

# **The Role of Insulin-like Growth Factor-type 1 Receptor (IGF-IR) Signalling in Tamoxifen- Resistant Breast Cancer**

by

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**Thesis submitted for the Degree of Doctor of Philosophy**

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This thesis was written in memory of my mother

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## SUMMARY

The aim of the first part of this thesis was to determine the role played by IGF-IR in mediating the growth of EGFR-positive tamoxifen-resistant variants of MCF-7 (Tam-R) and T47D (T47D-R) breast cancer cell lines. The results identify a general tamoxifen-resistant mechanism whereby the autocrine release and action of IGF-II, mediated through the IGF-IR, plays a significant and crucial supporting role in regulating basal EGFR/MAPK signalling and cell proliferation and this occurs via a c-SRC-dependent mechanism in both Tam-R and T47D-R cells. The latter aim of this thesis was to determine further mechanisms of cross-talk between EGFR and IGF-IR in a range of EGFR-positive cancer cell lines. These studies identified a novel physical interaction between the EGFR and IRS-1 in each of these cell lines. In Tam-R breast and LNCaP prostate cancer cells, recruitment of IRS-1 by EGFR limited the availability of IRS-1 to associate with IGF-1R, thus inhibiting IGF-IR signalling capacity. Blockade of EGFR activity with gefitinib allowed re-association of IRS-1 with IGF-1R and re-establishment of IGF-1R signalling, the dominant growth regulatory mechanism of gefitinib resistance in Tam-R cells. Thus, gefitinib played an active role in limiting its own efficacy in these cells by promoting activation of a resistance pathway. Importantly, induction of this pathway by gefitinib could be abrogated by co-treatment with an IGF-IR inhibitor. Such findings identify the IGF-IR as a potential therapeutic target for the treatment of both tamoxifen-resistant and gefitinib-resistant breast and prostate cancers.

## ABBREVIATIONS

4-OH-TAM	4-hydroxy-tamoxifen
ABDP	4-anilino-5-bromo-2-[4-(2-hydroxy-3-(N,N-dimethylamino) propoxy) anilino] pyrimidine
AF-1	ligand-independent activation function
AF-2	ligand-dependent activation function
$\alpha$	alpha
ALS	acid labile subunit
ANOVA	one-way analysis of variance
APS	ammonium persulphate
AR	amphiregulin
$\beta$	beta
BPB	bromophenol blue
BSA	Bovine Serum Albumen
CDK-1	cyclin dependent kinase 1
cDNA	complementary deoxyribonucleic acid
CT	cycle threshold
DAB	3,3'-diaminobenzidine
DBD	DNA binding domain
DMEM	Dulbecco's Minimum Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxy nucleotide triphosphates
DTT	dithiothreitol
ECM	extracellular matrix

EDTA	ethylene diamine tetra acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGTA	Ethyleneglycol- <i>bis</i> ( $\beta$ -aminoethyl)-N,N,N',N'-tetraacetic Acid
ER	oestrogen receptor
ERE	oestrogen response elements
ERK1/2	extracellular signal regulated kinase
FCS	foetal calf serum
GAB-1	Grb2 associated binder
GH	growth hormone
GPCR	G protein-coupled receptor
GRB2	growth factor receptor-bound protein 2
HB-EGF	heparin-binding-EGF
HCL	hydrochloric acid
HSP	heat shock proteins
ICA	immunocytochemical assay
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding proteins
IGF-IIR	type II insulin-like growth factor receptor
IGF-IR	insulin-like growth factor receptor
IR	insulin receptor
IRR	insulin receptor-related receptor
IRS	insulin receptor substrate
LBD	ligand binding domain

LTED	long-term oestrogen-deprived
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
Na <sub>3</sub> VO <sub>4</sub>	sodium orthovanadate
NaCl	sodium chloride
NaF	sodium flouride
NRG	neuregulin
NSCLC	non-small cell lung cancer
p70 <sup>S6K</sup>	p70 ribosomal protein S6 kinase
PBS	phosphate buffered saline
PDK-1	phosphoinositide-dependent kinase 1
PH	pleckstrin homology
PI3-K	phosphoinositide 3-kinase
PIP2	phosphatidylinositol 4,5-di-phosphate
PIP3	phosphatidylinositol 3,4,5-tri-phosphate
PK	protein kinase
PKCδ	protein kinase C delta
PLCγ	phospholipase C gamma
PMSF	phenylmethyl sulfonylflouride
PTB	phosphotyrosine binding domain
PTEN	phosphoinositide-specific phosphatase
RH	random hexamers
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription-polymerase chain reaction

SDS	sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SERM	selective oestrogen receptor modulators
SH2	SRC homology 2
SHC	src and collagen-homology protein
siRNA	small interfering RNA
T47D-R	tamoxifen-resistant T47D cell line
TAE	Tris-acetate EDTA
Tam-R MCF-7	tamoxifen-resistant MCF-7
Tam-R/Gef-R	Tam-R/gefitinib resistant MCF-7
TBS	tris buffered saline buffer
TEMED	N,N,N,N tetramethylethylenediamine
TGF $\alpha$	transforming growth factor alpha
TM	melting temperature
Tris-HCL	tris hydroxymethyl aminomethane hydrochloride
WT	wild-type
Y	tyrosine

## **1.0 Introduction:**

### ***1.1 The structure and function of the IGF system:***

The insulin-like growth factor (IGF) system is an evolutionarily-conserved signalling pathway that plays an essential role in the regulation of growth and development, from embryonic life continuing through adult life. It is comprised of a complex network of ligands, their cognate receptors, binding proteins (IGFBP) and IGFBP proteases (Adams *et al.*, 2000; LeRoith *et al.*, 1995).

#### ***1.1.1 IGF ligands:***

The IGF ligands are a family of growth factors with structural homologies to insulin (Rinderknecht and Humbel, 1978a and 1978b). They were first discovered during the search for factors that mediate the growth-stimulating effect of growth hormone (GH; Zapf and Froesch, 1986). It was found that the effects of GH on cartilage growth in the rat occurred through a serum factor subsequently called somatomedin, later shown to be IGF-I. Further research revealed that two IGFs exist, IGF-I (70 amino acids long) and IGF-II (67 amino acids long) which are single chain polypeptide growth factors (Rinderknecht and Humble, 1978a, and 1978b) that have potentially divergent roles in human physiology. In mammals, IGFs are widely expressed during foetal and pre-natal stages of growth and development. Animal studies indicate that the action of IGF-II on body development and growth occurs at a much earlier stage of life than that of IGF-I. Indeed, evidence suggests that IGF-II may play a key regulatory role during foetal growth (Schultz *et al.*, 1993; Lighten *et al.*, 1997). After birth, the role of IGF-II is gradually replaced by that of IGF-I where hepatic production of IGF-I, primarily under the regulation

of GH, becomes the major source of circulating IGF-I, although this growth factor has also been shown to be produced by numerous other organs, including kidney, lung and bone (Daughaday and Rotwein, 1989). Levels of circulating IGF-I change substantially over time where they increase slowly from birth to puberty, surge at puberty and decline thereafter with age. For IGF-II, however, the circulating levels remain relatively stable after puberty and GH has no regulatory effect on its expression. IGF-II can be synthesized by almost any tissue in the body and as such it is widely accepted that both IGF-I and IGF-II can work in an endocrine as well as paracrine/autocrine manner to influence cell growth and development (LeRoith *et al.*, 2001).

### ***1.1.2 The IGF receptors:***

The effects of both IGFs are exerted primarily through binding to a specific receptor on the cell membrane, namely the type I insulin-like growth factor receptor (IGF-IR), although IGF-I has been shown to bind the IGF-IR with a greater affinity than IGF-II (Rubin and Baserga, 1995). This receptor is a member of a larger family which also comprises the type II insulin-like growth factor receptors (IGF-IIR), the insulin receptor (IR) (Jones and Clemmons, 1995; Jerome *et al.*, 2003; Sepp-Lorrenzino, 1998; Adams *et al.*, 2000) and the insulin receptor-related (IRR) receptor, an orphan receptor for which no ligand has yet been identified (Shier and Watt, 1989).

#### ***1.1.2.1 The IGF-IR:***

Characterization of the IGF-IR was made possible in the early 1980s, when the receptor was purified from human placenta (Bhaumick *et al.*, 1981) and its

complementary deoxy ribonucleic acid (cDNA) and gene were cloned (Ullrich *et al.* 1986; Abbot *et al.*, 1992; Werner and LeRoith, 1996). It is a member of the type II receptor tyrosine kinase family, which also includes the IR and with which it shares 70% structural homology at both a protein and gene level. The gene, for both receptors consists of 21 exons spanning over 100kb of genomic DNA (Ullrich *et al.*, 1986). IGF-IR is synthesized as a single chain alpha beta ( $\alpha\beta$ )-pre-pro-receptor comprised of 1367 amino acids which is processed into the mature plasma membrane-bound glycoprotein receptor by proteolysis and glycosylation (Ullrich *et al.*, 1986). As a result of protease cleavage,  $\alpha$  and  $\beta$  subunits are released and subsequently linked by di-sulphide bonds in an  $\alpha_2\beta_2$  configuration to form a tetramer (Figure 1).

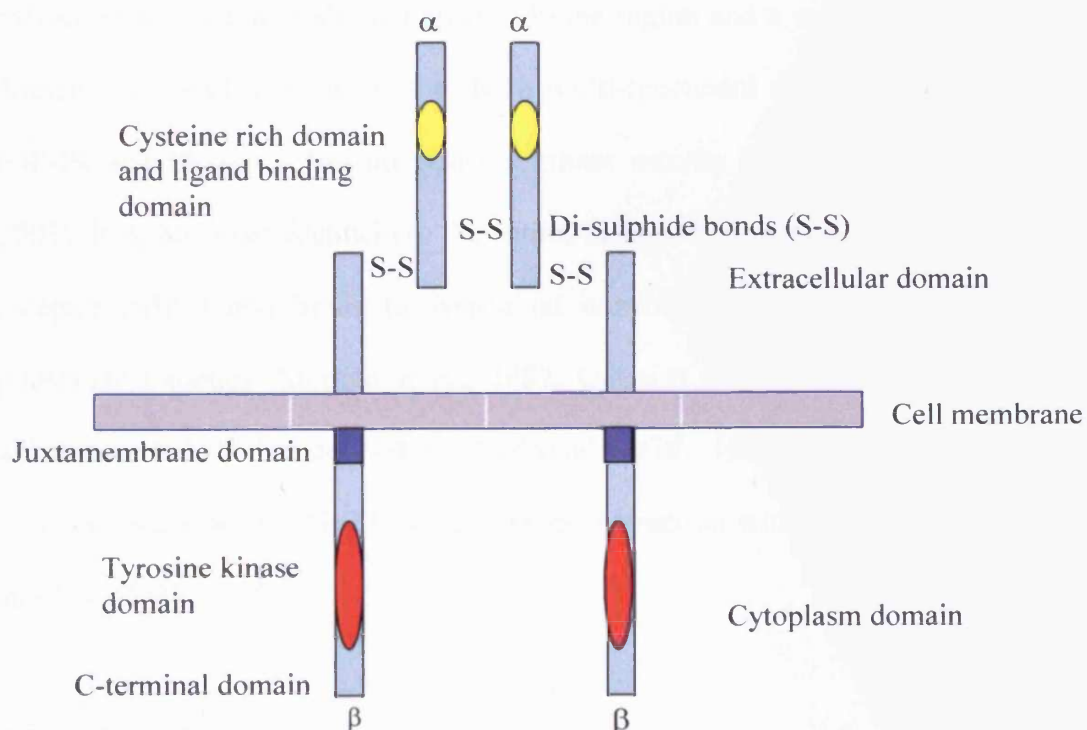


Figure 1. Major structural elements of IGF-IR



The  $\alpha$ -subunits (130-135 kDa) are entirely extracellular and contain a cysteine-rich domain responsible for ligand binding (Kjeldsen *et al.*, 1991). The  $\beta$ -subunits (90-95 kDa) display a highly hydrophobic 24 amino acid transmembrane domain that divides the  $\beta$ -subunit into an extracellular 195 amino acid region and an intracellular or cytoplasmic 408 amino acid portion. The cytoplasmic domain includes a tyrosine kinase domain (residues 973-1229) that becomes autophosphorylated following ligand binding to the  $\alpha$ -subunit (Jacobs and Cautrecasus, 1986, Kato *et al.*, 1993; Stewart and Rotwein, 1996; Werner and LeRoith, 1997).

#### **1.1.2.2. IGF-IIR**

Unlike the IGF-IR and IR, IGF-IIR is a single chain molecule with a large extracellular domain, a single transmembrane region and a small cytoplasmic domain. Although it is deemed to be a multi-functional receptor, unlike the IGF-IR and IR and it has no tyrosine kinase activity (Leroith and Roberts, 2003). It is, however, identical to the cation-independent mannose 6-phosphate receptor (MP6) and binds to lysosomal enzymes that carry mannose 6-phosphate moieties (Morgan *et al.*, 1987; Oates *et al.*, 1998). It has a high affinity for IGF-II, but not IGF-I (MacDonald *et al.*, 1988) and is believed to function as a sink for IGF-II to regulate its interaction with IGF-IR (Sachdev and Yee, 2001).

#### **1.1.2.3 IR isoforms:**

The IR exists in two isoforms (IR-A and IR-B) that arise from alternative splicing of exon 11 in the IR messenger ribonucleic acid (mRNA). This exon

codes for 12 amino acids that are inserted upstream of the third last residue of the extracellular  $\alpha$ -subunits of the IR-B isoform. The IR-A or (IR exon 11-) isoform lacks these 12 amino acids (Seino and Bell, 1989). The presence or absence of the exon 11-encoded peptide yields two different receptors with distinct biochemical properties. The IR-A is expressed predominantly in the central nervous system and haematopoietic cells while IR-B is expressed predominantly in adipose tissue, liver and muscle, the major target tissues for the metabolic effects of insulin (Moller *et al.*, 1989; Mosthaf *et al.*, 1990; White, 1997; Denley *et al.*, 2004). Importantly, although both isoforms have similar affinity for insulin, the IR-A binds IGF-II with high affinity and can predominantly mediate proliferative effects following activation. This serves to provide an additional way for IGF-II to enhance its own signalling potential compared to the principally metabolic effects elicited by insulin stimulation of the IR-B (Frasca *et al.*, 1999).

#### **1.1.2.4 IGF-IR hybrids:**

IGF-I does not show a significant level of cross-reactivity with the IR at physiological concentrations, since it has a relative affinity almost two orders of magnitude higher for the IGF-IR, as compared to the IR (Leroith and Roberts, 2003). However, because of the high sequence and structural similarity between IGF and insulin receptors, receptor hybrids can form which have biological relevance (LeRoith *et al.*, 1995). These chimeras consist of  $\alpha\beta$ -dimers of the IGF-IR linked to  $\alpha\beta$ -dimers of the insulin receptor (IR) by disulphide bonds (Soos *et al.*, 1993). Such chimeras can be found in a range of tissues and cell types and are likely to exist in all cells that express both IGF-IR

and IR (LeRoith *et al.*, 1995; Stewart and Rotwein, 1996). These hybrid receptors retain high affinity for IGF-I, but exhibit a dramatically decreased affinity for insulin (Soos *et al.*, 1993) and as such tend to behave like the IGF-IR. Thus binding of IGF-I to this hybrid receptor serves to provide an additional way for IGF-I to enhance its own signalling potential.

### **1.1.3 IGFBPs:**

The biological actions of the IGFs are modulated by a family of at least 6 binding proteins (IGFBPs) and these can be found in the circulation as well as in extracellular fluids and are produced by most tissues (Degeorges *et al.*, 2000; Duan and Xu, 2005). They have been shown to specifically associate with high affinity to IGF molecules where they act as carrier molecules for circulating IGFs, with more than 70% forming a ternary complex with IGFBP-3 (the largest and most abundant IGFBP) and the acid-labile subunit (ALS), a liver derived, GH-regulated glycoprotein (Oh, 1998). The principal role of IGFBP-3 has been postulated to be transporting IGFs and protecting them from both rapid clearance and degradation. IGFBP-3 also regulates IGFs bioavailability to their cognate receptors, thus serving to determine their effective physiological concentrations (Jones and Clemmons, 1995). The interactions of IGFBPs, such as IGFBP-3, with certain proteases, including cathepsin D, prostate-specific antigen (PSA) and plasmin, have also been shown to play an important regulatory role in facilitating the release of IGF ligand from the binding protein to receptor (Jones and Clemmons, 1995; Oh, 1998). Indeed, IGFBPs have been shown to both potentiate and inhibit IGF action '*in-vitro*'. Alongside its ability to modulate the biological action of IGFs

by preventing IGF binding to IGF receptors (IGF-dependent action) IGFBP3 can also exert its own biological action without interfering with the IGF-IGF-receptor axis (IGF-independent action) in targeted cells (Oh, 1998; Ricort, 2004). Such biological effects include, IGFBP-3's ability to bind specifically to the cell surface of certain human breast cancer cells where it can mediate the anti-proliferative effects of certain growth inhibitors (Gucev *et al.*, 1996) and also its ability to directly induce apoptosis in cells lacking the IGF-IR (Gill *et al.*, 1997).

#### ***1.1.4 IGF action and downstream signalling:***

Whilst the IR and IGF-IR belong to the same family, and share significant sequence and structural similarities, they mediate different effects on metabolism, cell proliferation, apoptosis and differentiation. The IR for example, is present mainly in metabolic tissues such as liver, muscle and adipose and following insulin stimulation, mediates glucose uptake and synthesis of glycogen and fat (White, 1997). The IGF-IR, however, plays a more central role in the control of cell proliferation, transformation and cell survival and is found to be ubiquitously expressed in most tissues types (Sepp-Lorenzino, 1998; Werner and LeRoith, 1997). Their close structural and sequence similarities and the ability of insulin and IGF ligands to bind each others receptors, as well as receptor hybrids, is permissive for a large degree of positive cross-talk and convergence between receptors and downstream signalling pathways (LeRoith and Roberts, 2003). However, for the purpose of this thesis, most attention will be focused on the role played by IGF-IR signalling on cell proliferation and survival as described below, although the

activation process of both IGF-IR and IR and the activation of their major down-stream signalling pathways are obviously overlapping (LeRoith and Roberts, 2003).

#### **1.1.4.1 IGF-IR phosphorylation and activation:**

IGF-IR activation results as a consequence of ligand binding to the extracellular  $\alpha$  domain of the IGF-IR (Figure 1). This results in a conformational change in the intracellular domain which induces receptor clustering and autophosphorylation of a number of key tyrosine residues within the cytoplasmic domain of the receptor (Sepp-lorenzino, 1998; Stewart and Rotwein, 1996; Adams *et al.*, 2000; LeRoith and Roberts, 2003; Jerome *et al.*, 2003).

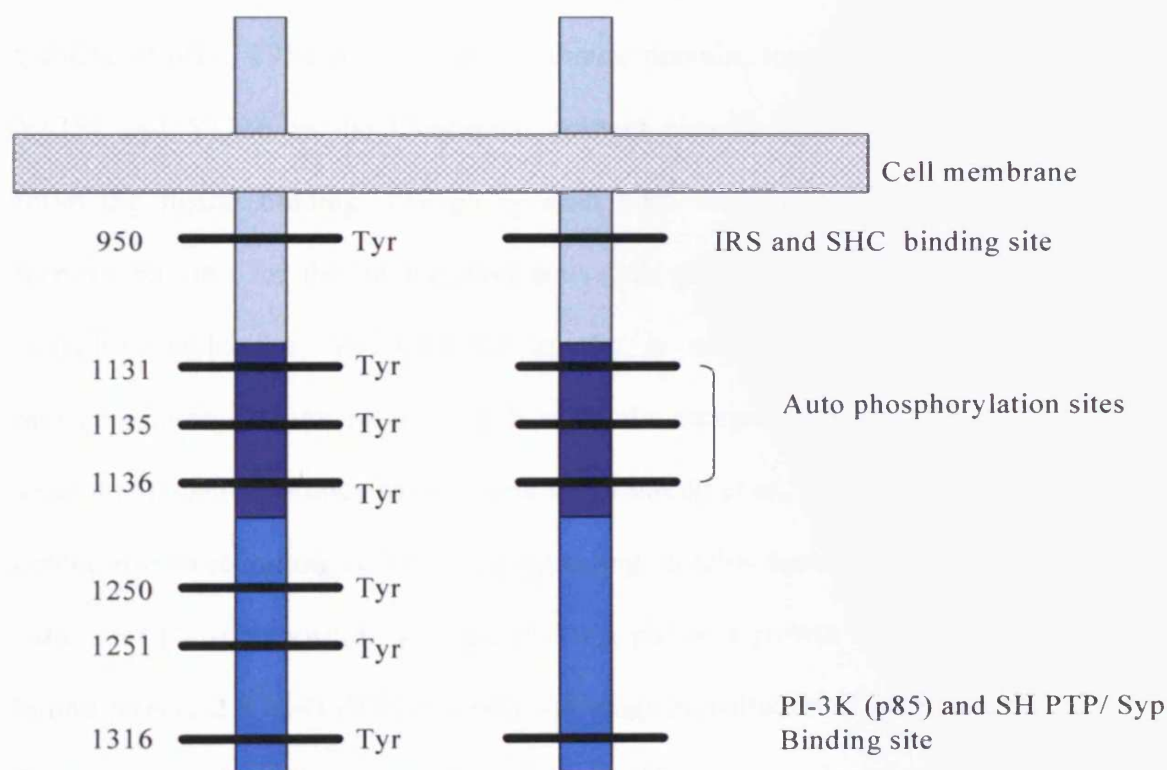


Figure 2. Illustrating the major tyrosine residues in the IGF-IR.

A number of tyrosine (Y) phosphorylation sites have been identified on the IGF-IR (as illustrated in Figure 2) including Y1131, Y1135, Y1136, Y1250, Y1251, Y1316 and Y950, predominantly by mutational analysis (Kato *et al.*, 1994; li *et al.*, 1994; Sepp-Lorrenzino, 1998). Indeed, mutational analyses have revealed that autophosphorylation of a cluster of tyrosines at positions 1131, 1135 and 1136 constitutes the initial phosphorylation event and accounts for the bulk of receptor phosphorylation with such phosphorylation required for full IGF-IR activation and for the biological activity of the receptor (Hernandez-Sanchez *et al.*, 1995; Sepp-Lorrenzino, 1998; Gronborg *et al.*, 1993). Mutation of all three tyrosines to phenylalanines inactivates the receptor, prevents receptor autophosphorylation, and abrogates mitogenesis, together with the capacity of the IGF-IR to transform normal cells to cancer cells (Kato *et al.*, 1994; li *et al.*, 1994; Gronborg *et al.*, 1993). Besides this tyrosine cluster, Y950 in the juxtamembrane domain, together with Y1250, Y1251 and Y1316 in the C-terminal domain also become phosphorylated following ligand binding. Phosphorylation sites such as these can act as recruitment sites for the binding and activation of a number of downstream signalling molecules. Y950 (Y960 in IR) is essential for binding and phosphorylating adaptor proteins such as insulin receptor substrate (IRS) and src and collagen-homology (SHC) protein (Yamasaki *et al.*, 1992) which play a central role in recruiting downstream signalling proteins such as the regulatory subunit of phosphoinositide 3-kinase (PI3-K), p85 and growth factor receptor-bound protein 2 (Grb2) (White, 1997). Although substitution of phenylalanine for tyrosine at the 950 amino acid site had no effect on autophosphorylation or

ligand-activated receptor internalization, it was shown to prevent IRS-1 phosphorylation (Miura *et al.*, 1995a; Yamasaki *et al.*, 1992).

Following mutational analysis, two tyrosine residues that lie within the C-terminal domain, Y1250 and Y1251 were shown to have a profound effect on the mitogenic and tumourigenic capacity in IGF-IR transfected fibroblast cells (Blakesley *et al.*, 1996; Miura *et al.*, 1995b). Moreover, deletion of the entire C-terminus from amino acid 1229 was shown to completely block tumour cell development (Surmacz *et al.*, 1995; Hongo *et al.*, 1996). Tyrosine 1316 is a direct binding site for p85 (Tartare-Deckert *et al.*, 1996), and tyrosine phosphatase SH-PTP-2/ Syp (Stewart and Rotwein, 1996). As such, mutation of Y1316 was shown to interrupt protein-protein interactions between the IGF-IR and these two molecules and was associated with impaired proliferation and tumourigenesis potential (Blakesley *et al.*, 1996). Importantly, as stated above, IGF-IR activation creates binding sites for several IGF-IR substrates. The best known substrates are the IRS and SHC adaptor proteins which have been shown to function as intermediates between the receptor and two major downstream signalling pathways, the extracellular signal regulated kinase (ERK1/2) mitogen-activated protein kinase (MAPK) and PI3-K cascades, which influence key cell signalling pathways (Myers *et al.*, 1993; White, 1997; Peruzzi *et al.*, 1999; Skolnik *et al.*, 1993; Valverde *et al.*, 2001; Giorgetti *et al.*, 1994). Figure 3 illustrates some of the key pathways involved in IGF-IR signalling.

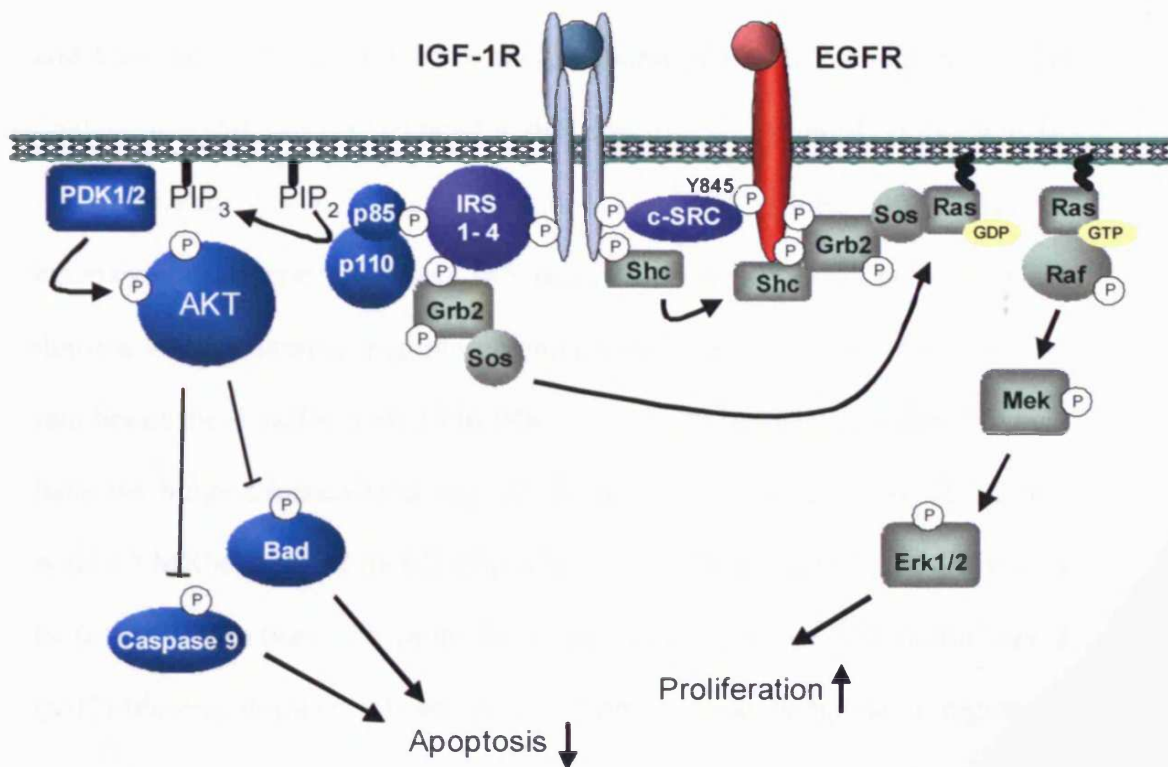


Figure 3. Key signalling pathways associated with IGF-IR.

