

# Expression of GnRH type II is regulated by the androgen receptor in prostate cancer

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## Abstract

GnRH II has important functional effects in steroid hormone-dependent tumours. Here we investigated the expression and regulation of GnRH II in prostate cancer. GnRH II protein was equally expressed in benign (73%) and malignant (78%) biopsies studied in a prostate tissue microarray ( $P=0.779$ ). There was no relationship between expression and clinical parameters in the cancer cohort. GnRH II was, however, significantly reduced in tumour biopsies following hormone ablation. This was further investigated in a prostate xenograft model where androgens increased GnRH II levels, while their withdrawal reduced it. In cell lines, we confirmed high levels of GnRH II in androgen receptor (AR)-positive LNCaP cells but low levels in AR-negative PC3 cells. In LNCaP cells, GnRH II induction by androgens was blocked by the AR inhibitor casodex, but not by cycloheximide treatment. Sequence analysis subsequently revealed a putative androgen response element in the upstream region of the *GnRH II* gene and direct interaction with the AR was confirmed in chromatin immunoprecipitation experiments. Finally, to test whether the effects of GnRH II were dependent on AR expression, LNCaP and PC3 cells were exposed to exogenous peptide. In both cell lines, GnRH II inhibited cell proliferation and migration, suggesting that its function is independent of AR status. These results provide evidence that GnRH II is widely expressed in prostate cancer and is an AR-regulated gene. Further studies are warranted to characterise the effects of GnRH II on prostate cancer cells and investigate its potential value as a novel therapy.

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## Introduction

Prostate cancer remains the most commonly diagnosed malignancy and the second leading cause of cancer-related deaths among men in the USA (Jemal *et al.* 2006). Gonadotrophin-releasing hormone I (GnRH I) analogue therapy is an important mode of treatment for men with locally advanced and metastatic disease. The GnRH–GnRH receptor (GnRHR) axis, however, may have a more direct anti-tumourigenic effect. In particular, GnRH I has been shown to have both anti-proliferative and anti-metastatic effects on prostate cancer cells (Dondi *et al.* 1998, Limonta *et al.* 1999). This effect may also be active in the hormone-refractory stages of clinical disease (Gnanapragasam *et al.* 2005).

A second form of GnRH, GnRH II, was first reported in humans by White *et al.* (1998). The gene is located on chromosome 20 and includes four exons and three introns coding for a 10-amino acid

sequence. In some mammals, GnRH II is involved in sexual function and reproduction, but its role in humans is less clear (Temple *et al.* 2003). In localisation studies, GnRH II has been found throughout the human central nervous system, but is also widely expressed in peripheral tissue. This suggests a hitherto unrecognised role for GnRH II in peripheral cell function (White *et al.* 1998). Intriguingly, expression levels in the prostate were found to be four times higher than in the brain. It has recently been shown that GnRH II has anti-proliferative effects on both ovarian and endometrial cancer cells (Choi *et al.* 2001, Grundker *et al.* 2002, 2004). Similar effects have also been reported following GnRH II treatment of breast cancer cells (Gunthert *et al.* 2005). These data suggest that GnRH II may have important anti-tumourigenic effects in steroid hormone-dependent malignancies. In this context, the role of GnRH II

in prostate cancer is of particular interest, especially in view of evidence of high expression in the prostate.

The expression and regulation of GnRH II in prostate cells is currently unknown. To investigate this, we undertook a study of GnRH II in a clinical cohort of benign and malignant prostate biopsies as well as in *in vitro* and *in vivo* models of prostate cancer.

## Materials and methods

### Cell culture

Prostate cancer cell lines were maintained in RPMI 1640 media (Sigma) containing 10% FCS (FM-full medium). Basal media (BM) was RPMI 1640 alone. For experiments requiring androgen-depleted medium, dextran-coated charcoal (DCC) was first used to remove endogenous steroids present in FCS (DCC medium). LNCaP cells (derived from a lymph node metastasis) were chosen for androgen induction studies as a model of hormone-sensitive prostate cancer (Horoszewicz *et al.* 1983). These cells display androgen-dependent growth characteristics and abundantly express the androgen receptor (AR). LNCaP cells grown in DCC media for 48 h were termed LNCaP-AD (androgen depleted). LNCaP-AI (androgen-independent) cells have been previously described (Halkidou *et al.* 2003).

### Quantitative real-time PCR (QPCR)

Quantitative mRNA expression was evaluated through real-time PCR using a 7900-HT sequence detection system (Applied Biosystems, Foster City, CA, USA). Total RNA extracted from cell lines was first treated with MMLV Reverse Transcriptase reaction cocktail (Promega) according to manufacturer's instructions. QPCR was performed by Jumpstart SYBR Green mastermix (Sigma) and primers designed using primer express 2.0 (Applied Biosystems) as follows: GnRH II forward 5' CAGAACTTC GGCTTCCGTTT 3' and reverse 5' CTGCTCACAGGTCTTTTCGCTTG 3', GAPDH forward 5' CGACCACTTTGTCAAGCTCA 3' and reverse 5' GGGTCTTACTCCTTG GAGGC 3', PSA forward 5' GGTGCATTACCGGAAGTGGAT 3' and reverse 5' TGGTCATTTCCAAGGTTCCAAG 3', F1R1 forward 5' GCTTTC TATGTTCTAATGAGAGGGATA 3' and reverse 5' TCCTGAAAAATCTAAACCAGA GCC 3', F2R2 forward 5' TAAACAGGCCTCTGACCCCC 3' and reverse 5' TACCTA CCCCAGGAGTGGCT 3' and ARE I forward 5' CCTAGATGAAGTCTCCATGAGC-TACA 3' and reverse 5' GGGAGGGAGAGCTAG-CACTTG 3'. The level of expression was evaluated using the absolute quantification method normalising

against the housekeeping gene *GAPDH* (ABI technical bulletin 2, www.appliedbiosystems.com).

