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Involvement of the glutamine RF-amide peptide (QRFP) and its cognate receptor GPR103 in prostate cancer

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Abstract
Glutamine RF-amide peptide (QRFP) belongs to the RFamide neuropeptide family, which is involved in a wide spectrum of biological activities, ranging from food intake and cardiovascular functioning to analgesia, aldosterone secretion, locomotor activity and reproduction. Recently, QRFP has been shown to exert its effects by activating the G protein coupled receptor GPR103. QRFP is expressed in the brain and peripherally in the adipose tissue, bladder, colon, testis, parathyroid and thyroid gland, as well as in the prostate gland. Following lung cancer, prostate cancer constitutes the second most frequently diagnosed cancer among men, whilst obesity appears to be a contributing factor for aggressive prostate cancer. In the present study we sought to investigate the role of QRFP in prostate cancer, using two androgen-independent human prostate cancer cells lines (PC3 and DU145) as in vitro experimental models and clinical human prostate cancer samples. Expression of both QRFP and GPR103 at gene and protein level was higher in human prostate cancer tissue samples compared to control and benign prostatic hyperplasia (BHP) samples. Furthermore, in both prostate cancer cell lines used in our study, QRFP treatment induced the phosphorylation of ERK1/2, p38, JNK and Akt. In addition, QRFP also increased cell migration and invasion in these in vitro models, with increased MMP2 expression. Moreover, we demonstrated that the pleiotropic adipokine leptin can increase the expression of QRFP and GPR103 in PC3 prostate cancer cells via a PI3K- and MAPK-dependent mechanism, suggesting a potential new link between adiposity and prostate cancer. Our present findings expand the existing evidence and provide novel insight into the implication of QRFP in prostate cancer.

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Dear Editors

We would like to submit to International Journal of Oncology the research manuscript with title: “Involvement of the glutamine RF-amide peptide (QRFP) and its cognate receptor GPR103 in prostate cancer”.

In this paper we provide novel evidence for the effects of QRFP in two androgen-independent cell lines, and also describe the expression of the peptide and its cognate receptor in clinical samples. This is a UK multicentre study, using an extensive repertoire of molecular, cellular and biochemical techniques and will be of great interest to a wide audience of basic scientists and clinicians.

We would be grateful if you consider this manuscript for publication at your journal.

Yours sincerely,
Dr Emmanouil Karteris
Involvement of the glutamine RF-amide peptide (QRFP) and its cognate receptor GPR103 in prostate cancer

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Abstract

Glutamine RF-amide peptide (QRFP) belongs to the RFamide neuropeptide family, which is involved in a wide spectrum of biological activities, ranging from food intake and cardiovascular functioning to analgesia, aldosterone secretion, locomotor activity and reproduction. Recently, QRFP has been shown to exert its effects by activating the G protein coupled receptor GPR103. QRFP is expressed in the brain and peripherally in the adipose tissue, bladder, colon, testis, parathyroid and thyroid gland, as well as in the prostate gland. Following lung cancer, prostate cancer constitutes the second most frequently diagnosed cancer among men, whilst obesity appears to be a contributing factor for aggressive prostate cancer. In the present study we sought to investigate the role of QRFP in prostate cancer, using two androgen-independent human prostate cancer cell lines (PC3 and DU145) as \textit{in vitro} experimental models and clinical human prostate cancer samples. Expression of both QRFP and GPR103 at gene and protein level was higher in human prostate cancer tissue samples compared to control and benign prostatic hyperplasia (BHP) samples. Furthermore, in both prostate cancer cell lines used in our study, QRFP treatment induced the phosphorylation of ERK1/2, p38, JNK and Akt. In addition, QRFP also increased cell migration and invasion in these \textit{in vitro} models, with increased MMP2 expression. Moreover, we demonstrated that the pleiotropic adipokine leptin can increase the expression of QRFP and GPR103 in PC3 prostate cancer cells via a PI3K- and MAPK-dependent mechanism, suggesting a potential new link between adiposity and prostate cancer. Our present findings expand the existing evidence and provide novel insight into the implication of QRFP in prostate cancer.
**Introduction**