#### 1.1.4.2 Insulin receptor substrate family:

IRS molecules were initially detected in insulin-stimulated Fao hepatoma cells as 185kda proteins (White *et al.*, 1985), after which their cDNA structures were also determined for various species, including human (Nishiyama and Wands, 1992). They are now known to constitute a family of structurally related adaptor 165-195Kda proteins (IRS-1-4) of which IRS-1 is the most extensively studied (White 1997; Butler *et al.*, 1998a). IRS molecules share similar features which include an N terminal pleckstrin homology (PH) domain adjacent to a phosphotyrosine binding (PTB) domain and a variable length carboxyl-terminal tail that contains numerous tyrosine and serine phosphorylation sites (Razzini *et al.*, 2000; Jacobs *et al.*, 2001; van der Geer



and Pawson, 1995). They bind to the  $\beta$ -subunit of IGF-IR through their PTB domain at Y950 which is situated within specific NPXY motifs in the IGF-IR juxtamembrane domain (Figure 2; Hsu *et al.*, 1994; White, 1997). There is extensive heterogeneity between IRS family members in the carboxyl-terminal domain which contains multiple potential phosphorylation docking sites. The number of these varies from 13 in IRS-3 to over 20 in IRS-1 and IRS-2, which have the longest intracellular regions. Many of these residues are also found within YMXM/ YXXM motifs (Sun *et al.*, 1993; White, 1997) that are known to favour interaction with proteins containing isoforms of SRC homology 2 (SH2)-binding domains (Ward *et al.*, 1996). These comprise a region of approximately 100 amino acids that share sequence similarity with a segment first identified in the cellular oncogene c-SRC (Stewart and Rotwein, 1996; Pawson and Schlessinger, 1993; Pawson, 2004).

#### ***1.1.4.3 Key signal transduction pathways recruited by IGF-IR/IRS-1:***

IRS interaction with SH2-containing proteins indicates a central role for IRS as a major recruitment factor for key downstream signalling proteins. Indeed IRS-1 has been shown to bind PI3-K (Myers *et al.*, 1994; Peruzzi *et al.*, 1999), SH2 domain containing tyrosine phosphatase (SH-PTP-2 or Syp), Fyn, Nck and Grb2 (Brodt *et al.*, 2000; White, 97). This interaction occurs as a result of IGF-IR-mediated phosphorylation of IRS-1 at a variety of tyrosine residues. These include Y612, Y941, Y608, Y632 (p85 binding sites; Esposito *et al.*, 2001), Y1172 (a SHPTP2/Syp binding site; Sun *et al.*, 1993) and Y895 (a Grb2 binding site; Valverde *et al.*, 2001; Hers *et al.*, 2002; Sun *et al.*, 1993). The

latter has shown to be an important binding site for initiating the MAPK pathway in some cells following insulin stimulation (Valverde *et al.*, 2001).

#### ***1.1.4.3.1 IGF/IGF-IR activation of the PI3-K pathway:***

The p85 regulatory subunit of PI3-K is known to interact with both phosphorylated IGF-IR directly and IRS molecules that have been phosphorylated as a result of IGF-IR receptor activation. Following interaction with these proteins, p85 promotes the activation of p110, the catalytic component of PI3-K. Phosphatidylinositol 4,5-di-phosphate (PIP<sub>2</sub>), the major substrate of PI3-K, is phosphorylated further to phosphatidylinositol 3,4,5-tri-phosphate (PIP<sub>3</sub>) by this kinase (Tartare-Deckert *et al.*, 1996). The best characterized substrates of PI3-K signalling are the Akt/PKB serine threonine kinases which comprise three known isoforms that are established targets of IGF-1 and insulin signalling (Chan *et al.*, 1999). Elevated PIP<sub>3</sub> binds to the PH domain of at least two proteins, Akt/PKB and serine-threonine kinase 3-phosphoinositide-dependent kinase 1 (PDK-1) (Shepherd, 2005). PDK-1 then phosphorylates Akt/PKB at two sites, threonine 308 and serine 473 and phosphorylation of both these residues is required for its activation (Alessi and Cohen, 1997). PDK-1 can also phosphorylate other proteins, including p70 ribosomal protein S6 kinase (p70<sup>S6K</sup>), a potent cell survival mediator (Pullen *et al.*, 1998), protein kinase C (PKC) and PKA (Moore *et al.*, 2002; Vincent and Feldman, 2002; Shepherd *et al.*, 2005). Following activation, Akt/PKB coordinates a range of biological effects including metabolic responses, proliferation and importantly, inhibition of apoptosis (Lawlor and Alessi, 2001). PI3-K signalling is negatively regulated by the phosphoinositide-

specific phosphatase PTEN, a tumour suppressor lipid that dephosphorylates the 3' and 5' positions of the inositol ring of phosphoinositides, including PIP3 (Maehama and Dixon, 1998).

#### ***1.1.4.3.2 IGF/IGF-IR activation of the MAPK pathway:***

The IGF-IR can activate the MAPK pathway via 2 independent routes converging at Grb2, an SH2 and SH3 containing adaptor protein. Firstly, IGF-IR activation can result in the recruitment of IRS-1 which when phosphorylated on sites such as Y896 can associate with Grb2 (Skolnik *et al.*, 1993; Valverde *et al.*, 1995). A second major adaptor protein recruited by the activated IGF-IR is SHC which binds to the receptor through its SH2 or PTB binding domain (Giorgetti *et al.*, 1994). This molecule is subsequently tyrosine phosphorylated enabling its association with Grb2. Following recruitment, Grb2 plays a central role in the regulation of the Ras-MAPK pathway by virtue of its interaction with the p21 Ras GTP-exchange factor SOS which promotes the exchange of GTP for GDP on p21 Ras resulting in the activation of Ras to its GTP state (Simon *et al.*, 1993; Benito *et al.*, 1996). P21 Ras protein binds guanyl nucleotides and has intrinsic GTPase activity resulting in hydrolysis of GTP back to GDP to restore the protein to its GDP-inactive state. Activation of p21 Ras at the cell membrane in turn phosphorylates MAPK kinase kinase (MEK1/2 kinase); also known as Raf which phosphorylates MEK1/2, which in its turn phosphorylates MAPK/ERK1/2. Critically, when phosphorylated, MAPK/ ERK1/2 is transported to the nucleus where it phosphorylates a variety of downstream targets, including transcription factors, which alter changes in

gene expression and enzymes involved in regulating cell proliferation, cell differentiation, survival and motility (Blenis, 1993; Davis, 1993).

#### ***1.1.4.3.3 IGF/IGF-IR activation of other signalling pathways:***

Other proteins which have been recruited as a result of IGF-IR activation in response to IGF include the proto-oncogenes c-Crk and also the p125 focal adhesion kinase (FAK) (Koval *et al.*, 1998; Baron *et al.*, 1998). The Crk family consist of proteins which include Crk I, Crk II, v-Crk and Crk-L (Matsuda *et al.*, 1992; Ten Hoeve *et al.*, 1993) They each contain SH2 and SH3 domains and despite the lack of a tyrosine kinase domain these proteins can be tyrosine phosphorylated (De Jong *et al.*, 1995). FAK, a tyrosine kinase molecule, Crk-II and Crk-L have been shown to be direct substrates of the IGF-IR (Koval *et al.*, 1998; Beitner-Johnson and Le-Roith, 1995; Baron *et al.*, 1998). Moreover, they have been shown to form a link between activation of the IGF-IR and the integrin-mediated signalling pathway and cytoskeleton by forming complexes with the focal adhesion adaptor proteins, paxillin and p130<sup>cas</sup> kinase, through a PI 3-kinase-dependent pathway, which in turn plays a central role in the regulation of cell shape and motility (Baron *et al.*, 1998; Casamassima and Rozengurt, 1998).

#### ***1.1.4.4 Cellular consequences of IGF-IR signalling:***

##### ***1.1.4.4.1 Survival:***

One of the most important roles for the IGF-IR facilitating its functions as a potent cell survival agent is its strong anti-apoptotic action and this is largely mediated through the PI3-K /Akt pathway (Vincent and Feldman, 2002). The

activation of PI3-K by IGF-I has emerged as the dominant route by which a variety of cell types are protected from apoptosis (Adams *et al.*, 2000). These anti-apoptotic effects are evident in cell types as diverse as osteoblast (Tumber *et al.*, 2000), melanoma cells (Ge *et al.*, 2000), cardiac myoblasts (Hong *et al.*, 2001; Fujio *et al.*, 2000), neuronal cells (van-Golen and Feldman, 2000) and epithelium (Ahmad *et al.*, 1999). Protection from apoptosis was also conferred by IGF-I to a number of haematopoietic and interleukin-3-dependent cells (Rodriguez-Tarduchy *et al.*, 1992). Moreover, the IGF-IR has been shown to play an important regulatory role in the mammary gland where apoptosis is a key feature of mammary gland development and re-modelling from the embryonic stages to pregnancy (Green and Streuli, 2004).

One mechanism by which Akt may promote survival is through the inhibition of a component of the cell death machinery. Among molecules central to the regulation of cell death in eukaryotes are members of the Bcl-2 family of proteins. Several members of the Bcl-2 family (including Bcl-2 and Bcl-X<sub>L</sub>) promote cell survival while other members (including BAD) promote cell death (Datta *et al.*, 1997; Gajewski and Thompson, 1996; Datta *et al.*, 1997; Lawlor and Alessi, 2001). Phosphorylation of Akt/PKB can result in the phosphorylation of Bad. In its non-phosphorylated state, Bad exerts its pro-apoptotic function by locating at the mitochondrial membrane where it forms heterodimers with Bcl-2 or Bcl-XL and prevents either of these molecules from performing their anti-apoptotic functions within the mitochondria (Yang *et al.*, 1995; Datta *et al.*, 1997). Once phosphorylated by Akt/PKB at 2 sites serine 136 or serine 112, Bad associates with the cytosolic protein 14.3.3 (Zha *et al.*, 1996) and becomes unable to interfere with Bcl-2 or Bcl-XL and thus

prevented from performing its role as a cell death-promoting protein (Vincent and Feldman, 2002; Peruzzi *et al.*, 1999; Adams *et al.*, 2000). Phosphorylated Akt/PKB can also prevent the initiation of the caspase cascade through phosphorylation and inactivation of caspase 9, a member of a large number of caspases which together trigger the cell death process (Kermer *et al.*, 2000).

#### **1.1.4.4.2 Proliferation:**

The IGF-IR has also been shown by many studies to play a pivotal role in cell cycle progression (Pietrzkowski *et al.*, 1992; Reiss *et al.*, 1992; Stull *et al.*, 2002; 2004). Indeed, a recent study by Stull *et al.*, (2002) showed that IGF-I action was essential for cell cycle progression in mammary epithelial cells and that it was required for epidermal growth factor (EGF)-mediated progression past the G (1)-S checkpoint in these cells. Additionally, the IGF system has been shown to regulate the expression and action of various molecules associated with cell cycle progression (Dupont and Holzenberger, 2003a). These include cyclin D1 and cyclin dependent kinase 1 (CDK-1) (Rosenthal and Cheng, 1995; Dupont *et al.*, 2003; Dufourney *et al.*, 1997), in addition to cyclins E, A and B1 in mammary epithelial cells (Stull *et al.*, 2002), and that these actions are largely mediated through PI-3K signalling (Dupont *et al.*, 2003; Dufourney *et al.*, 1997). Indeed, Akt/PKB activation has been shown to mediate cell cycle progression firstly by inducing the expression of cyclin D1 (Gille and Downward, 1999; Lawlor and Alessi, 2001) and secondly by phosphorylating and thus abrogating the activity of the CDK inhibitor (CKI) p27<sup>Kip1</sup> at threonine 157, as a result of its cytosolic retention (Shin *et al.*, 2002; Lawlor and Alessi, 2001).

The MAPK cascade, like PI3-K also plays a pivotal role in regulating cell cycle progression (Roovers and Assoian, 2000). Thus, several observations implicate the Ras/MAPK signalling pathway in cyclin D1 and p27<sup>Kip1</sup> regulation with its induction dependent on activation of this signalling cascade (Cheng *et al.*, 1998; Lavoie *et al.*, 1996; Aktas *et al.*, 1997). Moreover, such regulation of molecules associated with cell cycle progression mediated through Ras/MAPK signalling has been shown to be linked to IGF-IR activation of IRS-1 following IGF-I stimulation (Jackson *et al.*, 1998; Mawson *et al.*, 2005; Kuemmerle *et al.*, 2004).

#### ***1.1.4.4.3 Motility, migration and invasion:***

There is substantial evidence suggesting a role of IGF-IR in cell motility and migration (Guvakova, 2006). Indeed many cell types including, endothelial, osteoblast, neuroblasts, epithelial, breast cancer, lymphoid, smooth muscle and melanoma cells, have been shown to migrate towards a source of IGFs following activation of IGF-IR (Leventhal and Feldman, 1997; Leventhal *et al.*, 1997; Stracke *et al.*, 1989; Guvakova, 2006). Stimulation with IGF-I has been shown to cause cells to undergo dynamic morphological changes, leading to membrane ruffling and extension of cell protrusions such as lamellipodia, involved in the motile response (Kim and Feldman, 1998; Meyer *et al.*, 2001; Guvakova, 2006). Such IGF-I mediated cell motility has been shown to involve the activation of both the PI3-K and MAPK pathways (Imai and Clemmons, 1999; Meyer *et al.*, 2001; Kim and Feldman, 2000; Guvakova, 2006). In addition, the IGF-IR has been shown to play an important role in mediating tumour invasion and metastasis in a variety of cancer types including breast,

colon, lung, prostate and pancreas (Kubak and Dunn, 2003; Zhang and Brodt, 2003; Sekharam *et al.*, 2003; Bauer *et al.*, 2006, Long *et al.*, 1998a). Invasiveness of tumour cells is a complex process that requires the proteolytic cleavage or degradation of extracellular matrix (ECM) barriers, coupled with the migration of the cells through this modified region (Stetler-Stevenson *et al.*, 1993). Degradation of the ECM in malignant tumours is accomplished primarily by members of the matrix metallo proteinase (MMP) family of enzymes. Interestingly, numerous studies have identified the IGF-IR as a positive regulator of type IV collagenase (MMP-2) synthesis, known to play a critical role in tumour cell invasion by facilitating the degradation of basement membrane type IV collagen (Zhang *et al.*, 2003; Long *et al.*, 1998b; Grzmil *et al.*, 2004).

#### ***1.1.5 Physiological and pathological actions of IGF-IR:***

The IGF-IR and its components have been shown to be involved in the regulation of a number of key cellular events including cell proliferation, survival, motility and migration as described above and have been shown to be expressed abundantly in most tissues including nervous system, muscle, skin, lung, ovary, cartilage, bone and mammary gland (Dupont and Holzenberger, 2003b). As such, it comes as no surprise to learn that the IGF axis plays a critical role in the normal physiological actions associated with pre and post-natal growth and development (Peoples *et al.*, 1995; Daughaday and Rotwein, 1989; Powell-Braxton *et al.*, 1993; Liu *et al.*, 1993; Lupu *et al.*, 2001; LeRoith *et al.*, 2001; Stewart and Rotwein, 1996). For instance, in humans, loss of one copy of the IGF-IR gene was shown to result in patients presenting with severe growth retardation (Peoples *et al.*, 1995). Furthermore, extensive analyses in



mice have confirmed the critical role of IGF action in the normal development of the embryo and foetus (Powell-Braxton *et al.*, 1993; Liu *et al.*, 1993; Lupu *et al.*, 2001) and mice lacking the IGF-IR were small at full term, approximately 45% of wild-type size and were non-viable, dying of respiratory failure at birth (Liu *et al.*, 1993). In addition to the vital role played by the IGF-IR axis in normal growth and development, it is also essential for many physiological functions (Jones and Clemmons, 1995; LeRoith *et al.*, 2001). These include normal tissue repair, regeneration and turnover, functions that are typically required of tissues such as muscle and bone (Sims *et al.*, 2000; Powell-Braxton *et al.*, 1993; Saetrum Opgaard and Wang, 2005) and cell survival, proliferation and differentiation in tissues such as mammary gland, lung and nervous system (Richert and Wood, 1999, Wood *et al.*, 2000; Ruan and Kleinberg, 1999; Han *et al.*, 2003; D'ercole *et al.*, 1996).

An alteration or disruption in the balance of the IGF system has been shown to be associated with a variety of disease states. Indeed epidemiological and clinical studies have implicated low IGF-I levels in serum as a risk factor for myocardial infarction and other impairments of cardiac function (Kaplan *et al.*, 2005; Ren *et al.*, 1999). Similarly, conditions associated with old age such as dementia and Alzheimer's disease have also been shown to be linked to IGF-I deficiency, where patients with dementia present with significantly lower serum IGF-I concentrations than their matched controls (Watanabe *et al.*, 2005). Indeed, impaired expression of IGF components, normally produced by central nervous system neurons, have also been shown to be associated with Alzheimer's disease (Steen *et al.*, 2005). Such IGF imbalances are also evident in bone tissues, where a decline in bone IGF-I axis is correlated with the

increased risk of osteoporosis and fragility fractures in the elderly (Agnusdei and Gentilella, 2005). Another rare but disabling disorder that commonly affects the middle aged and often results in premature death is acromegaly. This disease is caused when the pituitary gland produces excess GH which can be secondary to a pituitary somatotroph tumour. Such elevation of GH stimulates the over production of IGF-I and the increased morbidity and mortality of acromegaly sufferers are the result of the actions of an excess of both GH and IGF-I secretion (Paisley and Trainer, 2003).

#### ***1.1.6 IGF action and cancer:***

In addition to its role in the patho/physiological processes described above, there is a huge body of evidence implicating a role for the IGF-IR axis in the development of many cancers (Baserga *et al.*, 2003; Yu and Rohan, 2000; Jerome *et al.*, 2003; Samani *et al.*, 2007). As described above, the IGF-IR system performs a fundamental role in the regulation of cellular proliferation, survival and motility, key oncogenic processes implicated in cancer progression (Yu and Rohan, 2000). Initially, the importance of IGF-IR in carcinogenesis was suggested by the observation that constitutive over-expression of IGF-IR and hyperactivation of its signalling potential often induced a transformed phenotype in cultured cell systems (Kaleko *et al.*, 1990; Pietrzkowski *et al.*, 1992). Conversely, experiments using cells derived from IGF-IR knockout mice provided evidence that different tumourigenic agents (Viral, chemical or genetic) were unable to induce transformation in the absence of IGF-IR, but were transforming when IGF-IR (but not IR) was re-expressed (Baserga, 1994; 1995). Furthermore, this transforming effect could

be prevented by the expression of truncated IGF-IR receptors lacking important residues in the tyrosine kinase domain (Hernandez-Sanchez *et al.*, 1995; Kato *et al.*, 1994), thus indicating that intact functional receptors are required to mediate tumourigenesis.

Various '*in vitro*' experiments with tumour cell lines, in addition to epidemiological studies have provided further evidence that activation of IGF-IR and its components are involved in the development of many common neoplastic diseases, including carcinoma of the pancreas (Korc, 1998); prostate (Djavan *et al.*, 2001; Chan *et al.*, 1998); liver (Scharf *et al.*, 2001); colon (Giovannucci, 2001); lung (Yu *et al.*, 1999); ovary (Kalli and Conover, 2003); endometrium (Oh *et al.*, 2004), skin (Kanter-Lewensohn *et al.*, 1998) and breast (Sachdev and Lee, 2001). For instance, it has been found that in some cancers, IGF-IR is over expressed compared to their normal counterparts (Burrow *et al.*, 1998; Hakam *et al.*, 1999; Kanter-Lewensohn *et al.*, 1998; Weber *et al.*, 2002; Jerome *et al.*, 2003). Moreover, IGF-I and IGF-II, potent mitogens in a wide variety of cancer cell lines (Oku *et al.*, 1991; Leroith *et al.*, 1995; Singh *et al.*, 1996; Yu and Rohan, 2000, Lee *et al.*, 1997), have also been shown to be produced by several tumours types (Cardillo *et al.*, 2003; Burrow *et al.*, 1998; Samani *et al.*, 2007). However, of particular interest, is the finding that IGF-II rather than IGF-I has been shown to be more commonly over-expressed in many cancers and in several cases found to be associated with tumour progression and poor patient survival (Sayer *et al.*, 2005; Mita *et al.*, 2000; Lu *et al.*, 2006; Kawamoto *et al.*, 1998; Weber *et al.*, 2002). Furthermore, high abundance of endocrine IGFs may also serve to influence IGF-IR signalling activity. In this context, it is important to note that elevated

plasma IGF-I and IGF-II concentrations have been linked with increased risk of several types of cancers (Oh *et al.*, 2004; Chan *et al.*, 1998; Yu *et al.*, 1999; LeRoith and Roberts, 2003; Pollak, 2000; Pollak 2004; Jerome *et al.*, 2003). Indeed, experimental evidence has shown that raised levels of IGF-I correlate with increased tumorigenicity in mice (Dunn *et al.*, 1997 and Butler *et al.*, 1998b).

Breast cancer has been one of the most extensively studied with respect to IGF-IR signalling as the IGF-IR has clearly been shown to be involved in the development of the disease. As the purpose of this thesis is to investigate the role of IGF-IR action in breast cancer, both breast cancer and its relationship to IGF-IR signalling are extensively reviewed below.

### **1.2 Breast cancer:**

Mammary growth occurs around puberty and is controlled by a series of intricate and complex events, which are co-ordinated at least in part by the ovarian steroids oestrogen and progesterone (Russo *et al.*, 2000; Dickson and Lippman, 1995). Disruption of the molecular events involved in the processes required for normal mammary function can ultimately result in transformation of the breast cells to the cancerous state (Schuchard *et al.*, 2003). Breast cancer alarmingly, accounts for almost one in three of all cancers in woman, with over 41,000 new cases each year in the UK alone. However, thanks to earlier detection and improved treatment regimes the breast cancer death rate in the UK has actually fallen by 20% in last 10 years as reported by the Cancer Research Campaign, (2005). Nevertheless, 1 in 9 women in the UK will sadly develop the disease during her lifetime. Although the precise events involved

in disease development are still not fully understood, many factors are known to contribute, including genetic, dietary, reproductive and environmental factors (Blackburn *et al.*, 2003; Coyle, 2004; Goodwin *et al.*, 2003; Chlebowski, 2005; Schuchard *et al.*, 2003; Bruning *et al.*, 1992; Ames *et al.*, 1995). While a family history of breast cancer is deemed a strong risk factor, with a first degree relative with breast cancer increasing the risk 1.5-3-fold (Willet, 1989) and germline mutations in the early onset familial breast cancer gene BRCA1 and BRCA2 are responsible for most hereditary breast cancer (Lux *et al.*, 2000; Palma *et al.*, 2006), hormonal events constitute the most significant factor (Clarke *et al.*, 2004). Thus since the breast is a hormone responsive tissue and since the finding that breast cancer is uncommon in males, many believe that most breast cancers originate as a result of hormone-dependent events. Indeed, exposure to endogenous and exogenous oestrogens have been shown to play a crucial role in regulating the development and growth of breast cancers (Russo and Russo, 1998; Shoker *et al.*, 1999; Colditz, 1998; Clarke *et al.*, 2004).

### ***1.2.1 Evidence for oestrogen dependency of breast cancer:***

As stated above, breast cancer growth often appears to be dependent on oestrogens and in particular 17 $\beta$ -oestradiol, the predominant circulating oestrogen and breast cancer cells can be shown to proliferate in response to this hormone (Nicholson *et al.*, 1992; Manning *et al.*, 1992; Lee *et al.*, 1997; Butt *et al.*, 2005). This dependency is perhaps reflected in the increased incidence of breast cancer in women who are subject to increased cumulative exposure to oestrogens, due for example to an early menarche or late menopause (Suschard

*et al.*, 2003). In contrast, an early pregnancy which is thought to mature the breast has been shown to reduce the lifetime incidence of breast cancer (Suschard *et al.*, 2003; Russo *et al.*, 2005).

The first indirect evidence that the growth of breast cancer may be under endocrine control was cited in 1889 by a German surgeon named Albert Schinzinger who noted that atrophy of the breast normally followed the cessation of ovarian function. This led to the suggestion that ovariectomy might lead to regression of breast cancer. This surgical procedure was later performed by Sir George Beatson who subsequently described the beneficial effects of castration in pre-menopausal women presenting with advanced breast cancer (Beatson, 1896). Importantly, eradication of the ovaries was used as an effective treatment, in addition to surgical breast tumour excision, for much of the 20<sup>th</sup> century. Based on this long experience it is now evident that ovariectomy provides benefit for about one third of pre-menopausal women with breast cancer through its capacity to reduce circulating oestrogens (Miller, 1990).

### ***1.2.2 Oestrogen receptor:***

Oestrogens exert their effect through their interaction with the oestrogen receptor (ER) leading ultimately to the transcriptional activation of a variety of genes, many of which have been shown to be important for normal cell physiology and growth of some breast tumours. These include the progesterone receptor (PgR), PS2, heat shock proteins, transforming growth factor alpha (TGF $\alpha$ ), IGF-II, IGF-IR, IRS-1, cyclin D1, and c-Myc (Frasor *et al.*, 2003);

Butt *et al.*, 2005; Manning *et al.*, 1993; Shao and Brown, 2004; Klinge, 2001; Tsai and O'Malley, 1994).

The ER was discovered by Elwood Jenson in 1958 and cloned in 1986 from rat uterine tissue by Green *et al.*, (1986). It is a 65kDa protein and its protein sequence, together with its 3-dimensional structure has since been determined (Green *et al.*, 1986). Although it was first thought to be a unique protein, a close family member has recently been discovered, ER $\beta$  (Kuiper *et al.*, 1996 and Mosselman *et al.*, 1996). The original ER is now commonly referred to as ER $\alpha$ . Both ER subtypes belong to a nuclear receptor superfamily, which also includes the P $\gamma$ R, glucocorticoid receptor and the androgen receptor (Mangelsdorf *et al.*, 1995). In each instance they function as hormone-inducible transcription factors to modulate the transcription of target genes (Beato and Klug, 2000; Shao and Brown, 2004; Mangelsdorf *et al.*, 1995). Molecular studies have revealed the existence of distinct functional domains in these receptor proteins. As such, both ER $\alpha$  and ER $\beta$  consist of 6 functional domains, including the DNA binding domain (DBD), which is the most conserved domain, the ligand binding domain (LBD), the ligand-independent activation function (AF-1) domain and the ligand-dependent activation function AF-2 domain (Kumar *et al.*, 1987; Osborne *et al.*, 2000; Schiff and Fuqua, 2002; Schiff *et al.*, 2004; Shao and Brown, 2004).

Both ER isoforms display different roles in the regulation of gene expression and show different affinities for various anti-oestrogens such as the SERM tamoxifen (Ogawa *et al.*, 1998). They also show different tissue distribution (Speirs *et al.*, 2002; Nilsson and Gustafsson, 2000) and are under different regulatory control (Katzenellenbogen and Katzenellenbogen, 2002).

Importantly, ER $\alpha$  rather than ER $\beta$ , appears to be the dominant regulator of oestrogen-induced genes in breast cancer (Palmieri *et al.*, 2002; Fuqua *et al.*, 2003) and is considered to be the predominant mediator of the mitogenic effects of oestrogen in the mammary gland (Hewitt and Korach, 2003). Extensive studies on clinical tissue have shown that ER $\alpha$  is expressed in a large proportion of breast tumours (McGuire *et al.*, 1978; Walker *et al.*, 1988) and in post-menopausal breast tumours as many as 70% of tumours show readily detectable quantities of the receptor (Robertson, 1996). Similarly, a recent immunohistochemical study by Fuqua *et al.*, (2003) demonstrated that in addition to ER $\alpha$ , ER $\beta$  protein is also co-expressed in a large percentage of breast tumours.

### ***1.2.3 Classical mode of ER action:***

Oestrogen exerts its effect by diffusing across both the plasma and nuclear membranes where it is retained in target cells by binding with high affinity to the ligand binding domain of ER (Fritsch *et al.*, 1992; Katzenellenbogen *et al.*, 1993). The binding of oestrogen to ER results in receptor phosphorylation, alteration in receptor conformation which then initiates the dissociation of chaperone proteins such as the heat shock proteins Hsp90 and Hsp70 (Pratt and Toft, 1997). Such events then trigger receptor dimerization, allowing binding of the receptor complex to promoter regions of target genes. The specific sequences of DNA recognised by ER are referred to as oestrogen response elements (EREs) and these reside within the promoter regions of target genes. The structure of the DBD of ER allows ER to recognise and interact with these ERE sequences. The ERE is a 13 nucleotide palindromic



sequence usually situated up-stream of oestrogen regulatory genes (Klein-Hitpass *et al.*, 1988; Kumar *et al.*, 1986; Klinge, 2001).

