THE ROLES OF THE PREPROGHRELIN-DERIVED PEPTIDES - GHRELIN, DESACYL GHRELIN AND OBESTATIN - IN PROSTATE CANCER

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KEYWORDS

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ABSTRACT

Prostate cancer is the second most common cause of cancer related deaths in Western men. Despite the significant improvements in current treatment techniques, there is no cure for advanced metastatic, castrate-resistant disease. Early detection and prevention of progression to a castrate-resistant state may provide new strategies to improve survival. A number of growth factors have been shown to act in an autocrine/paracrine manner to modulate prostate cancer tumour growth. Our laboratory has previously shown that ghrelin and its receptors (the functional GHS-R1a and the non-functional GHS-R1b) are expressed in prostate cancer specimens and cell lines. We have shown that ghrelin increases cell proliferation in the PC3 and LNCaP prostate cancer cell lines through activation of ERK1/2, suggesting that ghrelin could regulate prostate cancer cell growth and play a role in the progression of the disease. Ghrelin is a 28 amino-acid peptide hormone, identified to be the natural ligand of the growth hormone secretagogue receptor (GHS-R1a). It is well characterised as a growth hormone releasing and as an orexigenic peptide that stimulates appetite and feeding and regulates energy expenditure and bodyweight. In addition to its orexigenic properties, ghrelin has been shown to play a regulatory role in a number of systems, including the reproductive, immune and cardiovascular systems and may play a role in a number of pathological conditions such as chronic heart failure, anorexia, cachexia, obesity, diabetes and cancer. In cancer, ghrelin and its receptor are expressed in a range of tumours and cancer cell lines and ghrelin has been demonstrated to modulate cell proliferation, apoptosis, migration and invasion in some cell types.

The ghrelin gene (GHRL) encodes preproghrelin peptide, which is processed to produce three currently known functional peptides – ghrelin, desacyl ghrelin and obestatin. Prohormone convertases (PCs) have been shown to cleave the preproghrelin peptide into two primary products – the 28 amino acid peptide, ghrelin, and the remaining 117 amino acid C-terminal peptide, C-ghrelin. C-ghrelin can then be further processed to produce the 23 amino acid peptide, obestatin. Ghrelin circulates in two different forms – an octanoylated form (known as ghrelin) and a non-octanoylated form, desacyl ghrelin. The unique post-translational addition of
octanoic acid to the serine 3 residue of the propeptide chain to form acylated ghrelin is catalysed by ghrelin O-acyltransferase (GOAT). This modification is necessary for binding of ghrelin to its only known functional receptor, the GHS-R1a. As desacyl ghrelin cannot bind and activate the GHS-R1a, it was initially thought to be an inactive peptide, despite the fact that it circulates at much higher levels than ghrelin. Further research has demonstrated that desacyl ghrelin is biologically active and shares some of the actions of ghrelin, as well as having some opposing and distinct roles. Interestingly, both ghrelin and desacyl ghrelin have been shown to modulate apoptosis, cell differentiation and proliferation in some cell types, and to stimulate cell proliferation through activation of ERK1/2 and PI3K/Akt pathways.

The third known peptide product of the ghrelin preprohormone, obestatin, was initially thought to oppose the actions of ghrelin in appetite regulation and food intake and to mediate its effects through the G protein-coupled receptor 39 (GPR39). Subsequent research failed to reproduce the initial findings, however, and the possible anorexigenic effects of obestatin, as well as the identity of its receptor, remain unclear. Obestatin plays some important physiological roles, including roles in improving memory, the inhibition of thirst and anxiety, increased secretion of pancreatic juice, and regulation of cell proliferation, survival, apoptosis and differentiation. Preliminary studies have also shown that obestatin stimulates cell proliferation in some cell types through activation of ERK1/2, Akt and PKC pathways.

Overall, however, at the commencement of this PhD project, relatively little was known regarding the functions and mechanisms of action of the preproghrelin-derived functional peptides in modulating prostate cancer cell proliferation. The roles of obestatin, and desacyl ghrelin as potential growth factors had not previously been investigated, and the potential expression and regulation of the preproghrelin processing enzymes, GOAT and prohormone convertases was unknown in prostate cancer cell lines. Therefore, the overall objectives of this study were to:

1. investigate the effects of obestatin on cell proliferation and signaling in prostate cancer cell lines
2. compare the effects of desacyl ghrelin and ghrelin on cell proliferation and signaling in prostate cancer cell lines
3. investigate whether prostate cancer cell lines possess the necessary enzymatic machinery to produce ghrelin and desacyl ghrelin and if these peptides can regulate GOAT expression

Our laboratory has previously shown that ghrelin stimulates cell proliferation in the PC3 and LNCaP prostate cancer cell line through activation of the ERK1/2 pathway. In this study it has been demonstrated that treatments with either ghrelin, desacyl ghrelin or obestatin over 72 hours significantly increased cell proliferation in the PC3 prostate cancer cell line but had no significant effect in the RWPE-1 transformed normal prostate cell line. Ghrelin (1000nM) stimulated cell proliferation in the PC3 prostate cancer cell line by 31.66±6.68% (p<0.01) with the WST-1 method, and 13.55±5.68% (p<0.05) with the CyQUANT assay. Desacyl ghrelin (1000nM) increased cell proliferation in PC3 cells by 21.73±2.62% (p<0.01) (WST-1), and 15.46±7.05% (p<0.05) (CyQUANT) above untreated control. Obestatin (1000nM) induced a 28.37±7.47% (p<0.01) (WST-1) and 12.14±7.47% (p<0.05) (CyQUANT) significant increase in cell proliferation in the PC3 prostate cancer cell line. Ghrelin and desacyl ghrelin treatments stimulated Akt and ERK phosphorylation across a range of concentrations (p<0.01). Obestatin treatment significantly stimulated Akt, ERK and PKC phosphorylation (p<0.05). Through the use of specific inhibitors, the MAPK inhibitor U0126 and the Akt1/2 kinase inhibitor, it was demonstrated that ghrelin- and obestatin-induced cell proliferation in the PC3 prostate cancer cell line is mediated through activation of ERK1/2 and Akt pathways. Although desacyl ghrelin significantly stimulated Akt and ERK phosphorylation, U0126 failed to prevent desacyl ghrelin-induced cell proliferation suggesting ghrelin and desacyl ghrelin might act through different mechanisms to increase cell proliferation. Ghrelin and desacyl ghrelin have shown a proliferative effect in osteoblasts, pancreatic β-cells and cardiomyocytes through activation of ERK1/2 and PI3K/Akt pathways. Here it has been shown that ghrelin and its non-acylated form exert the same function and stimulate cell proliferation in the PC3 prostate cancer cell line through activation of the Akt pathway. Ghrelin-induced proliferation was also mediated through activation of the ERK1/2 pathway, however, desacyl ghrelin seems to stimulate cell proliferation in an ERK1/2-independent manner. As desacyl ghrelin does not bind and activate GHSR1a, the only known functional ghrelin receptor, the
finding that both ghrelin and desacyl ghrelin stimulate cell proliferation in the PC3 cell line suggests that these peptides could be acting through the yet unidentified alternative ghrelin receptor in this cell type. Obestatin treatment also stimulated PKC phosphorylation, however, a direct role for this pathway in stimulating cell proliferation could not be proven using available PKC pathway inhibitors, as they caused significant cell death over the extended timeframe of the cell proliferation assays. Obestatin has been shown to stimulate cell proliferation through activation of PKC isoforms in human retinal epithelial cells and in the human gastric cancer cell line KATO-III.

We have demonstrated that all of the prostate-derived cell lines examined (PC3, LNCaP, DU145, 22Rv1, RWPE-1 and RWPE-2) expressed GOAT and at least one of the prohormone convertases, which are known to cleave the proghrelin peptide, PC1/3, PC2 and furin, at the mRNA level. These cells, therefore, are likely to possess the necessary machinery to cleave the preproghrelin protein and to produce the mature ghrelin and desacyl ghrelin peptides. In addition to prohormone convertases, the presence of octanoic acid is essential for acylated ghrelin production. In this study octanoic acid supplementation significantly increased cell proliferation in the PC3 prostate cancer cell line by over 20% compared to untreated controls (p<0.01), but surprisingly, not in the DU145, LNCaP or 22Rv1 prostate cancer cell lines or in the RWPE-1 and RWPE-2 prostate-derived cell lines. In addition, we demonstrated that exogenous ghrelin induced a statistically significant two-fold decrease in GOAT mRNA expression in the PC3 cell line (p<0.05), suggesting that ghrelin could potentially downregulate its own acylation and, therefore, regulate the balance between ghrelin and desacyl ghrelin. This was not observed, however, in the DU145 and LNCaP prostate cancer cell lines. The GOAT-ghrelin system represents a direct link between ingested nutrients and regulation of ghrelin production and the ghrelin/desacyl ghrelin ratio. Regulation of ghrelin acylation is a potentially attractive and desirable tool for the development of better therapies for a number of pathological conditions where ghrelin has been shown to play a key role.

The finding that desacyl ghrelin stimulates cell proliferation in the PC3 prostate cancer cell line, and responds to ghrelin in the same way, suggests that this cell line expresses an alternative ghrelin receptor. Although all the cell lines examined
expressed both GHS-R1a and GHS-R1b mRNA, it remains uncertain whether these cell lines express the unidentified alternative ghrelin receptor. It is possible that the varied responses seen could be due to the expression of different ghrelin receptors in different cell lines. In addition to GOAT, prohormone convertases and octanoic acid availability may regulate the production of different peptides from the ghrelin preprohormone.

The studies presented in this thesis provide significant new information regarding the roles and mechanisms of action of the preproghrelin-derived peptides, ghrelin, desacyl ghrelin and obestatin, in modulating prostate cancer cell line proliferation. A number of key questions remain to be resolved, however, including the identification of the alternative ghrelin/desacyl ghrelin receptor, the identification of the obestatin receptor, a clarification of the signaling mechanisms which mediate cell proliferation in response to obestatin treatment and a better understanding of the regulation at both the gene and post-translational levels of functional peptide generation. Further studies investigating the role of the ghrelin axis using in vivo prostate cancer models may be warranted. Until these issues are determined, the potential for the ghrelin axis, to be recognised as a novel useful target for therapy for cancer or other pathologies will be uncertain.
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<thead>
<tr>
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<th>Definition</th>
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<tbody>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram(s)</td>
</tr>
<tr>
<td>µL</td>
<td>Microlitre(s)</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>µg/mL</td>
<td>Microgram per Milliliter</td>
</tr>
<tr>
<td>α-MSH</td>
<td>α-Melanocyte Stimulating Hormone</td>
</tr>
<tr>
<td>APT1</td>
<td>Acyl-protein Thioesterase 1</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein Kinase B (PKB)</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic Hormone</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>AGRF</td>
<td>Australian Genome Research Facility</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti-related peptide</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>AN</td>
<td>Anorexia Nervosa</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>ARC</td>
<td>Hypothalamic Arcuate Nucleus</td>
</tr>
<tr>
<td>AS</td>
<td>Antisense</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic Acid</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair(s)</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign Prostate Hyperplasia</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAPB</td>
<td>Cancer of the prostate and brain</td>
</tr>
<tr>
<td>CART</td>
<td>Cocaine amphetamine-related transcript</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
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<td>cDNA</td>
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<td>CHF</td>
<td>Chronic heart failure</td>
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<tr>
<td>CNS</td>
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<td>CRF</td>
<td>Corticotropin-releasing factor</td>
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<td>G-force</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GH</td>
<td>Growth Hormone</td>
</tr>
<tr>
<td>GHRL</td>
<td>Ghrelin</td>
</tr>
<tr>
<td>GHRH</td>
<td>Growth-hormone-releasing hormone</td>
</tr>
<tr>
<td>GHRP</td>
<td>Growth Hormone Releasing Peptide</td>
</tr>
<tr>
<td>GHS</td>
<td>Growth Hormone Secretagogue</td>
</tr>
<tr>
<td>GHS-R</td>
<td>Growth Hormone Secretagogue Receptor</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter type 4</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>GOAT</td>
<td>Ghrelin O-Acyl Transferase</td>
</tr>
<tr>
<td>GPR39</td>
<td>G Protein Coupled Receptor 39</td>
</tr>
<tr>
<td>HPC1</td>
<td>Hereditary prostate cancer on chromosome 1</td>
</tr>
<tr>
<td>HPC2</td>
<td>Hereditary prostate cancer on chromosome 2</td>
</tr>
<tr>
<td>HPC20</td>
<td>Hereditary prostate cancer on chromosome 20</td>
</tr>
<tr>
<td>HPCX</td>
<td>Hereditary prostate cancer on chromosome X</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>IL2A</td>
<td>Interleukin 12A</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IRS-1</td>
<td>Insulin receptor substrate 1</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton(s)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>KSFM</td>
<td>Keratinocyte Serum Free Medium</td>
</tr>
<tr>
<td>LysoPC</td>
<td>Lysophosphatidylcholine</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mA</td>
<td>Milliamp(s)</td>
</tr>
<tr>
<td>MAPK 1/2</td>
<td>Mitogen Activated Protein Kinases 1/2</td>
</tr>
<tr>
<td>MBOAT</td>
<td>Membrane-bound O-acyltransferase</td>
</tr>
<tr>
<td>MCFA</td>
<td>Medium-chain Fatty Acid</td>
</tr>
<tr>
<td>MCT</td>
<td>Medium-chain Triacylglycerols</td>
</tr>
<tr>
<td>MDS1</td>
<td>Myelodysplasia Syndrome-1</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre(s)</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram(s)</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometres</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>NST</td>
<td>Nucleus of Solitary Tract</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Phosphate Buffered Saline with Tween</td>
</tr>
<tr>
<td>PC</td>
<td>Prohormone Convertase</td>
</tr>
<tr>
<td>PCaP</td>
<td>Predisposing for Cancer of Prostate Gene</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>POMC</td>
<td>Pro-opiomelanocortin</td>
</tr>
<tr>
<td>PRL</td>
<td>Prolactin</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate Specific Antigen</td>
</tr>
<tr>
<td>PVN</td>
<td>Periventricular Nucleus</td>
</tr>
<tr>
<td>PWS</td>
<td>Prader-Willi Syndrome</td>
</tr>
<tr>
<td>PYY 3-36</td>
<td>Peptide YY 3-36</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>S</td>
<td>Sense</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SLC2A2</td>
<td>Solute Carrier Family 2, Member 2</td>
</tr>
<tr>
<td>SOX2</td>
<td>Sex Determining Region Y-box 2</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris Acetate EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris Buffered Saline with Tween</td>
</tr>
<tr>
<td>TE</td>
<td>Tris EDTA Buffer</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TrypLE</td>
<td>Trypsin-like Enzyme</td>
</tr>
<tr>
<td>U</td>
<td>Unit(s)</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volt(s)</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per Volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per Volume</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WHR</td>
<td>Waist-Hip Ratio</td>
</tr>
</tbody>
</table>
LIST OF PUBLICATIONS AND PRESENTATIONS

Publications


Presentations


Inge Seim, Laura Amorim, Penny Jeffery, Adrian Herington, Lisa Chopin (2011). Expression of ghrelin O-acyltransferase (GOAT) mRNA in prostate cancer tissue and cell lines and differential *in vitro* regulation of GOAT expression by ghrelin and des-ghrelin. *93rd Annual Meeting of the Endocrine Society (USA), Boston, USA.*
STATEMENT OF ORIGINAL AUTHORSHIP

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by any other person except where due reference is made.

Signed: ____________________________

Laura Miranda de Amorim

Date: ____________________________
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"A vontade de Deus nunca irá levá-lo aonde a Graça de Deus não irá protegê-lo."
Obrigada Senhor por ter tornado tudo isso possível!!
Chapter 1

Introduction and Literature Review
1 INTRODUCTION

Prostate cancer remains the most frequently diagnosed non-cutaneous malignancy in Western men (Hsing et al., 2007) and nearly three-quarters of registered cases occur in developed countries (Ferlay et al., 2004). It is a disease of the elderly and incidence rates increase with age, rising from 8.6% in men 45-54 of age, to 28% in men 55-64 years old, and 36.1% in men 65-74 years old (Brawley et al., 2009). The lifetime risk of developing invasive prostate cancer is one in six, and it is the second most lethal tumour among Western men (Hsing et al., 2007). At the early stages treatment options include hormonal therapy, radical prostatectomy, radiation therapy and watchful waiting. Treatment decisions must consider a patient’s life expectancy, quality of life, disease characteristics, potential benefits and negative side effects (Labrie, 2004; Gui-Zhong et al., 2011). During the late stages of the disease, following androgen-deprivation therapy, the disease progresses to an androgen independent or castrate-resistant form, with a poor prognosis and short survival expectancy. Despite improvements in current techniques and advances in the search for new therapies, there are no effective therapeutic options for metastatic, castrate-resistant disease (Steinberg et al., 1990; Narod et al., 1995; Gleave et al., 2003; Thompson et al., 2007; Jenks, 2010; Gui-Zhong et al., 2011). A better understanding of the factors that drive prostate cancer progression and the molecular mechanisms underlying these processes are required in order to identify better prognostic markers and to develop more efficient therapies.

We have previously demonstrated that the hormone, ghrelin, may be a growth factor in prostate cancer and that members of the ghrelin axis may be useful markers for prostate cancer (Jeffery et al., 2002; Yeh et al., 2005). Ghrelin is a 28 amino acid peptide that stimulates growth hormone (GH) release and food intake (Kojima et al., 1999; Wren et al., 2000). It is produced as a preprohormone, preproghrelin, and in addition to ghrelin its processing generates other derived peptides (Kojima et al., 1999; Pemberton et al., 2003; Zhang et al., 2005) that are expressed in a range of normal and malignant tissues (Table 1.3). In this chapter, the cell and molecular biology of prostate cancer is reviewed with particular attention to the ghrelin axis and ghrelin signalling pathways, which are further investigated in this PhD project.
1.1 PROSTATE CANCER

1.1.1 Epidemiology

Prostate cancer is a major health burden throughout the world, although there is a large variation in its incidence. The highest incidence rates are in the United States, Canada, Northern Europe and Australia, with intermediate rates in Western Europe and South American countries and the lowest rates in Asian countries (Hsing et al., 2000c; Baade et al., 2009) (Figure 1.1). One in nine Australian men will develop prostate cancer in their lifetime (Hekal and Ibrahiem, 2010) and according to the Australian Institute of Health and Welfare, prostate cancer incidence is increasing in Australia. Almost 20,000 new cases and over 3,300 deaths were expected in Australia in 2010 (Australian Institute of Health and Welfare and Australasian Association of Cancer Registries, 2008) (Table 1.1).

There has been a gradual increase in the incidence of prostate cancer since the 1960s in many countries (Etzioni et al., 1999; Gann, 2002; Quinn and Babb, 2002; Hsing et al., 2007) and survival from the disease has also improved during this period (Etzioni et al., 1999; Gann, 2002; Quinn and Babb, 2002). Changes in the international pattern of the disease may be largely a result of the increased availability of prostate specific antigen (PSA) testing during the early to mid 1990s, allowing an earlier or increased diagnosis of prostate cancer in asymptomatic or minimally symptomatic men (Etzioni et al., 1999; Gann, 2002; Quinn and Babb, 2002) and the initiation of therapy at more curable stages (Etzioni et al., 1999; Bauer et al., 2000; Crawford et al., 2001; Klein, 2002; Wei et al., 2010).

Among all cancers, prostate cancer has one of the largest differences between incidence and mortality. Although men in the United States and Canada had the highest incidence and mortality rates in the world, their mortality-incidence ratios were among the lowest in developed countries. Sweden and Australia on the other hand, had mortality-incidence ratios that were as high as those for low-risk countries (Hsing et al., 2000c). In Japan and China, where screening is less common, prostate cancers are diagnosed at more advanced stages with much lower survival rates (Prorok et al., 1996). Mortality rates in the United States, Canada and Australia have been decreasing since the early 1990s while mortality rates continue to increase significantly in Japan (Baade et al., 2009).
Figure 1.1 International estimated incidence rates (per 100,000 population) of prostate cancer in 2002 (Baade et al., 2009).

Table 1.1 Prostate cancer incidence and mortality projections in Australia

<table>
<thead>
<tr>
<th>Prostate cancer</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incidence</td>
<td>16,011</td>
<td>16,923</td>
<td>17,835</td>
<td>18,784</td>
<td>19,775</td>
</tr>
<tr>
<td>Mortality</td>
<td>3,028</td>
<td>3,124</td>
<td>3,203</td>
<td>3,283</td>
<td>3,366</td>
</tr>
</tbody>
</table>

Although PSA testing has been shown to increase prostate cancer detection by 81% compared to digital rectal examination alone (Catalona et al., 2000), its use as a screening tool remains debatable due to the lack of sensitivity and specificity for the detection of lethal prostate cancers at an early stage (Sharifi and Kramer, 2007). The commonly accepted clinical PSA threshold for the presence of prostate cancer is 4.0ng/mL in serum and the proposed upper limit has ranged between 2.5ng/mL to 10.0ng/mL (Thompson et al., 2004a; Schroder, 2009; Schroder and Roobol, 2009; Khan et al., 2010). About a third of men with a PSA concentration of 3-10ng/mL have prostate cancer (Thompson et al., 2004a). Interestingly, about 18% of men with a PSA concentration of 1-3ng/mL have intermediate- or high-grade disease (Catalona et al., 1997; Thompson et al., 2004a; Renehan et al., 2010). Lowering the PSA threshold increases the probability of identifying low risk cancer, but at the same time it is hard to define a lower limit at which no cancer is present (Fall et al., 2007; Brawley et al., 2009). Biopsy studies have shown that a substantial proportion of men have histological prostate cancer even though it may not cause symptoms or death (Thompson et al., 2004a; Konety et al., 2005; Delongchamps et al., 2007; Amling et al., 2010; Soto and Sonnenschein, 2010) and with the introduction of PSA testing more cases of this latent prostate cancer are being diagnosed (Etzioni et al., 2002). Some cancers detected by PSA screening are low grade and low volume (Bolenz et al., 2010) and more than one third of these cancers are unlikely to become clinically detectable (Draisma et al., 2009; Hoda et al., 2010).

With the increase in the incidence rates due to PSA screening, the number of patients undergoing treatment with radiation and surgery has dramatically increased (Parekh et al., 2010; Zilli et al., 2010). Accordingly, radiation therapy technology and surgical activity have evolved and the safety of these treatments improved (Wilt, 2000), however, undesirable side effects still occur (Potosky et al., 2004; Penson et al., 2005; Penson et al., 2008). The lack of sensitivity and specificity of PSA screening (Thompson et al., 2004a) has called attention to the over-diagnosis of prostate cancer and consequent unnecessary treatment of what could be indolent tumours with limited potential to cause mortality (Labrie, 2004; Brawley et al., 2009), subjecting patients to unnecessary side effects and psychological and social harm (Brawley et al., 2009) as well as substantial financial burdens for healthcare providers (Fitzpatrick et al., 2009). There is an urgent need for the identification of
better prognostic and diagnostic markers to allow detection of those tumours that need to be treated at a sufficiently early stage where cure is still possible.

1.1.2 Risk factors
Over the past 20 years, growing evidence points toward a complex genetic basis for prostate cancer. Although environmental and dietary risk factors are likely to be involved in the development and progression of the disease, familial aggregation and family-based linkage studies show a potential role for susceptibility genes and polymorphisms (Schaid, 2004).

Although hereditary cases of prostate cancer account for only 5%-10% of all prostate cancers (Klein, 2002), family history is one of the strongest known risk factors for the disease (Gallagher and Fleshner, 1998; Staples et al., 2003). Hereditary prostate cancer has led the search for prostate cancer susceptibility genes and linkage has been reported at a number of loci including: HPC1, for hereditary prostate cancer gene 1 (Smith et al., 1996), HPC2, for hereditary prostate cancer gene 2 (Tavtigian et al., 2001), PCaP, for predisposing for cancer of prostate (Berthon et al., 1998), CAPB, for cancer of prostate and brain (Gibbs et al., 1999), HPC20, located on chromosome 20 (Berry et al., 2000), and HPCX located on chromosome X (Xu et al., 1998). An increased risk of prostate cancer when a brother is affected with prostate cancer, compared with when a father is affected, has been a consistent finding across many studies. This suggests that these cancers could be caused either by X-linked or recessive genetic components (Steinberg et al., 1990; Monroe et al., 1995; Narod et al., 1995; Klein, 2002; Staples et al., 2003).

Prostate cancer has proven to be the most sensitive cancer to hormonal manipulation, and is particularly responsive to androgen deprivation (Labrie, 2004). The human androgen receptor (AR) gene is located on the X chromosome (Brown et al., 1989) and several studies have provided evidence of X-linked susceptibility genes for prostate cancer (Xu et al., 1998). Exon 1 of the AR contains several polymorphic repeats including: CAG (glutamine) repeats, GGN (glycine) repeats, and a GCA (proline) repeat (Edwards et al., 1992), and variations in these regions have been linked to prostate cancer risk. For example, men with shorter CAG repeats have an increased risk of prostate cancer (Ingles et al., 1997; Stanford et al., 1997; Kantoff et
al., 1998; Hsing et al., 2000b; Li et al., 2003). It is also not surprising that common genetic variants in genes that encode key proteins which are involved in androgen biosynthesis and actions are associated with a susceptibility to prostate cancer (Ross et al., 1999; Singh et al., 2000; Makridakis and Reichardt, 2001; Nwosu et al., 2001). Finally, and with particular relevance to this project, Sattler and colleagues reported a novel amplification unit on chromosome 3q25-27 in prostate carcinoma that is associated with progressive tumour growth (Sattler et al., 2000). Several genes (IL12A, MDS1, SLC2A2, SOX2) (Sattler et al., 2000) including ghrelin and its receptor (the growth hormone secretagogue receptor, GHS-R) (McKee et al., 1997b) are located in this unit and, thus may be important in the development of prostate cancer.

To date, however, no single locus has been consistently confirmed as being indicative of prostate cancer risk in studies in different populations. It is suggested that a number of loci may be involved in prostate cancer susceptibility and also that different loci may be involved in different populations (Ostrander and Stanford, 2000; Schaid, 2004). Although a number of rare, highly penetrant loci contribute to the Mendelian inheritance of prostate cancer, some of the familial risk may be due to shared environment and more specifically to common low-penetration genetic variants, which alter the predisposition to prostate cancer (reviewed in Simard et al., 2002).

The identification and mapping of prostate cancer susceptibility genes is likely to continue to be complicated due to genetic heterogeneity. There are a large number of men with sporadic disease in the population, and there seem to be no discernible differences in morbidity or mortality in genetic versus non-genetic cases, with the exception that hereditary cases tend to be diagnosed earlier in life (Hankey et al., 1999; Schaid, 2004).

Prostate cancer is a multifactorial disease. It is characterised by heterogeneous growth patterns that range from a slow-growing cancer with a long latency to very rapidly growing highly metastatic lesions. The disparity in incidence suggests a role for environmental or genetic factors in the progression from latent to clinically significant tumours (Hsing et al., 2000a). Despite the substantial morbidity from
prostate cancer world-wide, age, ethnicity and a family history of prostate cancer are the only well established risk factors (Carter et al., 1992; Gann, 2002).

A few studies have reported higher incidence of prostate cancer in African-Americans compared to European-Americans (Narain et al., 2002; Powell, 2007; Surveillance Epidemiology and End Results Program, 2007). In addition, African-Americans are more likely to be diagnosed at a younger age and have more aggressive forms of the disease (Narain et al., 2002; Powell, 2007). In contrast, low incidence rates are observed in Asian men (Sim and Cheng, 2005; Park et al., 2006). The observation that Chinese- and Japanese-Americans have rates much higher than their counterparts in China and Japan supports a role for environmental rather than genetic factors and suggests a role for diet components and life-style as a risk for the disease (Shimizu et al., 1991; Parkin, 1997; Hsing et al., 2000a). The extent to which ethnic differences are attributable to screening methods, environmental, hormonal and/or genetic factors is unknown.

An environmental factor thought to play a significant role in differential development of prostate cancer is diet. One of the most obvious differential characteristics of the Western diet is a high intake of total calories and fat. Although calorie restriction is a powerful inhibitor of cancer development in a variety of animal tumour models (Kolonel, 2001), epidemiological evidence to date does not suggest that total caloric intake itself plays an important aetiological role (Gann et al., 1994; Brawley and Parnes, 2000; Kolonel, 2001). Given the fact that dietary and behavioural risks are likely to cluster within families, discriminating genetic from non-genetic risks will be particularly challenging (Schaid, 2004). In spite of decades of epidemiological investigation, scientific evidence on the relationship between prostate cancer and some foods and nutrients is still insufficient or inconsistent (Gonzalez and Riboli, 2010). Data are also conflicting on the role of other potential risk factors like smoking (Plaskon et al., 2003; Platz et al., 2004), drinking alcohol (Platz et al., 2004; Schoonen et al., 2005), calcium intake (Chan et al., 1998a; Giovannucci et al., 1998), sexual behaviour (Strickler and Goedert, 2001) and occupational exposures (Alavanja et al., 2003; Rybicki et al., 2006). Previous studies showed no significant link between physical activity and risk for prostate cancer (Liu et al., 2000; Friedenreich et al., 2004), but recent evidence suggests that higher
physical activity levels may reduce overall mortality among patients with prostate cancer (Kenfield et al., 2011).

Although Asian populations have the lowest prostate cancer incidence rates in the world, numbers are rapidly increasing (Kurahashi et al., 2008; Matsuda and Saika, 2009). Reasons for the rapid increase remain unclear and increased detection through screening alone is unlikely to explain the rising numbers as screening for prostate cancer is relatively uncommon in this population (Hsing et al., 1998). It is possible that increased Westernisation may have contributed to this rapid rise (Tchernof et al., 1997; Rosmond et al., 1998). Westernisation has been linked to the increased prevalence of obesity and changes in societal factors such as the consumption of high fat and energy dense diets, as well as a more sedentary lifestyle (Tchernof et al., 1997; Rosmond et al., 1998; World Health Organisation, 2000). Obesity and its associated disorders are a growing epidemic in all age groups in both developed and developing countries (Kuskowska-Wolk and Bergstrom, 1993; Bennett and Magnus, 1994; Kuczmarski et al., 1994; Sichieri et al., 1994; Popkin et al., 1995; Prentice and Jebb, 1995; Seidell et al., 1995; Seidell and Flegal, 1997; Murphy et al., 2006; Wang et al., 2007a; International Obesity Task Force, 2010) (Figure 1.2).

Figure 1.2 Estimated overweight and obesity prevalence (body mass index ≥25Kg/m2) in males for 2010 (adapted from Ono et al., 2005).
The World Health Organization (WHO) estimates that by 2015 approximately 2.3 billion adults will be overweight and more than 700 million will be obese (World Health Organisation, 2010). Obesity is associated with several hormone related malignancies and various endocrine and metabolic changes (Tchernof et al., 1997; Rosmond et al., 1998; Hsing et al., 2000a), as well as increased risk of chronic diseases like diabetes mellitus, cardiovascular disease, stroke and hypertension, and consequently has a major health and economic cost worldwide (World Health Organisation, 2010).

Historically, obesity – largely measured by Body Mass Index (BMI – weight in kilograms divided by the square of the height in meters) – has been thought to be an imbalance between energy intake and energy expenditure. More recent research has suggested that obesity is a result of a complex interaction between a set of genetic, metabolic, physiological, dietary and behavioural factors (Jequier and Tappy, 1999; Achike et al., 2010; Dulloo et al., 2010; Kasuga, 2010; Oldham, 2011).

Obesity has been related with poorer prognostic outcomes of prostate cancer and decreased relapse-free survival (Potischman et al., 1996; MacInnis and English, 2006; Spangler et al., 2007). Some groups have reported a positive association between BMI and prostate cancer (Talamini et al., 1986; Moller et al., 1994; Gronberg et al., 1996; Andersson et al., 1997; Cerhan et al., 1997; Habel et al., 2000; Hsing et al., 2000a; MacInnis and English, 2006; Hsing et al., 2007; Freedland et al., 2008), while other groups failed to demonstrate any significant association (Nomura et al., 1985; Hayes et al., 1992; Walker et al., 1992; Chyou et al., 1994; Rohan et al., 1995; Ghadirian et al., 1996; Demark-Wahnefried et al., 1997; IARC, 2002; MacInnis and English, 2006; Skeen et al., 2006; Giovannucci et al., 2007). Other studies showed that when analysed separately by stage or tumour grade, obesity may be more strongly related to a higher risk of higher grade prostate cancer, but not to lower grade (MacInnis and English, 2006; Skeen et al., 2006; Rodriguez et al., 2007; Wright et al., 2007; Lee et al., 2011), suggesting that BMI may be more strongly related to prostate cancer mortality than incidence (Giovannucci et al., 2007; Rodriguez et al., 2007; Wright et al., 2007; El-Eshmawy et al., 2010; Labruna et al., 2011; Garner and Janda, 2011; Wang et al., 2011). A growing body of evidence suggests that abdominal obesity, rather than total body fat, is also a useful
independent predictor of various cardiovascular-related and cancer-related outcomes (Kumanyika et al., 2008). Although several studies failed to find an association between BMI and prostate cancer risk, some groups have shown a relationship between high waist-hip ratio (WHR) with advanced prostate cancer (Giovannucci et al., 1997; Hsing et al., 2000a; Giles and English, 2002; Pischon et al., 2008; Jackson et al., 2010), suggesting that abdominal fat may be a stronger predictor of obesity related health risks than BMI alone.

The association between excess body weight and cancer risk may be explained by alterations in metabolism caused by changes in endogenous hormones and growth factors including insulin, insulin-like growth factors (Allen et al., 2007; Roddam et al., 2008; Gonzalez and Riboli, 2010), sex steroids (Chan et al., 1998b; Bhaskar and Hay, 2007; Osorio-Costa et al., 2009), leptin (Considine et al., 1996; Heimburger et al., 1997) and ghrelin (Pinkney and Kopelman, 2004), each of which can also disturb the normal balance between cell proliferation, differentiation, and apoptosis. However, the pathophysiological and biological mechanisms underpinning these associations are only starting to be understood (Bhaskar and Hay, 2007; Osorio-Costa et al., 2009). Ghrelin is particularly known for potently stimulating appetite, promoting a positive energy balance and increasing body weight (Tschop et al., 2000; Wren et al., 2001; Small and Bloom, 2004; Hameed et al., 2009). It has also been shown to decrease the use of lipids and increase fat storage (Choi et al., 2003; Theander-Carrillo et al., 2006; Davies et al., 2009) and, therefore, the ghrelin axis has a potential role in obesity, although the relationship is highly complex (see Section 1.2.4.3).

1.1.3 Prostate cancer, androgens and growth factors

In an attempt to define the precise cellular and molecular mechanisms that initiate and progress into prostate cancer, many research groups have focused on investigating genetic alterations in tumour suppressor genes and oncogenes. Numerous population studies demonstrated that although cancers are caused by genetic alterations, these alterations alone are not sufficient to produce clinical cancers. While somatic mutations in oncogenes and tumour suppressor genes might occur throughout the whole body, the cancer only arises in specific tissues, suggesting a required tissue environment for the development of the disease (Green
and Evan, 2002; Vineis, 2003). The large variation in prostate cancer incidence in different populations and its high rates in Western societies, have suggested an important role for environmental factors and lifestyle in the development of prostate cancer (see Sections 1.1.1 & 1.1.2). As a result of a life style that favours a positive energy balance, obesity and other metabolic disorders are a growing epidemic (see Section 1.1.2), and many tissue morbidities associated with metabolic disturbance are a consequence of loss of endocrine homeostasis (Holly and Perks, 2008). Although many hormones and growth factors are produced at a primary site or primary endocrine gland, most hormones are widely expressed and can also be locally produced by neoplastic cells (Jeffery et al., 2002; Yeh et al., 2005; Meinbach and Lokeshwar, 2006). In addition, many of the endocrine pathways that regulate normal tissue growth and development can be reactivated by cancerous cells, conferring the necessary growth advantage to promote tumour progression (Byrne et al., 1996; Hellawell and Brewster, 2002).

