Novel therapeutic targets in advanced gastric cancer: the insulin-like growth factor signal transduction pathway

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Abstract

Patients with advanced gastric cancer have limited therapeutic options to which response rates are low. Very few targeted therapies are available. There is an urgent need for novel targeted inhibitors for patients who are either ineligible for the existing targeted therapies or have developed resistance. Several studies have supported the importance of the IGF signal transduction pathway in cancer cell survival and proliferation and inhibitors of the type I IGF and insulin receptors have reached clinical trials in various cancer types. To date, the role of the IGF signal transduction pathway in gastric cancer has not been investigated thoroughly.

The aim of the present study is to investigate the importance of the IGF signal transduction pathway in gastric cancer cells and the effect of inhibition of IGF signal transduction in cell survival and proliferation.

IGF-1 was protective against gastric cancer cell death induced by protein kinase inhibition and disruption of cell attachment to the extracellular matrix. The survival effect was mediated by the PI3K/Akt pathway. The survival effect of IGF-1 was even more prominent in *ex vivo* cultures of gastric cancer cells established from patient ascites. IGF-1 stimulated proliferation of gastric cancer cells, which was mediated by activation of the Ras/Raf/ERK pathway. IGF-2 and insulin induced also gastric cancer cell survival and proliferation.

Inhibition of the type I IGF receptor by siRNA knockdown reduced proliferation of gastric cancer cells and *ex vivo* cultures by inhibition of cell mitosis and DNA synthesis. Inhibition of the insulin receptor by siRNA knockdown resulted in the induction of apoptosis. Combined inhibition of the two receptors with a small molecule tyrosine kinase inhibitor reduced effectively cell growth and induced apoptosis.

Our results suggest that inhibition of the type I IGF and insulin receptors could be a valid therapeutic strategy for advanced gastric cancer patients who are not eligible for the currently available targeted treatments.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

ANOVA	Analysis of variance
Bad	Bcl-2 associated death promoter
BCA	Bicinchonic assay
Bcl-2	B cell lymphoma-2
Bcl-xL	B cell lymphoma-extra large
BrdU	5-bromo-2'-deoxyuridine
DAPI	4',6-diamidino-2-phenylindole
DCCS	Dextran-coated charcoal stripped serum
DMEM	Dullbeco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
FGFR2	Fibroblast growth factor receptor 2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GH	Growth hormone
HEPES	4-(2-hydroxyethyl)-1-piperazineeethanesulfonic acid
HER-(2,3,4)	Human epidermal growth factor (2,3,4)
HGF	Hepatocyte growth factor
IC_{50}	Half maximal inhibitory concentration
IGF-(1,2)	Insulin-like growth factor (1 or 2)
IGFBP	Insulin-like growth factor binding protein
IGF-IR	Type 1 insulin-like growth factor receptor
IGF-IIR	Type 2 insulin-like growth factor receptor
IR	Insulin receptor
IR-A	Insulin receptor isoform A
IR-B	Insulin receptor isoform B
IRS	Insulin receptor substrate
K_d	Dissociation constant
kDa	KiloDalton
MEK	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin
PARP	Poly-ADP ribose polymerase
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol-3-kinase
Poly-HEMA	Poly-(2-hydroxyethyl methacrylate)
RIPA	Radioimmunoprecipitation assay
Scr	Scrambled
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SFM	Serum-free medium
Shc	Src homology and collagen domain protein
ShRNA	Short hairpin RNA
siRNA	Short interfering ribonucleic acid
STS	Staurosporine
TBS	Tris-buffered saline
VEGF	Vascular endothelial growth factor
5-FU	5-fluorouracil
18S rRNA	18S ribosomal RNA

Chapter 1. Introduction

1.1 Cancer

Cancer is a disease that involves abnormal cell growth and has the potential to invade or spread to other parts of the body. The transformation of normal cells into cancer cells involves the acquisition of specific characteristics that give them malignant behaviour. In 2000, Hanahan and Weinberg described six traits that are shared by the majority of cancer cells, irrespective of the tumour type from which they originate (Hanahan and Weinberg, 2000). These characteristics include sustained proliferative signalling, resistance to cell death, evasion of growth suppressors, ability to invade and metastasise, replicative immortality and ability to induce angiogenesis (Figure 1.1).

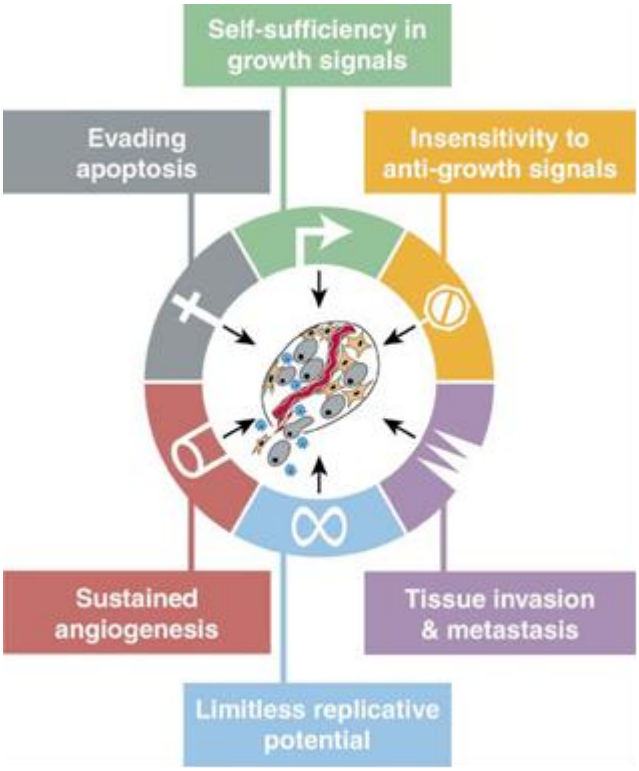

Figure 1.1. Acquired capabilities of cancer cells. Cancer cells tend to be self-sufficient in growth signals, insensitive to growth-inhibitory signals, capable of evading programmed cell death, have limitless replicative potential, sustained angiogenesis, and are able to invade and metastasise (Hanahan and Weinberg, 2000).

In 2011, the same authors described four additional hallmarks and enabling characteristics involved in the pathogenesis of cancer: the ability to modify cellular metabolism in order to support neoplastic proliferation, the ability to evade immunological destruction, genomic

instability and mutability and tumour-induced inflammation (Figure 1.2) (Hanahan and Weinberg, 2011). The acquisition of some of the above 10 hallmarks of cancer results from accumulation of loss-of-function mutations in oncosuppressor genes involved in apoptosis and suppression of proliferation or gain-of-function mutations in oncogenic genes involved in induction of survival and proliferation.

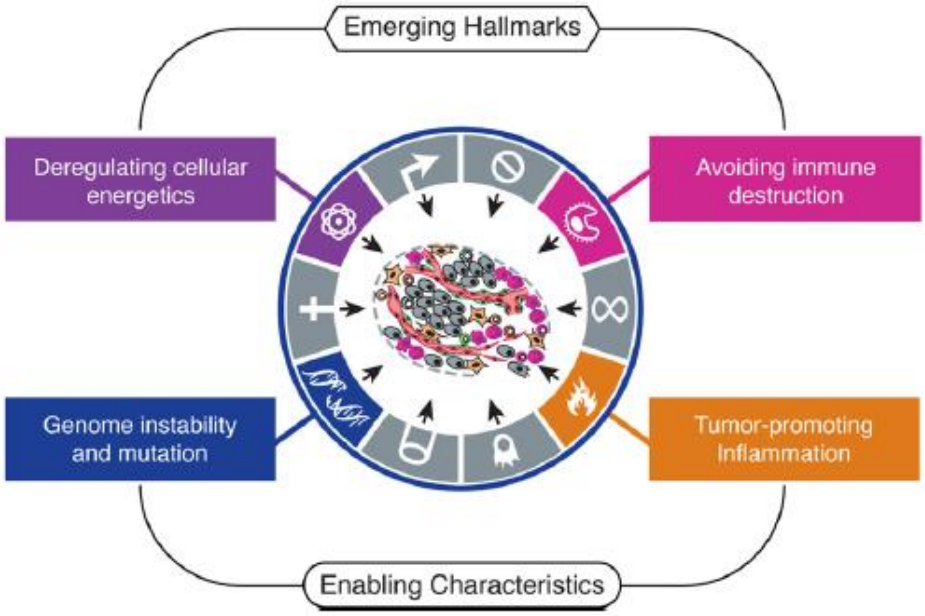

Figure 1.2. Emerging hallmarks and enabling characteristics. Cancer cells are able to deregulate cellular metabolism and evade immunological destruction. Cancer cells are enabled by genomic instability and tumour-promoting consequences of inflammatory responses (Hanahan and Weinberg, 2011).

In normal cells, growth-promoting signals are conveyed mostly by growth factors that bind to cell-surface receptors, which often contain intracellular tyrosine kinase domains. The receptors emit signals via branched intracellular signal transduction pathways that regulate progression through the cell cycle and, thus, growth. Cancer cells can acquire the capability to sustain proliferative signalling in various ways: they may produce growth factor ligands themselves, which bind to their cognate receptors, and act as autocrine stimulators of proliferation. Alternatively, signal transduction may be deregulated by elevation of the levels of receptor proteins, which makes the cells hyper-responsive to the normal amounts of growth factor-ligand. Finally, growth factor independence may derive from the constitutive activation of components of signal transduction pathways that operate downstream of these receptors, which obviates the need to stimulate these pathways by ligand-mediated receptor activation

(Hanahan and Weinberg, 2011). The mitogen-activated protein (MAP)-kinase and the PI3K/Akt pathways are important pathways that are often deregulated in tumour cells.

1.1 Gastric Cancer

1.1.1 Epidemiology

Despite a marked decrease in the incidence and mortality rates of gastric cancer over the past 50 years, it remains the fifth most common malignancy and the third leading cause of cancer-related deaths worldwide. There were approximately 723,000 deaths from gastric cancer in 2012, 8.8 % of the total number of cancer-related deaths. Approximately 70% of cases occur in developing countries and 50% of the cases, worldwide, occur in Eastern Asia, mainly China. Mortality rates are highest in Eastern Asia, followed by Central and Eastern Europe. Incidence rates, standardised for age, are twofold greater in men than women (Ferlay *et al.*, 2015). The chances of a patient surviving 5 years after diagnosis of gastric cancer are approximately 20%. This is remarkably low compared to more common cancer types, such as breast cancer, which has a 5-year survival rate of 80% and prostate cancer, with a 10-year survival rate of approximately 80%.

1.1.2 Risk factors

The main risk factor for the development of gastric cancer is infection with *Helicobacter pylori* (Xue *et al.*, 2001). Approximately 90% of patients with gastric cancer have previous or current *H. pylori* infection (Kato *et al.*, 2007; Yoon *et al.*, 2011). High consumption of salt and salt preserved foods has also been associated with increased risk of gastric cancer, however, the absolute increase was not very high (high salt intake group compared with low salt intake group, relative risk [RR], 1.68; 95% confidence interval, 1.17 to 2.41) (D'Elia *et al.*, 2012). Smoking and heavy alcohol intake have been linked to increased risk for gastric cancer. The odds ratio (OR) for gastric cancer in current smokers was 1.69 (95% confidence interval, 1.35 to 2.11) (La Torre *et al.*, 2009). A meta-analysis including a total of 34,557 gastric cancer cases reported an association between the risk of gastric cancer and heavy alcohol intake (four or more drinks per day) (Tramacere *et al.*, 2012). Obesity has been associated with increased risk of gastric cardiac cancer, which occurs in the top portion of the stomach near the junction of the oesophagus, but not non-cardiac cancer, which may be found in all other areas of the stomach (Chen *et al.*, 2013). Previously, development of gastric

cancer was attributed to the appearance of precancerous lesions, such as chronic superficial gastritis, atrophic gastritis, intestinal metaplasia and dysplasia, following infection with *H. pylori* (Correa, 1992). A more recent study showed that the risk of gastric cancer increased according to the severity of premalignant cancer lesions (de Vries *et al.*, 2008).

1.1.3 Histopathological classification

The inner lining of the stomach wall comprises four main layers. The mucosa is the first main layer that consists of the epithelium and the lamina propria, a thin layer of loose connective tissue. The submucosa lies under the mucosa and consists of fibrous connective tissue that joins the mucosa to the overlying smooth muscle. Muscularis is a thin layer of muscle, which is responsible for the digestion of food. The serosa is a smooth membrane which encloses the abdominal cavity and consists of layers of connective tissue continuous with the peritoneum (Figure 1.3).

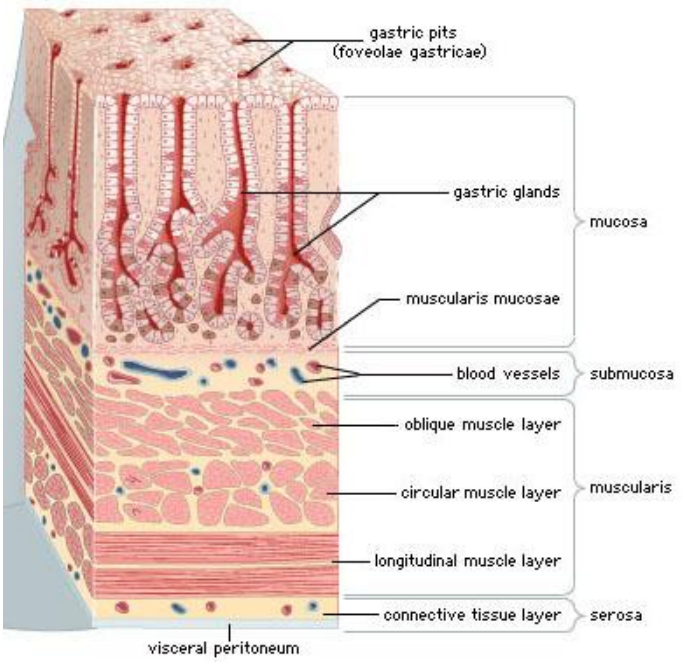

Figure 1.3. Structures of the human stomach. The stomach has three layers of muscle: an inner oblique layer, a middle circular layer and an outer longitudinal layer. The inner-lining consists of four layers: the mucosa, the submucosa, the muscularis and the serosa. The mucosa is densely packed with gastric glands, which contain cells that produce digestive enzymes, hydrochloric acid, and mucus (Encyclopaedia Britannica).

Malignant cells are confined initially to the mucosal layer and are called intra-mucosal cancers. As they develop, they infiltrate the other layers of the gastric wall and penetrate progressively through the submucosa, muscularis and serosa (Figure 1.4). When a cancer is

confined to the mucosa or submucosal layer, it is defined as early gastric cancer, whereas if it has invaded into the muscularis mucosae or beyond, at diagnosis, it is defined as advanced gastric cancer (Roukos *et al.*, 2002).

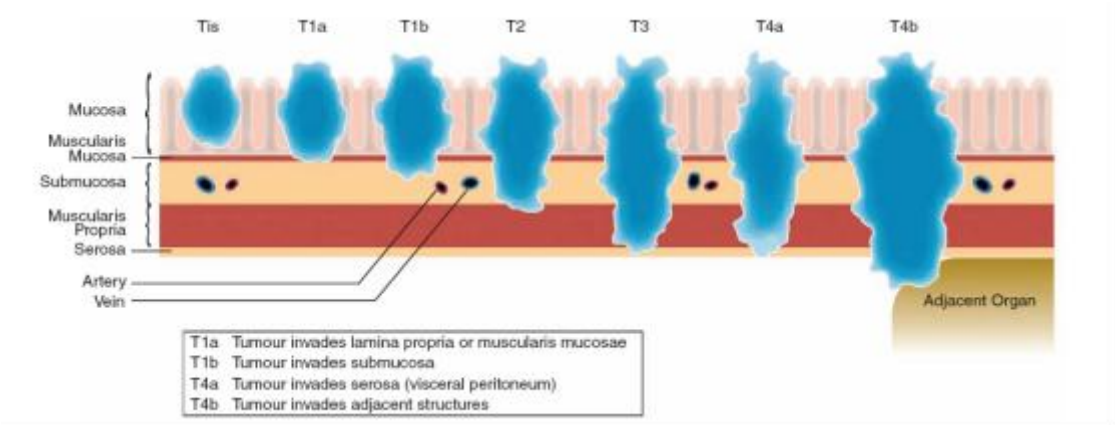

Figure 1.4. Schematic representation of pathological staging of tumour for gastric carcinoma. When the tumour invades the lamina propria or the muscularis mucosae it is staged as T1a. Cancer invasion in the submucosa is staged as T1b, in the serosa T4a, and invasion into adjacent organs is staged as T4b (Taken from Han and Lauwers, 2010).

Histologically, gastric malignancies are classified into five categories: adenocarcinomas, squamous cell cancers, lymphomas, gastrointestinal stromal tumours and neuroendocrine tumours. Adenocarcinomas are the most common form of gastric cancer and account for 90-95% of all malignant gastric tumours. They are malignant epithelial tumours which originate from the glandular epithelium of the gastric mucosa. Around 5% of gastric malignancies are lymphomas, whereas gastrointestinal stromal tumours and neuroendocrine tumours are extremely rare (Kumar *et al*., 2010).

The most successful and widely used classification of gastric adenocarcinoma has been described by Lauren in 1965. Lauren divided gastric adenocarcinomas into intestinal and diffuse types. Intestinal type adenocarcinomas are usually well-differentiated, associated with or developed from intestinal-type metaplastic epithelium and are characterised by cohesive neoplastic cells that form gland-like tubular structures. Diffuse-type adenocarcinomas are usually poorly differentiated or undifferentiated. In these tumours, cells are discohesive and secrete mucus which is delivered into the interstitium. When the mucus remains inside the tumour cells, it pushes the nucleus to the periphery and creates a "signet-ring cell" morphology (Lauren, 1965) (Figure 1.5).

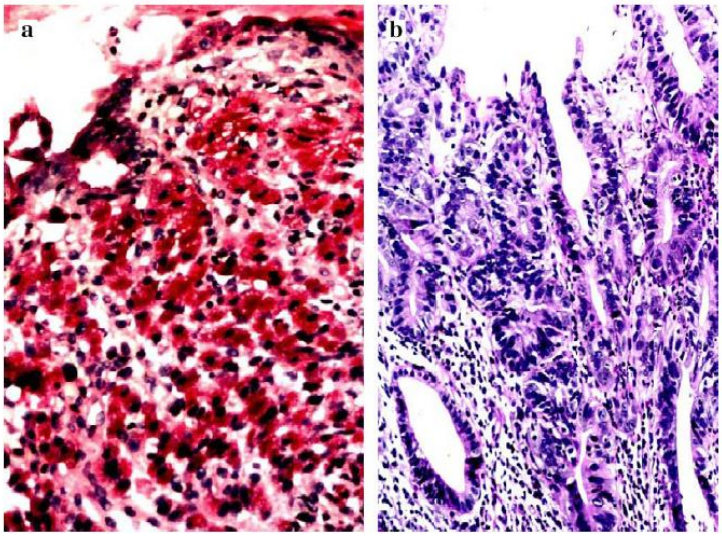

Figure 1.5. Gastric adenocarcinoma of diffuse (a) and intestinal (b) subtype. The most typical feature of the diffuse type is the appearance of a mass of "mucocellular" cells, some of which may resemble "signet-ring cells", which grow in the mucosa and in the gastric wall with poor or no glandular differentiation. The intestinal type is characterised by the appearance of gland-like tubular structures (Vauhkonen *et al.*, 2006).

Several studies have supported the idea that the development of intestinal-type gastric adenocarcinomas is a gradual, stepwise de-differentiation of the gastric epithelium into autonomously growing tumours that form glandular structures. Intestinal-type tumours may contain well-matured goblet cells and express enzymes typical of mature small bowel epithelium. These characteristics, however, can disappear gradually due to de-differentiation of tumours into more anaplastic forms of cancer. Atrophic gastritis and intestinal metaplasia are common lesions that underlie or accompany intestinal-type gastric cancer. Unlike intestinal-type tumours, there is little or no association between diffuse-type gastric tumours and intestinal-type epithelium. Infection with *H. Pylori* has been shown to be the only universal precursor for diffuse-type gastric cancer. These tumours lack glandular differentiation and grow as groups of single muco-cellular cells. In the best differentiated tumours, those muco-cellular cells have a singet-ring-cell type morphology (Vauhkonen *et al.*, 2006).

Epidemiologically, intestinal-type gastric cancer accounts for approximately 50% of all gastric adenocarcinomas diagnosed and that of the diffuse-type 35%. The remainder are characterised as "unclassified" or mixed-type cancers. In younger people, the diffuse-type is more prevalent than the intestinal-type. The intestinal-type is more common in patients older than 50 years of age (Munoz and Connelly, 1971; Roukos *et al.*, 1989; Lauren and

Nevalainen, 1993). Furthermore, patients with intestinal-type gastric cancer have significantly better survival than those with diffuse-type gastric cancer (Lee *et al.*, 2001).

1.1.4 Molecular classification

In a recent study, gastric adenocarcinomas have been classified molecularly, as part of The Cancer Genome Atlas (TCGA) project. Four subtypes of gastric adenocarcinoma have been described. The first subtype comprises tumours which are positive for Epstein-Barr virus and are characterised by recurrent mutations of the gene that encodes phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), extreme DNA hypermethylation and amplification of the genes that encode Janus kinase 2 (*JAK2*), programmed death-ligand 1 (*PD-L1*) and programmed cell death 1 ligand 2 (*PDCD1LG2*). The second subtype comprises microsatellite unstable tumours with elevated mutation rates of genes that encode oncogenic signal transduction proteins. The third subtype includes genomically stable tumours enriched for the diffuse histological variant and with mutations of the Ras homologue gene family member A (*RHOA*) or fusions involving RHO-family GTPase-activating proteins. The last group consists of tumours with chromosomal instability and marked aneuploidy, and is characterised by amplification of receptor tyrosine kinases (Cancer Genome Atlas Research, 2014).

1.1.5 Treatment

1.1.5.1 Symptoms

Gastric cancer may be asymptomatic in its early stages, which is why it is often diagnosed at an advanced stage. Inevitably, therapeutic options are limited. The most common symptoms of gastric cancer are unspecific e.g. abdominal pain, early satiety, reduced apetite or loss of weight. Clinical diagnosis requires upper gastrointestinal endoscopy and biopsy (Schwarz, 2015).

1.1.5.2 Surgical resection

Total or subtotal gastrectomy is the gold standard of gastric cancer management worldwide because complete surgical removal of macroscopic and microscopic tumour tissue will cure the disease. In Eastern Asia, the accepted standard treatment includes radical gastrectomy with a D2 lymphadenectomy, during which, distant perigastric nodes and the nodes along the

main arteries that supply the stomach are removed. In Western countries, radical gastrectomy with limited D1 lymphadenectomy of the perigastric nodes closest to the primary lesion, in combination with chemoradiotherapy, is more frequent (Figure 1.6) (Macdonald *et al.*, 2001). For advanced gastric cancers, surgical resection is palliative to relieve symptoms and, in some cases, prolong survival.

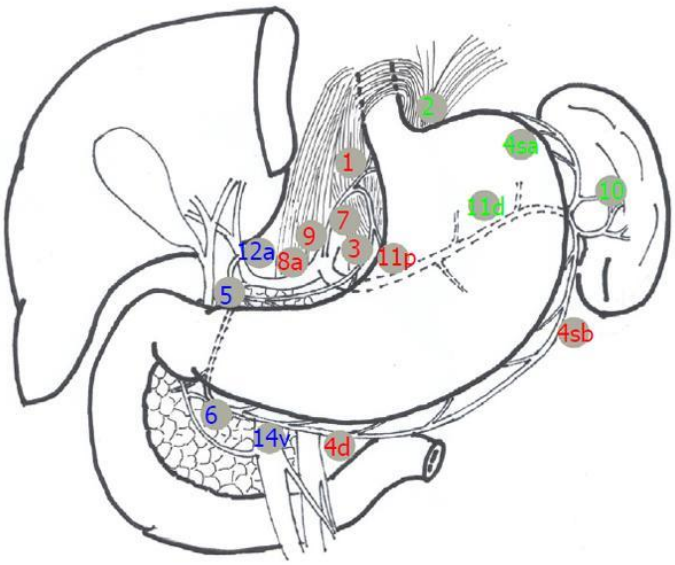

Figure 1.6. Surgical lymphadenectomy. The groups of lymph node in red, green and blue should be dissected in a D2 lymphadenectomy; the groups of lymph node in red and green should be dissected in a D1 lymphadenectomy (Cui *et al.*, 2012).

1.1.5.3 Chemotherapy

Approximately 40-60% of patients with advanced gastric cancer have recurrent disease after surgery (Gunderson, 2002). A number of phase III clinical trials have investigated the potential benefits of administering chemotherapy post-operatively or peri-operatively, to prolong disease-free survival.

1.1.5.3.1 Adjuvant chemotherapy

S-1 is an oral fluorouracil anticancer product that combines 3 pharmacological agents: tegafur, which is a pro-drug of 5 fluorouracil; gimeracil [5-chloro-2,4 dihydropyridine (CDHP)] which inhibits dihydropyrimidine dehydrogenase (DPD) enzyme activity; and oteracil [potassium oxonate (Oxo)] a gastrointestinal side effects corrector. During the Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer (ACTS-GC), a total of 1059 patients

with stage II or III gastric cancer, who underwent total gastrectomy with D2 lymphadenectomy, were randomized to either observation or one-year of oral S-1 adjuvant therapy. The 3-year overall survival was improved in the S-1 group (80.1% vs 70.1%) (Sakuramoto *et al.*, 2007). The positive results of this trial have made adjuvant S-1 therapy the standard treatment of choice for patients with advanced gastric cancer after radical resections with D2 lymphadenectomy in Japan.

A large meta-analysis of 3,838 individual patients from 17 clinical trials (Gastric Group), reported a small but significant benefit of adjuvant chemotherapy after curative resection of gastric cancer. According to the authors, postoperative adjuvant chemotherapy based on fluorouracil derivatives was associated with a statistically significant benefit in terms of overall survival and an increase from 49.6% to 55.3% in five-year overall survival (Group *et al.*, 2010).

An analysis of international (Korea, China, and Taiwan), multicentre, randomized, phase III trials (CLASSIC), compared a group of 520 patients, with locally advanced cancer, that received capecitabine plus oxaliplatin (CapOX) following gastrectomy with D2 lymphadenectomy, with an observation group of 515 patients that received surgery alone. Three-year disease-free survival increased significantly from 59% to 74% in the group that received a combination of capecitabine and oxaliplatin treatment compared to the surgery only group (Bang *et al.*, 2012).

1.1.5.3.2 Adjuvant radiochemotherapy

The intergroup 0116 trial (INT-0116) by the Southwest Oncology Group 9008, tested the application of adjuvant radiotherapy in combination with adjuvant 5-fluorouracil plus leucovorin after curative resection in 556 patients. The median overall survival in the surgery-only group was 27 months, as compared with 36 months in the chemoradiotherapy group (Smalley *et al.*, 2012).

The ARTIST trial (Adjuvant Chemoradiation Therapy in Stomach Cancer) tested the administration of postoperative treatment with capecitabine plus cisplatin (XP) versus capecitabine plus cisplatin (XP) plus radiotherapy with capecitabine (XP/XRT/XP). The addition of radiotherapy with capecitabine (XRT) to capecitabine plus cisplatin (XP) chemotherapy did not significantly prolong disease-free survival ($p = 0.0862$). However, in patients with lymph node metastasis at the time of surgery ($n = 396$), the addition of XRT

significantly increased three-year disease-free survival from 74 to 78% (p=0.0365) (Lee *et al.*, 2012).

1.1.5.3.3 Neoadjuvant therapy

The MAGIC trial in patients with gastric and gastroesophageal junctional cancer was designed to test the effect of perioperative chemotherapy consisting of epirubicin, cisplatin and 5-fluorouracil and surgery, compared to surgery alone. The resected tumours were significantly smaller and less advanced in the perioperative-chemotherapy group. As compared with the surgery alone group, the perioperative-chemotherapy group had a better pregression-free survival and overall survival; five-year survival rate was 36 percent vs. 23 percent (Cunningham *et al.*, 2006).

A phase III randomised trial by the Fédération Nationale des Centres de Luttecontre le Cancer (FNCLLCC) and the Fédération Francophone de Cancérologie Digestive (FFCD) in 28 French centres (ACCORD 07 trial) was also designed to evaluate the benefit in overall survival of perioperative fluorouracil plus cisplatin in resectable gastroesophageal adenocarcinoma. Compared with the surgery-alone group, the chemotherapy plus surgery group had a better 5-year overall survival rate of 38% vs 24% and a better 5-year disease-free survival rate of 34% vs 19% (Ychou *et al.*, 2011).

A summary of the clinical trials and the outcomes is shown in

Table 1.1.

Trial	Year	Cancer site	Patient number	Treatment	Outcomes	p value
INT-0116	2001	Gastric	556	postop. FL-RT vs S	48 vs 31 % 3 years RFS	0.005
1 ACTS-GC	2007	Gastric	1059	postop. S-1 vs S	80 vs 70 % 3 years OS	0.002
CLASSIC	2012	Gastric	1035	postop. CapOx vs S	75 vs 59 % 3 years DFS	<0.0001
ARTIST	2011	Gastric	458	postop. XP-RT vs XP	78 vs 74 % 3 years DFS	0.086
MAGIC	2006	Gastric	503	periop. ECF vs S	36 vs 23 % 5 years OS	0.009
ACCORD 07	2011	Gastro-oesophageal	224	periop. CF vs S	34 vs 24 % 5 years OS	0.02
GASTRIC	2010	Gastric	3838	Meta-analysis	55% vs 50% 5 years OS	<0.001

Table 1.1. Randomised phase III trials of therapies for resected gastric cancer (Modified from (Schwarz, 2015)). HR hazard ratio, RFS recurrence-free survival, OS overall survival, DFS disease-free survival, S surgical resection, FL 5-fluorouracil (5FU)/leukovorin, RT radiation therapy, CapOx capecitabine/oxaliplatin, XP capecitabine/cisplatin, ECF epirubicin/cisplatin/5FU, CF cisplatin/5FU.

As a result of the above clinical trials, a combination of a fluorouracil-based regimen, such as capecitabine or 5-fluorouracil, with cisplatin or oxaliplatin and epirubicin has become the standard of treatment for patients with advanced gastric cancer in the UK.

1.1.5.3.4 Mechanism of action of 5-florouracil and oxaliplatin

The fluoropyrimidine 5-fluorouracil (5-FU) is a uracil analogue with a fluorine atom at the C-5 position in place of hydrogen. It enters the cell using the same mechanism as uracil and is converted to active metabolites such as fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). The above active metabolites disrupt RNA synthesis as well as the action of thymidylate synthase. Thymidylate synthase catalyses the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), with the reduced folate 5,10-methylenetetrahydrofolate (CH2THF) as the methyl donor (Longley *et al.*, 2003). This reaction is necessary for the production of thymidylate, which is essential for DNA replication and repair. Depletion of dTMP levels, affects the levels of the other deoxynucleotides (dATP, dGTP, dCTP) through feedback mechanisms (Jackson *et al*., 1984). Disruption of the balance of the deoxynucleotide pool affects severely DNA synthesis and repair and results in lethal

DNA damage (Yoshioka *et al.*, 1987; Houghton *et al.*, 1995). Thymidylate synthase inhibition also causes accumulation of deoxyuridine monophosphate (dUMP), which leads to increased levels of deoxyuridine triphosphate (dUTP) (Mitrovski *et al.*, 1994; Aherne *et al.*, 1996). Both dUTP and the metabolite fluorodeoxyuridine triphosphate (FdUTP) can be misincorporated into DNA. High (F)dUTP/dTTP ratios cause ineffective repair of uracil and 5-FU-containing DNA by the nucleotide excision repair enzyme uracil-DNA-glycosylase (UDG), which results in additional false nucleotide incorporation (Lindahl, 1974). Repeated cycles of misincorporation, excision and repair lead to DNA strand breaks and, eventually, cell death.

Oxaliplatin (eloxatin; Sanofi-Synthelabo) is a third-generation platinum-based drug which was first approved for the treatment of colorectal cancer but is now used in several cancers, including gastric cancer. Similarly to cisplatin, oxaliplatin acts as an alkylating agent on DNA by forming platinated intrastrand cross-links between two adjacent guanine bases d(GpG) or two adjacent guanine-adenine bases d(GpA) (Misset *et al.*, 2000). The DACH-platinum DNA adducts formed by oxaliplatin are more bulky and hydrophobic than platinum adducts formed by cisplatin or carboplatin. Consequently, oxaliplatin inhibits DNA synthesis more effectively and is more cytotoxic than cisplatin or carboplatin (Schmidt and Chaney, 1993; Rixe *et al.*, 1996). Furthermore, damage recognition proteins e.g. the mismatch repair (MMR) enzyme complex can possibly be prevented from binding to oxaliplatin adducts because of the steric hindrance presented by the DACH ring. As a result, oxaliplatin causes primary and secondary DNA lesions that eventually lead to apoptosis in cancer cells (Faivre *et al.*, 2003).

1.1.5.3.5 Targeted therapy for advanced gastric cancer

In gastric cancer, the best studied molecular targets, so far, include human epidermal growth factor receptor-2 (HER-2), vascular endothelial growth factor (VEGF) and its receptors, fibroblast growth factor receptor 2 (FGFR2), hepatocyte growth factor receptor (c-Met) and mammalian target of rapamycin (mTOR).

HER2 is a transmembrane tyrosine kinase receptor and the preferred heterodimerization partner of the other HER family members, HER1 or EGFR, HER3 and HER4. The HER2-HER3 heterodimer plays a central role in oncogenic transformation in HER2-driven tumours (Hsieh and Moasser, 2007). Trastuzumab (Herceptin®) is a humanized monoclonal antibody that interacts with HER-2. The trastuzumab for Gastric Cancer (ToGA) trial, a pivotal randomised clinical trial of 594 patients with HER-2 positive, advanced, mostly metastatic, gastric cancer tested the efficacy of trastuzumab in combination with chemotherapy compared

to chemotherapy alone. Median overall survival was 13.8 months in those assigned to trastuzumab plus chemotherapy compared with 11.1 months in those assigned to chemotherapy alone (Bang *et al.*, 2010). After the above trial trastuzumab was the first targeted agent approved for patients with advanced gastric cancer.

VEGF is an endothelial cell-specific mitogen and the most potent driver of angiogenesis in tumourigenesis (Ferrara *et al.*, 2003). Bevacizumab (Avastin®) is a humanized monoclonal antibody against VEGF. AVAGAST was a multinational, randomised, placebo-controlled phase III clinical trial designed to evaluate bevacizumab in combination with standard chemotherapy with capecitabine plus cisplatin, as first-line therapy in patients diagnosed with inoperable, advanced gastric carcinoma. Fluorouracil was administered to patients unable to take oral medications. Median overall survival was 12.1 months with bevacizumab plus fluoropyrimidine-cisplatin and 10.1 months with placebo plus fluoropyrimidine-cisplatin. Median progression-free survival was longer at 6.7 versus 5.3 months and overall response rate was higher at 46.0% versus 37.4% with bevacizumab versus placebo. However, this trial did not reach its primary endpoint, which was to achieve a significant difference in overall survival (Ohtsu *et al.*, 2011).

Ramucirumab (IMC-1121B) is a fully human immunoglobulin G1 monoclonal antibody that binds with high affinity to the extracellular VEGF-binding domain of VEGF receptor-2 (VEGFR-2). REGARD study is a phase III, randomized, double-blinded trial of ramucirumab for the treatment of advanced gastric cancer or gastroesophageal junction adenocarcinoma. Patients received ramucirumab plus best supportive care or placebo plus best supportive care until progressive disease, unacceptable toxicity, or death. Ramucirumab conferred a statistically significant benefit in overall survival, 5.2 months vs 3.8 months, and progression-free survival, 2.1 months vs 1 month, compared to placebo in advanced gastric cancer and had an acceptable safety profile (Fuchs *et al.*, 2014).

Fibroblast growth factor receptor family members (FGFR1–4) are transmembrane receptor tyrosine kinases which activate both the phosphoinositide 3-kinase (PI3K-AKT) and the mitogen-activated protein kinase–extracellular signal-regulated kinase (MAPK-ERK) pathways (Matsumoto *et al.*, 2012). Preclinical work has suggested that FGFR2 amplified gastric cancer is sensitive to FGFR/VEGFR inhibition (Xie *et al.*, 2013). An ongoing phase II trial aims to test the FGFR inhibitor dovitinib as monotherapy in patients with metastatic or unresectable gastric cancer harbouring FGFR2 amplification (NCT01719549). Moreover, the efficacy of the FGFR1-3 inhibitor AZD4547 monotherapy is compared to paclitaxel in the

phase II randomised SHINE trial, in patients with advanced gastric adenocarcinoma with FGFR2 polysomy or gene amplification (NCT01457846).

MET is a proto-oncogene that encodes a protein known as hepatocyte growth factor receptor (HGFR). The receptor possesses tyrosine kinase activity and after binding with its ligand, hepatocyte growth factor, it activates signal transduction pathways involved in proliferation, motility, migration and invasion (Organ and Tsao, 2011). Rilotumumab (AMG 102) is a monoclonal antibody against hepatocyte growth factor (HGF), which binds c-Met. A phase II trial of rilotumumab in combination with epirubicin, cisplatin and capecitabine (ECX) in patients with advanced gastric or gastroesophageal junction cancers with high c-Met expression showed a trend toward improved progression-free survival and overall survival (Wadhwa *et al.*, 2013). A phase III, randomized, double-blind, placebo controlled trial of rilotumumab with cisplatin and capecitabine (CX) for untreated advanced c-Met-positive gastric or gastroesophageal junctional adenocarcinoma is currently ongoing (NCT02137343). The results from this trial will shed more light into the efficacy of rilotumumab in increasing progression-free and overall survival in advanced gastric cancer.

Mammalian target of rapamycin (m-TOR) is a serine/threonine protein kinase important for cell growth and proliferation, cellular metabolism and angiogenesis (Bjornsti and Houghton, 2004). Everolimus (RAD001) is an oral inhibitor of m-TOR. GRANITE-1 was a phase III trial of everolimus in 656 patients with advanced gastric cancer who had undergone gastrectomy received prior chemotherapy. Everolimus monotherapy did not improve significantly overall survival compared to placebo (5.39 months vs 4.34 months) (p=0.1244) (Ohtsu *et al.*, 2013).

1.1.5.3.6 Challenges

Despite the treatments available, the 5-year survival rate for gastric cancer remains dismal. Approximately 13 to 20% of patients with advanced gastric cancers have HER2 amplification or overexpression, 4-8% have FGFR2 amplification and 1.5-10% have MET amplification (Gravalos and Jimeno, 2008; Kawakami *et al.*, 2013; Xie *et al.*, 2013; Su *et al.*, 2014). There remain, therefore, a large proportion of patients whose tumour cells are not driven by one of these three tyrosine kinase receptors. Furthermore, approximately 50% of patients with HER-2 positive advanced gastric cancer did not respond to trastuzumab treatment and approximately 12% developed progressive disease (Bang *et al.*, 2010). It is, therefore, required to investigate alternative targeted therapies that might allow the introduction of

effective therapeutic regimens for patients who are ineligible or who have developed resistance to the above treatments.

1.2 The Insulin-Like Growth Factor (IGF) Signal Transduction Pathway

The insulin-like growth factor (IGF) signal transduction pathway comprises three ligands, insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) and insulin, which transduce their signals through two receptors, the type I IGF receptor (IGF-IR) and the insulin receptor (IR). The type I IGF and insulin receptors are heterotetrameric and contain two extracellular α-subunits and two transmembrane β-subunits, with intrinsic tyrosine kinase activity. The two receptors are transported to the membrane fully assembled in the heterotetrameric form (Ullrich *et al.*, 1986; Adams *et al.*, 2000). A third transmembrane protein, the type II IGF receptor (IGF-IIR), binds IGF-1 and IGF-2 but lacks tyrosine kinase activity and does not transduce a signal (MacDonald *et al.*, 1988). In the circulation, insulin-like growth factors (IGFs) bind to IGF-binding proteins (IGFBPs).

The insulin-like growth factor (IGF) system involves complex regulatory networks that operate at the whole organism, cellular and subcellular levels. IGF-1 acts both as a circulating hormone and as a tissue growth factor and is produced mainly in the liver. Hepatic IGF-1 production is regulated by hormonal and nutritional factors. Growth hormone (GH) is produced in the pituitary gland under control of the hypothalamic factors growth-hormone-releasing hormone (GHRH) and somatostatin (SMS) and stimulates IGF-1 production. IGF-2 production is not regulated by growth hormone. Several IGF-binding proteins (IGFBPs) are also produced in the liver. IGFs and IGFBPs can be delivered from the liver through the circulation to IGF-responsive tissues in an endocrine manner. IGFs and IGFBPs can also be locally produced in other organs in an autocrine and paracrine manner, often involving interactions between stromal and epithelial cells (Pollak *et al.*, 2004).

1.2.1 Signalling through the type I IGF receptor (IGF-IR)

Ligand binding of IGF-1 or IGF-2 to IGF-IR results in a conformational change that leads to transphosphorylation of one β subunit by the other. These phosphorylations create docking sites for receptor adaptor proteins, including Src homology 2 domain containing protein (Shc) and insulin receptor substrate proteins (IRS 1, 2 and 4). The adaptor proteins are able to initiate intracellular signalling by activating several pathways (Chitnis *et al.*, 2008)

Phosphorylation of IRS-1 in tyrosine residues recruits the regulatory (p85) and catalytic (p110) subunits of PI3K. Phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3) production activates PDK-1, which in turn phosphorylates the enzyme protein kinase B (Akt) on threonine 308. The mTORC2 complex, activated by an unknown mechanism by the IGF-IR, phosphorylates Akt on serine 473. Activated Akt promotes cell survival by negatively regulating the expression or function of Bcl-2 homology domain 3 (BH3)-only proteins, which exert their pro-apoptotic effects by binding to and inactivating pro-survival Bcl-2 family members. For example, Akt phosphorylates and inhibits the BH3-only protein BAD on S136 and this creates a binding site for 14-3-3 proteins, which causes release of BAD from its target proteins (Datta *et al.*, 2000). Another way of inhibiting BH3-only proteins by Akt is through effects on transcription factors, such as FOXO and p53. The BH3-only protein BIM is an important pro-apoptotic target of FOXO proteins which stimulates cell death in hematopoietic lineages following cytokine withdrawal (Dijkers *et al.*, 2002). MDM2 (or HDM2 in humans) is a third target of Akt, which promotes survival by inhibiting BH3-only proteins. It is an E3 ubiquitin ligase that triggers p53 degradation (Mayo and Donner, 2001). Also, Akt phosphorylates GSK3 isoforms on a N-terminal regulatory site (GSK3a-S21, GSK3β-S9), and inactivates the kinase (Cross *et al.*, 1995). A schematic summary of the interactions and activation of the downstream pathways is shown in Figure 1.7.

Concomitantly, phosphorylation of Shc or IRS proteins by IGF-IR leads to sequential activation of Ras and the mitogen-activated-protein-kinase (MAPK) pathway. There are 4 separate MAPK pathways: the classical pathway or MAPK/ERK pathway, the Big MAP kinase-1 (BMK-1) pathway, the c-Jun N-terminal kinase (JNK) pathway and the p38 signalling pathway (Cossa *et al.*, 2013). The above pathways share a similar organisation of 2 serine/threonine kinases and 1 dual specificity threonine/tyrosine kinase (Dhanasekaran and Premkumar Reddy, 1998). From upstream to downstream these kinases include a MAPK kinase-kinase (MAPKKK), e.g. Raf/MKKK/MEKK1, a MAPK kinase and a MAPK, e.g. ERK1/ERK5/p38 (De Luca *et al.*, 2012). The classical MAPK/ERK pathway comprises 3 types of MAPKKK: A-RAF, B-RAF and RAF-1 or c-RAF kinases. One level downstream are the MAPKKs, which are specified as MEK1 and MEK2. Finally, further downstream are the ERK1 and ERK2, which are the final effectors of the canonical MAPK pathway (Robinson and Cobb, 1997). ERK phosphorylation activates multiple substrates which are responsible for the stimulation of cell proliferation (Johnson and Lapadat, 2002).

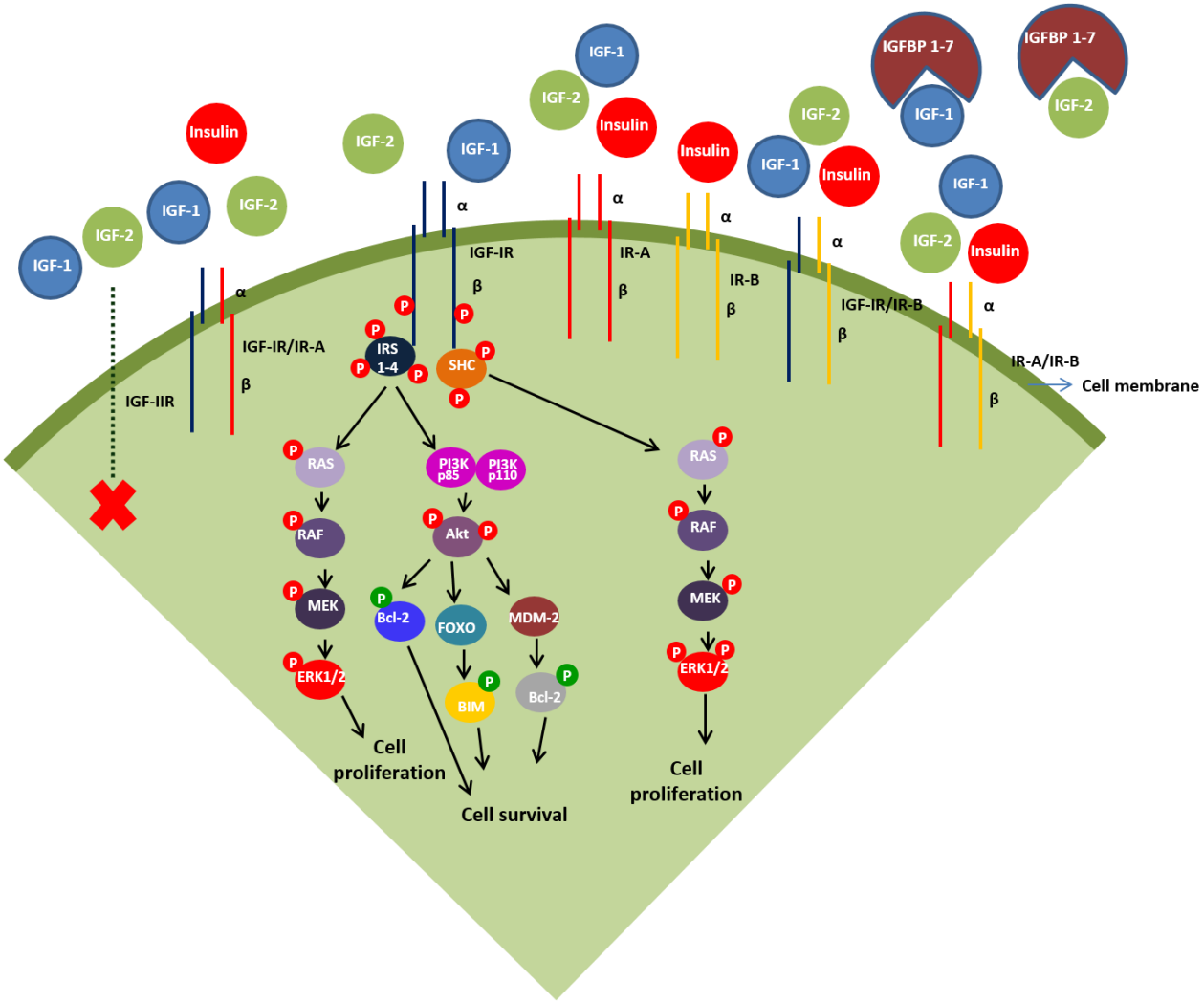

Figure 1.7. The insulin-like growth factor signal transduction pathway. Ligand binding to IGF-IR causes autophosphorylation of one β subunit by the other and creates docking sites for adaptor proteins (IRS1, 2 and 4, Shc). Phosphorylation of adaptor proteins results in the activation of two main pathways, the RAS/RAF/MEK/MAPK and the PI3K/Akt pathway. Both IR isoforms can transduce signal but the type II IGF receptor does not contain a tyrosine kinase domain. Hybrid receptors formed by insulin receptor isoforms and type I IGF receptor also contribute to downstream signalling. When in the circulation, IGFs are bound to IGF binding proteins, IGFBPs.

1.2.2 Type 2 IGF receptor (IGF-2R)

The type II IGF receptor (IGF-2R) or the cation-independent mannose-6-phosphate receptor binds IGF-1 and IGF-2 but lacks tyrosine kinase activity and does not transduce downstream signals. It has a higher affinity for IGF-2 compared to IGF-1 (Nissley *et al.*, 1985). IGF-2 binding to the type II IGF receptor results in IGF-2 downregulation via internalisation and degradation at lysosomes. IGF-2 binding to the type I IGF receptor is, consequently, precluded (Oates *et al.*, 1998). The type II IGF receptor is, therefore, considered a tumour suppressor, as it has antagonistic effects on cell growth. This is further supported by the loss-

of-heterozygosity and loss-of-function mutations in the type II IGF receptor detected in several cancers (Chappell *et al.*, 1997).

1.2.3 Insulin receptor (IR) and IGF-IR/IR hybrids

IGF-IR shares a high degree of homology to IR especially in the kinase domains which are 84% homologous (De Meyts, 1994). Two isoforms of IR are generated by alternative splicing of exon 11.The form that contains 12 amino acids encoded by exon 11 is called the B-isoform (IR-B) and the form that lacks the 12 amino acids encoded by exon 11 is called the A-isoform (IR-A). The two isoforms are expressed in a developmentally specific manner with IR-A expressed highly in foetal tissue and IR-B found predominantly in adult tissues. Overexpression of IR-A is observed in several human malignancies and is proposed to be a major mechanism of IGF system over-activation in cancer (Belfiore, 2007).

IR-A binds insulin with the highest affinity (K_d=0.25 nM), followed by IGF-2 (K_d=2 nM) and IGF-1 (K_d=10 nM) (Frasca *et al.*, 1999; Westley and May, 2013). Activation of IR also leads to phosphorylation of IRS adaptor proteins and activation of downstream phosphatidylinositol 3'-kinase and MAPK pathways. Evidence from gene deletion studies suggests that the functions of IR and IGF-IR, although physiologically distinct, overlap partially, with IR capable of stimulating growth and IGF-IR able to regulate a metabolic response (Ludwig *et al.*, 1996; Di Cola *et al.*, 1997).

Many cells and tissues have hybrid receptors assembled with one α and β chain of the IGF-IR and one α and β chain of the IR-A or IR-B (Pandini *et al.*, 1999; Frasca *et al.*, 2003). Both IGF-IR/IR-A and IGF-IR/IR-B hybrids have been described. It has been shown that IGF-IR/IR-A and IGF-IR/IR-B hybrids bind insulin with comparable and relatively low affinity, intermediate between that of homodimeric IR and homodimeric IGF-IR (Benyoucef *et al.*, 2007). The same group has also shown that both IGF-IR/IR-A and IGF-IR/IR-B hybrids bind IGF-1 and IGF-2 with high affinity, which is comparable to that of homodimeric IGF-IR. The existence of these hybrid receptors adds another level of complexity to the insulin and IGF signal transduction system. A representation of all the different receptor and possible interactions with the ligands is shown in Figure 1.7.

1.2.4 IGF binding proteins

The family of IGF binding proteins (IGFBPs) comprises six homologous multifunctional proteins, IGFBP1 to IGFBP6. IGF-binding proteins modulate the bioactivities of circulating IGFs (Clemmons, 1998). The IGFBPs may inhibit the signalling pathway by binding circulating IGFs, thus preventing their binding to the receptors, but they may also maintain IGFs in circulation and lengthen their circulating half-life (Jones and Clemmons, 1995). In this sense, they perform dual but opposing roles in regulating IGF bioavailability**.**

The IGFBPs bind to IGF-1 and IGF-2 with similar affinities, with the exception of IGFBP-6, which has a stronger affinity for IGF-2 (Headey *et al.*, 2004; Beattie *et al.*, 2008). The IGFs bind to the type I IGF receptor with similar affinities as their binding to IGFBPs. Therefore, pericellular IGFBPs can competitively inhibit IGF-IR binding and activation by IGFs. The above competition results from the fact that some IGF residues involved in IGFBP binding are also involved in the interaction with the type I IGF receptor (Sitar *et al.*, 2006; Forbes *et al.*, 2012).

1.2.5 The IGF pathway in normal development

Mouse models and other studies in animals have provided striking data regarding the role of IGFs in the development of somatic tissues. IGF-1 or IGF-2 null mice are viable, but approximately 40% smaller than littermates. Conversely, IGF-IR signalling is necessary for viability in mice; IGF-IR null mice die at birth and are approximately 55% smaller than littermates. Interestingly, IGF-1-IGF-2 doubly deficient mice remain viable, but 70% smaller than normal littermates (Liu *et al.*, 1993).

Data implicate IGF-1 signal transduction in variations in human growth and development. Rare IGF-1 deficient humans have been identified, who demonstrate short stature, delayed bone age, and low IGF-1 levels in the setting of increased levels of growth hormone. In the above syndrome called Laron syndrome, the growth hormone receptor is mutated or otherwise inactive (Savage *et al.*, 2006). A girl who was a compound heterozygote for point mutations in exon 2 of the IGF-IR gene, resulting in an alteration of the amino acid sequence of the α subunit of the receptor and in reduced binding of IGF-1 to the IGF-IR in fibroblasts, has been identified. The same group has also identified a boy with a nonsense mutation in exon 2 of the IGF-IR gene, that reduced the number of type I IGF receptors expressed by fibroblasts. Both children had intrauterine growth retardation and poor postnatal growth (Abuzzahab *et al.*, 2003).

1.2.6 Role of the IGF signal transduction pathway in tumourigenicity

Many tumours show altered expression of the type I IGF receptor, its ligands IGF-1 and IGF-2 and/or the IGFBPs. Alterations in IGF signal transduction pathway components might be early, possibly initiating, events in tumourigenesis; examples include loss of imprinting for IGF-2 expression and inactivation of the type 2 IGF receptor (Hebert, 2006; Cui, 2007). Furthermore, people with higher IGF-1 circulating levels are at increased risk of developing common solid tumours compared with those with levels at the lower end of the normal range, possibly because IGFs favour the neoplastic progression of small lesions that would otherwise remain occult (Pollak *et al.*, 2004).

At a molecular level, the IGF-IR mediates key features of malignancy. It is required for cellular transformation by most oncogenes, including Ras (Sell *et al.*, 1994), and mediates the combination of proliferation and survival signalling required for anchorage-independent growth (Resnicoff *et al.*, 1994). This property enables transformed cells to form macroscopic tumours, and to survive the process of detachment required for metastasis (Baserga *et al.*, 2003). Consistent with these functions, preclinical studies indicate that IGF-IR overexpression induces invasive and metastatic capability in a mouse model of pancreatic islet tumourigenesis and IGF-IR overexpression in transgenic mice induces mammary epithelial hyperplasia and tumour formation (Lopez and Hanahan, 2002; Jones *et al.*, 2007).

1.2.7 IGF-IR targeted therapy

Over the last decades preclinical data have supported the idea that inhibition of the IGF signal transduction pathway could be effective for cancer treatment. In a wide variety of *in vitro* and *in vivo* models, interruption of IGF signalling has been shown to inhibit tumour growth, block metastasis, and enhance the effects of other forms of cancer treatment (Baserga *et al.*, 2003; Samani *et al.*, 2007; Yuen and Macaulay, 2008). For example, IMC-A12, a monoclonal antibody against the type I IGF receptor, enhanced anti-tumour efficacy in combination with radiation in a non-small cell lung cancer model (Allen *et al.*, 2007). Furthermore, *in vivo* studies in musculoskeletal and prostate tumours supported the idea that blockade of the IGF-IR induced sensitisation to cytotoxic drugs (Scotlandi *et al.*, 2005; Wu *et al.*, 2006).

Genetic alterations resulting in reduced levels of the ligands, as well as knockdown of IGF-1 in mice decreased tumour incidence (Pollak *et al.*, 2001; Wu *et al.*, 2003). Multiple oncogenes, such as the simian virus 40 (SV40) large T antigen and the oncogenic ETV6-NTRK3 (EN) chimeric tyrosine kinase, required the presence of the type I IGF receptor to

achieve cellular transformation (Sell *et al.*, 1993; Martin *et al.*, 2006). Also, IGF-1 signalling was found to induce resistance to tamoxifen and trastuzumab in breast cancer cells (Wiseman *et al.*, 1993; Lu *et al.*, 2001). Other studies have provided evidence that inhibition of the type I IGF receptor can be useful in attenuating the malignancy of EGFR-overexpressing sarcomas and of *Kras* mutant ($Kras^{G12D}$) basal-like breast cancer (Huang *et al.*, 2009; Klinakis *et al.*, 2009). All of the above data have paved the way for the development of drugs targeting the IGF signal transduction pathway for cancer.

The three main classes of inhibitors of the IGF signal transduction pathway include, humanised monoclonal antibodies (mAb) directed specifically against the type I IGF receptor, small molecule inhibitors that target the tyrosine kinase domain of the IGF-IR (TKI) and monoclonal antibodies directed at the ligands, IGF-1 and IGF-2.

1.2.7.1 Monoclonal antibodies against the type I IGF receptor

The antibodies that have been developed are selective for the IGF-IR and do not bind the insulin receptor. They bind the extracellular domain of the IGF-IR, block ligand interaction with the receptor, then cause receptor internalisation and degradation (Gao *et al.*, 2012; King and Wong, 2012). Several monoclonal antibodies targeting the IGF-IR reached clinical trials, such as AMG-479, AVE-1642, MK-0646, CP-751,871 (figitumumab), BIIB-022, SCH-717454, R-1507 and IMC-A12 (cixutumumab). Phase I and II clinical trials of such inhibitors showed positive results in lung cancer, Ewing's sarcoma, breast cancer and adrenocortical carcinoma (Karp *et al.*, 2009; Haluska *et al.*, 2010; Olmos *et al.*, 2010; Pappo *et al.*, 2011; Gombos *et al.*, 2012).

1.2.7.2 Small molecule tyrosine kinase inhibitors

There are two categories of tyrosine kinase inhibitors against the type I IGF receptor. The most common are ATP antagonists, which compete for the ATP binding sites on the tyrosine kinase domains, thereby preventing receptor autophosphorylation. ATP-competitive tyrosine kinase inhibitors include OSI-906, BMS754807, BMS-536924, BMS-554417, NVP-ADW742, NVP-AEW541 and INSM-18. The second class of tyrosine kinase inhibitors includes non-ATP antagonists, such as BVP-51004 (picropodophyllin, PPP) and AG-1024 (Buck and Mulvihill, 2011; Haisa, 2013). Although small molecule tyrosine kinase inhibitors are highly effective, receptor specificity is poorer than that of monoclonal antibodies, and

concurrent insulin receptor inhibition usually occurs. Interestingly, this lack of specificity provides added benefit in the case of hybrid IGF-IR/IR receptors, which are more abundant in certain tumour types. BMS754807 (Bristol-Myers-Squibb, New York, NY, US7534792) is a dual inhibitor of IGF-IR and IR that has been shown to inhibit [^{3}H]-thymidine incorporation in pre-clinical studies of breast, lung, pancreatic and colon cancer cells as well as in multiple myeloma, leukaemia, and sarcomas (Carboni *et al.*, 2009).

1.2.7.3 Monoclonal antibodies against the ligands IGF-1 and IGF-2

A third class of inhibitors comprises monoclonal antibodies that neutralise selectively the bioactivity of IGF-1 and IGF-2 but do not affect insulin. Such inhibitors have reached phase I clinical trials (Friedbichler *et al.*, 2014; Haluska *et al.*, 2014).

1.2.7.4 Disappointing trials and future aims

After the promising outcomes of phase II clinical trials with IGF-IR specific antibodies and tyrosine kinase inhibitors, some anti-IGF-IR antibodies reached phase III clinical trials (Gao *et al.*, 2012; King *et al.*, 2014). However, an initial randomised phase III trial of figitumumab in non-small cell lung cancer (NSCLC) was disappointing compared to early phase trials and has documented low efficacy, with a combination of metabolic toxicity and hyperglycaemia (Langer *et al.*, 2014). The negative phase III results have initiated a debate, with some people in favour of abandoning any future attempt to investigate therapeutic targeting of the insulin and type I IGF receptor and others in favour of continuing the investigation but with selected patients or alternative IGF-IR inhibition methods.

According to Pollak, 2012, a few points need further investigation before IGF-IR targeted therapy is taken forward. Firstly, predictive biomarkers should be used to define a subset of patients that are most likely to benefit from IGF targeted therapy. For example, high IGF-1 circulating levels in some patients could indicate tumour dependency or addiction to IGF-IR activation, in which case it would be more likely for the tumour cells to respond to interruption of IGF signalling. Secondly, resistance mechanisms can often be observed as some cancers have evolved to such an extent that their behaviour is uninfluenced by growth signals. Furthermore, some cancers are driven by other receptors, e.g EGFR or HER-2, and any attempt to target the IGF pathway would be ineffective. Another possible resistance mechanism is observed when inhibition of IGF-IR leads to increased expression and

phosphorylation of another tyrorine kinase receptor, e.g HER-2 or EGFR. Likewise, EGFR inhibition with erlotinib results in increased IGF-IR expression and phosphorylation in ovarian and head and neck squamous cell carcinoma cells (Barr *et al.*, 2007). Thirdly, combination therapies used so far have not been selected on the basis of a specific synergy demonstrated in preclinical studies but on the basis of an approach involving the addition of a drug to an already standard regimen that has some activity but requires improved efficacy. It is, therefore, essential to establish appropriate combination therapies in a preclinical setting before bringing them to the clinic (Pollak, 2012).

1.2.8 Epidemiologic studies of IGF-IR expression in gastric cancer

Several epidemiologic studies support a link between IGF-IR expression and increased gastric cancer risk. Positive IGF-IR expression was detected in 87.5% gastric carcinoma tissues, 55% atypical hyperplasia tissues and 17.86% normal gastric mucosal membrane tissues, suggesting that IGF-IR expression might be related to the development of gastric carcinoma (Liu *et al.*, 2009). IGF-IR expression in gastric cancer has been associated with lymph node metastasis, worse prognosis and high histological malignancy grade (Gryko *et al.*, 2014). In particular, positive IGF-IR expression was found mostly in patients with moderately and poorly differentiated carcinoma and in 95.23% of patients with lymph node involvement, suggesting that the IGF signal transduction pathway might be important for gastric cancer progression. An additional study has demonstrated a correlation between IGF-IR overexpression and poor prognosis for gastric cancer (Ge *et al.*, 2009). Specifically, IGF-IR expression was associated with tumour size, stroma quantity, depth of invasion, lymph node involvement and stage of gastric cancer. The above suggest that IGF-IR expression in gastric cancer is more common in advanced stages and it is possibly involved in disease progression.

