AN INVESTIGATION INTO THE ROLE OF THE GHRELIN AXIS IN HORMONE-DEPENDENT CANCER AND CHARACTERISATION OF A NOVEL EXON 3-DELETED PREPROGHRELIN ISOFORM AND ITS MURINE HOMOLOGUE

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A thesis submitted for the degree of Doctor of Philosophy of the
Queensland University of Technology
2005
Abstract

Ghrelin is a 28 amino acid peptide hormone with a unique octanoic acid modification that has an extensive range of physiological effects, including stimulation of growth hormone (GH) release, appetite regulation, and modulation of reproductive functions. The cognate receptor for ghrelin is the growth hormone secretagogue receptor (GHS-R), a G protein-coupled receptor with two documented isoforms, the functional GHS-R type 1a and the C-terminally truncated GHS-R type 1b. Several ghrelin variants have also been identified in addition to the n-octanoylated form of ghrelin. In our laboratory, we have identified a novel exon 3-deleted preproghrelin variant that retains sequence for the mature ghrelin hormone and also encodes a novel C-terminal peptide (designated as C-terminal \( \triangle 3 \) peptide). There is emerging evidence to suggest that the ghrelin axis, encompassing ghrelin, several ghrelin variants and both forms of the GHS-R, is implicated in tumour growth. The objective of this project is to investigate the role of the ghrelin axis in hormone-dependent cancer and to further characterise the expression and function of the novel exon 3-deleted preproghrelin isoform.

Hormone-dependent cancers, including prostate and breast cancers, are significant causes of morbidity and mortality in the Western world. Improved diagnoses and treatments earlier in the progression of the disease are urgently required to improve patient outcomes. Growth factors play an integral role in prostate and breast cancer, particularly in the emergence of aggressive, hormone-refractory disease that is resistant to standard therapies. We have previously identified ghrelin as being a novel growth factor for prostate cancer cells \textit{in vitro} and have hypothesised that this may be extended to other hormone-dependent cancer types including breast cancer.

In the current study, techniques including real-time quantitative RT-PCR, Western blot analysis and immunohistochemistry have been used to determine and quantitate ghrelin, exon 3-deleted preproghrelin and GHS-R expression in prostate and breast cancer. Ghrelin and exon 3-deleted preproghrelin are highly expressed in prostate cancer tissues compared to expression levels in normal prostate glands. Similarly, breast carcinoma specimens display greater immunoreactivity for ghrelin
and exon 3-deleted preproghrelin than normal breast tissues. Expression of the exon 3-deleted preproghrelin mRNA isoform is upregulated in the oestrogen-independent, highly malignant MDA-MB-435 breast cancer cell line compared to the non-tumourigenic MCF-10A breast epithelial cell line, suggesting that augmented transcription of the isoform is associated with an increased malignant potential in breast cancer. The functional GHS-R type 1a is expressed in normal breast tissue and breast cancer specimens and cell lines. In contrast, the truncated GHS-R type 1b isoform is exclusively expressed in breast carcinoma. These data suggest that GHS-R type 1b, ghrelin and exon 3-deleted preproghrelin display potential as novel diagnostic markers for prostate and breast cancer.

These studies have been the first to demonstrate that ghrelin may have an important role in cell proliferation in breast and prostate cancer. Functional assays demonstrated that (10nM) ghrelin stimulated proliferation in the LNCaP prostate cancer cell lines (45.0 ± 1.7% above control, \( P < 0.01 \)) and rapidly activated the ERK 1/2 mitogen-activated kinase (MAPK) pathway in both PC3 and LNCaP cell lines. It does not, however, protect these cells from chemically-induced apoptosis. The MAPK inhibitors PD98059 and U0126 blocked ghrelin-induced MAPK activation, as well as cell proliferation, in both cell lines. Prostate cancer cells secrete mature ghrelin in vitro, and may therefore stimulate MAPK pathways in an autocrine manner. Ghrelin also appears to act as a growth factor in breast cancer cell proliferation, as the growth of MDA-MB-435 and MDA-MB-231 breast cancer cell lines is significantly increased by ghrelin treatment. Our findings suggest that the ghrelin axis could provide an important new target for adjunctive therapies for both breast and prostate cancer.

The C-terminal \( \Delta 3 \) peptide derived from exon 3-deleted preproghrelin may be an important new component of the ghrelin axis and studies into its function are currently in progress. Although it did not induce MAPK cascades or stimulate proliferation in prostate or breast cancer cell lines, the discovery of a murine counterpart, exon 4-deleted preproghrelin, indicates that it is highly conserved. Exon 4-deleted preproghrelin is expressed in all mouse tissues examined, with stomach being the predominant site of synthesis. Other components of the ghrelin axis were also found to be present in a wide-range of mouse tissues including brain, ovary and
prostate. This comprehensive report has paved the way for future work with in vivo mouse models of cancer.

This study has provided a substantial basis for the further evaluation of ghrelin, exon 3-deleted preproghrelin and the GHS-R type 1b as novel diagnostic/prognostic markers for prostate and breast cancer and supports the rationale for targeting the ghrelin axis for treatment of these tumours.

Keywords: Ghrelin, exon 3-deleted preproghrelin, GHS-R, growth factors, MAPK, ERK 1/2, hormone-dependent cancer, prostate, breast, diagnostic/prognostic marker, therapeutic targets.
List of publications, conference abstracts and patents

The following is a list of publications and submitted manuscripts, conference abstracts and patents that have been derived from the work performed for this thesis:


Yeh AH*, Jeffery PL*, Duncan RP, Herington AC, Chopin LK (2005) Ghrelin and a novel preproghrelin isoform are highly expressed in prostate cancer compared to normal prostate tissue and ghrelin-mediated prostate cancer cell line proliferation involves the MAPK pathway. Clinical Cancer Research, in press.

* These two authors contributed equally to the work as first authors

Other related publications:


Conference Abstracts


**Patents**

International Patent PCT/AU02/00582
‘Reproductive cancer diagnosis and therapy’
Chopin LK, **Jeffery PL**, Herington AC (14/11/2002)
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The work contained in this thesis has not been previously submitted for a degree or diploma at any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due references are made.

Signed:…………………………

Penelope Jeffery

Date:…………………………
Acknowledgements and Dedication

I would like to thank my supervisors, Dr Lisa Chopin and Professor Adrian Herington for their invaluable support, encouragement and friendship during my candidature. Many thanks also go to all of my colleagues in the Hormone-dependent cancer program for their advice and friendship. I would like to acknowledge the financial support I have received from the Australian Postgraduate Award PhD scholarship scheme and from the QUT Dean of Science top-up scholarship.