The normal development, the function of the prostate and prostate homeostasis are governed by androgenic steroid hormones, at all stages (Baskin et al., 1996; Cunha et al., 1996; Hayward et al., 1996; Thomson, 2001; Cunha and Baskin, 2004). At the embryonic stages, the early pulse of androgens is critical for initiating prostate development (Cunha, 1973; Lasnitzki and Mizuno, 1977; Thomson, 2001). By the third trimester of gestation, testosterone production declines and remains low after birth (Wilson et al., 1981; Xia et al., 1990; Thomson, 2001), until a sharp rise in androgens during puberty. This increase in androgens stimulates the growth and maturation of the tracts of epithelial cells into ducts and luminal glands (Donjacour and Cunha, 1988), which differentiate and begin to secrete a range of prostatic proteins (Aumuller and Seitz, 1990). Androgens are not only essential for prostate development and growth, but also for the secretory activity, homeostasis and architecture of the normal adult prostate (Mann, 1974; Montgomery et al., 1992; Thomson, 2001). Prostate cancer is primarily an androgen-dependent disease, and 95% of androgens circulating in the blood stream are testosterone of testicular origin (Lakey et al., 1979; Fowler and Whitmore, 1981). The balance between androgen-induced cell proliferation and apoptosis is thought to regulate the growth of normal and cancerous prostate (Soronen et al., 2004). In the normal prostate, androgen withdrawal results in apoptosis of epithelial cells and de-differentiation of stromal
cells (Sugimura et al., 1986; Isaacs et al., 1992; Hayward et al., 1996), through a process that involves a rapid increase in the transcription of multiple genes that are normally suppressed by testosterone (Montpetit et al., 1986). Similarly, prostate cancer patients treated for localised advanced or metastatic disease with hormone ablation therapy show an initial improvement with tumour reduction in response to low androgen levels. Unfortunately, the disease very often progresses to a castration-resistant phase when it fails to respond to anti-hormonal therapy and tumour growth can be driven by other factors.

Prostate cancer is a multifactorial disease that involves the activation of several pathways that increase and broaden androgen effects by upregulating androgen receptor (AR) expression, enhancing its activation, allowing ligand-independent activation and non-specific binding and activation of the AR, as well as activation of other pathways that bypass the AR via stimulation by other hormones, pro-inflammatory enzymes and various growth factors (Feldman and Feldman, 2001; Debes and Tindall, 2004).

Growth factors can act in an autocrine, paracrine and even intracrine manner to regulate cellular growth, differentiation, migration, apoptosis, angiogenesis and other cellular processes related to cancer progression. Research in the last decades has shown that prostate tissue produces a number of growth factors and hormones and homeostasis of the normal prostate relies on a balance between these stimulatory and inhibitory stimuli. Loss of balance between cell proliferation and cell death in a permissive environment that allows these cells to survive and expand confers the growth advantage necessary for cancer to develop and progress (Holly and Perks, 2008).

Stimulation and inhibition of prostate cancer cell growth is influenced by numerous growth factors, including transforming growth factor β (TGF-β), fibroblast growth factor (FGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), insulin-like growth factors I and II (IGF-I and IGF-II), interleukin-6 (IL-6) and leptin (reviewed in Hellawell and Brewster, 2002; Meinbach and Lokeshwar, 2006).
The hormone, ghrelin, plays a role in obesity, energy balance and metabolism, key factors in prostate cancer progression (see Section 1.1.2), and our laboratory has demonstrated that ghrelin may also be a growth factor in prostate cancer (Jeffery et al., 2002; Jeffery et al., 2005). Further investigation is needed to fully understand the complex roles of the ghrelin axis in cancer progression. The remainder of this thesis will focus on the role that the ghrelin axis could be playing in prostate cancer progression.

1.2 THE GHRELIN/GHRELIN RECEPTOR (GHS-R) AXIS

Ghrelin is a 28 amino acid peptide hormone, first isolated from the stomach as the endogenous ligand of the previously described Growth Hormone Secretagogue receptor (GHS-R1a) (Kojima et al., 1999). Initial studies generated great interest as it was found to stimulate GH release and food intake (Kojima et al., 1999; Wren et al., 2000). Since its discovery, it has been the focus of intensive research, and a range of other non-endocrine functions have also been described (Broglio et al., 2002a; Broglio et al., 2002b; Korbonits et al., 2004; Leontiou et al., 2007; Kojima and Kangawa, 2010; Ueno et al., 2010). It is widely expressed throughout the body and has been shown to play a role in the regulation of homeostasis in many normal tissues (Wang et al., 2002; Nogueiras et al., 2008; Lim et al., 2010), as well as in some pathological conditions such as chronic heart failure, anorexia, cachexia, obesity, diabetes and cancer (van der Lely et al., 2004; Leontiou et al., 2007; Ueno et al., 2010).

Ghrelin is produced as a preprohormone, preproghrelin, and is encoded by the ghrelin gene (GHRL). It has an important unusual post-translational modification that is necessary for its binding to the GHS-R1a and for some of its functions (Bednarek et al., 2000; Kojima et al., 2001b; Muccioli et al., 2002; Torsello et al., 2002; Broglio et al., 2006; Hosoda et al., 2006). The enzyme that catalyses the octanoylation of ghrelin was recently identified as membrane-bound O-acyltransferase 4 (MBOAT4), a member of the MBOAT family, and it was renamed ghrelin O-acyltransferase (GOAT) (Gutierrez et al., 2008; Yang et al., 2008a). A non-acylated form of ghrelin, desacyl ghrelin, is also biologically active and it is likely to act through an unidentified, alternative ghrelin receptor. The cleavage of the preproghrelin peptide gives rise to a few other molecules (Kojima et al., 1999;
Pemberton et al., 2003; Zhang et al., 2005), which are widely expressed and some have been demonstrated to be functionally active. In this review the components, distribution and functions of the ghrelin/GHS-R axis will be described in more detail.

1.2.1 The preproghrelin gene and peptides derived from preproghrelin

The discovery of ghrelin as the endogenous ligand of the GHS-R was a result of reverse pharmacology and started decades ago with the discovery of synthetic growth hormone secretagogues (GHSs) (Bowers et al., 1977; Bowers et al., 1980; Bowers et al., 1984; Smith et al., 1993; Patchett et al., 1995), which acted through the growth hormone secretagogue receptor (GHS-R) (Howard et al., 1996). Since its discovery, ghrelin has been the focus of extensive and intensive research in a number of biological systems.

Ghrelin is expressed in humans (Kojima et al., 1999) and many other mammalian and non-mammalian species, including the rhesus monkey (Angeloni et al., 2004), rat (Kojima et al., 1999), mouse (Tanaka et al., 2001), cat (Ida et al., 2007), Mongolian gerbil, cow, pig, sheep, dog, goat (Kaiya et al., 2001; Tomasetto et al., 2001; Kaiya et al., 2002; Kaiya et al., 2005; Unniappan and Peter, 2005), frog (Kaiya et al., 2001), goldfish (Unniappan et al., 2002), shark (Kaiya et al., 2003b), catfish (Kaiya et al., 2003a) and chicken (Kaiya et al., 2001; Tomasetto et al., 2001; Kaiya et al., 2002; Kaiya et al., 2005; Unniappan and Peter, 2005). The ghrelin amino acid sequence is well conserved throughout species and, in particular, the seven N-terminal amino acids (GSSFLSP) are identical in all mammals and birds and in most fish species examined (reviewed in Kojima and Kangawa, 2007). Rat and mouse ghrelin are identical and share 82.9% identity with human ghrelin, only differing by two amino acids (Kojima et al., 1999; Hosoda et al., 2003). This structural conservation reflects the fact that the N-terminal region is critical for ghrelin activity.

Ghrelin is a 28 amino acid peptide hormone derived from the ghrelin (GHRL) gene (Kojima et al., 1999). The mature ghrelin molecule is esterified by the recently identified enzyme, ghrelin O-acyltransferase (GOAT), which adds an octanoyl group to the serine 3 residue (Gutierrez et al., 2008; Yang et al., 2008a). This post-translational modification is conserved across species and takes place in the endoplasmic reticulum of ghrelin-producing cells (Yang et al., 2008a), and the
acylated peptide is secreted (Kojima et al., 1999; Hosoda et al., 2000a; Kojima et al., 2001b). The octanoylation increases the hydrophobicity of the ghrelin molecule and is essential to enable ghrelin to bind and activate the GHS-R1a and for some of ghrelin’s functions (Bednarek et al., 2000; Kojima et al., 2001b; Muccioli et al., 2002; Torsello et al., 2002; Broglio et al., 2006; Hosoda et al., 2006). Ghrelin and WNT3a protein are the only known naturally occurring peptides in mammals that have this serine acyl-group as a post-translational modification (Hosoda et al., 2000a; Kojima et al., 2001b; Takada et al., 2006).

In humans, ghrelin-derived molecules differing in the length of their acyl group (including molecules esterified with decanoyl or decenoyl groups) have also been isolated from the stomach, and found in the bloodstream (Hosoda et al., 2003). These acyl-modified ghrelin forms are capable of stimulating GH release in rats to a similar degree to the octanoylated ghrelin (Hosoda et al., 2003). In addition to acylated (octanoylated) ghrelin, a non-acylated form of ghrelin, desacyl ghrelin (or unacylated ghrelin), also exists in significant levels in both stomach and blood (Bednarek et al., 2000; Hosoda et al., 2000a; Kojima et al., 2001b; Banks et al., 2002; Kojima and Kangawa, 2005; Takada et al., 2006; Gutierrez et al., 2008; Yang et al., 2008a). In plasma, desacyl ghrelin circulates at higher levels than acylated ghrelin (with an acyl/desacyl ghrelin ratio of 1:4) (Hosoda et al., 2000a; Broglio et al., 2003b), which indicates its physiological relevance. Desacyl ghrelin does not bind to GHS-R1a (Hosoda et al., 2000a), or displace ghrelin binding to rat hypothalamus or pituitary membranes and is unable to stimulate GH release in vivo in rat and human pituitary cells (Hosoda et al., 2000a; Torsello et al., 2002) and it was, therefore, originally believed to be inactive. More recently, many studies have described functions of desacyl ghrelin through an unidentified alternative ghrelin receptor (Delhanty et al., 2006; Sato et al., 2006; Filigheddu et al., 2007; Granata et al., 2007) demonstrating that this peptide is biologically active.

The human ghrelin gene (GHRL) is located on chromosome 3p25-26 (Wajnrajch et al., 2000), the same chromosome as its receptor, the GHS-R (Smith et al., 2001). The human ghrelin gene was initially described as consisting of four exons (1-4). However, two more exons, (exon -1 and exon 0), and an extended exon1 have been described that encode additional 5’ untranslated sequence (Wajnrajch et al., 2000;
Kanamoto et al., 2004; Seim et al., 2007) (Figure 1.3). The gene is translated into a 117 amino acid sequence as a preprohormone, preproghrelin (Kojima et al., 1999). During preproghrelin processing, a 23 amino acid signal peptide is cleaved, generating a 94 amino acid proghrelin peptide. The prohormone convertase PC1/3 then cleaves proghrelin to produce mature ghrelin (Zhu et al., 2006) and a C-terminal protein with 66 amino acids, named C-ghrelin (Pemberton et al., 2003) (Figure 1.3). Recent studies in different cell lines have shown that PC2 and furin can also cleave proghrelin to generate ghrelin (Takahashi et al., 2009). Further processing of C-ghrelin produces an amidated, 23 amino acid peptide, obestatin (Zhang et al., 2005) and two other potential C-ghrelin derived peptides (Seim et al., 2007) (Figure 1.3). Although obestatin’s role in the regulation of energy balance and food intake remains controversial, many other functions have been described for this peptide (see Section 1.2.6).

In addition to ghrelin, desacyl ghrelin and obestatin, several alternative splice variants derived from the preproghrelin gene have also been described (Seim et al., 2007). To date, however, only des-Gln¹⁴ preproghrelin (Hosoda et al., 2000b; Hosoda et al., 2003) and exon 3-deleted preproghrelin (Jeffery et al., 2004; Yeh et al., 2005) have been shown to be translated into peptides. The splice variant, des-Gln¹⁴ preproghrelin, encodes a 116 amino acid peptide and produces a 27 amino acid, des-Gln¹⁴ ghrelin peptide that is identical to mature ghrelin except for the deletion of the Gln¹⁴ residue (Hosoda et al., 2000b; Hosoda et al., 2003). Des-Gln¹⁴ ghrelin is also modified by the addition of an n-octanoyl group at the serine 3 residue and exerts the same biological activities as ghrelin (Hosoda et al., 2000b; Bedendi et al., 2003). Although des-Gln¹⁴ ghrelin is found in the circulation, it is only present in small amounts in the stomach, indicating that ghrelin is the major physiological peptide released into circulation (Hosoda et al., 2000b; Bedendi et al., 2003). Further processing of the des-Gln¹⁴ proghrelin peptide also generates C-ghrelin and when cleaved, obestatin (Hosoda et al., 2000b; Hosoda et al., 2003). The exon 3-deleted preproghrelin encodes a 91 amino acid peptide (Jeffery et al., 2002), that when processed is cleaved into mature ghrelin and a novel 16 amino acid C-
Figure 1.3 The human preproghrelin gene and protein organisation. The gene comprises 6 exons and is translated into a 117 amino acid precursor, preproghrelin. Further processing of preproghrelin can produce mature ghrelin, desacyl ghrelin, obestatin and other potential C-ghrelin derived peptides (adapted from Seim et al., 2009).
terminal peptide (Jeffery et al., 2004). This transcript lacks the exon 3 sequence coding for obestatin and its expression may regulate the ratio between ghrelin and obestatin (Jeffery et al., 2004).

Although the required amino acid sequence for ghrelin action and activation of GHS-R1a appears to be the first 4-5 amino acids of the amino terminal end of the molecule, including the acylation at serine 3 (Bednarek et al., 2000; Matsumoto et al., 2001a; Matsumoto et al., 2001b), short ghrelin peptides consisting of residues 1-8 neither displace ghrelin binding to sites in the anterior pituitary and hypothalamus, nor stimulate GH release in vivo (Torsello et al., 2002). In addition, a recent study demonstrated that synthetic octanoylated pentapeptides containing only the N-terminal five amino acids of proghrelin (GSSFL) can inhibit GOAT in vitro (Yang et al., 2008b), suggesting that truncated ghrelin molecules might serve as endogenous regulators of GOAT expression and consequently regulate the balance between ghrelin and desacyl ghrelin.

1.2.2 The growth hormone secretagogue receptor (GHS-R) isoforms and tissue distribution

Several decades ago, growth hormone releasing peptides (GHRPs) and growth hormone secretagogues (GHSs), synthetic compounds with growth hormone-releasing properties (Momany et al., 1981; Leontiou et al., 2007), were discovered and demonstrated to stimulate GH release through a different receptor to the growth hormone releasing hormone (GHRH) (Leontiou et al., 2007). This led to the discovery of the orphan growth hormone secretagogue receptor (GHS-R) in 1996 (Howard et al., 1996), which mediates the release of GH by GHSs. The GHS-R is a G-protein coupled receptor that is mainly expressed in the hypothalamus and the pituitary (Howard et al., 1996). Its endogenous ligand was discovered a few years later and due to its growth hormone (GH) releasing properties, was named ghrelin, from the Proto-Indo-European word ghre, which means to grow (Kojima et al., 1999). Ghrelin was first isolated from the stomach in the acid-secreting oxyntic glands of the gastric fundus (Kojima et al., 1999; Date et al., 2000a; Kojima and Kangawa, 2005) and later was found to be expressed in a variety of tissues throughout the body (Korbonits et al., 2001a; Barreiro et al., 2002a; Gnanapavan et al., 2002; Jeffery et al., 2002; Tena-Sempere et al., 2002; Volante et al., 2002a;
The GHSR gene is located on chromosome 3q26.2 and produces two known isoforms, GHS-R1a and GHS-R1b (Howard et al., 1996; McKee et al., 1997a). The GHS-R1a is known to be the functional isoform, with seven transmembrane domains and it is 366 amino acids in length (Howard et al., 1996; McKee et al., 1997a). The GHS-R1b is a C-terminally truncated splice variant of the GHS-R1a, with only five transmembrane domains and 289 amino acids (Howard et al., 1996; McKee et al., 1997a). The GHS-R1b also has a unique 24-amino-acid tail, encoded by an unspliced intronic sequence (Smith et al., 1997). Expression studies in a human embryonic kidney cell line showed different cellular localisation for both GHS-R isoforms, with the GHS-R1a localised to the plasma membrane, whereas the GHS-R1b was also localised in the nucleus (Smith et al., 2005).

The growth hormone secretagogue receptor subtypes are widely distributed in almost all human tissues that express ghrelin (McKee et al., 1997a; Cassoni et al., 2001; Kojima et al., 2001b; Gnanapavan et al., 2002; Bedendi et al., 2003; Broglio et al., 2003b; Jeffery et al., 2003; Kojima and Kangawa, 2005; Camina, 2006; Cruz and Smith, 2008; Soares et al., 2008). Interestingly, the GHS-R1b is even more widely distributed than GHS-R1a (Cassoni et al., 2001; Bedendi et al., 2003; Broglio et al., 2003b), often at higher levels than the GHS-R1a isoform (Gnanapavan et al., 2002), and is also expressed in GHS-R1a-negative tissues (Gnanapavan et al., 2002; Jeffery et al., 2005; De Vriese and Delporte, 2007; Waseem et al., 2008). Interestingly, both receptor isoforms are expressed in a number of cancers, suggesting that these receptors might be involved in the pathogenesis and growth of these tumours (Adams et al., 1998; Skinner et al., 1998; Jeffery et al., 2002; Cassoni et al., 2004; Wang et al., 2007b). However, although the GHS-R1a isoform is expressed in normal breast tissues and breast cancer cell lines (Jeffery et al., 2005), only the GHS-R1b truncated isoform is expressed in breast carcinoma specimens (Jeffery et al., 2005). Furthermore, GHS-R1a expression is significantly reduced in malignant colorectal cells while expression of the GHS-R1b is enhanced (Waseem et al., 2008), suggesting the truncated isoform of the ghrelin receptor could be potentially used as a diagnostic marker for these cancers (Jeffery et al., 2005; Waseem et al., 2008).
Acylated ghrelin acts through the GHS-R1a to stimulate GH release, and to regulate appetite and energy balance (Kojima et al., 1999; Date et al., 2001; Nakazato et al., 2001; Wren et al., 2001; Inui et al., 2004; Schmid et al., 2005; Cummings, 2006; Date et al., 2006), mediate gastric acid secretion (Sibilia et al., 2006b), inhibit pancreatic insulin secretion (Dezaki et al., 2006; Sun et al., 2006), prevent oxidative stress (Suematsu et al., 2005; Kawczynska-Drozdz et al., 2006) and to control both pro- and anti-inflammatory and immune responses (Koo et al., 2001; Dixit et al., 2004; Dixit and Taub, 2005).

Signaling through the GHS-R1a is rather complex and still poorly understood (Muccioli et al., 2007). It has been shown that binding of the GHSs to the GHS-R1a triggers the phospholipase C (PLC) signaling pathway, leading to an increase in inositol phosphate turnover and protein kinase C (PKC) activation, resulting in the release of calcium from intracellular stores (Kojima et al., 2001b). GHS-R1a activation also inhibits K⁺ channels, allowing the entry of calcium through voltage-gated L- and T-type channels (Casanueva and Dieguez, 1999). A few reports have now demonstrated high levels of constitutive activity of the GHS-R1a by measuring inositol phosphate turnover or using a reporter assay for transcriptional activity (Holst et al., 2003; Holst et al., 2004; Wang et al., 2004; Holst et al., 2006; Pantel et al., 2006). Approximately 50% of GHS-R1a activity was seen in the absence of its peptide ligand (Holst et al., 2003) and further activation was seen upon ligand binding (Holst et al., 2003; Holst et al., 2004). Ligand binding to a receptor that displays a high constitutive signaling activity can either up regulate its signaling if it is an agonist, or down regulate if it is an inverse agonist (Nijenhuis et al., 2001; Holst et al., 2006). To date no endogenous inverse agonist has been described for the GHS-R1a (Holst et al., 2003; Holst et al., 2004).

1.2.3 The tissue distribution and function of ghrelin and desacyl ghrelin

Since its discovery, ghrelin has been the focus of numerous studies and a large body of evidence shows a range of physiological functions. Ghrelin is primarily produced in the stomach, in response to hunger and starvation, and acts through several mechanisms as a peripheral signal to the central nervous system (CNS) to stimulate food intake (Kojima et al., 1999; Ariyasu et al., 2001; Korbonits et al., 2001a; Korbonits et al., 2001b; Barreiro et al., 2002a; Barreiro et al., 2002b; Date et al.,
Although ghrelin is distributed in a variety of tissues, the majority of circulating ghrelin originates from the stomach (60-70%) and a smaller proportion originates from the small intestine (up to 30%) (Cowley et al., 2003; Leonetti et al., 2003; Kojima and Kangawa, 2005). This observation is supported by the fact that patients who undergo bariatric surgery exhibit a dramatic reduction in plasma ghrelin levels compared with healthy controls (Leonetti et al., 2003). Although ghrelin levels do not normalise, levels gradually return close to normal with time, suggesting that other sources of ghrelin compensate for the loss of the stomach as a major ghrelin producing organ (Sakata et al., 2002; Ariyasu et al., 2005). It is likely that the source of ghrelin in these patients is the small intestine, since it is the second major source of ghrelin synthesis and secretion (Sakata et al., 2002; Ariyasu et al., 2005).

Ghrelin is also produced in the central nervous system, and particularly in the hypothalamus, thalamus, and pituitary (Korbonits et al., 2001a; Cowley et al., 2003). It is also produced in a number of peripheral tissues such as the lung (Volante et al., 2002b), heart (Gnanapavan et al., 2002; Iwase et al., 2004), pancreas (Volante et al., 2002a; Wierup et al., 2002), thyroid gland (Papotti et al., 2000; Kanamoto et al., 2001; Barreiro et al., 2002a; Tena-Sempere et al., 2002; Caminos et al., 2003b; Gaytan et al., 2003; Volante et al., 2003; Rindi et al., 2004; Raghay et al., 2006), ovaries and testis (Barreiro et al., 2002a; Tena-Sempere et al., 2002; Caminos et al., 2003b; Gaytan et al., 2003), kidneys (Mori et al., 2000; Papotti et al., 2000; Gnanapavan et al., 2002), placenta (Gualillo et al., 2001) and lymphocytes (Hattori et al., 2001). The fact that ghrelin is secreted into the systemic circulation, but is also produced at peripheral organs suggests that this hormone is acting as an endocrine, paracrine and possibly autocrine and intracrine factor.

1.2.3.1 Desacyl ghrelin and the alternative ghrelin receptor
Desacyl ghrelin, the non-octanoylated form of ghrelin, is unable to bind and activate the GHS-R1a (Hosoda et al., 2000a; Broglio et al., 2003b) and was thought to be biologically inactive (Kojima et al., 1999). Although it lacks endocrine function through the GHS-R1a, desacyl ghrelin has been recently shown to exert a role in the cardiovascular system (Bedendi et al., 2003; Lear et al., 2010), cell proliferation (Delhanty et al., 2007), apoptosis (Cassoni et al., 2001; Granata et al., 2006;
Delhanty et al., 2007; Granata et al., 2007) and adipogenesis (Thompson et al., 2004b; Miegueu et al., 2011). Both ghrelin and desacyl ghrelin were shown to stimulate insulin release in insulinoma INS-1E cells, and while ghrelin’s action was completely abolished by the GHS-R1a inverse agonist D-lys\(^3\)GHRP-6, desacyl ghrelin’s effect persisted, suggesting that desacyl ghrelin can act through a different receptor to stimulate insulin secretion in INS-1E cells (Gauna et al., 2006). In addition, both ghrelin and desacyl ghrelin were shown to recognise common high affinity binding sites on H9c2 cardiomyocytes, a cell type that does not express GHS-R1a (Baldanzi et al., 2002). Ghrelin and desacyl ghrelin have also been shown to stimulate cell proliferation in HIT-T15 pancreatic β-cells (Granata et al., 2007) and in human osteoblast cells (Delhanty et al., 2006) in a GHS-R1a independent manner. Furthermore, acylated ghrelin was demonstrated to up-regulate phosphorylation of the insulin receptor substrate 1 (IRS-1) in HepG2 cells, a human hepatocellular carcinoma cell line that does not express the GHS-R1a (Thielemans et al., 2007). All of these findings suggest that the GHS-R1a is not the only ghrelin receptor, that desacyl ghrelin is biologically active, and that there is at least one yet unidentified receptor, distinct from GHS-R1a, that both ghrelin and desacyl ghrelin can bind to and activate (Cassoni et al., 2001; Baldanzi et al., 2002; Bedendi et al., 2003; Gauna et al., 2006; Granata et al., 2006; Delhanty et al., 2007; Granata et al., 2007; Thielemans et al., 2007; Lear et al., 2010).

### 1.2.3.2 Ghrelin and growth hormone (GH) release

The discovery of ghrelin established the existence of a novel independent pathway for the regulation of GH release. Growth hormone (GH) secretion from the anterior pituitary was initially believed to be primarily controlled via stimulation by growth hormone releasing hormone (GHRH) and inhibition by the hypothalamic hormone, somatostatin. Ghrelin is a potent stimulator of GH release both in rats and humans, but desacyl ghrelin does not stimulate GH release (Kojima et al., 1999; Seoane et al., 2000; Takaya et al., 2000; Arvat et al., 2001). Administration of peripheral ghrelin caused a dose-related increase in GH secretion in rodents (Kojima et al., 1999; Date et al., 2000b; Wren et al., 2000; Asakawa et al., 2001b) and this effect was more pronounced in humans (Smith et al., 1997; Kojima et al., 1999; Arvat et al., 2000; Peino et al., 2000; Seoane et al., 2000; Takaya et al., 2000; Arvat et al., 2001; Ghigo et al., 2001; Hataya et al., 2001). Importantly, unlike GHRH, ghrelin can only
stimulate the release of stored GH, while GHRH can stimulate both the release and synthesis of GH (van der Lely et al., 2004). Ghrelin and GHRH have been shown to act synergistically to stimulate GH secretion (van der Lely et al., 2004) indicating that they act, at least partially, via different mechanisms (Smith et al., 1997; Bluet-Pajot et al., 2001; Ghigo et al., 2001; Tannenbaum and Bowers, 2001).

1.2.3.3 Ghrelin and appetite regulation and energy balance

Ghrelin is the only known orexigenic hormone produced by the gastrointestinal tract (Hameed et al., 2009). Both intra-cerebroventricular and intravenous injection of ghrelin strongly stimulate feeding and increase body weight in rodents (Tschop et al., 2000), independent of its effect on GH secretion (Tschop et al., 2000; Nakazato et al., 2001). In addition, peripheral administration of ghrelin to humans stimulates appetite and increases food intake (Wren et al., 2001; Small and Bloom, 2004). Total ghrelin levels in human plasma increase during fasting and decrease post-prandially, supporting a role for ghrelin as a meal initiator (Cummings et al., 2001; Tschop et al., 2001a; Bellone et al., 2002; Cummings, 2006). Ghrelin levels also rise with voluntary meal initiation, in the absence of time- and food-related cues (Cummings et al., 2004). Although the mechanisms by which nutrients suppress ghrelin secretion are not well understood, some studies point towards a blood-borne signal rather than a gastrointestinal sensing system. Distension of the stomach does not appear to change plasma ghrelin levels (Tschop et al., 2000; Asakawa et al., 2001b; Cummings et al., 2001; Tschop et al., 2001a; Shiiya et al., 2002) and ghrelin levels only decrease after nutrients leave the stomach and are absorbed into the circulation (Williams et al., 2003). The post-prandial suppression of plasma ghrelin levels seems to be proportional to the ingested caloric load (Callahan et al., 2004) and macronutrient composition may represent one regulatory factor. High-fat and high-carbohydrate diets seem to significantly decrease plasma ghrelin levels both in rats and humans (Beck et al., 2002; Lee et al., 2002; Shiiya et al., 2002; Erdmann et al., 2003; Monteleone et al., 2003; Greenman et al., 2004; Heath et al., 2004; Overduin et al., 2005; Bowen et al., 2006; Foster-Schubert et al., 2008). Alterations in ghrelin levels work as a messenger to the brain to determine short-term and long-term body weight regulation (Kojima et al., 1999; Cummings and Shannon, 2003). In normal subjects, ghrelin levels increase after weight loss and decrease after weight gain (Cummings and Shannon, 2003) and changes can also be seen in pathological
conditions such as anorexia nervosa, cachexia and obesity (Otto et al., 2001; Hansen et al., 2002; Soriano-Guillen et al., 2004; Otto et al., 2005; Kotidis et al., 2006; de Luis et al., 2008; Liou et al., 2008; Pardina et al., 2009; Martos-Moreno et al., 2011; Rigamonti et al., 2010).

In humans, ghrelin reaches the brain through the systemic circulation where it crosses the blood-brain barrier through a saturable, bidirectional transport system (Banks et al., 2002) to stimulate appetite-regulating areas such as the hypothalamic arcuate nucleus (ARC), periventricular nucleus (PVN), dorsomedial regions, the central nucleus of amygdala and the nucleus of solitary tract (NTS) (Date et al., 2002a; Olszewski et al., 2003; Mano-Otagiri et al., 2006; Venkova and Greenwood-Van Meerveld, 2008). Desacyl ghrelin can also cross the blood-brain barrier (Banks et al., 2002). Ghrelin is also synthesised locally in the hypothalamus where it exerts paracrine effects (Korbonits and Grossman, 2004). The effects of stomach derived ghrelin on appetite are also mediated by vagal afferent neurones which supply the gut, providing a third mechanism through which ghrelin exerts actions of the central nervous system (Holst and Schwartz, 2004) (Figure 1.4). Ghrelin in the gut binds to GHS-R1a, which is expressed on vagal afferent nerve endings, and signals to the appetite centres via this neuronal route (Asakawa et al., 2001b; Date et al., 2002a; Sakata et al., 2003). The ARC nucleus is the main regulator of appetite and energy balance in the hypothalamus (Kohno et al., 2008). Neurones in the ARC nucleus contain the orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP), and other neurones express the anorexigenic peptides, cocaine amphetamine-related transcript (CART) and αMSH (melanocyte stimulating hormone), a product of pro-opiomelanocortin (POMC). Ghrelin activates arcuate NPY/AgRP neurons and inhibits POMC/CART neurons, leading to an increase in appetite and food intake (Cowley et al., 2003; Chen et al., 2004; Andrews et al., 2008) (Figure 1.4).

The role of desacyl ghrelin in appetite and food intake remains unclear and data are conflicting (Higgins et al., 2007). Intraperitoneal injection of desacyl ghrelin in rodents resulted in a reduction of food intake (Asakawa et al., 2005; Chen et al., 2005). However, in other studies, no change in feeding behaviour was observed (Neary et al., 2006; Toshinai et al., 2006). It has been shown that desacyl ghrelin...
Figure 1.4 Ghrelin’s regulation of energy balance, appetite and homeostasis. Ghrelin signals from the gastrointestinal tract to the hypothalamus to stimulate appetite and food intake, balancing against inhibitory signals to promote a positive energy balance. Ghrelin from the gut binds to GHS-R expressed on vagal afferents to signal to the central nervous system (CNS), via the neuronal route. Ghrelin also reaches the CNS as a circulating, endocrine hormone (via a hormonal route) and is produced in the CNS itself. Ghrelin stimulates orexigenic NPY/AgRP neurones to stimulate appetite and suppresses anorexigenic POMC/CART neurones in the arcuate nucleus (ARC). NPY, neuropeptide Y; AGRP, agouti related peptide; POMC, pro-opiomelanocortin; CART, cocaine and amphetamine-related transcript; PVN, periventricular nucleus; NTS, nucleus tractus solitarius; MCH, melanin-concentrating hormone; CRF, corticotropin-releasing factor; α-MSH, α-melanocyte stimulating hormone; CCK, cholecystokinin; PYY 3-36, peptide YY 3-36 (Holst and Schwartz, 2004).
does not have an effect on hypothalamic AMP-activated protein kinase (AMPK) activity (Kola et al., 2005), supporting a lack of effect of desacyl ghrelin on appetite (Neary et al., 2006). Transgenic mice over-expressing desacyl ghrelin have been reported to show lower body weight and body length when compared with controls (Ariyasu et al., 2005). Further studies are required in order to elucidate the relevance and possible pharmacological potential of this peptide on food intake and body weight.

The promotion of positive energy balance by ghrelin leads to body weight gain and this seems to be primarily a result of increases in fat mass (Tschop et al., 2000; Nakazato et al., 2001). Central administration of ghrelin decreases the use of lipids for the generation of energy (Choi et al., 2003; Theander-Carrillo et al., 2006) and increases fat storage in the white adipose tissue (Choi et al., 2003; Theander-Carrillo et al., 2006), consistent with the ghrelin-induced shift in food preference towards a high-fat diet (Shimbara et al., 2004). Leptin is an anorexigenic hormone produced by adipose tissue and has a direct effect on the same hypothalamic ARC neurons used by ghrelin to mediate its effect on food intake (Zigman and Elmquist, 2003). Accordingly, leptin directly activates POMC and CART neurons and inhibits NPY and AgRP neurons, directly opposing ghrelin’s effect on energy balance and stimulating a decrease in body weight (Friedman and Halaas, 1998; Kojima et al., 1999; Schwartz et al., 2000; Ahima and Osei, 2001; Kojima et al., 2001b; Tschop et al., 2001b; Zigman and Elmquist, 2003). Mice lacking leptin present with diabetes suggesting a possible role for leptin in glucose homeostasis through activation of the ARC neurons (Coleman, 1973).

Ghrelin has a role in the regulation of insulin secretion. In vitro, ghrelin inhibits glucose-stimulated insulin secretion in a dose-dependent manner from cultured pancreatic cells (Egido et al., 2002), isolated pancreatic islets (Colombo et al., 2003; Reimer et al., 2003) and immortalised β-cell lines (Colombo et al., 2003; Wierup et al., 2004). The effect of ghrelin in glucose regulation and insulin secretion in humans and rodents is controversial, however, as some studies suggest a stimulatory effect on insulin secretion (Ariyasu et al., 2001; Cummings et al., 2001; Adeghate and Ponery, 2002; Date et al., 2002b; Lee et al., 2002; Saad et al., 2002; Broglio et al., 2003c), while others found an inhibitory effect (Broglio et al., 2001; Egido et al., 2002;
Reimer et al., 2003; Akamizu et al., 2004). It is unclear whether ghrelin effects on insulin secretion occur at a physiological level or only at pharmacological doses. Studies in ghrelin or GHS-R knockout mice showed an increase in insulin sensitivity (Sun et al., 2003; Longo et al., 2008). However, another study failed to show any association between ghrelin and insulin sensitivity (Dezaki et al., 2006) while two other studies showed that administration of ghrelin led to impaired glucose tolerance in mice (Reimer et al., 2003; Salehi et al., 2004). This finding was supported in human studies where exogenous ghrelin administration induced insulin resistance in healthy males and post-gastrectomy patients (Damjanovic et al., 2006; Vestergaard et al., 2007).

1.2.3.4 Ghrelin functions in peripheral tissues

The extensive expression of the ghrelin axis in a great number of peripheral tissues partially reflects the wide range of functions of ghrelin (Figure 1.5). In addition to its two major neuroendocrine roles in stimulating GH release and regulating appetite and energy balance (Kojima et al., 1999; Date et al., 2001; Nakazato et al., 2001; Wren et al., 2001; Inui et al., 2004; Schmid et al., 2005; Cummings, 2006; Date et al., 2006), numerous other neuroendocrine effects have been attributed to ghrelin. In addition to stimulating the release of GH from the anterior pituitary, ghrelin also stimulates the secretion of corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and prolactin (PRL) from the anterior pituitary and inhibits gonadotropin-releasing hormone (GnRH) and gonadotropin release (Broglio et al., 2003a; Mozid et al., 2003; Korbonits and Grossman, 2004; van der Lely et al., 2004; Fernandez-Fernandez et al., 2005; Schmid et al., 2005; Leite-Moreira and Soares, 2007; Leontiou et al., 2007; Lorenzi et al., 2009). In addition to these endocrine functions, ghrelin has also been shown to exert diverse physiological effects in many peripheral tissues (Table 1.2) (Figure 5). It has been shown to improve cardiac function (Nagaya et al., 2001c; Bedendi et al., 2003; Tritos et al., 2004; Broglio et al., 2005), stimulate gastric motility and acid secretion (Date et al., 2001; Trudel et al., 2002; Inui et al., 2004; Charoenthongtrakul et al., 2009), regulate lipid metabolism (Tschop et al., 2000; Broglio et al., 2001; Murata et al., 2002; Poykko et al., 2003; Thompson et al., 2004b; Gauna et al., 2005; Dezaki et al., 2006; Heijboer et al., 2006; Sun et al., 2006; Miegueu et al., 2011), increase sleep duration (Weikel et al., 2003; Gluck et al., 2006; Steiger, 2007), enhance memory and
Figure 1.5 Ghrelin’s physiological functions in peripheral tissues throughout the body (De Vriese and Delporte, 2008).
Table 1.2 Ghrelin and desacyl ghrelin physiological roles in normal tissues and cell lines. GH, growth hormone; CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone; PRL, prolactin; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; MCFA, medium-chain fatty acid.

