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RESEARCH ARTICLE

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Follicle-stimulating hormone promotes growth of human prostate cancer cell line-derived tumor xenografts

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Abstract

Chemical castration in prostate cancer can be achieved with gonadotropin-releasing hormone (GnRH) agonists or antagonists. Their effects differ by the initial flare of gonadotropin and testosterone secretion with agonists and the immediate pituitary-testicular suppression by antagonists. While both suppress luteinizing hormone (LH) and follicle-stimulating hormone (FSH) initially, a rebound in FSH levels occurs during agonist treatment. This rebound is potentially harmful, taken the expression of FSH receptors (R) in prostate cancer tissue. We herein assessed the role of FSH in promoting the growth of androgen-independent (PC-3, DU145) and androgen-dependent (VCaP) human prostate cancer cell line xenografts in nude mice. Gonadotropins were suppressed with the GnRH antagonist degarelix, and effects of add-back human recombinant FSH were assessed on tumor growth. All tumors expressed GnRHR and FSHR, and degarelix treatment suppressed their growth. FSH supplementation reversed the degarelix-evoked suppression of PC-3 tumors, both in preventive (degarelix and FSH treatment started upon cell inoculation) and therapeutic (treatments initiated 3 weeks after cell inoculation) setting. A less marked, though significant FSH effect occurred in DU145, but not in VCaP xenografts. FSHR expression in the xenografts supports direct FSH stimulation of tumor growth. Testosterone supplementation, to maintain the VCaP xenografts, apparently masked the FSH effect on their growth. Treatment with the LH analogue hCG did not affect PC-3 tumor growth despite their expression of luteinizing hormone/choriongonadotropin receptor. In conclusion, FSH, but not LH, may directly stimulate the growth of androgen-independent prostate cancer, suggesting that persistent FSH suppression upon GnRH antagonist treatment offers a therapeutic advantage over agonist.

Abbreviations: BSA, Bovine serum albumin; CAS, castrated mice; DG, degarelix; FSH, Follicle-stimulating hormone; FSHR, Follicle-stimulating hormone receptor; GnRH, gonadotropin-releasing hormone; GnRHR, gonadotropin-releasing hormone receptor; hCG, human chorionic gonadotropin; IN, intact mice; ISH, in situ hybridization; i.t., intratumoral; LH, luteinizing hormone; LHCGR, luteinizing hormone/choriongonadotropin receptor; PBS, phosphate-buffered saline; PCa, prostate cancer; PSA, prostate-specific antigen; rhCG, recombinant choriongonadotropin; rhFSH, recombinant human follicle-stimulating hormone; s.c., subcutaneous.

KEYWORDS
follicle-stimulating hormone, follicle-stimulating hormone receptor, gonadectomy, gonadotropin-releasing hormone antagonist, prostate cancer

1 | INTRODUCTION

GnRH analogues are presently the standard hormonal treatment for prostate cancer (PCa).[1] While GnRH agonists have been in clinical use for nearly 40 years, the antagonists have been available only recently. Both suppress testosterone through pituitary downregulation. The agonists block gonadotropin secretion and release, and consequently testosterone production, through the downregulation of pituitary GnRH receptors (R), while the antagonists block GnRH action through competitive binding to GnRHR. Another mechanism for the antitumor action of GnRH analogues has been suggested to be their direct action on GnRHR expressing tumor cells.[2]

A potential drawback of GnRH agonists has been the initial flare of gonadotropin and androgen secretion, which can be controlled by antiandrogen at the beginning of treatment.[3] In contrast, the inhibition of gonadotropins and testosterone by antagonist treatment is immediate. Another fundamental difference in agonist and antagonist action is the permanent suppression of both LH and FSH by antagonist, while a rebound following initial suppression occurs in FSH levels during agonist treatment.[4,5]

The few comparative data available between agonist and antagonist treatments in the clinical outcome of PCa demonstrate no major differences in their efficacy.[6-8] However, very recently, a crossover study from GnRH agonist (leuprolide) to antagonist (degarelix) demonstrated better PCa control by the latter with a significant decrease in prostate-specific antigen (PSA) levels.[9] The improvement was ascribed to the concomitant FSH suppression, as suggested by earlier studies.[10-12]

The profound and long-term suppression of FSH by antagonist[5] offers a potential therapeutic advantage in view of the demonstrated FSHR expression in PCa parenchyma[13-15] and neo-vasculature,[16] and in vitro studies on stimulatory effects of FSH on signaling[15] and growth[13] of PCa cell lines. We therefore decided to study further the potential FSH effects on PCa growth using the athymic mouse in vivo model of human PCa cell xenografts in androgen-independent (PC-3, DU145) and androgen-dependent (VCaP) cell lines. The mice were treated with the GnRH antagonist degarelix to suppress LH and FSH secretion, and effects of supplementation with recombinant human (rh) FSH (rhFSH) and choriongonadotropin (CG) (rhCG) treatments were assessed on tumor growth. We provide evidence that FSH, but not LH, significantly stimulates the growth of androgen-independent PCa cell xenografts, possibly through direct action.

2 | MATERIALS AND METHODS

2.1 | Drugs and reagents

Degarelix was synthesized and supplied by Ferring Pharmaceuticals A/S, Denmark. rhFSH (#G16441, #H13284; 15 445 IU/mg) and rhCG (#13pd-84-Tox-2-DS-002, #16PD-8-DS-002; 21 717 IU/mg) were supplied by Bio-Technology General (Israel) Ltd., (BTG, Kiryat Malachi, Israel), a subsidiary of Ferring Pharmaceuticals. Osmotic continuous delivery pumps (Model #1004, 28-day delivery, 100 µL total volume with delivery rate of 0.11 µL/h = 2.64 µL/day) were purchased from DURECT Corporation (Cupertino, CA, USA). All other reagents, unless otherwise stated, were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2 | Preparation of drugs and treatments

Degarelix was dissolved in 5% mannitol solution and administered by subcutaneous (s.c.) injection of 10 mg/kg body weight (=250 µg/25 g mouse; 2500 µg/mL; 100 µL/mouse), adjusted for weight dependency. rhFSH (10 IU/kg/day) and rhCG (100 IU/kg/day) were administered as a continuous dose using micro-osmotic delivery pumps. Filling and preparation of the osmotic pumps were under aseptic conditions, according to the manufacturer's instructions. To ensure adequate filled volumes, pumps were weighed before and after filling. Filled pumps were primed by incubation in sterile saline solution at 37°C for 24 hours prior to the commencement of implantation. Depending on the experimental procedure, vehicle control was either 5% mannitol or 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS).