### Immunohistochemistry

A prostate tissue microarray (TMA) was constructed from 0.6 mm cores of benign ( $n=37$ ) and malignant samples ( $n=123$ ). Primary cancer biopsies were derived from hormone treatment in naïve patients either from transurethral resection of the prostate or radical prostatectomy. A further 35 patients had sequential biopsies available, of which 27 had received interim androgen ablation. In 13 of these patients, the second biopsy was obtained after the onset of hormone-refractory disease as defined by a sequential rise in the PSA. Immunohistochemistry was performed by GnRH II polyclonal antibody (Santz Cruz, Biotechnology Inc., Santa Cruz, CA, USA). The optimal antibody concentration was derived from dilution testing on composite tissue blocks (final concentration 1:100). Antigen retrieval was achieved by immersion in 0.01 M sodium citrate buffer (pH 6.0) and microwaving for 1 min (1000 W) in a pressure cooker. Sections incubated without primary antibody were employed as internal negative controls. Secondary antibody labelling was achieved using biotinylated antibodies (Dako, Ely, UK). All sections were counterstained with DAB and haematoxylin. Immunostained sections were studied without prior knowledge of the clinical details. Sample sections were viewed by two independent observers (VJ Gnanapragasam, MM Khan) and inter-observer agreement was obtained regarding a grading system. The level of GnRH II expression was correlated with the strength of the immunoreactivity signal and was scored as either absent (0), weak (+), moderate (++) or strong (+++). Where two or more signal intensities were present in one case, the predominant signal was taken as the score. The use of all tissue and patient data was in accordance with the approval granted by the local hospital's ethics committee. Statistical analysis was performed by correlating expression with clinical parameters using the  $\chi^2$  test.  $P<0.05$  was taken as being statistically significant.

### Case Western Reserve (CWR22) model

The CWR22 xenograft model is an androgen-dependent tumour derived from a high Gleason grade prostate cancer. AR expression is preserved, and tumour cell growth and PSA expression in this model are exquisitely androgen sensitive (Wainstein *et al.* 1994, Kim *et al.* 2002). The use of this model in our unit has been previously described (Gnanapragasam *et al.* 2002). Briefly, cell suspensions

were mixed with Matrigel (Collaborative Research, Bedford, MA, USA) before s.c. injection into 12- to 16-week-old CD 1 male nude mice (Charles Rivers, Wilmington, MA, USA). Following tumour growth for 6 weeks, mice were randomly allocated into three groups. The first group received s.c. implantation of sustained release testosterone pellets (Innovative Research of America, Sarasota, FL, USA). The second group was castrated by bilateral orchidectomy and the third group was sham anaesthetised only. Initial tumour volumes between the groups were not significantly different ( $P < 0.05$ ). Tumours were harvested at week 4, carefully dissected, denuded of host mouse tissue and fixed in paraffin. The 5  $\mu\text{m}$  sections were cut onto APES-coated slides for further study. GnRH II and PSA expressions were determined by immunohistochemistry using GnRH II and PSA antibodies (Santuz Cruz) diluted 1:100 as described previously.

### Androgen induction assays

LNCaP and LNCaP-AI cells were seeded at a density of  $5 \times 10^4$  cells/well in 6-well plates. Cells were grown in either DCC medium or DCC media containing the synthetic androgen 10 nm R1881 (Perkin–Elmer, Waltham, MA, USA). Where appropriate, cells were also pretreated with 10  $\mu\text{M}$  casodex. Androgen induction experiments were also repeated in the presence of 1  $\mu\text{M}$  cycloheximide. GnRH II immunofluorescence was performed by seeding  $5 \times 10^3$  cells/well in 8-well chamber slides (Becton–Dickinson, Oxford, UK) and growing them in either DCC medium, DCC media containing R1881 or full media in the absence or presence of casodex for 48 h. The cells were then fixed with 100% methanol for 20 min at  $-20^\circ\text{C}$  and were then incubated with the GnRH II polyclonal antibody overnight. They were washed in PBS and treated with secondary antibody diluted 1:200 for 1 h. Slides were mounted with Vectashield containing DAPI as a nuclear counterstain. Statistical analysis was performed by two-tailed Student's *t*-test.  $P < 0.05$  was taken as being statistically significant.

### Chromatin immunoprecipitation (CHIP) assays

LNCaP cells were grown in DCC media for 72 h and were then stimulated with 10 nM R1881 for 6 h. CHIP assays were performed as described previously (Gaughan *et al.* 2002). For immunoprecipitation, 2  $\mu\text{g}$  polyclonal AR antibody (Sigma) were used as indicated. Real-time PCR was performed using 10  $\mu\text{l}$  DNA from each sample against ARE I, F1R1 and F2R2 primers and compared with non-immunoprecipitated samples and input samples.