1.2.9 Preclinical studies of IGF-targeted therapeutic intervention in gastric cancer

Inhibition of IGF signal transduction has been investigated extensively in other cancer types, in which monoclonal antibodies that target the IGF-IR or tyrosine kinase inhibitors that target the IGF-IR and IR have reached phase III clinical trials. However, there have been limited studies in gastric cancer. In 2003, Pavelic et al., studied the expression of components of the IGF signal transduction pathway in primary gastric tumours. High stage diffuse-type tumours overexpressed IGF-2 but were moderately positive for the type 2 IGF receptor. IGF-IR mRNA expression correlated well with cell proliferation, assessed by thymidine

incorporation, in 12 diffuse-type gastric cancer samples, whereas aIR3, a monoclonal antibody against the IGF-IR, inhibited thymidine incorporation and decreased the number of colonies that formed in soft agar (Pavelic *et al.*, 2003).

In 2005, Min et al., showed that IGF-1 and IGF-2 induced cell proliferation in MKN45 gastric cancer cells and cell survival in MKN45 and MKN74 gastric cancer cells. IGF-1 induced Akt phosphorylation and transduction with an adenovirus that led to expression of a dominant negative type I IGF receptor, reduced this phosphorylation. Experiments with dominant negative receptors showed reduced proliferation, assessed by trypan blue staining, of MKN45, NUGC4 and MKN74 cells, compared to cells expressing the type I IGF receptor. Expression of the dominant negative receptor induced caspase-3 activity in MKN45 and MKN74 cells, which supported a role for IGF-IR on cell survival. Furthermore, expression of dominant negative IGF-IR enhanced the effects of radiotherapy and 5-fluorouracil-based chemotherapy both *in vitro*, in MKN45 cells and *in vivo* in gastric cancer xenografts (Min et al., 2005). In 2010, Wang et al., showed that a shRNA against the type I IGF receptor increased cell death in a tunnel assay and decreased colony formation in soft agar of MKN45 gastric cancer cells (Wang *et al.*, 2010a).

Despite the above encouraging results, there have been limited studies on the effectiveness of IGF targeted therapy combined with other agents in gastric cancer and only in 2011 Li investigated the potential of combining IGF-IR and VEGF inhibition in subcutaneous xenografts in mice. Transduction of MKN45 cells with adenovirus expressing dominant negative IGF-IR in combination with bevacizumab, a human VEGF targeted monoclonal antibody was more effective than either monotherapy and resulted in complete regression of 3 out of 7 subcutaneous MKN45 tumours in nude mice. Expression of dominant negative IGF-IR also reduced *in vitro* vascular formation and lymphangiogenesis (Li *et al.*, 2011).

The above studies provided encouraging data regarding the importance of the IGF pathway in gastric cancer progression and the potential of targeting IGF-IR. However, the number of studies is limited and further investigation into the importance of activation of the IGF-IR and downstream pathways in gastric cancer cell survival and proliferation is required. Also, most of the results obtained from the previous studies were based on the use of one particular cell line, MKN45 cells, as a model of gastric cancer. It is, therefore, important to test the response of different types of gastric cancer cells to increase the chances of getting positive results with IGF-IR inhibition in the clinic.

1.3 Apoptosis

Apoptosis is a type of programmed cell death that occurs in multicellular organisms to preserve their homeostasis or as part of their normal developmental programme. It is a tightly regulated process that results in the self-destruction of a particular unwanted or damaged cell, without harming its neighbouring cells. Apoptosis can be triggered after exposure to factors that induce different types of stress, such as nutrient deprivation, osmotic stress, cytotoxic agents and others.

Apoptosis is characterised by rounding-up of the cell, reduction of the cell volume, chromatin condensation, nuclear fragmentation, little or no ultrastructural modification of cytoplasmic organelles, pseudopode retraction, blebbing of the plasma membrane and engulfment of the cell by phagocytes (Kerr *et al.*, 1972).

Apoptosis can be regulated by an extrinsic and an intrinsic pathway. The extrinsic pathway is initiated by the activation of transmembrane death receptors by ligands released by other cells. Death receptors often include members of the tumour necrosis factor family (TNF). TNF receptors have a cysteine-rich extracellular subdomain that is responsible for recognising the ligands and a cytoplasmic domain, called the 'death domain', which is responsible for transmitting the death signal from the cell surface to intracellular pathways. Once the intracellular domain is activated it binds to the adaptor protein Fas-associated death domain (FADD) or TRADD (TNFR1-associated death domain protein) and forms the death inducing complex (DISC). The above complex recruits pro-caspase 8, which is cleaved proteolytically and acts as the 'initiator caspase', to activate downstream proteins such as caspases 3 and 7, which, in turn, initiate cell degradation resulting in apoptosis (Burz *et al.*, 2009).

The intrinsic or mitochondrial pathway is often activated by loss of growth factor signals or in response to death stimuli within the cell, e.g. DNA damage, oxidative stress, hypoxia or chemotherapeutic drugs. Lethal stimuli cause changes to the mitochondrial membrane and cause release of pro-apoptotic proteins, such as cytochrome C from the mitochondria to the cytoplasm. Cytochrome C stimulates formation of the apoptosome, a complex that includes apoptotic protease-activating factor [Apaf-1], dATP, cytochrome c and caspase 9. The apoptosome activates 'initiator' caspase 9, which, in turn, activates the 'executioner' caspases 3, 6 and 7. The 'executioner' caspases cleave vital proteins, such as the poly (ADP-ribose) polymerase (PARP), resulting in cell death (Burz *et al.*, 2009).

Interactions between proapoptotic and antiapoptotic proteins of the Bcl-2 protein family are important for controlling the intrinsic pathway. The members of the Bcl-2 family contain

conserved sequence motifs known as Bcl-2 homology domains (BH1 to BH4). Antiapoptotic Bcl-2 proteins contain all Bcl-2 homology domains and include Bcl-2, Mcl-1, Bcl-xL, and A1. Proapoptotic proteins are divided into two groups, the BH3-only group which comprises Bid, Bim, Bik, Bad, Bmf, Noxa, Puma, and Hrk and the BH1-3 group which comprises Bax, Bak and Bcl-xs. Antiapoptotic Bcl-2 proteins bind the proapototic proteins Bax and Bak and prevent mitochondrial damage (Scorrano and Korsmeyer, 2003). A schematic representation of the interactions involved in apoptosis is shown in Figure 1.8.

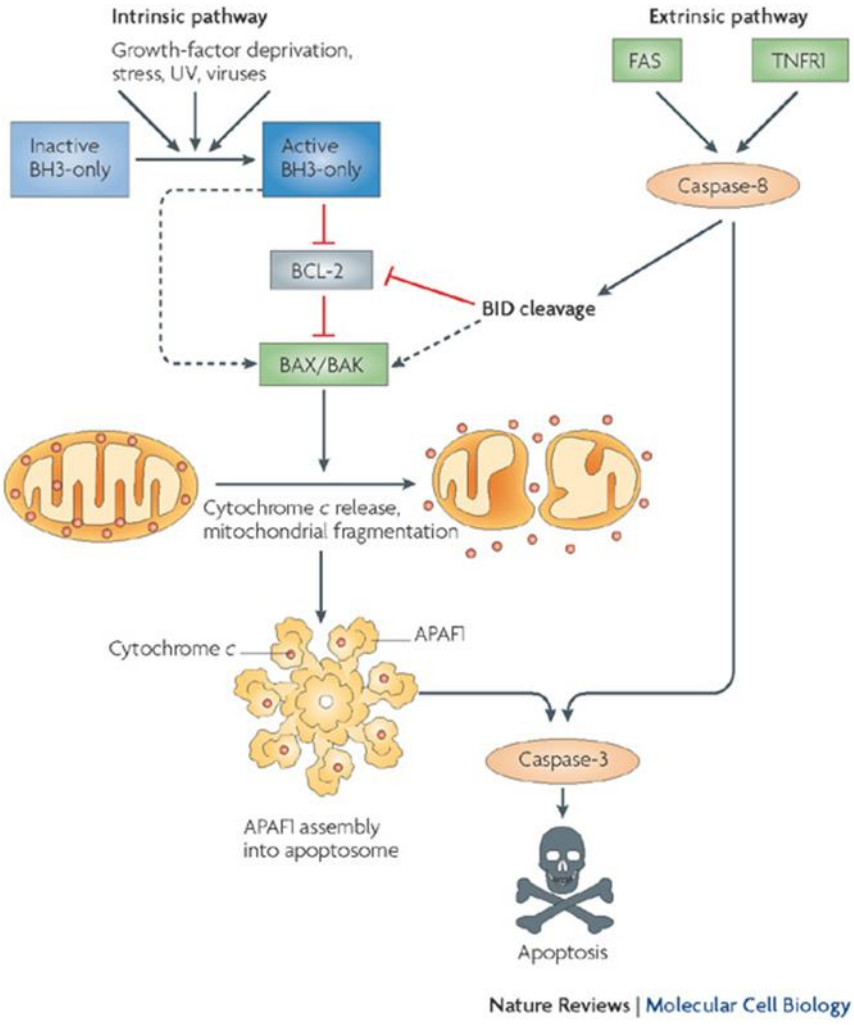

Figure 1.8. Intrinsic and extrinsic pathway of apoptosis. Apoptosis can be induced by the extrinsic pathway and the intrinsic pathway. The intrinsic pathway starts with induction of BH3-only proteins, which inactivates some BCL-2 family members. This activates BAX and BAK, which in turn results in apoptosis. Some BH3-only proteins, such as BIM and PUMA, also activate BAX and BAK. BAX and BAK promote cytochrome c release and mitochondrial fragmentation, which activates APAF1 into an apoptosome and activates caspase-9 to activate caspase-3. Caspase-3 then cleaves various substrates and leads to destruction of the cells. The extrinsic pathway bypasses the mitochondrial step by activating caspase-8 directly, resulting in caspase-3 activation and cell destruction. The BCL-2 family regulates the intrinsic pathway and modulates the extrinsic pathway when BID cleavage communicates between the two pathways (Youle and Strasser, 2008).

1.4 Cell Cycle

The cell cycle comprises four sequential phases. The most important are S phase, during which DNA is replicated and mitosis (M), during which the cell divides into two daughter cells. The two gap phases separating S phase and mitosis are called G_1 and G_2. G_1 follows mitosis and precedes S phase. During G_1 the cells are sensitive to positive or negative signals from growth signalling pathways. G_2 is the phase after S phase during which cells prepare for entry into mitosis (Murray *et al*., 1993). G_0 is a phase into which cells enter when they have withdrawn reversibly from the cell division cycle due to high cell density or absence of mitogens (Zetterberg and Larsson, 1985). Cells may also withdraw from the cell cycle irreversibly into terminally differentiated or senescent states.

Progression through the cell cycle and transition from one phase to the following are controlled by sensor mechanisms or checkpoints, which ensure the appropriate conditions and order of events (Hartwell and Weinert, 1989). When abnormal or incomplete cell cycle events, e.g. DNA damage, are detected by the sensor mechanisms, checkpoint pathways transmit the signal to effector proteins, which can induce cell cycle arrest until the abnormality is resolved (Bartek *et al.*, 2004; Musacchio and Salmon, 2007). Proteins responsible for regulating progression through the cell cycle comprise the cyclin-dependent kinase (CDK) family of serine/threonine kinases and the cyclins (Malumbres and Barbacid, 2006). Cyclin D-CDK4, cyclin D-CDK6 and cyclin E-CDK2 are responsible for driving G_1 progression through the restriction point, making the cell commit to the cycle (Planas-Silva and Weinberg, 1997). Cyclin A-CDK2 drives initiation of the S phase and cyclin B-CDK1 drives progression through G_2 and initiation of mitosis (Nigg, 2001). Effector proteins, which can reversibly stop cell cycle progression include the CDK inhibitors (CDKIs). G_1 arrest can be induced by the Ink4 family of CDKIs (p16, p15, p18 and p19), which inhibit CDK4 and CDK6 or by the Cip/Kip family of CDKIs (p21, p27 and p57), which inhibit CDK2 activity (Malumbres and Barbacid, 2009; Hanahan and Weinberg, 2011).

The malignant phenotype is characterised by deregulation of the cell cycle engine resulting in uncontrolled proliferation. Mitogens stimulate G_1-S CDK activities triggering phosphorylation of retinoblastoma proteins (pRB) proteins, which stops their interaction with the E2F family of transcription factors. In cancer cells, phosphorylation of pRB is often prevented, thus, causing E2F-induced G_1-S gene expression even in the absence of mitogens (Harbour and Dean, 2000). This can be caused by activating mutations in the mitogenic signalling pathways either in ligands and receptors, such as HER2/ErbB2/neu receptor

mutations or HER2 gene amplification, or in downstream signalling networks such as Ras-Raf-MAPK or PI3K/Akt signalling pathways.

1.5 Summary and Aims

The majority of gastric cancers are diagnosed at advanced stages, by which therapy has to be palliative rather than curative. Chemotherapy combined with targeted agents, such as trastuzumab for HER-2 positive cancers, is the standard of palliative therapy for patients with advanced inoperable gastric cancer. However, a large number of patients are ineligible or develop secondary resistance to the above agents. It is, therefore, essential to identify and validate alternative targets for patients with advanced gastric cancer, to establish the appropriate drug combinations. The utilisation of positive and negative predictive biomarkers could increase the likelihood for a particular targeted therapy to being effective.

Implication of the IGF signal transduction pathway in the progression of several cancer types and in the development of therapeutic resistance has been reported repeatedly. Monoclonal antibodies and tyrosine kinase inhibitors against the type I IGF receptor have reached clinical trials in various cancers. To date, the importance of the IGF signal transduction pathway in gastric cancer progression has not been investigated extensively. The selection of gastric cancer cell lines as models for demonstrating the importance of the IGF signal transduction pathway has not been based on a specific rational.

We hypothesise that activation of the IGF signal transduction pathway has important survival and proliferative effects in a subset of gastric cancer cells. Therefore, inhibition of IGF signalling could possibly confer a significant benefit in the growth and survival of the above gastric cancers.

The aim of this study is to test the effects of IGFs and insulin on survival and proliferation of gastric cancer cell lines and patient samples. The benefits of inhibiting the type I IGF and insulin receptors in gastric cancer cells will be examined by RNA interference methods and by the use of inhibitors which target the two receptors. The effect of combining IGF-IR/IR targeted inhibitors with cytotoxic drugs and other targeted agents will also be examined.

Chapter 2. Materials and Methods

2.1 Cell Culture

2.1.1 Cell lines

The human gastric cancer cell lines NCI-N87, KATO III, SNU-16, SNU-5, SNU-1, MKN74, NUGC3 and AGS were used in this study. Breast cancer cell lines SKBR3, MCF7 and MDA-MB-231 were used for comparative purposes. The origins of all cell lines and available clinico-pathological information about the cells and their amplification status are listed in Table 2.1.

Cell Line	**Reference**	**Origin**	**Differentiation status**	**Prior treatment**	**Gene Amplification**	**Age**	**Sex**	**Race**
NCI-N87	Park et al 1990	Liver metastasis of GA	Well differ.	None	*HER2*	?	M	USA
KATO III	Sekiguchi et al 1978	Pleural effusion	Poorly differ.	?	*FGFR2*	55	M	Asian
SNU-16	Park et al 1990	Ascites	Poorly differ.	None	*FGFR2*	33	F	Korea
SNU-5	Park et al 1990	Ascites	Poorly differ.	5-fluorouracil, doxorubicin, methotrexate	*MET*	33	F	Korea
SNU-1	Park et al 1990	Primary GA	Poorly differ.	None	-	44	M	Korea
MKN74	Motoyama et al 1986	Liver metastasis of GA	Well differ. (intestinal type)	?	-	?	?	?
NUGC3	Akiyama et al 1988	Metastasis in brachial muscle	Poorly differ.	?	-	?	?	?
AGS	Barranco et al 1983	Primary GA	Not known	None	-	54	F	Caucasian
SKBR3	Trempe and Old, 1970	BA; pleural effusion	Not known	Radiation, steroids, cytoxan, 5-fluorouracil	*HER2*	43	F	Caucasian
MCF7	Soule et al 1973	BA; pleural effusion	Well differ.	?	-	69	F	Caucasian

MDA-MB-231	Cailleau et al 1974	BA; pleural effusion	?	?	-	51	F	Caucasian

Table 2.1. Gastric and breast adenocarcinoma cell lines used. GA; gastric adenocarcinoma, BA; breast adenocarcinoma

2.1.2 Routine cell culture

NCI-N87, MKN74, NUGC3 and AGS were cultured as adherent monolayers. SNU-1, SNU-5 and SNU-16 cells were cultured in suspension and KATO III cells were cultured in mixed conditions, adherent and suspension. All cell lines except SNU-5 cells were sub-cultured in tissue culture flasks (Corning) in Dulbecco's modified Eagle's medium (DMEM) with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) modification (Sigma-Aldrich, Dorset, United Kingdom), supplemented with 10% foetal bovine serum (FBS), whereas SNU-5 cells were supplemented with 20% foetal bovine serum (FBS). When adherent cells reached 70-80% confluence they were washed twice with phosphate buffered saline (PBS) and detached from the flasks by incubation in trypsin-EDTA solution (Sigma-Aldrich, Dorset, United Kingdom). Cells were then collected by centrifugation at 300 x g for 3 minutes (Beckman Allegra X-12 R, rotor SX 4750) at room temperature and reseeded into fresh flasks at dilutions ranging from 1:5 to 1:20. Cells in suspension were collected by centrifugation at 300 x g for 3 minutes and reseeded into fresh flasks at dilutions ranging from 1:5 to 1:20. Cell lines were tested every 6 months to confirm the absence of Mycoplasma contamination. Long term storage of cells was in DMEM, 20% FBS and 10% dimethyl sulphoxide (DMSO) in 0.5 ml aliquots at -70 ^{o}C or in liquid nitrogen.

2.1.3 Establishment of primary cultures of gastric cancer cells from ascites

Ascites was collected from the peritoneal cavity of patients that were previously diagnosed with gastric adenocarcinoma. Ascites was diluted inside tissue culture flasks in a 1:1 ratio with Dulbecco's modified Eagle's medium (DMEM) with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) modification (Sigma), supplemented with 20% foetal bovine serum (FBS) and 1% penicillin/streptomycin (Sigma). The ascitic fluid was left inside the flasks for 5-7 days in order to enhance attachment of the epithelial cells. Subsequently, the supernatant was removed and the remaining adherent cells were supplemented with fresh medium supplemented with 20% FBS. Upon reaching 70-80% confluence, cells were trypsinised in a manner similar to the one described in section 2.1.2. Long term storage of

cells was in 90% FBS and 10% dimethyl sulphoxide (DMSO). Passage number 6 or lower was used for experiments as most primary cultures enter senescence around that stage.

2.1.4 Preparation of dextran-coated charcoal stripped serum (DCCS)

Dextran-coated charcoal was prepared by addition of 0.2 g dextran T70 (Pharmacia, Kent, United Kingdom) to 20 g charcoal (Sigma-Aldrich, Dorset, United Kingdom). The above was resuspended in 250 ml deionised water in 250 ml centrifuge bottles (Beckman Coulter). The suspension was allowed to stand for 10 minutes and then centrifuged at 7,000 rpm (Beckman Avanti J-26 XP centrifuge, JA 16.250 rotor) for 15 minutes at 4^{o}C. The supernatant was discarded and charcoal was resuspended in 250 ml of deionised water. After an additional centrifugation at 7,000 rpm for 15 minutes at 4^{o}C, the supernatant was discarded again and charcoal resuspended in 200 ml newborn calf serum (Invitrogen, Paisley, United Kingdom). The serum was transferred to a 500 ml conical flask and agitated rigorously in a shaking waterbath for 40 minutes at 55^{o}C. The serum was then transferred to new 250 ml centrifuge bottles and centrifuged twice at 10,000 rpm (JA 16.250 rotor) for 30 minutes at 4^{o}C. Finally, the supernatant was collected and filtered into sterile tissue culture glassware through a 0.45 µm filter (Corning). The serum was then aliquoted into 50 ml Falcon tubes and stored at -20^{o}C.

2.1.5 Withdrawal of cells from steroid hormones and growth factors

Withdrawal of cells from the effects of growth factors was achieved by culture in withdrawal medium which comprised phenol red-free DMEM (Sigma-Aldrich, Dorset, United Kingdom) supplemented with 10% dextran-coated charcoal treated calf serum (DCCS). During withdrawal, routine culture medium was removed by a pulled glass Pasteur pipette and cells were washed in phosphate buffered saline (PBS) before the addition of withdrawal medium. Medium was changed once or twice daily for 2 days, unless otherwise specified.

2.2 Stimulation of Protein Phosphorylation

Adherent cells were grown until ~70% confluence and detached from cell culture flasks by incubation with trypsin:EDTA. Cells were then resuspended in maintenance medium and counted using a haemocytometer. Cells were seeded into 12-well tissue culture plates in 1 ml maintenance medium at a concentration of 100,000 to 150,000 cells/well. Cells were left to

attach for ~24 hours and were withdrawn from growth factors by culturing in withdrawal medium for 2 days, as described above. Cells in suspension were grown until ~70% confluence and centrifuged at 300 x g for 3 minutes and resuspended in PBS. Cells were centrifuged again at 300 x g for 3 minutes and resuspended in DCCS medium. The above was repeated for 2 days. On the day of the assay, withdrawal medium was replaced with phenol-red free DMEM supplemented with 0.1% bovine serum albumin (BSA, Sigma-Aldrich, Dorset, United Kingdom) and cells were incubated at 37^{o}C for 2 hours. Then, medium was changed to phenol-red free DMEM with 0.1% bovine serum albumin and various concentrations of IGF-1, IGF-2 or insulin. Cells were incubated for 15 minutes prior to protein extraction with RIPA buffer plus inhibitors. The extent of phosphorylation of proteins in the IGF signal transduction pathway was analysed by Western transfer.

For experiments designed to investigate the effect of the BMS754807 tyrosine kinase inhibitor on the inhibition of receptor activation, the cells were plated and withdrawn as described above and were incubated with the appropriate concentrations of the BMS754807 for at least 30 minutes before the addition of IGF-1.

2.3 Apoptosis Assay

Adherent cells were grown until ~70% confluence and detached from cell culture flasks by incubation with trypsin:EDTA. Cells were resuspended in maintenance medium and counted using a haemocytometer. Cells were seeded into 12-well tissue culture plates in 1 ml maintenance medium at a concentration of 100,000 to 150,000 cells/well. Cells were left to attach for ~24 hours and were withdrawn from growth factors by culturing in withdrawal medium for 2 days, as described above. Cells in suspension were grown until ~70% confluence and centrifuged at 300 x g for 3 minutes and resuspended in PBS. Cells were centrifuged again at 300 x g for 3 minutes and resuspended in DCCS medium. The above was repeated for 2 days. In order to investigate the effect of IGF-1, IGF-2 or insulin on apoptosis, cells were incubated with various concentrations of the above growth factors for 15 minutes. Then, the medium was replaced with DCCS medium supplemented with 0.5-1 μM staurosporine (Sigma-Aldrich, Dorset, United Kingdom) with or without the growth factors. Cells were incubated in staurosporine for various times. At the end of each time point, protein extracts were prepared from the cells with RIPA buffer plus inhibitors. For experiments designed to investigate the effect of inhibiting the IGF-IR on the ability to induce apoptosis with staurosporine, cells were incubated with various concentrations of the BMS754807

inhibitor for 30 minutes prior to treatment with IGF-1. For experiments designed to test the effect of the LY294002 (New England Biolabs, Hitchin, United Kingdom) or U0126 inhibitor (Calbiochem) on the protective effect of IGF-1 against apoptosis, the cells were detached as before and incubated with the appropriate concentrations of the inhibitors for at least 30 minutes before the addition of IGF-1 and staurosporine.

2.4 Anoikis Assay

2.4.1 Preparation of poly (2-hydroxyethylmethacrylate) (poly-hema) coated plates

Poly (2-hydroxyethylmethacrylate) or poly-HEMA (Sigma-Aldrich, Dorset, United Kingdom) was dissolved in 97% ethanol at a concentration of 10 mg/ml and placed at 37^{o}C to prevent precipitation. Twelve-well tissue culture plates were coated with 0.5 ml of poly-HEMA solution per well, in a tissue culture hood. The plates were then incubated at 37^{o}C with gentle agitation, until dry. The procedure was repeated with the same amount of poly-HEMA solution and the plates were stored at 4^{o}C until use.

2.4.2 Anoikis assay

Adherent cells were grown until ~70% confluence and withdrawn from the effects of growth factors by culture in withdrawal medium for two days. Cells were detached from cell culture flasks by incubation with trypsin:EDTA. Cells were counted using a haemocytometer and diluted to the required density in the experimental medium. For experiments designed to investigate the effect of IGF-1 on cell survival, cells were resuspended in phenol red-free DMEM without serum (SFM), or SFM supplemented with various concentrations of IGF-1. The cell suspension was diluted to 100,000 cells/ml and wells were seeded with 2 ml/well. Cells were incubated in the poly-HEMA coated plates for 2-24 hours before lysis with RIPA buffer plus inhibitors.

2.5 Proliferation Assay

Adherent cells grown until ~70% confluence were detached from cell culture flasks by incubation with trypsin:EDTA. Cells were resuspended in maintenance medium and counted using a haemocytometer. Cells were seeded into 24-well plates at a density of 5,000 to 10,000 cells/well in 0.5 ml maintenance medium. After a 24-hour incubation, in order to allow

attachment, medium was removed gently using a pulled glass Pasteur pipette and replaced with DCCS or serum-free medium (SFM). Cells were cultured in withdrawal medium or serum-free medium for 24 hours. The next day the medium was replaced with DCCS medium containing 50 ng/ml IGF-1, IGF-2 or insulin. Withdrawal or serum-free medium with growth factors was replaced every 3 days. After 3, 6, and 9 days the medium was removed, cells were washed in cold PBS once and stored at -20^{o}C until analysis. The amount of DNA in each well was measured using a dsDNA quantification assay, as described in section 2.8.

For experiments which investigated the effect of the U0126 inhibitor on the proliferative effect of IGF-1, the cells were seeded into 24-well plates, as described above, and cultured in serum-free medium for 24 hours. The cells were then treated with the appropriate amounts of DMSO or the inhibitors for at least 30 minutes before the addition of the ligands. The medium containing the ligands and the inhibitors was replenished every 3 days and the experiments were stopped after 9 days of treatment.

For experiments designed to investigate the effect of the BMS754807 inhibitor on cell proliferation, adherent cells grown until ~70% confluence were detached from cell culture flasks by incubation with trypsin:EDTA. Cells were resuspended in maintenance medium and counted using a haemocytometer. Cells were seeded into 24-well plates at a density of 5,000 to 10,000 cells/well in 0.5 ml maintenance medium. The next day the medium was replenished with maintenance medium containing different concentrations of the BMS754807 inhibitor or DMSO. The medium was changed daily for up to 5 days and cells were stored at -20^{o}C for further analysis.

2.6 Protein Extraction and Quantification

2.6.1 Protein extraction

To extract proteins from attached cells, the medium was aspirated and cells were washed with cold PBS. The lysis buffer was radioimmunoprecipitation assay buffer (RIPA), which contained 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40 (v/v) and 0.25% sodium deoxycholate (w/v). Protease inhibitors were added at a final concentration of 1 μg/ml pepstatin, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM sodium orthovanadate, 2 mM sodium fluoride and 2 mM phenyl methyl sulphonyl fluoride. For 12-well plates, 80-100 μl of RIPA buffer plus inhibitors were added to each well and for 75 cm^2 cell culture flasks, 1 ml of RIPA buffer plus inhibitors was added per flask. Addition of the lysis buffer was followed by gentle

agitation on ice for 30 minutes. The lysis buffer was then pipetted repeatedly over the cell culture surface or the cell pellet and the lysate was transferred into 1.5 ml centrifuge tubes (Anachem). Cell lysates were centrifuged at 14,000 rpm (Thermo-scientific MicroCL 21R) for 10 minutes at 4^{o}C. The supernatant was then transferred to fresh centrifuge tubes and stored at -20^{o}C.

To extract proteins from non-attached cells cultured in poly-HEMA or cells that grow naturally in suspension, the medium containing the cells was collected into 15 ml Falcon tubes (Greiner Bio-one, Stroudwater, United Kingdom). The remaining cells were washed with cold PBS which was pipetted repeatedly over the flask or well surface. PBS was then collected and added to the cell suspension. Cells were centrifuged at 300 x g for 5 minutes at room temperature. The supernatant was removed and the cells were lysed with RIPA buffer plus inhibitors. Samples were agitated gently on ice for 30 minutes and then vortexed in order to ensure complete lysis. Samples were then transferred to 1.5 ml centrifuge tubes and centrifuged at 14,000 rpm for 10 minutes at 4^{o}C. The supernatant was transferred to fresh centrifuge tubes and stored at -20^{o}C.

2.6.2 Protein quantification

A binchinchonic acid assay (BCA, Pierce) was used for the measurement of protein concentrations of the cell lysates. Aliquots of 0.5 µl of each cell lysate were diluted in 4.5 µl sterile deionized water. Protein standards were prepared from bovine serum albumin (Sigma-Aldrich, Dorset, United Kingdom) in a 1:10 dilution of RIPA buffer in sterile deionized water. Standard concentrations ranged between 0.025 and 2 mg/ml. The assay reagents were mixed at a ratio of 50:1. For each standard and sample, 5 µl were mixed with 95 µl of BCA assay reagent mixture and incubated for 30 minutes at 37^{o}C. Samples were then transferred to ice and the absorbance at 562 nm was measured with a spectrophotometer (Beckman DU 640). The protein standards were used to generate a curve, with a correlation coefficient, R^2, greater than 0.99. The protein concentrations of the samples were interpolated from this standard curve.

2.7 Western Transfer Analysis

2.7.1 Polyacrylamide gel electrophoresis

Proteins were separated in sodium dodecyl sulphate (SDS) polyacrylamide gels. Separating gels comprised acrylamide and bisacrylamide in a 200:1 ratio, 0.5 M Tris-HCl pH 8.8, 0.1% sodium dodecyl sulphate (w/v), 0.05% ammonium persulphate (w/v) and 0.1% N, N, N', N'-tetramethylethylenediamine (TEMED) (v/v). Separating gels containing 20% acrylamide were used when the required proteins were smaller than 30 kDa, and gels containing 12% acrylamide were used for proteins larger than 30 kDa. Stacking gels contained 3% acrylamide and 0.15% bisacrylamide, 125 mM Tris-HCl pH 6.8, 0.1% SDS (w/v), 0.1% ammonium persulphate (w/v) and 0.5% TEMED. Both separating and stacking gels were poured in a vertical minigel apparatus (Hoeffer) and allowed to polymerise for 45 and 15 minutes, respectively.

Aliquots of the protein lysates containing equal amounts of protein were mixed with 124 mM Tris-HCl, 25 mM Na_2EDTA, 4% SDS (w/v), 20% glycerol (w/v), 0.01% bromophenol blue (w/v) and 10% β-mercaptoethanol (v/v) (pH 6.8) and heated at 95^{o}C for 10 minutes. Protein markers of known molecular mass were included in each polyacrylamide gel (Full Range Rainbow Marker: GE Life Sciences). Proteins were electrophoresed in a running buffer which comprised 0.5 M Tris-HCl, 0.38 M glycine and 0.1% SDS (w/v), at a constant current of 10 mA, in Mighty Small II electrophoresis chambers (Amersham Pharmacia).

2.7.2 Western transfer

After electrophoresis, proteins were transferred to a Westran 0.45 µM nitrocellulose membrane (VWR) in a semi-dry transfer apparatus (S&S CarboGlas, Peqlab PerfectBlue). The transfer sandwich comprised (from anode to cathode) two sheets of Whatman 3mm chromatography paper soaked in 0.3 M Tris, 20% methanol (Anode buffer 1), one sheet soaked in 25 mM Tris, 20% methanol (Anode buffer 2), the nitrocellulose membrane soaked in Anode buffer 2, the polyacrylamide gel, and three sheets of chromatography paper soaked in 25 mM Tris, 40 mM 6-amonicaproic acid, 20% methanol (Cathode buffer). Proteins were transferred at 100 mA for 10 to 30 minutes when the target proteins were smaller than 30 kDa, or for 45 to 60 minutes for proteins larger than 30 kDa. Following transfer, the membranes were dried overnight. The next day, the membranes were blocked in 5% milk (v/v) in 20 mM Tris-HCl, 140 mM NaCl, 0.1% Tween 20 (v/v) (TBS-Tween solution) for one

hour at room temperature. After three washes in TBS-Tween the membranes were incubated with primary antibody diluted in 5 ml of 5% milk in TBS-Tween, in 50 ml Falcon tubes and agitated on rollers overnight at 4^{o}C. Membranes were washed thrice in TBS-Tween before incubation in horse-radish peroxidase conjugated secondary antibody. The secondary antibody was diluted in 5 ml of 5% milk in TBS-Tween in 50 ml Falcon tubes and the membranes were incubated for 60 minutes agitated on rollers at room temperature. They were then washed again in TBS-Tween thrice and in TBS once. The membranes were incubated in a 1:1 mix of Supersignal West Dura extended duration substrate chemiluminescent solutions Luminol/Enhancer and Peroxide buffer, for 5 minutes at room temperature and exposed to X-ray film (Fuji, SuperRX).

A list of all the antibodies used and their origin is shown in Table 2.2.

Antibody	**Source**	**Concentration**	**Species**	**Antibody type**
Cleaved PARP (Asp 214)	Cell signaling technologies #9541	1:2000	Rabbit	Polyclonal
Cleaved caspase 3 (Asp175)	Cell signaling technologies #9661	1:1000	Rabbit	Polyclonal
IGF-IR-β	Cell signaling technologies #3027	1:2000	Rabbit	Polyclonal
IR-β	Cell signaling technologies #3025	1:1000	Rabbit	Monoclonal
IGF-IIR	Santa Cruz-25462	1:1000	Rabbit	Polyclonal
Phosphorylated HER-2/ErbB2 (Tyr1221/1222)	Cell signaling technologies #2243	1:1000	Rabbit	Monoclonal
HER-2	Cell signaling technologies #2165	1:1000	Rabbit	Monoclonal
Met	Cell signaling technologies #4560	1:2000	Rabbit	Polyclonal
FGFR-2	Fisher scientific- PA5-29426	1:1000	Rabbit	Polyclonal
Phosphorylated IGF-IR/IR (Tyr 1135/1136)	Cell signaling technologies #3024	1:1000	Rabbit	Monoclonal
IRS-1	Cell signaling technologies #3407	1:2000	Rabbit	Monoclonal
Akt	Cell signaling	1:2000	Rabbit	Polyclonal

	technologies #9272			
Phosphorylated Akt (Ser 473)	Cell signaling technologies #4060	1:2000	Rabbit	Monoclonal
ERK1/ERK2	Cell signaling technologies #9102	1:5000	Rabbit	Polyclonal
Phosphorylated ERK1/ERK2 (Thr202/Tyr204)	Cell signaling technologies #4370	1:5000	Rabbit	Monoclonal
Phosphorylated Bad (Ser136)	Cell signaling technologies #4366	1:1000	Rabbit	Monoclonal
GAPDH-HRP	Santa Cruz-25778	1:10000	Rabbit	Polyclonal
β-Tubulin (9F3)	Cell signaling technologies #5346	1:5000	Rabbit	Monoclonal
Anti-rabbit HRP	Cell signaling technologies #7074	1:5000	Goat	-
Anti-mouse HRP	Cell signaling technologies #7076	1:5000	Horse	-

Table 2.2. Origin of antibodies for western transfer analyses and dilutions used.

2.8 DNA Quantification

DNA was quantified using the Quanti-iT picogreen fluorimetric assay (Invitrogen). Prior to analysis, cells were frozen at -20^{o}C overnight. Cells were lysed in 0.15 M NaCl, 17 nM sodium citrate adjusted to pH 7.0, with 0.02% SDS (saline sodium citrate solution SSC:SDS) and incubated at 37^{o}C for 30 minutes with gentle agitation. DNA was sheared and homogenised by repeated pipetting of the lysis buffer through a 25g needle with a 2 ml syringe. DNA standards were prepared from calf thymus DNA (Invitrogen) in 0.02% SSC /SDS solution at a concentration range of 0.005-2 μg/ml. A volume of 50 μl of each sample and DNA standard were pipetted into a black 96-well plate (Greiner). Samples with high DNA concentration were diluted at a range of 1:2 to 1:10. The PicoGreen reagent was diluted 1:200 in 10 mM Tris-HCl, 1 mM Na_2EDTA adjusted to pH 7.5 (Tris-EDTA buffer) and 50 μl added to each well. The plates were covered in foil until measurement. The samples were excited at 485 nm and fluorescence emission intensity was measured at 520 nm, within 5 minutes of addition of the PicoGreen reagent. A standard curve was created from the DNA standards and the DNA concentrations of the samples were interpolated from the standard curve using MARS analysis software.

2.9 Immunofluorescence

Adherent cells grown on coverslips inside 6-well plates were fixed in methanol at -20^{o}C for 30 minutes, in 4% paraformaldehyde at room temperature for 20 minutes or in 70 % ethanol at room temperature for 20 minutes, depending on the protein of interest. Cells were blocked in 5% goat serum (Invitrogen, Paisley, United Kingdom), PBS, 0.3 % Triton-X for 1 hour and incubated with the appropriate concentration of primary antibody diluted in PBS with 0.3% Triton-X, at 4^{o}C overnight. The antibodies used and the different fixation methods corresponding to each antibody are shown in Table 2.3. Proteins were visualised by incubation in Alexa Fluorochrome-labelled antibodies (New England Biolabs, Hitchin, United Kingdom), followed by nuclear staining/mounting in Vectashield Mounting Medium with DAPI (Vector laboratories). A fluorescent microscope was used to take pictures of the cells (Leica). For quantification, five fields of view were analysed for each treatment.

For cells grown in suspension all incubations were in a 1.5 ml eppendorf tube, with repeated centrifugation steps at 500xg for 5 minutes. At the end of the procedure, the cells were resuspended in Vectashield Mounting Medium with DAPI, placed on top of microscope slides and mounted with coverslips.

Antibody	Source	Fixation method	Species	Antibody type
Cleaved PARP (Asp 214)	Cell signaling technologies #6894	Methanol	Rabbit	Monoclonal
Cleaved caspase 3 (Asp 175)	Cell signaling technologies #9603	Methanol	Rabbit	Monoclonal
Phospho-Akt	Cell signaling technologies #4060	Methanol	Rabbit	Monoclonal
Phospho-Histone H3 (Ser 10)	Cell signaling technologies #9701	4% paraformaldehyde	Rabbit	Polyclonal
BrdU	Cell signaling technologies #5292	70% ethanol, 1.5M HCl	Mouse	Monoclonal
Pan-Keratin (C11)	Cell signaling technologies #3478	Methanol	Mouse	Monoclonal
Anti-rabbit (AF 488)	Invitrogen #A-11034	-	Goat	-
Anti-mouse (AF 488)	Invitrogen #A-11001	-	Goat	-

Table 2.3. Antibodies for immunofluorescence analyses, their dilutions and the initial fixation conditions.

2.10 RNA Extraction

RNA was extracted with the Direct-zol™ RNA MiniPrep from Zymo Research. Cells were grown in 25 cm^2 tissue culture flasks until they were 80% confluent. They were washed in PBS once before the addition of 700 µl TRIzol (Invitrogen, Paisley, United Kingdom). TRIzol was pipetted repeatedly on top of the cell surface until cells were completely lysed. Lysed cells were transferred to an Eppendorf tube, vortexed vigorously and left to stand for 5 minutes at room temperature. After centrifugation at 12,000 x g for 1 minute, the supernatant was transferred to a fresh tube containing an equal volume of ethanol (95-100%). After vigorous vortexing, the lysate was loaded onto a Zymo-Spin™ IIC Column in a collection tube and centrifuged for 1 minute at 16,000 x g. The column was transferred into a new collection tube and 400 µl Direct-zol™ RNA PreWash buffer were added to the column, before centrifugation at 16,000 x g for 1 minute. This step was repeated once. Then, 700 µl RNA Wash Buffer were added to the column before another centrifugation at 16,000 x g for 1 minute. The flow-through was discarded and 25 µl of DNase/RNase-Free water were added directly to the column matrix. After a final step of centrifugation at 21,000 x g for 1 minute the eluted RNA was stored at -70^{o}C.

RNA was quantified by UV spectroscopy (Beckman DU 640). RNA samples were diluted 1:100 in 10 mM Tris, pH 8, 1 mM EDTA and the absorbance was measured at 260 and 280 nM. The A260/A280 ratio was used to assess the RNA purity.

2.11 Reverse Transcription and Polymerase Chain Reaction

Complementary DNA was synthesised from total RNA with the Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT) (Usb) in a 20 µl reaction. RNA aliquots of 1 µg were mixed with 0.5 µM Random Hexamers (Invitrogen) and sterile deionised water in a 0.5 ml Eppendorf tube and incubated at 70^{o}C for 10 minutes. The buffer was adjusted to pH 8.3 with Tris-HCl, 75 mm KCl, 3 mM MgCl2 and 10 mM DTT and 4mM dNTPs and 60 units of M-MLV RT were added. After vortexing, the sample was incubated at 25^{o}C for 10 minutes and at 37^{o}C for 60 minutes. To inactivate the enzyme, 7 mM EDTA pH 8 were added and the sample was incubated at 75^{o}C for 15 minutes. The sample was placed on ice to chill for 5 minutes and the evaporated liquid was recovered by centrifugation. Complementary DNA was stored at -20^{o}C.

Complementary DNA was amplified with the Red Hot DNA polymerase (Thermo-scientific) in a 10 µl PCR reaction. The standard protocol comprised 75 mM Tris-HCl (pH 8.8 at 25^{o}C), 20 mM (NH4)2SO4, 0.01% (v/v) Tween 20, 0.2 mM dNTPs, 0.5 µM forward primer, 0.5 µM reverse primer, 0.8 mg/ml bovine serum albumin, 1.5 mM $MgCl_2$, 0.625 units Red Hot DNA polymerase (Life technologies Ltd, Paisley, United Kingdom), 10 ng cDNA and sterile deionised water up to 10µl. The primers were obtained from Sigma-Aldrich and their sequences are shown in Table 2.4.

Polymerase chain reaction was in a thermocycler using the following conditions: initial denaturation for 2 minutes at 94^{o}C, two cycles of 90 seconds at 94^{o}C, 30 seconds at the annealing temperature and 60 seconds at 72^{o}C, 28-33 cycles of 20 seconds at 94^{o}C, 30 seconds at the annealing temperature and 60 seconds at 72^{o}C and final extension for 5 minutes at 72^{o}C. The annealing temperatures used for each pair of primers and the number of cycles for each reaction are shown in Table 2.4.

Primers	Nucleotide sequence (5'-3')	Annealing temperature	No of cycles
Insulin receptor (forward)	TGCTGCTCCTGTCCAAAGAC	61^{o}C	31
Insulin receptor (reverse)	TCACATTCCCAACATCGCCA		
Insulin receptor isoform A (forward)	GTTTTCGTCCCCAGGCCAT	61^{o}C	35
Insulin receptor isoform A (reverse)	GATGCGATAGCCCGTGAAGT		
Insulin receptor isoform B (forward)	CGTGGTTTTCGTCCCCAGAA	61^{o}C	35
Insulin receptor isoform B (reverse)	GACCAGCGACTCCTTGTTCA		
IRS-1 (forward)	AGAGGACCGTCAGTAGCTCA	57^{o}C	35
IRS-1 (reverse)	ACTGAAATGGATGCATCGTACC		
IRS-2 (forward)	GGCATTCCAGCCCCTATGTT	61^{o}C	35
IRS-2 (reverse)	AGCCCTCCAATCAAGTGTCG		
IRS-4 (forward)	GACCCCCGGAGAGAGAAGAT	57^{o}C	35
IRS-4 (reverse)	ACTGTAGACTGTAGCGCATCG		
18s rRNA (forward)	GCAATTATTCCCCATGAACG	61^{o}C	30
18s rRNA (reverse)	GGGACTTAATCAACGCAAGC		

Table 2.4. Primer sequences and PCR conditions

2.12 DNA Agarose Gels

DNA was electrophoresed in 3% agarose gels, which comprised 2% Nusieve agarose (FMC Bioproducts) and 1% Type I agarose (Sigma-Aldrich, Dorset, United Kingdom) in 89 mM Tris base, 89 mM Boric acid and 2 mM EDTA (pH 8.3) (TBE buffer). The agarose was stained with GelRed (Cambridge bioscience, Cambridge, United Kingdom) before casting the gels. Agarose gels were poured into a DNA gel chamber (Bioscience Services) and left to polymerise for 60 minutes. The PCR products were mixed with 0.2% Ficoll (w/v), 100 mM EDTA, 0.04% bromophenol blue (w/v) in TBE buffer. DNA was electrophoresed in TBE buffer, at 60 volts. DNA was visualised with Gel Doc (Bio-Rad).

2.13 Gene Silencing With Small Interfering RNA

Short interfering RNA sequences designed to target the type I IGF receptor and matched scrambled sequences were purchased from Sigma-Aldrich. The siRNA and scrambled

sequences are shown in Table 2.5. Cells were transfected with a reverse transfection procedure. The transfection medium was prepared by mixing serum-free DMEM with siRNA or scrambled oligonucleotides and lipofectamine (Invitrogen, Paisley, United Kingdom). Scaling of the transfection reagents according to the surface of tissue culture plasticware is shown in Table 2.6. The different concentrations of siRNAs used for each cell line are shown in Table 2.7. The transfection medium was incubated for 30 minutes at room temperature before the addition of cell suspension. Cells were incubated for 24 hours in 37^{o}C and were used in experiments, thereafter.

Oligonucleotide	**Sequence (5'-3')**
Scrambled 1 sense	UUCUCCGAACGUGUCACGU[dT][dT]
Scrambled 1 antisense	ACGUGACACGUUCGGAGAA[dT][dT]
Scrambled 2 sense	GCGCGAUAGCGCGAAUAUA[dT][dT]
Scrambled 2 antisense	UAUAUUCGCGCUAUCGCGC[dT][dT]
siRNA 2 (IGF-IR) sense	CUGACUACAGGGAUCUCAU[dT][dT]
siRNA 2 (IGF-IR) antisense	AUGAGAUCCCUGUAGUCAG[dT][dT]
siRNA 3 (IGF-IR) sense	GGAAUUGCAUGGUAGCCGA[dT][dT]
siRNA 3 (IGF-IR) antisense	UCGGCUACCAUGCAAUUCC[dT][dT]
siRNA 2 (IR) sense	GAAACUGCAUGGUCGCCCA[dT][dT]
siRNA 2 (IR) antisense	UGGGCGACCAUGCAGUUUC[dT][dT]
siRNA 3 (IR) sense	GAAACUCUUCUUCCACUAU[dT][dT]
siRNA 3 (IR) antisense	AUAGUGGAAGAAGAGUUUC[dT][dT]

Table 2.5. Oligonucleotide sequences.

Cell culture vessel	**Surface area (cm^2)**	**Volume of plating medium (ml)**	**Volume of transfection medium (µl)**	**Number of cells per well**	**siRNA (nM)**	**Lipofectamine (µl) (1:500)**
6-well plate	9.5	2.5	500	250.000	20-50	5 ul
12-well plate	3.8	1.25	250	100.000	20-50	2.5 ul
24-well plate	1.9	500	100	50.000	20-50	1 ul

Table 2.6. Scaling of transfection reagents.

Cell line	siRNA 2 (IGF-IR) (nM)	siRNA 3 (IGF-IR) (nM)	siRNA 2 (IR) (nM)	siRNA 3 (IR) (nM)
SNU-1	50	50	20	50
MKN74	40	20	-	-
NUGC3	40	20	20	50
AGS	40	20	-	-
GC	40	20	-	-
HC	20	20	-	-
NC	40	20	-	-
JW	40	20	-	-

Table 2.7. SiRNA concentration for each cell line.

2.14 Treatment of Cells with Drugs

Adherent cells were grown until ~70% confluence and detached from cell culture flasks by incubation with trypsin:EDTA. Cells were resuspended in maintenance medium and counted using a haemocytometer. For experiments designed to investigate the cytotoxic effect of oxaliplatin on gastric cancer cells, cells were seeded in 12-well tissue culture plates, in 1 ml maintenance medium at a concentration of 100,000 to 150,000 cells/well. Cells were left to attach for ~24 hours and the medium was changed to medium containing the appropriate amounts of oxaliplatin. Protein extracts were prepared after 1 to 3 days of incubation with the cytotoxic agents.

For experiments designed to investigate the effect of oxaliplatin, 5-fluorouracil (Sigma-Aldrich, Dorset, United Kingdom), lapatinib (LC laboratories, United States), AZD4547 (LC laboratories, United States), crizotinib (LC laboratories, United States) and BMS754807 (Bristol-Myers Squibb, Middlesex, United Kingdom) on cell number, cells were plated in 24-well plates at a concentration of 50000 cells/well or in 48-well plates at a concentration of 25000 cells/ well and treated with a range of concentrations for 3 days. Cells were then washed with PBS once and kept in -20^{o}C overnight before analysis with Quanti-iT picogreen fluorimetric assay (Invitrogen, Paisley, United Kingdom).

For the establishment of lapatinib resistant NCI-N87 cells, NCI-N87 cells were treated for a period of 6 months with gradually increasing concentrations of lapatinib ranging from 0.05 to 0.25 μM.

2.15 Statistical analysis

Experiments were replicated at least thrice. Differences between groups were tested by one-way or two-way analysis of variance (ANOVA) and paired or unpaired t test. P values < 0.05 were considered statistically significant.

Chapter 3. Expression and Activation of Components of the IGF Signal Transduction Pathway in Gastric Cancer Cells

3.1 Introduction

In advanced gastric cancer, trastuzumab is the first targeted agent approved for the treatment of HER-2-overexpressing tumours (Bang *et al.*, 2010). Inhibitors that target vascular endothelial growth factor, mammalian target of rapamycin, c-Met and FGFR2 are also under investigation (Qiu and Xu, 2013) (Su *et al.*, 2014). Approximately 13-22% of advanced gastric cancers have HER-2 amplification or overexpression, 2-10% have c-Met amplification and 4-8% have FGFR2 amplification (Gravalos and Jimeno, 2008) (Kawakami *et al.*, 2013) (Xie *et al.*, 2013) (Su *et al.*, 2014). There is, therefore, a large remaining population of patients that require alternative targeted treatments.

In order to identify tumours that are most likely to benefit from IGF-targeted therapies it is necessary to measure the expression of components of molecular pathways which are already targeted in advanced gastric cancer, in combination with components of the IGF-signal transduction pathway. Detection of a receptor amplification in a growth factor pathway different to the IGF-signal transduction pathway would automatically exclude the possibility of administering IGF-targeted monotherapy for this tumour type.

Unlike other growth factor pathways, it is quite uncommon for components of the IGF-signal transduction pathway to be amplified in cancer. However, detection of expression and activation of receptors and downstream molecules of the IGF-signal transduction pathway is of particular importance for elucidating the implication of the pathway in tumour progression.

3.1.1 Aim

The aim of the results reported in this chapter is to determine the expression of receptors and downstream molecules of the IGF-signal transduction pathway and other important growth factor pathways, in gastric cancer cells. Activation of the receptors and downstream molecules by IGF-1 was investigated in the same panel of gastric cancer cell lines. The effectiveness of a small molecule tyrosine kinase inhibitor in elimination of signal transduction in response to IGF-1 was also investigated.

3.2 Results

3.2.1 The relative expression of IGF-signal transduction pathway proteins in gastric cancer cells

The responsiveness of a particular cell type to IGFs might depend on the expression levels of the receptors and downstream signal transduction molecules. The type I IGF receptor binds both IGF-1 and IGF-2 with high affinity and is the main receptor that mediates activation of downstream signal transduction pathways. The ligands IGF-1 and IGF-2 bind also to the type II IGF receptor, which lacks a tyrosine kinase domain and cannot transduce a downstream signal. However, the type II IGF receptor is of particular importance in regulating the bioavailability of IGF-1 and IGF-2. For example, high expression levels of the type II IGF receptor result in reduced availability of IGF-1 and IGF-2. Insulin receptor binds insulin with the highest affinity but it also binds IGF-1 and IGF-2 with lower affinity. In particular, the foetal splice variant of the insulin receptor, IR-A, is highly expressed in many cancer types and has a high affinity for IGF-2 (Frasca *et al.*, 1999). Furthermore, the formation of hybrids between the type I IGF receptor and the insulin receptor attracts IGF-1 with high affinity and adds more complexity to the ligand-receptor interaction.

Activation of the type I IGF and insulin receptors attracts adaptor proteins such as the insulin receptor substrate proteins 1, 2 and 4 (IRS 1, 2 and 4). Activation and phosphorylation of the IRS proteins result in activation of the downstream PI3K/Akt and Ras/Raf/MAPK pathways.

Expression of the three receptors of the IGF-signal transduction pathway and of downstream molecules was investigated in a panel of gastric cancer cells lines. NCI-N87, KATO III, SNU-16, SNU-5, SNU-1, MKN74, NUGC3 and AGS cells were cultured until 80% confluence and protein extracts were prepared. The expression of the three receptors and downstream molecules in each cell line was determined by western transfer analysis or by RT-PCR.

NCI-N87 and AGS cells expressed the type I IGF and insulin receptors at lower levels compared to the other cells lines (Figure 3.1). KATO III and SNU-16 cells expressed higher levels of insulin receptor and type I IGF receptor and SNU-16 cells did not express the type II IGF receptor. SNU-5 cells expressed the insulin receptor and had much lower levels of the type I IGF receptor compared to the other cell lines. On the contrary, MKN74 cells expressed the highest levels of the type I IGF receptor among the cell lines, comparable with MCF7 breast cancer cells and SNU-16 cells, but had extremely lower levels of the insulin receptor in comparison with the remaining cell lines. Expression of the type I IGF and insulin receptors

was similar in SNU-1 and NUGC3 cells, with SNU-1 cells expressing slightly higher levels of type II IGF receptor.

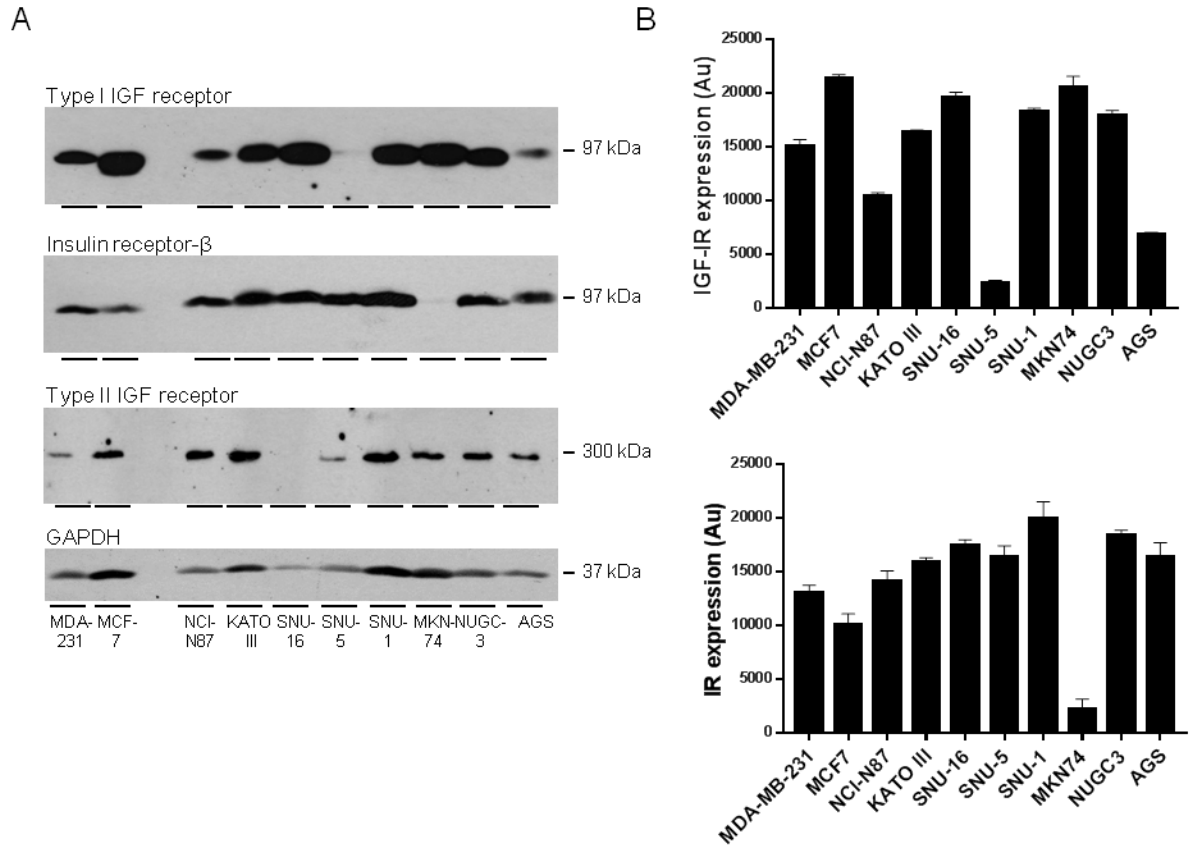

Figure 3.1. Expression of IGF-signal transduction pathway proteins in gastric cancer cells. NCI-N87, KATO III, SNU-16, SNU-5, SNU-1, MKN74, NUGC3 and AGS cells were cultured in 75 cm^2 tissue culture flasks in maintenance medium to ~80% confluence. Proteins were extracted with 1 ml per flask of RIPA buffer plus inhibitors, as described in the Materials and Methods section. Aliquots containing 10-20 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against the IGF-IR-β (1:2000), IR-β (1:1000), IGF-IIR (1:1000) and GAPDH (1:10000). All secondary antibodies were horseradish-peroxidase bound. Bands were visualised on Fuji X-ray film following incubation of membranes with Supersignal chemiluminescent solutions, as described in the Methods section. The images shown are representative of results from three separate experiments. The molecular masses of the proteins are indicated on the right hand side of the panels (**A**). Densitometric analysis of the bands for IGF-IR and IR was by Labworks 4.0 software. Measurements were adjusted for the background optical density of the X-ray film. Expression was not normalised to GAPDH, due to variation in GAPDH expression across different cell lines. Bars are the mean ±SEM (**B**).

Expression of IRS-1 was variable among the cell lines, with SNU-1 cells expressing the highest levels, followed by NCI-N87, MKN74 and AGS cells (Figure 3.2). IRS-1 was not detected in SNU-16 cells. The mRNA of IRS-1 was detected by PCR in all gastric cancer cell lines (Figure 3.3). The highest levels of IRS-1 mRNA were detected in SNU-1, SNU-5 and AGS cells, followed by NCI-N87, MKN74, KATO III, SNU-16 and NUGC3. Due to the lack of high quality commercially available antibodies for IRS-2 and IRS-4, their expression was detected only by PCR. IRS-2 was expressed in all the cell lines, with lowest expression

detected in KATO III, AGS and SNU-1 cells. IRS-4 was not expressed in NUGC3 cells and was detected at highest levels in SNU-5 and SNU-16 cells (Figure 3.3). All cell lines expressed Akt, with KATO III, MKN74 and AGS cells having the lowest levels of expression. Similarly, ERK1 and 2 were expressed in all of the cell lines and SNU-5 cells expressed the lowest levels (Figure 3.2).

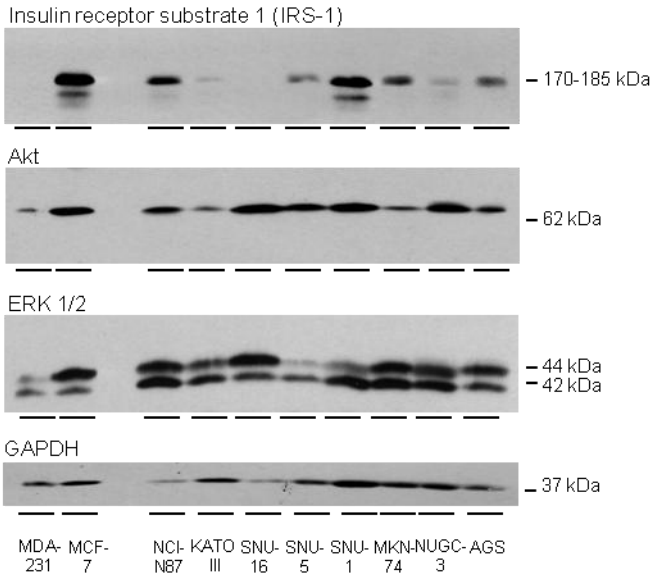

Figure 3.2. Expression of IGF-signal transduction pathway proteins in gastric cancer cells. NCI-N87, KATO III, SNU-16, SNU-5, SNU-1, MKN74, NUGC3 and AGS cells were cultured in 75 cm^2 tissue culture flasks in maintenance medium to ~80% confluence. Proteins were extracted with 1 ml per flask of RIPA buffer plus inhibitors, as described in the Materials and Methods section. Aliquots containing 10-20 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against IRS-1 (1:2000), Akt (1:3000) ERK1/2 (1:5000) and GAPDH (1:10000). All secondary antibodies were horseradish-peroxidase bound. Bands were visualised on Fuji X-ray film following incubation of membranes with Supersignal chemiluminescent solutions, as described in the Methods section. The images shown are representative of results from three separate experiments. The molecular masses of the proteins are indicated on the right hand side of the panels.

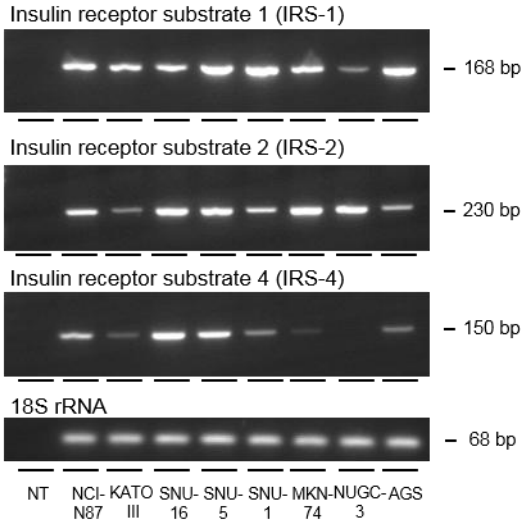

Figure 3.3. Expression of genes that encode for IGF-signal transduction pathway proteins in gastric cancer cells. NCI-N87, KATO III, SNU-16, SNU-5, SNU-1, MKN74, NUGC3 and AGS cells were cultured in 25 cm^2 tissue culture flasks in maintenance medium to ~80% confluence. Total RNA was extracted from the cells with the Direct-zol™ RNA MiniPrep, as described in the Materials and Methods section. Complementary DNA was synthesised from total RNA with the Moloney Murine Leukaemia Virus Reverse Transcriptase. Specific primer sequences were used to amplify IRS-1, IRS-2, IRS-4 and 18S rRNA. The amplified DNA was electrophoresed in a 3% agarose gel and bands were visualised with a UV transilluminator.

The level of IGF-IR expression in the gastric cancer cell lines was comparable to the level of expression in the breast cancer cell line MCF-7. MCF-7 cells are known to express high levels of the type I IGF receptor and IRS-1 and are particularly responsive to IGFs (Stewart *et al.*, 1990). SNU-1 cells, which also have relatively high levels of the type I IGF receptor and IRS-1, are expected to be highly responsive to stimulation with IGF-1 and IGF-2. SNU-5 cells are expected to be the least responsive to IGFs, since they express the type I IGF receptor and IRS-1 at extremely low levels.