RFamide peptides comprise a family of neuropeptides which are characterized by a common carboxy-terminal motif consisting of an arginine (R) and an amidated phenylalanine (F) (1). Vertebrate RFamides are categorized into five groups, namely the (i) neuropeptide FF (NPFF); (ii) prolactin-releasing peptide (PrRP); (iii) gonadotropin-inhibitory hormone (GnIH); (iv) kisspeptin (also known as metastin); and (v) 26RFa/QRFP group (2-3). The latter was initially identified in the frog brain (4), with the N-extended longest form of the glutamine RF-amide peptide (QRFP) consisting of 43-amino acids, while due to several processing sites of this peptide a 26 (26RFa), 6 (26RFa20-26) and 9 (9RFa) amino acid form can also be produced (4-7). QRFP has been identified as the cognate ligand of the previously identified human orphan G protein-coupled receptor (GPCR) GPR103 (8,9). Of note, GPR103 shares 48% and 47% protein sequence homology with the two orexin receptors, OX1R and OX2R, respectively (8).

In the human brain, the QRFP gene has been found to be almost exclusively expressed in certain hypothalamic areas/nuclei, such as the lateral hypothalamic area (LHA), the ventromedial hypothalamic nucleus (VMH), the arcuate nucleus (Arc) and the paraventricular nucleus (PVN), which are involved in the regulation of the feeding behaviour (5,7). Outside the central nervous system (CNS), in humans QRFP is also expressed in various endocrine glands (e.g. in the testis and the adrenal, thyroid and parathyroid glands), as well as in the prostate gland where its expression is higher compared to the hypothalamus (7,8,10). According to its widespread expression, QRFP appears to be implicated in a number of biological functions/systems, including the regulation of the feeding behaviour (11) and the control of the gonadotropic axis (12,13). Notably, in mice central administration of QRFP has been shown to result in increased arterial blood pressure (BP) and heart rate (HR), as well as increased stress activity levels based on grooming behaviour (6).

In addition, 26RFa has been also shown to be expressed in human prostate cancer and to stimulate the neuroendocrine differentiation and migration of the androgen-independent DU145 prostate cancer cells (14). Overall, prostate cancer is the fourth most common cancer globally, whilst it constitutes a leading cause of death from cancer and the second most frequently diagnosed cancer among men, following only lung cancer (15,16). Indeed, more than 1.1 million new prostate cancer cases were diagnosed worldwide in 2012 (17), whilst the global burden of prostate cancer is expected to keep increasing in the next decades in parallel to the increasingly ageing population (18). It should be also noted that, androgen deprivation/ablation therapy is the mainstay treatment option for advanced prostate cancer,
which is initially effective in slowing the disease progression since androgens stimulate the prostate cancer growth (19). However, prostate cancer often progresses eventually to an androgen-independent state which is characterized by poor prognosis (19).

Given the existing evidence showing that 26RFa and GPR103 are present in prostate carcinomatous foci which exhibit neuroendocrine differentiation (14), in the present study we aimed to further explore the role of both QRFP and GPR103 in prostate carcinogenesis by studying their expression in human prostate cancer samples and using two androgen-independent prostate cancer cells lines (PC3 and DU145) as in vitro experimental models.

**Methods**

**Prostate cancer cell lines cultures**

The human androgen-independent prostate cancer cell lines DU145 and PC3 were purchased from ATCC (Manassas, VA, USA) and were cultured in 75cm$^2$ cell culture flasks, in Hams-F12 and RPMI media, respectively, supplemented with 10% fetal calf serum (FCS) and 5 ml of 100x antibiotic-antimycotic. All flasks were incubated in a humidified incubator at 37°C in 5% CO$_2$, and were routinely passaged at approximately 70-80% confluence (examined by microscopic inspection).

**Prostate tissue samples**

Human prostate tissue samples were obtained from men undergoing various prostate procedures, such as radical retro-pubic prostatectomy (RRP), transurethral prostate resection (TURP) or transrectal ultrasound (TRUS) and prostate biopsy, at the University Hospital Coventry & Warwickshire (UHCW) NHS Trust. The study was approved by the National Research Ethics Committee and the hospital Research & Development department and was conducted according to the principles of good clinical practice and the recommendations of the Declaration of Helsinki. As such, all men recruited into the study provided informed signed consent. All collected prostate tissue samples for this study were immediately snap frozen in liquid nitrogen and stored at -80°C until use. Radical prostatectomy specimens were removed en bloc and were formalin-fixed, paraffin-embedded as per standard hospital practice. Tissue samples were available for use in this study after the hospital pathologist had issued a final pathology report on each specimen for grading/staging purposes.