ER mediated transcription is stimulated through at least 2 distinct transactivation domains called AF-1 and AF-2. These serve to recruit other proteins to transcription start sites such as transcriptional co-activators including the p160 family of co-activators, AIB1, GRIP-1 and SRC-1 (Osborne *et al.*, 2000; Shao and Brown, 2004). Ligand-independent AF-1 activity has been shown to be regulated by growth factors that act via the MAPK pathway (Kato *et al.*, 1995) whereas the AF-2 ligand dependent domain is activated by oestrogen (Kumar *et al.*, 1987). Often full transcriptional activation requires both AF-1 and AF-2 to be activated (Tzukerman *et al.*, 1994). AF-2, which lies within the ER-ligand binding domain, is believed to be the strongest transactivation function and acts by binding to short motifs (nuclear receptor boxes, NR boxes, consensus LXXLL) that are found repeated three or four times within the p160 proteins. Such AF-2/p160 contacts are thus oestrogen dependent (Webb *et al.*, 1998; Heery *et al.*, 1997).

#### ***1.2.4 Non classical mode of ER action:***

In addition to binding to a classic ERE element, the ER can also regulate transcription indirectly by interactions with other transcription factors such as c-Fos and c-Jun which act on promoters containing AP-1 or SP-1 recognition sites (Kushner *et al.*, 2000). It has been shown that ER interaction with AP-1-bound Fos and Jun proteins confers oestrogen responsiveness to the ovalbumin (Gaub *et al.*, 1990), collagenase (Webb *et al.*, 1995), and IGF-I (Umayahara *et al.*, 1994) genes and ER has been shown to interact with AP-1 and SP-1 sites in

breast cancer cell lines (Wang *et al.*, 1999; Rochefort, 1995; Philips, *et al.*, 1993; Webb *et al.*, 1995). In addition, a recent study by Glidewell-Kenney *et al.*, (2005) using micro-array analysis, identified oestrogen-responsive 'nonclassical' pathway ER $\alpha$  target genes that were differentially expressed in human breast tumours. Many of these genes were shown to be known AP-1 targets, implicating nonclassical ER $\alpha$  cross-talk with these transcription factors as important to breast cancer development.

These modes of action of ER described so far reside within the nuclear compartments of the cell and are due to the direct action on DNA and are therefore termed 'genomic effects'. However, recently, it has become clear that steroids such as oestrogens rapidly act on cells, in seconds to minutes, effects that are classified as 'nongenomic' (Falkenstein and Wehling, 2000). It has been shown that oestrogen-bound ERs can physically associate with several molecules including, the scaffold protein caveolin-1 and a variety of signalling molecules, including G proteins, c-SRC and Ras, leading to the activation of intracellular signalling cascades, all of which takes place at the plasma membrane (Razandi *et al.*, 2003; Razandi *et al.*, 2002; Migliaccio *et al.*, 2000; Migliaccio *et al.*, 1996). For instance, oestrogen-bound ERs when coupled to G proteins have been reported to rapidly activate the cell surface tyrosine kinase receptors such as epidermal growth factor receptor (EGFR) or IGF-IR resulting in signalling through Ras/MAPK and PI3-K/Akt pathways (Razandi *et al.*, 2003).

### ***1.3 IGF-IR action in breast cancer:***

While it is known that oestrogens are important for breast cancer cell growth, it is equally acknowledged that growth factors share this role and that the signalling pathways of oestrogens and growth factors are cross-reactive (Nicholson and Gee, 2000; Nicholson *et al.*, 2005). Pre-eminent within such growth factor signalling elements is the IGF-IR which not only can provide proliferative and anti-apoptotic signals to breast cancer cells (Karey and Sirbasku, 1988; Rubin and Baserga, 1995), but can promote the phosphorylation of the ER (Bunone *et al.*, 1996).

Although the IGF-IR has been shown to be an important factor in the biology of the normal mammary gland (Dickson and Lippman, 1995), studies on IGF-IR expression in breast cancers and its correlation with clinical-pathological parameters are fairly limited (Surmacz, 2000). Despite this, however, the IGF-IR has been shown to be expressed in a high percentage of breast tumours where its expression is positively correlated with ER status and usually co-expressed with markers of better overall prognosis (Surmacz *et al.*, 1998; Surmacz, 2000; Happerfield *et al.*, 1997; Gee *et al.*, 2005). Indeed, IGF-IR levels have been found to be over-expressed in primary tumours compared with normal epithelium or benign tumours (Pezzino *et al.*, 1996; Resnik *et al.*, 1998). The reason for high IGF-IR expression levels in breast cancer is not fully understood but it does not appear to be as a result of IGF-IR gene amplification since this event was only reported in 2% of cases analyzed (Berns *et al.*, 1992). However, it has been suggested that IGF-IR expression may be regulated by the tumour suppressor gene p53. This gene is frequently over-expressed in a mutated or non-functional form in human cancers

(Hollstein *et al.*, 1991). Because the IGF-IR gene is often over-expressed in breast tumours in which p53 is mutated (Horak *et al.*, 1991; Webster *et al.*, 1996) and since an in-active p53 de-represses the IGF-IR promoter it has been speculated, therefore, that in breast cancer, p53 loss of function may up-regulate the levels of IGF-IR (Werner *et al.*, 1996). Interestingly, the IR isoform IR-A also appears to be over-expressed in breast cancer cell lines and clinical samples, often alongside IGF-IR, and the high affinity interaction of the IR-A with IGF-II, has been shown to be important in cancer cell growth (Denley *et al.*, 2004). As a direct consequence of over-expression of the IR-A and IGF-IR in breast cancer samples, a high degree of receptor hybrid formation has also been demonstrated which has been shown to play an important role in further mediating the IGF-I signal in breast cancer (Pandini *et al.*, 1999; Frasca *et al.*, 1999).

In primary breast cancer over-expression of IGF-IR has also been shown to be hyper-phosphorylated compared with its status in normal mammary epithelia (Resnik *et al.*, 1998). As many tumours are known to produce IGF ligands, IGF-IR activation may thus simply occur as a result of autocrine or paracrine stimulation within the tumour mileu (Sachdev and Yee, 2001; Weber *et al.*, 2002). Additionally, however, epidemiology studies have found that high levels of circulating plasma IGF-I are associated with an increased risk of breast cancer in pre-menopausal women (Hankinson *et al.*, 1998; Yu *et al.*, 2002), implying an endocrine mediated IGF-IR activation.

Significantly, increased expression of other components of the IGF signalling axis have also been reported in clinical breast cancer specimens and linked to disease progression and recurrence. For instance, IRS-1 over expression was

shown to be constitutively phosphorylated in breast tumours (Chang *et al.*, 2002; Dearth *et al.*, 2006), associated with lymph node metastases (Koda *et al.*, 2005), confer poor patient prognosis (Lee *et al.*, 1999), as well as to be an indicator of early disease recurrence in patients with small tumours (Rocha *et al.*, 1997). Moreover, IGFBP expression has been correlated with ER status where high IGFBP-3 and IGFBP-4 levels are associated with poor patient outlook (Yee *et al.*, 1994; Rocha *et al.*, 1997).

In support of the above clinical evidence, several model systems have also demonstrated that IGF-IR plays a critical role in breast cancer development, survival and transformation (Yu and Rohan, 2000). For instance, *in vitro* inhibition of expression of the IGF-IR with an anti-sense IGF-IR mRNA or its function with anti IGF-IR antibodies resulted in growth inhibition and a reduced transforming potential in several different breast cancer cells (Arteaga, 1992; Neuenschwander *et al.*, 1995; Surmacz *et al.*, 1998). Similarly, animal studies also support a role for IGF function in the pathogenesis of breast cancer with MDA-231 xenograft tumour growth being significantly inhibited by blockade of IGF-IR with the monoclonal antibody  $\alpha$ IR3 (Arteaga, 1989).

Expression of the IGF-IR has been shown in most breast cancer cell lines, including hormone sensitive human MCF-7 cells (Stewart *et al.*, 1990; Lee *et al.*, 1999). In this cell line, the IGF-IR is not only a key receptor in mediating hormone sensitive growth, but shows significant cross-talk with ER (Hamelers and Steenbergh, 2003; Yee and Lee, 2000). Importantly, this leads to synergistic interactions between ER and IGF-IR signalling promoting efficient growth responses (Surmacz, 2000) where ER signalling stimulates IGF-IR expression and IGFs prime the activation of several kinases that are able to

phosphorylate ER at key AF-1 residues including serine 118 and serine 167. This increased phosphorylation is thought to occur through ERK1/2 MAPK (Bunone *et al.*, 1996) and initiate ERE-mediated gene expression (Hamelers and Steenbergh, 2003; Yee and Lee, 2000; Lee *et al.*, 1997).

In addition to an increase in IGF-IR expression following oestrogen treatment, other reports have also shown oestrogen to regulate the expression of other IGF axis elements including, IRS-1 and IRS-2 (Lee *et al.*, 1999, Salerno *et al.*, 1999; Molloy *et al.*, 2000; Bernard *et al.*, 2006), IGF-I (Umayahara *et al.*, 1994), IGF-II (Osborne *et al.*, 1989; Lee *et al.*, 1994), IGFBPs (McGuire *et al.*, 1992) and p85/p110 (Bernard *et al.*, 2006). Whilst these studies indicate that oestrogen induces expression of these proteins, whether this is a direct effect of ER upon each of their DNA promoters is unclear. Thus, although the IRS-1 promoter has four consensus half-EREs, supporting the possibility of direct ER regulation (Kato *et al.*, 1992), it also has several AP-1 and SP-1 sites (Araki *et al.*, 1995) which in other promoters have been shown to interact with the ER and activate transcription in a synergistic manner (Umayahara *et al.*, 1994). Significantly, the IGF-IR promoter also has several SP-1 sites (Beitner - Johnson *et al.*, 1995) and can be regulated by a wide range of hormones such as follicle stimulating hormone, GH, luteinizing hormone, thyroid hormone and glucocorticoids, as well as oestrogens (Sepp-Lorenzen, 1998).

#### ***1.4 Breast cancer therapy: Targeting ER signalling:***

Ovariectomy, which acts by reducing circulating oestrogens has proved to be an effective therapy for the treatment of breast cancer in about one third of premenopausal women (Miller, 1990). However, suppression of ovarian function

through surgical means is an irreversible procedure and as such this has led to the emergence and employment of hormonal therapies. In the 1980s, analogues of luteinising hormone-releasing hormone (LHRH) also known as gonadotrophin-releasing hormone (GnRH) agonists were introduced that produce suppression of ovarian function, yielding similar results to the surgical procedure but with the added benefit of being reversible (Robert *et al.*, 1989). These analogues function by downregulating pituitary GnRH receptors, thereby reducing the secretion of luteinising hormone and follicle-stimulating hormone, which, in turn, decrease the main source of oestradiol production in the ovaries (Rabaglio *et al.*, 2007). Such agonists, in particular, goserelin (Zoladex) has been shown to be as effective therapeutically as surgical ablation in both pre and peri-menopausal women with advanced breast cancer (Robertson and Blamey, 2003). Also, over the past two decades substantial effort has been directed toward developing potent inhibitors of aromatase, a cytochrome p450 enzyme which catalyzes the conversion of androgens (testosterone and androstenedione) to oestrogens (oestradiol and oestrone) respectively in peripheral tissues and breast tumours (Santen, 2003). Clinical studies initially demonstrated that administration of the first generation inhibitor, aminoglutethimide, caused regression of hormone-dependent breast cancer in women (Santen, 1990). This served as the impetus to develop highly potent and specific second and third generation inhibitors, such as anastrozole and letrozole, both of which have been shown to provide an effective first and second line therapeutic strategy in postmenopausal hormone dependent breast cancer (Simpson *et al.*, 2004; Santen, 2003; Brueggemeier, 2006). Moreover, drugs that antagonise the effects of oestrogen action at a cellular level have

been developed, such as the non-steroidal agents or selective oestrogen receptor modulators (SERMs) as reviewed in Howell *et al.*, 2004. One such compound, ICI46,474 also known as tamoxifen, was first re-invented as an anticancer agent from its origins as a failed contraceptive in the 1960s by Harper and Walpole (1967). At present, oestrogen antagonist therapy is still the most widely used treatment for woman with ER-positive breast cancer where tamoxifen promotes a significant improvement in survival for those patients with endocrine-responsive disease (Osborne, 1998; Osborne *et al.*, 2000; Shao and Brown, 2004).

Tamoxifen serves to competitively inhibit binding of oestrogen to its target, ER and induces a conformational change in the receptor that favours the recruitment of co-repressors, rather than co-activators, to target promoters that inhibit transcriptional activity (Graham *et al.*, 2000; Smith *et al.*, 1997; Shang and Brown, 2002). The degree to which anti-oestrogens block the cellular activities of the receptor depends on their molecular structure, and on the conformational state of the anti-hormone ER complex. Importantly, anti-oestrogenic effects can range from total inactivation of receptor function as seen for pure anti-oestrogens (typified by the compounds ICI 182,780, and ICI 164, 384), to substantial degrees of oestrogen-like activity for anti-oestrogens such as tamoxifen (Wakeling, 2000). In the latter case, although tamoxifen can inhibit AF-2 transcriptional activity, by completely blocking contacts between AF-2 and p160 co-activators, it fails to influence AF-1 activity (Berry *et al.*, 1990; Osborne *et al.*, 2001). Interestingly, the degree of oestrogen-agonist action exhibited appears also to be dependent on the cell type and promoter specificity of AF-1 (Berry *et al.*, 1990).



As well as blocking ER function, antihormonal treatment may prove potentially beneficial in its ability to influence other genes regulated by ER signalling. For instance, due to the close IGF-IR/ER signalling cross-talk demonstrated in hormone sensitive breast cancer cells and the regulation of IGF action by oestrogen, anti-hormonal treatment of endocrine responsive breast cancer cells commonly reduces IGF-signalling. Indeed, the effects exerted by tamoxifen or its active metabolite 4-OH-TAM on the IGF system in breast cancer cells include: inhibition of IGF-IR binding sites by 60% (Freiss *et al.*, 1990), reduced IGF-I induced tyrosine phosphorylation of IGF-IR (Guvakova and Surmacz, 1997), inhibition of IRS-1/PI3-K signalling (Guvakova and Surmacz, 1997), suppression of IGF-IR-dependent growth (Lee *et al.*, 1997; de-Cupis *et al.*, 1995) and down-regulation of IGF secretion (Huff *et al.*, 1988). These effects alongside the ability of anti-hormones to down-regulate plasma IGF-I levels in breast cancer patients are thought to significantly contribute to the anti-tumour effects of these agents (Colletti *et al.*, 1989).

### ***1.5 Endocrine resistance:***

The concept of therapeutic antagonists of oestrogen action was first suggested in the 1930s (Lacassagne, 1936), long before either the ER or anti-oestrogen-based drugs were identified. At present, oestrogen antagonist therapy is still the most effective treatment for woman with ER-positive breast cancer with tamoxifen currently remaining the most widely used endocrine agent for the treatment of ER positive breast cancer. However, a major problem that limits the effectiveness of this agent and ultimately confers poorer prognosis, is that

patients often present with primary *de novo* resistance to endocrine therapy, despite high tumour ER levels, and all patients with advanced disease that initially respond to tamoxifen treatment eventually relapse with acquired resistance (Howell *et al.*, 1993; Osborne and Fuqua, 1994). In general, acquired tamoxifen resistance is not always attributable to loss of or alteration in the ER as both tamoxifen-resistant tumours and breast cancer cell lines frequently retain the ER and remain responsive to pure anti-oestrogen therapy (Encarnacion *et al.*, 1993; Brunner *et al.*, 1993; Lykkesfeldt *et al.*, 1994; Robertson, 1996). Although the potential mechanisms for either *de novo* or acquired resistance are still poorly understood, several possible mechanisms have been investigated and could contribute, to some degree, to the development of these resistant phenotypes. These include, selection of ER $\alpha$  mutations; ER $\alpha$  splice variants; the role of ER $\beta$  and perturbation of the interactions between ER-co-regulatory proteins (Ali and Coombes, 2000; Ring and Dowsett, 2004). However, an increasing body of evidence now suggests that alterations/ aberrations of growth factor signalling networks that are highly interactive with the ER can readily promote anti-hormone resistance in breast cancer. The strongest evidence for this implicates members of the type 1 super family of receptor tyrosine kinases (RTKs), EGFR and c-erbB2 (Nicholson *et al.*, 2004; 2005).

#### ***1.5.1 The type 1 super family of RTKs:***

The type 1 super family of RTKs which consists of four genes termed c-erbB, encoding the EGF receptor (EGFR/c-erbB1/ HER1), c-erbB2/HER2, c-erbB3/HER3 and c-erbB4/HER4) (Salomon *et al.*, 1995; Nicholson *et al.*, 1997;

Olayioye *et al.*, 2000; Daly, 1999; Gullick, 2001; Bazley and Gullick, 2005; Normanno *et al.*, 2006). The c-erbB family of receptors are membrane spanning glycoproteins that share similar molecular structures to the type II RTKs. They are comprised of an extracellular ligand binding domain with two cysteine rich sequences which permit dimerization, a short transmembrane region and an intracellular tyrosine kinase domain, that is responsible for mediating transphosphorylation of carboxy-terminal tyrosine residues (Olayioye *et al.*, 2000; Bazley and Gullick, 2005). There are currently eleven genes encoding ligands for the receptor family, EGF, TGF $\alpha$ , heparin-binding (HB)-EGF, betacellulin, amphiregulin (AR), epiregulin, epigen, neuregulin (NRG)-1, NRG2, NRG3 and NRG4 (Olayioye *et al.*, 2000; Bazley and Gullick, 2005; Normanno *et al.*, 2006). Ligand binding of EGF-related growth factors, such as those mentioned above, results in EGF receptor homo and/or heterodimerization, activation and phosphorylation of the kinase domain at specific Y residues within the cytoplasmic region of the receptor. Several of these residues have been identified including Y845, Y1101, Y992, Y1173, Y1045, Y1068, Y1086 and Y1148 (Olayioye *et al.*, 2000). The latter six are known autophosphorylation sites (Hsuan *et al.*, 1989; Downward *et al.*, 1984; Margolis *et al.*, 1989; Walton *et al.*, 1990) whereas Y845 and Y1101 have been identified as non-receptor tyrosine kinase c-SRC dependent phosphorylation sites (Biscardi *et al.*, 1999). The adaptor protein Grb2 has been shown to bind directly to phosphorylated Y1068 and Y1086 residues or indirectly to phosphorylated Y1068 via phosphorylated SHC (Sorkin, 2001; Okutani *et al.*, 1994; Rojas *et al.*, 1996), whereas phosphorylated Y1173 and Y1148 residues can bind directly to SHC, all resulting in the activation of the MAPK pathway

(Sorkin, 2001; Okabayashi *et al.*, 1994). Tyrosine Y992 is a known binding site for phospholipase C gamma (PLC $\gamma$ ) and activation of this protein can also result in the activation of MAPK via PKC (Emlet *et al.*, 1997). Tyrosine Y1086 and Y1068 have also been shown to recruit Grb2 associated binder (GAB-1), an adaptor protein which acts as a docking site for a number of downstream signalling proteins including PI3-K (Rodrigues *et al.*, 2000).

### ***1.5.2 Role of c-erbB receptors in endocrine resistant breast cancer:***

An inverse relationship between ER activity and EGFR or c-erbB2 expression has been reported in clinical breast cancer, with over-expression of these RTKs being associated with decreased sensitivity to endocrine therapy and a poorer prognosis (Wright *et al.*, 1992; Nicholson *et al.*, 1993; Nicholson *et al.*, 1994). Transfection studies in hormone-sensitive breast cancer cell lines have also demonstrated that increased EGFR and c-erbB2 expression promotes hormone-independent growth (Van Agthoven *et al.*, 1992; Benz *et al.*, 1992; Miller *et al.*, 1994; Liu *et al.*, 1995; Kurokawa *et al.*, 2000), while cell models of acquired tamoxifen resistance have also suggested that increased levels of EGFR and c-erbB2 may contribute to increased proliferative activity (Long *et al.*, 1992, El-Zarruk and Van den Berg, 1999, Knowlden *et al.*, 2003). EGFR and c-erbB2 regulate cell proliferation via the activation of downstream signalling pathways such as the MAPK and PI3-K cascades (Schlessinger, 2000). Indeed, elevated levels of MAPK activity have been demonstrated in oestrogen non-responsive and long-term oestrogen deprived breast cancer cell lines (Coutts and Murphey, 1998; Shim *et al.*, 2000) and activation of this signalling pathway has been shown to contribute to anti-oestrogen resistance in

MCF-7 breast cancer cells (Knowlden *et al.*, 2003; El-Ashry *et al.*, 1997; Donovan *et al.* 2001; McClelland *et al.*, 2001). Importantly, these results support clinical observations in breast cancer samples with increased MAPK and Akt down-stream signalling being associated with reduced quality and duration of response to tamoxifen and shorter disease-free survival time which predict outcome in ER positive clinical breast cancer patients (Gee *et al.*, 2001; Mueller *et al.*, 2000; Perez-Tenorio *et al.*, 2002; Kirkegaard *et al.*, 2005; Tokunaga *et al.*, 2006).

Besides driving growth, it is now apparent that increased growth factor-induced kinase signalling can efficiently phosphorylate key regulatory serine residues (serine 118 and serine 167) within the AF-1 domain of the ER (Bunone *et al.*, 1996; Kato *et al.*, 1995, Joel *et al.*, 1998). Phosphorylation of these residues, especially serine 118, a known target for MAPK, can promote re-activation of ER $\alpha$  function in a ligand independent manner and lead to the re-expression of ERE containing genes and growth promotion in the presence of anti-hormone (Bunone *et al.*, 1996; kato *et al.*, 1995; Deblois and Giguere, 2003). As tamoxifen-resistant cell lines, like their clinical counterparts, continue to express ER $\alpha$  at a level equivalent to that observed in the parental cell lines (Encarnacion *et al.*, 1993; Brunner *et al.*, 1993; Lykkesfeldt *et al.*, 1994; Robertson, 1996, Hutcheson *et al.*, 2003), it is feasible that such ligand-independent activation of ER $\alpha$  may play a role in tamoxifen resistance. Indeed, elevated levels of serine 118 and serine 167 ER $\alpha$  phosphorylation have been demonstrated in breast cancer cell lines resistant to both tamoxifen and following long-term oestrogen deprivation (Martin *et al.*, 2003; Campbell *et al.*, 2001; Chan *et al.*, 2002; Vendrell *et al.*, 2005; Shou *et al.*, 2004; Britton *et*

*al.*, 2006; Glaros *et al.*, 2006) with re-expression of ER-regulated genes being reported (Britton *et al.*, 2006). Interestingly, in tamoxifen resistant MCF-7 cells, re-activation of ER $\alpha$  as a result of EGFR/MAPK-mediated phosphorylation of serine 118 has been shown to result in the re-expression of AR, generating a self-propagating autocrine growth regulatory loop in these cells (Britton *et al.*, 2006).

### ***1.5.3 Role of IGF-IR signalling in endocrine resistant breast cancer:***

More recent evidence has indicated that the c-erbB receptor family are not the only growth factor receptors involved in endocrine-resistant breast cancer. Indeed, despite the ability of anti-hormones to inhibit IGF-IR signalling a role for this receptor in resistance has been identified. Evidence for this was initially derived from gene transfer studies, where IGF-IR, IGF-II, or IRS-1 over-expression was shown to reduce sensitivity of MCF-7 and T47D breast cancer cells to oestradiol treatment (Daly *et al.*, 1991; Surmacz and Burgaud, 1995; Abdul-Wahab *et al.*, 1999). Indeed, it has also been demonstrated that IGF-IR expression levels are enhanced in MCF-7 cells resistant to long-term oestrogen deprivation and that growth of this oestrogen-independent MCF-7 cell variant was reduced by IGF-IR neutralising monoclonal antibody  $\alpha$ IR3 (Stephen *et al.*, 2001). Recent evidence further suggests a role for IGF-IR signalling in tamoxifen resistance and this has mainly been derived from experimental cell models that have acquired tamoxifen resistance. Indeed, increased sensitivity to the proliferative effects of IGF-I/II has been reported in tamoxifen-resistant MCF-7 cell lines following treatment with either oestradiol or tamoxifen (Parisot *et al.*, 1999; Wiseman *et al.*, 1993) respectively and once

again anti-IGF-IR monoclonal antibody  $\alpha$ IR-3 has been shown to block growth of tamoxifen-resistant MCF-7 cell variants (Parisot *et al.*, 1999). Interestingly, acquired resistance to tamoxifen has been reported to be accompanied by substantial increases in IGF-I binding in MCF-7 breast cancer cells (Wiseman *et al.*, 1993) while marked increases in IGF-IR expression *in vitro* was shown to reduce their oestrogen requirement (Guvakova and Surmacz, 1997).

Despite growing experimental evidence in support of a role for IGF-IR signalling in tamoxifen resistance, to date, no clinical studies have provided any support for this proposal. However, a recent immunocytochemical study in breast cancer specimens has observed readily detectable IGF-IR expression and activity in a small cohort of ER positive acquired tamoxifen resistant breast cancer samples (Gee *et al.*, 2005). It is therefore feasible that such IGF-IR signalling observed '*in-vivo*' may indeed be functional and play a role in supporting acquired tamoxifen resistance.

#### ***1.6 Role of IGF-IR cross-talk with EGFR in breast cancer:***

Interestingly, IGF-IR expression in samples from patients with acquired tamoxifen resistance was often observed in the presence of EGFR (Gee *et al.*, 2005). Furthermore, a substantial degree of cross-talk exists between members of the type I and type II RTK families and their ligands in both non-cancerous and cancerous cell models. For instance, studies have highlighted the presence of a direct association due to receptor heterodimerization between IGF-IR and EGFR in both MCF-7 and T47D breast cancer cell lines, which could be disrupted by the anti EGFR tyrosine kinase inhibitor gefitinib (Shou *et al.*, 2003), in non-small cell lung cancer (NSCLC) cells following treatment with

the tyrosine kinase inhibitor erlotinib (Morgillo *et al.*, 2006) and between IGF-IR and c-erbB2 in MCF-7 cells (Balana *et al.*, 2001) and SKBR3 anti-c-erbB2 (trastuzumab)-resistant breast cancer cells (Nahta *et al.*, 2005). Additional support for the presence of indirect mechanisms of cross-talk between IGF-IR and EGFR and their ligands have also been reported in a range of cancerous and non-cancerous or normal derived cell lines (Coppola *et al.*, 1994; Vardy *et al.*, 1995; Burgaud and Baserga, 1996, Swantek and Baserga, 1999; Roudabush *et al.*, 2000; Hurbin *et al.*, 2002; Wang *et al.*, 2002; Gilmore *et al.*, 2002; Desbois-Mouthon *et al.*, 2006). In mouse embryonic cells, prolonged ERK2 activation induced by EGF and other growth factors was shown to be largely dependent on IGF-IR signalling (Swantek and Baserga, 1999). Conversely, Vardy *et al.*, (1995) showed that the mitogenic effects of IGF-I were abolished by the presence of an EGFR monoclonal antibody inhibitor in keratinocytes.

Several mechanisms of receptor trans-activation have been suggested, although in all cases the effects have been reported to be rapid. In the case of IGF-I mediated trans-activation of EGFR in COS-7 cells and G protein-coupled receptor (GPCR) ligands in Rat-1 cells, MMP-dependent release of HB-EGF is required (Roudabush *et al.*, 2000; Prenzel *et al.*, 1999). This ligand is one of many endogenous ligands that bind and activate EGFR (Olayioye *et al.*, 2000). They are synthesized as trans-membrane precursors, which undergo controlled proteolysis to produce soluble growth factors that can bind and activate EGFR in an autocrine or paracrine manner. Support for the presence of indirect mechanisms of cross-talk between IGF-IR and EGFR and their ligands in breast cancer cell lines remains very limited, although a recent study by Gilmore *et al.*, (2002) demonstrated that IGF-I indirectly trans-activated EGFR



in mammary epithelial cells. Interestingly, similar actions in breast cancer cells may involve MMPs because it is not blocked with the broad-spectrum MMP inhibitor, Illomastat (Olayioye *et al.*, 2000). However, this area remains controversial, as more recent evidence suggests the large ADAM (a disintegrin and metalloproteinase) family of membrane glycoproteins maybe an important regulator of EGFR ligand membrane shedding in breast cancer cells and breast tumours (Gee and Knowlden, 2003).

### ***1.7 Aims of this study:***

As EGFR and IGF-IR have been shown to cross-talk in a variety of cancer cell lines and as both receptors have been implicated in endocrine resistant breast cancer, we have investigated whether such cross-talk exists in a cell model of endocrine resistance and what role this cross-talk may play in this disease state.

Our objectives were as follows:

1. To determine the role played by IGF-IR in mediating the growth of tamoxifen-resistant MCF-7 breast cancer cells (Tam-R), examining potential areas of cross-talk between IGF-IR and EGFR signalling components, with comparisons made with parental wild-type MCF-7 breast cancer cells.
2. To examine whether the possible mechanisms involved are also active in other tamoxifen-resistant breast cancer cells (T47D Tam-R) and other EGFR positive cancer cell lines.
3. To determine the effects of EGFR blockade on IGF-IR signalling in these cell lines.