<table>
<thead>
<tr>
<th>Site</th>
<th>Functions</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Pituitary</td>
<td>Stimulates secretion of GH, CRH, ACTH, PRL and inhibits GnRH</td>
<td>(Kojima et al., 1999; Takaya et al., 2000; Mozid et al., 2003; van der Lely et al., 2004; Fernandez-Fernandez et al., 2005; Schmid et al., 2005; Leontiou et al., 2007)</td>
</tr>
<tr>
<td>Stomach</td>
<td>Stimulates gastric motility and acid secretion</td>
<td>(Date et al., 2001; Inui et al., 2004)</td>
</tr>
<tr>
<td>Brain</td>
<td>Stimulates food intake and promotes a positive energy balance</td>
<td>(Tschope et al., 2000; Nakazato et al., 2001; Wren et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>Promotes slow-wave sleep</td>
<td>(Weikel et al., 2003; Steiger, 2007)</td>
</tr>
<tr>
<td></td>
<td>Improves behavioural response to stress</td>
<td>(Asakawa et al., 2001a; Lutter et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Enhances memory and learning</td>
<td>(Carlini et al., 2002; Carlini et al., 2004; Diano et al., 2006)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Some studies reported an inhibitory effect on glucose-stimulated insulin secretion, while some reported a stimulatory effect of ghrelin on insulin secretion</td>
<td>(Egido et al., 2002; Colombo et al., 2003; Reimer et al., 2003; Wierup et al., 2004)</td>
</tr>
<tr>
<td>Heart</td>
<td>Improves cardiac function</td>
<td>(Adeghate and Ponery, 2002; Date et al., 2002b; Lee et al., 2002; Broglio et al., 2003c)</td>
</tr>
<tr>
<td>Adipose Tissue</td>
<td>Increases fat storage and decreases the use of lipids as energy source</td>
<td>(Choi et al., 2003; Theander-Carrillo et al., 2006)</td>
</tr>
<tr>
<td>Lung</td>
<td>Stimulates pulmonary function</td>
<td>(Kodama et al., 2008; Nunes et al., 2008; Schwenge et al., 2008)</td>
</tr>
<tr>
<td>Bone</td>
<td>Stimulates bone formation</td>
<td>(Fukushima et al., 2005; Delhanty et al., 2006; Jurimae et al., 2009)</td>
</tr>
<tr>
<td>Immune system</td>
<td>Regulates immune function and possesses anti-inflammatory properties</td>
<td>(Hattori et al., 2001; Dixit and Taub, 2005; Ershahin et al., 2010; Jacob et al., 2010; Baatet et al., 2011; Cheyuo et al., 2011)</td>
</tr>
<tr>
<td>Reproductive system</td>
<td>Regulates reproductive functions by acting both at central and peripheral levels</td>
<td>(Tena-Sempere, 2005; Lorenzi et al., 2009; Repaci et al., 2011)</td>
</tr>
<tr>
<td>Cell lines</td>
<td>Ghrelin exerts a proliferative effect in cell lines derived from normal pituitary, hippocampus, cardiac, adipose and bone tissues</td>
<td>(Nanzer et al., 2004), Johansson et al., 2008, (Iglesias et al., 2004), (Zwirska-Korczala et al., 2007), (Kim et al., 2005)</td>
</tr>
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Ghrelin and Desacyl Ghrelin

<table>
<thead>
<tr>
<th>Site</th>
<th>Functions</th>
<th>References</th>
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<tbody>
<tr>
<td>Spinal cord</td>
<td>Stimulates neurogenesis</td>
<td>(Sato et al., 2006)</td>
</tr>
<tr>
<td>Bone</td>
<td>Augments proliferation during differentiation</td>
<td>(Delhanty et al., 2006)</td>
</tr>
<tr>
<td>Muscular tissue</td>
<td>Stimulates differentiation and fusion</td>
<td>(Filigheddu et al., 2007)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Modulates cell survival and proliferation</td>
<td>(Granata et al., 2006; Granata et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Desacyl ghrelin inhibits ghrelin-stimulated insulin secretion</td>
<td>(Qader et al., 2008)</td>
</tr>
<tr>
<td>Heart</td>
<td>Inhibits cell death</td>
<td>(Baldanzi et al., 2002; Lear et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>Desacyl ghrelin increases MCFAs uptake, while ghrelin has no effect</td>
<td>(Lear et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>Ghrelin inhibits insulin-induced glucose uptake, while desacyl ghrelin has no effect</td>
<td>(Lear et al., 2010)</td>
</tr>
<tr>
<td>Adipose Tissue</td>
<td>Increases adipogenesis and reduce lipolysis</td>
<td>(Muccioli et al., 2004; Thompson et al., 2004b)</td>
</tr>
<tr>
<td>Liver</td>
<td>Desacyl ghrelin inhibits ghrelin-stimulated glucose release</td>
<td>(Gauna et al., 2005)</td>
</tr>
<tr>
<td>Gonads</td>
<td>Inhibits luteinizing hormone(LH) secretion</td>
<td>(Martini et al., 2006)</td>
</tr>
</tbody>
</table>
Learning (Carlini et al., 2002; Carlini et al., 2004; Diano et al., 2006; Carlini et al., 2008), improve behavioural response to stress (Asakawa et al., 2001a; Carlini et al., 2004; Kanelisa et al., 2006; Kristensson et al., 2006; Lutter et al., 2008), stimulate pulmonary and immune functions (Hattori et al., 2001; Dixit and Taub, 2005), stimulate bone formation (Fukushima et al., 2005; Delhanty et al., 2006; Jurimae et al., 2009) and regulate cell proliferation (Cassoni et al., 2001; Baldanzi et al., 2002; Jeffery et al., 2002; Cassoni et al., 2004; Delhanty et al., 2006; Nakahara et al., 2006; Granata et al., 2007; Leite-Moreira and Soares, 2007; Leontiou et al., 2007) and differentiation (Filigheddu et al., 2007; Zhang et al., 2007a; Zhang et al., 2007b; Xu et al., 2008; Kim et al., 2009c; Miegueu et al., 2011) (Table 1.2) (Figure 1.5).

In the past few years, an increasing number of ghrelin responsive tissues and cell lines have been shown to be responsive to desacyl ghrelin (Table 1.2). Both ghrelin and desacyl ghrelin were shown to stimulate neurogenesis of rat foetal spinal cord (Sato et al., 2006), augment osteoblast proliferation during differentiation (Delhanty et al., 2006), induce differentiation and fusion in C2C12 skeletal myoblasts (Filigheddu et al., 2007), modulate cell survival and proliferation in pancreatic β cells and human islet cells (Granata et al., 2006; Granata et al., 2007) and stimulate growth of adrenocortical tumour cells (Delhanty et al., 2007). However, in the murine HL-1 cardiomyocyte cell line ghrelin and desacyl ghrelin seem to have opposing metabolic effects (Lear et al., 2010). Although both forms were equally protective against apoptosis in this cell type, only desacyl ghrelin was able to increase medium-chain fatty acid uptake and increase insulin-induced translocation of glucose transporter-4 from nuclear to cytoplasmic compartments (Lear et al., 2010) (Table 1.2).

1.2.4 Ghrelin in pathological conditions

1.2.4.1 Ghrelin in anorexia nervosa (AN) and cachexia

Ghrelin secretion is upregulated under conditions of negative energy balance and downregulated in the setting of positive energy balance. Constitutively high levels of ghrelin have been shown in lean patients with anorexia nervosa (AN) (Otto et al., 2001; Cuntz et al., 2002; Shiiya et al., 2002; Kojima et al., 2004). Interestingly ghrelin levels return to control levels following weight gain (Kojima et al., 2004).
Anorexia is a state of chronic under nutrition defined as a loss of desire to eat and is a frequent complication and vital contributor to cachexia. Cachexia is an involuntary loss of skeletal muscle and adipose tissue as a result of catabolic activity exceeding anabolic activity. Several chronic disorders including cancer, acquired immune deficiency syndrome (AIDS), congestive heart failure, chronic obstructive pulmonary disease (COPD), sepsis, chronic infectious disease, burns, trauma and starvation can lead to cachexia. Cachexia is associated with poor quality of life, slow recovery from diseases and poor survival, and reversion of the condition remains a therapeutic challenge (Vanhorebeek and Van den Berghe, 2004; Madeddu and Mantovani, 2009). Circulating ghrelin levels are also elevated in patients with cachexia and are inversely correlated with body mass index (BMI) (Nagaya et al., 2001b; Otto et al., 2001; Yoshimoto et al., 2002; Shimizu et al., 2003; Tacke et al., 2003). Increased ghrelin levels in anorexia and cachexia could represent a compensatory mechanism in response to a negative energy balance (Iglesias et al., 2004). Increased ghrelin levels with cachexia and anorexia nervosa could also reflect a ghrelin resistance syndrome similar to the leptin resistance syndrome in obesity (Considine et al., 1996), or the insulin resistance in type 2 diabetes mellitus (T2DM) (Owens et al., 2001). Ghrelin and analogues have been employed in the treatment of these conditions to try to reverse this negative energy balance state (Hanada et al., 2003; Broglio et al., 2004; Hanada et al., 2004; Neary et al., 2004; Miljic et al., 2006; Garcia and Polvino, 2007). Acute administration of ghrelin in cachectic patients led to a significant increase in food intake (Neary et al., 2004), although the short half-life of ghrelin limits its use as a therapeutic agent (Hosoda et al., 2000a; Nagaya et al., 2001a; Nagaya et al., 2001c; Tolle et al., 2002; Hosoda et al., 2003; Akamizu et al., 2004; Gauna et al., 2004; Vestergaard et al., 2007). Accordingly, ghrelin mimetic molecules have been developed and used to increase appetite and lean muscle mass in patients with anorexia and cachexia (Garcia and Polvino, 2007). Further studies are necessary to provide a better understanding of the ghrelin axis in these conditions and long-term application of its components as therapeutic targets.

1.2.4.2 Ghrelin in congestive heart failure
A positive outcome has been seen with ghrelin treatment of patients with congestive heart failure (CHF). CHF is very often a fatal condition where the heart muscles become weak and lack the strength to efficiently pump blood throughout the body.
CHF can lead to cachexia and is associated with increased morbidity and mortality (Murdoch and McMurray, 1999). Chronic ghrelin administration improved left-ventricular dysfunction and attenuated the development of cachexia in a rat model of CHF (Nagaya et al., 2001c; Nagaya et al., 2004). In humans, administration of ghrelin also decreased arterial blood pressure without increasing heart rate and increased cardiac output (Nagaya et al., 2001a). Ghrelin might be used as a potential therapeutic agent in the treatment of CHF and cardiac cachexia (Nagaya and Kangawa, 2006), although more studies are needed to further clarify the details of ghrelin mechanisms of action.

### 1.2.4.3 Ghrelin and obesity

Obesity is a complex disease that results from a chronic imbalance between energy intake and energy expenditure and it has genetic and environmental components. It is associated with the development of several chronic diseases including cardiovascular disease, diabetes mellitus, hypertension and cancer. Circulating ghrelin levels negatively correlate with body mass index (BMI), body fat mass, adipocyte size and plasma insulin levels (Muccioli et al., 2002; Krsek et al., 2003; Tritos et al., 2003). Obese patients have reduced serum ghrelin levels (Ravussin et al., 2001; Tschop et al., 2001b; Muccioli et al., 2002; Shiiya et al., 2002; Bunt et al., 2003; Krsek et al., 2003; Tritos et al., 2003; Helmling et al., 2004; Yildiz et al., 2004; Zorrilla et al., 2006), possibly indicating physiological adaptation to positive energy balance. Circulating ghrelin levels increase with weight loss, supporting this hypothesis (Hansen et al., 2002). One exception to the negative correlation between ghrelin levels and BMI occurs in Prader-Willi syndrome (PWS) patients. PWS is a complex genetic disorder, characterised by mild mental retardation, short stature, muscular hypotonia, obesity and hyperphagia (Nicholls and Knepper, 2001). PWS patients have high ghrelin levels, despite their high BMI (Cummings et al., 2002) and subjects are in a nearly constant state of hunger evidenced by disordered eating behaviours (Goldstone, 2004).

Genetic alterations in the ghrelin or GHS-R genes have been reported and might be associated with polygenic obesity (Kojima et al., 2004; Liu et al., 2007). Patients with the Leu72Met polymorphism in the ghrelin gene are phenotypically obese at an earlier age compared to homozygous Leu72 allele patients (Ukkola et al., 2001). In
addition, the Arg51Gln variant is seen in 6.3% of obese subjects (Ukkola et al., 2001). Moreover, four naturally occurring GHS-R mutations have been reported and seem to affect the constitutive activity of GHS-R1a (Liu et al., 2007).

Supporting the role of the ghrelin pathway in obesity, animal studies with ghrelin- and GHS-R1a-deficient mice showed that the absence of ghrelin or its receptor protects mice against rapid weight gain induced by a high-fat diet associated with decreased adiposity (Yoshimoto et al., 2002; Wortley et al., 2005). In addition, ghrelin immunisation in rats showed decreased feed efficiency and slower weight gain (Zorrilla et al., 2006). In vitro-generated biostable RNA-based compounds (spiegelmers) that specifically bind n-octanoyl ghrelin have been used successfully in experimental models, which aim to inhibit ghrelin-mediated GHS-R activation (Helmling et al., 2004). Given that the regulating mechanisms in the ghrelin axis are still poorly understood, further studies are necessary in order to elucidate the links between ghrelin and the development of obesity.

1.2.4.4 The Ghrelin/Ghrelin receptor (GHS-R) axis in cancer

The extensive expression of ghrelin and its receptors in a range of peripheral tissues helps to explain the wide range of endocrine and non-endocrine functions of ghrelin. The ghrelin axis is expressed not only in normal tissues, but also in many malignant tissues and cancer cell lines (Table 1.3). As ghrelin can be locally produced in a variety of cancer tissues, it could act as an autocrine/paracrine factor to regulate tumour growth (Jeffery et al., 2002). In vitro studies using a variety of cancer models demonstrated that ghrelin modulates processes that are relevant to cancer progression, including cell proliferation (Jeffery et al., 2002; Murata et al., 2002; Volante et al., 2003; Nanzer et al., 2004; Jeffery et al., 2005; Yeh et al., 2005; Cassoni et al., 2006; Fung et al., 2010), apoptosis (Cassoni et al., 2006; Fung et al., 2010), and migration and invasion (Bowers et al., 1984; Dixit et al., 2006) (Table 1.3).
Table 1.3 Ghrelin and the GHS-R expression in cancer tissues and cell lines (adapted from Nikolopoulos et al., 2010).

(+)= present; (-)= absent; ↑= increased; ↓= decreased

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>Ghrelin</th>
<th>GHS-R</th>
<th>Roles</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pituitary adenomas (somatotropinomas, prolactinomas, thyrotropinomas, corticotropinomas)</td>
<td>(+)</td>
<td>(+)</td>
<td>Highest levels of ghrelin mRNA expression in somatotropinomas</td>
<td>(Kim et al., 2001; Korbonits et al., 2001a; Wang et al., 2002)</td>
</tr>
<tr>
<td>GH3, somatomammotroph tumour cell line</td>
<td>(+)</td>
<td>(+)</td>
<td>Ghrelin ↑ cell proliferation</td>
<td>(Caminos et al., 2003a; Nanzer et al., 2004)</td>
</tr>
<tr>
<td>Gastro-entero-pancreatic tract cancer</td>
<td>(+)</td>
<td>(+)</td>
<td>↓Ghrelin levels compared to healthy controls; ↑GHS-R expression in well differentiated tumours; locally produced ghrelin positively correlates with colorectal malignancy and BMI Higher GHS-R expression compared with other pancreatic tumours</td>
<td>(Bowers et al., 1984)</td>
</tr>
<tr>
<td>Gastric carcinoids</td>
<td>(+)</td>
<td>(+)</td>
<td>Presence of ghrelin in well differentiated tumours and negative in poorly differentiated ones; possible marker in Leydig cell</td>
<td>(Gaytan et al., 2004; Barreiro et al., 2002a)</td>
</tr>
<tr>
<td>Gastric and colorectal cancers</td>
<td>(+)</td>
<td>(+)</td>
<td>Presence of ghrelin binding sites in N-PAP and ARO but absence of GHS-R mRNA, suggesting possible alternative ghrelin receptor</td>
<td>(Cassoni et al., 2000; Volante et al., 2003)</td>
</tr>
<tr>
<td>Pancreatic insulinomas</td>
<td>(+)</td>
<td>(+)</td>
<td>Ghrelin ↑ cell proliferation</td>
<td>(Bowers et al., 1984)</td>
</tr>
<tr>
<td>Pancreatic gastrinomas</td>
<td>(+)</td>
<td>(+)</td>
<td>Presence of ghrelin in well differentiated tumours and negative in poorly differentiated ones; possible marker in Leydig cell</td>
<td>(Gaytan et al., 2004; Barreiro et al., 2002a)</td>
</tr>
<tr>
<td>PANC1, MIA PaCa2, poorly differentiated pancreatic cancer cell lines</td>
<td>(+)</td>
<td>(+)</td>
<td>Presence of ghrelin in well differentiated tumours and negative in poorly differentiated ones; possible marker in Leydig cell</td>
<td>(Gaytan et al., 2004; Barreiro et al., 2002a)</td>
</tr>
<tr>
<td>BxPC3, Capan2, well differentiated pancreatic cancer cell lines</td>
<td>(+)</td>
<td>(+)</td>
<td>Presence of ghrelin in well differentiated tumours and negative in poorly differentiated ones; possible marker in Leydig cell</td>
<td>(Gaytan et al., 2004; Barreiro et al., 2002a)</td>
</tr>
<tr>
<td>HepG2, hepatoma cell line</td>
<td>(+)</td>
<td>(+)</td>
<td>Presence of ghrelin in well differentiated tumours and negative in poorly differentiated ones; possible marker in Leydig cell</td>
<td>(Gaytan et al., 2004; Barreiro et al., 2002a)</td>
</tr>
<tr>
<td>Lung carcinomas</td>
<td>(+)</td>
<td>(+)</td>
<td>Presence of ghrelin binding sites in N-PAP and ARO but absence of GHS-R mRNA, suggesting possible alternative ghrelin receptor</td>
<td>(Cassoni et al., 2000; Volante et al., 2003)</td>
</tr>
<tr>
<td>H345, small cell lung carcinoma (SCLC) cell line</td>
<td>(+)</td>
<td>(+)</td>
<td>Presence of ghrelin binding sites in N-PAP and ARO but absence of GHS-R mRNA, suggesting possible alternative ghrelin receptor</td>
<td>(Cassoni et al., 2000; Volante et al., 2003)</td>
</tr>
<tr>
<td>Non-small cell lung cancer (NSCLC)</td>
<td>(+)</td>
<td>(+)</td>
<td>Presence of ghrelin binding sites in N-PAP and ARO but absence of GHS-R mRNA, suggesting possible alternative ghrelin receptor</td>
<td>(Cassoni et al., 2000; Volante et al., 2003)</td>
</tr>
<tr>
<td>Thyroid carcinoma cell lines</td>
<td>(+)</td>
<td>(+)</td>
<td>Presence of ghrelin binding sites in N-PAP and ARO but absence of GHS-R mRNA, suggesting possible alternative ghrelin receptor</td>
<td>(Cassoni et al., 2000; Volante et al., 2003)</td>
</tr>
<tr>
<td>TT, parafollicular carcinoma</td>
<td>(+)</td>
<td>(+)</td>
<td>Presence of ghrelin binding sites in N-PAP and ARO but absence of GHS-R mRNA, suggesting possible alternative ghrelin receptor</td>
<td>(Cassoni et al., 2000; Volante et al., 2003)</td>
</tr>
<tr>
<td>NPA, human papillary</td>
<td>(+)</td>
<td>(+)</td>
<td>Presence of ghrelin binding sites in N-PAP and ARO but absence of GHS-R mRNA, suggesting possible alternative ghrelin receptor</td>
<td>(Cassoni et al., 2000; Volante et al., 2003)</td>
</tr>
<tr>
<td>WRO, ARO, N-PAP, follicular</td>
<td>(+)</td>
<td>(+)</td>
<td>Presence of ghrelin binding sites in N-PAP and ARO but absence of GHS-R mRNA, suggesting possible alternative ghrelin receptor</td>
<td>(Cassoni et al., 2000; Volante et al., 2003)</td>
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Sugita et al., 2000; Gaytan et al., 2005
Barreiro et al., 2002a; Barreiro et al., 2004; Gaytan et al., 2004
<table>
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<tr>
<th>Cell lines</th>
<th>Differentiation</th>
<th>Literature References</th>
</tr>
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<tbody>
<tr>
<td>Breast carcinoma and cell lines</td>
<td>(+) (+) Higher ghrelin expression in well differentiated invasive carcinomas; GHS-R1b expressed in breast carcinoma but not in normal breast tissue</td>
<td>(Cassoni et al., 2001; Jeffery et al., 2005)</td>
</tr>
<tr>
<td>MCF7, T47D, oestrogen-dependent cell lines</td>
<td>(+) (+) Absence of GHS-R1a; Ghrelin ↓ cell proliferation</td>
<td>(Cassoni et al., 2001)</td>
</tr>
<tr>
<td>MDA-MB-231, MDA-MB-435, oestrogen-independent cell lines</td>
<td>(+) (+) Ghrelin ↓ cell proliferation in MDA-MB-231 cells</td>
<td>(Cassoni et al., 2001; Jeffery et al., 2005)</td>
</tr>
<tr>
<td>Prostate Carcinoma</td>
<td>(+) N/A Absence of GHS-R1a and 1b mRNA</td>
<td>(Jeffery et al., 2002; Cassoni et al., 2004)</td>
</tr>
<tr>
<td>PC3, DU145, androgen-independent cell lines</td>
<td>(+) (+) Presence of GHS-R1b but not GHS-R1a</td>
<td>(Cassoni et al., 2004)</td>
</tr>
<tr>
<td>LNCaP, ALVA-41, androgen-dependent cell lines</td>
<td>(+) (+) See Section 1.2.4.4.1</td>
<td>(Jeffery et al., 2002; Cassoni et al., 2004; Yeh et al., 2005)</td>
</tr>
<tr>
<td>Astrocytoma cell lines</td>
<td>(+) (+) Ghrelin ↑ cell motility and invasion; Higher GHS-R expression compared to normal human astrocytes</td>
<td>(Dixit et al., 2006)</td>
</tr>
<tr>
<td>U-118, U-87, CCF-SSTG1, SW1088</td>
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</table>
Ghrelin and its receptor are co-expressed in various pituitary (Korbonits et al., 2001a; Caminos et al., 2003a), gastrointestinal (Papotti et al., 2001; An et al., 2007; D'Onghia et al., 2007; Wang et al., 2007b), lung (Rindi et al., 2002; Ghigo et al., 2005), pancreatic (Korbonits et al., 1998; Volante et al., 2002a), thyroid (Zhang et al., 2006), ovarian (Gaytan et al., 2005), breast (Cassoni et al., 2001; Jeffery et al., 2003), testicular (Gnanapavan et al., 2002; Gaytan et al., 2004) and prostate (Jeffery et al., 2002; Cassoni et al., 2004; Yeh et al., 2005) tumours (Table 1.3). Although the effects of ghrelin on tumour growth vary, it is still not clear whether ghrelin contributes to malignant transformation or has anti-cancer properties. A growing body of evidence supports a role for ghrelin in promoting cancer progression rather than protecting against it.

Ghrelin and desacyl ghrelin have been shown to confer growth advantage to a number of cancer cell lines and this is summarised in Table 1.3. In the GH-producing GH3 pituitary cancer cell line, ghrelin and its unacylated form (desacyl ghrelin), were shown to stimulate cell proliferation via activation of the mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) pathways and through tyrosine phosphatase (Nanzer et al., 2004). A similar proliferative effect was seen in the human hepatocellular carcinoma cell line HepG2 and in pancreatic adenocarcinoma cell lines treated with exogenous ghrelin; however, ghrelin-induced cell proliferation in the HepG2 cell line was mediated through ERK1/2 activation while in a pancreatic cancer cell line it was mediated through the Akt pathway (Duxbury et al., 2003). The HEL human erythroleukemic cell line has been found to secrete ghrelin and desacyl ghrelin into the culture medium (De Vriese et al., 2005). Exogenous ghrelin treatment in HEL cells showed no effect on cell proliferation, while treatment with anti-ghrelin antibodies significantly decreased cell proliferation, suggesting that endogenous ghrelin and desacyl ghrelin could be acting as autocrine factors in the HEL cell line to mediate cell growth (De Vriese et al., 2005). Moreover, the HEL cell line was found to express the GHS-R1b, but not the GHS-R1a, suggesting that ghrelin and desacyl could be modulating cell proliferation through the unidentified alternative receptor, which is distinct from the GHS-R1a (De Vriese et al., 2005). Interestingly, colorectal cancer cells over-express ghrelin and the GHS-R1b compared to normal colonocytes, although both normal and malignant cells expressed the GHS-R1a (Waseem et al., 2008). In these cell types, treatment with the
GHS-R inverse agonist, D-lys$^3$-GHRP-6, significantly decreased cell proliferation suggesting that the over-expression of endogenous ghrelin could be responsible for colorectal cancer cells proliferative and invasive behaviour (Waseem et al., 2008).

While several studies have demonstrated that ghrelin stimulates cell proliferation, it has also been shown to exert anti-proliferative effects in the thyroid carcinoma cell lines ARO and N-PAP (Volante et al., 2003), and the H345 small cell lung carcinoma cell line (Cassoni et al., 2006). In breast cancer cell lines, both stimulatory and inhibitory effects have been reported (Cassoni et al., 2001; Jeffery et al., 2005). Cassoni et al., (2001) showed a significant inhibitory effect in the MCF7, T47D and MDA-MB-231 breast carcinoma cell lines at 1000nM ghrelin, while Jeffery et al., (2005) showed a significant increase in cell proliferation in the MDA-MB-231 and MDA-MB-435 breast carcinoma cell lines at lower ghrelin concentrations (0.1-100nM) but no significant effect at 1000nM ghrelin. Data on the role of ghrelin in prostate cancer cell lines is conflicting (see Section 1.2.4.1).

In addition to ghrelin and desacyl ghrelin, another preproghrelin-derived peptide, obestatin, has also been shown to modulate cell proliferation in some cancer cell lines (Camina et al., 2007a; Pazos et al., 2007; Volante et al., 2009) (see Section 1.2.6).

1.2.4.4.1 The ghrelin axis in prostate cancer

Although ghrelin is mainly produced by the stomach, it is expressed in many normal and malignant tissues where it has been demonstrated to exert a proliferative effect (Jeffery et al., 2002; Yeh et al., 2005; Nogueiras et al., 2006). Ghrelin and its receptors are also expressed in human prostatic carcinomas and benign prostatic hyperplasia (BPH) specimens (Cassoni et al., 2004) and in the ALVA-41, LNCaP, DU145 and PC3 human prostate cancer cell lines (Jeffery et al., 2002; Yeh et al., 2005). Cassoni et al. (2004), however, only detected ghrelin in the PC3 androgen-independent cell line. In vitro studies performed by both groups demonstrated that these cell lines respond to ghrelin treatments (Jeffery et al., 2002; Cassoni et al., 2004; Yeh et al., 2005). Data on ghrelin’s mitogenic effects in prostate cancer cell lines are conflicting. Studies performed by our research group showed that 5 and 10nM ghrelin concentrations stimulate proliferation of the PC3 and LNCaP prostate
cancer cell lines (Jeffery et al., 2002; Yeh et al., 2005). However, another study demonstrated that ghrelin inhibits cell proliferation in the DU145 cell line, had no effect in LNCaP cells, and showed a biphasic effect in PC3 cells, stimulating proliferation at lower concentrations (10 and 100pM) and inhibiting at higher doses (1nM–1µM) (Cassoni et al., 2004). A more recent study found that ghrelin, at concentrations ranging from 10 to 50nM, significantly inhibited cell proliferation in PC3 prostate cancer cells (Diaz-Lezama et al., 2010). Possible reasons for these differences are unclear and could include the different range of ghrelin concentrations applied, as well as different methodological approaches. In addition, different cell lines could respond differently depending on androgen-dependent status and other regulating factors. Despite the conflicting data on the role of ghrelin in prostate cancer, it is clear that ghrelin regulates prostate cancer growth and pathogenesis (Lanfranco et al., 2008). A recent study showed that the binding of a fluorescein-labelled ghrelin analogue was 4.7 times higher to prostate cancer tissues compared to normal prostate tissue and benign prostatic hyperplasia (Lu et al., 2012). This probe may be clinically useful as a molecular probe for the diagnosis of prostate cancer and for distinguishing cancer from benign tissue (Lu et al., 2012).

1.2.5 Ghrelin O-Acyltransferase (GOAT)

The ghrelin/GHS-R axis is one of the most important mechanisms that regulates energy balance and metabolism (Kojima et al., 1999; Date et al., 2001; Wren et al., 2001; Inui et al., 2004; Date et al., 2006). Numerous functions have been described for ghrelin and its unacylated form, desacyl ghrelin, in several peripheral tissues (see Section 1.2.3) (Table 1.2). Ghrelin possesses a unique post-translational modification of its third residue that is required for activating its only known functional receptor, the GHS-R1a (Bednarek et al., 2000; Kojima et al., 2001b; Hosoda et al., 2006). Ghrelin’s octanoylation also is responsible for the hydrophobicity on the N-terminus of the ghrelin amino acid sequence (Banks et al., 2002). Recently, two different groups identified the enzyme responsible for ghrelin octanoylation (Gutierrez et al., 2008; Yang et al., 2008a). Ghrelin-O-acyltransferase (GOAT) is a porcupine-like enzyme that belongs to the membrane-bound O-acyl transferase (MBOAT) family and is also known as MBOAT4 (Gualillo et al., 2008; Yang et al., 2008a). All members of the MBOAT family share a region of high similarity and are able to transfer organic acids, typically long-chain fatty acids, to hydroxyl groups in the
membrane embedded substrates. However, GOAT was the only family member to transfer medium-chain fatty acids (MCFAs) and to acylate ghrelin, catalysing the addition of an octanoyl group to the serine 3 residue of the ghrelin molecule (Gutierrez et al., 2008; Yang et al., 2008a) (Figure 1.6). GOAT was also shown to acylate ghrelin with other fatty acids ranging from acetate (C2) to tetradecanoic acid (C14) (Gutierrez et al., 2008). Although the main bioactive form of acylated ghrelin is modified by n-octanoic acid, GOAT surprisingly showed a strong preference for n-hexanoic acid over n-octanoic acid in vitro (Ohgusu et al., 2009). Furthermore, short peptides containing only the five or four N-terminal amino acids of ghrelin were octanoylated by GOAT, suggesting that it may constitute the core motif for GOAT substrate recognition (Yang et al., 2008b; Ohgusu et al., 2009).

The GOAT-ghrelin system is highly conserved between species (Gutierrez et al., 2008). In human tissues GOAT was initially shown to be predominantly expressed in the stomach, intestine and pancreas (Gutierrez et al., 2008; Yang et al., 2008a). More recent studies showed that, like ghrelin, it is widely expressed and can also be found in lower levels in the breast, kidney, liver, lung, muscle, myocardium, pituitary, thyroid, ovary, testis and prostate (Lim et al., 2011). Interestingly, synthesis and release of acylated ghrelin are two independently regulated and occurring events (Liu et al., 2008; Kirchner et al., 2009; Romero et al., 2010). In addition to GOAT, prohormone convertases 1/3 (PC1/3), PC2 and furin have been shown to be involved in the production of mature acylated ghrelin (Zhu et al., 2006; Takahashi et al., 2009).
Figure 1.6 Diagrammatic illustration of the post-translational processing of the preproghrelin peptide to generate ghrelin’s acylated and unacylated forms. Cleavage of the signal peptide from the preproghrelin protein generates the proghrelin peptide. GOAT can modify the proghrelin peptide by adding an octanoyl group to its third residue (serine) to generate acylated ghrelin. Proghrelin is further processed by prohormone convertases to produce ghrelin and desacyl ghrelin (adapted from Romero et al., 2010).
In vitro studies demonstrated that expression of these enzymes was not sufficient to produce acylated ghrelin, and that the addition of n-octanoic acid to the culture medium was necessary for synthesis of octanoylated ghrelin (Takahashi et al., 2009). Interestingly, ingestion of MCFAs and medium-chain triacylglycerols (MCTs) were shown to increase the concentration of acylated ghrelin without changing total ghrelin levels (ghrelin and desacyl ghrelin) in normal mice (Nishi et al., 2005). In transgenic mice over-expressing human ghrelin and GOAT, animals fed a normal diet had reduced ghrelin levels, while when on an MCT-containing diet they produced higher levels of both ghrelin and desacyl ghrelin (Kirchner et al., 2009). Furthermore, a high-fat diet containing heptanoic acid, which cannot be naturally synthesised in mammalian cells, showed the production of an unnatural ghrelin form, heptanoylated ghrelin, in addition to octanoylated ghrelin (Nishi et al., 2005; Kirchner et al., 2009). This finding demonstrated that dietary lipids are directly used for ghrelin acylation and the GOAT-ghrelin system may function as a lipid sensor linking ingested nutrients with energy balance and endocrine homeostasis (Kirchner et al., 2009). However, little is known about GOAT function. While knockout models for ghrelin and GHS-R produced only mild phenotypes (Sun et al., 2003; Longo et al., 2008), mice with elimination of the GOAT gene showed significant changes in glucose homeostasis (Zhao et al., 2010). When subjected to normal or high fat diets, GOAT−/− mice showed normal growth and maintained the same weight as the wild type (WT) animals. On a 60% calorie restricted diet, both WT and GOAT−/− mice showed a 30% loss in body weight, 75% loss in body fat and a decrease in fasting blood glucose levels on day 4. However, after 4 days, blood glucose levels stabilised in WT mice, but continued to decrease in the GOAT−/− animals. In addition, WT animals showed normal physical activity while GOAT−/− animals become moribund (Zhao et al., 2010). Infusion of either GH or ghrelin reversed this phenotype and prevented death, suggesting a key role for the GOAT-ghrelin system in maintaining blood glucose (Zhao et al., 2010).

In addition to GOAT and prohormone convertases, other enzymes are likely to play a role in regulating the balance between acylated and unacylated ghrelin. The search for ghrelin desacylating enzymes reported a few candidates in the blood stream (De Vriese et al., 2004; Tham et al., 2009), however, only one enzyme was able to desacylate ghrelin (Satou et al., 2010). Acyl-protein thioesterase 1 (APT1) was
recently reported to demonstrate potent ghrelin desacylating activity both in vitro and in vivo (Satou et al., 2010). The balance between GOAT and APT1 could be crucial for regulation of acylated ghrelin availability and ghrelin/desacyl ghrelin ratio.

1.2.6 Obestatin

Six years after the discovery of ghrelin, obestatin, another preproghrelin-derived peptide, was identified. Obestatin is a 23 amino acid peptide, cleaved from the C-terminal domain of the preproghrelin preprohormone. It requires post-translational amidation of its carboxyl terminal for biological activity (Zhang et al., 2005). Recently, a structural study also showed that an α-helical structure at the obestatin C-terminal is critical for its in vivo activity (Subasinghage et al., 2010). Obestatin has been reported to be present in the circulation of mammals (Anderwald-Stadler et al., 2007; Butler and Bittel, 2007; Fontenot et al., 2007; Guo et al., 2007; Park et al., 2007; Qi et al., 2007; Gao et al., 2008a; Gao et al., 2008b; Ghanbari-Niaki et al., 2008; Guo et al., 2008; Harada et al., 2008; Huda et al., 2008; Koca et al., 2008; Lippl et al., 2008; Manshouri et al., 2008; Monteleone et al., 2008; Nakahara et al., 2008; Sedlackova et al., 2008; Wang et al., 2008; Zamrazilova et al., 2008; Beasley et al., 2009). However, a more detailed study failed to demonstrate obestatin’s presence in the plasma, but detected the presence of C-ghrelin, another product of the preproghrelin preprohormone and obestatin’s precursor molecule (Bang et al., 2007). Obestatin has been shown to degrade rapidly in serum and unlike ghrelin, is not able to cross the blood-brain- barrier (Pan et al., 2006).

Obestatin was first isolated from the stomach and was initially described to oppose ghrelin’s effect on food intake and body weight (Zhang et al., 2005), and therefore its name was derived from the Latin, obedere, meaning to devour, and statin meaning suppression (Zhang et al., 2005). Its expression has been described in a number of tissues including the pituitary, hypothalamus, gastrointestinal tract (Zhang et al., 2005), and pancreas (Chanoine et al., 2006), but its functions remain controversial. Although initial reports on obestatin’s anorexicogenic effects were successfully reproduced, at least partially, by some groups (Bresciani et al., 2006; Moechars et al., 2006; Green et al., 2007; Lagaud et al., 2007; Samson et al., 2007; Nagaraj et al., 2008; Zhang et al., 2008; Subasinghage et al., 2010) other groups failed to show any changes in food intake, body weight or gut motility (Gourcerol et al., 2006; Seoane
et al., 2006; Sibilia et al., 2006a; Bassil et al., 2007; De Smet et al., 2007; Gourcerol et al., 2007a; Gourcerol et al., 2007b; Nogueiras et al., 2007; Tremblay et al., 2007; Yamamoto et al., 2007; Chen et al., 2008a; Kobelt et al., 2008; Mondal et al., 2008).