**Western blot**
Protein samples were prepared by addition of 2x Laemmli buffer, and boiling for 5 min. The proteins were separated by SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes at 100 V for one hour in a transfer buffer containing 20 mM Tris, 150 mM glycine, and 20% methanol. PVDF membranes were blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% BSA for one or two hours, and were incubated with the relevant anti-rabbit primary antibodies overnight at 4 °C. On the following day, these membranes were washed thoroughly four times in 60 min with TBS-0.1% Tween, before incubation with the appropriate secondary antibody for one hour at room temperature. Antibody complexes were visualized using chemiluminescence ECL-Plus, as per the manufacturer’s instructions. The appropriate positive and negative controls were utilised. All densities were measured using a scanning densitometer coupled to scanning software ImageQuant™.

RNA isolation, cDNA synthesis and quantitative RT-PCR

Total RNA was extracted from human prostate cancer tissue samples and cell lines using Qiagen RNeasy plus Mini Kit. RNA samples were then treated by RNase-free DNase to eliminate genomic DNA contamination, and the concentration was quantified. The extracted RNA purity and quantity was measured by a Nano-Drop spectrophotometer. In addition, 1 microgram of total RNA was reverse transcribed into cDNA, by using Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase and random hexamers as primers.

The relative expression of the genes of interest was assessed by quantitative polymerase chain reaction (Q-PCR) on an ABI7500 instrument (Applied Biosystems) using SYBR® Green-PCR reaction mixture (Sigma Aldrich, UK). The primers used in this study were: for QRFP (sense) 5’-AGGCAGGACGAAGCAGTGA-3’; (antisense) 5’-GACCGAAGCGGAAGCTGAAG-3’; for GPR103 (sense) 5’-CCAGTCTACCGCTTGTGTA-3’; (antisense) 5’-GCCAGACCACACCTAGCATT-3’; and for GAPDH (sense) 5’-TGACCACACACTGTCTA-3’; (antisense) 5’-GATGCAGGGATGATGTTC-3’. Negative controls for all the reactions included preparations lacking cDNA or RNA-lacking reverse transcriptase in place of the cDNA. RNAs were assayed from two to three independent biological replicates. RNA levels were expressed as a “relative quantification” using the housekeeping gene GAPDH value. The “Delta Ct method” was employed for comparing relative expression results between treatments in qPCR (20).
**xCELLigence Migration and Invasion Assays**

Real time cell proliferation, migration and invasion experiments were performed using the ‘xCELLigence’ system (ACEA, Biosciences, San Diego, CA, USA), consisting of the Real Time Cell Analyzer Dual Purpose (RTCA-DP) instrument placed in a humidified incubator maintained at 5% CO$_2$ and 37 °C, and CIM (Cell Invasion-and-Migration) plates for cell migration and invasion; according to the manufacturer’s protocol. The RTCA software (Roche) monitors cell proliferation, reporting change in the Cell Index (CI) following the treatment of cells.

**Statistical Analysis**

Results are presented as mean values ± standard deviation (SD), unless stated otherwise. Student’s t-test was used to compare continuous variables between two groups. One-way analysis of variance (ANOVA) was used for comparisons between more than two independent groups. P value of less than 0.05 was considered statistically significant. All statistical analyses were performed using Graph Pad software (La Jolla, CA, USA).

**Results**

**QRFP and GPR103 expression in human prostate cancer clinical samples and cell lines**

QRFP and GPR103 mRNA levels in human prostate tissue samples were determined by RT-PCR which showed that the gene expression for both QRFP and GPR103 was significantly higher in prostate cancer samples (n=5) compared to the samples from benign prostate hyperplasia patients (BPH; n=5) (Figure 1A). Moreover, this statistically significant difference was also detected at the protein level, with both QRFP and GPR103 being significantly upregulated in prostate cancer tissue samples compared to BPH (Figure 1B). Using human prostate tissue lysate as a positive control, we also showed that both the androgen-insensitive prostate cancer cell lines (PC3 and DU145) which we used for *in vitro* experiments in our present study express QRFP and GPR103 at the protein level (Figure 1C).