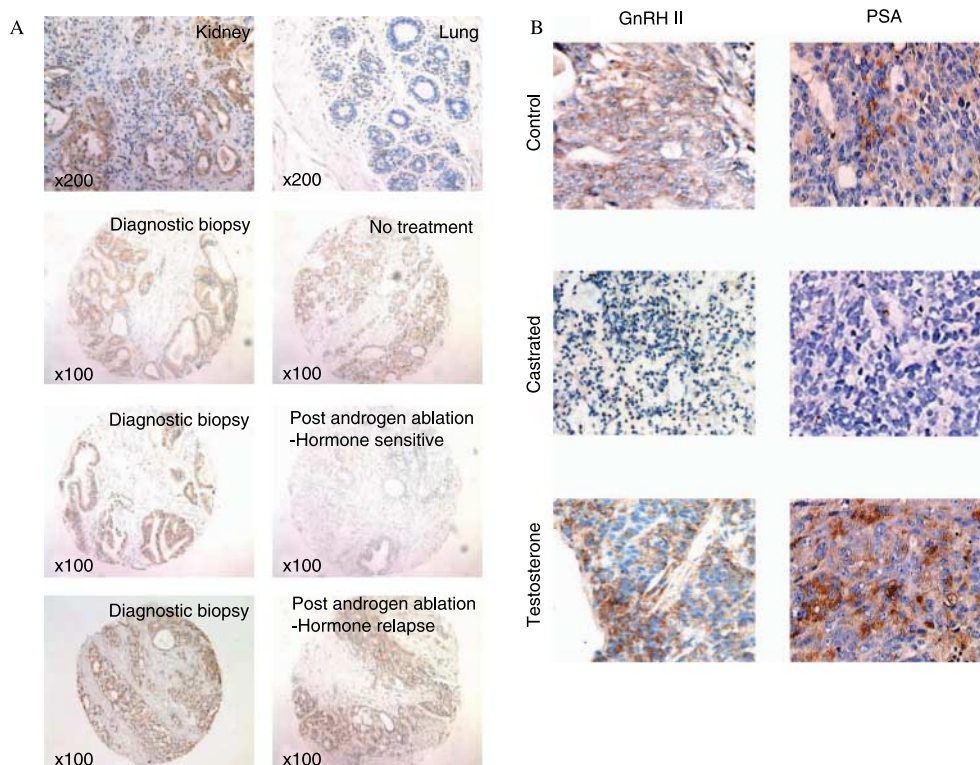
### Proliferation and migration assays

LNCaP and PC3 cells were seeded at a density of 3000 cells/well in 96-well plates (Corning). The cells were grown in full media containing 0–500 nM GnRH II (Bachem, GmbH, Weil am Rhein, Germany). WST 1 reagent (Roche) was then added in a 1:10 dilution and colorimetric change was measured at 410 nm on a Spectra Max 250 plate reader (Molecular Devices, Sunnyvale, CA, USA). In migration assays, LNCaP and PC3 cells were plated out at a density of 50 000 cells into BD Falcon migration chambers in basal media with 0–1  $\mu\text{M}$  GnRH II. Full media was used in the lower chamber as the chemoattractant. Cells were allowed to invade for 24 h in a humidified tissue culture incubator. Non-invading cells were removed from the upper chamber and invaded cells were fixed in methanol for 20 min at  $-20^\circ\text{C}$  and stained with haematoxylin. Membranes were removed with a sterile scalpel and mounted onto slides with DPX and counted using a bright field microscope (20 $\times$  magnification). A minimum of four different fields of view were used to obtain an average count per section. Statistical analysis was performed by two-tailed Student's *t*-test.  $P < 0.05$  was taken as being statistically significant.

## Results

### GnRH II protein expression in clinical benign and malignant prostates

GnRH II antibody was optimised for immunohistochemistry and tested against a panel of normal tissue. Strong signals were seen in sections of kidney (positive control), while the lung (negative control) failed to express the protein (Fig. 1A). This is consistent with previous reports of expression in human tissue (White *et al.* 1998). In prostate tissue, expression was seen predominantly in epithelial cells with no expression in stromal tissue (Fig. 1A). In the prostate TMA, GnRH II expression was seen in 73% (27/37) of benign biopsies and 78% (96/123) of primary cancer biopsies ( $P = 0.779$ ; Table 1). Expression of GnRH II in the cancer group was further stratified by the intensity of staining. The levels of GnRH II expression were variable in the group with no correlation of staining intensity with Gleason grade or clinical stage of the disease ( $P = 0.067$  and 0.334 respectively; Table 1). GnRH II expression was also not associated with PSA at presentation, metastasis or overall survival. In a subset of cancers, at least two sequential biopsies were available from the same patient (Table 1). In eight cancers, no treatment had been given in the interval between biopsies, while 14 patients had received androgen ablation therapy with the second biopsy taken during the hormone-sensitive



**Figure 1** *In vivo* levels of GnRH II protein is related to androgen status. (A) Strong positive signals in kidney with no expression in lung tissue. Immunohistochemistry in matched biopsy cores before and after androgen ablation. Representative biopsies from both hormone-sensitive and hormone-relapsed cases are shown. (B) Immunostaining in CWR22 prostate xenograft tumours following sham anaesthetic, surgical castration or testosterone implants.

stage of the disease. The effect of castration in the treatment group was confirmed by a significant reduction in serum PSA expression following the treatment (data not shown). In this cohort, we unexpectedly observed that in the post-treatment biopsies all initially positive tumours failed to express GnRH II (Table 1 and Fig. 1A). This result was independent of whether castration had been achieved medically ( $n=7$ ) or surgically ( $n=4$ ). In contrast, in the group that had not received treatment, there was no difference in GnRH II expression between the first and second biopsies. We next studied expression in a cohort of 13 hormone-refractory prostate cancers. These patients also had biopsies available from the point of diagnosis and were positive for GnRH II in eight cases. Among these, subsequent matched biopsies taken during the hormone-refractory stage of the disease was positive for GnRH II in 4/8 (50%) cases (Fig. 1A). These results suggest that GnRH II is frequently expressed in benign and malignant prostate glands. Expression is not dependent on disease grade or stage but is significantly reduced following hormone ablation. In hormone-refractory disease however, GnRH II is re-expressed in a significant number of cancers.