2.3 | Cell culture

The androgen-independent cells, PC-3 (ATCC CRL-1435) and DU145 (ATCC HTB-81) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The androgen-dependent VCaP cells (ATCC CRL-2876) was kindly provided by Dr Matti Poutanen (University of Turku, Finland). Cells were routinely cultured using appropriate media; RPMI 1640 (Sigma-Aldrich) for PC-3 and DU145 cells, and DMEM (Sigma-Aldrich) for VCaP cells. The media were supplemented with 10% fetal bovine serum (FBS) and 1% antimycotic/antibiotic agents, and regularly

tested for the absence of *Mycoplasma* contamination with standard protocols. Cell viability counts were done initially by manual method using a hemocytometer (Fisher Scientific, Loughborough, UK), and confirmed with Beckman Coulter Vi-CELLTM-XR 2.04 cell viability analyzer (Beckman Coulter, High Wycombe, UK). Prior to inoculation, PC-3 and DU145 cells were suspended in serum-free RPMI 1640, while the VCaP cells was suspended in a mixture containing equal volumes of DMEM and Matrigel (Corning, Bedford, MA, USA).

2.4 | Xenograft experiments

All experimental procedures were approved by the Central Biomedical Services, Imperial College London and conformed to the regulations and standards of the UK Home Office Animal Scientific Procedures Act (1986) and the European Union Directive (2010).

Adult 5-to 6-week-old male athymic-nude (Crl:NU(NCr)-*Foxn1*^{nu}) immunodeficient mice (Charles River, UK) were housed in pathogen-free environment, under a 12-hour light-dark cycle, with controlled humidity and temperature, and fed with irradiated rodent chow RM3 diet and autoclaved water ad libitum. Surgical procedures and injections were performed in sterile conditions, under isoflurane (Abbot Laboratories Ltd., Maidenhead, UK) anesthesia, and a follow-up post-operative analgesia treatment. Androgen ablation by castration was conducted by removal of both testes through midline incision of the skin and underlying tunica of the scrotal sac. Each testis, vas deferens and epididymal fat pad were carefully pulled out through the incision with blunt forceps. The blood vessels of the testis were clamped with a hemostat, and the testis dissected away. The vas deferens and the fat pad were cauterized and placed back into the scrotal sac. This procedure was repeated for the other testis, followed by the suture of the midline incision.

The micro-osmotic pump insertion for the administration of hormonal compounds and vehicle were implanted through an incision below the right dorsal skin, without impediment to vital organs, and suture sites were allowed to dry before commencement of tumor induction.

To minimize potential bias, mice were randomly distributed into four groups (n = 10/group) in each experiment. Groups 1 and 2 were treated with 5% mannitol vehicle. Groups 3 and 4, depending on the experiment, received either rhFSH or rhCG treatment. Groups 1 and 3 received 0.1% bovine serum albumin (BSA) in PBS vehicle, while groups 2 and 4 had degarelix treatment. Cells with viability >90% were used to induce tumors, by injection of 2×10^6 cells/100 μL media s.c. above the left flank. 100 μL of vehicle or degarelix, adjusted accordingly for weight dependency, were injected into the loose skin of the neck region over the shoulder area as a one-off 4-week treatment. Mice were monitored daily for abnormalities, including signs of pains, distress, infection, and local ulceration at implantation sites. Bi-weekly body weight assessment was further used to evaluate animal health status. Tumor volumes were also measured twice weekly with a digital caliper (VWR, Lutterworth, UK) and calculated with the formula: ½ (length × width^2), and data presented as mean ± SEM (mm^3).

In a separate xenograft experiment with PC-3 cells, we assessed the potential efficacy of intratumoral (i.t.) degarelix injection in the therapy of tumors. Intact (IN) mice were divided into four treatment groups (n = 10/group) of vehicle, i.t., s.c., and i.t./s.c. combined regimen. Cells were allowed to grow for 3 weeks, prior to treatment with 100 μL of vehicle or degarelix injection by i.t., s.c., i.t./s.c. combined route. Tumors were allowed to grow for a further 3 weeks, or until the license endpoint, of a mean superficial tumor diameter of >15 mm was reached, wherein they were culled immediately by a Schedule 1 method.

2.5 | Sample collection and preparation

Animals were euthanized at the end of each experiment by intraperitoneal injection of 2, 2, 2-tribromoethanol (Sigma-Aldrich).[17] Blood collection was by cardiac withdrawal followed by cervical dislocation. Serum was separated by centrifugation and stored at −20°C until required. At necropsy, tumors were excised from the flanks and stripped of non-tumoral tissues, and the mass of each tumor (tumor weight) determined. The tumor burden (tumor weight/body weight of animal) was also calculated to assess the tumor load on each animal. A portion of each graft was flash-frozen in liquid nitrogen and stored at −80°C for RNA isolation. Another portion was fixed in 10% formalin and processed for histological and immunohistochemical analyses.

In intact (IN) mice, the reproductive organs were exposed via an abdominal incision, and testes dissected from the scrotal sac as earlier indicated in the castration procedure. For the dissection of the seminal vesicle, the highest point of the urinary bladder was grasped with blunt forceps, lifted up, and cut along the ligament connecting the urinary bladder to the ventral body wall. The bladder was thereafter cut off, and the coagulation glands carefully separated along the edges without puncturing the seminal vesicle walls. The testes and seminal vesicle were separately weighed, and the mean value of the testes used for calculation.

2.6 | Hormonal measurements

Serum LH and FSH concentrations were measured by immunofluorometric assays (Delfia; Perkin-Elmer-Wallac,

Turku, Finland) as described previously.[18,19] Serum testosterone was measured using Waters Acquity UPLC and Waters TQS tandem mass spectrometer (Waters, Manchester, UK),[20] with lower limit of quantitation of 0.1 nmol/L.