**QRFP effects on phosphorylation status in prostate cancer cell lines**

To examine the signalling pathways which may be involved in the effects of QRFP on prostate cancer cells, the phosphorylation status of ERK1/2, p38, JNK and Akt was assessed in the two androgen-insensitive prostate cancer cell lines of our study following QRFP treatment. As such, PC3 and DU145 cells were treated with QRFP (100 nM) for up to 60 minutes (0, 5, 15, 30 and 60 min time-points). This QRFP treatment resulted in statistically
significant activation of ERK1/2 in the treated PC3 and DU145 cells at 5 minutes (P<0.01 and P<0.001, respectively) (Figure 2A-B). Significantly increased p38 phosphorylation was also observed at different time-points in these two cell lines. In PC3 cells the significant activation was noted at 30 minutes (P<0.01), as well as at 15 and 60 minutes (P<0.05), compared to basal levels. In DU145 cells, activation of p38 increased significantly at 60 minutes (P<0.01) compared to basal levels (Figure 2C-D). Moreover, maximal JNK phosphorylation occurred at 30-60 minutes in PC3 cells (P<0.05; Figure 2E), whilst in DU145 cells a biphasic response was noted at 5 and 60 minutes (P<0.01 and P<0.05, respectively; Figure 2F). Finally, Akt phosphorylation increased significantly in PC3 cells from 15 to 60 minutes of QRFP treatment (P<0.01; Figure 2G), whilst as similar trend was also noted in the QRFP-treated DU145 cells, with maximal Akt phosphorylation at 30 and 60 minutes (P<0.05 and P<0.01, respectively; Figure 2H).

To demonstrate the specificity of these responses, we have also employed a siRNA approach for the GPR103 receptor, using the PC3 cells and Akt phosphorylation as our experimental paradigm. Following siRNA transfection, the GPR103 mRNA levels significantly decreased in the transfected PC3 cells at increasing concentrations of siRNA, with maximum reduction observed at 10nM siRNA concentration. Moreover, QRFP-induced Akt phosphorylation was significantly decreased in PC3 cells transfected with GPR103 siRNA compared to control PC3 cells (data not shown).

**QRFP effects on cell migration and cell invasion in prostate cancer cell lines**

Following the effects of QRFP treatment on the phosphorylation of key kinases in the two prostate cancer cell lines of our study, we hypothesised that this will further impact on cell migration and invasion. To explore such effects, we utilized the xCELLigence system, and the epidermal growth factor (EGF) 50 ng/ml as a positive control.

Our experiments showed that there was a significant and concentration-dependent increase in cell migration (10 nM; P<0.05, and 100 nM; P<0.01, compared to basal) in QRFP-treated PC3 cells at 8 hours (hr) of treatment (Figure 3A). In DU145 cells statistical significance compared to basal levels was reached only at the highest used QRFP concentration (100 nM; P<0.01; Figure 3C). In both cell lines, only a PI3K inhibitor (LY294002; 10mM) significantly inhibited the effect of the applied QRFP treatment. Use of a MAPK inhibitor (U0126; 10 mM) showed a similar trend, but did not reach statistical significance in either PC3 (Figure 3B) or DU145 (Figure 3D) QRFP-treated cells.
Similarly, 8 hr of QRFP treatment increased significantly the invasion in QRFP-treated PC3 cells (10 nM; P<0.05, and 100 nM; P<0.01, compared to basal; Figure 4A). In DU145 cells a significant effect on invasion was noted only at the highest used QRFP concentration (100nM; P<0.01, compared to basal; Figure 4B).

Since there is strong evidence for the role of matrix metalloproteinases (MMPs) in the remodelling, including angiogenesis, of the extracellular matrix (ECM), in our present study we also tested the hypothesis that MMP2 is involved in the effects on cellular invasion which were noted in our previously described experiments. As such, PC3 and DUP145 cells were incubated with 100nM QRFP for up to 12 hr and MMP2 expression was assessed at regular time-points (1, 2, 4, 6, 10, 12 hr) using Western blotting. In PC3 cells, QRFP treatment significantly induced MMP2 protein expression at 1 to 6 hr (all P values <0.05, compared to basal; Figure 4C). In QRFP-treated DUP145 cells, the highest significant increase was noted at 1 hr (P<0.01), whilst significantly increased levels were also documented at 4 to 10 hr (all P values <0.05, compared to basal; Figure 4D).

**Leptin effects on QRFP and GPR103 gene expression in PC3 prostate cancer cells**

Several studies have shown an association between adipokines with prostate cancer progression. Thus, in our study we also investigated whether QRFP and GPR103 gene expression in PC3 cells is regulated by certain key adipokines, namely leptin, adiponectin, and chemerin. Based on these experiments, only leptin significantly induced the expression of QRFP and GPR103 (Figure 5A and 5C). This leptin-induced effect was time-dependent with significant increase at 12 and 24 hr (data not shown). Using specific inhibitors for PI3K (LY294002) and MAPK (U0126), we were also able to demonstrate that this leptin-induced effect is mediated via PI3K and MAPK pathways (Figure 5B and 5D).