3.2.2 The relative expression of HER-2, c-Met and FGFR2 in gastric cancer cells

Inhibition of the dependence of malignant cells on amplified or overexpressed tyrosine kinase receptors has proven an effective therapeutic strategy. The genes that encode the three tyrosine kinase receptors, HER-2, FGFR-2 and c-Met are known to be amplified in gastric cancer. As mentioned previously, approximately 13-22% of advanced gastric cancers have HER-2 amplification or overexpression, 2-10% have c-Met amplification and 4-8% have FGFR2 amplification (Gravalos and Jimeno, 2008; Kawakami *et al.*, 2013; Xie *et al.*, 2013; Su *et al.*, 2014).

In order to extend the expression profile of the gastric cancer cell lines, expression of the above three receptors was measured by western transfer (Figure 3.4). HER-2 was expressed at extremely high levels in NCI-N87 cells. Expression was even higher than in the HER-2 amplified breast cancer SKBR3 cells. No HER-2 expression was detected in the remaining cell lines by western transfer. Similarly, KATO III and SNU-16 were the only two cell lines that overexpressed FGFR2. SNU-5 cells expressed the highest levels of the hepatocyte growth factor receptor (c-Met), followed by KATO III, SNU-16 and NUGC3 cells (Figure 3.4).

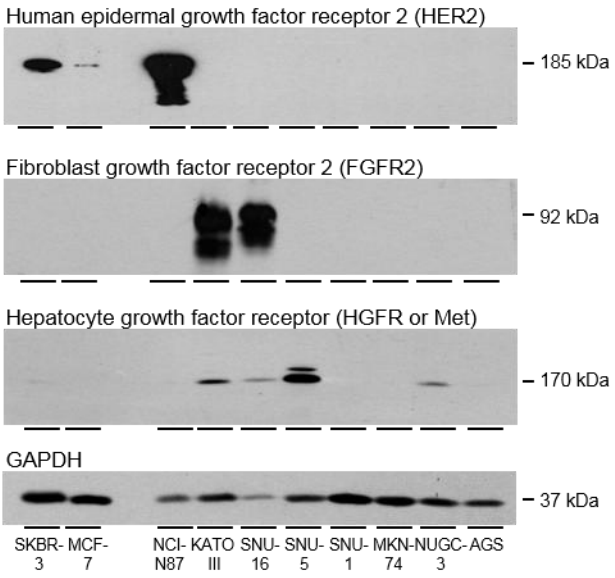

Figure 3.4. Expression of HER-2, FGFR2 and c-Met in gastric cancer cells. NCI-N87, KATO III, SNU-16, SNU-5, SNU-1, MKN74, NUGC3 and AGS cells were cultured in 75 cm^2 tissue culture flasks in maintenance medium to ~80% confluence. Proteins were extracted with 1 ml per flask of RIPA buffer plus inhibitors, as described in the Materials and Methods section. Aliquots containing 10-20 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against the HER-2 (1:2000), FGFR2 (1:1000) and Met (1:1000). All secondary antibodies were horseradish-peroxidase bound. Bands were visualised on Fuji X-ray film following incubation of membranes with Supersignal chemiluminescent solutions, as described in the Methods section. The molecular masses of the proteins are indicated on the right hand side of the panels.

Overexpression and/or amplification of a receptor which is not a member of the IGF-signal transduction pathway could affect the responsiveness of a particular cell type to IGFs. Cells with such amplification are expected to depend primarily on their amplified receptors, for activation of downstream pathways and induction of cell survival and proliferation. It is, therefore, likely that those cells will not be very sensitive to ligands from other pathways, such as IGF-1, IGF-2 and insulin. Consequently, NCI-N87, KATO III, SNU-16 and SNU-5 cells might not be as responsive to IGF stimulation as the remaining four cell lines, which don't have HER-2, FGFR2 or c-Met amplification.

3.2.3 The effect of IGF-1 stimulation on the activity of IGF-signal transduction pathway proteins

The effect of stimulation with IGF-1 on activation and phosphorylation of components of the IGF signal transduction pathway was tested in NCI-N87, KATO III, SNU-16, SNU-5, SNU-1, MKN74, NUGC3 and AGS cells. The cells were withdrawn from the effects of growth factors present in the serum by culture in medium containing dextran-coated charcoal stripped serum (DCCS). Phosphorylation of proteins of the IGF-signal transduction pathway in cells that had been treated with 50 ng/ml IGF-1 was determined by western transfer.

Phosphorylation of the type I IGF receptor and insulin receptor was not detected in any of the cell lines after culture in growth factor-depleted medium for 48 hours (Figure 3.5). Stimulation with IGF-1 resulted in the induction of phosphorylated IGF-IR and/or IR in all cell lines. The highest amount of phosphorylation was detected in MKN74 cells, which also expressed the highest levels of total IGF-IR. The lowest amount of phosphorylation was observed in SNU-5 cells, which expressed the lowest levels of total IGF-IR. The amount of total IGF-IR was not altered by stimulation with IGF-1 in any of the cell lines.

There were baseline levels of Akt phosphorylation in NCI-N87, MKN74, NUGC3 and AGS cells after withdrawal of growth factors from the culture medium. However, stimulation with IGF-1 increased Akt phosphorylation in all four cell lines. KATO III, SNU-16, SNU-5 and SNU-1 cells had no detectable baseline levels of Akt phosphorylation. IGF-1 stimulation induced Akt phosphorylation in all of these cell lines. Most effective induction of Akt phosphorylation in response to IGF-1 stimulation was observed in SNU-1 cells, which had no baseline levels of phosphorylated Akt and had the highest amount of phosphorylated Akt after stimulation with IGF-1. This suggests that activation of the Akt pathway might be highly dependent on IGF-1-induced phosphorylation of the type I IGF receptor in those cells. Total Akt levels were not affected by stimulation with IGF-1 in any of the cell lines (Figure 3.5).

Phosphorylated ERK2 was detected in the absence of IGF-1 in all the cell lines used here. There was no increase in ERK1 and ERK2 phosphorylation after stimulation with IGF-1 in KATO III and SNU-5 cells. Most effective induction of ERK2 phosphorylation was observed in MKN74, NUGC3, AGS and SNU-16 cells, followed by a smaller induction in NCI-N87 and SNU-1 cells. ERK1 phosphorylation was induced after stimulation with IGF-1 in NCI-N87, SNU-16, SNU-1 and MKN74 cells. The levels of total ERK1 and ERK2 remained unchanged after stimulation with IGF-1 in all cell lines, except NCI-N87 cells, where a small increase after IGF-1 stimulation was detected (Figure 3.5).

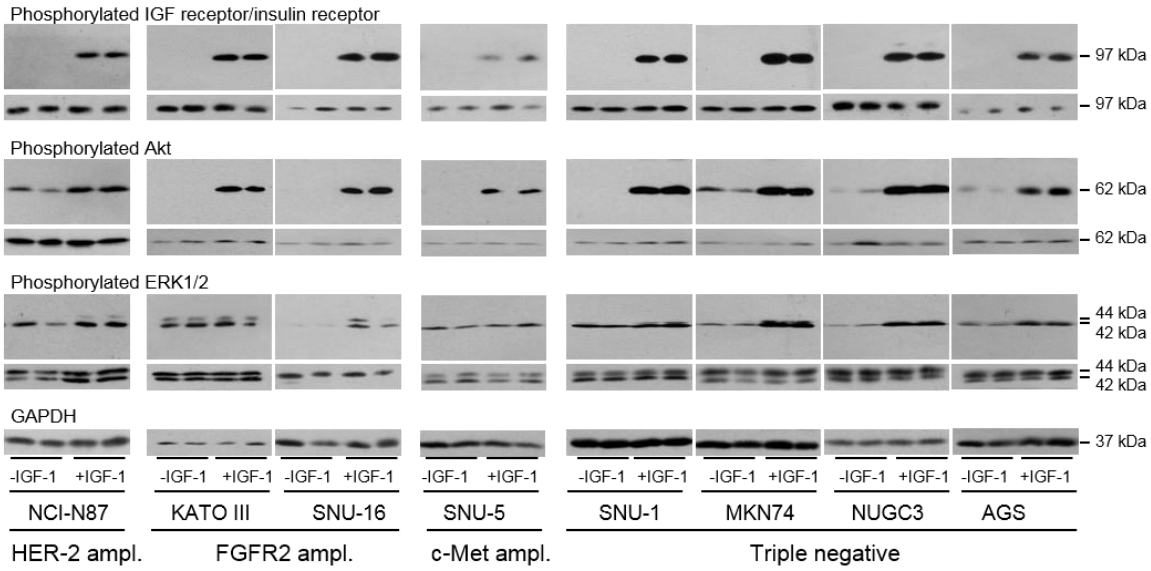

Figure 3.5. The effect of stimulation with IGF-1 on phosphorylation of IGF signal transduction pathway proteins in gastric cancer cell lines. SNU-16, SNU-5 and SNU-1 cells were centrifuged and resuspended in withdrawal medium and seeded into a 75 cm^2 flask at a concentration of $0.6x10^6$ cells per flask. After 24 hours, medium was replaced with 12 ml fresh withdrawal medium for another 24 hours. Cells were centrifuged and resuspended in serum-free medium with 0.1% BSA for 2 hours. Cells were then centrifuged and resuspended in serum-free medium with or without 50 ng/ml IGF-1 for 15 minutes. Cells were collected in a 15 ml Falcon tube and proteins were lysed with 50 µl RIPA buffer plus inhibitors. NCI-N87, KATO III, MKN74, NUGC3 and AGS cells were seeded into 12 well tissue culture plates at $15x10^4$ cells/well, in 1ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 0.5 ml PBS. Medium was replaced daily with 1 ml withdrawal medium for two days. Cells were incubated with serum-free medium which contained 0.1% BSA for two hours. Cells were incubated with 50 ng/ml IGF-1 for 15 minutes. Proteins were extracted with 80 µl per well RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against phosphorylated IGF-IR/IR (1:1000 dilution), IGF-IR (1:2000 dilution), phosphorylated Akt (1:3000 dilution), Akt (1:3000 dilution), phosphorylated ERK1/2 (1:5000 dilution), ERK1/2 (1:5000 dilution) and GAPDH (1:10000 dilution). Images obtained after incubation with antibodies against the total proteins are shown beneath those of the phosphorylated proteins. The images shown are representative of results from three separate triplicate experiments.

To investigate the concentration dependence of the IGF-1 activation effect of signal transduction proteins SNU-1 and NUGC3 cells were incubated with different IGF-1 concentrations up to 200 ng/ml. SNU-1 and NUGC3 were chosen as representative triple-negative gastric cancer cell lines that express relatively high levels of both the type I IGF and insulin receptors compared to other cell lines and that respond well to IGF-1 stimulation (Figure 3.5). Induction of IGF receptor phosphorylation was detected in SNU-1 cells, after stimulation with 5 ng/ml IGF-1 or higher (Figure 3.6). Maximal phosphorylation was achieved with 50 ng/ml IGF-1. Treatment with 200 ng/ml IGF-1 did not increase the amount of phosphorylated IGF-IR/IR, suggesting that the receptor(s) were saturated. Phosphorylated IGF-IR/IR was detected in NUGC3 cells after treatment with 2 ng/ml IGF-1 or higher. Again the maximal levels of phosphorylation were achieved with 50 ng/ml IGF-1.

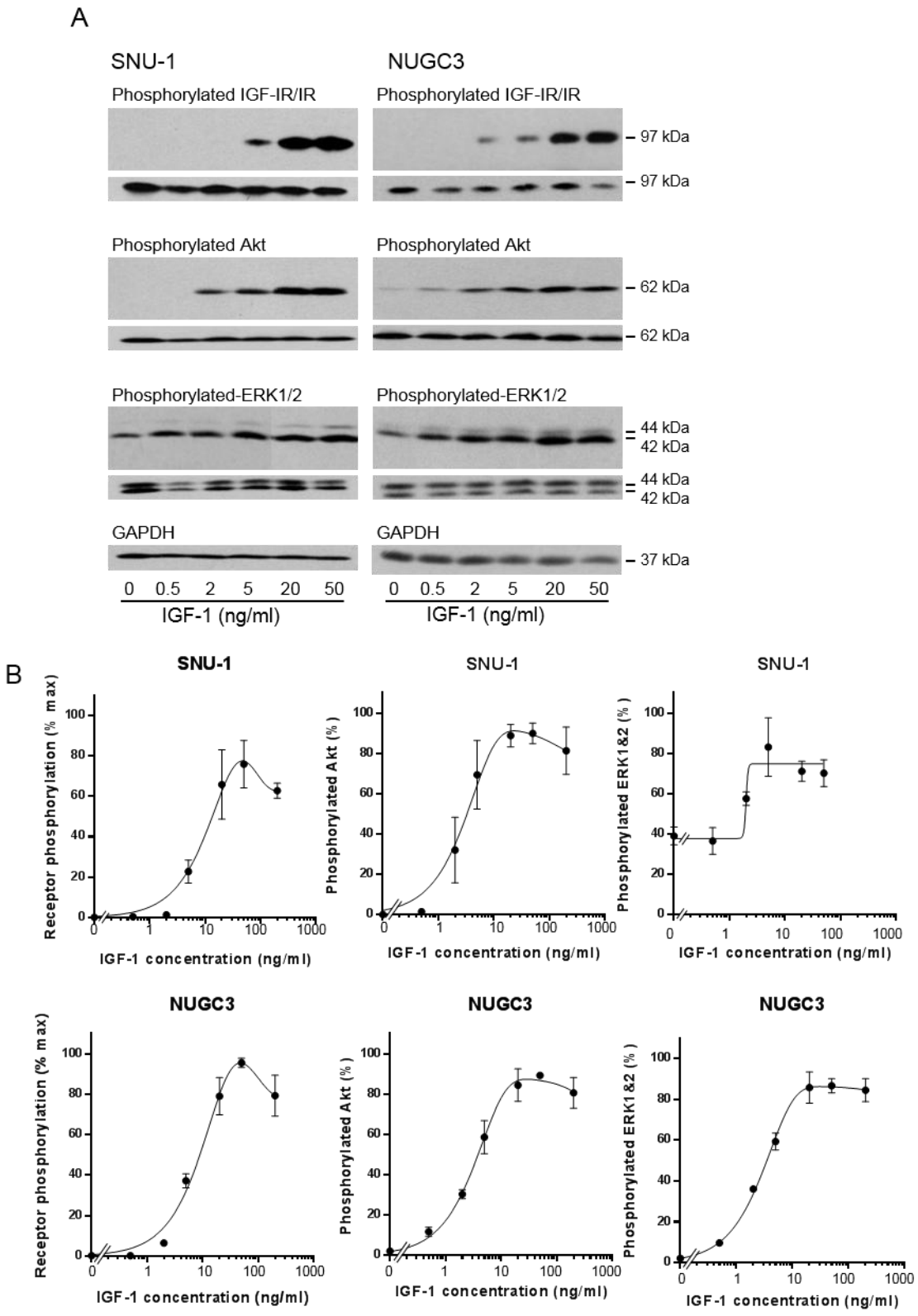

Figure 3.6. The effect of stimulation with different concentrations of IGF-1 on phosphorylation of IGF signal transduction proteins in gastric cancer cell lines. SNU-1 cells were centrifuged and resuspended in withdrawal medium and seeded into a 75 cm^2 flask at a concentration of $0.9x10^6$ cells per flask. After 24 hours, medium was replaced with 12 ml fresh withdrawal medium for another 24 hours. Cells were centrifuged and resuspended in serum-free medium with 0.1% BSA for 2 hours. Cells were then centrifuged and resuspended in serum-free medium with 0.1% BSA and 0, 0.5, 2, 5, 20, 50 ng/ml IGF-1 for 15 minutes. Cells were collected in a 15 ml Falcon tube and proteins were lysed with 50 µl RIPA buffer plus inhibitors. NUGC3 cells were seeded into 12-well tissue culture plates at $15x10^4$ cells/well, in 1 ml maintenance medium. Cells were incubated for 24

hours to allow attachment. Medium was aspirated and the cells washed with 0.5 ml PBS. Medium was replaced daily with 1 ml withdrawal medium for two days. Cells were incubated with serum-free medium which contained 0.1% BSA for two hours. Medium was changed to include IGF- 1 at final concentrations of 0, 0.5, 2, 5, 20, 50, 200 ng/ml. After incubation for 15 minutes proteins were extracted with 80 µl per well RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12 % polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against phosphorylated IGF-IR/IR (1:1000 dilution), IGF-IR (1:2000 dilution), phosphorylated Akt (1:3000 dilution), Akt (1:3000 dilution), phosphorylated ERK1/2 (1:5000 dilution), ERK1/2 (1:5000 dilution) and GAPDH (1:10000 dilution). Images obtained after incubation with antibodies against the total proteins are shown beneath those of the phosphorylated proteins. Images shown are representative of results from one triplicate experiment (**A**). Densitometric analysis of the bands was by Labworks 4.0 software. Measurements were adjusted for the background optical density of the X-ray film and normalised to the total proteins (**B**). Results are expressed as a percentage of the maximum observed phosphorylation in each case. Bars are the mean ±SEM.

Akt stimulation was detected in SNU-1 cells after treatment with 2 ng/ml IGF-1, whereas maximal Akt phosphorylation was achieved with 20 and 50 ng/ml IGF-1. In NUGC3 cells some levels of Akt phosphorylation were detected in the absence of IGF-1, but increased after IGF-1 stimulation in a concentration dependent manner. Again, maximal Akt phosphorylation was reached with 20 and 50 ng/ml IGF-1. Phosphorylated ERK1 and ERK2 were detected in both cell lines in the absence of IGF-1 but there was a concentration dependent increase after stimulation with IGF-1. Maximal phosphorylation was achieved with 5 ng/ml and above in SNU-1 cells and 20 ng/ml in NUGC3 cells (Figure 3.6).

Stimulation of most gastric cancer cell lines with IGF-1 resulted in phosphorylation of the type I IGF and/or insulin receptor and activation of downstream pathways, suggesting that signalling through the two receptors is functional in those cells. Detectable phosphorylation of IGF-IR/IR was achieved with a slightly lower IGF-1 concentration in NUGC3 cells compared to SNU-1 cells. Expression of the type II IGF receptor is slightly higher in SNU-1 cells compared to NUGC3 cells, which could contribute to reduced IGF-1 availability to the type I IGF receptor and result in a higher threshold for IGF-IR phosphorylation. Lower concentrations of IGF-1 were required to induce phosphorylation of Akt, ERK1 and ERK2 compared to phosphorylation of the type I IGF and insulin receptors in both SNU-1 and NUGC3 cells. Possibly, the sensitivity of the antibodies that detect phosphorylated Akt and ERK1 and 2 is higher compared to the antibody that detects phosphorylated IGF-IR/IR.

3.2.4 The effect of IGF-IR/IR inhibition with a small molecule tyrosine kinase inhibitor on activation of IGF-signal transduction pathway proteins in response to IGF-1

BMS754807 (Bristol-Myers-Squibb, New York, NY, US7534792) has been shown to inhibit [^{3}H]-thymidine incorporation in pre-clinical studies of breast, lung, pancreatic and colon

cancer cells as well as in multiple myeloma, leukaemia, and sarcomas (Carboni *et al.*, 2009). It prevents ATP interaction with the tyrosine kinase domain of the type I IGF receptor, which results in reduced receptor phosphorylation. Due to the high homology of the tyrosine kinase domain of the type I IGF receptor and the insulin receptor, BMS754807 inhibits both receptors. Phase I studies have investigated the safety and tolerability of BMS754807 alone in patients with advanced or metastatic solid tumours (NCT00569036 and NCT00898716) and the safety and tolerability of BMS754807 in combination with paclitaxel and carboplatin (NCT00793897). Phase I/II studies have investigated combinations of BMS754807 with cetuximab (EGFR inhibitor) in advanced or metastatic solid tumours (NCT00908024), with trastuzumab in advanced or metastatic HER-2 positive breast cancer (NCT00788333) and with letrozole in hormone receptor-positive breast cancer with resistance to non-steroidal aromatase inhibitors (NCT01225172). Results of the above trials are yet to be published.

The effectiveness of BMS754807 on the inhibition of activation of downstream pathways in response to IGF-1 was tested in NUGC3 and SNU-1 cells. The cells were withdrawn from the effects of growth factors present in the medium by culture in medium containing dextran-coated charcoal stripped serum (DCCS). Phosphorylation of proteins of the IGF-signal transduction pathway in cells that had been treated with different concentrations of BMS754807 prior to treatment with 50 ng/ml IGF-1 was determined by western transfer.

As mentioned previously, treatment of both SNU 1 and NUGC3 cells with 50 ng/ml IGF-1 resulted in maximal phosphorylation of the type I IGF and insulin receptors. Partial inhibition of IGF-IR/IR phosphorylation was achieved with 5 nM BMS754807 in SNU-1 cells and 5 and 50 nM in NUGC3 cells (Figure 3.7). Complete inhibition of IGF-IR/IR phosphorylation was achieved with 50 nM BMS754807 in SNU-1 cells and 500 nM in NUGC3 cells. The levels of total IGF-IR were not altered after treatment with BMS754807.

Akt phosphorylation was completely inhibited with 500 nM BMS754807 in SNU-1 cells. In NUGC3 cells phosphorylated Akt was detected in the absence of IGF-1 but increased after IGF-1 treatment. Complete restoration of phosphorylated Akt to baseline levels was achieved with 500 nM BMS754807. In SNU-1 cells there was some stimulation of ERK2 phosphorylation in response to IGF-1 but BMS754807 did not inhibit ERK2 phosphorylation at any concentration tested. In NUGC3 cells, stimulation of ERK1 and 2 phosphorylation in response to IGF-1 was much better compared to SNU-1 cells but BMS754807 reduced the levels of ERK1 and 2 phosphorylation to a very small extent (Figure 3.7).

BMS754807 inhibited effectively phosphorylation of the type I IGF and insulin receptor and Akt in response to IGF-1 at a concentration of 500 nM both in SNU-1 and NUGC3 cells. However, it was not equally effective in the inhibition of IGF-1-induced ERK1 and ERK2 phosphorylation.

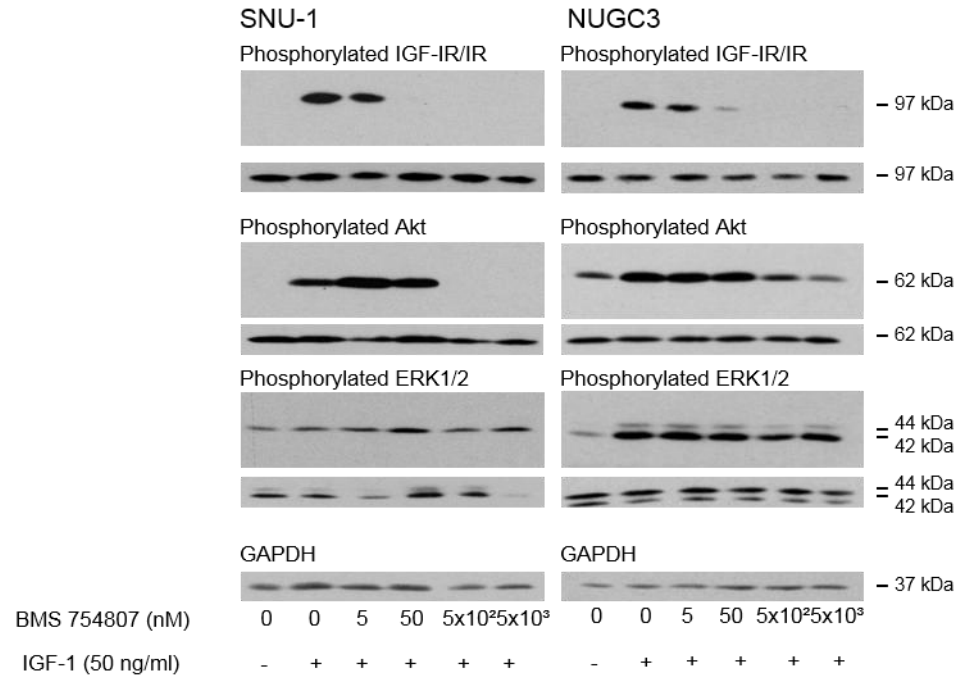

Figure 3.7. The effect of inhibition of IGF-IR/IR with BMS754807 on stimulation of SNU-1 and NUGC3 cells with IGF-1. SNU-1 cells were centrifuged and resuspended in withdrawal medium and seeded into a 75 cm^2 flask at a concentration of $0.9x10^6$ cells per flask. After 24 hours, medium was replaced with 12 ml fresh withdrawal medium for another 24 hours. Cells were centrifuged and resuspended in withdrawal medium containing 0, 5, 50, 500 or 5000 nM BMS754807 for 30 minutes. Cells were centrifuged and medium was changed to include 50ng/ml IGF-1. After incubation for 15 minutes cells were collected in a 15 ml Falcon tube and proteins were lysed with 50 µl RIPA buffer plus inhibitors. NUGC3 cells were seeded into 12 well tissue culture plates at $15x10^4$ cells/well, in 1 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 0.5 ml PBS. Medium was replaced daily with 1 ml withdrawal medium for two days. Cells were incubated with withdrawal medium with 0, 5, 50, 500 and 5000 nM BMS754807 for 30 minutes. Medium was then changed to include 50 ng/ml IGF-1. After incubation for 15 minutes proteins were extracted with 80 µl per well RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12 % polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against phosphorylated IGF-IR/IR (1:1000 dilution), IGF-IR (1:2000 dilution), phosphorylated Akt (1:3000 dilution), Akt (1:3000 dilution), phosphorylated ERK1/2 (1:5000 dilution), ERK1/2 (1:5000 dilution) and GAPDH (1:10000 dilution). Images obtained after incubation with antibodies against the total proteins are shown beneath those of the phosphorylated proteins.

3.3 Discussion

Liu et al., have investigated the correlation between IGF-IR expression and gastric cancer risk. Positive IGF-IR expression was detected in 87.5% of gastric carcinoma tissues, 55% of atypical hyperplasia tissues and 17.86% of normal gastric mucosal membrane tissues, suggesting that IGF-IR expression might be related to the development of gastric carcinoma (Liu *et al.*, 2009). An additional study has shown that IGF-IR expression in gastric cancer is

associated with lymph node metastasis, worse prognosis and high histological malignancy grade and is an independent predictor of survival (Gryko *et al.*, 2014). In a separate study, IGF-IR expression was associated with tumour size, stroma quantity, depth of invasion, lymph node involvement and stage of gastric cancer (Ge *et al.*, 2009).

Despite the above information linking IGF-IR expression to gastric cancer progression, it is known that IGF-IR expression alone is not a sufficient predictor of the responsiveness of a particular cell type to IGF-targeted treatment. Other proteins such as insulin receptor, type II IGF receptor or insulin receptor substrate proteins 1, 2 and 4 are also implicated and can affect the response of cells to IGFs, as well as the response to IGF-IR-targeted drugs. For example, SNU-1 and NUGC3 cells express similar amounts of the type I IGF receptor but SNU-1 cells express higher levels of IRS-1, which suggests that activation of the type I IGF receptor in those cells could have a more powerful biological effect. The above should be taken into consideration when designing targeted treatments.

Out of the eight gastric cancer cell lines used here, seven expressed IGF-IR and IR, one expressed HER-2, two expressed FGFR2 and four expressed c-Met. Despite the small sample size used, it seems that IGF-IR and IR expression is quite common in gastric cancer. This is also supported by the study of Liu et al., (2009), in which 87.5% of 56 gastric carcinomas were positive for IGF-IR and it suggests that a large proportion of gastric adenocarcinomas use the IGF signal transduction pathway for their growth and survival mechanisms.

Stimulation of all gastric cancer cell lines with IGF-1 resulted in phosphorylation of the type I IGF and insulin receptor and Akt, which demonstrates that the IGF signal transduction pathway is active in cells driven by overexpression of amplified HER2, FGFR2 or MET. However, the level of response varied amongst the cell lines. In general, cells without a known amplification, such as SNU-1, MKN74, NUGC3 and AGS responded well to IGF-1 stimulation. Best stimulation of ERK2 was achieved in MKN74, NUGC3, AGS and SNU-16 cells. Interestingly, NCI-N87 and SNU-16 cells, which overexpress HER-2 and FGFR2, respectively, responded quite well to IGF-1 stimulation by concomitant increase of Akt and ERK1 and ERK2 phosphorylation. Our results suggest that the IGF signal transduction pathway might be more important than previously appreciated in gastric cancer cells that are driven by amplification of other tyrosine kinase receptors and that consideration should be given to dual targeting to limit the onset of resistance.

BMS754807 is a small molecule tyrosine kinase inhibitor that inhibits activation of the type I IGF receptor and, due to the high homology of the two receptors, the insulin receptor.

Treatment of SNU-1 and NUGC3 cells, which express both receptors, with the BMS754807 inhibited completely phosphorylation of the two receptors in response to IGF-1. Furthermore, IGF-1-induced Akt phosphorylation was inhibited also by BMS754807 in both cell lines. However, the inhibitor did not reduce IGF-1-induced ERK1 and ERK2 phosphorylation in NUGC3 cells. This is in contrast with a study in which BMS754807 inhibited activation of both Akt and ERK1/2 phosphorylation by IGF-1 in mouse embryonic fibroblasts (Dinchuk *et al.*, 2010). In another study, IGF-1-induced Akt but not ERK1/2 phosphorylation, was inhibited by BMS754807 in rhabdomyosarcoma and colon cancer cells (Carboni *et al.*, 2009).

In summary, the above findings suggest that the IGF signal transduction pathway is active in the vast majority of gastric cancer cell lines used here. Importantly, the IGF signal transduction pathway can be activated even in cell lines with amplification of HER-2, FGFR2 and c-Met. It remains to be seen whether and how the biological effect of IGF-1 varies from one cell line to the other.

Chapter 4. The Effect of IGF-1 on Cell Survival in Gastric Cancer Cells

4.1 Introduction

Caspase-dependent programmed cell death has been evolved by multicellular organisms to eliminate unwanted or unhealthy cells, either as a part of normal development or following stress. It consists of multiple cellular events which lead to activation of a family of cysteine proteases called caspases. 'Initiator' caspases 2, 8, 9, and 10, which are activated in response to apoptotic stimuli, cleave and activate the 'executioner' caspases 3 and 7 (Fernald and Kurokawa, 2013). It has been proposed that apoptosis, serves as a natural barrier to cancer development. Unlike normal cells, cancer cells are constantly under stress, including oncogenic stress, genomic instability, and cellular hypoxia. In response to such stress, the intrinsic pathway of apoptosis would normally be activated. However, cancer cells can often establish the appropriate molecular machinery that will help them to evade apoptosis. This evasion may involve alteration of the balance between pro- and anti-apoptotic members of the Bcl-2 family of regulatory proteins (Adams and Cory, 2007).

Involvement of the IGF signal transduction pathway in evasion of apoptosis induced by chemotherapeutic agents and serum withdrawal has been reported in cancer cells. IGF-1 protected against apoptosis induced by 5-fluorouracil, methotrexate, tamoxifen and camptothecin in HBL-100 breast cancer cells (Dunn *et al.*, 1997), against doxorubicin-induced apoptosis in MCF-7 breast cancer cells (Gooch *et al.*, 1999), and against apoptosis induced by serum deficiency and doxorubicin in neuroblastoma cells (Gil-Ad *et al.*, 1999). IGF-1 inhibited apoptosis induced by IL-3 withdrawal in myeloid progenitors by maintaining high levels of Bcl-2 protein (Minshall *et al.*, 1997) and apoptosis induced by serum withdrawal in pheochromocytoma PC12 cells by increasing Bcl-XL levels (Parrizas and LeRoith, 1997). The importance of the type I IGF receptor in cell survival has also been reported. Inhibition of endogenous levels of IGF-IR increased sensitivity to UV radiation and proteasome inhibitors in A549 lung carcinoma cells (Jiang *et al.*, 1999), whereas insulin-induced IGF-IR activation protected against apoptosis caused by ultraviolet B irradiation in primary human keratinocytes (Kuhn *et al.*, 1999).

When displaced from the extracellular matrix, epithelial cells undergo a type of apoptosis, called anoikis (Folkman and Moscona, 1978). Anoikis prevents cells from surviving and colonising elsewhere when detached. Anoikis can be regulated by both the intrinsic and extrinsic pathways. In the intrinsic pathway, the Bcl-2 family of proteins controls the

formation of pores in the outer mitochondrial membrane, releasing pro-apoptotic factors, such as cytochrome C, which activate caspases. The extrinsic pathway consists of ligation of death receptors on the cell surface, resulting in the assembly of a death-inducing signalling complex (Gilmore, 2005).

IGF-1 has been reported to protect fibroblasts that overexpress IGF-IR from anoikis (Valentinis *et al.*, 1998), and Ewing's sarcoma cells carrying antisense IGF-IR had a significant increase in apoptosis induced by prevention of cell attachment (Scotlandi *et al.*, 2002).

One of the most common problems in cancer therapy involves the acquisition of resistance to apoptosis by cancer cells. Given the reported importance of the IGF signal transduction pathway in apoptosis evasion, it is possible that targeting this pathway could confer a significant benefit in gastric cancer patients.

4.1.1 Aim

The results presented in this chapter aim to elucidate the role of IGF-1 on survival of gastric cancer cells. Two cell survival assays were established, the apoptosis assay and the anoikis assay. The protective effect of IGF-1 against both apoptosis and anoikis was investigated. The role of the type I IGF and insulin receptors and of downstream molecules of the IGF-signal transduction pathway in the IGF-1 survival effect was investigated with a small molecule tyrosine kinase inhibitor that targets the type I IGF receptor and the insulin receptor and with inhibitors that target the PI3K/Akt and Raf/MAPK pathways.

4.2 Results

4.2.1 The effect of IGF-1 on staurosporine-induced apoptosis in gastric cancer cells

During apoptosis, caspase 3 is activated by proteolytic processing of its inactive zymogen into a 17 and a 12 kDa fragment (Nicholson *et al.*, 1995). Cleaved caspase 3 is responsible for the proteolytic cleavage of many key proteins, such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP) (Fernandes-Alnemri *et al.*, 1994). Cleavage of PARP from 113 to 89 kDa results in loss of its DNA binding domain. Staurosporine is an inhibitor of protein kinase C that prevents interaction of ATP with the active site on the kinase. At micromolar concentrations, staturosporine is broader-spectrum protein kinase inhibitor that induces

apoptosis in a wide variety of cells via activation of the caspase cascade (Meggio *et al.*, 1995; Omura *et al.*, 1995). The protective effect of IGF-1 against staurosporine-induced apoptosis has been demonstrated previously in triple-negative breast cancer cells (Davison *et al.*, 2011). To investigate whether IGF-1 can promote survival in gastric cancer cells, the ability of IGF-1 to inhibit staurosporine-induced cell death was assessed.

4.2.1.1 The effect of IGF-1 on staurosporine-induced apoptosis overtime

The protective effect of IGF-1 on gastric cancer cells was investigated over time. In SNU-1 cells, the 89 kDa PARP fragment was detected by western transfer analysis at very low levels after culture in withdrawal medium for two days. The amount of the 89 kDa PARP fragment increased 2, 4, 6 and 24 hours after the addition of staurosporine (Figure 4.1). Treatment with IGF-1 reduced significantly the amount of cleaved PARP in all time points tested. In NUGC3 cells, no cell death was detected after culture in withdrawal medium. Cell death was induced progressively after the addition of staurosporine for 2, 4, 6 and 24 hours. IGF-1 reduced the amount of cleaved PARP induced by staurosporine to 70% of the amount detected in the absence of IGF-1 after 24 hours of treatment (Figure 4.2).

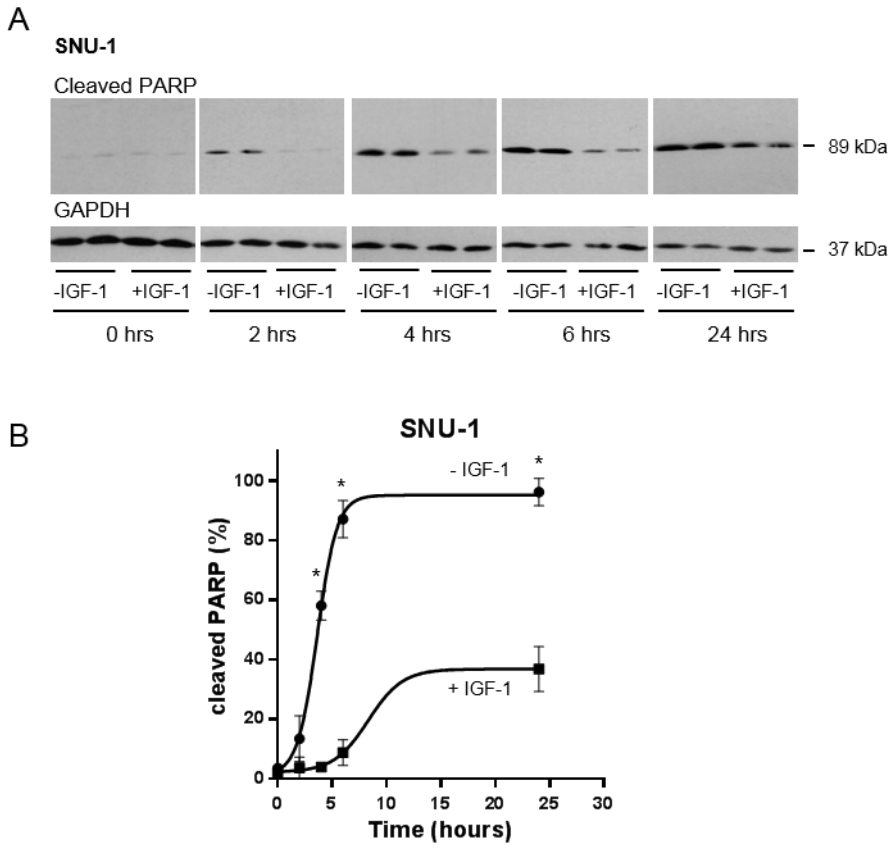

Figure 4.1. The effect of IGF-1 on staurosporine-induced apoptosis in SNU-1 cells. SNU-1 cells were centrifuged and resuspended in withdrawal medium and seeded into a 175 cm^2 flask at a concentration of $4.5x10^6$ cells per flask. After 24 hours, medium was replaced with 24 ml fresh withdrawal medium for another 24 hours. Cells were centrifuged and resuspended in withdrawal medium supplemented or not with 50 ng/ml IGF-1 for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of IGF-1 for different lengths of time. At the end of each time point, cells were collected in 15 ml Falcon tubes and lysed in 50 µl RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against cleaved PARP (1:2000 dilution) and GAPDH (1:10000 dilution). The images shown are representative of samples from triplicate experiments which have been repeated at least twice (**A**). Densitometry was with Labworks 4.0 software, adjusted for the background optical density of the X-ray film and normalised to GAPDH. Results are expressed as a percentage of the intensity of the cleaved PARP band observed in the absence of IGF-1. Bars represent the standard error of the mean (SEM). Asterisks show cleaved PARP levels that are statistically significantly lower in the presence of IGF-1 than in its absence (Two-way ANOVA; $p<0.0001$) (**B**).

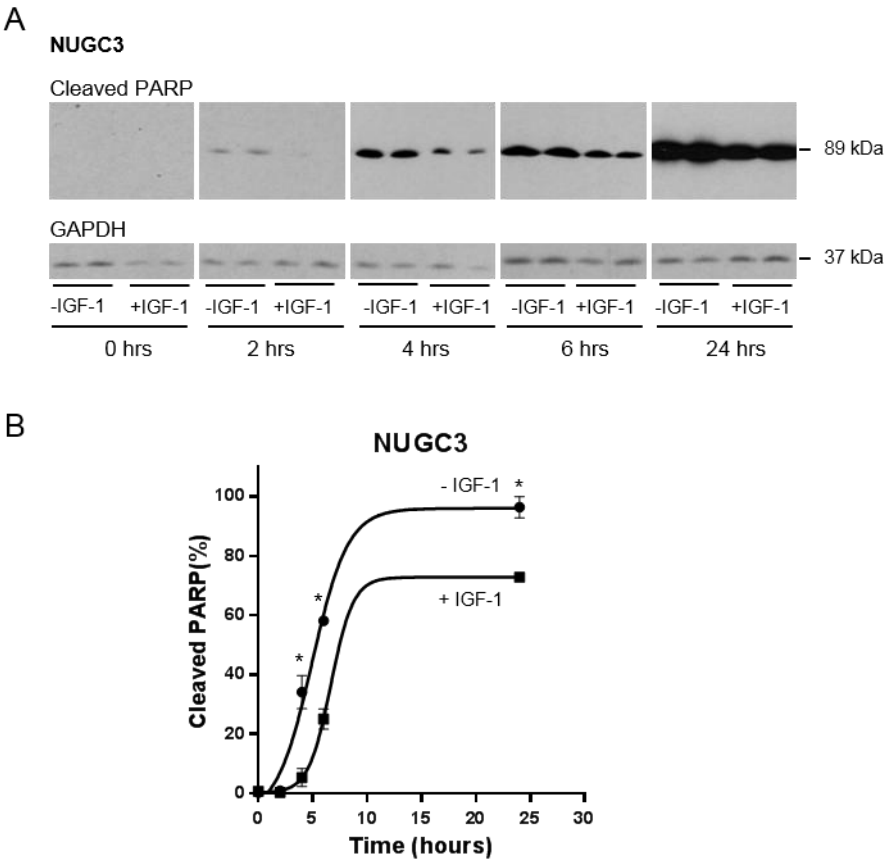

Figure 4.2. The effect of IGF-1 on staurosporine-induced apoptosis in NUGC3 cells. NUGC3 cells were seeded into 12 well tissue culture plates at 15 x 10^4 cells per well, in 1 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 0.5 ml PBS. Medium was replaced daily with 1 ml withdrawal medium for two days. Cells were incubated with withdrawal medium supplemented or not with 50 ng/ml IGF-1 for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of 50 ng/ml IGF-1 for different lengths of time. At the end of each time point, proteins were extracted with 80 µl of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against cleaved PARP (1:2000 dilution) and GAPDH (1:10000 dilution). The images shown are representative of samples from triplicate experiments which have been repeated at least twice (**A**). Densitometry was with Labworks 4.0 software, adjusted for the background optical density of the X-ray film and normalised to GAPDH. Results are expressed as a percentage of the intensity of the cleaved PARP band observed in the absence of IGF-1. Bars represent the standard error of the mean (SEM). Asterisks show cleaved PARP levels that are statistically significantly lower in the presence of IGF-1 than in its absence (Two-way ANOVA; $p<0.0001$) (**B**).

A protective effect of IGF-1 against apoptosis was also observed in AGS and NCI-N87 cells, after treatment with staurosporine for 2 and 5 hours, respectively. IGF-1 reduced the amount of the 89 kDa fragment of PARP to 30% in AGS cells and to 20% in NCI-N87 cells (Figure 4.3). No protective effect of IGF-1 against apoptosis was detected in KATO III cells after treatment with staurosporine at all the time points tested (Figure 4.4). A higher concentration of staurosporine (1µM) was required in order to induce cleavage of PARP in MKN74 cells. However, no protection was achieved in the presence of IGF-1 (Figure 4.4). The amount of cleaved PARP detected in SNU-16 and SNU-5 cells was relatively high, even in the absence

of staurosporine (Figure 4.4). Addition of staurosporine increased slightly the amount of cleaved PARP in SNU-16 cells but did not affect the amount of cleaved PARP in SNU-5 cells. Neither cell line was protected by IGF-1 at the time points tested.

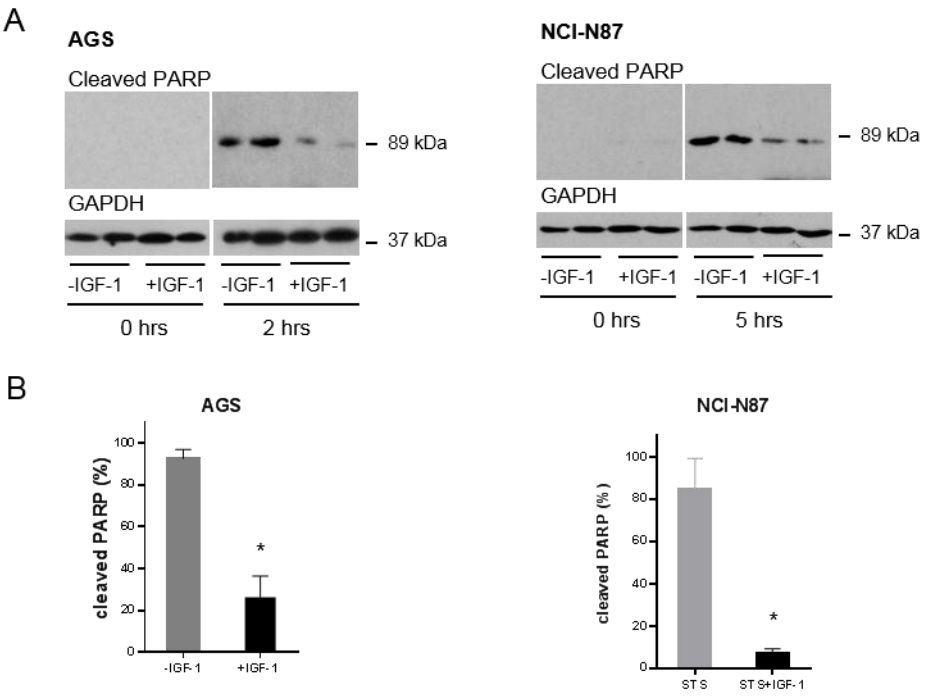

Figure 4.3. The effect of IGF-1 on staurosporine-induced apoptosis in AGS and NCI-N87 cells. AGS and NCI-N87 cells were seeded into 12 well tissue culture plates at 15 x 10^4 cells per well, in 1 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 0.5 ml PBS. Medium was replaced daily with 1 ml withdrawal medium for two days. Cells were incubated with withdrawal medium supplemented or not with 50 ng/ml IGF-1 for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of 50 ng/ml IGF-1 for different lengths of time. After two hours, proteins were extracted with 80 µl of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against cleaved PARP (1:2000 dilution) and GAPDH (1:10000 dilution). The images shown are representative of samples from triplicate experiments which have been repeated at least twice (**A**). Densitometry was with Labworks 4.0 software, adjusted for the background optical density of the X-ray film and normalised to GAPDH. Results are expressed as a percentage of the intensity of the cleaved PARP band observed in the absence of IGF-1. Bars represent the standard error of the mean (SEM). Asterisks show cleaved PARP levels that are statistically significantly lower in the presence of IGF-1 than in its absence (T-test; $p \leq 0.0002$) (**B**).

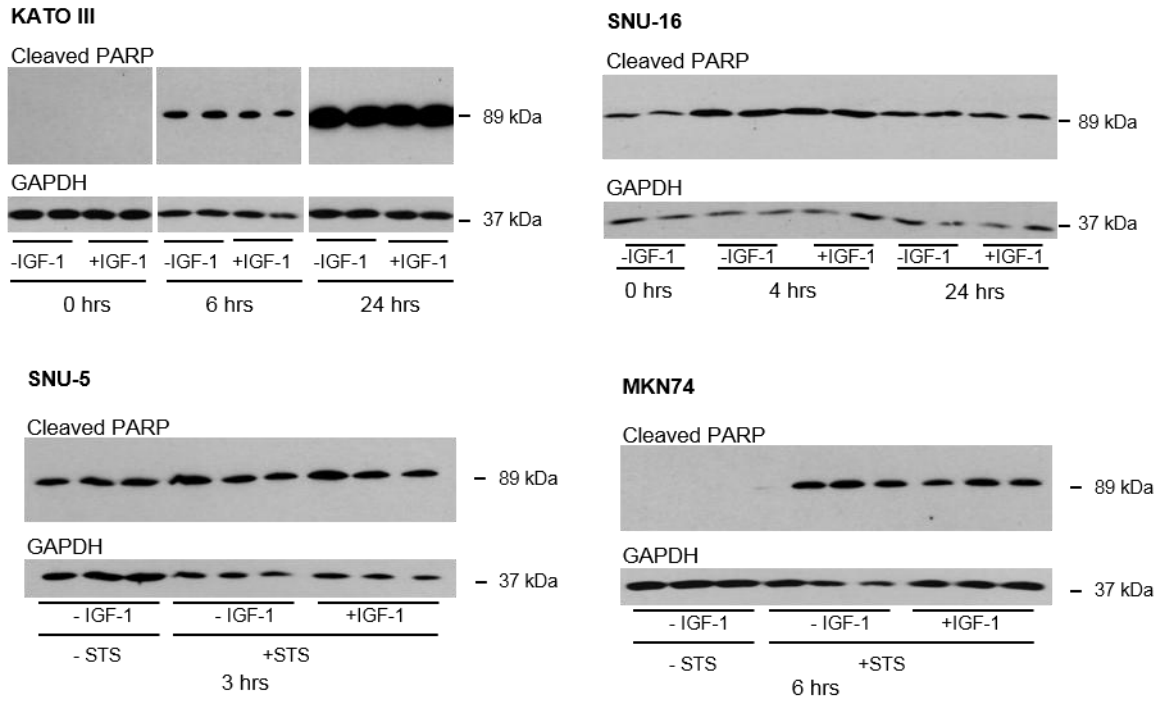

Figure 4.4. The effect of IGF-1 on staurosporine-induced apoptosis in KATO III, SNU-16, SNU-5 and MKN74 cells. SNU-16 and SNU-5 cells were centrifuged and resuspended in withdrawal medium and seeded into a 175 cm^2 flask at a concentration of $4.5x10^6$ cells per flask. After 24 hours, medium was replaced with 24 ml fresh withdrawal medium for another 24 hours. Cells were centrifuged and resuspended in withdrawal medium supplemented or not with different concentrations of IGF-1 for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of different concentrations of IGF-1 for 5 hours. Cells were collected in 15 ml Falcon tubes and lysed in 50 µl RIPA buffer plus inhibitors. KATO-III and MKN74 cells were seeded into 12 well tissue culture plates at $15x10^4$ cells per well, in 1 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 0.5 ml PBS. Medium was replaced daily with 1 ml withdrawal medium for two days. Cells were incubated with withdrawal medium supplemented or not with 50 ng/ml IGF-1 for 15 minutes. Cells were then incubated with 0.5 and 1 µM staurosporine, respectively, in the presence and absence of 50 ng/ml IGF-1 for 5 hours. Proteins were extracted with 80 µl of RIPA buffer plus inhibitors per well. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12 % polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against cleaved PARP (1:2000 dilution) and GAPDH (1:10000 dilution).

4.2.1.2 The effect of IGF-1 on caspase-dependent apoptosis in gastric cancer cells

To confirm that staurosporine activates caspase-dependent apoptosis and that IGF-1 is able to inhibit this activation, SNU-1 and NUGC3 cells were cultured in withdrawal medium and treated with STS in the presence or absence of 50 ng/ml IGF-1. The cells were then incubated with a fluorescent antibody that detects the activated cleaved form of caspase 3.

No cleavage of caspase 3 and PARP was detected in SNU-1 cells after culture in withdrawal medium for two days (Figure 4.5). Cleavage of caspase 3 was induced after 5 hours of treatment with staurosporine in 45% cells. When the cells were treated with staurosporine and IGF-1 combined, the above percentage was reduced to 15.6%. Treatment with staurosporine for 24 hours also resulted in detectable PARP cleavage in 52% cells. In cells that had been treated both with IGF-1 and staurosporine the above percentage was reduced to 25.3%.

Similarly, in NUGC3 cells there was no detectable cleavage of caspase 3 or PARP after culture in withdrawal medium for two days (Figure 4.5). Treatment with staurosporine for 24 hours resulted in cleavage of caspase 3 in 47.7% cells, whereas concomitant treatment with IGF-1 resulted in reduction of the number of cleaved caspase 3 positive cells to 27.1%. Cleavage of PARP was induced in 39.7% NUGC3 cells that had been treated with staurosporine for 24 hours, whereas IGF-1 reduced this percentage to 12.9%. These results indicate that IGF-1 can inhibit caspase-dependent cell death induced by staurosporine.

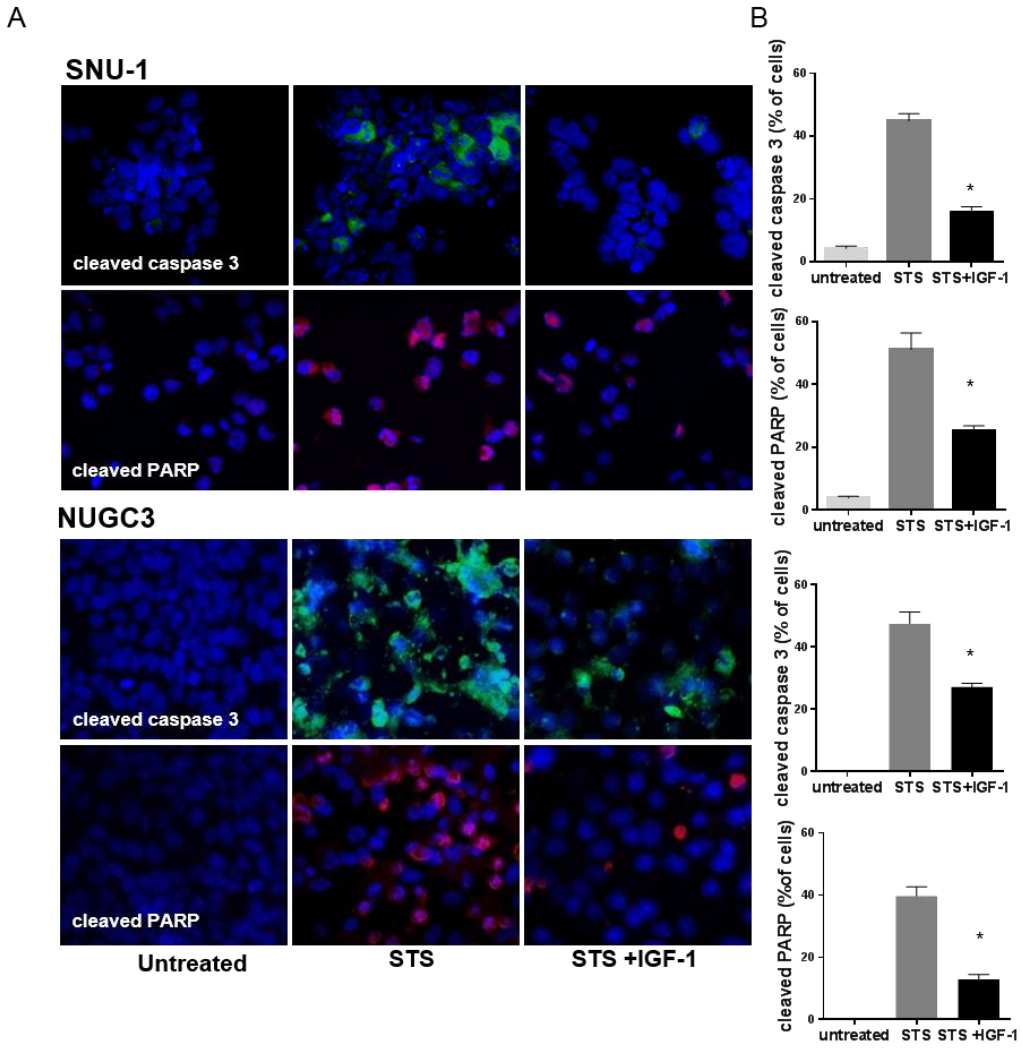

Figure 4.5. The effect of IGF-1 on caspase-dependent apoptosis induced by staurosporine in SNU-1 and NUGC3 cells. SNU-1 cells were centrifuged and resuspended in withdrawal medium and seeded into a 75 cm^2 flask at a concentration of $1.5x10^6$ cells per flask. After 24 hours, medium was replaced with 12 ml fresh withdrawal medium for another 24 hours. Cells were centrifuged and resuspended in withdrawal medium supplemented or not with 50 ng/ml IGF-1 for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of 50 ng/ml IGF-1 for 4 hours. Cells were collected in 1.5 ml Eppendorf tubes and fixed in 1 ml 4% paraformaldehyde. NUGC3 cells were seeded on top of coverslips in 6 well tissue culture plates at 30 x 10^4 cells per well, in 2.5 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 2 ml PBS. Medium was replaced daily with 2 ml withdrawal medium for two days. Cells were incubated in withdrawal medium supplemented or not with 50 ng/ml IGF-1 for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of 50 ng/ml IGF-1 for 24 hours. Cells were washed with 2 ml PBS and fixed in 2 ml ice-cold methano . Fixed cells were incubated with fluorescent antibodies against cleaved caspase 3 (1:50 dilution) and cleaved PARP (1:150

dilution). All secondary antibodies were Alexa-Fluor conjugated. Cells were mounted on top of slides in 20 µl DAPI X mounting medium. Pictures of the cells were taken in a Leica fluorescent microscope (**A**). Quantification of the number of cells with positive staining was done in five fields of view for each treatment. The results are shown as a percentage of the total number of cells. Bars, SEM. Asterisks show the number of apoptotic cells that is significantly smaller in the presence of IGF-1 than in its absence. (One-way ANOVA; SNU-1, cleaved caspase 3, $p<0.0001$; cleaved PARP, $p=0.0002$; NUGC3, cleaved caspase 3, $p<0.0001$; cleaved PARP, $p<0.0001$) (**B**).

4.2.1.3 The effect of different concentrations of IGF-1 on staurosporine-induced apoptosis

As mentioned previously, PARP cleavage induced by staurosporine is inhibited significantly after treatment with 50 ng/ml IGF-1 in SNU-1 and NUGC3 cells. To investigate the effect of different concentrations of IGF-1 on cell survival, SNU-1 and NUGC3 cells were withdrawn from the effects of growth factors and treated with staurosporine in the presence of IGF-1 concentrations ranging from 2 to 200 ng/ml.

Treatment with staurosporine resulted in the appearance of cleaved PARP in SNU-1 cells (Figure 4.6). The amount of cleaved PARP was reduced by IGF-1 in a concentration dependent manner. Best protection against cell death was achieved with 200 ng/ml IGF-1, which suggests that the receptor mediating the IGF-1 survival effect was not saturated even at relatively high IGF-1 concentrations. In NUGC3 cells, treatment with staurosporine also resulted in cleavage of PARP into its 89 kDa fragment (Figure 4.6). IGF-1 reduced the amount of cleaved PARP in a concentration dependent manner with maximum protection achieved with 50 and 200 ng/ml IGF-1. Treatment with 200 ng/ml IGF-1 did not reduce further the amount of cleaved PARP, suggesting that the receptor which mediates the IGF-1 survival effect was saturated.

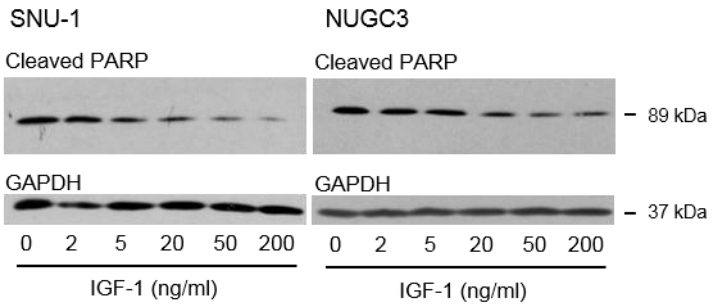

Figure 4.6. The protective effect of different concentrations of IGF-1 against staurosporine-induced apoptosis in SNU-1 and NUGC3 cells. SNU-1 cells were centrifuged and resuspended in withdrawal medium and seeded into a 75 cm^2 flask at a concentration of 0.9 x 10^6 cells per flask. After 24 hours, medium was replaced with 12 ml fresh withdrawal medium for another 24 hours. Cells were centrifuged and resuspended in withdrawal medium supplemented or not with different concentrations of IGF-1 for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of IGF-1 for 4 hours. Cells were collected in 15 ml Falcon tubes and lysed in 50 µl RIPA buffer plus inhibitors. NUGC3 cells were seeded into 12 well tissue culture plates at $15x10^4$ cells per well, in 1 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 0.5 ml PBS. Medium was replaced daily with 1 ml withdrawal medium for two days. Cells were incubated with withdrawal medium supplemented or not with

different concentrations of IGF-1 for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of IGF-1 for 5 hours. Proteins were extracted with 80 µl of RIPA buffer plus inhibitors per well. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against cleaved PARP (1:2000 dilution) and GAPDH (1:10000 dilution).

4.2.2 The effect of IGF-1 on cell death induced by anoikis

Frisch and Francis (1994) described anoikis as apoptosis induced by disruption of the interactions between normal epithelial cells and extracellular matrix. Disruption of the interactions may be achieved *in vitro* by culture of cells in plates coated with polyhydroxyethylmethacrylate (poly-HEMA). It has been suggested that circumvention of anoikis accompanies the acquisition of anchorage independence and cell motility (Frisch and Francis, 1994).

4.2.2.1 The effect of culture on poly-hema coated plates on cell attachment

Tissue culture plates were coated with poly-HEMA, as described in the Materials and Methods section. NUGC3 and AGS cells were seeded into poly-HEMA coated plates and cultured in serum-free medium for 24 hours. Photomicrographs of cells cultured in serum-free medium in poly-HEMA coated plates are shown in Figure 4.7. In comparison, cells that are cultured in medium supplemented with growth factors in attached conditions are shown. Inhibition of cell attachment by poly-HEMA resulted in the acquisition of a round shape in floating cells, which replaced the diagonal flat shape of adherent cells.

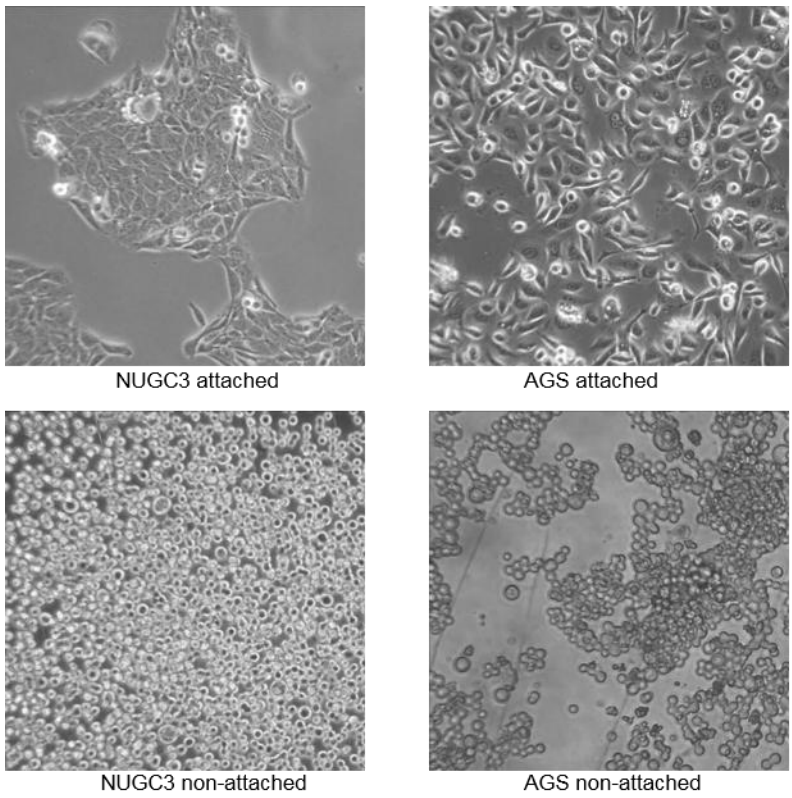

Figure 4.7. Morphological appearance of NUGC3 and AGS cells cultured in poly-HEMA coated plates. NUGC3 and AGS cells were seeded either into normal 12 well plates or into poly-HEMA coated 12 well plates at $20x10^4$ cells per well in 1 ml serum-free medium. After incubation for 4 hours, pictures were taken under the microscope.