Many thanks and love to my parents, Adrienne and Ross, and to my partner John, for their boundless patience, support and babysitting and to my daughter Cordelia, my greatest achievement of all.

This thesis is dedicated to my brother, Randall.
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>AEBSF</td>
<td>4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride</td>
</tr>
<tr>
<td>AGRF</td>
<td>Australian Genome Research Facility</td>
</tr>
<tr>
<td>AMV</td>
<td>Avian myeloblastosis virus</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxyribose nucleic acid</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
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<tr>
<td>dNTP</td>
<td>Deoxy-nucleotide triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetra acetic acid di-sodium salt</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal regulated kinase</td>
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<tr>
<td>EST</td>
<td>Expressed sequenced tag</td>
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<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
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<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
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<tr>
<td>g</td>
<td>Grams</td>
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<tr>
<td>GGDT</td>
<td>Ghrelin gene-derived transcript</td>
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<tr>
<td>GH</td>
<td>Growth hormone</td>
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<tr>
<td>GH-R</td>
<td>Growth hormone receptor</td>
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<td>GHS</td>
<td>Growth hormone secretagogue</td>
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<tr>
<td>GHS-R</td>
<td>Growth hormone secretagogue receptor</td>
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<td>h</td>
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<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
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<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
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<td>kDa</td>
<td>Kilodaltons</td>
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M                        Molarity (when referring to concentration)
MAPK                    Mitogen activated protein kinase
mg/ml                   Milligrams per millilitres
min                     Minute
ml                      Millilitre
mM                      Millimolar
mmol/L                  Millimoles per litre
mRNA                    Messenger ribose nucleic acid
MTT                     3, [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
mV                      Millivolts
MW                      Molecular weight marker
NCBI                    National centre for biotechnology information
PAGE                    Polyacrylamide gel electrophoresis
PBS                     Phosphate buffered saline
PCR                     Polymerase chain reaction
PKC                     Protein kinase C
PLC                     Phospholipase C
pmol                    Picomole
PSA                     Prostate specific antigen
RNA                     Ribose nucleic acid
RPM                     Revolutions per minute
RPMI                    Roswell park memorial institute
RT-PCR                  Reverse transcriptase polymerase chain reaction
s                       Seconds
SDS                     Sodium dodecyl sulphate
SEM                     Standard error of the mean
Ser-3                   3rd residue (serine)
SSC                     Tri-sodium citrate
Ta                      Annealing temperature
TBS                     Tris buffered saline
TBST                    Tris buffered saline/tween
TMD                     Transmembrane domain
U/ml                    Units per millilitre
<table>
<thead>
<tr>
<th>WST-1</th>
<th>2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
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<tr>
<td>μg</td>
<td>Micrograms</td>
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<td>μg/ml</td>
<td>Micrograms per millilitre</td>
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<td>μl</td>
<td>Microlitre</td>
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<td>μm</td>
<td>Micrometre</td>
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<td>μM</td>
<td>Micromolar</td>
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CHAPTER ONE

INTRODUCTION

Description of scientific problem investigated

Hormone-dependent cancer has a significant impact upon morbidity and mortality rates in the Western world. Extensive research is underway to identify new therapeutic targets and markers to treat and more effectively diagnose the wide-ranging spectrum of these cancers that include prostate, breast, endometrial and ovarian cancers. The most prevalent forms of hormone-dependent cancer, prostate and breast cancer, account for over 30% of newly diagnosed cancers in Western men and women respectively (Jemal et al. 2005). Prostate cancer is the second-leading cause of cancer mortality in men, with males from developed countries facing a lifetime risk of 1 in 6 of developing prostate cancer (Jemal et al. 2005). Prostate tumourigenesis is driven largely by steroid androgens and anti-androgen therapy is the mainstay of prostate cancer treatment (So et al. 2003, Van der Poel, 2004). There is now considerable evidence that up-regulated growth factor activity promotes an aggressive, androgen-independent prostate cancer phenotype that is resistant to traditional hormonal therapies and is associated with a poor prognosis (Feldman and Feldman, 2001). This highlights the need for adjunctive therapies that can prevent, or overcome, hormone-refractory disease. The activation of mitogen-activated protein kinase (MAPK) cascades by growth factors can enhance prostate cancer cell proliferation and survival (Maroni et al. 2004). Manipulation of MAPK signal transduction pathways is therefore a novel therapeutic strategy that has been proposed for the treatment of advanced prostate cancer (Sebolt-Leopold and Herrera, 2004). Diagnosis of prostate cancer has proven problematic due to the non-specificity of the most widely used tumour marker, serum prostate specific antigen (PSA) (Thompson et al. 2004). The PSA detection test is also unable to accurately predict pathological stage and new prognostic markers are clearly required to give a better profile of prostate tumour grade and severity (Watson and Schalken, 2004).
Breast cancer in women and prostate cancer in men share many common features including epidemiology, mortality rates and hormonal-dependence (Lopez-Otin and Diamandis, 1998). The effectiveness of selective oestrogen receptor modulators (SERMs) attests to the importance of oestrogen-withdrawal in the treatment of breast cancer. As is the case for prostate cancer, however, elevated growth factor signalling may allow for the emergence of hormone-refractory breast cancer which is associated with higher mortality rates, particularly in premenopausal women (Nicholson et al. 2004). Pharmacological molecules designed to block various growth factors and their receptors have displayed some success in the laboratory and the clinic, and this field of cancer treatment, including small molecule therapy, remains promising (Guillemard and Saragovi, 2004). The identification of new therapeutic targets as well as novel diagnostic and prognostic markers, particularly in hormone-refractory cancer, is imperative. Growth factors have the potential to fulfil these roles in the future.