### GnRH II in prostate cancer cells is regulated by androgen stimulation

An *in vivo* model of prostate cancer was used to test the interaction between androgens and GnRH II. The CWR22 human prostate xenograft model is particularly sensitive to androgen manipulation. GnRH II levels were investigated in tumours from surgically castrated mice and those that had been given testosterone supplements. GnRH II levels were significantly reduced in tumours from castrate mice compared with untreated controls (Fig. 1B). In contrast, expression was significantly higher in tumours from mice that had received testosterone supplements. PSA protein levels showed a similar pattern and acted as a control for the experiment (Fig. 1B). GnRH II was next studied in a panel of prostate cancer cell lines. Highest levels of mRNA expression were seen in AR-positive LNCaP cells with lower levels in AR-negative DU145 and even lower levels in AR-negative PC3 cells (Fig. 2A). We then tested the effect of androgen manipulation on LNCaP cells. In this experiment, GnRH II and PSA mRNA levels were significantly down-regulated in a time-dependent manner following androgen withdrawal (Fig. 2B).

**Table 1** Gonadotrophin-releasing hormone II (GnRH II) protein expression in a clinical cohort of benign and malignant prostates

	GnRH II negative		GnRH II positive		
All biopsies (n=165)					
Benign (n=37)	10		27		
Cancer (n=123)	27		96		P=0.779

	GnRH II score				
	Negative 0	+	Positive ++	+++	
Cancer biopsies (n=123)					
Gleason grade 3	9	5	16	3	
Gleason grade 4	4	17	21	8	
Gleason grade 5	14	10	13	3	P=0.067
Stage 1	11	6	20	6	
Stage 2	6	7	8	3	
Stage 3	6	15	12	2	
Stage 4	4	4	10	3	P=0.334

	GnRH II negative		GnRH II positive	
Matched cancer biopsies (n=22)				
Diagnostic TURP		3		5
Second TURP-no treatment		3		5
Diagnostic TURP		3		11
Second TURP-post-androgen ablation		14		0

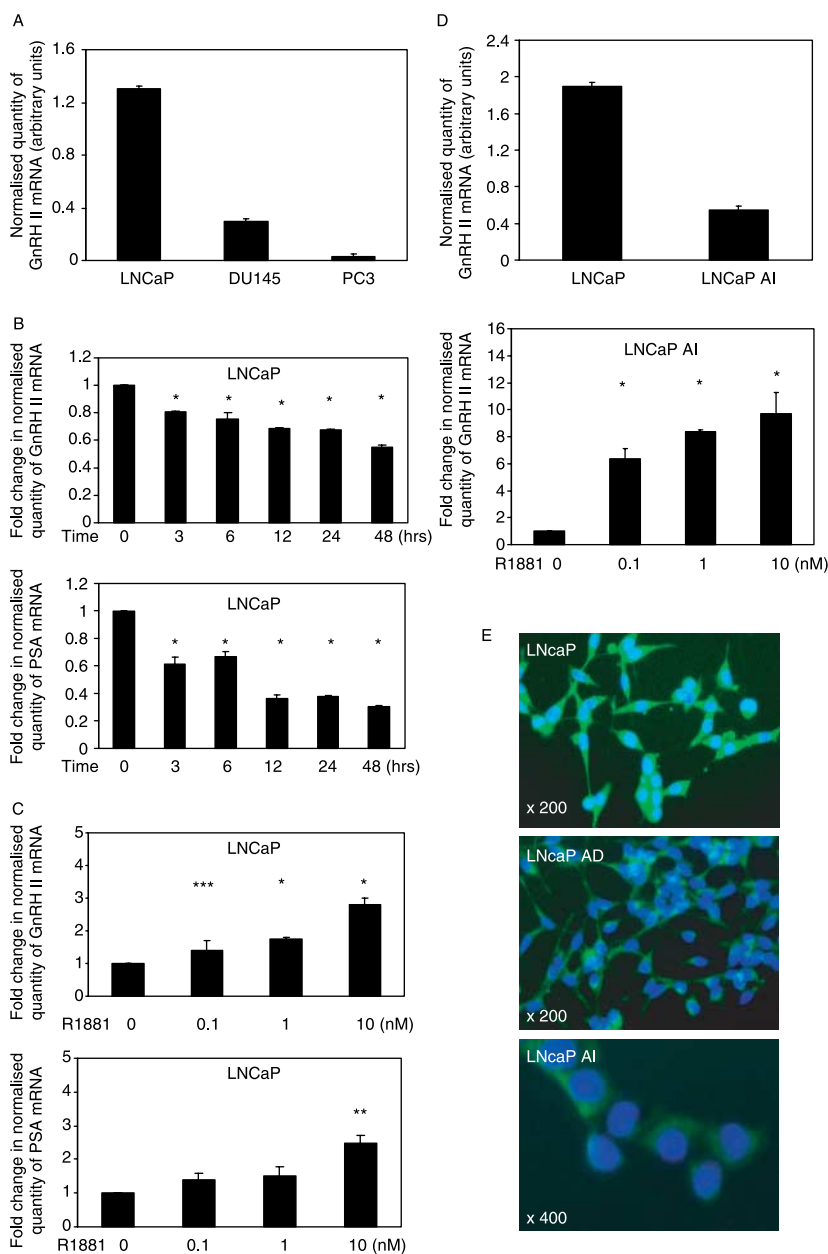
GnRH II was scored as either being absent (negative) or present (positive) in analysis of benign and malignant prostates. In the cancer cohort, intensity of signals were scored as negative (0), mild (+), moderate (++) or strong (+++). A *P* value of <0.05 was taken as significant. In matched cancers, the second biopsy was available from untreated patients or post-androgen ablation patients with either hormone-sensitive or hormone-relapsed disease as shown (stage – clinical stage).