## **2.0 Materials and Methods:**

All tissue culture medium and constituents were purchased from Gibco Europe Ltd, Paisley, UK, unless stated otherwise in the text and tissue culture plastics were obtained from Nunc, Roskilde, Denmark, supplied by Fisher Scientific, Loughborough, UK. All organic solvents were also purchased from Fisher Scientific. All general molecular grade chemicals and reagents used in all experimental procedures were purchased from Sigma Chemical Co Ltd, UK, unless stated otherwise.

### ***2.1 Role of IGF-IR signalling in tamoxifen-resistant MCF-7 (Tam-R) and T47D breast cancer cells:***

#### ***2.1.1 Routine cell culture of wild type MCF-7 and T47D breast cancer cells:***

Human wild-type (WT)-MCF-7, derived from a mammary gland adenocarcinoma (Astra Zeneca Pharmaceuticals, Cheshire, UK) and T47-D breast cancer cells (purchased from the American Tissue Culture Collection) were routinely cultured in phenol-red-free RPMI medium supplemented with penicillin/streptomycin (10 IU/ml-10µg/ml), L-glutamine (200mM) and fungizone (2.5 µg/ml), and 5% v/v foetal calf serum (FCS) and maintained in an incubator (BB16 Function Line from Heraeus Instruments, Hanau, Germany) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. The cells were replenished with fresh media every 48 hours and when confluent, were sub-cultured by trypsinisation. Briefly, the culture medium was removed from the flasks and the cells were washed three times with warm phosphate buffered saline (PBS). Trypsin solution containing ethylene diamine tetra acetic acid (EDTA; 0.02% w/v) and bovine trypsin (0.05% v/v) in PBS was added to the

flasks and the cells were incubated at 37°C until they became detached from the plastic surface. Culture medium pre-heated to 37°C was then added to the cells to inhibit residual trypsin activity and the cells were centrifuged using a Mistral 3000I (Sanyo Gallenkamp, Loughborough, UK) at 1000g for 5 minutes, after which time the cell pellet was resuspended in 10 ml culture medium. The cells were then passed up and down a syringe using a 25G (0.5 x 16) needle to remove any cell aggregates, and 100µl of cell suspension was then added to 10ml Isoton II solution (Beckman Coulter UK Ltd, High Wycombe, UK) in triplicate and counted using a Coulter Multisizer II, also from Beckman. Cells were routinely seeded at a density of 30,000 cells/cm<sup>2</sup>.

### ***2.1.2 Development of tamoxifen resistant cell lines:***

The tamoxifen resistant cell lines were developed by continually exposing WT-MCF-7 and T47D breast cancer cells to 4-hydroxy-tamoxifen (4-OH-TAM; 100nM in ethanol) over a period of 6 months. The cells were routinely passaged when necessary, by trypsinisation (see section 2.1.1). During the first two months, tamoxifen inhibited the growth of the cells, but after this period the growth rate gradually increased to that seen for the WT parental cells. Their routine tissue culture medium consisted of phenol-red-free RPMI medium supplemented with charcoal-stripped steroid-depleted FCS (5% v/v), antibiotics, L-glutamine (200 mM) and 4-OH-TAM (100nM). Briefly, the steroid-depleted FCS was obtained by first aliquoting FCS (100mL) and adjusting the pH to 4.2 with 5M hydrochloric acid (HCL) which was then left to equilibrate for 30 minutes at 4°C. A charcoal/dextran solution was prepared using distilled water with Norit A (charcoal, 11.1%) and dextran C (0.06%)

and was stirred vigorously for 1 hour. To each aliquot of FCS (100mL), 5ml charcoal solution was added and incubated with gentle agitation for 16 hour at 4°C. The charcoal was removed by centrifugation at 12,000 RPM for 40 minutes and the solutions were filtered to remove all traces of charcoal with Whatman filter paper N<sup>o</sup> 4 and then the pH was re-adjusted to pH 7.2. The solution was sterilised and re-filtered with 0.2µM membrane filter Supor Vacuap<sup>®</sup> 60 (Gellman Laboratory Pall, Ann Arbor, USA).

Upon achieving steady growth rates, the tamoxifen-resistant MCF-7 (Tam-R) and T47D (T47D-R) cell-lines were cultured for several months before any experimental work was started.

### ***2.1.3 Experimental procedure for WT-MCF-7 cells:***

To examine basal messenger ribonucleic acid (mRNA) and protein expression, WT-MCF-7 were seeded at a density of 30,000 cells/cm<sup>2</sup> and grown for 4 days until sub-confluent in phenol-red/steroid-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS (see section 2.1.2) before being transferred into phenol-red/steroid-free, serum growth factor-free DCCM medium [Biosynergy Europe, Cambridge, UK] for 24 hours prior to total protein and RNA extraction. Expression of protein and mRNA was assessed by Western blotting and reverse transcription-polymerase chain reaction (RT-PCR) technologies respectively. All experiments were performed at least three times.

To examine the effects of pharmacological agents, cells were lysed following a further incubation in DCCM medium supplemented with either IGF-II (10-100 ng/ml in 10 mM acetic acid/ 0.1% Bovine Serum Albumen {BSA}; R&D

Systems, Abingdon, UK) for either 5 or up to 30 minutes, an IGF-IR tyrosine kinase inhibitor AG1024 (5-20  $\mu$ M in dimethyl sulfoxide (DMSO; Calbiochem Ltd, UK) for 24 hours or the more specific IGF-IR tyrosine kinase inhibitor 4-anilino-5-bromo-2-[4-(2-hydroxy-3-(N,N-dimethylamino) propoxy) anilino] pyrimidine (ABDP; 0.1-1 $\mu$ M in DMSO; a kind gift from Astra Zeneca, Macclesfield, UK) for 7 days. Furthermore, WT-MCF-7 cells were also incubated for 2 and 6 weeks in phenol-red/steroid-free RPMI medium containing charcoal-stripped steroid-depleted FCS (5% v/v) supplemented with 4-OH-TAM (100nM). Controls in all cases were incubated for the same periods of time with the appropriate vehicle (see section 2.1.2 and 2.1.3). Protein expression and or mRNA expression was assessed by Western blotting and RT-PCR procedures. All experiments were performed at least three times unless stated otherwise.

#### ***2.1.4 Growth studies for WT-MCF- 7 cells:***

Cells were grown for 7 days in DCCM medium supplemented with increasing concentrations of either AG1024 (1-10  $\mu$ M), ABDP (0.1-1  $\mu$ M), both supplemented with IGF-II (100ng/ml). Cells were also grown for 7 days in DCCM medium supplemented with either a specific EGFR tyrosine kinase inhibitor gefitinib (1 $\mu$ M in ethanol; a gift from Astra Zeneca), IGF-II (100ng/ml) or a combination of the two agents. Controls were incubated for the same period of time with the appropriate vehicle (see section 2.1.3). Cell population growth was then evaluated by means of trypsin dispersion of the cell monolayers (performed in triplicate) as described in section 2.1.1. All cell culture experiments were performed at least 3 times.

### ***2.1.5 Experimental procedure for MCF-7 Tam-R and T47D breast cancer cells:***

To examine basal messenger ribonucleic acid (mRNA) and protein expression, cell lines were seeded at a density of 30,000 cells/cm<sup>2</sup> and grown for 4 days until sub-confluent in phenol-red/steroid-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS (see section 2.1.2) before being transferred into phenol-red/steroid-free, serum growth factor-free DCCM medium for 24 hours prior to protein and mRNA extraction. 4-OH-TAM (100mM) was included in the tamoxifen resistant cell-lines' culture medium at all times.

To examine the effects of pharmacological agents, tamoxifen resistant cells were incubated in DCCM medium supplemented with either IGF-II (10-100 ng/ml) for up to 30 minutes, EGF (10ng/ml) or TGF $\alpha$ ; (0.1-10 ng/ml) in PBS for 5 minutes, AG1024 (20  $\mu$ M) for 24 hours, ABDP (1 $\mu$ M) for 7 days, a pan MMP inhibitor MMP-II (0.01-1 $\mu$ M in DMSO; Calbiochem Ltd, UK) for 24 hours, the c-SRC inhibitor SU6656 (1  $\mu$ M in DMSO; Calbiochem Ltd, UK) for 24 hours, a more specific c-SRC inhibitor AZM 555 (1 $\mu$ M in DMSO) for 24 hours or finally gefitinib (1 $\mu$ M) for 1 hour, the latter two being gifts from Astra Zeneca. The above pharmacological inhibitor studies were performed in the presence or absence of IGF-II or TGF $\alpha$ . Cells were also incubated in DCCM medium supplemented with either increasing concentrations of ABDP (0.1-1 $\mu$ M) for 7 days or AG1024 (5-20  $\mu$ M) or SU6656 (0.01-1  $\mu$ M) for 24 hours. Finally, cells were incubated in DCCM medium supplemented with IGF-II, EGF, HB-EGF, TGF $\alpha$  or AR neutralising antibodies (ranging between 1-20  $\mu$ g/ml in PBS; R&D Systems, Abingdon, UK) for 1 hour, IGF-II for 20

minutes, or a combination of IGF-II (100ng/ml) with these neutralising antibodies. Controls were in all cases incubated for the same periods of time with or without the appropriate vehicle (see section 2.1.3, where appropriate). Protein expression was assessed by immunoprecipitation/Western blotting and immunocytochemical (ICA) procedures and the expression of mRNA was assessed by RT-PCR technologies. All experiments were performed at least three times unless stated otherwise.

#### ***2.1.6 Growth studies for MCF-7 Tam-R and T47D breast cancer cells:***

Cells were grown for 7 days in phenol-red/steroid-free RPMI medium containing charcoal-stripped steroid-depleted FCS medium supplemented with increasing concentrations of either AG1024 (1-20  $\mu$ M), ABDP (0.01-1 $\mu$ M) or SU6656 (0.01-1 $\mu$ M). Furthermore, cells were grown for 7 days in DCCM medium supplemented with either AG1024 (20  $\mu$ M), ABDP (1 $\mu$ M), SU6656 (1 $\mu$ M), AZM555 (1 $\mu$ M), gefitinib (1 $\mu$ M), IGF-II (100ng/ml), TGF $\alpha$  (10ng/ml) or IGF-II or TGF $\alpha$  in combination with one of each of these inhibitors. Controls were incubated for the same period of time with the appropriate vehicle (see sections 2.1.3 - 2.1.5). Cell population growth was then evaluated by means of trypsin dispersion of the cell monolayers (performed in triplicate) as described in section 2.1.1 All cell culture experiments were performed at least 3 times.

#### ***2.1.7 Total RNA isolation:***

Total RNA was isolated from basally grown sub-confluent cells in 60mm plastic tissue culture dishes using an RNA isolator kit (Tri Reagent, Sigma



Chemical Co Ltd, UK). One ml of solution was added to each dish at room temperature. The solution was pipetted up and down in each dish until it became viscous and then left to stand for 5 minutes. Each lysate was then transferred to a sterile eppendorf (Elkay, Galway, Ireland) to which 200µl of chloroform was added. The tubes were gently shaken for 15 seconds, left to stand at room temperature for 15 minutes and then centrifuged at 12,000 RPM for a further 15 minutes at 4°C. The colourless aqueous top phase was then carefully removed from each lysate and transferred to a fresh sterile eppendorf to which an equal volume of isopropanol was added. The tubes were then left at room temperature for 15 minutes and then centrifuged for a further 15 minutes at 4°C. The resultant RNA pellets were then washed with 1ml of ice cold ethanol (70% v/v made up in sterile RNA/DNase free water) and centrifuged again for a further 15 minutes at 4°C, after which the ethanol was removed carefully from each tube without disturbing the pellet. The tubes were briefly air-dried and then the RNA resuspended in 25µl sterile RNA/DNase free water and stored at -70°C until required.

#### ***2.1.8 Quantitation of nucleic acids:***

RNA samples were determined spectrophotometrically using a CECIL CE 2041 spectrophotometer (Cambridge, UK). Samples were diluted 1/500 in sterile water and the concentration of each was determined by reading the absorbance values at A260nm and A280nm. The concentration of deoxyribonucleic acid (DNA) or RNA was calculated from the following formula:

DNA or RNA concentration ( $\mu\text{g/ml}$ ) =  $[260] \times [\text{dilution factor}] \times [\text{extinction coefficient}]$

extinction coefficients: double stranded DNA= 50, single stranded RNA= 40 or oligonucleotides = 20.

The purity of the DNA and RNA was determined by 260nm:280nm ratio and both should be between 1.8 - 2.0.

To check the integrity of the RNA and to make sure that it was not degraded prior to RT-PCR analysis,  $1\mu\text{g}$  was run on a 2% w/v agarose gel (as described below in section 2.1.9).

### **2.1.9 Agarose gel electrophoresis:**

Agarose gel electrophoresis is a technique used to separate both DNA and RNA fragments. Separation occurs due to charge and so when an electric current is passed across the gel, DNA and RNA which are negatively charged will move towards the anode (Sambrook *et al.*, 1989). Electrophoresis was performed in 1 x TAE (Tris-acetate (40mM), EDTA (1mM), at pH 8.0) using a horizontal subcell system (Biorad laboratories, Ltd, Hemel Hempstead, UK). Gels of concentrations ranging between 2-3% w/v agarose were prepared by the addition of agarose to 1x TAE. The agarose was then dissolved by boiling in a microwave on full power for 1 minute followed by cooling at room temperature. Ethidium bromide solution was then added to give a final concentration of  $0.5\mu\text{g/ml}$ , after which the gel was poured into a gel tray with a suitable comb inserted and left to set. The comb was then removed and the gel placed into a tank containing 1x TAE. The samples (RNA/DNA and molecular weight markers (100 bp ladder, Promega, (Southampton, UK) were mixed with

approximately 1/3 volume loading buffer comprised of sucrose (40%) and bromophenol blue (BPB; 0.25% w/v) made up in sterile pure water and loaded onto the gel. Gels were run at 60V until the BPB tracking dye was 2/3 of the way down the gel. The gel was then visualised under UV transillumination and photographed using a polaroid DS-34 camera and polaroid 665 positive/negative black and white film (Polaroid Corporation, Cambridge, UK). Resultant PCR products were scanned and band quantitation was undertaken on a GS-700 densitometer connected to a microprocessor loaded with molecular analyst software (Biorad Laboratories, Watford, UK).

#### ***2.1.10 Preparation of cDNA templates by reverse transcription (RT):***

First strand complementary DNA (cDNA) synthesis of the total RNA was prepared by extension with random hexamers (RH), using the method of O'Brian *et al*, (1991). For each sample, 7µl of RNase/DNase free sterile water, containing 1µg of total RNA was added to a reaction mixture comprising RH (10µM; Pharmacia Biotechnologies, Herts, UK), 0.625mM of each deoxy nucleotide triphosphates (dNTPs; Gibco/BRL, Life Technologies, Basingstoke, UK), 1x PCR buffer (Tris-HCL; 10mM pH 8.3, potassium chloride (KCl; 50mM), magnesium chloride (MgCl<sub>2</sub>; 1.5mM), gelatine (0.001% v/v) and dithiothreitol (DTT; 0.01M). A negative reverse transcription (-RT) control tube (where sterile water was substituted for RNA) was also included to check for contamination. All mixes were denatured at 95°C for 5 minutes and cooled rapidly on ice, before the addition of RNase inhibitor (25 units; Promega Ltd, Southampton, UK), and MMLV reverse transcriptase (200 units; Gibco/BRL, Life Technologies, Basingstoke, UK), giving a final volume

of 20 $\mu$ l. Reaction mixes were then incubated at 22 $^{\circ}$ C for 10 minutes prior to reverse transcription, which was subsequently performed at 42 $^{\circ}$ C for 40 minutes. Finally the RNA was incubated at 95 $^{\circ}$ C for 5 minutes to terminate the reaction and resultant cDNA samples were stored at -20  $^{\circ}$ C until required.

#### **2.1.11 Semi-quantitative PCR:**

Semi-quantitative PCR was subsequently carried out on reverse transcribed cDNA samples, with either IGF-I, IGF-II, IGF-IR, IRS-1 or  $\beta$ -actin primers.  $\beta$ -actin (internal control) expression was assessed to check both for sample-to-sample variation under RT-PCR reaction conditions and also to monitor degradation and recovery of RNA. Two negative controls were included in each PCR run, a -RT control (see section 2.1.10) and a -PCR control (where sterile water was used in place of cDNA to check for PCR contamination). A positive control for IGF-I only was also included in all IGF-I PCR reactions using cDNA derived from MDA-231 breast cancer cells. RT-PCR conditions were optimised for each primer set using standard conditions as described previously (Knowlden *et al*, 1997). In brief, for highly expressed genes where detection was possible following 27 or fewer cycles of amplification, simultaneous PCR reactions were performed using primers sets both for the gene of interest and  $\beta$ -actin. For all other mRNA species where a higher cycle number was needed to permit detection, single PCRs for each primer pair were set up alongside, but independent of  $\beta$ -actin.

PCR amplification (using only one primer set) was performed in a total volume of 50 $\mu$ l (i.e. 1 $\mu$ l of RT product equivalent to 0.05 $\mu$ g cDNA and 49 $\mu$ l of 'master

mix' solution). The 'mastermix' solution comprised sterile pure RNase/DNase free water, 5'OH and 3'OH primers (0.5µM), dNTPs (200µM), 1x PCR buffer and Taq polymerase (2 units; Bioline Ltd, London, UK). For simultaneous PCR amplification, reactions were performed as described above except that β-actin primers were included in the reaction mix at a reduced concentration of 0.125µM. The tubes were overlaid with 100µl of sterile mineral oil (Sigma molecular biology grade) prior to thermal cycling.

Primer pairs for IGF-I and IGF-II (Swolin *et al.*, 1996) and β-actin (O'Brian *et al.*, 1989), were derived from published sequences and were synthesized on site. IGF-IR and IRS-1 primers were designed manually and specificity was checked using the EMBL-GenBank data base software using the Blast program.

5' β-actin	5' GGA GCA ATG ATC TTG ATC TT
3' β-actin	5' CCT TCC TGG GCA TGG AGT CCT
5' IGF-I	5' TGC TCT TCA GTT CGT GTG TG
3' IGF-I	5' TGG CAT GTC ACT CTT CAC TC
5'IGF-II	5' TGG GAA TCC CAA TGG GGA AG
3'IGF-II	5' CTT GCC CAC GGG GTA TCT
5'IGF-IR	5' AGC AGA TGA CAT TCC TGG G
3'IGF-IR	5' CTG GAC ATA GAA GAA CAC AG
5'IRS-1	5' TCA CGT TGG GTG GAG AGA GT
3'IRS-1	3' GAA TCA CCC ATC TCC TTC TG.

The PCR protocol subsequently employed was carried out on a PTC-100 thermocycler (Genetic Research Instrumentation Ltd, Essex, UK) with the following parameters:

A first cycle was performed for 2 minutes at 95°C (denaturing), at 55°C for 1 minute (annealing) and 72°C for 5 minutes (extension). This was followed by 20-40 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 30 seconds at 72°C for each of the samples to determine the optimal cycle number required for each primer set. Once conditions were optimised, PCR amplification was performed for 40 and 37 cycles for IGF-I and IGF-II respectively and 25 cycles for IRS-1, IGF-IR and  $\beta$ -actin. A final cycle was included of 1 minute at 95°C, 1 minute at 55°C and 10 minutes at 60°C. PCR products (7 $\mu$ l) were then loaded onto a 3% w/v agarose gel (see section 2.1.9) comprised of 2% w/v Nusieve GTG agarose (Flowgen Instruments Ltd, Sittingbourne, UK), 1% w/v normal agarose, containing ethidium bromide (0.5 $\mu$ g/ml) and separated by flat-bed gel electrophoresis alongside a 100bp DNA ladder as described in section 2.1.9.

#### **2.2.12 Real time PCR:**

Real Time PCR was performed to fully quantify IGF-II and  $\beta$ -actin mRNA levels in basally growing WT-MCF-7 and Tam-R cells. Total RNA was isolated and quantified as described in section 2.1.7-2.1.9 and RNA equivalent to 1 $\mu$ g was reverse transcribed using standard conditions as described in section 2.1.10. PCR reactions were set up using the Quantitect SYBR Green PCR Kit (Qiagen Ltd, Crawley, UK) using optimised conditions according to

the manufacturer's recommendations. Briefly each reaction mix contained Quantitect SYBR Green PCR Mastermix (HotStartaq DNA polymerase, 1x SYBR Green PCR buffer, SYBR Green 1, ROX, dNTPS, and  $\text{MgCl}_2$ ), forward and reverse specific primers ( $0.3\mu\text{M}$ ) of each (see section 2.2.11) and sterile pure RNase/DNase-free water. cDNA from three separate groups of experimental samples (equivalent to  $0.025\mu\text{g}$  RNA) or standard was added. DNA Standards for IGF-II and  $\beta$ -actin were initially prepared from freshly purified PCR specific products, purified using a PCR clean up kit (Qiagen Ltd, Crawley, UK). Each specific DNA standard was then diluted in sterile water to generate a range of concentrations of template to be included in each PCR reaction. Unknown test samples were then PCR amplified alongside a wide range of concentrations of each specific standard, in order to determine the most suitable range of standards that could be used for quantifying unknown target genes. After optimisation, final standard concentrations ranged from  $0.01\text{-}100\text{ pg}/\mu\text{l}$  and  $0.0001\text{-}0.1\text{ fg}/\mu\text{l}$  for  $\beta$ -actin and IGF-II respectively. All reactions were carried out in duplicate. The optimal cycling conditions were carried out as described in section 2.1.11 for semi-quantitative PCR, except that the initial denaturing step was increased to  $95^\circ\text{C}$  for 15 minutes to allow for the HotStarTaq enzyme to be fully activated. This was followed by subsequent cycling conditions of  $94^\circ\text{C}$  for 30 seconds,  $55^\circ\text{C}$  for 30 seconds and  $72^\circ\text{C}$  for 30 seconds, with fluorescence plate readings collected after each  $72^\circ\text{C}$  extension time. Reactions were carried out using the DNA engine Opticon 2 (Genetic Research Instrumentations Ltd, Essex, UK).

Prior to calculation of the template concentration, the position of the cycle threshold or ( $C_T$ ) line must be defined on a graph of fluorescence versus cycle

number. The  $C_T$  line is often positioned on a graph at a point where the fluorescent signals exceed background noise and begin to increase. The cycle threshold for an individual sample is defined as the cycle at which the sample's fluorescence trace crosses the  $C_T$  line, i.e. at which there is the first clearly detectable increase in fluorescence. By including quantitation standards with varying initial amounts of template in the run, a standard curve of Log Quantity versus  $C_T$  can be plotted. The quantity of initial template in unknowns can then be calculated by applying the sample's  $C_T$  value to the standard curve. To ensure accuracy of the results obtained for the test samples, standard curves, generated, were only used if they showed a linear regression analysis R square ( $R^2$ ) value of at least 0.996. This value indicates how well the fit of the standard curve describes the variation in the data. Values closer to 1, signify a good fit.

To verify the identity of specific products and the presence of un-desirable primer dimers, melting curves were performed following the cycling protocol, following the manufacturers instructions. Melting curves were run from 65-95°C for both IGF-II and  $\beta$ -actin. The fluorescence was measured continually, i.e., every 1°C/10 seconds as the temperature increases and these measurements were then plotted against temperature. At low temperatures, all PCR products are double stranded. SYBR Green binds to double stranded DNA and so under these conditions fluorescence will therefore be high, whereas at high temperatures, PCR products are denatured, resulting in rapid decreases in fluorescence. At a set temperature called the melting temperature ( $T_m$ ), non-specific, eg. primer dimers and specific PCR products are denatured, resulting in a rapid decrease in fluorescence, which is plotted as a sharp peak.



### **2.1.13 Protein cell lysis:**

Cell culture medium was removed and the cells were washed three times with warm PBS prior to cell lysis. Lysis buffer containing Tris (hydroxymethyl) aminomethane (Trizma) base (50mM), Ethyleneglycol-*bis*( $\beta$ -aminoethyl)-N,N,N',N'-tetraacetic Acid (EGTA; 5mM), sodium chloride (NaCl; 150mM), Triton X 100 (1% v/v), distilled water, pH 7.5 was stored on ice and immediately before cell lysis, a cocktail of protease and phosphatase inhibitors were added to give a final concentration of sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>; 2mM), sodium fluoride (NaF; 200mM), phenylmethyl sulfonylfluoride (PMSF; 1mM), phenylarsine oxide (20 $\mu$ M), sodium molybdate (10mM), leupeptin (10 $\mu$ g/ml), aprotinin (10 $\mu$ g/ml) and distilled water. Ice cold lysis buffer (250 $\mu$ l) was then added to each 60mm<sup>2</sup> dish of cells which were incubated on ice for 5 minutes. The cells were detached from the plastic cell surface using a cell scraper (Fisher Scientific, Loughborough, UK) and incubated for a further 10 minutes on ice. The cellular contents were transferred to eppendorf tubes (Elkay, Galway, Ireland) and clarified by centrifugation at 13,000 RPM for 15 minutes at 4°C. The supernatants were then aliquoted and stored at -20°C until required.

### **2.1.14 Lowry protein determination assay:**

To determine the protein content of the cells harvested, a modified method of Lowry *et al.*, (1951) was used. This involved the use of the DC protein assay Biorad Kit (Biorad Laboratories, Ltd, Hemel Hempstead, UK). Briefly, BSA protein concentration standards ranging from 0.25mg/ml-1.45mg/ml (diluted in

lysis buffer as described in section 2.1.13) and also test samples, diluted 1/10 in lysis buffer were prepared. Reagent A (250µl, containing 5µl Reagent S which was needed for detergent compatibility) was added to 50µl of each standard or test sample followed by 2ml Reagent B. After mixing and incubation at room temperature for 5 minutes, absorbances were measured at 750nm on a CECIL CE 2041 spectrophotometer (Cambridge, UK) using software to construct a standard curve from the BSA standards. Using the standard curve, sample test absorbances were assessed and respective protein concentrations were calculated.

#### ***2.2.15 Immunoprecipitation:***

Cell lysates containing 1mg protein were immunoprecipitated using 1µg specific total antibody and incubated on ice for 1 hour. A 30µl slurry of protein A agarose (Insight Biotechnology Ltd, Wembley, UK) was added to the mixture and the tubes were placed onto a rotary mixer (Thermo Life Sciences, UK) at 4°C for 2 hours. The immune complex was centrifuged at 3000 RPM at 4°C for 5 minutes and washed with ice cold lysis buffer. This procedure was repeated 2 more times and the resultant pellet resuspended in 20µl Laemmli sample loading buffer composed of SDS; 4% v/v), glycerol (0.4% v/v), Tris hydroxymethyl aminomethane hydrochloride (Tris-HCL; 120 mM pH 6.8), BPB; 0.01% w/v), DTT; 1.54% w/v) and distilled water. Samples were boiled at 100°C for 5 minutes to release the bound proteins prior to gel loading.

### ***2.1.16 Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE):***

Protein samples prepared from total cell lysates (20-100µg) and following immunoprecipitation (see section 2.1.15) were subjected to gel electrophoresis separation on a 7.5% v/v poly-acrylamide gel. The method of Laemmli (1970) was adapted using a Biorad mini-protein II apparatus (Biorad Laboratories, Hercules, USA). The lower resolving gel was comprised of acrylamide/bisacrylamide (7.5% w/v), Tris-HCL (375mM), pH 8.8, ammonium persulphate (APS; 0.1%w/v), sodium dodecyl sulphate (SDS; 0.1%v/v), N,N,N,N tetramethylethylenediamine (TEMED; 0.06% v/v) and distilled water. Each gel was allowed to polymerise between 2 glass plates for approximately 30 minutes, prior to making the upper gel. This consisted of acrylamide/bisacrylamide (4% v/v), Tris-HCL, pH 6.8 (125mM), SDS (0.1% v/v), APS (0.05% v/v) and TEMED (0.1% v/v). A suitable comb, used to form the wells, was placed in the upper gel which was allowed to set for 30 minutes. Protein cell lysate solutions were added to an equal volume of Laemmli sample loading buffer (see section 2.1.15) and boiled for 5 minutes. A rainbow marker molecular weight (10-250 Kda) protein standard (Amersham, UK) was loaded into the first well of the upper gel. The test samples were then loaded into the remaining wells. Gels were then placed into the gel tank, filled with 1 x running buffer (Trizma base (250mM), glycine (2M), SDS (40mM), in distilled water, pH 8.3) and run at 100-200 volts until the BPB tracker dye reached the base of the gel.