Growth hormone (GH) and its downstream mediators, the insulin-like growth factors (IGFs), have been implicated in both direct and indirect modulation of prostate and breast cancer cell function (Chopin and Herington, 1999, Laban et al. 2003). Components of the GH axis have therefore been presented as potential targets for anti-tumour therapy. In 1999, a 28 amino acid peptide hormone capable of potently stimulating GH release was purified from stomach tissue (Kojima et al. 1999). This hormone, named ghrelin (ghre: grow), was identified several years after its endogenous receptor, the growth hormone secretagogue receptor (GHS-R) (Howard et al. 1996). It is now widely accepted that ghrelin and its receptor are important new components of the GH axis. Ghrelin action is not restricted to GH release, however, and it possesses many potentially important physiological functions including appetite stimulation and energy homeostasis (Nakazato et al. 2001, Shintani et al. 2001), embryo implantation (Tanaka et al. 2003, Duncan et al. 2005) and cardiovascular actions (Benso et al. 2004). Several ghrelin isoforms have also been identified, including a novel exon 3-deleted preproghrelin variant discovered in our laboratory (Jeffery et al. 2002). Expression and function of this variant, along with that of its murine homologue, exon 4-deleted preproghrelin, was investigated over the course of this project. Ghrelin has also been shown to be a mitogen for cancer cell lines and our study of the proliferative effect of ghrelin on
prostate cancer cells was one of the first published in this field (Jeffery et al. 2002). The physiological role of ghrelin as a growth factor in hormone-dependent cancer requires further elucidation.

**Overall objectives of the study**

Preliminary data from our laboratory suggested that components of the ghrelin axis display potential as novel therapeutic targets and diagnostic markers for prostate cancer (Jeffery et al. 2002). The overall objective of this study was to investigate the role that the ghrelin axis, including the novel exon 3-deleted preproghrelin isoform, may play in prostate cancer, and to extend these studies to encompass breast cancer. This study also aimed to provide a better understanding of the physiology of the human ghrelin axis in general. Expression of the murine ghrelin axis was also investigated to prepare for the development of future mouse models.

**Specific aims of the study were:**

- **To investigate the expression of mRNA and protein for ghrelin, preproghrelin isoforms and the GHS-R** in prostate and breast cancer cell lines and tissues, as well as in normal tissues, using RT-PCR, real time quantitative RT-PCR, immunohistochemistry and Western blot analysis

- **To perform functional assays on prostate and breast cancer cell lines in vitro treated with synthetic ghrelin**
  Proliferation, migration and apoptosis assays were performed and the concentration of GH secreted into the conditioned media measured. The signalling mechanisms underlying ghrelin action in prostate cancer cells were also investigated in the laboratory, by studying the involvement of the mitogen-activated protein kinase (MAPK) pathways.

- **To investigate the expression of a mouse homologue of ghrelin and preproghrelin isoforms** in a wide range of mouse tissues using RT-PCR, real time quantitative RT-PCR and immunohistochemistry
Summary of scientific progress linking the journal manuscripts

This thesis is being presented “By Publication” based on Queensland University of Technology guidelines. Accordingly, each chapter represents a manuscript that has been published, or has been submitted for publication, in an international refereed journal.

Chapter 2: Literature Review: The potential autocrine/paracrine roles of ghrelin and its receptor in hormone-dependent cancer

This literature review was the first to be published regarding the role of the ghrelin axis in hormone-dependent cancer. This paper reviews the literature describing the expression, regulation and function of ghrelin and the GHS-R in cancer, as well as providing an updated assessment of studies exploring the role of GH and IGF-1 in cancer. The exon 3-deleted preproghrelin isoform was first discovered in prostate cancer cell lines (Jeffery et al. 2002), and this review contains a further analysis and schematic depiction of the exon deletion and putative protein sequence of this isoform. This paper was published in Cytokine and Growth Factor Reviews (2003) 14: 113-22. A brief literature update, encompassing recent studies on the ghrelin axis in cancer (2002-2005), is also included at the end of this chapter (Chapter 2, part B).

Chapter 3: Research article: Ghrelin and a novel preproghrelin isoform are highly expressed in prostate cancer compared to normal prostate tissue and ghrelin-mediated prostate cancer cell proliferation involves the MAPK pathway

As a natural progression following our studies in prostate cancer cell lines (Jeffery et al. 2002) we investigated the signalling pathways underlying ghrelin-stimulated prostate cancer cell proliferation. This paper contains novel data, derived from studies performed with the co-author Anthony Yeh, demonstrating that ghrelin increases prostate cancer cell proliferation via the MAPK pathway. The expression and function of the exon 3-deleted preproghrelin isoform discovered in our laboratory (Jeffery et al. 2002), and wild-type ghrelin, are more fully characterised in
prostate histopathological specimens and cell lines. This manuscript has been accepted for publication in Clinical Cancer Research (2005) and is currently in press.

 Chapter 4: Research article: Expression and function of the ghrelin axis, including a novel preproghrelin isoform, in human breast cancer tissues and cell lines

Breast cancer is the most commonly diagnosed malignancy in women in the Western world (Jemal et al. 2005), and like prostate cancer in men, is highly sensitive to the effects of growth factors. We therefore decided to investigate the role of the ghrelin axis in this hormone-dependent cancer. Ghrelin, exon 3-deleted preproghrelin, and GHS-R expression was demonstrated at the mRNA and protein level in breast cancer cell lines and tissues. Negligible expression of the GHS-R type 1b in normal breast tissue compared to expression in breast cancer tissues indicates that this receptor isoform may possess a unique role in breast cancer and has potential as a diagnostic marker. Ghrelin treatment elicited proliferation in breast cancer cell lines, as was previously observed in prostate cancer cell lines. Expression of the exon 3-deleted preproghrelin mRNA isoform was observed to be upregulated in breast cancer cell lines compared to expression in a non-tumourigenic breast epithelial cell line. This manuscript has been accepted for publication in Endocrine Related Cancer (2005) and is currently in press.

 Chapter 5: Research article: Expression of the ghrelin axis in the mouse: an exon 4-deleted proghrelin mouse variant encodes a novel C-terminal peptide

The data generated from the in vitro studies described in Chapters 3 and 4 has provided a substantial base for future work examining the role of the ghrelin axis in prostate and breast cancer in vivo. Mouse models will be important tools for this work, therefore, characterisation of the murine ghrelin axis was undertaken and the data derived from these studies are presented in Chapter 5. This paper also describes the expression of a murine homologue of exon 3-deleted preproghrelin and demonstrates that the novel isoform is conserved across species, implying physiological significance. The murine ghrelin isoform was shown to be expressed at the mRNA and protein levels by quantitative RT-PCR, Western blot and immunohistochemical analysis. This research article was the first comprehensive
report on the expression of the ghrelin axis and the ghrelin isoform in the mouse (Jeffery et al. 2005) and has been published in Endocrinology (2005, 146: 432-40).
REFERENCES


CHAPTER TWO

LITERATURE REVIEW

THE POTENTIAL AUTOCRINE/PARACRINE ROLES
OF GHRELIN AND ITS RECEPTOR IN
HORMONE-DEPENDENT CANCER

Penny L Jeffery, Adrian C Herington and Lisa K Chopin

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CHAPTER TWO (PART B)

LITERATURE REVIEW UPDATE
Literature Review Update

From the time that our review was published in early 2003, the body of research concerning the role of the ghrelin axis in cancer has expanded considerably; hence a brief update of the literature is required. This chapter contains a review of recent articles pertaining to ghrelin and the GHS-R in cancer, as this is the focus of subsequent chapters in this thesis.

