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Cloning of a novel insulin-regulated ghrelin transcript in prostate cancer

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Short title: A novel ghrelin isoform regulated by insulin in prostate cancer

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Abstract

Ghrelin is a multifunctional hormone, with roles in stimulating appetite and regulating energy balance, insulin secretion and glucose homeostasis. The ghrelin gene locus (*GHRL*) is highly complex and gives rise to a range of novel transcripts derived from alternative first exons and internally spliced exons. The wild-type transcript encodes a 117 amino acid preprohormone that is processed to yield the 28 amino acid peptide ghrelin. Here, we identified insulin-responsive transcription corresponding to cryptic exons in intron 2 of the human ghrelin gene. A transcript, termed in2c-ghrelin (intron 2-cryptic), was cloned from the testis and the LNCaP prostate cancer cell line. This transcript may encode an 83 AA preproghrelin isoform that codes for the ghrelin, but not obestatin. It is expressed in a limited number of normal tissues and in tumours of the prostate, testis, breast and ovary. Finally, we confirmed that in2c-ghrelin transcript expression, as well as the recently described in1-ghrelin transcript, is significantly upregulated by insulin in cultured prostate cancer cells. Metabolic syndrome and hyperinsulinaemia has been associated with prostate cancer risk and progression. This may be particularly significant after androgen deprivation therapy for prostate cancer, which induces hyperinsulinaemia, and this could contribute to castrate resistant prostate cancer growth. We have previously demonstrated that ghrelin stimulates prostate cancer cell line proliferation *in vitro*. This study is the first description of insulin regulation of a ghrelin transcript in cancer, and should provide further impetus for studies into the expression, regulation and function of ghrelin gene products.

Introduction

The 28 amino acid hormone, ghrelin, has emerged as a multifunctional peptide hormone with actions beyond its roles in growth hormone secretion, appetite regulation and energy balance (Kojima *et al.* 1999; Lim *et al.* 2011; Nakazato *et al.* 2001; Wren *et al.* 2000). In addition to its role in normal physiology, ghrelin has been associated with a number of disease processes, including obesity (Scerif *et al.* 2011; Yi *et al.* 2011), type 2 diabetes mellitus and hyperinsulinaemia (Ukkola 2011), gastrointestinal disease (Jeffery *et al.* 2011), cardiovascular disease (Isgaard and Granata 2011), reproductive disorders (Repaci *et al.* 2011), mental health (Steiger *et al.* 2011) and cancer (Chopin *et al.* 2011; Deboer 2011). Ghrelin may play a role in prostate cancer progression, and we have previously demonstrated that ghrelin mRNA and protein is expressed in prostate cancer cell lines and tissues and that ghrelin stimulates cell proliferation in prostate cancer cell lines (Jeffery *et al.* 2002; Yeh *et al.* 2005).

The ghrelin gene consists of four preproghrelin-coding exons (exons 1 to 4) that give rise to a 117 amino acid preproghrelin peptide (Kojima *et al.* 1999; Seim *et al.* 2011b). The first 23 amino acids are encoded by exon 1 and harbour a secretory signal peptide, while the remainder of exon 1 and part of exon 2 encode the 28 amino acid ghrelin peptide. Exon 3 encodes obestatin, a recently-discovered 23 amino acid peptide hormone with a range of novel functions, including regulation of adipogenesis, insulin secretion and pancreatic homeostasis and cancer cell progression (Seim *et al.* 2011b; Zhang *et al.* 2005).

Following cleavage of the signal peptide, the 94 amino acid proghrelin peptide is post-translationally modified in the endoplasmic reticulum where an octanoyl fatty acid group (acyl group) is added to the third residue of ghrelin, a serine, by the recently discovered enzyme ghrelin *O*-acyl transferase/GOAT (Gutierrez *et al.* 2008; Yang *et al.* 2008). Although both ghrelin and desacyl ghrelin circulate in the blood, the levels of desacyl ghrelin are significantly higher (Date *et al.* 2000; Holmes *et al.* 2009; Patterson *et al.* 2005). This may be the result of the rapid conversion of ghrelin to desacyl ghrelin by

esterases in the circulation (Beaumont *et al.* 2003; De Vriese *et al.* 2004; Eubanks *et al.* 2011; Satou *et al.* 2010). Alternatively desacyl ghrelin may be produced from proghrelin in the absence of GOAT expression, or when the fatty acid substrates for GOAT are unavailable (Takahashi *et al.* 2009). Only the acylated form of ghrelin is able to bind the cognate receptor, the growth hormone secretagogue receptor type 1a (GHSR1a) (Howard *et al.* 1996; Kojima *et al*. 1999), although alternative receptors for both acylated and desacyl ghrelin must also exist (Seim *et al.* 2011a). It has been demonstrated that GOAT and ghrelin play a crucial role in glucose balance , particularly in response to caloric restriction (Zhao *et al.* 2010), however, this finding has not been replicated in a number of mouse models (Yi *et al.* 2012). While the role of the ghrelin axis in insulin and glucose balance is currently unclear, ghrelin and GOAT may be useful therapeutic targets for treating insulin resistance (Kirchner *et al.* 2010; Lim *et al*. 2011; Romero *et al.* 2010). Insulin resistance is also common in prostate cancer patients after androgen deprivation therapy (Aggarwal *et al.* 2011).

It is now well established that the majority of genes generate alternative splice forms, many of which have novel functions (Nilsen and Graveley 2010). This is particularly true for preprohormones, which frequently harbour a range of bioactive peptides. The ghrelin gene (*GHRL*) is no exception, and it is now realised that the ghrelin locus encodes multiple bioactive molecules derived by alternative transcriptional splicing, proteolytic cleavage of larger precursor peptides and by other post-translational modifications (Seim *et al.* 2009; Seim *et al*. 2011a).

Here, we report a novel preproghrelin splice variant, termed in2c-ghrelin (intron 2-cryptic) that contains novel exon sequence derived from intron 2 of the ghrelin gene and investigate its expression in cancer. We demonstrate that this novel transcript, as well as the recently described in1-ghrelin transcript (Gahete *et al.* 2011; Gahete *et al.* 2010; Kineman *et al.* 2007), are upregulated in response to insulin treatment in the 22Rv1 and LNCaP prostate cancer cell lines and, therefore, they may have a role in prostate cancer progression, particularly in cases of hyperinsulinaemia.