4.2.2.2 The effect of IGF-1 on anoikis in gastric cancer cells

To test the hypothesis that IGF-1 is important for resistance to anoikis, NUGC3 and AGS cells were cultured in non-attached conditions in the presence or in the absence of IGF-1. No cleaved PARP was detected in NUGC3 cells before culture in poly-HEMA coated plates. Cleaved PARP was induced after 4 hours and progressively increased 8 and 24 hours after culture in non-attached conditions. IGF-1 reduced effectively the amount of cleaved PARP induced by anoikis approximately four fold (Figure 4.8). The amount of cleaved PARP remained low in the presence of IGF-1 throughout the course of 24 hours of culture in non-attached conditions.

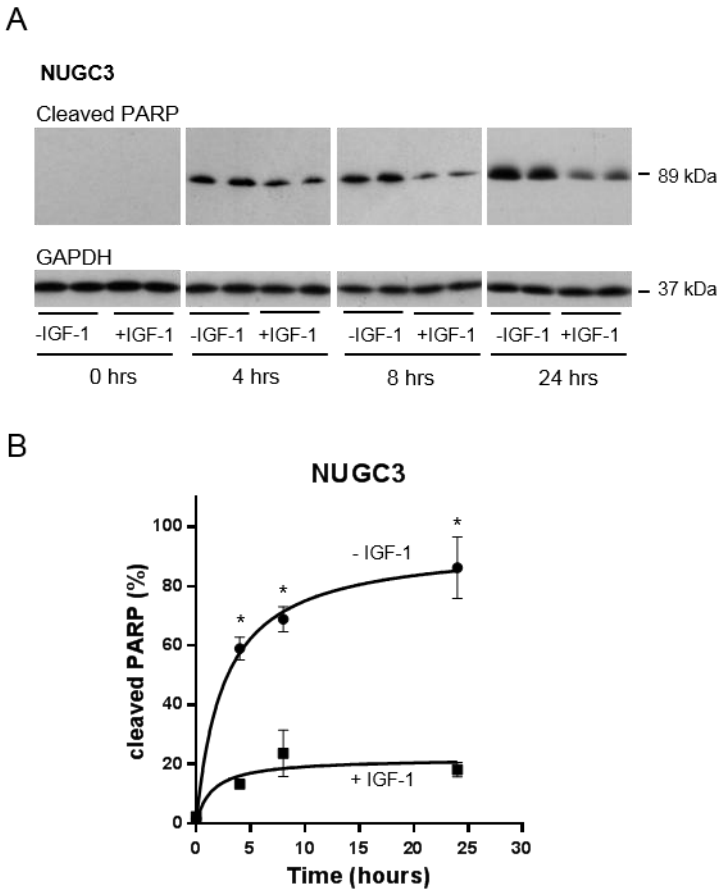

Figure 4.8. The effect of IGF-1 on PARP cleavage in non-attached conditions in NUGC3 cells. NUGC3 cells were withdrawn from the effects of growth factors by culture in withdrawal medium for 2 days. Cells were seeded into poly-HEMA coated 12 well plates at $20x10^4$ cells per well in 1 ml serum-free medium, in the presence or not of 50 ng/ml IGF-1. After incubation for various times, proteins were extracted with 50 µl per well of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against cleaved PARP (1:2000 dilution) and GAPDH (1:10000 dilution). The images shown are representative of samples from triplicate experiments which have been repeated at least twice (**A**). Densitometry was with Labworks 4.0 software, adjusted for the background optical density of the X-ray film and normalised to GAPDH. Results are expressed as a percentage of the intensity of the cleaved PARP band observed in the absence of IGF-1. Bars are the mean ±SEM, * denotes statistically significantly less cleaved PARP in the presence of IGF-1 compared with in the absence of IGF-1 (Two-way ANOVA; $p<0.0001$) (**B**).

A significant effect of IGF-1 was observed also in AGS cells (Figure 4.9). Induction of cleaved PARP was detected 2 hours after culture in poly-HEMA and increased after 24 hours. In cells that had been treated with IGF-1 the amount of cleaved PARP remained unchanged from 2 to 24 hours to approximately 20% of the amount detected in cells that had not been treated with IGF-1. In KATO III cells, PARP cleavage was increased after culture in non-attached conditions, however, no protection of IGF-1 was detected at any time tested (Figure 4.10).

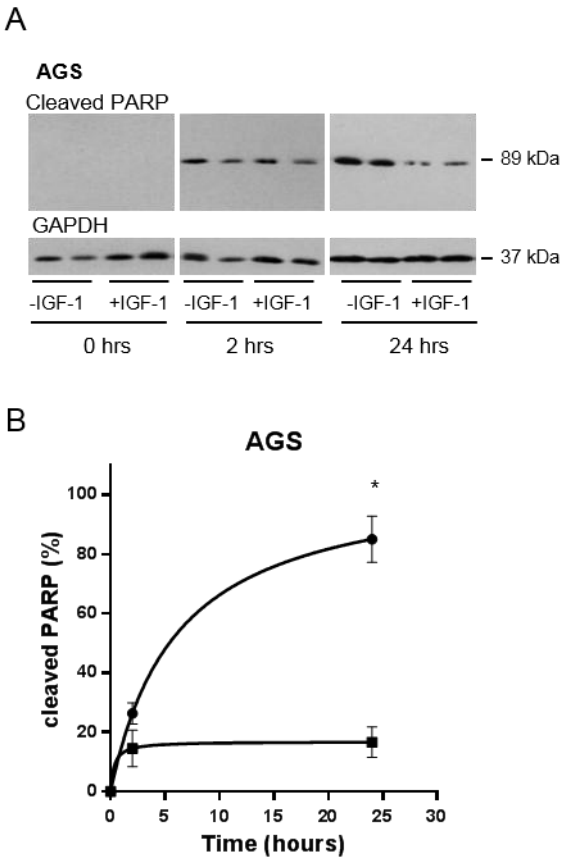

Figure 4.9. The effect of IGF-1 on PARP cleavage in non-attached conditions in AGS cells. AGS cells were withdrawn from the effects of growth factors by culture in withdrawal medium for 2 days. Cells were seeded into poly-HEMA coated 12 well plates at $20x10^4$ cells per well in 1 ml serum-free medium, in the presence or not of 50 ng/ml IGF-1. After incubation for various times, proteins were extracted with 50 µl per well of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against cleaved PARP (1:2000 dilution) and GAPDH (1:10000 dilution). The images shown are representative of samples from triplicate experiments which have been repeated at least twice (**A**). Densitometry was with Labworks 4.0 software, adjusted for the background optical density of the X-ray film and normalised to GAPDH. Results are expressed as a percentage of the intensity of the cleaved PARP band observed in the absence of IGF-1. Bars are the mean ±SEM, * denotes statistically significantly less cleaved PARP in the presence of IGF-1 compared with in the absence of IGF-1 (Two-way ANOVA, $p<0.0001$) (**B**).

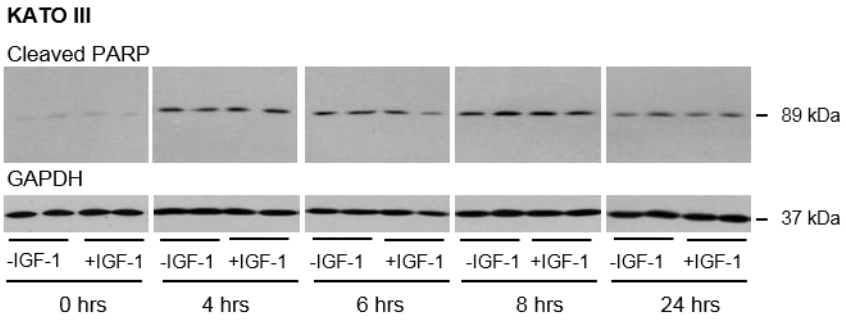

Figure 4.10. The effect of IGF-1 on PARP cleavage in non-attached conditions in KATO III cells. KATO III cells were withdrawn from the effects of growth factors by culture in withdrawal medium for 2 days. Cells were seeded into poly-HEMA coated 12 well plates at $20x10^4$ cells per well in 1 ml serum-free medium, in the presence or not of 50 ng/ml IGF-1. After incubation for various times, proteins were extracted with 50 µl per well of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against cleaved PARP (1:2000 dilution) and GAPDH (1:10000 dilution).

Cell lines that grow in suspension, e.g. SNU-1, SNU-5 and SNU-16 are inherently resistant to anoikis. No PARP cleavage was detected in MKN74 cells cultured in non-attached conditions (data not shown).

To investigate whether cell death induced by anoikis is caspase-dependent and whether IGF-1 can inhibit caspase activation, NUGC3 cells were cultured in the presence or absence of different concentrations of IGF-1 for 24 hours and the extent of caspase and PARP cleavage was analysed by western transfer. Cleaved caspase 3 was detected by western transfer analysis in cells that had been cultured in non-attached conditions in the absence of IGF-1. The amount of cleaved caspase 3 was reduced with IGF-1 in a concentration dependent manner. A similar effect was detected for cleaved PARP (Figure 4.11).

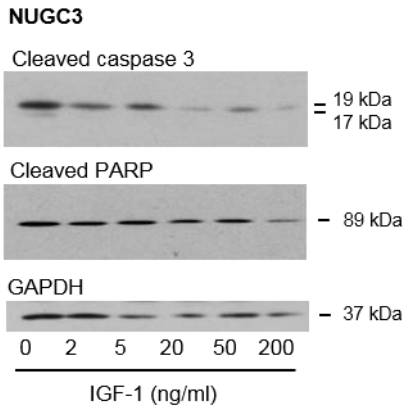

Figure 4.11. The protective effect of different concentrations of IGF-1 against caspase-dependent cell death induced by anoikis in NUGC3 cells. NUGC3 cells were withdrawn from the effects of growth factors by culture in withdrawal medium for 2 days. Cells were seeded into poly-HEMA coated 12 well plates at $20x10^4$ cells per well in 1 ml serum-free medium, in the presence or not of different concentrations of IGF-1. After incubation for various times, proteins were extracted with 50 µl per well of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against cleaved caspase 3 (1:1000 dilution), cleaved PARP (1:2000 dilution) and GAPDH (1:10000 dilution).

4.2.3 The effect of inhibiting IGF-IR/IR with BMS754807 on the IGF-1 survival effect in controlled conditions

The ability of the small molecule tyrosine kinase inhibitor BMS754807 to inhibit the survival effect of IGF-1, was investigated in SNU-1 and NUGC3 cells. As mentioned previously, BMS754807 prevents ATP interaction with the tyrosine kinase domain of the type I IGF receptor, resulting in reduced receptor phosphorylation. Due to the high homology of the tyrosine kinase domain of the type I IGF receptor and the insulin receptor, BMS754807 inhibits both receptors.

SNU-1 cells were withdrawn from the effects of growth factors by culture in withdrawal medium for two days and treated with staurosporine in the presence or absence of IGF-1 and in the presence or absence of BMS754807. The concentration of BMS754807 used abolishes completely phosphorylation of the type I IGF and insulin receptors and of Akt (Figure 3.7). Cleaved PARP was detected after treatment of SNU-1 cells with staurosporine for 4 hours. Treatment with IGF-1 reduced significantly the amount of cleaved PARP, as previously. Treatment of SNU-1 cells with BMS754807 did not induce additional cell death compared to untreated cells. In cells that were treated with the BMS754807 inhibitor, the survival effect of IGF-1 was abrogated (Figure 4.12).

The ability of BMS754807 to inhibit the IGF-1 survival effect was investigated in NUGC3 cells in the context of anoikis. NUGC3 cells were cultured in withdrawal medium for 2 days in order to withdraw them from the effect of growth factors. The cells were seeded into poly-HEMA coated plates in the presence or absence of BMS754807 and in the presence or absence of IGF-1. The cells were cultured in non-attached conditions for 24 hours before protein lysis. Culture in poly-HEMA coated plates induced anoikis and resulted in PARP cleavage. IGF-1 protected NUGC3 cells significantly from the effects of anoikis, as previously (Figure 4.12). Treatment with BMS754807 for 24 hours increased the amount of cleaved PARP in NUGC3 cells. Treatment with the BMS754807 inhibitor obliterated the survival effect of IGF-1 against anoikis.

The above results suggest that treatment with BMS754807, which inhibits both the type I IGF receptor and the insulin receptor, abolishes the survival effect of IGF-1, both in the context of apoptosis and anoikis. This further suggests that either or both the type I IGF and insulin receptors are the mediators of the IGF-1 survival effect.

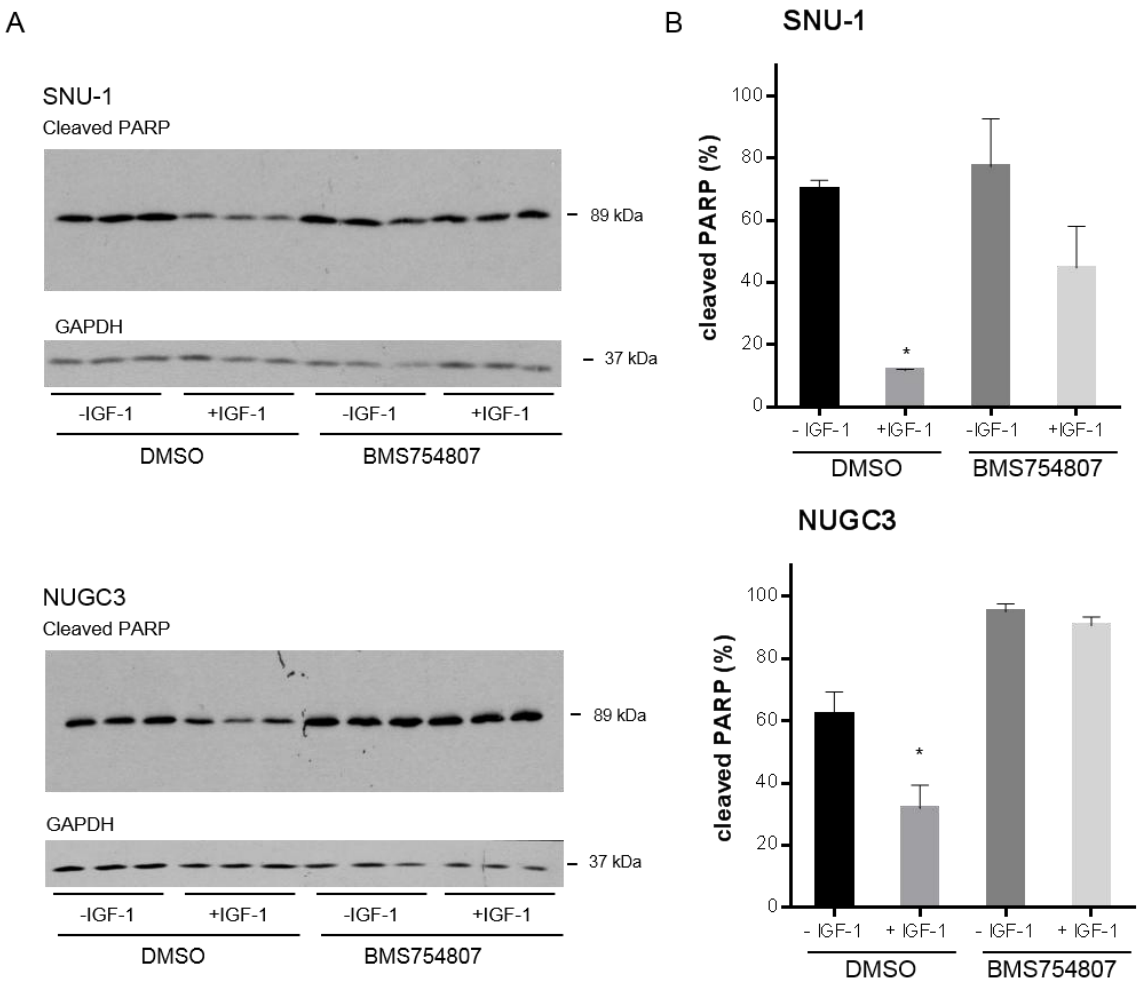

Figure 4.12. The effect of inhibition of IGF-IR/IR with BMS754807 on the survival effect of IGF-1 in SNU-1 and NUGC3 cells. SNU-1 cells were centrifuged and resuspended in withdrawal medium and seeded into a 75 cm^2 flask at a concentration of $1.8x10^6$ cells per flask. After 24 hours, medium was replaced with 12 ml fresh withdrawal medium for another 24 hours. Cells were centrifuged and resuspended in withdrawal medium containing DMSO or 500 nM BMS754807 for 30 minutes. Cells were centrifuged and medium was changed to include 50 ng/ml IGF-1 for 15 minutes. Medium was then changed to include 0.5 µM staurosporine for 4 hours. Cells were collected in a 15 ml Flacon tube and proteins were lysed with 50 µl RIPA buffer plus inhibitors. NUGC3 cells were detached from tissue culture flasks and resuspended in serum free medium at a concentration of 200000 cells/ml. Six ml of the cell suspension were incubated in DMSO or 500 nM BMS754807 for 30 minutes. The cells were then seeded into poly-HEMA coated 12 well plates at $20x10^4$ cells per well in 1 ml serum-free medium, in the presence or not of 50 ng/ml IGF-1. After incubation for 24 hours, proteins were extracted with 50 µl per well of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against cleaved PARP (1:2000 dilution) and GAPDH (1:10000 dilution) (**A**). Densitometry was with Labworks 4.0 software, adjusted for the background optical density of the X-ray film and normalised to GAPDH. The images shown are representative of samples from triplicate experiments which have been repeated at least twice. Bars are the mean ±SEM, * denotes statistically significantly less cleaved PARP in the presence of IGF-1 compared with in the absence of IGF-1 (One-way ANOVA; SNU-1, p=0.0041; NUGC3, p=0.0212) (**B**).

4.2.4 The importance of the PI3K/Akt and RAS/RAF/ERK signal transduction pathways in the IGF-1 survival effect

The PI3K/Akt pathway is important for transmission of the effects of external growth factor stimuli through tyrosine kinase receptors. The PI3K family consists of a number of lipid and serine/threonine kinases, which, upon activation, phosphorylate phosphatidylinositol-4,5-bisphosphate PtdIns(4,5)P2 to produce PtdIns(3,4,5)P3, a second messenger that binds and recruits a subset of pleckstrin-homology (PH), FYVE, Phox (PX), C1, C2 and other lipid-binding domains in downstream targets. Akt is the primary downstream mediator of the effects of PI3K. Akt signalling inhibits several pro-apoptotic factors such as BAD, procaspase-9 and Forkhead (FKHR) transcription factors (Hennessy *et al.*, 2005).

The Ras/Raf/ERK pathway is activated preferentially by mitogenic factors, differentiation stimuli and cytokines. Ligand binding to the respective cell surface receptor induces the activation of the small GTPase Ras, which recruits the MAP kinase kinase kinase Raf to the membrane. Activated Raf phosphorylates and activates the MAP kinase kinases MEK1 and MEK2, which in turn activate the effector MAP kinases ERK1 and ERK2. ERK1 and ERK2 are multifunctional serine/threonine kinases that phosphorylate a vast array of substrates, such as protein kinases, signalling effectors, receptors, cytoskeletal proteins and nuclear transcriptional regulators (Meloche and Pouyssegur, 2007).

4.2.4.1 The effect of IGF-1 on activation of the PI3K/Akt and RAS/RAF/ERK signal transduction pathways in the context of apoptosis and anoikis

Activation of downstream signal transduction pathways in response to IGF-1 was investigated both in the apoptosis and in the anoikis assay. SNU-1 and NUGC3 cells were withdrawn from the effects of growth factors by culture in withdrawal medium for 2 days. The cells were then treated with staurosporine in the presence or absence of different concentrations of IGF-1.

No activated Akt was detected in SNU-1 cells treated with staurosporine, in the absence of IGF-1 (Figure 4.13). Treatment with increasing concentrations of IGF-1 resulted in a concentration dependent increase in Akt phosphorylation, whereas total Akt levels were slightly reduced in a concentration dependent manner. ERK2 phosphorylation was detected in SNU-1 cells treated with staurosporine and addition of IGF-1 did not increase those levels. Total ERK1 and ERK2 levels also remained unchanged.

Similar results were obtained with NUGC3 cells. Low levels of Akt phosphorylation were detected in the absence of IGF-1. In response to IGF-1, Akt phosphorylation was increased in a concentration dependent manner, while total Akt levels decreased in a concentration dependent manner. ERK1 and ERK2 activation was detected in the absence of IGF-1 and did not increase with any concentration of IGF-1. Total ERK1 and ERK2 levels were not affected by different concentrations of IGF-1.

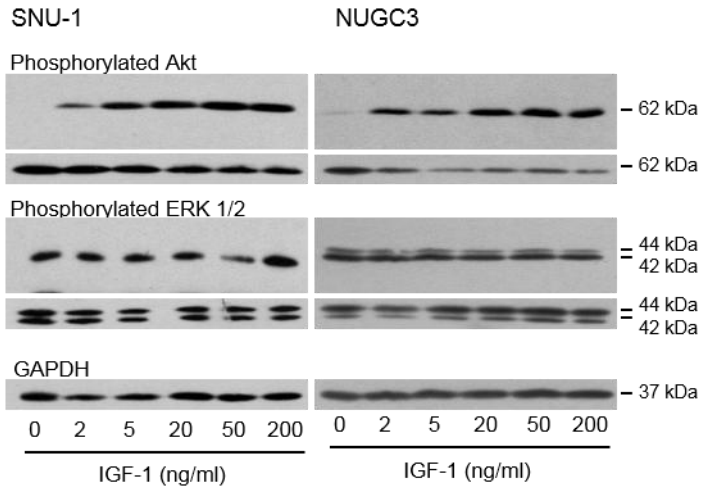

Figure 4.13. The effect of IGF-1 stimulation on activation of the PI3K/Akt and RAS/RAF/ERK signal transduction pathways in apoptosis. SNU-1 cells were centrifuged and resuspended in withdrawal medium and seeded into a 175 cm^2 flask at a concentration of $4.5x10^6$ cells per flask. After 24 hours, medium was replaced with 24 ml fresh withdrawal medium for another 24 hours. Cells were centrifuged and resuspended in withdrawal medium supplemented or not with different concentrations of IGF-1 for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of different concentrations of IGF-1 for 4 hours. Cells were collected in 15 ml Falcon tubes and lysed in 50 µl RIPA buffer plus inhibitors. NUGC3 cells were seeded into 12 well tissue culture plates at 15 x 10^4 cells per well, in 1 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 0.5 ml PBS. Medium was replaced daily with 1 ml withdrawal medium for two days. Cells were incubated with withdrawal medium supplemented or not with 50 ng/ml IGF-1 for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of different concentrations of IGF-1 for 4 hours. Proteins were extracted with 80 µl of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against phosphorylated Akt (1:3000 dilution), total Akt (1:3000 dilution), phosphorylated ERK1/ERK2 (1:5000 dilution), total ERK1/ERK2 (1:5000 dilution) and GAPDH (1:10000 dilution). Densitometry was with Labworks 4.0 software, adjusted for the background optical density of the X-ray film and normalised to GAPDH. The images shown are representative of samples from triplicate experiments which have been repeated at least twice. Images obtained after incubation with antibodies against the total proteins are shown beneath those of the phosphorylated proteins.

In the context of anoikis, there was no activated Akt in NUGC3 cells, when cultured in poly-HEMA coated plates in the absence of IGF-1 (Figure 4.14). Akt phosphorylation was induced by IGF-1 and increased in a concentration dependent manner. Total Akt levels decreased inversely proportional to phosphorylated levels. Activated ERK1 and ERK2 were detected in the context of anoikis even in the absence of IGF-1, and a very small induction was seen

when the cells were treated with 5 ng/ml and higher IGF-1 concentrations. Total ERK1 and ERK2 levels were not altered.

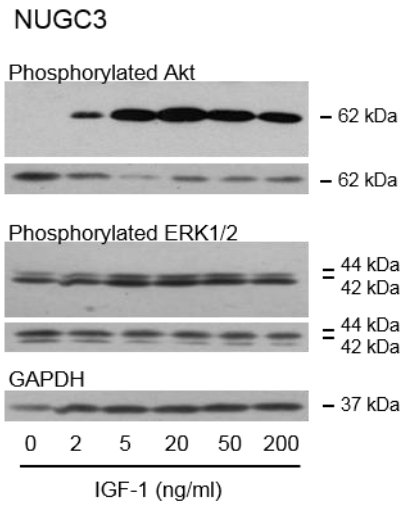

Figure 4.14. The effect of IGF-1 stimulation on activation of the PI3K/Akt and MAPK signal transduction pathways in anoikis. NUGC3 cells were withdrawn from the effects of growth factors by culture in withdrawal medium for 2 days. Cells were seeded into poly-HEMA coated 12 well plates at $20x10^4$ cells per well in 1 ml serum-free medium, in the presence or not of 50 ng/ml IGF-1. After incubation for various times, proteins were extracted with 50 µl per well of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against phosphorylated Akt (1:2000 dilution), Akt (1:2000 dilution), phospho-ERK1/2 (1:5000 dilution), ERK1/2 (1:5000 dilution) and GAPDH (1:10000 dilution).

To quantify the amount of Akt phosphorylation in response to IGF-1 in the context of apoptosis, SNU-1 and NUGC3 cells were withdrawn from the effects of growth factors and cultured in the presence or absence of staurosporine and IGF-1, before incubation with an antibody against phosphorylated Akt and a fluorescently-labelled secondary antibody. Akt was not phosphorylated in SNU-1 and NUGC3 cells cultured in withdrawal medium for two days, or in cells treated with staurosporine (Figure 4.15). However, IGF-1 caused a remarkable induction of Akt phosphorylation in both cell lines. Approximately 80% of SNU-1 cells and 55% of NUGC3 cells treated with IGF-1 in the presence of staurosporine were positive for Akt phosphorylation. Phosphorylated Akt was primarily localised in the cytoplasm, surrounding the nuclei.

The above results suggest that the PI3K/Akt pathway might be more involved in the IGF-1 survival effect compared to the Ras/Raf/MAPK pathway.

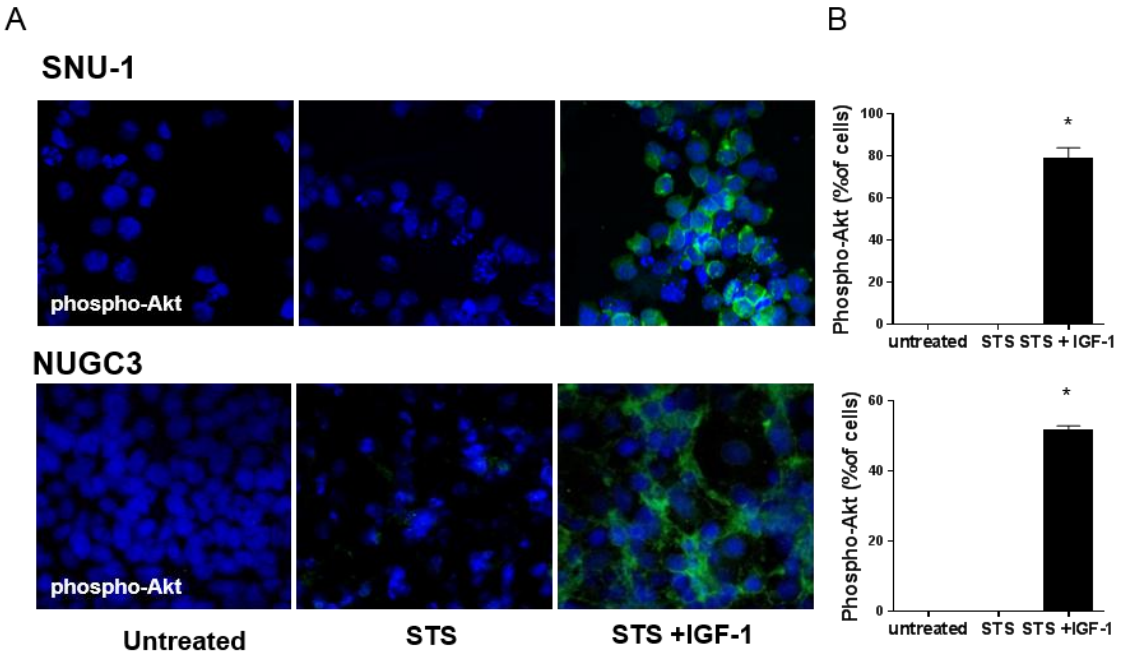

Figure 4.15. The effect of IGF-1 stimulation on activation of the PI3K/Akt signal transduction pathway in apoptosis. SNU-1 cells were centrifuged and resuspended in withdrawal medium and seeded into a 75 cm^2 flask at a concentration of $1.5x10^6$ cells per flask. After 24 hours, medium was replaced with 12 ml fresh withdrawal medium for another 24 hours. Cells were centrifuged and resuspended in withdrawal medium supplemented or not with 50 ng/ml IGF-1 for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of 50 ng/ml IGF-1 for 4 hours. Cells were collected in 1.5 ml Eppendorf tubes and fixed in 1 ml 4% paraformaldehyde. NUGC3 cells were seeded on top of coverslips in 6 well tissue culture plates at 30 x 10^4 cells per well, in 2.5 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 2 ml PBS. Medium was replaced daily with 2 ml withdrawal medium for two days. Cells were incubated with withdrawal medium supplemented or not with 50 ng/ml IGF-1 for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of 50 ng/ml IGF-1 for 24 hours. Cells were washed with 2 ml PBS and fixed in 2 ml ice-cold methanol. Fixed cells were incubated with an antibody against phosphorylated Akt. The secondary antibody was Alexa-Fluor conjugated. Cells were mounted on top of slides in 20 µl DAPI X mounting medium. Pictures of the cells were taken in a Leica fluorescent microscope (**A**). Quantification of the number of cells with positive staining was done in five fields of view for each treatment. The results are shown as a percentage of the total number of cells. Bars, SEM. Asterisks show the significant increase in the number of cells with phosphorylated Akt after treatment with IGF-1. (One-way ANOVA; SNU-1, $p<0.0001$; NUGC3, $p<0.0001$) (**B**).

4.2.4.2 The effect of inhibiting the PI3K/Akt signal transduction pathway on the survival effect of IGF-1

2-(4-morpholinyl)-8-phenyl-4H-l-benzopyran-4-one, also known as 2-(4-morpholinyl)-8-phenylchromone, LY294002, is a compound that abolishes selectively PI3K activity, by acting as a competitive inhibitor for its ATP binding site (Vlahos *et al.*, 1994). The importance of the PI3K/Akt pathway in mediation of the IGF-1 anti-apoptotic effect was investigated using LY294002. SNU-1 cells were withdrawn from the effects of growth factors for 2 days and then cultured with 0.5 µM staurosporine and in the presence or absence of 60 µM LY294002 and 50 ng/ml IGF-1.

Akt is the primary downstream target of PI3K, therefore, PI3K inhibition is expected to reduce Akt activation. Akt was not phosphorylated in the absence of IGF-1 and LY294002 in SNU-1 cells (Figure 4.16). IGF-1 induced significantly Akt phosphorylation. No Akt phosphorylation was detected in SNU-1 cells treated with LY294002 when IGF-1 was not present. When IGF-1 was present, LY294002 reduced the amount of Akt phosphorylation to 5% of the amount detected when the drug was not present. This suggests that LY294002 inhibits effectively the activity of the PI3K/Akt pathway.

SNU-1 cells which had been withdrawn and treated with staurosporine underwent apoptosis, evaluated by detection of the 89 kDa fragment of PARP (Figure 4.16). IGF-1 reduced significantly the amount of cleaved PARP. Inhibition of PI3K/Akt by LY294002 increased slightly the amount of cleaved PARP in the absence of IGF-1, confirming that inhibition of the pathway affects the survival of the cells. IGF-1 was ineffective at inhibiting staurosporine-induced PARP cleavage when LY294002 was present. This result suggests that activation of PI3K/Akt is important for mediating the IGF-1 survival effect.

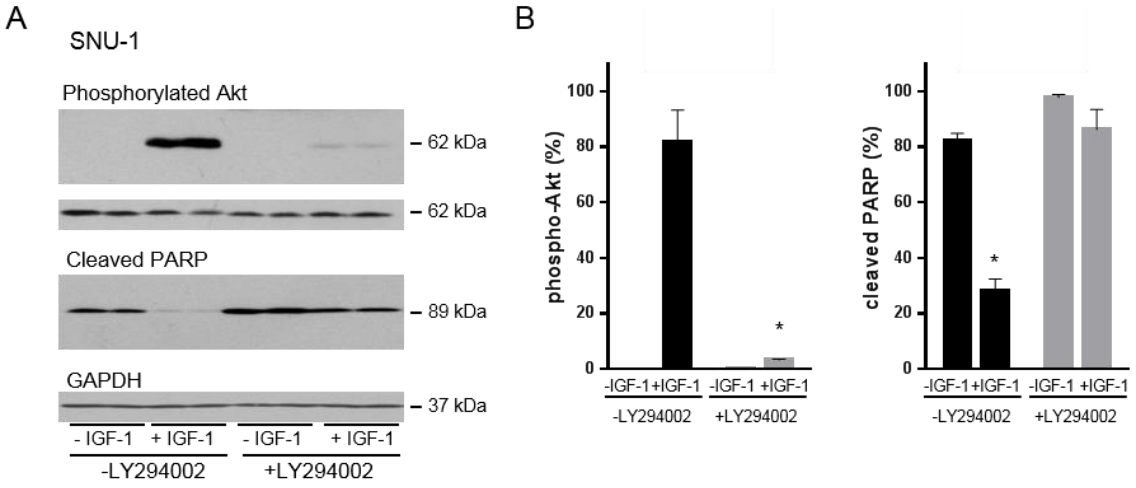

Figure 4.16. The effect of PI3K inhibition with LY294002 on the anti-apoptotic effect of IGF-1 in SNU-1 cells. SNU-1 cells were centrifuged and resuspended in withdrawal medium and seeded into a 75 cm^2 flask at a concentration of $1.8x10^6$ cells per flask. After 24 hours, medium was replaced with 24 ml fresh withdrawal medium for another 24 hours. Cells were centrifuged and resuspended in withdrawal medium supplemented or not with 60 µM LY294002 for one hour and with or without 50 ng/ml IGF-1 for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of LY294002 and IGF-1 for 4 hours. Proteins were extracted with 80 µl of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against phospho-Akt (1:3000), Akt (1:3000), cleaved PARP (1:2000 dilution) and GAPDH (1:10000 dilution). The images shown are representative of samples from triplicate experiments (**A**). Densitometry was with Labworks 4.0 software, adjusted for the background optical density of the X-ray film and normalised to GAPDH. Results are expressed as a percentage of the intensity of the cleaved PARP band observed in the absence of IGF-1 and in the presence of LY294002. Bars are the mean ±SEM, *denotes phosphorylated Akt levels that are statistically significantly lower in the presence of LY294002 (Two-way ANOVA; $p<0.0001$) and cleaved PARP levels that are statistically significantly lower in the presence of IGF-1 than in its absence (Two-way ANOVA; $p=0.0001$) (**B**).

4.2.4.3 The effect of inhibiting the Ras/Raf/ERK signal transduction pathway on the survival effect of IGF-1

U0126 is a compound that inhibits selectively the mitogen-activated protein kinase kinase family members, MEK-1 and MEK-2 (Favata *et al.*, 1998). The importance of the Ras/Raf/MAPK pathway in the survival effect of IGF-1 was investigated in SNU-1 cells. SNU-1 cells were withdrawn from the effects of growth factors for 2 days and then cultured in the presence of 0.5 μM staurosporine and in the presence or absence of 6 μM U0126 and 50 ng/ml IGF-1.

ERK1 and ERK2 are downstream targets of MEK-1 and MEK-2. Inhibition of MEK-1 and MEK-2 with the U0126 inhibitor is, therefore, expected to reduce activation of ERK1 and ERK2. There was ERK1 and ERK2 phosphorylation in the absence of IGF-1 and U0126 (Figure 4.17). Addition of IGF-1 did not increase the basal levels of ERK1 and ERK2 phosphorylation. U0126 inhibited completely ERK1 and ERK2 phosphorylation, both in the absence and in the presence of IGF-1.

SNU-1 cells were treated with staurosporine and underwent apoptosis, assessed by detection of the 89 kDa fragment of PARP (Figure 4.17). IGF-1 reduced significantly the amount of cleaved PARP from 100% to less than 20%. Inhibition of MEK1 and MEK2 by U0126 did not increase the amount of cleaved PARP in the absence of IGF-1, suggesting that inhibition of the pathway does not affect cell survival, under these conditions. IGF-1 retained its ability to inhibit staurosporine-induced PARP cleavage with the same effectiveness as when U0126 was not present. This suggests that an active Ras/Raf/ERK pathway is not essential for mediating the IGF-1 survival effect.

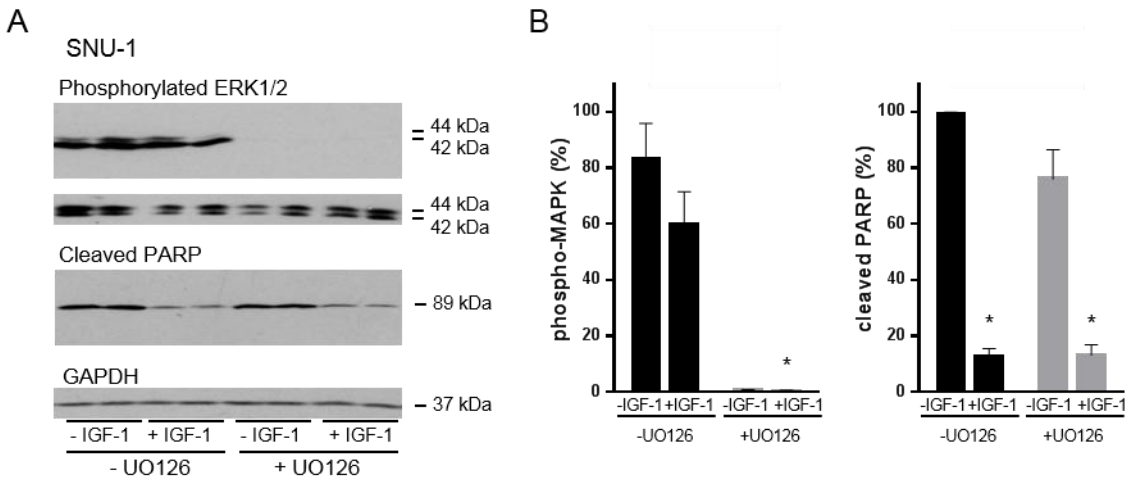

Figure 4.17. The effect of MEK inhibition with U0126 on the anti-apoptotic effect of IGF-1 in SNU-1 cells. SNU-1 cells were centrifuged and resuspended in withdrawal medium and seeded into a 75 cm^2 flask at a concentration of $1.8x10^6$ cells per flask. After 24 hours, medium was replaced with 24 ml fresh withdrawal medium for another 24 hours. Cells were centrifuged and resuspended in withdrawal medium supplemented or not with 6 µM U0126 for one hour and with or without 50 ng/ml IGF-1 for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of UO06 and IGF-1 for 4 hours. Proteins were extracted with 80 µl of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against phospho-ERK1/2 (1:5000), ERK1/2 (1:5000), cleaved PARP (1:2000 dilution) and GAPDH (1:10000 dilution). The images shown are representative of samples from triplicate experiments (**A**). Densitometry was with Labworks 4.0 software, adjusted for the background optical density of the X-ray film and normalised to GAPDH. Results are expressed as a percentage of the intensity of the cleaved PARP band observed in the absence of IGF-1 and in the presence of U0126. Bars are the mean ±SEM, *denotes phosphorylated ERK1/2 levels that are statistically significantly lower in the presence of U0126 (Two-way ANOVA; p=0.0006) and cleaved PARP levels that are statistically significantly lower in the presence of IGF-1 than in its absence (Two-way ANOVA; p<0.0001) (**B**).

4.3 Discussion

The principal aim of the experiments presented in this chapter was to test the hypothesis that the IGF signal transduction pathway is important for cell survival and hence a viable therapeutic target in gastric cancer. For this reason, the survival effect of IGF-1 was tested in gastric cancer cells, using survival assays based on induction of apoptosis by staurosporine and by disruption of epithelial cell-matrix interaction. We demonstrated a significant protective effect of IGF-1 on staurosporine-induced apoptosis in gastric cancer cell lines SNU-1, NUGC3 and AGS. The protective effect of IGF-1 against the broad-spectrum kinase inhibitor staurosporine emphasises the potency of IGF-1 as a pro-survival factor. Interestingly, the above cell lines did not express or expressed extremely low levels of three tyrosine kinase receptors, HER-2, FGFR2 and c-Met, which are being inhibited in the clinic. This suggests that they represent patients who would be ineligible for the above treatments and could, possibly benefit from IGF-targeted treatment. NCI-N87 cells, which overexpress HER-2, were, also, protected effectively from staurosporine-induced cell death by IGF-1. This

is supported by a recent study which suggests that activity of the IGF signal transduction pathway in NCI-N87 cells provides a conduit for resistance to lapatinib, a dual EGFR/HER2 targeted inhibitor (Zhang *et al.*, 2014).

A small number of studies have reported the survival effect mediated by the IGF signal transduction pathway in gastric cancer cells. IGF-1 protected against ethanol-induced caspase 3 activation in MET-amplified MKN45 cells (Min *et al.*, 2005; Smolen *et al.*, 2006). The same study showed that expression of a truncated IGF-IR receptor increased cleavage of caspase 3 induced by ethanol, 5-fluorouracil and radiation in MKN45 cells. In a separate study, reduction of IGF-IR expression by recombinant adenovirus increased DNA fragmentation in response to apoptosis induced by UV radiation and cisplatin (Wang *et al.*, 2010a).

Despite the significant protective effect of IGF-1 against staurosporine-induced apoptosis in SNU-1, NUGC3, AGS and NCI-N87 cells, the ligand was not as effective in KATO III and MKN74 cells. As mentioned in Chapter 3, KATO III cells overexpress FGFR2, and possibly depend on activation of this pathway in order to sustain their survival and growth potential. Under those circumstances, activation of the IGF signal transduction pathway might not suffice to promote survival. MKN74 cells have no known amplification and express the highest levels of the type I IGF receptor amongst all the gastric cancer cell lines tested. Furthermore, they respond to IGF-1 stimulation, with induction of both Akt and ERK1 and ERK2 phosphorylation. The lack of IGF-1 protection against apoptosis in this cell line is, therefore, unexpected. It is possible, though, that different experimental settings could have contributed to the manifestation of the IGF-1 survival effect in those cells.

Relatively high levels of cleaved PARP were detected in SNU-16 and SNU-5 cells prior to staurosporine treatment. Staurosporine increased only slightly the levels of cleaved PARP in SNU-16 cells but IGF-1 had no protective effect. Staurosporine did not increase cleaved PARP levels in SNU-5 cells. The relatively high basal levels of cleaved PARP in control conditions suggest that the cells were not healthy and, therefore, any investigation of the IGF-1 survival effect would be invalid.

Activation of caspase 3 and PARP by proteolytic cleavage was detected in SNU-1 and NUGC3 cells after treatment with staurosporine. This result suggests that staurosporine induces cell death in a caspase-dependent manner. Activation of caspase 3 and 7 and cleavage of PARP in response to staurosporine has been reported previously in LNCaP prostate cancer cells (Marcelli *et al.*, 1999). Also, staurosporine activated the caspase cascade and cleavage of

PARP in T47D and MCF7 breast cancer cells (Mooney *et al.*, 2002) and in macrophages (Son *et al.*, 2014). However, activation of both caspase-dependent and independent cell death by staurosporine has also been reported (Belmokhtar *et al.*, 2001).

Inhibition of cell attachment to the extracellular matrix after culture in plastic coated with poly-HEMA in the absence of growth factors, induced a type of apoptosis described as anoikis in NUGC3, AGS and KATO III cells. IGF-1 protected NUGC3 and AGS cells from anoikis, overtime. This protection against anoikis proposes a role for IGF-1 in promoting survival of anchorage-deprived gastric cancer cells and, possibly, providing a stimulus for a migratory and invasive phenotype. As mentioned previously, circumvention of anoikis accompanies the acquisition of anchorage independence and cell motility, traits necessary for invasion and metastasis. In agreement with the results obtained from the staurosporine assay, KATO III cells were not protected from anoikis by IGF-1, suggesting that those cells do not respond to activation of the IGF signal transduction pathway for their survival mechanisms.

Treatment of SNU-1 and NUGC3 cells with BMS754807 abrogated the protective effect of IGF-1 against staurosporine-induced apoptosis. BMS754807 may inhibit both the type I IGF receptor and the insulin receptor due to the high similarity of their tyrosine kinase domains. It is, therefore, possible that either or both of the above two receptors are responsible for mediating the IGF-1 survival effect in those cells. Selective inhibition of the type I IGF receptor or the insulin receptor would clarify which of the two is involved in mediation of the IGF-1 survival signal.

The marked increase of Akt but not ERK1 and ERK2 phosphorylation in response to IGF-1 in SNU-1 and NUGC3 cells, both in the context of apoptosis and anoikis, suggests that the Akt pathway might be more involved in mediating the IGF-1 survival effect. Inhibition of PI3K with the LY294002 inhibitor in SNU-1 cells reduced significantly the amount of phosphorylated Akt and abrogated partially the IGF-1 anti-apoptotic effect. In contrast, inhibition of MEK1 and MEK2 with the U0126 inhibitor reduced significantly the amount of phosphorylated ERK1 and ERK2 but did not alter the anti-apoptotic effect of IGF-1. It could be concluded, therefore, that in the context of staurosporine-induced apoptosis, activation of the PI3K/Akt pathway and not the Raf/ERK mediates the survival effect of IGF-1.

A more prominent effect of the PI3K/Akt compared to the Raf/ERK pathway in mediation of the IGF-1 survival effect is supported by several studies. Inhibition of the PI3K/Akt pathway with the LY294002 inhibitor abrogated the protective effect of IGF-1 against apoptosis induced by serum deprivation in Schwann cells (Delaney *et al.*, 1999), 3T3-L1 preadipocytes

(Gagnon *et al.*, 2001) and pancreatic beta cells (Liu *et al.*, 2002). Also, PI3K activity was more important for the protective effect of IGF-1 against paclitaxel and doxorubicin-induced apoptosis in MCF-7 breast cancer cells (Gooch *et al.*, 1999).

However, a dual role for the PI3K/Akt and the Raf/MAPK pathways in transduction of the IGF-1 survival effect has also been described. Activation of both pathways was necessary for the protective effect of IGF-1 against apoptosis induced by serum deprivation in immortalised brown adipocytes (Navarro *et al.*, 1998), whereas IGF-1 protection against oxygen-glucose deprivation-induced apoptosis was blocked by PI3K/Akt and Raf/ERK signal transduction pathway inhibition in pheochromocytoma cells (Chung *et al.*, 2007). Inhibition of PI3K and ERK1 and ERK2 activation partially inhibited the survival effect of IGF-1 in osteoblastic cells (Grey *et al.*, 2003). Attenuation of the IGF-1 survival effect by inhibition of PI3K/Akt and ERK1 and ERK2 was also described in skeletal muscle cells subjected to oxidative stress and in retinal ganglion cells in hypoxic conditions (Yang *et al.*, 2010; Yang *et al.*, 2013).

In summary, the findings reported in this chapter suggest that the IGF signal transduction pathway is important for the survival of as subset of gastric cancer cells against caspase-dependent apoptosis induced by inhibition of kinase activity and by loss of cell-matrix attachment. Therefore, targeting the pathway may be useful in controlling the spread of gastric cancer cells.

Chapter 5. The Effect of IGF Signal Transduction Pathway Activation on Proliferation of Gastric Cancer Cells

5.1 Introduction

One of the most important traits of a cancer cell is the ability to sustain proliferation in the absence of appropriate external signals. This can be achieved by mutations in oncogenes or tumour suppressor genes or by increased expression or activation of growth factor pathways that stimulate cell proliferation.

The mitogenic properties of the insulin-like growth factor signal transduction pathway have been described in various studies. IGF-1 and IGF-2 stimulated the growth of an oesophageal squamous cancer cell line (Oku *et al.*, 1991), whereas IGF-1 enhanced the proliferation of acute myelogenous leukaemia blasts alone and in combination with exogenous cytokines (Frostad and Bruserud, 1999). In a human osteosarcoma cell line IGF-1 stimulated cyclin D1 expression during G1 phase, cyclin B (G2 cyclin) and two cyclin-dependent kinases, cdc2 and cdk2 (Furlanetto *et al.*, 1994). Stimulation of cyclin D1 synthesis, DNA synthesis and cell division was stimulated by IGF-1 in MCF7 cells (Dufourny *et al.*, 1997). Increased proliferation of MDA-MB-231 breast cancer cells after treatment with IGF-1, IGF-2 and insulin has been described (Stewart *et al.*, 1990). The involvement of IGF-2 in cell proliferation has been reported in human colon cancer cells, in which an anti IGF-2 antibody significantly retarded cell growth (Singh *et al.*, 1996).

The growth of prostatic cancer cell lines was inhibited by an antisense oligodeoxynucleotide to the type I IGF receptor or by peptide analogues of IGF-1 that compete with IGF-1 binding to its receptor (Pietrzkowski *et al.*, 1993). Transfection of MCF-7 breast cancer cells with a construct encoding an antisense RNA complementary to the region surrounding the translation initiation site of the type I IGF receptor mRNA reduced both IGF-1- and serum-stimulated proliferation (Neuenschwander *et al.*, 1995). Furthermore, inhibition of the endogenous type I insulin-like growth factor receptor by stable expression of a dominant-negative IGF-IR repressed the tumourigenicity of a human lung carcinoma cell line (Jiang *et al.*, 1999).

Since increased proliferation is one of the main drivers of cancer progression, inhibition of the IGF signal transduction pathway could possibly, delay the growth of certain tumour types and prolong the survival of patients.

5.1.1 Aim

The results reported in this chapter aim to elucidate the role of the IGF-signal transduction pathway in the proliferation of gastric cancer cells without receptor tyrosine kinase amplifications. These cells are models for gastric tumour cells of patients who would not be eligible for the currently available targeted treatments. The role of IGF-1 in cell proliferation was investigated in cells cultured in controlled conditions. The importance of the type I IGF receptor in the induction of cell proliferation was investigated in controlled conditions and in a growth factor enriched environment, by transient silencing of the type I IGF receptor with a short interfering RNA sequence. The effect of IGF-IR knockdown on cell cycle progression was also investigated. Finally, the efficiency of the small molecule tyrosine kinase inhibitor BMS754807 in reduction of cell proliferation was tested in a growth factor enriched environment.

5.2 Results

5.2.1 The effect of IGF-1 on cell proliferation in gastric cancer cells

The effect of IGF-1 on gastric cancer cell growth was investigated. MKN74 and NUGC3 cells were cultured in withdrawal medium which is depleted from growth factors or serum-free medium, respectively, in the presence or absence of 50 ng/ml IGF-1. The amount of DNA in the presence and absence of IGF-1 was measured with the PicoGreen fluorimetric assay. During the cell cycle, each cell doubles its DNA content and subsequently divides into two new cells. Therefore, the amount of double stranded DNA in a given sample is directly proportional to the number of cells.

The amount of DNA increased progressively in MKN74 cells cultured in withdrawal medium over the course of 9 days, when IGF-1 was not present (Figure 5.1). This suggests that the cells were not withdrawn completely from the effects of growth factors. When IGF-1 was present in the medium, the amount of DNA was higher at all times tested and this increase was statistically significant after 9 days. NUGC3 cells proliferated when cultured in serum-free medium, until day 3 when they started to reach a plateau (Figure 5.1). The amount of DNA in cells grown in the presence of IGF-1 was increased significantly after 3 days and continued to increase until day 9. Interestingly, cell numbers almost doubled in the presence of IGF-1 compared with in the absence of IGF-1. This suggests that NUGC3 cells were more responsive to IGF-1 compared to MKN74 cells, in terms of cell growth.

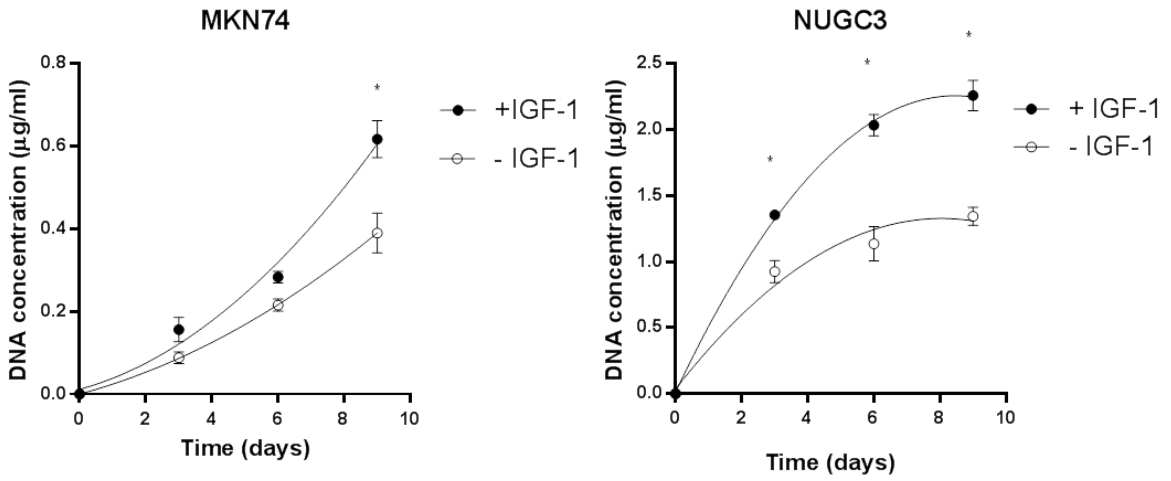

Figure 5.1. The effect of IGF-1 on cell proliferation in MKN74 and NUGC3 cells. MKN74 and NUGC3 cells were seeded into 24 well tissue culture plates at $1x10^4$ cells per well, in 0.5 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 0.5 ml PBS. Medium was replaced every 3 days with 0.5 ml serum-free medium (NUGC3 cells) or 0.5 ml withdrawal medium supplemented or not with 50 ng/ml IGF-1. Medium was aspirated and the cells washed with 0.5 ml ice cold PBS. PBS was aspirated and the cells stored in -20^{o}C. The cells were lysed in SSC/SDS solution and the amount of DNA in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM, * denotes statistically significantly increased DNA content in the presence of IGF-1 compared with in the absence of IGF-1 (Two-way ANOVA; MKN74, $p<0.0001$; NUGC3, $p<0.0001$).

5.2.2 Effect of siRNA knockdown of the type I IGF receptor on the proliferative effect of IGF-1 in NUGC3 cells

To test the hypothesis that the proliferative effect of IGF-1 in NUGC3 cells is mediated by the type I IGF receptor, the effect of IGF-1 on cell proliferation was tested after transient knockdown of the type I IGF receptor with siRNA.

First, the efficiency of the siRNA oligonucleotide against the type I IGF receptor was tested. NUGC3 cells were reverse transfected with 3 siRNA sequences designed to target the type I IGF receptor and with a scrambled sequence, at different concentrations. Cells were incubated with the transfection mix, containing the siRNA sequences and lipofectamine, for 72 hours before protein lysis. Expression of the type I IGF receptor was analysed by western transfer.

The type I IGF receptor was detected in NUGC3 cells that had been transfected with the scrambled sequence (Figure 5.2). Incubation for 72 hours with the first siRNA sequence designed to target the type I IGF receptor, resulted in reduced IGF-IR expression at 20, 30 and 40 nM. The second siRNA sequence reduced IGF-IR expression at 20 and 30 nM and it completely counteracted IGF-IR expression at 40 nM. Finally, the third siRNA sequence completely inhibited IGF-IR expression at 20, 30 and 40 nM. Since siRNA duplexes 2 and 3 were more effective, they were selected for the following experiments. A concentration of 40

nM was used for siRNA 2 and 20 nM for siRNA 3. SiRNA 2 was slightly more effective than siRNA 3 at reduction of cell proliferation (see later, Figure 5.8), and was used for the following experiments, unless otherwise specified.

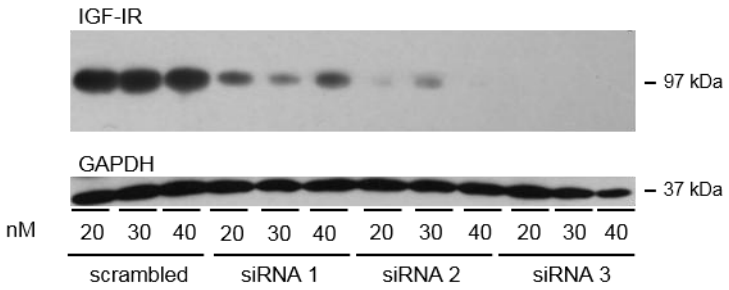

Figure 5.2. The effect of siRNA knockdown on expression of the IGF-IR in NUGC3 cells. Transfection medium was prepared by mixing either scrambled (scr) or IGF-IR targeted (IGF-IR) siRNA duplexes at a final concentration of 20, 30 and 40 nM and Lipofectamine RNAiMAX at a final concentration of 0.2% (v/v), in 0.25 ml DMEM without serum. The transfection medium was then incubated at room temperature for 30 minutes. NUGC3 cells grown in maintenance medium were detached using trypsin:EDTA and resuspended in maintenance medium at 15 x 10^4 cells /ml. One ml of the cell suspension was mixed with 0.25 ml of the transfection medium, containing scrambled or IGF-IR targeted sequences, in 12 well tissue culture plates. After incubation for 72 hours, protein extracts were prepared with 80 µl per well of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose for at 100 mA for 45 minutes. Membranes were incubated with antibodies against the IGF-IR (1:2000 dilution) and GAPDH (1:10000 dilution).

The effect of reducing the expression of the type I IGF receptor on IGF-1–stimulated cell proliferation was tested. NUGC3 cells were reverse transfected with siRNA 2, which targets the type I IGF receptor or with scrambled sequence and cultured in serum-free medium, in the presence or absence of IGF-1 for 9 days. In the absence of IGF-1, there was a small reduction in cell number after transfection with siRNA against the type I IGF receptor (Figure 5.3). This could be attributed to the lack of withdrawal of the cells from growth factors prior to transfection. The proliferative response of NUGC3 cells to IGF-1 was reduced significantly in cells in which endogenous expression of the type I IGF receptor had been lowered (Figure 5.3). Therefore, the type I IGF receptor mediates, at least in part, the IGF-1 proliferative effect.

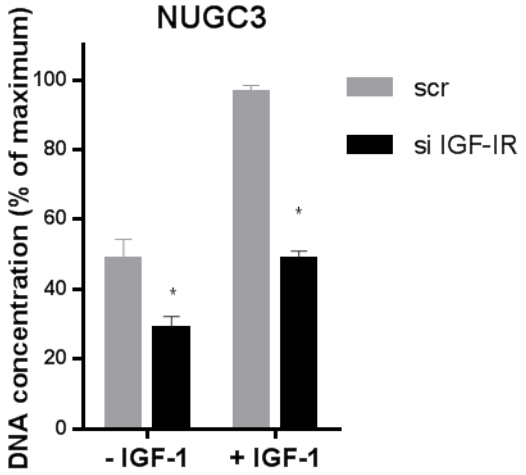

Figure 5.3. The effect of knockdown of the IGF-IR with siRNA on the proliferative effect of IGF-1 in NUGC3 cells. Transfection medium was prepared by mixing either scrambled (scr) or IGF-IR targeted (IGF-IR) siRNA duplexes at a final concentration of 40 nM and Lipofectamine RNAiMAX at a final concentration of 0.2% (v/v), in 0.1 ml DMEM without serum. The transfection medium was then incubated at room temperature for 30 minutes. NUGC3 cells grown in maintenance medium were detached using trypsin:EDTA and resuspended in maintenance medium at $12.5x10^4$ cells /ml. Four hundred µl of the cell suspension were mixed with 0.1 ml of the transfection medium, containing scrambled or IGF-IR targeted sequences, in 24 well tissue culture plates. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells were washed with PBS. Medium was replaced every 3 days with serum-free medium supplemented or not with 50 ng/ml IGF-1. After treatment with IGF-1 for 9 days, medium was aspirated and cells were washed once with ice cold PBS. PBS was aspirated and the cells stored in -20^o C. The cells were lysed in SSC/SDS solution and the amount of DNA present in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM. Asterisks indicate significant reduction in IGF-stimulated proliferation after reduction in type I IGF receptor expression (Two-way ANOVA; $p<0.01$).

5.2.3 Effect of siRNA knockdown of the type I IGF receptor on downstream pathway activation in gastric cancer cells

To test the effect of reduction of the expression of the IGF-IR by siRNA knockdown on activation of downstream signalling pathways, SNU-1 and MKN74 cells, which express relatively high levels of the type I IGF receptor compared to the other cell lines, were transfected with siRNA that targets the IGF-IR or with a scrambled oligonucleotide and proteins were extracted every day over a course of 6 days.

In SNU-1 cells grown in full medium, expression of the IGF-IR was reduced 24 hours after transfection with si-IGF-IR compared with the scrambled oligonucleotide (Figure 5.4). Complete knockdown was achieved 48 hours after transfection and remained stable until day 6. Very little IRS-1 expression was detected 24 and 48 hours after transfection. IRS-1 expression was detected 72 hours after transfection. There was a clear increase in IRS-1 levels in cells in which IGF-IR expression had been lowered, 96 and 120 hours after transfection. Akt phosphorylation was detected 72 hours after transfection. IGF-IR knockdown resulted in

a reduction in Akt phosphorylation 72, 120 and 144 hours after transfection. In contrast, total Akt levels increased in cells with IGF-IR knockdown, 120 and 144 hours after transfection. ERK2 phosphorylation was detected in SNU-1 cells 24 hours after transfection. Reduction of IGF-IR expression resulted in reduced ERK2 phosphorylation at all times tested. Similarly, total ERK2 levels were reduced in cells transfected with siRNA against the type I IGF receptor. ERK1 levels were also reduced in cells with IGF-IR knockdown but to a smaller degree compared to ERK2.