### **2.1.17 Western blotting:**

Prior to protein transfer, gels were pre-soaked in a transfer buffer comprised of Tris (25mM), glycine (192mM), methanol (20% v/v) and distilled water, pH 8.3, alongside 4 pieces of 3mm Whatman filter paper, 2 transfer blot fibre pads and 2 pieces of nitrocellulose membrane (0.2µm pore size; Schleicher and Schuell, Dassel, Germany), all cut to the size of the gels. The transfer apparatus was then put together in the following order inside a plastic transfer cassette [fibre pad, filter paper, gel, nitrocellulose, filter paper, fibre pad]. Any trapped air bubbles between the layers were carefully removed by a gentle rubbing movement before the layers were clamped inside the plastic transfer cassette and then placed inside the transfer cassette holder, fixed inside the electrophoresis tank, which was filled with transfer buffer. The apparatus containing the gels was then subjected to 100V for 1 hour to allow the proteins to be trans-blotted onto the surface of the nitrocellulose membranes.

Following transfer, the membranes were removed and incubated in Ponceau S (Ponceau S; 1% w/v made up in 5% v/v acetic acid in distilled water) for 1 minute, a protein stain used to check that the proteins had transferred successfully and that the samples were loaded equally. Blots were then washed 3 times for 5 minutes in tris buffered saline buffer (TBS)-Tween (0.05% v/v) which was comprised of Trizma base (10mM) pH 7.6, NaCl, (100mM), Tween 20 (0.05% v/v) and then blocked at room temperature for 1 hour in 5% w/v non-fat dried milk made up in TBS-Tween (0.05% v/v) on a platform rocker STR6 (Stuart Scientific Bibby Sterilin Ltd, Stone, UK). The blots were then incubated for a minimum of 2 hours in primary antibody diluted 1/1000 in 5% v/v Western Blocking Reagent (Roche Diagnostics, Mannheim, Germany) in

TBS Tween (0.05% v/v) for all antibodies except for phosphorylated and total EGFR which were incubated for a minimum of 24 hours. Furthermore, total EGFR antibody was diluted in 1% w/v non-fat dried milk made up in TBS-Tween (0.05% v/v) instead of Western Blocking Reagent as this was found to reduce background noise. The membranes were washed three times for 5 minutes in TBS-Tween (0.05% v/v) and then incubated for 1 hour with secondary IgG horse radish peroxidase labelled donkey anti-rabbit antibody (Amersham, UK), diluted 1/10,000 in Western Block Reagent (5% v/v) /TBS Tween (0.05% v/v). The membranes were washed five times for 5 minutes in TBS-Tween (0.05% v/v) with agitation.

Detection was performed by applying a small volume of SuperSignal West Dura long duration or SuperSignal West Femto sensitive chemiluminescent detection reagent (Pierce and Warriner Ltd, Cheshire, UK), using enough to produce a thin film across each membrane for 5 minutes. ECL film (Genetic Research Instrumentation (GRI), Rayne, UK) was then exposed to each membrane inside an autoradiography cassette for varying time periods, typically ranging from 1-60 minutes. The film was removed and placed inside an X-o-graph compact X2 film developer (X-o-graph imaging systems Ltd, Gloucestershire, UK). Image acquisition and band quantitation was undertaken on a GS-700 densitometer connected to a microprocessor loaded with molecular analyst software (Biorad Laboratories, Hercules, USA).

Antibodies used were directed against total EGFR SC-03, total IGF-IR SC-712, total IRS-1 SC-7200 (Insight Biotechnology Ltd, Wembley, UK), phosphorylated EGFR Y1068, total and phosphorylated ERK1/2 and AKT, (Cell signalling Technology, Hertfordshire, UK), phosphorylated IGF-IR/IR

Y1158, IRS-1 Y612, IRS-1 Y896 and total and phosphorylated c-SRC Y418; Biosource International, USA),  $\beta$ -actin (Sigma, UK) and specific IGF-IR (Y1316), a gift from AstraZeneca, Macclesfield, UK.

#### ***2.1.18. Immunocytochemical studies:***

Cells were grown on sterile 3-aminopropyltriethoxysilane-coated coverslips at  $1 \times 10^4$  cells/cm<sup>2</sup> according to the routine cell culture conditions described above in section 2.1.1. and 2.1.2. Total and phosphorylated IGF-IR expression was, however, assessed by incubation of cell monolayers for 24 hours in DCCM prior to a PBS wash and an appropriate cell fixation procedure (see below).

#### ***2.1.19 Total IGF-IR immunocytochemical assay (ICA):***

Cover slips were fixed for 5 minutes in formal saline (3.7% v/v) containing phenol (2.5% v/v) at room temperature, rinsed once with ethanol (70% v/v) and then washed two times in PBS-Tween 20 (0.02% v/v) for 5 minutes. This was followed by incubation overnight at 23°C in total IGF-IR primary antibody (SC-712; Insight Biotechnology Ltd, Wembley, UK) diluted 1/125 in PBS. Coverslips were subsequently washed three times for 1 minute in PBS, two times for 5 minutes in PBS-Tween 20 (0.02% v/v) and then incubated for 2 hours at room temperature in Rabbit enVision peroxidase labelled polymer secondary antibody (Dako Ltd, Ely, UK). They were then washed three times for 1 minute in PBS, two times for 5 minutes in PBS-Tween 20 (0.02% v/v), incubated for 6 minutes at room temperature in en Vision DAB chromagen solution (Dako Ltd) and subsequently rinsed three times for 3 minutes in

distilled water. The coverslips were then incubated in methyl green (0.02% v/v) for 20 seconds as a counter-stain, rinsed in distilled water and allowed to air dry before mountant was applied.

#### ***2.1.20 Phosphorylated IGF-IR Y1131 ICA:***

Cover slips were fixed in methanol/vanadate/acetone solution. Briefly, coverslips were immersed in methanol containing sodium orthovanadate ( $\text{Na}_3\text{VO}_4$ ; 2mM) for 5 minutes at -10 to -30°C, followed by acetone for 5 minutes at -10 to -30°C and then air dried for 20-30 minutes. Coverslips were then rinsed once with ethanol (70% v/v), washed two times for 5 minutes in PBS and quickly dipped in PBS-Tween 20 (0.02% v/v). This was followed by incubation overnight at 37°C in anti phospho IGF-IR/IR (Y1131) primary antibody (Cell Signalling Technology, Hertfordshire, UK), diluted 1/20 in PBS-Triton (0.4% v/v). Coverslips were then washed three times for 1 minute in PBS, two times for 5 minutes in PBS-Tween 20 (0.02% v/v) and incubated for 2 hours at room temperature in Rabbit enVision peroxidase labelled polymer secondary antibody (Dako Ltd). They were subsequently washed three times for 1 minute in PBS, two times for 5 minutes in PBS-Tween 20 (0.02% v/v), incubated for 10 minutes at room temperature in en Vision DAB chromagen solution (Dako Ltd) and rinsed three times for 3 minutes in distilled water. The coverslips were then counterstained as described above

#### ***2.1.21 Phosphorylated IGF-IR Y1316 ICA***

Cover slips were fixed in paraformaldehyde/vanadate solution. Briefly, coverslips were immersed in paraformaldehyde (2%) containing  $\text{Na}_3\text{VO}_4$

(2mM) for 20 minutes at room temperature, followed by two times 5 minutes washes in PBS and then quickly dipped in PBS-Tween 20 (0.02%). This was followed by incubation overnight at 23°C in anti phospho IGF-IR (Y1316) primary antibody (gift from AstraZeneca), diluted 1/50 in PBS-Triton (0.4% v/v). Coverslips were then washed in PBS, incubated in secondary antibody and detected as described above for IGF-IR Y1131. Evaluation of immunostaining was carried out by two people on an Olympus BH-2 light microscope using a dual-viewing attachment. Membrane and cytoplasmic staining was assessed for total and phosphorylated IGF-IR.

#### ***2.1.22 Small interfering RNA (siRNA) transfection: Silencing of IGF-IR expression:***

Tam-R cells were grown in 60mm dishes for 4 days until approximately 60-70% confluent in phenol-red/steroid-free RPMI medium containing 5% charcoal-stripped steroid free FCS (see section 2.1.2) before being transferred into the same medium containing no antibiotics or fungicides for 24 hours prior to siRNA transfection. Transfections were performed following the Manufacturer's instructions (Dharmacon RNA Technologies, Lafayette, CO, USA). Briefly, Dharmacon SMARTpool siRNA reagents (four pooled SMART selection-designed siRNAs) specific for IGF-IR were diluted to a working stock concentration of 20µM in sterile Dharmacon RNase free 1x siRNA buffer. SMARTpool siRNA were then mixed with Dharmafect 1 transfection reagent at a ratio of 10µl siRNA: 0.75µl reagent and incubated at room temperature for 20 minutes. The transfection reagent mix was added to the appropriate dish of Tam-R cells, which were maintained in fresh antibiotic-



free phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS, to give a final siRNA concentration of 100 nM per dish. Control experiments consisted of transfection with the non-targeting siRNA scrambled control (100 nM), medium only (non-transfected cells), or Dharmafect 1 reagent only (lipid). All experimental arms were set up in duplicate. Cells were then incubated for a period of 4 days prior to cell lysis as described in section (2.1.13) and protein extracts subjected to Western analysis as described in sections (2.1.16 and 2.1.7).

Dishes were also set up at the same time using the same experimental conditions, except that the cells were incubated for only 2 days post transfection after which they were subjected to RNA isolation and RT-PCR analysis as described in sections (2.1.7-2.1.11). RT-PCR was performed on the resultant cDNA samples using specific primers for IGF-IR, each co-amplified with  $\beta$ -actin for 25 cycles as described in these sections.

### ***2.1.23 Statistical analysis:***

Overall differences between control and treatment groups were determined by one-way analysis of variance (ANOVA). Direct comparisons between control and treatment effects were assessed in cells using a post-hoc t-test with the Bonferroni adjustment factor. For real time PCR analysis an unpaired Student t-test was performed to compare mRNA levels in both WT-MCF-7 and Tam-R cells. Differences were considered significant at the  $P < 0.05$  level.

## **2.2 Role of IRS-1 involvement in EGFR and IGF-1R signalling:**

### **2.2.1 Cell culture:**

WT-MCF-7 and Tam-R MCF-7 cells were routinely cultured as described in section 2.1.1 and 2.1.2 respectively. The Tam-R/gefitinib resistant MCF-7 derived cell line (Tam-R/Gef-R) was developed by growing Tam-R cells in a phenol-red-free RPMI medium containing 5% v/v charcoal-stripped steroid-depleted FCS (see section 2.1.2), antibiotics, glutamine (200mM), supplemented continuously with 4-OH TAM (100 nM in ethanol) and gefitinib (1µM in ethanol). The cells were grown over a period of 6 months until gefitinib resistance developed (Jones *et al.*, 2004). These resistant cells were then routinely cultured for several months in this medium.

T47D breast cancer and LNCaP prostate cancer cells, purchased from the American Tissue Culture Collection, were routinely cultured in phenol-red-free RPMI medium supplemented with penicillin-streptomycin (10 IU/ml-10 µg/ml), fungizone (2.5 µg/ml), glutamine 200mM) and either 5% or 10% v/v FCS respectively. Du145 prostate cancer cells and A549 non small cell lung carcinoma cells (a gift from Astra Zeneca Pharmaceuticals, Cheshire, UK) were routinely cultured in Dulbecco's Minimum Eagle's Medium (DMEM) supplemented with 10% v/v FCS plus penicillin-streptomycin (10 IU/ml-10 µg/ml), fungizone (2.5 µg/ml) and glutamine 200mM). All cell lines were maintained at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

### **2.2.2 Experimental procedures:**

To examine basal protein expression, each cell line was grown for 4 days until sub-confluent in their routine culture medium as described in section 2.2.1

before being transferred into phenol-red/steroid-and serum free DCCM medium for 24 hours, except for the A549 cells where the medium was supplemented with 0.5% FCS. 4-OH-TAM (100nM in ethanol) was included in both the Tam-R and Tam-R/gef-R MCF-7 cell culture medium at all times, with the latter also containing gefitinib (1 $\mu$ M in ethanol).

To examine the effects of pharmacological agents, cells were incubated in DCCM medium supplemented with either IGF-II growth factor (100 ng/ml), EGF (10 ng/ml) or a combination of IGF-II (100ng/ml) and EGF (10ng/ml) for between 5 and 20 minutes. Cells were also incubated in phenol-red-free RPMI medium supplemented with charcoal-stripped 5% FCS (see section 2.1.2) supplemented with gefitinib (0.01-1 $\mu$ M) or ABDP (0.01-1 $\mu$ M) for 7 days. Additionally, cells were incubated in DCCM medium supplemented with gefitinib (1 $\mu$ M) for 7 days, IGF-II (100ng/ml) for 5 minutes, or IGF-II (100ng/ml) in combination with gefitinib (1 $\mu$ M). Finally, cells were incubated in DCCM medium supplemented with gefitinib (1 $\mu$ M), gefitinib (1 $\mu$ M) and ABDP (1 $\mu$ M) in combination for 4 days or both treated with IGF-II (100ng/ml) for 5 minutes. Controls in all cases were incubated for the same periods of time with or without the appropriate vehicle (see section 2.1.3 and 2.1.5). Expression of protein was assessed by immunoprecipitation/Western blotting and ICA technologies with all experiments performed at least three times.

### **2.2.3 Growth Studies:**

Tam-R cells were grown in phenol-red-free RPMI medium containing charcoal-stripped 5% FCS supplemented with gefitinib alone (1 $\mu$ M) or gefitinib (1 $\mu$ M) and ABDP (1  $\mu$ M) for 7 days. Controls were incubated for the

same period of time with the appropriate vehicle (see section 2.1.3). Cell population growth was then evaluated by means of trypsin dispersion of the cell monolayers (performed in triplicate) as described in section 2.1.1 All cell culture experiments were performed at least 3 times. Tam-R cells were also grown continuously over a period of 23 weeks in phenol-red-free RPMI medium containing charcoal-stripped 5% FCS supplemented with gefitinib alone (1 $\mu$ M) or ABDP alone (0.25 $\mu$ M) or gefitinib (1 $\mu$ M) and ABDP (0.25 $\mu$ M) in combination. Controls were incubated for the same period of time with the appropriate vehicle (see section 2.1.3).

#### ***2.2.4 Immunocytochemical studies:***

Cells were seeded onto TESPA-coated slides at  $1 \times 10^4$  cells/cm<sup>2</sup> using routine cell culture conditions as described in see section 2.1.1. and 2.1.2 and then incubated in serum free DCCM medium for either 24 hours or in phenol-red-free RPMI medium containing charcoal-stripped steroid-depleted FCS 5% for 7 days supplemented with either gefitinib (1 $\mu$ M), IGF-II growth factor (100 ng/ml) for 5 minutes, EGF (10ng/ml) for 5 minutes or a combination of IGF-II and EGF, prior to PBS wash and cell fixation according to the immunocytochemical assay being performed. Controls were incubated for the same period of time with the appropriate vehicle (see section 2.1.5).

#### ***2.2.5 Phosphorylated IRS-1 Y612 and IRS-1 Y896 ICA:***

Cover slips were fixed in paraformaldehyde/vanadate solution as described in section 2.1.21. This was followed by incubation overnight at room temperature in either IRS-1 Y612 or IRS-1 Y896 rabbit primary antibodies (Biosource,

International, USA), diluted 1/75 in BSA (2%). Coverslips were washed for 3 minutes in PBS, washed two times for 5 minutes in PBS-Tween 20 (0.02%) and then incubated for 2 hours at room temperature in Rabbit enVision peroxidase labelled polymer secondary antibody (Dako Ltd, Ely, UK). They were then washed for 3 minutes in PBS, two times for 5 minutes in PBS-Tween 20 (0.02%), incubated for 10 minutes at room temperature in en Vision DAB chromagen solution (Dako Ltd, Ely, UK) and then rinsed two times for 2 minutes in distilled water. The coverslips were then incubated in methyl green (0.5%) for 45 seconds as a counterstain, rinsed in distilled water and allowed to air dry before DPX mountant was applied.

Evaluation of immunostaining was carried out by two people on an Olympus BH-2 light microscope using a dual-viewing attachment. Combined membrane and cytoplasmic staining intensity and percentage staining was assessed by HScore analysis as shown below (McCarty *et al.*, 1985).

$$\begin{aligned} \text{HScore} = & (\% \text{ cells staining weakly}/100) \times 1 + \\ & (\% \text{ cells staining moderately}/100) \times 2 + \\ & (\% \text{ cells staining strongly}/100) \times 3. \end{aligned}$$

#### **2.2.6 Protein cell lysis:**

Cell monolayers were washed three times with warm PBS prior to cell lysis as described in section 2.1.13. Resultant protein cell extracts were then quantified using the Lowry protein determination assay as described in section (2.1.14).

### ***2.2.7 Immunoprecipitation:***

Cell lysates were immunoprecipitated using 1µg specific total antibody and the resultant pellets resuspended in 20µl Laemelli sample loading buffer ready for SDS-PAGE as described in section 2.2.15.

### ***2.2.8 SDS-PAGE /Western blotting:***

Protein samples prepared from total cell lysates (20-100µg) and following immunoprecipitation were subjected to gel electrophoresis separation on a 7.5% v/v poly-acrylamide gel, prior to transfer onto nitrocellulose membranes as described in section (2.1.16 and 2.1.17) respectively. Antibodies used were as described previously (see section 2.1.17).

### ***2.2.9. siRNA transfection: Silencing of IGF-IR and / or EGFR expression:***

Tam-R cells were grown in preparation for transfection as described in section 2.1.22. Dharmacon SMARTpool siRNA reagents (four pooled SMART selection-designed siRNAs) specific for either IGF-IR or EGFR were diluted to a working stock concentration of 20µM and then mixed with Dharmafect 1 transfection reagent (see section 2.1.22), prior to addition to the cells. Cells were then incubated for a period of 4 days prior to treatment with either IGF-II (100 ng/ml) or vehicle alone for 5 minutes (see section 2.1.2). The cells were then lysed (see section 2.1.13) and protein extracts subjected to Western analysis as described in sections 2.1.16 and 2.1.17 respectively.

Dishes were also set up at the same time for RT-PCR analysis (see sections 2.1.22). RT-PCR was performed on the resultant cDNA samples using specific primers for EGFR or IGF-IR, each co-amplified with β-actin for 28, 25 and 25

cycles respectively as described in sections 2.1.10-2.1.12. Primers for EGFR were designed manually and specificity was checked using the EMBL-GenBank data base software using the Blast program.

5' EGFR 5' CAA CAT CTC CGA AAG CCA  
3' EGFR 5' CGG AAC TTT GGG CGA CTA T

#### ***2.2.10 Statistical analysis:***

For immunocytochemical analysis, comparisons of H-scores were determined using the Mann-Whitney 'U' test for non-parametric data. Overall differences between control and treatment groups were determined by one-way ANOVA. Direct comparisons between control and treatment effects were assessed using a Student's *t* test with the Bonferroni adjustment factor. Differences were considered significant at the  $P < 0.05$  level.

### **3.0 Results:**

#### **3.1 Section 1:**

##### **3.1.1 IGF-IR signalling in MCF-7 Tam-R and in T47D-R breast cancer cells:**

Expression of the IGF-IR has been shown in most breast cancer cell lines including the MCF-7 human breast cancer cell line and a number of reports have demonstrated that the IGF-IR is a key receptor in mediating the growth of these cells (Stewart *et al.*, 1990; Lee *et al.*, 1999). Moreover, numerous reports have also demonstrated that the IGF-IR can also significantly cross-talk with ER in endocrine responsive breast cancer cells (Hamelers and Steenbergh, 2003; Yee and Lee, 2000). As such, oestrogens have been shown to favour synergistic interactions with IGFs resulting in an increase in both expression of the IGF-IR and growth, whereas anti-oestrogens have been shown to down-regulate IGF-IR mediated action. Indeed, numerous studies have demonstrated that the anti-oestrogen tamoxifen can significantly interfere with IGF-IR signalling in WT-MCF-7 cells, as part of its growth inhibitory action (Surmacz, 2000). Nonetheless, such signalling has been shown to be restored and play an active role in tamoxifen resistance (Parisot *et al.*, 1999; Wiseman *et al.*, 1993). Our 'in-house' MCF-7 breast cancer cells that have become resistant to long-term tamoxifen treatment (Tam-R) display increased expression levels of EGFR and MAPK and these proteins play a key role in mediating their growth (Knowlden *et al.*, 2003). The aim of the first part of this study was to examine whether the IGF-IR and its signalling components, notably IGF-I and IGF-II, play a continuing role in supporting the growth of these Tam-R cells (with comparisons made where appropriate to their wild type parental counterparts),



in addition to another tamoxifen-resistant T47D breast cancer cell line (T47D-R) and to investigate potential areas of cross-talk between IGF-IR and EGFR in these cell lines.

***3.1.2 Development of a real time PCR assay to detect the expression of IGF-I and IGF-II mRNA in WT-MCF-7 and Tam-R breast cancer cells:***

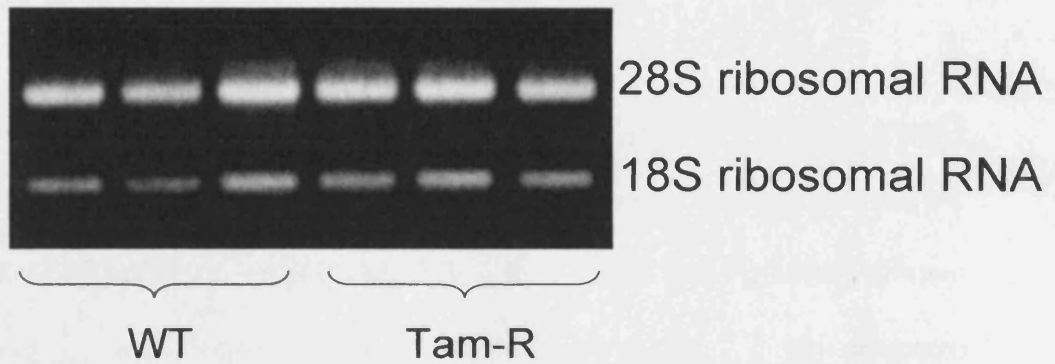
The integrity and constancy of the RNA prior to RT-PCR was assessed following agarose gel electrophoresis of 1µg total RNA prepared from 3 sets of WT-MCF-7 and Tam-R cells (Figure 1A). Amounts of 18S and 28S RNA were found to be relatively constant and the RNA was of good integrity. Semi-quantitative RT-PCR amplification was first performed using specific primers for both IGF-I and IGF-II, in addition to the house keeping gene  $\beta$ -actin. PCR conditions were optimised as described in the Materials and Methods Section 2.1.11. Under basal growth conditions, IGF-II (336bp), but not IGF-I (325bp) mRNA, was shown to be expressed in both cell lines albeit at low levels and these levels appeared higher in Tam-R cells (Figure 1B). Indeed, this increase observed in Tam-R cells was more evident when the IGF-II levels were corrected for  $\beta$ -actin (204bp; Figure 3A). As also shown in Figure 1B, IGF-I mRNA (325bp) expression was detected in another breast cancer cell line, MDA-231 cells, under the same PCR conditions (Figure 1B), which served as a positive control for this gene in this experiment. The inclusion of two negative controls, a -RT and -PCR (included as a marker of contamination for the RT-PCR protocol) revealed no evidence of any non-specific or contaminating DNA (Figure 1B).

To validate the above IGF-II mRNA findings, real time quantitative PCR was performed on basally growing WT-MCF-7 and Tam-R cell lines using OPTICON 2 technology. Conditions were optimised as described in the Materials and Methods Sections 2.1.11 and 2.1.12 and were based upon those used for semi-quantitative PCR. Experiments were also set up to quantify  $\beta$ -actin, in order to normalise IGF-II mRNA expression levels. As described in the Materials and Methods Section 2.1.12, prior to calculation of the IGF-II and  $\beta$ -actin template concentration following amplification, the position of the cycle threshold or  $C_T$  line must be defined on a graph of fluorescence versus cycle number. Figure 2A and Figure 2B shows representative graphs obtained of fluorescence versus cycle number for IGF-II and  $\beta$ -actin mRNA respectively. The threshold cycle for an individual sample is then defined as the cycle at which the sample's fluorescence trace first crosses the  $C_T$  line, as also shown in Figure 2A and Figure 2B. By including quantitation standards with varying initial amounts of template in the run, a standard curve of Log Quantity versus  $C_T$  Cycle can be plotted as illustrated in Figure 2C and Figure 2D for IGF-II and  $\beta$ -actin respectively and this was used to calculate the quantity of initial template in the unknowns (see Materials and Methods Section 2.1.12). To verify the identity of specific products and to rule out the presence of un-desirable primer dimers, melting curves were also performed following the cycling protocol as described in the Materials and Methods Section 2.1.12. As shown in Figure 2E and Figure 2F, the resultant PCR products obtained, were shown to be specific, generating single fluorescent peaks at 87 °C and 84 °C for IGF-II and  $\beta$ -actin respectively.

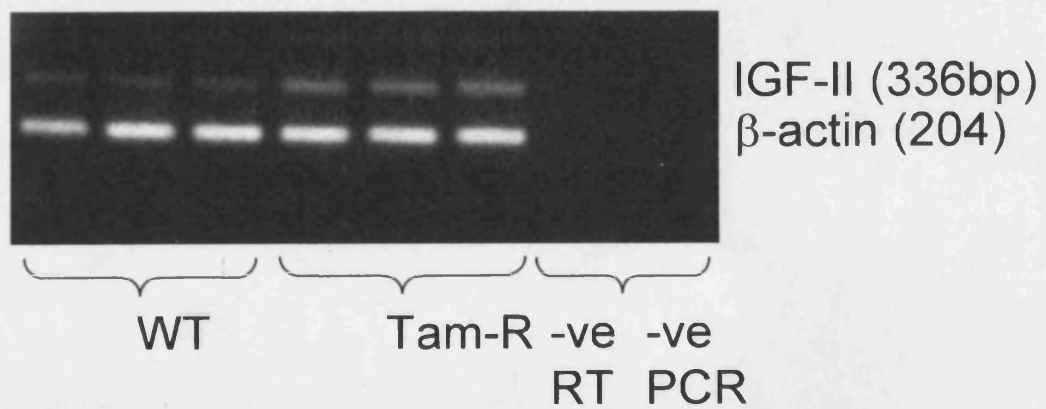
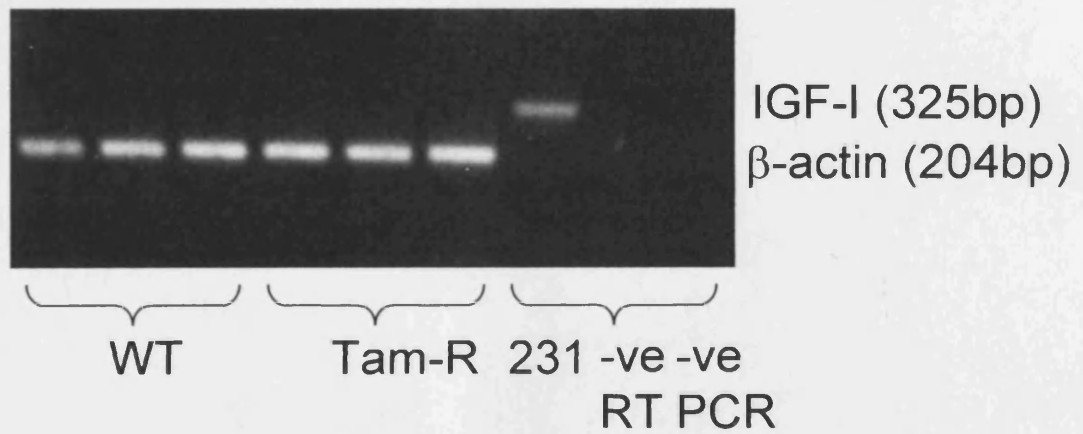
IGF-II mRNA levels when corrected for  $\beta$ -actin, were found to be increased significantly by approximately 3 fold in the Tam-R cells compared to the WT-MCF-7 cells ( $7.6 \times 10^{-4} \pm 1.2 \times 10^{-4}$  fg/ $\mu$ l versus  $2.6 \times 10^{-4} \pm 4.5 \times 10^{-5}$  fg/ $\mu$ l respectively;  $p= 0.017$ ) as illustrated in Figure 3B, thus validating the semi-quantitative PCR results.