Materials and Methods

Cell Culture

Cell lines were originally obtained from the American Type Culture Collection (ATCC, Rockville, MD). The PC3 (ATCC CRL-1435), DU145 (ATCC HTB-81), LNCaP (ATCC CRL-1740) and 22Rv1 (ATCC CRL-2505) prostate cancer cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Carlsbad, CA) with 10% New Zealand Cosmic Calf Serum (FCS, HyClone, South Logan, UT) supplemented with 100 U/mL penicillin G and 100 µg/mL streptomycin (Invitrogen). All cell lines were passaged at two to three day intervals at 70% confluency using 0.25% Trypsin/EDTA (380 mg/ml or 0.913 mM EDTA, and 2500 mg/ml or 0.105 mM trypsin) (Invitrogen). Cell morphology and viability was monitored by microscopic observation and regular testing was performed by PCR (Universal Mycoplasma Detection Kit, ATCC) to ensure that cells were not contaminated with *Mycoplasma*.

Microarray profiling of insulin-treated LNCaP prostate cancer cell line

To identify RNAs expressed in the prostate that could be developed as disease-specific targets for prostate cancer treatment, we employed a custom-designed microarray from the Vancouver Prostate Centre (VPC). The microarray, an Agilent custom 2x105K array (Agilent Technologies, Wilmington, DE), combines the probes from the Agilent 44K (v1) human gene expression set with probes targeting alternative protein isoforms and non-coding RNAs. All probes on the microarray were classified based on their relationship to the RNAs in the NCBI RefSeq database (http://www.ncbi.nlm.nih.gov/RefSeq; December 2010).

LNCaP prostate cancer cells were seeded on 10 cm tissue culture flasks (Nunc, International) in phenol-red free RPMI1640 medium with 5% FCS (Invitrogen) and left overnight to attach. The following day, serum was replaced with 5% charcoal-stripped serum (Invitrogen) for 48 hours, followed by a 24 hour incubation in serum-free medium and treatment with 10 nM insulin (Sigma-

Aldrich, St. Louis, MO) or an equal volume of vehicle (70% ethanol) for 10 hours. All treatments were performed in triplicate. Total RNA was isolated using TRIzol (Invitrogen), One-colour gene expression analysis was performed following Agilent labeling (Quick Amp Labeling Kit), hybridisation, and scanning (Agilent Feature Extraction version 10.5.1) protocols using the custom Agilent 2x105K microarray. Data were normalised using the quantile normalisation method in Linear Models for Microarray Data (LIMMA), an open-source R-based software package for the analysis of gene expression data (Smyth 2005). Differential gene expression was determined using LIMMA linear models with an FDR adjusted p-value ≤ 0.05 and fold change ≥ 1.5.

In silico and RT-PCR verification of putative ghrelin exons identified by microarray analysis

To investigate whether novel exons identified by microarray analysis correspond to splice variants of the preproghrelin gene (*GHRL*), and to determine the open reading frame of any resulting full-length transcript, we performed BLAST queries (Johnson *et al.* 2008), UCSC Genome Browser multigenome alignments (specifically, the 17-way conservation track) (Fujita *et al.* 2011), and RT-PCR with primers spanning the putative exons (In2c-ghrelin-ORF-F/R, Table 1). Briefly, for RT-PCR analyses 2 µg total RNA from human testis and pancreas (FirstChoice, Ambion, Austin, TX), and from the LNCaP prostate cancer cell line were subjected to *DNase I* digestion (amplification grade, Invitrogen), and cDNA synthesis with SuperScript III and oligo$(dT)_{18}$ primers (Invitrogen) according to the manufacturer's instructions. RT-PCR was performed in a total reaction volume of 50 µl containing 1 × PCR buffer, 0.2 mM deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, 0.2 µM primers (In2c-ghrelin-ORF-F/R, Table 1), 1 µl cDNA and 1 unit of Platinum *Taq* DNA Polymerase (Invitrogen) on a PTC-200 thermal cycler (MJ Research, Watertown, MA) according to the manufacturer's instructions. Negative, no-template controls were performed.

Quantitative real time RT-PCR of cultured cells and tissues

Unless otherwise noted, total RNA was harvested from cultured cells using QIAshredder and RNEasy Plus Mini kits (QIAGEN, Australia), according to the manufacturer's instructions, and stored frozen at

-80°C. RNA concentration and purity were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Australia) to ensure adequate RNA quality. Contaminating genomic DNA was removed by *DNase I* digestion (amplification grade, Invitrogen), and 2 μg total RNA was reverse transcribed with oligo$(dT)_{18}$ primers using SuperScript III (Invitrogen). The prostate cancer cell lines described above, as well as RNA previously isolated from cell lines derived from normal lung (Beas-2B, ATCC CRL-9609), non-small cell lung cancer (A549, ATCC CCL-185 and NCI-H1299, ATCC CRL-5803), normal ovary (hOSE17.1), ovarian cancer (SKOV-3, ATCC HTB77) and breast cancer (MDA-MB231, ATCC HTB-26) were examined. Quantitative real-time RT-PCR was performed using primers In2c-real-F/R (Table 1) with 2 × SYBR green master mix (Applied Biosystems; AB, Foster City, CA) on a PRISM 7000 Sequence-Detection System (AB). Data were analysed using the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Expression in different samples is shown as fold change (relative values) compared to a calibrator sample (value denoted as 1) after normalisation to the *18S* ribosomal RNA housekeeping gene. Primers are shown in Table 1.

Normal human tissue (HMRT102) and cancer (CSRT103) cDNA panels were purchased from OriGene (Rockville, MD). HMRT102 consists of 48 normal tissues (adrenal gland, bone marrow, brain, breast, cervix, colon, duodenum, epididymis, fat, heart, small intestine, intracranial artery, kidney, liver, lung, lymph node, peripheral blood leukocytes, muscle, nasal mucosa, oesophagus, optic nerve, ovary, oviduct, pancreas, penis, pericardium, pituitary, placenta, prostate, rectum, retina, seminal vesicles, skin, spinal cord, spleen, stomach, testis, thymus, thyroid, tongue, tonsil, trachea, urethra, urinary bladder, uterus, uvula, vagina, and vena cava), while CSRT103 consists of 2-6 normal and approximately 20 tumour samples from a range of tissues. In2c-ghrelin was interrogated as detailed above using the AB PRISM 7000 Sequence Detection System in a total reaction volume of 20 μl using 2× SYBR green master mix (AB) on separate, identical array plates, which were loaded with equal amounts of cDNA per well.

Quantitative real time RT-PCR analysis of the effect of insulin on in2c-ghrelin and in1-ghrelin expression in cultured prostate cancer cells

In order to verify the microarray data, which indicated that the novel in2c-ghrelin transcript is upregulated by insulin, we performed qRT-PCR on RNA extracted from the LNCaP and 22Rv1 prostate cancer cell lines. The LNCaP and 22Rv1 prostate cancer cell lines were cultured in six-well plates in phenol red-free RPMI 1640 (Invitrogen), as outlined above, except that cells were treated with 10 nM insulin (Sigma-Aldrich) for 48 hours. Insulin was refreshed after 24 hours. RNA extractions and qRT-PCR were performed as outlined above. In1-ghrelin (In1-real-F/R, Table 1) and wild-type ghrelin (wt-real-F/R, Table 1) were detected using primer sequences published by Gahete and colleagues (Gahete *et al*. 2011; Gahete *et al*. 2010). All treatments were performed in triplicate. Data was analysed using the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Ribosomal protein, L32 (RPL32F/R, Table 1), which is particularly suitable for cultured prostate cancer cells (Lubik *et al.* 2011), was employed as a housekeeping gene for qRT-PCRs.