**Discussion**

In our present study we show that QRFP and its cognate receptor GPR103 are expressed in two androgen-independent human prostate cancer cell lines (PC3 and DU145), and that their expression is upregulated in human prostate cancer tissue samples compared to that in samples from benign prostate hyperplasia patients. These findings are in line with the data from Alonzeau *et al.* showing that 26RFa is expressed in human prostate cancer, stimulating the neuroendocrine differentiation and migration of the DU145 cells (14).

Our data also show that QRFP induces phosphorylation of ERK1/2, p38, JNK and Akt in both prostate cancer cell lines used in our study. It is already known that the
mitogen-activated protein kinase family (MAPK) regulates different cellular processes in prostate cancer, and that this signalling pathway is over-expressed in human prostate cancer compared to normal prostate tissue (21,22). Autocrine and paracrine-acting growth factors could be inducing increased expression of the Ras/Raf/MEK/ERK pathway, which has been associated with progressive prostate cancer (21). Moreover, Ras signalling is implicated in cancer cell invasion and metastasis, whilst activation of the EGFR-ERK1/2 pathway promotes the migration and invasion of prostate cancer cells (23). On the other hand, there are data showing that activate ERK dependent apoptosis in prostate cancer cell lines (24). We should also note that, Ras/Raf/MEK/ERK is shown to be expressed at low levels in androgen-independent prostate cancer lines. Hence, while it is plausible that activation of the Ras/Raf/MEK/ERK cascade could contribute to prostate cancer development, further research is required to elucidate these mechanisms in androgen-independent prostate cancer lines which exhibit low expression levels of this cascade (25).

Unlike ERK1/2, p38 exhibits weak activation by mitogens, although it is strongly activated in response to various stressors, including inflammatory cytokines, UV radiation, and osmotic and heat shock (26). Interestingly, TNFα (a pro-inflammatory cytokine which is known to activate the MAPK stress response) induces apoptosis in the androgen-dependent LNCaP prostatic cancer cell line, but not in the androgen-independent PC3 cells, while p38 appears to protect against this TNFα-induced apoptosis in LNCaP cells (27). Of note, activation of p38 in prostate cancer may be a result of up-regulated upstream kinases (MKK3/6) combined with down-regulated MAPK phosphatases (28-30). Furthermore, p38 phosphorylation has been demonstrated in prostate cancer cell lines exposed to toxic agents, and this activation is implicated in apoptosis (31,32). Moreover, it has been shown that prostate cancer cell invasion is mediated via the p38 MAPK pathway, leading to phosphorylation of the heat shock protein 27 (HSP27) that in turn regulates MMP2 activation and cell invasion (33). In addition, Chen et al. have shown that stimulation of the G protein-coupled P2Y purinoceptor (metabotropic GPCR family) can induce prostate cancer cell invasion, which is regulated via the activation of the p38 pathway (34). It becomes evident that p38 activation plays a significant role in prostate cancer, and additional research is clearly needed to further explore the exact implications of the QRFP-induced p38 phosphorylation we noted in the two androgen-independent prostatic cancer cell lines in our study.

Moreover, herein we showed that QRFP mediates also the activation of the JNK pathway in androgen-independent prostate cancer cells (PC3 and DU145). It is known that,
JNK is activated by certain growth factors and stressors (e.g. UV radiation) (35). In turn, this JNK activation frequently results in cell death via activation of the mitochondrial apoptotic pathway in various cell types, including in prostate cancer PC3 and DU145 cells where JNK-initiated Fas-mediated apoptotic signals are considered to have a significant role in chemo-sensitivity (35,36). It has also been shown that, depending on the cell type and stimulus, JNK can activate several transcription factors (e.g. c-Jun, c-fos, Elk-1, c-My, ATF-2 and p53), as well as various members of the Bcl-2 family (37,38). Indeed, JNK appears to regulate apoptosis via two distinct mechanisms, namely (i) by promoting the phosphorylation of c-Jun and ATF-2 which results in AP-1 activation and the expression of Fas/FasL signalling pathway-related proteins which further mediates the activation of certain caspases (caspases 8 and 3) that trigger apoptosis; and (ii) by mediating the phosphorylation of the anti-apoptotic proteins Bcl-2/Bcl-xL, thus changing the mitochondrial membrane potential and resulting in the release of cytochrome C and the activation of caspases 9 and 3 to induce apoptosis (39).

