen deprivation therapy, FSH = follicle-stimulating hormone, FSHR = follicle-stimulating hormone receptor, LH = Luteinizing hormone.

Conflict of interest

The authors have no conflicts of interest to disclose.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.urolonc.2018.12.011.

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