RT-PCR product purification and sequencing

All RT-PCR products were, unless noted, purified using a MinElute (QIAGEN) PCR Purification Kit, cloned into *pGEM-T Easy* (Promega, Madison, WI), transformed into One Shot MAX Efficiency *DH5α-T1R* chemically-competent cells (Invitrogen) and sequenced at the Australian Genome Research Facility (AGRF, Brisbane, Australia) using BigDye III (Applied Biosystems).

Statistical analyses

Data are expressed as means ± standard deviation. Statistical significance was determined using Student's t-test using Graphpad Prism (La Jolla, CA) or Microsoft Excel (Seattle, WA) software with a P value <0.05 considered to be statistically significant.

Results

Identification and cloning of novel, cryptic exons of the ghrelin gene (GHRL)

Using human exon-junction microarray probes, novel exons (named I and II) located in intron 2 of *GHRL* were found to be upregulated 3.9-fold by treatment with 10 nM insulin in the LNCaP prostate cancer cell line (Figure 1). These exons match four GenBank entries (EF139854-57) that partially overlap the ghrelin coding-exons and contain novel exon sequence in intron 2 (Figure 2). Interestingly, the GenBank entries are previously unpublished RT-PCR validations of testis-specific exons which were identified in a large-scale study by Clark and colleagues (Clark *et al.* 2007). Their study employed exon arrays to locate tissue-specific exons in 16 normal tissues, but focused on novel, brain-enriched exons in the published manuscript (Clark *et al*. 2007). To verify whether the novel exons correspond to splice variants of the ghrelin gene and to determine the open reading frame of these transcripts, RT-PCRs were performed (using cDNA reverse transcribed with oligo$(dT)_{18}$ primers) using RNA from the LNCaP prostate cancer cell line, human testis and pancreas (with a forward primer in exon 1 of ghrelin and a reverse primer in the novel exon II, which is common to all of the testis-derived GenBank entries). A single, major amplicon was obtained in the testis and LNCaP cell line, representing exons 1, 2 and II of *GHRL* (Figure 1; Supplementary Figure 1) (GenBank: JN243773). We have termed the identified full-length splice variant in2c-ghrelin (intron 2 cryptic-ghrelin). The exons for In2c-ghrelin have canonical GT/AG splice junctions, with the exception of the intron flanking a 5' truncated exon II, exon IIb, which harbours a rare GT/AC junction (Burset *et al.* 2000) (Figure 2). The cryptic exons in the human gene are not conserved in the mouse or rat, which would, therefore, be unlikely to express the in2c-ghrelin isoform (data not shown). These analyses demonstrate that the human *GHRL* gene contains additional exons within intron 2 that are *bona fide* internal exons of preproghrelin splice variants that harbour exons 1, 2, 3 and 4.

The novel In2c-ghrelin isoform includes cryptic exons that lead to a frameshift in the translation of preproghrelin. The 83 amino acid in2c-ghrelin isoform harbours the coding region for the 28 amino acid mature ghrelin peptide (Figure 3). While the C-terminal (C-ghrelin) region of wild-type ghrelin

encodes a 66 amino acid peptide, the open reading frame of in2c-ghrelin encodes the first 24 amino acids of the C-ghrelin sequence and 8 unique C-terminal residues, before the introduction of a stop codon. As a result of the frameshift, it lacks the coding sequence for obestatin (Figure 3). The 3' untranslated region of the mRNA is otherwise identical to wild-type ghrelin, and has the same polyadenylation signal.

Quantification of in2c-ghrelin in normal tissues

To selectively amplify the in2c-variant and to avoid co-amplification of ghrelin pre-mRNA or genomic DNA, we designed a reverse qRT-PCR primer that spans the exon II-exon 3 junction (Supplementary Figure 2). Quantitative real-time RT-PCR analyses of 48 tissues show that in2c-ghrelin mRNA, in contrast to the wild-type ghrelin transcript (Gnanapavan *et al.* 2002; Ueberberg *et al.* 2009), is expressed in a small number of tissues, including the stomach, lymph node, peripheral blood leukocytes, testis, epididymis, skin and adrenal gland (Figure 4).

Quantification of in2c-ghrelin in cell lines

Next, we examined the expression of in2c-ghrelin in cultured cells using qRT-PCR. In2c-ghrelin was highly expressed in the PC3, LNCaP and 22Rv1 prostate cancer cell lines, while the DU145 prostate cancer cell line did not express this transcript (Figure 5). In2c-ghrelin expression was low in the lung, ovarian and breast cell lines examined. Interestingly, in2c-ghrelin is expressed in the SKOV-3 ovarian cancer cell line, but not in the hOSE1.7 normal ovarian cell line (Figure 5).

In2c-ghrelin expression in normal versus tumour samples

To further explore the role of in2c-ghrelin in cancer, we analysed the expression of the variant using commercial cDNA arrays from OriGene. In the testis cDNA tissue panel, in2c-ghrelin was expressed in normal testicular tissue, but is absent or present at low levels in testicular tumour samples (Figure 6A). Conversely, prostate tumours exhibited high levels of in2c-ghrelin compared to normal prostate tissue (with 9/19 expressing higher levels than normal tissue, and 14/19 prostate tumours expressing

the variant) (Figure 6B). Finally, normal breast (Figure 6C) and ovary (Figure 6D) samples did not express in2c-ghrelin, while the transcript was expressed in 13/20 breast tumours and 7/21 ovarian tumours examined. Together, these results demonstrate that in2c-ghrelin is expressed at high levels in prostate cancer cell lines and prostate tumour tissue. It is expressed in all normal testis samples examined, while its expression is low or absent in testicular tumours; and it is expressed in breast and ovarian tumour, but not normal tissue.

In2c-ghrelin expression is regulated by insulin in prostate cancer cells

The microarray experiment revealed that in2c-ghrelin was regulated by insulin in LNCaP prostate cancer cells, therefore, we confirmed that this transcript is insulin regulated using quantitative RT-PCR of the LNCaP and 22Rv1 prostate cancer cell lines. As shown in Figure 7A, treatment with 10 nM insulin significantly increased the expression of in2c-ghrelin in the LNCaP (1.3 fold, $P<0.05$) and 22Rv1 (4.4-fold, $P<0.05$) prostate cancer cell lines. Interestingly, insulin also significantly upregulated the expression of a recently-reported intron 1-retained transcript, in1-ghrelin (Gahete *et al*. 2011; Gahete *et al*. 2010; Kineman *et al*. 2007). A 2.2-fold increase in expression was observed in LNCaP cells and a 2.8 fold increase in 22Rv1 cells (Figure 7B).