Conversely, treating androgen-deprived cells with increasing doses of a synthetic androgen (R1881) resulted in a strong induction of GnRH II and PSA mRNA levels (Fig. 2C). The LNCaP AI cell line has been previously described and is serially maintained in androgen-free media. AR expression in these cells is preserved and remains highly responsive to ligand stimulation (data not shown). The cells, however, do not rely on androgens for survival. In these cells, GnRH II mRNA was found to be significantly reduced in comparison to parental LNCaP cells (Fig. 2D). Treating these cells with R1881 (synthetic androgen), however, restored expression in a dose-dependent manner (Fig. 2D). We confirmed our findings at the protein level using immunofluorescence. In keeping with our mRNA data, we found high levels of GnRH II protein in LNCaP cells (Fig. 2E). We similarly observed very low GnRH II levels in PC3 cells (data not shown). Both short-term and long-term androgen deprivation (LNCaP AD and LNCaP AI respectively) significantly reduced endogenous GnRH II protein levels (Fig. 2E). These data suggest that androgens have an important role in regulating GnRH II expression in the prostate cancer cell. This effect is unlikely to be cell type specific as it is

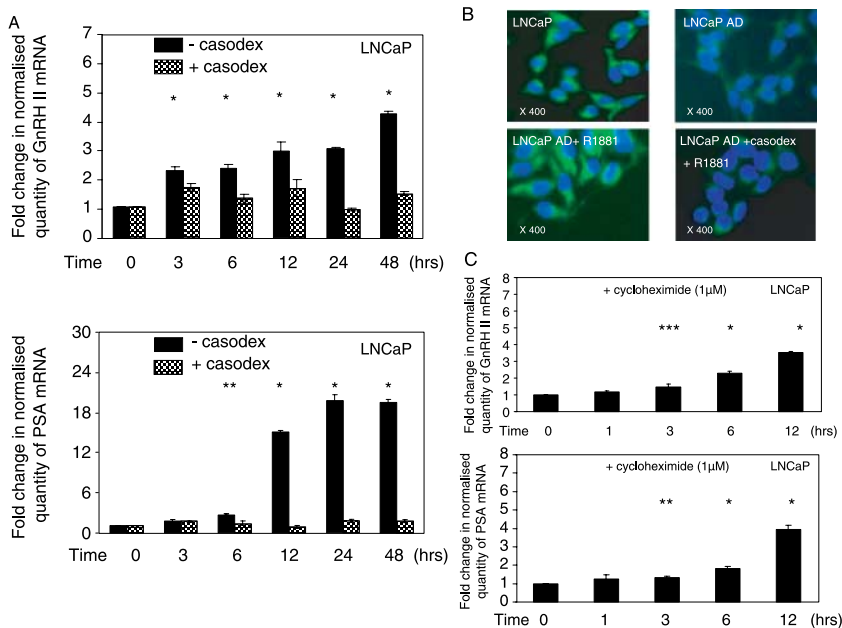
observed in clinical samples, prostate xenografts and cultured cell lines.

### Androgen induction of GnRH II occurs through the AR

We next tested whether androgen regulation was a direct or indirect effect. To investigate this LNCaP cells were exposed to androgens and in the presence or absence of the non-steroidal AR antagonist (casodex). In this experiment, R1881 again significantly induced GnRH II mRNA expression. This induction, however, was effectively blocked by co-treatment with casodex (Fig. 3A). A similar result was seen with PSA as a control. These findings were subsequently confirmed at the protein levels. In immunofluorescence studies, GnRH II protein was reduced by androgen depletion (LNCaP AD) but increased with the addition of R1881. This effect was lost when cells were co-incubated with casodex (Fig. 3B). Induction may, however, occur through an intermediary protein. To test this, we again exposed LNCaP cells to androgens following pretreatment with the peptidyl-transferase inhibitor cycloheximide. In these studies, the addition of cycloheximide had no effect on the induction of GnRH II by androgens. There was also



**Figure 2** Expression of GnRH II is regulated by the presence or absence of androgens. (A) GnRH II mRNA expression in a panel of prostate cancer cells. (B) LNCaP cells were deprived of androgens and assayed for GnRH II and PSA mRNA at the displayed times. In both experiments, results shown are the mean of three experiments done in triplicate with error bars representing s.d.s. (C) Initially androgen-deprived LNCaP cells were treated with synthetic androgens (R1881) at increasing doses as shown before mRNA extraction at 24 h and measurement of GnRH II and PSA levels. (D) LNCaP AI cells were obtained by serial culture in an androgen-free environment for at least 8 months. mRNA was extracted and assayed for GnRH II expression and compared with LNCaP controls. LNCaP AI cells were then treated with increasing doses of R1881 for 24 h before mRNA extraction and measurement of GnRH II. Results shown are the mean of three experiments each done in triplicate and is expressed as a fold change over un-induced cells (given a value of 1) with error bars representing s.d.s. (E) Corresponding changes in LNCaP GnRH II expression at the protein level were confirmed using immunofluorescence and under the different treatment conditions (green – GnRH II protein, blue – DAPI nuclear stain). One representative of three different experiments is shown here. LNCaP AD, LNCaP androgen depleted for 48 h; LNCaP AI, LNCaP androgen independent as described above. \* $P < 0.001$ , \*\* $P < 0.005$ , \*\*\* $P < 0.05$ .