**Figure 1.** Levels of total RNA and expression of IGF-I, IGF-II and  $\beta$ -actin mRNA in WT-MCF-7 and Tam-R cells. Cells were grown for 24 hours in serum-free DCCM medium. Following cell lysis, total RNA and mRNA expression was assessed by (A) ethidium bromide stained agarose gel and (B) semi-quantitative RT-PCR analysis. Data are representative of three separate experiments.

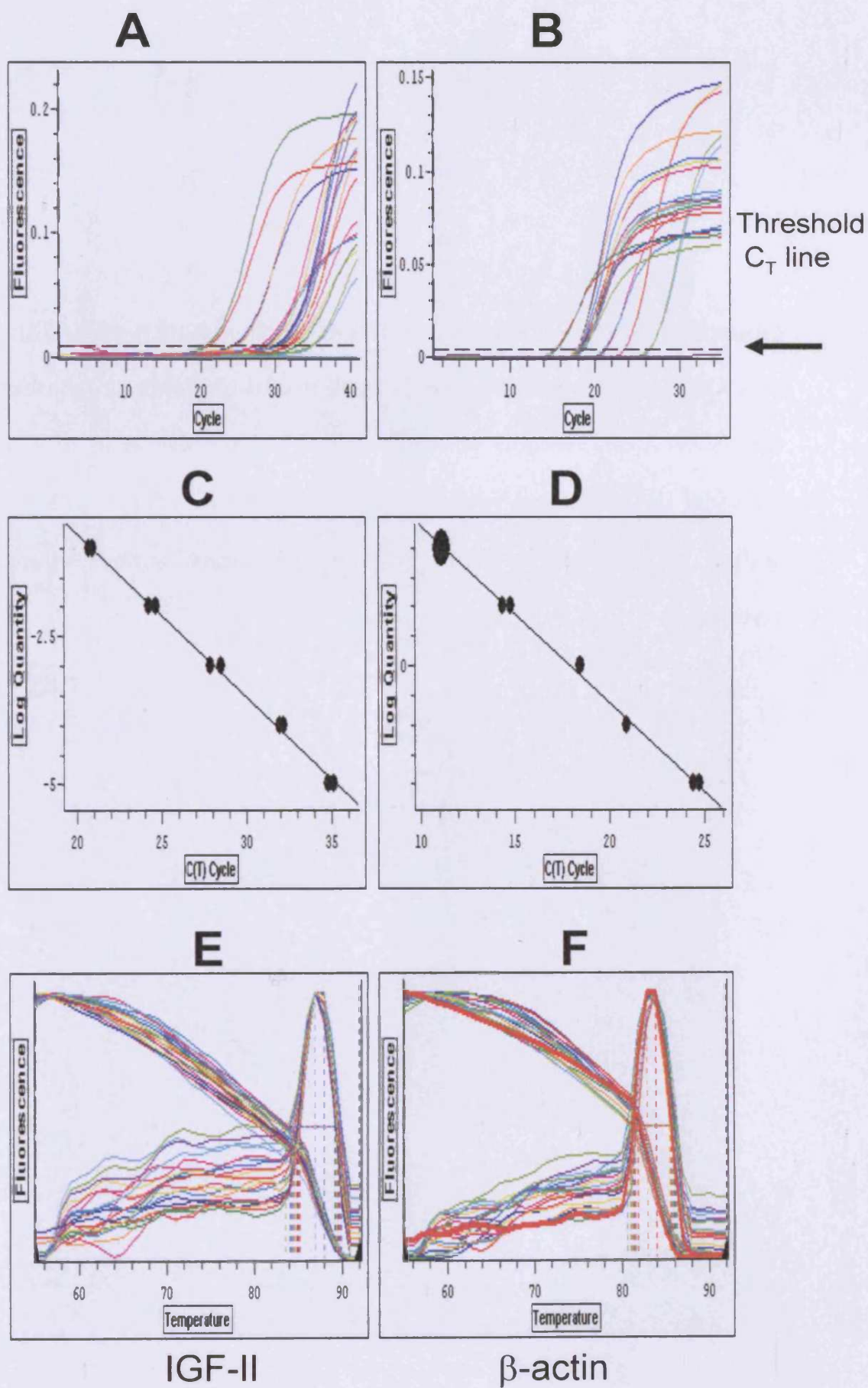
**A**



**B**

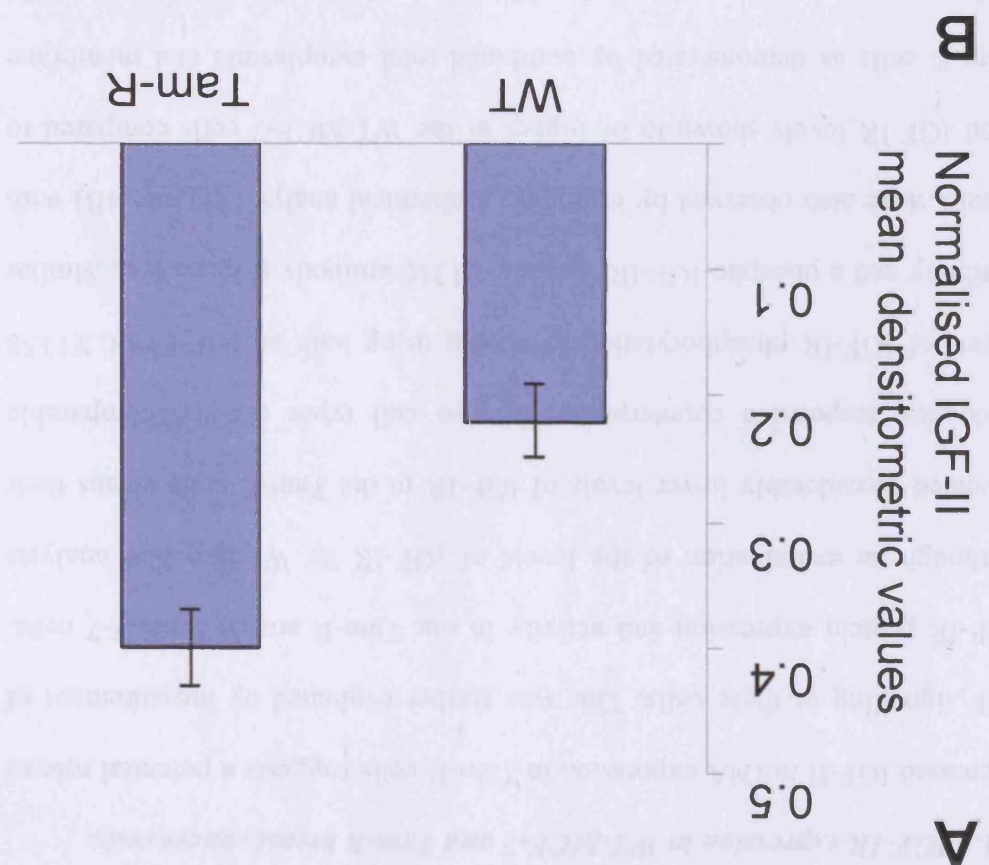
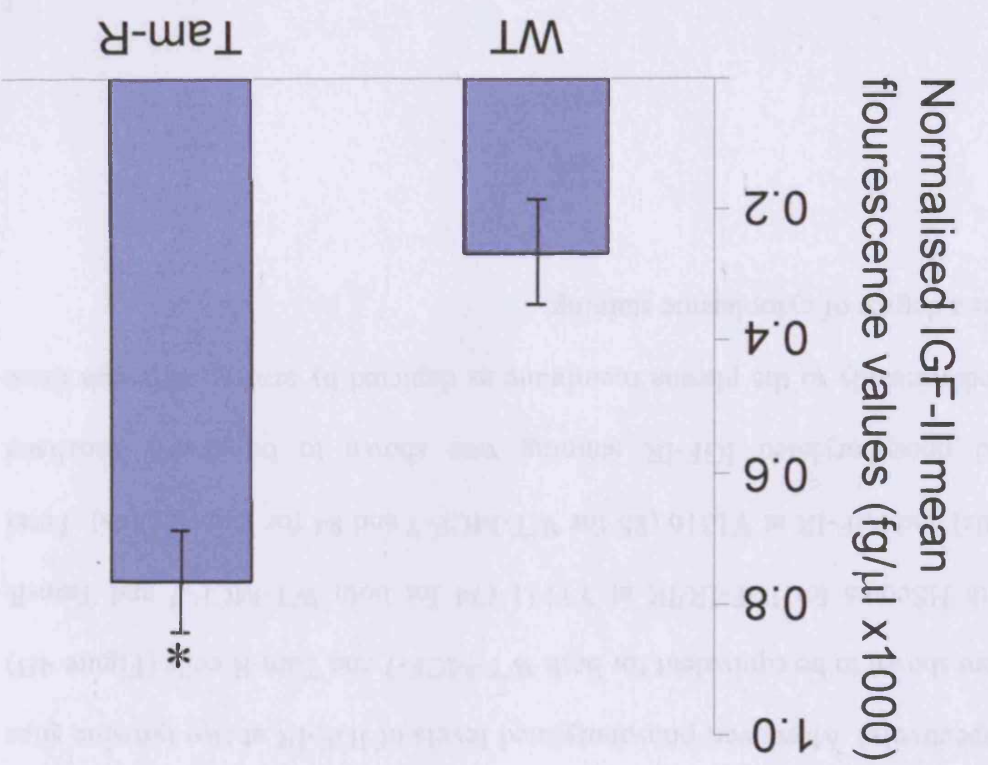


**Figure 2.** Representative graphs obtained of fluorescence versus cycle number (A and B), standard curve of Log Quantity versus  $C_T$  cycle (C and D) and melting curves (E and F) for both IGF-II and  $\beta$ -actin respectively. Data are representative of three separate experimental groups.



**Figure 3.** Basal expression levels of IGF-II mRNA in WT-MCF-7 and Tam-R cells. Cells were grown for 24 hours in serum-free DCCM medium. Following cell lysis, mRNA expression was assessed by (A) semi-quantitative RT-PCR analysis with densitometric values corrected for  $\beta$ -actin and (B) real time PCR analysis, also corrected for  $\beta$ -actin. Data are representative of three separate experiments.

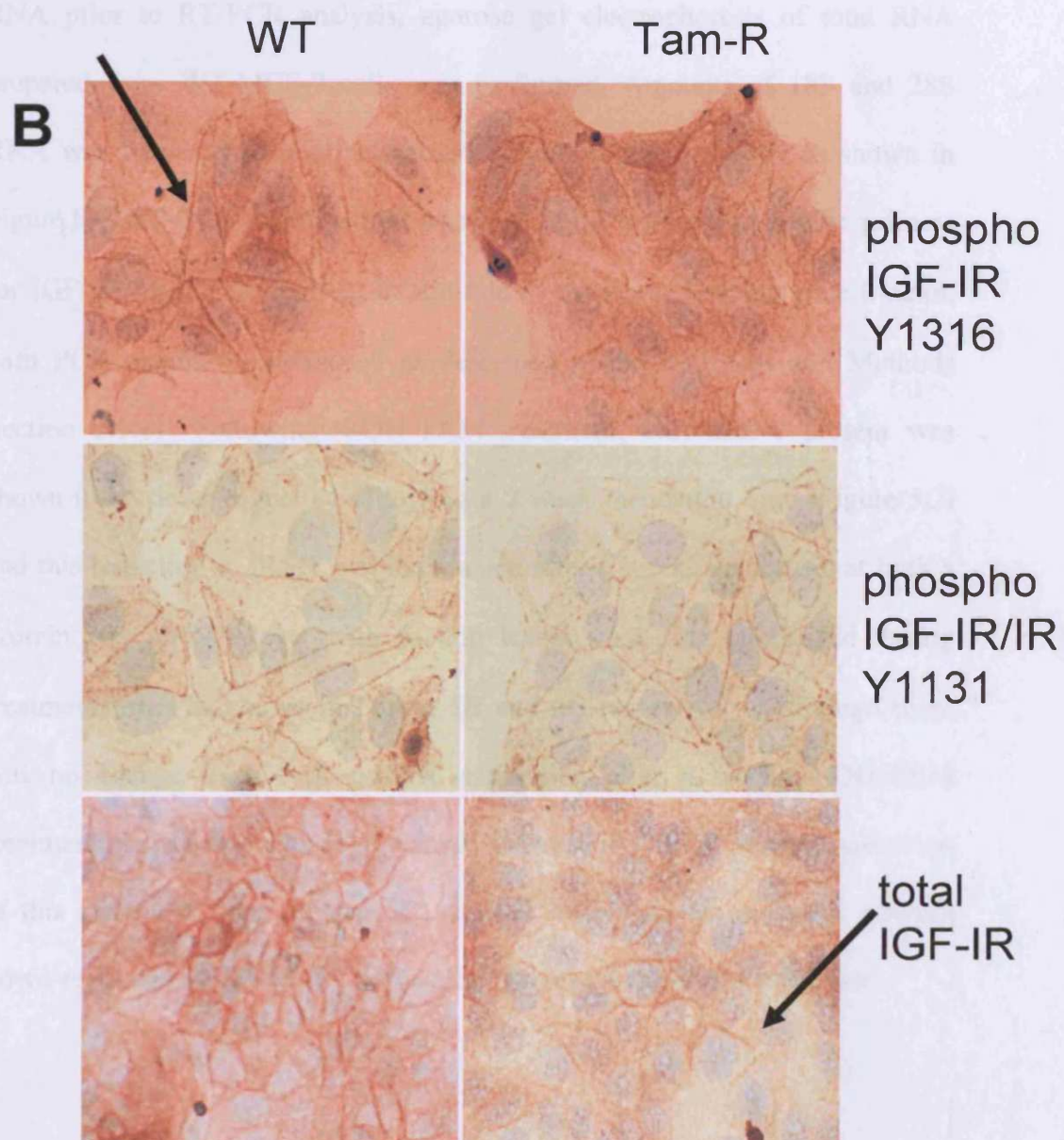
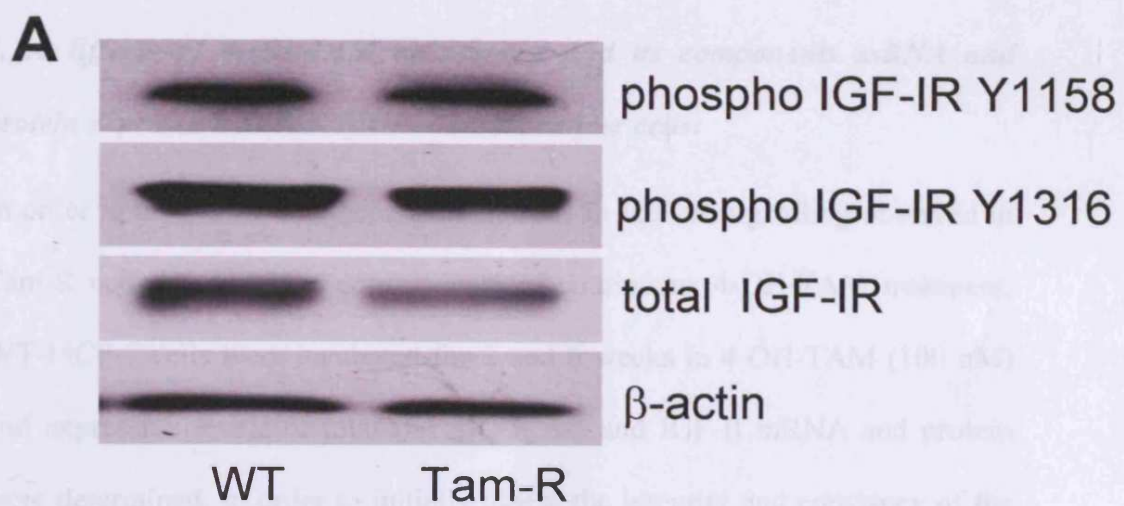




### ***3.1.3 IGF-IR expression in WT-MCF-7 and Tam-R breast cancer cells:***

Increased IGF-II mRNA expression in Tam-R cells suggests a potential role of IGF signalling in these cells. This was further evaluated by measurement of IGF-IR protein expression and activity in our Tam-R and WT MCF-7 cells. Although an examination of the levels of IGF-IR by Western blot analysis revealed considerably lower levels of IGF-IR in the Tam-R cells versus their endocrine responsive counterparts, the two cell types showed comparable levels of IGF-IR phosphorylation as shown using both an IGF-IR/IR Y1158 antibody and a phospho-IGF-IR specific Y1316 antibody (Figure 4A). Similar results were also observed by immunocytochemical analysis (Figure 4B) with total IGF-IR levels shown to be higher in the WT-MCF-7 cells compared to Tam-R cells as demonstrated by combined total cytoplasmic and membrane staining intensity as assessed by HScore analysis (79 compared to 59 respectively). Moreover, phosphorylated levels of IGF-IR at two tyrosine sites were shown to be equivalent for both WT-MCF-7 and Tam-R cells (Figure 4B) with HScores for IGF-IR/IR at Y1131 (74 for both WT-MCF-7 and Tam-R cells) and IGF-IR at Y1316 (85 for WT-MCF-7 and 84 for Tam-R cells). Total and phosphorylated IGF-IR staining was shown to be clearly localised predominantly to the plasma membrane as depicted by arrows, although there was a degree of cytoplasmic staining.

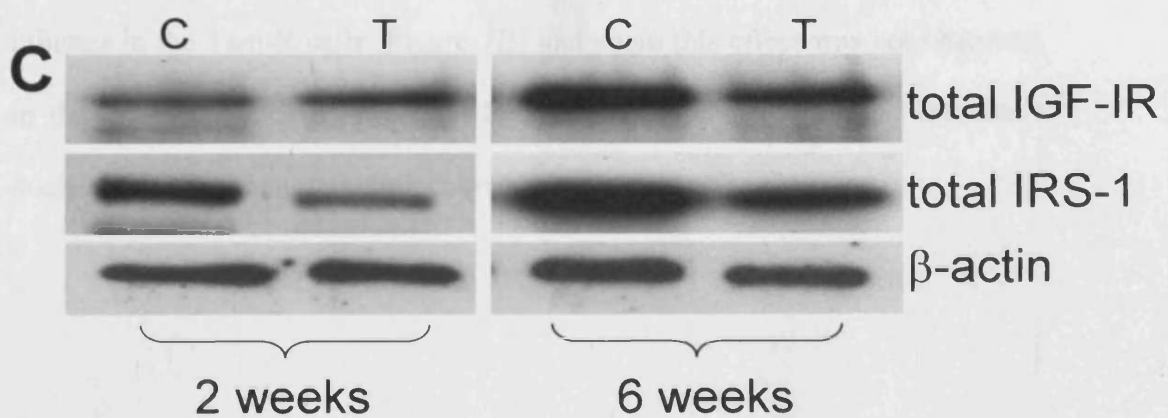
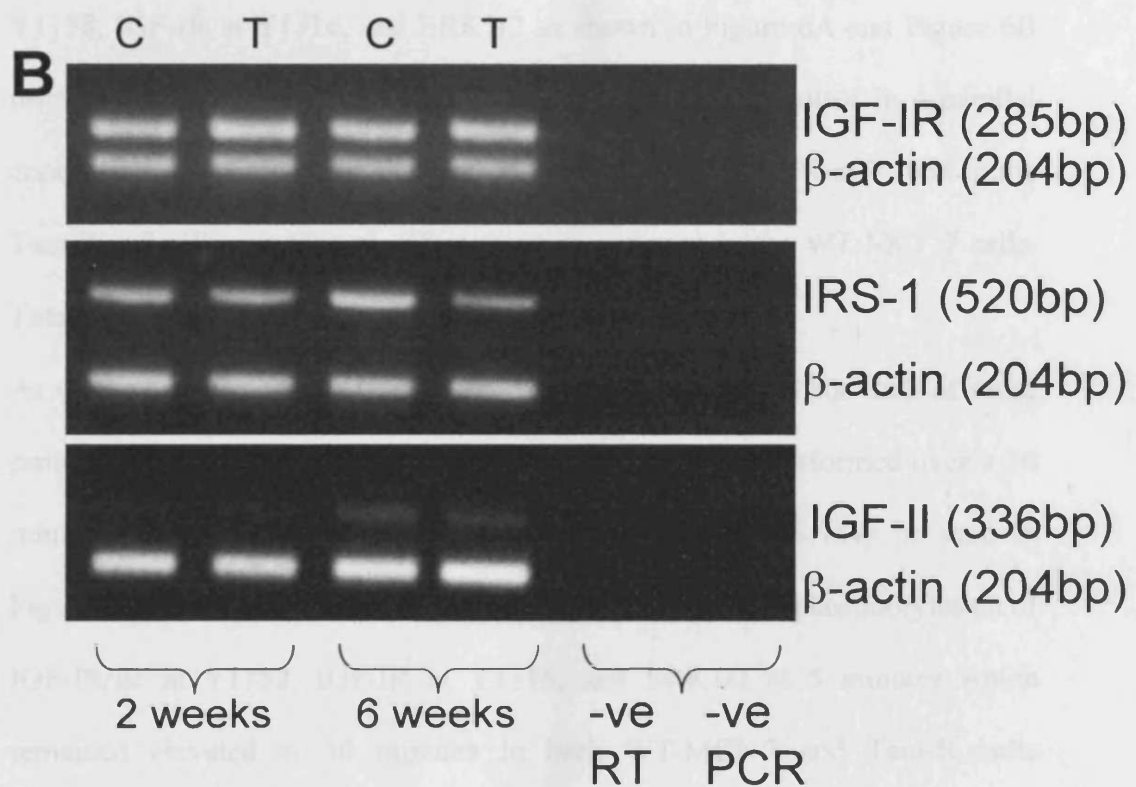
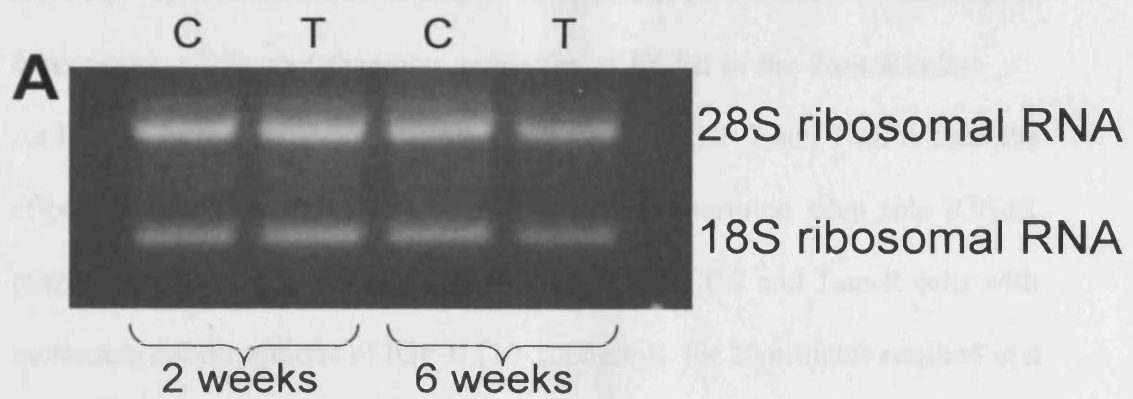
**Figure 4.** Basal protein expression levels of phosphorylated and total IGF-IR in WT-MCF-7 and TAM-R cells. Cells were grown for 24 hours in serum-free DCCM medium and assessed by (A) Western blot and (B) immunocytochemical analysis. Data are representative of three separate experiments for each methodology. Original magnification (x40) for immunocytochemical analysis.



#### ***3.1.4 Effects of 4-OH-TAM on IGF-IR and its components mRNA and protein expression in WT- MCF-7 breast cancer cells:***

In order to determine whether the alterations in IGF-IR signalling observed in Tam-R cells are solely a consequence of continuous 4-OH-TAM treatment, WT-MCF-7 cells were incubated for 2 and 6 weeks in 4-OH-TAM (100 nM) and expression levels of total IGF-IR, IRS-1 and IGF-II mRNA and protein were determined. In order to initially assess the integrity and constancy of the RNA prior to RT-PCR analysis, agarose gel electrophoresis of total RNA prepared from WT-MCF-7 cells was performed. Amounts of 18S and 28S RNA was found to be relatively constant and of good integrity as shown in Figure 5A. RT-PCR amplification was then performed using specific primers for IGF-IR, IRS-1 and IGF-II, in addition to the house keeping gene  $\beta$ -actin, with PCR conditions optimised as described in the Materials and Methods Section 2.1.11. Following 4-OH-TAM treatment, only IRS-1 protein was shown to be down-regulated following a 2 week incubation time (Figure 5C) and this reduction in IRS-1 was maintained after 6 weeks incubation at both a protein and mRNA level with  $\beta$ -actin levels remaining unchanged during treatment times as shown in Figure 5B and 5C respectively. Although there was no change in IGF-IR mRNA expression after 6 weeks 4-OH-TAM treatment, there was evidence of a small decrease in IGF-IR protein expression at this treatment time (Figure 5C). IGF-II mRNA levels appeared slightly down-regulated in WT-MCF-7 cells after 6 weeks 4-OH-TAM treatment.

**Figure 5.** Levels of total RNA, expression of IGF-IR, IRS-1, IGF-II and  $\beta$ -actin mRNA and total IGF-IR, IRS-1 and  $\beta$ -actin protein in WT-MCF-7 cells grown in 4-OH-TAM treatment. WT-MCF-7 were grown in phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS supplemented with 4-OH-TAM (T; 100nM) or vehicle control (C) for 2 and 6 weeks. Following cell lysis, total RNA, mRNA and protein expression was assessed by (A) ethidium bromide stained agarose gel (B), semi-quantitative RT-PCR and (C) Western blot analysis. Data are representative of two separate experiments.