Discussion

We have demonstrated a new ghrelin isoform that is regulated by insulin treatment in prostate cancer cell lines. Cryptic exon sequence in the in2c-ghrelin variant leads to a frame-shift in the translation of preproghrelin and leads to an open reading frame that would encode an 83 amino acid preproghrelin peptide, which is considerably shorter than the 117 amino acid wild-type form. This isoform contains the 28 amino acid ghrelin, but lacks the coding sequence for obestatin.

This study provides further evidence that microarrays, and in particular exon arrays where probes are designed to detect individual exons, are excellent tools to discover novel alternative exons (Clark *et al*. 2007; French *et al.* 2007; Menghi *et al.* 2011; Rahman *et al.* 2010; Skotheim and Nees 2007). The

exon-junction probe in our custom array was designed to target GenBank sequences deposited by Affymetrix as a result of RT-PCR validation of testis-enriched exon array data (Clark *et al*. 2007). Alternative splicing results in cell- or tissue-specific expression of exons, and is frequently observed in cancer (He *et al.* 2009; Nilsen and Graveley 2010; Skotheim and Nees 2007).

We have previously demonstrated that ghrelin mRNA and protein is expressed in prostate cancer cell lines, using RT-PCR and Western blotting (Jeffery *et al*., 2002; Yeh *et al*., 2005). Expression of the ghrelin peptide has been demonstrated in prostate cancer cell lines using Western analysis and in clinical specimens of prostate cancer using immunohistochemistry. Using these methods, it is not possible to discriminate between the expression of wild-type ghrelin and the in2c ghrelin peptide isoforms at the peptide level. The in2c-ghrelin mRNA isoform appears to be particularly highly expressed in prostate cancer cell lines and tissues, but is expressed at low levels or is absent in normal prostate tissue, which may indicate that the variant could be a useful diagnostic or prognostic marker for this cancer. A recent *in vitro* binding study using a fluorescein labelled ghrelin-derived probe (consisting of the first 18 amino acids of ghrelin) has demonstrated that ghrelin may have potential as a diagnostic or prognostic marker for prostate cancer (Lu *et al.* 2012). Ghrelin binding was significantly higher in clinical samples of prostate cancer compared to normal tissue and samples of benign prostate hyperplasia in a study with 13 patients undergoing radical prostatectomy (Lu *et al*. 2012). If this probe is effective *in vivo,* it could be combined with PET (positron emission tomography) scanning and be useful for discriminating between benign disease and cancer, and possibly for detecting micrometastases (Lu *et al*. 2012).

Treatment with acylated ghrelin stimulates cell proliferation through the ERK1/2 pathway in the PC3 and LNCaP prostate cancer cell lines (Jeffery *et al*., 2002, Yeh *et al*., 2005). Ghrelin may stimulate cell proliferation by acting through the GHSR1a, which is expressed in prostate cancer cell lines (Jeffery *et al*., 2002; Yeh *et al*., 2005), although it is also possible that ghrelin may act through the unidentified, widely hypothesised alternative ghrelin receptor (Chopin *et al*., 2012). We have

demonstrated that GOAT is expressed in prostate cancer cell lines (I Seim, P Jeffery, L Amorim, A Herington and L Chopin, 2012, unpublished observation), indicating that ghrelin may be acylated in these cells. The role of ghrelin in prostate cancer growth remains uncertain, however. A similar increase in cell proliferation in response to ghrelin treatment was seen in the PC3 cell line in another study using lower concentrations of ghrelin, while supraphysiological levels (1000 nM) inhibited proliferation in this study (Cassoni *et al.* 2004). Recently, a third laboratory demonstrated that ghrelin decreases proliferation and promotes apoptosis of PC3 cells (Diaz-Lezama *et al.* 2010). In this study, however, the investigators measured tritiated thymidine incorporation (DNA synthesis) in the last 6 hours of a 72 hour ghrelin treatment, and it is unlikely that ghrelin would been intact at this point of the experiment. Ghrelin is unstable in culture, with a short half-life, with the octanoyl group (acylation) being rapidly lost and the peptide rapidly cleaved (De Vriese *et al*. 2004; Hosoda *et al.* 2004). Contrasting observations have also been observed for the LNCaP and DU145 cell lines (Cassoni *et al*. 2004; Yeh *et al*. 2005). We propose that the observed discrepancies may arise from the use of different forms of the cell lines, the different types of assays used, and the handling and stability of the acylated ghrelin peptide.

In2c-ghrelin is expressed in all normal testis samples examined, while it is expressed in a small number of testicular tumours. This correlates well with previous studies, which have shown that the ghrelin peptide is present in normal human testis (Gaytan *et al.* 2004; Ishikawa *et al.* 2007) and in a subset of testicular tumours (Gaytan *et al*. 2004). Employing a small number of normal samples, we also demonstrate that in2c-ghrelin is expressed in breast and ovarian tumours, but not in normal breast or ovary. The SKOV-3 ovarian cancer cell line expresses the in2c-ghrelin variant, but no expression was detected in the hOSE17.1 normal ovary-derived cell line. Ghrelin has previously been shown to be expressed in breast cancer and, as is the case for prostate cancer, studies from other laboratories report different responses in cell proliferation assays when cell lines are treated with exogenous ghrelin peptide (Cassoni *et al.* 2001; Jeffery *et al.* 2005).

The in2c-ghrelin isoform is expressed in a limited number of normal tissues (including the testis) and at higher levels in many tumours. In normal tissues, in2c-ghrelin expression was most abundantly expressed in lymph node tissue. Interestingly, the LNCaP cell line (Lymph Node Carcinoma of the Prostate), which demonstrated very high levels of in2c-ghrelin, is derived from a lymph node metastasis (Yu *et al.* 2009). Furthermore, in2c-ghrelin is expressed in peripheral blood leukocytes, indicating that it may play a role in immune regulation. Indeed, the ghrelin peptide has been shown to play an immunoregulatory role (Baatar *et al.* 2011).

A number of other ghrelin isoforms have also been described previously (Seim *et al*. 2009; Seim *et al*. 2011a), including exon 3-deleted preproghrelin (Jeffery *et al*. 2005; Yeh *et al*. 2005) and the intron 1-retained transcript (originally termed in2-ghrelin, but renamed in1-ghrelin using the current ghrelin gene nomenclature) (Gahete *et al*. 2011; Gahete *et al*. 2010; Kineman *et al*. 2007). In1-ghrelin would encode a 117 amino acid polypeptide that includes the signal peptide of preproghrelin, the first 12 amino acids of the 28 amino acid ghrelin peptide sequence and a novel 81 amino acid C-terminal peptide, but not obestatin (Gahete *et al*. 2011). In1-ghrelin is regulated in response to metabolic stress in mice (Kineman *et al*. 2007), and is down-regulated in particular brain regions in Alzheimer's disease in humans (Gahete *et al*. 2010) and upregulated in breast cancer (Gahete *et al*., 2011).