In the present study we have also noted a marked QRFP-induced effect in increasing the activation of Akt signalling in both used human prostate cell lines, with a higher degree of phosphorylation in PC3 compared to DU145 cells. The latter finding could be attributed to higher-expression of Akt in the PC3 cells (40). Using siRNA for the cognate GPR103 receptor, we have further demonstrated that the QRFP-induced response is receptor-specific. Notably, the Akt signalling pathway can be activated by several cytokines, growth factors and oncogenes (41), whilst PI3K-Akt phosphorylation may contribute to the induction of tumour invasiveness and cancer development. Indeed, PI3K/Akt pathway activation is more frequently associated with prostate cancer progression toward resistant/metastatic disease (42). As such, it is considered that the PI3K/Akt pathway plays a role in the progression of prostate cancer, with inhibition of the Akt pathway significantly affecting the EGFR-induced prostate cancer cell migration (43).

Overall, the PI3K/Akt/mTOR signalling pathway has been shown to regulate multiple cellular processes, such as cell survival, metabolism, proliferation, migration, and angiogenesis. Accordingly, the ERK and PI3K/Akt pathways are critical for the regulation of cancer cell survival and proliferation. Indeed, in prostate cancer activated ERK and Akt translocate to the nucleus, inducing various downstream effects (e.g. cell proliferation, migration, invasion and angiogenesis) (44). Moreover, activation of ERK1/2 promotes cell migration and invasion in prostate cancer cells (37,45). Interestingly, QRFP significantly induced both migration and invasion in PC3 and DU145 cells. Inhibition of the PI3K/Akt
pathway reduced significantly the effect of QRFP (100nM) on migration in both PC3 and DU145 cells. On the other hand, MAPK/ERK inhibition with U0126 inhibited only partially the effect of QRFP, without reaching significance.

It is also noteworthy that MMPs expression is associated with the migration, invasiveness and metastatic potential of prostate cancer cell lines (46). Recent data have also indicated a connection between the ERK1/2 signalling activation and MMPs (47). Indeed, upregulated MMPs expression has been linked to MAPK (ERK, p38 JNK) and Akt pathways (48). Furthermore, it has been also shown that the p38 MAPK pathway is required for the TGF-β-mediated MMP-2 induction and increased cell invasion in prostate cancer (49). As such, it is plausible that MMP2 activation and the subsequent induction of cell invasion involves the MAPK/PI3K-Akt signalling pathways in accord with the findings of the cell migration and invasion experiments in our present study.

Finally, there are data suggesting that obesity may be associated with higher risk of advanced/aggressive prostate cancer, potentially through the effects of adipose-tissue derived factors/hormones, collectively termed adipokines (e.g. leptin which constitutes the prototype adipokine) (50-53). Indeed, although the available data on leptin and leptin receptor expression in the human prostate and relevant prostate cell lines are contradictory, the existing evidence suggests that this pleiotropic, pro-inflammatory adipokine may exert varying effects on prostate cancer at different stages of its progression (52,54). Of note, it has been shown that in the androgen-resistant PC3 and DU145 prostate cancer cell lines leptin can increase cell growth in a dose-dependent manner and induce ERK1/2 phosphorylation and JNK activation, whereas these leptin-induced effects were less prominent or absent in the androgen-sensitive LNCaP prostate cancer cells (55,56). In the experiments of our study, only leptin, but neither adiponectin nor chemerin, was shown to significantly induce the gene expression of both QRFP and its cognate GP103 receptor in the PC3 androgen-independent prostate cancer cell line. Interestingly, recent data suggest that the leptin-induced stimulatory effects on the proliferative activity of prostate cancer cell lines depend on the expression of the variant 1 isoform of the leptin receptor (LEPR var 1; OB-R) (54). Previous data have also indicated that JNK mediates the leptin-stimulated cell proliferation of androgen-independent prostate cancer cells through STAT3 and Akt (57). In our experiments, we demonstrated that inhibition of both MAPK/ERK and PI3K-Akt pathways significantly abated the leptin-induced effect of on QRFP and GPR103 expression in the androgen-independent PC3 prostate cancer cell line.
Collectively, our present data provide novel insight into the effects of QRFP in human prostate cancer. Our present findings also indicate that the adipokine leptin can modulate the expression of QRFP and GPR103 in an androgen-independent human prostate cancer cell line via a PI3K and a MAPK-dependent mechanism, thus providing a potential link between adiposity and prostate cancer.
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Figure legends