Another controversial area in obestatin research is the identity of its natural receptor. Obestatin was initially reported to mediate its anorexigenic effects through the orphan G-protein coupled receptor 39 (GPR39), a member of the ghrelin receptor subfamily (Zhang et al., 2005; Moechars et al., 2006; Zhang et al., 2008). GPR39 transcripts are expressed throughout the body, particularly in the central nervous system and gastrointestinal system (McKee et al., 1997b). However, subsequent studies have been unable to confirm that obestatin is the natural ligand of the GPR39 (Lauwers et al., 2006; Chartrel et al., 2007; Holst et al., 2007; Tremblay et al., 2007). GPR39 is activated by high concentrations of zinc ions (Zn$^{2+}$), but not by obestatin (Lauwers et al., 2006; Holst et al., 2007; Zhang et al., 2011).

Although the effects of obestatin on food intake and body weight remain controversial, obestatin does have a number of functions. Obestatin opposes the effects of ghrelin on sleep in rats (Bodosi et al., 2004; Szentirmai and Krueger, 2006) increasing the number of non-rapid-eye-movement sleep episodes, but not slow wave sleep (Szentirmai and Krueger, 2006). In addition, obestatin has been shown to improve memory performance (Carlini et al., 2007), inhibit thirst and anxiety (Samson et al., 2007), control fluid homeostasis (Samson et al., 2008), and increase the secretion of pancreatic juice enzymes (Kapica et al., 2007). Recently, three different groups investigated the effects of obestatin in the 3T3-L1 preadipocyte cell line. Zhang et al. showed that obestatin treatment stimulated c-fos expression and ERK1/2 phosphorylation (Zhang et al., 2008). Gurriaran-Rodriguez et al. demonstrated that obestatin stimulates adipogenesis through phosphorylation of Akt and a range of its downstream targets and through inactivation of AMPK, and regulates metabolism through translocation of glucose transporter type 4 (GLUT4) and glucose uptake (Gurriaran-Rodriguez et al., 2011). Miegueu et al. demonstrated that obestatin stimulates pre-adipocyte differentiation, fatty acid uptake and inhibition of lipolysis (Miegueu et al., 2011). In addition, preproghrelin mRNA and protein expression increases throughout adipogenesis, consistent with the findings that both ghrelin and obestatin could be playing a relevant role in the process (Zhang et al., 2008; Gurriaran-Rodriguez et al., 2011; Miegueu et al., 2011).
In pancreatic β-cells, obestatin stimulated proliferation, cell survival and inhibited apoptosis via activation of ERK1/2 and PI3K/Akt pathways (Granata et al., 2008). Obestatin also stimulates cell proliferation in human epithelial retinal cells (Camina et al., 2007a) and human gastric cancer cells (Pazos et al., 2007) via activation of ERK1/2 and PKC pathways (Camina et al., 2007a; Pazos et al., 2007). In contrast, obestatin showed anti-proliferative properties in the TT medullary thyroid carcinoma cell line, and in the pancreatic neuroendocrine tumour cell line, BON-1 (Volante et al., 2009).

1.3 SUMMARY AND RELEVANCE TO PROJECT
Prostate cancer is the second most common cause of cancer-related death in Western men. Current therapeutic options are limited and there are no effective treatment alternatives for advanced metastatic castrate-resistant disease, and diagnostic and prognostic markers lack sensitivity and specificity. A better understanding of the mechanisms leading to the progression to a castrate-resistant state and comprehending how tumour growth is driven in those conditions could be a useful tool to support the development of improved adjunctive therapies and identification of more accurate markers for this disease. Recent research has shown that growth factors are likely to play a crucial role in stimulating tumour growth during the castrate-resistant state. The ghrelin axis is expressed in many normal and cancerous tissues and has been demonstrated to act as a growth factor and modulate cell proliferation and survival in a number of cell types. Interestingly, the ghrelin gene produces a preproprotein precursor that is cleaved by prohormone convertases and/or furin to yield not only acylated and non-acylated ghrelin (desacyl ghrelin) but also obestatin, a novel peptide hormone that despite the initial controversy in its potential as an anorexigenic molecule, has recently been shown to exert other relevant physiological roles. Previous studies in our laboratory showed that ghrelin stimulates proliferation in prostate cancer cell lines through activation of ERK1/2. However, the role of desacyl ghrelin in prostate cancer has not yet been investigated. Although desacyl ghrelin was initially thought to be an inactive peptide and unable to bind and activate the only known ghrelin receptor, the GHS-R1a, recent research has shown it shares some actions with ghrelin as well as exerting some opposing and distinct roles. These effects are likely to be mediated through a yet unidentified receptor
shared by both ghrelin forms. The initial finding that obestatin opposed the effects of ghrelin on food intake and body weight and the identification of an exon 3-deleted preproghrelin transcript lacking the obestatin sequence in prostate cancer specimens led to the question of whether obestatin could oppose the effect of ghrelin on prostate cancer cell proliferation.

The mechanisms and conditions regulating ghrelin gene expression and production and secretion of its derivative peptides remain largely unknown. A recent study revising the ghrelin gene structure identified the presence of several sense and anti-sense transcripts and mRNA splice variants demonstrating the regulation of expression of the preproghrelin-derived peptides is far more complex than anticipated. The identification of GOAT, the enzyme that utilises medium chain fatty acids (including octanoic acid) to acylate ghrelin, represented an important step to understand the mechanisms regulating the balance between acylated and unacylated ghrelin and possibly preproghrelin protein production.

Therefore, the overall objective of this study was to gain a better understanding of the ghrelin axis and explore the potential role of the preproghrelin-derived peptide hormones in modulating prostate cancer cell proliferation as well as the possible intra-cellular mechanisms involved in this process.

1.4 HYPOTHESES

The underlying hypotheses explored in my PhD studies were:

1. Obestatin is expressed in prostate cancer and stimulates cell proliferation in prostate cancer cell lines, signaling through ERK1/2, Akt and PKC pathways

2. Ghrelin and desacyl ghrelin act as growth factors and stimulate cell proliferation in prostate cancer cells through the alternative ghrelin receptor signaling through ERK1/2 and Akt pathways

3. As prostate cancer cells produce and secrete ghrelin, we hypothesise that GOAT and prohormone convertases are expressed in prostate cancer cell lines and octanoic acid supplementation can stimulate cell proliferation in prostate cancer cells through production of endogenous ghrelin.

4. Ghrelin and desacyl ghrelin regulate GOAT expression
1.5 AIMS

The aims of this PhD study were to:

1. investigate the functional effects and signaling mechanisms of obestatin in cell proliferation in prostate cancer cell lines.

2. investigate whether ghrelin and desacyl ghrelin exert the same effect and signaling mechanisms in prostate cancer cell line proliferation and whether this action is mediated through the GHS-R1a.

3. investigate whether prostate cancer cell lines possess the necessary enzymatic machinery to produce ghrelin and desacyl ghrelin and if these peptides can regulate GOAT expression.
Chapter 2

General Materials and Methods
2 INTRODUCTION
This chapter will describe the materials and methods for common techniques used throughout this thesis. More specific materials and methods will be described in each subsequent chapter.

2.1 GENERAL REAGENTS AND CHEMICALS
All general reagents and chemicals of analytical grade were obtained from Ajax Chemicals (Melbourne, Australia), BDH Chemicals (Kilsyth, Australia) or Sigma-Aldrich Chemical Company (Castle Hill, Australia), unless otherwise stated. Acylated ghrelin was obtained from Mimotopes (Clayton, Victoria, Australia) and obestatin was obtained from Auspep (Parkville, Victoria, Australia).

2.2 CELL LINES
The human prostate cell lines RWPE-1, RWPE-2, LNCaP, 22Rv1, PC3 and DU145, were obtained from the American Type Culture Collection (ATCC, Manassas, USA). RWPE-1 cells were originally isolated from the peripheral zone of a histologically normal adult human prostate and immortalised with the human papilloma virus 18 (HPV-18), while RWPE-2 cells are a clonal derivative of RWPE-1 cells transformed by Ki-ras using the Kirstin murine sarcoma virus (Ki-MuSV) (Bello et al., 1997). RWPE-1 cells exhibit many normal cell characteristics while RWPE-2 cells form colonies in agar and tumours in nude mice revealing a more invasive phenotype (Bello et al., 1997). The LNCaP cell line was established from a metastatic lesion of human prostatic adenocarcinoma (Horoszewicz et al., 1980). Although they attach only lightly to the substrate and grow in clusters, LNCaP cells develop tumours when injected into nude mice and show an androgen-dependent growth (Horoszewicz et al., 1980; Horoszewicz et al., 1983). A single point mutation in the ligand binding domain of the androgen receptor (AR) in LNCaP cells facilitates its activation by non-androgenic steroid hormones and anti-androgens. Despite the absence of receptors for progesterone and estradiol, these hormones as well as anti-androgen drugs can compete with androgens for binding to the androgen receptor and stimulate both cell growth and secretion of prostate specific acid phosphatase (Schuurmans et al., 1990; Veldscholte et al., 1990; Veldscholte et al., 1994).
22Rv1 is a human prostate carcinoma epithelial cell line derived from a xenograft after castration induced regression and relapse in mice (Sramkoski et al., 1999). 22Rv1 cells secrete low levels of prostate specific antigen (PSA) and although they express the AR, growth is weakly stimulated by dihydroxytestosterone (Sramkoski et al., 1999). The cell line displays both androgen-responsive and androgen-insensitive features and in androgen depleted conditions does not express or secrete PSA at detectable levels (Tepper et al., 2002). As in LNCaP cells, the AR ligand binding domain is mutated and has promiscuous affinity for other natural and synthetic steroid hormones (Tepper et al., 2002; Attardi et al., 2004). Furthermore, a tandem duplication of the exon 3 of the DNA-binding domain of the AR is present in this cell line (Tepper et al., 2002). 22Rv1 cells also express two additional AR isoforms, lacking the COOH terminal domain, that are constitutively active and promote the expression of endogenous AR-dependent genes, as well as the proliferation of 22Rv1 cells in a ligand-independent manner (Tepper et al., 2002; van Bokhoven et al., 2003; Dehm et al., 2008). More recently, two novel AR variants resulting from aberrant splicing of AR pre-mRNA were also shown to be constitutively active and another AR mutant lacking exon 3 tandem duplication was described (Marcias et al., 2010).

The PC3 cell line was derived from a bone metastasis of a grade IV prostatic adenocarcinoma and was shown to produce subcutaneous tumours in nude mice. Cultured cells showed anchorage-independent growth in both monolayers and in soft agar suspension (Kaighn et al., 1979). PC3 cells do not respond to androgens, glucocorticoids, or epidermal or fibroblast growth factors (Kaighn et al., 1979), do not produce PSA (Pang et al., 1995) and do not express the AR (Chlenski et al., 2001). The PC3 cell line has functional and morphologic characteristics of a poorly-differentiated adenocarcinoma with many features common to neoplastic cells of epithelial origin (Kaighn et al., 1979) and should be a good model to investigate changes in advanced prostate cancer.

The DU145 cell line is derived from a human prostate adenocarcinoma that had metastasised to the brain (Stone et al., 1978). The cells have an epithelial origin, form colonies in soft agar, are androgen independent, androgen insensitive (Stone et al., 1978) and do not produce PSA (Stone et al., 1978; Pang et al., 1995) or express
the AR (Chlenski et al., 2001). Although some DU145 and PC3 sublines expressing low levels of AR have been reported, the receptor does not stimulate androgen-regulated gene expression in these cases (Buchanan et al., 2004; Alimirah et al., 2006). The heterogeneity of prostate cancer cell lines reaffirms the heterogeneity of the disease and the many obstacles to be overcome to elucidate the mechanism underlying prostate cancer development and progression.

2.3 CELL CULTURE

2.3.1 Cell maintenance
All cell lines were maintained at 37°C with 5% CO₂ in a Sanyo IR Sensor Incubator (Quantum Scientific, Brisbane, Australia). The PC3, DU145, LNCaP and 22Rv1 prostate cancer cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Eugene, USA) with 10% New Zealand Cosmic Calf Serum (HyClone, South Logan, USA) supplemented with 100 U/mL penicillin G and 100 µg/mL streptomycin (Invitrogen). The RWPE-1 and RWPE-2 transformed normal prostate cell lines were cultured in Keratinocyte Serum Free Medium (KSFM) (Invitrogen) supplemented with 50µg/mL bovine pituitary extract and 5ng/mL epidermal growth factor (Invitrogen). All cell lines were passaged at two to three day intervals at 70% confluency using either 0.25% Trypsin/EDTA (Invitrogen) for PC3, DU145, LNCaP and 22Rv1 or Stable Trypsin-like Enzyme (TrypLE Express) (Invitrogen) for RWPE-1 and RWPE-2. Cell morphology and viability was monitored by microscopic observation and regular Mycoplasma testing was performed using PCR. All general disposable cell culture labware was from Nagle Nunc International (Roskilde, Denmark).

2.3.2 Cell counting
Cell suspensions of detached cells were counted using a NucleoCounter (ChemoMetic, Allerød, Denmark) according to the manufacturer’s instructions. Cells were grown to confluency, washed once with 1X Phosphate Buffered Saline (PBS) (Life Technologies, Melbourne, Australia) and detached using 1mL of Trypsin/EDTA or TrypLE Express. The detached cell suspension was resuspended in growth medium and mixed with lysis and stabilising buffers, then loaded into NucleoCassettes (ChemoMetic) containing propidium iodide to stain cell nuclei and measure total cell concentration.
2.4 CELL PROLIFERATION ASSAYS

Cell proliferation assays were performed using the Cell Proliferation Reagent WST-1 – a metabolic assay (Roche, Castle Hill, Australia) – and CyQUANT NF Cell Proliferation Assay kit – a DNA based assay (Invitrogen). A seeding test with the cell lines selected was performed to determine the number of cells that would reach the desired confluence by the fifth day, allowing for linear cell growth. Cells were resuspended in the appropriate culture medium, seeded into 96-well microplates (Nagle Nunc International) and allowed to attach overnight. After 24 hours, the medium was replaced, treatments and control were added and the cells were cultured for 72 hours. Medium was replaced every 24 hours for both treated and untreated cells. After 72 hours, the medium was aspirated and the cells incubated in either WST-1 or CyQUANT reagent according to the manufacturer’s instructions. Absorbance was measured using the multi-well plate reader Benchmark Plus Microplate Spectrophotometer (BioRad, Sydney, Australia) at 440nm with a reference wavelength of 600nm for the WST-1 assay and fluorescence was measured using the POLAR Optima Microplate reader (BMG labtech, Mount Eliza, Australia) for the CyQUANT assay with excitation at 485nm and emission detection at 530nm. At least three independent experiments were performed, with n=16 for each treatment, unless otherwise stated.

2.5 SIGNALING ASSAYS

2.5.1 Protein extraction

Soluble protein was isolated by the addition of 200µL of ice-cold lysis solution [(10mM Tris-HCl pH 8, 150mM NaCl, 1% Triton X-100, 5mM EDTA, 10mM NaF, 1% Phosphatase Inhibitor Cocktail 2 (Sigma, Castle Hill, Australia)], and the addition of one protease inhibitor tablet [(Complete EDTA-free Protease inhibitor cocktail (Roche)] per 25mL of lysis solution. The lysates were then collected, vortexed for 2-3 minutes and centrifuged for 20 minutes at 14,000 x g at 4°C in a bench top centrifuge. The supernatant was collected and the total protein concentration determined in duplicates using the Bicinchoninic Acid (BCA) protein assay (Pierce, Rockford, USA) as described below (Chapter 2.5.2).

2.5.2 Protein quantification by Bicinchoninic Acid (BCA) assay

The BCA assay was performed essentially as described by the manufacturer (Pierce,
Rockford, USA) in 96-well microplates. Using 2mg/mL Bovine Serum Albumin (BSA) stock, a set of protein standards was prepared (0.125-1mg/mL) by serial dilution in Tris-EDTA (TE, 10mM Tris-HCl, 1mM EDTA, pH 8.0). Then, 200µL working reagent (supplied in the Pierce kit) was added to each well. Twenty-five µL of each BSA standard, blank control (TE) and protein test samples were added to each well, mixed gently and then incubated for 30 minutes at 37°C. Once cooled to room temperature (RT), the samples were measured at 560nm in a microplate spectrophotometer (BioRad, Sydney, Australia).

2.5.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels were made using the BioRad Mini-PROTEAN3 system (BioRad) according to the manufacturer’s instructions. Gels were prepared containing a 4% acrylamide stacking gel layer to enhance band quality and a 10% resolving gel to separate total protein. Fresh protein lysates were added to gel loading buffer (250mM Tris-HCl, 2% SDS, 10% glycerol, 20mM β-mercaptoethanol, 0.01% bromophenol blue) before samples were incubated for 10 minutes at 95°C. PAGE gels were electrophoresed in 1X Tris-glycine running buffer (25mM Tris-HCl, 0.25M glycine, 0.1% w/v SDS, pH 8.3) at 80 volts (V) until the loading dye had reached the end of the stacking gel, and then at 100V until the proteins were sufficiently resolved. A pre-stained molecular weight protein marker between 10 and 250 kilo Daltons (kDa) in size (Precision Dual Colour Marker, BioRad) was used to identify appropriate electrophoresis time and to estimate cell protein sizes.

2.5.4 Western immunoblotting

The proteins separated by SDS-PAGE were transferred to a BioTrace NT nitrocellulose membrane (Pall Life Sciences, Pensacola, USA) in a Tris-Glycine buffer (250mM Tris-HCl, 400mM Glycine and 20% methanol) for 90 minutes at 4°C using a constant current of 200 milliamps (mA). To monitor transfer efficiency and equal protein loading, the membrane was stained with Ponceau S (Sigma-Aldrich) for 5 minutes, rinsed in tap water and the resulting proteins visualised. Following the Ponceau S staining, non-specific protein binding sites were blocked by incubating the membrane in 5% w/v skim milk powder diluted in 0.05% TBS-T (Tris-buffered saline-Tween-20, 10mM Tris-HCl, 0.5M NaCl, 0.05% Tween-20, pH 7.4) at room
temperature for 1 hour. Primary antibodies were diluted in blocking buffer or 5% BSA (Sigma-Aldrich)/TBS-T and incubated with the membrane, with agitation, at 4°C overnight. All primary antibodies are listed in the subsequent chapters. Membranes were then washed with 0.05% TBS-T (4 x 5 minutes), incubated with appropriate secondary antibodies diluted in 5% BSA/TBS-T and incubated with agitation at room temperature for 1 hour. Membranes were then washed again in 0.05% TBS-T (4 x 5 minutes) before incubation in a chemiluminescent substrate (SuperSignal West Femto, Pierce, Thermo Scientific, Melbourne, Australia) for 3 minutes. Signal was detected by exposing membranes to X-ray film (Fujifilm, Kyoto, Japan) for the appropriate time to produce the best image. X-ray film was developed using an Agfa CP1000 automatic film processor.

2.5.5 Densitometry analysis
Western immunobLOTS were quantitated by scanning the X-ray film and analysing images using the Syngene Gene Tools Software program. The Syngene software generates a histogram based on the intensity of the band of interest and quantitates the area under the curve. Values were then used for comparisons of band intensity.

2.6 RNA EXTRACTION AND QUANTIFICATION
Cells were grown to 80% confluency, washed once with 1X Phosphate Buffered Saline (PBS) (Life Technologies) and detached using 1mL of Trypsin/EDTA or TripLE Express. The detached cell suspension was resuspended in growth medium and washed twice in 1X PBS. RNA was extracted using QIAshredder and RNeasy Mini Kits according to the manufacturer’s instructions (QIAGEN, Hilden, Germany). RNA concentrations and purity were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) where an A260 optical density (OD) of 1 represented 40 μg/mL RNA. Purity was assessed using the A260/280 ratios, which were generally between 1.8 and 2.1. Contaminating genomic DNA was removed by DNase I digestion (Amplification grade, Invitrogen, Carlsbad, USA). RNA was aliquoted and stored at -80°C.

2.7 COMPLEMENTARY DNA SYNTHESIS
One μg of total RNA was reverse transcribed to complementary DNA (cDNA) using 200 units (U) of SuperScript III (Invitrogen) and random hexamers or oligodT in a final volume of 20μL according to the manufacturer's instructions.
2.8 POLYMERASE CHAIN REACTION (PCR)

PCRs were performed in a final volume of 50µL with a final concentration of 1X PCR buffer (Invitrogen), 1.5mM MgCl₂ (Invitrogen), 0.2mM dNTPs (Invitrogen), 2µM forward and reverse primers and 1U Platinum Taq (Invitrogen) and 10-100ng of DNA template or sterile distilled water (no template negative control). Thermal cycling (PTC-200 Thermal Cycler, MJ Research, Watertown, USA) consisted of initial denaturation for 2 minutes at 94°C, followed by 35 cycles of denaturation (30 seconds at 94°C), annealing (30 seconds at 60°C), extension (30 seconds at 72°C) and a final extension (3 minutes) at 72°C. Annealing temperatures are primer specific and modifications of this protocol are indicated in the Results chapters.

2.8.1 DNA electrophoresis

All PCR products were electrophoresed and separated by molecular weight on 1-3% (w/v) agarose gels. The agarose was dissolved in 1X TAE (Tris-acetate-EDTA) buffer and microwaved at high temperature until the agarose was completely dissolved then cooled to approximately 50°C. Once cooled, ethidium bromide (0.5µg/mL) was added to the melted agarose and the gel was poured and left to set at room temperature. The samples were added to 6X gel loading buffer consisting of 1:1 food dye: 80% glycerol (Rose Pink food dye, Brisbane, Australia) mixed and applied to the gel. The electrophoresis was carried out at 100V in a BioRad Minigel System (BioRad) for 20 to 40 minutes. Approximately 0.5 µg of DNA Marker IX (Roche) was used to compare DNA sizes. Images were captured under UV illumination using a Syngene UV System (Geneworks, Adelaide, Australia).

2.8.2 DNA sequencing

DNA sequencing was carried out on purified DNA using the sequencing service at the Australian Genome Research Facility (AGRF), University of Queensland, Brisbane, Australia. Sequencing reactions were performed according to the AGRF guidelines using ABI Big Dye Terminator technology. Sequence alignments were performed using the Blast program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

2.9 STATISTICAL ANALYSIS

The statistical significance was tested using a one-way analysis of variance (ANOVA), followed by Tukey’s test post hoc comparisons. P-values <0.05 were considered statistically significant.
Chapter 3

Investigation of the Effects of Obestatin on Prostate Cancer Cell Line Proliferation
INTRODUCTION

Obestatin is a recently discovered peptide hormone, encoded by the ghrelin gene, which also codes for ghrelin (Zhang et al., 2005). Like ghrelin, obestatin was also first isolated from the stomach and requires a post-translational modification for its activity, the amidation of its carboxyl terminal (Zhang et al., 2005). Obestatin has been shown to be widely expressed throughout the body (Zhang et al., 2005) and obestatin only mRNA transcripts have been described (Seim et al., 2007). It remains unclear whether these transcripts can be translated into peptides and whether obestatin can be produced independently of ghrelin (Seim et al., 2007; Seim et al., 2009). Interestingly, in addition to full length preproghrelin mRNA, a transcript lacking the coding sequence for obestatin has been found in prostate (Jeffery et al., 2002) and breast (Jeffery et al., 2005) cancer cell lines and when processed it produces mature ghrelin and a novel C-terminal peptide (Jeffery et al., 2005).

Obestatin was initially described to oppose ghrelin’s effect on food intake and energy balance (Zhang et al., 2005) and although some groups successfully reproduced these initial findings (Bresciani et al., 2006; Moechars et al., 2006; Green et al., 2007; Lagaud et al., 2007; Nagaraj et al., 2008; Zhang et al., 2008; Subasinghage et al., 2010) other groups failed to confirm its anorexigenic effects (Gourcerol et al., 2006; Seoane et al., 2006; Sibilia et al., 2006a; Bassil et al., 2007; De Smet et al., 2007; Gourcerol et al., 2007b; Gourcerol and Tache, 2007; Nogueiras et al., 2007; Tremblay et al., 2007; Yamamoto et al., 2007; Chen et al., 2008a; Kobelt et al., 2008; Mondal et al., 2008). Its functions in the gastrointestinal tract and energy homeostasis remain unclear. Another area of controversy in obestatin research is the identity of its natural receptor. Obestatin was initially reported to mediate its anorexigenic effects through the orphan G-protein coupled receptor 39 (GPR39) (Zhang et al., 2005; Moechars et al., 2006; Zhang et al., 2008). However, subsequent studies have been unable to confirm that obestatin is its natural ligand (Lauwers et al., 2006; Chartrel et al., 2007; Holst et al., 2007; Tremblay et al., 2007) and showed that high concentrations of zinc ions (Zn$^{2+}$), but not obestatin, could activate the GPR39 (Lauwers et al., 2006; Holst et al., 2007). In addition, obestatin has been shown to participate in other physiological processes such as sleep (Szentirmai and Krueger, 2006), memory performance (Carlini et al., 2007), thirst and anxiety...
(Samson et al., 2007), fluid homeostasis (Samson et al., 2008) and secretion of pancreatic juice enzymes (Kapica et al., 2007). Moreover, obestatin has been recently shown to play a role in adipose tissue, increasing fatty acid uptake, inhibiting lipolysis (Miegueu et al., 2011) and stimulating pre-adipocyte differentiation (Gurriaran-Rodriguez et al., 2011; Miegueu et al., 2011), the latter via activation of the Akt pathway (Gurriaran-Rodriguez et al., 2011). More importantly, obestatin has also been shown to participate in processes relevant to cancer progression. It stimulates proliferation in human epithelial retinal cells (Camina et al., 2007a) and human gastric cancer cells (Pazos et al., 2007) via activation of ERK1/2 and PKC pathways (Camina et al., 2007a; Pazos et al., 2007) and showed a proliferative, survival and anti-apoptotic effect in pancreatic β-cells via activation of ERK1/2 and PI3K/Akt pathways (Granata et al., 2008). In contrast, it demonstrated an anti-proliferative effect in the TT medullary thyroid carcinoma cell line, and in the pancreatic neuroendocrine tumour cell line, BON-1 (Volante et al., 2009).

Mitogen-activated protein kinase (MAPK), Akt and protein kinase C (PKC) pathways are signaling cascades that transmit extracellular signals to their intracellular targets by phosphorylating and activating downstream components to initiate the required physiological process. These pathways have been demonstrated to regulate cellular processes such as proliferation, translation, differentiation, migration and apoptosis and are necessary for normal growth and tissue homeostasis. Disruption of the normal balance of stimulatory and inhibitory effects can result in abnormal growth. Hyperactivation of these pathways has been reported in a number of human cancers (Camina et al., 2007a; McCubrey et al., 2007; Pazos et al., 2007; Carnero et al., 2008; Granata et al., 2008; Keyse, 2008; Arnoldussen and Saatcioglu, 2009; Gorin and Pan, 2009; Reyland, 2009; Carnero, 2010).

Prostate cancer is a heterogeneous disease and in addition to androgens, many growth factors have been shown to stimulate prostate cancer cell growth. Obestatin has been shown to act as a growth factor in human cancer cell lines and stimulate cell proliferation via activation of ERK1/2, Akt and PKC pathways. Preliminary results performed in our laboratory showed that obestatin is expressed in benign prostatic hyperplasia (BPH) and prostate cancer specimens (Figure 3.1) and this observation has led to our current hypothesis that obestatin may play a role in prostate cancer.
progression. In this study we investigate a possible role for obestatin as a growth factor in prostate cancer cell lines and the possible pathways involved in this process. A preliminary analysis of the presence of the G-protein coupled receptor GPR39 was also undertaken as at the time it was believed to be a putative receptor for obestatin.

Figure 3.1 Representative immunohistochemical localisation of obestatin in benign prostatic hyperplasia (BPH) and prostate cancer specimens. Preliminary data showing expression of obestatin (brown staining) in (BPH) and prostate cancer tissue. Non-immunoreactive nuclei are counterstained with haematoxylin. (Performed by Ms Rachael Murray, Ghrelin Research Group, Queensland University of Technology).

3.1 MATERIALS AND METHODS
General Materials and Methods are outlined in detail in Chapter 2. Experimental procedures which are specific to this chapter are described below.

3.1.1 Cell culture
Cells were maintained in culture medium, as previously described (see Section 2.3.1). The PC3, DU145, LNCaP and 22Rv1 prostate cancer cell lines and RWPE-1 and RWPE-2 transformed normal prostate cell lines were assessed for the presence of the putative obestatin receptor, GPR39. The PC3, androgen-independent metastasis derived prostate cancer cell line and the RWPE-1 cell line, were selected to represent a model of advanced prostate cancer versus a normal model of prostate cells. Both cell lines were treated with exogenous obestatin ± signaling pathway inhibitors and used in functional and signaling assays as outlined below.
3.1.2 RNA extraction and GPR39 RT-PCR

RNA extraction of the cultured cell lines was performed as previously described (see Section 2.6). Normal prostate total RNA was obtained from Ambion (Houston, USA). PCR primers (Table 3.1) that spanned the intron 1 region of the GPR39 gene were designed by Dr. Peter Cunningham for amplification of GPR39 transcript. Complementary DNA synthesis and PCR was performed as previously described (see Sections 2.7 & 2.8), in a 50µL reaction using 1µL template cDNA or sterile distilled water (no template negative control) with 1U Platinum Taq. Thermal cycling (PTC-200 Thermal Cycler, MJ Research, Watertown, USA) consisted of an initial denaturation of 2 minutes at 94°C, followed by 35 cycles of 10 seconds at 94°C for denaturation, 30 seconds at 60°C for annealing, 1 minute at 72°C for extension and a final extension for 10 min at 72°C. PCR products were electrophoresed and images were captured (as described in Section 2.8.1). In order to confirm the purity and quality of the cDNA, reactions for the housekeeping gene β-actin (Table 3.1) were performed. The PCR reactions were purified and sequenced to confirm the specificity of the reaction according to Section 2.8.2.

Table 3.1 PCR primer sequences for GPR39

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 5’→3’</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPR39</td>
<td>S</td>
<td>gctcatgaaaagccagaagg</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>catgatcctcgaatctggt</td>
</tr>
<tr>
<td>β-actin*</td>
<td>S</td>
<td>cgtagggccgcctaggaccaa</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>tgtgccttaggttcaagggg</td>
</tr>
</tbody>
</table>

*S, sense primer; AS, antisense primer
* (Ponte et al., 1984)

3.1.3 Cell proliferation assays

Proliferation assays were performed on PC3 and RWPE-1 prostate cancer cell lines as previously described (see Section 2.4). Two standard cell proliferation assays were employed, a metabolism based assay, Cell Proliferation Reagent WST-1 (Roche, Castle Hill, Australia) and a DNA based assay, CyQUANT (Invitrogen, Eugene, USA). Both methods were performed concurrently with a range of obestatin concentrations (0-1000nM)±10nM ghrelin. Cells were seeded in 200µL of medium (5000 cells/well for PC3 and 12000 cells/well for RWPE-1) in 96-well microplates and allowed to attach overnight. After 24 hours, medium was replaced and
treatments were added. The cells were incubated for 72 hours with fresh treatments added every 24 hours. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and 24 hours after the last treatment, cell proliferation rate was measured using either the WST-1 or CyQUANT reagents according to the manufacturer’s instructions (see Section 2.4). Each assay was performed with 16 replicates of each concentration and each experiment repeated independently at least 3 times. Statistical analysis was performed as described in Section 2.9.

3.1.4 Signaling assays for MAPK, Akt and PKC pathways

PC3 cells were plated in six-well plates (Nagle Nunc International) and when reaching 70% to 80% confluency were serum starved overnight. Cells were treated with various concentrations of obestatin (0, 1, 10, 100, and 1000nM) in phenol red-free 1640 RPMI in serum-free conditions for 5, 15, 30, and 60 minutes at 37°C in a humidified atmosphere containing 5% CO₂. As a positive control, PC3 cells were also treated with 10% fetal bovine serum (FBS) for 15 minutes. Medium and treatments were added and wells then aspirated immediately (0 min) or at 5, 15, 30 and 60 minutes after treatment. Cells were lysed by the addition of 200μL of ice cold lysis solution containing protease and phosphatase inhibitors as described in Section 2.5.1. Protein concentration was determined by Bicinchoninic acid (BCA) protein assay as previously described (see Section 2.5.2) and polyacrylamide gels (10%) were prepared and electrophoresed as described in Section 2.5.3. Western immunoblots were performed as detailed in Section 2.5.4. Membranes were incubated with primary antibodies (Table 3.2) overnight at 4°C. After four brief washes in 0.05% TBST, the membranes were incubated with the appropriate horseradish peroxidase (HRP) conjugated antibodies (Table 3.2) at room temperature for 1 hour. After washing (4 x 5 minutes), chemiluminescent SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Thermo Scientific, Melbourne, Australia) was applied and the membrane then exposed to X-ray film and developed.

Developed films were scanned and image densitometry was performed, as described in Section 2.5.5. The band density of phosphorylated protein was corrected for protein loading by measuring the density of GAPDH, total ERK1/2 or total Akt bands. Each experiment was performed at least 3 independent times. Statistical analysis was performed as described in Section 2.9.
Table 3.2 Antibodies used for Western immunoblotting

<table>
<thead>
<tr>
<th>Target</th>
<th>Antibody</th>
<th>WB dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary Antibodies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAkt</td>
<td>rabbit polyclonal anti-phospho-Akt (Thr308)</td>
<td>1:1000</td>
<td>Cell Signaling, Genesearch, Arundel, Australia</td>
</tr>
<tr>
<td>tAkt</td>
<td>rabbit polyclonal anti-Akt</td>
<td>1:2000</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>pERK 1/2</td>
<td>mouse monoclonal anti-phospho-p44/42 MAPK</td>
<td>1:1000</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td></td>
<td>(Thr202/Tyr204)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tERK 1/2</td>
<td>rabbit polyclonal anti-p44/42 MAPK</td>
<td>1:2000</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>pPKC</td>
<td>rabbit polyclonal anti-phospho-PKC (pan) ((\gamma) Thr 514)</td>
<td>1:1000</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>GAPDH</td>
<td>mouse monoclonal anti-GAPDH</td>
<td>1:20000</td>
<td>Chemicon, Millipore, Sydney, Australia</td>
</tr>
<tr>
<td><strong>Secondary Antibodies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>anti-mouse IgG, HRP-linked antibody</td>
<td>1:2000</td>
<td>Pierce, Thermo Scientific, Melbourne, Australia</td>
</tr>
<tr>
<td></td>
<td>anti-rabbit IgG, HRP-linked antibody</td>
<td>1:10000</td>
<td>Cell Signaling</td>
</tr>
</tbody>
</table>
3.1.5 Signaling inhibition assays

To investigate whether obestatin-induced phosphorylation was specific to the pathways studied, widely used inhibitors were selected for each pathway: U0126 (Sigma) for MAPK (ERK); Akt1/2 kinase inhibitor (Sigma) for Akt; and for PKC, due to the lack of a specific optimised inhibitor, 3 inhibitors were selected: i) staurosporine (Sigma), an inhibitor of a variety of kinases including PKA, PKG, MLCK, CaMK, and PKC; ii) LY294002 (Sigma), an inhibitor of PKC through inhibition of phosphoinositide 3-kinase (PI3K); and iii) Ro320432 (Sigma) a reportedly more specific PKC inhibitor. Each inhibitor was tested over a different range of concentrations in order to determine the lowest concentration to significantly suppress protein phosphorylation. PC3 cells were plated in six-well plates and at 70% to 80% confluency were serum starved overnight. Cells were pre-treated for 30-120 minutes with either 1-10µM of the ERK inhibitor U0126, 1-10µM Akt1/2 kinase inhibitor, 0.25-1µM staurosporine, 10-30µM LY294002 or 0.25-1µM Ro320432, then stimulated with obestatin at 1000nM for 15 minutes. Control incubations included no treatment (culture medium only), vehicle (DMSO, Sigma), inhibitor alone and 10% FBS. Each experiment was performed at least three independent times. Statistical analysis was performed as described in Section 2.9.