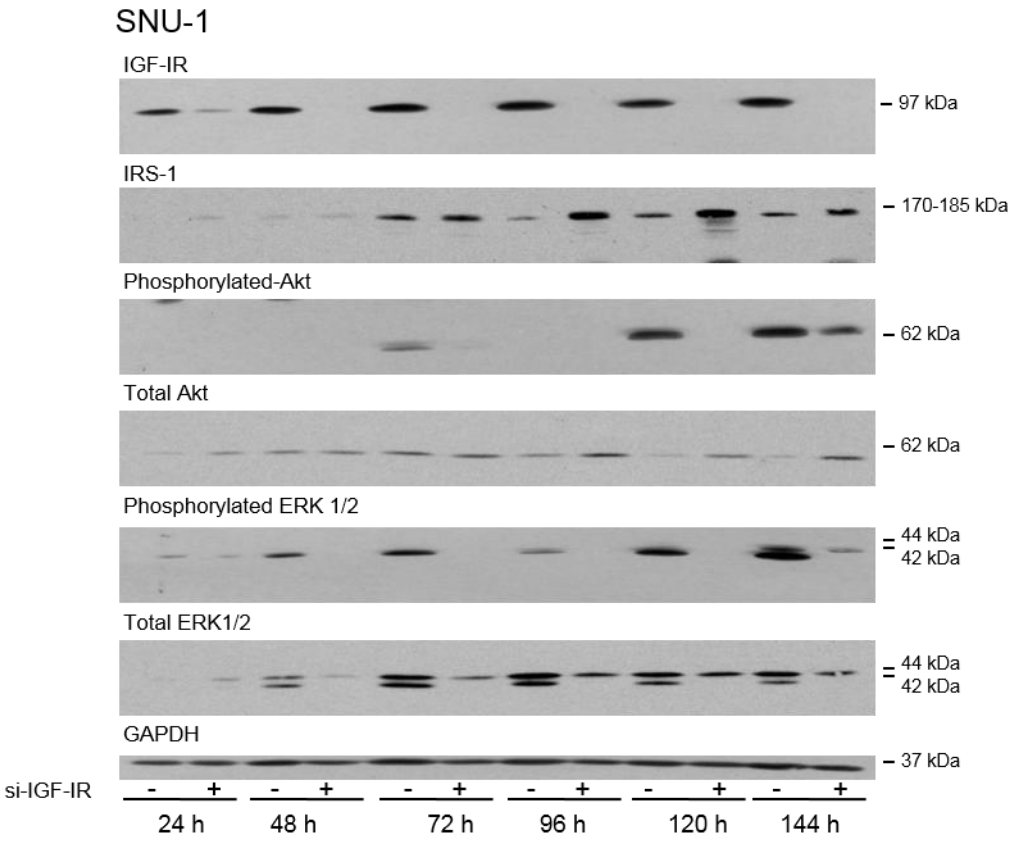

Figure 5.4. The effect of knockdown of the IGF-IR with siRNA on downstream signalling in medium supplemented with serum in SNU-1 cells. Transfection medium was prepared by mixing either scrambled (scr) or IGF-IR targeted (IGF-IR) siRNA duplexes at a final concentration of 50 nM and Lipofectamine RNAiMAX at a final concentration of 0.2% (v/v), in 0.2 ml DMEM without serum. The transfection medium was then incubated at room temperature for 30 minutes. SNU-1 cells were centrifuged and diluted in medium supplemented with 10% foetal calf serum at 10 x 10^4 cells/ml. One ml of the cell suspension was mixed with 0.25 ml of the transfection medium, containing scrambled or IGF-IR targeted sequences, in 12 well tissue culture plates. After 24, 48, 72, 96, 120 and 144 hours, protein extracts were prepared with 80 µl per well of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose for at 100 mA for 45 minutes. Membranes were incubated with antibodies against the IGF-IR (1:2000 dilution), IRS-1 (1:2000 dilution), phosphorylated Akt (1:3000 dilution), Akt (1:3000 dilution), phosphorylated ERK1/2 (1:5000 dilution), ERK1/2 (1:5000 dilution) and GAPDH (1:10000 dilution).

In MKN74 cells, knockdown of the IGF-IR was achieved 24 hours after transfection and remained stable for 6 days after transfection (Figure 5.5). An increase in IRS-1 levels,

following IGF-IR knockdown was observed, 48, 72 and 96 hours after transfection. Akt phosphorylation was reduced after IGF-IR knockdown at all times, except 24 hours. On the contrary, total Akt levels increased following transfection with si IGF-IR. Both phosphorylated and total ERK2 levels were reduced following IGF-IR knockdown at all times analysed. ERK1 phosphorylation was reduced to a small extent after IGF-IR knockdown, whereas total ERK1 was not affected by IGF-IR knockdown.

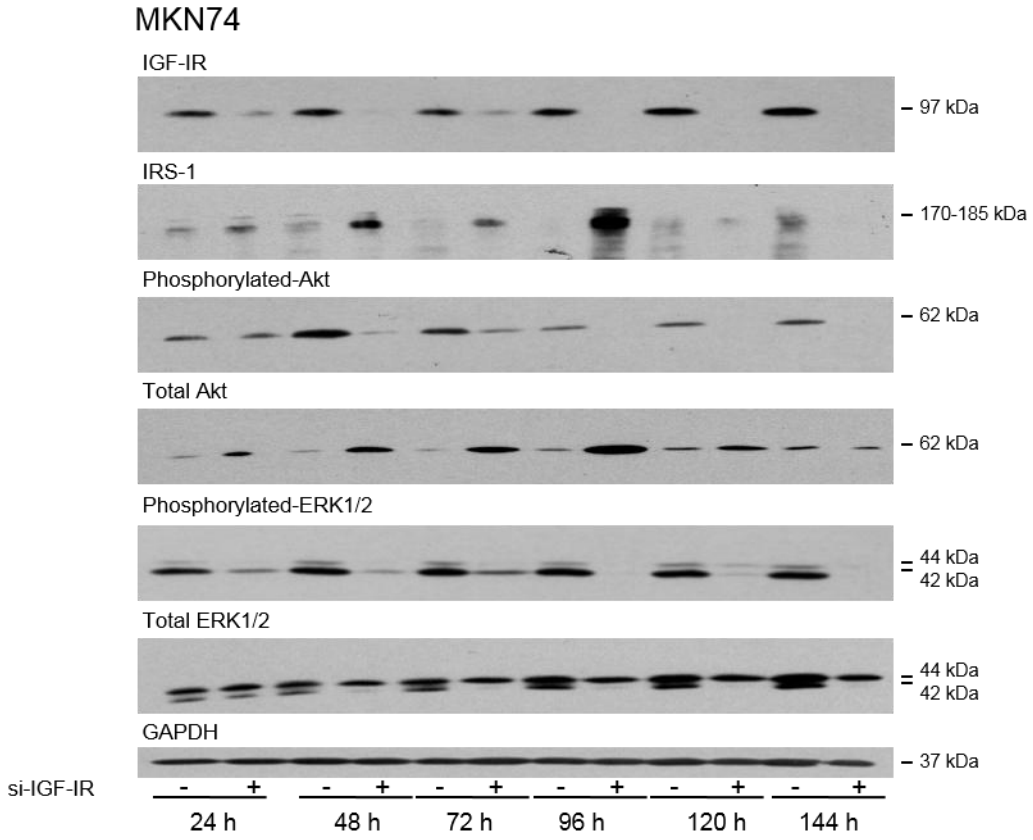

Figure 5.5. The effect of knockdown of the IGF-IR with siRNA on downstream signalling in medium supplemented with serum in MKN74 cells. Transfection medium was prepared by mixing either scrambled (scr) or IGF-IR targeted (IGF-IR) siRNA duplexes at a final concentration of 40 nM and Lipofectamine RNAiMAX at a final concentration of 0.2% (v/v), in 0.2 ml DMEM without serum. The transfection medium was then incubated at room temperature for 30 minutes. MKN74 cells grown in maintenance medium were detached using trypsin:EDTA and resuspended in medium supplemented with 10% foetal calf serum at 10 x 10^4 cells /ml. One ml of the cell suspension was mixed with 0.2 ml of the transfection medium, containing scrambled or IGF-IR targeted sequences, in 12 well tissue culture plates. After 24, 48, 72, 96, 120 and 144 hours, protein extracts were prepared with 80 µl per well of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose for at 100 mA for 45 minutes. Membranes were incubated with antibodies against the IGF-IR (1:2000 dilution), IRS-1 (1:2000 dilution), phosphorylated Akt (1:3000 dilution), Akt (1:3000 dilution), phosphorylated ERK1/2 (1:5000 dilution), ERK1/2 (1:5000 dilution) and GAPDH (1:10000 dilution).

In NUGC3 cells, complete knockdown of the IGF-IR was achieved 48 hours after transfection and remained stable thereafter (Figure 5.6Figure 5.5). Total IRS-1 was reduced in cells with reduced IGF-IR expression after 72 and 120 hours. The amount of phosphorylated and total

Akt was not reduced after IGF-IR knockdown at all times tested. ERK2 phosphorylation was reduced following IGF-IR knockdown at 72, 96, 120 and 144 hours, whereas the amount of phosphorylated ERK1 was not altered. Both ERK1 and ERK2 levels were reduced in cells with IGF-IR knockdown.

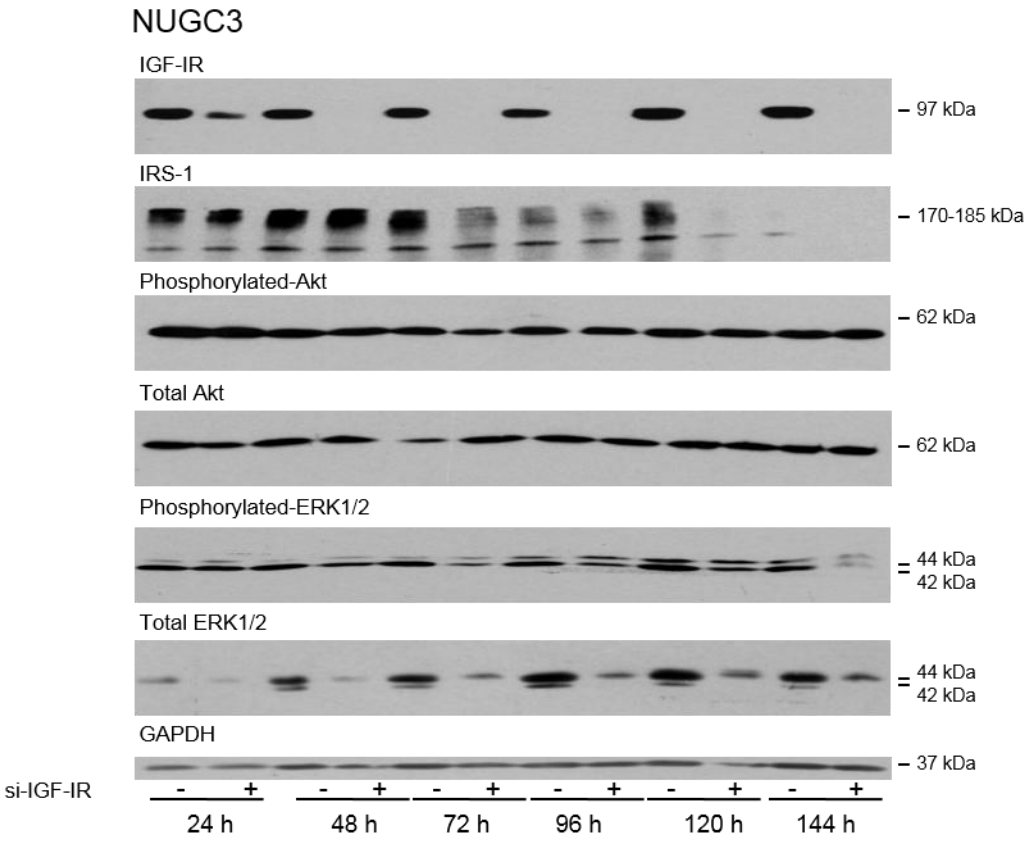

Figure 5.6. The effect of knockdown of the IGF-IR with siRNA on downstream signalling in medium supplemented with serum in NUGC3 cells. Transfection medium was prepared by mixing either scrambled (scr) or IGF-IR targeted (IGF-IR) siRNA duplexes at a final concentration of 40 nM and Lipofectamine RNAiMAX at a final concentration of 0.2% (v/v), in 0.2 ml DMEM without serum. The transfection medium was then incubated at room temperature for 30 minutes. NUGC3 cells grown in maintenance medium were detached using trypsin:EDTA and resuspended in medium supplemented with 10% foetal calf serum at 10 x 10^4 cells /ml. One ml of the cell suspension was mixed with 0.2 ml of the transfection medium, containing scrambled or IGF-IR targeted sequences, in 12 well tissue culture plates. After 24, 48, 72, 96, 120 and 144 hours, protein extracts were prepared with 80 µl per well of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose for at 100 mA for 45 minutes. Membranes were incubated with antibodies against the IGF-IR (1:2000 dilution), IRS-1 (1:2000 dilution), phosphorylated Akt (1:3000 dilution), Akt (1:3000 dilution), phosphorylated ERK1/2 (1:5000 dilution), ERK1/2 (1:5000 dilution) and GAPDH (1:10000 dilution).

The above results suggest that reduction in IGF-IR expression affects directly activation of the RAS/RAF/ERK pathway in SNU-1, MKN74 and NUGC3 cells and of the PI3K/Akt pathway in SNU-1 and MKN74 cells.

5.2.4 Effect of MEK1 and MEK2 inhibition on the proliferative effect of IGF-1 in NUGC3 cells

As mentioned previously, reduction of IGF-IR expression with siRNA transfection resulted in reduced phosphorylation of ERK1 and 2, as well as reduced total levels of ERK1 and 2 in medium supplemented with serum in SNU-1, MKN74 and NUGC3 cells. This suggests that an active IGF-IR is required for optimal activation of the ERK pathway. To investigate whether the proliferative effect of IGF-1 is mediated by ERK1 and 2 activation, NUGC3 cells were cultured in serum-free medium, in the presence and absence of 6µM U0126 and 50 ng/ml IGF-1 for 9 days. U0126 inhibits MEK1 and MEK2 and, as a result, prevents downstream activation of ERK1 and 2. The amount of DNA in each sample was measured with the PicoGreen fluorimetric assay.

In the absence of IGF-1, treatment with U0126 resulted in a small reduction in cell growth, compared with untreated cells (Figure 5.7). When U0126 was not present, treatment with IGF-1 increased significantly the number of cells by 30% after 9 days. However, in the presence of U0126, IGF-1 did not induce a significant increase in cell number. This suggests that activation of ERK1 and ERK2 is required for transmission of the proliferative effect of IGF-1 in NUGC3 cells.

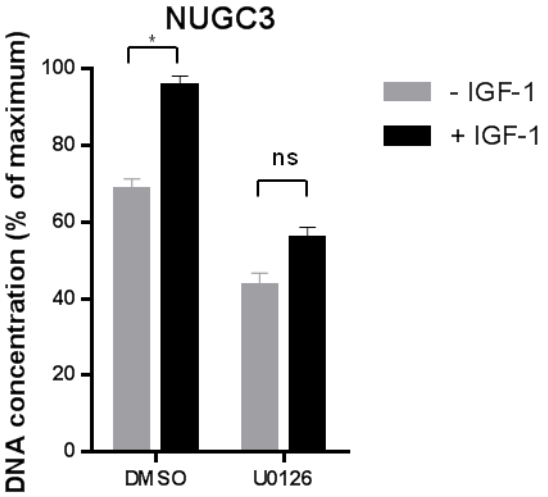

Figure 5.7. The effect of inhibition of MEK1/2 with the U0126 inhibitor on the proliferative effect of IGF-1 in NUGC3 cells. NUGC3 cells were seeded into 24 well tissue culture plates at $1x10^4$ cells per well, in 0.5 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 0.5 ml PBS. Medium was replaced every 3 days with 0.5 ml serum-free medium, supplemented or not with 6 µM U0126 and 50 ng/ml IGF-1. After 9 days, medium was aspirated and the cells washed with 0.5 ml ice cold PBS. PBS was aspirated and the cells stored in -20^{o}C. The cells were lysed in SSC/SDS solution and the amount of DNA in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM. Asterisk indicates significant increase in cell number after treatment with IGF-1 (Two-way ANOVA; p=0.0007).

5.2.5 Effect of siRNA knockdown of the type I IGF receptor on proliferation of gastric cancer cells in a growth factor enriched environment

As mentioned previously, in normal conditions, cancer cells exist in an environment enriched with a variety of growth factors, which may have survival and growth stimulatory effects. It is, therefore, of particular importance to test the effectiveness of a targeted agent in such an environment. For this reason, MKN74 and NUGC3 cells were transfected with siRNA 2 and siRNA 3, which target the type I IGF receptor or with a scrambled sequence and cultured in medium supplemented with 10% foetal calf serum for 3 days. The number of cells was measured by detecting the amount of DNA in each sample with the PicoGreen fluorimetric assay.

In MKN74 cells, transfection with siRNA 2 and siRNA 3 reduced the amount of DNA after 3 days, to 50% and 60% of the amount of DNA detected after transfection with the scrambled oligonucleotide (Figure 5.8). Similarly, in NUGC3 cells, siRNA 2 reduced the amount of DNA to 50% and siRNA 3 to 75%. Both siRNA sequences reduced significantly cell number in both cell lines but siRNA 2 was more effective and was used in the following experiments.

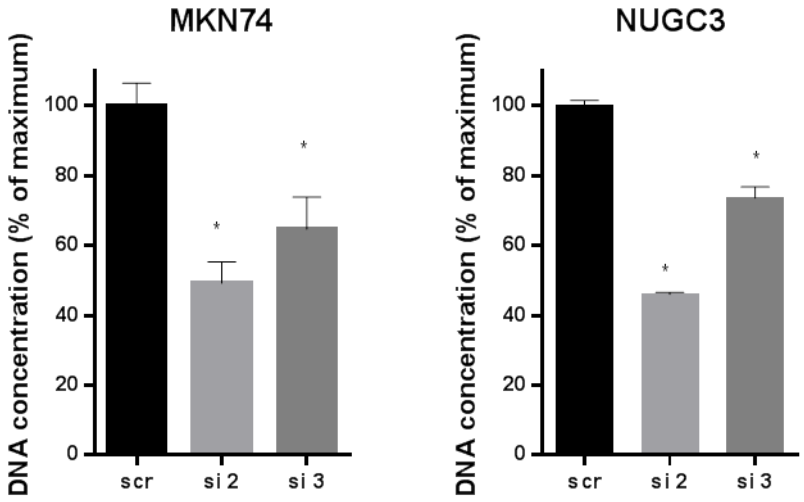

Figure 5.8. The effect of knockdown of the IGF-IR with siRNA on cell number in MKN74 and NUGC3 cells in full medium. Transfection medium was prepared by mixing either scrambled (scr) or IGF-IR targeted (IGF-IR) siRNA duplexes 2 and 3 at a final concentration of 40 and 20 nM, respectively, and Lipofectamine RNAiMAX at a final concentration of 0.2% (v/v), in 0.1 ml DMEM without serum. The transfection medium was then incubated at room temperature for 30 minutes. MKN74 and NUGC3 cells grown in maintenance medium were detached using trypsin:EDTA and resuspended in medium supplemented with 10% foetal calf serum at 12.5 x 10^4 cells /ml. Four hundred µl of the cell suspension were mixed with 0.1 ml of the transfection medium, containing scrambled or IGF-IR targeted sequences, in 24 well tissue culture plates. After 72 hours, the cells were washed with 0.5 ml ice cold PBS and stored in -20^{o}C. The cells were lysed in SSC/SDS solution and the amount of DNA present in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM. Asterisks indicate significant reduction in cell number after transfection with a siRNA against the type I IGF receptor compared to transfection with the scrambled oligonucleotide (One-way ANOVA; MKN74, siRNA 2 p=0.005, siRNA 3 p=0.0261; NUGC3, siRNA 2 p<0.0001, siRNA 3 p=0.0002).

To investigate the effect of reducing the expression of the type I IGF receptor on cell proliferation over a period of time, SNU-1, MKN74, NUGC3 and AGS cells were transfected with siRNA 2 or with a scrambled sequence and cultured in medium supplemented with 2-10% foetal calf serum for 1 to 4 days. The number of cells at the end of each time point was measured by detecting the amount of DNA in each sample with the PicoGreen fluorimetric assay.

Cell proliferation was reduced significantly in SNU-1 cells after transfection with siRNA against the IGF-IR, compared to cells transfected with scrambled sequence (Figure 5.9). Interestingly, although cells with intact IGF-IR expression were actively proliferating at a fast rate until day 5, cells with reduced IGF-IR expression stopped proliferating completely after day 2. This suggests that the type I IGF receptor is one of the main receptors driving cell proliferation in this cell line.

In MKN74 cells, reduction of IGF-IR expression by siRNA knockdown reduced the proliferation rate compared to cells transfected with scrambled oligonucleotide (Figure 5.9). Cell number was reduced significantly two days after transfection and remained low until day 4. A similar effect was seen in NUGC3 cells. Reduced IGF-IR expression following transfection resulted in a significant reduction in cell number 3 days after transfection. The number of AGS cells was reduced significantly 2 days after transfection with the siRNA against the type I IGF receptor and remained low over the course of 4 days.

Cell proliferation was reduced in all four cell lines tested after knockdown of the type I IGF receptor. This suggests that even in an environment with various growth factors and stimuli, targeting of the type I IGF receptor can have detrimental effects on gastric cancer cell growth.

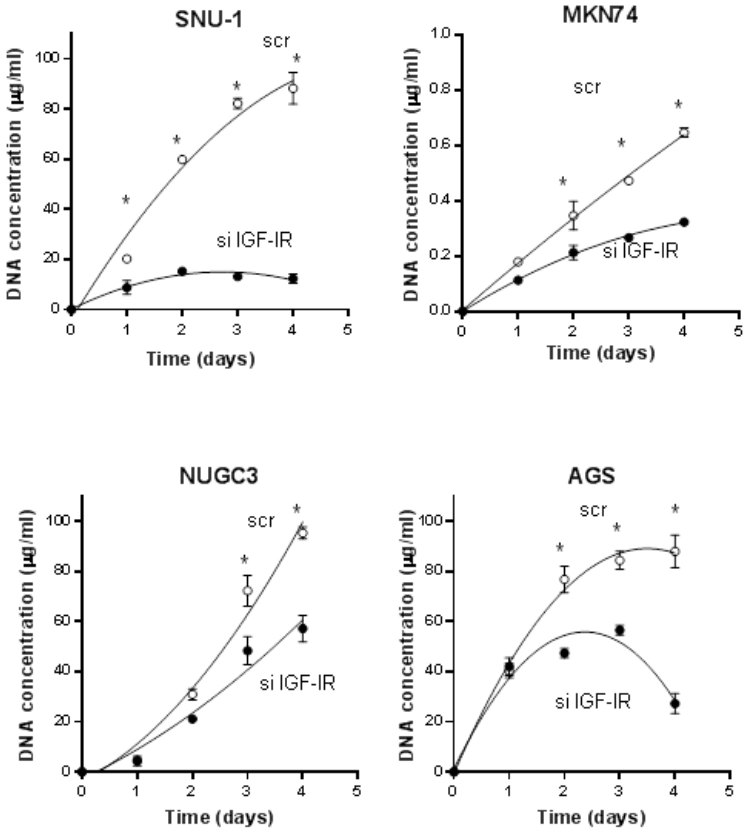

Figure 5.9. The effect of knockdown of the IGF-IR with siRNA on proliferation of SNU-1, MKN74, NUGC3 and AGS cells in full medium. Transfection medium was prepared by mixing either scrambled (scr) or IGF-IR targeted (IGF-IR) siRNA duplexes at a final concentration of 50 nM (SNU-1) and 40 nM (MKN74, NUGC3 and AGS) and Lipofectamine RNAiMAX at a final concentration of 0.2% (v/v), in 0.1 ml DMEM without serum. The transfection medium was then incubated at room temperature for 30 minutes. SNU-1 cells were centrifuged and diluted in medium supplemented with 2% foetal calf serum at 10 x 10^4 cells/ml. MKN74, NUGC3 and AGS cells grown in maintenance medium were detached using trypsin:EDTA and resuspended in medium supplememented with 10%, 2% and 10% foetal calf serum, respectively, at 12.5 x 10^4 cells /ml. Four hundred µl of the cell suspension were mixed with 0.1 ml of the transfection medium, containing scrambled or IGF-IR targeted sequences, in 24 well tissue culture plates. After 24, 48, 72 and 96 hours, the cells were washed with 0.5 ml ice cold PBS and stored in -20^o C. The cells were lysed in SSC/SDS solution and the amount of DNA present in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM. Asterisks show times at which there were significantly fewer cells after transfection with the si IGF-IR2 than after transfection with the scrambled oligonucleotide (Two-way ANOVA; SNU-1, $p<0.0001$; MKN74, $p<0.0001$; NUGC3, $p<0.0001$; AGS, $p<0.0001$).

5.2.6 Effect of siRNA knockdown of the type I IGF receptor on cell cycle progression in gastric cancer cells in a growth factor enriched environment

To confirm that the reduction in cell number observed after knockdown of the type I IGF receptor is a result of decreased cell proliferation, as opposed to increased cell death, two markers of cell cycle progression were measured in gastric cancer cells that had been transfected with a siRNA against the type I IGF receptor.

Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) is a synthetic nucleoside that is an analogue of thymidine and was first described in the 1950s (Kit *et al.*, 1958). BrdU can be

incorporated in the place of thymidine into the newly synthesized DNA of replicating cells, during the S phase of the cell cycle. It allows the detection of proliferating cells in living tissues. To test the effect of decreased IGF-IR expression on DNA synthesis, SNU-1, MKN74, NUGC3 and AGS cells were transfected with siRNA 2, which targets the type I IGF receptor or with a scrambled sequence and cultured in medium supplemented with 2-10% foetal calf serum for 72 hours. The cells were incubated with BrdU for 2 hours before fixation and incubation with an antibody that detects BrdU.

Approximately 15% of SNU-1 cells transfected with scrambled oligonucleotide were actively synthesizing DNA (Figure 5.10). Reduction of IGF-IR expression by transfection with siRNA 2 reduced significantly the above percentage to 7%. Similarly, reduced IGF-IR expression by siRNA knockdown reduced significantly the percentage of MKN74 cells in S phase from 21% to 8%. In NUGC3 and AGS cells, the percentage of cells that were synthesizing DNA was reduced from 13% to 4% and from 43% to 9%, respectively.

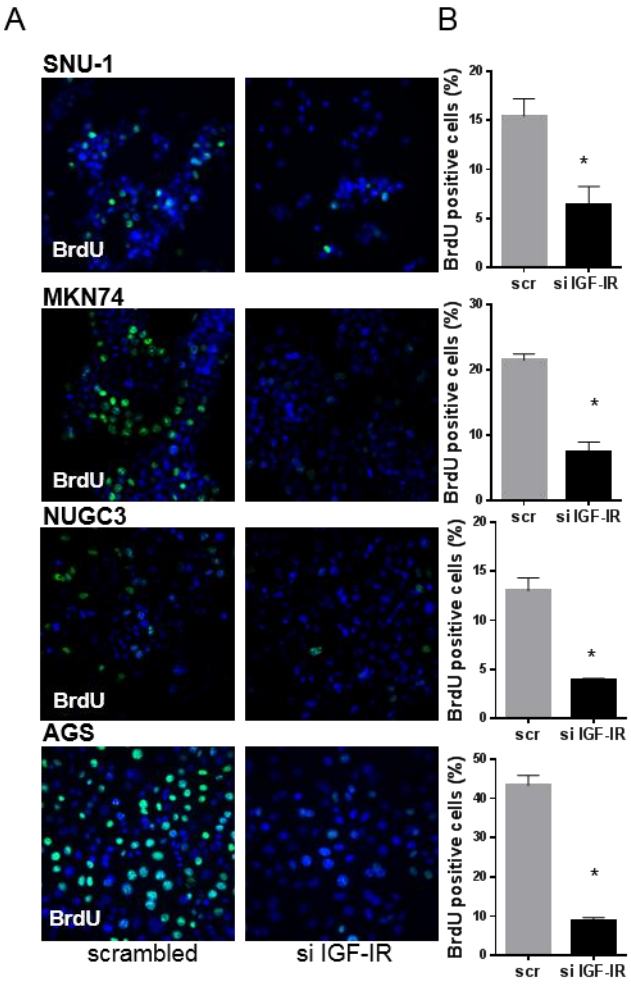

Figure 5.10. The effect of knockdown of the IGF-IR with siRNA on DNA synthesis in SNU-1, MKN74, NUGC3 and AGS cells in full medium. Transfection medium was prepared by mixing either scrambled (scr) or IGF-IR targeted (IGF-IR) siRNA duplexes at a final concentration of 50 nM (SNU-1 cells) and 40 nM (MKN74, NUGC3 and AGS cells) and Lipofectamine RNAiMAX at a final concentration of 0.2% (v/v), in 0.5 ml DMEM without serum. The transfection medium was then incubated at room temperature for 30 minutes. SNU-1 cells were centrifuged and resuspended in medium supplemented with 2% foetal calf serum at $25x10^4$ cells/ml. MKN74, NUGC3 and AGS cells grown in maintenance medium were detached using trypsin:EDTA and resuspended in medium supplemented with 2%, 10% and 2% foetal calf serum at $25x10^4$ cells/ml. Two ml of the cell suspension were mixed with 0.5 ml of the transfection medium, containing scrambled or IGF-IR targeted sequences, inside the wells of 6 well plates. After 72 hours, cells were incubated with 0.03 mg/ml bromodeoxyuridine for 2 hours. Then the cells were fixed in 4% paraformaldehyde. Fixed cells were incubated with a fluorescent antibody against BrdU (1:1400). The secondary antibody was Alexa-Fluor conjugated. Cells were mounted on top of slides in DAPI X mounting medium. Pictures of the cells were taken in a Leica fluorescent microscope (**A**). Quantification of the number of cells with positive staining was done in five fields of view for each treatment. The results are shown as a percentage of the total number of cells. Bars, SEM. Asterisks indicate that the proportion of cells in S phase is significantly lower after transfection with the si IGF-IR2 than after transfection with the scrambled oligonucleotide. Unpaired t-test; S-phase: SNU-1, p=0.0078; MKN74, p=0.0001; NUGC3, p=0.0001; AGS, p=0.0004) (**B**).

Phosphorylation of histone H3 at Ser10 is tightly correlated with chromosome condensation during mitosis (Goto *et al.*, 1999). To test the effect of decreased IGF-IR expression on mitosis, SNU-1, MKN74, NUGC3 and AGS cells were transfected with siRNA 2, which targets the type I IGF receptor or with a scrambled sequence and cultured in medium

supplemented with 2-10% foetal calf serum for 72 hours. The cells were fixed and incubated with an antibody that detects phosphorylated histone H3 at Ser10.

Approximately 4% of SNU-1 cells that had been transfected with a scrambled sequence were undergoing mitosis (Figure 5.11). Decreased IGF-IR expression resulted in a significant reduction of the percentage of the actively dividing cells to 1.5%. Similar results were obtained with the remaining three cell lines, with knockdown of the type I IGF receptor resulting in a significant reduction of the number of cells undergoing mitosis from 2.8 to 0.8% for MKN74 cells, from 6 to 2% for NUGC3 cells and from 2.4 to 1.1% for AGS cells.

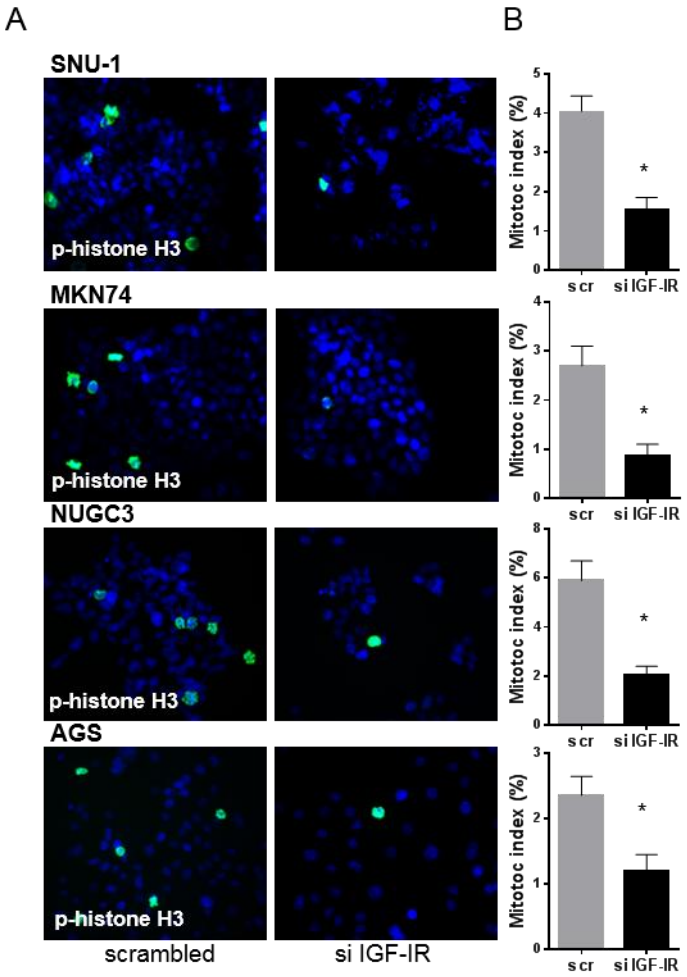

Figure 5.11. The effect of knockdown of the IGF-IR with siRNA on mitosis in SNU-1, MKN74, NUGC3 and AGS cells in full medium. Transfection medium was prepared by mixing either scrambled (scr) or IGF-IR targeted (IGF-IR) siRNA duplexes at a final concentration of 50 nM (SNU-1 cells) and 40 nM (MKN74, NUGC3 and AGS cells) and Lipofectamine RNAiMAX at a final concentration of 0.2% (v/v), in 0.5 ml DMEM without serum. The transfection medium was then incubated at room temperature for 30 minutes. SNU-1 cells were centrifuged and resuspended in medium supplemented with 2% foetal calf serum at $25x10^4$ cells/ml. MKN74, NUGC3 and AGS cells grown in maintenance medium were detached using trypsin:EDTA and resuspended in medium supplemented with 2%, 10% and 2% foetal calf serum at $25x10^4$ cells/ml. Two ml of the cell suspension were mixed with 0.5 ml of the transfection medium, containing scrambled or IGF-IR targeted sequences, inside the wells of 6 well plates. After 72 hours, cells were fixed in 4% paraformaldehyde. Fixed cells were incubated with a fluorescent antibody against phosphorylated histone H3 (1:100 dilution). The secondary antibody was Alexa-Fluor conjugated. Cells were mounted on top of slides in DAPI X mountant medium.

Pictures of the cells were taken in a Leica fluorescent microscope (**A**). Quantification of the number of cells with positive staining was done in five fields of view for each treatment. The results are shown as a percentage of the total number of cells. Bars, SEM. Asterisks indicate that the proportion of cells in mitosis is significantly lower after transfection with the si IGF-IR2 than after transfection with the scrambled oligonucleotide. (Unpaired t-test; SNU-1, p=0.0009; MKN74, p=0.0046; NUGC3, p=0.0023; AGS, p=0.017) (**B**).

Decreased IGF-IR expression resulted in a reduction of the number of cells in S phase and in mitosis in all four cell lines tested here. This suggests that activation of the type I IGF receptor transmits signals that trigger both DNA synthesis and mitosis.

5.2.7 Effect of IGF-IR/IR inhibition by BMS754807 on cell proliferation in a growth factor enriched environment

As mentioned previously, reduction of IGF-IR expression with siRNA knockdown resulted in decreased proliferation rates in SNU-1, MKN74, NUGC3 and AGS cells. We, therefore, tested if the small molecule tyrosine kinase inhibitor, BMS754807, which targets both the type I IGF receptor and the insulin receptor would inhibit proliferation. SNU-1, MKN74, NUGC3 and AGS cells were cultured in medium supplemented with 10% foetal calf serum and various concentrations of BMS754807, ranging from 0 to 10 μM for 3 days. The cells were lysed and the amount of DNA present in each sample was measured with the PicoGreen fluorimetric assay.

In SNU-1 cells, the amount of DNA was reduced by BMS754807 in a concentration dependent manner (Figure 5.12). Treatment with 10 μM BMS754807 reduced the number of cells by 60%. The concentration of BMS754807 required to reduce the number of cells by 50% (IC_{50}) was approximately 2 μM. A similar profile in response to BMS754807 was observed also in MKN74 and NUGC3 cells, with IC_{50} close to 2 μM. AGS cells were slightly more sensitive to BMS754807 compared to the other cells lines, as 10 μM of the inhibitor reduced the amount of cells by 80%. The IC_{50} of the BMS754807 in this cell line was approximately 0.8 μM (Figure 5.12).

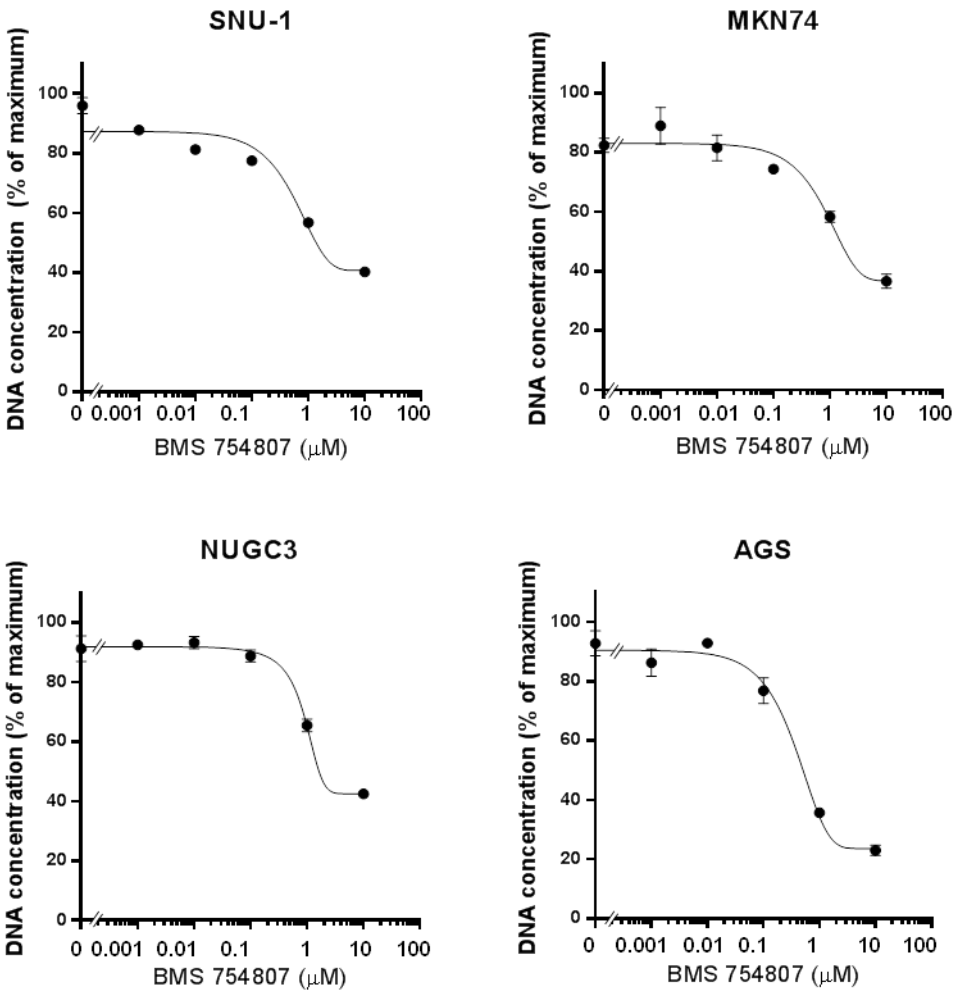

Figure 5.12. The effect of treatment with different concentrations of BMS754807 on cell number. SNU-1, MKN74, NUGC3 and AGS cells were seeded into 24 well tissue culture plates at $1x10^4$ cells per well, in 0.5 ml maintenance medium. Adherent cells were incubated for 24 hours to allow attachment. Medium was replaced to maintenance medium which contained DMSO, 0.001, 0.01, 0.1, 1 or 10 μM BMS754807. After 3 days, medium was aspirated and the cells washed with 0.5 ml ice cold PBS. PBS was aspirated and the cells stored in -20°C. The cells were lysed in SSC/SDS solution and the amount of DNA in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM.

To test the effect of the BMS754807 inhibitor overtime, SNU-1, MKN74, NUGC3 and AGS cells were cultured in medium supplemented with 10% foetal calf serum in the presence or absence of 5 μM BMS754807 for 1 to 5 days (Figure 5.13). In the absence of BMS754807, SNU-1 cells proliferated quite fast and doubled their number from day 2 to day 3. In the presence of BMS754807, their growth rates were reduced significantly and there was a 60% reduction in cell number on day 3 compared to untreated cells. The growth of MKN74 cells was inhibited completely by BMS754807. In NUGC3 cells, treatment with 5 μM BMS754807 resulted in a reduction of cell number by 50% after 3 days. In AGS cells, growth was completely abolished after treatment with 5 μM BMS754807.

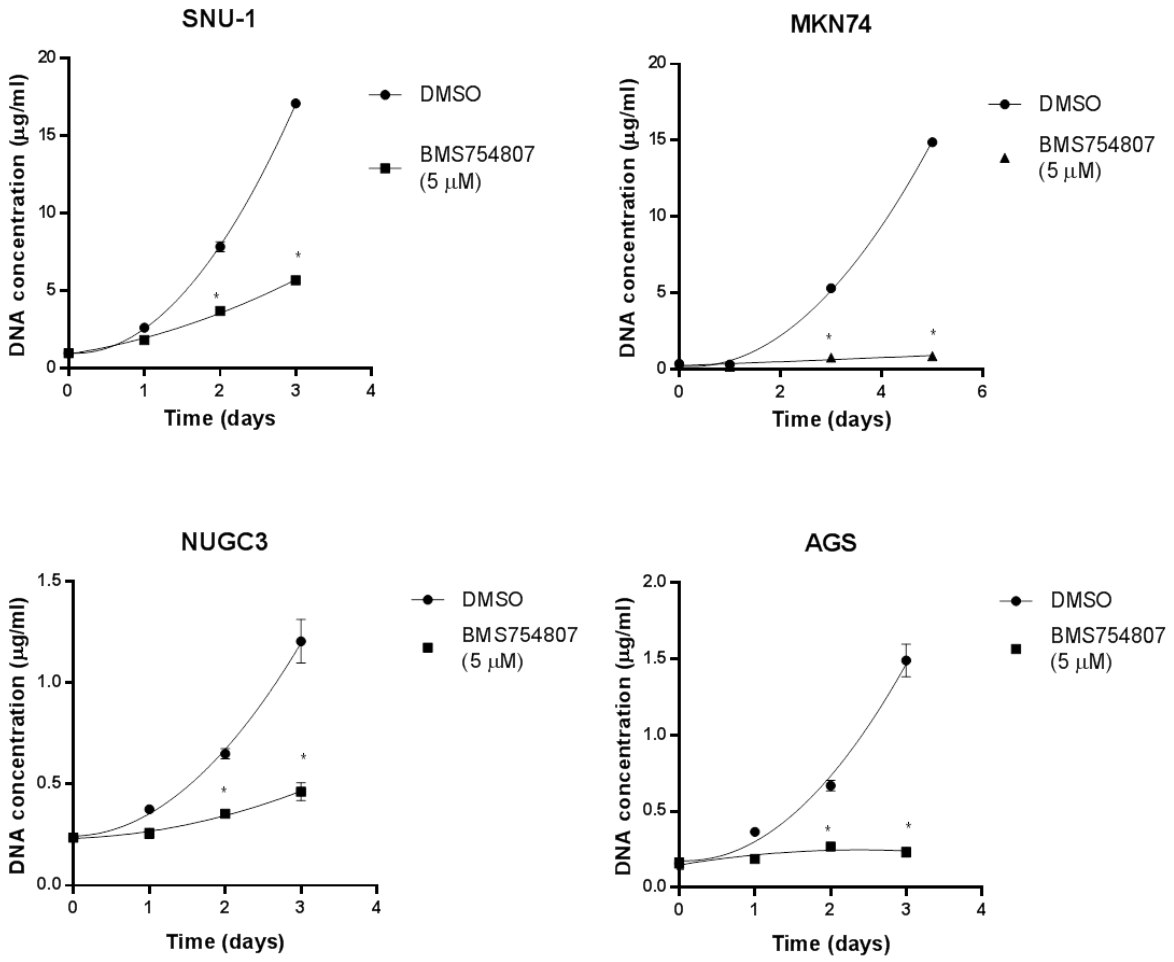

Figure 5.13. The effect of BMS754807 on proliferation in SNU-1, MKN74 and NUGC3 cells cultured in maintenance medium. MKN74, NUGC3 and AGS cells were seeded into 24 well tissue culture plates at $1x10^4$ cells per well, in 0.5 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was replaced to maintenance medium which contained DMSO, 0.5 µM or 5 µM BMS754807. SNU-1 cells were seeded into 24 well tissue culture plates at $1x10^4$ cells per well, in 0.5 ml maintenance medium which contained DMSO, 0.5 µM or 5 µM BMS754807. Medium was replaced every 3 days. After 0, 1, 2, and 3 days (SNU-1, NUGC3 and AGS cells) and 0, 1, 3 and 5 days (MKN74 cells) medium was aspirated and the cells washed with 0.5 ml ice cold PBS. PBS was aspirated and the cells stored in -20^{o}C. The cells were lysed in SSC/SDS solution and the amount of DNA in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM, * denotes statistically significantly lower DNA content in the presence of BMS754807 compared with in the absence of BMS754807 (Two-way ANOVA; SNU-1, $p<0.0001$; MKN74, $p<0.0001$; NUGC3, $p\leq0.0002$; AGS, $p<0.0001$).

The above results suggest that BMS754807 effectively inhibits growth of gastric cancer cells, even in an environment enriched with growth factors. This underlines the importance of the type I IGF receptor and possibly the insulin receptor as mediators of mitogenic stimuli.

5.3 Discussion

The aim of the results reported in this chapter was to investigate the proliferative effect of the IGF signal transduction pathway and to evaluate the potential of blocking this proliferative effect in gastric cancer cells. IGF-1 increased effectively the proliferation of MKN74 and NUGC3 cells, in the absence of other growth factors. The proliferative effect of IGF-1 was reduced significantly in NUGC3 cells when expression of the type I IGF receptor was reduced and with an inhibitor against MEK1 and MEK2. Our demonstration that ERK1 and ERK2 activation is essential for the induction of the IGF-1 proliferative effect suggests distinct roles for Akt and ERK1 and ERK2 activation in response to IGF-1. Activation of the PI3K/Akt, and not the Raf/MAPK pathway, is necessary for the induction of the IGF-1 survival effect (chapter 4), whereas ERK1 and ERK2 phosphorylation is necessary for mediating the IGF-1 proliferative effect. Further, our results suggest that the type I IGF receptor is the main mediator of the IGF-1 proliferative effect.

The importance of the type I IGF receptor in the growth of four gastric cancer cell lines without HER-2, FGFR2 and c-Met amplification, cultured in the presence of growth factors, was demonstrated by significant reduction in cell proliferation after knockdown of the type I IGF receptor. In particular, SNU-1 cells were dependent on the type I IGF receptor for proliferation, as knockdown of the receptor resulted in cessation of growth in those cells. Previously, reduced colony formation has been shown after inhibition of the type I IGF receptor with shRNA and the αIR3 antibody for MET-amplified MKN45 cells and tumour explants, respectively (Pavelic *et al.*, 2003; Wang *et al.*, 2010a). Infection with an adenovirus that expresses truncated IGF-IR reduced proliferation of MKN74 cells, as well as two other gastric cancer cell lines, NUGC4 and the MET-amplified cell line MKN45 (Min *et al.*, 2005).

We confirmed that the reduction in cell growth after knockdown of the type I IGF receptor in SNU-1, MKN74, NUGC3 and AGS cells was reflected in reduced proportion of cells in the S-phase and mitotic-phase of the cell cycle. Therefore, rather than inducing cell death, inhibition of IGF-IR expression resulted in reduced cell proliferation by inhibiting progression through the cell cycle. This is supported by a previous study in which induction of cyclin D1 synthesis, DNA synthesis and cell division was detected in MCF-7 breast cancer cells after treatment with IGF-1 (Dufourny *et al.*, 1997).

IGF-IR knockdown resulted in reduced phosphorylation of Akt in SNU-1 and MKN74 cells and reduced phosphorylation of ERK1 and ERK2 in SNU-1, MKN74 and NUGC3 cells. This suggests that even in the presence of other growth factors and activated receptors, the type I

IGF receptor is primarily important for stimulating Akt and ERK1 and ERK2 and, consequently, cell survival and proliferation. The reduction of phosphorylated Akt was accompanied by an accumulation of total Akt, in cells with decreased IGF-IR expression. On the contrary, reduction of phosphorylated ERK2 levels resulted in a reduction of total ERK2 levels. In MKN74 cells, inhibition of ERK2 phosphorylation preceded the reduction of total ERK2 levels. This suggests that there is a negative feedback loop that reduces the expression of ERK2 in cells in which ERK2 phosphorylation is inhibited. There was a clear increase in IRS-1 levels in SNU-1 and MKN74 cells with reduced IGF-IR expression 96 hours after siRNA transfection. Due to the lack of good quality commercially available antibodies for phosphorylated IRS-1, it was not possible to investigate differences in IRS-1 phosphorylation after IGF-IR knockdown. It could be hypothesised that accumulation of total IRS-1 is a result of reduction in IRS-1 phosphorylation after loss of IGF-IR activation.

Treatment with the BMS754807 inhibitor, which prevents phosphorylation of the type IGF and insulin receptors, inhibited the growth of SNU-1, MKN74, NUGC3 and AGS cells. Inhibition of cell growth with BMS754807, evaluated by ^{3}H-thymidine incorporation into the newly synthesised DNA, has been reported in Ewing's sarcoma, rhabdomyosarcoma, neuroblastoma, liposarcoma, breast, lung, pancreatic, colon cancer, multiple myeloma and leukaemia cell lines (Carboni *et al.*, 2009).

According to experts in the field, small-molecule tyrosine kinase inhibitors, such as BMS754807, that target both IGF1R and the insulin receptor would yield better results in the clinic compared to monoclonal antibodies against the type I IGF receptor, as they would prevent interaction of IGFs with the insulin receptor. However, Bristol-Myers Squibb stopped enrolment into trials of BMS754807 due to disappointing phase III trials (Guha, 2013). The negative results obtained from phase III trials suggest that the establishment of predictive biomarkers is essential to select patients more likely to respond to treatment. Also, the combination of IGF-IR and IR targeted inhibitors with inhibitors used in the clinic should be tested preclinically. Furthermore, some cancer cells are driven by other receptors to such an extent that insulin and IGF signalling become less relevant and these cells would also be predicted to be resistant (Pollak, 2012).

In summary, the results reported in this chapter suggest that the type I IGF receptor is important for cell proliferation in "triple negative" gastric cancer cells. Mediation of the proliferative effect by the type I IGF receptor requires activation of the Raf/ERK pathway. Inhibition of the type I IGF receptor results in reduction of the number of cells in the S-phase

and mitotic-phase of the cell cycle. Finally, BMS754807 inhibits effectively the growth of “triple negative“ gastric cancer cells.

Chapter 6. Responsiveness of Gastric Cancer Cells to IGF-2 and Insulin and the Role of Type I IGF and Insulin Receptors in Survival and Proliferation

6.1 Introduction

Activation of the type I IGF receptor by IGF-1 and IGF-2 has been considered the main mechanism of stimulation of downstream IGF signal transduction pathways involved in cell survival and proliferation, whereas signalling through the insulin receptor has been linked traditionally to regulation of metabolic processes. However, there is a considerable amount of preclinical data which indicate that signalling through the insulin receptor could be more involved in cancer progression than thought previously.

Two isoforms of the insulin receptor are generated by alternative splicing of exon 11. The form that contains 12 amino acids encoded by exon 11 is called the B-isoform (IR-B) and the form that lacks the 12 amino acids encoded by exon 11 is called the A-isoform (IR-A) (Seino and Bell, 1989). IR-A binds insulin with the highest affinity (K_d=0.25 nM), followed by IGF-2 (K_d=2 nM) and IGF-1 (K_d=10 nM) (Westley and May, 2013). It is overexpressed in several human cancers and it has been proposed that its overexpression provides a mechanism for over-activation of the IGF signal transduction pathway in cancer (Belfiore, 2007). Another way in which the insulin receptor can contribute to IGF-1 and IGF-2 signal transduction is by the formation of hybrid receptors assembled with one α and β chain of the IGF-IR and one α and β chain of the IR-A or IR-B (Pandini *et al.*, 1999; Frasca *et al.*, 2003). These hybrid receptors bind IGF-1 and IGF-2 with very high affinity (K_d=0.3-0.7 nM) and insulin with lower affinity (K_d=70-80 nM). Hybrid receptors are thought to contribute to downstream signalling that leads to cell survival and proliferation.

Insulin has been shown to stimulate effectively growth of breast, lung and prostate cancer cells (Sciacca *et al.*, 2014). Both IGF-IR and isoform A of the insulin receptor mediated increased tumour growth in a mouse mammary model and it has been proposed that inhibition of both receptors may provide additional efficacy compared to inhibition of IGF-1R alone (Buck *et al.*, 2010). Downregulation of the insulin receptor with shRNA reduced growth, angiogenesis, lymphangiogenesis and metastasis of LCC6 breast xenograft tumours in mice (Zhang *et al.*, 2010). The above results from preclinical studies in combination with negative results from clinical studies of monoclonal antibodies which inhibit the IGF-IR selectively

(King and Wong, 2012; Langer *et al.*, 2014), suggest that there is a rationale for co-targeting the type I IGF and insulin receptor in cancer.

6.1.1 Aims

The aim of the results reported in this chapter is to investigate the biological effect of interactions between IGF-2 and insulin with the type I IGF and insulin receptors in gastric cancer cells. Activation of the receptors and downstream molecules by IGF-2 and insulin was investigated. The effect of IGF-2 and insulin on cell survival and proliferation was also analysed. The importance of the type I IGF and insulin receptors in gastric cancer cell survival was investigated by transient silencing of the two receptors with a short interfering RNA sequence and by tyrosine kinase inhibition with the small molecule inhibitor BMS754807. Finally, expression of the two insulin receptor isoforms was measured in gastric cancer cell lines.

6.2 Results

6.2.1 The effect of IGF-2 on gastric cancer cells

6.2.1.1 The effect of different concentrations of IGF-2 on activation of IGF-signal transduction pathway proteins

IGF-2 is known to bind the type I IGF receptor with very high affinity (K_d=0.5 nM) but can also bind isoform A and isoform B of the insulin receptor with slightly lower affinities (K_d=2 and 10 nM, respectively).

To investigate the effect of stimulation with different concentrations of IGF-2 on downstream signalling, SNU-1 and NUGC3 cells were selected as representative cell lines. SNU-1 and NUGC3 cells have no HER-2, FGFR2 or c-Met amplification and they express relatively high amounts of the type I IGF and insulin receptors. Therefore, all possible interactions between the ligands and the receptors of the IGF signal transduction pathway may take place in those cells. SNU-1 and NUGC3 cells were withdrawn from the effects of growth factors by culture in withdrawal medium for 2 days and, then, treated with different concentrations of IGF-2 up to 50 $ngml^{-1}$ for 15 minutes prior to protein lysis.

Induction of IGF-IR and IR phosphorylation was detected in SNU-1 cells, after stimulation with 20 ng/ml IGF-2 or higher (Figure 6.1). Maximal phosphorylation was achieved with 50 ng/ml IGF-2. Phosphorylated IGF-IR and IR were detected in NUGC3 cells also after treatment with 20 ng/ml IGF-2 or higher. Again, the maximal levels of phosphorylation were achieved with 50 ng/ml IGF-2. The levels of total IGF-IR and IR remained unchanged after stimulation with IGF-2.

Akt stimulation was detected in SNU-1 cells after treatment with 20 ng/ml IGF-2, whereas a higher IGF-2 concentration did not increase phosphorylated Akt levels (Figure 6.1). In NUGC3 cells some Akt phosphorylation was detected in the absence of IGF-2, but it increased after IGF-2 stimulation in a concentration-dependent manner. Maximal Akt phosphorylation was reached with 20 and 50 ng/ml IGF-2. Phosphorylated ERK1 and 2 was detected in both cell lines in the absence of IGF-2 and there was a concentration-dependent increase after stimulation with IGF-2, only in NUGC3 cells. Maximal phosphorylation was achieved with 50 ng/ml IGF-2 in NUGC3 cells. In SNU-1 cells, IGF-2 did not increase ERK1 and 2 phosphorylation at any concentration. Total Akt and ERK1 and 2 levels were not affected by stimulation with IGF-2.

Stimulation of SNU-1 and NUGC3 cells with IGF-2 resulted in phosphorylation of the type I IGF and/or insulin receptor and activation of downstream pathways. Detectable phosphorylation of the type I IGF and/or insulin receptor required higher IGF-2 concentrations compared to IGF-1 concentrations in SNU-1 and NUGC3 cells (20 ng/ml vs 2-5 ng/ml, see chapter 3). Also, in SNU-1 cells higher IGF-2 concentrations were required to induce Akt phosphorylation compared to IGF-1 (20 ng/ml vs 2 ng/ml, chapter 3).

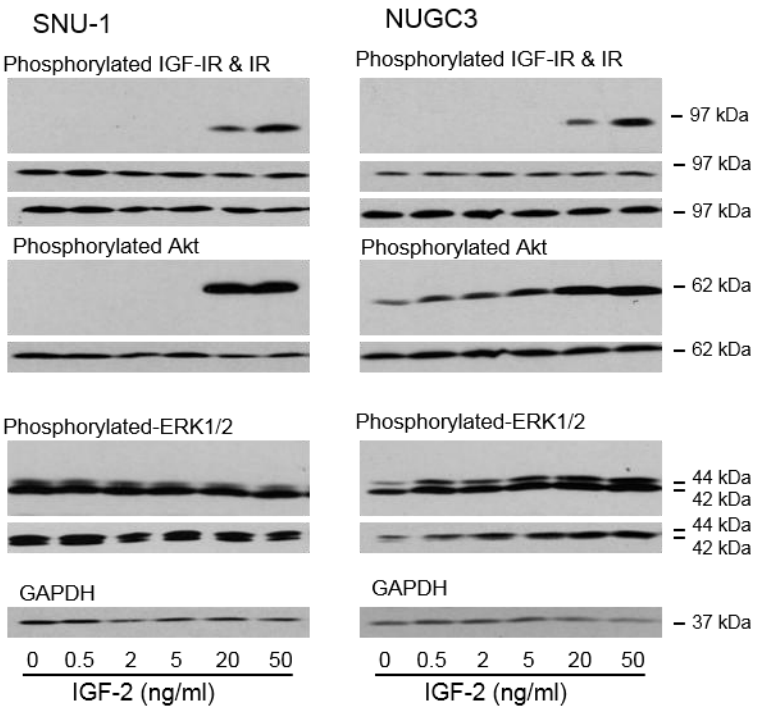

Figure 6.1. The effect of stimulation with different concentrations of IGF-2 on phosphorylation of IGF signal transduction proteins in SNU-1 and NUGC3 cells. SNU-1 cells were centrifuged and resuspended in DCCS medium and seeded into a 75 cm^2 flask at a concentration of 0.9 x 10^6 cells per flask. After 24 hours, medium was replaced with 12 ml fresh DCCS medium for another 24 hours. Cells were centrifuged and resuspended in serum-free medium with 0.1% BSA for 2 hours. Cells were then centrifuged and resuspended in serum-free medium with 0.1% BSA and 0, 0.5, 2, 5, 20, 50 ng/ml IGF-2 for 15 minutes. Cells were collected in a 15 ml Flacon tube and proteins were lysed with 50 µl RIPA buffer plus inhibitors. NUGC3 cells were seeded into 12 well tissue culture plates at 15x10^4 cells/well, in 1 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 0.5 ml PBS. Medium was replaced daily with 1 ml DCCS medium for two days. Cells were incubated with serum-free medium which contained 0.1% BSA for two hours. Medium was changed to include IGF-2 at final concentrations of 0, 0.5, 2, 5, 20, 50 ng/ml. After incubation for 15 minutes proteins were extracted with 80 µl per well RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against phosphorylated IGF-IR/IR (1:1000 dilution), IGF-IR (1:2000 dilution), IR (1:1000 dilution), phosphorylated Akt (1:3000 dilution), Akt (1:3000 dilution), phosphorylated ERK1/2 (1:5000 dilution), ERK1/2 (1:5000 dilution) and GAPDH (1:10000 dilution). Images shown are representative of results from one triplicate experiment. Images obtained after incubation with antibodies against the total proteins are shown beneath those of the phosphorylated proteins. Total insulin receptor is shown below the total type I IGF receptor.

6.2.1.2 The effect of IGF-2 on survival of gastric cancer cells

To investigate whether IGF-2 can protect against staurosporine-induced apoptosis, SNU-1 and NUGC3 cells were withdrawn from the effects of growth factors and treated with concentrations of IGF-2 ranging from 2 to 200 ng/ml in the presence of 0.5 µM staurosporine. Treatment with staurosporine resulted in PARP cleavage in SNU-1 cells. The amount of cleaved PARP was reduced by IGF-2 in a concentration-dependent manner (Figure 6.2). Best protection against cell death was achieved with 50 and 200 ng/ml IGF-2. In NUGC3 cells, treatment with staurosporine also resulted in PARP cleavage. IGF-2 reduced the amount of

cleaved PARP in a concentration-dependent manner with maximal protection achieved with 200 ng/ml.

Higher concentration of IGF-2 were required to reduce PARP cleavage in SNU-1 and NUGC3 cells compared to IGF-1 (SNU-1; 20 vs 5 ng/ml, NUGC3; 50 vs 20 ng/ml, see chapter 3). The above results suggest that IGF-2 can protect gastric cancer cells against cell death caused by tyrosine kinase inhibition.

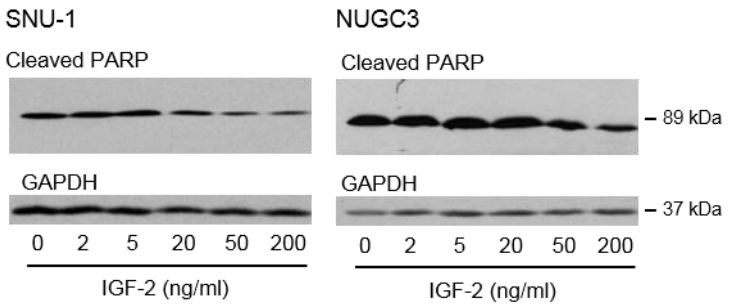

Figure 6.2. The protective effect of different concentrations of IGF-2 against staurosporine-induced apoptosis in SNU-1 and NUGC3 cells. SNU-1 cells were centrifuged and resuspended in DCCS medium and seeded into a 75 cm^2 flask at a concentration of 0.9 x 10^6 cells per flask. After 24 hours, medium was replaced with 12 ml fresh DCCS medium for another 24 hours. Cells were centrifuged and resuspended in DCCS medium supplemented or not with different concentrations of IGF-2 for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of IGF-2 for 4 hours. Cells were collected in 15 ml Falcon tubes and lysed in 50 µl RIPA buffer plus inhibitors. NUGC3 cells were seeded into 12 well tissue culture plates at 15 x 10^4 cells per well, in 1 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 0.5 ml PBS. Medium was replaced daily with 1 ml DCCS medium for two days. Cells were incubated with DCCS medium supplemented or not with different concentrations of IGF-2 for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of IGF-2 for 5 hours. Proteins were extracted with 80 µl of RIPA buffer plus inhibitors per well. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against cleaved PARP (1:2000 dilution) and GAPDH (1:10000 dilution). Densitometry was with Labworks 4.0 software, adjusted for the background optical density of the X-ray film and normalised to GAPDH.