We speculate that the in2c-ghrelin variant is another example that illustrates an emerging theme where mRNA transcripts encode ghrelin, but not obestatin, as is the case for exon 3-deleted ghrelin and in1-ghrelin. Indeed, evidence is emerging that obestatin, the peptide derived from exon 3 of preproghrelin, may be a growth factor that could promote or inhibit cancer progression, depending on the cancer type (Pazos *et al.* 2009; Pazos *et al.* 2007; Volante *et al.* 2009). Similarly, putative obestatin-coding transcripts that lack the ghrelin coding region are also expressed in prostate cancer cell lines (Seim *et al.* 2007). These studies suggest that cells can regulate the production of ghrelin gene-derived peptides independently by means of alternative splicing and this could partly explain the observed lack of correlation between plasma ghrelin and obestatin peptide levels (Pemberton *et al*.,

2002). One small study has reported that the ratio of acylated ghrelin to total ghrelin (acyl and desacyl ghrelin combined) in the circulation was higher in prostate cancer patients compared to subjects with benign prostatic hyperplasia (BPH) (Malendowicz *et al.* 2009). In contrast, obestatin was not elevated in these patients (Malendowicz *et al*. 2009).

Insulin regulates a large number of genes (Hartmann *et al.* 2009; O'Brien and Granner 1996; Rome *et al.* 2003), and in this study, we have demonstrated that insulin increases the expression of in2c-ghrelin and in1-ghrelin in the LNCaP and 22Rv1 prostate cancer cell lines. Insulin treatment also upregulates ghrelin expression in mouse stomach oxyntic glands (which are the major site of ghrelin expression) (Toshinai *et al.* 2001), and in adipocytes (Gurriaran-Rodriguez *et al.* 2011). Conversely, insulin downregulates the expression of wild-type and in1-ghrelin mRNA in the mouse hypothalamus, where it has an effect on appetite regulation (Fick *et al.* 2009; Kineman *et al*. 2007). The regulation of ghrelin in cancer has yet to be established, and this is the first study to demonstrate regulation of ghrelin transcripts by insulin in cancer.

Although in2c-ghrelin is insulin-regulated in the prostate, we failed to detect the in2c-ghrelin isoform in the pancreas itself, despite the fact that the wild-type ghrelin transcript and the ghrelin peptide are abundantly expressed in the endocrine pancreas and ghrelin appears to plays an important role in glucose homeostasis and insulin regulation (Granata *et al.* 2010). The importance of the ghrelin axis in the regulation of blood glucose was recently illustrated in a study of the ghrelin acylation enzyme, GOAT, knockout mouse, which is unable to produce acylated ghrelin. Under severe caloric restriction (60%), GOAT knockout mice are unable to maintain glucose homeostasis and die, however, animals can be rescued with the administration of acylated ghrelin or growth hormone (Zhao *et al*. 2010). This finding has been contradicted, however, by a comprehensive study using a range of mouse models, suggesting that the ghrelin axis may not play an important role in insulin regulation in response to caloric restriction (Yi *et al*. 2012).

Ghrelin has been linked directly with obesity given its clear role in appetite, energy balance and metabolic regulation, and ghrelin and GOAT are important targets for the treatment of obesity (Lim *et al*. 2011). In addition to being a risk factor for cancer, obesity is associated with metabolic syndrome, which is also typified by hyperinsulinaemia (Hammarsten and Peeker 2011). There is mounting evidence that elevated circulating insulin levels are positively associated with increased cancer risk and particularly with breast and prostate cancer (Amling 2005; Bao *et al.* 2011; Buschemeyer and Freedland 2007; Gunter *et al.* 2009; Hammarsten *et al.* 2010; Hammarsten and Hogstedt 2005; Hammarsten and Peeker 2011; Healy *et al.* 2010; LeRoith 2010; Ma *et al.* 2008; Suissa *et al.* 2011; Wilson 2011). Although there have been few studies at the cellular level, insulin appears to play a role in the progression of prostate cancer. Insulin stimulates cell proliferation in cultured breast and prostate cancer cell lines (Lann and LeRoith 2008; Pollak 2008), and promotes tumour progression by stimulating *de novo* steroidogenesis in prostate cancer (Lubik *et al*. 2011). Interestingly, diet-induced hyperinsulinaemia results in increased cell proliferation of LNCaP prostate cancer cell xenografts in mice (Venkateswaran *et al.* 2007). We show that in2c-ghrelin is expressed in the PC3 prostate cancer cell line, which is derived from a bone metastasis (Yu *et al*. 2009). Men with hyperinsulinaemia are more likely to develop bone metastases and progress faster to terminal, castrate-resistant prostate cancer (Lubik *et al*. 2011). While hyperinsulinaemia has been associated with prostate cancer progression, androgen deprivation therapy, the standard treatment for advanced prostate cancer, frequently gives rise to metabolic syndrome and insulin resistance, with hyperinsulinaemia, elevated fasting blood glucose and elevated C-reactive peptide (Aggarwal *et al*. 2011). As insulin treatment leads to increased ghrelin expression in the LNCaP prostate cancer cell line, this may also increase cell proliferation in prostate cancer.

In conclusion, in the present study we have identified *bona fide* exons within intron 2 of the ghrelin gene locus (*GHRL*) and described a novel ghrelin isoform that, together with the recently described in1-ghrelin isoform, is upregulated by insulin in prostate cancer cell lines. In2c-ghrelin includes cryptic exon sequence and would result in the translation of an 83 amino acid preproghrelin isoform

that encodes ghrelin, but not obestatin. In2c-ghrelin is expressed in a range of tumour samples derived from the prostate, breast and ovary, but in a limited number of normal tissues, including lymph nodes, stomach and testis. Further studies are warranted to elucidate the role of the ghrelin gene products in normal physiology and in conditions associated with hyperinsulinaemia and in cancer.

Declarations of interest

The authors declare that they have no conflicts of interest.

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Figures and Tables

Table 1. Designations and sequences of RT-PCR primers. Annealing temperatures (T_a) of oligonucleotide primers employed in RT-PCR and the expected product sizes are shown.