3.1.6 Cell proliferation inhibition assays

To investigate whether the obestatin-induced effect on proliferation was related to particular pathway activation, cell proliferation assays were also performed in the presence of U0126, Akt1/2 kinase inhibitor, staurosporine, LY294002 and Ro320432 at the same concentrations used for the signalling inhibition assay. The assays were performed as previously described above (see Section 3.1.4) and the cells were incubated either with 10µM U0126, 10µM Akt1/2 kinase inhibitor, 0.25-1µM staurosporine, 10-30µM LY294002 and 0.25-1µM Ro320432, with or without obestatin at 100 and 1000nM. WST-1 assays were performed to assess cell viability and cell proliferation rate. Three independent experiments were performed with n=8 for each treatment. Statistical analysis was performed as described in Section 2.9.
3.2 RESULTS

3.2.1 GPR39 mRNA expression in prostate-derived cell lines and normal prostate

GPR39 mRNA transcripts of the predicted size (172bp) were detected in PC3, RWPE-1, LNCaP, RWPE-2 and DU145 prostate-derived cell lines but not in 22Rv1 prostate cancer cells (Figure 3.2A), using non-quantitative reverse transcription-PCR. This amplicon was sequenced and was confirmed as GPR39. All samples were positive for the β-actin housekeeping gene (243bp) (Figure 3.2B).

3.2.2 Obestatin stimulates cell proliferation in the PC3 prostate cancer cell line

To investigate the effects of obestatin on cell proliferation, two prostate cell lines were selected, the PC3 androgen-independent prostate cancer cell line, and RWPE-1, a transformed normal prostate cell line. Two different types of assays were performed in order to confirm any results obtained – WST-1, a metabolism based assay and CyQUANT, a DNA based assay. PC3 cells incubated for 72 hours with obestatin (0-1000nM) showed a significant increase in cell proliferation compared with untreated controls using both WST-1 and CyQUANT methods (Figure 3.3). This dose-dependent increase in proliferation was greatest with 1000nM obestatin treatments with viable cell numbers increasing to 28.37±7.47% (p<0.01) above untreated controls with WST-1 reagent (Figure 3.3A) and to 12.14±2.61% (p<0.05) with CyQUANT (Figure 3.3B). Significant increases in cell proliferation (15.97±3.33% above untreated controls, p<0.05) were also observed at 100nM obestatin with WST-1 reagent (Figure 3.3A). Combined treatment of 10nM ghrelin ± 0.1-100nM obestatin showed an 18.93±2.07% (p<0.01) significant increase in cell proliferation at 10nM ghrelin/10nM obestatin with WST-1 reagent (Figure 3.4aA) and 16.37±2.75% (p<0.01) with CyQUANT (Figure 3.4aB). However, when cells were treated with 10nM ghrelin/100nM obestatin no significant change was observed with WST-1 but a significant 22.64±3.27% increase was seen with CyQUANT (p<0.01). No significant changes were seen in RWPE-1 transformed normal prostate cells after 72 hours of treatment with a range of obestatin concentrations (Figure 3.4).
Figure 3.2 Ethidium bromide-stained agarose gels showing mRNA expression of the GPR39 receptor in prostate cell lines. Total RNA was extracted and used for RT-PCR using specific primers for the GPR39 receptor (A) and β-actin (B) transcripts. Amplicons of the correct size were observed (172bp for GPR39 and 243bp for β-actin) and no product was generated in the no template negative control (-ve). GPR39 constructs were used as a positive control (+ve). A) GPR39 is expressed in PC3, RWPE-1, LNCaP, RWPE-2 and DU145 but not in the 22Rv1 prostate cancer cell line. B) β-actin screening confirmed purity and quality of the cDNA.
Figure 3.3 Obestatin treatments significantly increase cell proliferation rate of the PC3 prostate cancer cell line. PC3 cells were treated with obestatin (0-1000nM) for 72 hours and cell proliferation rate was measured with WST-1 and CyQUANT assays. Obestatin significantly increased cell proliferation in the PC3 prostate cancer cells with both WST-1 (A) and CyQUANT (B) methods at 1000nM (p<0.05). Obestatin also significantly stimulated cell proliferation at 100nM with WST-1 assay (A). Data are expressed as percentage above control and represent the mean ± SEM of three independent experiments with 16 replicates for each obestatin concentration in a single experiment. Statistical analysis was performed on combined data. *p<0.05 and **p<0.01 compared with untreated controls (one-way ANOVA with Tukey’s test post hoc analysis).
Figure 3.4 Obestatin treatments had no effects on proliferation of the RWPE-1 transformed normal prostate cell line. RWPE-1 cells were treated with obestatin (0-1000nM) for 72 hours and cell proliferation rate was measured with WST-1 and CyQUANT assays. There were no significant changes with any obestatin concentration with both WST-1 (A) and CyQUANT (B) methods. Data are expressed as percentage above control and represent the mean ± SEM of three independent experiments with 16 replicates for each obestatin concentration in a single experiment. Statistical analysis was performed on combined data by one-way ANOVA with Tukey’s test post hoc analysis.
3.2.3 Obestatin-induced phosphorylation of Akt, ERK 1/2 and PKC in the PC3 prostate cancer cell line

To investigate the signaling pathways that could be involved in the obestatin mitogenic effect, PC3 cells were treated with a range of obestatin concentrations (0-1000nM) for 0, 5, 15, 30 and 60 minutes and the level of protein activation (phosphorylation) was assessed by Western blot. Obestatin treatments activated Akt, ERK1/2 and PKC in a dose dependent manner and significantly increased Akt (4.8±3.01 fold, p<0.05) (Figure 3.5A) and ERK1/2 (6.01±3.27 fold, p<0.05) (Figure 3.5C) phosphorylation at 1000nM and PKC (1.85±0.47 fold, p<0.05) (Figure 3.6A) phosphorylation at 100nM. Obestatin significantly phosphorylated Akt at 15 minutes, and this activation peaked at 30 minutes remaining high for 60 minutes after treatment (p<0.01) (Figure 3.5B). ERK1/2 activation was intense but more transient, peaking at 15 minutes after treatments but no phosphorylation was seen 30 minutes after treatment (p<0.01) (Figure 3.5D). Obestatin-induced PKC phosphorylation showed a different pattern compared to Akt and ERK1/2. Activation levels peaked 5 minutes after 100nM obestatin treatment and were sustained for at least 60 minutes after treatment (p<0.05) (Figure 3.6B). Phosphorylated protein was normalised to GAPDH, a housekeeping protein, as a loading control. However, to assess whether total Akt and ERK1/2 levels were changing when cells were treated with a range of obestatin concentrations, total Akt and ERK1/2 levels were also investigated. No significant changes in total Akt and ERK1/2 were observed (Figure 3.5a). In addition, phosphorylated protein showed the same pattern when normalised to either GAPDH or total Akt or ERK1/2 (Figure 3.5a). Therefore, phosphorylated protein levels in subsequent assays were normalised to GAPDH only as a loading control.
Figure 3.5 Obestatin stimulates Akt and ERK1/2 phosphorylation in the PC3 prostate cancer cell line. PC3 cells were serum starved overnight then stimulated with obestatin (0-1000nM) in serum free conditions for 0, 5, 15, 30, and 60 minutes. Obestatin stimulated Akt (A) and ERK1/2 (C) phosphorylation in a dose dependent manner and was statistically significant at 1000nM (p<0.05, compared to untreated control). Obestatin-induced (1000nM) Akt activation reached significant levels at 15 minutes, peaked at 30 and remained high for 60 minutes after treatment (p<0.01) (B) while obestatin-induced (1000nM) ERK1/2 phosphorylation peaked at 15 minutes and no phosphorylation was seen 30 minutes after treatment (p<0.01) (D). Phosphorylated protein was corrected for protein loading by normalising to GAPDH. Blots are representative of three independent identical experiments. Data are expressed as fold change compared to untreated control and represent the mean densitometry ± SEM of three independent experiments. Statistical analysis was performed on combined data. *p<0.05 and **p<0.01 compared with untreated controls (one-way ANOVA with Tukey’s test post hoc analysis). 0nM Obestatin = untreated control; 10% FBS stimulation (15 minutes) was used as a positive control.
Figure 3.5a Obestatin does not alter total Akt and Erk levels in the PC3 prostate cancer cell line. PC3 cells were serum starved overnight then stimulated with obestatin (0-1000nM) in serum free conditions for 0, 5, 15, 30, and 60 minutes. Obestatin stimulated Akt (A) and ERK1/2 (C) phosphorylation in a dose dependent manner but did not alter total Akt and Erk levels. Obestatin-induced (1000nM) Akt activation at 15 minutes, peaked at 30 and remained high for 60 minutes after treatment (B) while obestatin-induced (1000nM) ERK1/2 phosphorylation peaked at 15 minutes and no phosphorylation was seen 30 minutes after treatment (D). Phosphorylated protein was corrected for protein loading by normalising to total Akt and total Erk. Data are expressed as fold change compared to untreated control. n=1. 0nM Obestatin = untreated control; 10% FBS stimulation (15 minutes) was used as a positive control.
Figure 3.6 Obestatin stimulates PKC phosphorylation in the PC3 prostate cancer cell line. PC3 cells were serum starved overnight then stimulated with obestatin (0-1000nM) in serum free conditions for 0, 5, 15, 30, and 60 minutes. A) Obestatin statistically significantly stimulated PKC phosphorylation in the PC3 prostate cancer cell line at 100nM (p<0.05, compared to untreated control). B) Obestatin-induced (100nM) PKC activation reached significant levels at 5 minutes (p<0.05) and remained high for at least 60 minutes after treatment (p<0.05). Phosphorylated protein was corrected for protein loading by normalising to GAPDH. Blots are representative of three independent identical experiments. Data are expressed as fold change compared to untreated control and represent the mean densitometry ± SEM of three independent experiments. Statistical analysis was performed on combined data. *p<0.05 and **p<0.01 compared with untreated controls (one-way ANOVA with Tukey’s test post hoc analysis). 0nM Obestatin = untreated control; 10% FBS stimulation for 15 minutes was used as a positive control.
3.2.4 ERK1/2, Akt and PKC inhibition assays

Obestatin treatment caused a significant proliferative effect in the PC3 prostate cancer cell line at 100-1000nM (p<0.05) and significantly increased Akt, ERK1/2 and PKC phosphorylation (p<0.05) (Figures 3.3, 3.5 and 3.6). To confirm the specificity of Akt, ERK1/2 and PKC activation by obestatin, kinase inhibitors for each pathway were employed. Obestatin significantly stimulated Akt and ERK1/2 phosphorylation but when cells were pre-treated with Akt1/2 kinase inhibitor and U0126, no phosphorylation was detected (Figure 3.7). To investigate whether obestatin-induced Akt and ERK1/2 phosphorylation was related to obestatin-induced proliferation, cell proliferation assays using the WST-1 reagent were performed in the presence of obestatin with and without Akt1/2 kinase inhibitor (Figure 3.8A) or U0126 (Figure 3.8B). Obestatin treatment significantly stimulated proliferation at 1000nM (over 20% above control and vehicle) over 72 hours (p<0.05), but combined treatment with obestatin and either the Akt1/2 kinase inhibitor or U0126 completely prevented obestatin-stimulated cell proliferation (Figure 3.8). Inhibitor alone or vehicle (DMSO) had no significant effect showing the cells were viable at the inhibitor concentration employed (Figure 3.8). These results suggest that activation of Akt and ERK1/2 are necessary for obestatin-induced cell proliferation in the PC3 prostate cancer cell line.

To investigate whether obestatin-induced PKC activation was related to obestatin stimulation of cell proliferation, first a widely employed PI3K inhibitor, LY294002, and a potent protein kinase inhibitor, staurosporine were used. To assess cell viability in the presence of these inhibitors and their effects in obestatin-induced cell proliferation, WST-1 cell proliferation assays were performed during 72 hours in the presence of obestatin ± 0.25-1µM staurosporine (Figure 3.9) or 10-30µM LY294002 (Figure 3.10). Obestatin treatment significantly increased cell proliferation at 1000nM compared to untreated control and vehicle (DMSO) (p<0.01) (Figures 3.9 & 3.10). A range of concentrations of inhibitors were employed, however, both inhibitors induced significant cell death (over 60% compared to controls) at all concentrations used, and almost 100% at the highest concentrations applied (Figures 3.9 and 3.10). Moreover, signaling inhibition assays, using the lowest inhibitor concentration used in the cell proliferation and viability assay, failed to significantly inhibit obestatin-induced PKC phosphorylation (Figure 3.11).
Figure 3.7 Obestatin-induced cell proliferation in the PC3 prostate cancer cell line is mediated through activation of ERK1/2 and Akt pathways. PC3 cells were serum starved overnight then pre-treated for 30 minutes with either 10µM U0126 (A) or 10µM Akt 1/2 kinase inhibitor (B) then stimulated with 1000nM obestatin for 15 minutes. Obestatin significantly stimulated ERK1/2 (A) and Akt (B) phosphorylation (p<0.05, compared to untreated control and vehicle) and this effect was completely abolished when combined with U0126 (A) or Akt 1/2 kinase inhibitor (B).

Phosphorylated protein was corrected for protein loading by normalising to total ERK1/2 (A) or GAPDH (B). Blots are representative of three independent identical experiments. Data are expressed as fold change compared to untreated control and represent the mean densitometry ± SEM of three independent experiments. Statistical analysis was performed on combined data. a = p<0.01 compared to untreated control, b = p<0.01 compared to vehicle and c = p<0.01 compared 1000nM obestatin (one-way ANOVA with Tukey’s test post hoc analysis). Vehicle = DMSO; Akt ½ Inh = Akt 1/2 kinase inhibitor; 10% FBS was used as a positive control.
Figure 3.8 Inhibition of obestatin-induced proliferation in the PC3 prostate cancer cell line by specific ERK1/2 and Akt inhibitors. PC3 cells were treated with either 10µM U0126 (A) or 10µM Akt 1/2 kinase inhibitor (B) ± obestatin at 100 and 1000nM for 72 hours. Cell viability and proliferation rate were measured with WST-1 assays. Obestatin significantly increased cell proliferation in the PC3 prostate cancer cell line at 1000nM (p<0.05, compared to untreated control and vehicle) and this effect was completely abolished when combined with U0126 (A) or Akt 1/2 kinase inhibitor (B). Untreated control (0nM Obes), Vehicle (DMSO) and inhibitor alone were used as controls. Data are expressed as percentage above control and represent the mean ± SEM of three independent experiments with eight replicates for each treatment in a single experiment. Statistical analysis was performed on combined data. a = p<0.01 compared to untreated control, b = p<0.01 compared to vehicle and c = p<0.01 compared to 1000nM obestatin (one-way ANOVA with Tukey’s test post hoc analysis). Obes = obestatin; A) Inh = U0126; B) Inh = Akt 1/2 kinase inhibitor.
Staurosporine, a broad protein kinase inhibitor, induces cell death in the PC3 prostate cancer cell line. PC3 cells were treated with 0.25-1µM staurosporine ± obestatin at 100 and 1000nM for 72 hours. Cell viability and proliferation rate were measured with WST-1 assays. 1µM staurosporine induced over 90% cell death in PC3 prostate cancer cells (p<0.01, compared to vehicle) and even at the lowest concentration used (0.25µM) staurosporine induced almost 70% cell death (p<0.01, compared to vehicle). Obestatin significantly increased cell proliferation in the PC3 prostate cancer cell line at 1000nM (p<0.01). Untreated control (0nM Obes), Vehicle (DMSO) and inhibitor alone were used as controls. Data are expressed as percentage above control and represent the mean ± SEM of three independent experiments with eight replicates for each treatment in a single experiment. Statistical analysis was performed on combined data. a = p<0.01 compared to untreated control, b = p<0.05 compared to untreated control, c = p<0.01 compared to vehicle, d = p<0.01 compared to 1000nM obestatin (one-way ANOVA with Tukey’s test post hoc analysis). Obes = obestatin; Inh = Staurosporine.
**Figure 3.10** LY294002, a PI3K inhibitor, induces cell death in the PC3 prostate cancer cell line. PC3 cells were treated with 10-30µM LY294002 ± obestatin at 100 and 1000nM for 72 hours. Cell viability and proliferation rate were measured with WST-1 assays. 30µM LY294002 induced more than 80% cell death in PC3 prostate cancer cells (p<0.01, compared to vehicle) and even at the lowest concentration used (10µM) LY294002 caused more than 60% cell death (p<0.01, compared to vehicle). Obestatin significantly increased cell proliferation in the PC3 prostate cancer cell line at 1000nM (p<0.01). Untreated control (0nM Obes), Vehicle (DMSO) and inhibitor alone were used as controls. Data are expressed as percentage above control and represent the mean ± SEM of three independent experiments with eight replicates for each treatment in a single experiment. Statistical analysis was performed on combined data. a = p<0.01 compared to untreated control, b = p<0.01 compared to vehicle and c = p<0.01 compared to 1000nM obestatin (one-way ANOVA with Tukey’s test post hoc analysis). Obes = obestatin; Inh = LY294002.
Figure 3.11 Staurosporine and LY294002, two broad spectrum PKC inhibitors, did not inhibit obestatin-induced PKC phosphorylation in the PC3 prostate cancer cell line at a concentration where cells were viable. PC3 cells were serum starved overnight then pre-treated for 30 minutes with either 0.25-1μM Staurosporine or 10-30μM LY294002 then stimulated with 100 or 1000nM obestatin for 15 minutes. Obestatin stimulated PKC phosphorylation but this effect was not inhibited when combined with either 0.25μM Staurosporine (A) or 10μM LY294002 (B). 0.5-1μM Staurosporine and 20-30μM LY294002 significantly caused cell death in the PC3 prostate cancer cell line (Figures 3.9 & 3.10). Blots are representative of two independent experiments. Phosphorylated protein was corrected for protein loading by normalising to GAPDH. Vehicle = DMSO; * =100nM Obestatin; ** = 1000nM Obestatin, A) Inhibitor = Staurosporine; B) Inhibitor = LY294002; 10% FBS was used a positive control.
A second approach to try to inhibit obestatin-induced PKC phosphorylation and to investigate whether PKC activation is involved in obestatin-induced cell proliferation was the use of a reportedly more specific PKC inhibitor, Ro320432. Using the WST-1 cell proliferation assay, PC3 cells were treated with obestatin ± 0.5µM Ro3204032 for 72 hours (Figure 3.12). Obestatin significantly increased cell proliferation at 1000nM compared to untreated control and vehicle (DMSO) (p<0.05) and combined treatment of obestatin with Ro3204032 completely prevented obestatin-induced cell proliferation (Figure 3.12). Inhibitor or vehicle alone (DMSO) had no significant effect showing the cells were viable at the inhibitor concentration used (Figure 3.12).

To verify that obestatin-induced PKC phosphorylation was inhibited in the PC3 prostate cancer cell line when treated with 0.5µM Ro320432, Western blot analysis was performed in the presence of 100nM obestatin ± 0.5µM Ro3204032 (Figure 3.13). Obestatin alone stimulated PKC phosphorylation but pre-treatment with Ro320432 failed to inhibit obestatin-induced PKC phosphorylation at 0.5µM (Figure 3.13) and at higher doses (0.5-3µM) (Figure 3.14), suggesting that Ro320432 could be inhibiting pathways other than PKC to prevent obestatin-induced cell proliferation. Preliminary results showed that stimulation with 100nM obestatin after pre-treatment with 0.5µM Ro320432 for 30 minutes prevented obestatin-induced ERK1/2 phosphorylation but not obestatin-induced activation of Akt or PKC (Figure 3.15).
Figure 3.12 Inhibition of obestatin-induced proliferation in the PC3 prostate cancer cell line by Ro320432, a specific PKC inhibitor. PC3 cells were treated with 0.5µM Ro320432 ± obestatin at 100 and 1000nM for 72 hours. Cell viability and proliferation rate were measured with WST-1 assays. Obestatin significantly increased cell proliferation in the PC3 prostate cancer cell line at 1000nM (p<0.05, compared to untreated control and vehicle) and this effect was completely abolished when combined with Ro320432. Untreated control (0nM Obes), Vehicle (DMSO) and inhibitor alone were used as controls. Data are expressed as percentage above control and represent the mean ± SEM of four independent experiments with eight replicates for each treatment in a single experiment. Statistical analysis was performed on combined data. a = p<0.05 compared to untreated control, b = p<0.05 compared to vehicle and c = p<0.01 compared to 1000nM obestatin (one-way ANOVA with Tukey’s test post hoc analysis). Obes = obestatin; Inh = Ro320432.
Figure 3.13 Ro320432, a reportedly more specific PKC inhibitor, did not inhibit obestatin-induced PKC phosphorylation in the PC3 prostate cancer cell line. PC3 cells were serum starved overnight then pre-treated for 30 minutes with 0.5µM Ro320432 before stimulation with 100nM obestatin for 15 minutes. Preliminary results show obestatin stimulated PKC phosphorylation when compared to untreated control and vehicle but this effect was not suppressed when combined with the inhibitor Ro320432. Blots are representative of two independent experiments. Phosphorylated protein was corrected for protein loading by normalising to total GAPDH. Vehicle = DMSO; Inhibitor = Ro320432; 10% FBS was used as a positive control.
Figure 3.14 Ro320432, a reportedly more specific PKC inhibitor, did not inhibit obestatin-induced PKC phosphorylation in the PC3 prostate cancer cell line at a range of concentrations. PC3 cells were serum starved overnight then pre-treated for 30 minutes with 0.5-3µM Ro320432 before stimulation with 100nM obestatin for 15 minutes. Preliminary results show obestatin stimulated PKC phosphorylation when compared to untreated control but phosphorylation was not prevented when obestatin was combined with the inhibitor Ro320432 at different concentrations. Blots are representative of two independent experiments. Phosphorylated protein was corrected for protein loading by normalising to total GAPDH. Vehicle = DMSO; Inh = Ro320432; 10% FBS was used as a positive control; a = 0.5µM Inh; b = 1µM Inh; c = 3µM Inh.
Figure 3.15 Ro320432, a reportedly more specific PKC inhibitor, inhibited obestatin-induced ERK1/2 phosphorylation but not obestatin-induced Akt or PKC phosphorylation. PC3 cells were serum starved overnight then pre-treated for 30 minutes with 0.5µM Ro320432 before stimulation with 100nM obestatin for 15 minutes. Obestatin stimulated ERK1/2, Akt and PKC phosphorylation when compared to untreated controls. Preliminary results show combined treatment of obestatin and Ro320432 inhibited ERK1/2 phosphorylation but did not inhibit obestatin-induced Akt and PKC phosphorylation. Phosphorylated protein was corrected for protein loading by normalising to GAPDH (pAkt and pPKC) or total ERK1/2 (pERK). n=1; Vehicle = DMSO; Inhibitor = Ro320432; 10% FBS was used as a positive control.
3.3 DISCUSSION

This study has demonstrated that the peptide hormone, obestatin, stimulates cell proliferation in the PC3 prostate cancer cell line, by activating the Akt and ERK1/2 signaling pathways. Intensive research since the discovery of ghrelin has expanded our knowledge of its wide range of functions, and also highlighted the complexity of the ghrelin axis. Processing of ghrelin precursor protein, preproghrelin, not only produces ghrelin in its acylated and unacylated forms (Kojima et al., 1999), but also gives rise to the peptide hormone, obestatin (Zhang et al., 2005). Other peptides may also be produced from mRNA isoforms (Seim et al., 2007). Expression of mRNA for the coding region of obestatin is widely expressed throughout the body (Zhang et al., 2005) and an isoform coding obestatin alone has also been described (Seim et al., 2007).

Preliminary results in our laboratory suggested that obestatin is expressed in benign prostatic hyperplasia (BPH) and prostate cancer specimens at the protein level. Studies showing obestatin protein expression in human tissues are limited, however. Obestatin antibodies would also detect proghrelin and C-ghrelin, making it impossible to determine the exact form of obestatin expressed using antibodies and immunohistochemical methods. Obestatin protein expression has been previously reported in the pituitary, pancreatic islets, bronchial tree and gastrointestinal tract, using immunohistochemistry, while immunoreactivity was absent in the thyroid, parathyroid, adrenal, liver, thymus, lymph node, kidney, testis and ovary (Volante et al., 2009). In human cancers, immunohistochemistry studies showed obestatin protein expression in thyroid and gastro-enteropancreatic tumours (Volante et al., 2009) and in oral squamous cell carcinoma (Alnema et al., 2010). The preproghrelin mRNA has been previously shown to be expressed in prostate cancer cell lines and the mature ghrelin peptide was detected in prostate cancer specimens (Yeh et al., 2005). Interestingly, obestatin has recently been demonstrated at higher levels in seminal plasma than in plasma and concentrations (measured using radioimmunoassay) are correlated with sperm concentration and motility (Moretti et al., 2011). These authors hypothesised that obestatin could play a role in cell proliferation in the testes as it does in other cell types (Moretti et al., 2011). It is unclear if prostatic fluid contributes to this elevated level of obestatin, however. A previous study, using an ELISA, reported obestatin levels were lower in the seminal
plasma compared to circulating levels (Panidis et al., 2008).

Antibody-based assays, including immunohistochemical studies, ELISAs and RIAs, are difficult to interpret, however, as they are unable to distinguish between obestatin and its precursor molecule, proghrelin (Bang et al., 2007; Mondal et al., 2008). The protein expression of obestatin requires further study using techniques that can recognise the size of the peptide, including Western immunoblotting and mass spectrometry.

Initially it was reported that obestatin binds and activates GPR39, a receptor in the small ghrelin receptor family (Zhang et al., 2005). Numerous studies have since demonstrated that obestatin does not bind and activate the GPR39 (Lauwers et al., 2006; Chartrel et al., 2007; Holst et al., 2007; Tremblay et al., 2007) and therefore GPR39 is unlikely to be the obestatin receptor (see Section 1.2.6). On the basis of initial reports, the expression of GPR39 was investigated in prostate-derived cell lines and normal prostate in this study. GPR39 mRNA is expressed in the PC3, DU145, LNCaP, RWPE-1, RWPE-2 prostate cell lines and in normal prostate, but not in the 22Rv1 prostate cancer cell line. In addition, preliminary results in our laboratory demonstrated that GPR39 protein is expressed in prostate cancer specimens (personal communication Ms Rachael Murray, Ghrelin Research Group, QUT) and in the PC3 prostate cancer cell line (personal communication Dr. Peter Cunningham) (data not shown). Although the GPR39 is unlikely to be the obestatin cognate receptor, it is a member of the ghrelin receptor family and has been shown to be expressed in different regions of the mouse prostate (Zhang et al., 2011) and respond to high concentrations of zinc (Lauwers et al., 2006; Holst et al., 2007; Zhang et al., 2011). Therefore, the GPR39 could play an important role in mediating zinc function in the prostate (Costello et al., 2005; Zhang et al., 2011).

Initial studies into the function of obestatin have proven controversial and further investigation is needed to gain a better understanding of its biological function and mechanisms of action (Bresciani et al., 2006; Gourcerol et al., 2006; Moechars et al., 2006; Seoane et al., 2006; Sibilia et al., 2006a; Bassil et al., 2007; De Smet et al., 2007; Gourcerol et al., 2007a; Gourcerol et al., 2007b; Green et al., 2007; Lagaud et al., 2007; Nogueiras et al., 2007; Samson et al., 2007; Tremblay et al., 2007;
Yamamoto et al., 2007; Chen et al., 2008a; Kobelt et al., 2008; Mondal et al., 2008; Nagaraj et al., 2008; Zhang et al., 2008; Subasinghage et al., 2010)(see Section 1.2.6). Although the potential for obestatin as an anorexigenic peptide remains unclear and its natural receptor unknown (see Section 1.2.6), recent reports have confirmed that obestatin is an active peptide, that it is expressed in normal and pathological conditions (Huda et al., 2008; Alexandridis et al., 2009; Kim et al., 2009b; Volante et al., 2009; Alnema et al., 2010), and that it has some relevant physiological roles (Bodosi et al., 2004; Szentirmai and Krueger, 2006; Camina et al., 2007a; Carlini et al., 2007; Kapica et al., 2007; Pazos et al., 2007; Granata et al., 2008; Samson et al., 2008; Gurriaran-Rodriguez et al., 2011; Miegueu et al., 2011). Obestatin has been shown to stimulate cell proliferation in a number of other different cell lines, including pancreatic islet cells and human retinal pigment epithelial cell lines (Camina et al., 2007a; Granata et al., 2008). Exogenous obestatin stimulates cell proliferation in the KATO-III gastric cancer cell line (Pazos et al., 2007), but not the AGS gastric cancer cell line (Pazos et al., 2007). In this study it was demonstrated that obestatin also acts as a growth factor stimulating cell proliferation in the PC3 prostate cancer cell line through activation of ERK1/2, Akt and possibly PKC pathways, while no effect was seen in the RWPE-1 transformed normal prostate cell line. Employment of specific inhibitors for ERK1/2 and Akt phosphorylation demonstrated that obestatin-induced ERK1/2 and Akt phosphorylation is directly linked to its effect on cell proliferation. Similar doses of exogenous obestatin (100-500nM) have been shown to stimulate cell proliferation through activation of ERK1/2 and PI3K/Akt pathways in pancreatic β-cells and isolated human pancreatic islet cells (Granata et al., 2008). Obestatin also stimulates cell proliferation and ERK1/2 and PKC isoform phosphorylation in two distinct cell types, pancreatic β-cells and human retinal epithelial cells (Camina et al., 2007a; Pazos et al., 2007). These studies suggested the signaling model for obestatin-induced proliferation in these cell types was through activation of PI3K upon obestatin binding to a G-protein coupled receptor, and subsequent activation of PKC, then ERK1/2 (Camina et al., 2007a; Pazos et al., 2007).

In contrast, obestatin has been demonstrated to inhibit cell proliferation in a number of cell lines and cell types. An anti-proliferative effect was seen with 100nM obestatin treatments in the TT medullary thyroid carcinoma cell line and BON-1
pancreatic neuroendocrine tumour cell line (Volante et al., 2009), suggesting that the response to obestatin might be cell-type specific. Interestingly, obestatin expression is downregulated in thyroid and pancreatic tumour tissue (Volante et al., 2009). Obestatin also inhibits proliferation (using a metabolic assay) in the human C28-I2 rib chondrocyte cell line, and in the ATDC5 mouse embryonic carcinoma-derived cell line after it has been differentiated into chondrocytes (Lago et al., 2007). In regenerating adrenocortical cells it reduced the mitotic index (Rucinski et al., 2010).

In this study obestatin treatment in the PC3 prostate cancer cell line significantly induced phosphorylation of protein kinase C (PKC), however, it is not clear if obestatin stimulates cell proliferation through this pathway, as we were unable to specifically inhibit PKC phosphorylation. Obestatin has previously been shown to stimulate PKC phosphorylation in the human retinal epithelial and human gastric cancer cell line KATO-III (Camina et al., 2007a; Pazos et al., 2007). Treatment with widely used inhibitors LY294002, a potent inhibitor and competitive agonist of PI3K (Vlahos et al., 1994), and staurosporine, a potent, but non-selective inhibitor of a wide range of serine/threonine- and tyrosine- specific protein kinases (Tamaoki, 1991; Yamamoto et al., 1992; Ozawa et al., 1999), failed to inhibit obestatin-induced PKC phosphorylation. In addition, treatment with a range of concentrations of LY294002 or staurosporine caused significant cell death in the PC3 prostate cancer. In other studies concentrations of 10-50µM LY294002 are used and many studies have only used LY294002 in signaling assays, where incubation times are much shorter (20 minutes to three hours compared with 72 hours in the current study) (Vlahos et al., 1994; Hughes-Fulford et al., 2006; Liu et al., 2006; MacManus et al., 2007; Chen-Roetling et al., 2008; Kim et al., 2009a). Staurosporine has been used in similar assays, as most studies show its use in signaling assays rather than in vitro functional assays and concentrations used range from 1-10µM (Ogata et al., 2007; Pazos et al., 2007; Carraway et al., 2008; Kim et al., 2009a), which are potentially cytotoxic to the cells. In addition to the potential cytotoxic effects of these inhibitors, it is recognised that they lack specificity for PI3K and PKC. LY294002 has been shown to inhibit multiple other signaling molecules to the same extent as it inhibits PI3K (Brunn et al., 1996; Stein, 2001; Vanhaesebroeck et al., 2001; El-Kholy et al., 2003; Pasapera Limon et al., 2003) and although staurosporine has been shown to target and inhibit PKC phosphorylation, it is a non-selective inhibitor and able to
target a variety of protein kinases (Tamaoki, 1991; Yamamoto et al., 1992; Ozawa et al., 1999). These observations led us to use a second approach with a reportedly more specific PKC inhibitor, Ro320432 (Bis-XI), which is a bisindolylmaleimide (Bis) derivative (Wilkinson et al., 1993). Currently there are ten bisindolylmaleimides (Bis-I to Bis-XI) and numerous reports have shown that bisindolylmaleimides influence PKC (Basu, 1998; Denning et al., 1998; Brehmer et al., 2004; Szanda et al., 2008), although some studies have shown that different Bis derivatives can exert distinct effects to each other (Pajak et al., 2008). In this study we showed that 0.5µM Ro320432 successfully prevented obestatin-induced proliferation, but like LY294002 and staurosporine failed to suppress obestatin-induced phosphorylation of PKC at the same concentration used in the cell proliferation assay (0.5µM Ro320432), or at higher concentrations (0.5-3µM Ro320432). Surprisingly pre-treatment with 0.5µM Ro320432 significantly prevented obestatin-induced ERK1/2 phosphorylation, and, therefore, the inhibition of obestatin-induced cell proliferation by Ro320432 may be mediated by ERK1/2 inactivation rather than PKC inhibition. Interestingly, this result is consistent with a recent report that demonstrated that some of the bisindolylmaleimides derivatives are able to modulate other PKC-independent signaling pathways (Pajak et al., 2008). It is, therefore, currently unclear if obestatin-induced PKC phosphorylation is related to obestatin-induced proliferation in the PC3 prostate cancer cell line.

The activation of the ERK1/2 (MAPK), Akt and PKC pathways has been shown to be related to cancer progression in several cancer models (Henttu and Vihko, 1998; Tanaka et al., 2003; Ghosh et al., 2005; Gonzalez-Guerrico et al., 2005; Gao et al., 2006; McCubrey et al., 2006; Mulholland et al., 2006; Xin et al., 2006; Camina et al., 2007a; Camina et al., 2007b; Fabregat et al., 2007; McCubrey et al., 2007; Pazos et al., 2007; Roberts and Der, 2007; Shen and Abate-Shen, 2007; Berry et al., 2008; Grant, 2008; Keyse, 2008; Kinkade et al., 2008; Ali et al., 2009; Alvarez et al., 2009; Antico Arciuch et al., 2009; Tatsuda et al., 2010; Hafeez et al., 2011; Toton et al., 2011), including in advanced prostate cancer (Cornford et al., 1999; Ghosh et al., 2003; Gao et al., 2006; Kinkade et al., 2008; Ishiguro et al., 2009; Ishiguro et al., 2011). Increased levels of ERK1/2 activation have been reported in a number of androgen-independent, metastatic late-stage prostate cancers, suggesting a critical role for the MAPK signal transduction pathway in malignant transformation (Price et
Akt has often been implicated in prostate cancer disease and studies in prostate cancer cell lines revealed that Akt activation is important for the progression of prostate cancer to a castrate-resistant state (Ghosh et al., 2003; Ghosh et al., 2005; Gao et al., 2006; Mulholland et al., 2006; Shen and Abate-Shen, 2007; Chen et al., 2008b; Kinkade et al., 2008) and poorly differentiated tumours exhibit increased expression of a phosphorylated Akt compared to normal tissue, prostatic intraepithelial neoplasia or well-differentiated prostate cancer (Ghosh et al., 2003).