6.2.2 The effect of insulin on gastric cancer cells

6.2.2.1 The effect of different concentrations of insulin on activation of IGF-signal transduction pathway proteins

Insulin binds the insulin receptor with very high affinity (K_d=0.25-0.5 nM) and it has a much lower affinity for the type I IGF receptor (K_d=100 nM). However, it has an intermediate affinity for hybrid receptors formed by the assembly of one α and β chain of the IGF-IR and one α and β chain of the IR-A or IR-B (K_d=70-80 nM) (Westley and May, 2013).

The effect of stimulation with different concentrations of insulin on downstream signalling was investigated in SNU-1 and NUGC3 cells. SNU-1 and NUGC3 cells were withdrawn from

the effects of growth factors by culture in withdrawal medium for 2 days and, then, treated with different concentrations of insulin ranging from 2 to 50 $ngml^{-1}$ for 15 minutes prior to protein lysis.

Induction of IGF-IR and IR phosphorylation was detected in SNU-1 cells, after stimulation with 5 ng/ml insulin or higher (Figure 6.3). Maximal phosphorylation was achieved with 50 ng/ml. Phosphorylated IGF-IR and IR were detected in NUGC3 cells after treatment with 2 ng/ml insulin or higher. Again, the maximal levels of phosphorylation were achieved with 50 ng/ml insulin. The levels of total IGF-IR and IR remained unchanged after stimulation with insulin.

Akt phosphorylation was detected in SNU-1 cells after treatment with 5 ng/ml insulin, and maximal phosphorylation was achieved with 50 ng/ml (Figure 6.3). In NUGC3 cells, some levels of Akt phosphorylation were detected in the absence of insulin but increased after insulin stimulation in a concentration-dependent manner. Maximal Akt phosphorylation was reached with 20 and 50 ng/ml insulin. Phosphorylated ERK1 and 2 were detected in both cell lines in the absence of insulin and there was a concentration-dependent increase after stimulation with insulin, only in NUGC3 cells. Maximal phosphorylation was achieved with 20 and 50 ng/ml in NUGC3 cells. In SNU-1 cells, insulin did not increase ERK1 and 2 phosphorylation at any concentration. Total Akt and ERK1 and 2 levels were not affected by stimulation with insulin.

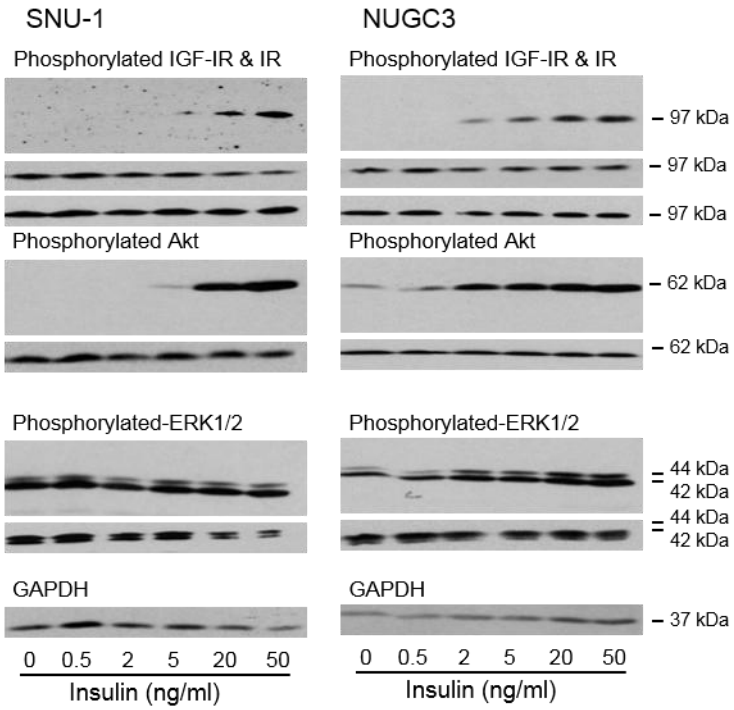

Figure 6.3. The effect of stimulation with different concentrations of insulin on phosphorylation of IGF signal transduction proteins in SNU-1 and NUGC3 cells. SNU-1 cells were centrifuged and resuspended in DCCS medium and seeded into a 75 cm^2 flask at a concentration of $0.9x10^6$ cells per flask. After 24 hours, medium was replaced with 12 ml fresh DCCS medium for another 24 hours. Cells were centrifuged and resuspended in serum-free medium with 0.1% BSA for 2 hours. Cells were then centrifuged and resuspended in serum-free medium with 0.1% BSA and 0, 0.5, 2, 5, 20, 50 ng/ml insulin for 15 minutes. Cells were collected in a 15 ml Falcon tube and proteins were lysed with 50 µl RIPA buffer plus inhibitors. NUGC3 cells were seeded into 12 well tissue culture plates at $15x10^4$ cells/well, in 1 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 0.5 ml PBS. Medium was replaced daily with 1 ml DCCS medium for two days. Cells were incubated with serum-free medium which contained 0.1% BSA for two hours. Medium was changed to include insulin at final concentrations of 0, 0.5, 2, 5, 20, 50 ng/ml. After incubation for 15 minutes proteins were extracted with 80 µl per well RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against phosphorylated IGF-IR/IR (1:1000 dilution), IGF-IR (1:2000 dilution), phosphorylated Akt (1:3000 dilution), Akt (1:3000 dilution), phosphorylated ERK1/2 (1:5000 dilution), ERK1/2 (1:5000 dilution) and GAPDH (1:10000 dilution). Images shown are representative of results from one triplicate experiment. Images obtained after incubation with antibodies against the total proteins are shown beneath those of the phosphorylated proteins. Total insulin receptor is shown below the total type I IGF receptor.

Stimulation of both SNU-1 and NUGC3 cells with insulin resulted in phosphorylation of the type I IGF and insulin receptors and activation of the PI3K/Akt pathway in SNU-1 and NUGC3 cells and the Ras/Raf/MAPK pathway in NUGC3 cells. Phosphorylation of the type I IGF and/or insulin receptor in SNU-1 and NUGC3 cells was detected with similar concentrations of insulin and IGF-1 (SNU-1 cells; 5 ng/ml, NUGC3 cells; 2 ng/ml, see chapter 3). Also, a slightly higher concentration of insulin was required to induce Akt phosphorylation in SNU-1 cells compared to IGF-1 (5 ng/ml vs 2 ng/ml, chapter 3).

6.2.2.2 The effect of insulin on survival of gastric cancer cells

The effect of insulin on staurosporine-induced apoptosis was investigated. SNU-1 and NUGC3 cells were withdrawn from the effects of growth factors and treated with concentrations of insulin ranging from 2 to 200 ng/ml in the presence of 0.5 µM staurosporine. Cleaved PARP was detected in SNU-1 cells treated with staurosporine. The amount of cleaved PARP was reduced by insulin in a concentration-dependent manner (Figure 6.4). Highest protection against cell death was achieved with 200 ng/ml. In NUGC3 cells, treatment with staurosporine also resulted in cleavage of PARP into its 89 kDa fragment. Insulin reduced the amount of cleaved PARP in a concentration-dependent manner with maximal protection achieved with 200 ng/ml.

The above results suggest that, like IGF-1 and IGF-2, insulin can also afford protection against cell death induced by tyrosine kinase inhibition.

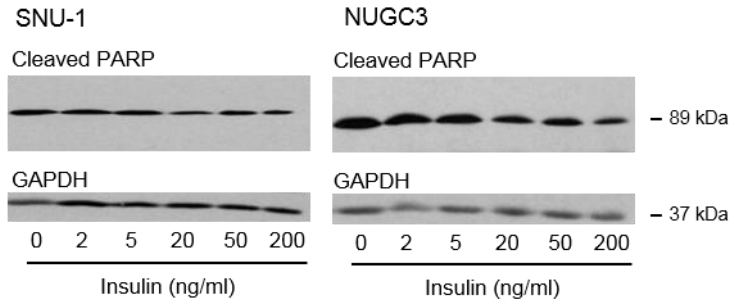

Figure 6.4. The protective effect of different concentrations of insulin against staurosporine-induced apoptosis in SNU-1 and NUGC3 cells. SNU-1 cells were centrifuged and resuspended in DCCS medium and seeded into a 75 cm^2 flask at a concentration of $0.9x10^6$ cells per flask. After 24 hours, medium was replaced with 12 ml fresh DCCS medium for another 24 hours. Cells were centrifuged and resuspended in DCCS medium supplemented or not with different concentrations of insulin for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of insulin for 4 hours. Cells were collected in 15 ml Falcon tubes and lysed in 50 µl RIPA buffer plus inhibitors. NUGC3 cells were seeded into 12 well tissue culture plates at $15x10^4$ cells per well, in 1 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 0.5 ml PBS. Medium was replaced daily with 1 ml DCCS medium for two days. Cells were incubated with DCCS medium supplemented or not with different concentrations of insulin for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of insulin for 5 hours. Proteins were extracted with 80 µl of RIPA buffer plus inhibitors per well. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against cleaved PARP (1:2000 dilution) and GAPDH (1:10000 dilution). Densitometry was with Labworks 4.0 software, adjusted for the background optical density of the X-ray film and normalised to GAPDH.

6.2.3 The effect of siRNA knockdown of the type I IGF receptor on survival of gastric cancer cells in the presence of serum

Our results suggest that all three growth factors, IGF-1, IGF-2 and insulin, promote survival of gastric cancer cells. To determine which of the two receptors of the IGF signal transduction pathway is involved in mediating survival signals, expression of the type I IGF receptor was

reduced in SNU-1 and NUGC3 cells and the effect on cell survival was investigated, in culture medium enriched with growth factors. The levels of cleaved PARP were measured in SNU-1 and NUGC3 cells that had been transfected with the siRNA oligonucleotide against the type I IGF receptor and compared to the levels detected after transfection with scrambled oligonucleotide.

Expression of the type I IGF receptor was detected readily in SNU-1 cells transfected with the scrambled oligonucleotide (Figure 6.5). Following transfection with siRNA 2 against the type I IGF receptor, expression of the receptor was reduced to non-detectable levels. Low levels of cleaved PARP were detected in cells transfected with the scrambled oligonucleotide (Figure 6.5). Knockdown of the type I IGF receptor with siRNA 2 did not alter significantly the amount of PARP cleavage. In NUGC3 cells, siRNA 2 against the type I IGF receptor reduced also receptor expression to non-detectable levels (Figure 6.5). Reduction of type I IGF receptor expression was followed by a very small induction of PARP cleavage.

The above results suggest that signalling through the type I IGF receptor is not critical for the survival of SNU-1 cells when many ligands are available, whereas it might be slightly more important in mediation of survival in NUGC3 cells.

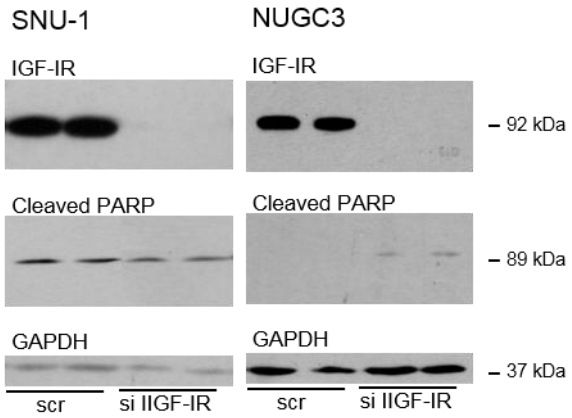

Figure 6.5. The effect of inhibition of the type I IGF receptor with siRNA knockdown on apoptosis in SNU-1 and NUGC3 cells cultured in maintenance medium. Transfection medium was prepared by mixing either scrambled (scr) or IGF-IR targeted (siIGF-IR) siRNA duplex 2 at final concentrations of 50 and 40 nM for SNU-1 and NUGC3 cells, respectively, and Lipofectamine RNAiMAX at a final concentration of 0.2% (v/v), in 0.25 ml DMEM without serum. The transfection medium was then incubated at room temperature for 30 minutes. SNU-1 cells were centrifuged and diluted in medium supplemented with 10% foetal calf serum at 10 x 10^4 cells/ml. NUGC3 cells grown in maintenance medium were detached using trypsin:EDTA and resuspended in maintenance medium at 10 x 10^4 cells /ml. One ml of the cell suspension was mixed with 0.25 ml of the transfection medium, containing scrambled or IGF-IR targeted sequences, in 12 well tissue culture plates. After incubation for 72 hours, protein extracts were prepared with 80 µl per well of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose for at 100 mA for 45 minutes. Membranes were incubated with antibodies against the IGF-IR (1:2000 dilution), cleaved PARP (1:2000) and GAPDH (1:10000 dilution).

6.2.4 The effect of siRNA knockdown of the insulin receptor on survival of gastric cancer cells in the presence of serum

The importance of the insulin receptor in cell survival was investigated by transient knockdown of the insulin receptor, in culture medium enriched with growth factors. The levels of cleaved PARP were measured in SNU-1 and NUGC3 cells that had been transfected with the siRNA oligonucleotides against the insulin receptor and compared to the levels detected after transfection with scrambled oligonucleotide.

Insulin receptor expression was detected readily in SNU-1 cells transfected with the scrambled oligonucleotide (Figure 6.6A). Following transfection with siRNA 2 against the insulin receptor, expression of the receptor was reduced to non-detectable levels. Transfection with siRNA 3 reduced insulin receptor expression by 60% (Figure 6.6B). Low levels of cleaved PARP were detected in cells transfected with the scrambled oligonucleotide (Figure 6.6A). Knockdown of the insulin receptor with siRNA 2 induced significantly PARP cleavage. There was no increase in the levels of cleaved PARP after transfection with siRNA 3 (Figure 6.6B), probably, due to inefficient knockdown of the insulin receptor with the above siRNA.

In NUGC3 cells, both siRNAs against the insulin receptor reduced insulin receptor expression to non-detectable levels (Figure 6.6A and B). Reduction of insulin receptor expression was followed by significantly increased PARP cleavage with both siRNAs (Figure 6.6A and B).

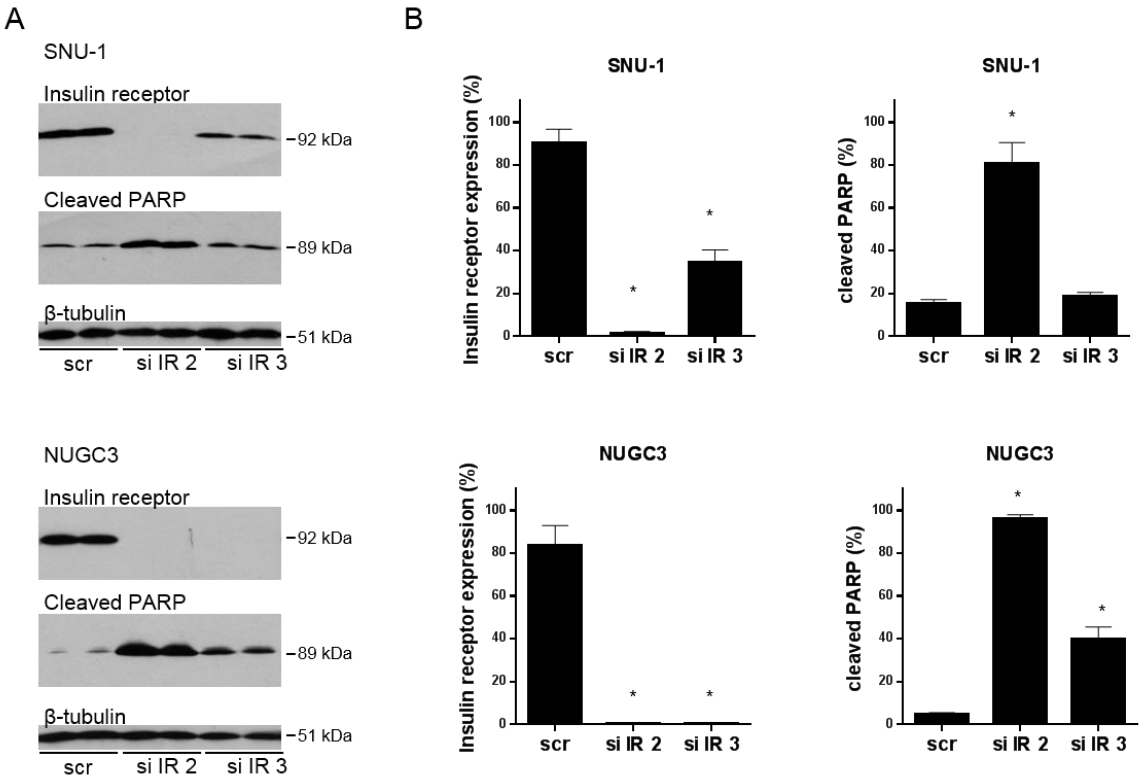

Figure 6.6. The effect of inhibition of the insulin receptor with siRNA knockdown on apoptosis in SNU-1 and NUGC3 cells cultured in maintenance medium. Transfection medium was prepared by mixing either scrambled (scr) or IR targeted (InsR) siRNA duplexes 2 and 3 at a final concentration of 50, 20 and 50 nM, respectively, and Lipofectamine RNAiMAX at a final concentration of 0.2% (v/v), in 0.25 ml DMEM without serum. The transfection medium was then incubated at room temperature for 30 minutes. SNU-1 cells were centrifuged and diluted in medium supplemented with 10% foetal calf serum at 10 x 10^4 cells/ml. NUGC3 cells grown in maintenance medium were detached using trypsin:EDTA and resuspended in maintenance medium at 10 x 10^4 cells /ml. One ml of the cell suspension was mixed with 0.25 ml of the transfection medium, containing scrambled or IR targeted sequences, in 12 well tissue culture plates. After incubation for 72 hours, protein extracts were prepared with 80 µl per well of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose for at 100 mA for 45 minutes. Membranes were incubated with antibodies against the IR (1:1000 dilution), cleaved PARP (1:2000) and GAPDH (1:10000 dilution) (**A**). Densitometry was with Labworks 4.0 software, adjusted for the background optical density of the X-ray film and normalised to β-tubulin. Results are expressed as a percentage of the intensity of the cleaved PARP band observed after transfection with siRNA 2. Bars are the mean ±SEM, * denotes statistically significantly lower insulin receptor expression and higher amount of cleaved PARP after transfection with the siRNA against insulin receptor (One-way ANOVA; SNU-1, IR, $p \leq 0.0005$, cleaved PARP, $p < 0.0001$; NUGC3, IR, $p < 0.0001$, cleaved PARP, $p \leq 0.0001$) (**B**).

The above results suggest that signalling through the insulin receptor is important for mediating survival signals and preventing apoptosis in SNU-1 and NUGC3 cells, even in the presence of other growth factors and, presumably, active signal transduction pathways.

6.2.5 The effect of concomitant knockdown of the type I IGF receptor and insulin receptor on survival of gastric cancer cells in the presence of serum

The relative importance of the type I IGF and insulin receptors in cell survival was investigated by reduction of the two receptors' expression separately and concomitantly, in medium supplemented with growth factors. NUGC3 cells were transfected with scrambled siRNA, with siRNA-2 against the type I IGF receptor (si-IGF-IR), with siRNA-2 against the insulin receptor (si-IR) and with a combination of si-IGF-IR 2 and si-IR 2. Cells were incubated with the transfection mix, containing the siRNA sequences and lipofectamine and cultured for 72 hours before protein lysis. Expression of the two receptors and cleavage of PARP were analysed by western transfer.

Both the type I IGF and insulin receptors were detected in cells that had been transfected with scrambled siRNA (Figure 6.7). No type I IGF receptor was detected in cells that had been transfected with si-IGF-IR 2. Very low levels of insulin receptor were detected in cells that had been transfected with si-IR 2. Combination of si-IGF-IR 2 and si-IR 2 resulted in effective knockdown of both receptors, simultaneously. Reduction in the expression of either receptor did not alter the expression levels of the other receptor.

There was no cleaved PARP in cells that had been transfected with scrambled siRNA. Reduced IGF-IR expression resulted in appearance of a small amount of the 89 kDa fragment of PARP (Figure 6.7). Reduced IR expression induced 4x higher levels of cleaved PARP compared to reduced IGF-IR expression. Finally, combination of the two siRNAs did not induce a significant increase in cell death, compared to transfection with the si-IR alone. These results suggest that the insulin receptor might be more important for cell survival than the type I IGF receptor in NUGC3 cells.

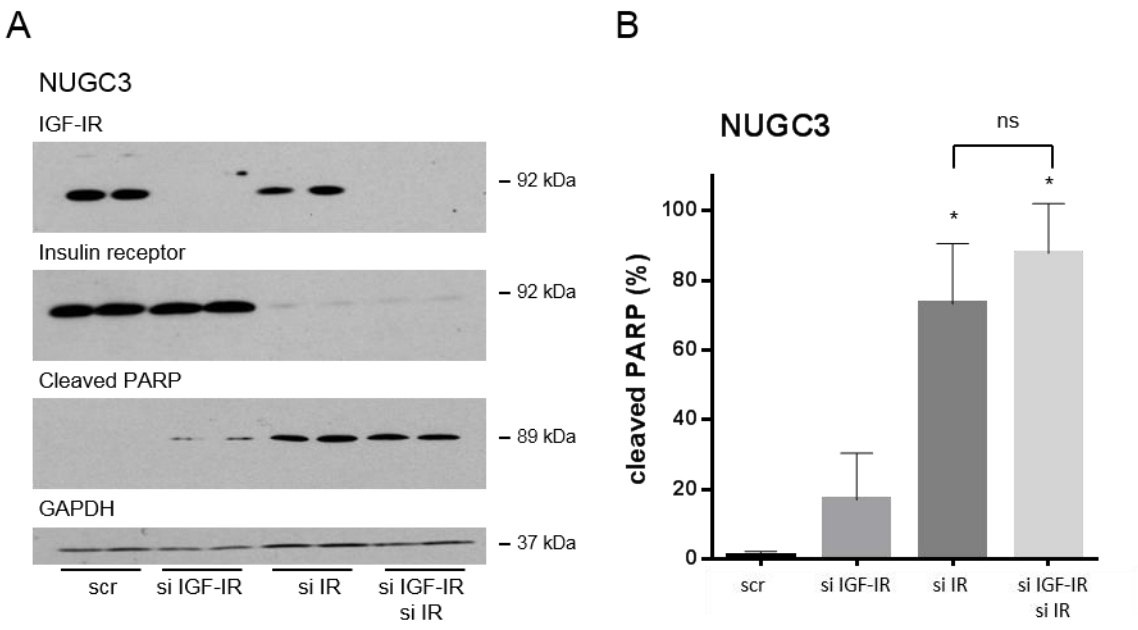

Figure 6.7. The effect of concomitant inhibition of the type I IGF and insulin receptors with siRNA knockdown on apoptosis in NUGC3 cells cultured in maintenance medium. Transfection medium was prepared by mixing scrambled (60 nM), IGF-IR targeted (40 nM), IR targeted (20 nM), or IGF-IR targeted plus IR targeted (40+20 nM) siRNA duplexes and Lipofectamine RNAiMAX at a final concentration of 0.2% (v/v), in 0.25 ml DMEM without serum. The transfection medium was then incubated at room temperature for 30 minutes. NUGC3 cells grown in maintenance medium were detached using trypsin:EDTA and resuspended in maintenance medium at 15 x 10^4 cells /ml. One ml of the cell suspension was mixed with 0.25 ml of the transfection medium, containing scrambled, IR or IGF-IR targeted sequences, in 12 well tissue culture plates. After incubation for 72 hours, protein extracts were prepared with 80 µl per well of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against the IGF-IR (1:2000 dilution), IR (1:1000), cleaved PARP (1:2000) and GAPDH (1:10000 dilution). The results shown are from an experiment that has been duplicated (**A**). Densitometry was with Labworks 4.0 software, adjusted for the background optical density of the X-ray film and normalised to GAPDH. Results are expressed as a percentage of the intensity of the cleaved PARP band observed after transfection with si IR and si IGF-IR. Bars are the mean ±SEM, * denotes statistically significantly higher amount of cleaved PARP after transfection with si IR (One-way ANOVA; p≤0.0027) (**B**).

6.2.6 The effect of treatment with BMS754807 in the presence of serum

The effect of treatment with the BMS754807 inhibitor, which targets both the type I IGF and insulin receptor, was investigated in SNU-1 and NUGC3 cells in the presence of growth factors. SNU-1 and NUGC3 cells were cultured with and without 0.5 µM BMS754807 in maintenance medium supplemented with 10% foetal calf serum for 3 days. A small amount of cleaved PARP was detected in SNU-1 cells after 3 days of culture in maintenance medium (Figure 6.8). Treatment with BMS754807 increased significantly the amount of cleaved PARP (p=0.001). Similarly, a relatively small amount of cleaved PARP was detected in NUGC3 cells after culture in medium supplemented with 10% foetal calf serum for 3 days (Figure 6.8). Treatment of cells with the BMS754807 inhibitor increased significantly the amount of cleaved PARP (p=0.0002).

The above results suggest that treatment with the BMS754807 in the presence of other growth factors and functioning pathways can affect viability of SNU-1 and NUGC3 cells. This result is in agreement with the data obtained from siRNA knockdown of the type I IGF and insulin receptors (Figure 6.7).

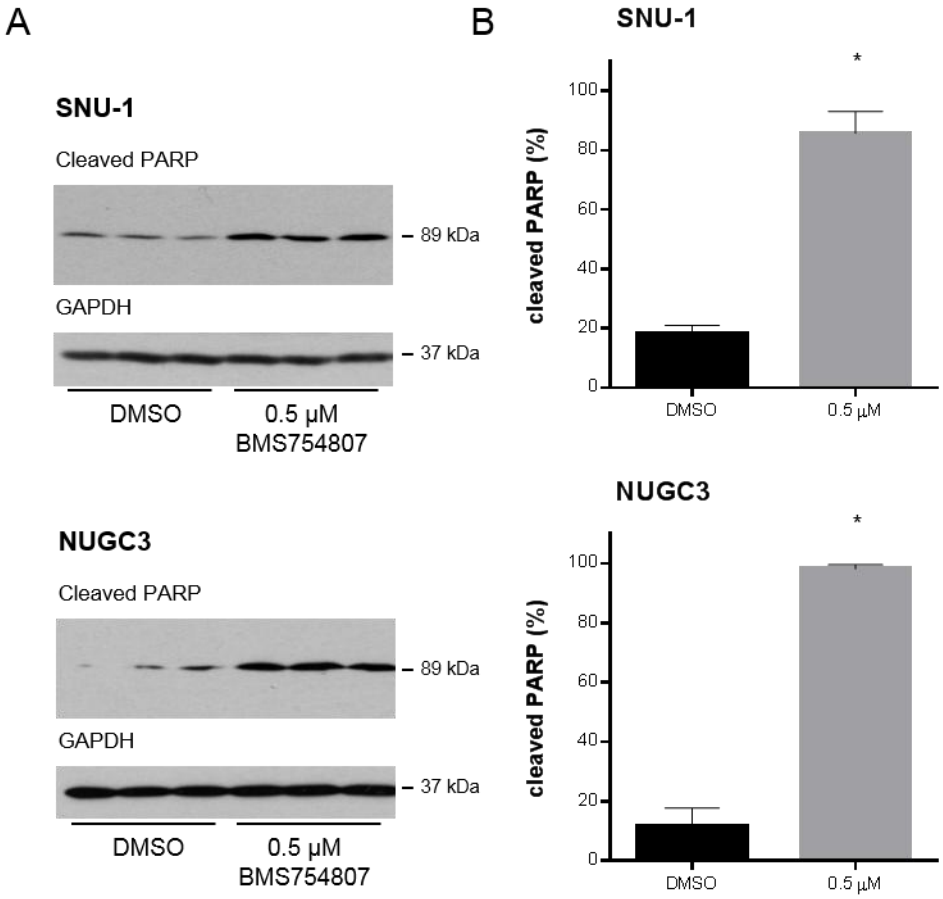

Figure 6.8. The effect of inhibition of IGF-IR/IR with BMS754807 on apoptosis in SNU-1 and NUGC3 cells cultured in maintenance medium. SNU-1 and NUGC3 cells were seeded into 12 well tissue culture plates at a concentration of 150000 cells/well, in 1 ml maintenance medium which contained DMSO, 0.5 µM or 5 µM BMS754807. The cells were left for 72 hours and protein extracts were prepared with 90 µl per well RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against cleaved PARP (1:2000 dilution) and GAPDH (1:10000 dilution) (**A**). Densitometry was with Labworks 4.0 software, adjusted for the background optical density of the X-ray film and normalised to GAPDH. Results are expressed as a percentage of the intensity of the cleaved PARP band observed in the presence of the BMS754807 inhibitor. Bars are the mean ±SEM, * denotes statistically significantly increased cleaved PARP levels in the presence of BMS54807 compared with in the absence of BMS754807 (Unpaired t-test; SNU-1, p=0.001; NUGC3, p=0.0002) (**B**).

6.2.7 The effect of IGF-2 on proliferation

So far our results suggest that IGF-2 and insulin mediate survival signals in SNU-1 and NUGC3 cells and the insulin receptor is more involved in mediation of survival signals than the type I IGF receptor. To investigate the effect of IGF-2 on gastric cancer cell growth,

NUGC3 cells were cultured in serum-free medium over several days, in the presence or absence of 50 ng/ml IGF-2. The amount of DNA in the presence and in the absence of IGF-2 was measured with the PicoGreen fluorimetric assay. The amount of DNA increased progressively in NUGC3 cells cultured in serum-free medium over the course of 9 days, when IGF-2 was not present (Figure 6.9). This could be attributed to the lack of withdrawal of NUGC3 cells from the effects of growth factors prior to treatment. When IGF-2 was present in the medium, the amount of DNA was increased at all time-points tested and this increase was statistically significant after 6 and 9 days of treatment. Importantly, in NUGC3 cells grown in serum-free medium, there was no significant increase in cell number from day 3 to day 6 (Two-way ANOVA; p=0.2714). However, in the presence of IGF-2, the number of cells increased significantly from day 3 to day 6 (Two-way ANOVA; p=0.0002). There was no increase in cell number both in the presence and in the absence of IGF-2 from day 6 to day 9, which suggests that the cells were confluent.

The above result suggests that IGF-2 can promote increased proliferation in NUGC3 cells.

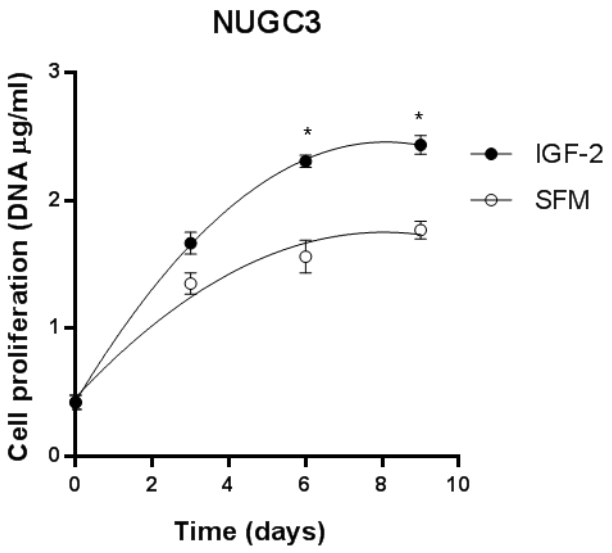

Figure 6.9. The effect of IGF-2 on cell proliferation in NUGC3 cells. NUGC3 cells were seeded into 24 well tissue culture plates at $1x10^4$ cells per well, in 0.5 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 0.5 ml PBS. Medium was replaced every 3 days with 0.5 ml serum-free medium (NUGC3 cells) or 0.5 ml DCCS medium, supplemented or not with 50 ng/ml IGF-2. At the end of each time point, medium was aspirated and the cells washed with 0.5 ml ice cold PBS. PBS was aspirated and the cells stored in -20^{o}C. The cells were lysed in SSC/SDS solution and the amount of DNA in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM, * denotes statistically significantly higher DNA content in the presence of IGF-2 compared with in the absence of IGF-2 (Two-way ANOVA; p<0.0001).

6.2.8 The effect of insulin on proliferation

The effect of insulin on gastric cancer cell growth was investigated over time. NUGC3 cells were cultured in serum-free medium, in the presence or absence of 50 ng/ml insulin for 9 days. The amount of DNA in the presence and in the absence of insulin was measured with the PicoGreen fluorimetric assay. The amount of DNA increased progressively in NUGC3 cells cultured in serum-free medium over the course of 9 days, when insulin was not present (Figure 6.10). Again, increase in cell number could be attributed to the lack of withdrawal of the cells from the growth factors present in the serum prior to treatment. When insulin was present in the medium, the amount of DNA was increased at all time-points tested and the increase was statistically significant after 6 and 9 days of treatment. In the absence of insulin, there was no significant increase in cell number from day 3 to day 6 (Two-way ANOVA; p=0.5473), whereas when insulin was present cell number increased significantly (Two-way ANOVA; p=0.0002).

This suggests that, apart from inducing cell survival, insulin can also promote increased proliferation in NUGC3 cells.

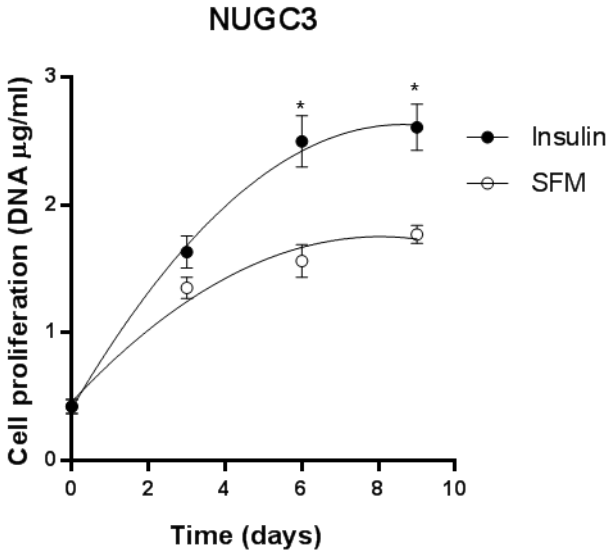

Figure 6.10. The effect of insulin on cell proliferation in NUGC3 cells. NUGC3 cells were seeded into 24 well tissue culture plates at $1x10^4$ cells per well, in 0.5 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 0.5 ml PBS. Medium was replaced every 3 days with 0.5 ml serum-free medium (NUGC3 cells) or 0.5 ml DCCS medium, supplemented or not with 50 ng/ml insulin. At the end of each time point, medium was aspirated and the cells washed with 0.5 ml ice cold PBS. PBS was aspirated and the cells stored in -20^{o}C. The cells were lysed in SSC/SDS solution and the amount of DNA in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM, * denotes statistically significantly higher DNA content in the presence of insulin compared with in the absence of insulin (Two-way ANOVA; p≤0.0002).

6.2.9 The effect of IGF-IR knockdown on the proliferative effect of IGF-2 and insulin

To investigate whether the type I IGF receptor mediates the IGF-2 proliferative effect in NUGC3 cells, the effect of IGF-2 on cell proliferation was tested after transient knockdown of the type I IGF receptor with siRNA.

NUGC3 cells were reverse transfected with siRNA 2, which targets the type I IGF receptor or with scrambled sequence and cultured in serum-free medium, in the presence or absence of IGF-2 for 9 days. In the absence of IGF-2, transfection with siRNA 2 resulted in a small reduction in cell growth, compared with transfection with scrambled sequence (Figure 6.11). This suggests that, as mentioned in section 6.2.7, NUGC3 cells were not completely withdrawn from the effects of growth factors prior to transfection, therefore, IGF-IR knockdown might have retarded their growth. The proliferative response of NUGC3 cells to IGF-2 was reduced significantly in cells in which endogenous expression of the type I IGF receptor had been lowered (Figure 6.11). This suggests that the type I IGF receptor mediates, at least in part, the IGF-2 proliferative effect in NUGC3 cells.

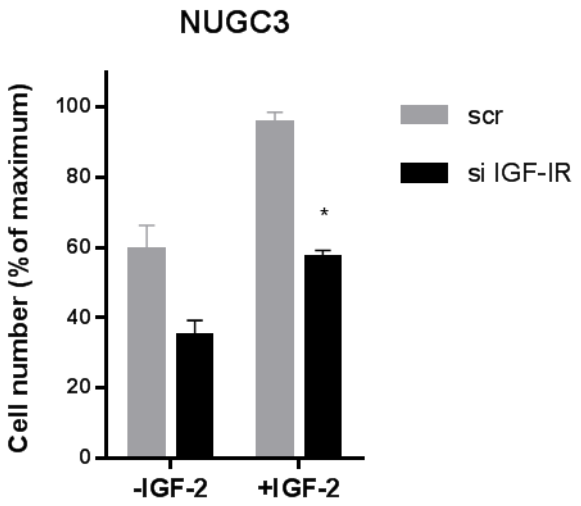

Figure 6.11. The effect of inhibition of the type I IGF receptor on the IGF-2 proliferative effect. Transfection medium was prepared by mixing either scrambled (scr) or IGF-IR targeted (IGF-IR) siRNA duplexes at a final concentration of 40 nM and Lipofectamine RNAiMAX at a final concentration of 0.2% (v/v), in 0.1 ml DMEM without serum. The transfection medium was then incubated at room temperature for 30 minutes. NUGC3 cells grown in maintenance medium were detached using trypsin:EDTA and resuspended in maintenance medium at $12.5x10^4$ cells /ml. Four hundred µl of the cell suspension were mixed with 0.1 ml of the transfection medium, containing scrambled or IGF-IR targeted sequences, in 24 well tissue culture plates. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells were washed with PBS. Medium was replaced every 3 days with serum-free medium supplemented or not with 50 ng/ml IGF-2. After treatment with IGF-2 for 9 days, medium was aspirated and cells were washed once with ice cold PBS. PBS was aspirated and the cells stored in -20^o C. The cells were lysed in SSC/SDS solution and the amount of DNA present in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM. Asterisks indicate significant reduction in IGF-2-stimulated proliferation after reduction in type I IGF receptor expression (Two-way ANOVA; p=0.0007).

The importance of the type I IGF receptor in transmission of the proliferative effect of insulin was investigated in NUGC3 cells, by testing the effect of insulin on cell proliferation after transient knockdown of the type I IGF receptor with siRNA.

NUGC3 cells were reverse transfected with siRNA 2, which targets the type I IGF receptor or with scrambled sequence and cultured in serum-free medium, in the presence or absence of insulin for 9 days (Figure 6.12). In the absence of insulin, transfection with siRNA 2 resulted in a small reduction of cell growth, compared with transfection with scrambled sequence, due to the absence of withdrawal of the cells from growth factors prior to transfection. The proliferative response of NUGC3 cells to insulin was reduced significantly in cells in which endogenous expression of the type I IGF receptor had been lowered (Figure 6.11). This suggests that the type I IGF receptor mediates, at least in part, the proliferative effect of insulin in NUGC3 cells.

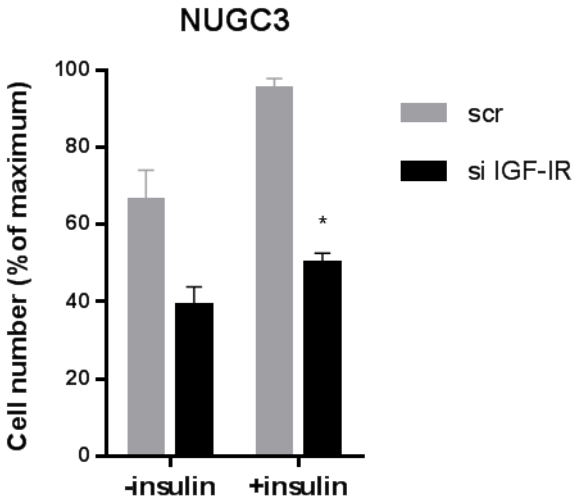

Figure 6.12. The effect of inhibition of the type I IGF receptor on the insulin proliferative effect.
Transfection medium was prepared by mixing either scrambled (scr) or IGF-IR targeted (IGF-IR) siRNA duplexes at a final concentration of 40 nM and Lipofectamine RNAiMAX at a final concentration of 0.2% (v/v), in 0.1 ml DMEM without serum. The transfection medium was then incubated at room temperature for 30 minutes. NUGC3 cells grown in maintenance medium were detached using trypsin:EDTA and resuspended in maintenance medium at $12.5x10^4$ cells /ml. Four hundred µl of the cell suspension were mixed with 0.1 ml of the transfection medium, containing scrambled or IGF-IR targeted sequences, in 24 well tissue culture plates. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells were washed with PBS. Medium was replaced every 3 days with serum-free medium supplemented or not with 50 ng/ml insulin. After treatment with insulin for 9 days, medium was aspirated and cells were washed once with ice cold PBS. PBS was aspirated and the cells stored in -20^o C. The cells were lysed in SSC/SDS solution and the amount of DNA present in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM. Asterisks indicate significant reduction in insulin-stimulated proliferation after reduction in type I IGF receptor expression (Two-way ANOVA; p=0.0005).

6.2.10 The effect of IGF-IR and IR knockdown on cell number in the presence of serum

To compare the effect of reduced expression of the type I IGF and insulin receptor on cell number in the presence of growth factors, NUGC3 cells were reverse transfected with siRNA 2 against the type I IGF receptor, siRNA 2 against the insulin receptor or scrambled sequence and the number of cells was measured three days after transfection.

There was a significant reduction in the amount of DNA after knockdown of either the type I IGF receptor or the insulin receptor in NUGC3 cells (Figure 6.13). Knockdown of the type I IGF receptor did not induce significant levels of PARP cleavage (Figure 6.5) and as seen in chapter 5, loss of IGF-IR reduced the number of cells synthesizing DNA and undergoing mitosis (Figure 5.10 and Figure 5.11). Therefore, we can conclude that the reduction in cell number after IGF-IR knockdown is a result of reduced cell proliferation rather than increased cell death. On the contrary, reduction in IR expression induced significant amount of cell death (Figure 6.6), which has led to detachment of the cells from tissue culture plates (data not shown) and subsequent reduction in cell number. Therefore, it cannot be excluded that reduction in cell number after IR knockdown is a result of increased cell death rather than reduced cell proliferation.

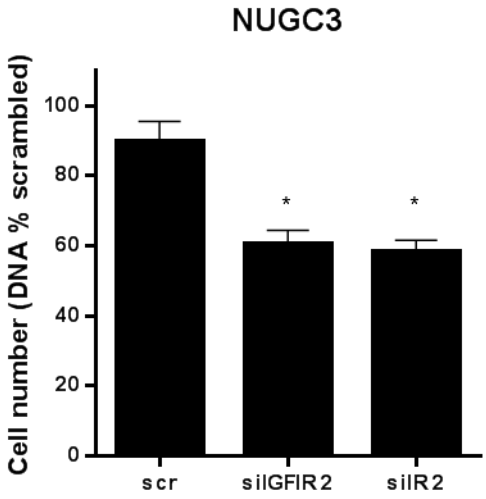

Figure 6.13. The effect of inhibition of the type I IGF and insulin receptor on cell number. Transfection medium was prepared by mixing scrambled (scr), IGF-IR targeted (si IGF-IR 2) or IR targeted (si IR 2) siRNA duplexes at a final concentration of 40, 40 and 20 nM, respectively, and Lipofectamine RNAiMAX at a final concentration of 0.2% (v/v), in 0.1 ml DMEM without serum. The transfection medium was then incubated at room temperature for 30 minutes. NUGC3 cells grown in maintenance medium were detached using trypsin:EDTA and resuspended in maintenance medium at $12.5x10^4$ cells /ml. Four hundred µl of the cell suspension were mixed with 0.1 ml of the transfection medium, containing scrambled or IGF-IR targeted sequences, in 24 well tissue culture plates. Cells were incubated for 72 hours. Medium was aspirated and cells were washed once with ice cold PBS. PBS was aspirated and the cells stored in -20^o C. The cells were lysed in SSC/SDS solution and the amount of DNA present in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM. Asterisks indicate significant reduction in cell number after reduction in type I IGF and insulin receptor expression (One-way ANOVA; $p \leq 0.0057$).

6.2.11 Expression of insulin receptor isoforms in gastric cancer cells

As mentioned previously, there are two insulin receptor isoforms, which are generated by alternative splicing of exon 11. The form that contains 12 amino acids encoded by exon 11 is called the B-isoform (IR-B) and the form that lacks the 12 amino acids encoded by exon 11 is called the A-isoform (IR-A). Isoform A of the insulin receptor has 2x higher affinity for insulin, 5x higher affinity for IGF-2 and 10x higher affinity for IGF-1 compared to isoform B (Westley and May, 2013). Given the observed importance of the insulin receptor in survival of SNU-1 and NUGC3 cells (sections 6.2.4 and 6.2.5), it is of interest to analyse expression of the two isoforms in gastric cancer cells.

To determine the expression of the two insulin receptor isoforms in gastric cancer cells, polymerase chain reactions were designed to amplify specifically isoform A of the insulin receptor or isoform B. A relatively high number of PCR cycles was used to ensure detection of the two isoforms in gastric cancer cells. Isoform A of the insulin receptor was detected in all cell lines, with MKN74 cells expressing the lowest amount (Figure 6.14). Similarly,

isoform B of the insulin receptor was detected in all cell lines tested, with MKN74 cells expressing again the lowest levels.

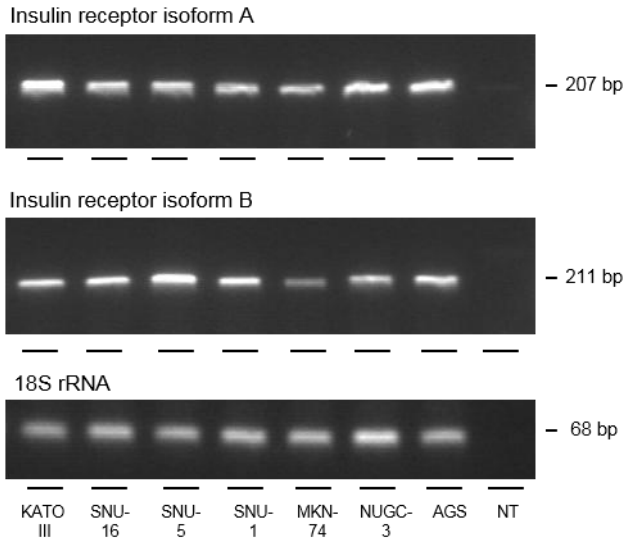

Figure 6.14. Expression of insulin receptor isoform A and insulin receptor isoform B in gastric cancer cells. KATO III, SNU-16, SNU-5, SNU-1, MKN74, NUGC3 and AGS cells were cultured in 25 cm^2 tissue culture flasks in maintenance medium to ~80% confluence. Total RNA was extracted from the cells with the Direct-zol™ RNA MiniPrep, as described in the Materials and Methods section. Complementary DNA was synthesised from total RNA with the Moloney Murine Leukaemia Virus Reverse Transcriptase. Specific primer sequences were used to amplify isosoform A and isoform B of the insulin receptor and 18S rRNA. A high number of PCR cycles (35) was used for detection of the two isoforms. The amplified DNA was electrophoresed in a 3% agarose gel, stained with GelRed, and bands were visualised with a UV transilluminator.

To determine the relative abundance of the two insulin receptor isoforms in gastric cancer cells, a specific polymerase chain reaction was designed to amplify both isoforms simultaneously. The amplicon corresponding to insulin receptor isoform B is 187 bp in length, whereas the one corresponding to isoform A is 151 bp. A lower number of PCR cycles was performed to detect any potential differences in the expression of the two isoforms. SNU-16, NCI-N87 and MKN74 cells expressed lower levels of insulin receptor compared to KATO III, SNU-5, SNU-1, NUGC3 and AGS cells (Figure 6.15). The two isoforms were expressed equally in SNU-16, NCI-N87 and SNU-5 cells. The remaining cell lines, KATO III, SNU-1, MKN74, NUGC3 and AGS had higher IRA:IRB ratios compared to the previous cell lines. In the above four cell lines, isoform A of the insulin receptor was the predominantly expressed isoform.

It is of interest that SNU-1 and NUGC3 cells, in which knockdown of the insulin receptor induced cell death, expressed considerably higher levels of isoform A of the insulin receptor than isoform B. It could, therefore, be concluded that isoform A is primarily responsible for the mediation of survival signals in the above cells.

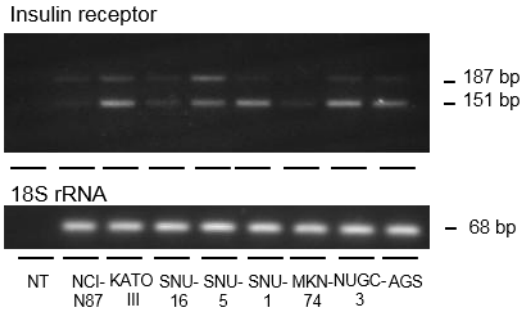

Figure 6.15. Relative expression of insulin receptor isoforms A and B in gastric cancer cells. NCI-N87, KATO III, SNU-16, SNU-5, SNU-1, MKN74, NUGC3 and AGS cells were cultured in 25 cm^2 tissue culture flasks in maintenance medium to ~80% confluence. Total RNA was extracted from the cells with the Direct-zol™ RNA MiniPrep, as described in the Materials and Methods section. Complementary DNA was synthesised from total RNA with the Moloney Murine Leukaemia Virus Reverse Transcriptase. Specific primer sequences were used to amplify both insulin receptor isoforms and 18S rRNA. A relatively low number of PCR cycles was used (31). The amplified DNA was electrophoresed in a 3% agarose gel, stained with GelRed, and bands were visualised with a UV transilluminator.

6.3 Discussion

The aim of the experiments reported in this chapter was to investigate the responsiveness of gastric cancer cells to IGF-2 and insulin and the relative importance of the type I IGF and insulin receptor in cell survival and proliferation. Both IGF-2 and insulin stimulated phosphorylation of the type I IGF and insulin receptors and activated the PI3K/Akt pathway in SNU-1 and NUGC3 cells and the Ras/MAPK pathway in NUGC3 cells. Consequently, apart from IGF-1 binding to the type I IGF receptor, it is evident that other ligand-receptor interactions may take place and contribute to IGF signal transduction and biological effects. Exogenous IGF-2 and insulin afforded protection against staurosporine-induced PARP cleavage in SNU-1 and NUGC3 cells. Also, both growth factors stimulated proliferation of NUGC3 cells.

Previously, IGF-2 protected MKN74 and Met-amplified MKN45 gastric cancer cells against apoptosis which had been induced by treatment with 5% ethanol (Min *et al.*, 2005). The same study has shown that IGF-2 stimulates proliferation of MKN45 gastric cancer cells. The role of IGF-2 in cell proliferation has been demonstrated in MCF-7 breast cancer cells (Stewart *et al.*, 1990) and in colon cancer cells, in which an anti IGF-2 antibody reduced effectively cell growth (Singh *et al.*, 1996). Furthermore, the mitogenic effect of 29 ng/ml insulin, lower to the concentration of 50 ng/ml used in the present study, has been shown in a variety of cancer cell lines, including breast, lung and prostate cancer cells (Sciacca *et al.*, 2014).

Transient knockdown of the insulin receptor with two different siRNA sequences, in an environment enriched with growth factors, resulted in increased levels of apoptosis in SNU-1

and NUGC3 cells. This suggests that gastric cancer cells depend on signalling through the insulin receptor in order to evade apoptosis and that other growth factors present in the serum are not sufficient to substitute its survival effects. A cell survival effect mediated by the insulin receptor has been shown previously in glioblastoma cells, in which, knockdown of the insulin receptor increased caspase 3/7 activity (Gong *et al.*, 2015).

The effect of reducing the expression of the insulin receptor was compared to the effect of reducing the expression of the type I IGF receptor on cell survival. Reduction of IGF-IR expression did not induce significant levels of cleaved PARP in NUGC3 cells, whereas reduction of IR expression increased cleaved PARP levels significantly. Targeting both receptors at the same time did not increase further the amount of cleaved PARP, which suggests that the insulin receptor is mainly responsible for evasion of apoptosis. It was not possible to investigate the role of insulin receptor on cell proliferation in this study, as knockdown of the insulin receptor resulted in increased detachment of the cells from tissue culture plates, due to cell death, which led to reduction of the amount of DNA. Consequently, quantification of the amount of DNA as a measure of cell proliferation would not have been applicable in this case. Detection of proliferation markers, such as BrdU incorporation or histone H3 phosphorylation could specify the role of insulin receptor in cell proliferation.

Treatment of SNU-1 and NUGC3 cells with the tyrosine kinase inhibitor BMS754807, which targets both the type I IGF and insulin receptors, induced PARP cleavage in SNU-1 and NUGC3 cells grown in the presence of growth factors. Also, treatment with BMS754807 reduced cell proliferation in SNU-1, MKN74, NUGC3 and AGS cells (section 5.2.7). This suggests that the BMS754807 inhibitor targets effectively both the proliferative effect mediated by the type I IGF receptor and the survival effect mediated by the insulin receptor.

Several studies have reported apoptosis induction by BMS754807 alone or in combination with other drugs, in other cancer types. Treatment with BMS754807 increased PARP and caspase 3 cleavage in a rhabdomyosarcoma cell line, Rh41 (Carboni *et al*., 2009). Combination of the BMS754807 inhibitor with docetaxel in a tumourgraft model of human triple negative breast cancer, resulted in tumour regression associated with increased apoptosis (Litzenburger *et al*., 2011). Combined use of BMS754807 with gemcitabine increased caspase 3 and PARP cleavage in pancreatic ductal adenocarcinoma cell lines (Awasthi *et al*., 2012).

Our results suggest that the type I IGF receptor mediates cell proliferation and the insulin receptor is important for cell survival. It is, therefore, expected that inhibition of both

receptors simultaneously might be a more effective therapeutic strategy than inhibition of either receptor alone. In prostate cancer cells, signalling through the insulin receptor compensated for and mediated IGF-1 mitogenic signals, following inhibition of the type I IGF receptor with cixutumumab, a monoclonal antibody against the type I IGF receptor (Weinstein *et al.*, 2014). In a mouse mammary tumour model, inhibition of both the type I IGF receptor and insulin receptor with OSI-906, a small-molecule tyrosine kinase inhibitor, was more effective at reducing tumour volume compared to inhibition of the type I IGF receptor alone (Buck *et al.*, 2010). Co-targeting of the type I IGF and insulin receptors with AZ12253801 and NVPAWD742 tyrosine kinase inhibitors was more effective at reducing non-small cell lung cancer cell proliferation than targeting the type I IGF receptor alone with the monoclonal antibody aIR3 (Vincent *et al.*, 2013). Given the reported importance of the insulin receptor in survival and proliferation, it could be concluded that the lack of survival benefit in phase III clinical trials of figitumumab, an anti-IGF-IR antibody, in non-small cell lung cancer patients, could be attributed, partly, to insulin receptor signalling, which might have circumvented IGF-IR inhibition (Langer *et al.*, 2014).

Interestingly, knockdown of the type I IGF receptor resulted in significant reduction of the proliferative effect of IGF-2 and insulin in NUGC3 cells. This suggests that the type I IGF receptor is activated by IGF-2 and insulin and stimulates downstream pathways that result in cell growth. Previously, the proliferative effect of insulin in MCF-7 and 184B5 breast cancer cells was inhibited by 50-60%, after treatment with a monoclonal antibody which targets the type I IGF receptor (Milazzo *et al.*, 1992). However, the same study showed that in two breast cancer cell lines, T-47D and ZR-75-1, the proliferative effect of insulin was not inhibited by the same monoclonal antibody against the type I IGF receptor, which suggests that in these cells insulin acts only through its own receptor.

Expression of the two insulin receptor isoforms was detected in the gastric cancer cell lines, with 5 out of 8 cell lines expressing higher levels of isoform A than isoform B. This is consistent with the finding that isoform A of the insulin receptor is highly expressed in several human malignancies (Belfiore, 2007). KATO III, SNU-1, NUGC3 and AGS cells expressed highly isoform A of the insulin receptor and expressed very small amounts of the isoform B.

In summary, the findings in this chapter have confirmed that apart from IGF-1-mediated activation of the type I IGF receptor, other ligand-receptor interactions take place and contribute to increased survival and proliferation. Both IGF-2 and insulin activated

downstream signal transduction pathways, protected cells against apoptosis induced by tyrosine kinase inhibition and increased cell proliferation. Also, the insulin receptor was more important than the type I IGF receptor in mediation of survival signals. Our results suggest that simultaneous inhibition of the type I IGF and insulin receptors might be a more effective therapeutic strategy for gastric cancer than inhibition of the type I IGF receptor alone.

Chapter 7. Combination of IGF signal transduction pathway inhibition with cytotoxic and targeted therapy in gastric cancer cells

7.1 Introduction

Preclinical analysis of the effects of combining a targeted inhibitor with cytotoxic drugs, helps to determine the effective drug combinations to be tested in the clinic. In the UK, the standard cytotoxic treatment for patients with advanced gastric cancer comprises a fluorouracil-based regimen, such as capecitabine or 5-fluorouracil, with cisplatin or oxaliplatin and epirubicin. It is, therefore, important to investigate the effect of combining anti-IGF-IR/IR targeted treatment to the above cytotoxic agents in a pre-clinical setting.

As mentioned previously, inhibitors against FGFR2 and c-Met are currently being evaluated in clinical trials for patients with advanced gastric cancer. In preclinical models, it has already been demonstrated that activation of several receptor tyrosine kinases, including EGFR, HER3 and Met contributed to reduced sensitivity to AZD4547, a selective FGFR2 inhibitor, in FGFR2 amplified gastric cancer cells (Chang *et al.*, 2015). The rescue effect exerted by the above tyrosine kinases was abrogated by inhibiting them with their targeted tyrosine kinase inhibitors. Concomitant inhibition of IGF-IR with FGFR2 or Met has not been investigated in gastric cancer cells.

Advanced gastric cancer patients with HER2 positive cancer receive trastuzumab as well as cytotoxic drugs as part of their standard treatment. Approximately 50% of patients with HER-2 positive advanced gastric cancer do not respond to trastuzumab treatment and 12% develop progressive disease within 18 months (Bang *et al.*, 2010). The effect of IGF-IR/IR inhibition has yet to be investigated in HER2 amplified gastric cancer cells, which have acquired resistance to a HER2 targeted inhibitor.

7.1.1 Aims

The aim of the results reported in this chapter is to investigate the effect of combining cytotoxic agents currently used in the clinic for advanced gastric cancer patients with IGF-IR/IR inhibition in gastric cancer cells. Furthermore, the potential of combining IGF-IR/IR inhibition with FGFR2 and Met inhibitors, which are currently in clinical trials for advanced gastric cancer, will be investigated in gastric cancer cells. Finally, inhibition of the IGF signal

transduction pathway will be investigated as a potential way of circumventing secondary resistance of HER-2 amplified gastric cancer cells to a HER-2 targeted inhibitor.

7.2 Results

7.2.1 The effect of treatment with oxaliplatin on viability of gastric cancer cells

Oxaliplatin is a third-generation platinum-based drug with cytotoxic activity (Misset *et al.*, 2000). It is currently included in the chemotherapeutic regime administered to patients with advanced gastric cancer and other cancers. To investigate the effect of oxaliplatin on the viability of gastric cancer cells *in vitro,* SNU-1 and NUGC3 cells were treated with increasing concentrations of oxaliplatin and the amount of DNA in each sample was measured after 3 days.

Oxaliplatin reduced significantly the number of SNU-1 cells at concentrations equal to or higher than 1 μM (Figure 7.1). The IC_{50} of oxaliplatin in the above cell line was approximately 0.8 μM and the IC_{90} was approximately 10 μM. In NUGC3 cells, oxaliplatin reduced significantly the number of cells at concentrations equal to or higher that 0.1 μM. An IC_{50} of approximately 0.8 μM and an IC_{90} of 3 μM was detected in those cells.

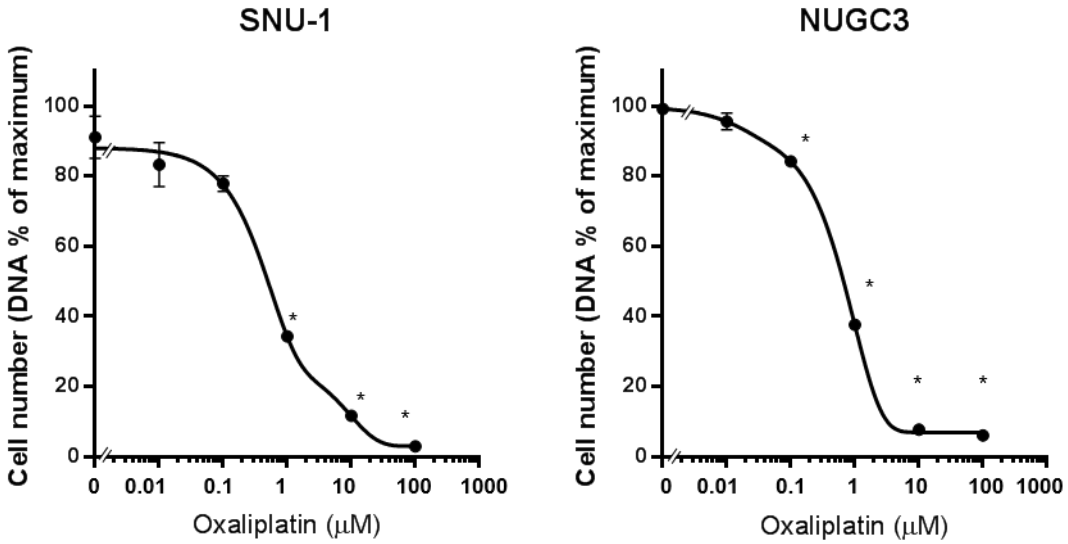

Figure 7.1. The effect of treatment with different concentrations of oxaliplatin on viability of SNU-1 and NUGC3 cells. SNU-1 and NUGC3 cells were seeded into 24 well tissue culture plates at $5x10^4$ cells per well, in 0.5 ml maintenance medium. Adherent cells were incubated for 24 hours to allow attachment. Medium was replaced to maintenance medium which contained 0, 0.01, 0.1, 1, 10 or 100 μM oxaliplatin. After 3 days, medium was aspirated and the cells washed with 0.5 ml ice cold PBS. PBS was aspirated and the cells stored in -20°C. The cells were lysed in SSC/SDS solution and the amount of DNA in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM, * denotes statistically significantly lower amount of DNA after treatment with oxaliplatin compared to untreated cells (One-way ANOVA; $p<0.0001$).

To investigate the effect of different concentrations of oxaliplatin on cell survival, NUGC3 cells were treated with a range of concentrations of oxaliplatin for 24 hours and cell death was measured by detecting the amount of cleaved PARP. No cleaved PARP was detected in NUGC3 cells after culture in maintenance medium in the absence of oxaliplatin (Figure 7.2). Oxaliplatin increased the amount of cleaved PARP in a concentration dependent manner and maximum levels were achieved with 20 μM.

