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Interplay between Hypoxia and Gastrin in Gastrointestinal Cancer

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Thesis submitted to the University of Nottingham
for the degree of Doctor of Philosophy

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Abstract

Tumour hypoxia has been linked to increased resistance to both radiotherapy and chemotherapy, especially in solid metastatic GI tumours. Under hypoxic conditions, genes that promote tumour growth and survival are up-regulated, via the transcription factor hypoxia-inducible factor-1 (HIF-1). The digestive hormone gastrin, which is often over-expressed in GI cancers, has also been shown to act as a pro-survival factor, up-regulating processes such as tumour proliferation, angiogenesis and migration, and down-regulating apoptosis. Due to the high level of similarity between the downstream events mediated by the two proteins, the relationship between gastrin and HIF-1 was investigated.

HIF-1α nuclear protein expression was inducible under hypoxic conditions, which led to an expected increase in VEGF gene expression, followed by a 12-50 fold increase in hypoxic gastrin mRNA expression. HIF-1α expression and transcriptional activity were not consistently affected by exogenous gastrin. RNA-interference-mediated knockdown of HIF-1α resulted in a 40-60% down-regulation of gastrin gene expression under hypoxic conditions suggesting that HIF-1α is partially responsible for gastrin up-regulation in hypoxia. Potential hypoxia-response elements (HREs) were identified within the gastrin promoter, but were only partially responsive to hypoxic incubation in GI carcinoma cells in luciferase-reporter assays. Other possible mechanisms that may account for the increased gastrin gene expression induced under hypoxic conditions include interactions of gastrin with other transcriptional regulators, either in synergy with or independent from HIF-1, or the sequestration of gastrin within the cell by ‘P’-bodies or RNA-binding proteins.
These findings may indicate that the addition of anti-gastrin agents such as CCK-2 receptor antagonists or gastrin immunogens to the treatment regime of patients with solid GI tumours may be clinically beneficial, especially if combined with agents used to reduce radiotherapy and chemotherapy resistance.
Acknowledgements

Firstly I would like to say a huge thank you to my project supervisors, Professor Sue Watson and Dr Anna Grabowska for all their help, guidance and support throughout my PhD.

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Outside the department, I would like to thank Dr Thilo Hagen for his guidance with the hypoxia assays and plasmid transfections.

On a more personal note, I’d like to thank my family for all their love and support now and always. I’d like to take this opportunity to especially thank my parents, for being there for me during the difficult times and for giving me the much needed pep-talk when it needed telling!!

Finally, I’d like to thank everyone at Wilkinson Avenue, both past and present for making the last three years an enjoyable and rewarding experience.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>4E-BP1</td>
<td>Eukaryotic Initiation Factor 4 – Inhibiting Binding Protein-1</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous Polyposis Coli</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>ARNT</td>
<td>Aryl Hydrocarbon Receptor Nuclear Translocator</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic Helix-Loop-Helix</td>
</tr>
<tr>
<td>BNIP3</td>
<td>Bcl-2/Adenovirus EIB 19kD-Interacting Protein-3</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>C-terminus/terminal</td>
<td>Carboxy-(COOH)-Terminus of a Protein</td>
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<td>CBP</td>
<td>CREB (cAMP-Response Element Binding Protein) – Binding Protein</td>
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<td>Cysteine-Histidine 1</td>
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<td>Description</td>
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<td>CXCR4</td>
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<td>Gastrointestinal</td>
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<td>Glucose Transporter-1</td>
</tr>
<tr>
<td>Gly-G17/-G34</td>
<td>Glycine-Extended Gastrin (Either 17 or 34 Amino Acids in Length)</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>Gly-gastrin</td>
<td>Glycine-Extended Gastrin</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth Factor Receptor-Bound Protein 2</td>
</tr>
<tr>
<td>H⁺/K⁺-ATPase</td>
<td>Hydrogen / Potassium Adenosine Triphosphate</td>
</tr>
<tr>
<td>H. pylori</td>
<td>Helicobacter pylori</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>Heparin-Binding Epithelial Growth Factor</td>
</tr>
<tr>
<td>HER2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-Inducible Factor</td>
</tr>
<tr>
<td>HIF-1/2</td>
<td>Hypoxia-Inducible Factor-1 / -2</td>
</tr>
<tr>
<td>HIF-1α/2α/3α</td>
<td>Hypoxia-Inducible Factor-1 / -2 / -3 Alpha Subunit</td>
</tr>
<tr>
<td>HIF-1β</td>
<td>Hypoxia-Inducible Factor-1 Beta Subunit</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine Phosphoribosyl Transferase</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia Response Element</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat Shock Protein</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cells</td>
</tr>
<tr>
<td>IAP-2</td>
<td>Inhibitor of Apoptosis Protein-2</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-Like Growth Factor</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitor of NF-κB (Nuclear Factor Kappa B)</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB (Inhibitor of Nuclear Factor Kappa B) Kinase</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 Beta</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>INS-GAS</td>
<td>Insulin-Gastrin</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK (Mitogen Activated Protein Kinase) – ERK (Extracellular Kinase) Kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium Chloride</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro-Ribonucleic Acid</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix Metalloproteinases</td>
</tr>
<tr>
<td>MMP-2/-3/-9</td>
<td>Matrix Metalloproteinase-2 / -3 / -9</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>mSOS</td>
<td>Mammalian Son of Sevenless</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>N-terminus/terminal</td>
<td>Amino-(NH₂)-Terminus of a Protein</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa B</td>
</tr>
<tr>
<td>NT</td>
<td>Non-Targeting</td>
</tr>
<tr>
<td>ODD domain</td>
<td>Oxygen-Dependent Degradation Domain</td>
</tr>
<tr>
<td>Opti-MEM</td>
<td>Optimum Minimal Essential Media</td>
</tr>
<tr>
<td>P bodies</td>
<td>Processing Bodies</td>
</tr>
<tr>
<td>p70^{S6K}</td>
<td>Ribosomal Protein p70 S6 Kinase-1</td>
</tr>
<tr>
<td>PAM</td>
<td>Peptidyl-Alpha-Amidating Mono-Oxygenase</td>
</tr>
<tr>
<td>PAS</td>
<td>(PER (Period) – ARNT (Aryl Hydrocarbon Receptor Nuclear Translocator) – SIM (Single-Minded))</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome-Proliferator-Activated Receptor-γ Coactivator-1α</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PI3-kinase</td>
<td>Phosphatidylinositol 3-Kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homologue Deleted on Chromosome 10</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Difluoride</td>
</tr>
<tr>
<td>pVHL</td>
<td>Von Hippel-Lindau Tumour Suppressor Protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA (Ribonucleic Acid)-Induced Silencing Complex</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>S.D</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SDSC</td>
<td>San Diego Supercomputer Centre</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SHC</td>
<td>Src Homology Collagen-Like Protein</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small-Interfering Ribonucleic Acid</td>
</tr>
<tr>
<td>SOC</td>
<td>Super Optimum Broth, with Catabolite Repression</td>
</tr>
<tr>
<td>Sp-1</td>
<td>Specificity Protein 1</td>
</tr>
<tr>
<td>Strept/ABComplex HRP</td>
<td>Streptomyein/Avidin-Biotin Complex Horseradish Peroxidase</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian Virus 40</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris Borate EDTA (Ethylenediamine Tetra-Acetic Acid)</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-Buffered Saline, with Tween</td>
</tr>
<tr>
<td>TCF</td>
<td>T Cell Factor</td>
</tr>
<tr>
<td>TIA-1</td>
<td>T-Cell Intracellular Antigen</td>
</tr>
<tr>
<td>Tm</td>
<td>Annealing Temperature</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor-Alpha</td>
</tr>
<tr>
<td>UNG</td>
<td>Uracil DNA (Deoxyribonucleic Acid) Glycosylase</td>
</tr>
<tr>
<td>uPAR</td>
<td>Urokinase Plasminogen Activator Receptor</td>
</tr>
<tr>
<td>USF-1/-2</td>
<td>Upstream Stimulatory Factor-1 / -2</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VMAT2</td>
<td>Vesicular Monoamine Transporter type-2</td>
</tr>
</tbody>
</table>
Chapter One
INTRODUCTION
1.1 Background

Cancer is considered as one of the leading causes of death in the developed world, second only to circulatory disorders such as heart disease and strokes, and therefore is a major burden on public health services in these countries. In 2005, there were 153,491 cancer deaths in the UK alone, which accounted for approximately 26% of the total deaths for that year (CancerStats Mortality – UK, 2008; Jemal, 2008). There were approximately 284,560 cases of cancer diagnosed in 2004 in the UK (CancerStats Incidence – UK, 2008), with males and females being approximately equally affected, which was also seen in the cancer mortality data. It has now been established that over one in three people will develop cancer at some point in their lives and approximately one in four will die from it (CancerStats Incidence – UK, 2008; Jemal, 2008). Factors influencing cancer risk involve both genetic and environmental components, such as family history and genetic inheritance, diet and smoking patterns (CancerStats Incidence – UK, 2008).

There are over 200 known forms of cancer, although there are four types which account for approximately half of all cancer diagnosis (54%) and mortality (47%) in the UK and other developed countries; Lung, Colorectal, Breast and Prostate cancers (CancerStats Incidence – UK, 2008; CancerStats Mortality – UK, 2008). Cancer is considered an age-dependent disease, with 64% of all cases occurring in patients aged 65 or over and approximately 76% of all cancer mortalities occurring in patients from the same age group (CancerStats Mortality – UK, 2008). Cancer does however affect the younger patient as well, as approximately 1 in 10 cases of cancer are diagnosed in patients...
between the ages of 25-50. The overall risk of an individual developing cancer during their lifetime is one in three. For patients under the age of 50 however, this risk is reduced to 1 in 27 (CancerStats Incidence – UK, 2008). Cancer is responsible for a third of deaths in those under 65, outnumbering those caused by the circulatory system (heart disease / stroke) and respiratory system combined (CancerStats Mortality – UK, 2008). The majority of tumours diagnosed in adults were solid in nature (CancerStats Incidence – UK, 2008).

The overall incidence of cancer has increased by approximately 25% between the years of 1975 to 2004 (CancerStats Incidence – UK, 2008). As the average life expectancy of a country in the Western world keeps increasing, the chance of developing cancer also increases. It has been suggested that by 2025, the number of patients diagnosed with cancer will increase by around 100,000 new cases per year (CancerStats Incidence – UK, 2005). The numbers of cancer deaths however are slowly decreasing, despite the increase in its incidence. During the thirty year period between 1976 and 2005, the number of cancer mortalities per 100,000 people dropped by approximately 20%, from 218 to 180 (CancerStats Mortality – UK, 2008), with the majority of the decrease in cancer mortality occurring in the final ten years (i.e. 1996 to 2005).

Tables 1.1 and 1.2 below describe the number of patients who were diagnosed with and died from cancers of the gastrointestinal (GI) tract in 2004 and 2005 respectively. Colorectal cancer was the most common form of GI cancer, accounting for between 12-14% of all cancer incidences and 10-11% of mortalities. Colorectal cancer was responsible for over 16,000 deaths in 2005,
slightly affecting more men than women (23 per 100,000 deaths versus 14 per 100,000 deaths respectively) (CancerStats Mortality – UK, 2008). The actual incidence of colorectal carcinoma however has decreased by approximately 10% since 1996, with mortality rates down by approximately 20%. This is probably due to an improved diet in Western countries and the earlier diagnosis of the condition (CancerStats Incidence – UK, 2005; CancerStats Mortality – UK, 2008). The current lifetime risk of developing colorectal cancer is still approximately 1 in 19 (Table 1.1).

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Lifetime risk of developing cancer</th>
<th>Number of patients diagnosed in 2004</th>
<th>Gender-specific cancer incidences</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
<td>Males</td>
</tr>
<tr>
<td>Colorectal</td>
<td>1 in 18</td>
<td>1 in 20</td>
<td>36,109</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(3rd most common)</td>
</tr>
<tr>
<td>Oesophagus</td>
<td>1 in 75</td>
<td>1 in 75</td>
<td>7,654</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(9th most common)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>1 in 96</td>
<td>1 in 96</td>
<td>7,654</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(10th most common)</td>
</tr>
<tr>
<td>Stomach</td>
<td>1 in 44</td>
<td>1 in 86</td>
<td>8,178</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(8th most common)</td>
</tr>
</tbody>
</table>

Table 1.1 – UK incidence rates and lifetime risk of common GI cancers (Information taken from CancerStats Incidence – UK, 2008).

Stomach cancer is another common GI cancer, making up approximately 3% of all cancer diagnoses and 4% of cancer mortalities (CancerStats Incidence – UK, 2008; CancerStats Mortality – UK, 2008). The incidence of this cancer however has decreased by 25% since 1992 and mortality by over 30%, which
is one of the largest decreases seen in both cancer incidence and mortality, and continues the trend seen over the last fifty years (CancerStats Incidence – UK, 2005; CancerStats Mortality – UK, 2008; Jemal 2008). Despite the decrease in both incidence and mortality, the lifetime risk of developing stomach cancer is 1 in 44 and 1 in 86 for males and females respectively (Table 1.1).

Despite the number of cancer-related deaths being on the decrease, due to earlier detection and advances in cancer treatment, the actual numbers of cancer cases are still increasing, and with an ever-aging population, cancer incidence will continue to rise. The five-year survival rates for the GI cancers discussed above are all under 50%, and apart from colorectal cancer are considerably lower than the average cancer five-year survival rate of 43% (men) and 56% (women) (CancerStats Survival – UK, 2008). Table 1.3 details the overall five-year survival rates for the four GI cancers in England and Wales from 2001, which considered cancers diagnosed between the years 1996-1999 (CancerStats Survival – UK, 2008). This data in Table 1.3 does not consider the ultimate cause of death of the patients, or any recent advancement in cancer treatment, but does highlight how the battle against GI cancer is not

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Number of mortalities in 2005</th>
<th>Gender-specific cancer mortalities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Males (X% of total)</td>
</tr>
<tr>
<td>Colorectal</td>
<td>16,092 (2nd most common)</td>
<td>8,637 deaths</td>
</tr>
<tr>
<td>Oesophagus</td>
<td>7,419 (5th most common)</td>
<td>4,847 deaths (6% of total)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>7,238 (6th most common)</td>
<td>3,455 deaths (4% of total)</td>
</tr>
<tr>
<td>Stomach</td>
<td>5,672 (7th most common)</td>
<td>3,523 deaths (5% of total)</td>
</tr>
</tbody>
</table>

Table 1.2 – UK mortality rates in common GI cancers (Information taken from CancerStats Mortality – UK, 2008).
yet over.

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Five year survival rate in 2001</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
</tr>
<tr>
<td>Colorectal</td>
<td>47%</td>
</tr>
<tr>
<td>Oesophagus</td>
<td>7%</td>
</tr>
<tr>
<td>Pancreas</td>
<td>2%</td>
</tr>
<tr>
<td>Stomach</td>
<td>13%</td>
</tr>
</tbody>
</table>

Table 1.3 – England and Wales five-year survival rates in common GI cancers (Information taken from CancerStats Survival – UK, 2008).

For a cancer to become fully metastatic and invasive, it must acquire six characteristic phenotypes (Hanahan and Weinberg, 2000), which are as follows:

- Self-sufficient in providing growth signals (e.g. mutation in oncogenes produces a constitutively active component of a growth-promoting signalling pathway, such as Akt (also known as Protein kinase B)).

- Show insensitivity to inhibiting signals (e.g. mutation in tumour suppressor genes, such as PTEN (phosphatase and tensin homologue deleted on chromosome 10) gene, which normally suppressors phosphatidylinositol 3-kinase (PI3-kinase) /Akt pathways).

- Reduction in cell apoptosis (e.g. down-regulation of pro-apoptotic signals, or up-regulation of anti-apoptotic signals).

- Able to continue with DNA synthesis (e.g. up-regulation of telomerase, allows for preservation of the full sequence).
• Initiates angiogenesis (e.g. up-regulation of vascular endothelial growth factor (VEGF)).

• Show metastatic properties allowing for tissue invasion.

The mutations that transform the cells lining the normal mucosa of a tissue through hyperplasia, into dysplasia and eventually into a fully formed cancer, occur over a long period of time (Hanahan and Weinberg, 2000). There are mutations that occur early during carcinogenesis and are often specific to a particular cancer, such as p16 and p53 in oesophageal cancer (reviewed in Jenkins et al, 2002) and APC (adenomatous polyposis coli), K-ras and p53 in colorectal cancer (Ichii et al, 1993). Tumours generally require three or more mutations in different key pathways to induce a malignant phenotype (Vogelstein and Kinzler, 2004).

1.2 Gastrin
The initial idea that gastric acid secretion was controlled by both nervous and hormonal signals arose in 1902, when Bayliss and Starling suggested the hormone secretin influenced the levels of pancreatic secretions. Evidence for the same style of control occurring in the stomach was proposed in 1905 by Edkins, who suggested that the digestive hormone gastrin played a role in stomach acid secretion (Reviewed in Modlin et al, 1997).

The digestive hormone gastrin is produced and released into the circulation by G cells, which are located in the antrum of the stomach lining, in the base of
Gastrin is mainly responsible for the production of gastric acid from parietal cells and the proliferation of enterochromaffin-like (ECL) cells. The parietal cells are located in the neck of the glands in the fundus of the stomach, whereas the ECL cells are located in the base of the glands throughout the entire stomach (reviewed in Watson et al, 2006). G cells are mainly located in the antrum of the stomach and in the duodenum, but negligible expression has also been described in the pituitary gland and pancreas (reviewed in Dockray, 2004). The ECL cells are stimulated by the serum gastrin to proliferate. This induces the cells to secrete histamine, which then acts at histamine receptors on the surface of parietal cells, initiating gastric acid secretion (reviewed in Watson et al, 2006).

Gastrin also causes the proliferation of the parietal cells located in the glands of the stomach lining. The use of a gastrin-knockout mouse model revealed that the loss of gastrin gene diminished both parietal and ECL cell development and number, abolishing gastric acid secretion (Friis-Hansen et al, 1998). The introduction of exogenous gastrin reversed these effects, restoring the integrity of the stomach lining (Friis-Hanson et al, 1998).

Normal fasting plasma gastrin levels are approximately 30-50pmol/l; however, the presence of food in the stomach increases the levels by three-fold (Dockray, 2004). The presence of food is detected by microvilli on the surface of G cells, initiating the gastrin-histamine-gastric acid secretion pathway. To prevent the overproduction of gastric acid, a negative-feedback loop is also activated. Histamine, produced by the ECL cells also induces the release of
somatostatin from D cells, which are located throughout the stomach, at the base of the glands (reviewed in Watson et al, 2006). The somatostatin inhibits G cell function in a paracrine fashion via reducing gene transcription. Inhibiting gastric acid secretion via the gastric $\text{H}^+/\text{K}^+$-ATPase inhibitor omeprazole decreased somatostatin mRNA expression 3-fold and as a result increased plasma gastrin expression 4-fold. This effect was reversed by exogenous treatment with a somatostatin analogue, therefore confirming its inhibitory effect on gastrin expression (Brand and Stone, 1998). Therefore, the release of gastrin from G cells is inhibited in a negative feedback loop (Brand and Stone, 1998).

Gastrin release from the G cell is also positively modulated via gastrin-releasing peptide, which is released by neurones present in the stomach, acting on its receptor located on the surface of the G cell (Nishi et al, 1985).

### 1.3 CCK-2 receptor
Gastrin acts via cholecystokinin-2 receptors (CCK-2 or CCK$_B$-receptors), which are 74kD seven-transmembrane G protein-coupled receptors expressed within the baso-lateral border of ECL and parietal cells (Figure 1.1) (Haigh et al, 2003). Low CCK-2 receptor expression can also be found on smooth muscle, the pancreas and the brain (Innis et al 1980; Menozzi et al, 1989). Gastrin binds to its receptor via a small five amino acid region within the second extracellular loop (Silvente-Poirot and Wank, 1996), which induces its proliferative effect. The C-terminal amide group found on the mature gastrin peptide is required for it to act at the CCK-2 receptor. If it is removed, the
proliferative and secretory effects normally seen are no longer detectable (reviewed in Dockray, 2004).

![CCK-2 receptor structure](image)

Figure 1.1 – The structure of the CCK-2 receptor, which is a classic seven transmembrane domain G protein-coupled receptor.

There is also a CCK-1 isoform of the receptor, which has approximately 50% homology to the CCK-2 receptor isoform, but which gastrin can only bind to with low affinity (Silvente-Poirot and Wank, 1996).

Gastrin shares a similar structure to cholecystokinin, as both molecules express the C-terminal pentapeptide amide group required for receptor activation. Cholecystokinin can bind to both receptors with high affinity, whereas gastrin is selective for the CCK-2 receptor (Reubi and Waser, 1996). As the plasma gastrin concentrations increase following the presence of food in the stomach, its expression is often becomes 5-10 fold higher than that of cholecystokinin (reviewed in Dockray, 2004).
1.4 Gastrin synthesis

The gastrin gene is located on chromosome 17q21. It is 4kb long and consists as a single gene of three exons, with two introns (Ito et al, 1984). Only the second and third exons are transcribed to produce the final gastrin protein (Ito et al, 1984). The expression of this gene is regulated at both the transcriptional and translational level.

Post-translational cleavage and peptide modifications allow for multiple peptides to be produced from the same gastrin gene and released into the plasma as individual biologically active proteins. These proteins include the processing-intermediates progastrin and C-terminal glycine-extended gastrin (gly-gastrin) and the final mature amidated gastrin hormones. The fully processed gastrin molecules can either be found as 17 and 34 amino acid peptides, with an amide group attached at their C-terminus; they are known as G17 and G34 respectively. It is this C-terminus amidation that is vital for gastric acid secretion (Morley et al, 1965).

Within the G cells, the original gastrin transcript is formed from two of the three exons of the gastrin gene as a 0.7kb mRNA product. The transcript passes through into the endoplasmic reticulum, where its N-terminal sequence is removed to yield progastrin (see Figure 1.2), via the cleavage between amino acid residues 21 and 22 (Wang et al, 1996).

This intermediate then continues through into the Golgi apparatus, where it is stored in secretory vesicles (Varro et al, 1994). Here, it is cleaved into gly-
gastrin via the actions of two enzymes. Subtilisin-like prohormone convertases, a family of endopeptidases, cleave at pairs of basic residues (Arg57/58 and Arg94/95) (Bishop et al., 1998; Dickinson et al., 1993; Varro et al., 1994), before carboxypeptidase E removes further basic residues from the C-terminus, revealing the glycine group. This gly-gastrin intermediate is 34 amino acids in length and is often modified further via processes such as serine residue-96 phosphorylation and sulphation of tyrosine residues, which occur within the Golgi apparatus (Varro et al., 1994).

![Pathway of gastrin synthesis](image)

Figure 1.2 – The pathway of gastrin synthesis (information taken from Dockray, 2004 and Stepan et al., 2002).

The G34 gly-gastrin is then either hydroxylated at the N-terminus, before being amidated via the enzyme peptidyl-alpha-amidating mono-oxygenase (PAM) to form the G34 final gastrin product, or cleaved into a 17 amino acid gly-gastrin (Stepan et al., 2002; Varro et al., 1995), which then can be amidated via PAM. Only once this processing has occurred, are the G17 or G34 gastrin
peptides released from the vesicles. Amidated G34 and G17 share a common C-terminal peptide sequence, which is required for binding to the CCK-2 receptor (reviewed in Dockray, 2004).

Under optimum conditions, the production of the fully mature amidated gastrin (G17) takes approximately 2hrs. The progastrin intermediate is produced within 30mins. The 34-amino acid amidated or gly-gastrin is detectable within 60mins and the final G17 gastrin molecules (either the amidated or gly-gastrin isoform) by 120mins (Varro et al, 1995).

The final amidated gastrin produced is regulated by the pH of the processing vesicle. The pH of the stomach is approximately 5.6. Under these conditions, the cleavage of the G34 molecule into the G17 gastrin products is favoured, as the prohormone convertase enzymes involved are pH sensitive. (Blackmore et al, 2001). Any increase of pH, via the uptake of amines into the vesicle inhibits the cleavage of G34 into G17, resulting in the accumulation of G34 peptide. Physiologically, G34 is often produced in duodenal G cells as the pH is higher than that found in the antral G cells, where G17 molecule is preferentially secreted (reviewed in Dockray, 2004). G34 peptide has a lower metabolic clearance rate than G17 and therefore is held in the plasma for longer, leading to increased concentrations of total plasma gastrin levels.
1.5 Expression of gastrin in GI tumours

The digestive hormone gastrin has been suggested to play a role in the development of GI cancers, although its exact role is still being elucidated, and is a matter of some debate.

There is evidence that suggests that the presence of gastrin is important for carcinoma progression in the early stages of carcinogenesis. The expression of both gastrin and its CCK-2 receptor were found within non-metastatic gastric carcinoma samples (Henwood et al, 2001), which ranged in nature from atrophic gastritis samples, through intestinal metaplasia to epithelial dysplasia samples. Pre-carcinogenic colonic polyps were also found to co-express the gastrin and CCK-2 receptor genes (Smith and Watson, 2000). Only half the polyps that expressed the gastrin gene however translated it into the fully mature amidated gastrin, which would induce CCK-2-receptor-mediated tumour progression.

In gastric and colonic tumour xenograft mouse models, exogenous treatment with gastrin increased tumour volume and DNA content (Sumoyoshi et al, 1984; Winsett et al, 1986).

Gastrin was shown to be expressed in several human gastric and colorectal carcinoma cells (McWilliams et al, 1998), pancreatic carcinoma cells (Harris et al, 2004) and oesophageal carcinoma cells (Harris et al, 20042).

Increased amidated gastrin levels have also been linked to increased incidence of colorectal carcinoma cases in a number of human studies (Ciccotosto et al,
1995; Seitz et al, 1991; Thorburn et al, 1998), and has even been linked to increasing histological grade of the tumours (Mao et al, 2008). Gastrin protein was shown to be expressed in colonic tumours and their associated resection margins (Hartwich et al, 2001), but not in normal colonic mucosa (Hartwich et al, 2001; Smith and Watson, 2000). This evidence however has been disputed by further clinical and animal studies in a number of GI carcinomas (Graffner et al, 1992; Pinson et al, 1995, Siddheshwar et al, 2001; Yuan et al, 2008). The exact association between hypergastrinaemia and colorectal cancer is still under investigation. The initial research into the expression of gastrin in colorectal cancers did not correct for confounding factors, such as Helicobacter pylori (H. pylori) infection (Ciccotosto et al, 1995) and only considered the amidated versions of gastrin and not its processing intermediates. So far, these transitional gastrin peptides have been detected in all colorectal tumours tested, often being favoured over the fully mature peptide, and are up-regulated when compared to control samples (Ciccotosto et al, 1995; Siddheshwar et al, 2001; Thorburn et al, 1998).

Gastrointestinal tumours show increased growth in response to an application of gastrin via the CCK-2 receptor. The majority also express the gastrin gene, and therefore can produce both amidated, progastrin and occasionally gly-gastrin (40-50% of tumours), which could encourage further proliferation of the tumour in an autocrine or paracrine fashion (Ciccotosto et al, 1995; Wang et al, 1996). An example of autocrine growth has been demonstrated with the rat pancreatic cell line, AR42J, which endogenously expresses both gastrin and its CCK-2 receptor, leading to high basal activation (Harris et al, 2004).
The presence of excess gastrin would increase the rate of cellular proliferation, therefore increasing the chance of spontaneous mutations within the cells. As a result, the excess gastrin would aid the progression of a pre-cancerous lesion into full malignancy (reviewed in Smith and Watson, 2000; Watson and Smith, 2001).

A number of non-cancerous medical conditions can also induce an increase in serum gastrin concentrations. These include:

1) An infection of the antrum by *H. pylori*, a bacterium which is linked with increased risk of the host developing a gastric ulcer. Infection with *H. pylori* increases the number and activity of G cells and reduces the inhibitory effects of D cells (Liu *et al.*, 2005; Zavros *et al.*, 2002). As a result, infection with *H. pylori*, especially CagA positive strains, induces an increase in both gastric acid production (which would lead to peptic ulcers) and serum gastrin levels, which may up-regulate gastric cell proliferation, therefore increasing the chance of carcinoma development.

2) Zollinger-Ellison syndrome, which induces acid hypersecretion and stomach ulceration, leads to high plasma concentrations of the amidated gastrin peptide (both G17 and G34) (Gregory *et al.*, 1969). Tumours from patients with this condition were the first to link gastrin with mucosal transformation of normal tissue to a hyperplasia state during the 1960s (Gregory *et al.*, 1960).
3) Pernicious anaemia, where gastric acid secretion is reduced to a level where somatostatin-inhibition of the G cells becomes redundant. This allows free transcription and translation of the gastrin gene, inducing an increase in serum gastrin levels, in an attempt to induce gastric acid secretion (reviewed in Dockray, 2004).

4) Prolonged treatment with proton pump inhibitors, which are the standard treatment of stomach ulcers (reviewed in Dockray, 2004). They reduce the level of gastric acid production, therefore removing the feedback inhibition of G cell activity, allowing free transcription and translation of the gastrin gene.

1.6 Expression of CCK-2 receptors in GI tumours

A number of epithelial cell tumours have been shown to express the CCK-2 receptor. These include gastric, colonic and pancreatic tumours, where 75-90% expressed both amidated gastrin and the CCK-2 receptor, as identified in both human tumour samples and carcinoma cell lines (Goetze et al, 2000; McWilliams et al, 1998). This data however has also been disputed by some studies, which suggest that only a low percentage of gastric and colorectal tumours expressed the CCK-2 receptor (Ahmed et al, 2004).

Within oesophageal carcinomas, approximately 60% of tumours show an increased level of CCK-2 receptor expression (Haigh et al, 2003), however, patients with the pre-cancerous condition Barrett’s oesophagus, where the oesophagus was damaged due to reflux of stomach acid, leading to chronic
pain and inflammation, revealed CCK-2 receptors to be expressed in all samples tested (Haigh et al, 2003). Gastrin would act to increase cellular proliferation in an attempt to repair the damaged tissue, but may be less important for more advanced stages of carcinogenesis.

Further isoforms of the gastrin receptor have also been demonstrated to exist, formed from the original CCK-2 receptor gene as alternate splice variants (Biagini et al, 1997; McWilliams et al, 1998; Miyake et al, 1995). A truncated CCK-2 receptor isoform (ΔCCK-2) has been isolated from human stomach, being derived from the original CCK-2 gene using a novel exon 1 splice variant. This produces a receptor with no extracellular N-terminus (Miyake et al, 1995). In one study, the ΔCCK-2 receptor was shown to be expressed in 75.6% of colonic tumours and 100% of resultant metastatic samples, compared to 23% and 43% expression for the original CCK-2 receptor isoform in colonic tumours and metastases respectively (Biagini et al, 1997). It has also been found to be expressed on gastric carcinoma cell lines (McWilliams et al, 1998), being co-expressed with gastrin, suggesting that the truncated isoform may have a role in the progression and invasion of carcinoma.

Retention of intron IV of the CCK-2 receptor gene gives rise to a further isoform of the gastrin receptor, the CCK-C (Cancer) receptor (Smith et al, 2002). CCK-C receptor mRNA is expressed within pancreatic carcinomas, but not within normal tissue (Smith et al, 2002), suggesting a role in the progression of carcinogenesis. Unlike the normal CCK-2 receptor, the CCK-C receptor can bind both the processing intermediates and the final, mature
gastrin molecule (Biagini et al, 1997). It has been suggested that this receptor may result in autocrine up-regulation of cell proliferation (Biagini et al, 1997).

A further CCK-2 receptor splice variant is the CCK_{2R4} receptor, which also has retained intron IV of the CCK-2 receptor gene, giving it 69 extra amino acids within the third intracellular loop of the receptor (Hellmich et al, 2000).

Expression of this receptor in a HEK-293 xenograft model induced constitutive tumour growth, in a Src-dependent manner (Chao et al, 2006).

### 1.7 Gastrin gene regulation

A number of growth factors and cytokines are responsible for up-regulation of gastrin at the transcriptional level (Marks et al, 1996; Shiotani and Merchant, 1995; Suzuki et al, 2001). These include epidermal growth factor (EGF), tumour necrosis factor-1α (TNF-α), and interleukins 1β and 8 (IL-1β, IL-8), which are all frequently expressed in tumours (Hartwich et al, 2001; Konturek et al, 2006). EGF interacts within the gastrin promoter (from -68bp to -54bp) at a GC-rich EGF-response element (5’ – GGGCGGGGTG GGGGG – 3’) via the transcription factor Sp-1 (Specificity protein 1) (Merchant et al, 1995), which up-regulates gastrin mRNA expression (Merchant et al, 1991).

These growth factors have also been suggested to up-regulate the expression of the transcription factor, c-Fos, by a mitogen-activated protein kinase (MAPK)-dependent or protein kinase C (PKC)-dependent signalling pathway, allowing it to bind to the gastrin promoter at a separate site to that of EGF and again induce gastrin expression (Marks et al, 1996; Suzuki et al, 2001).
Gastrin gene expression is also regulated by the β-catenin/TCF (T-cell factor) pathway, especially in colorectal carcinomas (Koh et al., 2000). A mutation in the APC gene, which is normally responsible for the degradation of β-catenin, removes its ability to inhibit β-catenin activity. This allows β-catenin binding to the TCF transcription factor, translocation to the nucleus, binding to target sequences within the promoter of target genes and as a result, increased transcription of gastrin. Transfection of a constitutively active β-catenin protein up-regulates the gastrin promoter activity three-fold (Koh et al., 2000).

Gastrin gene expression is also highly regulated at the level of translation into protein therefore its abundance within the cell is very tightly controlled (Bate et al., 1996). A recent study by Grabowska et al. (2008) has identified an alternative gastrin transcript, which originates within intron one of the gastrin gene and is probably driven by a promoter that is present further upstream within intron one. The alternative transcript showed increased activity, when inserted into a luciferase-expressing construct, which was further increased in conditions of ‘stress’, such as treatment with cytotoxic agents (Grabowska et al., 2008).

1.8 Gastrin – Cell survival and proliferation
Once gastrin has bound to the CCK-2 receptor, PKC becomes activated, and via a signal transduction pathway utilising both calcium and MAPK, the receptors are internalised and translocated to the nucleus, where they may play a role in gene transcription via up-regulating expression of transcription factors such as c-fos and Elk-1 (ETS-like transcription factor 1) (Wroblewski et al.,
2002; Todisco et al, 1997; Todisco et al, 1997^2). Inhibition of PKC or MAPK activity via specific inhibitors initiated an 80% decrease in gastrin activity, as shown in rat AR42J cells (Todisco et al, 1997). Similar results were achieved via the use of gastrin-specific inhibitor CI-988 (Moody and Jensen, 2001). The actions of gastrin allow the production of growth factors, such as heparin-binding growth factor (HB-EGF) and amphiregulin. The mRNA expression of both of these growth factors was up-regulated in male Sprague-Dawley rats within hours of a gastrin infusion. They act at the EGF receptor, enhancing the proliferative effects of gastrin on the gastric mucosa (Tsutsui et al, 1997) and possibly even of tissues that do not express the CCK-2 receptor. *H. pylori* infection further increases the expression and shedding of HB-EGF in the gastric mucosa, in a gastrin / CCK-2 receptor-dependent fashion (Dickson et al, 2006), which would further increase the transactivation capacity of gastrin.

Within the ECL cells, gastrin up-regulates the expression of genes involved in histamine synthesis and secretion, via initiating cellular signalling pathways that up-regulate transcription factors such as Sp-1 and CREB (cAMP-Response Element Binding Protein) (Höcker et al, 1998), which then activate target genes. These include histidine decarboxylase (responsible for the conversion of histidine to histamine) and vesicular monoamine transporter type 2 (VMAT2) (allows storage of freshly manufactured histamine) (reviewed in Dockray, 2004), which would lead to increased gastric acid production. The promoter of VMAT2 has been shown to express a gastrin response element (5’ – CCGCCCCCTC – 3’) from -56bp to -47bp (Catlow et al, 2007). Similar gastrin response elements have been identified in the promoters of histidine
decarboxylase (Raychowdhury et al, 1999) and the Reg1 growth factor (Steele et al, 2007), which are responsible for histamine production and ECL proliferation respectively.

Amidated gastrin induced cell proliferation and DNA synthesis in a number of human carcinoma cell lines, including colonic (Colucci et al, 2005), pancreatic (Smith et al, 1995), oesophageal (Haigh et al, 2003) and thyroid medulla (Bläker et al, 2004). In the human gastrin-transgenic mouse model (INS-GAS (Insulin-gastrin)), the presence of hypergastrinemia resulted in increased proliferation and thickening of the gastric mucosa, when compared to the control animals (Wang et al, 1996; Wang et al, 2000), which eventually led to the development of gastric carcinoma (Wang et al, 2000). The INS-GAS mouse over-expresses human amidated gastrin due to the insertion of the human gastrin gene within the promoter of the insulin gene; this allows for human gastrin to be produced from the β-cells of the INS-GAS mouse pancreas (Wang et al, 1996). The excess amidated gastrin would act via the CCK-2 receptor, which has also been shown to be over-expressed in the INS-GAS mouse model (Ottewell et al, 2006). The effects induced by the over-expression of human gastrin in the INS-GAS were further accelerated by *H. pylori* infection or treatment with proton pump inhibitors, which are both known to induce hypergastrinemia (Wang et al, 1996; Wang et al, 2000). The presence of excess gastrin was also shown to aid gastric mucosal regeneration after damage with cytotoxic agents, suggesting that gastrin is also responsible for maintaining the integrity of the GI tract (Ottewell et al, 2006). Similar positive growth effects of gastrin were also seen in colonic and pancreatic
xenograft mouse models, where exogenous gastrin treatment significantly increased tumour volume (Smith and Solomon, 1988; Smith et al, 1995).