This is the first study to demonstrate that obestatin may act as a growth factor in prostate cancer cells and could play some part in the pathogenesis of the disease. Obestatin has been shown to increase cell proliferation in a number of cell lines, but decreases cell proliferation in the BON-1 pancreatic neuroendocrine tumour cell line (Volante et al., 2009) and in the human C28-I2 rib chondrocyte cell line (Lago et al., 2007). Exogenous obestatin treatments stimulate cell proliferation in the castrate-resistant PC3 prostate cancer cell line, but not in the RWPE-1 transformed normal prostate cell line, and acts through the ERK1/2 and Akt signalling pathways. Although obestatin stimulates PKC phosphorylation, it is unclear if this pathway mediates cell proliferation in response to obestatin. Obestatin may exert a mitogenic effect in castrate resistant prostate cancer and may stimulate tumour progression. Studies using a wider range of cell lines and other functional in vitro assays such as migration, invasion and apoptosis assays as well as in vivo assays are required to evaluate obestatin’s potential role in prostate cancer progression, however. Further studies using cell lines that do not express the GPR39 receptor (eg 22Rv1 cells) or siRNA GPR39 knockdown would clarify whether this receptor is involved in obestatin signaling to promote cell proliferation. Furthermore, the identification of the receptor for obestatin and a better understanding of the mechanism of action of obestatin should facilitate studies into the role of obestatin in prostate cancer. To date, it remains unclear whether obestatin plays any role in metabolism, however, in this study obestatin stimulated an approximately 30% increase in cell proliferation with the WST-1 metabolic assay, while only a 12-14% increase with the DNA based CyQUANT assay in the PC3 prostate cancer cell line. The use of DNA synthesis inhibitors would be helpful to clarify whether the results seen in his study are due to
an increase in cell proliferation only or whether obestatin is stimulating metabolism rate as well as cell proliferation in the PC3 prostate cancer cell line. Although PC3 cells respond to exogenous obestatin treatment, its endogenous obestatin levels remain currently unknown. Current techniques are limiting and unable to distinguish between obestatin and its precursor molecule, proghrelin. Development of standard mass spectrophotometry assays would be an important tool to assess endogenous obestatin levels in cell lines and tissues. Quantitative expression studies, in a larger number of clinical samples, would be useful in assessing the potential clinical significance of obestatin expression in prostate cancer and could possibly reveal its potential as a diagnostic and therapeutic target.
Chapter 4

The Effects of Ghrelin and Desacyl Ghrelin on Prostate Cancer Cell Line Proliferation
INTRODUCTION

Ghrelin is a 28 amino acid peptide hormone, derived from the preproghrelin precursor protein (Kojima et al., 1999) and well known for its functions in appetite, gut motility and GH release (Kojima et al., 1999; Wren et al., 2001). It possesses an unusual post-translational modification, an octanoylation in the third serine residue, which is necessary for binding and activating its only known functional receptor, the GHS-R1a (Bednarek et al., 2000; Kojima et al., 2001b; Muccioli et al., 2002; Torsello et al., 2002; Broglia et al., 2006; Hosoda et al., 2006). Another ghrelin receptor isoform has been described, the GHS-R1b isoform, which is a C-terminally truncated splice variant of the GHS-R gene (Howard et al., 1996; McKee et al., 1997a) and is a non-functional ghrelin receptor (Howard et al., 1996; Kojima et al., 1999). Ghrelin circulates in the plasma in two major forms: octanoylated ghrelin (ghrelin) and a non-octanoylated form, termed desacyl ghrelin or unacylated ghrelin. Desacyl ghrelin circulates at much higher concentrations (approx 4-fold) in the plasma than acylated ghrelin (Hosoda et al., 2000a; Broglia et al., 2003b). Although the stomach is the major source of ghrelin (Cowley et al., 2003; Leonetti et al., 2003; Kojima and Kangawa, 2005), both ghrelin and its receptors are widely expressed throughout the body (McKee et al., 1997a; Kojima et al., 1999; Date et al., 2000a; Cassoni et al., 2001; Kojima et al., 2001a; Korbonits et al., 2001a; Barreiro et al., 2002a; Gnanapavan et al., 2002; Jeffery et al., 2002; Tena-Sempere et al., 2002; Volante et al., 2002a; Volante et al., 2002b; Wierup et al., 2002; Cowley et al., 2003; Gaytan et al., 2003; Volante et al., 2003; Iwase et al., 2004; Rindi et al., 2004; Kojima and Kangawa, 2005; Raghay et al., 2006; Cruz and Smith, 2008; Soares et al., 2008) and ghrelin can also be locally produced in many peripheral tissues (Jeffery et al., 2002; Yeh et al., 2005; D’Onghia et al., 2007; Wang et al., 2007b). Ghrelin’s functions have been extensively investigated and it has been shown to exert a range of physiological roles (Kojima et al., 1999; Nagaya et al., 2001c; Weikel et al., 2003; Broglia et al., 2005; Heijboer et al., 2006; Charoenthongtrakul et al., 2009; Miegueu et al., 2011) (Table 1.2; Figures 1.4 &1.5). Although desacyl ghrelin was originally thought to be inactive, as it does not bind to or activate GHS-R1a (Kojima et al., 1999; Hosoda et al., 2000a; Torsello et al., 2002; Broglia et al., 2003b), recent studies have shown that ghrelin and desacyl ghrelin share some functions (Delhanty et al., 2006; Sato et al., 2006; Filigheddu et al., 2007; Granata et
al., 2007), as well as exert some opposing roles (Gauna et al., 2005; Qader et al., 2008; Hirayama et al., 2010; Lear et al., 2010) (Table 1.2). This suggests the existence of at least one unidentified receptor which is shared by both ghrelin and desacyl ghrelin (Baldanzi et al., 2002; Delhanty et al., 2006; Gauna et al., 2006; Delhanty et al., 2007; Granata et al., 2007; Thielemans et al., 2007; Lear et al., 2010).

The ghrelin axis has also been shown to play a role in pathological conditions. Ghrelin levels are inversely correlated with BMI, being elevated in anorexia nervosa (Otto et al., 2001; Cuntz et al., 2002; Shiiya et al., 2002; Kojima et al., 2004) and cachexia (Nagaya et al., 2001b; Otto et al., 2001; Yoshimoto et al., 2002; Shimizu et al., 2003; Tacke et al., 2003) and generally decreased in obese patients (Ravussin et al., 2001; Tschop et al., 2001b; Muccioli et al., 2002; Shiiya et al., 2002; Bunt et al., 2003; Krsek et al., 2003; Tritos et al., 2003; Helmling et al., 2004; Yildiz et al., 2004; Zorrilla et al., 2006), with the exception of obese patients with Prader Willi Syndrome (Nicholls and Knepper, 2001; Cummings et al., 2002; Goldstone et al., 2004). Ghrelin also has a protective effect in chronic heart failure (Nagaya et al., 2001a; Nagaya et al., 2001c; Nagaya et al., 2004) and inflammation (Smith et al., 2005; Hattori, 2009; Taub et al., 2010) and it improves cardiac function and regulates the immune response. Furthermore, ghrelin and its receptors are expressed in malignant tissues and cancer cell lines and have been shown to be locally produced in tumours (Table 1.3). Ghrelin is involved in a number of processes that are relevant to cancer development and progression and could be acting in a paracrine/autocrine manner to drive abnormal tissue growth (Jeffery et al., 2002; Murata et al., 2002; Volante et al., 2003; Nanzer et al., 2004; Jeffery et al., 2005; Yeh et al., 2005; Cassoni et al., 2006; Dixit et al., 2006; Fung et al., 2010)(Table 1.3). Although the possible roles of desacyl ghrelin in cancer have been less extensively explored, some studies showed that both ghrelin and desacyl ghrelin can modulate cell differentiation (Thompson et al., 2004b; Sato et al., 2006; Filigheddu et al., 2007), proliferation and cell survival (Delhanty et al., 2006; Granata et al., 2006; Delhanty et al., 2007; Granata et al., 2007). The effects of ghrelin and desacyl ghrelin in cell proliferation seem to be mediated through activation of ERK1/2 and PI3K/Akt pathways (Delhanty et al., 2006; Granata et al., 2007), which are known to be involved in cell proliferation and survival (Cairns et al., 1997; Cantley and
Ghrelin has been shown to act as a growth factor in a range of cancer models (Table 1.3). Although ghrelin’s role in prostate cancer remains controversial (Jeffery et al., 2002; Cassoni et al., 2004; Yeh et al., 2005; Diaz-Lezama et al., 2010), we have previously shown that ghrelin and its receptor are present in prostate cancer specimens (Figure 4.1) and cell lines (Yeh et al., 2005) and that ghrelin is locally produced in prostate cells (Jeffery et al., 2002; Yeh et al., 2005) (Figure 4.2 A). Furthermore, prostate cancer cell lines secrete ghrelin into the medium (Jeffery et al., 2002; Yeh et al., 2005) (Figure 4.2 B), and it could act as a paracrine/autocrine growth factor.

In this study, the potential role of desacyl ghrelin as a growth factor in prostate cancer cell lines, and the possible pathways mediating these actions, have been investigated and contrasted with those of ghrelin itself.

![Figure 4.1](image)

**Figure 4.1** Mature ghrelin peptide immunostaining in human prostatectomy tissue specimens. i) negative control using preabsorbed antibody shows no immunoreactivity; ii) low levels of positive ghrelin expression in normal prostate epithelial glands (arrow); iii) ghrelin expression in the glands of a grade 1, well-differentiated prostate cancer specimen (brown staining). Bar, 100 µm (i); 50 µm (ii and iii) (adapted from Yeh et al., 2005).
Figure 4.2 Western immunoblot analysis for detection of ghrelin. A) cell lysates from LNCaP (L), PC3 (P), ALVA41 (A) and DU145 (D) prostate cancer cell lines using anti-human ghrelin antibody showing bands of approximately 3 KDa, the expected size for octanoylated ghrelin (1 μg, lane G) (adapted from (Jeffery et al., 2002); B) detection of ghrelin (3 kDa) in conditioned media from PC3 prostate cancer cells using a human anti-ghrelin antibody showing a protein identical in size to a band with positive control, 1,000 nmol/L synthetic n-octanoylated ghrelin (+). No band was detected in serum-free RPMI 1640, which was used as a negative control (-) (adapted from Yeh et al., 2005).

4.1 MATERIALS AND METHODS
General Materials and Methods are outlined in detail in Chapter 2. Experimental procedures which are specific to this chapter are described below.

4.1.1 Cell culture
Cells were maintained in culture medium, as described in Section 2.3.1. The PC3, DU145, LNCaP and 22Rv1 prostate cancer cell lines and RWPE-1 and RWPE-2 transformed normal prostate cell lines were investigated for the presence of the ghrelin receptors, GHSR-1a and GHSR-1b. The PC3 androgen-independent, prostate cancer cell line (derived from a metastatic tumour) and the RWPE-1 transformed normal cell line were selected to represent a model of advanced prostate cancer compared to a normal model of prostate cells. Both cell lines were treated with exogenous ghrelin and desacyl ghrelin with and without pathway inhibitors and used in functional and signaling assays.
4.1.2 RNA extraction and GHS-R1a and GHS-R1b RT-PCR

RNA extraction of the cell lines PC3, DU145, 22Rv1, LNCaP, RWPE-1 and RWPE-2 was performed as previously described (see Section 2.6). PCR primers (Table 4.1) for the GHS-R1a and the GHS-R1b transcripts were described previously (Jeffery et al., 2002).

Table 4.1 PCR primer sequences for GHS-R. A = sense primer, AS = antisense primer.

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence 5’→3’</th>
<th>Size(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHS-R1a</td>
<td>S accaccaacctctacctgtc</td>
<td>435</td>
</tr>
<tr>
<td></td>
<td>AS gaagaagatgtggacaccc</td>
<td></td>
</tr>
<tr>
<td>GHS-R1b</td>
<td>S ttctctctctctttgtg</td>
<td>179</td>
</tr>
<tr>
<td></td>
<td>AS gataggacccgcagagaaa</td>
<td></td>
</tr>
</tbody>
</table>

Reverse transcription and PCR were performed as previously described (see Sections 2.7 & 2.8), in a 50µL reaction using 1µL template cDNA or sterile distilled water (no template negative control) with 1U Platinum Taq. In order to confirm the purity and quality of the cDNA, reactions for the housekeeping gene β-actin were performed as a positive control (Table 3.1) and no RT-controls were performed to test for genomic DNA (gDNA) contamination, critical for the detection of the GHS-R1b, which contains intronic sequence. Thermal cycling (PTC-200 Thermal Cycler, MJ Research, Watertown, USA) consisted of an initial denaturation of 2 minutes at 94°C, followed by 35 cycles of 10 seconds at 94°C for denaturation, 30 seconds at 62°C for annealing, 1 minute at 72°C for extension and a final extension for 10 min at 72°C. PCR products were electrophoresed and images were captured as described in Section 2.8.1. Only one band of the expected size resulted from the reaction (Table 4.1). The PCRs were purified and sequenced to confirm the specificity of the reaction according to Section 2.8.2.

4.1.3 Cell proliferation assays

Proliferation assays (WST-1 and CyQUANT) were performed on PC3 and RWPE-1 prostate cell lines as previously described (see Section 2.4). Both methods were
performed concurrently with different concentrations of ghrelin and desacyl ghrelin (0-1000nM). Cells were seeded in 200µL medium at the optimal cell number (5000 cells/well for PC3 and 12000 cells/well for RWPE-1) in 96-well microplates and allowed to attach overnight. After 24 hours, medium was replaced and treatments were added. The cells were treated for 72 hours and fresh treatments were added every 24 hours. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO₂. 24 hours after the last treatment, the rate of cell proliferation was measured using the WST-1 or CyQUANT reagents according to the manufacturer’s instructions (see Section 2.4). Each assay was performed with 16 replicates for each concentration and each experiment repeated independently at least 3 times. Statistical analysis was performed as described in Section 2.9.

4.1.4 Signaling assays for MAPK and Akt pathways
Signaling assays were performed on PC3 cell line lysate as described above in Section 2.5. PC3 cells were treated with a range of concentrations of ghrelin and desacyl ghrelin (0, 1, 10, 100, and 1000nM) in phenol red-free 1640 RPMI in serum free conditions for 5, 15, 30, and 60 minutes at 37°C in a humidified atmosphere containing 5% CO₂. As a positive control, PC3 cells were also treated with 10% foetal bovine serum (FBS) for 15 minutes. Cell lysates were collected using a standard ice-cold lysis buffer according to Section 2.5.1 and quantified using BCA protein assay according to Section 2.5.2. Protein samples were resolved in a SDS-PAGE gel (see Section 2.5.3), transferred to a nitrocellulose membrane, and Western immunoblotting was performed as described in Section 2.5.4. Membranes were incubated with antibodies to detect phosphorylated (p)Akt, total (t)Akt, pERK 1/2, tERK 1/2 and GAPDH (Table 3.2) overnight at 4°C. After four brief washes in 0.05% TBST, the membranes were incubated with the horseradish peroxidase (HRP) conjugated secondary antibody (Table 3.2) at room temperature for 1 hour. After washing, chemiluminescent SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Thermo Scientific, Melbourne, Australia) was applied and the membrane was exposed to X-ray film and developed. Densitometry and statistical analyses were performed as described in Sections 2.5.5 and 2.9, respectively.

4.1.5 Signaling inhibition
To investigate whether ghrelin and desacyl ghrelin-induced phosphorylation was
specific to the pathways studied, previously selected and optimised concentrations of the ERK1/2 inhibitor, U0126, and Akt 1/2 kinase inhibitor were employed (see Section 3.1.5). PC3 cells were plated in six-well plates and at 70% to 80% confluency were serum starved overnight. Cells were pre-treated for 30 minutes with either 10µM U0126 or 10µM Akt1/2 kinase inhibitor, then stimulated with maximally-active doses of either ghrelin (100nM) or desacyl ghrelin (10nM) for 15 minutes. Control incubations included no treatment (culture medium only), vehicle (DMSO, Sigma), inhibitor alone, or 10% FBS. Each experiment was performed at least 3 independent times. Statistical analysis was performed as described in Section 2.9.

4.1.6 Cell proliferation inhibition assays
To investigate whether the effect of ghrelin and desacyl ghrelin-induced on cell proliferation was related to the activation of the ERK1/2 or Akt pathways, cell proliferation assays were also performed in the presence of U0126 or Akt1/2 kinase inhibitor. These assays were performed at the same concentrations that were shown to inhibit phosphorylation using Western immunoblot analysis. The assays were performed as previously described (see Section 3.1.6) and the cells were incubated either with 10µM U0126 or 10µM Akt1/2 kinase inhibitor, with or without ghrelin or desacyl ghrelin (100 and 1000nM). WST-1 assays were performed to assess cell viability and cell proliferation rate. At least three independent experiments were performed with eight replicates for each treatment. Statistical analysis was performed as described in Section 2.9.

4.1.7 Cell proliferation assays performed using the GHS-R1a inverse agonist, D-Lys³-GHRP-6
To investigate whether the ghrelin and desacyl ghrelin-induced effect on proliferation was occurring through the GHS-R1a, cell proliferation assays were also performed in the presence of the GHS-R1a inverse agonist, D-Lys³-GHRP-6 (Chan et al., 2004). The assays were performed as described above (see Section 4.1.6) and the cells were incubated with a combination of either ghrelin or desacyl ghrelin at 100 and 1000nM, with or without a range of D-Lys³-GHRP-6 concentrations (0.1µM-1µM) (Demers et al., 2009). WST-1 assays were performed to assess cell viability and cell proliferation rate. Three independent experiments were performed
with eight replicates for each treatment. Statistical analysis was performed as described in Section 2.9.
4.2 RESULTS

4.2.1 GHS-R1a and GHS-R1b mRNA expression in prostate-derived cell lines and normal prostate

To investigate the expression of the two known ghrelin receptor isoforms in the PC3, RWPE-1, LNCaP, RWPE-2, DU145, and 22Rv1 prostate cell lines, RT-PCR was performed using specific primers to GHS-R1a and GHS-R1b transcripts. The reactions resulted in products of the expected size (345bp for GHS-R1a and 179bp for GHS-R1b) in all cell lines examined (Figure 4.3A & B). The GHS-R1a and 1b bands were sequenced from the positive control samples to verify their identity (data not shown). All samples were positive for β-actin (243bp) (Figure 4.3C) confirming the integrity of the cDNA used.

4.2.2 Exogenous ghrelin and desacyl ghrelin stimulate cell proliferation in the PC3 prostate cancer cell line

The PC3 prostate cancer cell line, a model of advanced prostate cancer, and the RWPE-1 transformed normal prostate cell line were used in the two cell proliferation assays, WST-1 (metabolic assay) and CyQUANT (DNA based assay) with the addition of a range of concentrations of either ghrelin or desacyl ghrelin (0-1000nM). PC3 cells treated with ghrelin or desacyl ghrelin showed a significant increase in cell proliferation at 1000nM compared with untreated controls using both methods (p<0.05) (Figures 4.4 & 4.5). In the PC3 cell line, ghrelin significantly stimulated cell proliferation in a dose-dependent manner with WST-1 assay (p<0.05) showing statistically significant increases of 31.66±6.68% (p<0.01) above untreated control at 1000nM, 15.65±2.03% (p<0.05) at 100nM, and 12.49±3.04% (p<0.05) at 10nM (Figure 4.4A). Using the CyQUANT assay, 1000nM ghrelin significantly stimulated a 13.55±5.68% (p<0.05) increase in cell proliferation, in the PC3 cell line, when compared to the untreated control (p<0.05) (Figure 4.4B). Desacyl ghrelin also stimulated cell proliferation in a dose-dependent manner in the PC3 cell line, although significant levels were only achieved at 1000nM with both WST-1 (21.73±2.62%, p<0.01) and CyQUANT (15.46±7.05%, p<0.05) methods (Figure 4.5). No significant changes were seen in RWPE-1 transformed normal prostate cells after 72 hours of treatment with a range of concentrations (0-1000nM) of either ghrelin or desacyl ghrelin (Figures 4.6 & 4.7).
Figure 4.3 Ethidium bromide-stained agarose gels showing mRNA expression of GHS-R1a and GHS-R1b in prostate cell lines. Total RNA was extracted and used for RT-PCR using specific primers for the GHS-R1a (A), GHS-R1b (B) and β-actin (C) transcripts. Amplicons of the correct size were observed (345bp for GHS-R1a, 179bp for GHS-R1b and 243bp for β-actin) and no product was generated in the no template negative control (-ve). GHS-R1a and GHS-R1b constructs were used as positive controls (+ve). A) GHS-R1a and B) GHS-R1b are expressed in PC3, RWPE-1, LNCaP, RWPE-2, DU145 and 22Rv1 prostate cell lines C) β-actin screening confirmed purity and quality of the cDNA.
Figure 4.4 Exogenously applied ghrelin increases cell proliferation in the PC3 prostate cancer cell line in a dose-dependent manner. PC3 cells were treated with ghrelin (0-1000nM) for 72 hours and cell proliferation rate was measured with WST-1 and CyQUANT assays. Ghrelin significantly increased cell proliferation in PC3 cells at 10, 100 and 1000nM using a WST-1 assay (p<0.05) (A) and 1000nM using a CyQUANT assay (p<0.05) (B). Data are expressed as percentage above control (% above control) and represent the mean ± SEM of three independent experiments with 16 replicates for each ghrelin concentration in a single experiment. Statistical analysis was performed on combined data. *p<0.05 and **p<0.01 compared with untreated controls (one-way ANOVA with Tukey’s test post hoc analysis).
Figure 4.5 Desacyl ghrelin increases cell proliferation in the PC3 prostate cancer cell line. PC3 cells were treated with desacyl ghrelin (0-1000nM) for 72 hours and cell proliferation rate was measured with WST-1 and CyQUANT assays. Desacyl ghrelin significantly increased cell proliferation in the PC3 prostate cancer cells with both WST-1 (A) and CyQUANT (B) methods at 1000nM (p<0.05). Data are expressed as percentage above control (% above control) and represent the mean ± SEM of three independent experiments with 16 replicates for each desacyl ghrelin concentration in a single experiment. Statistical analysis was performed on combined data. *p<0.05 and **p<0.01 compared with untreated controls (one-way ANOVA with Tukey’s test post hoc analysis).
Figure 4.6 Ghrelin had no effect on cell proliferation in the RWPE-1 transformed normal prostate cell line. RWPE-1 cells were treated with ghrelin (0-1000nM) for 72 hours and cell proliferation rate was measured with WST-1 and CyQUANT assays. There were no significant changes with any ghrelin concentration with either WST-1 (A) or CyQUANT (B) methods. Data are expressed as percentage above control (% above control) and represent the mean ± SEM of three independent experiments with 16 replicates for each ghrelin concentration in a single experiment. Statistical analysis was performed on combined data by one-way ANOVA with Tukey’s test post hoc analysis.
Figure 4.7 Desacyl ghrelin had no effect on RWPE-1 transformed normal prostate cell line proliferation. RWPE-1 cells were treated with desacyl ghrelin (0-1000nM) for 72 hours and cell proliferation rate was measured with WST-1 and CyQUANT assays. There were no significant changes with any desacyl ghrelin concentration with either WST-1 (A) or CyQUANT (B) methods. Data are expressed as percentage above control (% above control) and represent the mean ± SEM of three independent experiments with 16 replicates for each desacyl ghrelin concentration in a single experiment. Statistical analysis was performed on combined data by one-way ANOVA with Tukey’s test post hoc analysis.
4.2.3 Activation of Akt and ERK1/2 by ghrelin and desacyl ghrelin in the PC3 prostate cancer cell line

PC3 cells were treated with either ghrelin (0-1000nM) or desacyl ghrelin (0-1000nM) for 0, 5, 15, 30 and 60 minutes to investigate the signaling pathways that could be involved in the mitogenic effect of these hormones in the PC3 prostate cancer cell line. Western blot analysis revealed a significant increase in Akt and ERK1/2 phosphorylation at 10nM (3.90±2.61 fold, p<0.05), 100nM (4.04±2.66 fold, p<0.05) and 1000nM (3.46±1.88 fold, p<0.05) ghrelin for Akt (p<0.05) (Figure 4.8A) and 10nM (2.33±0.18 fold, p<0.05) and 100nM (2.71±0.18 fold, p<0.01) ghrelin for ERK1/2 (p<0.05) (Figure 4.8C). Akt activation peaked at 15 minutes and remained 30 minutes after treatment but decreased 60 minutes after treatment (p<0.01) (Figure 4.8B) while ERK1/2 activation peaked at 10 minutes and no phosphorylation was seen 30 minutes after ghrelin stimulation (p<0.01) (Figure 4.8D).

Desacyl ghrelin significantly stimulated Akt phosphorylation at all concentrations used - 1nM (2.39±0.41 fold, p<0.05), 10nM (2.43±0.45 fold, p<0.05), 100nM (2.61±0.68 fold, p<0.05) and 1000nM (2.51±0.31 fold, p<0.01) (Figure 4.9A). Desacyl ghrelin treatment also stimulated ERK1/2 phosphorylation at 1nM (3.10±0.27 fold, p<0.01), 10nM (4.11±0.27 fold, p<0.01), 100nM (3.10±0.10 fold, p<0.01) and 1000nM (3.43±0.20 fold, p<0.01) (Figure 4.9C). Akt activation peaked at 15 minutes, and was still statistically significant 30 minutes after treatment but decreased 60 minutes after treatment (p<0.05) (Figure 4.9B), while ERK1/2 activation peaked at 10 minutes and no phosphorylation was seen 30 minutes after stimulation with desacyl ghrelin (p<0.01) (Figure 4.9D).
Figure 4.8 Ghrelin stimulates Akt and ERK1/2 phosphorylation in the PC3 prostate cancer cell line. PC3 cells were serum starved overnight then stimulated with ghrelin (0-1000nM) in serum free conditions for 0, 5, 10, 15, 30, and 60 minutes. Ghrelin significantly stimulated Akt (A) and ERK1/2 (C) phosphorylation at 10 and 100nM in the PC3 prostate cancer cell line (p<0.05, compared to untreated control). Akt activation peaked at 15 minutes and remained high 30 minutes after treatment but decreased after 60 minutes (p<0.01) (B) while ERK1/2 phosphorylation peaked at 15 minutes and no phosphorylation was seen 30 minutes after treatment (p<0.01) (D). Phosphorylated protein was corrected for protein loading by normalising to GAPDH. Blots are representative of three independent identical experiments. Data are expressed as fold change compared to untreated control and represent the mean densitometry ± SEM of three independent experiments. Statistical analysis was performed on combined data. *p<0.05 and **p<0.01 compared with untreated controls (one-way ANOVA with Tukey’s test post hoc analysis). 0nM ghrelin = untreated control; 10% FBS stimulation for 15 mins was used as a positive control.
Figure 4.9 Desacyl ghrelin stimulates Akt and ERK1/2 phosphorylation in the PC3 prostate cancer cell line. PC3 cells were serum starved overnight then stimulated with desacyl ghrelin (0-1000nM) in serum free conditions for 0, 5, 10, 30, and 60 minutes. Desacyl ghrelin significantly stimulated Akt (p<0.05, compared to untreated control) (A) and ERK1/2 (p<0.01, compared to untreated control) (C) phosphorylation in the PC3 prostate cancer cell line. Akt activation peaked at 15 minutes and remained high 30 minutes after treatment but decreased after 60 minutes (p<0.05) (B) while ERK1/2 phosphorylation peaked at 15 minutes and no phosphorylation was seen 30 minutes after treatment (p<0.01) (D). Phosphorylated protein was corrected for protein loading by normalising to GAPDH. Blots are representative of three independent identical experiments. Data are expressed as fold change compared to untreated control and represent the mean densitometry ± SEM of three independent experiments. Statistical analysis was performed on combined data. *p<0.05 and **p<0.01 compared with untreated controls (one-way ANOVA with Tukey’s test post hoc analysis). 0nM desacyl ghrelin = untreated control; 10% FBS stimulation for 15 minutes was used as a positive control.
4.2.4 Ghrelin and desacyl ghrelin stimulate cell proliferation in the PC3 prostate cancer cell line through phosphorylation of Akt and ERK1/2

Ghrelin and desacyl ghrelin showed a significant increase in PC3 prostate cancer cell line proliferation at 1000nM (p<0.05) with both WST-1 and CyQUANT assays (p<0.05) (Figures 4.4 & 4.5) and significantly increased Akt and ERK1/2 phosphorylation (p<0.05) (Figures 4.8 & 4.9). To assess whether Akt and ERK1/2 activation were responsible for the mitogenic effects seen in the cell proliferation assay, specific inhibitors for Akt and ERK1/2 were employed. Using Western blot analysis, ghrelin and desacyl ghrelin significantly stimulated Akt and ERK1/2 but pre-treatment with either Akt kinase inhibitor or U0126 completely abolished ghrelin- and desacyl ghrelin-induced phosphorylation of Akt and ERK1/2 (Figures 4.10 & 4.11). To further investigate whether ghrelin and desacyl ghrelin-induced Akt and ERK1/2 activation was related to their mitogenic effect in the PC3 prostate cancer cell line, cell proliferation assays using the WST-1 reagent were performed in the presence of either ghrelin or desacyl ghrelin ± Akt1/2 kinase inhibitor (Figure 4.12) or U0126 (Figure 4.13). Inhibitor or vehicle alone (DMSO) had no significant effect compared to the 0nM control, showing that the cells were viable at the inhibitor concentration employed (Figures 4.12 & 4.13). Ghrelin and desacyl ghrelin treatment significantly stimulated proliferation at 1000nM (over 15% above control and vehicle) during 72 hours (p<0.01). When combined with 1000nM ghrelin or desacyl ghrelin treatments, Akt1/2 kinase inhibitor completely prevented the stimulation of proliferation seen in cells treated with 1000nM ghrelin or desacyl ghrelin alone (p<0.01) (Figure 4.12). Treatment with U0126 completely prevented ghrelin-induced cell proliferation (1000nM ghrelin, p<0.01) (Figure 4.13A), but not cell proliferation in response to 1000nM desacyl ghrelin (Figure 4.13B), suggesting that these different forms of ghrelin could be acting through different signaling mechanisms to stimulate cell proliferation in the PC3 prostate cancer cell line. These results suggest that ghrelin- and desacyl ghrelin-induced cell proliferation is mediated through activation of Akt and ERK1/2 in the PC3 prostate cancer cell line, although the signaling mechanisms of these two peptide hormones might differ to each other.
Figure 4.10 Ghrelin activates ERK1/2 and Akt pathways in PC3 prostate cancer cell lines. PC3 cells were serum starved overnight then pre-treated for 30 minutes with either 10µM Akt 1/2 kinase inhibitor (A) or 10µM U0126 (B) then stimulated with 100nM ghrelin for 10 minutes. Ghrelin significantly stimulated Akt (A) and ERK1/2 (B) phosphorylation (p<0.01, compared to untreated control and vehicle) and this effect was completely abolished when combined with Akt 1/2 kinase inhibitor (A) or U0126 (B). Phosphorylated protein was corrected for protein loading by normalising to GAPDH. Blots are representative of three independent identical experiments. Data are expressed as fold change compared to untreated control and represent the mean densitometry ± SEM of three independent experiments. Statistical analysis was performed on combined data. a = p<0.01 compared to untreated control, b = p<0.01 compared to vehicle, c = p<0.05 compared to vehicle and d = p<0.01 compared to 100nM ghrelin (one-way ANOVA with Tukey’s test post hoc analysis). Vehicle = DMSO; G = ghrelin; Akt ½ Inh = Akt 1/2 kinase inhibitor; 10% FBS was used as a positive control.
Figure 4.11 U0126 and Akt1/2 kinase inhibitor prevent desacyl ghrelin-induced phosphorylation of ERK1/2 and Akt pathways in the PC3 prostate cancer cell line, respectively. PC3 cells were serum starved overnight then pre-treated for 30 minutes with either 10µM Akt 1/2 kinase inhibitor (A) or 10µM U0126 (B) then stimulated with 10nM desacyl ghrelin for 15 minutes. Desacyl ghrelin significantly stimulated Akt (A) and ERK1/2 (B) phosphorylation (p<0.05, compared to untreated control and vehicle) and this effect was completely abolished when combined with Akt 1/2 kinase inhibitor (A) or U0126 (B). Phosphorylated protein was corrected for protein loading by normalising to GAPDH. Blots are representative of three independent identical experiments. Data are expressed as fold change compared to untreated control and represent the mean densitometry ± SEM of three independent experiments. Statistical analysis was performed on combined data. a = p<0.05 compared to untreated control, b = p<0.01 compared to untreated control, c = p<0.05 compared to vehicle, d = p<0.01 compared to vehicle, e = p<0.01 compared to 10nM desacyl ghrelin (one-way ANOVA with Tukey’s test post hoc analysis). Vehicle = DMSO; DesG = desacyl ghrelin; Akt ½ Inh = Akt 1/2 kinase inhibitor; 10% FBS was used as a positive control.
**Figure 4.12** Inhibition of ghrelin and desacyl ghrelin-induced proliferation in the PC3 prostate cancer cell line by Akt 1/2 kinase inhibitor. PC3 cells were treated with either ghrelin (A) or desacyl ghrelin (B) at 100 and 1000nM ± 10µM Akt 1/2 kinase inhibitor for 72 hours. Cell viability and proliferation rate were measured with WST-1 assays. Both ghrelin (A) and desacyl ghrelin (B) significantly increased cell proliferation in the PC3 prostate cancer cell line at 1000nM (p<0.01, compared to untreated control and vehicle) and this effect was completely abolished when combined with Akt 1/2 kinase inhibitor. Untreated control (0nM G or 0nM DesG), Vehicle (DMSO) and inhibitor alone were used as controls. Data are expressed as percentage above control (% above control) and represent the mean ± SEM of three independent experiments with eight replicates for each treatment in a single experiment. Statistical analysis was performed on combined data. a = p<0.01 compared to untreated control, b = p<0.01 compared to vehicle and c = p<0.01 compared to 1000nM ghrelin (A) or 1000nM desacyl ghrelin (B) (one-way ANOVA with Tukey’s test *post hoc* analysis). G = ghrelin; DesG = desacyl ghrelin; Inh = Akt 1/2 kinase inhibitor.
Figure 4.13 Inhibition of ghrelin and desacyl ghrelin-induced proliferation in the PC3 prostate cancer cell line using U0126. PC3 cells were treated with either ghrelin (A) or desacyl ghrelin (B) at 100 and 1000nM ± 10µM U0126 for 72 hours. Cell viability and proliferation rate were measured with WST-1 assays. Both ghrelin (A) and desacyl ghrelin (B) significantly increased cell proliferation in the PC3 prostate cancer cell line at 1000nM (p<0.01, compared to untreated control and vehicle) and this effect was completely abolished when ghrelin was combined with U0126 (A) but not when desacyl ghrelin was combined with U0126 (B) suggesting that ghrelin and desacyl ghrelin could be acting through different mechanisms to stimulate cell proliferation in the PC3 prostate cancer cell line and that desacyl ghrelin effects could be occurring independently of the MAPK pathway. Untreated control (0nM G or 0nM DesG), Vehicle (DMSO) and inhibitor alone were used as controls. Data are expressed as percentage above control (% above control) and represent the mean ± SEM of three independent experiments with eight replicates for each treatment in a single experiment. Statistical analysis was performed on combined data. a = p<0.01 compared to untreated control, b = p<0.01 compared to vehicle, c = p<0.01 compared to 1000nM ghrelin (A) or 1000nM desacyl ghrelin (B) (one-way ANOVA with Tukey’s test post hoc analysis). G = ghrelin; DesG = desacyl ghrelin; Inh = U0126.
4.2.5 Cell proliferation assays using the GHS-R1a receptor inverse agonist

In order to determine if ghrelin stimulated cell proliferation through the GHS-R1a and to demonstrate that desacyl ghrelin was likely to be acting through an alternative ghrelin receptor, proliferation assays were performed using treatment with either ghrelin and desacyl ghrelin, and 0.1µM D-Lys$^3$-GHRP-6, a well-characterised GHS-R1a inverse agonist (Duxbury et al., 2003; Kim et al., 2005; Gauna et al., 2006; Sibilia et al., 2006b; Delhanty et al., 2007; Rossi et al., 2008; Miegueu et al., 2011; Zhang et al., 2010). PC3 cells were treated with either ghrelin or desacyl ghrelin with and without D-Lys$^3$-GHRP-6 for 72 hours and cell viability and cell proliferation rate were measured using the WST-1 assay. Both ghrelin and desacyl ghrelin significantly stimulated cell proliferation in the PC3 prostate cancer cell line (over 20% above control and vehicle) (p<0.01) (Figure 4.14). Combined treatment with D-Lys$^3$-GHRP-6 inhibited both (100 and 1000nM) ghrelin (Figure 4.14A) and (100 and 1000nM) desacyl ghrelin-induced proliferation (Figure 4.14B) in the PC3 prostate cancer cell line. D-Lys$^3$-GHRP-6 or vehicle alone (DMSO) had no significant effect on PC3 cells in this assay.
A

Ghrelin

% Above Control

0nM G  100nM G  1000nM G  Vehicle  Compound  100nM G + Compound  1000nM G + Compound

B

Desacyl Ghrelin

% Above Control

0nM Des G  100nM Des G  1000nM Des G  Vehicle  Compound  100nM Des G + Compound  1000nM Des G + Compound
Figure 4.14 D-Lys³-GHRP-6, a GHS-R1a inverse agonist, inhibited ghrelin and desacyl ghrelin-induced proliferation in the PC3 prostate cancer cell line. PC3 cells were treated with either ghrelin (A) or desacyl ghrelin (B) at 100 and 1000nM ± 0.1µM D-Lys³-GHRP-6 for 72 hours. Cell viability and proliferation rate were measured with WST-1 assays. Both ghrelin (A) and desacyl ghrelin (B) significantly increased cell proliferation in the PC3 prostate cancer cell line at 1000nM (p<0.01, compared to untreated control and vehicle) and this effect was completely abolished when combined with the GHS-R1a inverse agonist D-Lys³-GHRP-6. Untreated control (0nM G or 0nM DesG), Vehicle (DMSO) and D-Lys³-GHRP-6 were used as controls. Data are expressed as percentage above control (% above control) and represent the mean ± SEM of four independent experiments with eight replicates for each treatment in a single experiment. Statistical analysis was performed on combined data. a = p<0.01 compared to untreated control, b = p<0.01 compared to vehicle and c = p<0.01 compared to 1000nM ghrelin (A) or 1000nM desacyl ghrelin (B) (one-way ANOVA with Tukey’s test post hoc analysis). G = ghrelin; DesG = desacyl ghrelin; Compound = D-Lys³-GHRP-6.
4.3 DISCUSSION

This study has demonstrated that desacyl ghrelin stimulates cell proliferation in prostate cancer cells via stimulated Akt phosphorylation, as does acylated ghrelin, but unlike ghrelin did not stimulate cell proliferation through the ERK1/2 signaling pathway. Recent research exploring the possible roles of desacyl ghrelin changed the initial notion that it is an inactive peptide and raised many questions about the broad range of functions of the ghrelin axis. Although desacyl ghrelin is unable to bind and activate ghrelin’s only known functional receptor, the GHS-R1a (Hosoda et al., 2000a; Broglio et al., 2003b), desacyl ghrelin circulates at higher levels than ghrelin itself and desacyl ghrelin has been shown to exert a role in the cardiovascular system, and on adipogenesis, insulin secretion, cell proliferation and apoptosis (see Section 1.2.3.1; Table 1.2). In some cell types desacyl ghrelin exhibits similar effects to ghrelin (Baldanzi et al., 2002; Muccioli et al., 2004; Thompson et al., 2004b; Delhanty et al., 2006; Granata et al., 2006; Sato et al., 2006; Filigheddu et al., 2007; Granata et al., 2007) and in some cases appears to have opposing actions to the acylated form (Gauna et al., 2005; Qader et al., 2008; Lear et al., 2010).