2.7 | Histology and in situ hybridization

Grafts were fixed in 10% formalin overnight and subsequently dehydrated in 2-3 changes each of graduated ethanol solutions until absolute water-free ethanol, cleared in histoclear (National Diagnostics, Hessle Hull, UK), and embedded in paraffin. Sections of 5 μm were cut and mounted on polylysine microscope slides (VWR, Lutterworth, UK), dried at 37°C for 1 hour, and stored until required. Sections for histology were stained with the routine hematoxylin and eosin protocol.

In situ hybridization (ISH) of mRNA in graft sections was performed using predesigned probes for human *FSHR* (*POLR2A*), mouse *Fshr* (*Polr2a*), and nonsense (dapB) according to the manufacturer's protocol and as previously described,[21] using RNAScope 2.5 HD Reagent Kit-BROWN (Advanced Cell Diagnostics, Newark, CA, USA). Hybridization with the predesigned probes was performed in HybEZTM Oven (Advanced Cell Diagnostics, Newark, CA, USA). Sections were scanned with Pannoramic Midi FL slide scanner (3DHISTECH Ltd., Budapest, Hungary) and pictures taken using Pannoramic Viewer (3DHISTECH Ltd.).

2.8 | Quantitative real-time PCR analyses of target genes

Total RNA (tRNA) was purified from xenografts with TRI Reagent (Sigma-Aldrich). 1 μg of tRNA was reverse-transcribed to complimentary DNA (cDNA) using SuperScript II First Strand Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Quantitative PCR (qPCR) of gene expression was performed on diluted cDNA as described previously,[22,23] using SYBR Green JumpStart Taq ReadyMix reagent in ABI Step One Plus QPCR system (Applied Biosystems, Carlsbad, CA, USA) with human and mouse gene-specific primer sequences (Table S1), using the program: 95°C-10 minutes, 40 cycles at 95°C-15 seconds, 60°C-30 seconds, and 72°C-30 seconds. A minimum of three samples from each group were quantified, with each gene analyzed three times in duplicates per assay. Mean results of all three assays were analyzed using the $2^{-\Delta\Delta Ct}$ method.[24] β-actin gene was used as endogenous control for the expression of all human genes while the mouse gene expression was normalized against the geometric mean of ribosomal protein L19 (RpL19) and mouse β-actin.

2.9 | Statistical analysis

Statistics were performed with GraphPad Prism 8.3 software (GraphPad Software, Inc, San Diego, CA, USA). All data sets are presented as mean ± SEM, unless otherwise stated. Statistical significance between means was assessed using Student's t tests, and multiple comparisons using ANOVA, and Newman-Keuls post hoc test. For pooled data analysis, the 2-way ANOVA was used to ascertain the effect of independent variables. Difference between two data subsets was considered significant at P at least $\leq .05$. GraphPad StatMate 2.0 was used for power analysis to determine the numbers required for the groups under comparison, at a significance level of 0.05, and a power of 80%. With an expected 40% effect size and 30%-40% SD, the n needed per group was estimated as 7-10.

3 | RESULTS

3.1 | FSH treatment reverses the growth inhibition of degarelix in androgen-independent PC-3 and DU145 cell xenografts

The preliminary assessment of PC-3 cell tumors over a 4-week period demonstrated reduced growth with degarelix treatment, by 33% and 35%, in intact (IN) and castrated (CAS) mice (Figure S1A; $P < .0001$), respectively, with a concomitant decrease of about 50% in tumor weights (Figure S1B; $P < .0001$). No significant differences in body weights were observed during the experiments (Figure S2A,B). The definitive PC-3 cell experiment in degarelix-treated mice, with or without concomitant rhFSH supplementation replicated the preliminary degarelix effects on tumor volume (39% reduction in IN and 34% in CAS) (Figure 1A,B; $P < .0001$), tumor weight (Figure 2A,B; $P < .05$), and tumor burden (Figure S3A,B; $P < .001$). rhFSH significantly reversed the inhibitory effect of degarelix on PC-3 tumor volume (Figure 1A,B; $P < .05$). The FSH effect was, however, not seen in tumor volume of non-degarelix-treated mice (Figure 1A,B; $P > .05$). Hence, whether assessed by tumor weight or burden, or in IN or CAS mice, the same conclusion could be drawn, that rhFSH partially reversed the growth inhibition of PC-3 cell xenografts by degarelix.

In a clinically more relevant therapy-based setting, with hormone treatments of IN mice starting 3 weeks after tumor inoculation, and a subsequent follow-up for another 3 weeks, degarelix again suppressed tumor volume (Figure 1C; $P < .05$) and weight (Figure 2C; $P < .05$). rhFSH treatment, with and without degarelix, also significantly increased the tumor weights, with no significant differences observed in body weights (Figure S2C) or tumor burdens (Figure S3C).