Primer name	Primer sequence	T_a (°C)	Expected size (bp)
In2c-ghrelin-ORF-F	CTTCCTGAGCCCTGAACACC	64	-
In2c-ghrelin-ORF-R	TCAGCCTTAGCCTCTAATGGGA		-
In2c-real-F	AGAGGCATGAGAGTGCAAGTTC	64	131
In2c-real-R	GATGTCCTGAAGAAACTTCCCC		
In1-real-F	TCTGGGCTTCAGTCTTCTCC	60	215
In1-real-R	GTTCATCCTCTGCCCCTTCT		
wt-real-F	TCAGGGGTTCAGTACCAGCA	60	158
wt-real-R	CAAGCGAAAAGCCAGATGAC		
RPL32-F	CCCCTTGTGAAGCCCAAGA	60	59
RPL32-R	GACTGGTGCCGGATGAACTT		
18S-F	TTCGGAACTGAGGCCATGAT	60	151
18S-R	CGAACCTCCGACTTTCGTTCT		

Figure legends

Figure 1 Image depicting mRNA transcript architecture in the *GHRL* locus was obtained through the UCSC Genome Browser. Microarray analysis detected a 3.9-fold increase in the expression of novel exons (indicated in pink) in intron 2 of the ghrelin gene (*GHRL*). Wild-type-preproghrelin coding exons 2 to 4 are indicated in blue font, while the cryptic exons are indicated in red font. GenBank entries corresponding to the novel cryptic exons are indicated by a red box.

Figure 2(A) Overview of the ghrelin locus showing the ghrelin gene, *GHRL*, with wild-type preproghrelin-coding exons in blue and the cryptic exons in intron 2 as red boxes (IIa, and the 5' truncated exon IIb). Exons are represented as boxes and introns as horizontal lines. (B) Mapping of the novel cryptic exon ghrelin transcripts. The exon structure of amplicons is derived from the human testis and LNCaP cells. Exons are represented as boxes, and RT-PCR primers are indicated as arrows above exons.

Figure 3 Multiple sequence alignment of wild-type preproghrelin and putative In2c-preproghrelin. Secretion signal peptides are shown in purple, ghrelin in blue, obestatin in red, and C-ghrelin is highlighted in grey.

Figure 4 Relative expression of in2c-ghrelin in human tissues by quantitative real-time RT–PCR. Data were normalised to β-actin and are represented as fold changes relative to the expression in the adrenal gland (1.0).

Figure 5 Relative expression of in2c-ghrelin in human cell lines using quantitative real-time RT-PCR. Data is represented as means and standard error of mean from two technical replicates of two

733 independent replicate experiments (n=2). The housekeeping gene, *18S* ribosomal RNA, was used as a
734 reference for normalisation. Data are represented as fold changes relative to expression of transcripts
735 in the Beas-2B normal lung cell line (set at 1).

736

737 **Figure 6** Relative expression of in2c-ghrelin in cancer determined using real-time quantitative RT-
738 PCR. The labels in X-axis indicate the clinical status of the subjects (N denotes normal, Roman
739 numerals indicate disease stages). Data represented as fold changes relative to the expression in a
740 normal tissue sample (set at 1.0), or in the absence of expression in a normal tissue a tumour sample.
741 (A) Testis (B) Prostate (C) Breast (D) Ovary.

742

743 **Figure 7** Real-time quantitative RT-PCR analysis of (A) in2-ghrelin and (B) in1-ghrelin gene
744 expression in 22Rv1 and LNCaP prostate cancer cell lines in response to treatment with 10nM insulin
745 over 48 hours. The housekeeping gene, Ribosomal protein L32, was used as a reference for
746 normalisation. Data is represented as means and standard error of the mean with three technical
747 replicates of three independent replicate experiments (n=3). **P*<0.05 = significantly different from the
748 vehicle-treated control (set at 1-fold) using Student's t test.

749

750 **Supplementary Figure 1** Ethidium bromide-stained agarose electrophoresis gel of *GHRL* exon 1 to II
751 (non-quantitative) RT-PCR amplicons in the LNCaP prostate cancer cell line, testis and pancreas.
752 NTC = no template control (water). M = 100 bp DNA molecular weight marker (New England
753 Biolabs).

754

755 **Supplementary Figure 2** (A) Overview of In2c-ghrelin real-time, quantitative RT-PCR assay design
756 where the reverse PCR primer spans the exon II-exon 3 junction to eliminate gDNA and pre-mRNA

757 amplification and amplify In2c-ghrelin transcripts only. (B) Agarose gel separation of quantitative
758 RT-PCR products generated by interrogating the pancreas and testis with the in2c-ghrelin primers. (C)
759 Dissociation curves of quantitative RT-PCR products (annealing at 64°C) generated from the
760 pancreas and testis using the in2c-ghrelin primers.

761

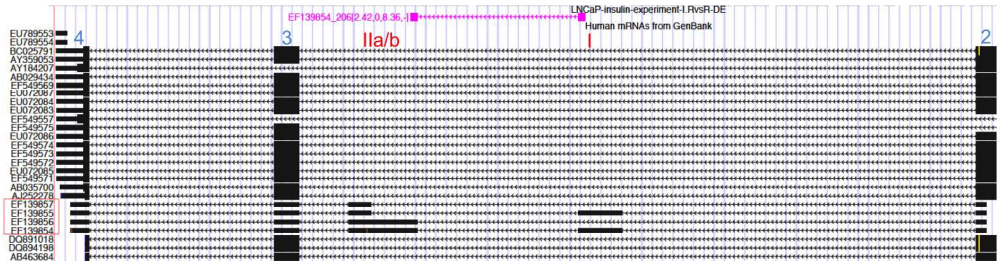

Figure 1 Image depicting mRNA transcript architecture in the GHRL locus was obtained through the UCSC Genome Browser. Microarray analysis detected a 3.9-fold increase in the expression of novel exons (indicated in pink) in intron 2 of the ghrelin gene (GHRL). Wild-type-preproghrelin coding exons 2 to 4 are indicated in blue font, while the cryptic exons are indicated in red font. GenBank entries corresponding to the novel cryptic exons are indicated by a red box.
1057x269mm (72 x 72 DPI)