The growth responses induced by gastrin were specifically inhibited by the use of gastrin antisense, gastrin-specific siRNA (small-interfering ribonucleic acid) and CCK-2 receptor antagonists (but not CCK-1 receptor antagonists) in both in vitro and in vivo models (Bläker et al, 2004; Grabowska et al, 2007; Haigh et al, 2003; Harris et al, 2004; Moody and Jensen, 2001; Smith 1995), confirming the idea that gastrin plays a role as a growth factor. Treatment of GI carcinoma cells with gastrin siRNA reduced cell growth by approximately 60%. Cells that expressed highest levels of endogenous gastrin, such as the colonic C170HM2 carcinoma cells (Grabowska et al, 2007) and pancreatic PANC1 carcinoma cells (Smith et al, 1995) were most affected by growth inhibition treatment, suggesting that gastrin acts in both an autocrine and endocrine manner.

The up-regulation of β-catenin has been shown to increase the transcription of the gastrin gene. There is also evidence however that suggests that gastrin plays a role in regulating β-catenin expression (Cao et al, 2006), leading to increased tumour growth and survival, via the up-regulation of β-catenin/TCF target gene expression. Gastrin was shown to increase the expression and translocation of β-catenin in the colorectal cell line Colo320WT in a CCK-2 receptor-dependent fashion (Cao et al, 2006). Exogenous treatment with gastrin induced the translocation of β-catenin into the nucleus in a p21-activated kinase-dependent fashion (He et al, 2008), inducing the expression of
β-catenin/TCF transcription factor target genes including c-myc and cyclin D1, which have roles in cell proliferation, migration and cell cycle regulation (Cao et al, 2006; He et al, 2008). Mutation of the p21-activated kinase inhibited gastrin-induced β-catenin translocation from the cell membrane into the nucleus, and its resultant association with TCF (He et al, 2008).

1.9 Gastrin - Apoptosis

The evasion of apoptosis (or programmed cell death) is a further property that is acquired by tumours, to prevent targeted and controlled destruction of cells that contain pro-survival mutations. In a recent study, the levels of apoptosis in colorectal cancer were correlated to the level of gastrin expression. High gastrin expression was associated with low levels of apoptosis, whereas low gastrin expression suggested high levels of apoptosis (Mao et al, 2008).

Gastrin is responsible for the induction of cyclooxygenase-2 (COX-2) expression in a CCK-2 receptor-dependent mechanism. COX-2 is the membrane-associated enzyme that catalyses the rate-limiting step in the production of prostaglandins, such as prostaglandin E\textsubscript{2}. As a result of prostaglandin production, tumour proliferation is induced (Wang and DuBois, 2006). Colorectal and gastric carcinomas have been shown to express COX-2, which is also expressed in pre-malignant conditions, such as Barrett’s oesophagus (Abdalla et al, 2004; Hartwich et al, 2001). In one study, 90% colorectal tumour samples were shown to express COX-2 mRNA, whereas the adjacent normal mucosa lacked COX-2 expression (Hartwich et al, 2001).
COX-2 is responsible for the propagation of many pro-survival processes, which includes the inhibition of apoptosis. The up-regulation of COX-2 expression can lead to the increased expression of anti-apoptotic proteins, such as Bcl-2 and the decreased expression of the pro-apoptotic proteins, such as Bax (Hartwich et al., 2001). The resultant increase in the ratio between anti- and pro-apoptotic proteins reduces the risk of the tumour cell entering apoptosis, as cytochrome c is prevented from being released from the mitochondria and therefore cannot initiate the effector caspase cascade, required for the organised destruction of the cell organelles and proteins (reviewed in Hanahan and Weinberg, 2000).

The activity of the Bcl-2 anti-apoptotic protein was also increased after treatment with gastrin, via signalling through the Rac/cdc42 G protein, which in turn activates the p21-activated kinase (He et al., 2008). The p21 kinase then interacts with β-catenin, enhancing gene transcription via association with the TCF transcription factor (He et al., 2008), as previously described above, further inducing the inhibition of apoptosis. Gastrin has also been associated with the death receptor ligand Fas in colorectal cancer, which regulates apoptosis via inducing the caspase cascade (Mao et al., 2008).

The up-regulation of COX-2 protein by gastrin also involved the PI3-kinase /Akt pro-survival signalling pathway. In rat AR42J cells, which show high levels of endogenous gastrin, there were also high levels of phosphorylated (activated) Akt (Harris et al., 2004). Akt was also shown to be phosphorylated
in oesophageal carcinoma cell lines, after exogenous treatment with gastrin (Harris et al, 2004; Todisco et al, 2001).

Inhibition of gastrin signalling using gastrin antisense, gastrin siRNA, CCK-2 receptor antagonist or simply removing the serum from the growth media increased the level of apoptosis, and more specifically of cytotoxic caspase expression, whilst decreasing the level of Akt phosphorylation (Harris et al, 2004; Grabowska et al, 2007; Todisco et al, 2001). This confirms that gastrin also plays a role as an anti-apoptotic protein.

1.10 Gastrin - Invasion and malignancy

Gastrin is known to play a role in tumour cell invasion and extracellular membrane remodelling. The presence of hypergastrinemia has previously been associated with liver metastasis in colorectal cancer patients (Kameyama et al, 1993).

Gastrin actions via the CCK-2 receptor cause a number of genes to be up-regulated, such as matrix metalloproteinases (MMPs) and extracellular protease inhibitors, which are responsible for the degradation of the extracellular matrix and the remodelling of tissue. Gastrin was shown to stimulate the invasion of AGS cells through an artificial basement membrane, via the activation of MMP-9 (Wroblewski et al, 2002). The up-regulation of MMPs, such as MMP-2, MMP-3 and MMP-9 also aids tumour malignancy via increasing the shedding of HB-EGF, allowing for the induction of further signalling pathways (Clarke et al, 2006). Therefore, gastrin may be involved
in the processes of invasion and metastasis in carcinogenesis. Gastrin also induced the expression of focal adhesion kinase, a key molecule in tumour malignancy in a CCK-2 receptor-dependent manner (Yu et al, 2006). The expression of CCK-2 receptor has also been suggested to be involved in tissue repair after injury, as the receptor was associated with areas of regeneration (Schmassmann et al, 2000). The induction of hypergastrinemia increased the rate of ulcer healing in a rat oxyntic mucosa model (Schmassmann et al, 2000).

1.11 Gastrin - Angiogenesis

Angiogenesis, the development of new blood vessels, is another key property required for the survival of solid tumours. This process is highly regulated in adults and is normally only required in wound healing / reproduction (reviewed in Folkman and Shing, 1992). Angiogenesis is a major component of several pathological conditions, such as cancer, and is often seen in advanced tumours where it enhances growth and the ability to metastasise via ensuring a constant blood supply, due to the increased microvessel density.

The effects of gastrin on angiogenesis have been investigated using both in vitro and in vivo models. Amidated gastrin was shown to induce human umbilical vein endothelial cell (HUVEC) tubule and node formation in two separate studies, in a CCK-2 receptor-dependent manner (Clarke et al, 2006; LeFranc et al, 2004). The use of CCK-2 receptor antagonists, such as L360,260 significantly inhibited the HUVEC cell angiogenesis (Clarke et al, 2006; LeFranc et al, 2004). The pro-angiogenic effect was also suggested to be transmitted in a PI-3 kinase/Akt-dependent signal transduction pathway
(LeFranc et al., 2004). The HUVEC cells were also shown to express the cancer-specific CCK-C receptor (LeFranc et al., 2004) and increased HB-EGF expression / shedding (Clarke et al., 2006), suggesting the possibility that gastrin was also inducing other pro-survival processes within the cells. Exogenous gastrin treatment was also shown to induce the migration of HUVEC cells when grown on gelatine (LeFranc et al., 2004); further suggesting that gastrin plays a role in tumour invasion and malignancy.

Microvessels derived from human glioblastomas were also shown to express CCK-2 and CCK-C receptors mRNA (LeFranc et al., 2004), and again were found to be responsive to gastrin treatment in a pro-angiogenic manner. Similar results were also observed via the use of a chorioallantonic membrane assay (Clarke et al., 2004), where angiogenesis was promoted after treatment with gastrin.

Finally, in a study involving the pre-malignant APC^Min mouse model, tumour microvessel density was increased after treatment with gastrin (Clarke et al., 2006), again confirming the early involvement of gastrin in the process of carcinogenesis, promoting not only tumour growth, but also angiogenesis in a CCK-2 receptor-dependent manner.

Another key molecule involved in the regulation of angiogenesis is COX-2, whose expression has been suggested to be induced by the actions of gastrin (Ansorge et al., 2007), which, via the stimulation of the CCK-2 receptors activates both PI3-kinase/Akt and MAPK pathways, enhancing COX-2
transcription and activity (Abdalla et al, 2004; Colucci et al, 2005). The signalling is mediated via the binding of USF-1 (upstream stimulatory factor-1) and USF-2 transcription factors to a cAMP response element-E box at -56bp to -48bp, within the COX-2 promoter (Ansorge et al, 2007). The level of gastrin expression can be positively correlated to the degree of COX-2 present (Abdalla et al, 2004). As a result of gastrin-induced COX-2 expression, a number of cell survival processes are up-regulated, including angiogenesis, cellular proliferation and invasion, whilst apoptosis is inhibited.

Although normal colonic mucosa does not express COX-2 mRNA, the mucosa directly surrounding the tumour has been shown to express both COX-2 and the CCK-2 receptor (Hartwich et al, 2001). The expression of both these proteins in non-tumourigenic tissue is another indication that gastrin plays a role in the initiation and progression of tumours. The effects of COX-2 activity were abolished by the use of CCK-2 receptors antagonists, such as YM022 and L-365,260 (Abdalla et al, 2004; Colucci et al, 2005), which suggested that gastrin was responsible for the cellular proliferation seen in the colon carcinoma cells. The use of COX-2-specific non-steroidal anti-inflammatory drugs however, which prevent the actions of the COX-2 enzyme only partially inhibited the effects of gastrin. This indicated that gastrin acts via a number of mechanisms (Abdalla et al, 2004; Colucci et al, 2005).

1.12 Hypoxia

The pro-survival properties induced by gastrin in GI carcinomas are vital for the growth and viability of the tumour. Problems occur however when the rate
of cell proliferation exceeds tumour angiogenesis. The established tumour vasculature becomes unable to maintain sufficient oxygen and nutrient supply to the tumour, due to the increasing molecular diffusion distances being generated. Any pre-existing vasculatures that do evolve become malformed and irregular in nature, resulting in an unbalanced oxygen supply to the tumour (reviewed in Vaupel et al., 1989). This leads to the generation of regions of low oxygen tension, or hypoxia; a key feature of solid tumours. Therefore, the cancer must adapt to survive under low oxygen conditions.

The presence of hypoxia is responsible for modulating gene expression in solid tumours, inducing both tumour growth and angiogenesis (Maxwell et al., 1997), via the activity of the transcription factor ‘Hypoxia-Inducible Factor-1’ (HIF-1), which was originally postulated as the regulator of hypoxic erythropoietin expression (Wang and Semenza, 1993).

A side effect of tumour hypoxia is the development of resistance to both radiotherapy and chemotherapy regimes. Radiotherapy requires oxygen to induce DNA breaks (Roots and Smith, 1974), whereas most chemotherapy regimes use existing vasculature to reach the internal tumour cells, which in large solid tumours is usually inadequate (reviewed in Vaupel et al., 1989). The idea that the success of radiotherapy was dependent on the level of tumour oxygenation was first described by Gray et al in 1953 (reviewed in Brown, 1999). The higher the level of tumour oxygenation, the more likely it is to be susceptible to radiotherapy (Brizel et al., 1997; Nordsmark et al., 1996) and chemotherapy (Hussein et al., 2006; Teicher et al., 1990). Teicher et al (1990)
tested a panel of chemotherapeutic agents and radiation on both ‘normally oxygenated’ and hypoxic tumour cells. They determined that the majority of agents/treatments tested were 2-6-fold more cytotoxic towards the oxygenated cells, when compared to the hypoxic equivalents. Only a few of drugs tested had any affect on the hypoxic cells, suggesting that the cells originating the furthest away from the vasculature would be the least affected by chemotherapeutic agents.

1.13 Hypoxia-inducible factors (HIFs)

The transcription factor, Hypoxia-Inducible Factor-1 (HIF-1) plays a fundamental role in the cellular adaptation to reduced physiological oxygen levels (hypoxia). Its expression was first determined in 1995 in Hep3B cells (Wang et al., 1995), as a protein present under reduced oxygen tension that regulated the expression of the erythropoietin gene. It has since been established however that HIF-1 is expressed throughout the majority of mammalian cells, regulating the expression of key target genes.

HIF-1 is composed of two distinct subunits, the 120kDa inducible alpha subunit (HIF-1α) and the 91-94kDa constitutively expressed beta subunit (HIF-1β), which is also known as ARNT (aryl hydrocarbon receptor nuclear translocator) (Wang and Semenza, 1995; Huang et al., 1996). Both subunits are members of the basic helix-loop-helix (bHLH) PER-ARNT-SIM (PAS) family of proteins, (Wang et al., 1995), with HIF-1α sharing more homology with the drosophila ‘singled-minded’ (SIM) protein, over that of ARNT (Wang et al., 1995). The HIF-1β subunit can form dimers with other bHLH-PAS
proteins, whilst HIF-1α is unique to HIF-1.

There are two other isoforms of the HIF-α subunit, HIF-2α and HIF-3α. The HIF-2α isoform also plays a role in regulating gene transcription via undergoing dimerisation with the HIF-1β subunit and binding to the promoters region of target genes under hypoxic conditions (Tian et al, 1997). Whilst the expression of the HIF-1α subunit is ubiquitous amongst mammalian cells, the HIF-2α subunit is limited to vascular endothelial cells, the kidney, heart, lung, astrocytes and the epithelium of the small intestine (reviewed in Gordan and Simon, 2007). HIF-1α and HIF-2α proteins share similar structural motifs, such as the ODD (oxygen-dependent degradation) domain, dimerisation domain, DNA binding motifs and conserved regulatory elements, and both bind to the same sequence within the promoter regions of target genes.

The HIF-3α isoform is the least understood of the three proteins. It is believed however that HIF-3α is responsible for the antagonism of HIF-1 or HIF-2-dependent gene expression (reviewed in Heidbreder et al, 2003; Ratcliffe, 2007), by reducing the ability of the other isoforms to bind to the DNA and therefore reducing their transcriptional activity (Maynard et al, 2007).

1.14 HIF-1α regulation under normal physiological conditions

The HIF-1 transcription factor is responsible for the up-regulation of the expression of a number of pro-survival target genes, which are not required for normal cellular processes; therefore, the expression of the HIF-1α subunit is down-regulated at physiological oxygen concentrations (i.e. between 20-
100mmHg, which relates to approximately 7% oxygen (Adams et al., 1999)), via proteomic degradation, but is highly inducible under hypoxic conditions. The HIF-1α protein is constitutively transcribed and translated, however, is subjected to rapid degradation (i.e. has a half life of less than 5mins) via an ubiquitin-proteasomal-dependent pathway (Wang et al., 1995).

The inducible subunit HIF-1α becomes hydroxylated on proline residues 402 and 564, within its ODD domain (Chan et al., 2002; Ivan et al., 2001; Jaakkola et al., 2001). This hydroxylation occurs rapidly via the actions of three oxygen (plus iron, 2-oxoglutarate and ascorbate)-dependent HIF-1α prolyl hydroxylase enzymes, which are active only under physiological oxygen concentrations (Jaakkola et al., 2001). Each proline hydroxylation is considered as an independent event (i.e. the hydroxylation of one proline residue does not affect the hydroxylation of the other) (Chan et al., 2000).

Prolyl hydroxylase enzymes are homologous to egl-9 (egg-laying defect gene) in C. elegans, where they were first identified. They are also known as ‘HIF prolyl hydroxylases’ (Bruick and McKnight, 2001).

The hydroxylation of HIF-1α allows for the von-Hippel-Lindau tumour suppressor protein (pVHL), in complex with several other proteins, including Cul2 and Elongin C and B, to bind to the HIF-1α. The binding of pVHL was confirmed via precipitation studies, where both HIF-1α and pVHL were shown to co-immunoprecipitate together under normoxic conditions (Maxwell et al., 1999). pVHL is the recognition component of an E3 ubiquitin ligase, therefore
causing the polyubiquitination of HIF-1α, which is then degraded via the 26S proteasome (Huang et al., 1998; Maxwell et al., 1999; Salceda and Caro, 1997) (Figure 1.3).

The regulation of the HIF-1α subunit under normoxic conditions was determined using mutational analysis and inhibition studies. Mutation of either of the proline residues (402 or 564), or the inhibition of either the ubiquitination or proteasomal degradation processes allowed for the accumulation of HIF-1α expression under normoxic conditions (Chan et al., 2002; Chachami et al., 2004).

The inactivation of pVHL activity under normoxic conditions causes HIF-1α accumulation, as seen in in vitro models using renal carcinoma cells. HIF-1α becomes constitutively expressed and therefore promotes unregulated growth, even if prolyl enzymes are present and functional (Gunaratnam et al., 2003). If the pVHL becomes inactivated, the HIF-1α protein is still subjected to...
hydroxylation. HIF-1α can also become over-expressed under normoxic conditions due to the presence of other oncogenic mutations, other than those that inactivate pVHL. This would allow HIF-1α to become functionally active, despite the presence of the fully operational degradation pathway (Hofer et al, 2001).

HIF-1α expression is also induced by CoCl₂ (cobaltous ions), DFO (defereroxamine) and dimethyloxalylglycine (both iron chelators) under normoxic conditions (Semenza et al, 1995, Chan et al, 2002; Chachami et al, 2004). All three molecules act via pharmacologically competing with prolyl hydroxylases, which are iron-dependent enzymes (Chan et al, 2002).

The inhibition of HIF-1α under normoxic oxygen concentrations is also regulated via an oxygen-dependent asparagine hydroxylase, known as ‘factor-inhibiting HIF-1 (FIH-1), which acts as a second oxygen sensor in the HIF-1α regulation pathway (Lando et al, 2002¹). This enzyme was originally identified in 2002 and is responsible for hydroxylating asparagine residue 803, in the COOH-terminal transactivation domain of HIF-1α (Lando et al, 2002²; McNeill et al, 2002). This hydroxylation reduces the ability of HIF-1α to bind to its co-activator p300/CBP (CREB-binding protein) at its CH1 (cysteine-histidine) domain (McNeill et al, 2002). FIH-1 prevents HIF-1α accumulation in the nucleus under hypoxic conditions, therefore inhibiting its activity, although it does not affect its degradation (Lando et al, 2002¹). Under hypoxic conditions however, FIH-1 activity is inhibited, allowing HIF-1α to form a transcriptional complex with p300/CBP and other accessory co-activators,
such as Redox-factor 1 (Lando et al, 2002). Mutation of the conserved asparagine induces constitutive p300 interaction, which leads to increased transcriptional activation of the HIF-1 transcription factor (Lando et al, 2002).

As a result, the expression of HIF-1α under normoxic conditions is tightly regulated via two different mechanisms; degradation by prolyl hydroxylase enzymes and inhibition of transcriptional activity by FIH-1. Therefore the expression of HIF-1α is very unstable at physiological oxygen concentrations, having a half-life of less than 5mins (Wang et al, 1995). HIF-1α mRNA is constitutively expressed under normoxia within the cytoplasm, to ensure rapid up-regulation of the protein if the cell enters hypoxic conditions (Heidbreder et al, 2003; Huang et al, 1996).

1.15 HIF-1α and cancer
HIF-1α is over-expressed in over 70% of human tumours, including those found in the brain (especially the cerebral cortex and hippocampus), breast, cervical, colon, gastric, lung, ovarian, pancreatic, prostate and renal cancers, as shown using multiple in vitro and in vivo models, as well as within primary biopsies from patients, when compared to the equivalent normal control (Palayoor et al, 2003; Zhong et al, 2002; Zhong et al, 1999). HIF-1α expression in the heart, liver and kidney under hypoxic conditions was low, suggesting further mechanisms were required to allow the cell to survive in hypoxia (Heidbreder et al, 2003). HIF-1α protein was mainly expressed in the nucleus of tumour cells, often concentrated around areas of neovascularisation,
on the periphery of necrotic regions and at invading edges of tumours (Acs et al, 2003; Beasely et al, 2002; Büchler et al, 2003; Griffiths et al 2007; Jiang et al, 2004; Kuwai et al, 2003; Zagzag et al; 2000; Zhong et al, 1999). HIF-1α has been suggested to play a role in cancer progression, as a number of studies investigating different tumour types have linked increasing HIF-1α expression with increasing histological tumour grade. These studies included breast (Bos et al, 2001; Bos et al, 2003; Zhong et al, 1999), colorectal (Jiang et al, 2004; Kuwai et al, 2003), gastric (Griffiths et al, 2007; Mizokami et al, 2006) and ovarian (Nakayama et al, 2002) human carcinoma samples. Higher HIF-1α expression was observed in more advanced tumour samples (i.e. poorly-differentiated tumours), compared to those of a well-differentiated phenotype. Its full role in cancer progression however is still under debate, as the expression of HIF-1α within human tumour samples has been correlated with both increased and decreased treatment success and overall patient survival. For example, in breast, cervical and gastric carcinomas, the expression of HIF-1α has been associated with increased disease recurrence and shorter overall patient survival (Birner et al, 2000; Bos et al, 2003; Griffiths et al, 2007; Mizokami et al, 2006; Oh et al, 2008; Vleugel et al, 2005). Improved treatment outcome and reduced patient mortality was however noted in head and neck, lung and oesophageal tumour samples (Beasely et al, 2002; Koukourakis et al, 2001; Lee et al, 2003; Volm et al, 2000). This may suggest that the role of HIF-1α is dependent on the location of the tumour within the body and therefore further research is required to determine the full role of HIF-1α in carcinogenesis.
The constitutive expression of HIF-1 causes the tumours to show a more aggressive phenotype, with increased rates of proliferation, angiogenesis (shown by increased microvessel density) and invasion / malignancy (Kuwai et al, 2003; Oh et al, 2008; Zhong et al, 1999). This allows the cancer to be more resistant to treatment, both using chemotherapy and radiotherapy (Hussein et al, 2006) and is therefore linked to a poor prognosis. The expression of HIF-1α is more prominent in solid tumours, which have taken time to establish, increasing their rate of glycolysis and neovascularisation, therefore ensuring their survival (Zhong et al, 2000). Often, the expression of HIF-1α protein increases as the pathological stage increases (i.e. higher expression is present in poorly differentiated tumour samples, compared to well differentiated tumours) (Bos et al, 2001).

The early expression of HIF-1α also protects cells against apoptosis initially as HIF-1 is responsible for the transcription of genes that are anti-apoptotic. As hypoxia continues however, the expression of anti-apoptotic genes is replaced by pro-apoptotic versions, such as IAP-2 (inhibitor of apoptosis protein-2) (Dong et al, 2001).

1.16 HIF-1α regulation under hypoxic conditions
Under hypoxic conditions, (where tissue oxygen levels range between 0-10mmHg, which equals approximately 1.5% oxygen concentration (Adam et al, 1999)) the actions of the HIF-1 transcription factor are initiated via the inhibition of the regulatory ubiquitin-proteasomal signalling pathway that controls the expression of normoxic HIF-1α protein. The inhibition of prolyl
hydroxylases allows for the stabilisation of HIF-1α; therefore, pVHL is prevented from binding, allowing the cell to survive with the stress exerted on it by hypoxia (Chan et al., 2002) (Figure 1.4).

Figure 1.4 – Hypoxic regulation of Hypoxia-Inducible Factor-1.

HIF-1α protein levels are dramatically increased under hypoxic conditions, whereas the mRNA levels remained approximately the same under both normoxic and hypoxic conditions (Heidbreder et al., 2003; Huang et al., 1996; Kuwai et al., 2003). The induction of HIF-1α under hypoxic conditions requires de novo protein translation, but not transcription (Chachami et al., 2004). HIF-1α protein is detectable within 30mins and becomes stabilised for several hours (Büchler et al., 2003; Wang et al., 1995).

Under hypoxic conditions, the non-hydroxylated HIF-1α protein translocates to the nucleus (Chan et al., 2002). HIF-1α protein contains a nuclear translocation signal within the C-terminus, allowing entry through the nuclear membrane
(Kallio et al., 1998). A single amino acid mutation within this signal was shown to prevent HIF-1α from entering the nucleus, and prevented p300/CBP binding (Kallio et al., 1998; McNeill, 2002).

Nuclear localisation of HIF-1α has been confirmed on several occasions using cell immunofluorescent staining (Mabjeesh et al., 2003, Mottet et al., 2003). Once in the nucleus, HIF-1α forms a dimer with the HIF-1β subunit (Figure 1.4). The bHLH domain of both HIF-1α and HIF-1β, located at the N-terminus of the peptides is required for both heterodimerisation and DNA binding (Wang et al., 1995).

The fully formed HIF-1 transcription factor can then bind within the major groove of the DNA (Wang and Semenza, 1995). Both subunits make contact with the DNA, with HIF-1α forming the strongest bond (Wang and Semenza, 1995). HIF-1 binds to conserved hypoxia-response elements (HRE) within the promoter region of target genes, along with further regulatory sequences (reviewed in Wenger et al., 2005).

Under hypoxic conditions, the HIF-1α subunit is no longer hydroxylated at asparagine-803 and is able to interact with its co-activator p300/CBP, at its COOH-terminal transactivation domain (Lando et al., 2002). The interaction with p300/CBP increases the transcriptional activity of the HIF-1 transcription factor, via recruitment of co-activating protein complexes to the promoter of the target gene (Lando et al., 2002).
1.17 Intracellular signal transduction control of HIF-1α expression

The expression of the transcription factor HIF-1 is also regulated at the protein level via intracellular signalling pathways. This normally occurs under normoxic conditions, although the exact pathway involved appears to be both cell-type-specific and dependent on mutations in oncogenes, unlike the hypoxic response which is ubiquitous (Hudson et al, 2002; Laughner et al, 2001). The cell signalling often leads to HIF-1α phosphorylation, causing its stabilisation (Richard et al, 1999). HIF-1α expression is also stabilised via nitric oxide and reactive oxygen species, generated in an inflammatory environment (Chachami et al, 2004; Griffiths et al, 2007). Despite the suggestion that normoxic signalling pathways can induce HIF-1α accumulation, the actual expression levels achieved are lower than that induced via hypoxia (Li et al, 2005).

A number of growth factors and small molecules are responsible for activating the signalling pathways that regulate HIF-1α expression. These include insulin, EGF, TNF-α, insulin-like growth factors (IGFs), IL-1β, prostaglandins, angiotensin II, endothelin and thrombin (reviewed in Wenger et al, 2005). There is a level of redundancy in the signalling induced by these molecules, as they seem to converge onto two main signalling pathways; the PI3-kinase/Akt pathway and MAPK pathway, after binding to their tyrosine kinase receptors (e.g. HER2 (human epidermal growth factor receptor 2) /neu receptor). The inhibition of these receptors removes the ability to stimulate HIF-1α stabilisation and therefore function (Gunaratnam et al, 2003).
The PI3-kinase/Akt pathway is involved in the activation of HIF-1, via stabilising HIF-1\(\alpha\) expression (Chachami et al., 2004), but its exact role has not been fully elucidated, although it is understood that HIF-1\(\alpha\) is not a direct substrate for Akt, but is a downstream target of Akt signalling (Mottet et al., 2003). PI3-kinase is responsible for converting phosphatidylinositol-4, 5-bisphosphate to phosphatidylinositol-3, 4, 5-trisphosphate, which activates phosphoinositide-dependent kinase-1. This then phosphorylates Akt on Serine-473 (on the regulatory domain) and Threonine-308 (on the catalytic domain), causing its activation and its ability to phosphorylate further proteins on serine or threonine residues (reviewed in Vara et al., 2004). Inhibition of PI3-kinase using LY294002 decreased the expression of normoxic HIF-1\(\alpha\) protein (Zhou et al., 2004).

A suggested target of the PI3-kinase /Akt signalling involved in the stabilisation of HIF-1\(\alpha\) is mTOR (mammalian target of rapamycin). mTOR is responsible for phosphorylating 4E-BP1 (eukaryotic initiation factor 4 – inhibiting binding protein-1), causing the dissociation of the eukaryotic initiation factor, which leads to the stabilisation of HIF-1\(\alpha\) mRNA translation (Lang et al., 2007) and the up-regulation of transcription of target genes. Treating cells with the mTOR inhibitor rapamycin would inhibit the mTOR activity and as a result, prevent the accumulation of HIF-1\(\alpha\), even under hypoxic conditions (Hudson et al., 2002; Treins et al., 2002).

A further target of Akt phosphorylation is FRAP (FKBP (FK506-binding protein)-rapamycin associated protein), which also acts on multiple targets,
such as the ribosomal protein p70^{S6K} (p70 S6 kinase 1) (Laughner et al, 2001, Zhong et al, 2000). Autocrine activation of FRAP was shown to up-regulate HIF-1α protein expression via inducing its translocation to the nucleus, as seen in the myeloma cell line A431 (Fukuda et al, 2003; Zhong et al, 2002). Using rapamycin, an inhibitor of FRAP, hypoxic induction of HIF-1α was shown to be greatly reduced *in vitro*, whilst tumour growth and angiogenesis was inhibited *in vivo*, suggesting FRAP plays an important role in the activation of HIF-1 via intracellular signalling (Zhong et al, 2000). FRAP was shown to up-regulate HIF-1α protein expression only, as the mRNA levels were not affected by rapamycin inhibition (Fukuda et al, 2003).

The PI3-kinase/ Akt pathway is also responsible for the up-regulation of heat shock protein (Hsp) expression, which also aids the stabilisation of the HIF-1α protein under normoxic conditions (Zhou et al, 2004). Hsp70 has been shown to interact directly with ODD domain of the HIF-1α subunit, whereas Hsp90 interacts with the bHLH domain (Zhou et al, 2004). Targeting Hsp90 for inhibition also decreased the expression of HIF-1α protein (Zhou et al, 2004).

Hypoxia can induce the PI3-kinase pathway via an autocrine mechanism, as growth factors transcribed by HIF-1 can act at tyrosine kinase receptors on the cell surface (Mottet et al, 2003).

The MAPK signalling pathways (e.g. MEK-ERK pathway (MAPK-ERK kinase – extracellular-regulated kinase pathway)) have also been shown to up-regulate the HIF-1α protein, suggesting a further, non-hypoxic mechanism of
HIF-1 regulation (Fukuda et al, 2003). The MAPK pathway, as with the PI3-kinase pathway, can be activated via growth factors that act at tyrosine kinase receptors, such as the IGF-receptor, causing their autophosphorylation. This attracts the protein SHC (Src homology collagen-like protein), which through interaction with Grb2 (Growth factor receptor-bound protein 2) and mSOS (mammalian sons of sevenless), initiates the phosphorylation of the G protein Ras. This then phosphorylates Raf-1, which in turn phosphorylates MEK (reviewed in Fang and Richardson, 2005). The kinase MEK phosphorylates ERK, which then directly phosphorylates the HIF-1α subunit (Sang et al, 2002). It has been suggested that when HIF-1α becomes phosphorylated via MAPK signalling, then it can form a dimer with HIF-1β and up-regulate gene transcription. A dephosphorylated version of HIF-1α promotes apoptosis, due to its ability to bind to p53 (Suzuki et al, 2001). The actions of the HIF-1α co-activator p300 also require the presence of an active MAPK, which indirectly stimulates the transactivation of HIF-1α (Sang et al, 2003).

Inhibition of MEK using PD98059 prevents ERK phosphorylation and therefore blocks HIF-1α expression, whereas a constitutively active ERK pathway continually induces HIF-1α protein. The inhibition of other MAPKs, such as p38 had no effect on HIF-1α expression (Sang et al, 2002; Richard et al, 1999). The inhibition of MAPK also prevented the p300 protein from associating with HIF-1α, reducing its ability to translocate to the nucleus and up-regulate gene expression (Sang et al, 2003). As a result, the transcription of HIF-1α target genes is dependent on MAPK signalling (Hofer et al, 2001).
As a result of receptor activation and intracellular signalling, the activity of multiple transcription factor activities, such as c-fos and NF-κB (nuclear factor κB) are up-regulated (Fukuda et al, 2003; Gunaratnam et al, 2003; Zhong et al, 2002), which would increase both cell survival and proliferation.

These normoxic signalling pathways are up-regulated within a number of cancers, due to mutations that occur within either key oncogenes, such as c-Src, Ras or HER2/neu receptor, or key tumour suppressor genes, such as pVHL, PTEN or p53. Oncogenes only require a mutation in one allele before they become constitutively active, whereas tumour suppressor genes require the mutation in both alleles before their expression is knocked out. The up-regulation of oncogenes or down-regulation of tumour suppressor genes allows for the constitutive activity of a number of growth promoting signalling pathways, including the PI3-kinase/Akt pathway (reviewed in Hanahan and Weinberg, 2000; Vogelstein and Kinsler, 2004). This induces an over-expression of HIF-1α, even under normoxic conditions (Chan et al, 2002). As a result, key pro-survival processes are induced, such as increased rate of tumour proliferation, induction of angiogenesis or inhibition of apoptosis (Maxwell et al, 1997; Zhong et al, 1999). The inactivation of PTEN, a phosphatase that reverses the actions of PI3-kinase, is often seen in cancer, to aid its progression and the process of angiogenesis. The expression of PTEN decreases as the tumour advances. The over-expression of HIF-1α has been significantly linked to PTEN inactivation. HIF-1α expression has been shown to increase as the levels of PTEN decreases (Jiang et al, 2003; Zundel et al, 2000), allowing the PI3-kinase/Akt pathway to up-regulate both expression
and activation of HIF-1 (Li et al., 2005). The over-expression of HER2/neu receptor leads to the constitutive expression of Akt, even in the presence of a functional PTEN protein (Li et al., 2005). This also leads to the induction of HIF-1α protein under normoxic conditions. Inhibitors of PI3-kinase, Akt and FRAP, or the reintroduction of PTEN, removed HIF-1α expression from the cell, reducing both growth and angiogenesis (Jiang et al., 2003; Treins et al. 2002; Zhong et al., 2000), making them all possible targets for cancer therapies.

1.18 Downstream transcriptional effects of HIF-1 activation

HIF-1 is responsible for the induction of multiple target genes, which aid both tumour survival and progression in a hypoxic environment, producing a more metastatic phenotype. Once bound HIF-1 causes the transcription of multiple target genes, responsible for a whole range of biological activities such as cell proliferation, metabolism and angiogenesis / oxygen transport (See Table 1.4 for a list of key target genes and list of functions.) The expression of these genes enables the cell to adapt and endure hypoxic conditions. HIF-1 is suggested to regulate the expression of up to 200 genes in hypoxia (Wenger et al., 2005), accounting for 1-5% of the entire human genome (Semenza, 2003).

HIF-1 binds to the DNA at promoter regions of target genes at consensus hypoxia-response elements (HRE), ranging from -50bp to -5000bp upstream of the coding sequence (reviewed in Wenger et al., 2005). HIF-1 recognises the sequence 5’ – (C/G/T)RCGTGC(G/T) – 3’, where R is any purine residue. In approximately 75% of HRE sequences, the purine is an adenine residue, with
the rest being guanine residues (reviewed in Wenger et al, 2005). The HIF-1α subunit binds tightly to the entire HRE sequence, whereas the HIF-1β subunit only recognises the final GTG residues (Wenger et al, 2005). The HRE sequence was originally determined in the erythropoietin promoter, prior to the discovery of the HIF-1 transcription factor (Semenza et al, 1994).

One of the major targets of HIF-1 transcriptional activity is VEGF, which is one of the most investigated genes in relation to HIF-1 activity. VEGF plays a major role in neovascularisation and cellular proliferation, acting via one of three VEGF receptors. Under physiological conditions, VEGF signalling is required for both wound healing and the reproductive cycle; however, it has been implicated in a number of pathological conditions, such as cancer, rheumatoid arthritis and diabetes (reviewed in Byrne et al, 2005). VEGF expression is predominantly up-regulated by hypoxia; however, its expression can also be regulated via growth factor signalling, such as IGF-1 and EGF under normoxic conditions (reviewed in Mikhopadhyay and Datta, 2004), via a NF-κB-dependent manner (reviewed in Pradeep et al, 2005). VEGF expression was associated with HIF-1α expression in a number of cancer phenotypes, including colon, pancreatic and breast cancer (Bos et al, 2001; Büchler et al, 2003; Currie et al, 2004; Kuwai et al, 2003), which was further correlated with increased microvessel density and reduced patient survival (Bos et al, 2001; Kuwai et al, 2003). VEGF was co-expressed with HIF-1α within tumour cells, but in the cytoplasm instead of the nucleus (Büchler et al, 2003; Griffiths et al, 2007). Inhibition of VEGF via HIF-1α down-regulation resulted in reduced tumour weight and vascularisation, where tumours were
smaller, weaker and less branched (Stoeltzing et al, 2004), suggesting that VEGF plays a role in angiogenesis and vessel maturation.