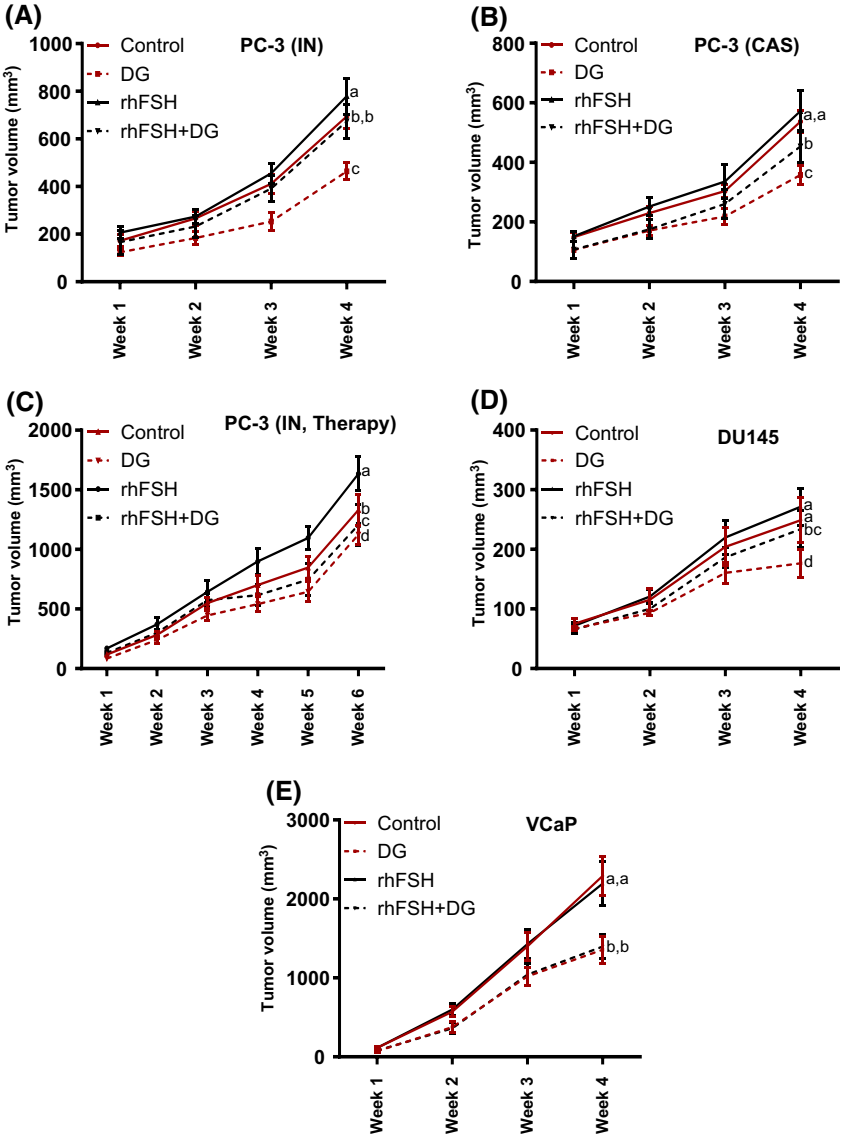

FIGURE 1 Effects of gonadectomy, degarelix (DG), and rhFSH on prostate cancer xenograft growth, as monitored by tumor volume, during the 4-week (6 weeks in panel C) experiments. A, PC-3 xenografts in intact mice (IN) in preventive setting. B, PC-3 xenografts in castrated mice (CAS) in preventive setting. C, PC-3 xenografts in IN mice in therapeutic setting. D, DU145 xenografts in IN mice in preventive setting, and (E) VCaP xenografts in IN mice in preventive setting. Different letters represent significant differences between the groups at the end of the experiment, n = 7-10 mice/group. Data are represented as mean ± SEM. *$P < .05$; (ANOVA/ Newman-Keuls)

We next assessed with IN mice the effect of degarelix and rhFSH on another androgen-independent prostate cancer cell line, DU145. A 30% reduction in tumor growth was found upon degarelix treatment (Figure 1D; $P < .001$). Non-significant increasing effects of rhFSH on tumor weight (Figure 2D) were found with and without degarelix treatment. A two-way analysis of variance (ANOVA), however, demonstrated a statistically significant rhFSH effect (Figure 2D; $P = .05$). No significant differences in the body weights (Figure S2D), and tumor burdens (Figure S3D) were found.

3.2 | Androgen-dependent VCaP xenografts responded to degarelix, but not to FSH treatment

The maintenance of VCaP cells require testosterone supplementation, which attained serum concentrations between 11.7 ± 1.4 and 14.8 ± 1.7 nmol/L in the treatment groups. Degarelix treatment decreased VCaP tumor growth by about 40% (Figure 1E; $P < .0001$), and tumor weight by about 33% (Figure 2E; $P < .001$), but no effect of rhFSH supplementation was observed. No differences were observed in body weights (Figure S2E), and in tumor burden (Figure S3E).

3.3 | Responses of hormones and hormonal effects to degarelix and FSH treatments

In PC-3 cell xenografts, degarelix suppressed LH and FSH levels (Figure 3A,B), while castration increased their levels (Figure S1C,D). In DU145 xenografts, we observed an unexpected moderate increase of gonadotropins level in the rhFSH treatment group (Figure 3A,B). Because of the exogenous testosterone treatment of mice carrying VCaP cell xenografts, their gonadotropin levels became very low (0.04–0.07 ng/L) (Figure 3A,B). FSH was suppressed