2B.1 Ghrelin/GHS-R expression in cancer

The number of tumour types that have been demonstrated to express ghrelin and the GHS-R has increased in recent years. Endocrine tumours that produce and secrete high quantities of ghrelin into the circulation (named ghrelinomas), have been identified. A grossly elevated serum ghrelin concentration was measured in a patient with a highly malignant gastric ghrelinoma (Tsolakis et al. 2004) and the occurrence of a rare pancreatic ghrelinoma has been reported (Corbetta et al. 2003). A subset of pancreatic islet cell tumours associated with multiple endocrine neoplasia-1 (MEN-1) show ghrelin immunoreactivity (Iwakura et al. 2002). Ghrelin and both isoforms of the GHS-R are expressed in a bronchial neuroendocrine tumor, but not in adjacent normal lung tissue, suggesting a modulatory role for the ghrelin axis in this tumour type (Arnaldi et al. 2003). Ghrelin and the functional GHS-R type 1a are also expressed in reproductive system malignancies including highly differentiated testicular tumours (Gaytan et al. 2004) and ovarian tumours (Gaytan et al. 2005).

2B.2 Interaction of the ghrelin axis with sex steroid hormones

A plethora of experimental and epidemiological studies has demonstrated that sex steroids, including oestrogens and androgens, are the prime modulators of hormone-dependent cancers, particularly those of the breast and prostate (Haslam and Woodward, 2003, So et al. 2003). Recent research has indicated that ghrelin and its cognate receptor (GHSR 1a) may interact with sex steroid hormones including androgens, oestrogens and progesterone. Ovarian thecal and granulosa cells are prime sites of oestrogen biosynthesis in the pre-menopausal female (Ryan and Petro,
1966), and these cell types express ghrelin and the GHSR 1a in a variable pattern across the normal menstrual cycle, suggesting that ghrelin may modulate oestrogen function (Gaytan et al. 2003). This may only be relevant to autocrine/paracrine oestrogen physiology, as ghrelin administration in rats did not perturb serum estradiol levels (Fernandez-Fernandez et al. 2005), and surprisingly, there are no available data regarding the impact of ghrelin treatment on oestrogen levels in humans.

Conversely, oestrogen may regulate ghrelin expression, as in rats oophorectomy significantly increases plasma ghrelin levels and the number of ghrelin-immunoreactive cells in the stomach (Matsubara et al. 2004). This effect is reversed upon administration of 17-β-estradiol indicating that oestrogen negatively regulates ghrelin production in the rat (Matsubara et al. 2004). In humans studies however, oestrogen supplementation increased plasma ghrelin concentrations in severely-undernourished female patients (Grinspoon et al. 2004) and in postmenopausal women receiving HRT (hormone replacement therapy), although the mechanisms behind these effects are unknown (Kellokoski et al. 2005). Postmenopausal HRT use has been associated with an increased risk of developing breast cancer (Chlebowski et al. 2003), although studies in this field are contradictory (Creasman, 2005). Oestrogen also potentiates ghrelin-induced pulsatile GH secretion (Veldhuis and Bowers, 2003), and is an important modulator of GH function (Leung et al. 2004). GH is emerging as an important oncogenic hormone in breast cancer (Waters and Conway-Campbell, 2004). No research has been published to date regarding the potential interaction of ghrelin with oestrogen in the context of breast cancer.

Other sex hormones appear to influence ghrelin secretion. Androgen levels in women with polycystic ovary disease are elevated and may inhibit ghrelin secretion, resulting in lower circulating ghrelin levels compared to normal controls (Pagotto et al. 2002, Panidis et al. 2005). Ghrelin levels increase to normal concentrations in these patients after androgen-blockade (Gambineri et al. 2003). Ghrelin and GHSR 1a co-immunoreactivity is present in ovarian hilus interstitial cells which are capable of testosterone secretion, under the control of luteinising hormone (Gaytan et al. 2003, Erickson et al. 1985). This research indicates that
ghrelin may regulate androgen secretion and/or function in the ovary (Gaytan et al. 2003). Ghrelin and the GHSR 1a are also co-expressed in the steroidogenic Leydig cells of the testis and ghrelin inhibits testosterone secretion from testicular tissue in vitro, potentially by downregulating the expression of key enzymes in the steroidogenic pathway (Gaytan et al. 2004).

The synthetic progestin molecule, cyproterone acetate, is the most frequently used steroidal anti-androgen in prostate cancer therapy (Culig et al. 2004). The recent finding that cyproterone acetate significantly elevates total ghrelin levels in healthy men (Gambineri et al. 2005) may hold significance for the treatment of men with advanced prostate cancer, as cell lines derived from metastatic prostate tumours proliferate in response to ghrelin treatment (Jeffery et al. 2002).