**Figure 3** Androgen induction of GnRH II is a direct effect through the androgen receptor. (A) LNCaP cells with and without pretreatment with the AR antagonist casodex (10  $\mu$ M) before the addition of androgens. GnRH II and PSA mRNA were assayed at the displayed times. (B) Protein expression of GnRH II was assessed by immunofluorescence in LNCaP cells, following androgen deprivation (LNCaP AD) and androgen stimulation with or without casodex treatment. (C) LNCaP cells were treated with androgens as described and with prior administration of cycloheximide. Cells were harvested for mRNA at the defined time points. Results shown are the mean of three experiments each done in triplicate and is expressed as a fold change over un-induced cells (given a value of 1) with error bars representing s.d.s. For immunofluorescence, one representative of three different experiments is shown (green – GnRH II protein, blue – DAPI nuclear stain). \* $P < 0.001$ , \*\* $P < 0.01$ , \*\*\* $P < 0.05$ .

no effect seen on the induction of PSA used as a positive control (Fig. 3C). These results suggest that androgen-dependent regulation of GnRH II requires the AR and is not a secondary effect of AR activation by an intermediary protein.

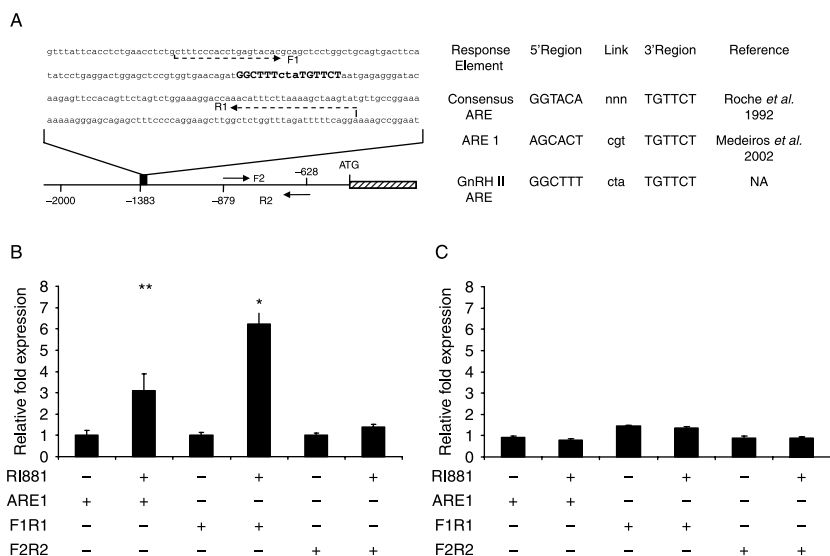
### The GnRH II upstream sequence contains a putative AR binding site

Our findings raised the possibility of a direct interaction between the AR and the *GnRH II* genes. Analysis of the *GnRH II* gene sequence revealed a possible androgen regulatory element (ARE) at position –1383 to –1369 upstream of the translation start codon (Fig. 4A). This motif was found to have a high degree of sequence homology with the consensus DNA binding site for the AR (14) (Fig. 4A). To confirm *in vivo* interaction between the AR and this identified sequence, we performed CHIP experiments using an AR antibody followed by QPCR. The primer set F1R1 was designed to cover the sequence of interest, while a second primer set, F2R2, spanned an adjacent region downstream and acted as a negative control. A known ARE sequence (ARE 1 derived from the PSA promoter) was used as a positive control. With

primer set F2R2, there was no difference in the amplicon signal using DNA from LNCaP cells cultured in the presence or absence of androgens (Fig. 4B). QPCR with F1R1, however, produced a significantly stronger signal with template from androgen-treated LNCaP cells as compared with untreated controls (Fig. 4B). Similar high amplicon signals were obtained using primers for the ARE1-positive control in androgen-treated cells. There was no difference in the signal strength when primer sets F1R1, F2R2 or ARE1 were amplified in input samples isolated prior to immunoprecipitation (Fig. 4C). These results suggest that the presence of an AR binding site in the region spanned by F1R1 and correspond to the presence of identified putative ARE in this sequence.

### Exogenous GnRH II exerts similar effects on both androgen-dependent and androgen-independent prostate cancer cells

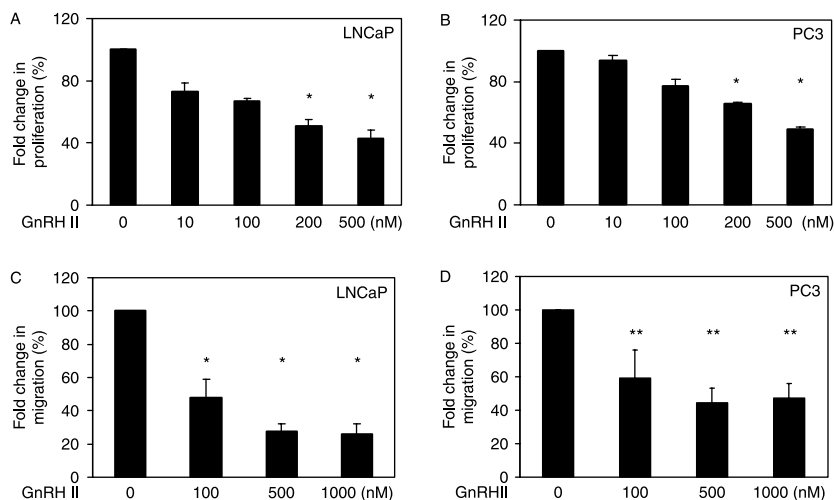
GnRH II has been shown to have anti-tumourigenic effects in a number of steroid-dependent tumours. We therefore asked whether this effect occurred in prostate cancer and was influenced by the AR status of the cell. This was investigated by adding exogenous GnRH II to