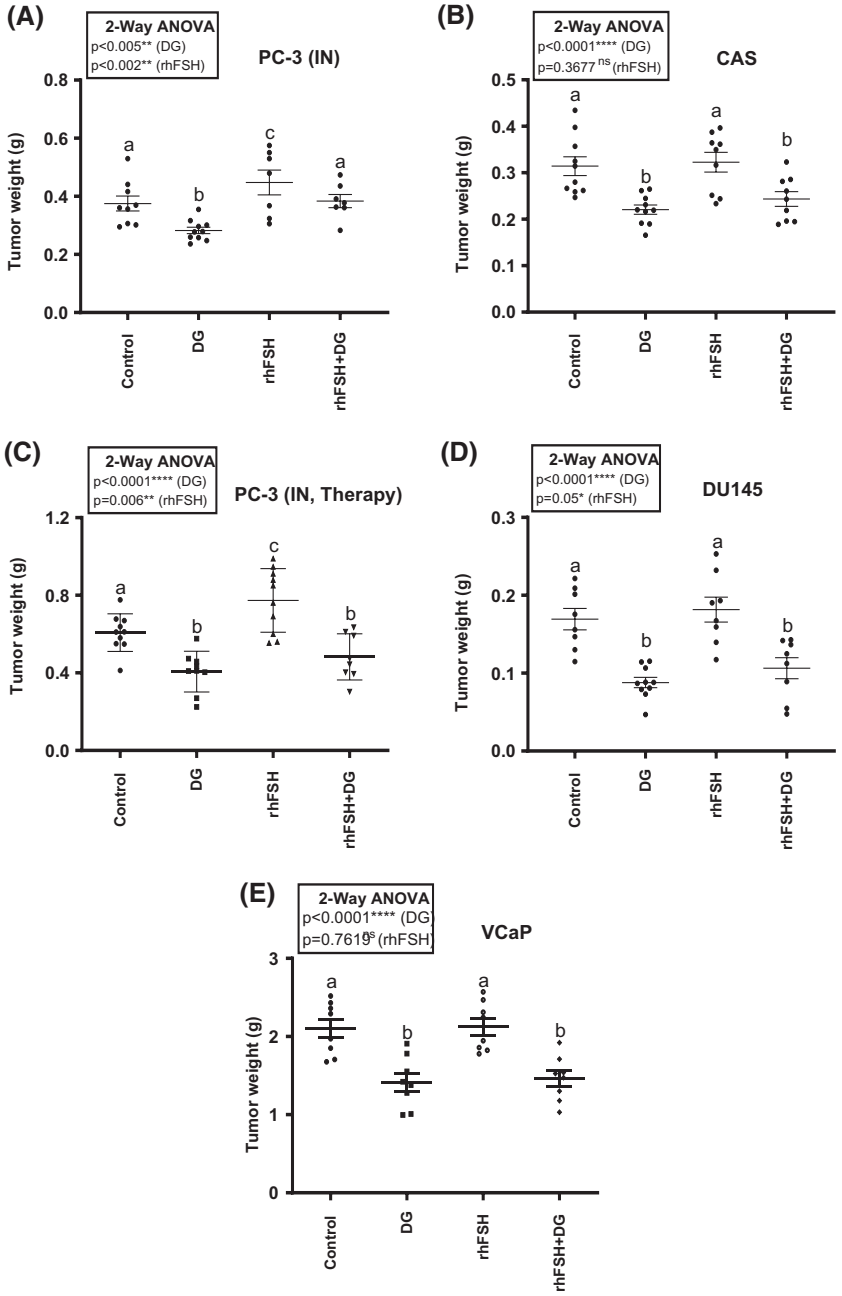

FIGURE 2 Effects of gonadectomy, degarelix (DG), and rhFSH on final weights of tumors at the end of the 4-week (6 weeks in panel C) experiments. A, PC-3 tumors in intact (IN) mice. B, PC-3 tumors castrated (CAS) mice. C, PC-3 tumors in a therapeutic setting (IN mice). D, DU145 tumors (IN mice), and E, VCaP tumors (IN mice). Data are mean ± SEM, n = 7-10 mice/group. Groups with different superscript letters differ significantly from each other (*P* at least < .05; ANOVA/Newman-Keuls)

by degarelix in the absence and presence of rhFSH supplementation (Figure 3B), but was somewhat higher in the latter, possibly due to the 5.6% cross-reactivity of human FSH in the murine FSH immunoassay.[19] Testosterone levels were non-detectable in all degarelix-treated androgen-independent mice (Figure 3C; Figure S1E, S5E, S6E), while FSH treatment increased testosterone levels in IN mice (Figure 3C). There was a clear decrease in testis and seminal vesicle weights in all degarelix-treated groups (Figure S4A-D), and FSH did not influence these weights. The level of human FSH in the rhFSH-treated mice was 5.91 ± 1.12 IU/L (n = 6).

3.4 | hCG had no effect on the growth of PC-3 cell xenografts

Because the PC-3 xenografts expressed both hLHCGR and mLhr (Figure 4A,D), and degarelix suppressed both gonadotropins, we assessed whether treatment with the LH analogue hCG would affect xenograft growth. As before, degarelix suppressed tumor weights and burden (Figure S5A,B) and gonadotropin levels (Figure S5C,D). However, hCG reversed the degarelix-suppressed testosterone levels (Figure S5E) with no effect on tumor growth, providing further evidence for their androgen independence.

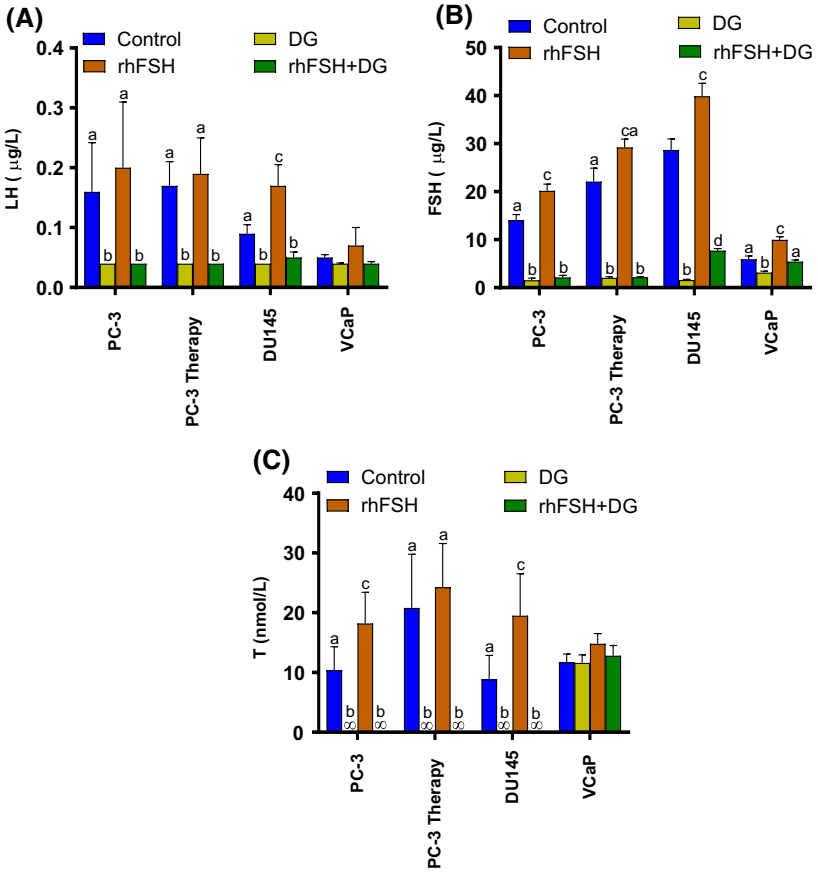

FIGURE 3 Serum gonadotropins and testosterone levels in mice with different prostatic cancer cell xenografts. A, LH. B, FSH, and C, testosterone. Data represent mean ± SEM; n = 7-10 individual samples/group. Bars with different superscript letters differ significantly from each other (*P* at least < .05; ANOVA/Newman-Keuls). ∞ = undetectable, ascribed the minimum detectable concentration (0.10 nmol/L) for statistical calculation