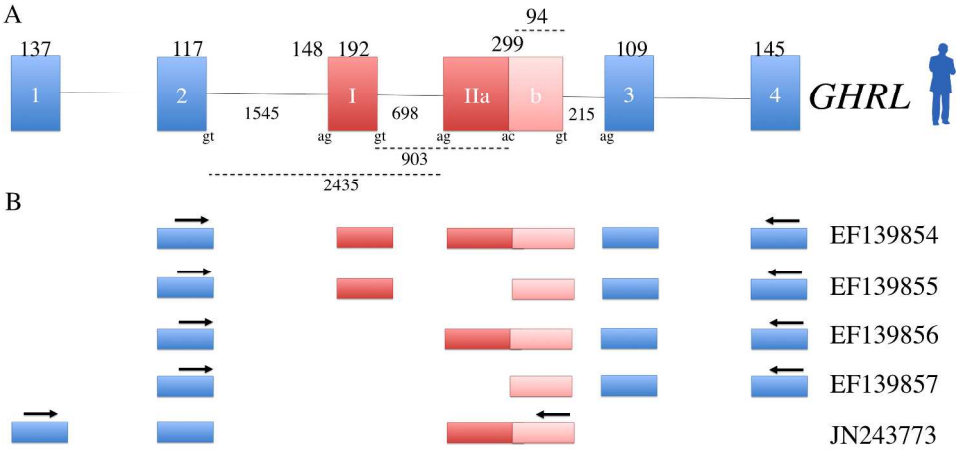

Figure 2(A) Overview of the ghrelin locus showing the ghrelin gene, GHRL, with wild-type preproghrelin-coding exons in blue and the cryptic exons in intron 2 as red boxes (IIa, and the 5′ truncated exon IIb). Exons are represented as boxes and introns as horizontal lines. (B) Mapping of the novel cryptic exon ghrelin transcripts. The exon structure of amplicons is derived from the human testis and LNCaP cells. Exons are represented as boxes, and RT-PCR primers are indicated as arrows above exons.
1057x472mm (72 x 72 DPI)

```
Wildtype-preproghrelin    1 MPSPGTVCSLLLLGMLWLDLAMAGSSFLSPEHQRVQQRKESKKPPAKLQPR
In2c-preproghrelin        1 MPSPGTVCSLLLLGMLWLDLAMAGSSFLSPEHQRVQQRKESKKPPAKLQPR

Wildtype-preproghrelin   52 ALAGWLRPEDGGQAEGAEDELEVRFNAPFDVGIKLSGVQYQQHSQALGKF
In2c-preproghrelin       52 ALAGWLRPEDGGQAEGAEDELEVRASKKGLCI------------------

Wildtype-preproghrelin  102 LQDILWEEAKEAPADK
In2c-preproghrelin          ----------------
```

Figure 3 Multiple sequence alignment of wild-type preproghrelin and putative In2c-preproghrelin. Secretion signal peptides are shown in purple, ghrelin in blue, obestatin in red, and C-ghrelin is highlighted in grey.
1057x249mm (72 x 72 DPI)

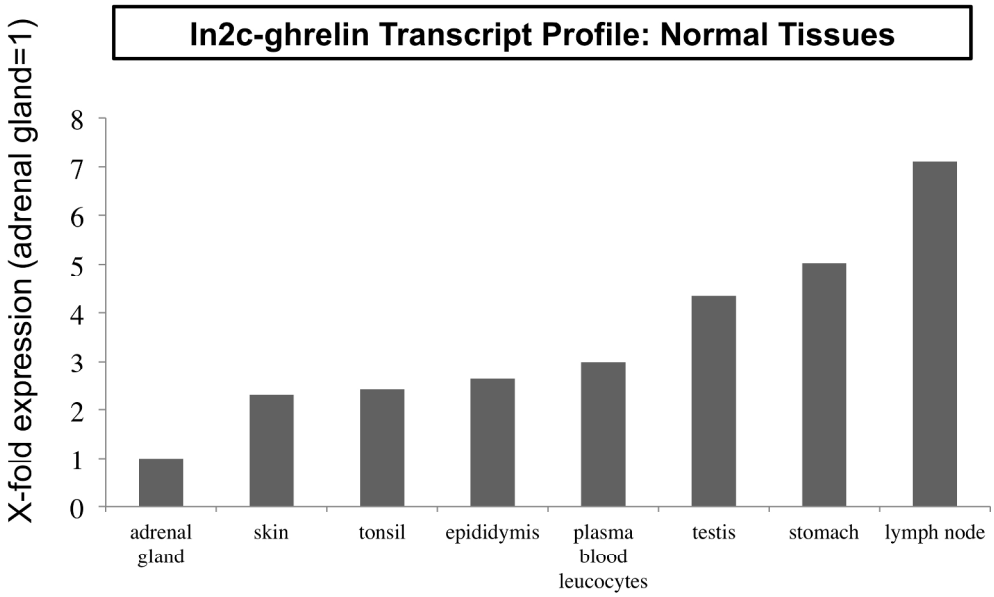

Figure 4 Relative expression of in2c-ghrelin in human tissues by quantitative real-time RT–PCR. Data were normalised to β-actin and are represented as fold changes relative to the expression in the adrenal gland (1.0).
1057x688mm (72 x 72 DPI)

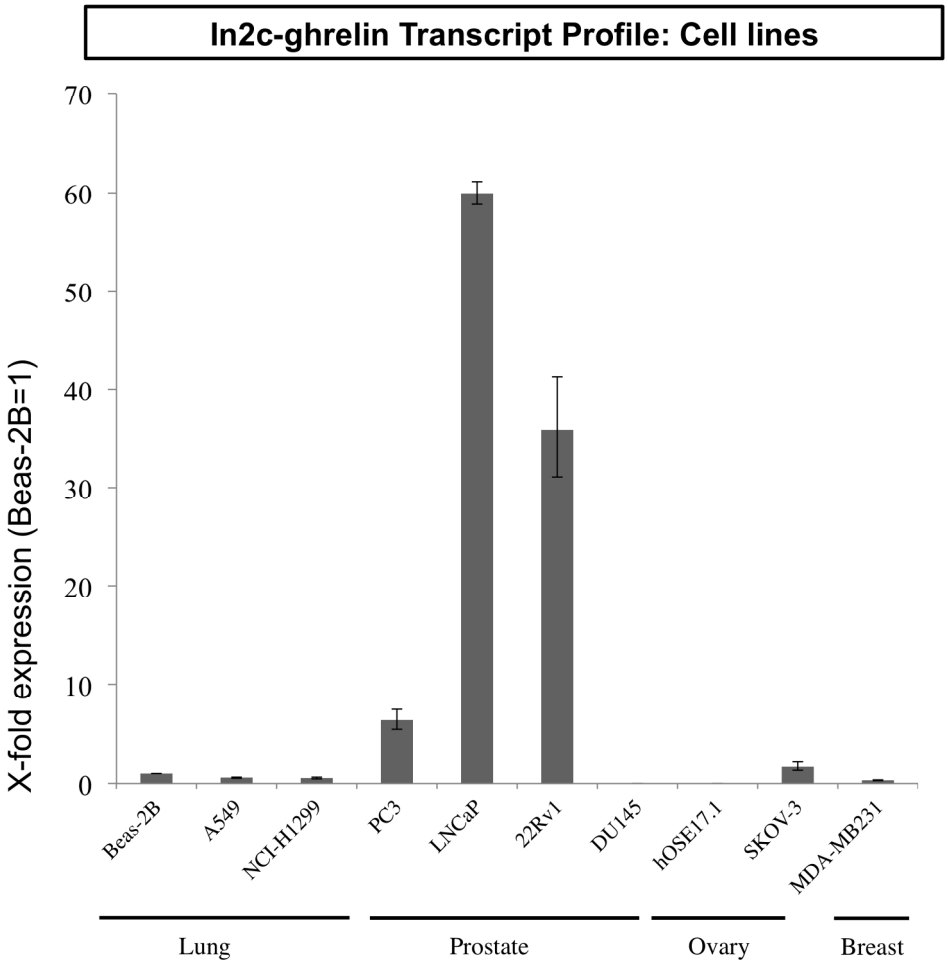

Figure 5 Relative expression of in2c-ghrelin in human cell lines using quantitative real-time RT-PCR. Data is represented as means and standard error of mean from two technical replicates of two independent replicate experiments (n=2). The housekeeping gene, 18S ribosomal RNA, was used as a reference for normalisation. Data are represented as fold changes relative to expression of transcripts in the Beas-2B normal lung cell line (set at 1).
793x793mm (72 x 72 DPI)