2B.3 Functional studies on the role of ghrelin/GHS-R in cancer

Enhanced proliferation in response to ghrelin treatment has now been demonstrated in numerous cell lines and many studies have investigated the signal transduction pathways underlying this response. Exogenous ghrelin increases the proliferative, invasive and migratory potential of pancreatic adenocarcinoma cells in vitro, via augmented Akt phosphorylation (Duxbury et al. 2003). Ghrelin activates the ERK1/2 MAPK pathway and thereby stimulates growth in adrenocortical cells (Andreis et al. 2003, Mazzocchi et al. 2004), 3T3-L1 adipocytes (Kim et al. 2004) and the rodent somatotroph pituitary tumour cell line GH3 (Nanzer et al. 2004). Proliferation of primary cultures of rodent osteoblasts also increases after treatment with physiological concentrations of ghrelin (Maccarinelli et al. 2005), whilst rodent neuronal proliferation is stimulated by ghrelin both in vivo and in vitro (Zhang et al. 2004). Endogenous ghrelin may be part of an autocrine loop sustaining growth of human erythroleukemic (HEL) cells, as blocking antibodies inhibit proliferation of this cell line in vitro (De Vriese et al. 2005). The growth-promoting role of ghrelin may also be attributed to its anti-apoptotic actions in many cell lines (Baldanzi et al. 2002, Kim et al. 2004, Mazzocchi et al. 2004, Kim et al. 2005). Apoptosis plays an integral role in tumour growth; therefore ghrelin may contribute to tumourigenesis by enhancing tumour cell survival. Some conflicting studies, however, have reported an inhibitory role for ghrelin in tumour biology. For example, apoptosis is increased in
aldosteronoma cell lines after exposure to exogenous ghrelin (Belloni et al. 2004). In agreement with their previous work in breast cancer cells (Cassoni et al. 2001), Volante and co-workers have demonstrated that ghrelin inhibits proliferation of thyroid carcinoma cell lines in vitro, an effect that may be mediated by a receptor other than the GHS-R (Volante et al. 2003). This research group has also recently published data that agreed with our own studies (Jeffery et al. 2002) regarding the mitogenic effect of ghrelin in the PC3 prostate cancer cell line (Cassoni et al. 2004). The non-acylated form of ghrelin may have a negative impact upon cell growth, as the des-acyl ghrelin overexpressing transgenic mouse displays a small phenotype and lowered GH/IGF-I levels (Ariyasu et al. 2005). The physiological effect of ghrelin on cultured cells in vitro is clearly cell type-specific and may be reliant upon GHS-R isoform expression, intracellular signalling crosstalk, and ghrelin acylation status; however, this phenomenon is yet to be fully explored.
REFERENCES


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CHAPTER THREE

GHRELIN AND A NOVEL PREPROGHRELIN ISOFORM ARE HIGHLY EXPRESSED IN PROSTATE CANCER COMPARED TO NORMAL PROSTATE TISSUE AND GHRELIN-MEDIATED PROSTATE CANCER CELL LINE PROLIFERATION INVOLVES THE MAPK PATHWAY

Anthony H. Yeh*, Penelope L. Jeffery*, Russell P. Duncan, Adrian C. Herington and Lisa K. Chopin

* These two authors contributed equally to the work as first authors.

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Clinical Cancer Research (2005), in press
CHAPTER FOUR

EXPRESSION AND FUNCTION OF THE GHRELIN AXIS, INCLUDING A NOVEL PREPROGHRELIN ISOFORM, IN HUMAN BREAST CANCER TISSUES AND CELL LINES

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Endocrine-Related Cancer (2005), in press
CHAPTER FIVE

EXPRESSION OF THE GHRELIN AXIS IN THE MOUSE: AN EXON 4-DELETED MOUSE PROGHRELIN VARIANT ENCODES A NOVEL C-TERMINAL PEPTIDE

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CHAPTER SIX

GENERAL DISCUSSION

The discovery of the peptide hormone ghrelin in 1999, several years after its endogenous receptor, the growth hormone secretagogue receptor (GHS-R) was identified, has had a substantial impact upon the field of endocrinology. The ubiquitous expression of ghrelin in the body, as well as its significant concentration in serum (Kojima et al. 1999), suggests a wide range of physiological roles for this unique hormone, in addition to growth hormone (GH) release. There is an expanding body of research supporting a role for ghrelin and its receptor in cancer. Our previous work published in 2002, was the first to report the expression of ghrelin, an exon 3-deleted preproghrelin mRNA variant, and the GHS-R in prostate cancer cell lines and also reported the growth-promoting effect of ghrelin on PC3 prostate cancer cells in vitro (Jeffery et al. 2002). This work led to our hypothesis that ghrelin is a growth factor in prostate cancer and, potentially, other cancers. Prostate and breast cancer are hormone-dependent cancers that are significant causes of cancer mortality in Western men and women respectively (Jemal et al. 2005) and are sensitive to the effects of growth factors and cytokines. Therefore, a logical extension of my initial studies in prostate cancer cell lines (Jeffery et al. 2002) was to investigate the role of the ghrelin axis in breast cancer.

Our hypothesis that ghrelin is a growth factor for cancer required further elucidation and therefore the objectives of this thesis were to investigate the expression and function of the ghrelin axis in human hormone-dependent cancers, specifically prostate and breast cancer. These studies include the description of a novel ghrelin isoform, exon 3-deleted preproghrelin. The salient findings of this project include the full description of exon 3-deleted preproghrelin expression in prostate and breast cancer, the identification of ghrelin and exon 3-deleted preproghrelin as potential diagnostic markers for cancer and the identification of the ghrelin axis as a novel target for therapeutics. A full characterisation of the murine ghrelin axis, as a prelude to future in vivo studies using mouse models, has also been performed.
Characterisation of the exon 3-deleted preproghrelin isoform

The expression of the human exon 3-deleted preproghrelin mRNA variant was first identified in prostate cancer cell lines (Jeffery et al. 2002) and in this thesis I have characterised its expression and explored the potential functions of this novel isoform in prostate cancer and breast cancer tissues and cell lines. Translation of the full-length human preproghrelin transcript results in a 117 amino acid peptide that is processed to produce the 28 amino acid mature ghrelin hormone (Kojima et al. 1999). Low molecular weight peptides derived from the carboxy terminus of proghrelin, which may possess cardiovascular activity, are also present in human serum (Pemberton et al. 2003). Exclusion of exon 3 from the preproghrelin transcript results in a truncated preproghrelin peptide of 91 amino acids, with a unique 16 amino acid C-terminal peptide sequence (C terminal \( \Delta 3 \) peptide). The C-terminal peptide begins with a pair of arginine residues, known to be a classic protease cleavage signal for prohormones (Docherty and Steiner, 1982, Seidah and Chretien, 1997). Bioinformatic analysis of the dibasic sequence demonstrates that it is a trypsin cleavage site (Expasy Proteomics Server, Swiss Institute of Bioinformatics), indicating that C-terminal \( \Delta 3 \) peptide may be cleaved and exist independently from the rest of exon 3-deleted preproghrelin. Using Western blot analysis, we were able to determine that cleavage of C-terminal \( \Delta 3 \) peptide from the rest of the exon 3-deleted preproghrelin peptide does occur in human stomach tissue and in LNCaP prostate cancer cell lysate (Chapter 3). Importantly, the exon 3-deleted preproghrelin isoform retains sequence coding for the mature ghrelin peptide (Figure 1). Using primers specific for the exon 3-deleted preproghrelin transcript, I have now demonstrated the expression of the isoform in prostate (Chapter 3) and breast cancer specimens and cell lines (Chapter 4), and in a range of other human tissues including anterior pituitary and placenta (Jeffery et al. in preparation). The antibody used to detect protein expression of the exon 3-deleted preproghrelin was raised against the C-terminal peptide and displayed specificity for the isoform. This is the only antibody currently available that can distinguish the unique isoform from full-length preproghrelin protein and has enabled detection of exon 3-deleted preproghrelin expression in prostate and breast tissue specimens by immunohistochemistry. Measurement of C-ghrelin peptide concentrations in human blood has recently been successfully performed (Pemberton et al. 2003) and a similar
approach using HPLC coupled with RIA could be used in the future to quantitate C-terminal \( \Delta 3 \) peptide immunoreactivity in serum taken from prostate and breast cancer patients.