3.5 | Combined subcutaneous and intratumoral degarelix treatment did not improve suppression of tumor growth

Here, we assessed whether higher local concentration of degarelix by direct intratumoral (i.t.) injection would amplify its antitumoral effect. This was not the case and, contrarily, we observed that i.t. degarelix was less effective in suppressing tumor growth than a similar s.c. dose. The i.t. degarelix-treated xenografts grew at the same rate with vehicle-treated mice (Figure S6A,B; $P \geq .05$). Augmentation of the drug effect by a combined i.t./s.c. dose showed similar antitumor response obtained with s.c. treatment only, both in tumor weight and burden (Figure S6A,B; $P \geq .05$). Despite these findings, the different modes of degarelix administration showed similar suppression of serum gonadotropins and testosterone (Figure S6C-E).

3.6 | Tumor xenografts express both human and mouse receptors

hFSHR and *hLHCGR* were expressed in PC-3 tumor xenografts, but not in the original cells from culture (Figure 4A). Highest *hFSHR* expression was found in PC-3 xenografts in control and FSH-treated mice, and degarelix suppressed the expression in both groups, while FSH treatment brought about a small increase in the xenografts of degarelix-treated mice. *hLHCGR* expression was found in all xenografts, and unlike FSHR, it was upregulated by degarelix treatment. *GnRH1*, *GnRH2,* and *hGnRHR* were also expressed in all samples, with no clear difference between the groups (Figure 4A). Unlike gonadotropin receptors, these genes were also expressed in cultured PC-3 cells. Low levels of mouse *Fshr*, *Lhr,* and *Gnrh* expression were detected in the tumors (Figure 4D).

As with PC-3 cells, the cultured DU145 cells did not express either human or mouse receptors for LH or FSH, but these receptors were expressed in the xenografts. Both cell and tumor samples also expressed *hGnRH1*, *hGnRH2,* and *hGnRHR* (Figure 4B). As in PC-3 cells, degarelix treatment suppressed *hFSHR* expression, while the rhFSH effect on *hLHCGR* did not reach significance in these cells. As with PC-3 cells, the receptor expression in DU145 cells was too low to allow detailed conclusions about the differences between treatments. The DU145 xenografts also expressed variable levels of mouse *Fshr*, *Lhr*, and *Gnrh* (Figure 4E).

Like the other xenografts, parental VCaP cells did not express either *hFSHR* or *hLHCGR*. Unlike the other tumors, VCaP xenografts did not express *hLHCGR* (Figure 4C). Also, only mouse *Fshr* and *Lhr*, but not *Gnrh*, were expressed in the VCaP xenografts (Figure 4F).