<table>
<thead>
<tr>
<th>Gene:</th>
<th>Cellular function:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelin-1</td>
<td>Vasoconstrictive peptide</td>
</tr>
<tr>
<td>Erythropoietin</td>
<td>Glycoprotein hormone responsible for red blood cell production</td>
</tr>
<tr>
<td>Heme oxygenase</td>
<td>Inhibits the production of reactive oxygen species and regulates vasodilation</td>
</tr>
<tr>
<td>Vascular Endothelial Growth Factor (VEGF)</td>
<td>Growth factor responsible for blood vessel formation</td>
</tr>
<tr>
<td>Inducible and Endothelial Nitric Oxide Synthase</td>
<td>Enzymes responsible for synthesis of Nitric oxide, a vasoactive compound</td>
</tr>
</tbody>
</table>

Glycolytic enzymes:
- Aldose A and C (muscle and brain isoforms)
- Enolase-α
- Phosphofructokinase (liver and platelet isoforms)
- Hexokinase
- Lactate dehydrogenase (LDH)
- Phosphoglycerate kinase
- Glucose phosphate isomerase I
- Triosephosphate isomerase
- Pyruvate kinase
- Glyceraldehyde-3-phosphate dehydrogenase

Provide energy source for cells via anaerobic respiration

Glucose transporter 1 (GLUT1) Transmembranal glucose transporter, which helps regulate glucose uptake into cells

BNIP3 (Bcl-2/adenovirus EIB 19kD-interacting protein 3) Member of the Bcl2 apoptosis protein family, which up-regulates apoptosis

Galactokinase Enzyme involved in maintaining energy levels within cells

Galectin-3 Anti-apoptotic lectin which promotes cell survival and migration

RhoA Causes F-actin polymerisation and cytoskeletal rearrangement

Glycogen synthase 1 Responsible for the synthesis of energy stores from glucose

Human telomerase reverse transcriptase A telomerase

Transferrin and its receptor An iron-binding protein

Matrix metalloproteinases (MMPs) Proteases that are required for invasion

Table 1.4 – Key target genes transcribed as a result of HIF-1 activity (Information taken from Greijer et al, 2005; Wenger et al, 2005).
HIF-1 is also responsible for transcribing genes involved in glycolysis and glucose transport (Semenza et al., 1994). The lack of oxygen prevents cells from performing normal metabolic processes; however, the transcriptional activity of HIF-1 results in an energy source (adenosine triphosphate (ATP)) being provided for the tumour, even under low oxygen tension, via anaerobic respiration (Semenza et al., 1994). Examples of metabolic genes that are induced by HIF-1 include glucose transporter 1 (GLUT1) and lactate dehydrogenase (LDH), acting to increase glucose entry into cells and to maintain cellular energy levels (see Table 1.4 for further examples).

Initially, HIF-1 also transcribes anti-apoptotic genes, such as Galectin-3 to enhance cell survival, but as hypoxia progresses, gene expression alters towards expression of pro-apoptotic molecules, such as BNIP3 (Bel-2/adenovirus E1B 19kD-interacting protein 3) (Greijer et al., 2005). The induction of hypoxia is only expected to be temporary, therefore after a period of extended hypoxia the cells are unable to sustain the non-physiological conditions, therefore undergo apoptosis. However, after longer periods of hypoxia, invasive genes are also up-regulated, which would alter cell differentiation and migration properties, enhancing chances of survival (Greijer et al., 2005; Zhong et al., 2002).

A number of genes are also down-regulated by hypoxic conditions as shown by Greijer et al (2005) via microarray (a total of 114 genes, which is approximately 15% of total genome). These include genes involved in cytoskeletal motility (e.g. RhoA kinase), mRNA processing, DNA repair and
histone interactions but only a small proportion (<20%) are regulated by HIF-1 directly (Greijer et al, 2005).

Surprisingly, genes encoding components of prolyl hydroxylases are also transcribed under hypoxic conditions (Greijer et al, 2005), suggesting an autoregulatory mechanism, anticipating the presence of oxygen, and therefore initiating HIF-1α degradation. Once normoxia resumes, the prolyl hydroxylase enzymes reassemble and target HIF-1α to the proteasome (Mottet et al, 2003).

The removal of cells from hypoxic conditions will result in the degradation of HIF-1α after only a few minutes, but the effects of hypoxia will last longer as the target genes, such as VEGF and GLUT1 continue to be translated (Palayoor et al, 2003).

The central role that HIF-1 plays in tumour development makes it a possible target of anti-cancer therapy. As HIF-1β is constitutively expressed within the cell, targeting the inducible HIF-1α subunit would be more successful at inhibiting the carcinogenic properties of the HIF-1 transcription factor. Targeting HIF-1α expression has been previously been shown to inhibit cancer progression in vivo. For example, HIF-1α inhibition via the use of dominant-negative HIF-1α constructs or HIF-1α-null cell lines in xenograft mouse models inhibited the expression of HIF-1 target genes, the level of tumour vascularisation and overall tumour growth (Ryan et al, 2000; Stoeltzing et al, 2004).
1.19 Project hypothesis and objectives

In normoxia, the effect of gastrin on angiogenesis is well-defined to be indirectly mediated through (i) COX-2 and (ii) amphiregulin/HB-EGF via VEGF. We hypothesise that in hypoxia to maintain this pro-angiogenic role, gastrin-HIF interactions occur to either:

- maintain the expression of gastrin to act on GI cancer cascades,

- or for gastrin to have an inherent ability to switch to increasing HIF-1α expression (see Figure 1.5).

The objectives for this project were therefore,

1) Investigation into the effect of exogenous gastrin treatment of GI carcinoma cell lines on HIF-1α protein expression and transcriptional activity under both normoxia and hypoxia.

2) Investigation into the effect of hypoxia-induction on gastrin mRNA expression.

3) Effect of gastrin depletion on HIF-1α and downstream biological events linked to hypoxia.
GI Malignancy

Normoxia
- Gastrin
- Growth factor signalling
- COX-2
- Angiogenesis
- VEGF
- HIF

Hypoxia – no Gastrin
- COX-2
- Angiogenesis
- VEGF
- HIF
- Growth factor signalling

Hypoxia – with Gastrin
- Gastrin
- Growth factor signalling
- COX-2
- Angiogenesis
- VEGF
- HIF

Figure 1.5 – Project hypothesis investigating the relationship between gastrin and HIF-1.
Chapter Two
MATERIALS AND METHODS
2.1 Basic cell culture

Human GI carcinoma cell lines were routinely cultured in RPMI-1640 medium (Sigma Aldrich, Poole, UK), supplemented with heat-inactivated foetal bovine serum (FBS) and 2mM L-glutamine (both Sigma Aldrich). The cell lines were grown at 37°C, in a humidified 5% CO₂ (carbon dioxide) atmosphere.

The epithelial cell monolayers were routinely sub-cultured when they reached 80% confluency in T75cm² flasks (Corning, Fisher Scientific, Loughborough, UK), using 0.025% ethylenediamine tetra-acetic acid (EDTA) in phosphate-buffered saline (PBS) (Oxoid, Basingstoke, UK), at a ratio of 1:6 through the addition of fresh cell culture medium. The GI carcinoma cell lines that were used are shown in Table 2.1.

The majority of experiments were conducted in three key cell lines; HCT116, MGLVA1 and PAN1 carcinoma cells. The key characteristics for these carcinoma cell lines are described in Table 2.2.

2.2 Induction of hypoxia

Hypoxia was induced in the GI carcinoma cell lines listed in Table 2.1, using the following methods:

1) Cobalt chloride (CoCl₂ (Sigma Aldrich)). CoCl₂, an iron antagonist, was used to inhibit prolyl hydroxylase activity. Growth medium was aspirated from the cell lines and replaced with fresh medium containing 150µM CoCl₂. Cells were incubated at 37°C, in a humidified 5% CO₂ atmosphere for between 2-24hr.
<table>
<thead>
<tr>
<th>Cell line:</th>
<th>Tissue of origin:</th>
<th>ECACC number:</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGS</td>
<td>Human gastric adenocarcinoma</td>
<td>89090402</td>
</tr>
<tr>
<td>BxPC3</td>
<td>Human pancreatic adenocarcinoma</td>
<td>93120816</td>
</tr>
<tr>
<td>C170HM2</td>
<td>Human colonic adenocarcinoma</td>
<td>Derived from parental cell line C170 (ECACC number 97071507).</td>
</tr>
<tr>
<td>HCT116</td>
<td>Human colonic adenocarcinoma</td>
<td>91091005</td>
</tr>
<tr>
<td>MGLVA1</td>
<td>Human gastric adenocarcinoma</td>
<td>Derived within the Division of Pre-Clinical Oncology</td>
</tr>
<tr>
<td>OE19</td>
<td>Human oesophageal adenocarcinoma</td>
<td>96071721</td>
</tr>
<tr>
<td>OE21</td>
<td>Human oesophageal squamous carcinoma</td>
<td>96062201</td>
</tr>
<tr>
<td>OE33</td>
<td>Human oesophageal adenocarcinoma</td>
<td>96070808</td>
</tr>
<tr>
<td>PAN1</td>
<td>Human pancreatic adenocarcinoma</td>
<td>Derived within the Division of Pre-Clinical Oncology</td>
</tr>
<tr>
<td>PANC1</td>
<td>Human pancreatic adenocarcinoma</td>
<td>87092802</td>
</tr>
<tr>
<td>ST16</td>
<td>Human gastric adenocarcinoma</td>
<td>Derived within the University of Nottingham.</td>
</tr>
</tbody>
</table>

Table 2.1 – Human GI carcinoma cells lines used to determine HIF-1α protein expression, HIF-1α gene expression and gastrin gene expression.

2) Deferoxamine (DFO (Sigma Aldrich)). DFO, an iron chelator was used to inhibit prolyl hydroxylase activity. Growth medium was aspirated from the cell lines and replaced with fresh medium containing 200µM DFO. Cells were incubated at 37°C, in a humidified 5% CO₂ atmosphere for between 2-24hr.

3) Atmospheric hypoxia. True hypoxic conditions were maintained using the Invivo₂ 400 Hypoxia workstation (Biotrace Fred Baker, Bridgend, UK), to facilitate the stabilisation and up-regulation of the HIF-1α subunit. The hypoxia workstation was able to maintain a humidified 1% oxygen, 5% CO₂, 94% nitrogen environment, at a constant 37°C. Cells and equipment
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell line characteristics</th>
</tr>
</thead>
</table>
| HCT116 cells | • Derived from a poorly-differentiated human colonic carcinoma.  
• High level of endogenous gastrin gene expression.  
• Expresses both the standard and alternative transcripts of the gastrin gene.  
• Inhibition of gastrin gene expression resulted in reduced cell survival and increased apoptosis.  
• Cell growth was inhibited after exogenous gastrin treatment.  
• Gastrin treatment induces cAMP expression.  
• Does not endogenously express the CCK-2 receptor.  
• Treatment with glycine-extended gastrin induced cell proliferation. |
| MGLVA1 cells | • An ascitic variant of the gastric cell line MGN45G.  
• Relatively low level of basal gastrin gene expression.  
• Expresses both the standard and alternative transcripts of the gastrin gene.  
• Inhibition of gastrin gene expression resulted in reduced cell survival, which was restored after treatment with exogenous amidated gastrin and glycine-extended gastrin.  
• Does not endogenously express the CCK-2 receptor.  
• Secretes both progastrin and glycine-extended gastrin, but not amidated gastrin. |
| PAN1 cells | • Derived from a poorly-differentiated pancreatic adenocarcinoma.  
• Relatively low level of basal gastrin gene expression.  
• Expresses both the standard and alternative transcripts of the gastrin gene.  
• Expresses gastrin at both the gene and protein level, at levels similar to those found in pancreatic tumours.  
• Inhibition of gastrin gene expression resulted in reduced cell survival and increased apoptosis.  
• CCK-2 receptor status unknown. |

Table 2.2 – Key gastrin-related cell characteristics for the HCT116, MGLVA1 and PAN1 carcinoma cell lines. (Information taken from Bold et al, 1994; Colucci et al, 2008; Ferrand et al, 2006; Grabowska et al, 2007; Grabowska et al, 2008; Harris et al, 2004; Ishizuka et al, 1994; Watson et al, 1998; Watson et al, 1999).

were placed into the hypoxia workstation via an air-lock system. Cells were harvested between 1-16hr hypoxic incubation (for Western blotting, RT-PCR (reverse transcriptase polymerase chain reaction) and luciferase
assays) and lysed (for Western blotting and luciferase assays) within the workstation, before the assays were continued within a class II cell culture hood. When possible, the reagents required for harvesting the assays were placed in the hypoxia workstation for 30-60mins prior to use, to allow them to equilibrate to the hypoxic environment and remove any traces of oxygen from the solutions. Equivalent normoxic controls remained at 37°C in a humidified 5% CO₂ environment.

2.3 Exogenous treatment of cells with amidated gastrin
The GI carcinoma cell lines were treated with exogenous gastrin, prior to being harvested for Western blotting, RT-PCR and luciferase assays. Serum-starved cells were treated with 1nM, 10nM or 100nM exogenous amidated human gastrin (G17) (Aphton Corporation, Philadelphia, USA), 1nM, 10nM or 100nM AK-80 scrambled control (Aphton Corporation), or untreated control (serum-free medium only). The scrambled control contained the same first five amino acids as the amidated gastrin (methionine-glutamine-arginine-leucine-cysteine), but in a randomised sequence. The exogenous gastrin-treated carcinoma cells were incubated under normoxic or hypoxic conditions for a known time point (4-72hr), before being harvested as described below.

2.4 Exogenous treatment with epithelial growth factor (EGF)
The GI carcinoma cell lines were treated with exogenous EGF, prior to being harvested for luciferase assays. The EGF would bind to known EGF reporter
elements within the transfected plasmid and therefore act as a positive control, up-regulating the luciferase activity.

Approximately 24hr after plasmid transfection, the growth medium was aspirated and replaced with fresh serum-free medium +/- 10µg/ml EGF (Sigma Aldrich). The GI carcinoma cell lines were incubated under normoxic conditions for 24-48hr, with the EGF treatment being refreshed every 12hr, before being harvested as described below.

2.5 Western blotting

Specific expression of nuclear HIF-1α and HIF-1β protein after the induction of hypoxia or exogenous amidated gastrin treatment (HIF-1α expression only) was determined using target-specific primary antibodies by the process of Western blotting.

2.5.1 Nuclear lysate preparation

Carcinoma cell lines were plated into six-well plates at a density of 3 x 10^5 – 5 x 10^5 cells/well (cell line-dependent), to achieve a 50-60% confluency after 24hr incubation at 37ºC, in a humidified 5% CO₂ atmosphere. Cells were then serum-starved, by replacing the normal growth media with fresh serum-free equivalent for approximately 24hr, before being exposed to hypoxia or treated with exogenous gastrin as described above, for a known time point.

Nuclear lysates were prepared using BioVision Nuclear/Cytosol Fractionation Kit (Cambridge Bioscience, Cambridge, UK). Briefly, cells were harvested
using 0.25% trypsin/EDTA and centrifuged to form a pellet, which was then resuspended in ice-cold ‘cytosol extraction buffer A’. The extraction buffer contained additional protease inhibitors and DTT (Dithiothreitol), to prevent protein degradation and aid disulphide-bond reduction respectively. The cells were incubated on ice for 10mins, before ice-cold ‘cytosol extraction buffer B’ was added. After further incubation on ice, the samples were centrifuged at 14000g for 5mins at 4°C, to separate the cytoplasmic fraction from the nuclear cell pellets. The cytoplasmic supernatant was removed and the remaining cell pellets resuspended in ice-cold ‘nuclear extraction buffer’, with additional protease inhibitors and DTT. The lysates were incubated on ice for 40mins, with occasional agitation, before being centrifuged at 14000g for 10mins at 4°C. The resultant supernatant contained the nuclear fraction of the samples. The protein concentrations of the nuclear lysates were determined using the Nanodrop ND-100 spectrophotometer according to the manufacturer’s instructions. The samples were stored at -80°C until required.

2.5.2 Protein gel electrophoresis

10µg protein per nuclear sample was separated using SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). The nuclear protein samples were diluted in a 1:1 ratio with Tris-Glycine 2X SDS (sodium dodecyl sulphate) sample buffer (Invitrogen, Paisley, UK), prior to being denatured by heating at 99°C for 5mins.

Precast 8-16% gradient Tris-Glycine gels (Invitrogen) were used for gel electrophoresis. After rinsing in distilled water and 1X Tris-Glycine SDS
running buffer (25mM Tris Base, 192mM Glycine and 3.5mM SDS (all Sigma Aldrich)), the gels were locked into the XCell SureLock Novex Mini-Cell electrophoresis system (Invitrogen). The inner chamber of the Mini-Cell electrophoresis system (containing the electrode) and half of the outer chamber were filled with 1X SDS running buffer, before 10µg nuclear protein samples were loaded per gel lane. The gel was also loaded with two protein markers; 5µl SeeBlue Plus2 pre-stained (1X) protein standards (Invitrogen) and 2µl MagicMark XP (1X) Western protein standards (Invitrogen), which provided an accurate molecular weight scale, to aid with protein size determination. The SeeBlue Plus2 was visible throughout the protocol, whereas the MagicMark XP was only visible during the development stage. The gels were run for 90mins at 125V.

2.5.3 Gel transfer onto PVDF membrane

The separated nuclear proteins were transferred onto polyvinylidene difluoride (PVDF) 0.2µm pore membranes (Invitrogen), which were pre-soaked in 100% methanol (Fisher Scientific) and 1X Tris-Glycine transfer buffer (12mM Tris Base and 96mM Glycine). The proteins were transferred from the Tris-Glycine gels to the membranes using the XCell II Blot Module (Invitrogen), which was designed to fit into the XCell SureLock Novex Mini-Cell electrophoresis system. The Tris-Glycine gels and PVDF membranes were assembled into the XCell II Blot module, alongside pre-soaked filter paper and blotting pads (Invitrogen), according to the manufacturer’s instructions (as detailed in Figure 2.1). The XCell II Blot module was locked into the XCell SureLock Novex Mini-Cell electrophoresis system. The blot module and half
of the outer chamber were filled with fresh 1X transfer buffer, before the gels were transferred for 90mins at 30V.

The PVDF membranes were briefly rinsed in 1X TBST (Tris-buffered saline (20mM Tris Base (pH 7.6) and 137mM sodium chloride), with 0.05% Tween-20 (all from Sigma Aldrich)), before non-specific protein binding was blocked for 2hr at room temperature, using 2% ECL (Enhanced Chemi-Luminescence) Advance blocking buffer (Amersham Bioscience, Buckinghamshire, UK), prepared in 1X TBST.

2.5.4 Probing with antibodies

The PVDF membranes were then probed with a target-specific primary antibody, diluted in 2% ECL Advance blocking buffer overnight, at 4ºC (see Table 2.3 for a list of the primary antibodies used, and their specifications).
The β-actin was used to confirm equal loading of the proteins, whilst the mouse anti-bromodeoxyuridine antibody was used as a negative control, to ensure the immune complexes generated after being probed with the test antibody were specific.

<table>
<thead>
<tr>
<th>Target protein detected:</th>
<th>Target molecular weight:</th>
<th>Antibody dilution required:</th>
<th>Antibody raised in:</th>
<th>Supplier:</th>
<th>Notes:</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>120kDa</td>
<td>1:250</td>
<td>Mouse (monoclonal)</td>
<td>BD transduction labs</td>
<td>Test antibody</td>
</tr>
<tr>
<td>HIF-1β</td>
<td>92kDa</td>
<td>1:2000</td>
<td>Rabbit (polyclonal)</td>
<td>Novus Biologicals</td>
<td>Test antibody</td>
</tr>
<tr>
<td>Bromodeoxyuridine</td>
<td>Dependent on test antibody</td>
<td>1:1000</td>
<td>Mouse (monoclonal)</td>
<td>DAKO Cytomation</td>
<td>Negative control</td>
</tr>
<tr>
<td>β-Actin</td>
<td>42kDa</td>
<td>1:5000</td>
<td>Mouse (monoclonal)</td>
<td>Abcam</td>
<td>Loading control</td>
</tr>
</tbody>
</table>

Table 2.3 – Specification for the target-specific primary antibodies for Western blotting.

The PVDF membranes were thoroughly washed with 1X TSBT, before being probed for 1hr at room temperature with a biotin-conjugated secondary antibody, raised against the species of the primary antibody:

- Rabbit α-mouse for HIF-1α, actin and bromodeoxyuridine mouse negative control (DAKOCytomation, Ely, UK).

- Swine α-mouse for HIF-1β (DAKOCytomation).

The secondary antibodies were used at a 1:1000 dilution and were also prepared in 2% ECL Advance blocking buffer.
The PVDF membranes were thoroughly washed with 1X TBST, before they were probed with Strept/ABComplex HRP (Streptomycin/avidin-biotin complex horseradish peroxidase)-conjugated (DAKOCytomation), for 30mins at room temperature. The Strept/ABComplex HRP was prepared in 1X TBST, at a 1:20 dilution, approximately 60mins prior to use, to allow the avidin/biotin complexes to form.

The PVDF membranes were developed using ECL Advance Western blotting detection kit, following the manufacturer’s instructions. Briefly, equal amounts of ECL Advance solutions A and B were mixed together, before being poured onto the freshly washed PVDF membranes. The immune complexes were visualised and densitometrically-analysed using the Syngene ChemiGenius BioImaging system GeneTools and GeneSnap programs.

Equal loading was confirmed by stripping the PVDF membranes to remove the test antibody, and then reprobing with a β-actin loading control antibody. The membranes were sealed into glass tubes, containing 30ml of Western blotting stripping buffer (62.5mM Tris-HCl (pH 6.7), 70mM SDS and 100mM 2-mercaptoethanol). The PVDF membranes were incubated in the stripping buffer for 30mins at 50ºC, with constant agitation to ensure the removal of all previous immune complexes from the membrane. The membranes were then thoroughly washed with 1x TBST wash buffer, before being blocked with 2% ECL Advance blocking buffer for 2hr and probed with the primary antibody as previously described.
2.6 Immunofluorescent cell staining

Specific expression of nuclear HIF-1α protein or gastrin protein after the
induction of hypoxia was determined using target-specific primary antibodies,
by the process of immunofluorescent cell staining.

Carcinoma cell lines were plated into eight-well chamber slides at a density of
5 x 10^4 cells/well, to achieve a 60-80% confluency after 24hr incubation at
37ºC, in a humidified 5% CO_2 atmosphere. Cells were then serum-starved, by
replacing the normal growth medium with fresh serum-free equivalent for
approximately 24hr, before being exposed to hypoxia (as described above) for
approximately 6hr.

The carcinoma cell lines were washed in PBS (Oxoid), before being fixed in
4% paraformaldehyde (diluted from 16% stock (Science Services, London,
UK)) for 10mins at room temperature. The aldehyde was quenched with
10mM 2-aminoethanol (Sigma Aldrich) (prepared in PBS) for 5mins, before
the cells were washed in PBS. The cells were then permeabilised in 0.1%
Triton X-100 (Sigma Aldrich) (prepared in PBS) for 1min, before being rinsed
in PBS.

Non-specific antibody binding was blocked by incubating the cells in PBS +
1% BSA (bovine serum albumin) for 30mins, followed by a second 30min
incubation in 10% goat serum (diluted in PBS + 1% BSA
(DAKOCytomation)). The carcinoma cells were then incubated in their
primary antibody as described below:
• 1:250 mouse anti-HIF-1α (BD Transduction Labs) overnight at 4°C, with mouse anti-bromodeoxyuridine (DAKOCytomation) as a negative control.

• 1:200 rabbit anti-gastrin (DAKOCytomation) for 10mins at room temperature, with universal rabbit negative control (DAKOCytomation) as a negative control.

The carcinoma cells were washed thoroughly in PBS + 1% BSA, before being incubated in a 1:500 dilution of the secondary antibody; Alexa-Fluor Goat α-mouse IgG 488 (HIF-1α expression) or Alexa-Fluor Goat α-rabbit IgG 488 (gastrin expression) (Molecular probes, Invitrogen) for 1hr at room temperature. The chambers were then removed, before the cells were thoroughly washed in PBS. The nuclei were then counterstained using 0.5µg/ml Hoescht dye (Molecular Probes, Invitrogen) for 10mins, before being washed in PBS. The slides were then coverslipped using CitiFluor (Science Services). The slides were viewed and images taken using a Leica DMLB fluorescent microscope.

2.7 Gene expression assays
Specific expression of HIF-1α, VEGF and gastrin genes, after the induction of hypoxia, specific target mRNA inhibition, or exogenous amidated gastrin treatment was determined using quantitative real time RT-PCR.
2.7.1 RNA extraction

Carcinoma cell lines were plated into six-well plates at a density of $3 \times 10^5 - 5 \times 10^5$ cells/well (cell line-dependent), to achieve a 50-60% confluency after 24hr incubation at 37ºC, in a humidified 5% CO$_2$ atmosphere. Cells were then serum-starved, by replacing the normal growth media with fresh serum-free media for approximately 24hr, before being exposed to hypoxia or treated with exogenous gastrin as described above, for a known time point. Cells were harvested using 0.25% trypsin/EDTA and centrifuged to form a pellet. The cell pellets were stored at -80ºC until required.

RNA was then extracted from the frozen cell pellets, using one of two reagents; RNABee (Biogenesis, Poole, UK) or TRI-reagent (Sigma Aldrich). Initial experiments (e.g. hypoxic time course studies) used the RNABee reagent for RNA extraction, however this reagent was discontinued during the course of this study, so was replaced by TRI-reagent for the later experiments (e.g. siRNA transfection studies). Both reagents use the phenol/chloroform method of RNA extraction, and had virtually identical protocols.

Frozen cell pellets prepared after the induction of hypoxia or exogenous amidated gastrin treatment, which contained approximately $3 \times 10^5 - 5 \times 10^5$ cells, were resuspended in 1ml RNABee or TRI-reagent, before 200µl chloroform (Sigma Aldrich) was added and the cell suspension shaken for several seconds. An extraction control was prepared alongside the RNA. This contained RNABee or TRI-reagent and chloroform, but no cells, to ensure that the gene expression produced was derived from the RNA and not from any
genomic contamination. Frozen cell pellets prepared after transfection with siRNA only contained up to 5 x 10^4 cells. Therefore, the RNA was extracted from these samples using half volumes of reagents.

The samples were incubated on ice or at room temperature for 5mins (for RNABee and TRI-reagent respectively), before being centrifuged at 13,000g for 15-30mins at 4°C.

The resultant clear, aqueous phase, which contained the RNA was transferred into 500µl isopropanol (Sigma Aldrich) and incubated for 10mins at room temperature (for TRI-reagent samples) or 20mins at -20°C (for RNABee samples). This allowed for RNA precipitation, which was collected by centrifugation at 13000g for 10-15mins at 4°C. The isopropanol was removed and the remaining RNA pellet was washed in 1ml 70% ethanol (Sigma Aldrich, diluted using RNase (ribonuclease)-free distilled water from absolute stock), before being centrifuged at 13000g for 5mins at 4°C. The ethanol was removed and the RNA pellet was allowed to air dry, before being resuspended in RNase-free distilled water (Sigma Aldrich).

### 2.7.2 cDNA synthesis

Complementary DNA (cDNA) was synthesised from the RNA samples using the Superscript II RNase H Reverse Transcriptase kit (Invitrogen). Briefly, 10µl RNA was incubated with 1.2µl random hexamers (pd(N)_6 Amersham Biosciences) at 70°C for 10mins, before the samples were immediately transferred to ice, to stop the reaction.
Control samples were also prepared, to ensure that any signal produced from each cDNA positive sample was specific to the RNA, and not as a result of genomic contamination or from primer dimers. The controls included:

- cDNA negatives, which used water in the place of the random hexamers and reverse transcriptase.

- Reagent control, which contained no RNA, but all other reagents to ensure that they did not generate any signal in their own right.

Table 2.4 shows the reagents required for the cDNA mastermix for one RNA sample. All reagents for the cDNA mastermix were provided in the reverse transcriptase kit, except for the deoxynucleotides (dNTPs) (Sigma Aldrich).

<table>
<thead>
<tr>
<th>Reagent:</th>
<th>Volume required per sample:</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase / RNase-free distilled water</td>
<td>3µl</td>
</tr>
<tr>
<td>5X buffer</td>
<td>4µl</td>
</tr>
<tr>
<td>0.1M DTT</td>
<td>2µl</td>
</tr>
<tr>
<td>dNTPs</td>
<td>0.6µl</td>
</tr>
<tr>
<td>Reverse transcriptase</td>
<td>0.4µl</td>
</tr>
</tbody>
</table>

Table 2.4 – The amount of reagents required per sample (cDNA mastermix).

10µl cDNA mastermix was added to each RNA/primer sample and the cDNA was synthesised using the following programme: 10mins at 25ºC, 60mins at 42ºC, followed by 5mins at 95ºC. The resultant cDNA was diluted 1:5 using RNase-free distilled water and stored at -20ºC until required.
2.7.3 Real time RT-PCR

Quantitative real time RT-PCR was performed on each cDNA sample. Table 2.5 shows the reagents required for the RT-PCR mastermix for each cDNA sample. All reagents for the mastermix were obtained from the qPCR Core kit for Sybr Green I, except for the UNG (Uracil DNA Glycosylase) and primers (all from Eurogentec).

<table>
<thead>
<tr>
<th>Reagent:</th>
<th>Volume required per sample:</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase / RNase – free distilled water</td>
<td>12.6µl</td>
</tr>
<tr>
<td>10X reaction buffer</td>
<td>2.5µl</td>
</tr>
<tr>
<td>50mM MgCl₂ (magnesium chloride)</td>
<td>1.75µl</td>
</tr>
<tr>
<td>5mM dNTPs</td>
<td>1µl</td>
</tr>
<tr>
<td>1:2000 dilution of Sybr Green</td>
<td>0.75µl</td>
</tr>
<tr>
<td>HotGoldStar Taq (5U/µl)</td>
<td>0.125µl</td>
</tr>
<tr>
<td>UNG (1U/µl)</td>
<td>0.25µl</td>
</tr>
<tr>
<td>Primer (1:20 dilution of 100µM stock)</td>
<td>1µl</td>
</tr>
</tbody>
</table>

Table 2.5 – The amount of reagents required per sample (RT-PCR mastermix).

Gene-specific primers were designed using the Applied Biosystems Primer Express program (version 2.0). The primers were complimentary to regions with the gene cDNA sequence, and were restricted by the following parameters:

- Have a Tm (annealing temperature) between 58-60ºC.
- Be approximately 20 nucleotides in length.
- Have a guanine / cytosine content of 30-80% (and not contain more guanine bases than cytosine bases).
The DNA sequences of the test and endogenous control primers used are shown in Table 2.6.

<table>
<thead>
<tr>
<th>Primer:</th>
<th>Sequence:</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>Forward: CCTCTGTGATGAGGCTTACCATC</td>
</tr>
<tr>
<td></td>
<td>Reverse: CATCTGTGCTTTTCATGTCATCTTCC</td>
</tr>
<tr>
<td>VEGF</td>
<td>Forward: ACGAGGCGGCTGGAGTGTGT</td>
</tr>
<tr>
<td></td>
<td>Reverse: TTTGTGGTGCTGTAGGAAGCTCAT</td>
</tr>
<tr>
<td>HPRT (endogenous control)</td>
<td>Forward: ATTATGCTGAGGATTTGGAAAGGG</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCCTCCCATCTCTTCATCAC</td>
</tr>
<tr>
<td>Gastrin</td>
<td>Forward: CCACACCTCGTGGCAGAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCCATCCATCCATAGGCTTC</td>
</tr>
<tr>
<td>HPRT (for use with gastrin primers)</td>
<td>Forward: GACCAGTCAACAGGGGACAT</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGACCTTGACCATCTTTGGA</td>
</tr>
<tr>
<td>ARNT (HIF-1β)</td>
<td>Forward: AACTTCGTGAGCAGCTTTCCA</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCTGACCTTCCTTTTCACTGTTC</td>
</tr>
</tbody>
</table>

Table 2.6 – Oligonucleotide primer pairs used during real-time RT-PCR.

20µl of the RT-PCR mastermix was added to each well of a 96-well optical plate (Applied Biosystems, Warrington, UK), before 5µl of the diluted cDNA samples were added per well. Each cDNA sample was run in triplicate. The plate was then sealed with an optical adhesive cover (Applied Biosystems) and run using the Applied Biosystems 7500 Real Time PCR System, with the following protocol; 50ºC for 2mins (annealing stage), 95ºC for 10mins (denaturing stage) and then 40 cycles of 95ºC for 15secs (denaturing stage), followed by 60ºC for 1min (annealing/extension stage). The program finished with a dissociation step, which lasted 45mins.
Alongside the test cDNA samples, the negative control samples generated from each stage of their synthesis were included in the RT-PCR (i.e. extraction control, cDNA negative samples, cDNA reagent control and also RT-PCR water control), to ensure that the signal generated were specific to the RNA / cDNA samples, and not from other genomic contamination, protocol reagents or primer dimers.

The level of gene expression was quantified by the level of Sybr Green present in the sample. The RT-PCR data was analysed by the relative quantification method using the \(2^{-\Delta C_t}\) equation (from Livak and Schmittgen, 2001). This method allows the relative change in gene expression to be compared between the test gene and the endogenous housekeeping gene, HPRT (hypoxanthine phosphoribosyl transferase). The \(C_t\) value of each gene (i.e. the number of cycles required to reach a Sybr Green fluorescence threshold, during the exponential phase of the RT-PCR), was determined for both the test and HPRT gene.

The \(2^{-\Delta C_t}\) for the samples could then be calculated using the following equation:

\[
2^{-\Delta C_t} = 2^{[C_t (test
gene) – C_t (HPRT)]}
\]

The \(\Delta C_t\) can be defined as the difference in the threshold cycles between the test gene and the endogenous control, from the same sample. This allowed for a direct comparison of gene expression between the test gene and its control. For each cycle, there is a doubling in the amount of template generated;
therefore, a single $C_t$ difference represents a two-fold difference in the gene expression. Converting the $\Delta C_t$ to $2^{-\Delta C_t}$, allowed for the differences seen in expression levels to be compared between different cell lines (Livak and Schmittgen, 2001).

2.8 siRNA transfection

Target-specific mRNA expression was down-regulated after the transfection of small-interfering RNA (siRNA). These are short (21-25 nucleotides in length), double-stranded RNA molecules with a two nucleotide overhang, which utilise a naturally-occurring mechanism to repress gene expression (reviewed in Gong et al., 2005; Sklan and Glenn, 2007). During transfection, siRNA molecules are taken up into cells and are incorporated into RISC (RNA-induced silencing complex), which contains an endonuclease. The ‘sense’ strand of the siRNA is degraded, leaving the ‘antisense’ strand associated with RISC, which is complementary to the target mRNA. The siRNA-RISC complex is then free to target and degrade specific mRNA sequences.

Carcinoma cell lines were plated into 24-well plates at a density of $5 \times 10^4$ cells/well, to achieve 30-80% confluency after 24hr incubation at 37ºC, in a humidified 5% CO$_2$ atmosphere. The following day, the growth medium was removed and replaced with 200µl fresh medium. For each well that was transfected, 4µl siPORT Amine transfection reagent (Ambion, Warrington, UK) was mixed with 45µl Opti-MEM (minimal essential media) 1 medium (Invitrogen), before being incubated for 20mins at room temperature. The siPORT Amine/ Opti-MEM 1 mixture was added to 40µM target-specific
siRNA (see Table 2.7 for details) and gently mixed via pipetting, before being incubated for a further 20mins at room temperature. 50µl of siRNA/siPORT Amine complex was added per well in a drop-wise fashion, with constant rocking. The final concentration of siRNA per well was 20nM. All transfections were completed in duplicate.

<table>
<thead>
<tr>
<th>siRNA target:</th>
<th>siRNA target sequence:</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARNT (HIF-1β)</td>
<td>GAGACUUGGCCAUAAGAUUU</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>AAUGUGAGUUCGCAUCUUGAU</td>
</tr>
<tr>
<td>Gastrin</td>
<td>AAGAAAGCCUAUGGAUGGA</td>
</tr>
<tr>
<td>Scrambled gastrin control</td>
<td>AAGCGAAGAAACGAGGUGUAU</td>
</tr>
</tbody>
</table>

Table 2.7 – Sequence-specific mRNA targets for siRNA repression.