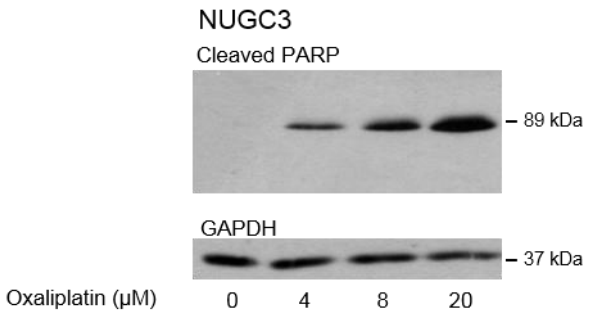

Figure 7.2. The effect of treatment with oxaliplatin on survival of NUGC3 cells. NUGC3 cells were seeded into 12 well tissue culture plates at $1x10^5$ cells per well, in 1 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was replaced to maintenance medium which contained 0, 4, 8, or 20 μM oxaliplatin. After 24 hours, proteins were extracted with 80 μl of RIPA buffer plus inhibitors per well. Aliquots containing 10 μg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against cleaved PARP (1:2000 dilution) and GAPDH (1:10000 dilution).

The above results suggest that oxaliplatin effectively reduces the number of SNU-1 and NUGC3 gastric cancer cells, in a concentration-dependent manner. Also, oxaliplatin effectively induces cell death in NUGC3 cells, as measured by the induction of cleaved PARP.

7.2.2 The effect of IGF-1 on gastric cancer cells treated with oxaliplatin in the presence of serum

Treatment with oxaliplatin reduced the number of SNU-1 and NUGC3 cells, in a concentration-dependent manner and induced apoptosis. To investigate whether IGF-1 can protect gastric cancer cells from the cytotoxic effects of oxaliplatin, SNU-1 and NUGC3 cells were treated with different concentrations of oxaliplatin in the presence or absence of 50 ng/ml IGF-1 and the amount of DNA in the presence or absence of IGF-1 was measured.

In SNU-1 cells, oxaliplatin reduced significantly the number of cells after three days of treatment, however, concomitant treatment with IGF-1 did not alter the amount of DNA at

any concentration of oxaliplatin tested (Figure 7.3). A similar result was obtained in NUGC3 cells, with IGF-1 having no effect on the amount of DNA present after treatment with different concentrations of oxaliplatin. Since the cells were not withdrawn from the effects of growth factors prior to treatment, it is possible that some IGF-1 was already present in the serum and, therefore, the externally added IGF-1 did not induce an additional effect.

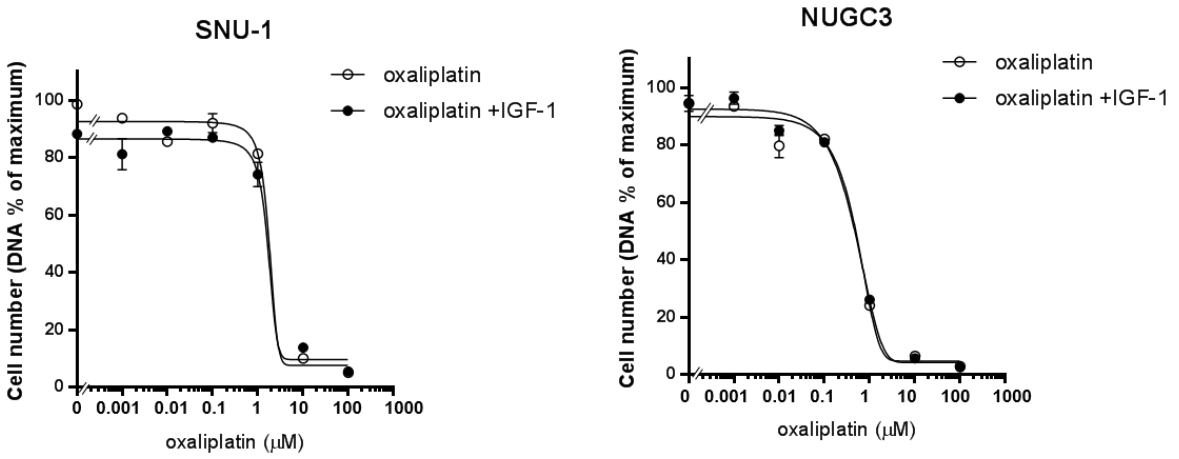

Figure 7.3. The effect of IGF-1 on treatment with oxaliplatin in SNU-1 and NUGC3 cells. SNU-1 and NUGC3 cells were seeded into 24 well tissue culture plates at $5x10^4$ cells per well, in 0.5 ml maintenance medium. Adherent cells were incubated for 24 hours to allow attachment. Medium was replaced to maintenance medium which contained 0, 0.001, 0.01, 0.1, 1, 10 or 100 μM oxaliplatin, in the presence or absence of 50 ng/ml IGF-1. After 3 days, medium was aspirated and the cells washed with 0.5 ml ice cold PBS. PBS was aspirated and the cells stored in -20^{o}C. The cells were lysed in SSC/SDS solution and the amount of DNA in each well was quantified with the Quanti-iT picogreen fluorimetric assay.

7.2.3 The effect of inhibition of the IGF signal transduction pathway on cell death induced by oxaliplatin in gastric cancer cells

To investigate whether inhibition of the IGF signal transduction pathway can intensify the amount of cell death induced by oxaliplatin, NUGC3 cells were transfected with a siRNA against the type I IGF receptor and treated with 8 μM oxaliplatin for 3 days and the amount of cleaved PARP was measured by western transfer analysis.

No reduction of IGF-IR expression was detected in NUGC3 cells that had been transfected with the scrambled oligonucleotide (Figure 7.4). Transfection with the siRNA against the type I IGF receptor resulted in effective reduction of IGF-IR expression. No cleaved PARP was detected in cells that had been transfected with the scrambled oligonucleotide in the absence of oxaliplatin. Treatment with 8 μM oxaliplatin for three days induced cleaved PARP in NUGC3 cells. Transfection of NUGC3 cells with siRNA against the type I IGF receptor in the absence of oxaliplatin, also induced cleaved PARP. When treatment with oxaliplatin was

combined with inhibition of IGF-IR by siRNA knockdown, the amount of cleaved PARP was higher than in any of the above conditions alone (Figure 7.4A). Interestingly, the effect observed with the combined treatment was higher than the additive effect of the two treatments (Figure 7.4B). This suggests that inhibition of IGF-IR might potentiate induction of cell death by oxaliplatin in NUGC3 cells.

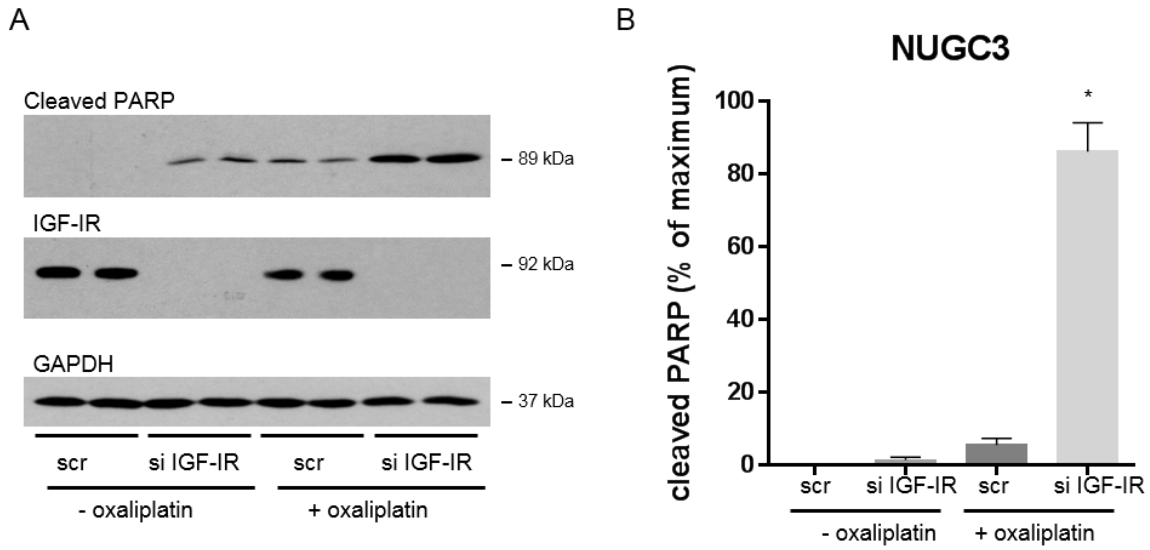

Figure 7.4. The effect of treatment with oxaliplatin in combination with reduction of IGF-IR expression on survival of NUGC3 cells. Transfection medium was prepared by mixing either scrambled (scr) or IGF-IR targeted (IGF-IR) siRNA duplexes at a final concentration of 20 nM and Lipofectamine RNAiMAX at a final concentration of 0.2% (v/v), in 0.25 ml DMEM without serum. The transfection medium was then incubated at room temperature for 30 minutes. NUGC3 cells grown in maintenance medium were detached using trypsin:EDTA and resuspended in medium supplemented with 10% foetal calf serum at 10 x 10^4 cells /ml. One ml of the cell suspension was mixed with 0.25 ml of the transfection medium, containing scrambled or IGF-IR targeted sequences, in 12 well tissue culture plates. After 24 hours the medium was replaced with maintenance medium, which contained 8 µM oxaliplatin. After 72 hours of treatment with oxaliplatin, protein extracts were prepared with 80 µl per well of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose for at 100 mA for 45 minutes. Membranes were incubated with antibodies against cleaved PARP (1:2000 dilution), IGF-IR (1:2000 dilution) and GAPDH (1:10000 dilution) (**A**). Densitometry was with Labworks 4.0 software, adjusted for the background optical density of the X-ray film and normalised to GAPDH. Results are expressed as a percentage of the intensity of the cleaved PARP band observed in cells transfected with siRNA against the IGF-IR and treated with oxaliplatin. Bars are the mean ±SEM, * denotes statistically significantly higher amount of cleaved PARP in the presence of oxaliplatin and in the absence of IGF-IR (One-way ANOVA; $p<0.0001$) (**B**).

7.2.4 The effect of combining cytotoxic agents with BMS754807 on viability of gastric cancer cells

To investigate the effect of combining cytotoxic agents currently used in the clinic for advanced gastric cancer patients, such as oxaliplatin and 5-fluorouracil with inhibition of the IGF signal transduction pathway, SNU-1 and NUGC3 cells were treated with different concentrations of the above cytotoxic agents, in the presence and absence of BMS754807.

In SNU-1 cells, treatment with oxaliplatin resulted in significant reduction of the number of cells at concentrations ranging from 1 to 100 μM, whereas concentrations ranging from 0 to 0.1 μM did not have any significant cytotoxic effect (Figure 7.5). Treatment with 5 μM BMS754807 within the non-cytotoxic range of oxaliplatin concentrations (0 to 0.1 μM), reduced significantly the number of cells. However, at higher concentrations of oxaliplatin, which were cytotoxic for the cells, treatment with the BMS754807 did not confer any additional benefit. A similar result was observed in NUGC3 cells, in which BMS754807 reduced significantly the number of cells within the non-cytotoxic range of oxaliplatin concentrations, but did not confer any additional benefit at higher cytotoxic concentrations (Figure 7.5). The above result suggests that inhibition of the IGF signal transduction pathway with BMS754807 does not aggravate the detrimental effect of oxaliplatin on cell viability.

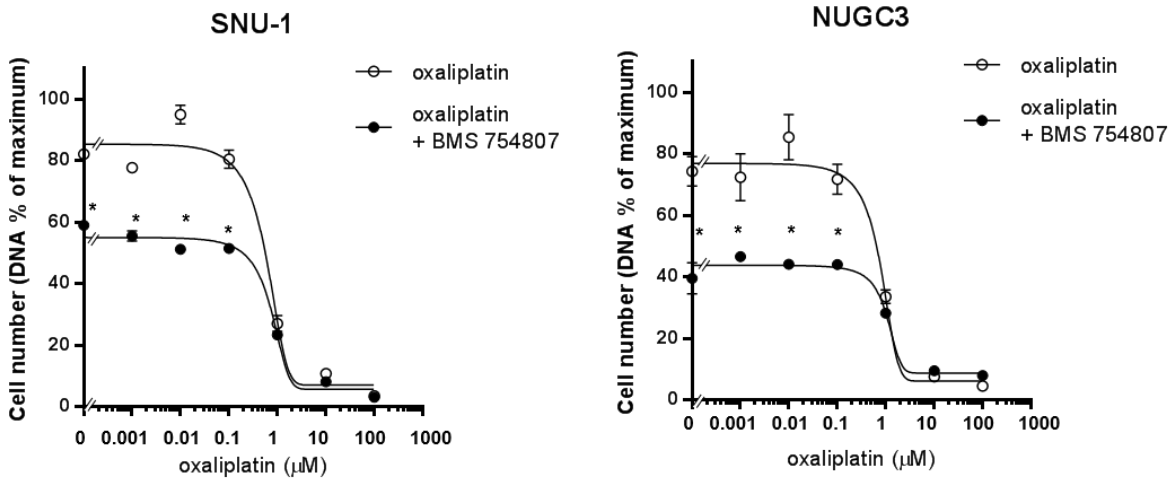

Figure 7.5. The effect of treatment of SNU-1 and NUGC3 cells with different concentrations of oxaliplatin in combination with the BMS754807 inhibitor. SNU-1 and NUGC3 cells were seeded into 24 well tissue culture plates at $5x10^4$ cells per well, in 0.5 ml maintenance medium. Adherent cells were incubated for 24 hours to allow attachment. Medium was replaced to maintenance medium which contained 0, 0.0001, 0.001, 0.01, 0.1, 1, 10 or 100 μM oxaliplatin with 5 μM BMS754807 or DMSO. After 3 days, medium was aspirated and the cells washed with 0.5 ml ice cold PBS. PBS was aspirated and the cells stored in -20^{o}C. The cells were lysed in SSC/SDS solution and the amount of DNA in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM, * denotes statistically significantly lower amount of DNA in the presence of BMS754807 inhibitor and oxaliplatin compared to treatment with oxaliplatin alone (Two-way ANOVA; SNU-1, $p<0.0001$, NUGC3, $p\leq0.0001$).

The fluoropyrimidine 5-fluorouracil (5-FU) is a uracil analogue, which enters the cell in a similar manner to uracil and leads to creation of DNA strand breaks, which eventually cause cell death (Lindahl, 1974). To investigate the effect of 5-fluorouracil on the viability of gastric

cancer cells *in vitro*, SNU-1 and NUGC3 cells were treated with increasing concentrations of the drug and the amount of DNA in each sample was measured after 3 days.

In SNU-1 cells treatment with 5-fluorouracil reduced significantly the number of cells at concentrations ranging from 10 to 100 μM, whereas concentrations up to 1 μM did not have any significant cytotoxic effect (Figure 7.5). The IC_{50} of 5-fluorouracil in SNU-1 cells was close to 10 μM, which suggests that SNU-1 cells are less sensitive to 5-fluorouracil than to oxaliplatin. As seen with oxaliplatin, treatment with 5 μM BMS754807 within the non-cytotoxic range of 5-fluorouracil concentrations (0 to 0.1 μM), reduced significantly the number of SNU-1 cells. Interestingly, treatment with BMS754807 reduced further the number of cells which had been treated with 10 and 100 μM 5-fluorouracil, concentrations which kill approximately 60% and 80% of SNU-1 cells, respectively. In NUGC3 cells, 5-fluorouracil was cytotoxic at concentrations starting from 1 μM and higher, with an IC_{50} of approximately 2 μM. Treatment with 5 μM BMS754807 reduced even further the number of cells at all concentrations of 5-fluorouracil used, both in the non-cytotoxic and in the cytotoxic range (Figure 7.6).

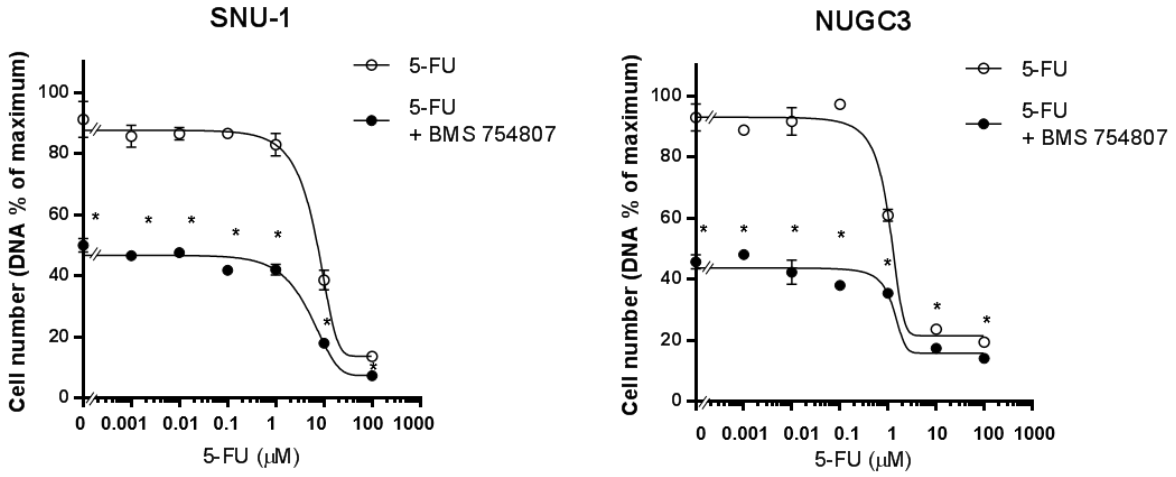

Figure 7.6. The effect of treatment of SNU-1 and NUGC3 cells with different concentrations of 5-fluorouracil in combination with the BMS754807 inhibitor. SNU-1 and NUGC3 cells were seeded into 24 well tissue culture plates at $5x10^4$ cells per well, in 0.5 ml maintenance medium. Adherent cells were incubated for 24 hours to allow attachment. Medium was replaced to maintenance medium which contained 0, 0.0001, 0.001, 0.01, 0.1, 1, 10 or 100 μM 5-fluorouracil, with 5 μM BMS754807 or DMSO. After 3 days, medium was aspirated and the cells washed with 0.5 ml ice cold PBS. PBS was aspirated and the cells stored in -20°C. The cells were lysed in SSC/SDS solution and the amount of DNA in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM, * denotes statistically significantly lower amount of DNA in the presence of BMS754807 inhibitor and 5-fluorouracil compared to treatment with 5-fluorouracil alone (Two-way ANOVA; SNU-1, $p \leq 0.0435$, NUGC3, $p \leq 0.0015$).

The above data suggests that there is a small potentiation of the cytotoxic effect of 5-fluorouracil by BMS754807 in SNU-1 and NUGC3 cells.

7.2.5 The effect of IGF-IR and IR inhibition on viability of FGFR2-amplified cells treated with AZD4547

As mentioned previously, inhibitors that target FGFR2 and Met are currently in clinical trials for advanced gastric cancer. We tested whether or not such inhibitors inhibit effectively gastric cancer cell growth *in vitro* and whether IGF-1 might provide some protection against those inhibitors.

AZD4547 is a selective inhibitor of the FGFR1, 2 and 3 tyrosine kinases (Gavine *et al.*, 2012) and it is currently in clinical trials for advanced gastric cancer. To test the efficacy of AZD4547, FGFR-2 amplified cell lines KATO III and SNU-16 were treated with different concentrations of AZD4547 and the amount of DNA was measured after three days of treatment. In KATO III cells, treatment with 0.003 μM AZD4547 or higher, reduced significantly the amount of DNA, compared to untreated cells (Figure 7.7A). SNU-16 cells were even more sensitive to AZD4547; cell number was reduced significantly with 0.001 μM AZD4547 and was reduced even further with higher concentrations (Figure 7.7B).

The effect of IGF-1 was tested in KATO III and SNU-16 cells which had been treated with AZD4547. KATO III cells were not protected against AZD4547 by IGF-1 (Figure 7.7C). However, in SNU-16 cells there was a small trend of IGF-1 protection against AZD4547, with a significant increase in cell number after treatment with IGF-1 in the presence of 0.3 μM AZD4547 (Figure 7.7D).

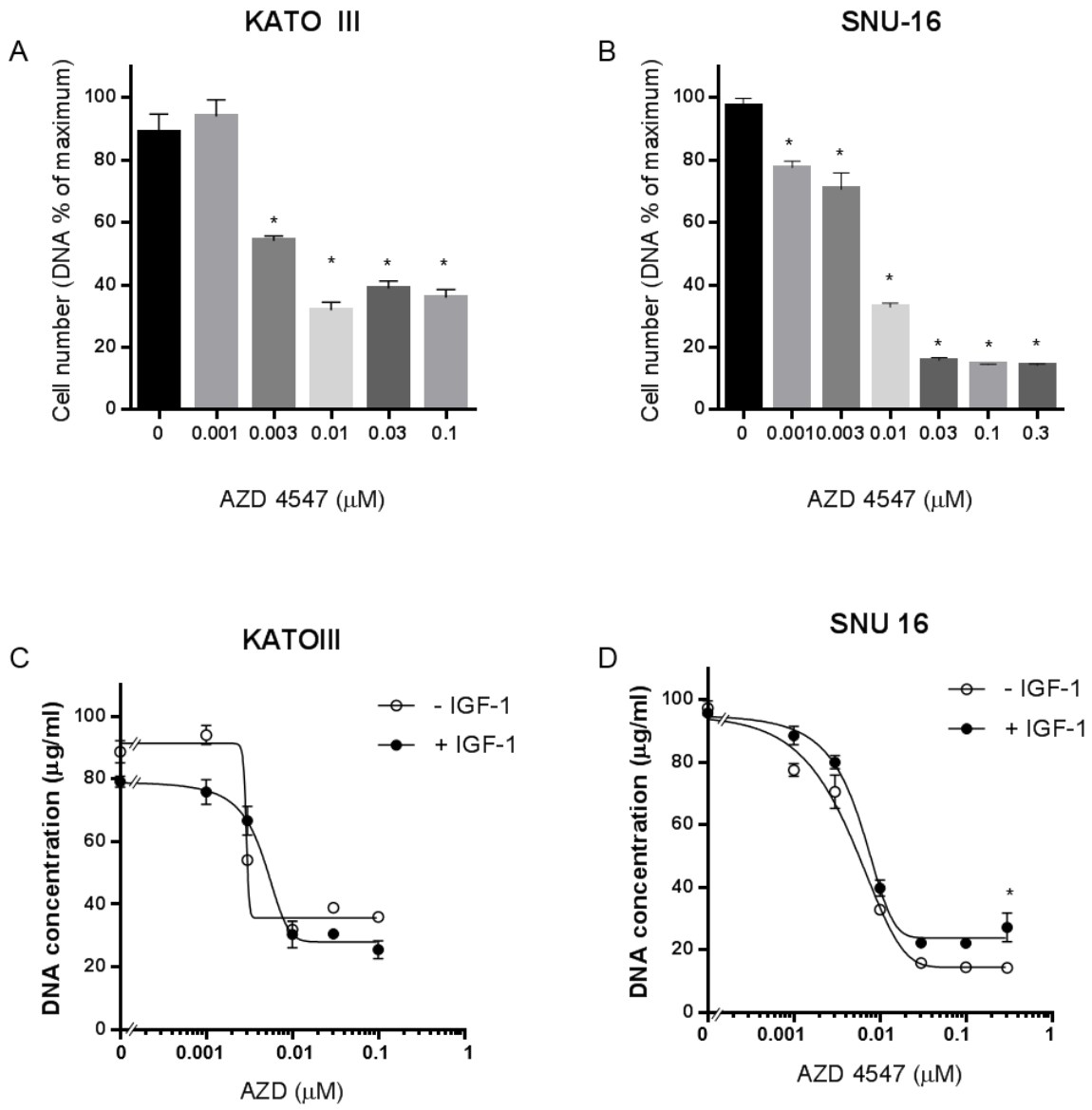

Figure 7.7. The effect of IGF-1 on treatment with AZD4547 in FGR-2 amplified cells. KATO III and SNU-16 cells were seeded into 24 well tissue culture plates at $5x10^4$ cells per well, in 0.5 ml maintenance medium. Adherent cells were incubated for 24 hours to allow attachment. Medium was replaced to maintenance medium which contained 0, 0.001, 0.003, 0.01, 0.03, 0.1 or 0.3 μM AZD4547. After 3 days, medium was aspirated and the cells washed with 0.5 ml ice cold PBS. PBS was aspirated and the cells stored in -20^{o}C. The cells were lysed in SSC/SDS solution and the amount of DNA in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM, * denotes statistically significantly lower levels of DNA in cells treated with AZD4547 compared with untreated cells (One-way ANOVA; p≤0.0003) (**A, B).** KATO III and SNU-16 cells were seeded into 24 well tissue culture plates at $5x10^4$ cells per well, in 0.5 ml maintenance medium. Adherent cells were incubated for 24 hours to allow attachment. Medium was replaced to maintenance medium which contained 0, 0.001, 0.003, 0.01, 0.03, 0.1 or 0.3 μM AZD4547 in the presence or absence of 50 ng/ml IGF-1. After 3 days, medium was aspirated and the cells washed with 0.5 ml ice cold PBS. PBS was aspirated and the cells stored in -20^{o}C. The cells were lysed in SSC/SDS solution and the amount of DNA in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM, * denotes statistically significantly higher levels of DNA in the presence of IGF-1 compared with in the absence of IGF-1 (Two-way ANOVA; p=0.0064) (**C, D**).

To test whether inhibition of the IGF signal transduction pathway affects the response of SNU-16 cells to AZD4547, SNU-16 cells were treated with different concentrations of AZD4547 in the presence or absence of BMS754807 and the amount of DNA was measured. Treatment with 0.5 µM BMS754807 did not reduce the number of SNU-16 cells in the absence of AZD4547 (Figure 7.8A). Combination of BMS754807 with AZD4547 reduced the number of cells significantly at all concentrations of AZD4547 tested. Additionally, the IC_{50} of AZD4547 was reduced from 0.01 µM to approximately 0.005 µM in the presence of 0.5 µM BMS754807. Treatment with a higher concentration of BMS754807 (5 µM) reduced the number of cells to 15% in the absence of AZD4547 and combined treatment with AZD4547 did not reduce further the number of cells (Figure 7.8B). This suggests that at lower concentrations, BMS754807 enhances the growth inhibitory effect of AZD4547 in SNU-16 cells, whereas higher concentrations of BMS754807 overpower the growth inhibitory effect of AZD4547.

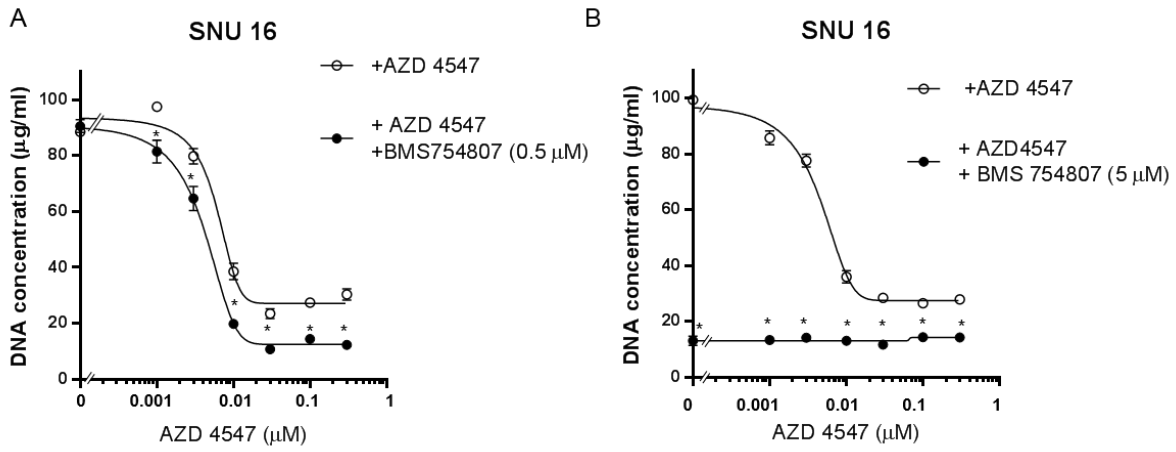

Figure 7.8. The effect of combining BMS754807 with different concentrations of AZD4547 in SNU-16 cells. SNU-16 cells were seeded into 24 well tissue culture plates at $5x10^4$ cells per well, in 0.5 ml maintenance medium, which contained 0, 0.001, 0.003, 0.01, 0.03, 0.1 or 0.3 µM AZD4547 in the presence or absence of 0.5 µM (**A**) or 5 µM BMS754807 (**B**). After 3 days, medium was aspirated and the cells washed with 0.5 ml ice cold PBS. PBS was aspirated and the cells stored in -20^{o}C. The cells were lysed in SSC/SDS solution and the amount of DNA in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM, * denotes statistically significantly lower levels of DNA in cells treated with BMS754807 and AZD4547 compared to cells treated with AZD4547 alone. (Two-way ANOVA; (**A**) $p \leq 0.0057$, (**B**) $p < 0.0001$).

The above experiments suggest that SNU-16 cells are responsive to inhibition of the IGF signal transduction pathway and combination of IGF-IR/IR inhibition with FGFR2 inhibition may be more effective than inhibition of either receptor alone. However, optimal selection of the concentrations of the two inhibitors is important.

7.2.6 The effect of IGF-IR and IR inhibition on viability of MET-amplified cells treated with crizotinib

Crizotinib is a small-molecule tyrosine kinase inhibitor of MET, ALK and ROS1 kinases (Christensen *et al.*, 2007). In Met-amplified SNU-5 cells, crizotinib reduced significantly cell number at 0.002 µM and higher (Figure 7.9A). There was a small but non-significant increase in the number of SNU-5 cells treated with IGF-1 in the presence of 0.002 and 0.06 µM crizotinib (Figure 7.9B). At higher concentrations of crizotinib, IGF-1 did not confer any protective effect (Figure 7.9B).

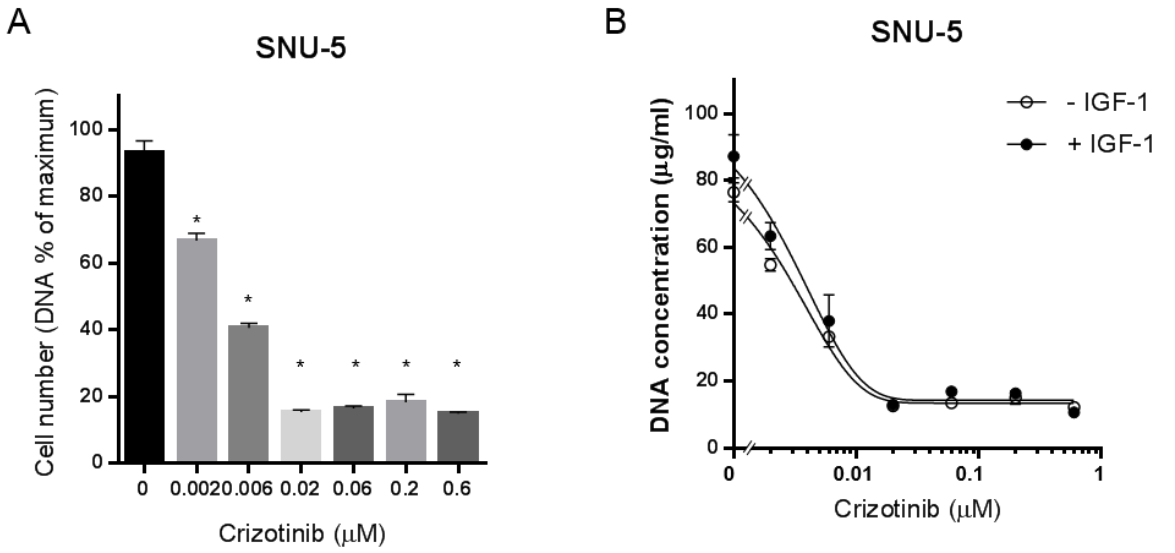

Figure 7.9. The effect of IGF-1 on treatment with crizotinib in Met-amplified cells. SNU-5 cells were seeded into 24 well tissue culture plates at $5x10^4$ cells per well, in 0.5 ml maintenance medium, which contained 0, 0.002, 0.006, 0.02, 0.06, 0.2 or 0.6 µM crizotinib. After 3 days, medium was aspirated and the cells washed with 0.5 ml ice cold PBS. PBS was aspirated and the cells stored in -20^{o}C. The cells were lysed in SSC/SDS solution and the amount of DNA in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM, * denotes statistically significantly higher levels of DNA in cells treated with crizotinib compared to untreated cells (Two-way ANOVA; $p<0.0001$) (**A**). SNU-5 cells were seeded into 24 well tissue culture plates at $5x10^4$ cells per well, in 0.5 ml maintenance medium, which contained 0, 0.002, 0.006, 0.02, 0.06, 0.2 or 0.6 µM crizotinib in the presence or absence of 50 ng/ml IGF-1. After 3 days, medium was aspirated and the cells washed with 0.5 ml ice cold PBS. PBS was aspirated and the cells stored in -20^{o}C. The cells were lysed in SSC/SDS solution and the amount of DNA in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM (**B**).

To test whether inhibition of the IGF signal transduction pathway affects the response of SNU-5 cells to crizotinib, SNU-5 cells were treated with different concentrations of crizotinib in the presence or absence of BMS754807 and the amount of DNA was measured. Treatment with 0.5 µM BMS754807 did not reduce the number of SNU-5 cells in the absence of crizotinib (Figure 7.10A). In the presence of different concentrations of crizotinib, treatment with 0.5 µM BMS754807 did not reduce further the amount of DNA (Figure 7.10A).

Treatment of SNU-5 cells with 5 μM BMS754807 reduced the number of SNU-5 cells to 35% in the absence of crizotinib (Figure 7.10B). Treatment with 5 μM BMS754807 in combination with low concentrations of crizotinib (up to 0.006 μM), reduced significantly the number of cells (Figure 7.10). At higher concentrations of crizotinib ranging from 0.02 to 0.6 μM, the above benefit was neutralised (Figure 7.10B). The above results suggest that addition of BMS754807 does not augment the growth inhibitory effect of high concentrations of crizotinib in SNU-5 cells.

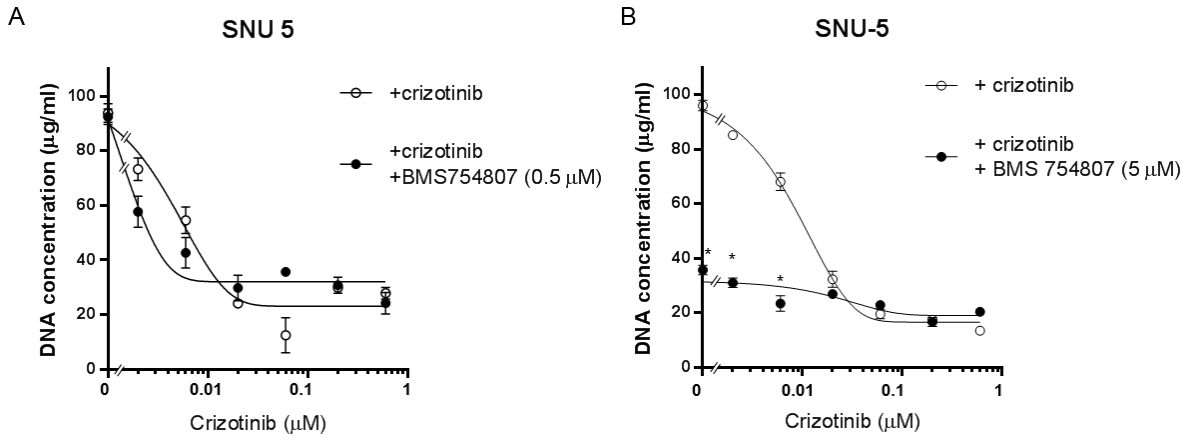

Figure 7.10. The effect of combining BMS754807 with different concentrations of crizotinib in SNU-5 cells. SNU-5 cells were seeded into 24 well tissue culture plates at $5x10^4$ cells per well, in 0.5 ml maintenance medium, which contained 0, 0.002, 0.006, 0.02, 0.06, 0.2 or 0.6 μM crizotinib in the presence or absence of 0.5 μM (**A**) or 5 μM (**B**) BMS754807. After 3 days, medium was aspirated and the cells washed with 0.5 ml ice cold PBS. PBS was aspirated and the cells stored in -20^{o}C. The cells were lysed in SSC/SDS solution and the amount of DNA in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM, * denotes statistically significantly lower levels of DNA in cells treated with BMS754807 and crizotinib compared to cells treated with crizotinib alone. (Two-way ANOVA; (**B**) $p<0.0001$).

7.2.7 The effect of EGF and IGF-1 on survival of HER-2 amplified cells

As mentioned in chapter 4, HER-2-amplified NCI-N87 gastric cancer cells are protected by IGF-1 against cell death induced by protein kinase inhibition (Figure 4.3). To investigate the response of NCI-N87 cells to combined treatment with IGF-1 and epidermal growth factor, NCI-N87 cells were treated with staurosporine in the presence or absence of IGF-1 and EGF, separately and in combination.

Cleavage of PARP was detected in NCI-N87 cells treated with staurosporine in the absence of IGF-1 and EGF (Figure 7.11). Treatment of the cells with EGF reduced significantly the amount of cleaved PARP to approximately 40% and a similar effect was observed with IGF-1. Most effective protection against staurosporine-induced PARP cleavage was achieved after concomitant treatment with EGF and IGF-1 (Figure 7.11). Double treatment resulted in

reduction of cleaved PARP amount to 10% of the amount detected in the absence of the two growth factors.

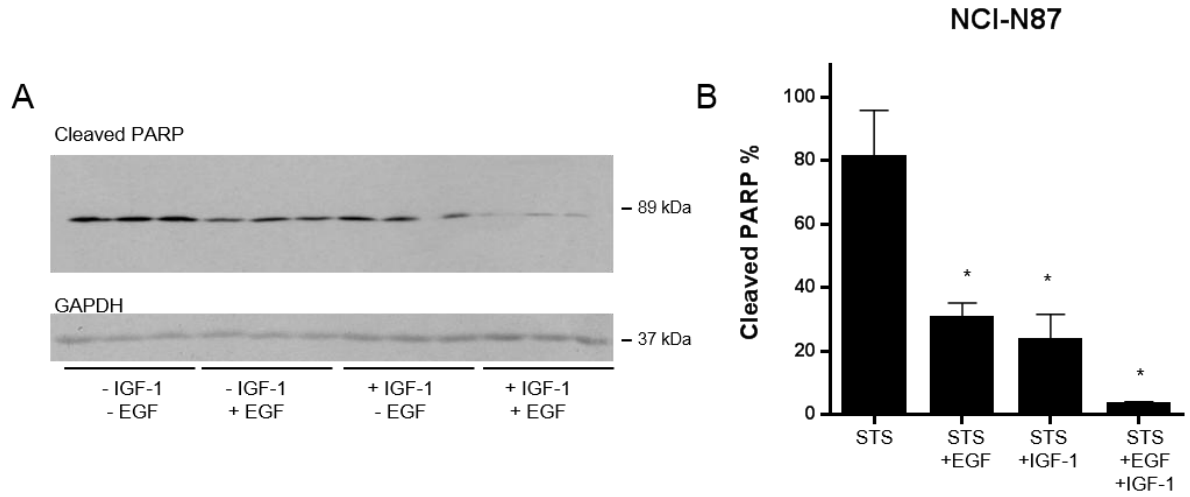

Figure 7.11. The effect of EGF and IGF-1 on cell survival in HER-2-amplified NCI-N87 cells. NCI-N87 cells were seeded into 12 well tissue culture plates at 15 x 10^4 cells per well, in 1 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 0.5 ml PBS. Medium was replaced daily with 1 ml withdrawal medium for two days. Cells were incubated with withdrawal medium supplemented or not with 50 ng/ml EGF, 50 ng/ml IGF-1 or both, for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of 50 ng/ml EGF, 50 ng/ml IGF-1 or both for 4 hours. Proteins were extracted with 80 µl of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against cleaved PARP (1:2000 dilution) and GAPDH (1:10000 dilution) (**A**). Densitometry was with Labworks 4.0 software, adjusted for the background optical density of the X-ray film and normalised to GAPDH. Results are expressed as a percentage of the intensity of the cleaved PARP band observed in the absence of EGF and IGF-1. Bars are the mean ±SEM, * denotes statistically significantly less cleaved PARP in the presence of EGF, IGF-1 or both than in their absence (One-way ANOVA; $p \leq 0.0002$) (**B**).

The above result suggests that despite the amplification of human epidermal growth factor receptor (HER-2) in NCI-N87 cells, they are still responsive to IGF-1. Also, treatment with both growth factors is more effective at inhibition of cell death than treatment with each growth factor alone. It would, therefore, be interesting to investigate the response of NCI-N87 cells to inhibition of the IGF signal transduction pathway in the presence of a HER-2 inhibitor.

7.2.8 The effect of treatment with lapatinib on viability of HER-2 amplified cells with and without lapatinib resistance

As mentioned previously, approximately 50% of patients with HER-2 positive advanced gastric cancer do not respond to trastuzumab treatment and approximately 12% develop progressive disease (Bang *et al.*, 2010). Involvement of other signal transduction pathways,

such as Met, HER3 and the IGF pathways, in primary resistance to lapatinib, a dual tyrosine kinase inhibitor against HER2 and EGFR, has been reported previously (Zhang *et al.*, 2014). We wanted to test whether the IGF signal transduction pathway is involved in acquired resistance of HER-2 amplified gastric cancer cells to lapatinib.

Resistance to lapatinib was achieved by continuous treatment of NCI-N87 cells with increasing concentrations of lapatinib for 6 months. The sensitivity of non-resistant and resistant cells to lapatinib was analysed by quantification of the amount of DNA after treatment with different concentrations of lapatinib. In non-resistant NCI-N87 cells, treatment with lapatinib reduced the amount of DNA in a concentration dependent manner (Figure 7.12). The IC_{50} of lapatinib for those cells was close to 90 nM. In NCI-N87/lapatinib resistant cells, the effectiveness of lapatinib in reduction of cell number was reduced significantly. In those cells, the IC_{50} of lapatinib was increased to approximately 9 µM. This suggests that the cells had lost their sensitivity to the drug and could therefore be used as models of resistant disease.

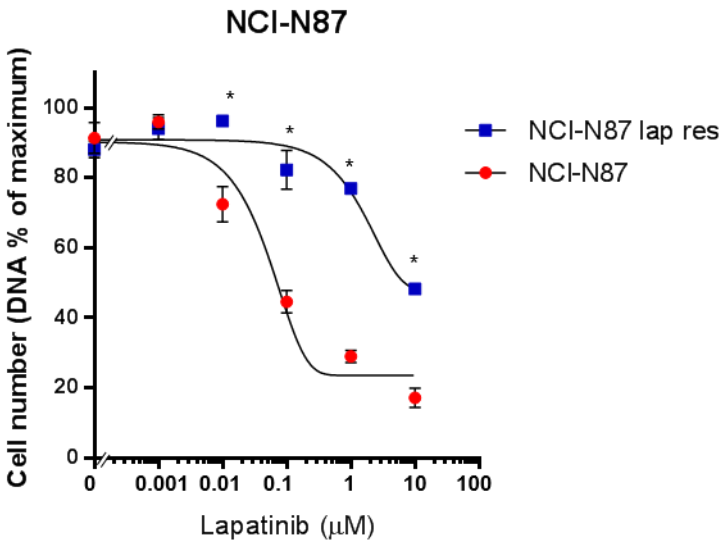

Figure 7.12. Effect of treatment with different concentrations of lapatinib on parental NCI-N87 cells and on NCI-N87 cells which have developed a secondary resistance to lapatinib. NCI-N87 and NCI-N87/lapatinib resistant cells were seeded into 24 well tissue culture plates at $5x10^4$ cells per well, in 0.5 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was replaced to maintenance medium which contained 0, 0.001, 0.01, 0.1, 1 or 10 µM lapatinib. After 3 days, medium was aspirated and the cells washed with 0.5 ml ice cold PBS. PBS was aspirated and the cells stored in -20°C. The cells were lysed in SSC/SDS solution and the amount of DNA in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM, * denotes statistically significantly higher amount of DNA in lapatinib resistant cells compared to non-resistant cells (Two-way ANOVA; $p \leq 0.0001$).

7.2.9 The effect of treatment with lapatinib on signal transduction in HER-2 amplified cells with and without resistance to lapatinib

To test the effect of lapatinib on lapatinib-resistant and non-resistant NCI-N87 cells, both cell lines were cultured in the presence or absence of lapatinib and phosphorylation of key proteins was analysed.

In non-resistant NCI-N87 cells, treatment with lapatinib reduced effectively HER-2 phosphorylation (Figure 7.13). In lapatinib-resistant NCI-N87 cells, the basal levels of HER-2 phosphorylation were reduced compared to non-resistant cells. Moreover, treatment with lapatinib did not seem to affect the amount of phosphorylated HER-2. The amount of total HER-2 was not affected by acquisition of resistance to lapatinib or by treatment with lapatinib.

To investigate potential involvement of the IGF signal transduction pathway in acquisition of resistance to lapatinib, phosphorylation of IGF-IR and IR was analysed in resistant and non-resistant cells. There were no differences between IGF-IR and IR phosphorylation levels in resistant and non-resistant cells (Figure 7.13). Also, the amount of total IGF-IR was not affected by acquisition of resistance to lapatinib or by treatment with lapatinib.

Activation of downstream signalling pathways in response to lapatinib treatment was also investigated. In non-resistant NCI-N87 cells, treatment with lapatinib reduced effectively the amount of phosphorylated Akt. However, in cells with resistance to lapatinib, Akt phosphorylation was not reduced after lapatinib treatment (Figure 7.13). A similar result was obtained with ERK1 and ERK2 phosphorylation. Both total Akt and total ERK1 and ERK2 levels were not altered following acquisition of resistance to lapatinib.

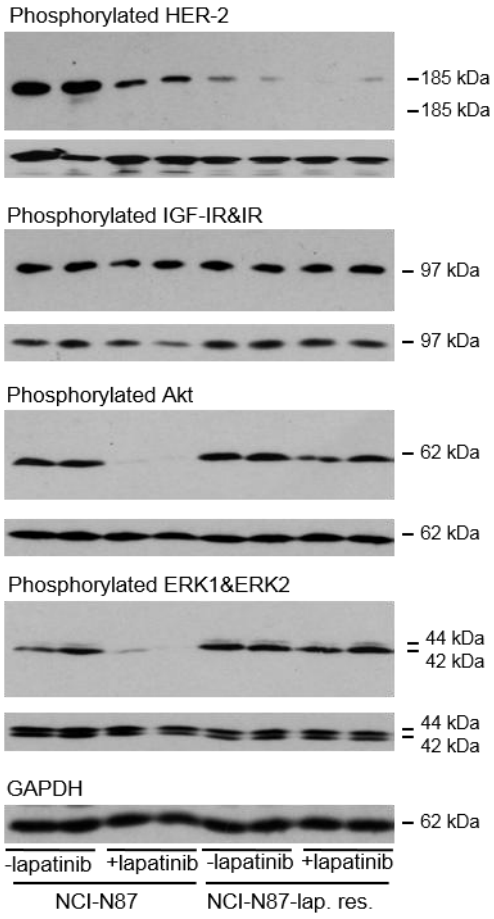

Figure 7.13. The effect of lapatinib on signal transduction in NCI-N87 cells and NCI-N87- lapatinib-resistant cells. NCI-N87 and NCI-N87/lapatinib resistant cells were seeded into 12 well tissue culture plates at 1.5 x 10^5 cells per well, in 1 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was replaced to maintenance medium which contained 0.2 µM lapatinib. After 24 hours, proteins were extracted with 80 µl of RIPA buffer plus inhibitors per well. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against phosphorylated HER-2 (1:1000 dilution), HER-2 (1:1000 dilution), phosphorylated IGF-IR&IR (1:1000 dilution), IGF-IR (1:2000 dilution), phosphorylated Akt (1:2000 dilution), Akt (1:2000 dilution), phosphorylated ERK1&ERK2 (1:5000), ERK1&ERK2 (1:5000 dilution) and GAPDH (1:10000 dilution). Images obtained after incubation with antibodies against the total proteins are shown beneath those of the phosphorylated proteins.

The above results suggest that NCI-N87 cells with an acquired resistance to lapatinib have lost their dependency on the HER-2 receptor and are able to stimulate the PI3K/Akt and Ras/Raf/MAPK pathway independent of HER-2 activation. Resistance to lapatinib-induced inhibition of Akt and ERK1 and ERK2 activation is not mediated by increased IGF-IR and IR phosphorylation.

7.2.10 *The effect of treatment with BMS754807 on viability of HER-2 amplified cells with and without resistance to lapatinib*

To investigate if inhibition of the IGF-signal transduction pathway is effective in HER-2 amplified cells, with and without secondary resistance to lapatinib, NCI-N87 and NCI-N87/lapatinib-resistant cells were cultured with different concentrations of BMS754807 and the amount of DNA was measured. In NCI-N87 cells, treatment with BMS754807 reduced the number of cells in a concentration-dependent manner. Significant reduction in cell number was achieved with 1 and 10 µM BMS754807 (Figure 7.14). In NCI-N87/lapatinib-resistant cells, the cytotoxic effect of BMS754807 was identical to non-resistant cells, at 1 µM BMS754807. When the cells were treated with 10 µM BMS754807, the amount of DNA was reduced significantly in lapatinib-resistant cells compared to non-resistant cells (p=0.0029). This suggests that NCI-N87 cells with acquired resistance to lapatinib, are more sensitive to high concentrations of the BMS754807 inhibitor than non-resistant cells.

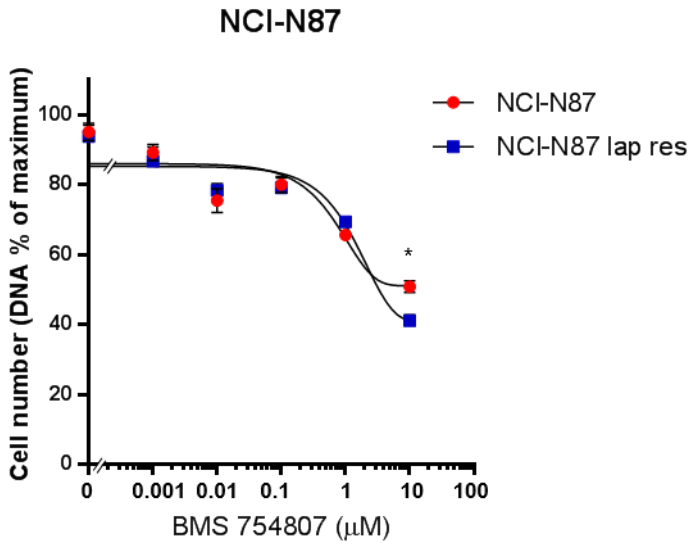

Figure 7.14. Effect of treatment with different concentrations of BMS754807 on viability of NCI-N87 cells and NCI-N87 lapatinib resistant cells. NCI-N87 and NCI-N87/lapatinib-resistant cells were seeded into 24 well tissue culture plates at $5x10^4$ cells per well, in 0.5 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was replaced to maintenance medium which contained 0, 0.001, 0.01, 0.1, 1 or 10 µM BMS754807. After 3 days, medium was aspirated and the cells washed with 0.5 ml ice cold PBS. PBS was aspirated and the cells stored in -20°C. The cells were lysed in SSC/SDS solution and the amount of DNA in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM, * denotes statistically significantly lower amount of DNA in lapatinib-resistant cells that had been treated with BMS754807compared to non-resistant cells (Two-way ANOVA; p=0.0029).

7.3 Discussion

SNU-1 and NUGC3 cells were sensitive to treatment with oxaliplatin and 5-fluorouracil, as indicated by the reduction of cell number and induction of PARP cleavage. The IC_{50}s for both

cell lines were higher for 5-fluorouracil than for oxaliplatin, which suggests that oxaliplatin had a more potent cytotoxic effect. Treatment with IGF-1 did not rescue the cells from the cytotoxic effect of oxaliplatin, in the presence of other growth factors found in the serum. It is possible that some IGF-1 was already present in the serum and the externally added IGF-1 did not confer any additional changes in cell growth. Alternatively, it could be concluded that IGF-1 is unable to rescue gastric cancer cells from the cytotoxic effects of oxaliplatin in a growth factor enriched environment.

Combined inhibition of the type I IGF receptor by siRNA knockdown with oxaliplatin treatment resulted in higher amounts of cleaved PARP, compared to the additive effect of the two treatments. However, when SNU-1 and NUGC3 cells were treated with different concentrations of oxaliplatin, addition of BMS754807 did not reduce further the number of cells. There is, therefore, an inconsistency between the results obtained in the genetic level and the pharmacological level. It is possible that different time-frames are required for the treatments to show an effect at each level. For example, induction of PARP cleavage might precede the reduction in cell number which could be detectable at later time points. It would be useful to investigate different time-frames of treatment with oxaliplatin in combination with siRNA knockdown or BMS754807.

Addition of BMS754807 to SNU-1 and NUGC3 cells treated with different concentrations of 5-fluorouracil, reduced further the number of cells even at the higher range of 5-fluorouracil concentrations. Combined use of IGF-IR inhibition with cytotoxic drugs has proven beneficial in preclinical models of various cancer types. Combination of PQIP, a tyrosine kinase inhibitor which targets IGF-IR/IR, with oxaliplatin and fluorouracil, showed synergistic inhibition of colorectal cancer cell proliferation (Flanigan *et al.*, 2010). Combination of robatumumab (SCH 717454), an IGF-IR targeted antibody, with cisplatin was significantly more effective than either agent alone at reducing the volume of paediatric tumour xenografts (Wang *et al.*, 2010b). Downregulation of IGF-IR with antisense oligonucleotide treatment increased significantly chemosensitivity of central nervous system atypical teratoid/rhabdoid tumours cells to cisplatin (D'Cunja *et al.*, 2007) and of human colon cancer cells to 5-fluorouracil (Yavari *et al.*, 2010).

In gastric cancer, there is a very limited number of preclinical studies which have investigated the potential of combined inhibition of the IGF signal transduction pathway with chemotherapy. Wang et al., have shown that reduction of IGF-IR expression with a dominant negative adenovirus increases cisplatin-induced apoptosis in MKN45, Met-amplified, gastric

cancer cells (Wang *et al.*, 2010a). Min et al., found that reduction of IGF-IR expression with a dominant negative adenovirus, increased 5-fluorouracil-induced apoptosis in MKN45 cells. Combination of the two treatments was more effective than either therapy alone, at inhibiting growth of tumours, which were induced by subcutaneous injection of MKN45 cells in mice (Min *et al.*, 2005). Our results are, therefore, the first to investigate the potential of combining IGF signal transduction pathway inhibition with chemotherapy in un-amplified gastric cancer cells.

FGFR2-amplified gastric cancer cell lines KATO III and SNU-16 were extremely sensitive to the AZD4547 inhibitor, with IC_{50} values lower than 10 nM. The effectiveness of AZD4547 in inhibition of KATO III and SNU-16 cell growth has been shown previously (Xie *et al.*, 2013). The same group have reported a significant concentration-dependent tumour growth inhibition in FGFR2-amplified xenografts treated with AZD4547.

Our results show that IGF-1 afforded a small protection against AZD4547 treatment in SNU-16 cells, but not KATO III cells, even in the presence of other growth factors. Further, treatment of SNU-16 cells with low concentrations of BMS754807 equal to 0.5 μM, enhanced the growth inhibitory effects of AZD4547, which suggests that SNU-16 cells are more responsive to activation of the IGF signal transduction pathway compared to KATO III, possibly due to higher IGF-IR expression in those cells (Figure 3.1). Involvement of other tyrosine kinase receptors, such as EGFR, HER3 and MET in attenuation of the efficacy of AZD4547 on SNU-16 cells has been reported previously (Chang *et al.*, 2015). However, our results are the first to suggest that combined inhibition of FGFR2 and IGF-IR/IR might be more effective in SNU-16 cells than either treatment alone.

The growth inhibitory effect of crizotinib on Met-amplified, SNU-5 cells was potent. This is supported by a study in which treatment with crizotinib reduced the volume of tumour xenografts established by subcutaneous injection of SNU-5 cells (Okamoto *et al.*, 2012). IGF-1 did not protect SNU-5 cells against the growth inhibitory effect of crizotinib and treatment with the BMS754807 inhibitor did not enhance the effect of crizotinib. SNU-5 cells express very low amounts of the type I IGF receptor, which might explain the lack of their responsiveness to IGF pathway inhibition.

Combined inhibition of IGF-IR and HER-2 has been shown previously to have synergistic effects in breast cancer cells. Combination of trastuzumab with NVP-AEW541, a tyrosine kinase inhibitor which targets IGF-IR and IR, was more effective at inhibiting BT474 cell proliferation than either agent alone (Esparis-Ogando *et al.*, 2008). A bispecific antibody

which targeted both HER-2 and IGF-IR was more effective at inhibiting proliferation of breast cancer cells and tumour growth in mice compared to trastuzumab alone or trastuzumab in combination with m590, a monoclonal antibody against the type I IGF receptor (Chen *et al.*, 2014).

In gastric cancer, the IGF signal transduction pathway has been reported to be involved in mediating primary resistance of HER-2-amplified-gastric cancer cells, NCI-N87, to lapatinib and combined treatment with lapatinib and AEW-541, a tyrosine kinase inhibitor against IGF-IR and IR, resulted in reduced cell viability and reduced tumour volume in mice compared to either inhibitor alone (Zhang *et al.*, 2014). In trastuzumab-resistant NCI-N87 cells, there was increased IGF-IR activation, which suggested that the type I IGF receptor might be involved in acquisition of resistance to trastuzumab (Zuo *et al.*, 2015).

In our study, acquisition of resistance to lapatinib was accompanied by a reduction in phosphorylated HER-2. Also, lapatinib had lost its ability to inhibit Akt and ERK1 and ERK2 phosphorylation in resistant cells. Activation of the type I IGF and insulin receptors was not altered in NCI-N87/lapatinib-resistant cells. However, treatment with high concentrations of BMS754807 inhibitor, which targets the type I IGF and insulin receptors, was more effective at inhibiting growth of NCI-N87/lapatinib-resistant cells compared to non-resistant cells. This suggests that NCI-N87/lapatinib resistant cells are more susceptible to inhibition of the IGF signal transduction pathway, although IGF-IR/IR phosphorylated and total levels are not increased. Alterations in adaptor protein expression and activation e.g. IRS 1, 2, 4 in resistant cells, may have increased the dependency of the cells on the IGF signal transduction pathway. Investigation of expression and activation of adaptor proteins in resistant cells could clarify the above hypothesis. Also, loss of HER-2 phosphorylation in lapatinib resistant cells might have made more PI3K subunits or Ras molecules available to the IGF signal transduction pathway, resulting in increased sensitivity of the phosphorylated IGF-IR, although the levels of phosphorylation were not affected.

In summary, the results reported in this chapter suggest that there may be potential for concomitant treatment of SNU-1 and NUGC3 cells with 5-fluorouracil and BMS754807, as demonstrated by the reduction of cell number after treatment with the combination of the two drugs. Also, the small molecule tyrosine kinase inhibitor BMS754807 improves the efficiency of the anti-FGFR2 inhibitor AZD4547, in FGFR2-amplified SNU-16 cells. Finally, NCI-N87/lapatinib-resistant cells may be more sensitive to IGF-IR/IR inhibition than non-resistant cells.

Chapter 8. Importance of the IGF signal transduction pathway in primary cultures of gastric cancer cells

8.1 Introduction

The term ascites describes the pathological accumulation of excessive fluid inside the peritoneal cavity (Parsons *et al.*, 1996b). Approximately 10% of all ascites cases are caused by malignant disease. Malignant ascites presents as an end-stage event in a variety of cancers and is associated with significant morbidity (Saif *et al.*, 2009). The most common cancer types that may result in development of ascites are ovarian, endometrial, breast, oesophageal, gastric, colorectal, lung, pancreatic, hepatobillary and primary peritoneal carcinomas (Runyon, 1994). Approximately 50% of patients with malignant ascites present with it at the initial diagnosis of their cancer (Garrison *et al.*, 1986; Parsons *et al.*, 1996a).

Management of patients with ascites ranges from drainage procedures to chemotherapy. The quality of life and, sometimes, the survival of patients with malignant ascites can be improved with appropriate combination chemotherapy (Buckman *et al.*, 1992).

Established, immortalised cancer cell lines are an invaluable tool for the study of molecular and cellular biology underpinning cancer. However, it can be argued that established cell lines represent a simplified model for the study of cancer, as they comprise isolated cells, which are no longer in contact with their surrounding microenvironment and have been growing in culture indefinitely. In this sense, *ex vivo* primary cultures are one step closer to the organism level and could provide valuable models for testing the efficiency of targeted therapies. Previously, primary cultures of cancer cells have been established from ascites isolated from patients with ovarian cancer (Shepherd *et al.*, 2006; Theriault *et al.*, 2013).

8.1.1 Aims

The aim of the results reported in this chapter is the establishment of a model of advanced gastric cancer, based on the utilisation of primary cultures of gastric cancer cells derived from ascites of patients with advanced gastric adenocarcinoma. Expression and activation of receptors and downstream molecules of the IGF-signal transduction pathway was investigated in the above primary cultures. The role of IGF-1 and the type I IGF receptor in cell survival and proliferation was tested. The effectiveness of the small molecule tyrosine kinase inhibitor BMS754807 in reduction of cell viability was also investigated.

8.2 Results

8.2.1 The relative expression of IGF-signal transduction pathway proteins in primary cultures of gastric cancer cells

Four primary cultures of gastric cancer cells were established from the ascitic fluid collected from four patients diagnosed with advanced gastric adenocarcinoma. The appearance of cells in culture within two weeks of isolation from patients is shown in Figure 8.1. Tumour cells had varied morphology but most grew with a characteristic epithelial cell, pavement-like appearance as monolayers in culture. Their epithelial origin was confirmed by expression of total keratins 4, 5, 6, 8, 10, 13 and 18 (Figure 8.2). The epithelial cells proliferated until passage 5-6, with a doubling time of approximately 7 days. All experiments were performed with passage numbers lower than 6.

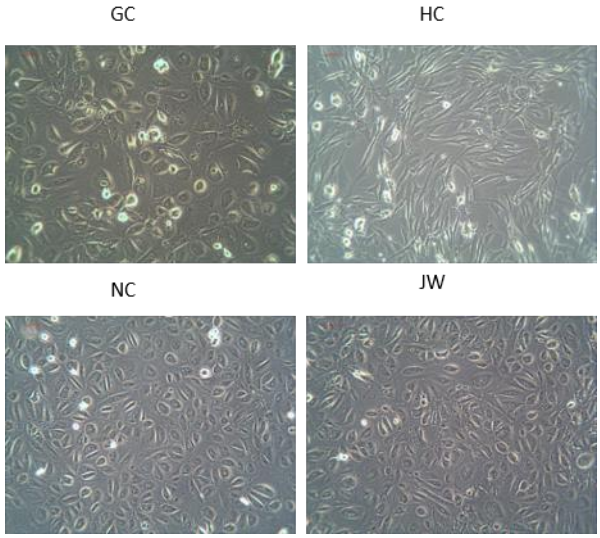

Figure 8.1. Primary cultures of gastric cancer cells established from ascites of patients with gastric adenocarcinoma. Ascitic fluid was collected from patients with advanced gastric adenocarcinoma and was diluted 1:1 in medium supplemented with 20% foetal calf serum. After 5-7 days the medium was changed to maintenance medium with 20% foetal calf serum and the adherent cells were either kept in culture or frozen. Four primary cultures were established, GC, HC, NC and JW. The primary cells were seeded on top of coverslips in 6 well tissue culture plates at 30 x 10^4 cells per well, in 2.5 ml maintenance medium. Cells were incubated for 7 days. Medium was replaced every two to three days. Cells were washed with 2 ml PBS and fixed in 2 ml ice-cold methanol. Fixed cells were incubated with a fluorescent pan-keratin antibody (1:50 dilution) and cleaved PARP (1:50 dilution). Cells were mounted on top of slides in 20 µl DAPI X mounting medium. Pictures of the cells were taken with a Leica fluorescent microscope.