Ghrelin and its receptors have been shown to be expressed in many cancer tissues and cell lines and to modulate cell growth (Section 1.2.4.4; Table 1.3). Interestingly, recent reports showed that both ghrelin and desacyl ghrelin can act as a growth factor and stimulate proliferation in the SW-13 adrenocortical carcinoma cell line (Delhanty et al., 2007), in HIT-T15 pancreatic β-cells (Granata et al., 2006; Granata et al., 2007), and in human osteoblasts (Delhanty et al., 2006). Ghrelin has previously been shown to be expressed in prostate cancer specimens and cell lines (Jeffery et al., 2002; Cassoni et al., 2004; Yeh et al., 2005) and play a role in cell proliferation. Consequently, it was of interest to investigate whether desacyl ghrelin, the more abundant isoform of ghrelin in the circulation (Hosoda et al., 2000a; Broglio et al., 2003b), could also act as a growth factor in prostate cancer. In this study the potential of ghrelin and desacyl ghrelin as growth factors in prostate-derived cell lines and the possible mechanisms involved were investigated. Using RT-PCR, it was confirmed that both ghrelin receptor mRNA isoforms, GHS-R1a and GHS-R1b, are expressed in the PC3, RWPE-1, LNCaP, RWPE-2, DU145 and 22Rv1, but not in normal prostate. Previous studies showed that ghrelin was expressed in prostate cancer cell lines and exogenous acylated ghrelin treatments...
increased cell proliferation in the PC3 and LNCaP prostate cancer cell lines (Jeffery et al., 2002; Yeh et al., 2005). However, another group reported an inhibitory effect in the DU145 cell line, while a biphasic effect was seen in the PC3 cell line, where low doses stimulated cell proliferation and higher doses inhibited (Cassoni et al., 2004). A more recent study showed that ghrelin inhibited proliferation in the PC3 cell line and increased apoptosis by regulating T-type Ca\textsuperscript{2+} channel expression (Diaz-Lezama et al., 2010). Using functional in vitro assays, in this study it has been demonstrated that exogenous ghrelin significantly increased cell proliferation at 1000nM in the PC3 androgen-independent prostate cancer cell line using two different methods, WST-1 and CyQUANT. A significant effect was seen at lower ghrelin doses (10 & 100nM) with WST-1 metabolic assay, but not with CyQUANT, suggesting that ghrelin could not only be stimulating cell proliferation in the PC3 cell line, but also metabolism. This result could also indicate a difference in sensitivity between the two methods used, however. In addition, desacyl ghrelin also significantly increased cell proliferation at 1000nM in PC3 cells with both methods employed, suggesting that this form of ghrelin could also be playing a role in prostate cancer cell proliferation. No significant effect was seen in the RWPE-1 transformed normal cell line with both forms of ghrelin. Although there is conflicting data on the effect of ghrelin in prostate cancer cell proliferation, it is clear that the ghrelin axis plays a role in prostate cancer growth and further studies are justified.

Transformation of a normal to cancerous cell often results from an imbalance between stimulatory and inhibitory stimuli, leading to hyper-activation or inactivation of kinases and/or their effectors, ultimately resulting in a growth advantage characterised by enhanced proliferation and survival and/or inhibition of apoptosis (Hanahan and Weinberg, 2000; Evan and Vousden, 2001). As previously discussed in Chapter 3, the Akt and MAPK pathways are frequently altered in prostate cancer and could be involved in malignant transformation and progression to castrate-resistant disease (see Section 3.3). In human osteoblasts, ghrelin and desacyl ghrelin have been shown to have mitogenic effects which are mediated by the MAPK and PI3K pathways (Delhanty et al., 2006), while in the HIT-T15 pancreatic β-cells proliferation is mediated through activation of cAMP/PKA, ERK1/2 and PI3K/Akt (Granata et al., 2006; Granata et al., 2007). In this study it was demonstrated that both ghrelin and desacyl ghrelin significantly stimulated Akt and ERK1/2
phosphorylation in the PC3 prostate cancer cell line. Ghrelin activated Akt and ERK1/2 phosphorylation, at the same concentrations that significantly increased cell proliferation in WST-1 metabolic assays (10, 100nM ghrelin). Interestingly, the minimum concentration of desacyl ghrelin necessary to activate Akt and ERK1/2 was 1000-fold less than required to significantly increase cell proliferation. Specific inhibitors for Akt and ERK1/2 pathways were used to investigate whether ghrelin and desacyl ghrelin effects on cell proliferation were directly linked to their induction of Akt and ERK1/2 phosphorylation. Treatment with Akt1/2 kinase inhibitor inhibited ghrelin- and desacyl ghrelin-induced proliferation in PC3 cells. Treatment with U0126 inhibited ghrelin-induced cell proliferation but not desacyl ghrelin-induced proliferation, suggesting that both ghrelin and desacyl ghrelin could act through different signaling pathways (and possibly different receptors) to increase cell proliferation in the PC3 androgen-independent cell line.

As discussed, there is a growing body of evidence that supports the existence of an alternative, yet unidentified ghrelin receptor. This is supported by the finding that although desacyl ghrelin is unable to bind and activate the GHS-R1a, it exerts some relevant physiological roles (see Section 1.2.3.1) (Table 1.2). Moreover, some cell types that do not express the GHS-R1a are responsive to exogenous ghrelin treatment (Baldanzi et al., 2002; Muccioli et al., 2004; Granata et al., 2007; Thielemans et al., 2007). In addition, ghrelin and desacyl ghrelin share common high affinity binding sites in some cell types (Cassoni et al., 2001; Muccioli et al., 2004; Lear et al., 2010), suggesting the existence of at least one as yet unidentified receptor shared by ghrelin and desacyl ghrelin. It has been demonstrated in this study that ghrelin and desacyl ghrelin exert similar effects in the PC3 prostate cancer cell line and both activate Akt and ERK1/2 phosphorylation. To try to determine whether ghrelin’s effects in the PC3 cell line were mediated through the GHS-R1a, functional assays were performed in the presence of D-Lys³-GHRP-6, a GHS-R1a inverse agonist. Surprisingly, co-incubation with D-Lys³-GHRP-6 inhibited proliferation in response to desacyl ghrelin as well as in response to ghrelin. Similarly, ghrelin and desacyl ghrelin were shown to stimulate growth of the SW-13 adrenocortical carcinoma cell line (Delhanty et al., 2007), a cell type that expresses extremely low levels of GHS-R1a mRNA (Barzon et al., 2005). Interestingly, the use of D-Lys³-GHRP-6 in the SW-13 cells also prevented cell proliferation in response to desacyl ghrelin in
addition to the response to acylated ghrelin (Delhanty et al., 2007). A possible explanation for this is that the GHS-R1a inverse agonist D-Lys³-GHRP-6 could be also preventing the activity of the unidentified alternative receptor, as the degree of similarity between these receptors is currently not known. D-Lys³-GHRP-6 is known to be a non-specific inverse agonist for the GHS-R1a and other receptors may also be downregulated by this compound (Schioth et al., 1997; Zizzari et al., 2011). Ghrelin and desacyl ghrelin have also been demonstrated to exert similar effects in the insulinoma cell line, INS-1E (Gauna et al., 2006). Although ghrelin-induced insulin release was completely abolished by D-Lys³-GHRP-6, desacyl ghrelin effects persisted when co-incubated with the GHS-R1a inverse agonist. Interestingly, Lear et al., (2010) reported distinct roles for ghrelin and desacyl ghrelin in the murine cardiomyocyte cell line, HL-1. Moreover, two independent specific binding sites for desacyl ghrelin but not recognised by ghrelin were identified (Lear et al., 2010), suggesting the existence of more than one unidentified receptor. A recent study showed that a fluorescein-labelled ghrelin analogue has higher binding affinity to prostate cancer tissues compared to normal prostate tissue and benign prostatic hyperplasia (Lu et al., 2012) and may be clinically useful as a molecular probe for the diagnosis of prostate cancer (Lu et al., 2012). However, this probe is unlikely to be specific for the GHS-R1a as ghrelin high affinity binding sites have been identified in cell lines that do not express the GHS-R1a (Baldanzi et al., 2002; Thielemans et al., 2007). Identification of ghrelin and desacyl ghrelin alternative receptor(s) and studies using more specific inhibitors for GHS-R1a, or siRNA knockdown of the GHS-R1a, would be useful in clarifying the role of the GHS-R1a in mediating cell proliferation in response to ghrelin treatment in the prostate.

Ghrelin is modified by the addition of an octanoyl group to its third serine residue and this post-translational modification is necessary for binding and activating the GHS-R1a (Bednarek et al., 2000; Kojima et al., 2001b; Muccioli et al., 2002; Torsello et al., 2002; Broglio et al., 2006; Hosoda et al., 2006), while desacyl ghrelin lacks this modification. The enzyme that catalyses this reaction was recently identified to be the ghrelin O-acyltransferase (GOAT) (Gutierrez et al., 2008; Yang et al., 2008a) and it seems to act in concert with prohormone convertases to regulate ghrelin processing and production (Zhu et al., 2006; Takahashi et al., 2009). In addition, it has been recently reported that acyl-protein thioesterase 1 (APT1), an
enzyme present in the gastric mucosa (Sunaga et al., 1995; Shanado et al., 2004), liver (Sugimoto et al., 1996) and serum (Satou et al., 2010), is able to de-acylate ghrelin (Satou et al., 2010) and could be regulating the ghrelin/desacyl ghrelin ratio. Other enzymes which de-acetylate ghrelin in the circulation have also been described (De Vriese et al., 2004; De Vriese et al., 2007; Satou et al., 2010; Satou et al., 2011). Although ghrelin and desacyl ghrelin promote growth in the PC3 prostate cancer cell line, ghrelin serum levels did not significantly differ in a study which compared patients with BPH and prostate cancer (Mungan et al., 2008). However, another study that differentiated between acylated and unacylated ghrelin serum levels, reported that the ratio between acylated/unacylated ghrelin was significantly higher in patients with prostate cancer compared to patients with BPH, although total ghrelin levels did not change (Malendowicz et al., 2009). This highlights the importance of a better understanding of the mechanisms that regulate ghrelin and desacyl ghrelin production and secretion. Ghrelin has been shown to be locally produced in a number of tissues and cell types (see Section 1.2.3), including prostate cancer cell lines (Jeffery et al., 2002; Cassoni et al., 2004; Yeh et al., 2005), therefore, ghrelin serum levels might not reflect the local concentrations of ghrelin that the prostate cells are exposed to. Despite progress in trying to elucidate the regulating mechanisms of ghrelin and desacyl ghrelin expression and secretion, recent studies reinforce the view that it is a complicated yet precise process. The mechanisms regulating GOAT expression and therefore ghrelin and desacyl ghrelin expression and production are explored in Chapter 5.

This study has demonstrated that ghrelin and desacyl ghrelin act as growth factors in the PC3 androgen-independent prostate cancer cell line and could act in an autocrine/paracrine manner to promote cell proliferation through activation of Akt and potentially ERK1/2 pathways. Although the GHS-R1a is expressed in PC3 cells, it is unlikely that the mitogenic effects of ghrelin and desacyl ghrelin are mediated through this receptor. Further investigation is needed to clarify whether the GHS-R1a plays a role in ghrelin-induced cell proliferation and signaling. Inhibition of the ghrelin axis could, therefore, be a useful adjuvant approach for inhibiting castrate-resistant prostate cancer progression. Further investigation is needed to gain better understanding of the mechanisms of action of ghrelin and desacyl ghrelin and their potential roles as tissue diagnostic markers for advanced prostate cancer.
Chapter 5

Expression of GOAT and its Regulation by Ghrelin and Desacyl Ghrelin in Prostate Cancer Cell Lines
5 INTRODUCTION

Since its discovery, ghrelin has been the focus of extensive research to explore its diverse functions. More recent research has called attention to its unacylated form, which demonstrates a number of relevant physiological functions (Bedendi et al., 2003; Granata et al., 2006; Delhanty et al., 2007; Granata et al., 2007; Lear et al., 2010; Miegueu et al., 2011) (see Section 1.2.3.1; Table 1.2). Ghrelin is widely expressed throughout the body and circulates in the plasma in two major forms: ghrelin and desacyl ghrelin, although the latter is present in far greater amounts (Hosoda et al., 2000a; Broglio et al., 2003b). Both ghrelin and desacyl ghrelin are derived from a precursor protein, preproghrelin, and are locally produced in many peripheral tissues (Jeffery et al., 2002; Yeh et al., 2005) (see Sections 1.2.1 & 1.2.3). Unacylated ghrelin (or desacyl ghrelin) is post-translationally modified to form acylated ghrelin by the addition of an octanoyl group to its third residue (serine) (Kojima et al., 1999) (Figure 1.6). The enzyme that catalyses this reaction was recently simultaneously identified, by two different groups. This enzyme was the membrane-bound transferase 4 (MBOAT4), a member of the MBOAT family and was renamed ghrelin O-acyltransferase (GOAT) (Gutierrez et al., 2008; Yang et al., 2008a). All members of the MBOAT family are able to transfer organic acids of varying lengths to their substrates, but only GOAT was able to transfer medium-chain fatty acids (MCFAs) and to acylate ghrelin (Gutierrez et al., 2008; Yang et al., 2008a). GOAT is also able to acylate shorter peptides containing the first four or five N-terminal amino acids of ghrelin, which constitutes its substrate recognition site (Yang et al., 2008b; Ohgusu et al., 2009).

In addition to GOAT, prohormone convertases 1/3 (PC1/3), PC2 and furin have been shown to be involved in the processing and production of acylated ghrelin (Zhu et al., 2006; Takahashi et al., 2009). However, expression of these enzymes is not sufficient to produce acylated ghrelin in vitro and addition of n-octanoic acid is crucial for synthesis of its octanoylated form (Takahashi et al., 2009). Another study showed that ingestion of MCFAs and medium-chain triacylglycerols (MCTs) increased circulating levels of acylated ghrelin without changing total ghrelin levels (Nishi et al., 2005). A diet containing heptanoic acid generated heptanoylated ghrelin, a form of ghrelin that is not normally produced (Nishi et al., 2005).
demonstrating that dietary lipids are directly used for ghrelin acylation (Nishi et al., 2005; Kirchner et al., 2009).

Prostate cancer is a multifactorial disease and the large variation in its incidence suggests an important role for environmental or ethnic factors in the development and progression of the disease (see Section 1.1.2). An environmental factor thought to play a significant role in prostate cancer is diet. A distinctive characteristic of the Western diet is a high intake of calories and fat and the Westernisation process may be changing the incidence pattern of prostate cancer and metabolic disorders in many countries (Tchernof et al., 1997; Rosmond et al., 1998; World Health Organisation, 2000) (see Section 1.1.2). The finding that the GOAT-ghrelin system directly uses dietary lipids for ghrelin acylation suggests a link between ingested nutrients and regulation of acylated ghrelin production (Nishi et al., 2005; Kirchner et al., 2009). In this study we have demonstrated that both ghrelin and desacyl ghrelin function as growth factors to stimulate cell proliferation in the PC3 prostate cancer cell line through stimulation of ERK1/2 and Akt pathways. In this chapter we investigate whether prostate-derived tissues and cell lines express the necessary enzymes required for acylated ghrelin production and we investigate whether acylated ghrelin and desacylated ghrelin may regulate GOAT expression in the prostate.

5.1 MATERIALS AND METHODS

General Materials and Methods are outlined in detail in Chapter 2. Experimental procedures which are specific to this chapter are described below.

5.1.1 Statement of Contribution

The practical work described in this Chapter has been produced jointly through a collaboration between the candidate, Laura de Amorim, and Dr Inge Seim. Dr Seim performed the quantitative real-time PCR (Results 5.2.1; 5.2.2; 5.2.5) whereas Laura de Amorim performed all of the cell culture, cell RNA extractions, cell proliferation experiments and cell treatments for real-time PCR (Results 5.2.2; 5.2.3; 5.2.4; 5.2.5).

5.1.2 Cell culture

Cell lines were maintained in culture medium, as described in Section 2.3.1. The LNCaP, DU145, 22Rv1, PC3, RWPE-1 and RWPE-2 prostate cell lines were
investigated for the presence of GOAT, PC1/3, PC2 and furin and treated with octanoic acid to investigate its potential effect on cell proliferation. The LNCaP, DU145 and PC3 prostate cancer cell lines were also treated with ghrelin and desacyl ghrelin to investigate the regulation of GOAT expression.

5.1.3 Non-quantitative RT-PCR for GOAT, PC1/3, PC2 and furin

RNA extraction of the prostate cell lines PC3, DU145, 22Rv1, LNCaP, RWPE-1 and RWPE-2 and cDNA synthesis was performed as previously described (see Sections 2.6 & 2.7). RT-PCRs using primers for GOAT and processing proteases necessary for ghrelin acylation (PC1/3, PC2 and furin) (Takahashi et al., 2009) (Table 5.1) were performed (see Section 2.8) in a 50µL reaction using 1µL template cDNA or sterile distilled water (no template negative control) with 1U Platinum Taq. In order to verify the integrity of the cDNA samples, RT-PCR for the housekeeping gene GAPDH was performed (Takahashi et al., 2009) (Table 5.1). Thermal cycling (PTC-200 Thermal Cycler, MJ Research, Watertown, USA) consisted of an initial denaturation of 2 minutes at 94°C, followed by 35 cycles of 10 seconds at 94°C for denaturation, 30 seconds at 60°C for annealing, 1 minute at 72°C for extension and a final extension for 10 min at 72°C. PCR products were separated by electrophoresis in a 2% TAE agarose gel and images were captured as previously described (see Section 2.8.1).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer 5’→3’</th>
<th>Reverse Primer 5’→3’</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC1/3</td>
<td>catgtggaatcagcaatggt</td>
<td>ctgtgtagccacacagtcaca</td>
<td>599</td>
</tr>
<tr>
<td>PC2</td>
<td>cctggccttgatttgaatgt</td>
<td>gtctgtggctgtgatgtga</td>
<td>588</td>
</tr>
<tr>
<td>Furin</td>
<td>cagaccccaagttcctcag</td>
<td>gcagttgcagtgctagtt</td>
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<td>311</td>
</tr>
<tr>
<td>GAPDH</td>
<td>gcaccgtcaaggtgagaac</td>
<td>atgtggtgaagacgcag</td>
<td>142</td>
</tr>
</tbody>
</table>
5.1.4 Cell proliferation assays

Proliferation assays have been performed using LNCaP, DU145, 22Rv1, RWPE-2, PC3 and RWPE-1 prostate cell lines as previously described (see Section 2.4). Both the WST-1 and CyQUANT methods were performed concurrently with different concentrations of added octanoic acid (0, 0.01, 0.02%) (Takahashi et al., 2009). Cells were seeded in 200µL of medium in the desired cell density (5000 cells/well for LNCaP, DU145 and PC3; 10000 cells/well for 22Rv1; and 12000 cells/well for RWPE-1 and RWPE-2) in 96-well microplates and allowed to attach overnight. After 24 hours, cell medium was replaced and the plates were incubated at 37°C in a humidified atmosphere containing 5% CO₂. The cells were treated for 72 hours and cell proliferation rate was measured using the WST-1 or CyQUANT reagents according to the manufacturer’s instructions (see Section 2.4). Each assay was performed with 16 replicates of each concentration and each experiment repeated independently at least three times. Statistical analysis was performed using a one-way ANOVA followed by Tukey’s test post hoc comparisons as described in Section 2.9.

5.1.5 Ghrelin and desacyl ghrelin treatments

LNCaP, DU145 and PC3 cells were cultured in six-well plates (Nagle Nunc International, Roskilde, Denmark) and when reaching 70% to 80% confluency, cells were serum starved overnight. Cells were treated with ghrelin (0, 100 and 1000nM) and desacyl ghrelin (0, 100 and 1000nM) in phenol red-free 1640 RPMI, in serum-free conditions for 0, 1 and 6 hours at 37°C in a humidified atmosphere containing 5% CO₂. Cells were scraped from plates and pellets stored at -80°C for RNA extraction.

5.1.6 Quantification of GOAT mRNA levels in prostate cancer cell lines by real time RT-PCR

Total RNA was extracted from prostate cancer cells as previously described (see Section 2.6) and 1.5µg of total RNA was reverse transcribed using the SuperArray RT² First Strand Kit (SABiosciences, Frederick, USA). Quantitative real-time RT-PCRs were performed using the AB 7000 sequence detection system (Applied Biosystems, AB, Foster City, USA) in a total reaction volume of 20µL using 2x SYBR green master mix (AB). Primers for GOAT were obtained from SABiosciences and expression levels were normalised to 18S ribosomal RNA.
(primers: forward 5’-TTCGGAACCTGAGGCCATGAT-3’ and reverse 5’-CGAACCTCCGACTTTCTTGTTCT-3’). Fold changes were quantified as $2^{\Delta \Delta C_{\text{t}}}$, as described previously (Livak and Schmittgen, 2001). Statistical data analysis was performed using the Student’s t-test, with duplicates from two independent experiments, or one-way ANOVA with Tukey’s post-hoc analysis for multiple group analysis, with P-value <0.05 considered to be statistically significant.

5.1.7 Quantification of GOAT mRNA levels in tissue specimens
Real-time RT-PCR was used to determine GOAT mRNA levels in samples from tissues of normal prostate and prostate cancer patients using OriGene TissueScan qPCR Prostate Cancer panels (panel II, OriGene, Rockville, USA). The cDNA panels, derived from eight normal prostate and a range of tumour tissue samples, were interrogated by real-time RT-PCR as described above (see Section 5.1.5). Primers for β-actin were supplied with the cDNA panel (OriGene), while the primers for GOAT were supplied by SABiosciences. Each gene was evaluated on separate, identical array plates, which were loaded with equal amounts of cDNA per well, as described by the manufacturer. GOAT expression levels were normalised against β-actin. Fold changes were quantified as $2^{\Delta \Delta C_{\text{t}}}$ (Livak and Schmittgen, 2001) and statistical data analysis was performed as described above (see Section 5.1.5).

5.2 RESULTS
5.2.1 GOAT mRNA is expressed in normal prostate and prostate cancer tissues
Using quantitative real time RT-PCR, normal prostate and prostate cancer tissues were examined for the presence of GOAT mRNA. GOAT is expressed in most normal and cancerous tissues but was not expressed at higher levels in prostate cancer tissue compared to normal prostate specimens, regardless of cancer stage (Figure 5.1).

5.2.2 Quantification of GOAT mRNA expression in prostate cell lines
Having confirmed GOAT mRNA is expressed in prostate tissues (Figure 5.1), three
prostate cancer cell lines - the androgen-dependent LNCaP prostate cancer cell line and the androgen-independent, DU145 and PC3 prostate cancer cell lines - and two transformed normal prostate cell lines (RWPE-1 and RWPE-2) were used to investigate the expression of ghrelin O-acyltransferase (GOAT) using real-time RT-PCR. GOAT is differentially expressed in prostate cell lines, with the lowest expression levels in the RWPE-1 cell line and the greatest in the LNCaP cell line (Figure 5.2). GOAT mRNA expression was significantly higher in the LNCaP (13.4 fold), DU145 (2.9 fold) and PC3 (2.6 fold) prostate cancer cell lines compared to the transformed normal prostate cell line RWPE-1 (p<0.05) (Figure 5.2). Although the RWPE-2 cell line exhibits a more invasive phenotype compared to its parental cell line RWPE-1 (Bello et al., 1997), GOAT expression levels were not significantly different in the two transformed normal cell lines (Figure 5.2). While all three prostate cancer cell lines examined showed a statistically significant higher level of GOAT mRNA expression, the LNCaP androgen-dependent cell line displayed the highest level compared to all cell lines examined (p<0.05) (Figure 5.2).
Figure 5.1 Real-time quantitative RT-PCR showing relative GOAT mRNA expression levels in normal prostate (N) and prostate cancer (stage II to IV) tissue. Data were normalised to β-actin and are expressed as fold change relative to expression of transcripts in a normal prostate sample (1.0).
Figure 5.2 Real-time quantitative RT-PCR showing relative mRNA expression levels of *GOAT* in the prostate cell lines RWPE-1, RWPE-2, DU145, LNCaP and PC3. Data were normalised to 18S ribosomal RNA and are expressed relative to the RWPE-1 cell line mRNA levels (set as 1) and represent the mean ± SEM of three independent experiments performed in duplicate. Statistical analysis was performed on combined data. *p*<0.05 compared to the RWPE-1 cell line, and #*p*<0.05 compared to the LNCaP cell line (one-way ANOVA with Tukey’s test *post hoc* analysis).
5.2.3 GOAT, PC1/3, PC2 and furin expression in prostate cell lines

In order to determine if the prostate cell lines used in this study possess the enzymatic machinery necessary to synthesise acylated ghrelin, in addition to GOAT, the cell lines were also examined for the expression of PC1/3, PC2 and furin, three members of the prohormone convertase family that are responsible for the protease processing of proghrelin to the 28-amino acid ghrelin peptide (Takahashi et al., 2009). RT-PCRs showed that all the prostate-derived cell lines examined expressed GOAT and at least one of the processing enzymes necessary to produce acylated ghrelin (Figure 5.3). PC1/3 and PC2 expression varied but all cell lines expressed furin and GOAT. PC1/3 was expressed in all cell lines except the DU145 androgen-independent prostate cancer cell line. PC2 was only expressed in the LNCaP androgen-dependent prostate cancer cell line (Figure 5.3). These data demonstrate that the prostate cell lines investigated possess the minimal enzymatic machinery to cleave preproghrelin and produce mature acylated ghrelin.

5.2.4 Effects of octanoic acid on proliferation in prostate cell lines

In addition to GOAT and prohormone convertases, it has recently been shown that supplementation of octanoic acid is essential for octanoylated ghrelin production (Takahashi et al., 2009). Octanoic acid (0, 0.01, 0.02%) was added to prostate-derived cell lines and the cell proliferation rate was assessed using WST-1 and CyQUANT assays. Similar results were seen with both techniques at all concentrations (p<0.01). The PC3 androgen-independent prostate cancer cell line showed a 19.05±2.05% (p<0.01)(WST-1) and 23.15±3.29% (p<0.01) (CyQUANT) statistically significant increase in cell proliferation with 0.01% octanoic acid supplementation (p<0.01), while no significant change was seen with 0.02% octanoic acid treatments (Figure 5.4). In contrast, addition of 0.01% octanoic acid concentration resulted in a significant decrease in cell proliferation in the androgen-dependent LNCaP prostate cancer cell line of approximately 16.62±2.26% (p<0.01) with WST-1 and 19.64±0.95% (p<0.01) with CyQUANT and treatment with 0.02% octanoic acid caused a decrease in cell proliferation of over 30% (p<0.01) with both techniques (Figure 5.4). No changes in cell proliferation were seen in the DU145 and 22Rv1 prostate cancer cell lines when treated with 0.01% octanoic acid, while over 20% (p<0.01) significant decrease in cell proliferation was seen with both techniques.
when cells were treated with 0.02% octanoic acid (Figure 5.4). No significant changes were seen in the RWPE-2 and RWPE-1 transformed normal prostate cell lines at either octanoic acid concentrations tested (Figure 5.4).
Figure 5.3 mRNA expression of PC1/3, PC2, furin and GOAT in prostate cell lines. Ethidium bromide stained agarose gel showing the RT-PCR products for GOAT and furin, which are expressed in LNCaP, DU145, 22Rv1, RWPE-2, PC3 and RWPE-1 prostate cell lines. PC1/3 is expressed in all cell lines except the DU145 androgen-independent prostate cancer cell line. PC2 is only expressed in the LNCaP androgen-dependent prostate cancer cell line.
**Figure 5.4** Effects of octanoic acid treatment on cell proliferation in prostate-derived cell lines. Cells were treated with octanoic acid (0-0.02%) for 72 hours and cell proliferation rate was measured with WST-1 (A) and CyQUANT (B) assays. Octanoic acid at 0.01% significantly decreased cell proliferation in the LNCaP cells and increased proliferation in the PC3 cells (p<0.01) while having no significant effect in DU145, 22Rv1, RWPE-2 and RWPE-1 cells. At 0.02%, octanoic acid significantly decreased cell proliferation rate in LNCaP, DU145 and 22Rv1 prostate cancer cell lines (p<0.01) while having no effect in RWPE-2, RWPE-1 and PC3 cell lines. Data are expressed as percentage above control (% above control) and represent the mean ± SEM of at least three independent experiments with 16 replicates for each octanoic acid concentration in a single experiment. Statistical analysis was performed on combined data. **p<0.01 compared with untreated controls (one-way ANOVA with Tukey’s test post hoc analysis).
5.2.5 Regulation of GOAT expression by ghrelin and desacyl ghrelin in prostate cancer cell lines

Having confirmed that the PC3, DU145 and LNCaP prostate cancer cell lines possess the necessary enzymatic system to produce acylated and unacylated ghrelin (Figures 5.2 & 5.3), the PC3, DU145 and LNCaP cell lines were treated with both ghrelin and desacyl ghrelin to investigate whether these hormones can regulate GOAT mRNA expression in prostate cancer cell lines. Desacyl ghrelin treatments did not alter the expression of GOAT mRNA in the PC3, DU145 and LNCaP prostate cancer cell lines (Figure 5.5B, D & F). Acylated ghrelin treatments had no significant effect on GOAT mRNA levels in the DU145 and LNCaP prostate cancer cell lines (Figure 5.5 C & E). However, treatment with 100nM (Figure 5.5A), or 1000nM (data not shown) acylated ghrelin for six hours induced a statistically significant, two-fold decrease in GOAT mRNA expression in the PC3 prostate cancer cell line compared to untreated controls (p<0.05). No change in GOAT mRNA expression was seen after one hour treatment with either ghrelin or desacyl ghrelin (data not shown).
Figure 5.5 Real-time quantitative RT-PCR analysis of GOAT gene expression in response to ghrelin and desacyl ghrelin treatments over six hours. Ghrelin-treated PC3 (A), DU145 (C) and LNCaP cells (E) and desacyl ghrelin-treated PC3 (B), DU145 (D) and LNCaP cells (F). Data is represented as mean ± SEM of two independent experiments performed in duplicates. *p<0.05 indicate values that differ significantly (Student's t-test) from vehicle-treated control (set at 1-fold).
5.3 DISCUSSION
In this study we have demonstrated that prostate-derived cell lines express mRNA for GOAT and prohormone convertases and could potentially possess the necessary machinery to produce ghrelin and desacyl ghrelin. We have demonstrated that octanoic acid supplementation in the culture medium significantly stimulates cell proliferation in the PC3 prostate cancer cell line but not in the DU145, LNCaP, 22Rv1, RWPE-1 and RWPE-2 prostate-derived cell lines. In addition, we showed that ghrelin decreases GOAT mRNA expression in the PC3 cell line but not in the DU145 and LNCaP prostate cancer cell lines.

The addition of an octanoyl group to the third serine residue of ghrelin is an unusual modification which is necessary for binding and activating its only known functional receptor, the GHS-R1a (Bednarek et al., 2000; Kojima et al., 2001b; Muccioli et al., 2002; Torsello et al., 2002; Broglio et al., 2006; Hosoda et al., 2006). The two major ghrelin forms - octanoylated (ghrelin) and non-octanoylated ghrelin (desacyl ghrelin) - are present in the stomach and bloodstream (Hosoda et al., 2000a; Broglio et al., 2003b) and can be locally produced in many peripheral normal and tumour tissues and in cancer cell lines (Jeffery et al., 2002; Yeh et al., 2005) (see Section 1.2.3 & Tables 1.2 and 1.3). The enzyme that catalyses the addition of the octanoyl group to ghrelin’s amino acid chain was recently identified as the membrane bound O-acyltransferase 4 (MBOAT4), also named ghrelin O-acyltransferase, GOAT (Gutierrez et al., 2008; Yang et al., 2008a). It is highly conserved among species (Gutierrez et al., 2008) and its tissue expression pattern mirrors that of ghrelin throughout the body (Gutierrez et al., 2008; Yang et al., 2008a; Sakata et al., 2009; Lim et al., 2011).

A great amount of interest has been raised with the discovery of GOAT and recent studies have examined its potential as an important key step in regulating ghrelin’s diverse functions and the ghrelin/desacyl ghrelin ratio. GOAT knockout mice have been produced (Kirchner et al., 2009; Zhao et al., 2010) and examined for phenotypic changes. Under normal dietary conditions, few developmental or gross anatomical changes were observed (Kirchner et al., 2009; Zhao et al., 2010), and this was consistent with the minor changes seen with ghrelin knockout mice (Sun et al., 2003; Wortley et al., 2004; De Smet et al., 2006; Dezaki et al., 2006). Under high fat
dietary challenge several studies suggest that both ghrelin and GOAT deficient mice have decreased body weight, lower fat mass and increased energy expenditure (Sun et al., 2003; Wortley et al., 2004; De Smet et al., 2006; Dezaki et al., 2006; Kirchner et al., 2009; Zhao et al., 2010). Chemical inhibition of GOAT was shown to reduce plasma levels of acylated ghrelin and to promote weight gain (Barnett et al., 2010). The strongest evidence for the importance of GOAT has come from studies of GOAT knockout mice challenged with 60% caloric restriction (Zhao et al., 2010). These mice rapidly become moribund due to hypoglycaemia and abnormal insulin balance, and are rescued from this phenotype by acylated ghrelin or by growth hormone replacement (Zhao et al., 2010). GOAT and acylated ghrelin may therefore have more important roles during caloric restriction rather than during dietary excess. These observations reinforce the close link between GOAT and ghrelin function and clearly indicate that mechanisms controlling GOAT expression could be major physiological and/or pathophysiological regulators of ghrelin function and the ghrelin/desacyl ghrelin ratio. However, little is currently known regarding the regulation of GOAT expression.

In addition to GOAT, other enzymes have been described as important contributors to the production of mature ghrelin. The processing of the preproghrelin peptide to produce acylated ghrelin requires a sequence of steps that requires the action of specific enzymes. Following cleavage of the signal peptide, proghrelin can be post-translationally acylated by GOAT to generate acylated ghrelin (Gutierrez et al., 2008; Yang et al., 2008a). Both acylated and non-acylated proghrelin are then cleaved by one of a number of enzymes, including the members of the prohormone convertases (PCs) family PC1/3, PC2 and furin, to produce either the mature ghrelin or desacyl ghrelin peptides (Zhu et al., 2006; Takahashi et al., 2009). PC1/3, PC2 and furin are members of the prohormone convertase family, a family of neuroendocrine serine proteases responsible for the tissue specific processing of a number of peptide hormones (Seidah et al., 1999) and have been demonstrated to be involved in processing of the proghrelin peptide into ghrelin (Zhu et al., 2006; Takahashi et al., 2009). More importantly, a detailed in vitro study showed that in addition to the PCs or furin and GOAT, the presence of octanoic acid is crucial for production of acylated ghrelin (Takahashi et al., 2009). In this study we found that GOAT is expressed in both normal and prostate cancer specimens. However,
expression levels in clinical prostate cancer tissue samples were not significantly different to levels in the normal prostate tissues, regardless of cancer stage. Moreover, all the prostate-derived cell lines examined express GOAT and at least one of the processing enzymes necessary to produce acylated ghrelin.