To confirm that the down-regulation of the mRNA was specific to the target siRNA, the carcinoma cell lines were also transfected with one of three controls:

- Non-targeting siRNA control (Eurogentec, Southampton, UK), as a negative control (for both the HIF-1α and ARNT siRNA). Sequence unavailable.

- Mock control, which contained the siPORT Amine transfection reagent, but no siRNA.

- Untreated control, which only contained the Opti-MEM I, and no siRNA or transfection reagent.
For the gastrin siRNA, a target-specific scrambled control siRNA was available, which contained the same nucleotides, but in a randomised sequence. This was transfected in place of the non-targeting siRNA control.

The transfected cells were incubated for approximately 6hr at 37°C, in a humidified 5% CO₂ atmosphere, before a further 1ml growth medium was added per well. At this point, the transfected cells were either exposed to hypoxia, or retained under normoxia for approximately 16hr, before being harvested using 0.25% trypsin/EDTA and centrifuged to form a pellet. The cell pellets were stored at -80°C until required.

2.9 Construction of hypoxia-response element sequence-expressing reporter plasmid

2.9.1 Sequence analysis

The DNA sequence upstream of both the Homo sapiens gastrin and VEGF gene coding regions (-3500bp to + 77bp and -2000bp to + 50bp respectively) were obtained from the Ensembl genome browser (http://www.ensembl.org, exon information section, genes ENSG00000184502 and ENSG00000112715 respectively). The two sequences were compared for any consensus sequences using the ClustalW Multiple Alignment Program within the SDSC (San Diego Supercomputer Centre) Biology WorkBench software (http://workbench.sdsc.edu).
2.9.2 DNA extraction

A gastrin luciferase reporter plasmid was synthesised using a PGL4-basic luciferase reporter vector (Promega, Southampton, UK), which was a 4242bp plasmid that contained a synthetic firefly luciferase reporter construct, but lacked any promoter or enhancer elements (Figure 2.2). The DNA insert that included the putative gastrin HRE sequence was prepared from freshly extracted HCT116 carcinoma cell DNA.

The DNA was extracted from the HCT116 carcinoma cell line using the Wizard SV Genomic DNA purification system (Promega). Briefly, the HCT116 cells were plated into six-well plates at a density of $5 \times 10^6$ cells/well. After an overnight incubation at 37°C, in a humidified 5% CO$_2$ atmosphere, the growth medium was aspirated and the cells were rinsed in PBS (Oxoid). The cells were harvested using 150µl Wizard SV lysis buffer, before being applied to a Wizard SV Minicolumn assembly. The sample was centrifuged at 13,000g for 3mins, to collect the DNA sample into the column ‘binding matrix’. The DNA was then washed four times using 650µl Wizard SV wash solution (which contained additional ethanol (Sigma Aldrich)) per wash, followed by centrifugation at 13,000g for 1min. The binding matrix was dried, before the DNA was eluted into DNase (deoxyribonuclease) / RNase-free distilled water. The DNA concentration was determined using the Nanodrop ND-100 spectrophotometer according to the manufacturer’s instructions. The DNA was stored at -20°C until required.
2.9.3 Polymerase chain reaction (PCR)

Primers were designed using PRIMER3 software from the SDSC Biology WorkBench software. The forward primer was complimentary to a sequence upstream of both the putative gastrin HRE sequence and an endogenous XhoI restriction enzyme site. The reverse primer was complimentary to a sequence in the gastrin coding region and was designed to contain a BglIII restriction enzyme site. Both primers were restricted by the following parameters:

- Have a Tm between 57-63°C.
- Be approximately 20 nucleotides in length.
- Have a guanine / cytosine content of 20-80%.

The DNA sequences of the primers used to prepare the putative gastrin HRE sequence are as follows:

- Forward primer – ATCAGTTCTGGTACACGGC

Figure 2.2 – A plasmid map of the PGL4-basic luciferase reporter vector, detailing key constructs and restriction enzyme sites.
• Reverse primer –
  
  CCTCCTCCTTCTAGAGTTTTCTCACCTGCAGAGC

2.9.4 PCR amplification

Table 2.8 showed the reagents required for the PCR mastermix. All reagents for the mastermix were obtained from the HotStarTaq MasterMix Kit (Qiagen, Crawley, UK), except for the deoxynucleotides (Sigma Aldrich) and primers (Eurogentec).

17µl of the PCR mastermix was added to every 8µl of HCT116 DNA or water negative control. The PCR was run on the PTC-200 Peltier Thermal Cycler (MJ Research, GRI, Braintree, UK) using the following protocols:

• Original PCR protocol; 40 cycles of 95°C for 1min (denaturing stage), 60°C for 1min (annealing stage) and 72°C for 3mins (extension stage).

• Putative gastrin HRE long-product PCR protocol; 94°C for 15mins, then 35 cycles of 94°C for 30secs (denaturing stage), 55°C for 1min (annealing stage) and 68°C for 3.5mins (extension stage), for the first 10 cycles, plus an additional 10secs per cycle for the remaining 25 cycles.

The negative control sample ensured that the PCR product was generated from the specific DNA added to the sample and not from other genomic contamination.
Table 2.8 – The amount of reagents required per sample (PCR mastermix).

<table>
<thead>
<tr>
<th>Reagent:</th>
<th>Volume required per sample:</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase / RNase – free distilled water</td>
<td>6.75µl</td>
</tr>
<tr>
<td>10X reaction buffer</td>
<td>2.5µl</td>
</tr>
<tr>
<td>5X Q-Solution</td>
<td>5µl</td>
</tr>
<tr>
<td>10mM dNTPs</td>
<td>0.5µl</td>
</tr>
<tr>
<td>HotStarTaq (5U/µl)</td>
<td>0.25µl</td>
</tr>
<tr>
<td>Forward primer (5µM)</td>
<td>1µl</td>
</tr>
<tr>
<td>Reverse primer (5µM)</td>
<td>1µl</td>
</tr>
</tbody>
</table>

2.9.5 Agarose gel purification of PCR product

The resultant PCR product was purified using agarose gel electrophoresis. A 0.8% agarose gel was prepared by melting 0.8g agarose (Eurogentec) into 100ml 1X TBE (Tris Borate EDTA) buffer, which was diluted from a 10X stock (Sigma Aldrich). The buffer was heated in a microwave for approximately 2mins on medium, until the agarose was molten. Ethidium bromide was then added to the agarose solution, to give a final dilution of 0.5µg/ml, before the agarose gel was poured into the gel electrophoresis casting and left to set. Two 16-well gel-combs were inserted into the agarose gel at this point.

Once set, the agarose gel was placed in a gel tank, filled with 1X TBE buffer + 0.5µg/ml ethidium bromide. Approximately 10-20µl PCR product was loaded per well, after being diluted 5:1 with Blue Juice sample buffer (Invitrogen). The gel was also loaded with 5µl of a 1:20 diluted 2-log DNA ladder (New
England Biolabs, Hitchin, UK), which provided an accurate molecular weight scale, to aid with DNA size quantification. The gel was run at 120V for approximately 60mins. The DNA molecules ran towards the cathode, being separated according to their molecular weight. The mutagen ethidium bromide intercalated itself into the double-stranded DNA, allowing it to be visualised under ultra-violet (UV) light, either via the Syngene ChemiGenius BioImaging system, or a UV lamp.

The DNA was purified from the agarose gel using the QIAquick Gel Extraction kit (Qiagen). Briefly, the DNA fragments of interest were excised from the gel using a clean scalpel and weighed. The gel slices were dissolved in 3X volume of Buffer QG, with occasional agitation, before the DNA was precipitated using isopropanol. The DNA samples were applied to QIAquick columns and collected onto the filter by centrifugation at 13,000g for 1min. The DNA was then washed by adding Buffer PE and centrifuging the samples at 13,000g for 1min. The DNA was eluted into DNase / RNase free distilled water and its concentration was determined using the Nanodrop ND-100 spectrophotometer according to the manufacturer’s instructions. The DNA PCR product was stored at -20ºC until required.

2.9.6 Restriction digestion of DNA

Prior to the ligation and bacterial transformation, the vector and PCR product were digested using sequence-specific restriction enzymes, to produce compatible ends within the DNA. The restriction enzymes targeted specific
sequences in the DNA, which are listed in Table 2.9, alongside the concentration of enzyme and 10X buffer used.

<table>
<thead>
<tr>
<th>Restriction enzyme:</th>
<th>Target sequence in DNA:</th>
<th>Concentration of enzyme used:</th>
<th>10X Buffer required:</th>
<th>Supplier:</th>
</tr>
</thead>
<tbody>
<tr>
<td>XhoI</td>
<td>5’-C*TGGAG-3’  3’-GAGGT_C-5’</td>
<td>20units/µg DNA</td>
<td>Buffer H (with BglII) Buffer B (with HindIII)</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>BglII</td>
<td>5’-A*GATCT-3’  3’-TCTAG_A-5’</td>
<td>5units/µg DNA</td>
<td>Buffer M (alone) Buffer H (with XhoI)</td>
<td>Roche Applied Science</td>
</tr>
<tr>
<td>HindIII</td>
<td>5’-A*AGCTT-3’  3’-TTCGA_A-5’</td>
<td>4units/µg DNA</td>
<td>Buffer B (with XhoI)</td>
<td>Roche Applied Science</td>
</tr>
<tr>
<td>ScaI</td>
<td>5’-AGT*ACT-3’  3’-TCA_TGA-5’</td>
<td>4units/µg DNA</td>
<td>Buffer B (with HindIII and XhoI)</td>
<td>Roche Applied Science</td>
</tr>
</tbody>
</table>

Table 2.9 – Specification of the restriction endonucleases used to digest DNA (cleaved at the points * and _).

A 10µl reaction volume contained 1µl 10X buffer, 4-20units restriction enzyme and 1µg DNA (as determined using the Nanodrop concentrations), with the difference made up using DNase / RNase-free distilled water. The DNA was digested overnight, at 37ºC, with the resulting PCR products run out on a 0.8% agarose gel, as described above to confirm that the restriction digest had been successful.

2.9.7 TA cloning and preparation of DNA plasmids

To further amplify the stocks of putative gastrin HRE PCR product, the freshly-synthesised DNA was cloned into pCRII-TOPO vector (Invitrogen), using the TOPO TA cloning kit (Invitrogen). The linearised pCRII-TOPO
vector was supplied with a single 3’ thymidine residue overhang, which allowed for the direct insertion of the PCR product into the vector. The PCR product had to be amplified using a non-proofreading Taq polymerase, as the resultant DNA would then always end in a single 3’ adenosine residue overhang, due to the activity of an endogenous terminal transferase enzyme. This allowed for the PCR product to be bound into the pCRII-TOPO vector, via the activity of an endogenous topoisomerase enzyme.

2µl of the putative gastrin HRE PCR product was mixed with 1µl of the pCRII-TOPO vector and salt solution and incubated for 10 mins at room temperature. The TOPO vector (containing the PCR product) was then transformed into TOP10F’ competent cells (Invitrogen), using the ‘One shot’ chemical transformation protocol. Briefly, 1µl of the TOPO cloning reaction was gently added to a vial of ‘One shot’ chemically competent Escherichia coli (E. coli), which were then incubated on ice for 30 mins. The cells were heat-shocked for 30 secs at 42°C, before being transferred immediately to ice, which allowed the TOPO vector to be taken up into the bacteria. 125µl SOC (Super optimum broth, with catabolite repression) medium was added to the sample, which was then incubated at 37°C for 1 hour, with constant agitation.

Agar plates (containing ampicillin), were prepared using FastMedia LB Amp powder (Fermantas, York, UK). Briefly, the powdered agar was melted into 200ml distilled water by heating in a microwave for approximately 4 mins on medium (with occasional agitation), until the agar was molten. The agar was cooled slightly, before being divided between 10-12 sterile Petri dishes and left
to set. 50µl of the TOPO vector (plus putative gastrin HRE) transformation was spread over the agar, before being incubated overnight at 37°C.

To ensure that the colonies obtained were from a successful transformation, a negative control was also prepared, replacing the TOPO cloning reaction DNA with water. If colonies were obtained from this sample, then the colonies obtained on the TOPO cloning plate were less likely to contain the putative gastrin HRE PCR product.

Twelve bacterial colonies were selected from the agar plate and grown up overnight at 37°C in 5ml NZY medium (86mM sodium chloride, 17mM magnesium sulphate, 5g yeast extract, 10g casein hydrosylate) (all Sigma Aldrich, except for magnesium sulphate (Fisher Scientific)) containing 50µg/ml ampicillin (Sigma), with constant agitation.

DNA plasmids were prepared using Genelute plasmid miniprep kit (Sigma). Briefly, the *E. coli* bacteria were centrifuged at 13,000g for 2mins at room temperature to form a pellet, before being resuspended in the ‘resuspension’ buffer (which contained RNase A solution) and lysed using the ‘lysis’ buffer. The resulting lysate was ‘cleared’ using the alkali-based neutralisation buffer, before the cell waste was collected via centrifuging at 13,000g for 10mins at room temperature. The cleared lysate was then applied into a ‘mini-spin column’. The sample was centrifuged at 13,000g for 1min, to collect the DNA sample into the column filter. The DNA was then washed using the wash solution provided (which contained additional ethanol (Sigma Aldrich)),
followed by centrifugation at 13,000g for 1min. The column filter was dried, before the DNA was eluted into DNase / RNase-free distilled water. The DNA concentration was determined using the Nanodrop ND-100 spectrophotometer according to the manufacturer’s instructions. Test restriction digests were performed as described above to confirm that the TOPO vector contained the PCR product insert. The DNA was stored at -20ºC until required.

2.9.8 Ligation and transformation of the gastrin luciferase reporter plasmid

PGL4-basic luciferase plasmid and TOPO vector (+ putative gastrin HRE PCR product) were digested using XhoI and HindIII restriction enzymes, before being gel purified, as described above, to produce molecules with compatible ends for ligation. The use of XhoI and HindIII enzymes cleaved the amplified gastrin PCR product out of the TOPO vector and also ensured the correct orientation of the PCR product when ligated into the PGL4-basic luciferase vector.

For the ligation, the vector and PCR insert was added to the reaction in a 1:3 molar ratio. The 10µl reaction volume contained 1µl 10X buffer, ATP and T4 ligase enzyme (Stratagene, Amsterdam), 100ng PGL4-basic luciferase vector and approximately 200ng putative gastrin HRE PCR product insert, with the difference made up using DNase / RNase-free distilled water. The amount of insert required for the ligation was determined using the following equation (when using 100ng vector):

Insert (ng) = 3 x 100 x Y/X (where X is the size of the vector (in base pairs) and Y is the size of the insert (in base pairs))
A second ligation reaction was set up, which lacked the PCR product insert and therefore acted as a negative ligation control. The two ligations were incubated overnight at 4°C, to allow for the compatible ends of the vector and PCR product insert to combine.

The ligated gastrin luciferase reporter plasmid was then transformed into XL1-Blue Supercompetent (*E. coli*) cells (Stratagene). The supercompetent cells were thawed on ice, before 3.4µl β-mercaptoethanol (Stratagene) was gently added. The supercompetent cells were incubated on ice for 10mins, with occasional agitation, before being equally divided into five pre-chilled 14ml tubes. Between 0.1-50ng ligated plasmid was added to two samples of the *E. coli* cells, which were then incubated on ice for 30mins. Two different concentrations of ligation reaction were added to the supercompetent cells, in an attempt to obtain bacterial colonies. If there was too much DNA present in the sample, it actually inhibited the process of transformation. Therefore, the use of two different concentrations of the ligated DNA would increase the chance of obtaining colonies. To ensure that the colonies obtained actually contained the ligated plasmid; three control transformations were also set up:

- **Ligation negative control**, which lacked the PCR product insert. If colonies were obtained from this sample, they were probably due to vector self-ligation.

- **Transformation negative control**, which lacked any DNA. If colonies were obtained from this sample, they were probably due to genomic contamination.
Transformation positive control, which contained a ligated single-digested PGL4-basic plasmid. If the transformation experiment was successful, bacterial colonies would be present.

After the 30mins incubation, the *E. coli* cells were ‘heat-shocked’ at 42°C for 45secs, which allowed the ligated plasmid to be taken up into the bacteria. The duration of the heat shock was critical for maximum efficiency. The supercompetent cells were then incubated on ice for 2mins, before 450μl SOC medium was added to each sample. The tubes were incubated at 37°C for 1hour, with constant agitation.

Approximately 50μl of each transformation sample were spread over ampicillin-containing agar plates, which were then incubated overnight at 37°C. Twenty-four bacterial colonies were selected from the resultant positive gastrin luciferase reporter plasmid agar plate and were cultured overnight in 5ml NZY-ampicillin medium, at 37°C with constant agitation. The gastrin luciferase reporter plasmids were prepared using Genelute plasmid miniprep kit (Sigma), as described above.

### 2.9.9 Sequencing of plasmid

Selected clones of the gastrin luciferase reporter plasmid and HRE-multimer plasmid were sent to the Biopolymer Synthesis and Analysis Unit, in the Queen’s Medical Centre, Nottingham for sequencing. The sequencing reaction used the primer ‘RVPrimer3’ (CTAGCAAAATAGGCTGTCCC), which was complementary to 4191-4210bp of the PGL4-basic luciferase plasmid. The
sequencing reaction consisted of 2µl Big Dye reaction mix, 2µl 5X ABI sequencing buffer, 1µl plasmid clone, 50ng RVPrimer3 and 4µl distilled water, which was run using the following GC-rich PCR program; 25 cycles of 98°C for 30secs (denaturing stage), 50°C for 15secs (annealing stage) and 56.5°C for 4mins (extension stage). The resultant samples were then analysed using the ABI 310 Genetic Analyser (Applied Biosystems, Warrington, UK).

2.9.10 HRE multimer oligonucleotide annealing and plasmid preparation

Gastrin HRE multimer luciferase reporter plasmids were synthesised using a PGL4-basic luciferase reporter vector, which also contained a SV40 (Simian virus 40) minimal promoter element. The DNA sequence upstream of gastrin gene transcript was further screened for the presence of the HRE consensus sequence (A/G)CGTG as previously described using the SDSC Biology WorkBench software. The putative HRE sequences (plus surrounding nucleotides) were used to construct oligonucleotide sequences, which are listed in Table 2.10. The oligonucleotides were synthesised by Sigma-Genosys, and were resuspended in RNase-free distilled water to a final concentration of 50µM. 20µl of each oligonucleotide pair (i.e. both forward and reverse constructs) were annealed together using the following programme; 2mins at 100°C, 10mins at 65°C, followed by 60mins at 55°C. The annealed oligonucleotides were then allowed to cool naturally within the heating block to room temperature (took approximately 3-4hr), before being ligated into BglIII-digested PGL4-basic luciferase reporter vector as previously described.
<table>
<thead>
<tr>
<th>Oligonucleotide construct name:</th>
<th>Repeated sequence (containing putative gastrin HREs)</th>
<th>Full oligonucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligonucleotide 1</td>
<td>Forward: AGTGTATAAAGCG TGTCGACACAGAC &lt;br&gt; Reverse: TCACATATTTTCGCA C ACGTGTCTG</td>
<td>Forward: 5’ GATCTAGTGTATAAAGCGTGTGACACAGAC CACAGACAGAAGCGTGTCTGTCGACACAGACCA 3’ &lt;br&gt; Reverse: 5’ GATCTGTCGACACAGAC CTTTATACAGTCTGTGACACAGAC GCTTTATACAGTCTGTGACACAGACCTTTTATACACTAGACAA 3’</td>
</tr>
<tr>
<td>Oligonucleotide 2</td>
<td>Forward: TGGCTCACGTCTG &lt;br&gt; Reverse: ACCGAGTGCAGAC</td>
<td>Forward: 5’ GATCTTGGCTCACGTCTG TGGCTCACGTCTGTTGGCTCACGTCTG &lt;br&gt; Reverse: 5’ GATCTGAGCAGACGTGAGCCA CAGACGTGAGCCA 3’</td>
</tr>
<tr>
<td>Oligonucleotide 3</td>
<td>Forward: TTACAGACGTGAG &lt;br&gt; Reverse: AATGTCTGCACTC</td>
<td>Forward: 5’ GATCTTTACAGACGTGAGTTACAGACGTGAG &lt;br&gt; Reverse: 5’ GATCTTTACAGACGTGAGTAACACGTGAGGCA 3’</td>
</tr>
<tr>
<td>Oligonucleotide 4</td>
<td>Forward: GCGCACACGTGGC &lt;br&gt; Reverse: CGCGTGTGCACCG</td>
<td>Forward: 5’ GATCTGCGCACACGTGGC GCGCACACGTGGCGCGCACACGTGGC &lt;br&gt; Reverse: 5’ GATCTTTACGTCCTGGGCCTCACGTCCTGGG &lt;br&gt; Reverse: 5’ GATCTTTACGTCCTGGGCCTCACGTCCTGGG</td>
</tr>
<tr>
<td>Oligonucleotide 5</td>
<td>Forward: CCCAGGACGTGAG &lt;br&gt; Reverse: GGGTCTCTGCACTC</td>
<td>Forward: 5’ GATCTCCCAGGACGTGAG &lt;br&gt; Reverse: 5’ GATCTCCCAGGACGTGAGGGTCTCTGCACTC</td>
</tr>
</tbody>
</table>

Table 2.10 – HRE multimer sequences derived from the DNA upstream of the gastrin gene transcript, plus the oligonucleotides used to synthesize the plasmids.

### 2.10 Transfections

Carcinoma cell lines were plated into 24-well plates at a density of 2 x 10⁵ cells/well (in 500µl of cell growth medium), to achieve an 80-90% confluency after 24hr incubation at 37°C, in a humidified 5% CO₂ atmosphere. The following day, the cells were transfected with 250-1000ng DNA luciferase reporter plasmids, which are detailed in Table 2.11.
Table 2.11 – Firefly luciferase reporter plasmids used for transient transfections.

<table>
<thead>
<tr>
<th>Plasmid:</th>
<th>Function</th>
<th>Plasmid information:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrin luciferase reporter plasmids (plus gastrin HRE multimer luciferase reporter plasmids)</td>
<td>Main test plasmids</td>
<td>pTA GasProm 1-4 contained full gastrin-HRE PCR product. PGL4-(gastrin)-HRE 1-2 contained 3-4x gastrin-HRE sequence (AGTGTATAAAGCGTGTGCACAGAC) PGL4-(gastrin)-HRE 3-8 contained 3-4x gastrin-HRE sequence with an SV40 minimal promoter element</td>
</tr>
<tr>
<td>PGL4-(LDH)-HRE reporter plasmid</td>
<td>Positive control plasmid</td>
<td>PGL4-basic luciferase reporter plasmid, with an SV40 minimal promoter element and 3x LDH-HRE sequences (GCGGACGTGCGGGAACCCAC)</td>
</tr>
<tr>
<td>VEGF-HRE luciferase reporter plasmid</td>
<td>Positive control plasmid</td>
<td>Luciferase reporter plasmid with VEGF promoter insert (-2018bp to +50bp)</td>
</tr>
<tr>
<td>PGL4-basic control plasmid</td>
<td>Negative control plasmid</td>
<td>Basic luciferase reporter plasmid, with no promoter or enhancer elements</td>
</tr>
<tr>
<td>SV40 promoter control plasmid</td>
<td>Negative control plasmid</td>
<td>PGL4-basic luciferase reporter plasmid, with an SV40 minimal promoter element</td>
</tr>
</tbody>
</table>

For each well that was transfected, 2µl Lipofectamine 2000 transfection reagent (Invitrogen) was mixed with 50µl Opti-MEM I medium (Invitrogen), before being incubated for 5mins at room temperature. 250-1000ng test or control plasmid DNA, plus 100ng β-Galactosidase control reporter plasmid were also diluted to 50µl in volume, using Opti-MEM I medium. The lipofectamine 2000 reaction mix was added to the DNA and incubated for 20mins at room temperature. 100µl of plasmid DNA/Lipofectamine 2000 complex was added per well, in a drop-wise fashion, with constant rocking. Each condition was transfected in duplicate, and the data was pooled together once the samples were analysed.
The transfected cells were incubated for 24hr at 37°C, in a humidified 5% CO₂ atmosphere. After approximately 6hr incubation, a further 1ml growth media was added per well, before the cells were returned to the incubator. After the full 24hr incubation, the DNA reporter plasmid-transfected carcinoma cells were exposed to hypoxia, or serum-starved, ready to be treated with exogenous amidated gastrin or EGF, as described above.

2.10.1 Plasmids

The following plasmids and cell line were used, alongside the gastrin-luciferase reporter plasmids:

1) A lactate dehydrogenase (LDH) expressing PGL3-luciferase reporter plasmid, which was kindly donated by Dr Kaye Williams (University of Manchester) (Figure 2.3).

2) A VEGF-HRE luciferase reporter plasmid was kindly donated by Prof Alan Knox (University of Nottingham) (Figure 2.4).

3) A HCT116 dual-luciferase cell line was obtained from Prof Ian Stratford (University of Manchester). It contained a stably-transfected HRE-linked firefly luciferase gene and an endogenous control renilla luciferase gene.
**PGL3 LDH-HRE luciferase reporter vector**

Figure 2.3 – A plasmid map of the PGL3-LDH HRE luciferase reporter vector, detailing key constructs.

**VEGF promoter luciferase reporter vector**

Figure 2.4 – A plasmid map of the VEGF promoter luciferase reporter vector, detailing key constructs.
2.10.2 Dual luciferase reporter plasmid and siRNA transfection

For the dual transfection, the amount of siRNA and DNA reporter plasmid transfected were kept at the normal amounts, whereas the volumes of transfection reagent and Opti-MEM medium were reduced by half.

GI carcinoma cell lines were plated as before at a density of $8 \times 10^4$ cells/well, and incubated for 24hr at 37°C, in a humidified 5% CO$_2$ atmosphere. The following day, the growth medium was removed and replaced with 200μl of fresh growth medium. For each well that was transfected, 2μl siPORT Amine transfection reagent was mixed with 22μl Opti-MEM 1 medium and incubated for 20mins as before. Meanwhile, 1μl Lipofectamine 2000 transfection reagent was mixed with 25μl Opti-MEM I medium. The siPORT Amine / Opti-MEM 1 mixture was added to 1μl HIF-1α siRNA, whilst the Opti-MEM-diluted 500ng PGL4-(LDH)-HRE plasmid and 100ng β-Galactosidase control were combined with the Lipofectamine 2000 reagent, before both were incubated for a further 20mins at room temperature.

25μl of the siRNA/siPORT Amine complex was added per well in a drop-wise fashion with constant rocking, alongside 50μl of the plasmid DNA/Lipofectamine 2000 complex. Each condition was transfected in duplicate, and the data was pooled together once the samples were analysed.

The transfected cells were incubated for 24hr at 37°C, in a humidified 5% CO$_2$ atmosphere. After approximately 6hr incubation, a further 1ml growth medium was added per well, before the cells were returned to the incubator. After the
full 24hr incubation, the dual siRNA/DNA plasmid-transfected carcinoma cells were then exposed to hypoxia, as described above.

To confirm that the down-regulation of the luciferase-reporter activity was down to the target-specific siRNA transfected, the carcinoma cell lines were also transfected with:

- Non-targeting siRNA control (Eurogentec), plus the luciferase reporter plasmid.

- Mock control, which contained both transfection reagents, but only the luciferase reporter plasmid, and no siRNA.

- Untreated control, which only had been transfected with the luciferase reporter plasmid.

2.11 Firefly luciferase reporter assay

Cells transfected with DNA luciferase reporter plasmids were harvested using Promega’s passive lysis buffer. Briefly, the cell growth medium was aspirated and the cells washed once in PBS (Oxoid). The cells were then lysed in 100µl 1X passive lysis buffer (diluted from a 5X stock), before being incubated at room temperature for 20mins with constant agitation. The resultant lysates could be analysed immediately or stored at -20°C until required.

Single Firefly luciferase activity was determined using the Luciferase Reporter Assay System (Promega). 5µl of each cell lysate was mixed with 25µl
luciferase assay reagent in a black 96-well plate, before the luminescence was analysed for 1sec/well, using a BMG labtech FLUOstar Optima luminometer. Each sample was analysed in triplicate. The luminescence from the luciferase assay reagent alone was also determined for each run, to ensure that the luminescence produced from the cell lysates was due to specific firefly luciferase expression.

For each sample, the firefly luciferase activity was expressed relative to the equivalent β-Galactosidase control, to ensure the data obtained was a true representation of the results, rather than just being due to differences in cell densities.

2.11.1 β-Galactosidase activity assay
The control β-Galactosidase activity from the same samples was measured using the Galacto-Light Plus system (Applied Biosystems). Briefly, enough Galacton-plus Substrate for the immediate assay was diluted with the Reaction Buffer Diluent, to form the Reaction Buffer. 5μl of each cell lysate was mixed with 17.5μl reaction buffer, before being incubated for 1hr at room temperature. Each sample was analysed in triplicate. Immediately prior to reading the luminescence, 25μl Accelerator II buffer was added per well, which were then read for 1sec/well.

2.12 Dual luciferase reporter assay
The HCT116 dual-luciferase cells were plated at a density of 2 x 10^5 cells/well and were incubated at 37°C, in a humidified 5% CO₂ atmosphere for 24hr.
They were then transfected with gastrin siRNA or equivalent scrambled control, before being harvested using the passive lysis buffer, as previously described.

The luciferase activity was determined using the Stop and Glo Luciferase assay System (Promega). To determine the firefly luciferase activity, 5µl of each cell lysate was mixed with 25µl luciferase assay reagent (II) in a black 96-well plate, before the luminescence was analysed for 1sec/well, using a BMG labtech FLUOstar Optima luminometer.

To determine the corresponding endogenous renilla luciferase activity, a 1X solution of the ‘Stop and Glo’ reagent was prepared by diluting the 50X Stop and Glo substrate with the Stop and Glo buffer. 25µl of the Stop and Glo reagent was added to each well of the plate, immediately after the firefly luciferase was read, before the renilla luminescence was analysed for 1sec/well.

Each sample was analysed in triplicate, with the firefly luciferase activity being expressed relative to the equivalent renilla luciferase control.

### 2.13 Statistical analysis

The significance of differences in mRNA expression, when investigated as a time course was calculated using a one-way ANOVA (Analysis of variance), with Bonferroni multiple comparison tests. The significance of differences in mRNA expression, when analysing individual data samples was measured...
using a student’s t-test. Data correlation was analysed using a linear regression test. The differences were considered significant if $p<0.05$. 
Chapter Three
VALIDATION OF TARGET
3.1 Background

Under hypoxic conditions, the enzymes responsible for the hydroxylation of HIF-1α are inhibited and therefore the degradation of HIF-1α is prevented. The non-hydroxylated HIF-1α is free to translocate to the nucleus and dimerise with the constitutively expressed HIF-1β subunit, forming the complete transcription factor, HIF-1. This then binds to conserved HREs within promoter regions of target genes, resulting in the transcription of genes that promote cell growth, angiogenesis and metabolism, including erythropoietin, VEGF and GLUT1. The expression of HIF-1-induced target genes enables the cell to adapt to and endure the stress exerted on it by hypoxia.

The aim of this chapter was to confirm that the HIF-1 transcription factor was up-regulated under hypoxic conditions in GI carcinoma cell lines, both in expression and function. Firstly, the expression of the HIF-1α protein was investigated, using both Western blotting and immunofluorescent techniques to confirm that it was translocated to the nucleus under hypoxic conditions. Then the expression of the key HIF-1 target gene, VEGF was further investigated, to determine whether this HIF-1 transcription factor was functionally active.

3.2 Nuclear expression of HIF-1α protein after chemical induction – The accumulation of protein under hypoxic conditions

To investigate the response of GI carcinoma cell lines after treatment with chemical agents that mimic hypoxia, and to optimise both the induction time and antibody concentration required, HIF-1α protein was induced in cell lines using either 150µM CoCl2 (an iron antagonist) or 200µM DFO (an iron
chelator). Both compounds inhibit the activity of the prolyl hydroxylase enzymes, therefore preventing the degradation of the HIF-1α protein under normal physiological oxygen tension (See Materials and Methods section 2.2).

The nuclear expression of HIF-1α protein in GI carcinoma cell-lines, under both ‘normal’ physiological oxygen concentrations of 20.9% (normoxia) and chemically-induced hypoxic conditions was investigated using Western blotting (See Materials and Methods section 2.5).

Expression of HIF-1α had been previously investigated in the HCT116 carcinoma cells by other groups, who found that it was highly inducible under hypoxic conditions, but not under normoxia (Krishnamachary et al, 2003). The expression of HIF-1α has not been previously investigated in either the MGLVA1 or PAN1 carcinoma cell lines.

The nuclear expression of HIF-1α protein under hypoxic conditions was found to be increased in the HCT116, MGLVA1 and PAN1 carcinoma cell lines, after treatment with either DFO or CoCl₂ at all time points investigated. The results for DFO treatment are shown in Figures 3.1, 3.2 and 3.3 (for HCT116, MGLVA1 and PAN1 cell lines respectively). The results for CoCl₂ treatment are shown in Figures 3.4, 3.5 and 3.6 (for HCT116, MGLVA1 and PAN1 cell lines respectively). DFO treatment induced maximal nuclear accumulation of HIF-1α protein after 16hr incubation in all three cell lines tested. It induced a 12-fold increase of nuclear expression of HIF-1α protein in the HCT116 cells at this time point, and a 50-fold increase in both the MGLVA1 and PAN1
cells, with the protein expression reducing after this time point. CoCl$_2$ treatment induced a rapid increase in nuclear expression of HIF-1$\alpha$ protein in both the HCT116 and MGLVA1 cell lines, with an approximate 18-fold and 2-fold increase seen in the nuclear expression of HIF-1$\alpha$ protein after treatment with the hypoxic mimetic respectively. The PAN1 cells followed a similar pattern to that seen after DFO treatment, with nuclear expression of HIF-1$\alpha$ protein under hypoxic conditions peaking after 6-16hr treatment, producing a 19-fold increase ($p=0.0294$) in the nuclear expression of HIF-1$\alpha$ protein after CoCl$_2$ treatment.

### HIF-1$\alpha$ nuclear protein expression in HCT116 carcinoma cell lines.

<table>
<thead>
<tr>
<th>Time point:</th>
<th>2hr</th>
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<td>DFO</td>
<td>Norm</td>
<td>DFO</td>
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<td>HIF-1$\alpha$</td>
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<tr>
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</tbody>
</table>

Figure 3.1 – HIF-1$\alpha$ nuclear protein expression in HCT116 carcinoma cells, after treatment with DFO, relative to $\beta$-actin control (Norm = Untreated). n=2 (data representative of replicate experiments).

### HIF-1$\alpha$ nuclear protein expression in MGLVA1 carcinoma cell lines.

<table>
<thead>
<tr>
<th>Time point:</th>
<th>2hr</th>
<th>6hr</th>
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<tbody>
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<td>DFO</td>
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<td>$\beta$-actin</td>
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<td><img src="image19.png" alt="Image" /></td>
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</tbody>
</table>

Figure 3.2 – HIF-1$\alpha$ nuclear protein expression in MGLVA1 carcinoma cells, after treatment with DFO, relative to $\beta$-actin control (Norm = Untreated). n=2 (data representative of replicate experiments).
**HIF-1α nuclear protein expression in PAN1 carcinoma cell lines.**

<table>
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<tr>
<th>Time point:</th>
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<tr>
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<td>DFO</td>
<td>Norm</td>
<td>DFO</td>
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<tr>
<td>HIF-1α</td>
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<td>β-actin</td>
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<tr>
<td>Bromo-deoxyuridine</td>
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</tbody>
</table>

Figure 3.3 – HIF-1α nuclear protein expression in PAN1 carcinoma cells, after treatment with DFO, relative to β-actin control (Norm = Untreated). n=2 (data representative of replicate experiments).

**HIF-1α nuclear protein expression in HCT116 carcinoma cell lines.**

<table>
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<tr>
<th>Time point:</th>
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<tr>
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<td>CoCl₂</td>
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<td>Bromo-deoxyuridine</td>
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</table>

Figure 3.4 – HIF-1α nuclear protein expression in HCT116 carcinoma cells, after treatment with CoCl₂, relative to β-actin control (Norm = Untreated). n=2 (data representative of replicate experiments).

**HIF-1α nuclear protein expression in MGLVA1 carcinoma cell lines.**

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<thead>
<tr>
<th>Time point:</th>
<th>2hr</th>
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<tr>
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<td>CoCl₂</td>
<td>Norm</td>
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<td>Bromo-deoxyuridine</td>
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</table>

Figure 3.5 – HIF-1α nuclear protein expression in MGLVA1 carcinoma cells, after treatment with CoCl₂, relative to β-actin control (Norm = Untreated). n=2 (data representative of replicate experiments).
Within both HCT116 and PAN1 cells after treatment with either DFO or CoCl₂, nuclear expression of HIF-1α protein under normoxic conditions was negligible at all time points investigated (Figures 3.1, 3.3, 3.4 and 3.6). In MGLVA1 cells, nuclear expression of HIF-1α protein under normoxic conditions was consistently detected at low levels throughout the experiment (Figures 3.2 and 3.5), which differed from the other cell lines tested. This could be due to MGLVA1 expressing an oncogenic mutation, such as a constitutively active Akt protein, or an inactive pVHL, which would allow for the endogenous expression of the HIF-1α protein, even under normoxic conditions.