A comparison of the exon 3-deleted preproghrelin sequence with those in EST databases (Blast, NCBI) unearthed an homologous preproghrelin sequence in a mixed mouse pancreas library (GenBank accession # BM054332), leading to the discovery and characterisation of the murine homologue (Chapter 5). This isoform was named exon 4-deleted preproghrelin because the mouse ghrelin gene was known to consist of 5 exons (Tanaka et al. 2001). It has since been demonstrated that human ghrelin gene also consists of five exons (Kanamoto et al. 2004), although the human exon 3-deleted preproghrelin has been named according to coding exons only. Along with murine GHS-R and ghrelin, exon 4-deleted preproghrelin was detected in a wide-range of mouse tissues, with stomach being the predominant site of exon 4-deleted preproghrelin synthesis (Jeffery et al. 2005; Chapter 5). The unique C-terminal peptide of mouse exon 4-deleted preproghrelin exhibits significant homology (approximately 60\%) with its human counterpart. This peptide sequence conservation suggests a functional role for the novel isoform.

**Figure 1. Schematic depiction of full-length and exon 3-deleted preproghrelin proteins.**

Full-length preproghrelin is a 117 amino acid prohormone which is processed to produce the mature ghrelin hormone. Deletion of exon 3 from the preproghrelin transcript generates a premature stop codon and therefore a truncated C-peptide with a novel pro-fragment (C-terminal \( \Delta 3 \) peptide). The introduction of a dibasic cleavage site (RR) may allow cleavage of this C-terminal pro-fragment *in vivo.*
The identification of ghrelin and exon 3-deleted preproghrelin as potential diagnostic markers for hormone-dependent cancer

Better indicators of prostatic disease and tumour progression are urgently required. The widely used serum prostate-specific antigen (PSA) test for prostate malignancy is limited by poor specificity (Thompson et al. 2004), and there are currently no reliable prognostic markers for prostate cancer (Watson and Schalke, 2004). Similarly, there are few validated prognostic markers for breast cancer, although expression of growth factors and their receptors (for example HER-2/neu) are often included in diagnostic profiles of breast disease (Coradini and Daidone, 2004). Immunohistochemistry is commonly employed for detection and localization of protein expression in histological sections; however, it is also emerging as an important tool in the diagnosis of malignant changes in tissue, as well as for grading tumour severity. Prognostication aided by immunohistochemical markers has the potential to better predict prostate cancer behaviour than traditional histological grading alone (Sivridis et al. 2002, Epstein, 2004). The immunohistochemical studies performed in this project revealed that both ghrelin and the novel preproghrelin isoform are more abundantly expressed in malignant tissues when compared to levels of expression in normal tissues. At the protein level, greater immunoreactivity for ghrelin and exon 3-deleted preproghrelin was found in human prostatectomy tumour specimens than normal prostate tissue by immunohistochemistry (Chapter 3). This was also found to be the case in breast cancer specimens when compared to normal breast tissue (Chapter 4) and this suggests that ghrelin and exon 3-deleted preproghrelin may represent novel diagnostic or prognostic immunohistochemical markers for prostate and breast cancer.

Real-time RT-PCR is an effective, sensitive technique for the detection and quantitation of low levels of mRNA expression (Pfaffl et al. 2002). Ghrelin mRNA is known to be a low abundance mRNA species, therefore, we utilised this technique to quantitate full-length preproghrelin and exon 3-deleted preproghrelin transcripts in breast cancer cell lines of varying oncogenic phenotypes. Two methods of quantitation are possible: absolute and relative quantitation. In this project, the relative quantitation strategy was employed. This method is based on the expression ratio of a target transcript versus a reference transcript (in this case 18S ribosomal
RNA). This technique is effective for detecting changes in the mRNA levels between different samples and has been used successfully to investigate potential prognostic markers in breast cancer (Peirce and Chen, 2001). Importantly, real time RT-PCR demonstrated that exon 3-deleted preproghrelin transcript expression is significantly up-regulated in highly malignant breast cancer cell lines (MDA-MB-231 and MDA-MB-435) when compared to a benign breast epithelial cell line (MCF-10A). This reflects our data generated from immunohistochemical studies on breast tissues and provides more evidence for a role for the unique isoform in breast cancer, including its potential as a novel diagnostic marker (Chapter 4).

Current mathematical models used to analyse data generated with the relative quantitation strategy allow for only one transcript to be quantitated at a time – that is, relative expression levels of a single transcript can be compared between samples, however, multiple transcripts cannot. Software that can compare the relative expression levels of up to four target genes in a sample has been developed to circumvent this problem (Pfaffl et al. 2002) and could be useful for future studies. This would allow the direct comparison of full-length and exon 3-deleted preproghrelin transcript levels in one tissue and would allow us to determine if transcription of the exon 3-deleted preproghrelin isoform is favoured over full-length preproghrelin, as is the case for many alternatively-spliced transcripts in tumour tissues (Venables, 2004). During the course of this project, real time RT-PCR analysis of prostate cancer specimens for ghrelin and exon 3-deleted preproghrelin expression was commenced; however, difficulty in obtaining prostate tumour samples did not allow completion of this task. Full evaluation and validation of ghrelin and exon 3-deleted preproghrelin as novel prognostic markers for prostate and breast cancer requires further experiments with a larger number of histopathological specimens. This work is clearly warranted given the data presented in this thesis.