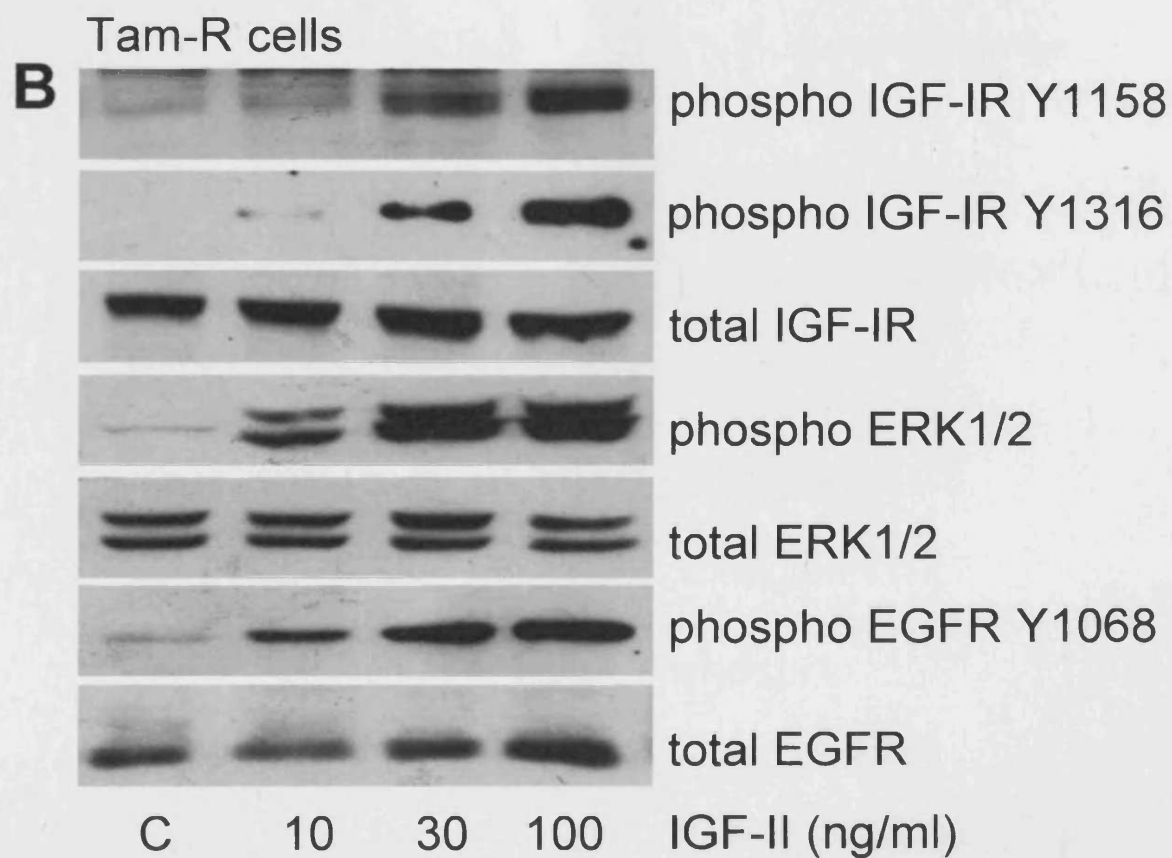
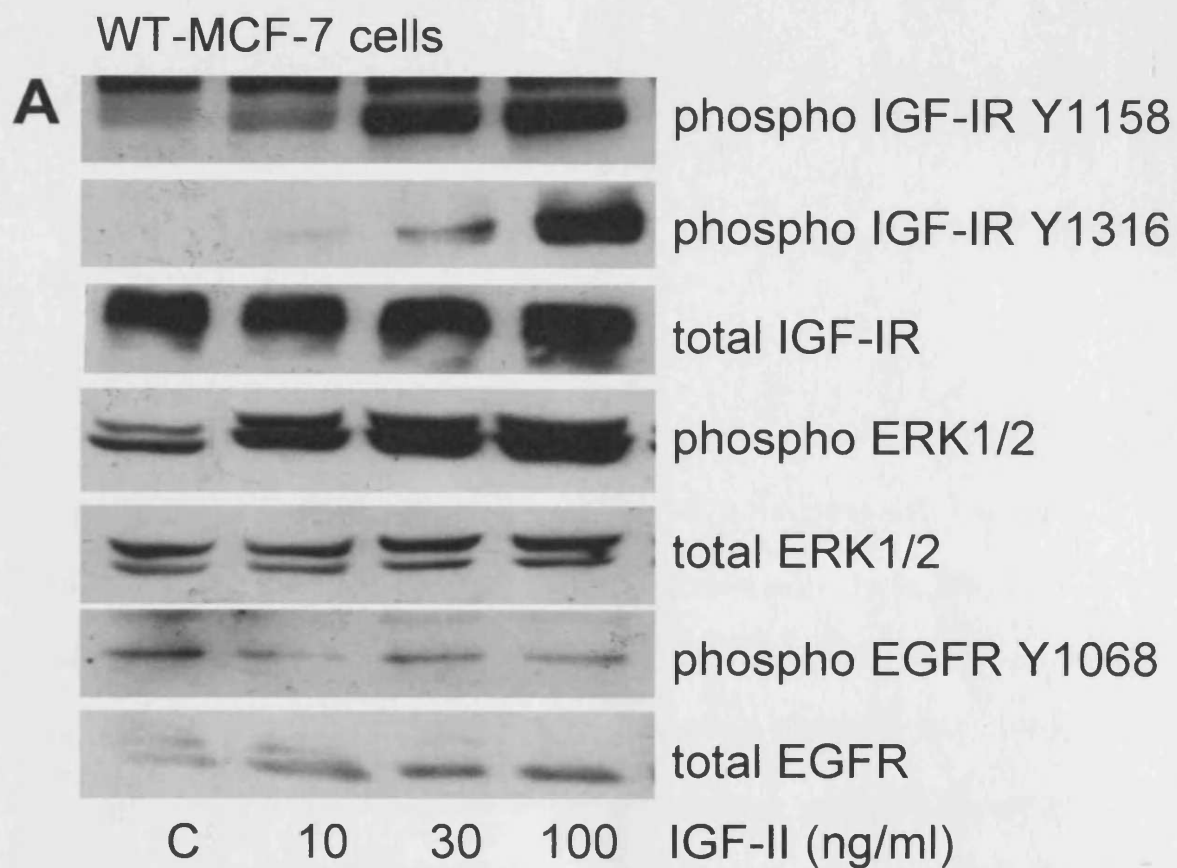
### ***3.1.5 IGF-II activates IGF-IR and its components in WT-MCF-7 and Tam-R breast cancer cells and promotes activation of EGFR in the Tam-R cells:***

As IGF-IR is expressed and is active in both WT-MCF-7 and Tam-R cells the effect of IGF-II treatment was next assessed to determine what role IGF-IR may be playing in these cells. Incubation of WT-MCF-7 and Tam-R cells with increasing concentrations of IGF-II (10-100ng/ml), for 20 minutes resulted in a concentration-dependent increase in basal phosphorylation of IGF-IR/IR at Y1158, IGF-IR at Y1316, and ERK1/2 as shown in Figure 6A and Figure 6B respectively. Interestingly, IGF-IR activation by IGF-II resulted in a parallel concentration-dependent increase in phosphorylation of EGFR at Y1068 in the Tam-R cells (Figure 6B), an effect that was not seen in the WT-MCF-7 cells. Total levels of each of these proteins remained unchanged.

As maximum stimulation was observed at 100ng/ml IGF-II for each of these proteins in both cell lines, a time course experiment was performed over a 30 minute time period using this concentration of IGF-II. As may be seen in Figure 7A and Figure 7B, this resulted in a rapid increase in phosphorylation of IGF-IR/IR at Y1158, IGF-IR at Y1316, and ERK1/2 at 5 minutes which remained elevated at 30 minutes in both WT-MCF-7 and Tam-R cells respectively. In contrast, EGFR Y1068 phosphorylation levels peaked at 20 minutes in the Tam-R cells (Figure 7B) and again this effect was not observed in the WT-MCF-7 cells. The total levels of each of these proteins remained unchanged throughout this time course.

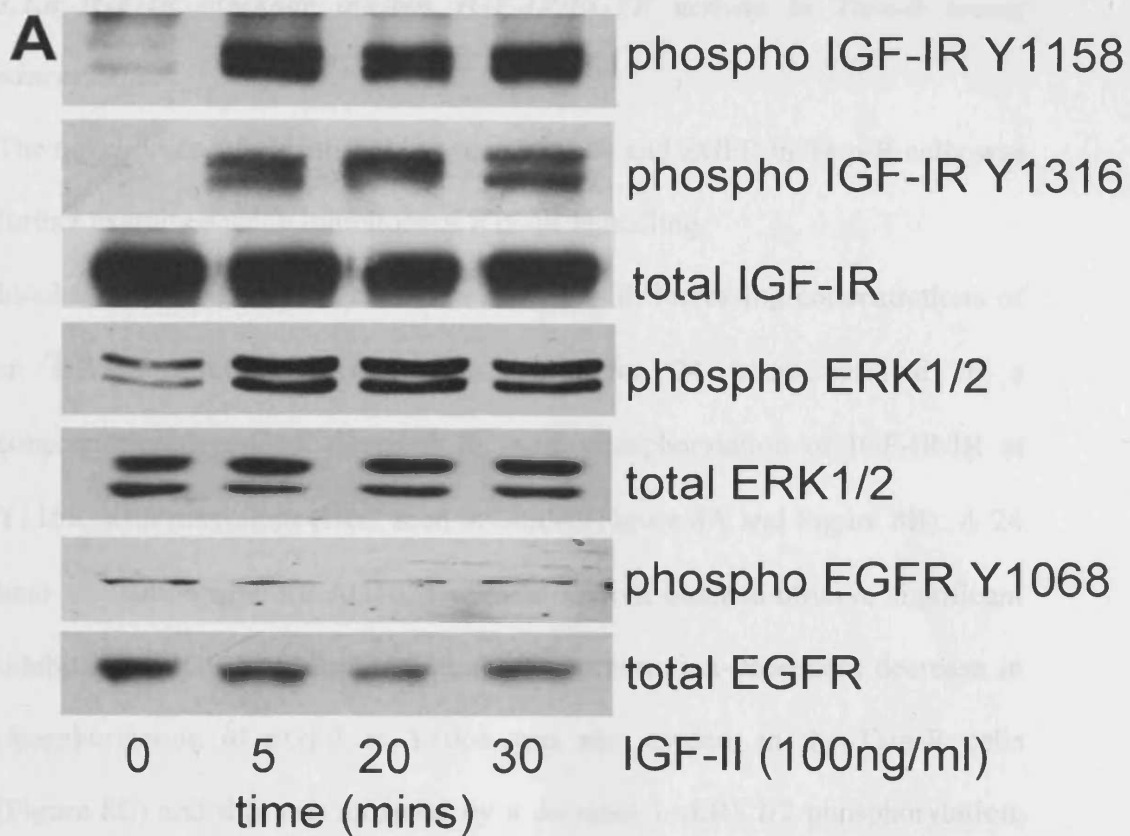


**Figure 6.** The effects of IGF-II concentration on phosphorylated and total IGF-IR, ERK1/2 and EGFR protein expression in WT-MCF-7 and Tam-R cells. WT-MCF-7 and Tam-R cells were incubated in serum-free DCCM medium supplemented with increasing concentrations of IGF-II (10-100ng/ml) or appropriate vehicle control for 20 minutes. Following cell lysis, protein expression was assessed in (A) WT-MCF-7 and (B) Tam-R cells by Western blot analysis. Data are representative of three separate experiments.

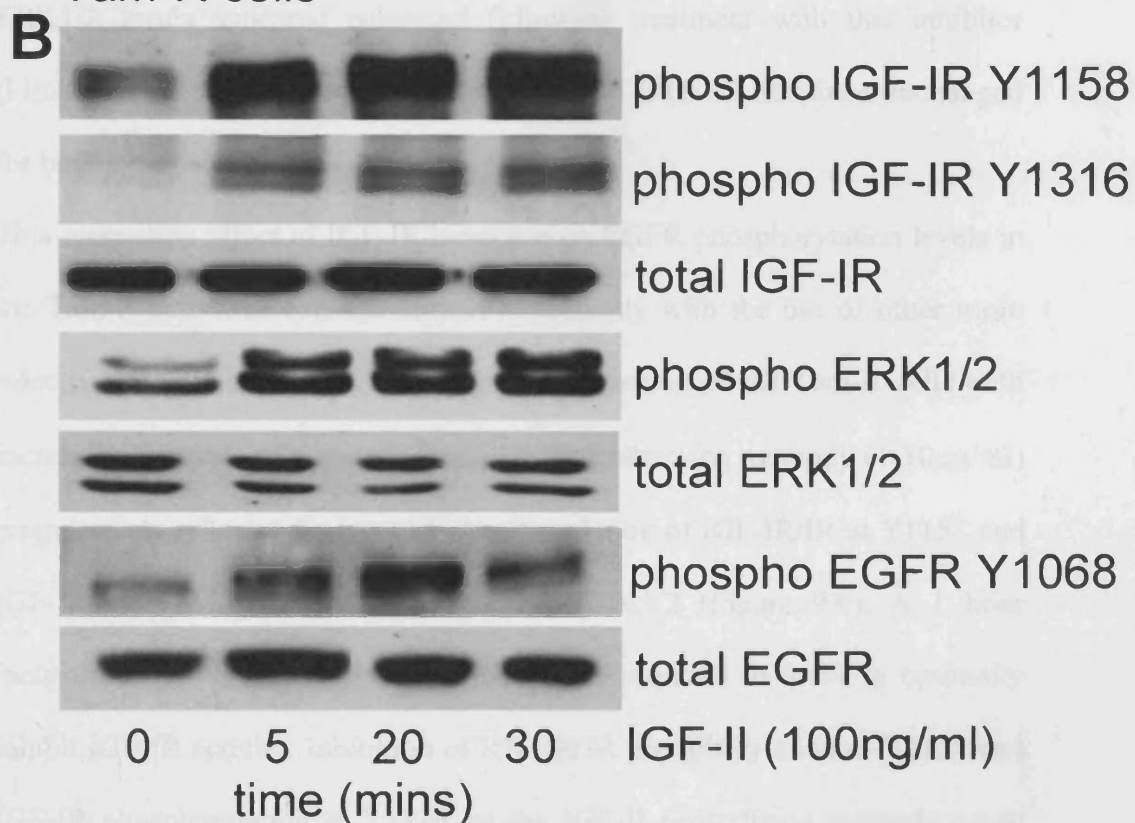


**Figure 7.** The effects of IGF-II on phosphorylated and total IGF-IR, ERK1/2 and EGFR protein expression in WT-MCF-7 and Tam-R cells. WT-MCF-7 and Tam-R cells were incubated in serum-free DCCM medium, supplemented with IGF-II (100ng/ml) or appropriate vehicle control for up to 30 minutes. Following cell lysis, protein expression was assessed in (A) WT-MCF-7 and (B) Tam-R cells by Western blot analysis. Data are representative of three separate experiments.

# WT-MCF-7 cells



# Tam-R cells



### ***3.1.6 IGF-IR blockade inhibits IGF-IR/EGFR activity in Tam-R breast cancer cells:***

The novel cross-talk identified between IGF-IR and EGFR in Tam-R cells was further examined using inhibitors of IGF-IR signalling.

Incubation of WT-MCF-7 and Tam-R cells with increasing concentrations of an IGF-IR inhibitor AG1024 (1-20 $\mu$ M) for 24 hours resulted in a concentration-dependent decrease in basal phosphorylation of IGF-IR/IR at Y1158, with maximum effect seen at 20 $\mu$ M (Figure 8A and Figure 8B). A 24 hour incubation time for AG1024 was required in order to observe significant inhibition of IGF-IR phosphorylation. A concentration-dependent decrease in phosphorylation of EGFR at Y1068 was also evident in the Tam-R cells (Figure 8B) and this was mirrored by a decrease in ERK1/2 phosphorylation. AG1024 was without effect on EGFR phosphorylation in WT-MCF-7 cells and ERK1/2 levels appeared enhanced following treatment with this inhibitor (Figure 8A). Total levels of IGF-IR, EGFR and ERK1/2 remained unchanged for both treatment groups.

This interesting effect of IGF-IR blockade on EGFR phosphorylation levels in the Tam-R cells was examined more extensively with the use of other more selective IGF-IR inhibitors. A 1 hour pre-incubation of the Tam-R cells with increasing amounts of a specific anti IGF-II neutralising antibody (1-10 $\mu$ g/ml) progressively reduced the level of phosphorylation of IGF-IR/IR at Y1158 and IGF-IR at Y1316, EGFR at Y1068 and ERK1/2 (Figure 9A). A 1 hour incubation with the neutralising antibody was required in order to optimally inhibit IGF-IR activity. Inhibition of IGF-IR/IR phosphorylation at Y1158 and IGF-IR phosphorylation at Y1316 by the IGF-II neutralising antibody could

not be overcome by the inclusion of IGF-II (Figure 9B). The antibody had no effect on the total expression levels of these proteins. Similar inhibitory effects were also seen with the IGF-IR tyrosine kinase inhibitor ABDP. Incubation of Tam-R cells with increasing concentrations of ABDP (0.1-1 $\mu$ M) for 7 days resulted in a concentration-dependent decrease in basal phosphorylation of IGF-IR/IR at Y1158, IGF-IR at Y1316, EGFR at Y1068 and ERK1/2 in Tam-R cells with maximum effect seen at 1 $\mu$ M (Figure 10). A 7 day incubation time for ABDP was required in order to observe significant inhibition of basal IGF-IR phosphorylation. Total levels of IGF-IR, EGFR and ERK1/2 remained unchanged for each treatment groups. Furthermore, AG1024 (20 $\mu$ M) and ABDP (1 $\mu$ M) were both shown to also inhibit IGF-II- induced phosphorylation of IGF-IR/IR at Y1158 and IGF-IR at Y1316, EGFR at Y1068 and ERK1/2 (Figure 11A and Figure 11B) in Tam-R cells. The inhibitory effects of AG1024 on EGFR Y1068 and ERK1/2 phosphorylation could be partially overcome by EGF stimulation (Figure 12A), while, EGF treatment was without effect on phosphorylation of IGF-IR/IR at Y1158 and IGF-IR at Y1316 in this cell line (Figure 12B).

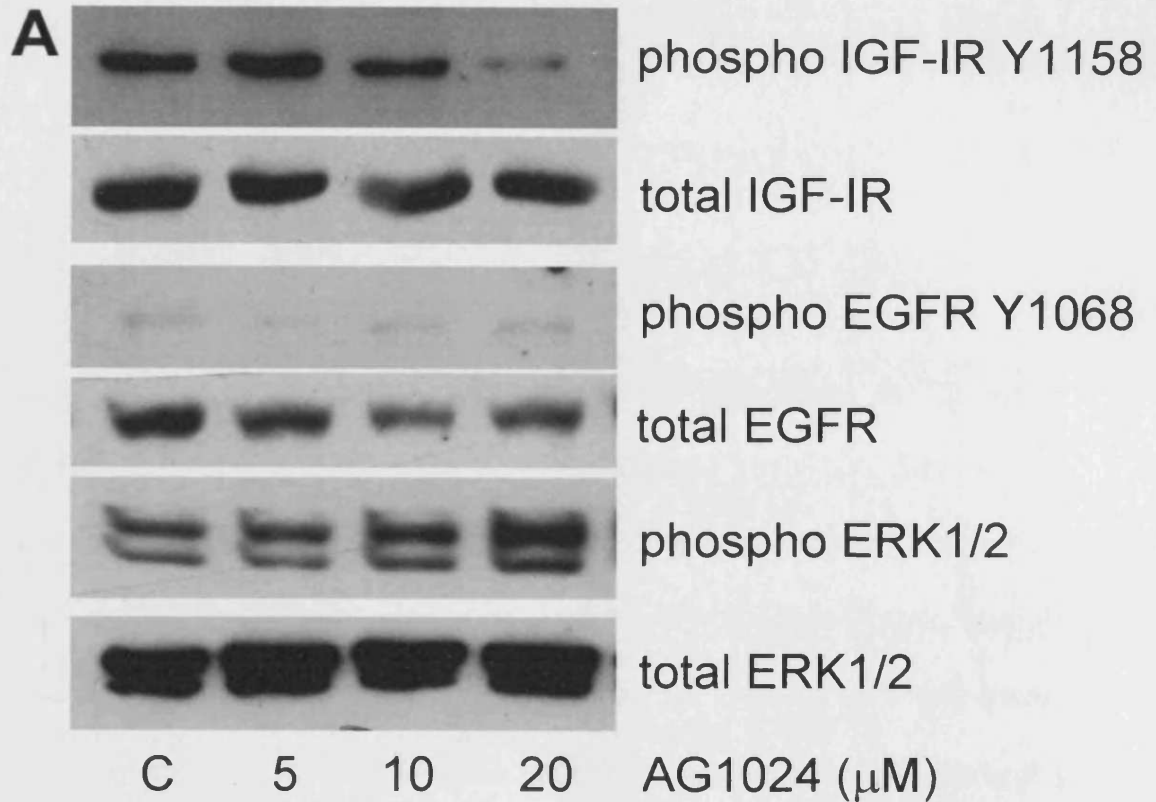
Similar findings to those for AG1024, ABDP and neutralising anti- IGF-II antibodies were also observed following the silencing of the IGF-IR gene in Tam-R cells using siRNA technology. The siRNA was firstly shown to specifically down-regulate total IGF-IR mRNA expression following semi-quantitative RT-PCR analysis (Figure 13B) with the integrity and constancy of the RNA prior to RT-PCR analysis found to be relatively constant and of good integrity (Figure 13A). As a direct consequence of down-regulation of mRNA expression, IGF-IR protein expression was also markedly and specifically

reduced compared to the medium only, non-targeting siRNA scrambled or Dharmafect 1 reagent (lipid) only controls as shown by Western analysis (Figure 13C), with total levels of all other proteins remaining unchanged. Levels of phosphorylated IGF-IR at Y1316, EGFR at Y1068 and ERK1/2 were also reduced quite substantially 4 days post transfection compared to the experimental controls (Figure 13C).

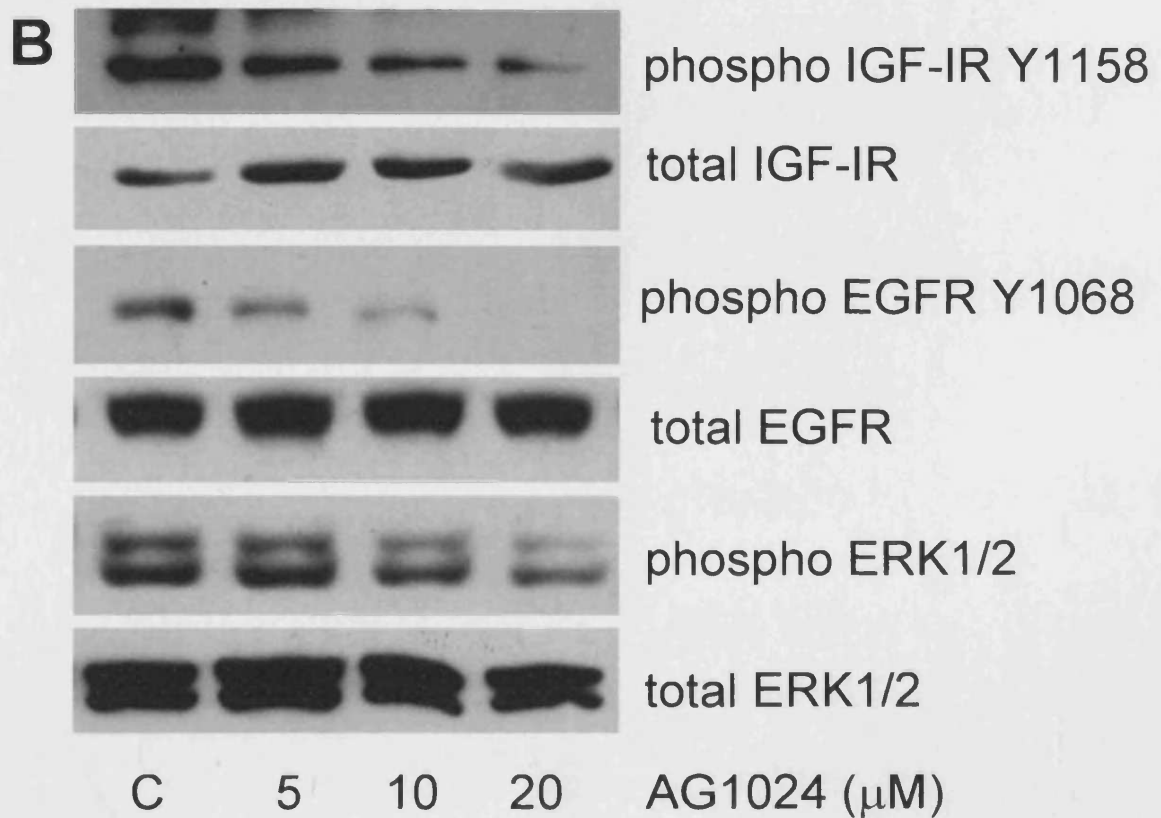
**Figure 8.** The effects of AG1024 on phosphorylated and total IGF-IR, EGFR and ERK1/2 protein expression in WT-MCF-7 and Tam-R cells. WT-MCF-7 and Tam-R cells were incubated in serum-free DCCM medium, supplemented with increasing concentrations of AG1024 (5-20 $\mu$ M) or appropriate vehicle control for 24 hours. Following cell lysis, protein expression was assessed in (A) WT-MCF-7 and (B) Tam-R cells by Western blot analysis. Data are representative of three separate experiments.



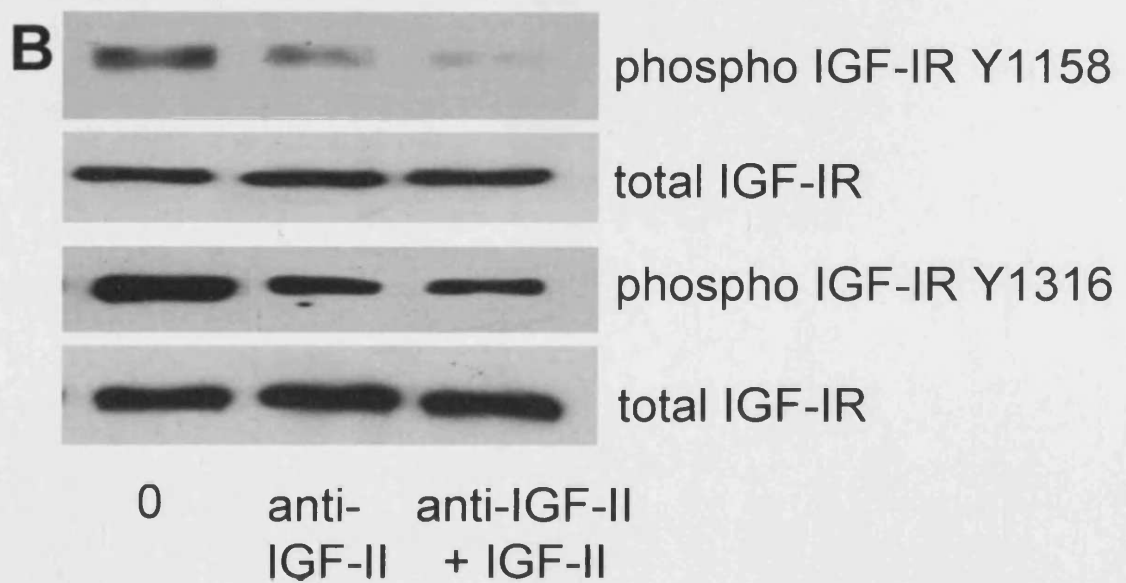
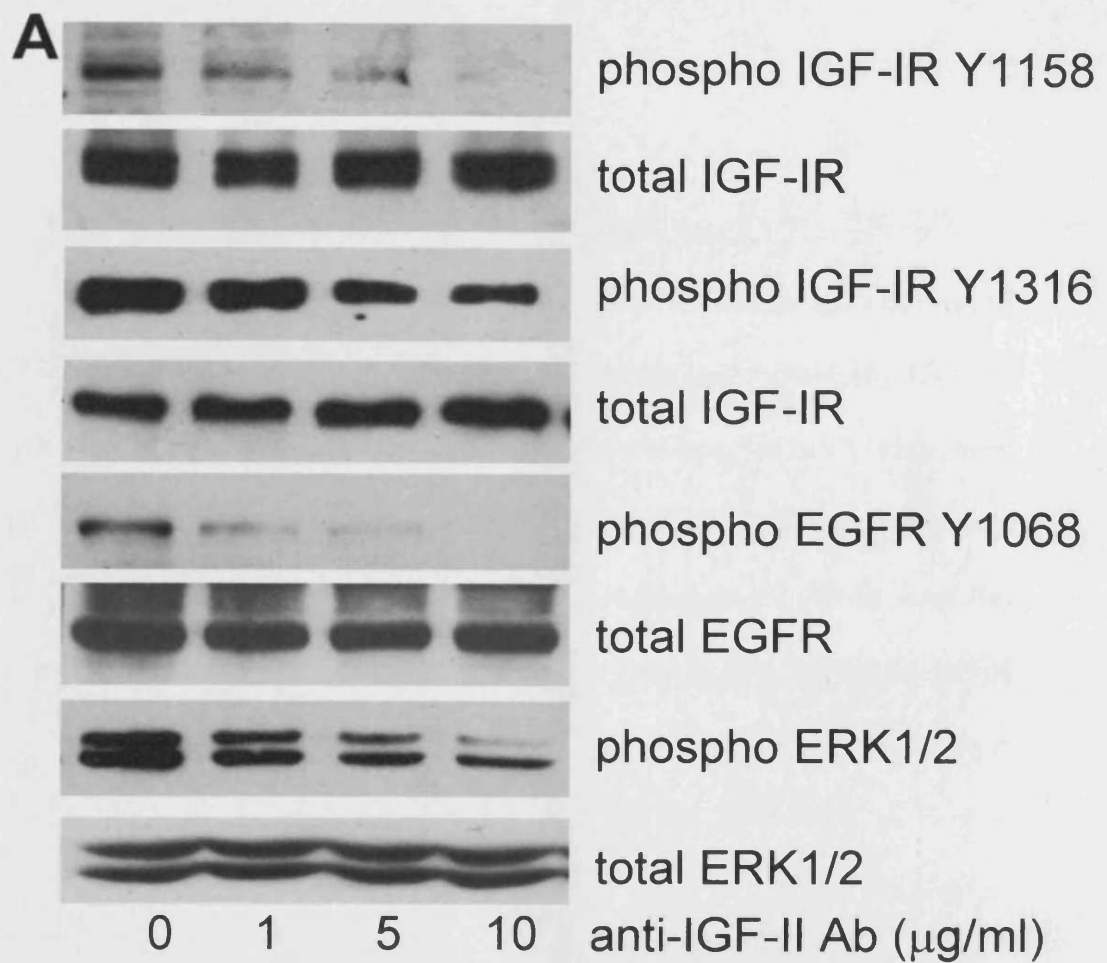
WT-MCF-7 cells