The level of protein expression (both HIF-1α and Actin) varied depending on the GI carcinoma cell line investigated. For example, the MGLVA1 cells produced very intense protein bands (Figures 3.2 and 3.5), whereas the HCT116 cells produced lower levels of protein expression (Figures 3.1 and 3.4). The differences were endogenous to the cell lines, as the cell lysates were normalised to the same protein concentration prior to loading into the wells.
This suggested that the MGLVA1 cells expressed higher levels of HIF-1α and β-actin protein, compared to the HCT116 and PAN1 cell lines.

The nuclear expression of HIF-1α protein under hypoxic conditions was shown to be specific, as no bands were produced at the equivalent weight of HIF-1α protein (120kb) when the samples were probed with a non-specific mouse bromodeoxyuridine antibody (Figures 3.1, 3.3, 3.4, 3.5 and 3.6).

The expression of HIF-1α protein was also investigated within the corresponding cytoplasmic lysates of the carcinoma cell lines. As the expression was shown to be negligible under both normoxic and hypoxic conditions in each cell line tested (data not shown); no further experiments were carried out using the cytoplasmic lysates.

The expression of the HIF-2α protein was also investigated under both normoxic and hypoxic conditions, after treatment of the GI carcinoma cell lines with either CoCl₂ or DFO. No HIF-2α protein was detected at any time point tested in any cell line (data not shown). This was to be expected, as the HIF-2 transcription factor is normally only expressed in specific tissues, such as endothelial cells, the kidneys and the lungs, whereas the expression of HIF-1 is ubiquitous. Therefore, no further work investigating the expression of HIF-2α was undertaken in this project.
3.3 Nuclear expression of HIF-1β protein after chemical induction – The accumulation of protein under hypoxic conditions

The corresponding nuclear protein expression of the constitutively expressed HIF-1β subunit was also investigated using Western blotting. In all cell lines tested, the nuclear expression of the HIF-1β protein under normoxic conditions was relatively low, but constitutively expressed (Figures 3.7 and 3.8).

![HIF-1β nuclear protein expression in HCT116 carcinoma cell lines.](image1)

Figure 3.7 – HIF-1β nuclear protein expression in HCT116 carcinoma cells, after treatment with CoCl₂, relative to β-actin control (Norm = Untreated). n=1

![HIF-1β nuclear protein expression in MGLVA1 carcinoma cell lines.](image2)

Figure 3.8 – HIF-1β nuclear protein expression in MGLVA1 carcinoma cells, after treatment with CoCl₂, relative to β-actin control (Norm = Untreated). n=1

On induction of hypoxic conditions by CoCl₂, there was a consistent increase in the nuclear expression of HIF-1β protein throughout the time course in all cell lines tested. In the HCT116 and MGLVA1 cell lines, the expression of nuclear HIF-1β protein increased by a consistent 2-4 fold and 2-5 fold respectively under hypoxic conditions (Figures 3.7 and 3.8). Induction of
hypoxic conditions by DFO produced a 1-4 fold increase in the expression of nuclear HIF-1β protein at the later time points only (data not shown), which may suggest that CoCl₂ is more efficient at inducing hypoxia (via chemical induction). In these initial experiments, HIF-1β expression was undetectable in PAN1 cells.

These results suggest that the HIF-1β protein was stabilised in the nucleus once hypoxia had been established, ready to initiate target gene transcription. As the HIF-1β protein is constitutively expressed within the majority of carcinoma cell lines, the increased hypoxic expression of HIF-1β may suggest that HIF-1α and HIF-1β proteins dimerise within the cytoplasm, translocating to the nucleus as the fully formed transcription factor, rather than dimerising within the nucleus itself.

3.4 Nuclear expression of HIF-1α protein under hypoxic conditions – The accumulation of protein under hypoxic conditions

After the initial studies were performed using chemical treatments to induce hypoxia, the Invivo² 400 hypoxic workstation became available for use. This maintains atmospheric hypoxia, as the cells are incubated in 1% oxygen environment. This generates ‘true’ hypoxia within cells, instead of inhibiting the enzymes responsible for the degradation of the HIF-1α protein. Slight modifications to the Western lysate preparation assay were required to ensure that the HIF-1α protein was not broken down during its brief, but inevitable exposure to normoxia (See Materials and Methods section 2.2).
The nuclear expression of HIF-1α protein in GI carcinoma cell-lines, under both ‘normal’ physiological oxygen concentrations of 20.9% (normoxia) and atmospheric hypoxic conditions was investigated using Western blotting. These experiments continued to use a similar time-frame of hypoxic induction and antibody concentration (1µg/ml) as performed in the chemical induction experiments.

Within the HCT116, MGLVA1 and PAN1 carcinoma cell lines, the nuclear expression of HIF-1α protein was highly inducible under hypoxic conditions (Figures 3.9, 3.10 and 3.11 respectively). Maximal nuclear accumulation of HIF-1α protein was reached after 8-12hr hypoxic incubation, in the three cell lines tested, with up to a 15-fold increase in nuclear protein expression seen in the HCT116 and PAN1 cell lines at this time point, and a 10-fold increase in the MGLVA1 cell line. The maximal nuclear expression of the HIF-1α protein induced at 1% oxygen concentration occurred at an earlier time point than when induced via chemical treatment. Under normoxic conditions, nuclear expression of HIF-1α protein was negligible at all time points investigated in the HCT116 and PAN1 cell lines, but consistently expressed at low levels in the MGLVA1 cell line (Figure 3.10). This could be due to the MGLVA1 carcinoma cells expressing a carcinogenic mutation, such as a constitutively active Akt or an inactive pVHL, which could induce the expression of HIF-1α even under normoxic conditions.
The expression of HIF-1α protein was also investigated within the corresponding cytoplasmic lysates. As seen in chemically induced hypoxia, its
expression was shown to be negligible under both normoxic and hypoxic conditions in each cell line tested (data not shown).

### 3.5 Nuclear expression of HIF-1α protein under hypoxic conditions – The accumulation of protein under hypoxic conditions using cell immunofluorescence

To verify the cellular location of the HIF-1α protein under hypoxic conditions, HCT116, MGLVA1 and PAN1 cells were incubated at 1% oxygen concentration, before the expression of HIF-1α was determined via immunofluorescence staining (See Materials and Methods section 2.6). Under hypoxic conditions, the HIF-1α protein was highly expressed within the cell nucleus, but not within the nucleoli in each cell line tested (Figures 3.12, 3.13 and 3.14 for HCT116, MGLVA1 and PAN1 cell lines respectively). No distinguishable HIF-1α protein was identified within the nucleus under normoxic conditions within the HCT116 and PAN1 carcinoma cell lines (data not shown). This therefore corroborates the data seen within the Western blots, confirming that incubation under hypoxic conditions induces the expression and translocation of the HIF-1α protein to the nucleus. The MGLVA1 cells however did express the HIF-1α protein under normoxic conditions and the protein appeared to surround the nucleus, without actually entering it (Figure 3.15). The negative control image however also showed a level of non-specific staining, of a similar pattern, but at a lower intensity. This data corroborates that seen within the MGLVA1 Western blot, where HIF-1α protein was expressed under normoxic conditions, at low concentrations.
**HIF-1α nuclear protein localisation in HCT116 carcinoma cell line.**

![Image of HIF-1α protein expression](image1)

**Hoechst nuclear counterstain**

**Hoechst nuclear counterstain**

Figure 3.12 – HIF-1α protein expression in HCT116 carcinoma cells, after incubation under hypoxic conditions, compared to non-specific mouse bromodeoxyuridine control. Nuclear HIF-1α expression was confirmed by counterstaining cells with Hoechst nuclear stain. n=3 (data representative of replicate experiments).

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**HIF-1α nuclear protein localisation in MGLVA1 carcinoma cell line.**

![Image of HIF-1α protein expression](image2)

**Hoechst nuclear counterstain**

**Hoechst nuclear counterstain**

Figure 3.13 – HIF-1α protein expression in MGLVA1 carcinoma cells, after incubation under hypoxic conditions, compared to non-specific mouse bromodeoxyuridine control. Nuclear HIF-1α expression was confirmed by counterstaining cells with Hoechst nuclear stain. n=2 (data representative of replicate experiments).
HIF-1α nuclear protein localisation in PAN1 carcinoma cell line.

Figure 3.14 – HIF-1α protein expression in PAN1 carcinoma cells, after incubation under hypoxic conditions, compared to non-specific mouse bromodeoxyuridine control. Nuclear HIF-1α expression was confirmed by counterstaining cells with Hoescht nuclear stain. n=2 (data representative of replicate experiments).

HIF-1α nuclear protein localisation in MGLVA1 carcinoma cell line.

Figure 3.15 – HIF-1α protein expression in MGLVA1 carcinoma cells, after incubation under normoxic conditions, compared to non-specific mouse bromodeoxyuridine control. Nuclear HIF-1α expression was confirmed by counterstaining cells with Hoescht nuclear stain. n=1
3.6 HIF-1α gene expression after chemical induction – down-regulation of the HIF-1α gene expression under hypoxic conditions

The induction of hypoxia, either via incubation in 1% oxygen or after chemical treatment, induced nuclear expression of HIF-1α protein, at all time points tested, reaching maximal expression between 8-16hr, depending on the cell line and the hypoxic induction method used.

The corresponding gene expression of HIF-1α under both normoxic and hypoxic conditions was also investigated, using quantitative real-time RT-PCR (See Materials and Methods section 2.7). Initially, the gene expression of HIF-1α was determined after chemically-inducing hypoxia using 150μM CoCl₂ or 200μM DFO in MGLVA1 and PAN1 carcinoma cell lines (Figures 3.16).

Chemical induction of hypoxia by DFO caused a significant decrease in the gene expression of HIF-1α in both carcinoma cell lines investigated (Figure 3.16). After 6hr hypoxic induction, treatment with DFO decreased the gene expression of HIF-1α by 85% and 40% for MGLVA1 and PAN1 cells respectively (p<0.0001 for both cell lines). After 16hr chemical treatment, DFO again induced a significant decrease in gene expression of HIF-1α by 70% and 50% in MGLVA1 and PAN1 cells respectively (Figure 3.16, p<0.0001 for both cell lines).
HIF-1α gene expression after chemical-induction of hypoxia

Figure 3.16 – HIF-1α gene expression in MGLVA1 carcinoma cells (top graph) and PAN1 carcinoma cells (middle graph), after treatment with either CoCl₂ or DFO, relative to the HPRT control (*p=0.0281 / ***p<0.0001 (analysed using a student’s t-test), + 95% confidence interval). HPRT expression in PAN1 and MGLVA1 cells (bottom graphs), after treatment with either CoCl₂ or DFO. (PAN1 and MGLVA1 cells, n=2 (data representative of replicate experiments)).
Mimetic induction of hypoxia by CoCl$_2$ had no obvious effect on the gene expression of HIF-1$\alpha$ in PAN1 cells, but significantly decreased gene expression in MGLVA1 cells by 25% after 6hr incubation (p<0.0001). After 16hr hypoxic induction, CoCl$_2$ had no effect on the gene expression of HIF-1$\alpha$ in the MGLVA1 cells, and induced an increase in the gene expression of HIF-1$\alpha$ in the PAN1 cells (p=0.0281) (Figure 3.16).

The differences seen in HIF-1$\alpha$ gene expression in the MGLVA1 and PAN1 carcinoma cell lines, after treatment with either CoCl$_2$ or DFO were not due to changes in the HPRT control gene expression. Figure 3.16 shows the mean HPRT expression under each condition, at both the 6hr and 16hr time points. There was no significant difference in HPRT expression after treatment with the hypoxia mimetics, when compared to the normoxic control, suggesting that the decrease in HIF-1$\alpha$ gene expression is induced by DFO treatment.

**3.7 HIF-1$\alpha$ gene expression after hypoxic induction – down-regulation of the HIF-1$\alpha$ expression under hypoxic conditions**

After the initial studies were performed using chemical treatments to induce hypoxia, the assay was performed in 1% oxygen and the expression of the HIF-1$\alpha$ gene was investigated in HCT116, MGLVA1 and PAN1 carcinoma cell lines, at a number of different time points, under both normoxic and hypoxic conditions, using quantitative real-time RT-PCR.

In HCT116, MGLVA1 and PAN1 cell lines, incubation under hypoxic conditions induced a significant decrease in the HIF-1$\alpha$ gene expression over
the first 2-8hr of the experiment (Figures 3.17, p<0.0001). The expression of the HIF-1α gene then levelled off over the remaining part of the time course, resulting in it being between 50-80% (cell line-dependent) lower than the equivalent normoxic control expression. Under normoxic conditions, there was a slight increase in the expression of the HIF-1α gene (5-40%, cell line-dependent) over the time points investigated (Figures 3.17).

The expression of the HIF-1α gene under hypoxic conditions displayed the opposite behaviour to the equivalent HIF-1α protein expression, when the RT-PCR data was compared with its corresponding Western blotting data. As the HIF-1α protein levels increased over the first 1-8hr, the equivalent HIF-1α gene expression decreased, before levelling off over the rest of the experiment. The expression of the HIF-1α protein however, started to decrease once it had reached its maximal expression after 8-12hr hypoxic incubation. Despite the noticeable differences between the HIF-1α nuclear protein and gene expression under hypoxic conditions, statistical correlation analysis proved inconclusive, possibly due to the limited number of HIF-1α gene expression data points available for analysis.
Figure 3.17 – HIF-1α gene expression in HCT116 carcinoma cells (top graph), MGLVA1 carcinoma cells (middle graph) and PAN1 carcinoma cells (bottom graph), after incubation under normoxic or hypoxic conditions, relative to the HPRT control (p<0.0001 (analysed using a one-way ANOVA), individual data points; ***p<0.0001, + 95% confidence interval). (HCT116 and MGLVA1 cells, n=2 (data representative of replicate experiments), PAN1 cells n=4 (data representative of replicate experiments)).
3.8 VEGF gene expression after hypoxic induction - Up-regulation of the VEGF gene expression

To determine whether the nuclear HIF-1α protein induced during hypoxia was transcriptionally active, the gene expression of VEGF, a known target gene of HIF-1-regulated transcription was investigated in the HCT116, MGLVA1 and PAN1 carcinoma cell lines, under both normoxic and hypoxic conditions, using quantitative real-time RT-PCR.

VEGF gene expression was shown to significantly increase after 6-8hr hypoxic incubation, continuing to increase throughout the rest of the time course in HCT116, MGLVA1 and PAN1 cell lines (Figures 3.18). The VEGF gene expression under hypoxic conditions increased by 4 to 5-fold (cell line-dependent, p<0.0001), compared to the equivalent normoxic gene expression, which remained virtually consistent throughout the experiment, only slightly increasing after 16hr incubation (Figures 3.18).

The increase seen in the VEGF gene expression under hypoxic conditions correlated (p=0.0324, PAN1 cells) with the increase in HIF-1α nuclear protein expression. These results suggested that the HIF-1α protein that was up-regulated under hypoxic conditions was forming a functional transcription factor and initiating the transcription of target genes.
Figure 3.18 – VEGF gene expression in HCT116 carcinoma cells (top graph), MGLVA1 carcinoma cells (middle graph) and PAN1 carcinoma cells (bottom graph), after incubation under normoxic or hypoxic conditions, relative to the HPRT control (p<0.0001 (analysed using a one-way ANOVA), individual data points; ***p<0.0001, + 95% confidence interval). (HCT116 and MGLVA1 cells, n=2 (data representative of replicate experiments), PAN1 cells n=4 (data representative of replicate experiments)).
3.9 Chapter Summary and Conclusions

The translocation of the HIF-1α protein to the nucleus under hypoxic conditions is required for the activation of the HIF-1 transcription factor, alongside dimerisation with the HIF-1β subunit. Specific nuclear expression of the HIF-1α protein was highly inducible under hypoxic conditions in the three carcinoma cell lines investigated (HCT116, MGLVA1 and PAN1 cells), when compared to the normoxic equivalent. HIF-1α protein was inducible either via treatment with CoCl₂ or DFO, or under atmospheric hypoxic conditions (i.e. 1% oxygen). The generation of hypoxia inhibits the activity of the prolyl hydroxylase enzymes, which are oxygen, iron and 2-oxoglutarate-dependent enzymes, responsible for targeting the HIF-1α subunit for degradation (Bruick and McKnight, 2001). The iron chelator CoCl₂ and iron antagonist DFO inhibit the availability of iron to the prolyl hydroxylase, therefore inducing a hypoxic state, similar to that induced by reduced oxygen tension (Wang et al, 1995).

The pattern and expression levels of nuclear HIF-1α protein, after either incubation under atmospheric hypoxic conditions or chemical induction with CoCl₂ or DFO, were very similar in the three carcinoma cell lines investigated; the expression mainly varied in the timescale of induction. The prolyl hydroxylase enzymes are active under normoxic conditions, resulting in the rapid degradation of the HIF-1α protein. Also, restoring normoxia from previously hypoxic conditions produces a HIF-1α protein with a half-life of less than five minutes (Wang et al, 1995)…When inhibiting the prolyl hydroxylases however, using iron chelators or antagonists, the resultant effects
will last as long as it takes for the cell to metabolise the compound and therefore stabilising the expression of HIF-1α protein even under normoxic conditions. The concentration of CoCl$_2$ used to induce HIF-1α expression was quite high (150µM) and seemed to be cytotoxic to the MGLVA1 cells after 16-24hr incubation, as it reduced the β-actin expression of the cells, despite the levels of HIF-1α protein increasing. Wang and Semenza (1995) showed that HIF-1α protein expression was inducible after treatment with only 25µM CoCl$_2$. Therefore, if the hypoxia chamber had not become available during this project, the concentration of CoCl$_2$ used to induce HIF-1α expression would have been reduced for subsequent experiments, and additional loading controls would be used, such as tubulin, to identify if the cytotoxic effect of CoCl$_2$ may have been β-actin-specific.

Other GI carcinoma cell lines where HIF-1α was shown to be inducible after 4-24 incubation under hypoxic conditions include the colorectal carcinoma cells Colo201, DLD-1 and HCT116 (Krishnamachary _et al_, 2003; Kuwai _et al_, 2003), the gastric carcinoma cell line TMK-1 (Stoelzing _et al_, 2004) and the pancreatic carcinoma cells Capan1 and PANC1 (Büchler _et al_, 2003) at the single time points investigated. The hypoxic induction of HIF-1α protein in the HCT116, MGLVA1 and PAN1 carcinoma cell lines follow a similar pattern to that originally described in Hep3B cells (Wang _et al_, 1995), where the nuclear HIF-1α protein expression was rapidly inducible under hypoxic conditions, before increasing to a maximal expression after 4-8hr incubation.
Nuclear translocation of the HIF-1α protein under hypoxic conditions was confirmed in the HCT116, MGLVA1 and PAN1 carcinoma cell lines using HIF-1α cell immunofluorescent staining. The HIF-1α protein produced a distinctive pattern of accumulation within the nucleus, which excluded the nucleolus; a structure that is involved in ribosome synthesis and mitosis regulation (reviewed in Boisvert et al., 2000), rather than gene transcription. The pattern of HIF-1α accumulation in the three cell lines investigated was identical to that produced in HepG2 cells after exposure to 5 hours hypoxia, as shown by Mottet et al (2003).

Under normoxic conditions, there was only negligible expression of the HIF-1α protein in the HCT116 and PAN1 carcinoma cell lines, which was likely to be due to the hydroxylation, ubiquitination and degradation of HIF-1α that normally occurs under physiological oxygen concentrations. In the MGLVA1 cells however, there was a consistently low level of active HIF-1α protein expressed under normoxic conditions. This could be due to mutations that may have occurred in growth factor signalling pathways, which would allow for the stabilisation of the HIF-1α protein under normoxic conditions, in a cell-specific manner. For example, the downstream targets of PI3-kinase/Akt signalling pathway mTOR and FRAP have been linked to the stabilisation of HIF-1α protein under normoxic conditions (Hudson et al, 2002; Laughner et al, 2001; Treins et al, 2002), as has the loss of PTEN inhibitory signalling (Zundel et al, 2000). Further investigations are now required to determine the exact nature of the mutations within the signalling pathways of the MGLVA1 cells, using target-specific inhibitors such as rapamycin (for mTOR and
FRAP). As gastrin also signals via the PI3-kinase/Akt pathway (Harris et al., 2004), the role of gastrin in MGLVA1 cell signalling and HIF-1α expression could also be investigated.

The nuclear expression of the HIF-1β protein was also increased under hypoxic condition (via treatment with CoCl2) in both the HCT116 and MGLVA1 carcinoma cell lines, compared to the normoxic expression. Unlike the HIF-1α protein however, the HIF-1β subunit was consistently detectable under normoxic conditions. This result confirms that of Wang et al. (1995), which suggested that the nuclear HIF-1β protein expression was increased under hypoxic conditions, in parallel with the nuclear HIF-1α protein expression. These results suggested that the HIF-1β protein also is translocated to the nucleus under hypoxic conditions, or becomes stabilised within the nucleus through binding to the HIF-1α subunit.

HIF-1α gene is generally considered to be constitutively expressed throughout the majority of mammalian cell and tissue types. It has been shown to be expressed in ‘normal’ tissue, such as the lung, heart, liver, spleen, kidney and brain as well as the corresponding tumour tissue (Wiesener et al., 2001). Its expression has been shown to be unaffected by the presence of hypoxia, in both colorectal (HCT116 cells) and gastric carcinoma cell lines (SGC7901 cells) (Kuwai et al., 2003; Liu et al., 2008). However, in the HCT116, MGLVA1 and PAN1 carcinoma cell lines investigated in this study, the HIF-1α gene expression was shown to decrease under hypoxic conditions (either induced by atmospheric incubation or chemical treatment), when compared to
the normoxic expression of the HIF-1α gene. The decrease in HIF-1α gene expression was not due to increased cell death, as the HPRT control levels were unaffected by hypoxic incubation.

A similar observation was made in an early study into HIF-1α gene expression, which was conducted by Wang et al (1995). This study suggested that under hypoxic conditions, the HIF-1α mRNA declined in expression from 2hr onwards, reaching minimal expression levels after 8hr hypoxic incubation. The HIF-1α gene then recovered its expression over the rest of the time course. The decrease in HIF-1α mRNA expression was suggested to be due to the presence of eight RNA instability elements (5’ – UUAUUAUAWW – 3’) in the 3’ untranslated region of the HIF-1α gene (Wang et al, 1995). RNA instability elements are often found in transiently-expressed mRNA sequences and are often possessed by oncogenes, resulting in the destabilisation of their expression (Shaw and Kamen, 1986). As the expression of the HIF-1α gene under hypoxic conditions recovered over time in the Hep3B cells used by Wang et al (1995), it would be reasonable to consider whether the same event would have occurred in the HCT116, MGLVA1 and PAN1 carcinoma cell lines, if incubated under hypoxic conditions for a longer time point.

To determine whether the HIF-1α protein induced under hypoxic conditions was transcriptionally functional, the expression of VEGF, a known target gene of HIF-1 was investigated in a panel of GI carcinoma cell lines. VEGF gene expression was significantly increased under hypoxic conditions, suggesting that HIF-1 was functionally active. The VEGF gene expression up-regulation
occurred slightly downstream of the HIF-1α protein induction, which could suggest that time was required to synthesise the HIF-1α protein, before it translocated to the nucleus, ready to up-regulate target gene transcription. Similar results were observed in further GI carcinoma cell lines, including colorectal carcinoma cells, CaCo2, HT29 and DLD-1 (Mizukami et al, 2004) and pancreatic carcinoma cells, MIA PaCa-2 (Büchler et al, 2003), where VEGF gene expression was up-regulated by up to 10-fold after a 12-24hr incubation.
Chapter Four

REGULATION OF HYPOXIA-INDUCIBLE FACTOR-1A BY GASTRIN
4.1 Background
Gastrin has been shown to play a role in tumourigenesis, influencing cancer proliferation and metastasis, as well as possessing both pro-angiogenic and anti-apoptotic capabilities, which would further aid tumour progression. By binding to its CCK-2 receptor, gastrin is able to initiate cellular signalling pathways that can up-regulate the actions of transcription factors, such as c-fos. This leads to the production of growth factors, such as HB-EGF and amphiregulin, which are able to signal via the EGF receptor, enhancing any proliferative effect.

The resultant cellular effects induced by activation of the HIF-1 transcription factor are similar to those induced by gastrin, especially the process of angiogenesis; therefore the aim of this chapter was to determine whether gastrin signals through HIF-1α when initiating its pro-carcinogenic effects. Firstly, the effect of exogenous gastrin on HIF-1α expression was determined, before the expression of the pro-angiogenic target gene VEGF was investigated after gastrin treatment. Finally, the effect of endogenous gastrin inhibition on HIF-1α was examined.

4.2 Effects of exogenous gastrin treatment on HIF-1α expression and transcriptional activity

4.2.1 Nuclear expression of HIF-1α protein after treatment with exogenous amidated gastrin
HIF-1α nuclear protein expression was investigated after the treatment of carcinoma cell lines with 1nM, 10nM and 100nM exogenous amidated gastrin
or 10nM peptide control, under both normoxic and hypoxic conditions (1% oxygen) via Western blotting.

Initial experiments in PAN1 carcinoma cells revealed that treatment with exogenous gastrin increased the expression of HIF-1α under both normoxic and hypoxic conditions (Figure 4.1).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Gastrin conc: Control 1nM 10nM 100nM</th>
<th>4hr normoxic</th>
<th>24hr normoxic</th>
<th>4hr hypoxic</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>nuclear protein expression in PAN1 carcinoma cell lines.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Figure 4.1 – HIF-1α nuclear protein expression in PAN1 carcinoma cells, after treatment with exogenous amidated gastrin under normoxic and hypoxic conditions, relative to β-actin control. n=3 (data representative of replicate experiments).</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Under normoxic conditions, exogenous gastrin had little effect on HIF-1α expression after only 4hr treatment. After 24hr normoxic incubation however, there was a noticeable increase in nuclear HIF-1α after 1nM and 10nM amidated gastrin treatment. Treatment with the peptide control failed to induce nuclear HIF-1α protein expression to the levels seen after whole molecule amidated gastrin treatment, under normoxic conditions. Under hypoxic conditions, treatment with exogenous gastrin only slightly augmented the expression of HIF-1α protein that already existed, due to the reduced oxygen concentrations.

The expression of HIF-1α nuclear protein after treatment with increasing concentrations of exogenous amidated gastrin was also investigated in other
carcinoma cell lines of different GI origins, to determine whether the effects seen in the PAN1 cells were replicated in other cell lines. Treatment of colonic HCT116 and gastric MGLVA1 carcinoma cell lines with increasing concentrations of exogenous gastrin failed to induce expression of HIF-1α protein under both normoxic and hypoxic conditions (data not shown). Both the HCT116 and MGLVA1 cells are known to not to endogenously express the CCK-2 receptor (Colucci et al, 2008; McWilliams et al, 1998), so were not expected to respond to exogenous gastrin treatment, despite expressing high levels of endogenous gastrin gene (Grabowska et al, 2008). The CCK-2 receptor status of the PAN1 cells is currently unknown.

As exogenous gastrin treatment had only induced an effect in the PAN1 carcinoma cell line and not cells from different GI origins, the effect of amidated gastrin on the expression of nuclear HIF-1α protein was then investigated in further pancreatic carcinoma cell lines, as PAN1 was pancreatic in origin.

Treatment of BxPC3 and PANC1 carcinoma cells with increasing concentrations of exogenous amidated gastrin under normoxic conditions had negligible effect on the expression of nuclear HIF-1α at any concentration tested, in either cell line (data not shown). Under hypoxic conditions, the exogenous gastrin treatment failed to significantly induce the existing expression of the nuclear HIF-1α protein in the BxPC3 and PANC1 cell lines (Figure 4.2), above that already induced by hypoxia itself. The slight decrease seen in the HIF-1α protein expression after 4hr hypoxia was probably due to
non-specific affects of the treatment with exogenous gastrin, or the scrambled control peptide.

### HIF-1α nuclear protein expression in BxPC3 and PANC1 carcinoma cell lines.

**BxPC3 cells**

<table>
<thead>
<tr>
<th>Condition:</th>
<th>Gastrin conc:</th>
<th>4hr hypoxic</th>
<th>8hr hypoxic</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Untr. Scr. 1nM 10nM</td>
<td>Untr. Scr. 1nM 10nM</td>
<td></td>
</tr>
<tr>
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<tr>
<td>β-actin</td>
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<td><img src="image4.png" alt="Image" /></td>
<td></td>
</tr>
</tbody>
</table>

**PANC1 cells**

<table>
<thead>
<tr>
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<th>Gastrin conc:</th>
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<th>8hr hypoxic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untr. Scr. 1nM 10nM</td>
<td>Untr. Scr. 1nM 10nM</td>
<td></td>
</tr>
<tr>
<td>HIF-1α</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.2 – HIF-1α nuclear protein expression in BxPC3 and PANC1 carcinoma cells, after treatment with exogenous amidated gastrin under hypoxic conditions, relative to β-actin control. n=2 (for both cell lines) (data representative of replicate experiments).

### 4.2.2 HIF-1α target gene expression after normoxic treatment with exogenous amidated gastrin

Whilst the treatment of PANC1 cells with exogenous amidated gastrin had no significant affect on nuclear HIF-1α protein expression under hypoxic conditions, treatment with both 1nM and 10nM amidated gastrin induced the expression of HIF-1α under normoxic conditions. To further investigate whether exogenous gastrin treatment had any affect on HIF-1α in normoxia, emphasis was changed from HIF-1α expression to HIF-1α activity. The transcription factor HIF-1 is responsible for up-regulating the expression of a number of target genes under hypoxic conditions. Therefore, the effect of 1nM...
and 10nM exogenous gastrin treatment on the expression of the HIF-1 target gene, VEGF was investigated under normoxic conditions.

After both 24hr and 48hr treatment of PAN1 carcinoma cells with increasing concentrations of exogenous amidated gastrin under normoxic conditions, the expression of the VEGF gene increased by 20-105%, when compared to the VEGF expression obtained after 10nM scrambled control treatment (Figure 4.3, p<0.02). These data suggested that exogenous gastrin may up-regulate HIF-1 transcriptional activity under normoxic conditions.

### 4.2.3 Cell reporter luciferase expression after normoxic treatment with exogenous amidated gastrin

Under normoxic conditions, HIF-1 may be responsible for up-regulating VEGF gene expression, as its protein expression can be stabilised by several oncogenic signalling pathways that override the usual normoxic HIF-1α degradation system. Other growth-factor regulated signalling pathways however, are known to play a role in regulating VEGF gene expression in a HIF-1-independent manner.

Therefore, to determine whether the increase in VEGF gene expression induced by exogenous amidated gastrin treatment was HIF-1-dependent, cells were transfected with a range of luciferase-reporter plasmids that contained the HRE sequence, which the HIF-1 transcription factor binds to within the promoter region of the target genes, to initiate their transcription.
Figure 4.3 – VEGF gene expression in PAN1 carcinoma cells, after 24hr (top graph) and 48hr (bottom graph) treatment with exogenous amidated gastrin under normoxic conditions, relative to the HPRT control (*p=0.0106 / ***p<0.003 (analysed using students t-test), + 95% confidence interval). n=3 (for 24hr time point) N=2 (for 48hr time point) (data representative of replicate experiments).

To determine whether treatment with exogenous amidated gastrin allowed HIF-1 to initiate transcription, two different luciferase models were used, as described below:
1) VEGF-HRE luciferase reporter plasmid, which contains the DNA promoter sequence from upstream of the VEGF coding region (-2018bp to +50bp), attached to a firefly-luciferase reporter construct. After test transfections of the VEGF-HRE luciferase reporter plasmid, there was a significant 2-3 fold increase in luciferase expression, under hypoxic conditions, compared to that obtained in the equivalent normoxic control (Figure 4.4, p<0.02) (See Materials and Methods section 2.11).

2) PGL4-(LDH)-HRE luciferase reporter plasmid, which contains multiple copies of the HRE sequence expressed in the promoter of the LDH gene, a known target gene of HIF-1. The PGL4-(LDH)-HRE reporter plasmid was derived from a LDH-expressing PGL3-luciferase reporter plasmid, which was kindly donated by Dr Kaye Williams (University of Manchester). The LDH-HRE construct and a SV40 minimal promoter were originally excised from a PGL3 luciferase reporter plasmid and ligated into the
PGL4-basic luciferase reporter plasmid (Promega), to help eliminate background luminescence. Test transfections of the PGL4-(LDH)-HRE luciferase reporter plasmid induced a significant 8.5 fold increase in luciferase expression under hypoxic conditions, compared to that obtained under normoxic conditions (Figure 4.5, p<0.0001).

**Firefly luciferase expression in PAN1 cells transfected with PGL4-(LDH)-HRE luciferase reporter plasmid**

![Graph showing firefly luciferase expression in PAN1 cells under normoxic and hypoxic conditions](image)

Figure 4.5 – Firefly luciferase expression in PAN1 carcinoma cells under both normoxic and hypoxic conditions, after transfection with PGL4-(LDH)-HRE luciferase reporter plasmid (**p<0.0001 (analysed using a student’s t-test), + S.D). Each bar represents combined data from duplicate transfections. n=10 (data representative of replicate experiments).

**VEGF-HRE luciferase reporter plasmid**

Treatment of PAN1 cells with increasing concentrations of exogenous amidated gastrin for both 24hr and 48hr under normoxic conditions, after being transiently transfected with the VEGF-HRE luciferase reporter plasmid also had no significant effect on the expression of the firefly luciferase gene (Figure 4.6) at any concentration tested, when compared to the expression obtained after treatment with the scrambled control. Similar results were obtained in replicate experiments, whether the cells were only treated once with exogenous...
gastrin, or repeatedly dosed with fresh gastrin every 12hr. Similar results were also obtained after 72hr exogenous gastrin treatment (data not shown).

Firefly luciferase expression after transfection with VEGF-HRE luciferase reporter plasmid.

Figure 4.6 – Firefly luciferase expression in PAN1 carcinoma cells, after transfection with VEGF-HRE luciferase reporter plasmid, prior to 24hr treatment (top graph) or 48hr treatment (bottom graph) with exogenous amidated gastrin that was dosed every 12hr, under normoxic conditions (non-significant (analysed using a student’s t-test), + S.D). Each bar represents combined data from duplicate transfections. n=2 (for both time points) (data representative of replicate experiments).

PGL4-(LDH)-HRE luciferase reporter plasmid

Treatment of PAN1 cells with exogenous amidated gastrin for 24hr under normoxic conditions, after transfection with the PGL4-(LDH)-HRE luciferase
reporter plasmid had no significant effect on the firefly luciferase expression, at any concentration, when compared to the luciferase expression induced via treatment with the equivalent scrambled control (Figures 4.7). Similar results were also replicated at all time points investigated (i.e. 48hr and 72hr, data not shown).

![Firefly luciferase expression after transfection with PGL4-(LDH)-HRE luciferase reporter plasmid.](image)

Figure 4.7 – Firefly luciferase expression in PAN1 carcinoma cells, after transfection with PGL4-(LDH)-HRE luciferase reporter plasmid, prior to 24hr treatment with exogenous amidated gastrin that was dosed every 12hr, under normoxic conditions (non-significant (analysed using a student’s t-test), + S.D). Each bar represents combined data from duplicate transfections. n=3 (data representative of replicate experiments).

The use of the two HRE-reporter systems (i.e. the VEGF-HRE and PGL4-(LDH)-HRE luciferase reporter plasmids) suggested that the increase in VEGF gene expression induced after exogenous gastrin treatment was probably not HIF-1-dependent or mediated through the VEGF promoter. As a result, investigations into the affect of exogenous amidated gastrin treatment on HIF-1α expression and activity were discontinued.
4.3 Effects of endogenous gastrin activity on HIF-1α expression and transcriptional activity

4.3.1 Effect of gastrin siRNA transfection on HIF-1α gene expression

In an attempt to determine whether endogenous gastrin plays a role in the regulation of HIF-1α expression and/or function, carcinoma cell lines were transfected with a gastrin-specific siRNA to knock down any endogenous gastrin expression (See Materials and Methods section 2.8).

PAN1 carcinoma cells were transfected with either 20nM gastrin-specific siRNA or 20nM scrambled control siRNA (i.e. consists of identical nucleotides to those found in the gastrin-specific siRNA, but whose order had been scrambled), under both normoxic and hypoxic conditions.