Identification of the ghrelin axis as a potential target for therapeutics in prostate and breast cancer

The mechanism behind the proliferative effect of ghrelin in the LNCaP and PC3 prostate cancer cell lines (Jeffery et al. 2002) was further explored in this study.
Ghrelin is the endogenous ligand for the GHS-R; a G protein-coupled receptor (GPCR) (Kojima et al. 1999, Sun et al. 2004) that is expressed in prostate cancer cell lines and tissues (Jeffery et al. 2002, Chapter 3) and may be involved in an autocrine/paracrine loop in ghrelin-mediated prostate cancer cell proliferation (Jeffery et al. 2002). GPCR signalling by various ligands can generate a mitogenic response in prostate cell lines, often via crosstalk with mitogen activated protein kinase (MAPK) pathways (Daaka, 2004). Phosphorylated (activated) MAPKs, specifically the extracellular-signal regulated kinases (ERK 1 and 2), have been implicated in prostate tumourigenesis, and growth factor-induced activation of MAPK pathways results in increased cell proliferation and metastasis (Maroni et al. 2004). Murata and co-workers reported that ghrelin upregulates MAPK activity and cell proliferation in the hepatoma cell line HepG2 (Murata et al. 2002) and therefore, we speculated that this might also occur in prostate cancer cell lines (Figure 2). In studies carried out primarily by an Honours student in our laboratory (Anthony Yeh), it was shown that ghrelin did indeed rapidly activate the extracellular-signal regulated kinases (ERKs) in the LNCaP and PC3 prostate cancer cell lines, an effect that was completely abolished with specific MAPK inhibitors (Chapter 3, Yeh et al, in press). The alternative MAPK pathway, the p38 kinase pathway, was also activated (phosphorylated) in the LNCaP cell line after ghrelin treatment (Chapter 3). Phosphorylated p38 MAPK activates transcription factors and cell-cycle regulators which in turn influence cell proliferation, apoptosis and splicing events (Olson and Hallahan, 2004). In the MCF-10A breast epithelial cell line, the p38 MAPK pathway also interacts with the ERK 1/2 cascade resulting in increased cell migration and invasion (Kim et al. 2003). Phospho-p38 MAPK can also activate the pro-survival PI3K/AKT pathway (Horowitz et al. 2004) and ghrelin has also been shown to signal through the PI3/AKT pathway in some cell types (Duxbury et al. 2003); therefore, further work focusing on the downstream effects ghrelin-induced p38 activation in prostate cancer is required.

Although ghrelin did not appear to protect prostate cancer cells from chemically-induced apoptosis, the potential effect of ghrelin on apoptosis and cell survival could be more accurately quantified using a Cell Death ELISA™ PLUS (Roche) to measure DNA fragmentation and a Caspase 3 Activity Assay (Roche), both using a fluorescence microplate reader (PolarSTAR, BMG). Direct observation of DNA
fragmentation could also be achieved by fluorescent microscopy using a TUNEL assay (*In situ* cell death detection kit, Roche) on cells grown on coverslips. The influence of ghrelin treatment on the regulation of genes that are anti-apoptotic (Bcl-2/BclXL, NF-κB, IGF-I, caveolin and Akt) or pro-apoptotic (PTEN, p53, Bin1, TGF-beta, and Par-4) in prostate cancer (Gurumurthy *et al.* 2001) could be determined using real-time PCR, although not all of these genes are present in all of the cell lines used in this study.

A number of studies have since been published demonstrating that ghrelin augments cell proliferation via MAPK activation in a range of cell lines (Andreis *et al.* 2003, Kim *et al.* 2004, Mazzocchi *et al.* 2004, Nanzer *et al.* 2004, Zhang *et al.* 2004). The finding that the proliferative effect of ghrelin in prostate cancer cells is primarily a result of ghrelin-induced MAPK pathways is extremely significant given that new treatments for hormone-refractory prostate cancer are urgently required, with MAPK cascades being a promising new target for anti-cancer therapy (Maroni *et al.* 2004) (Figure 2).
Figure 2. A proposed model of ghrelin-mediated cell proliferation in prostate cancer cells.

Ghrelin is produced by prostate cancer cells and is secreted into culture medium. Secreted ghrelin may then act in an autocrine/paracrine fashion to stimulate MAPK cascades via the GHS-R, which is also expressed in prostate cancer. Phosphorylation of ERK 1 and 2 by exogenous ghrelin treatment stimulates prostate cancer cell proliferation. Activated ERK 1 and 2 may also bind transcription factors (TF) including ELK-1. Binding sites for ELK-1 exist on the ghrelin promoter (Kanamoto et al. 2004); hence, autocrine/paracrine ghrelin action may also augment ghrelin production, further driving cell proliferation. The novel exon 3-deleted preproghrelin isoform is also expressed by prostate cancer cells, although its precise function is as yet unknown. MAPK inhibitors decrease cancer cell proliferation (blue arrows) and blocking of ghrelin production, secretion or signalling (red arrows) may also antagonize MAPK cascades, thereby representing a novel therapeutic approach for cancer in the future. This model may also be applicable to breast cancer cells and other hormone-dependent cancer cell types.
Ghrelin stimulates cultured pituitary cells to secrete growth hormone (GH) (Kojima et al. 1999). GH is a potent mitogen for prostate cancer cells in vitro (Untergasser et al. 1999), and has been identified as being an important oncogene in breast cancer (Zhu et al. 2005). As prostate cancer cells have been shown to express the GH axis (Chopin et al. 2002a), a commercial GH ELISA (Roche) was used to determine if the addition of ghrelin to prostate cancer cells induced the secretion of prostatic GH in vitro. Neither ghrelin nor C-terminal△3 peptide induced measurable GH release from LNCaP or PC3 prostate cancer cell lines (data not shown). However, investigating the effect of ghrelin treatment on expression of GH, and its downstream mediator insulin-like growth factor I (IGF-I), in the prostate in vivo is an important area of future study.