Although ghrelin and GOAT are co-expressed in normal tissues (Sakata et al., 2009), the expression of GOAT in cancer has not been widely investigated. A recent study that reported a novel human ghrelin variant, In1-ghrelin, showed that GOAT is overexpressed in breast cancer specimens (Gahete et al., 2011), but GOAT expression has not been reported in other cancers (to our knowledge). GOAT mRNA levels were the lowest in the RWPE-1 transformed normal cell line, while significantly higher levels were found in the PC3 (2.6 fold), DU145 (2.9 fold) and LNCaP (13.4 fold) prostate cancer cell lines when compared to RWPE-1 cells. The RWPE-2 cell line is a clonal derivative from the RWPE-1 cell line transformed by Ki-ras using the Kirstin murine sarcoma virus (Ki-MuSV) (Bello et al., 1997). Although the RWPE-2 cell line presents a more invasive and tumourigenic phenotype than the RWPE-1 cell line, GOAT expression levels were not significantly different in these two transformed normal cell lines. Although in prostate tissues GOAT expression was not significantly different between normal and cancer specimens, expression did differ between transformed normal and prostate cancer cell lines. GOAT expression levels also varied according to the status of androgen responsiveness and the highest levels were seen in the LNCaP androgen-dependent prostate cancer cell line (13.4 fold), while lower levels were seen in the PC3 (2.6 fold) and DU145 (2.9 fold) androgen-independent prostate cancer cell lines.

Our group previously showed that ghrelin is expressed in normal prostate and prostate cancer tissues and is secreted by the PC3 and LNCaP prostate cancer cell lines (Yeh et al., 2005). We have confirmed that the prostate cell lines examined in this study express the necessary enzymatic machinery to produce acylated ghrelin. While GOAT and furin mRNA are expressed in all cell lines, PC1/3mRNA is expressed in all cell lines except the DU145, and PC2 mRNA is only expressed in the LNCaP cell line. In addition to this enzymatic system, it has recently been reported that supplementation with the substrate octanoic acid is crucial for octanoylated ghrelin production in cell culture (Takahashi et al., 2009). Takahashi et
al., (2009) showed that the COS-7 green monkey kidney cell line expresses low levels of furin mRNA, but does not express mRNA for GOAT, PC1/3 or PC2. When co-transfected with a preproghrelin construct and either one of the prohormone convertases (PC1/3, PC2 or furin), all three PCs were able to process proghrelin into mature desacyl ghrelin. When cells were triple co-transfected with ghrelin, PCs and GOAT, no octanoylated ghrelin was produced unless octanoic acid was supplied in the medium (0.01%) (Takahashi et al., 2009). Previously, in this study it was demonstrated that exogenous ghrelin and desacyl ghrelin treatments stimulate cell proliferation in the PC3 androgen-independent prostate cancer cell line through activation of Akt and possibly MAPK pathways (Chapter 4). Consistent with these previous results, the PC3 cell line also showed a 20% significant increase in cell proliferation rate in response to 0.01% octanoic acid treatment (p<0.01) with both WST-1 and CyQUANT methods, suggesting that endogenous ghrelin was being produced and stimulating cell proliferation in this cancer cell line. Interestingly, no significant change was seen at a higher octanoic acid dose (0.02%), perhaps reflecting an increasing toxicity of this treatment in the PC3 cell line. Surprisingly, in the LNCaP prostate cancer cell line, both 0.01% and 0.02% octanoic acid concentrations resulted in a significant decrease in cell proliferation. No changes were seen in the DU145 and 22Rv1 prostate cancer cell lines when treated with 0.01% octanoic acid, and a significant decrease in cell proliferation was seen when cells were treated with 0.02% octanoic acid. No significant changes were seen in the RWPE-2 and RWPE-1 transformed normal prostate cell lines at either octanoic acid concentrations tested. In this study equivalent (0.01%) and higher (0.02%) concentrations of octanoic acid were used compared to other studies (0.01%, Takahashi et al., 2009; 0.0125%, Gutierrez et al., 2008; and 0.001%, Ohgusu et al., 2009). The varied cellular responses could reflect a varied cellular sensitivity to octanoic acid, which, due to a subtle pH effect, could be having a cytotoxic effect in the cell lines at the higher concentrations. Neutralisation of the media may reduce these effects. Although the addition of octanoic acid to the medium would allow cells to endogenously produce acylated ghrelin, octanoic acid may have effects that are not related to ghrelin in these cell lines. To determine if octanoic acid treatments are having their effect through octanoylated ghrelin we would need to perform further studies to determine if octanoylated ghrelin is produced in response to these treatments. By knocking down the expression of ghrelin using siRNA, we would also
be able to determine if the effects of octanoic acid were mediated through ghrelin octanoylation.

Nutritional status is a factor that is known to influence ghrelin levels (Takahashi et al., 2009), and ghrelin plasma levels increase during fasting and before meals and decrease post-prandially (Cummings et al., 2001; Tschop et al., 2001a; Bellone et al., 2002; Cummings, 2006). The mechanisms regulating ghrelin suppression after food intake remain unclear and might depend on the macronutrient composition of the diet (Beck et al., 2002; Lee et al., 2002; Shiiya et al., 2002; Erdmann et al., 2003; Monteleone et al., 2003; Greenman et al., 2004; Heath et al., 2004; Overduin et al., 2005; Bowen et al., 2006; Foster-Schubert et al., 2008). It has been suggested the GOAT-ghrelin system may function as a nutrient sensor by using readily absorbable medium-chain fatty acids to inform the brain of the presence of dietary calories (Kirchner et al., 2009). In addition to ghrelin, furin mRNA levels have also been shown to be significantly increased after 48 hours of fasting in rats, but other prohormone convertases showed no significant increase when compared to controls (Takahashi et al., 2009). Both ghrelin and furin levels returned to normal after feeding, while PC1/3, PC2 and GOAT levels were significantly decreased (Takahashi et al., 2009), suggesting these enzymes are differentially regulated.

The mechanisms regulating GOAT expression and ghrelin acylation remain poorly understood. In the insulinoma INS-1 cell line, insulin inhibits GOAT mRNA expression and GOAT promoter activity through activation of the mammalian target of rapamycin (mTOR) (An et al., 2010). In addition to insulin, bacterial lipopolysaccharide (LPS) has been shown to modulate GOAT expression. A decrease of plasma acylated and unacylated ghrelin was seen after 2, 5 and 7 hours of intraperitoneal LPS. A more pronounced decrease in acylated than unacylated ghrelin was seen after 2 hours when ghrelin levels dropped 53% compared to 28% for desacyl ghrelin. This rapid reduction in acylated ghrelin was associated with a 38% decrease in GOAT serum levels, however, GOAT mRNA expression remained unchanged (Stengel et al., 2010). In vivo studies showed two mice strains showed different responses in ghrelin secretion in fasting conditions. CD1 mice had increased total ghrelin and obestatin serum levels but no change in preproghrelin mRNA after 24 hours fasting, while C57BL/6 mice showed an increase in preproghrelin mRNA
expression, stomach acylated ghrelin peptide levels but no change in plasma obestatin levels (Morash et al., 2010). Interestingly, neither GOAT nor PC1/3 mRNA levels were affected by fasting, suggesting the reduction in acylated ghrelin levels is not through modulation of proghrelin acylation and processing in these models (Morash et al., 2010).

In this study we have demonstrated that treatment with desacyl ghrelin did not alter the levels of GOAT mRNA expression in the PC3, DU145 and LNCaP prostate cancer cell lines. Interestingly, acylated ghrelin significantly reduced GOAT mRNA expression in the PC3 prostate cancer cell line, thereby suggesting a selective negative feedback loop. In the PC3 prostate cancer cell line, ghrelin may downregulate GOAT expression, therefore, downregulating its octanoylation. This could change the balance between ghrelin and desacyl ghrelin in the prostate. In agreement with our findings, synthetic octanoylated pentapeptides containing only the N-terminal five amino acids of proghrelin (GSSFL), the minimal sequence required for activation of the GHS-R1a (Bednarek et al., 2000; Matsumoto et al., 2001a; Matsumoto et al., 2001b) have been demonstrated to inhibit GOAT in vitro (Yang et al., 2008b). Although ghrelin and desacyl ghrelin appear to exert the same effect on prostate cancer cell proliferation (see Chapter 4), both peptide forms may exert additional but different roles in the prostate. Therefore, changing the balance between ghrelin and desacyl ghrelin may change the physiological response in the prostate. Ghrelin and desacyl ghrelin have been shown to share some common high affinity binding sites (Baldanzi et al., 2002; Delhanty et al., 2006; Granata et al., 2007; Thielemans et al., 2007) and functions, but can also have different functions in other cell types (Gauna et al., 2005; Qader et al., 2008; Lear et al., 2010). The cell proliferation studies here indicate that somewhat differing signalling pathways (Akt vs ERK1/2) may be used by ghrelin and desacyl ghrelin. Microarray studies would be useful for determining if ghrelin and desacyl ghrelin activate different pathways, and therefore, have different functions in prostate cancer cells. Although all prostate cancer cell lines express the GHS-R1a, it is likely both ghrelin and desacyl ghrelin function in the prostate through the yet unidentified alternative receptor.

During the last decade the importance of ghrelin has been well investigated in vitro and in vivo. Although desacyl ghrelin functions have been the subject of less study, it
is the most abundant form of ghrelin measured in the bloodstream (Hosoda et al., 2000a; Broglio et al., 2003b) and a range of different functions have been described (see Section 1.2.3.1). Ghrelin and desacyl ghrelin have been shown to share some functions including stimulating cell differentiation (Thompson et al., 2004b; Sato et al., 2006; Filigheddu et al., 2007), increasing cell proliferation (Delhanty et al., 2006; Granata et al., 2007) and inhibiting cell death (Baldanzi et al., 2002; Granata et al., 2006; Lear et al., 2010) as well as exerting some opposing roles in insulin and glucose metabolism (Gauna et al., 2005; Qader et al., 2008; Lear et al., 2010) (Table 1.3). Several groups have investigated acylated ghrelin stability in serum and reported that the acyl group is rapidly hydrolysed while the C-terminal ghrelin is enzymatically and physically stable for several hours (De Vriese et al., 2004; Hosoda et al., 2004; Satou et al., 2010). Furthermore, De Vriese et al. (2004) also showed that homogenates from the rat stomach, kidney and liver have high endopeptidase activity against the ghrelin N-terminal. Interestingly, an additional ghrelin desacylation enzyme, acyl-protein thioesterase 1 (APT1), has been very recently reported (Satou et al., 2010). APT1 was originally identified as a lysophospholipase that can hydrolyse lysophosphatidylcholine (lysoPC) to free fatty acid and was purified from gastric mucosa (Sunaga et al., 1995; Shanado et al., 2004) and rat liver (Sugimoto et al., 1996). Although other ghrelin desacylation enzymes may be present in the circulation, APT1 is the only known enzyme in the serum to desacylate ghrelin both in vitro and in vivo (Satou et al., 2010). The balance between GOAT and APT1 could be crucial for regulation of acylated ghrelin availability and ghrelin/desacyl ghrelin ratio.

In this study we showed that GOAT mRNA is expressed in normal and prostate cancer specimens and that GOAT, PC1/3, PC2 and furin are expressed in prostate-derived cell lines at mRNA level. Moreover, we showed that exogenous ghrelin treatment decreases GOAT mRNA expression in the PC3 prostate cancer cell line but not in the DU145 and LNCaP prostate cancer cell lines and that octanoic acid supplementation in the cell culture medium increases cell proliferation in the PC3 prostate cancer cell line. Although these results suggest that these cell lines possess the necessary enzymatic machinery to produce acylated ghrelin, further studies are needed to assess GOAT protein and activity levels and whether changes in GOAT mRNA expression correlates with GOAT activity. A large number of studies have
shown that ghrelin acylation is essential for some of its functions and binding and activating the GHS-R1a. The identification of GOAT and its participation in the processing of the preproghrelin peptide into the mature acylated ghrelin peptide is an important regulating step in ghrelin’s production and could represent an important tool in regulating the balance between ghrelin and desacyl ghrelin. Furthermore, in addition to the enzymatic machinery, the presence of octanoic acid is crucial for ghrelin production, showing a direct link between dietary lipids and ghrelin levels and highlighting the importance of elucidating the regulating mechanism of the GOAT-ghrelin system in order to understand the key regulating steps of ghrelin production.
Chapter 6

General Discussion
In the last two decades the detection rate of prostate cancer has increased, accompanied by a decrease in the incidence of high-grade cancer and prostate cancer specific mortality rates. It has been suggested that these decreases have resulted from the enhanced detection of prostate cancer during more curable stages (Pienta, 2009). Despite the progress stemming from introduction of wider prostate-specific antigen (PSA) screening, this test lacks sensitivity and specificity (Thompson et al., 2004a; Sharifi and Kramer, 2007; Brawley et al., 2009; Amling et al., 2010; Renehan et al., 2010) and there is a need for a more accurate prognostic marker. Prostate cancer remains the most commonly diagnosed cancer in Western men and the second leading cause of cancer mortality in Western men and this is mainly attributed to castrate-resistant disease (Jemal, 2009). Early detection and prevention of progression to a castrate-resistant state may provide a new strategy to improve survival of patients diagnosed with prostate cancer (Bonkhoff and Berges, 2010).

Growth factors may play a crucial role in the progression to a hormone refractory stage (Ishii, 2009) and a better understanding of the mechanisms involved in this progression could be a useful tool for developing better prognostic markers and more successful therapies.

Several groups have been trying to understand the complex mechanisms that initiate the disease and support its progression to a castrate resistant phase. Investigation of genetic alterations in prostate cancer has suggested that mutations alone are unlikely to produce clinical cancers and that a favorable tissue environment is necessary for the cancer to arise (Green and Evan, 2002; Vineis, 2003). Prostate cancer is a very heterogeneous disease and highly responsive to hormonal alterations (Labrie, 2004). Importantly, several growth factors have been shown to act in an autocrine/paracrine and even intracrine manner to influence prostate cancer cell growth (reviewed in Hellawell and Brewster, 2002; Meinbach and Lokeshwar, 2006) and this could be a key to better understand the mechanisms driving tumour growth in castrate-resistant prostate cancer.

Ghrelin is a growth factor that has been shown, in some studies, to increase cell proliferation in vitro in some cancer cell lines, including prostate cancer cell lines (Jeffery et al., 2002; Yeh et al., 2005), through activation of MAPK, Akt/PI3K and PKC pathways (Jeffery et al., 2002; Yeh et al., 2005; Delhanty et al., 2006; Granata

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et al., 2006; Camina et al., 2007; Granata et al., 2007; Pazos et al., 2007), although other studies have suggested it may actually suppress prostate cancer cell growth (Cassoni et al., 2004; Diaz-Lezama et al., 2010). In this study we have confirmed our previous studies showing that ghrelin stimulates cell proliferation in the PC3 prostate cancer cell line using a DNA based proliferation assay, in addition to the metabolic based WST-1 assay. Despite conflicting findings in different studies, it has been suggested that the ghrelin/ghrelin receptor axis may be a promising potential target in this disease (Jeffery et al., 2002; Cassoni et al., 2004; Yeh et al., 2005). The ghrelin gene (GHRL) encodes the complex preproghrelin peptide, which can be subsequently processed into three currently known functional peptides - ghrelin, desacyl ghrelin and obestatin – and a number of other potential peptides (Seim et al., 2007). Post-translational processing of preproghrelin occurs through the actions of the prohormone convertases (PCs) PC1/3, PC2 and furin (Takahashi et al., 2009). A second important post-translational event impacting on the function of ghrelin is the addition of an octanoyl group to the third residue (serine) of the preproghrelin peptide. The discovery of GOAT, the enzyme that catalyses this octanoylation step (Gutierrez et al., 2008; Yang et al., 2008), shed new light on the regulation of ghrelin acylation and, therefore, the regulation of ghrelin/desacyl ghrelin balance. GOAT expression mirrors ghrelin throughout the body (Lim et al., 2011) and higher levels are found in the stomach, intestine and pancreas (Gutierrez et al., 2008; Yang et al., 2008). In this study we demonstrated that GOAT is expressed in prostate cancer cell lines and tissues, indicating that the prostate is capable of producing octanoylated ghrelin. The octanoylation is critical for ghrelin’s interaction with its known receptor, GHSR-1a, and particularly for the stimulation of growth hormone secretion and appetite regulation (Bednarek et al., 2000; Kojima et al., 2001b; Broglio et al., 2006; Hosoda et al., 2006). For other effects, such as modulation of cell proliferation, survival and differentiation, the octanoylation may not be specifically required, as both acylated and non-acylated ghrelin exert similar effects (see Table 1.2) and this is most likely mediated through an as yet undefined alternative receptor. In some circumstances, ghrelin and desacyl ghrelin exert differential effects – therefore, emphasising the important role of GOAT and des-acylation enzymes, such as APT1, in maintaining or actively regulating the balance between ghrelin and desacyl ghrelin.
This study has examined the expression of GOAT at the mRNA level. Further studies are required in order to investigate whether changes in mRNA expression influence expression at the protein level and if they also correlate with the enzymatic activity of GOAT. However, such studies into the newly discovered ghrelin acylation enzyme are difficult, due to the absence of well developed methodologies to measure GOAT protein levels and enzymic activity. Although some antibodies are currently available, our group has experienced difficulty in extracting the highly hydrophobic GOAT enzyme (which is bound to the endoplasmic reticulum) and detecting it using Western immunoblotting (Chopin and Jeffery, personal communications).

Obestatin is the third known peptide product of the ghrelin gene and although its functional repertoire is not fully known, there has been some controversy over its potential anorexigenic and metabolic effects (Zhang et al., 2005; Moechars et al., 2006; Gourcerol et al., 2006; Seoane et al., 2006; Sibilia et al., 2006a; Bassil et al., 2007; De Smet et al., 2007; Gourcerol et al., 2007a; Gourcerol et al., 2007b), however, it has been reported to also act as a growth factor in some cell lines (Camina et al., 2007; Pazos et al., 2007; Granata et al., 2008).

In this study I have investigated the effects of the three preproghrelin-derived peptides, obestatin, ghrelin and desacyl ghrelin, in prostate-derived cell line proliferation and the possible intracellular pathways involved. Furthermore, we investigated the expression of GOAT and prohormone convertases (PCs) mRNA in prostate cancer cell lines and whether ghrelin and desacyl ghrelin could regulate GOAT expression in these systems. Obestatin, ghrelin and desacyl ghrelin significantly increased cell proliferation in the PC3 androgen-independent prostate cancer cell line, but not in the transformed normal cell line, RWPE-1, through activation of the MAPK and Akt pathways. Their role in utilising the PKC pathway can be suggested but requires further studies. Moreover, we showed that prostate-derived cell lines express the necessary enzymatic machinery to produce and secrete ghrelin and desacyl ghrelin suggesting ghrelin forms could be acting in an autocrine/paracrine manner to confer a growth advantage to prostate cancer cells. These findings both support and extend previous studies in various cancer cell lines and provide significant further evidence that ghrelin can indeed stimulate proliferation, not inhibit it as reported by one group (Diaz-Lezama et al., 2010), at
least in the context of prostate cancer cell lines. Furthermore, we have shown for the first time that ghrelin treatment decreases GOAT mRNA expression in the PC3 androgen-independent prostate cancer cell line suggesting a negative feedback loop where ghrelin’s presence inhibits its own acylation.

While I have shown that each of the three recognised preproghrelin products can act in a similar way in stimulating prostate cancer cell line proliferation, a key element to consider is the physiological or pathophysiological environment in which these peptides are produced and may act – together in combination, or independently. The exact mechanisms regulating the expression and secretion of these peptides remain unclear. Are they always produced in concert, in equivalent amounts, are they produced independently and therefore exert independent effects, are there external factors that might impact the post-translational processing and modifications and therefore independently regulate peptide function? Recent observations suggest each of these may be operational mechanisms.

A recent revised study of the ghrelin gene structure revealed that the regulation of its expression is far more complex than anticipated. The study demonstrated the presence of several ghrelin gene derived mRNA splice variants and several antisense transcripts in a number of human tissues and cell lines (Seim et al., 2007). Interestingly, a transcript coding for obestatin but not ghrelin was identified, suggesting obestatin could be potentially produced independently from ghrelin. However, it is not yet known whether this novel transcript coding for obestatin and not ghrelin is translated into protein. Similarly, the novel antisense transcripts are unlikely to be translated into proteins but may function as non-coding regulatory RNA (Seim et al., 2007). In contrast, a transcript lacking the obestatin coding sequence but coding for ghrelin (exon 3-deleted preproghrelin) has been previously reported at both mRNA and protein levels in prostate (Yeh et al., 2005) and breast cancer specimens (Jeffery et al., 2005) and in the PC3 prostate cancer cell line (Yeh et al., 2005). These observations raise critical questions as to the regulation of gene expression and translation and the impact on cell function. Studies are currently in progress to ascertain the importance and role of these novel sense and antisense transcripts.
Additional questions are raised with respect to the effect of external factors, specifically diet, in regulating functional consequences of ghrelin gene expression and translation. While the presence of the cellular enzymatic machinery for acylating ghrelin has been demonstrated, previous studies in vivo have shown that this is not sufficient and supplementation of medium-chain fatty acids (MCFAs) in the diet or in cell culture medium was essential for acylated ghrelin production (Nishi et al., 2005; Kirchner et al., 2009; Takahashi et al., 2009). This finding demonstrated that dietary lipids are directly used for ghrelin acylation and that its production is highly dependent on nutritional status. The observation that the ingestion of MCFAs or medium-chain triglycerides (MCTs) increases acylated ghrelin levels without changing total ghrelin levels (Nishi et al., 2005) highlights the importance of the GOAT-ghrelin system and the nutritional status in the regulation of ghrelin/desacyl ghrelin ratio. Given some of the recognised differences between ghrelin and desacyl ghrelin actions, and specifically the differential ability to interact with the one known receptor for ghrelin, GHSR-1a, regulation of ghrelin acylation may be an attractive mechanism for the development of better therapies for a number of pathological conditions where ghrelin has been shown to play a key role. On the basis of the studies presented in this thesis, this is not likely to impact on therapies for prostate cancer, as ghrelin and desacyl ghrelin show similar effects, however, regulating acylation could be of considerable interest in those conditions involving energy metabolism, appetite regulation and adipose tissue function. Ghrelin serum levels generally mirror nutritional status, being inversely correlated with BMI. Elevated ghrelin levels have been shown in patients with anorexia nervosa (Otto et al., 2001; Cuntz et al., 2002; Shiiya et al., 2002; Kojima et al., 2004) and cachexia (Nagaya et al., 2001; Otto et al., 2001; Yoshimoto et al., 2002; Shimizu et al., 2003; Tacke et al., 2003), while reduced ghrelin levels are seen in obese patients (Ravussin et al., 2001; Tschop et al., 2001; Muccioli et al., 2002; Shiiya et al., 2002; Bunt et al., 2003; Krsek et al., 2003; Tritos et al., 2003; Helmling et al., 2004; Yildiz et al., 2004; Zorrilla et al., 2006) and patients with metabolic syndrome (Ukkola et al., 2006). Not surprisingly, ghrelin levels decrease after weight gain (Kojima et al., 2004) and increase after weight loss (Hansen et al., 2002) suggesting ghrelin’s production adapts to promote a positive energy balance. One exception to this correlation between ghrelin and BMI is the elevated level of ghrelin in patients with Prader-Willi Syndrome (PWS) despite their increased body weight (Cummings et al.,
PWS is a complex genetic disorder characterised by obesity and hyperphagia among other traits (Nicholls and Knepper, 2001) and whether ghrelin plays a role in this condition needs further investigation.

Ghrelin and desacyl ghrelin have been shown to exert multiple functions in different tissues (Table 1.2). More importantly, both forms have been shown to participate in some processes relevant to cancer progression such as cell differentiation, proliferation, survival and cell death (Baldanzi et al., 2002; Muccioli et al., 2004; Thompson et al., 2004b; Delhanty et al., 2006; Granata et al., 2006; Sato et al., 2006; Filigheddu et al., 2007; Granata et al., 2007). Ghrelin’s role as a growth factor has been more widely investigated and ghrelin and GHS-R1a expression has been detected in a number of cell types (Table 1.3). The finding that both ghrelin and desacyl ghrelin can exert the same role in some cell types (Muccioli et al., 2004; Thompson et al., 2004b; Delhanty et al., 2006; Granata et al., 2006; Sato et al., 2006; Filigheddu et al., 2007; Granata et al., 2007) suggested the existence of an alternative yet unidentified receptor shared by both forms. Moreover, the fact that ghrelin and desacyl ghrelin can exert opposing or distinct roles in the same cell types (Gauna et al., 2005; Qader et al., 2008; Lear et al., 2010) raises the question whether desacyl ghrelin possesses its own receptor and both acylated and unacylated forms could be acting through different mechanisms. Both ghrelin and desacyl ghrelin were shown to stimulate insulin release in the INS-1E insulinoma cell line, however, ghrelin’s action seems to be mediated through the GHS-R1a and ghrelin’s effect was completely abolished by the GHS-R1a inverse agonist D-lys³-GHRP-6, while desacyl ghrelin effects persisted (Gauna et al., 2006) suggesting both forms are acting through different receptors to induce the same cellular response in this cell type. Common high affinity binding sites for both ghrelin and desacyl ghrelin were identified in the H9c2 cardiomyocytes (Baldanzi et al., 2002) and in rat epididymal adipocytes (Muccioli et al., 2004), while distinct binding sites were detected in the HL-1 cardiomyocyte cell line (Lear et al., 2010), suggesting there is an unknown receptor that can be shared by both ghrelin forms and a possible receptor specific just for desacyl ghrelin.

In this study we showed that both ghrelin and desacyl ghrelin significantly stimulated cell proliferation in the PC3 prostate cancer cell line through activation of ERK1/2
and Akt pathways but had no significant effect in the transformed normal cell line, RWPE-1. It remains unclear if the effects of ghrelin in the prostate are mediated through the GHSR1a, the alternative receptor, or both, however. In this study we used the GHSR1a inverse agonist, D-lys\(^3\)-GHRP-6, in an attempt to inhibit GHSR1a signaling in response to ghrelin. While D-lys\(^3\)-GHRP-6 prevented cell proliferation in response to acylated ghrelin in this study, surprisingly it also inhibited the effects of desacyl ghrelin, which is understood not to activate GHSR1a. Interestingly, Delhanty et al., (2007) have also shown that D-lys\(^3\)-GHRP-6 prevented both ghrelin- and desacyl ghrelin-induced cell proliferation in the SW-13 adrenocortical carcinoma cell line. Since the ghrelin/desacyl ghrelin receptor remains unknown, a feasible explanation for this is the possible high degree of similarity between these receptors, and therefore, it is possible D-lys\(^3\)-GHRP-6 could be actually antagonising both receptor isoforms. In addition, it remains currently unknown whether exogenously applied desacyl ghrelin could be converted into acylated ghrelin and bind and activate the GHS-R1a, as well as the yet unidentified receptor, to exert its proliferative effect. To determine if the effects of ghrelin and desacyl ghrelin are mediated through the alternative ghrelin receptor, further studies are required. Identification of the alternative receptor and studies using knockdown of GHSR1a expression using siRNA techniques are likely to provide more conclusive results. Further investigation on GOAT mechanisms is needed to assess whether GOAT can acylate the mature desacyl ghrelin peptide or the proghrelin precursor only.

Although ghrelin and desacyl ghrelin have been shown to exert the same effect in prostate cancer cells and stimulate cell proliferation, ghrelin’s value as a diagnostic marker for this disease is still unclear. While one group found no statistical significance in ghrelin serum levels between patients with BPH and prostate cancer (Mungan et al., 2008), another group suggested a link between ghrelin/desacyl ghrelin serum levels and prostate cancer when compared with BPH and control individuals (Malendowicz et al., 2009). The first however, did not differentiate between acylated and unacylated ghrelin. The use of ghrelin as a diagnostic marker and/or therapeutic agent is challenging. Most of the current reports do not distinguish between total ghrelin and acylated ghrelin due to the limiting methodology to detect and differentiate between acylated and unacylated forms (Yin et al., 2009). Furthermore, in vitro studies have shown that acylated ghrelin has a relatively short
half-life in serum (Tschop et al., 2000; Kanamoto et al., 2001; De Vriese et al., 2004; Gauna et al., 2004; Muccioli et al., 2004; De Vriese et al., 2005), therefore adding to the complexity of accurately determining acylated ghrelin levels. The recent findings that desacyl ghrelin, the most abundant ghrelin isoform in the circulation, shares some functions with acylated ghrelin, as well as exerting some opposing and distinct roles of its own, highlights the importance of more specific techniques that differentiate between the two forms. Although the link between ghrelin and ghrelin/desacyl ghrelin serum levels and prostate cancer remains unclear, both forms can be locally produced in the prostate (Jeffery et al., 2002; Yeh et al., 2005) and circulating levels might not reflect the tissue microenvironment. Accurately measuring ghrelin plasma levels has proven to be rather challenging, as there is a lack of international standard testing and results from different laboratories can vary widely. Ghrelin has a short half-life in plasma due to rapid deacylation and degradation into smaller fragments (Tschop et al., 2000; Kanamoto et al., 2001; De Vriese et al., 2004; Gauna et al., 2004; Muccioli et al., 2004; De Vriese et al., 2005), therefore collecting time and conditions as well as processing conditions are crucial for accurate results. The development of standard techniques to measure acylated and unacylated ghrelin as well as distinguish between the two types, will be an important tool and allow for comparisons between circulating and local levels of the peptides as well as comparing between normal and cancerous tissues.

The discovery that the preproghrelin gene generates not only ghrelin and desacyl ghrelin, but also obestatin, was a very exciting and promising finding. Although initial studies showed obestatin did not alter GH secretion (Zhang et al., 2005), obestatin was reported to oppose ghrelin’s effect on food intake and body weight (Zhang et al., 2005; Bresciani et al., 2006; Moechars et al., 2006; Green et al., 2007; Lagaud et al., 2007; Samson et al., 2007; Nagaraj et al., 2008; Zhang et al., 2008; Subasinghage et al., 2010) and this generated a lot of interest and suggested great potential as a novel therapeutic target for obesity and metabolic disorders. When this project started therefore, the focus of our attention was based on these initial findings suggesting that obestatin could trigger opposite effects to ghrelin. Furthermore, it was believed obestatin’s actions were mediated through the GPR39. Our laboratory had previously demonstrated that ghrelin acted as a growth factor to stimulate cell proliferation in the PC3 and LNCaP prostate cancer cell lines (Jeffery et al., 2002;
Yeh et al., 2005) and that a novel exon 3-deleted preproghrelin transcript, a transcript lacking the coding sequence for obestatin, was overexpressed in prostate cancer specimens when compared with normal prostate tissues (Yeh et al., 2005). Following our previous findings, the initial hypothesis, therefore, was that exogenously applied obestatin would have an inhibitory effect in prostate cancer cell proliferation mediated via the GPR39. All cell lines examined expressed GPR39 mRNA, however, our results demonstrated that obestatin stimulates cell proliferation in the PC3 prostate cancer cell line and further investigation showed this action is mediated through activation of the MAPK and Akt pathways. During the course of this study, however, the original reports of obestatin opposing ghrelin actions could not be faithfully reproduced (Gourcerol et al., 2006; Seoane et al., 2006; Sibilia et al., 2006a; Bassil et al., 2007; De Smet et al., 2007; Gourcerol et al., 2007a; Gourcerol et al., 2007b; Nogueiras et al., 2007; Tremblay et al., 2007; Yamamoto et al., 2007; Chen et al., 2008a; Kobelt et al., 2008; Mondal et al., 2008) and the obestatin field has been subject to a lot of controversy, not only in regard to its functions but also its receptor. Obestatin was originally thought to bind and activate GPR39 to exert its anorexigenic effects, however, subsequent studies showed that Zn+2 but not obestatin could activate this receptor (Lauwers et al., 2006; Holst et al., 2007; Zhang et al., 2011) and until now the obestatin natural receptor remains unknown. The data reported in this thesis clearly demonstrates that obestatin can act as a growth factor to promote cell proliferation in the PC3 prostate cancer cell line and this was consistent with other findings that showed obestatin could stimulate cell proliferation in human retinal epithelial cells (Camina et al., 2007), human gastric cancer cells (Pazos et al., 2007) and in pancreatic β-cells (Granata et al., 2008) through activation of ERK1/2 (Camina et al., 2007; Pazos et al., 2007; Granata et al., 2008) and Akt (Granata et al., 2008). Future studies are required to determine the cellular receptor through which obestatin exerts its proliferative effect.

Due to the accumulating controversy regarding obestatin’s biology, recent work investigating its structure and stability tried to address possible reasons for that inconsistency. One study showed that the quality and purity of obestatin peptides obtained from different manufacturers may have compromised experimental results and in some cases the peptides employed were insufficient to elicit in vitro and in vivo effects (De Spiegeleer et al., 2008). A recent study on the human obestatin
structure showed that in some, but not all, solvents analysed obestatin presents a secondary structure, an α-helix between residues Val¹⁴ and Ser²⁰, which was crucial for in vivo activity (Subasinghage et al., 2010). Furthermore, some groups have investigated possible roles for obestatin fragments (1-10) and (11-23) (Subasinghage et al., 2010; Agnew et al., 2012) and it remains to be elucidated whether obestatin is naturally further processed into smaller molecules and whether these fragments are biologically active. Recent evidence highlights the importance of the experimental conditions to obtain consistent results in obestatin research (De Spiegeleer et al., 2008; Subasinghage et al., 2010) and might help explain some of the inconsistencies reported in the obestatin field.

In addition to the controversies in obestatin research, the number of studies showing it possesses a similar proliferative effect to ghrelin and could act as an autocrine/paracrine factor in some cell types is growing, supporting a possible role for obestatin as well as other components of the ghrelin axis, in cancer progression. This thesis has addressed these issues with respect to cell proliferation, principally in the PC3 cell line, and the key findings are summarised and illustrated in figure 6.1.

Key questions that still remain to be resolved include identifying the alternative ghrelin/desacyl ghrelin receptor, identifying the obestatin receptor, clarifying the role of the PKC pathway in obestatin action, expanding the scope of the study to other prostate-derived cell lines, investigating other functional endpoints such as migration, invasion and apoptosis, better understanding of GOAT and APT1 mechanisms of action, better understanding of the regulation at both the gene and post-translational levels of functional peptide generation, quantitative expression studies in clinical samples to assess the clinical significance of the preproghrelin derived peptides in prostate cancer and its potential as a diagnostic and therapeutic target. Until these issues are determined, the potential for the ghrelin axis, and perhaps obestatin in particular, to be recognised as novel useful targets for therapy for cancer or other pathologies will be uncertain.
Figure 6.1 A proposed model for the effects of preproghrelin-derived peptides – ghrelin, desacyl ghrelin and obestatin – on cell proliferation in the PC3 prostate cancer cell line. PC3 cells express the ghrelin gene and its protein product preproghrelin, as well as the preproghrelin processing enzymes - the prohormone convertases PC1/3, PC2 and furin. Post-translational processing of preproghrelin occurs sequentially through the actions of PC1/3, PC2 and/or furin and further addition of the Ser\(^3\) n-octanoyl group is catalysed by ghrelin O-acyltransferase (GOAT), which is also expressed. Octanoic acid treatments significantly increase cell proliferation in PC3 cells suggesting a direct link between acylated ghrelin and cell proliferation. In addition, exogenous ghrelin significantly decreases GOAT expression in PC3 cells suggesting a negative feedback loop where ghrelin downregulates its own octanoylation. Exogenous ghrelin, desacyl ghrelin and obestatin significantly stimulate cell proliferation in the PC3 prostate cancer cell line, through phosphorylation of ERK1/2 and Akt. Obestatin-induced cell proliferation may also exert its effects through activation of the PKC pathway, however, further investigation is needed to clarify the role of this pathway in response to obestatin. GPR39 is expressed in prostate-derived cell lines, however, it is unlikely that obestatin binds and activates the GPR39 and its natural receptor (R-Z) remains to be identified. Although PC3 cells express the GHS-R1a at mRNA and protein levels, it remains unclear whether this receptor is involved in ghrelin-induced cell proliferation and it is likely that both ghrelin and desacyl ghrelin mediate their effects on cell proliferation through a yet unidentified alternative receptor (R-X). Furthermore, ghrelin and desacyl ghrelin act through different signaling mechanisms to stimulate cell proliferation in the PC3 prostate cancer cell line and it is possible that desacyl ghrelin could also bind and activate a desacyl ghrelin-only receptor (R-Y) to mediate its effects.
Chapter 7

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