Under normoxic conditions, transfection of gastrin-specific siRNA resulted in a 75-95% down-regulation in the expression of the gastrin gene, compared to the scrambled control (Figure 4.8, p<0.0001). Similar results were also seen under hypoxic conditions, where gastrin-specific siRNA transfection resulted in 75-85% down-regulation in gastrin gene expression (Figure 4.8, p<0.0001). The gastrin siRNA inhibits the production of endogenous gastrin, which would usually be secreted into the surrounding environment, where it could act in an autocrine fashion, up-regulating gastrin signalling via the CCK-2 receptor. It is currently unknown whether the PAN1 carcinoma cell line expresses the CCK-2 receptor.
Exogenous gastrin was shown to up-regulate the expression of HIF-1α protein in the PAN1 carcinoma cell line. Therefore to determine whether endogenous gastrin played a role in the regulation of HIF-1α under normoxic conditions, target-specific gastrin siRNA was used to determine the effect on HIF-1α gene expression. Transfection of PAN1 carcinoma cells with gastrin-specific siRNA resulted in an approximate 30% decrease in HIF-1α gene expression.
under normoxic conditions (Figure 4.9, p<0.0001), when compared to the HIF-1α expression achieved after transfection with the scrambled control siRNA. Under hypoxic conditions however, transfection with gastrin siRNA had no significant effect on the level HIF-1α gene expression (data not shown). This data suggests that endogenous gastrin is partially responsible for the regulation of HIF-1α gene expression under normoxic conditions, possibly acting via its CCK-2 receptor located on the cell surface.

![HIF-1α gene expression after gastrin siRNA transfection under normoxic conditions.](image)

Figure 4.9 – HIF-1α gene expression in PAN1 carcinoma cells after transfection with gastrin siRNA or scrambled control siRNA, under normoxic conditions, relative to the HPRT control (***p<0.0001 (analysed using student’s t-test), + 95% confidence interval). Each bar represents combined data from duplicate transfections. n=2 (data representative of replicate experiments).

The down-regulation of HIF-1α gene expression may lead to a reduction in HIF-1α protein expression and as a result reduce the overall level of downstream HIF-1 transcriptional activity. To determine whether the inhibition of HIF-1α gene expression has an effect on the level of HIF-1 transcriptional activation, HCT116 dual luciferase carcinoma cells (that had been stably transfected with an HRE-luciferase construct) were transiently
transfected with 20nM gastrin siRNA or its scrambled control under both normoxic and hypoxic conditions.

HCT116 dual luciferase cells contain a stably-expressed HRE-luciferase (firefly) reporter, alongside an endogenous renilla-luciferase control. Pilot study data revealed that there was a significant six-fold increase in luciferase expression, under hypoxic conditions, compared to the normoxic equivalent (Figure 4.10, p<0.01) (See Materials and Methods section 2.12).

![Firefly luciferase expression in HCT116 dual-luciferase reporter cells.](image)

Figure 4.10 – Firefly luciferase expression in HCT116 dual-luciferase reporter carcinoma cells under both normoxic and hypoxic conditions (**p<0.01 (analysed using a student’s t-test), + S.D (Standard deviation)). Each bar represents combined data from duplicate transfections (n=1).

Transfection of the HCT116 dual luciferase cells with gastrin siRNA had no significant effect on the level of firefly luciferase expression under either normoxic or hypoxic conditions (Figure 4.11), when compared to luciferase expression obtained after scrambled control siRNA transfection. This data suggested that even if gastrin played a role in regulating the expression of HIF-1α gene and protein, it does not affect the transcriptional activity of the transcription factor (i.e. it does not act through an HRE sequence).
Figure 4.11 – Firefly luciferase expression in HCT116 dual luciferase carcinoma cells, after transfection with gastrin siRNA or scrambled control siRNA, under both normoxic and hypoxic conditions (non-significant (analysed using a student’s t-test), + S.D). Each bar represents combined data from duplicate transfections (n=1).

4.4 Chapter Summary and Conclusions

Treatment with exogenous amidated gastrin only induced an increase in nuclear HIF-1α protein expression in the PAN1 carcinoma cell line, despite several other carcinoma cell lines being investigated, including those from both similar and different tissues of origin. The increase of the HIF-1α protein under normoxic conditions within the PAN1 cell line was often low and therefore quite difficult to visualise and interpret. This may be explained by the suggestion that only a subset of the PAN1 cells were responsive to exogenous gastrin treatment. In a study by Chao et al, (2006) the presence of the CCK-2 receptor splice variant CCK\textsubscript{2i4} increased the normoxic basal expression of HIF-1α protein in HEK-293 cells, compared to those transfected with wild-type CCK-2 receptors. The CCK\textsubscript{2i4}-expressing cells were shown to be more responsive to the proliferative effects of gastrin, using a xenograft
The authors did not however, investigate whether exogenous gastrin treatment using either the stably CCK-2i4-transfected cell line or xenograft mouse model further enhanced the HIF-1α protein expression. A subset of the PAN1 carcinoma cells used in the nuclear HIF-1α protein expression studies could have expressed the CCK2i4 receptor, making them more responsive to exogenous gastrin treatment. To determine whether the increase in HIF-1α protein expression seen in PAN1 cells was due to the affects of gastrin treatment, the cells should be pre-treated with a CCK-2 receptor or CCK2i4 receptor-specific antagonist, prior to HIF-1α expression being investigated.

Exogenous gastrin treatment of the pancreatic carcinoma cell lines (i.e. PAN1, BxPC3 and PANC1) under hypoxic conditions failed to significantly augment the already high levels of nuclear HIF-1α protein that had been induced by the presence of low oxygen tension. Any real effect that the exogenous amidated gastrin treatment had on the HIF-1α protein expression was probably masked by the high levels of HIF-1α and therefore is only considered as trivial when inducing the final downstream activities of the HIF-1 transcription factor.

Investigations into the effect of exogenous amidated gastrin treatment on HIF-1α were transferred from HIF-1α protein expression studies to HIF-1α protein activity assays (i.e. RT-PCR and luciferase reporter assays), in a final attempt to elucidate whether gastrin played a role in regulating the HIF-1 transcription factor. The previously partially-responsive PAN1 carcinoma cell line was transfected with one of two different HRE-luciferase reporter plasmids, the activity of which were dependent on the up-regulation of HIF-1 transcriptional
activity. The HRE-transfected cells were then treated with increasing concentrations of exogenous amidated gastrin. This failed to significantly induce the luciferase expression in cells transfected with either the VEGF-HRE plasmid or PGL4-(LDH)-HRE plasmids at any time point or concentration investigated. Therefore, it was concluded that exogenous amidated gastrin treatment had no affect on the activity of the HIF-1 transcription factor and that this part of the hypothesis should not be continued any further and instead replaced with investigations into the role of hypoxia on gastrin mRNA expression.

Conversely, treatment with exogenous amidated gastrin significantly increased the VEGF gene expression after both 24hr and 48hr incubation. VEGF is a known target gene of the HIF-1 transcription factor and has been previously shown to be increased under hypoxic conditions (see Figure 3.17). However, despite the lack of involvement of HIF-1α in gastrin signalling, as shown using both Western blotting and luciferase reporter assays, the VEGF gene expression was still inducible under normoxic conditions. This increase in expression may be explained by growth factor signalling pathways, induced by molecules such as EGF and IGF-1, which are also known to be active under normoxia (reviewed in Mikhopadhyay and Datta, 2004). Gastrin known to up-regulate the expression of growth factors, such as amphiregulin and HB-EGF, which act at the EGF receptor (Tsutsui et al, 1997), and therefore may up-regulate the normoxic VEGF gene expression in a HIF-1-independent fashion. To be completely certain that HIF-1α is not involved in the up-regulation of VEGF gene expression by gastrin, the carcinoma cells should be transfected
with a HIF-1α-specific siRNA prior to exogenous gastrin treatment, to remove any endogenous HIF-1α activity that may up-regulate the expression of the VEGF gene.

Transfection of PAN1 cells with gastrin specific siRNA induced a high level of endogenous gastrin gene knock-down under both normoxic and hypoxic conditions. The levels of gene knock-down obtained matched those previously described in a study by Grabowska et al (2007), which used the same siRNA constructs. In this study, the loss of the gastrin gene led to a reduction of gastrin-regulated events, such as decreased cell growth and increased apoptosis. The large level of gastrin gene knock-down may suggest that endogenous gastrin plays a role in cell proliferation and survival. Endogenous gastrin would be secreted into the surrounding environment, where it would act in an autocrine manner, up-regulating gastrin-specific signalling pathways via the CCK-2 receptor. Treatment with exogenous gastrin may further enhance the endogenous actions of gastrin, which may result in up-regulating HIF-1α expression, as seen in the PAN1 carcinoma cell line. To determine whether endogenous gastrin plays a role in cell signalling, carcinoma cell lines could be treated with a CCK-2 receptor antagonist, alongside being transfected with gastrin siRNA.

Transfection of gastrin siRNA also induced a partial decrease in HIF-1α gene expression, suggesting that gastrin plays a partial role in the regulation of HIF-1α at the gene expression level under normoxic conditions only. Generally, the level of HIF-1α gene remains constant in carcinoma cells, even under hypoxic...
conditions (Kuwai et al, 2003; Liu et al, 2008) and it is the induction of the HIF-1α protein which is responsible for the activation of the HIF-1 transcription factor. If gastrin is however partially responsible for the regulation of the HIF-1α gene expression, it may suggest that the HIF-1 transcription factor is partly responsible in transmitting the downstream affects of gastrin signalling, such as increased angiogenesis and cell survival. The transfection of a HRE-expressing cell line with the gastrin siRNA had negligible effect on the transcriptional activity of HIF-1, which suggests that any affect that gastrin has on the HIF-1 transcription factor is not regulated through a HRE sequence. This data further supports the idea that investigations into the role of gastrin on HIF-1α activity should be discontinued in favour of investigating the role of hypoxia on gastrin expression.
Chapter Five

REGULATION OF GASTRIN EXPRESSION BY HYPOXIA
5.1 Background
Gastrin has been shown to play a role in cancer cell proliferation, as well as possessing pro-angiogenic and anti-apoptotic capabilities. The induction of HIF-1 activation also has been linked to increased tumour growth, angiogenesis and loss of apoptosis; therefore the aim of this chapter was to determine whether gastrin was a target gene of the HIF-1 transcription factor. Firstly, it was established whether gastrin expression was regulated by hypoxia, and then more specifically, whether it was regulated by HIF-1α itself.

5.2 Gastrin gene expression after hypoxic induction - Up-regulation of gastrin gene expression
Gastrin gene expression was investigated after exposure of GI carcinoma cell lines to hypoxia (1% oxygen), using quantitative real-time RT-PCR. Gastrin gene expression was shown to significantly increase after 16hr hypoxic incubation in the HCT116, MGLVA1 and PAN1 carcinoma cell lines (Figure 5.1, p<0.0001). The gastrin gene expression under hypoxic conditions increased by 17-50 fold (cell line-dependent, p<0.0001), compared to the corresponding normoxic gene expression, which remained at a consistently low level throughout, in these cell lines. In the MGLVA1 cells, the gastrin gene expression was always higher under hypoxic conditions, when compared to the equivalent normoxic controls at each time point tested (between 1-12hr) (Figure 5.1).
Figure 5.1 – Gastrin gene expression in HCT116 carcinoma cells (top graph), MGLVA1 carcinoma cells (middle graph) and PAN1 carcinoma cells (bottom graph), after incubation under normoxic or hypoxic conditions, relative to the HPRT control (p<0.0001 (analysed using a one-way ANOVA), individual data points: *p<0.05 / **p<0.001 / ***p<0.0001, + 95% confidence interval). n=2 (HCT116 cells) / n=3 (MGLVA1 cells) / n=8 (PAN1 cells) (data representative of replicate experiments).
The large induction of gastrin gene expression occurred only after 16hr hypoxic incubation in the GI carcinoma cell lines and not at any earlier time points, with the exception of PAN1 cells, in which, after 12hr hypoxic incubation, there was a consistent two-fold increase in gastrin gene expression, compared to the normoxic equivalent (data not shown, p<0.002).

5.3 Gastrin gene expression after hypoxic induction - Expression of the gastrin gene in other GI carcinoma cell lines

In the previous section, incubation under hypoxic conditions induced the up-regulation of gastrin gene within the HCT116, MGLVA1 and PAN1 carcinoma cell lines (see Figure 5.1). These three cell lines were originally derived from different locations within the GI tract. To determine whether the increase of gastrin gene induced under hypoxic conditions was reproducible throughout the entire digestive system, the expression of gastrin gene was investigated in a panel of GI carcinoma cells, whose origins ranged from oesophageal, gastric, pancreatic and colonic in nature. Whilst the majority of the GI carcinoma cell lines did show an increase in gastrin gene expression under hypoxic conditions, other different expression patterns were also identified.

5.3.1 Up-regulation of gastrin gene expression under hypoxic conditions

In the majority of additional GI carcinoma cell lines investigated, gastrin gene expression was significantly increased by 3-4-fold (cell line-dependent) under hypoxic conditions, compared to the equivalent normoxic control, as well as
the 0hr control samples (i.e. the initial gastrin gene expression prior to hypoxic induction). The cell lines studied included AGS cells, a gastric cell line (Figure 5.2, p<0.0001), BxPC3 cells, a pancreatic cell line (Figure 5.2, p<0.0004), OE19 and OE33 cells, which were both oesophageal cell lines (Figures 5.2, p<0.0004).

With the majority of the cell lines tested, the gastrin gene expression after 16hr incubation under normoxic conditions slightly decreased, when compared to the 0hr control. This proved to be significant in the BxPC3 and OE19 cells (see Figures 5.2 p<0.0004). The other cell lines, such as the OE33, the gastrin gene expression increased slightly after 16hr normoxic incubation, compared to the 0hr control, (see Figure 5.2, p<0.0001). These observations may actually reflect the level of cell confluency within the cell culture well, as opposed to a genuine result. Increased cell number may have induced gastrin expression, due to the increased ‘stresses’ of their environment. However, the slight increase in the normoxic gastrin expression in the OE33 carcinoma cells after 16hr incubation may also be due to endogenous gastrin activity, up-regulating its expression by signalling via its CCK-2 receptor in an autocrine manner.

**5.3.2 Reduction of basal gastrin gene expression**

In the majority of GI carcinoma cell lines investigated, gastrin gene expression was significantly increased under hypoxic conditions, however, the gastric carcinoma cell line ST16 consistently showed a different pattern of gastrin gene expression, when compared to other GI carcinoma cells (Figure 5.3).
Figure 5.2 – Gastrin gene expression in AGS carcinoma cells (top graph), BxPC3 carcinoma cells (middle graph), OE19 carcinoma cells (middle graph) and OE33 carcinoma cells (bottom graph), after 16hr incubation under normoxic or hypoxic conditions, relative to the HPRT control (**p=0013/ ***p<0.0004 (analysed using a student’s t-test), + 95% confidence interval). n=2 (BxPC3) / n=1 (AGS/OE19/OE33) (data representative of replicate experiments).
Gastrin gene expression significantly decreased under both normoxic and hypoxic conditions, when compared to the basal gastrin expression (i.e. 0hr control) (Figure 5.3, p<0.0001 (normoxic cells) p<0.02 (hypoxic cells)). The ST16 cell line showed the highest level of endogenous gastrin gene expression of any of the carcinoma cell lines tested. Investigations into the gastrin gene expression after the 16hr hypoxic incubation, resulted in an 11-fold increase in expression, when compared to the equivalent normoxic control (Figure 5.3, p<0.0001). This may be due to the affects of endogenous gastrin signalling within the ST16 carcinoma cells. At the 0hr time point, there could be a high level of endogenous gastrin signalling, which in turn up-regulates the transcription of the gastrin gene via acting at the CCK-2 receptor. After the 16hr normoxic incubation however, a negative-feedback mechanism may have been induced, to prevent over-expression of the gastrin gene.

Figure 5.3 – Gastrin gene expression in ST16 carcinoma cells, after 16hr incubation under normoxic or hypoxic conditions, relative to the HPRT control (*p=0.0182/ ***p<0.0001 (analysed using a student’s t-test), + 95% confidence interval). n=3 (data representative of replicate experiments).
5.3.3 Up-regulation of gastrin gene expression under normoxic conditions

In the majority of cell lines investigated, there was a significant increase in the gastrin gene expression under hypoxic conditions, compared to the equivalent normoxic control and the normoxic expression of the gastrin gene remained at a consistently low level throughout the experiments.

In the colonic C170HM2 and oesophageal OE21 carcinoma cell lines however, 16hr incubation under both normoxic and hypoxic conditions induced a significant increase in gastrin gene expression (Figure 5.4, p<0.0001), with the gastrin gene expression under normoxic conditions being approximately two-fold higher than that seen under hypoxic incubation. This also could be due to the effects of endogenous gastrin on both the C170HM2 and OE21 carcinoma cell lines. Production of high levels of endogenous gastrin over the time course could induce the expression of the gastrin gene, in a CCK-2 receptor-dependent manner, increasing the relatively low gastrin gene expression at 0hr, to the high expression observed after 16hr normoxic incubation. Under hypoxic conditions at the same time point, either a negative-feedback mechanism has been induced; preventing the build-up of large amounts of gastrin gene, or part of the gastrin transcription-signalling mechanism is intolerant to hypoxia, reducing the level of overall gastrin gene expression.
Figure 5.4 – Gastrin gene expression in C170HM2 carcinoma cells (top graph) and OE21 carcinoma cells (bottom graph), after 16hr incubation under normoxic or hypoxic conditions, relative to the HPRT control (***(p<0.0001 (analysed using a student’s t-test), + 95% confidence interval). n=2 (C170HM2 cells) / n=1 (OE21 cells) (data representative of replicate experiments).

Under hypoxic conditions, gastrin gene expression increased by 2.7- and 2.9-fold in C170HM2 and OE21 cell lines respectively (Figure 5.4, p<0.0001), however, the increase in gastrin gene expression under normoxic conditions was approximately 5-fold in both cell lines (Figures 5.4, p<0.0001). Therefore, the effect of hypoxia on gastrin gene expression can be considered cell line dependent-effect.
5.4 Gastrin protein expression after hypoxic induction -
Up-regulation of gastrin expression

As gastrin gene expression was shown to increase under hypoxic conditions in
a number of GI carcinoma cell lines, the effect of hypoxia (i.e. 1% oxygen) on
gastrin protein expression was also investigated, using both Western blotting
and cell immunofluorescence.

The detection of the gastrin protein by Western blotting was unsuccessful. The
gastrin-specific antibody, which targeted the C-terminus of the gastrin protein
failed to detect any protein expression at the correct molecular weight
(~8.8kDa), but generated a high level of non-specific protein binding, which
was identical to that produced in the negative control Western blots (data not
shown). However, pilot study data from cell immunofluorescence experiments
suggested that gastrin protein expression was specifically increased under
hypoxic conditions, in the HCT116 and PAN1 carcinoma cells (Figures 5.5
and 5.6 respectively), compared to the negative control. The gastrin protein
expression also appeared to be nuclear in nature. Under normoxic conditions,
gastrin protein was detectable, but only at low levels (Figure 5.7, HCT116
cells only) when compared to the hypoxic equivalents. These data indicate that
the gastrin protein was also up-regulated under hypoxic conditions, although
further repeats are required before the data can be considered as significant.
Figure 5.5 – Gastrin protein expression in HCT116 carcinoma cells, after incubation under hypoxic conditions, compared to universal rabbit negative control (n=1).

Figure 5.6 – Gastrin protein expression in PAN1 carcinoma cells, after incubation under hypoxic conditions, compared to universal rabbit negative control (n=1).
5.5 Effect of HIF-1α siRNA transfection on HIF-1α gene expression and protein function

To determine whether the HIF-1 transcription factor was involved in the up-regulation of the gastrin gene under hypoxic conditions, target-specific siRNA was used to knock-down HIF-1 gene expression, and as a result, its transcriptional activity.

5.5.1 Optimisation of transfection – HIF-1α gene expression

The initial aim was to use HIF-1α and ARNT (HIF-1β) siRNAs in combination to knock-down total HIF-1 expression and the resultant protein activity, however, pilot experiments using the HIF-1β siRNA failed to induce any significant knock-down of the HIF-1β gene (data not shown). As HIF-1β is known to dimerise with several proteins, and therefore will not specifically inhibit the activity of the HIF-1 transcription factor, all subsequent siRNA-
inhibition experiments concentrated on the knock-down of the HIF-1α subunit, rather than HIF-1β, to down-regulate HIF-1 expression and its resulting functional activity.

Pilot HIF-1α siRNA transfection experiments used the PAN1 carcinoma cell lines. The cells were originally transfected with 20nM HIF-1α under both normoxic and hypoxic conditions. Transfection with HIF-1α siRNA knocked down the HIF-1α gene expression by over 80% under normoxic conditions and 55-60% under hypoxic conditions (Figure 5.8. p<0.0001), when compared to the HIF-1α expression in non-targeting control siRNA-transfected cells. In the majority of transfection experiments, transfection with the non-targeting control siRNA increased the basal gene expression by approximately 15-35%, under both normoxic and hypoxic conditions, when compared the data obtained from untreated cells (data not shown). This increase in gene expression was probably a non-specific effect caused by the transfection reagent, as similar levels of HIF-1α gene expression were obtained from cells treated with the transfection reagent alone, when compared to the results obtained from cells treated with non-targeting control siRNA (data not shown). Any knock-down of the HIF-1α gene after HIF-1α siRNA transfection was therefore considered a genuine affect.
Figure 5.8 – HIF-1α gene expression in PAN1 carcinoma cells under normoxic conditions (top graph) and hypoxic conditions (bottom graph), after transfection with HIF-1α siRNA, or non-targeting (NT) control siRNA (***p<0.0001 (analysed using student’s t-test), + 95% confidence interval). Each bar represents combined data from duplicate transfections (n=1).

In an attempt to increase the knock-down efficiency of the HIF-1α gene under hypoxic conditions, the concentration of siRNA transfected was doubled from a 20nM to 40nM per well. 20nM HIF-1α siRNA induced a 46% decrease in HIF-1α gene expression under hypoxic conditions, compared to the HIF-1α expression from the non-targeting control siRNA transfection, whereas 40nM increased the HIF-1α gene knock down to 65% (Figure 5.9, p<0.0001). However, the increase in HIF-1α gene knock-down could be explained by an
increase in HIF-1α gene expression in non-targeting control siRNA-treated cells rather than a decrease in overall HIF-1α gene expression after transfection with the target-specific siRNA. As a result, subsequent transfections were performed using 20nM siRNA concentration of the siRNA.

![Graph showing HIF-1α gene expression after HIF-1α siRNA transfection under hypoxic conditions.](image)

Figure 5.9 – HIF-1α gene expression in PAN1 carcinoma cells under hypoxic conditions, after transfection with either 20nM or 40nM HIF-1α siRNA or non-targeting (NT) control siRNA (**p<0.0001 (analysed using student’s t-test), + 95% confidence interval). Each bar represents combined data from duplicate transfections. n=2 (data representative of replicate experiments).

### 5.5.2 Effect of HIF-1α siRNA transfection on HIF-1α gene expression and protein function in a panel of GI carcinoma cell lines

To confirm that the HIF-1α siRNA specifically down-regulates HIF-1α gene expression throughout the GI tract, rather than just being a cell type-dependent event, the affect of HIF-1α siRNA on gene expression was investigated in a panel of GI carcinoma cell lines. HCT116, MGLVA1 and PAN1 carcinoma cells were transfected with 20nM HIF-1α siRNA or non-targeting control siRNA, before being incubated under normoxic or hypoxic conditions.
In all three cell lines investigated, transfection with HIF-1α siRNA resulted in down-regulation of HIF-1α gene expression under normoxic (70-90%) and hypoxic (60-80%) conditions (Figures 5.10 and 5.11, cell line-dependent, all p<0.0001), when compared to the HIF-1α expression obtained from non-targeting control siRNA transfection. These results were similar to those obtained in the pilot siRNA transfection studies (see Figure 5.8), suggesting that the HIF-1α siRNA affects HIF-1α gene expression throughout the entire GI tract. Despite the differences seen in the level of down-regulation of HIF-1α gene expression under both normoxic and hypoxic conditions, the final level of HIF-1α gene expression obtained was approximately equal (e.g. 0.1 for HCT116 cells, 0.6-0.8 for MGLVA1 cells and 0.1 for PAN1 cells) (see Figures 5.10 and 5.11).

Again, transfection with the non-targeting control (random) siRNA increased the base line HIF-1α gene expression by approximately 20-35%, under both normoxic and hypoxic conditions, when compared to data obtained from untreated cells (data not shown). Similar results were also obtained from cells treated with the transfection reagent alone, and therefore the increase can be considered a non-specific affect induced by the reagent.

Transfection with either the HIF-1α or non-targeting control siRNA also slightly decreased the expression of the control gene HPRT, under both normoxic and hypoxic conditions, when comparing the data obtained from untreated cells (data not shown). However, the decrease in HPRT expression was consistent throughout all transfected cells.
HIF-1α gene expression after HIF-1α siRNA transfection under normoxic conditions.

Figure 5.10 – HIF-1α gene expression in HCT116 carcinoma cells (top graph) MGLVA1 carcinoma cells (middle graph) and PAN1 carcinoma cells (bottom graph) under normoxic conditions, after transfection with HIF-1α siRNA or non-targeting (NT) control siRNA (**p<0.0001 (analysed using student’s t-test), + 95% confidence interval). Each bar represents combined data from duplicate transfections. n=2 (for each cell line) (data representative of replicate experiments). Transfection-reagent induced HIF-1α gene expression was approximately equal to that induced by the non-targeting control siRNA.
HIF-1α gene expression after HIF-1α siRNA transfection under hypoxic conditions.

Figure 5.11 – HIF-1α gene expression in HCT116 carcinoma cells (top graph), MGLVA1 carcinoma cells (middle graph) and PAN1 carcinoma cells (bottom graph) under hypoxic conditions, after transfection with HIF-1α siRNA or non-targeting (NT) control siRNA (***p<0.0001 (analysed using student’s t-test), + 95% confidence interval). Each bar represents combined data from duplicate transfections. n=2 (for each cell line) (data representative of replicate experiments). Transfection-reagent induced HIF-1α gene expression was approximately equal to that induced by the non-targeting control siRNA.
To confirm that using the HIF-1α-specific siRNA to down-regulate HIF-1α expression also led to the inhibition of HIF-1 transcriptional activity, cells were dual-transfected with the HIF-1α siRNA and a HIF-1 reporter plasmid PGL4-(LDH)-HRE, which contained the HRE sequence expressed in the promoter of the LDH gene, a known target gene of the HIF-1 transcription factor (See Materials and Methods section 2.12).

HIF-1α siRNA transfection produced a significant 95% down-regulation of HIF-1 transcriptional activity under hypoxic conditions, in all three cell lines, (Figure 5.12, p=0.0244 (HCT116 cells), p=0.0008 (MGLVA1 cells) and p=0.0014 (PAN1 cells)), when compared to the firefly luciferase expression obtained from non-targeting control siRNA transfection.

Despite the low expression levels of the HIF-1α protein under normoxic conditions, transfection with both HIF-1α siRNA and PGL4-(LDH)-HRE luciferase reporter plasmid also significantly down-regulated HIF-1 transcriptional activity in the HCT116 and MGLVA1 carcinoma cell lines, by 80% and 95% respectively, under normoxic conditions (Figure 5.13, p=0.0097 (HCT116 cells) and p=0.0232 (MGLVA1 cells)), when compared to the firefly luciferase expression obtained after non-targeting control siRNA transfection. The HIF-1α siRNA transfection had no significant effect on normoxic PAN1 HIF-1 transcriptional activity, when compared to that obtained in the non-targeting control siRNA transfected cells (Figure 5.13). Although, to confirm whether these observations were genuine, the data needs to be correlated to the protein concentration of the luciferase samples.
Firefly luciferase expression after HIF-1α siRNA transfection under hypoxic conditions.

![Graph for HCT116 cells](image)

![Graph for MGLVA1 cells](image)

![Graph for PAN1 cells](image)

Figure 5.12 – Firefly luciferase expression in HCT116 carcinoma cells (top graph), MGLVA1 carcinoma cells (middle graph) and PAN1 carcinoma cells (bottom graph) under hypoxic conditions, after dual-transfection with PGL4-(LDH)-HRE reporter plasmid and HIF-1α siRNA or non-targeting (NT) control siRNA (*p=0.0244 / **p=0.0014 / ***p=0.0008 (analysed using student’s t-test), + S.D). Each bar represents combined data from duplicate transfections. n=1 (for HCT116 / MGLVA1 cells) n=2 (PAN1 cells) (data representative of replicate experiments). Transfection reagent induced HIF-1α gene expression was approximately equal to that induced by the non-targeting control siRNA.
Figure 5.13 – Firefly luciferase expression in HCT116 carcinoma cells (top graph), MGLVA1 carcinoma cells (middle graph) and PAN1 carcinoma cells (bottom graph) under normoxic conditions, after dual-transfection with PGL4-(LDH)-HRE reporter plasmid and HIF-1α siRNA or non-targeting (NT) control siRNA (*p=0.0232 / **p=0.0097 (analysed using student’s t-test), + S.D). Each bar represents combined data from duplicate transfections. n=1 (for HCT116 / MGLVA1 cells) n=2 (PAN1 cells) (data representative of replicate experiments). Transfection reagent-induced HIF-1α gene expression was approximately equal to that induced by the non-targeting control siRNA.
The raw Firefly luciferase expression data of HCT116 and MGLVA1 cells was 10-fold higher than that seen in the PAN1 cells (data not shown), suggesting a higher level of HIF-1 transcriptional activity within these cell lines. This correlated with the nuclear Western blots of these cell lines, which showed a greater intensity of HIF-1α expression in the HCT116 and MGLVA1 cells, compared to the PAN1 cells. These results could suggest that the HCT116 and MGLVA1 carcinoma cell lines express a level of growth factor signalling, which induces HIF-1 transcriptional activity under normoxic conditions, whereas the PAN1 carcinoma cell line lacked this signalling.

5.6 Effect of HIF-1α siRNA transfection on gastrin gene expression

To determine whether the HIF-1 transcription factor was involved in the up-regulation of the gastrin gene under hypoxic conditions, HIF-1α siRNA was used to specifically knock-down HIF-1α gene expression, and its resultant transcriptional activation. As shown above, test transfections using HIF-1α siRNA were shown to significantly down-regulate both HIF-1α gene expression and the resultant protein activity of the HIF-1 transcription factor. Gastrin gene expression was investigated in a panel of GI carcinoma cell lines, after transfection with either 20nM HIF-1α siRNA or non-targeting control siRNA, before being incubated under hypoxic conditions.

In the HCT116 and PAN1 carcinoma cell lines, transfection with HIF-1α siRNA under hypoxic conditions resulted in a 45% and 65% decrease in gastrin gene expression respectively (Figure 5.14, p<0.0001), when compared
to the gastrin gene expression obtained after non-targeting control siRNA transfection.

The expression of the gastrin gene in the presence of HIF-1α siRNA under hypoxic conditions was reduced to the level of gastrin expression normally seen under normoxic conditions at the same time point in the HCT116 and PAN1 carcinoma cells (after transfection with the non-targeting control siRNA (data not shown)). As previously seen in the HIF-1α gene expression studies, transfection with the non-targeting control siRNA increased the baseline gastrin gene expression, when comparing the data obtained from untreated cells, which was probably caused by the transfection reagent (data not shown).

Inhibition of gastrin gene expression after HIF-1α siRNA transfection was not replicated in the MGLVA1 carcinoma cells, as transfection with HIF-1α siRNA induced a 30% increase in the hypoxic gastrin gene expression, when compared to the expression obtained in the non-targeting control siRNA-treated cells (Figure 5.15, p=0.0002). These results suggest that HIF-1α plays a role in regulating gastrin gene expression under hypoxic conditions, but in a cell-dependent manner.
Gastrin gene expression after HIF-1α siRNA transfection under hypoxic conditions.

Figure 5.14 – Gastrin gene expression in HCT116 carcinoma cells (top graph) and PAN1 carcinoma cells (bottom graph) under hypoxic conditions, after transfection with HIF-1α siRNA or non-targeting (NT) control siRNA (***p<0.0001 (analysed using student’s t-test), + 95% confidence interval). Each bar represents combined data from duplicate transfections. n=2 (for both cell lines) (data representative of replicate experiments).

Gastrin gene expression after HIF-1α siRNA transfection under hypoxic conditions.

Figure 5.15 – Gastrin gene expression in MGLVA1 carcinoma cells under hypoxic conditions, after transfection with HIF-1α siRNA, non-targeting (NT) control siRNA, or untreated control (**p=0.0002 (analysed using student’s t-test), + 95% confidence interval). Each bar represents combined data from duplicate transfections. n=2 (data representative of replicate experiments).
5.7 Chapter Summary and Conclusions

Specific expression of the gastrin gene was up-regulated under hypoxic conditions in a number of GI carcinoma cell lines, derived from different origins throughout the GI tract. These included colorectal carcinoma cells (HCT116 cells), gastric carcinoma cell lines (MGLVA1 and ST16 cells), oesophageal carcinoma cells (OE19, OE21 and OE33) and pancreatic carcinoma cells (BxPC3 and PAN1). The up-regulation of gastrin expression under hypoxic conditions may help cells endure the harsh conditions found in hypoxia, by up-regulating gastrin-dependent pro-survival mechanisms, such as increased cell proliferation (Colucci et al., 2005; Smith et al., 2005), angiogenesis (Clarke et al., 2006; LeFranc et al., 2004) and invasion (Wroblewski et al., 2002). Gastrin expression may also lead to the down-regulation of anti-survival mechanisms, such as apoptosis (Hartwich et al., 2000).

The up-regulation of gastrin expression under hypoxic conditions was further confirmed by gastrin cell immunofluorescent staining, which appeared to be nuclear in nature.

The up-regulation of the gastrin gene under hypoxic conditions consistently occurred downstream of hypoxic VEGF gene induction. Generally, target genes of HIF-1 are noticeably up-regulated within the first 4-8hr of hypoxic incubation, although this figure can vary. A study by Semenza et al. (1994) investigated the role of hypoxia on the expression of a number of glycolytic enzymes. They showed that some enzymes (e.g. aldolase A) were inducible
within 1hr hypoxic incubation, whereas the induction of other enzymes took much longer, such as phosphoglycerate kinase, which took over 8hr before the enzyme was detectable. Therefore, the next step was to determine whether the gastrin gene was a target gene of HIF-1 or whether its induction was due to a secondary indirect mechanism. Therefore, the effect of HIF-1 inhibition on gastrin expression was investigated using target-specific siRNA.

Transient transfection of HIF-1α siRNA proved to be very successful at down-regulation the expression of the HIF-1α gene, and as a result, the transcriptional activity of the HIF-1 transcription factor, under both normoxic and hypoxic conditions, in all carcinoma cell lines investigated. The original plan was to co-transfect both HIF-1α and HIF-1β siRNA into the carcinoma cell lines, to fully inhibit the activity of HIF-1. However, the HIF-1β-specific siRNA had failed to induce a significant affect on the expression of the HIF-1β gene. Targeting the HIF-1β gene would not have provided specific inhibition for the HIF-1 transcription factor, as HIF-1β or ARNT is a common subunit of multiple bHLH-PAS proteins, such as the aryl hydrocarbon receptor (Wang et al, 1995) and therefore the siRNA would have targeted more than one gene for knockdown. This would have meant that any downstream events as a result of HIF-1β knockdown could not be completely associated to the HIF-1 transcription factor.

The up-regulation of the gastrin gene expression was partially inhibited by a HIF-1α-specific siRNA in a cell line-dependent manner. This suggested that the gastrin gene up-regulation under hypoxic conditions was partially mediated
via HIF-1 and as a result, its promoter may include a 'hypoxia-response element' (HRE).
Chapter Six
CONSTRUCTION AND EVALUATION OF A LUCIFERASE REPORTER PLASMID CONTAINING A PUTATIVE GASTRIN HRE SEQUENCE
6.1 Background

The transcription factor HIF-1 is responsible for the up-regulation of several key target genes under hypoxic conditions. These genes have roles in cell growth and survival, cell proliferation, angiogenesis and metabolism, and include examples such as VEGF, GLUT1 and the glycolytic enzymes. HIF-1 is often up-regulated in cancers, which leads to the increased expression of its target genes, driving progression through the carcinoma sequence. A key feature of the HIF-1 target genes is the expression of a HRE upstream of their transcription start site. This element is required for HIF-1 to bind and up-regulate transcription, and is encoded by five key bases (A/G)CGTG.

In the previous chapter, hypoxia was shown to significantly up-regulate the expression of the gastrin gene in a panel of GI carcinoma cell lines. The gastrin gene was also shown to be partially responsive to HIF-1α siRNA transfection, suggesting that gastrin is partially mediated via HIF-1. As a result its promoter may contain a HRE sequence. Both gastrin and HIF-1α have been independently shown to possess similar cell proliferative, angiogenic and anti-apoptotic properties; therefore the aim of this chapter was to determine whether gastrin was a target gene of the HIF-1 transcription factor. Firstly, the DNA upstream of the gastrin gene transcription start site was screened for the presence of a HRE sequence. As this proved successful, the putative gastrin HRE sequence was cloned into a luciferase reporter vector to determine whether it was directed regulated by HIF-1.
6.2 Identification of a putative hypoxia-response element upstream of the gastrin gene transcript

To determine whether up-regulation of gastrin gene expression under hypoxic conditions was due to the activity of the HIF-1 transcription factor itself, or a secondary effect of a HIF-1 target gene activity, the DNA upstream of the gastrin gene transcript was examined for the presence of a hypoxia-response element (See Materials and Methods section 2.9).