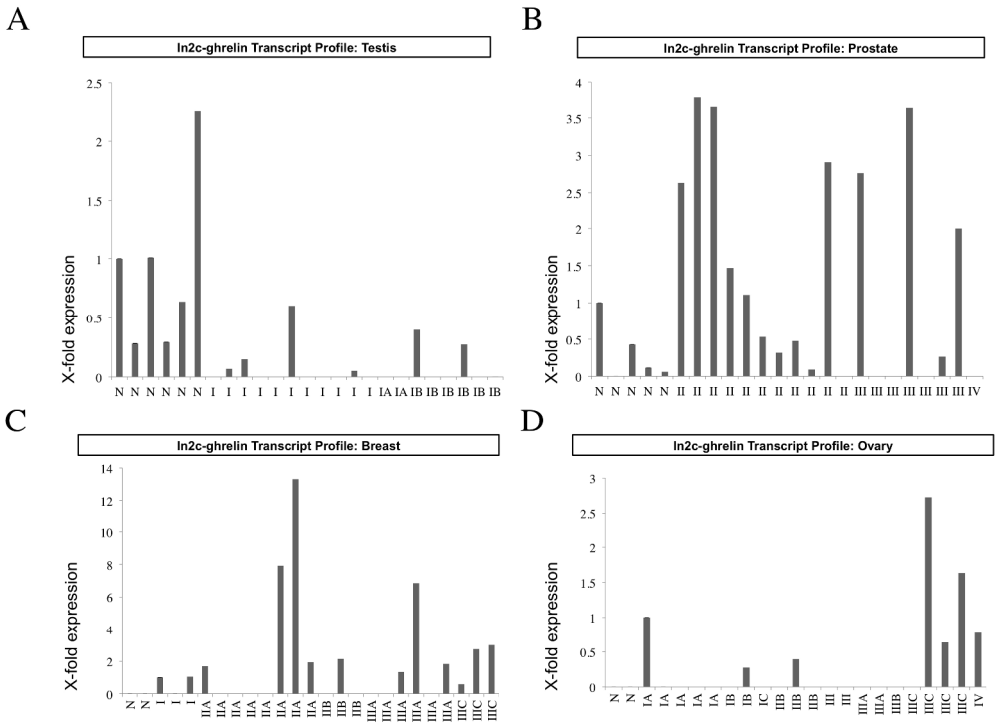

Figure 6 Relative expression of in2c-ghrelin in cancer determined using real-time quantitative RT-PCR. The labels in X-axis indicate the clinical status of the subjects (N denotes normal, Roman numerals indicate disease stages). Data represented as fold changes relative to the expression in a normal tissue sample (set at 1.0), or in the absence of expression in a normal tissue a tumour sample. (A) Testis (B) Prostate (C) Breast (D) Ovary.
1057x767mm (72 x 72 DPI)

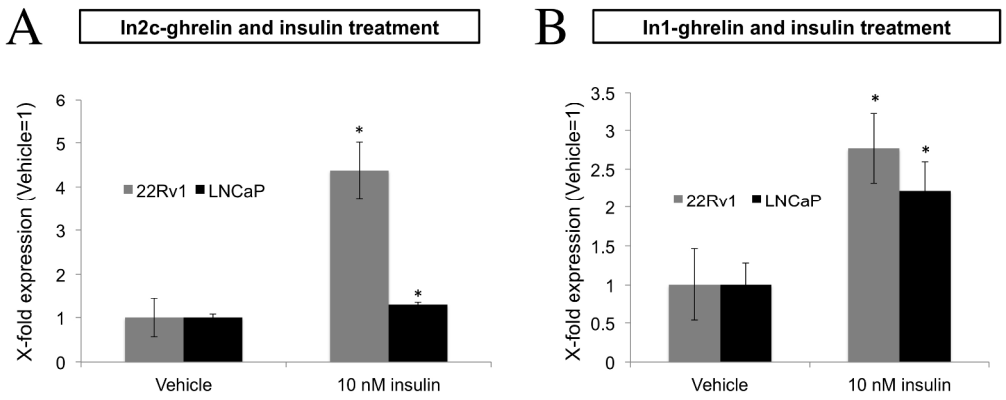

Figure 7 Real-time quantitative RT-PCR analysis of (A) in2-ghrelin and (B) in1-ghrelin gene expression in 22Rv1 and LNCaP prostate cancer cell lines in response to treatment with 10nM insulin over 48 hours. The housekeeping gene, Ribosomal protein L32, was used as a reference for normalisation. Data is represented as means and standard error of the mean with three technical replicates of three independent replicate experiments (n=3). *P<0.05 = significantly different from the vehicle-treated control (set at 1-fold) using Student's t test.
1057x413mm (72 x 72 DPI)

Table 1. Designations and sequences of RT-PCR primers. Annealing temperatures (T_a) of oligonucleotide primers employed in RT-PCR and the expected product sizes are shown.

Primer name	**Primer sequence**	**T_a (°C)**	**Expected size (bp)**
In2c-ghrelin-ORF-F	CTTCCTGAGCCCTGAACACC	64	-
In2c-ghrelin-ORF-R	TCAGCCTTAGCCTCTAATGGGA		-
In2c-real-F	AGAGGCATGAGAGTGCAAGTTC	64	131
In2c-real-R	GATGTCCTGAAGAAACTTCCCC		
In1-real-F	TCTGGGCTTCAGTCTTCTCC	60	215
In1-real-R	GTTCATCCTCTGCCCCTTCT		
wt-real-F	TCAGGGGTTCAGTACCAGCA	60	158
wt-real-R	CAAGCGAAAAGCCAGATGAC		
RPL32-F	CCCCTTGTGAAGCCCAAGA	60	59
RPL32-R	GACTGGTGCCGGATGAACTT		
18S-F	TTCGGAACTGAGGCCATGAT	60	151
18S-R	CGAACCTCCGACTTTCGTTCT		