**Figure 1.** Panel A: Glutamine RF-amide peptide (QRFP) and its cognate receptor GPR103 mRNA expression in benign prostatic hyperplasia (BHP) and prostate cancer (malignant) samples (**P<0.001, for n=5 per group, mean ± standard deviation, SD). Panel B: Quantification of western blots showing significant protein expression levels of QRFP (14 kDa) and GPR103 (49 kDa) in human prostate cancer tissue (malignant) compared to BHP (**P<0.01, *P<0.5; n = 5 per group; mean ± SD). Panel C: Western blotting demonstrating that both the androgen-independent human prostate cancer cell lines (PC3 and DU145) used in the *in vitro* experiments of our study express QRFP and GPR103 at protein level. Human prostate tissue lysate was used as a positive control.

**Figure 2.** Representative western blots in androgen-independent PC3 and DU145 prostate cancer cells, demonstrating significantly increased phosphorylation status of ERK1/2 (Panels A-B), p38 (Panels C-D), JNK (Panels E-F), and Akt (Panels G-H). Cells were treated for 5, 15, 30 and 60 minutes (min) with 100nM QRFP.

**Figure 3.** The effect of QRFP treatment on the cell migration of PC3 (Panel A) and DU145 (Panel C) using the xCELLigence system. Both cell lines were treated in a concentration-dependent manner (1, 10 and 100 nM) for 8 hours (hr). Treatment with epidermal growth factor (EGF; 50 ng/ml) was used as positive control. Results were calculated by the xCELLigence Real Time Cell Analyzer (RTCA) software (*P<0.05 and **P<0.01 compared to basal, n= 5; mean ± standard deviation, SD). The effects of QRFP treatment (100nM for 8hr) in the presence or absence of PI3K (PI3Ki: LY294002; 10mM) and MAPK/ERK (UO126; 10mM) inhibitors on migration signalling pathways in PC3 (Panel B) and DU145 cells (Panel D). Data were evaluated using the xCELLigence RTCA software (#P <0.05 compared to basal; *P<0.05 and **P<0.01 compared to QRFP treatment; n= 5; mean ± SD).

**Figure 4.** Effect of QRFP treatment on the cell invasion of PC3 (Panel A) and DUP145 cells (Panel B) using the xCELLigence system. Both cell lines were treated in a concentration-dependent manner (1, 10 and 100 nM) for 8 hours (hr). Treatment with epidermal growth factor (EGF; 50 ng/ml) was used as positive control. Results were calculated by the xCELLigence Real Time Cell Analyzer (RTCA) software (*P<0.05, **P<0.01, n= 5; mean ± standard deviation, SD). The effect of treatment with 100 nM QRFP
at varying time points (1, 2, 4, 6, 10 and 12 hr) on MMP2 protein expression in PC3 (Panel C) and DUP145 prostate cancer cells (Panel D). Data presented as mean ± SD from three independent experiments. *P<0.05, **P<0.01 compared to basal expression. Inserts; representative western blots; GAPDH used as a loading control.

**Figure 5.** Effects on QRFP (Panel A) and GPR103 (Panel C) gene expression in the PC3 prostate cancer cell line following 24 hours (hr) of treatment with leptin (100 nM) or adiponectin (10 nM) or chemerin (1 nM). Data presented as mean ± standard deviation (SD). Only the applied leptin treatment significantly increased QRFP (Panel A) and GPR103 (Panel C) mRNA levels at 24 hours (**P<0.01, compared to basal; experiment performed in triplicate). Effects of PI3K (PI3Ki: LY294002) and MAPK (UO126) inhibitors in the presence or absence of leptin on QRFP (Panel B) and GPR103 (Panel D) gene expression (data presented as mean ± SD; *P<0.05, **P<0.01 compared with basal levels; #P<0.05 and ##P<0.01 compared to leptin treatment; experiment was performed in triplicate).
Figure 1. Expression of QRFP and GPR103
Figure 2. Effect of QRFP on kinase phosphorylation
Figure 3. Effect of QRFP on cell migration
Figure 4. Effect of QRFP on cell invasion
Figure 5. Effect of QRFP on adipokine expression