Approximately 4000bp of the DNA sequence upstream of the gastrin transcription start site was downloaded from the Ensembl genome browser (www.ensembl.org, Ensembl file number ENSG00000184502). Homology between the 4000bp DNA sequence (from upstream of the gastrin transcript) and the VEGF HRE sequence (from -985bp to -939bp within the VEGF promoter (Forsythe et al, 1996)) was investigated using the ClustalW multiple alignment program within the SDSC Biology WorkBench software. The resulting alignment between the two sequences is shown in Figure 6.1. The DNA sequence -2985bp and -2933bp upstream of the gastrin transcription start site shared approximately 66% homology with the VEGF HRE sequence.

The DNA upstream of the gastrin gene transcript also contained a five base pair HRE consensus sequence GCGTG within this region of homology (Wenger et al, 2005, Figure 6.1, red box). The presence of this putative HRE sequence upstream of the gastrin transcript may explain the induction of gastrin gene under hypoxic conditions and the ability of HIF-1α siRNA to inhibit gastrin gene expression.
Potential HRE sequence upstream of the gastrin transcription start site.

VEGF sequence

Putative gastrin promoter

Consensus

- CAGACTAATA ---GTGATACAC ---AGGTCTTTTCGTCAAGC---

TCCGCAAGTTAAAAATTTTTAGAGCTTGTCAATTTTCGCTGACTGCTCCCTATGAC

- CC-AGTCTAAAACTTCGGC-3AC---T---CCCTCCCTTTGCA---TGCA---

<table>
<thead>
<tr>
<th>VEGF HRE sequence</th>
<th>Possible Gastrin HRE sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>-985bp ➔ -939bp upstream of VEGF coding sequence</td>
<td>-2965bp ➔ -2933bp upstream of Gastrin coding sequence</td>
</tr>
</tbody>
</table>

Homology between VEGF and Gastrin sequences highlighted in green

Figure 6.1 – The location and sequence of the putative hypoxia response element within the DNA upstream of the gastrin gene.

To determine whether this putative HRE sequence was responsive to hypoxia, and more specifically the HIF-1 transcription factor, luciferase reporter plasmids containing the DNA sequence upstream of the gastrin gene transcript, including the putative gastrin HRE sequence (AGTGTATAAAGCGTGTCGCA CAGAC) were generated.

The DNA sequence from upstream of the gastrin transcription start site, exon one of the gastrin gene transcript and part of intron one (i.e. from -3500 base pairs to +77 base pairs) was screened for the presence of restriction enzyme digest sites, using the TACG program from the SDSC Biology WorkBench software. The restriction sites present within this DNA were then compared to those found in the multiple cloning region of the PGL4-basic luciferase reporter vector (Figure 6.2), which was the vector used in the preparation of the gastrin luciferase reporter plasmids.
The PGL4-basic luciferase reporter vector (Promega) was a 4242bp vector that contained a synthetic firefly luciferase gene, but lacked any additional promoter or enhancer elements (Figure 6.2).

![PGL4-basic luciferase reporter vector](image)

Figure 6.2 – A plasmid map of the PGL4-basic luciferase reporter vector (from Promega), detailing key constructs and restriction enzyme digest sites.

The results of the TACG screening program revealed an endogenous XhoI restriction digest site (CTCGAG) 246-252bp upstream of the putative gastrin HRE sequence (Figure 6.3). The DNA sequence however did not contain any further digest sites from the multiple cloning region of the PGL4-basic luciferase reporter vector (i.e. for the enzymes EcoRV, BglIII, BglII/SfiI and HindIII (see Figure 6.2)). Therefore, to ensure that the DNA upstream of the gastrin gene transcript was inserted into the plasmid in the correct orientation, an extra restriction enzyme site was added to the end of the reverse primer.

To allow for the DNA upstream of the gastrin gene transcript to be amplified using PCR, forward and reverse primers were designed using the Primer3 program from the SDSC Biology WorkBench software. The forward primer
(sequence 5’-ATCAGTTTCTGGTGACACGC-3’) and reverse primer (sequence GGTGGTTTCTCACCTGCAGAGC-3’) (Figure 6.3) were located at -3362bp to -3342bp and +25bp to +42bp of the DNA sequence respectively, producing a final promoter fragment of 3320bp. A BglII restriction enzyme site (AGATCT) was added to the end of the reverse primer (new sequence 5’-CCTCCTCCTTCTAGAGTTTTTCTCACCTGCAGAGC-3’, incorporating the BglII restriction digest site), to ensure that the DNA was inserted into the plasmid in the correct orientation.

6.3 Preparation of gastrin luciferase reporter plasmid for use in luciferase reporter assays

6.3.1 Optimisation of restriction enzymes

To determine the optimum concentration of restriction enzyme required, 1µg of PGL4-basic plasmid was digested overnight with 5-10units/µg BglII or XhoI. The BglII restriction enzyme successfully digested the entire PGL4-basic vector added (Figure 6.4A), therefore for all subsequent digestions, the BglII restriction enzyme was used at this concentration. The XhoI restriction enzyme failed to digest the entire vector at this concentration (Figure 6.4A). Therefore, the concentration of XhoI restriction enzyme was increased to 20units/µg DNA, which was shown to be successful at completely digesting the PGL4-basic plasmid (Figure 6.4B).
Figure 6.3 – The DNA sequence -3460bp to -2560bp (top) and -70bp to +77bp (bottom) upstream of the gastrin gene transcript, highlighting the locations of the putative gastrin HRE sequence, the endogenous XhoI restriction enzyme site and annealing site of the forward and reverse primers.

Figure 6.4 – A. Restriction digest of PGL4-basic plasmid with 5units/µg BgIII or 10units/µg XhoI. B. Restriction digest of PGL4-basic plasmid with 20units/µg XhoI.
6.3.2 Optimisation of PCR protocol

All PCR experiments performed during the preparation of the gastrin luciferase reporter plasmid used DNA extracted from HCT116 carcinoma cells, as gastrin mRNA was inducible under hypoxic conditions and was responsive to HIF-1α siRNA transfection in these cells.

The first few attempts at amplifying the DNA upstream of the gastrin gene transcript used standard PCR protocols; 40 cycles of 95°C for 1min, 60°C for 1min and 72°C for 3mins. These were however unsuccessful at amplifying the DNA for use in the gastrin luciferase reporter plasmid, despite the DNA successfully producing bands when amplified using other PCR protocols and primers (data not shown).

Reducing the annealing temperature of the standard PCR protocol (e.g. to 50°C or 55°C) had no effect on the success rate of the PCR in amplifying the DNA upstream of the gastrin gene transcript.

Therefore, it was decided to use a PCR protocol specifically designed to amplify long DNA fragments (i.e. up to 10kb in length). This protocol consisted of 35 cycles of 94°C for 30 seconds, 55°C for 1min and 68°C for 3.5mins (for the first 10 cycles), plus an additional 10 seconds per cycle for the remaining 25 cycles. The initial PCR protocol for long DNA fragments also failed (data not shown), however, the addition of Q-solution to the reaction resulted in the DNA fragment being successfully amplified, producing a single DNA band approximately 3.3kb in size (Figure 6.5). Q-solution helps to
increase the success-rate of DNA amplification of sequences that are GC-rich in nature or have a high level of secondary structure, both of which are common features found in promoter regions of DNA. Q-solution changes the melting behaviour of the DNA, allowing the Taq enzyme to make contact with DNA that has profound secondary structures.

**Confirmation of successful amplification of the DNA upstream of the gastrin gene transcript.**

Figure 6.5 – Successful amplification of the DNA upstream of the gastrin gene transcript using DNA from HCT116 cells. PCR protocol consisted of 94°C for 15mins, then 35 cycles of 94°C for 30 seconds, 55°C for 1min and 68°C for 3.5mins (for the first 10 cycles), plus an additional 10 seconds per cycle for the remaining 25 cycles.

### 6.3.3 Issues with promoter fragment concentration

The next stage of the gastrin luciferase reporter plasmid construction required the preparation of large batches of XhoI and BglII restriction enzyme-digested PGL4-basic vector and HCT116 promoter fragment ready for ligation. This required a minimum of 100ng vector and approximately 220ng promoter fragment to set up. Therefore, approximately 5μg of both the vector and DNA fragment prepared via PCR were purified via gel extraction, using the QIAquick gel extraction kit (Qiagen). These were then digested with the XhoI and BglII restriction enzymes (see Materials and Methods section 2.9.6), gel
purifying in between each digestion, due to the close proximity of the restriction digest sites. The images in Figure 6.6 show a batch of promoter fragment undergoing gel purification, prior to restriction digest by XhoI and BglII respectively. After each gel purification step, it was noted that a considerable amount of promoter fragment had been lost (Figure 6.6). Whilst the PGL4-basic vector was clearly visible (4.2kb in size), following the restriction digest and gel purification, the promoter fragment, which was expected to be 3.3kb in size, was undetectable alongside the vector.

Several repeats of DNA amplification and restriction digestion were performed in an attempt to prepare enough XhoI and BglII-digested promoter fragment for use in ligation. Altering the purification process from gel extraction to ethanol precipitation, increasing the number of cycles of the PCR program or increasing the initial concentration of DNA in the PCR reaction all failed to have a significant effect on increasing the yield of promoter fragment obtained.
Loss of promoter fragment after gel purification.

Figure 6.6 – Loss of HCT116 promoter fragment after gel purification and overnight restriction digestion with either XhoI or BglII.
6.3.4 Generation of gastrin luciferase reporter plasmid

In order to increase the yield of promoter fragment, for ligation into the PGL4-basic luciferase reporter vector, the whole PCR product was successfully subcloned into a TA cloning vector, pCRII-TOPO (Figure 6.7, Invitrogen), before being transformed into *E. coli*.

Ampicillin-resistant individual transformants (pTA Gasprom (i.e. contains the DNA upstream of the gastrin transcript)) were picked, grown up and plasmid DNA extracted using the Genelute plasmid miniprep kit. Linearisation of the pTA Gasprom transformants with the restriction enzyme HindIII confirmed the
insertion of the entire promoter fragment (3320bp in size) in the TA cloning vector, forming a plasmid of 7320bp in size (data not shown). The promoter fragment could have been inserted in one of two orientations; clockwise or anticlockwise (Figure 6.8).

![Possible plasmid maps of the pTA Gasprom transformants](image)

Figure 6.8 – Plasmid maps of the pTA Gasprom transformants. The promoter fragment could have been inserted in a clockwise (top) or anti-clockwise fashion (bottom).

Digestion of four of the clones with BglIII to determine insert orientation was found to only linearise the plasmid, suggesting the BglIII site with in the primer had been lost during PCR or cloning.
The pTA Gasprom transformants were therefore digested with both XhoI and HindIII, alongside the PGL4-basic vector to ensure the correct orientation of the promoter fragment within the luciferase reporter plasmid. The addition of the restriction enzyme ScaI further cleaved the TA cloning vector, allowing for easy identification of the promoter fragment. The triple digestion of the pTA Gasprom with XhoI, HindIII and ScaI produced three bands of approximately 3.4kb, 2.1kb and 1.5kb in size (data not shown).

The 3.4kb band contained the gastrin gene transcript required for the luciferase reporter plasmid and was ligated into the PGL4-basic vector, prior to being transformed into supercompetent TOPO10F’ E. coli bacteria (See Materials and Methods section 2.9.7).

This methodology resulted in the successful ligation of the 3.4kb promoter fragment into the PGL4 basic luciferase-reporter plasmid. Figure 6.9 shows a test restriction digest using the XhoI and HindIII enzymes, of four clones of the gastrin luciferase reporter plasmid, chosen at random. All four plasmids contained the PGL4-basic vector (4.2kb in size) and the inserted DNA, derived from upstream of the gastrin transcription start site (3.4kb in size). A simplified map of the gastrin luciferase reporter plasmid is also shown in Figure 6.9.
Confirmation of a successful plasmid via the restriction digest of plasmid with XhoI and HindIII.

Figure 6.9 – The plasmid map and restriction digest of gastrin luciferase reporter plasmids using restriction enzymes XhoI and HindIII, confirming the expression of the 3.4kb promoter fragment within the PGL4-basic plasmid.

6.3.5 Sequencing of the gastrin luciferase reporter plasmid

Gastrin luciferase reporter plasmid clones 1, 2 and 4 were sent away for sequencing at the Biopolymer Synthesis and Analysis Unit, in the Queen’s Medical Centre, Nottingham. The sequencing used the RV Primer3 (CTAGCAAAATAGGCTGTCCC), which anneals to the PGL4-basic plasmid at 4191-4210, approximately 60 base pairs upstream of the XhoI restriction digest site (i.e. the start of the DNA sequence from upstream of the gastrin gene transcript). There was a high level of homology seen between the DNA sequence from upstream of the gastrin gene transcript (line 4 of sequence) and clones 1, 2 and 4 (Lines 1, 2 and 3 respectively) (Figure 6.10).
The green boxes represent conserved residues within the sequence, whereas blue residues suggest identical residues and red residues suggest bases that are similar in shape, but not identical. The putative gastrin HRE sequence is highlighted by the clear black box in the centre of the sequence and is completely conserved throughout all four sequences. The shaded box at the top of the figure represents the location of the forward primer and highlights an area where the sequences are similar, but not identical.

6.4 Initial test transfections of gastrin luciferase reporter plasmids

From the batch of plasmids that were suggested to contain the DNA upstream of the gastrin gene transcript, four were chosen for further investigation, including the three clones that were sequenced. The four clones of the gastrin luciferase reporter plasmid were transfected into HCT116 cells at a concentration of 500ng/well, alongside the PGL4-(LDH)-HRE multimer luciferase reporter as a positive control and PGL4- basic as a negative control.

After 16hr hypoxic incubation, there was a significant 20-30% increase in the firefly luciferase expression in the HCT116 cells transfected with the four plasmid clones (Figure 6.11, p<0.03), when compared to the equivalent normoxic luciferase expression. However, transfection with the PGL4-(LDH)-HRE positive control plasmid induced an 800% increase in luciferase expression under hypoxic conditions (Figure 6.11, p<0.03), which was approximately 9-fold higher than that obtained after transfection with the gastrin luciferase reporter plasmids.
Figure 6.10 – Initial sequencing of gastrin luciferase reporter plasmids clones 1, 2 and 4 (lines 1, 2 and 3 respectively), in comparison with the DNA sequence of the DNA upstream of the gastrin gene transcript (line 4). Green represents conserved residues, blue represents identical residues and red represents similar residues (in shape).
Firefly luciferase expression after transfection with gastrin luciferase reporter plasmids.

HCT116 cells

In an attempt to optimise the hypoxic luciferase expression, HCT116 cells were transfected with increasing concentrations of gastrin luciferase reporter plasmid clone 4 (250ng/well, 500ng/well and 1000ng/well), alongside PGL4-(LDH)-HRE and PGL4-basic plasmids, before being exposed to hypoxia for increasing lengths of time.

Incubation under hypoxic conditions induced a significant 20-35% increase in the firefly luciferase expression within the HCT116 cells transfected with 500ng/well gastrin luciferase reporter plasmid (16hr and 24hr incubation) or
100ng/well (24hr incubation time point only), when compared to the equivalent normoxic luciferase expression (Figure 6.12, p<0.05).

Transfection with the PGL4-(LDH)-HRE positive control plasmid however, induced a 200-700% (time point-dependent) increase in luciferase expression under hypoxic conditions (Figure 6.12, p<0.05), which was up to 24-fold higher than that obtained after transfection with the gastrin luciferase reporter plasmid.

Figure 6.12 – Firefly luciferase expression in HCT116 carcinoma cells under both normoxic and hypoxic conditions, after transfection with increasing concentrations of gastrin luciferase reporter plasmid (GasProm4), PGL4-(LDH)-HRE or PGL4-basic control plasmid (*p<0.05 / **p<0.007 (analysed via student’s t-test), + S.D). Each bar represents combined data from duplicate transfections. n=1.
Further attempts at optimising the gastrin luciferase reporter plasmid transfection in an effort to increase the luciferase expression under hypoxic conditions included altering:

1) The carcinoma cell line transfected (e.g. changed from HCT116 to PAN1 carcinoma cells).

2) The media conditions (e.g. changed from FBS-containing media to serum-free media).

3) The endogenous control transfected (e.g. changed from β-Galactosidase to Renilla luciferase).

The introduction of any or all of these alterations to the transfection protocol failed to significantly improve the induction of luciferase expression under hypoxic conditions (data not shown). Therefore, all subsequent transfections using the gastrin luciferase reporter plasmid were performed using 500ng/well, with a 16hr hypoxic incubation.

### 6.5 Responsiveness of gastrin luciferase reporter plasmid transfection to exogenous EGF treatment

The sequencing of the gastrin luciferase reporter plasmids suggested that they contained the DNA sequence upstream of the gastrin gene transcript, however, to confirm the responsiveness of the gastrin promoter, HCT116 cells were transfected with the gastrin luciferase reporter plasmid (clone 4), prior to exogenous treatment with 10µg/ml EGF. The gastrin promoter is known to express an EGF-response element, upstream of the gastrin transcription start
site (Merchant et al., 1995). Therefore, treatment with EGF was expected to increase the activity of the gastrin luciferase reporter plasmid.

Exogenous treatment of EGF for 24hr resulted in an increase in luciferase expression by 30% under normoxic condition (Figure 6.13, p=0.0182) and 68% under hypoxic conditions (Figure 6.13, p=0.0141), when compared to the untreated PGL4-basic control. This data suggested that the gastrin luciferase reporter plasmids are partially responsive to EGF and therefore may contain the EGF-response element expressed in the gastrin promoter sequence.

![Firefly luciferase expression after transfection with gastrin luciferase reporter plasmid.](image)

Figure 6.13 – Firefly luciferase expression in HCT116 carcinoma cells under normoxic (left) and hypoxic conditions (right), after transfection with 500ng/well gastrin luciferase reporter plasmid (GasProm), or PGL4-basic control plasmid, prior to endogenous EGF treatment (16hr incubation) (*p<0.02 (analysed via a student’s t-test) + S.D). Each bar represents combined data from duplicate transfections. n=3 (for normoxic incubation) / n=2 (for hypoxic incubation) (data representative of replicate experiments).

### 6.6 Preparation and test transfections of Gastrin-HRE multimer reporter plasmid

In an attempt to further enhance the luciferase expression induced under hypoxic conditions after transfection with the gastrin luciferase reporter...
plasmid, 3-4 copies of the putative gastrin HRE sequence (5’-AGTGTATAAAGCGTGTGCACAGAC-3’, see Figure 6.1 for further information) were cloned into PGL4-(LDH)-HRE reporter plasmid, which expressed a luciferase gene and a SV40 minimal promoter. Essentially, the LDH HRE-multimer sequence was cleaved from the plasmid via BglII restriction digest, prior to the oligonucleotide expressing 3-4 copies of the putative gastrin HRE sequence being ligated in its place. The PGL4-(gastrin)-HRE plasmid were sequenced prior to use, to confirm that they expressed the putative gastrin HRE sequence (Figure 6.14).

### PGL4-(gastrin)-HRE 1 sequence (contains 3x putative Gastrin HRE sequence)

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTCTTTCTTATCCAGAAAAATTCTCTTGGCCTAACTGGCCGGTACCTGAGCTCCTAGGCTC</td>
<td>BglII</td>
</tr>
<tr>
<td>AGATCTAGTGTATAAAGCGTGTGCACAGACAGTGTATAAAGCGTGTGCACAGACAGTGTATAAAGCGTGTGCACAGACAGTGTATAAAGCGTGTGCACAGACAGTGTATAAAGCGTGTGCACAGACAGATCT</td>
<td>BglII</td>
</tr>
<tr>
<td>GCGATCTGCATCTGAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCTAACTCCGCCCATCCCGCCCATCCCGCCCATCGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAA</td>
<td>HindIII</td>
</tr>
</tbody>
</table>

### PGL4-(gastrin)-HRE 2 sequence (contains 4x putative Gastrin HRE sequence)

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGCCTCTTTTGATAGAAAAATTTTTCTCTTGGCCAACTGGCCGGTACCTGAGCTGCTAGGCTCCTGCAAGCTCTTATCCAGAAAAATTCTCTTGGCCTAACTGGCCGGTACCTGAGCTCCTAGGCTC</td>
<td>BglII</td>
</tr>
<tr>
<td>CATCTAGTGTATAAAGCGTGTGCACAGACAGTGTATAAAGCGTGTGCACAGACAGTGTATAAAGCGTGTGCACAGACAGTGTATAAAGCGTGTGCACAGACAGTGTATAAAGCGTGTGCACAGACAGATCT</td>
<td>BglII</td>
</tr>
<tr>
<td>GCGATCTGCATCTGAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCATCCCGCCCATCCCGCCCATCCCGCCCATCCCGCCCATCGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAA</td>
<td>HindIII</td>
</tr>
</tbody>
</table>

Figure 6.14 – initial sequencing of the PGL4-(gastrin)-HRE luciferase reporter plasmids 1 and 2, highlighting the expression of the putative gastrin HRE sequence.
Two clones of the PGL4-(gastrin)-HRE luciferase reporter plasmid were transfected into HCT116 cells, alongside the PGL4-(LDH)-HRE multimer luciferase reporter as a positive control and SV40 negative control plasmid as a negative control.

After 16hr hypoxic incubation there was a 20-30% decrease in the luciferase expression of cells transfected with the PGL4-(gastrin)-HRE plasmids, compared to the equivalent normoxic control expression (Figure 6.15, p=0.0006). Transfection with the PGL4-(LDH)-HRE positive control induced an approximate 2-fold increase under hypoxic conditions (Figure 6.15, p=0.0117). This data suggested that the putative HRE sequence located upstream of the gastrin gene transcript was not responsible for the up-regulation of the gastrin gene expression under hypoxic conditions.

![Firefly luciferase expression after transfection with gastrin-(HRE) reporter plasmid.](firefly_luciferase_expression.png)

Figure 6.15 – Firefly luciferase expression in HCT116 carcinoma cells under both normoxic and hypoxic conditions (16hr incubation), after transfection with 500ng/well PGL4-(gastrin)-HRE luciferase reporter plasmids, PGL4-(LDH)-HRE or SV40 negative control plasmid (*p=0.0117 / ***p=0.0006 (analysed via student’s t-test), + S.D). Each bar represents combined data from duplicate transfections. n=2 (data representative of replicate experiments).
6.7 Preparation and test transfection of further HRE multimer reporter plasmids derived from the DNA upstream of the gastrin gene

Incubation of a number of carcinoma cell lines investigated under hypoxic conditions induced a significant increase in gastrin gene expression. It has been suggested above that this increase was dependent on the activity of HIF-1α; however it was not dependent on the presence of the HRE sequence approximately 3000bp upstream of the gastrin transcription start site. Further screening of the DNA upstream of the gastrin gene was undertaken, using the Ensembl software to compare the DNA with the known HRE consensus sequence. This revealed four additional possible HRE sequences that could be responsible for the up-regulation of gastrin gene expression under hypoxic conditions (Table 6.1).

To determine whether any of the above sequences possessed a functional HRE sequence, multiple copies of the motifs were cloned into a PGL4 luciferase reporter plasmid, which expressed both a luciferase element and a SV40 minimal promoter. The six successful clones of the PGL-4-(gastrin)-HRE luciferase reporter plasmids (described in Table 6.1) were transfected into HCT116 cells, alongside the PGL4-(LDH)-HRE multimer luciferase reporter as a positive control and the SV40 negative control plasmid.

After 16hr hypoxic incubation, there was a 40-180% increase in the expression of the luciferase gene in all plasmid clones tested (Figure 6.16, p<0.02), when compared to the normoxic luciferase expression.
<table>
<thead>
<tr>
<th>Oligonucleotide construct name:</th>
<th>Repeated sequence (containing gastrin HRE)</th>
<th>Full oligonucleotide sequence</th>
</tr>
</thead>
</table>
| Oligonucleotide 2 (PGL4-(Gastrin)-HRE 3) | Forward: TGGCTCACGTCTG  
Reverse: ACCGAGTGCAGAC | Forward: 5’ GATCTTGGCTCACGTCTG  
TGGCTCACGTCTGTTGGCTCACGTCTG  
TGGCTCACGTCTG  
Reverse: 5’ GATCTCAGACGTGAGCCA  
CAGACGTGAGCCA  
CAGACGTGAGCCA  
CAGACGTGAGCCA  
CAGACGTGAGCCA 3’ |
| Oligonucleotide 3 (PGL4-(Gastrin)-HRE 4 and 5) | Forward: TTACAGACGTGAG  
Reverse: AATGTCTGCACTC | Forward: 5’ GATCTTTACAGACGTGAG  
TTACAGACGTGAGTTACAGACGTGAG  
TTACAGACGTGAG  
Reverse: 5’ GATCTCTCACGTCTGTAAA  
CTCACGTCTGTAA  
CTCACGTCTGTAA  
CTCACGTCTGTAAA  
CTCACGTCTGTAAA 3’ |
| Oligonucleotide 4 (PGL4-(Gastrin)-HRE 6 and 7) | Forward: GCCCACACGTGGCC  
Reverse: GGCACGTGAGCCA  
GGCACACGTGAGCCA  
GGCACACGTGAGCCA  
GGCACACGTGAGCCA 3’ | Forward: 5’ GATCTTGCGACACGTGAGCC  
GCCACGTGAGCCA  
GCCACGTGAGCCA  
GCCACGTGAGCCA  
GCCACGTGAGCCA 3’ |
| Oligonucleotide 5 (PGL4-(Gastrin)-HRE 8) | Forward: CCCAGGACGTGAG  
Reverse: GGGTCTGCACTC | Forward: 5’ GATCTCCCAGGACGTGAG  
CCCAGGACGTGAGCCCAGGACGTGAG  
CCCAGGACGTGAGCCCAGGACGTGAG  
CCCAGGACGTGAGCCCAGGACGTGAG 3’ |

Table 6.1 – Additional HRE sequences derived from the DNA upstream of the gastrin gene transcript, plus the oligonucleotides used to synthesise the plasmids.

Only half the increases however, induced under hypoxic conditions proved to be significant, and the majority of the luciferase expression induced by the PGL4-(gastrin)-HRE luciferase reporter plasmids were lower than that caused by the SV40 negative control plasmid (Figure 6.16).

This data suggested that the PGL4-(gastrin)-HRE reporter plasmids (and therefore the putative gastrin hypoxia-response elements) were only partially responsive to HIF-1α regulation. The data produced was inconclusive whether these new putative HRE sequences were responsible for the increase seen in the gastrin gene expression under hypoxic conditions. Further investigation is
required to determine whether one of these putative HRE sequences is responsible for the up-regulation of gastrin gene under hypoxic conditions.

Figure 6.16 – Firefly luciferase expression in HCT116 carcinoma cells under both normoxic and hypoxic conditions (16hr incubation), after transfection with 500ng/well PGL4-(gastrin)-HRE luciferase reporter plasmids, PGL4-(LDH)-HRE or SV40 negative control plasmid (*p<0.02 / **p<0.01 / ***p<0.0009 (analysed via student’s t-test), + S.D). Each bar represents combined data from duplicate transfections (n=1).

6.8 Chapter Summary and Conclusions

Screening of the DNA upstream of the gastrin gene transcript resulted in a putative gastrin HRE sequence being identified -2985bp to -2933bp upstream of the transcription start site. Within this sequence was a HRE-consensus sequence GCGTG (Wenger et al, 2005) required for the binding of the HIF-1 transcription factor into the major groove of target genes (Semenza et al, 1994; Wang and Semenza, 1995).

The DNA upstream of the gastrin gene transcript that contained the putative gastrin HRE sequence was successfully cloned into the PGL4-basic plasmid.
Exogenous treatment with epidermal growth factor induced a small increase in luciferase production under both normoxic and hypoxic conditions, suggesting that the putative gastrin-promoter (pTA Gasprom) plasmid contained the DNA upstream of the gastrin gene transcript. The gastrin promoter is known to contain an EGF-response element -54bp to -68bp upstream of the gastrin transcription start site (Merchant et al., 1991). The location of the EGF-response element in the PCR product was very close to the reverse primer site. At this location, it would be more likely to develop mutations within the sequence during the PCR process, which may account for some of the lower than expected responses to hypoxic conditions, although sequencing the current EGF-response element would confirm this. Future experiments would aim to amplify and clone the EGF response element with a longer region between the HRE and the primer site to determine if that affects the level of induction observed.

The pTA Gasprom plasmid only induced a small, but significant increase in luciferase expression under hypoxic conditions, suggesting the presence of an HRE sequence upstream of the gastrin coding region. Under the same conditions, the positive control plasmid induced a 9-fold greater response to hypoxia, when compared to that induced by the pTA Gasprom plasmids, although the positive control plasmid contained four HRE sequences versus the single putative gastrin HRE sequence and therefore was expected to produce a slightly larger response to hypoxia.
Comparison of the pTA Gasprom plasmid luciferase induction with an idealised PGL4-(LDH)-HRE luciferase reporter plasmid may also not have been appropriate. The PGL4-(LDH)-HRE consists of an optimised HRE 20bp multimer, whereas the pTA Gasprom plasmid consists of an endogenous promoter fragment. Therefore, repeating the transfections, using another endogenous promoter fragment, such as the VEGF-promoter plasmid, as a positive control may confirm the HRE sequence upstream of the gastrin coding region as a true hypoxia response element.

The isolation of the putative gastrin HRE sequence into PGL4-(gastrin)-HRE multimer luciferase reporter plasmids confirmed that the hypoxic induction of gastrin gene was not regulated by the HIF-1 transcription factor at this site. The addition of an Sp-1 binding site to the gastrin HRE sequence may also enhance the hypoxic induction of the gastrin gene, to produce a positive response. Welford et al (2006) have shown in mutational analysis experiments that an Sp-1 site adjacent to the HRE sequence caused an increase in HIF-1 transcriptional activity in a mouse embryonic fibroblast model, which were transfected with putative HRE constructs.

Further possible HRE sequences derived from the DNA sequence upstream of the gastrin gene transcript have now been discovered and are currently under investigation as to whether they are responsible for the up-regulation of the gastrin gene under hypoxic conditions. Screening these sequences for the presence of a Sp-1 binding site may aid in the selection of possible constructs for transfection.
Recently an alternative transcript has been discovered for the gastrin gene, which originates within intron one of the gastrin transcript (Grabowska et al., 2008). Screening of the DNA upstream of the new transcript has revealed the presence of the HRE consensus sequence, ACGTG, which will also be cloned into a luciferase reporter plasmid.

Therefore, it is currently inconclusive as to whether the gastrin gene is a true target of the HIF-1 transcription factor, or whether its expression is increased by a secondary indirect mechanism.
Chapter Seven
DISCUSSION
7.1 Study aims and objectives

The aim of this study was to investigate the relationship between the digestive hormone gastrin and hypoxia within GI carcinoma cells. The main finding from this study was that gastrin mRNA was significantly up-regulated under hypoxic conditions. This result led to the generation of a further objective, to investigate whether gastrin was a downstream transcriptional target of the HIF-1 transcription factor.

7.2 Gastrin and hypoxia – a putative link in GI cancers

Gastrin is a known pro-survival factor in the development of GI cancers, with roles in cell proliferation / tumour growth (Colucci et al, 2005; Haigh et al, 2003; Smith et al, 2004; Tsutsui et al, 1997), angiogenesis (Clarke et al, 2006; LeFranc et al, 2004), and invasion (Clarke et al, 2006; Wroblewski et al, 2002; Yu et al, 2006) and has also been linked with reduced apoptosis (Hartwich et al, 2001; Mao et al, 2008). The presence of increased serum gastrin concentrations (hypergastrinemia) is common occurrence in patients with GI cancers (Bombski et al, 2003; D’Agostino et al, 1995; Hartwich et al, 2001; Konturek et al, 2000; Mihas et al, 1995; Seitz et al, 1991; Thorburn et al, 1998), which would aid increased tumour growth and survival (Chu et al, 1995; McGregor et al, 1982; Watson and Smith, 2001). As the tumour expands, aided by the pro-survival actions of gastrin, the existing vasculature is unable to supply the entire tumour with its oxygen and nutrient requirements (reviewed in Vaupel et al, 1989), which would lead to the generation of areas of hypoxia. The presence of tumour hypoxia is known to increase resistance to both radiotherapy (Brizel et al, 1997; Nordsmark et al, 1996) and
chemotherapy (Hussein et al., 2006; Sermeus et al., 2008; Teicher et al., 1981; Teicher et al., 1990) in cancer patients.

The main finding of this study was that gastrin gene expression was up-regulated under hypoxic conditions. The increased presence of gastrin within hypoxic regions of tumours may aid cell survival, which may further stabilise the treatment resistance to both chemotherapy and radiotherapy. Under hypoxic conditions, the expression of the HIF-1α protein is stabilised (Wang and Semenza, 1993; Wang and Semenza, 1995; Wang et al., 1995), leading to the up-regulation of target gene transcription in solid tumours, which aids tumour growth and angiogenesis (Maxwell et al., 1997). The downstream signalling effects of both HIF-1 and gastrin show a level of redundancy, leading to the suggestion that gastrin may be a downstream transcriptional target of the HIF-1 transcription factor.

### 7.3 Alternate isoforms of hypoxia-inducible factors

The main focus of this study was the nuclear expression and function of the HIF-1α protein. There are however, three known isoforms of the hypoxia-inducible factor-alpha subunits; HIF-1α, HIF-2α and HIF-3α.

HIF-3α isoform is the least understood of the three proteins. It is believed that HIF-3α is responsible for the antagonism of HRE-dependent gene expression (reviewed in Ratcliffe, 2007), acting via different DNA binding elements, not found within either HIF-1α or HIF-2α (Maynard et al., 2007). HIF-3α reduces the ability of the other isoforms to bind to the HRE sequences and therefore
reduces their transcriptional activity (Maynard et al, 2007). Its expression is often down-regulated in cancers (Maynard et al, 2007) and therefore was not investigated in this study.

The HIF-2α isoform does play a role in target gene transcription via also forming a dimer with the HIF-1β subunit, and subsequently binding to the HRE sequences within promoter regions of target genes under hypoxic conditions (Tian et al, 1997), alongside the HIF-1 transcription factor, as highlighted in gastric, ovarian and glioblastoma cell lines (Koizume et al, 2008). Whilst the expression of the HIF-1α subunit is ubiquitous amongst mammalian cells, the HIF-2α subunit is limited to vascular endothelial cells, the kidney, heart, lung, astrocytes and the epithelium of the small intestine (reviewed in Gordan and Simon, 2007). HIF-2α protein is not the dominant isoform expressed throughout the GI tract. For example, in a study investigating the expression of HIF-alpha isoforms in oesophageal carcinomas, over 50% of the tumour samples showed strong expression of the HIF-1α protein, whereas only 13% of samples had HIF-2α staining (Koukourakis et al, 2001). In initial investigations via Western blotting into the expression of HIF-1α protein in the panel of GI carcinoma cells, HIF-2α expression was also investigated, however, as no HIF-2α protein was detectable (data not shown), no further work in this area was undertaken.
7.4 Effects of hypoxia on gastrin gene expression

The key finding of the study was that the expression of gastrin mRNA was significantly increased by 2.5-20 fold after 16 hr hypoxic incubation in the majority of GI carcinoma cell lines investigated. Within the panel of GI carcinoma cells, the HIF-1α nuclear protein was shown to be expressed (at low levels) after only 1 hr hypoxic incubation. The HIF-1 transcription factor was shown to be functionally active soon after, by inducing a significant increase in VEGF expression within 6-8 hr hypoxic incubation. The increase in gastrin mRNA expression however, was delayed compared to that of the VEGF expression, only being induced after 16 hr hypoxic incubation. This could suggest that the hypoxic induction of gastrin is induced by a downstream target of the HIF-1 transcription factor, such as VEGF or even another, currently unidentified transcription factor, rather than HIF-1 itself. The transcription factors Sp-1, c-fos and NF-κB are known to be responsible for the up-regulation of gastrin gene (Marks et al; 1996; Merchant et al, 1995; Ogasa et al, 2003). Further investigation is required to determine whether either is regulated by the HIF-1 transcription factor.

Investigations were designed to interrogate whether the increase seen in the gastrin mRNA expression was regulated via the HIF-1 transcription factor, as a result of a HIF-1-target gene activity, or a HIF-1-independent mechanism. The induction of HIF-1 target gene expression under hypoxic conditions is generally variable and is not necessarily dependent on the distance of the HRE sequence from the target gene transcript (Wenger et al, 2005). For example, the expression of the glycolytic enzyme aldolase A was inducible after only...
1hr under hypoxic conditions, whereas the expression of phosphoglycerate kinase 1 was only induced after 8hr hypoxic incubation (Semenza et al, 1994). The locations of their HRE sequences however, were both approximately 200bp upstream of the gene transcript (Wenger et al, 2005).