**Figure 4** The GnRH II upstream sequence contains a putative ARE. (A) The 5' upstream region was identified and analysed for AR binding sites. The sequence was compared with the published consensus ARE and the positive control ARE 1. (B) QPCR was performed on LNCaP chromatin immunoprecipitated with the AR antibody in the presence or absence of androgens. Primers were used for F1R1, F2R2 and the control ARE 1. Non-androgen-stimulated samples were assigned the value of 1 and androgen-stimulated samples compared as a fold change with error bars representing s.d.s. All values were normalised against input samples as a control. (C) Similar experiments on input chromatin were used to ensure equal DNA loading. In both experiments, results shown are one of three repeat tests with androgen exposure for 24 h. \* $P < 0.001$ , \*\* $P < 0.01$ .

AR-positive (LNCaP) and AR-negative (PC3) prostate cancer cell lines. GnRH II significantly inhibited LNCaP cell proliferation by up to 50% in a dose-dependent manner ( $P < 0.005$ ; Fig. 5A). In PC3 cells, GnRH II treatment again resulted in an up to 40% reduction in cell proliferation ( $P < 0.005$ ; Fig. 5B). We

next tested, the effect of GnRH II on cell migration using full media as a stimulus. In these experiments, exogenous GnRH II efficiently reduced the migration of LNCaP cells through a porous membrane by up to 50% ( $P < 0.005$ ; Fig. 5C). This effect was most pronounced at higher treatment doses, but it was also



**Figure 5** Effect of exogenous GnRH II on AR-positive and AR-negative prostate cancer cells. (A and B) LNCaP and PC3 cells were cultured in full media with increasing doses of GnRH II as shown. Cell proliferation was measured using WST 1 assays. (C and D) LNCaP and PC3 cells were cultured in migration chambers with full media as a chemoattractant. GnRH II was then added in increasing doses as shown. In all studies, results shown are the mean of three experiments each done in triplicate and is expressed as a fold change over un-induced cells (given a value of 100%) with error bars representing s.d.s. \* $P < 0.005$ , \*\* $P < 0.001$ .



effective at a dose of 100 nM. In PC3 cells, a similar inhibitory effect was seen with an up to 75% reduction in cell migration in the presence of GnRH II ( $P < 0.001$ ; Fig. 5D). These results show evidence that exogenous GnRH II exerts both anti-proliferative and anti-migratory effects on prostate cancer cells. Of note, this effect appears to be independent of whether cells were AR positive or negative suggesting that GnRH II signalling mechanism are likely to be functionally intact in both.

## Discussion

A number of studies have suggested that GnRH I and GnRH II as well as the GnRHR are regulated by gonadotrophins and steroid hormones. Choi *et al.* (2006) have recently shown that gonadotrophins follicle-stimulating hormone and luteinizing hormone (FSH and LH) are able to reduce the levels of GnRH II but not GnRH I mRNA in ovarian surface epithelium and cancer cell lines. Gonadotrophin treatment also reduced the expression of the type I GnRHIR in these cells. Nathwani *et al.* (2000) have shown that oestrogens can down-regulate GnRH I expression in ovarian granulosa–luteal cells. Conversely, oestrogens actually increase the expression of GnRH II in these cells and in human neuronal cells (Chen *et al.* 2002a, Khosravi & Leung 2003). Oestrogens also appear to have a suppressive effect on GnRHIR expression possibly by a direct effect on the gene promoter (Nathwani *et al.* 2000, Kang *et al.* 2001). Progesterone acting through specific receptor isoforms has been shown to be a potent regulator of GnRH I and GnRHIR expressions but appears to have no effect on GnRH II (An *et al.* 2005). Androgen treatment has been shown to down-regulate GnRH I expression in a time-dependent fashion in hypothalamic neuronal cells (Belsham *et al.* 1998). GnRHIR, however, is not influenced by androgen treatment in work done in prostate cells (Gnanapragasam *et al.* 2005).

In this study, we found GnRH II protein expression in the majority of both normal and malignant prostate glands. There was no association between the expression and the tumour grade or stage in the sub-analysis of the cancer cohort. This is in contrast to breast tissue where expression has been found in both normal and neoplastic cells but is significantly higher in malignant tissue (Chen *et al.* 2002b). We did observe, however, that GnRH II protein expression was significantly down-regulated in patients who had been treated by androgen withdrawal therapy. We further confirmed this observation of androgen responsiveness in a human prostate xenograft model. A possible direct effect of GnRH analogues on

GnRH II expression is unlikely, as androgen withdrawal was achieved in the animal model and in some clinical samples by surgical castration. Androgen induction was further tested using *in vitro* models. Here both mRNA and protein expressions of GnRH II were induced by androgens acting directly through the AR. Conversely, androgen withdrawal significantly reduced GnRH II levels. This is in contrast to the inhibitory effects of androgens on hypothalamic GnRH I expression and the lack of effect on GnRHIR expression.