Functional assays demonstrating that ghrelin promotes proliferation in breast cancer cell lines also supports previous work performed in prostate cancer cell lines (Jeffery et al. 2002, Chapter 3). However, this contrasts with reports published by other groups. In particular, ghrelin has been shown to inhibit cell proliferation of breast and thyroid carcinoma cell lines in vitro (Cassoni et al. 2001, Volante et al. 2003). The proliferative actions of ghrelin appear to be cell-specific, possibly due to GHS-R subtype expression and crosstalk between signalling pathways (Duxbury et al. 2003, Maroni et al. 2004). The acylation status of ghrelin also clearly influences the function of the peptide (Ariyasu et al. 2004) and the octanoic acid modification of ghrelin is lost rapidly with non-optimum storage conditions (Hosoda et al. 2004). Des-acyl ghrelin has recently been shown to have a negative impact on tissue growth in an over-expressing mouse model, potentially via the modulation of octanoylated ghrelin-induced GH secretion (Ariyasu et al. 2005). Evidence to support a clear role for ghrelin in the mitogenesis of a range of cell lines in vitro now exists (see Literature update, Chapter 2). The moderate yet significant increase in cell growth post-ghrelin treatment may be due to the fact that autocrine production of endogenous ghrelin (Figure 2) is sufficient to stimulate proliferation, as is postulated to be the case with the human erythroleukemic cell line HEL (DeVriese et al. 2005). The use of anti-sense technology to downregulate ghrelin expression could help to clarify this issue in the future. Indeed, inhibition of ghrelin signalling may have therapeutic potential in prostate and breast (Chopin et al. 2002b); however, effective ghrelin/GHS-R antagonists were not available during the course of this project.
Potent and selective ghrelin analogues that bind to the GHS-R and inhibit ghrelin signalling have recently been developed (Halem et al. 2004, Liu et al. 2004) and may prove useful for inhibition of the ghrelin axis in prostate and breast cancer.

Treatment of prostate and breast cancer cell lines with the C-terminal peptide of exon 3-deleted preproghrelin (C-terminal△3 peptide) had no effect on proliferation or MAPK phosphorylation, and the primary role of the isoform may only be to produce the mature ghrelin peptide (Figure 1). I have established a continuous cell line derived from normal epithelial prostate cells (RWPE-1, Bello et al. 1997) and the PC3 prostate cancer cell lines, transfected with exon 3-deleted preproghrelin mRNA using a tetracycline-regulated expression system for mammalian cells (T-Rex, Invitrogen). The phenotype of these cell lines is currently being investigated and will allow a more thorough functional analysis of the novel isoform in the future. Prostate cancer cell lines overexpressing full-length preproghrelin have also been generated in our laboratory, although due to time constraints, their phenotypes have yet to be examined. Migration of RWPE-1 cells towards a chemoattractant (bovine calf serum) is increased in the presence of exogenous ghrelin (data not shown) and therefore it would be interesting to examine the migratory properties of these overexpressing cell lines and the behaviour of breast cancer cell lines transfected with these constructs. Future studies involving the subcutaneous or subcapsular renal injection of the transfected cells into nude or SCID mice would also be valuable in assessing the metastatic potential of prostate or breast cancer cells overexpressing ghrelin and or exon 3-deleted preproghrelin in an in vivo model.

This project has relied heavily on the use of experimentally immortalized cell lines for in vitro functional assays and for extraction of RNA and protein. It is a regular criticism that cell-based assays are not physiologically relevant and that cell line models are not valid representations of disease in vivo. The immortalization of cells, essential to circumvent natural cell senescence, often results in karyotypic change, a transformed phenotype, and selection of non-representative cell populations (Navone et al. 1999, Gudjonsson et al. 2004). However, transformed cell lines are relatively inexpensive to maintain, safe to manipulate and provide valuable preliminary in vitro data, often providing the justification for more expensive and
difficult *in vivo* investigations. Although the availability of human tumour tissue samples has been limited over the course of this project, data generated from cell lines and *in vitro* cell assays have allowed us to establish excellent collaborations with the medical fraternity, thereby providing substantial resources of clinical samples for future work. In addition, the future use of primary cell cultures, multicellular spheroids (three-dimensional aggregates of cells) and anchorage-independent cell culture (via soft agar assays) may also provide a better model of *in vivo* tumour behaviour than traditional monolayer cell culture. For example, spheroid cell culture has been shown to more accurately reflect tumour microenvironment and stromal-epithelial interactions (Hamilton, 1998, Bates *et al.* 2000). A more accurate depiction of the role of the ghrelin axis in hormone-dependent cancer is likely to be generated, however, through the future use of *in vivo* mouse models.

*The characterisation of the ghrelin axis in the mouse for the future development of mouse models of cancer.*

Mouse models of prostate and breast cancer are important tools for studying the mechanisms behind early and late carcinogenesis (Abate-Shen and Shen, 2002, Cardiff *et al.* 2003), particularly when there are difficulties in accessing human tissue specimens. They also allow *in vivo* investigations into the physiological effects of administration of compounds, as well as chemoprevention studies, which would be otherwise unfeasible. In the TRAMP (Transgenic Adenocarcinoma Mouse Prostate) mouse model, specific expression of the oncogene SV40 T antigen in prostate epithelia is driven by the prostate-specific probasin promoter and results in the development of prostate cancer and subsequent distant site metastases (Greenberg *et al.* 1995). Interrogation of signalling pathways in TRAMP mice has demonstrated the importance of growth factors including IGF-I in prostate cancer progression (Foster *et al.* 1999) and it would be interesting to investigate *in vivo* ghrelin/GHS-R expression and signalling in this manner. To ensure that future studies of the ghrelin axis in this and other mouse models of cancer are warranted, we examined the expression of the axis in a wide range of mouse tissues (Chapter 5, Jeffery *et al.* 2005). This report is the first to fully characterise the expression of ghrelin, a murine homologue of exon 3-deleted preproghrelin, and the GHS-R type 1a in the mouse. This will serve as an important reference for the examination of the role of the
ghrelin axis in development/progression of prostate cancer and other hormone-dependent cancers, including breast cancer. This work has also generated important collaborations with international research groups. Ongoing work includes in vivo mouse studies to investigate the potential function of the exon 3-deleted preproghrelin variant via injection of the novel isoform into mice and binding assays investigating its affinity for orphan G protein-coupled receptors.

In summary, this study has provided new and compelling evidence that supports a role for the ghrelin axis in hormone-dependent cancer and specifically in prostate and breast cancer. We have identified several potential diagnostic markers for prostate and breast cancer within the ghrelin axis. Ghrelin stimulates proliferation of hormone-dependent cancer cells and therefore the ghrelin axis represents a novel target for the treatment of such cancers (Figure 2). This project has also provided a basis for future in vivo work in mouse models, which will potentially aid in the development of new adjunctive therapies for prostate and breast cancer.
REFERENCES


Yeh AH, Jeffery PL, Duncan RP, Herington AC, Chopin LK (2005) Ghrelin and a novel preproghrelin isoform are highly expressed in prostate cancer compared to normal prostate tissue and ghrelin-mediated prostate cancer cell line proliferation involves the MAPK pathway. Clinical Cancer Research 2005 in press