In an attempt to determine whether the increase in gastrin mRNA expression under hypoxic conditions was dependent on HIF-1 functional activity, three GI carcinoma cell lines were transfected with HIF-1α siRNA, before the expression of gastrin mRNA was investigated. Importantly, the HIF-1α siRNA had been shown to down-regulate both HIF-1α gene expression and resultant transcriptional activity by approximately 70-95% under both normoxic and hypoxic conditions. The affect of HIF-1α siRNA on HIF-1α protein expression was also investigated; however, the siRNA transfection produced a high level of cytotoxicity within the cells, resulting in a very low yield of protein being obtained. Therefore, the transcriptional activity of the HIF-1 transcription factor was investigated instead. Gastrin mRNA expression was down-regulated by 45-65% after transfection of HIF-1α siRNA, in the HCT116 and PAN1 cells only. The HIF-1α siRNA had no effect on gastrin mRNA of the gastric MGLVA1 cells, and in fact induced a small, but significant increase in the hypoxic gastrin mRNA expression. This suggested that the HIF-1 transcription factor only played a partial role in hypoxic gastrin gene induction, in a cell line-dependent manner.
7.5 Role of HIF in mediating the up-regulation of the gastrin gene

The original putative gastrin HRE sequence was located -2985bp to -2933bp upstream of the gastrin gene transcript, and shared a high level of homology with the VEGF HRE sequence, located approximately -985 to -939 upstream of the VEGF transcription start site (Forsythe et al, 1996). Whilst the putative gastrin HRE sequence appeared a large distance away from the transcription start site of the gene, other known functional HRE sequences at a similar distance from the start codon have been described. For example, the HRE sequences of connective tissue growth factor and endothelial nitric oxide synthase are located over 3000bp and 5000bp respectively upstream of the transcription start site (Wenger et al, 2005).

Using the gastrin promoter construct, a small, but significant increase in firefly luciferase expression was induced after 16hr hypoxic incubation, suggesting that the HIF-1 transcription factor was partially responsible for the induction of gastrin gene expression under hypoxic conditions. The luciferase expression induced using the gastrin promoter construct however, was still approximately nine-fold less than the positive control LDH-HRE construct tested.

In an attempt to enhance the hypoxic firefly luciferase expression, multimer putative gastrin HRE constructs were prepared, containing either three or four repeats of the gastrin HRE sequence. These constructs however, failed to induce luciferase expression, suggesting that the HRE sequence at -2985bp to -2933bp was not responsible for the induction of the gastrin mRNA expression under hypoxic conditions. The previous induction of luciferase was lost when
the HRE sequence was extracted from the full promoter. This could suggest that the induction of HIF-1 transcriptional activity needs more than just HIF-1 binding to a HRE sequence, for example interactions with possible co-factors or other transcription factors, such as Sp-1.

Further putative gastrin multimer HRE constructs were then prepared from alternate HRE sequences located upstream of the gastrin gene transcript. All six clones tested induced a small increase in hypoxic firefly luciferase expression, however, not all were significant, and none induced the 10-fold increase as seen in the positive control LDH positive control HRE construct. This again suggested that these alternative gastrin HRE sequences were not responsible for the induction of the gastrin mRNA expression under hypoxic conditions or were unable to act in isolation.

In a review of 108 genes proposed to be regulated by HIF-1 under hypoxic conditions, Wenger et al (2005) proposed the sequence of the ‘optimum’ hypoxia-response element, taken from the promoter elements of the HIF-1 target gene sequences. Figure 7.1 summarises the central region of the proposed HRE sequence, consisting of eight nucleotides. The core CGTG residues were conserved in all HRE sequences investigated, whereas there was a slight degree of flexibility either side of this consensus region. From Figure 7.1, the size of the residues in positions -2, -1, +5 and +6 indicates the probability of appearing in the consensus HRE sequence.
Table 7.1 lists the putative gastrin HRE sequences and their level of homology with the ‘optimum’ HRE sequence, as described in Figure 7.1. The original putative gastrin HRE sequence shares 63% homology with the ideal construct. The other four alternative HRE sequence all shared 88% homology with the ‘optimum’ HRE sequence. All five putative gastrin HRE sequences contained the central ‘CGTG’ motif. In theory, all five constructs should have induced a hypoxic response and yet none induced a strong luciferase induction. This response may however be considered as a positive effect, if it was compared to a more relevant positive control, such as the VEGF promoter plasmid. This plasmid contains the DNA upstream of the VEGF gene, and is therefore more comparable to the gastrin promoter plasmid, rather than the idealised PGL4-(LDH)-HRE reporter plasmid. Also, the addition of a Sp-1 site adjacent to the putative gastrin HRE sites may also up-regulate HIF-1 transcription (Welford et al, 2006).

<table>
<thead>
<tr>
<th>Plasmid clone</th>
<th>Putative HRE sequence</th>
<th>Level of homology with optimum HRE sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGL4-(gastrin)-1/2</td>
<td>AGCGTGCG</td>
<td>5/8 (63%)</td>
</tr>
<tr>
<td>PGL4-(gastrin)-3</td>
<td>GACGTGAG</td>
<td>7/8 (88%)</td>
</tr>
<tr>
<td>PGL4-(gastrin)-4/5</td>
<td>GACGTGAG</td>
<td>7/8 (88%)</td>
</tr>
<tr>
<td>PGL4-(gastrin)-6/7</td>
<td>CACGTGGC</td>
<td>7/8 (88%)</td>
</tr>
<tr>
<td>PGL4-(gastrin)-8</td>
<td>GACGTGAG</td>
<td>7/8 (88%)</td>
</tr>
</tbody>
</table>

Table 7.1 – Putative gastrin HRE sequences in comparison with the ‘optimum’ HRE sequence.
A recent study by Grabowska *et al* (2008) has identified an alternative gastrin transcript within the PAN1 and other GI cell lines, which differed from that listed in the Ensembl database. The new, alternative transcript of the gastrin gene originated within intron one of the gastrin transcript and was probably driven by a promoter that is present further upstream within intron one of the gastrin gene. Translation of alternative transcript is increased in conditions of ‘stress’, which included hypoxia (Grabowska *et al*, 2008).

Using the Ensembl software, the non-coding gastrin intron 1 sequence was scanned for the presence of the core HRE sequence. A putative gastrin HRE sequence (ACGTG) was identified within intron 1 of the gastrin gene, from +524bp to +528bp. The identification of a further putative gastrin HRE sequence upstream of the new transcript could provide an explanation for the increase in gastrin mRNA expression induced under hypoxic conditions. Therefore, an intron 1 gastrin HRE luciferase reporter plasmid is currently being constructed and investigated for the induction of firefly luciferase expression under hypoxic conditions. No data is available as of yet.

### 7.6 Alternative mechanisms for up-regulation of gastrin under hypoxia

If the hypoxic gastrin mRNA expression was only partially regulated via the HIF-1 transcription factor, other mechanisms may also be involved in the regulation of gastrin expression, either working in combination with HIF-1 or are HIF-1-independent.
If the HIF-1 transcription factor failed to induce transcriptional activation of the gastrin gene, we should consider the possibility that HIF-2 plays a role in the regulation of gastrin expression, despite the known lack of HIF-2α expression within the GI tract; it could still play an important role within emerging tumour cells. The HIF-1 and HIF-2 transcription factors have been shown to regulate both distinct and overlapping groups of genes, in a cell-type-dependent manner (Koizume et al, 2008; Lau et al, 2007 and reviewed in Taylor, 2008), suggesting that they play different roles in tumour progression. HIF-1α is mainly responsible for activating genes required for glycolysis (i.e. the glycolytic enzymes, such as lactate dehydrogenase and phosphoglycerate kinase) and apoptosis (e.g. BNIP3), whereas HIF-2α is solely responsible for the expression of key proliferative genes, such as transforming growth factor-α and cyclin-D1, and the de-differentiation protein Oct4 (Hu et al, 2007; Lau et al, 2007; Raval et al, 2005 and reviewed in Gordan and Simon, 2007). Both HIF-1α and HIF-2α proteins however, are responsible for the activity of pro-angiogenic genes, (such as VEGF, platelet-derived growth factor and Angiopoietin-2) and pro-metastatic and invasion genes (such as CXCR4 (CXC chemokine receptor 4), MMP-2 and lysyl oxidase) (Raval et al, 2005 and reviewed in Gordan and Simon, 2007). Generally, tissue that has high HIF-2α expression shows lower HIF-1α expression and vice versa (Koizume et al, 2008; Koukourakis et al, 2001), therefore, the expression of the overlapping genes is generally controlled by the dominant HIF-alpha isoform present in the cell (Carroll and Ashcroft, 2006; Koizume et al, 2008; Sowter et al, 2003).
Both HIF-1α and HIF-2α isoforms bind to the same HRE sequence within the promoter region of target genes under hypoxic conditions and share similar structural motifs, such as the ODD domain, dimerisation domain, DNA binding motifs and conserved regulatory elements (i.e. prolyl and asparagyl hydroxylation sites) (Lau et al, 2007; Tian et al, 1997). Unlike the HIF-1 transcription factor, which simply has to bind to the HRE sequence within the promoters of target genes to initiate transcription, HIF-2 requires the involvement of the N-terminal and C-terminal transactivation domains to differentially bind to the promoter to initiate any downstream effects (Koizume et al, 2008; Lau et al, 2007). Lau et al (2007) showed that if the C-terminal domain of the HIF-1α protein was replaced with the C-terminal domain of HIF-2α, the HIF-1 transcription factor gained the ability to regulate HIF-2α-specific target genes. Similar results were also induced via the replacement of the HIF-1α N-terminal transactivation domain with the HIF-2α equivalent (Hu et al, 2007). Therefore, just expressing the target gene HRE sequence within a luciferase construct would not allow for HIF-2 transcriptional activation, as the sequence would lack the additional regulatory elements. Further elements of the promoter region would also have to be added to the luciferase reporter construct to determine what effect HIF-2α had on target gene transcription.

Gastrin has previously been linked to cell proliferation, angiogenesis and invasion in GI carcinoma cells, which are similar properties to those induced by HIF-1/ HIF-2 transcription factors. Therefore it could be suggested that the increase hypoxic gastrin gene expression was regulated via HIF-1 and HIF-2, acting alone or possibly in cooperation with each other. The use of HIF-1α
siRNA only partially inhibited the induction of gastrin mRNA expression under hypoxic conditions, in both PAN1 and HCT116 cell lines. To investigate whether the HIF-2 transcription factor plays a role in the regulation of the gastrin gene, the effect of HIF-2α siRNA transfection on gastrin gene expression should be determined. Also, the gastrin promoter luciferase reporter construct could be transfected into a HIF-2α-dominant carcinoma cell line, such as RCC4, to determine whether the HIF-2 transcription factor could further increase the induction of luciferase expression, compared to that achieved via HIF-1.

The HIF-1 transcription factor was only partially responsible for the large increase seen in hypoxic gastrin gene in a cell-line specific manner. This may imply the need for another transcription factor, acting in synergy with HIF-1 to increase target gene expression. For example, under hypoxic conditions, the key tumourigenic protein β-catenin is competitively-inhibited from binding to the TCF transcription factor, therefore preventing the up-regulation of β-catenin/TCF target genes, such as c-myc and cyclin D1 (Kaidi et al, 2007). Instead, β-catenin is suggested to form a complex with HIF-1α, which then binds to promoter regions of HIF-1 target genes, increasing HIF-1-dependent transcription (Kaidi et al, 2007), which may include the gastrin gene. To determine whether the addition of β-catenin further augments the transcriptional activity of HIF-1, the induction of luciferase expression after transfection with the gastrin promoter construct should be investigated in the presence of increasing concentrations of β-catenin.
Alternatively, HIF-1α has been shown to interact with the Notch transcription factor, specifically at its intracellular domain, which leads to enhanced recruitment of HIF-1 to the promoters of target genes, such as GLUT1 and lysyl oxidase, and therefore increasing its transcriptional activity (Sahlgren et al, 2008; Zheng et al, 2008). The Notch transcription factor has a role in cell development and proliferation, and is especially important in the differentiation of stem cells (reviewed in Sahlgren et al, 2008). Again, to determine whether the Notch transcription factor played a role in augmenting the hypoxic expression of the gastrin gene, the induction of luciferase expression after transfection with the gastrin promoter construct should be investigated in the presence of a dominant-negative Notch construct. Similar findings have also been identified with the HIF-2 transcription factor, as HIF-2α has been shown to act in cooperation with the transcription factor Elk-1, upregulating the expression of HIF-2α-specific target genes, such as cited-2 (CBP/p300-interacting transactivator, with glutamic acid (E) and aspartic acid (D)-rich tail-2) and erythropoietin (Hu et al, 2007), both of which may also be linked to gastrin gene expression.

If the increase in gastrin gene expression induced under hypoxic conditions partially involved HIF-1α or HIF-2α and alternative HIF-independent mechanisms, potential mechanisms may involve the transcriptional coactivator PGC-1α (peroxisome-proliferator-activated receptor-γ coactivator-1α), which alongside HIF-1α, is induced under hypoxic conditions and is responsible for the induction of VEGF expression and the resultant angiogenesis in skeletal muscle cells (Arany et al, 2008). PGC-1α was shown to induce angiogenesis
in a HIF-1α-independent fashion, via forming a transcriptional complex with the orphan nuclear receptor ERR-α (Estrogen receptor-related receptor-alpha), that binds to ERR-α binding sites (AAGGTCA) within the promoter of target genes, instead of an HRE sequence (Arany et al., 2008). It is possible that PGC-1α may also regulate the pro-angiogenic gastrin gene and therefore, the gastrin promoter should be investigated for the presence of ERR-α binding sites.

Another possible indirect mechanism behind the hypoxia-induced gastrin expression could involve targeted RNA sequestration via miRNA (Micro-ribonucleic acid)-dependent inhibition of mRNA translation. miRNA are formed when hair-pin RNA molecules are cleaved into 21-23 nucleotide molecules (with a 2-3 nucleotide overhang) via Dicer enzymes (reviewed in Farazi et al., 2008; Liu et al., 2005), before being incorporated into ‘RISC’ (RNA-induced silencing complex), which also recruit Argonaute proteins (reviewed in Hutvagner and Simard, 2008; Meister, 2007). The Argonaute proteins, associated with the miRNA-RISC complex binds to the 3’-untranslated region of the mRNA strand, allowing the Argonaute proteins to compete with the eukaryotic translation initiation factor eIF4E for the cap binding, therefore preventing ribosomal association and the initiation of translation of the mRNA (reviewed in Hutvagner and Simard, 2008; Meister, 2007). The Argonaute proteins are also required for untranslated mRNA translocation to specific cytoplasmic ‘processing’ (P) bodies, where the mRNA can be either stored or degraded (Liu et al., 2005). The increase in gastrin mRNA expression under hypoxic conditions could be due to increased
sequestration of untranslated gastrin mRNA within the P bodies. This theory suggests that gastrin is not involved in tumour progression under hypoxic conditions, but may suggest that it is needed for recovery from hypoxia.

Alternatively, the expression of the gastrin mRNA may be stabilised under hypoxic conditions via binding to ‘RNA binding proteins’ at its 3’-untranslated region. Previous studies using VEGF mRNA revealed that the VEGF transcript binds to the double-stranded RNA-binding proteins, such as HuR, TIA-1 (T-cell intracellular antigen) and 76/NF90 (Levy et al, 1998; Nabors et al, 2001; Suswam et al, 2005; Vumbaca et al, 2008), which led to increased hypoxic VEGF mRNA stability and therefore increased VEGF protein secretion, in a HIF-1-independent fashion. The binding protein targets AU-rich elements at the 3’-untranslated region of the target mRNA transcript (Nabors et al, 2001; Suswam et al, 2005). Repression of RNA binding proteins reduced both mRNA stability and ribosomal association, leading to a reduction of hypoxic VEGF expression and resultant angiogenesis within breast carcinoma orthotopic mouse model (Levy et al, 1998; Vumbaca et al, 2008). RNA binding proteins were consistently expressed in tumour models, especially in areas adjacent to necrotic regions, where pro-angiogenic factors, such as VEGF were expressed (Nabors et al, 2001). Stabilisation of the gastrin transcript, via RNA-binding proteins would increase tumour progression; therefore, the 3’-untranslated region of the gastrin mRNA should be screened for RNA-binding protein elements.
7.7 Effects of gastrin on HIF-1α expression

Exogenous amidated gastrin treatment of GI carcinoma cells only induced an increase in nuclear expression of the HIF-1α protein in the PAN1 carcinoma cell line, under normoxic conditions, despite several other carcinoma cell lines being investigated, including those from both similar and different tissues of origin. The effect seen in the PAN1 cells was induced with either the 1nM or 10nM gastrin concentration, however, was extremely weak and often difficult to visualise and interpret.

Hypoxic HIF-1α protein expression was also investigated after exogenous treatment with amidated gastrin and again there was a slight indication that 1nM gastrin induced HIF-1α expression in the PAN1 cells, although this was not significant. The data was often difficult to quantify, due to the high levels of nuclear HIF-1α protein expression that were naturally induced under hypoxic conditions. Any effect of the gastrin treatment would be masked by the strong HIF-1α induction. Therefore, despite the evidence suggesting that normoxic signalling pathways, such as those involving PI3-kinase / Akt, MAPK and possibly gastrin signalling can induce nuclear HIF-1α accumulation, the actual expression levels achieved are lower than those induced via hypoxia (Li et al, 2005). The increase induced by these pathways under hypoxic conditions probably had little cumulative effect on the total HIF-1α expression, compared to the amount induced via true hypoxia alone.

Despite the lack of clear evidence of stabilisation of HIF-1α protein as a result of gastrin signalling, the resultant mRNA expression of its target gene VEGF
was significantly increased after treatment with 1nM or 10nM exogenous gastrin, under normoxic conditions. To determine whether the increase in VEGF expression was a HIF-1α-mediated effect, cells transiently-transfected with either the VEGF-HRE or PGL4-(LDH)-HRE luciferase reporter plasmids were treated with either 1nM or 10nM exogenous gastrin.

The treatment with amidated gastrin had no significant effect on the induction of firefly-luciferase expression, when compared to that induced by the scrambled control, which suggested that gastrin did not induce HIF-1α expression or activity. This also indicates that the significant increase in VEGF mRNA expression induced after exogenous gastrin treatment was not dependent on HIF-1α expression, but other growth factor signalling pathways, such as NF-κB-signalling pathway (reviewed in Miknopadhyay and Datta, 2004; Pradeep et al, 2005).

Treatment with amidated gastrin had no significant effect on firefly luciferase induction or nuclear HIF-1α protein expression, with the exception of PAN1 carcinoma cells. It is not however, only the final amidated products of the gastrin gene that induce biological effects; processing intermediates, such as progastrin and gly-gastrin have been speculated to have their own independent biological roles. Both progastrin and gly-gastrin have been shown to induce proliferation and development of the colonic mucosa (Cobb et al, 2004; Hollande et al, 1997; Koh et al, 1999; Koh et al, 2004; Ogunwobi and Beales, 2006; Siddheshwar et al, 2001; Singh et al, 2003; Wang et al, 1996). Some studies have suggested that colorectal carcinomas express progastrin and gly-
gastrin over that of amidated gastrin (Hollande et al 1997; Thorburn et al, 1997). It is possible that exogenous treatment of GI carcinoma cells with either progastrin or gly-gastrin may up-regulate the expression or transcriptional activity of the HIF-1 transcription factor, instead of amidated gastrin.

The data produced after treatment with exogenous amidated gastrin does not agree with a study conducted by Chao et al (2006), which used mice xenograft models, generated using HEK-293 cells, transfected with either the wild-type CCK-2 receptor or its splice variant CCK\textsubscript{2i4}, which retains intron 4 that is normally excised from the wild-type receptor. Expression of the splice variant CCK\textsubscript{2i4} receptor increased xenograft tumour growth as a result of endogenous gastrin signalling. It acted via up-regulation of HIF-1α and its target gene VEGF in a Src-dependent fashion, leading to increased tumour microvessel density. Expression of the wild-type CCK-2 receptor however, failed to induce similar increases in tumour proliferation and angiogenesis. This data suggests that the increase in HIF-1α protein expression induced in the PAN1 carcinoma cells under normoxic conditions may have been a genuine result, which failed to reach significance as it only involved a sub-set of cells that possibly expressed the CCK-2 receptor splice variant. Over-expressing the CCK\textsubscript{2i4} receptor in a panel of GI carcinoma cells would help determine whether HIF-1α protein expression and transcriptional activity was induced via gastrin signalling through this alternative receptor.
7.8 Effects of gastrin on HIF-1α expression – possible mechanisms of action

There are two main pathways that gastrin could utilise, to induce HIF-1α protein stabilisation; the PI3-kinase / Akt signalling pathway or the NF-κB signalling pathway. Gastrin has been shown to up-regulate the expression of activated (i.e. phosphorylated) Akt protein, in a CCK-2 receptor-dependent fashion (Harris et al., 2004; Todisco et al., 2001), which enhanced cell survival in a pro-angiogenic and anti-apoptotic manner. The activated Akt protein has not been directly linked to the stabilisation of HIF-1α protein. Instead, downstream targets of Akt signalling, such as mTOR and FRAP have been suggested to stabilise HIF-1α expression under normoxic conditions, as shown using PI3-kinase, FRAP or mTOR target-specific inhibitors (Hudson et al., 2002; Lang et al., 2007; Laughner et al., 2001; Treins et al., 2002; Zhong et al., 2000). To investigate whether gastrin induced HIF-1α expression via the PI3-kinase / Akt signalling pathway, the GI carcinoma cells could be pre-treated with either Akt pathway inhibitors (e.g. LY294002 (for PI3-kinase) or rapamycin (for mTOR/FRAP)) or pathway activators (e.g. dominant-negative Akt construct) before the effect on HIF-1α expression was determined, following exogenous gastrin treatment.

Gastrin has also been shown to activate NF-κB signalling, via initiating the degradation of the inhibitory protein IκB (inhibitor of NF-κB) by its kinase, IκB-kinase (IKK), leading to IκB ubiquitination and resultant proteasomal degradation, via a CCK-2-receptor-PKC-dependent signalling mechanism (Ogasa et al., 2003). NF-κB is normally sequestered in the cytoplasm via the actions of IκB, but is released upon IκB phosphorylation and is then free to
translocate to the nucleus, up-regulating target gene expression. The activation of IKK (especially the \( \beta \)-isoform) in macrophages has also been linked to NF-\( \kappa \)B nuclear translocation, which leads to increased levels of HIF-1\( \alpha \) protein accumulation and increased target gene expression, such as VEGF, COX-2 and GLUT1, as well as the usual increase in cytokine and chemokine expression (Rius et al, 2008), as it induces the degradation of I\( \kappa \)B, therefore up-regulating NF-\( \kappa \)B activity. In this study, NF-\( \kappa \)B activity was also linked to increased HIF-1\( \alpha \) mRNA expression under normoxic conditions, as well as increased HIF-1\( \alpha \) protein expression under hypoxic conditions, as the HIF-1\( \alpha \) promoter contains a NF-\( \kappa \)B response element, located at -197bp to -188bp (Rius et al, 2008). NF-\( \kappa \)B translocation was shown to occur prior to HIF-1\( \alpha \) protein accumulation, suggesting that in macrophages, NF-\( \kappa \)B may control HIF-1\( \alpha \) expression under hypoxic conditions (Rius et al, 2008).

If gastrin was responsible for the up-regulation of HIF-1\( \alpha \) expression, it would induce the expression of several pro-survival characteristics, which would be beneficial for solid tumour progression. Alongside the increase in VEGF, stem cell factor and glycolytic enzyme activity (Forsythe et al, 1996; Han et al, 2008; Semenza et al, 1994), which would aid neovascularisation (in a VEGF-receptor and EGF-receptor-dependent mechanism) and anaerobic respiration, an increase in HIF-1\( \alpha \) activity would also aid tumour progression, metastasis and invasion. Therefore, the up-regulation of the HIF-1 transcription factor could be the mechanism behind some of pro-malignant effects induced by gastrin.
In a study investigating the significance of HIF-1α in the progression of gastric cancer, HIF-1α protein expression was shown to increase in both density and intensity as tumourigenesis progressed from *Helicobacter pylori*-associated gastritis, through intestinal metaplasia and epithelial dysplasia to full gastric adenocarcinoma (Griffiths *et al.*, 2007). The HIF-1α expression was again significantly associated with the invading tumour margin and areas of necrosis, as seen in previous studies (Mizokami *et al.*, 2006; Zhong *et al.*, 1999), as well as macrophages, indicating a role of HIF-1α in an inflammatory response. In another study, HIF-1α protein expression was also significantly correlated with the depth of tumour invasion and therefore was considered as a poor patient prognostic factor, despite only being expressed in 16% of gastric tumour samples tested (Oh *et al.*, 2008). HIF-1α was responsible for the up-regulation of pro-invasion target genes, such as MMP-2, extracellular matrix proteases and uPAR (urokinase plasminogen activator receptor), as highlighted in HCT116 carcinoma cells (Krishnamachary *et al.*, 2003). HIF-1α was also responsible for up-regulating the expression of pro-metastatic proteins TWIST and lysyl oxidase (Erler *et al.*, 2006; Yang *et al.*, 2008), aiding tumour metastasis and therefore increasing levels of treatment failure and patient mortality. TWIST is another bHLH transcription factor, which is normally required during development for gastrulation and mesoderm-specification, but has been implicated in cancer metastasis, being associated with aggressive tumour progression and poor patient outcome (reviewed in Peinado and Cano, 2008). HIF-1 regulates the expression of both TWIST and lysyl oxidase via binding to an HRE sequence within their promoters (Erler *et al.*, 2006; Yang *et al.*, 2008). The induction of lysyl oxidase enhanced epithelial-to-mesenchymal
transition, a key process behind tumour cell motility and invasion (Higgins et al, 2007; Sahlgren et al, 2008).

A similar metastatic phenotype is also generated as a result of gastrin signalling, which is known to play a role in tumour cell invasion and extracellular membrane remodelling. For example, gastrin has been shown to up-regulate the expression of molecules responsible for the degradation of the extracellular matrix, such as MMP-9 in both cell and patient models (Wroblewski et al, 2002). As a result, gastrin induced the invasion of basement membranes in a MMP-9-dependent fashion, acting via a CCK-2 receptor-PKC-Raf-MEK-dependent signalling pathway (Wroblewski et al, 2002). Gastrin also induced the expression of focal adhesion kinase, a key molecule in tumour malignancy, in a CCK-2 receptor-dependent manner (Yu et al, 2006), which resulted in increased colon cancer invasion and lymph node metastasis. Finally, the presence of hypergastrinemia has been associated with liver metastasis in colorectal cancer patients (Kameyama et al, 1993). The up-regulation of a metastatic and invasive phenotype, induced by gastrin signalling could have been transmitted via the HIF-1 transcription factor, which would result in the up-regulation of the expression of MMPs and pro-metastatic proteins to promote tumour invasion.
7.9 Biological and clinical implications of the up-regulation of gastrin in GI carcinoma under hypoxic conditions

The induction of the gastrin gene under hypoxic conditions would have implications throughout all stages of tumour progression, from its initiation, through to development of metastatic phenotype.

The expression of the gastrin gene has been shown to be regulated by the β-Catenin/TCF-dependent pathway (Koh et al, 2000), which would allow for increased gastrin signalling acting via its CCK-2 receptor. However, in what could be described as a positive feedback pathway, gastrin has also been shown to up-regulate the expression of the β-catenin gene, which leads to increased colorectal tumour growth and survival, by up-regulating β-catenin/TCF target gene expression (Cao et al, 2006; Pannequin et al, 2007), which includes cyclin D1 and c-myc.

Under physiological conditions, HIF-1α protein has been shown to sequester Max, the dimerisation partner of c-myc, either directly or via the up-regulation of the alternate binding partner MXI-1, therefore preventing c-myc transactivation (reviewed in Dang et al, 2008; Corn et al, 2005; Koshiji et al, 2004). HIF-1α also directly blocks the activity of c-myc, either by directly competing for binding to the promoter (as both HIF-1α and c-myc use the consensus sequence, ACGTG) (Koshiji et al, 2004), or via competing with TCF for interaction with β-catenin (Kaidi et al, 2007). Under pathological conditions, which may include hypoxia, c-myc can become de-regulated. This deregulation of c-myc has been described in approximately 30% of cancers,
including colorectal carcinomas (reviewed in Dang et al, 2008), and even may be as a result of gastrin signalling up-regulating the β-catenin signalling pathway.

HIF-1α has been suggested to act in co-operation with the deregulated c-myc protein, promoting key cellular functions, such as glycolysis and angiogenesis via up-regulating hexokinase 2 and VEGF expression respectively (Kim et al, 2007; Robey et al, 2008). There is a level of redundancy between the transcriptional targets of the c-myc and HIF-1 transcription factors (possibly due to the similarity in both their recognition sequences), as they both lead to the induction of key metabolic and angiogenic gene expression, which would further aid tumour survival and neovascularisation. Whilst the expression of hexokinase 2, pyruvate dehydrogenase and VEGF have been shown to be induced via the two transcription factors working in co-operation (Kim et al, 2007), the expression of other target genes such as lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase show no such co-operation and therefore are up-regulated independently by the two transcription factors (reviewed in Dang et al, 2008). The up-regulation of respiration induced via deregulated c-myc activity (in co-operation with HIF-1α), leads to the increased uptake of glucose and corresponding production of lactate, even in the presence of oxygen. This is known as ‘The Warburg Effect’, which is defined as “the propensity for cancer cells to convert glucose to lactate in the presence of adequate oxygen levels” (Dang et al, 2008). Tumour cells take up higher levels of glucose in an attempt to keep up with their increased metabolic demands. This altered glycolysis can be further enhanced via over-expression
of either c-myc or HIF-1α (as a result of oncogenic mutations), and would result in increased tumour survival. To determine whether the increase of deregulated c-myc was gastrin-dependent, the expression of c-myc under hypoxic condition should be investigated, in the presence of anti-gastrin agents, such as gastrin siRNA, CCK-2 receptor antagonists or gastrin immunogens.

The presence of hypergastrinemia is a common occurrence in patients with GI cancer (Bombski et al, 2003; D’Agostino et al, 1995; Hartwich et al, 2001; Konturek et al, 2000; Mihas et al, 1995; Seitz et al, 1991; Thorburn et al, 1998), which would aid increased tumour growth and survival (Chu et al, 1995; McGregor et al, 1982; Watson and Smith, 2001), by inducing pro-survival characteristics, including cell growth (Colucci et al, 2005; Haigh et al, 2003; Smith et al, 2004; Tsutsui et al, 1997), angiogenesis (Clarke et al, 2006; LeFranc et al, 2004), and invasion (Clarke et al, 2006; Wroblewski et al, 2002; Yu et al, 2006), producing a tumour that is solid in nature.

As the tumour expands, aided by the pro-survival actions of gastrin, the existing vasculature is unable to supply the entire tumour with its oxygen and nutrient requirements (reviewed in Vaupel et al, 1989), which would lead to the generation of areas of hypoxia. The induction of hypoxia would further increase the expression of gastrin, as shown using both gene and protein expression, which could further aid cell survival and possibly induce metastasis of the tumour to other sites.
The induction of tumour hypoxia is known to increase resistance to both radiotherapy (Brizel et al., 1997; Nordsmark et al., 1996) and chemotherapy (Hussein et al., 2006; Sermeus et al., 2008; Teicher et al., 1981; Teicher et al., 1990) in cancer patients. The increased presence of gastrin within hypoxic regions of tumours may further increase resistance to both chemotherapy and radiotherapy (Wang and Semenza, 1993; Wang et al., 1995).

As gastrin may play a role in the development and progression of GI tumours, it may be beneficial to include anti-gastrin therapies in the treatment strategies of patients. These could include CCK-2-receptor antagonists, such as YM022 (Kitano et al., 2000) or Z-360 (Grabowska et al., 2008; Kawasaki et al., 2008), which would act at the gastrin receptor, blocking the transmission of any pro-survival processes. Alternatively, the addition of an immunogen that induces the production of antibodies against gastrin, such as G17DT (Gilliam et al., 2004), would allow for gastrin to be sequestered (via binding at their N-terminus (Watson et al., 1999)), therefore neutralising any trophic effects it may induce. The addition of a bioreductive, such as tirapazamine, would sensitise the hypoxic component of the metastatic tumour to both radiotherapy and chemotherapy (Adams et al., 2008; Masunaga et al., 2000) and therefore may increase the efficiency of the anti-gastrin agent.

Thus in conclusion, this study has further clarified the prevalence and potential importance of hypoxia in GI cancer, and shows a link to a common deregulated hormone implicated in GI carcinogenesis. The study has therefore identified a new therapeutic approach for the treatment of GI cancer patients,
(by combining anti-gastrin agents with bioreductive drugs), which is urgently required, and includes a possible mechanisms to overcome radio and chemotherapeutic resistance.

7.10 Summary of future work

Firstly, the hypoxic expression of gastrin would be examined, using either mouse models or human tumour samples, to confirm that gastrin is up-regulated under hypoxic conditions within a true pathological situation. Regions of hypoxia / HIF-1α expression would be correlated with gastrin expression (using in situ hybridisation), ensuring that the samples were fixed quickly after tissue harvesting to preserve the presence of HIF-1α, which has a half-life of less than 5 minutes under normoxic conditions (Wang et al, 1995).

Next, the putative gastrin HRE sequence DNA should be rescreened for the presence of an adjacent, downstream Sp-1 binding site, as this is suggested to augment HIF-1 transcriptional activity (Welford et al, 2006). The presence of a Sp-1 site, plus the use of an alternative positive control (i.e. the VEGF-promoter plasmid) may prove one or more of the putative gastrin HRE constructs successful. Also the HRE sequence within the alternative gastrin transcript should be cloned into a luciferase reporter plasmid and investigated. Multiple carcinoma cell lines have been shown to express this alternate transcript (Grabowska et al, 2008) and therefore would express the alternative putative gastrin HRE sequence.
Next, the other possible mechanisms that may explain the increase in gastrin gene under hypoxic conditions require investigation. These included:

- The role of the HIF-2 transcription factor. As the HIF-1 transcription factor was shown to be only partially responsible for the induction of gastrin under hypoxic conditions, it is possible that the gastrin gene is at least partially regulated by the HIF-2 transcription factor. Whilst the HIF-1 and HIF-2 transcription factor share a level of redundancy in their target gene expression, they are also responsible for the regulation of unique genes. For example, HIF-2 is solely responsible for cyclin D1 and Oct4 expression regulation under hypoxic conditions (reviewed in Gordan and Simon, 2007). Therefore, gastrin gene expression should be investigated after transfection of HIF-2α siRNA (alone and in combination with HIF-1α siRNA). Also, hypoxic gastrin expression should be investigated in a HIF-2α-dominant cell line, such as RCC4.

- The need for a second, unrelated transcription factor. The protein β-catenin was suggested to act in synergy with HIF-1α to up-regulate target gene expression (Kaidi et al, 2006) and has also been implicated in the regulation of gastrin gene expression itself (Koh et al, 2000). Therefore, to investigate whether HIF-1α and β-catenin dually regulate the expression of gastrin gene under hypoxic conditions, GI carcinoma cell lines could be either transfected with a constitutively active β-catenin or treated with a β-catenin inhibitor, such as glycogen-synthase kinase 3β, prior to gastrin expression being determined.
Also, the Notch transcription factor has been shown to interact directly with HIF-1, further augmenting the expression of HIF-1 target genes (Sahlgren et al, 2008 Zheng et al, 2008). To determine whether Notch plays a role in the up-regulation of gastrin gene expression, GI carcinoma cells could be transfected with a dominant-negative Notch, prior to incubation under hypoxic conditions.

- The role of the transcriptional co-activator PGC-1α should also be investigated. PGC-1α independently up-regulated the expression of VEGF under hypoxic conditions, without any interaction with the HIF-1 transcription factor (Arany et al, 2008). Therefore, it may be responsible for the up-regulation of the gastrin gene via a similar mechanism. Firstly, the DNA upstream of the coding region should be screened for the presence of the PGC-1α-dependent ERR-α binding site (Arany et al, 2008). If this proves successful, GI carcinoma cell lines could be pre-treated with a PGC-1α inhibitor prior to exposure to hypoxia to determine its affect on gastrin gene expression.

- The role of P-bodies and RNA-binding proteins. The expression of both P-bodies and RNA binding proteins should be determined under hypoxic conditions, before being correlated with the gastrin gene expression. The 3’-untranslated region of the gastrin gene should also be screened for AU-rich regions, which is a key feature for RNA sequestration by RNA binding proteins. RNA binding proteins have previously been shown to be expressed adjacent to areas of necrosis and have been correlated with
expression of pro-angiogenic factors (Nabors et al., 2001), therefore hold similar properties to the HIF-1 transcription factor.

Finally, the relationship between gastrin, HIF-1α, β-catenin and c-myc requires further investigation. The actions of gastrin may increase the level of c-myc expression, by acting via the β-catenin/TCF signalling pathway. Whereas, HIF-1α has been linked to c-myc inhibition under physiological conditions, via both direct and indirect mechanisms (reviewed in Dang et al., 2008). Under pathological conditions, such as cancer however, HIF-1α has been suggested to interact with c-myc, and as a result enhances glycolysis (reviewed in Dang et al., 2008). This c-myc may have been induced via the actions of gastrin signalling, whose expression was increased under hypoxic conditions.

The full role of gastrin in this relationship has not been investigated; by determining the role of gastrin, answers may be found that could solve this conundrum of the c-myc/HIF-1α interaction.
Chapter Eight

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