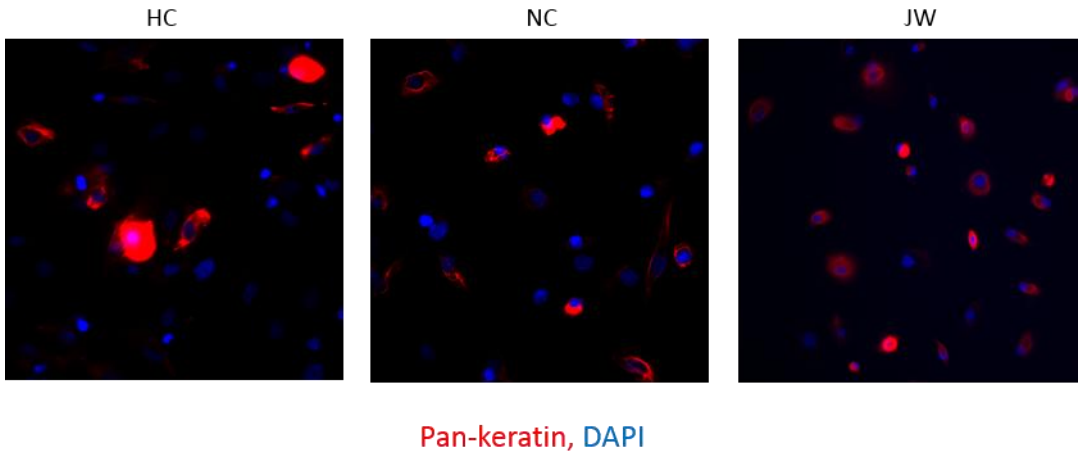

Figure 8.2. Expression of keratins in primary cultures of gastric cancer cells. HC, NC and JW cells were seeded on top of coverslips in 6 well tissue culture plates at 15 x 10^4 cells per well, in 2.5 ml maintenance medium. Cells were incubated for 5-7 days. Cells were washed with 2 ml PBS and fixed in 2 ml ice-cold methanol. Fixed cells were incubated with a fluorescent pan-keratin antibody that detects total keratins 4, 5, 6, 8, 10, 13 and 18 (1:50 dilution). Cells were mounted on top of slides in 20 µl DAPI X mounting medium. Pictures of the cells were taken in a Leica fluorescent microscope.

Expression of the receptors of the IGF-signal transduction pathway and of downstream molecules was measured in the four primary cultures by western transfer analysis. GC, HC, NC and JW cells were cultured until 80% confluence and protein extracts were prepared.

Highest IGF-IR expression was detected in NC cells, followed by JW, GC and HC (Figure 8.3). In general, the levels of IGF-IR expression in the primary cultures were similar to the levels detected in AGS cells. A small amount of insulin receptor was detected in all primary cultures; NC cells expressed the highest amount, followed by HC, JW and GC. HC cells expressed the highest amount of type II IGF receptor, whereas NC, JW and GC cells did not express much IGF-IIR (Figure 8.3). The receptors of the IGF signal transduction pathway were expressed in those cells, which suggests that the pathway might be active.

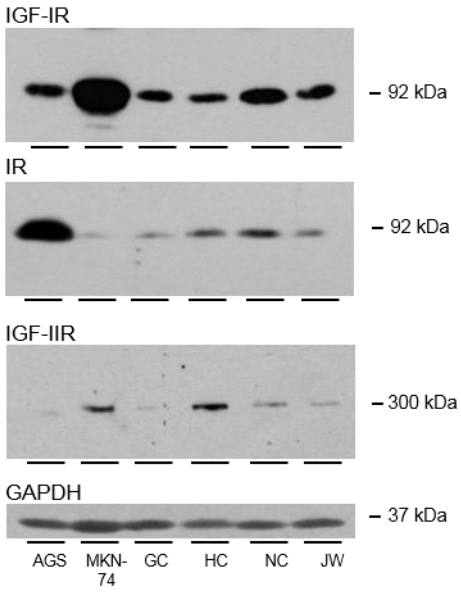

Figure 8.3. Expression of IGF-signal transduction pathway proteins in primary cultures of gastric cancer cells. GC, HC, NC and JW cells were cultured in 75 cm^2-tissue culture flasks in maintenance medium to ~80% confluence. Proteins were extracted with 1 ml per flask of RIPA buffer plus inhibitors, as described in the Materials and Methods section. Aliquots containing 10-20 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against the IGF-IR (1:2000), IR (1:1000), IGF-IIR (1:1000), and GAPDH (1:10000). All secondary antibodies were horseradish-peroxidase bound. Bands were visualised on Fuji X-ray film following incubation of membranes with Supersignal chemiluminescent solutions, as described in the Methods section. The images shown are representative of results from three separate experiments. The molecular masses of the proteins are indicated on the right hand side of the panels.

To detect the amount of the two insulin receptor isoforms present in the primary cultures of gastric cancer cells, specific DNA primers were designed to amplify both the A and B isoform of the receptor. Both isoforms of the insulin receptor were detected in GC, HC and NC cells, and all three primary cultures expressed higher levels of the B isoform compared to the A isoform (Figure 8.4). Also, in comparison with SNU-16 and KATO III cells, the IRA:IRB ratio was much lower in the primary cultures.

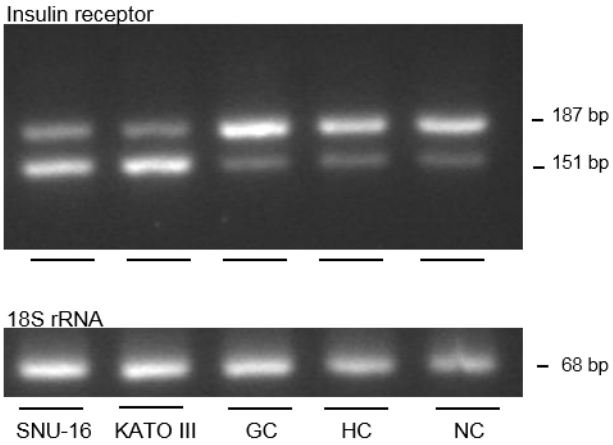

Figure 8.4. Expression of insulin receptor isoforms in primary cultures of gastric cancer cells. SNU-16, KATO III, GC, HC and NC cells were cultured in 25 cm^2 tissue culture flasks in maintenance medium to ~80% confluence. Total RNA was extracted from the cells with the Direct-zol™ RNA MiniPrep, as described in the Materials and Methods section. Complementary DNA was synthesised from total RNA with the Moloney Murine Leukaemia Virus Reverse Transcriptase. Specific primer sequences were used to amplify both insulin receptor isoforms and 18S rRNA. The amplified DNA was electrophoresed in a 3% agarose gel and bands were visualised with a UV transilluminator.

There was lower expression of IRS-1 in GC, HC, NC and JW cells, compared to AGS and MKN74 cells (Figure 8.5). Akt, ERK1 and ERK2 were expressed in all four primary cultures in similar levels to those detected in the gastric cancer cell lines (Figure 8.5). Expression of IRS-1 mRNA was detected in all four primary cultures and was similar to the AGS cell line (Figure 8.6). This suggests that there are either differences between the mRNA and protein expression of IRS-1 in the primary cultures, possibly due to post-translational modifications in the IRS-1 protein, or there is a difference in the sensitivity of the two assays.

Due to the lack of high quality commercially available antibodies against IRS-2 and IRS-4 proteins, expression of the above genes was analysed by PCR (Figure 8.6). Expression of both IRS-2 and IRS-4 was detected in all primary cultures, with HC cells expressing slightly higher levels of IRS-4 compared to the other two primary cultures.

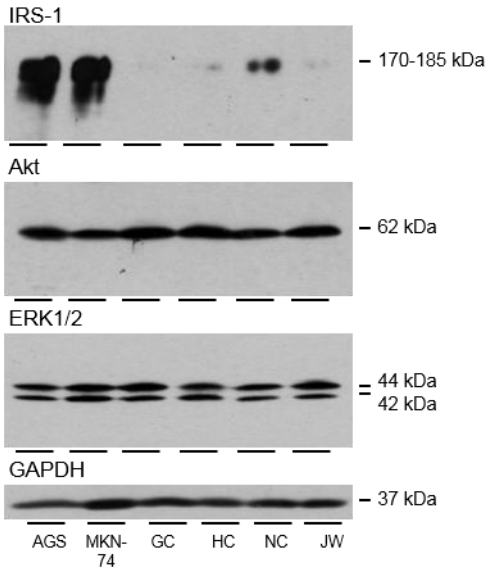

Figure 8.5. Expression of IGF-signal transduction pathway downstream proteins in primary cultures of gastric cancer cells. GC, HC, NC and JW cells were cultured in 75 cm^2-tissue culture flasks in maintenance medium to ~80% confluence. Proteins were extracted with 1 ml per flask of RIPA buffer plus inhibitors, as described in the Materials and Methods section. Aliquots containing 10-20 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against IRS-1 (1:2000), Akt (1:3000) ERK1/2 (1:5000) and GAPDH (1:10000). All secondary antibodies were horseradish-peroxidase bound. Bands were visualised on Fuji X-ray film following incubation of membranes with Supersignal chemiluminescent solutions, as described in the Methods section. The images shown are representative of results from three separate experiments. The molecular masses of the proteins are indicated on the right hand side of the panels.

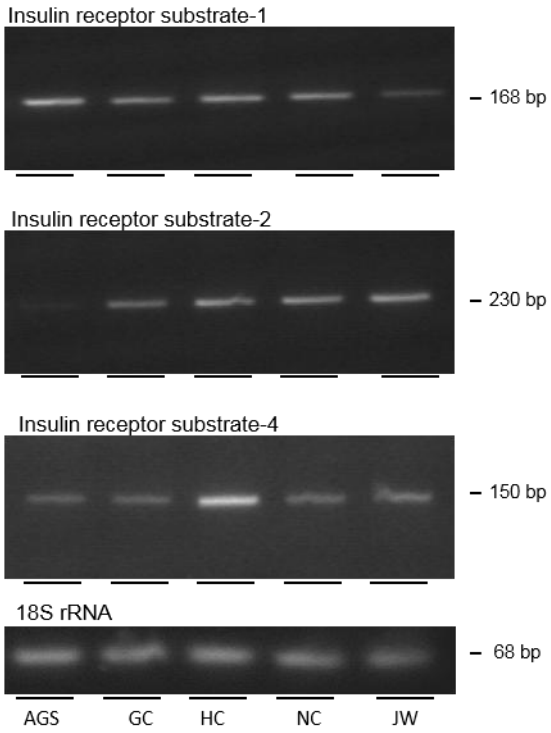

Figure 8.6. Expression of insulin-receptor substrate proteins (IRS) in primary cultures of gastric cancer cells. AGS, GC, HC, NC and JW cells were cultured in 25 cm^2 tissue culture flasks in maintenance medium to ~80% confluence. Total RNA was extracted from the cells with the Direct-zol™ RNA MiniPrep, as described in

the Materials and Methods section. Complementary DNA was synthesised from total RNA with the Moloney Murine Leukaemia Virus Reverse Transcriptase. Specific primer sequences were used to amplify IRS-1, IRS-2, IRS-4 and 18S rRNA. The amplified DNA was electrophoresed in a 3% agarose gel and bands were visualised with a UV transilluminator.

8.2.2 The relative expression of HER-2, FGFR2 and c-Met in primary cultures of gastric cancer cells

To identify potential amplification or overexpression that could affect responsiveness to IGF ligands, expression of the three most frequently amplified growth factor receptors in gastric cancer, HER-2, FGFR2 and c-Met, was measured in the four primary cultures of gastric cancer cells. None of those three receptors were detected by western transfer analysis in the four primary cultures (Figure 8.7).

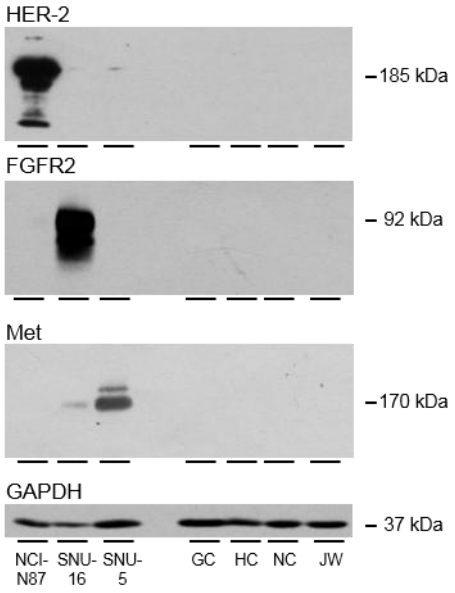

Figure 8.7. Expression of HER-2, FGFR2 and c-Met in primary cultures of gastric cancer cells. GC, HC, NC and JW cells were cultured in 75 cm^2 tissue culture flasks in maintenance medium to ~80% confluence. Proteins were extracted with 1 ml per flask of RIPA buffer plus inhibitors, as described in the Materials and Methods section. Aliquots containing 10-20 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against the HER-2 (1:2000), FGFR2 (1:1000) and c-Met (1:1000). All secondary antibodies were horseradish-peroxidase bound. Bands were visualised on Fuji X-ray film following incubation of membranes with Supersignal chemiluminescent solutions, as described in the Methods section. The molecular masses of the proteins are indicated on the right hand side of the panels.

The four primary cultures expressed the type I IGF receptor but not HER-2, FGFR2 and c-Met. This suggests that they could possibly be responsive to stimulation with IGF ligands and that the IGF-signal transduction pathway might be important for the survival and growth of these cells.

8.2.3 The effect of IGF-1 stimulation on the activity of IGF-signal transduction pathway proteins in primary cultures of gastric cancer cells

The effect of stimulation with IGF-1 on activation and phosphorylation of components of the IGF signal transduction pathway was tested in GC, HC, NC and JW cells. The cells were withdrawn from the effects of growth factors present in the serum by culture in medium supplemented with dextran-coated charcoal stripped calf serum (DCCS). Phosphorylation of proteins in the IGF signal transduction pathway in cells that had been treated with 50 ng/ml IGF-1 was determined by western transfer.

Phosphorylation of the type I IGF and/or insulin receptor was not detected in any of the primary cultures after culture in growth factor-depleted medium for 48 hours (Figure 8.8). Stimulation with IGF-1 resulted in IGF-IR/IR phosphorylation in all primary cultures. The amount of total IGF-IR was not altered by stimulation with IGF-1 for 15 minutes.

Akt phosphorylation was detected only in JW cells after withdrawal of cells by culture in growth-factor-deprived culture medium. However, stimulation with IGF-1 increased Akt phosphorylation in those cells. GC, HC and NC cells had no detectable levels of Akt phosphorylation after withdrawal. IGF-1 stimulated Akt phosphorylation in all these cells. Total Akt levels were not affected by incubation with IGF-1 for 15 minutes. Phosphorylated ERK2 was detected in the absence of IGF-1 in all the primary cultures, except GC cells. There was no increase in ERK1 or ERK2 phosphorylation after stimulation with IGF-1 in JW cells. Most effective induction of ERK2 phosphorylation was observed in GC cells. The levels of total ERK1 and ERK2 remained unchanged after stimulation with IGF-1 in all primary cultures.

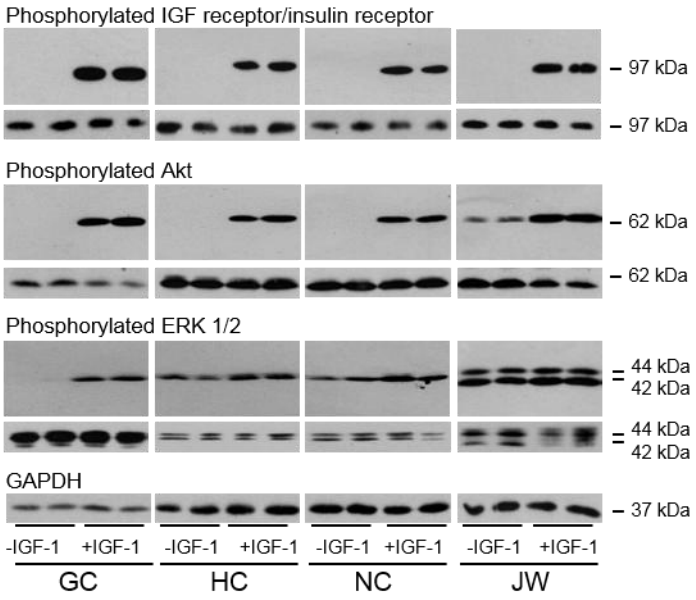

Figure 8.8. The effect of stimulation with IGF-1 on phosphorylation of IGF-signal transduction pathway proteins in primary cultures of gastric cancer cells. GC, HC, NC and JW cells were seeded into 12 well tissue culture plates at $15x10^4$ cells/well, in 1 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 0.5 ml PBS. Medium was replaced daily with 1 ml withdrawal medium for two days. Cells were incubated with serum-free medium which contained 0.1% BSA for two hours. Cells were incubated with 50 ng/ml IGF-1 for 15 minutes. Proteins were extracted with 80 µl per well RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against phosphorylated IGF-IR/IR (1:1000 dilution), IGF-IR (1:2000 dilution), phosphorylated Akt (1:3000 dilution), Akt (1:3000 dilution), phosphorylated ERK1/2 (1:5000 dilution), ERK1/2 (1:5000 dilution) and GAPDH (1:10000 dilution). Images obtained after incubation with antibodies against the total proteins are shown beneath those of the phosphorylated proteins.

8.2.4 The effect of IGF-1 on staurosporine-induced apoptosis in primary cultures of gastric cancer cells

To investigate whether IGF-1 can promote survival in the primary cultures of gastric cancer cells, the ability of IGF-1 to inhibit staurosporine-induced cell death was assessed. GC, HC, NC and JW cells were withdrawn from the effects of growth factors for 2 days and treated with staurosporine in the presence and absence of IGF-1 for 5 hours.

In GC cells, the 89 kDa PARP fragment was detected by western transfer analysis after culture in 0.5 µM staurosporine, in the absence of IGF-1 (Figure 8.9). Treatment with IGF-1 completely inhibited cleavage of PARP to its 89 kDa fragment ($p<0.0001$), suggesting that IGF-1 rescued those cells from apoptosis. Identical results were obtained with HC and NC cells. In JW cells, IGF-1 reduced potently the amount of the 89 kDa fragment of PARP but did not inhibit it completely.

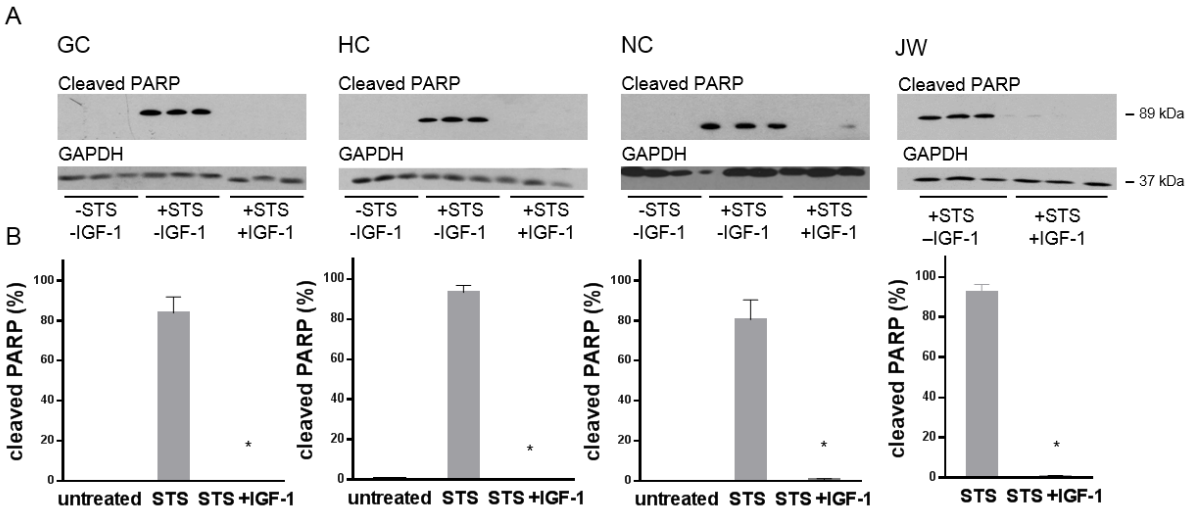

Figure 8.9. The effect of IGF-1 on staurosporine-induced apoptosis in primary cultures of gastric cancer cells. GC , HC, NC and JW cells were seeded into 12 well tissue culture plates at $15x10^4$ cells per well, in 1 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 0.5 ml PBS. Medium was replaced daily with 1 ml withdrawal medium for two days. Cells were incubated with withdrawal medium supplemented or not with 50 ng/ml IGF-1 for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of 50 ng/ml IGF-1 for 6 hours. Proteins were extracted with 80 µl of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against cleaved PARP (1:1000 dilution) and GAPDH (1:10000 dilution) (**A**). Densitometry was with Labworks 4.0 software, adjusted for the background optical density of the X-ray film and normalised to GAPDH. Results are expressed as a percentage of the intensity of the cleaved PARP band observed in the presence of staurosporine and in the absence of IGF-1. Asterisks show cleaved PARP levels that are statistically significantly lower in the presence of IGF-1 than in its absence (One-way ANOVA; GC, $p<0.0001$; HC, $p<0.0001$; NC, $p<0.0001$; JW, $p<0.0001$) (**B**).

8.2.5 The effect of IGF-1 on cell death induced by anoikis in primary cultures of gastric cancer cells

As mentioned previously, anoikis is a form of apoptosis induced by disruption of the interactions between normal epithelial cells and extracellular matrix. Disruption of the interactions can be achieved *in vitro* by culture of cells in plates coated with polyhydroxyethylmethacrylate (poly-HEMA).

To test the hypothesis that IGF-1 is important for resistance to anoikis, GC, HC, NC and JW cells were cultured in non-attached conditions in the presence or in the absence of IGF-1. The 89 kDa PARP fragment was detected in cells cultured in poly-HEMA coated plates in the absence of IGF-1 (Figure 8.10). IGF-1 reduced the amount of cleaved PARP in all four primary cultures, suggesting that it promotes resistance to anoikis. In particular, cleaved PARP was completely inhibited in NC cells by IGF-1 ($p=0.0008$), whereas very low levels of cleaved PARP were detected in HC ($p=0.0003$), JW ($p=0.0004$) and GC cells ($p=0.0470$).

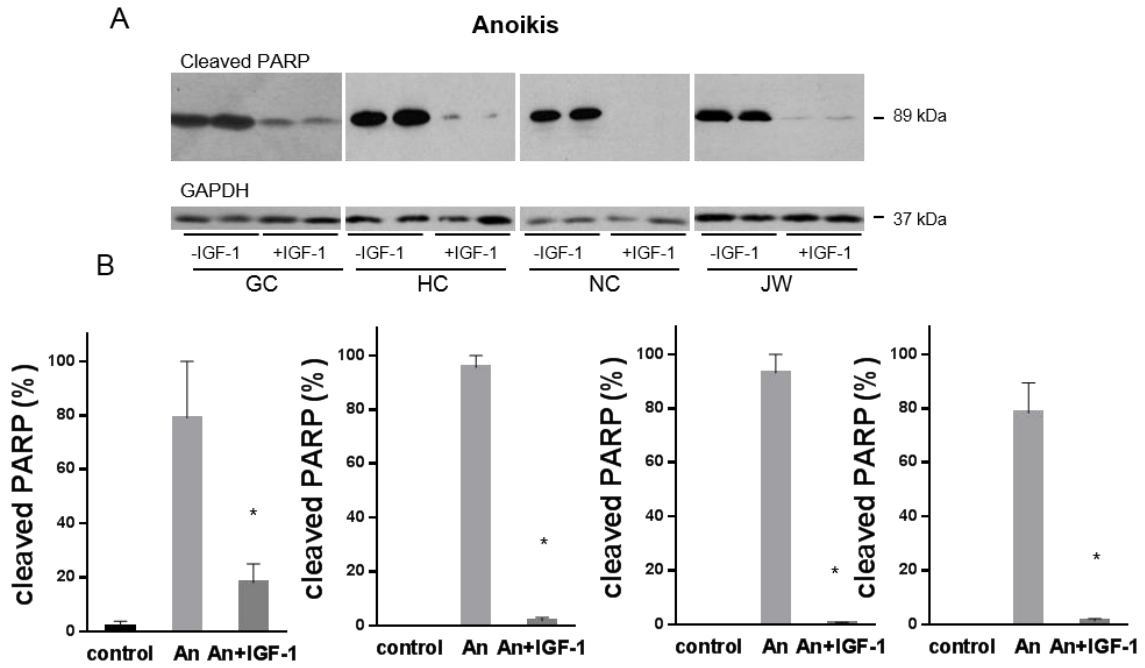

Figure 8.10. The effect of IGF-1 on PARP cleavage in non-attached conditions in primary cultures of gastric cancer cells. GC, HC, NC and JW cells were seeded into poly-HEMA coated 12 well plates at $20x10^4$ cells per well in 1 ml serum-free medium, in the presence or not of 50 ng/ml IGF-1. After incubation for 6 hours, proteins were extracted with 50 µl per well of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against cleaved PARP (1:1000 dilution) and GAPDH (1:10000 dilution) (**A**). Densitometry was with Labworks 4.0 software, adjusted for the background optical density of the X-ray film and normalised to GAPDH. Results are expressed as a percentage of the intensity of the cleaved PARP band observed in non-attached conditions and in the absence of IGF-1. Asterisks show cleaved PARP levels that are statistically significantly lower in the presence of IGF-1 than in its absence (One-way ANOVA; GC, p=0.0470; HC, p=0.0003; NC, p=0.0008; JW, p=0.0004) (**B**).

8.2.6 The effect of IGF-1 on activation of the PI3K/Akt and RAS/RAF/ERK signal transduction pathways in the context of apoptosis

Activation of downstream signal transduction pathways in response to IGF-1 was investigated in the apoptosis assay. GC, HC and NC cells were withdrawn from the effects of growth factors by culture in withdrawal medium for 2 days. The cells were then treated with staurosporine in the presence or absence of 50 $ngml^{-1}$ IGF-1.

In GC, HC and NC cells, cultured in withdrawal medium, very low amount of phosphorylated Akt was detected. Staurosporine inhibited slightly phosphorylation of Akt, particularly in HC cells (Figure 8.11). Treatment with IGF-1 resulted in a significant increase in Akt phosphorylation in all three primary cultures ($p \leq 0.0033$) (Figure 8.11). ERK1 and ERK2 phosphorylation was detected in GC, HC and NC cells cultured in withdrawal medium. Treatment with staurosporine reduced the amount of phosphorylated ERK1 and ERK2 in all

three primary cultures. However, IGF-1 did not increase ERK2 phosphorylation in any of the primary cultures (Figure 8.11).

The above results corroborate the results obtained in established gastric cancer cell lines, in which increased Akt rather than ERK1 and ERK2 phosphorylation was detected after treatment with IGF-1 in the context of apoptosis (Figure 4.13).

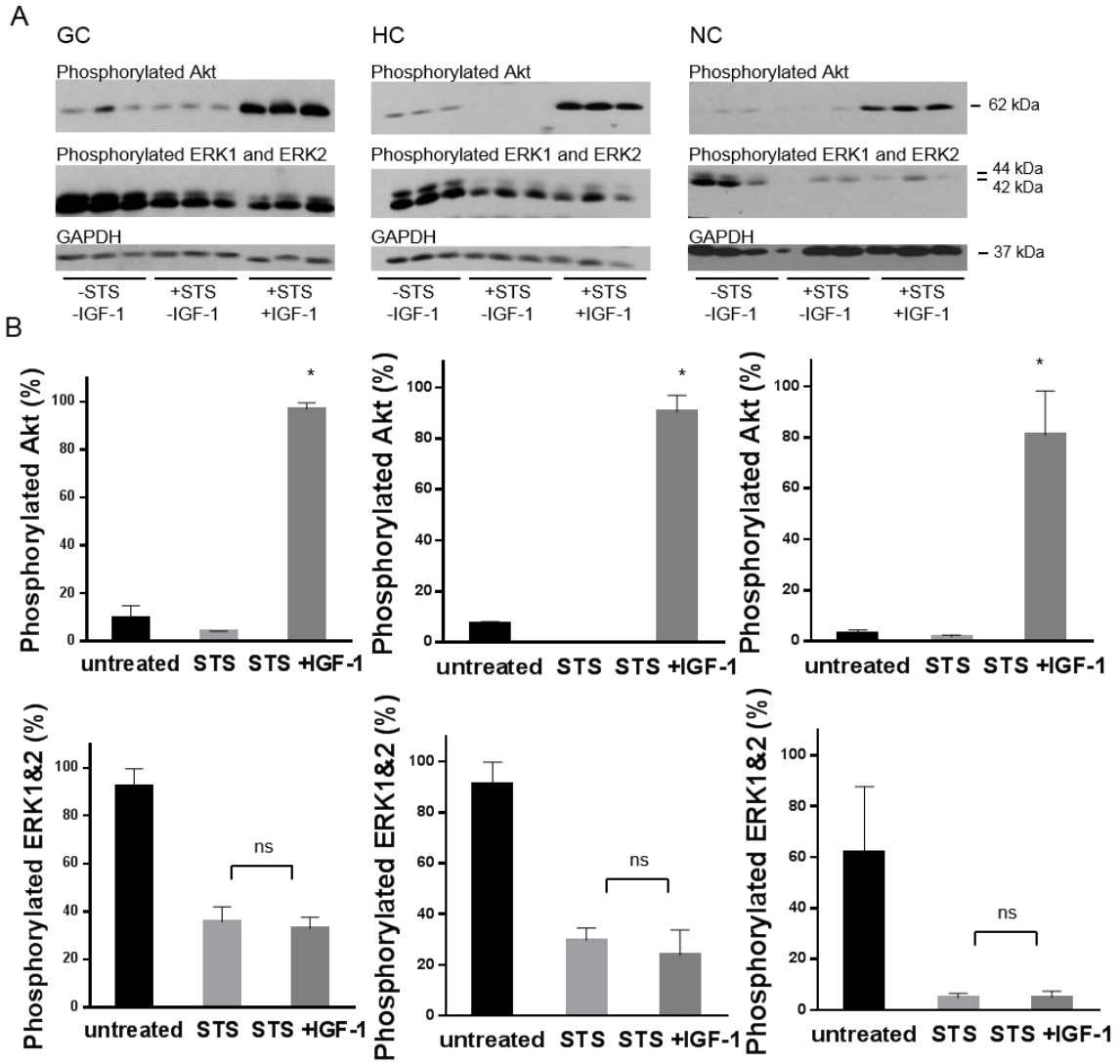

Figure 8.11. The effect of IGF-1 stimulation on activation of the PI3K/Akt and RAS/RAF/ERK signal transduction pathways in apoptosis. GC , HC and NC cells were seeded into 12 well tissue culture plates at 15x104 cells per well, in 1 ml maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and the cells washed with 0.5 ml PBS. Medium was replaced daily with 1 ml withdrawal medium for two days. Cells were incubated with withdrawal medium supplemented or not with 50 ng/ml IGF-1 for 15 minutes. Cells were then incubated with 0.5 µM staurosporine in the presence and absence of 50 ng/ml IGF-1 for 6 hours. Proteins were extracted with 80 µl of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against phosphorylated Akt (1:3000 dilution), phosphorylated ERK1/ERK2 (1:5000 dilution) and GAPDH (1:10000 dilution) (**A**). Densitometry was with Labworks 4.0 software, adjusted for the background optical density of the X-ray film and normalised to GAPDH. Results are expressed as a percentage of the intensity of the phosphorylated Akt band observed in the presence of IGF-1 or as percentage of the intensity of the phosphorylated ERK1 and 2 band observed in the absence of staurosporine and IGF-1. Asterisks show phosphorylated Akt levels that are statistically significantly

higher in the presence of IGF-1 than in its absence (One-way ANOVA; GC, $p<0.001$; HC, $p<0.0001$; NC, $p=0.0033$) (**B**).

8.2.7 The effect of siRNA knockdown of the type I IGF receptor on survival of primary cultures of gastric cancer cells

To investigate the importance of the type I IGF receptor in cell survival, expression of the receptor was lowered with transient knockdown, in an environment enriched with growth factors.

The type I IGF receptor was detected in HC and NC cells that had been transfected with scrambled siRNA (Figure 8.12). Incubation for 72 hours with siRNA 2, designed to target the type I IGF receptor, inhibited completely IGF-IR expression. The importance of type I IGF receptor on cell survival was investigated by measuring the levels of cleaved PARP in HC and NC cells that had been transfected with siRNA against the IGF-IR or with scrambled sequence, in medium supplemented with foetal calf serum. No cleaved PARP was detected in HC cells after transfection with the scrambled sequence. Furthermore, no cleavage of PARP into its 89 kDa fragment was induced after reduction of IGF-IR expression. Low levels of cleaved PARP were detected after transfection with the scrambled sequence in NC cells. Those levels were not increased when IGF-IR was absent.

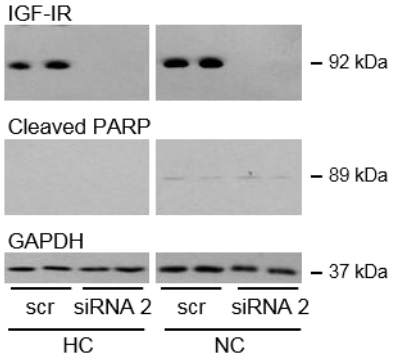

Figure 8.12. The effect of knockdown of the IGF-IR with siRNA on cell death in HC and NC cells in full medium. Transfection medium was prepared by mixing either scrambled (scr) or IGF-IR targeted (IGF-IR) siRNA duplexes at a final concentration of 40 nM and Lipofectamine RNAiMAX at a final concentration of 0.2% (v/v), in 0.1 ml DMEM without serum. The transfection medium was then incubated at room temperature for 30 minutes. Cells grown in maintenance medium were detached using trypsin:EDTA and resuspended in maintenance medium at $10x10^4$ cells /ml. Four hundred µl of the cell suspension were mixed with 0.1 ml of the transfection medium, containing scrambled or IGF-IR targeted sequences, in 24 well tissue culture plates. After 3 days, proteins were extracted with 50 µl per well of RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against IGF-IR (1:2000), cleaved PARP (1:1000 dilution) and GAPDH (1:10000 dilution).

The above results suggest that knockdown of the type I IGF receptor in HC and NC cells, does not activate apoptotic pathways which lead to PARP cleavage. Either the cells do not depend on the type I IGF receptor for their survival, or they undergo cell death through a different mechanism, independent of PARP.

8.2.8 The effect of siRNA knockdown of the type I IGF receptor on growth of primary cultures of gastric cancer cells

Proliferation of primary cultures of gastric cancer cells that do or do not express the type I IGF receptor was investigated in a growth-factor-enriched-environment. GC, HC, NC and JW cells were transfected with siRNA 2 or siRNA 3, which target the type I IGF receptor or with a scrambled sequence and cultured in medium supplemented with 20% foetal calf serum for 7-10 days. The number of cells at the end of each time point was measured by detecting the amount of DNA in each sample with the PicoGreen fluorimetric assay.

In GC cells, there was a 20% growth inhibition after transfection with siRNA 2 against the type I IGF receptor, compared to transfection with the scrambled oligonucleotide (p=0.0322) (Figure 8.13). Transfection with siRNA 3 resulted in 10% reduction in cell growth, which was not statistically significant. In HC cells, both siRNAs reduced cell growth by 25%, and this reduction was statistically significant (siRNA 2; p=0.001, siRNA 3; p=0.008). There was a 35% reduction in growth in NC cells after transfection with siRNA 2 (p=0.0001) and 25% reduction with siRNA 3 (p=0.0006). In JW cells, siRNA 2 reduced significantly cell growth by 20% (p=0.0411) and siRNA 3 by 45% (p=0.006).

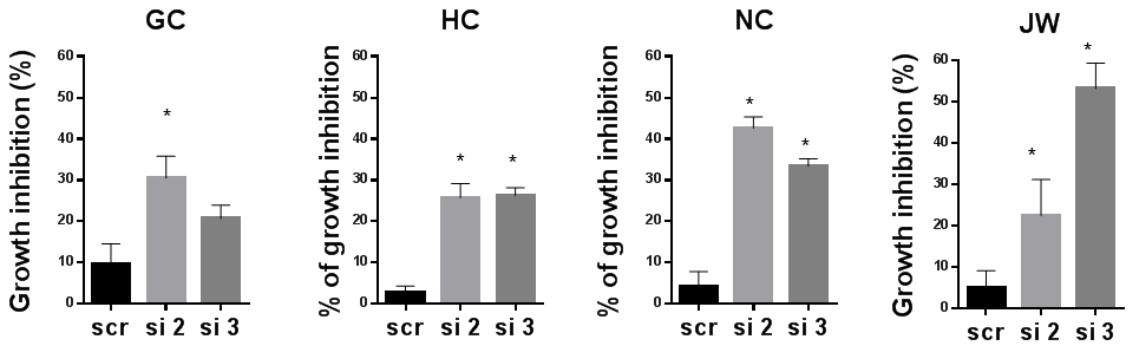

Figure 8.13. The effect of knockdown of the IGF-IR with siRNA on cell number in GC, HC, NC and JW cells in full medium. Transfection medium was prepared by mixing either scrambled (scr) or IGF-IR targeted (IGF-IR) siRNA duplexes at a final concentration of 40 nM and Lipofectamine RNAiMAX at a final concentration of 0.2% (v/v), in 0.1 ml DMEM without serum. The transfection medium was then incubated at room temperature for 30 minutes. Cells grown in maintenance medium were detached using trypsin:EDTA and resuspended in maintenance medium at $10x10^4$ cells /ml. Four hundred µl of the cell suspension were mixed with 0.1 ml of the transfection medium, containing scrambled or IGF-IR targeted sequences, in 24 well tissue

culture plates. After 7 days, the cells were washed with 0.5 ml ice cold PBS and stored in -20^{o} C. The cells were lysed in SSC/SDS solution and the amount of DNA present in each well was quantified with the Quanti-iT picogreen fluorimetric assay. Bars are the mean ±SEM. Asterisks indicate significant growth inhibition after reduction in type I IGF receptor expression (One-way ANOVA; GC, p=0.0322; HC, p=0.001, p=0.0008; NC, p=0.0001, p=0.0006; JW, p=0.0411, p=0.0006).

Reduction of IGF-IR expression affects growth of the primary cultures, suggesting that the above receptor is important for cell proliferation.

8.2.9 The effect of IGF-IR/IR inhibition by BMS754807 on cell survival in a growth factor-enriched environment

The effectiveness of the small molecule tyrosine kinase inhibitor BMS754807 on induction of cell death was tested by measuring the levels of cleaved PARP after treatment of the cells with the inhibitor, in the presence of 20% foetal calf serum.

Low levels of cleaved PARP were detected in untreated HC and NC cells (Figure 8.14). Addition of 0.5 μM BMS754807 increased slightly the amount of cleaved PARP in both primary cultures but in a non-significant manner. A higher concentration of BMS (5 μM) induced a significant increase of the amount of cleaved PARP ($p<0.0001$). It is noteworthy that cell death was induced in the primary cultures after inhibition of the type I IGF and insulin receptors by BMS754807, even in the presence of a high concentration of foetal calf serum, which contains a variety of growth factors. This underlines the importance of these receptors for the survival of gastric cancer cells present in ascites.

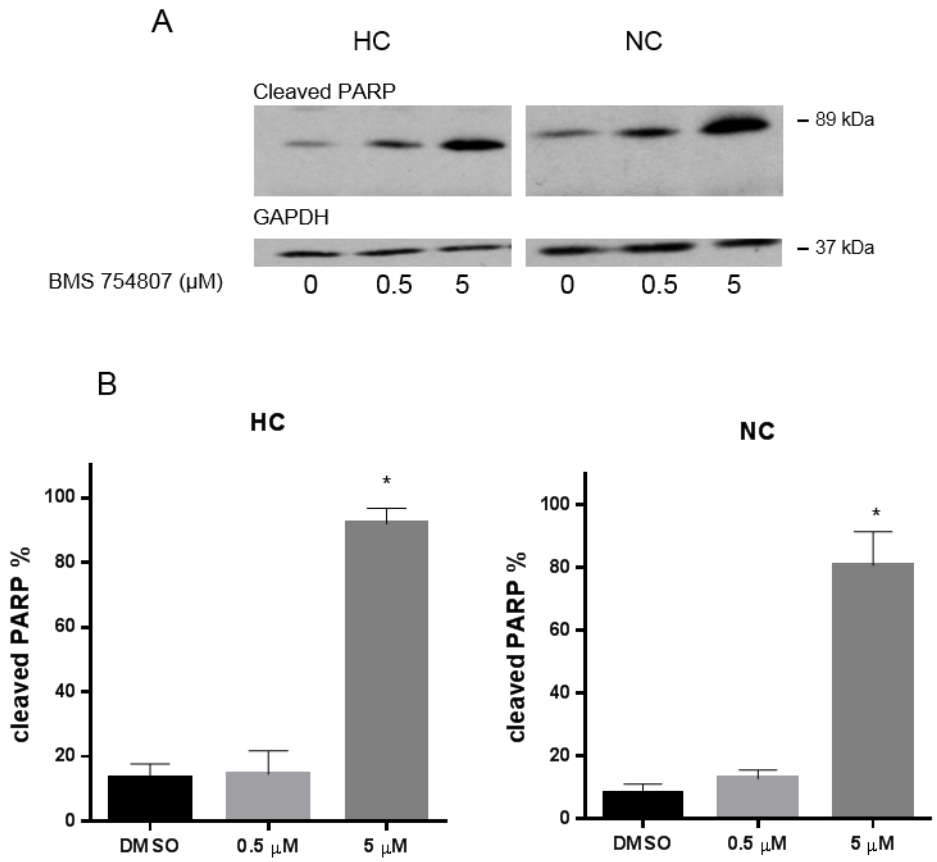

Figure 8.14. The effect of inhibition of IGF-IR/IR with BMS754807 on apoptosis in primary cultures of gastric cancer cells cultured in maintenance medium. HC and NC cells were seeded into 12 well tissue culture plates at a concentration of 150000 cells/well, in 1 ml maintenance medium which contained DMSO, 0.5 µM or 5 µM BMS754807. The cells were left for 7 days and protein extracts were prepared with 80 µl per well RIPA buffer plus inhibitors. Aliquots containing 10 µg of protein were electrophoresed on denaturing 12% polyacrylamide gels and transferred to nitrocellulose at 100 mA for 45 minutes. Membranes were incubated with antibodies against cleaved PARP (1:2000 dilution) and GAPDH (1:10000 dilution) (**A**). Densitometry was with Labworks 4.0 software, adjusted for the background optical density of the X-ray film and normalised to GAPDH. Results are expressed as a percentage of the intensity of the cleaved PARP band observed after treatment with 5 µM BMS754807. Bars are the mean ±SEM. Asterisks indicate significant induction of cleaved PARP after treatment with the BMS754807 inhibitor (One-way ANOVA test; HC, $p<0.0001$; NC, $p<0.0001$).

8.3 Discussion

Four primary cultures of gastric cancer cells, GC, HC, NC and JW, were established from ascites removed from patients with advanced gastric adenocarcinoma. To our knowledge, this is the first study to establish primary cultures of gastric cancer cells from ascites. Primary cultures of ovarian cells derived from ascitic fluid have been described previously (Theriault *et al.*, 2013).

The gastric cancer cells in culture had a variable morphology but mostly had an epithelial appearance and were positive for keratin expression. The epithelial origin of ovarian cancer cells established from ascites has been reported previously by expression of cytokeratins and

EPCAM (ODonnell *et al.*, 2014). In the molecular level, the four primary cultures did not express any of the three receptors, HER-2, FGFR2 and c-Met, which are currently targeted in the clinic in patients with gastric cancer. However, type I IGF receptor and low levels of insulin receptor expression were demonstrated readily in all primary cultures.

IGF-1 treatment in a growth-factor-depleted environment induced IGF-IR/IR phosphorylation in the primary cultures. In GC gastric cancer cells, IGF-1 stimulated particularly strong activation of the PI3K/Akt and Ras/ERK pathways. In the other gastric cancer cells from patients, activation of the PI3K/Akt pathway was more marked than of the Ras/ERK pathway. The above results are in agreement with the data obtained from the established gastric cancer cell lines, in which IGF-IR/IR and Akt activation in response to IGF-1 were universal, whereas stimulation of ERK1 and ERK2 activation was not equally effective in all of the cell lines.

It was noteworthy that cell death could be induced in gastric cancer cells isolated from patients with very advanced gastric cancer either by inhibition of kinase activity or by prevention of cell attachment. Remarkably, the survival effect of IGF-1 for these gastric cancer cells was more pronounced than for the established gastric cancer cell lines. The concomitant activation of the PI3K/Akt but not the Ras/ERK pathway indicates that the cell survival signal is transmitted via the former pathway and supports the data obtained from the established cell lines. The role of IGF-1 in protection against anoikis is of particular importance, as it suggests that circulating IGF-1 could promote the survival of cancer cells present in the ascitic fluid and increase their migratory and invasive potential.

Reduction of IGF-IR expression with siRNA knockdown resulted in inhibition of cell growth in all four primary gastric cancer cultures. This confirms the result obtained in gastric cancer cell lines and strengthens the hypothesis that inhibition of IGF-IR could be a valid treatment option for patients with gastric cancer. The primary cultures of gastric cancer cells have a significantly slower growth rate compared to the immortalised cell lines, with a doubling time of approximately 5-7 days, versus 1-2 days for cell lines. It is, therefore, very encouraging that inhibition of the IGF-IR affected the growth rate of the primary cultures only 7 days after knockdown of the IGF-IR and it suggests that the receptor is important for their growth.

The use of RNA interference methods in ovarian cancer cells derived from ascites has been described previously (Ingram *et al.*, 2010). In the above study, primary cultures of ovarian cancer cells were transduced with a recombinant adenovirus and the level of transduction was

much higher than in the established cell lines. This suggests that it is possible to use RNA interference methods as a method of gene therapy in cells present in the ascitic fluid.

In summary, the findings in this chapter suggest that epithelial cells present in the ascitic fluid of patients can be used as models of advanced gastric cancer. In the four primary cultures established here the importance of the IGF signal transduction pathway in cell survival was manifested by the protective effect of IGF-1 against apoptosis induced by tyrosine kinase inhibition and prevention of cell attachment. Also, knockdown of the type I IGF receptor and inhibition of the IGF-IR and IR by the small molecule inhibitor, BMS754807, reduced proliferation and increased cell death, respectively.

Chapter 9. Discussion

The aim of the present study was to investigate the importance of the IGF signal transduction pathway in gastric cancer cells and to explore the potential of targeting the above pathway as a therapeutic option for advanced gastric cancer.

Gastric cancer is the third-leading cause of cancer-related death, worldwide, with a median survival of 11.2 months (Cunningham *et al.*, 2008; Ferlay *et al.*, 2015). Standard therapy includes total or subtotal gastrectomy combined with removal of the perigastric lymph nodes and chemotherapeutic agents, such as 5-fluorouracil/capecitabine, cisplatin/oxaliplatin and epirubicin. The 5-year survival rate of patients diagnosed with gastric cancer is very low, close to 20%. Approximately 70% of advanced gastric cancer patients would be ineligible for the currently available targeted treatments, such as anti-HER-2, anti-c-Met and anti-FGFR-2 treatment (Gravalos and Jimeno, 2008; Kawakami *et al.*, 2013; Xie *et al.*, 2013; Su *et al.*, 2014). There is, therefore, an urgent need for the introduction of new targeted agents into the existing therapeutic regime, to prolong survival and alleviate symptoms.

A number of studies have reported the importance of the IGF signal transduction pathway in development and progression of several cancer types (reviewed by (Pollak, 2008)). Phase I and II trials of monoclonal antibodies against the type I IGF receptor or small molecule tyrosine kinase inhibitors against the type I IGF and insulin receptors have shown positive results for a variety of cancers (Karp *et al.*, 2009; Haluska *et al.*, 2010; Olmos *et al.*, 2010). Very few preclinical studies have attempted to investigate the involvement of the IGF signal transduction pathway in the development and progression of advanced gastric cancer.

Among the gastric cancer cell lines analysed here, NCI-N87 cells expressed high levels of HER2, KATO III and SNU-16 expressed high levels of FGFR2 and SNU-5 overexpressed c-Met, consistent with reported amplification of the genes that encode these receptors (Liu *et al.*, 2014). SNU-1, MKN74, NUGC3 and AGS cells represent the majority of gastric cancers that do not overexpress the three tyrosine kinase receptors against which drugs are licensed or in clinical trial (triple negative gastric cancer). Importantly, they expressed the type I IGF receptor and all but MKN74 expressed high levels of isoform A of the insulin receptor.

IGF-1 stimulated phosphorylation of the type I IGF and/or insulin receptor and Akt in all the gastric cancer cell lines analysed which demonstrates that the IGF signal transduction pathway is active even in cells that overexpress HER2, FGFR2 or MET. In particular, NCI-

N87 and SNU-16 cells, with amplification of HER-2 and FGFR2, respectively, were very responsive to IGF-1 stimulation with concomitant increase in Akt and ERK1 and ERK2 phosphorylation. It is possible that despite the dependence of those cells on their amplified receptors, the IGF signal transduction pathway also contributes, to a certain extent, to their growth and survival mechanisms.

Previously, IGF-1 protected MET-amplified MKN45 cells from ethanol-induced caspase 3 activation (Min *et al.*, 2005). Our study is the first to demonstrate a significant survival effect of IGF-1 against caspase-dependent apoptosis induced by protein kinase inhibition or anchorage deprivation in triple negative gastric cancer cells. That IGF-1 protects against apoptosis induced by the kinase inhibitor staurosporine, emphasizes the potency of IGF-1 as a pro-survival factor and suggests that it may protect gastric cancer cells from therapeutic kinase inhibitors. Also, IGF-1 protection against anoikis underlines the importance of IGF-1 for the survival of cells grown in anchorage-independent conditions and suggests that it could facilitate the migration of cancer cells from their initial site to another location. Overall, that the survival effect of IGF-1 was manifested in two different survival assays strengthens the conclusion that it can promote survival in various settings and under different conditions.

Inhibition of the type I IGF and insulin receptors with the small molecule tyrosine kinase inhibitor BMS754807 (Carboni *et al.*, 2009), abrogated the IGF-1 survival effect, which suggests that in the absence of active type I IGF and insulin receptors, IGF-1 is no longer able to transduce survival signals. Furthermore, the protective effect of IGF-1 was associated with activation of the PI3-kinase/Akt pathway but not the Ras/MAP-kinase pathway, and was prevented by inhibitors of the former but not the latter pathway. Involvement of the PI3-kinase/Akt pathway in mediation of the IGF-1 survival effect has been shown in MCF-7 breast cancer cells against cell death induced by chemotherapy (Gooch *et al.*, 1999).

Proliferation of gastric cancer cells was stimulated by IGF-1 and the stimulation was decreased by inhibition of the Ras/MAP-kinase pathway. Our results demonstrate that the type I IGF receptor mediates the IGF-1 proliferative effect. The overall importance of the type I IGF receptor in gastric cancer cell proliferation was demonstrated by the significant reductions in cell-growth in serum-containing medium and in the proportion of cells in S- and mitotic-phases of the cell cycle after knockdown of receptor expression. In previous studies, inhibition of the type I IGF receptor with shRNA and the αIR3 antibody reduced colony formation in MET-amplified MKN45 cells and tumour explants, respectively (Pavelic *et al.*, 2003; Wang *et al.*, 2010a). Further, infection with an adenovirus that expressed truncated

IGF-IR reduced proliferation of MKN74, NUGC4 and MET-amplified MKN45 cells (Min *et al.*, 2005).

IGF-2 and insulin protected SNU-1 and NUGC3 cells from apoptosis induced by the protein kinase inhibitor staurosporine and stimulated proliferation of NUGC3 cells. The overall importance of the insulin receptor in gastric cancer cell survival was demonstrated by a significant increase in cleaved PARP levels after knockdown of insulin receptor expression, in growth-factor-enriched medium. Importantly, when individual and concomitant knockdown of the type I IGF and insulin receptors was attempted, only loss of the insulin receptor resulted in significant induction of apoptosis. Our results suggest that the insulin receptor is primarily important for the mediation of cell survival. To elucidate the role of the insulin receptor in cell proliferation, markers of cell proliferation e.g. BrdU incorporation, could be measured in cells transfected with siRNA against the insulin receptor.

Treatment of triple-negative gastric cancer cells with the small-molecule tyrosine kinase inhibitor BMS754807, which targets both the type I IGF and insulin receptors, induced apoptosis and inhibited effectively proliferation in serum-containing medium. It has been argued that inhibition of both receptors might be more beneficial in the clinic than inhibition of the type I IGF receptor alone. Several pre-clinical studies have reported that combined inhibition of the type I IGF and insulin receptors is more efficient than inhibition of either receptor alone (Buck *et al.*, 2010; Vincent *et al.*, 2013). In this respect, small molecule inhibitors which target both receptors might be superior to monoclonal antibodies which target the type I IGF receptor, selectively.

Our results suggest that treatment of SNU-1 and NUGC3 cells with the cytotoxic agents oxaliplatin and 5-fluorouracil, at concentrations equal to or higher than 1μM, reduced their viability. Concomitant treatment with 5-fluorouracil and BMS754807 was more effective than treatment with either agent alone in triple-negative SNU-1 and NUGC3 cells. To date, a remarkably small number of preclinical studies have investigated the effect of combined treatment with cytotoxic agents and IGF-IR/IR inhibitors in gastric cancer cells. Inhibition of the type I IGF receptor enhanced chemotherapy-induced apoptosis in Met-amplified MKN45 gastric cancer cells (Min *et al.*, 2005; Wang *et al.*, 2010a). Our study is the first to investigate the effect of concomitant inhibition of the type I IGF and insulin receptors with oxaliplatin and 5-fluorouracil treatment in triple-negative gastric cancer cells. It is encouraging that a small potentiation of the cytotoxic effect of 5-fluorouracil was detected after treatment with BMS754807 in SNU-1 and NUGC3 cells, however, an extensive concentration range for the

two drugs should be analysed and the effects of combined treatment with the two drugs should be investigated in *in vivo* models.

Whether or not resistance to cytotoxic therapy can be subverted by inhibition of the IGF signal transduction pathway has not been determined here. Previously, it has been shown that colorectal cancer cells which have acquired resistance to 5-fluorouracil and oxaliplatin had increased phosphorylated and total levels of the type I IGF receptor (Dallas *et al.*, 2009). The same study has shown that inhibition of the type I IGF receptor with a monoclonal antibody was more efficient at reduction of growth and induction of apoptosis in chemoresistant tumours than in non-resistant tumours. It would be useful to create gastric cancer cell lines with resistance to cytotoxic agents, currently used in the clinic, and measure the levels of phosphorylated and total type I IGF and insulin receptors.

Consistent with previous studies, FGFR2-amplified KATO III and SNU-16 cells and c-Met-amplified SNU-5 cells were highly sensitive to FGFR2 and c-Met inhibitors, AZD4547 and crizotinib, respectively (Okamoto *et al.*, 2012; Xie *et al.*, 2013). In FGFR2 amplified SNU-16 cells, which express high levels of the type I IGF receptor, addition of external IGF-1 afforded a small protection against treatment with AZD4547. In addition, inhibition of the type I IGF and insulin receptors with BMS754807 aggravated the cytotoxic effect of AZD4547. On the contrary, no significant benefit was achieved after combined treatment of KATO III and SNU-5 cells with BMS754807 and AZD4547 or crizotinib, respectively. This study is the first to investigate the effect of combining a FGFR2 inhibitor with an IGF-IR/IR inhibitor in gastric cancer cells. Our results suggest that dual targeting may be a valid therapeutic option for a subset of gastric cancers. The effect of IGF-IR/IR inhibition on cells with resistance to FGFR2 and c-Met targeted inhibitors should also be investigated.

In a previous study, signalling through both the type I IGF and insulin receptor was involved in primary resistance of NCI-N87 cells to lapatinib (Zhang *et al.*, 2014). Lapatinib-resistant HER2-amplified NCI-N87 cells were established in this study. NCI-N87/lapatinib-resistant cells lost their sensitivity to the dual, anti-HER2 and anti-EGFR inhibitor lapatinib, suggesting that the cells had lost their dependency on HER2 signalling and relied more on other growth factor signal transduction pathways for sustaining their growth and survival. Involvement of the IGF signal transduction pathway in acquisition of lapatinib-resistance has not been demonstrated here, since there was no alteration in phosphorylated or total IGF-IR levels in resistant cells. Detection of expression and activation levels of downstream molecules of the IGF signal transduction pathway, as wells as other receptors of the HER

signalling pathway, e.g. HER3 and HER4, could shed some light on the mechanisms of resistance developed in these cells.

This study is the first to report establishment of *ex vivo* cultures of gastric cancer cells from ascites removed from patients with advanced gastric cancer. The type I IGF receptor and insulin receptor were expressed in these cells. There was no expression of HER2, FGFR2 or c-Met, which suggests that the patients would not have benefited from targeted therapies currently in the clinic. Remarkably, the survival effect of IGF-1 against caspase-dependent apoptosis induced by anchorage deprivation or by protein kinase inhibition was more pronounced in patient-derived gastric cancer cells than in established cell lines, which indicates that IGF-dependence is characteristic of gastric cancer cells and not derived during culture. The role of IGF-1 in protection against anoikis is of particular importance because circulating IGFs could promote the survival of cancer cells present in serum or ascitic fluid and increase their migratory and invasive potential.

Consistent with the findings from gastric cancer cell lines, knockdown of the type I IGF receptor in triple-negative gastric cancer cells isolated from patients inhibited their growth. Treatment with BMS754807 induced apoptosis even in the presence of a high concentration of growth-factor-containing serum. Confirmation of the results obtained from gastric cancer cell lines in *ex vivo* cultures corroborates the importance of the IGF signal transduction pathway in progression of gastric cancer and strengthens the idea that it should be considered as a potential therapeutic target in gastric tumours without known amplifications.

Conclusions

Our results suggest that the gastric cancer cells analysed in this study could be classified in two separate categories, cells with amplification of *HER-2*, *FGFR2* or *MET* and cells without amplification or triple-negative cells. All triple-negative gastric cancer cells, apart from MKN74, were protected from apoptosis by IGF-1. Amongst the amplified cell lines, an IGF-1 survival effect was detected in NCI-N87 cells; for the remaining amplified cells, either apoptosis was not induced or IGF-1 was not protective against apoptosis (Table 8.1).

The proliferative effect of IGF-1, in controlled conditions, was demonstrated in MKN74 and NUGC3 triple-negative gastric cancer cells. All triple-negative gastric cancer cells responded with reduced proliferation upon IGF-IR/IR inhibition. The effect of IGF-IR/IR inhibition was not investigated directly in amplified cells. However, experiments in which SNU-16 and

SNU-5 cells were treated with the BMS754807 inhibitor showed that there was no effect on cell growth after treatment with 0.5 μM of the inhibitor, but that there was significant reduction in cell number after treatment with 5 μM of the inhibitor (Figure 7.8 and Figure 7.10). Finally, IGF-IR/IR inhibition with BMS754807 induced apoptosis in SNU-1, NUGC3, HC and NC triple-negative gastric cancer cells (Table 8.1.

Cell type	**Amplification**	**IGF-mediated survival effect**	**IGF-mediated proliferative effect**	**Reduced proliferation in response to IGF-IR/IR inhibition**	**Increased cell death in response to IGF-IR/IR inhibition**
NCI-N87	*HER-2*	+	Not tested	Not tested	Not tested
KATO-III	*FGFR2*	-	Not tested	Not tested	Not tested
SNU-16	*FGFR2*	No apoptosis	Not tested	Not tested	Not tested
SNU-5	*MET*	No apoptosis	Not tested	Not tested	Not tested
SNU-1	-	+	Not tested	+	+
MKN74	-	No apoptosis	+	+	Not tested
NUGC3	-	+	+	+	+
AGS	-	+	Not tested	+	Not tested
GC	-	+	Not tested	+	Not tested
HC	-	+	Not tested	+	+
NC	-	+	Not tested	+	+
JW	-	+	Not tested	+	Not tested

Table 8.1. Response of amplified and triple-negative gastric cancer cells to IGFs and IGF receptor-targeted therapy.

It could be concluded that triple-negative gastric cancer cells represent distinct gastric cancer populations which could potentially benefit from inhibition of the IGF signal transduction pathway in the clinic. The lack of alternative targeted treatments available currently for patients with these types of gastric cancer further supports the idea of investigating the effect of IGF-IR/IR inhibition in a clinical setting.

Future work

Introduction of IGF-targeted inhibitors into the clinic would require the identification of effective biomarkers with which to select patients most likely to respond. Expression of the type I IGF and insulin receptors is not considered a sufficient predictor of IGF responsiveness. For instance, gastric cancer cells with amplified genes that encode tyrosine kinase receptors were shown in the present study to express receptors of the IGF signal transduction pathway. The presence of amplification of other tyrosine kinase receptor genes could provide a contraindication of response to IGF-targeted therapy because those tumour cells are likely to be driven by the amplified tyrosine kinase receptor pathways. Expression of IGF signal transduction pathway receptors in combination with downstream insulin receptor substrate proteins (IRS-1, 2 and 4) might be indicative of potential positive response to IGF-targeted therapy.

The standard cytotoxic chemotherapeutic regimen in the UK for advanced gastric cancer patients comprises cisplatin/oxaliplatin, epirubicin and capecitabine/5-fluorouracil. Combined efficacy of cytotoxic therapy with IGF-targeted inhibitors should be demonstrated preclinically prior to introduction into the clinic. Validation of results in *in vivo* models or in tumour explants could offer another level of validation of the above therapeutic combination. Similarly, combination of IGF-targeted drugs with HER2, FGFR2 and c-Met inhibitors could be tested pre-clinically, as a way of reducing onset of resistance. As shown in our results, NCI-N87 cells, which have *HER2* amplification, were responsive to IGFs which suggests that some *HER2*-amplified gastric cancer cells might benefit from concomitant HER2 and IGF-IR/IR inhibition.

Acquisition of resistance to cytotoxic drugs or targeted inhibitors is a common problem in oncology that is encountered also in gastric cancer patients. It would be of value to test the involvement of the IGF signal transduction pathway in acquisition of resistance and the potential benefit of inhibiting the pathway in resistant cancers.

Appendix-published work

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