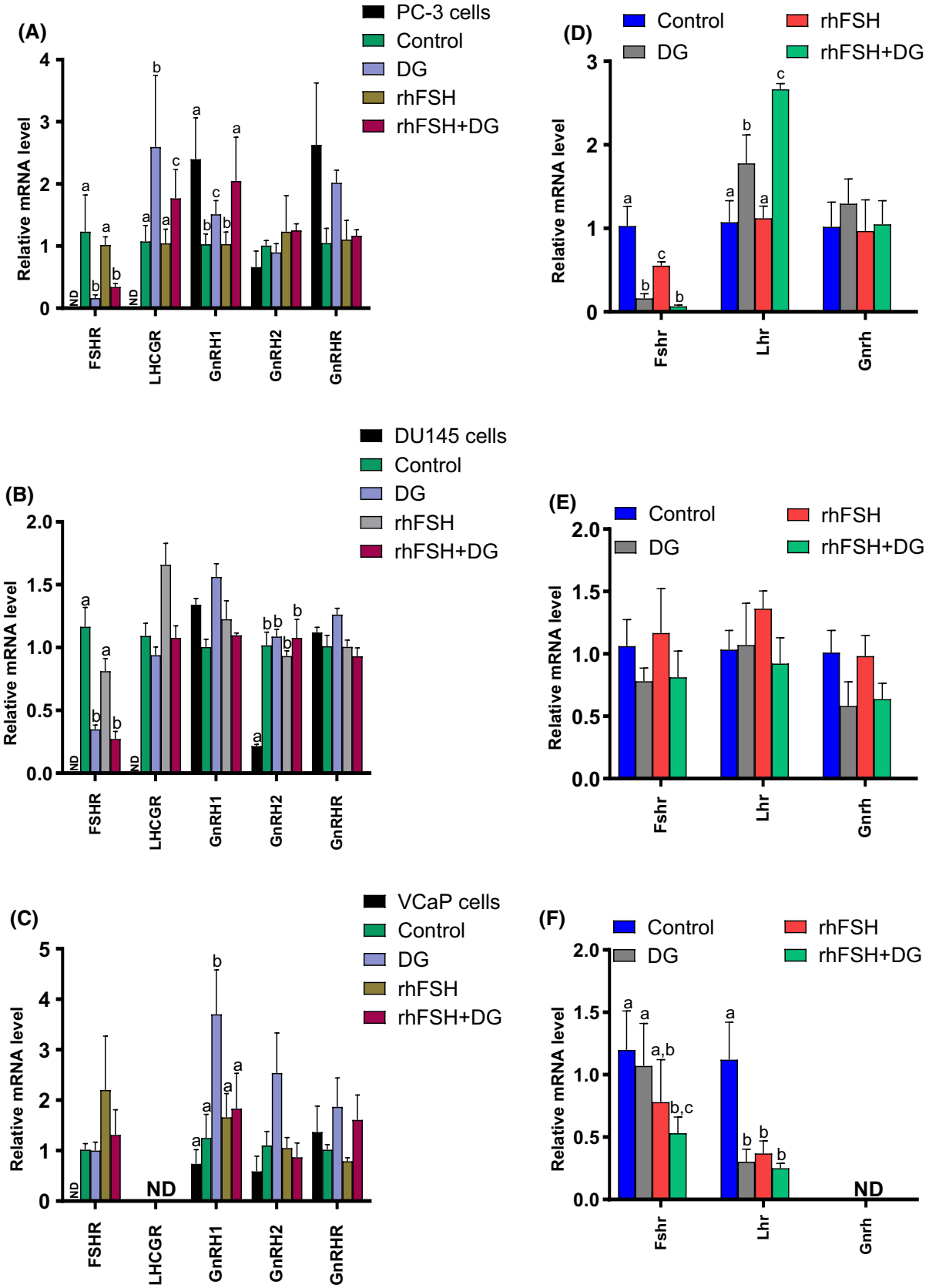

FIGURE 4 Gonadotropins, GnRH, and their receptor mRNA expression in prostate cancer cell xenografts. A-C, Expression of human hormone receptors (*FSHR, LHCGR*), and gonadotropin-releasing hormone genes and receptor (*GnRH1*, *GnRH2,* and *GnRHR*) in intact (IN) and degarelix (DG) treated PC-3, DU145 and VCaP xenografts. D-F, Mouse hormone receptors (*Fshr, Lhr*) and gonadotropin-releasing hormone (*Gnrh*) in IN and degarelix (DG) treated PC-3, DU145, and VCaP xenografts, respectively. Data represent mean ± SEM; n = 4 samples/group. Bars with different superscript letters differ significantly from each other ($P < .05$; ANOVA/Newman-Keuls)

3.7 | FSH receptors were present in PC-3 xenograft parenchyma

RNAScope in situ RNA hybridization of paraffin-embedded sections from PC-3 xenografts showed a low, although clearly detectable transcripts of *hFSHR* mRNA expression (Figure 5B) confined to tumor parenchyma. No mFshr mRNA (Figure 5C) expression was observed.

4 | DISCUSSION

GnRH antagonist degarelix reduced the tumor growth in both androgen-dependent and androgen-independent human PCa tumor xenograft models tested, in line with earlier reports.[2,25,26] Both GnRH agonists and antagonists have been shown to inhibit PCa growth, which has been interpreted to indicate that they share similar mechanisms of action in cancer cells. This is different from their opposite actions in the pituitary gonadotrophs, that is, stimulation and inhibition, respectively, of gonadotropin synthesis and secretion. The difference is best explained by their biased mechanisms of action in tumor cells, both probably activating the same signaling pathways, which appear to include inhibition of the epidermal growth factor and plasminogen activator systems and activation of caspase 8-mediated apoptosis.[2]

Besides direct inhibition of xenograft growth, the degarelix effect can also be due to concomitant inhibition of gonadotropin secretion, which may have a direct action on the prostate. Accordingly, we detected both *LHCGR* and *FSHR* expression in the xenograft parenchyma. Interestingly, no expression of either gonadotropin receptor was found in the cultured cells, indicating that the in vitro conditions were unable to replicate the in vivo situation to induce and sustain the receptor expression. The uncharacterized induction mechanisms on gonadotropin receptor expression are apparently functional in the tumor cells only in vivo.

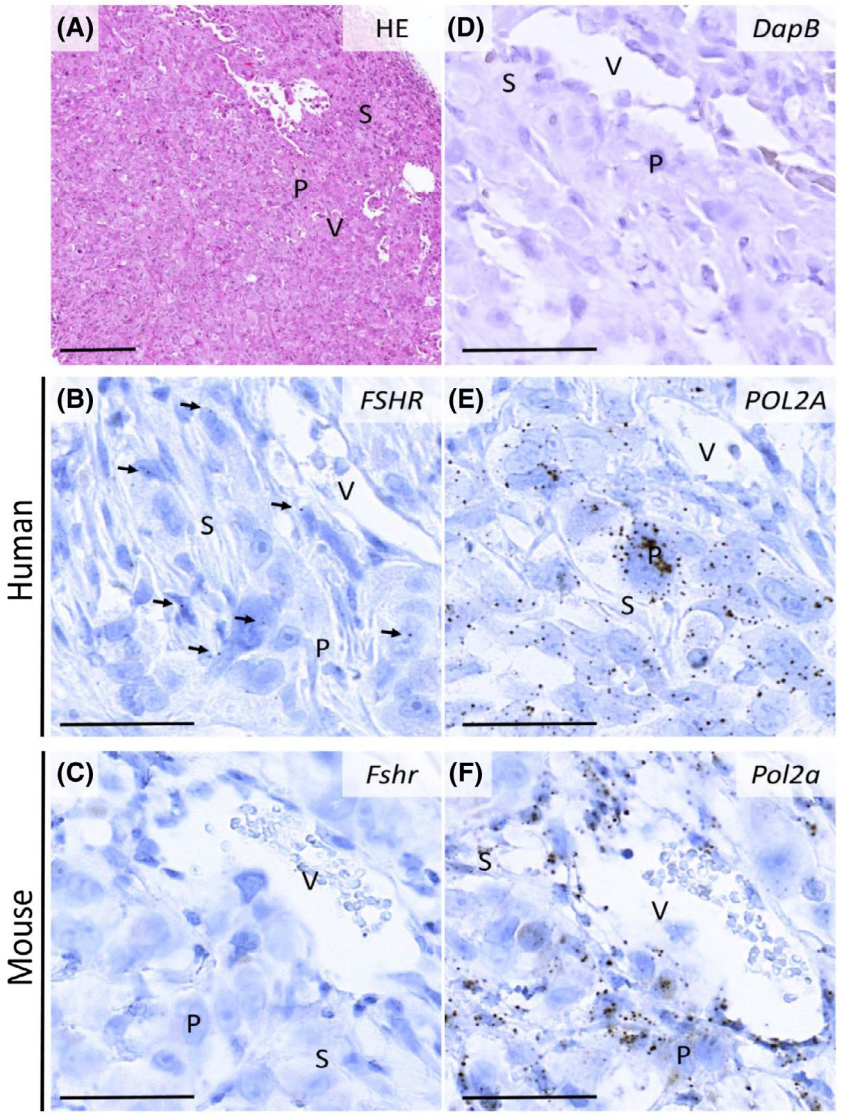

FIGURE 5 Localization of FSHR mRNA in PC-3 xenografts. A, Representative image of tumor with hematoxylin and eosin staining. B, Corresponding in situ hybridization localization of human *FSHR* mRNA (arrows). C, Mouse *Fshr* mRNA (negative) (V). D, Negative control (*DapB*), and E and F, Positive controls (*POL2A* and *Pol2a*). The panels are representative images of n > 3 samples. Bar = 50 μm. P, tumor parenchyma; S, smooth muscle; V, vasculature