The human GnRH II promoter has been previously characterised (Cheng *et al.* 2003). The minimal promoter is located between  $-1124$  and  $-750$  bp upstream of the translation start codon. The untranslated first exon is also thought to be important for full promoter activity (acting as an enhancer). Our analysis of the sequence proximal to the ATG revealed a putative androgen response element (ARE) which is further upstream of this minimal promoter region. We have previously observed a similar arrangement with the androgen-sensitive *FGF8* gene promoter where the hormone response region is distinct from the active promoter (Gnanapragasam *et al.* 2002). Furthermore, Chen *et al.* (2001) have previously identified a cAMP response element at positions  $-67$  to  $-60$  upstream of the GnRH II start codon and hence separate from the active promoter. It is possible that conformational changes in the gene following steroid hormone binding might bring the ARE into closer proximity with the active promoter and may also involve recruitment of the enhancer region in exon 1. Such an arrangement has been recently proposed for the PSA promoter (Wang *et al.* 2005). In CHIP experiments, the sequence incorporating this putative ARE bound to the AR protein at comparable levels to a known ARE in cells stimulated with androgens. These observations in clinical, *in vivo* and *in vitro*, models provide compelling evidence that GnRH II is an androgen-regulated gene. We also found evidence that in contrast to androgen ablated hormone-sensitive cancers, GnRH II was expressed in a subset of biopsies from patients with androgen-independent prostate cancer. Based on our findings, we hypothesise that this is likely to be related to a reactivated AR signalling pathway, which is an important feature in the development of hormone-refractory disease (Grossmann *et al.* 2001, Cronauer *et al.* 2003).

In this study, GnRH II treatment inhibited prostate cancer cell proliferation regardless of the AR status. Furthermore, GnRH II was also capable of reducing the migratory ability of these cells, a key step in tumourigenesis and metastasis. The fact that GnRH II is induced by the AR and yet has inhibitory effects in prostate cells is indeed paradoxical. The AR, however, has been shown to have growth inhibitory effects in

differentiated benign epithelial cells (Rossi *et al.* 1996, 1998). In the normal prostate, it is also thought to have tumour suppressive effect and inhibit excess proliferation of luminal cells (Ling *et al.* 2001, Whitacre *et al.* 2002, Litvinov *et al.* 2006). We hypothesise that the induction of GnRH II by the AR may be a part of this homeostatic mechanism to regulate the level of prostate cell stimulation. This homeostatic mechanism, however, is unlikely to be effective in preventing the development of hormone-sensitive cancer. GnRH II may therefore not have a role in hormone-sensitive cancers at physiological levels of expression. Expression may also be further lowered by androgen withdrawal therapy which might further facilitate overactivity in kinase pathways because of the loss of an endogenous regulatory mechanism. In this context, exogenous treatment with non-physiological doses of GnRH II might prove a useful therapy to directly inhibit androgen-dependent cancers perhaps, as an adjunct to androgen withdrawal. Similarly, although we have detected endogenous GnRH II in androgen-independent cancers the levels of expression may be insufficient to effectively inhibit tumour cells. GnRH II may therefore also have a role in hormone-refractory disease and this is supported by our observation of its anti-tumourigenic effect on both androgen-dependent and androgen-independent prostate cancer cells.

The mechanism by which GnRH II inhibits tumourigenesis is not yet fully understood. The mammalian type II GnRHIIIR is not translated in humans because of a premature stop codon in its sequence (Neill 2002). It has therefore been suggested that GnRH II might signal through the type I receptor in humans. Lu *et al.* (2005) have shown that both GnRH I and GnRH II can bind to the GnRHIR. Binding of each may stabilise different receptor active conformations and allow ligand-specific selective signalling. Silencing of GnRHIR in ovarian cancer cells has certainly been shown to reverse the anti-proliferative effects of GnRH II (Kim *et al.* 2006). In contrast, Grundker *et al.* (2004) working in ovarian and endometrial cells have reported that the GnRHIR is not required for the inhibitory effects of GnRH II. One possibility is that the human GnRHIIIR gene might produce incomplete transcripts and subsequently partial peptides that can form a GnRH II-responsive complex which may also involve GnRHIR (Neill *et al.* 2004). In support of this notion, Eicke *et al.* (2005) have identified a GnRHIIIR-like protein in endometrial and ovarian cancer cells, while Maiti *et al.* (2005) have identified a novel GnRH II binding protein in prostate cancer cells. The intracellular effects of GnRH are mediated through G protein-coupled receptors (Millar

*et al.* 2004). These in turn can recruit MAPKs that have a crucial role in GnRH intracellular signalling. The effects of GnRH II inhibition in ovarian cancer cells for instance have been shown to involve both ERK 1/2 and p38 MAPK (Kim *et al.* 2004, 2005). Protein kinase C is also thought to be involved in this process (Kim *et al.* 2006). In breast cancer cells, GnRH II has been reported to block epidermal growth factor (EGF)-dependent phosphorylation of the EGF receptor and subsequent recruitment of ERK1/2 (Gunthert *et al.* 2005). A novel mechanism of GnRH function was proposed by Chen *et al.* (2002b). In this study, GnRH II and GnRH I inhibited the expression of ribosomal phosphoproteins in human cancer cells. These are key regulators of protein elongation and suggest a role in regulating translation. It is possible that the mechanisms of GnRH II signalling may be cell and tissue type specific. The specific mechanisms involved in GnRH II binding and signalling in prostate cancer cells warrant further directed investigations, particularly in the light of the findings in this study, and are the subject of our current investigations.

In conclusion, in this report, we show first evidence that GnRH II is expressed in prostate cancer cells and is regulated by androgens. This induction requires the AR that binds to a cognate response element in the gene. Our ongoing work is focused on further characterisation of this androgen response element, defining the mechanism of GnRH II binding and signalling in prostate cancer cells, the significance of the inhibitory effects of GnRH II in benign as well as androgen-dependent and androgen-independent prostate cancer cells and its potential value as a novel therapeutic agent.

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