In support of our findings, FSH has been localized[14,27-29] and shown to be synthesized[30] in normal and malignant prostatic tissue. FSHR expression has been demonstrated in normal and tumorous prostate tissue,[13,14] as well as in vascular endothelial cells of tumors from human prostate cancer.[16] Due to the low but detectable hFSHR expression together with the low expression of GnRH and its receptor in the current study, these findings should be considered semi-quantitative, not allowing detailed conclusions about the effect of different treatments on their quantities.

We can exclude the direct LH/hCG stimulation of human prostate cell xenograft growth, at least in those originating from PC-3 cells, because the treatment of degarelix-suppressed PC-3 xenograft carrying mice with hCG demonstrated no effect on tumor growth. *LHCGR* expression has been demonstrated in human PCa tissue,[14,31] and it has been shown that silencing of LHCGR by the siRNA technique blocks LH-stimulated LNCaP and LAPC-3 cell proliferation.[32] Our findings on PC-3 cell xenografts, however, did not reproduce these findings.

In contrast to LH/hCG, direct FSH action on the PC-3 tumor growth was observed. In all PC-3 cell experiments, both in IN and CAS mice, in preventive and therapeutic approach, as well as with and without degarelix suppression, FSH treatment increased tumor size. The effect was clearest in IN mice of the preventive experiment, apparently due to greater FSH gradient between FSH-treated and non-treated animals. The interference of indirect gonadotropin effects through stimulation of testicular testosterone production can be excluded due to the androgen non-responsiveness of PC-3 cells.[33]

A recent study demonstrated FSHR expression at mRNA and protein level in several human PCa cell lines including C4-2, LNCaP, and PC-3.[34] The findings are at variance with our data, where FSHR expression, at mRNA level, was only found in the tumor xenografts but not in the original in vitro-cultured cells. Several explanations may be offered for the difference, including the well-known heterogeneity of the same cell type between laboratories, subtle differences in culture conditions, and sensitivity of the methods used. The agreement between the in vitro data of Dizeyi et al[34] and our in vivo data was that FSH stimulated tumor cell growth in both studies. The in vitro data, in addition, demonstrated that FSH stimulation of prostate cancer cells was able to activate the PIK3/Akt pathway and increase β-catenin expression.

In the other androgen-independent DU145 cell line tested, cell growth was slower and the xenografts much smaller than those of PC-3 cells, which was not surprising in view of their lower tumorigenicity,[35] resulting therefore in a less marked, even though still significant effect of FSH on tumor growth during the 4-week experiment. A longer time of tumor growth could probably have demonstrated a more marked FSH effect in this cell line, which unfortunately was not permitted by our experimental license. Likewise, even greater FSH effects would likely have occurred in PC-3 cell xenografts, had the experiments lasted longer. In this respect the short xenograft experiments may understate the effects of the chronic situation of years in human cancer.

Direct FSH effect on tumor growth was observed in the two androgen-independent cell lines, PC-3 and DU145, but not in the androgen-dependent VCaP cells, despite similar FSHR expression in the xenografts of all cell lines. We have recently observed in the testis[22] that FSH and androgen actions are partly overlapping, stimulating the expression of the same genes. There is also evidence that these two structurally and functionally different hormones share partly the same signaling mechanisms, which can explain their unexpected overlap of actions.[36] Concerning the current findings, we hypothesize that the stimulatory FSH effect on PCa growth is more readily detected in the absence of the much stronger androgen action, as is the case with PC-3 cells. In contrast, when the cells are strongly androgen-responsive and maintained by physiological testosterone concentration, as the VCaP cells, the androgen effect is so strong that it masks the smaller, but probably still persistent FSH effect. This could explain why the FSH action was detected in the androgen-independent PC-3 and DU145 cells, but not in the androgen-dependent VCaP cells. Furthermore, no LHCGR expression was found in the VCaP cells, possibly due to the testosterone supplementation and the dynamics of LH and its receptor regulation in the mouse. Moreover, the testosterone supplementation must have significantly suppressed GnRH-stimulated LH release.

If the above mechanistic explanation holds true, the finding is of translational significance. PCa growth may be simultaneously stimulated by a quantitatively strong androgen action and a lesser FSH action, which may be initially masked by the androgen effect. When the cells lose their androgen dependence the FSH dependency may remain and become detectable, as is the case with PC-3 cells. In this scenario, the difference in the actions of GnRH agonists and antagonists could become clinically significant. While agonists bring about permanent suppression of only LH, both LH and FSH are permanently suppressed by antagonist. The latter therefore offers a therapeutic advantage, especially at later stages of the cancer progression when the cells have attained androgen independence, by suppressing the remaining FSH component in the hormonal stimulation of PCa growth. Clinical data supporting this contention are indeed starting to emerge.[9]

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CONFLICT OF INTEREST

The authors declared that no conflict of interest exists.

AUTHOR CONTRIBUTIONS

O.O. Oduwole, N.A. Rahman, C.L. Bevan, W. Koechling, and I.T. Huhtaniemi designed research; O.O. Oduwole, A. Poliandri, A. Okolo, and G. Serrano de Almeida performed in vitro experiments; O.O. Oduwole, A. Okolo, and P. Rawson performed in vivo experiments; M. Doroszko and M. Chrusciel performed in situ hybridization experiments; O.O. Oduwole, A. Poliandri, A. Okolo, M. Doroszko, M. Chrusciel, N.A. Rahman, C.L. Bevan, W. Koechling, and I.T. Huhtaniemi analyzed data; O.O. Oduwole and I.T. Huhtaniemi wrote the manuscript, with final editing from all authors.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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