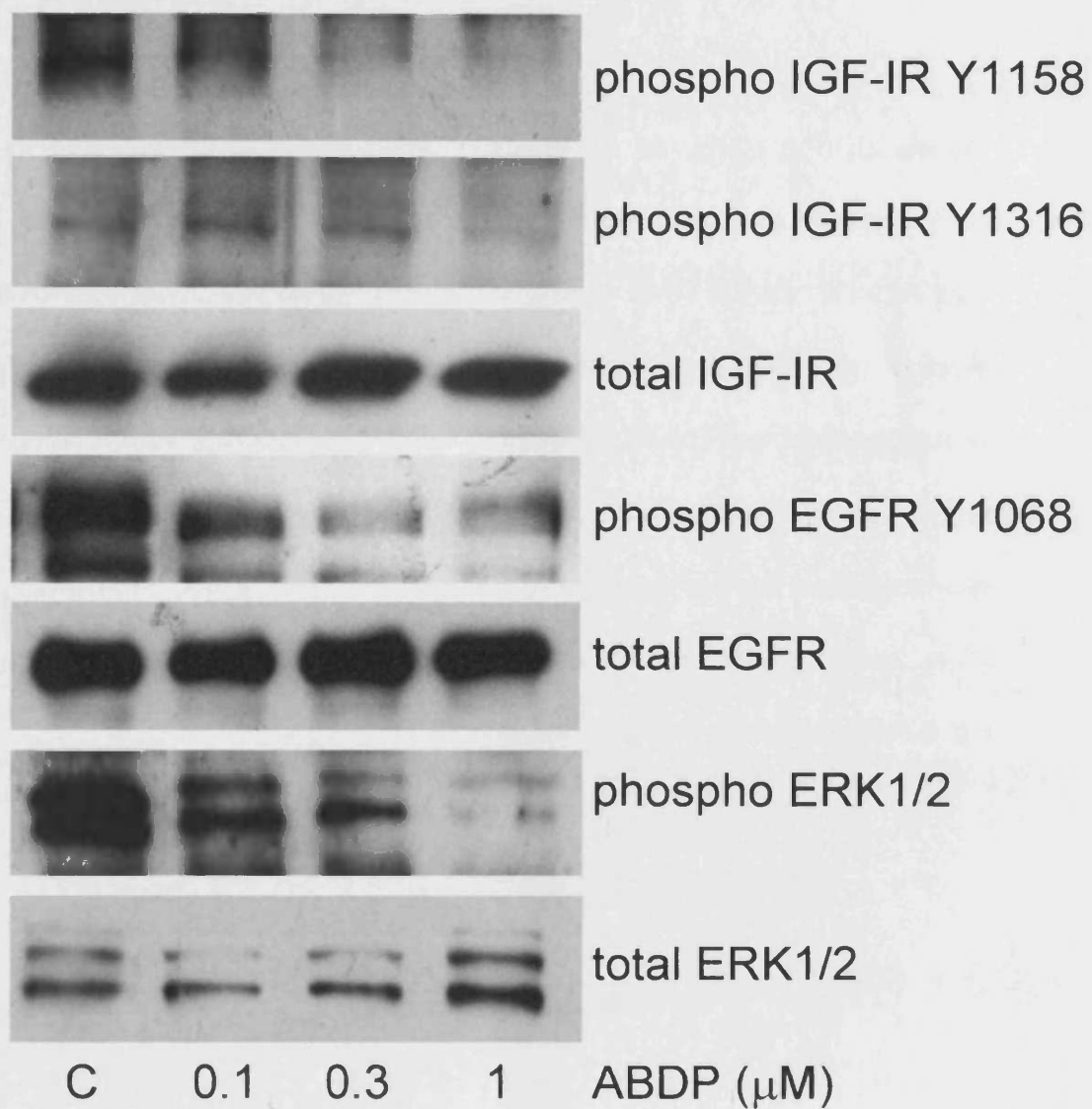
Tam-R cells



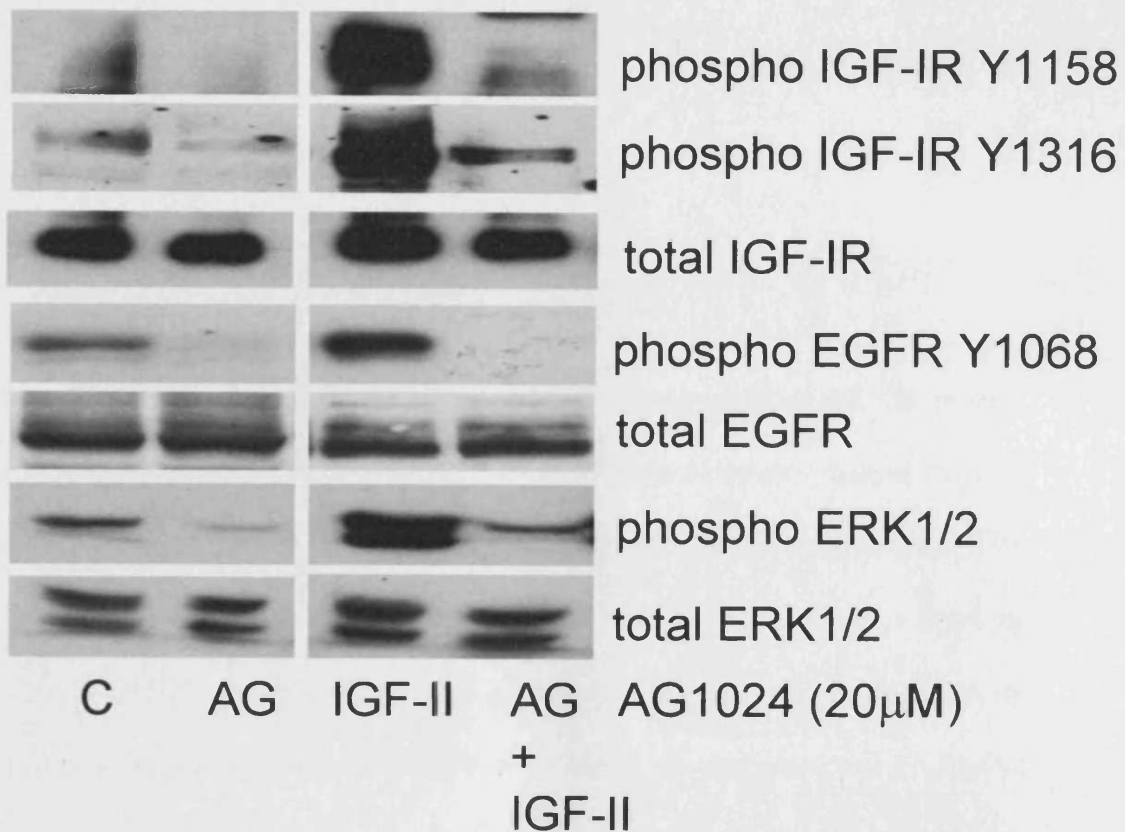
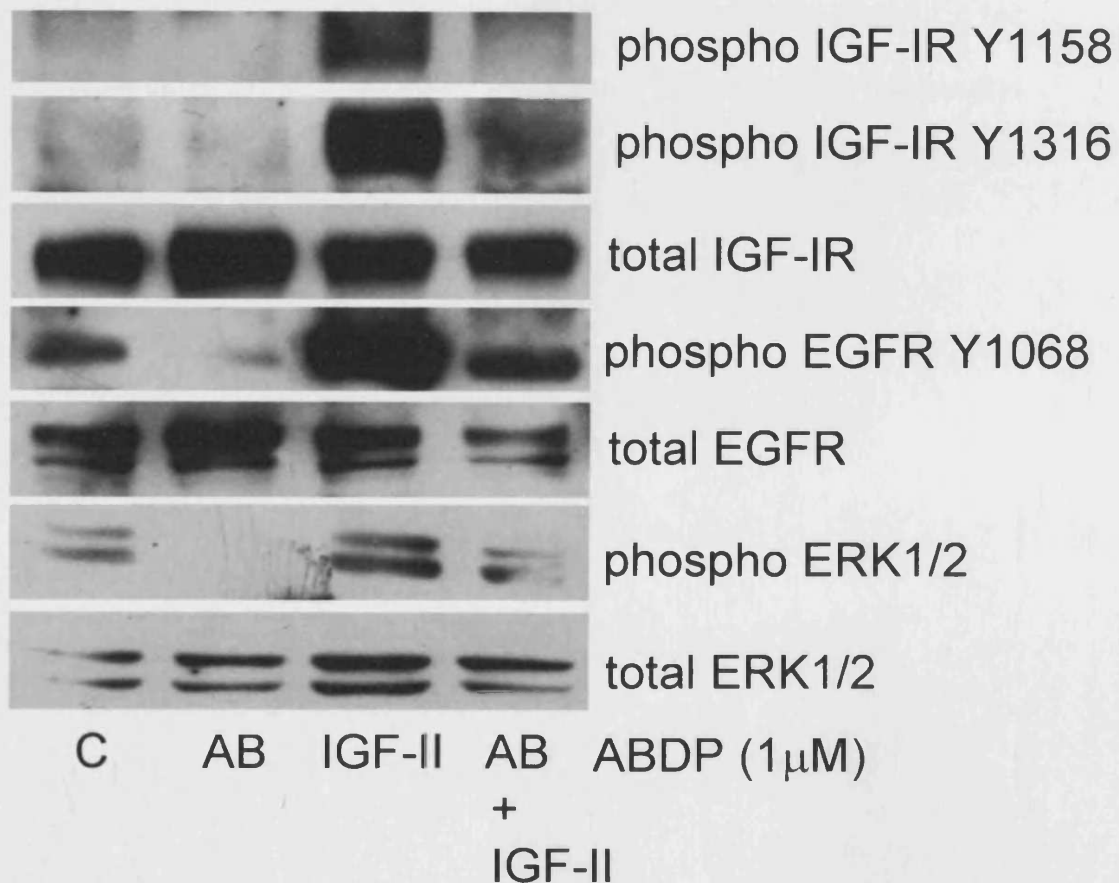
**Figure 9.** The effects of IGF-II neutralising antibody on phosphorylated and total IGF-IR, EGFR and ERK1/2 protein expression in Tam-R cells. Tam-R cells were incubated in serum-free DCCM medium, supplemented with (A) increasing concentrations of an IGF-II neutralising antibody (1-10 $\mu$ g/ml) or appropriate vehicle control for 1 hour and (B) anti-IGF-II neutralising antibody (10 $\mu$ g/ml for 1 hour) in either the presence or absence of IGF-II (100ng/ml) or appropriate vehicle control for 20 minutes. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of three separate experiments.



**Figure 10.** The effects of ABDP on phosphorylated and total IGF-IR, EGFR and ERK1/2 protein expression in Tam-R cells. Tam-R cells were incubated in serum-free DCCM medium for 7 days, supplemented with increasing concentrations of ABDP (0.01-1 $\mu$ M) or appropriate vehicle control. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of three separate experiments.

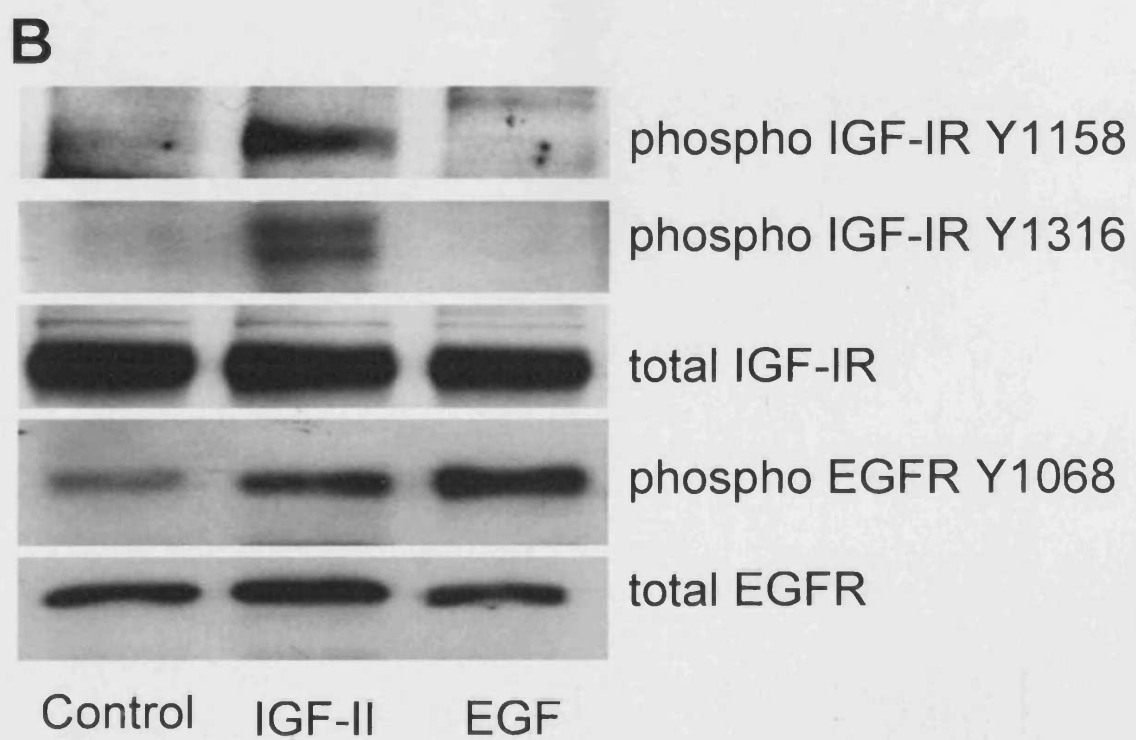
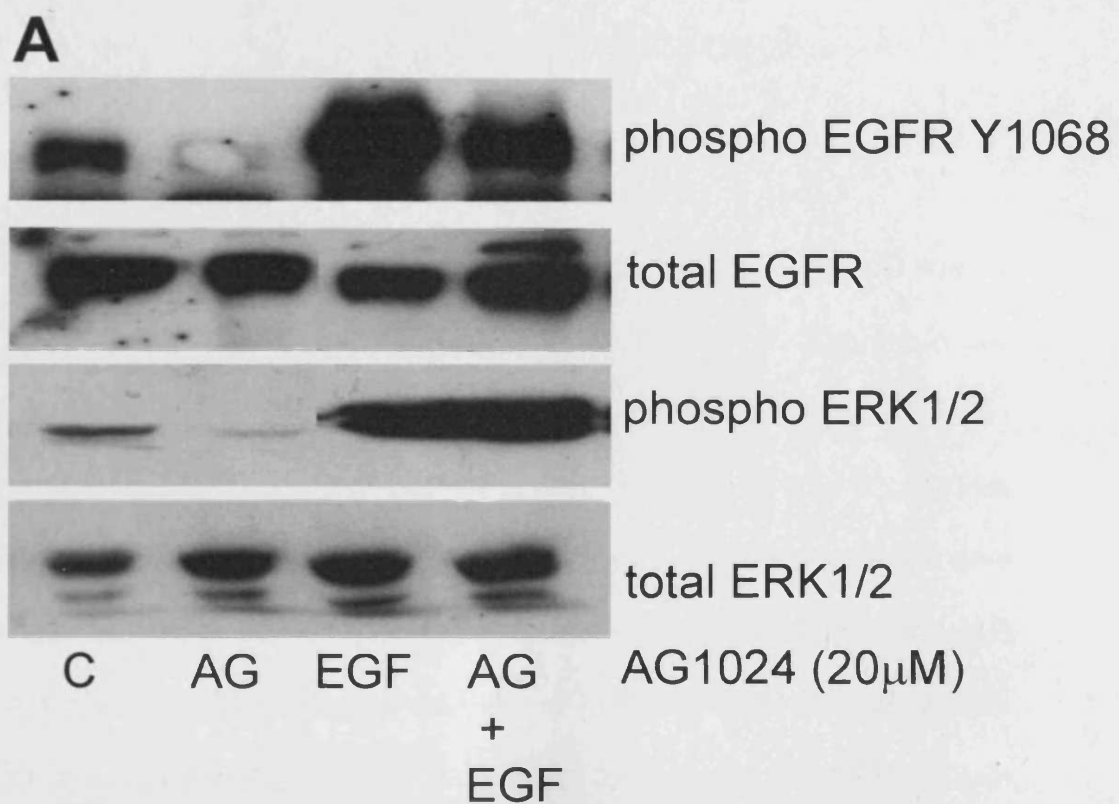


**Figure 11.** The effects of AG1024 or ABDP on basal and IGF-II-primed phosphorylated and total IGF-IR, EGFR and ERK1/2 protein expression in Tam-R cells. Tam-R cells were incubated in serum-free DCCM medium, supplemented either (A) AG1024 (20 $\mu$ M) for 24 hours, IGF-II (100ng/ml) for 20 minutes or a combination of these two agents or appropriate vehicle controls or (B) ABDP (1 $\mu$ M) for 7 days, IGF-II (100ng/ml) for 20 minutes or a combination of the two agents or appropriate vehicle controls. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of three separate experiments.

**A****B**

**Figure 12.** The effects of AG1024 on phosphorylated and total EGFR and ERK1/2 protein expression following EGF treatment, in addition to IGF-II or EGF treatment on phosphorylated and total IGF-IR and EGFR protein expression in Tam-R cells. Tam-R cells were incubated in serum-free DCCM medium, supplemented with either (A) AG1024 (20 $\mu$ M) for 24 hours, EGF (10ng/ml) for 5 minutes or a combination of the two agents or appropriate vehicle controls and (B) IGF-II (100ng/ml) for 20 minutes or EGF (10ng/ml) for 5 minutes or appropriate vehicle controls. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of three separate experiments.



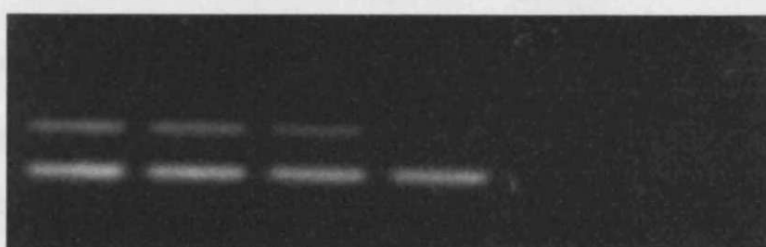


**Figure 13.** The effects of IGF-IR siRNA on total RNA, expression of IGF-IR and  $\beta$ -actin mRNA and total and phosphorylated IGF-IR, EGFR and ERK1/2 protein in Tam-R cells. Tam-R cells were incubated in phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS supplemented with either culture media alone (C), Dharmafect 1 transfection reagent alone (D), Dharmafect 1 transfection reagent and non-targeting scrambled siRNA mix (S; 100nM) or Dharmafect 1 transfection reagent and IGF-IR (I<sub>SI</sub>) siRNA mix (100nM) for 2 days (for RNA and RT-PCR analysis) or 4 days (for Western analysis). Following cell lysis, total RNA, mRNA and protein expression was assessed by (A) ethidium bromide stained agarose gel, (B) semi-quantitative RT-PCR and (C) Western blot analysis. Data are representative of two separate experiments.

**A**

28S ribosomal RNA

18S ribosomal RNA

**B**

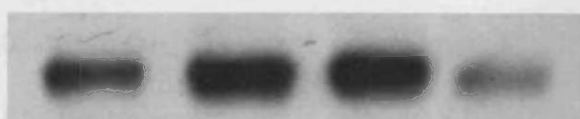
IGF-IR (285bp)

β-actin (204bp)

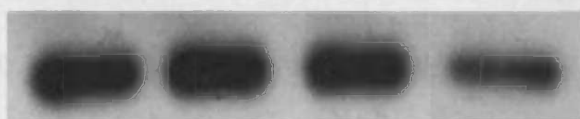
C

D

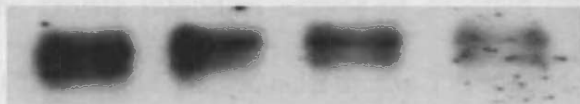
S

I<sub>sl</sub>-ve  
RT-ve  
PCR**C**

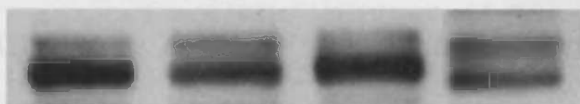
phospho IGF-IR Y 1316



total IGF-IR



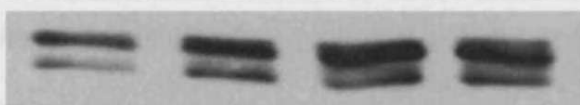
phospho EGFR Y1068



total EGFR



phospho ERK1/2



total ERK1/2

C

D

S

I<sub>sl</sub>

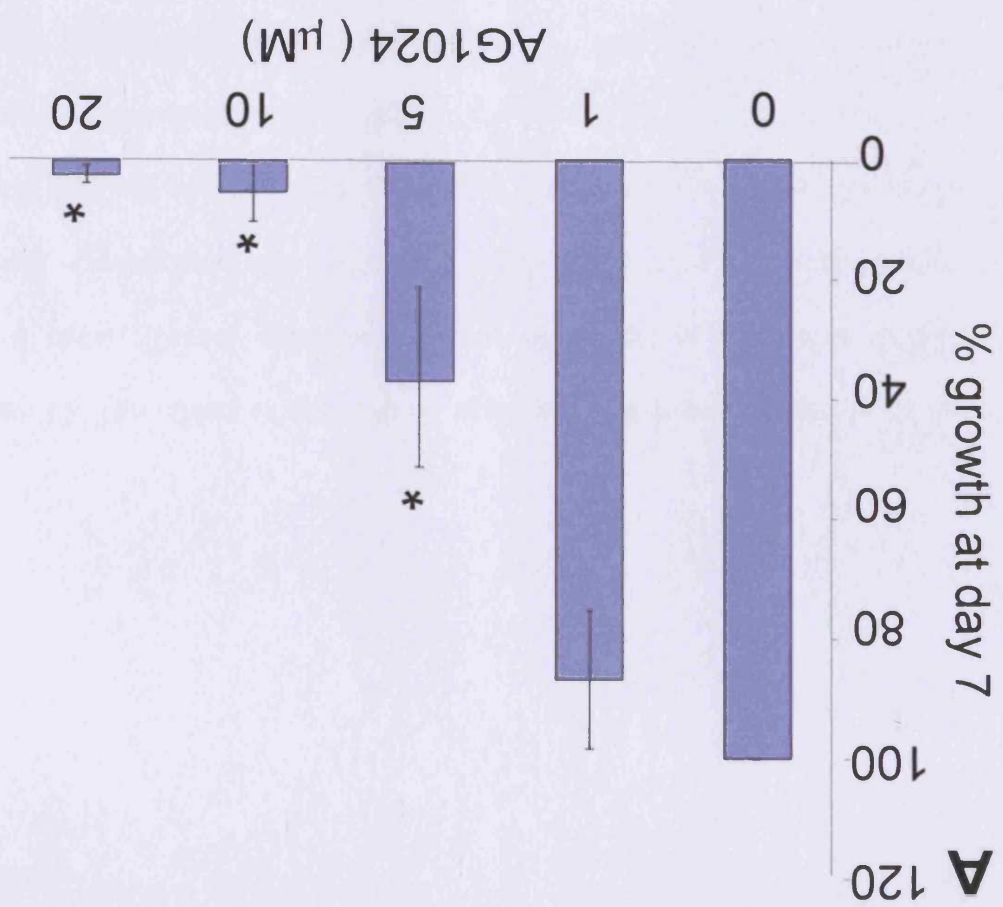
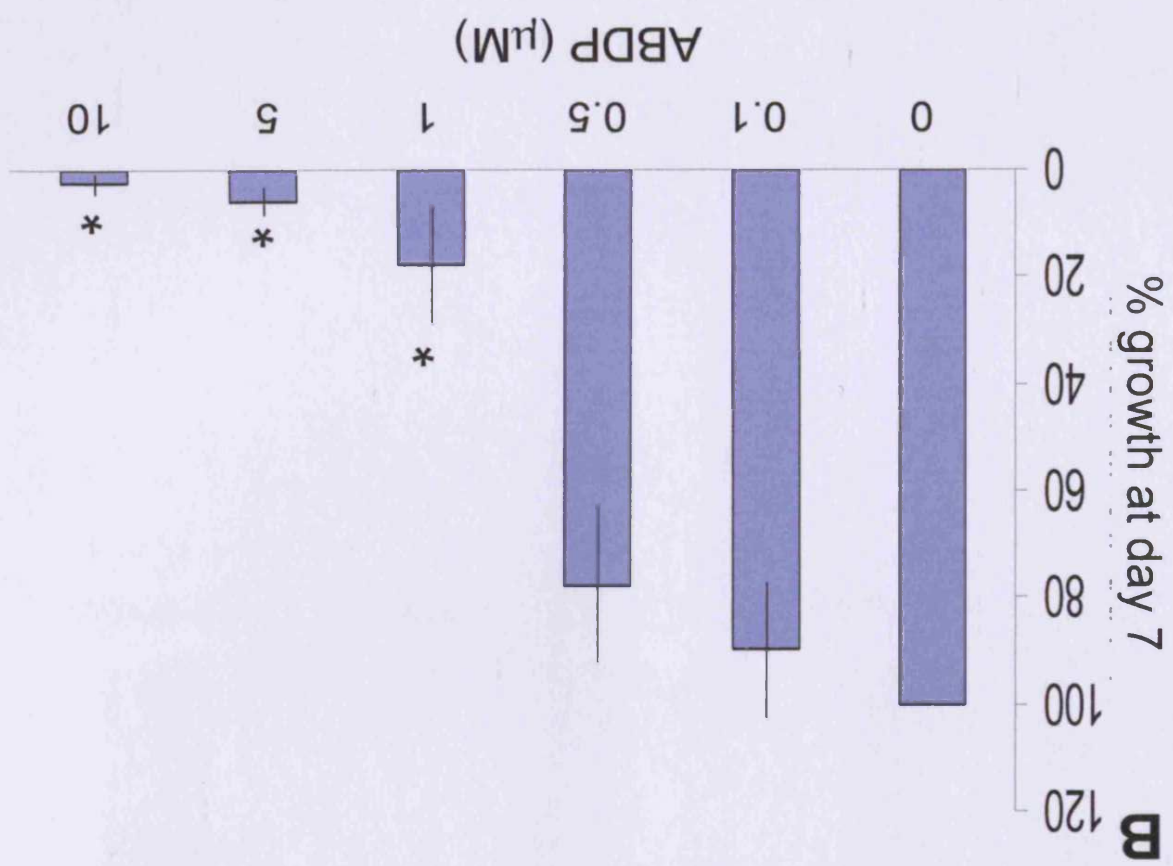
### ***3.1.7 The effects of IGF-IR and EGFR blockade on basal and IGF-II-primed growth of WT-MCF-7 and Tam-R breast cancer cells:***

As IGF-IR inhibition potentially reduced EGFR signalling, which is key to mediating growth in Tam-R cells, the effect of IGF-IR blockade on Tam-R and WT-MCF-7 cell growth was assessed.

Treatment of Tam-R cells with increasing concentrations of AG1024 (1-20 $\mu$ M) or ABDP (0.1-10 $\mu$ M) was shown to significantly reduce basal Tam-R cell growth in a concentration-dependent manner with approximately a 95% and 80% reduction in cell growth observed at 20 $\mu$ M ( $P \leq 0.001$  vs control,  $n=3$ ) and 1 $\mu$ M ( $P \leq 0.001$  vs control,  $n=3$ ) respectively at day 7 (Figure 14A and Figure 14B). Further treatment of Tam-R cells with the selective EGFR inhibitor gefitinib also significantly reduced basal Tam-R cell growth by approximately 80% ( $P \leq 0.001$  vs control,  $n=6$ , Figure 15). In addition, as illustrated in Figure 16A, treatment of Tam-R cells with IGF-II (100ng/ml) alone promoted growth by approximately 15-20% at day 7. However, IGF-II was unable to overcome the growth inhibitory effects of AG1024 (20 $\mu$ M), ABDP (1 $\mu$ M) or gefitinib (1  $\mu$ M) in the Tam-R cells at day 7 ( $P \leq 0.001$  vs IGF-II alone,  $n=4$ ;  $P \leq 0.001$  vs IGF-II alone,  $n=3$  and  $P \leq 0.001$  vs IGF-II alone,  $n=6$ ) respectively (Figure 16A, Figure 16B and Figure 15). Treatment of WT-MCF-7 cells with increasing concentrations of AG1024 (1-10 $\mu$ M) or ABDP (0.1-1 $\mu$ M) for 7 days was also shown to significantly reduce IGF-II-primed cell growth in a concentration-dependent manner as shown in Figure 17A and Figure 17B with approximately a 90% reduction in cell growth observed at 10 $\mu$ M AG1024 and 1 $\mu$ M ABDP ( $P \leq 0.001$  vs IGF-II only,  $n=3$ ) and ( $P \leq 0.001$  vs IGF-II only,  $n=3$ ) respectively. However, despite the finding that treatment of WT-MCF-7 cells with gefitinib

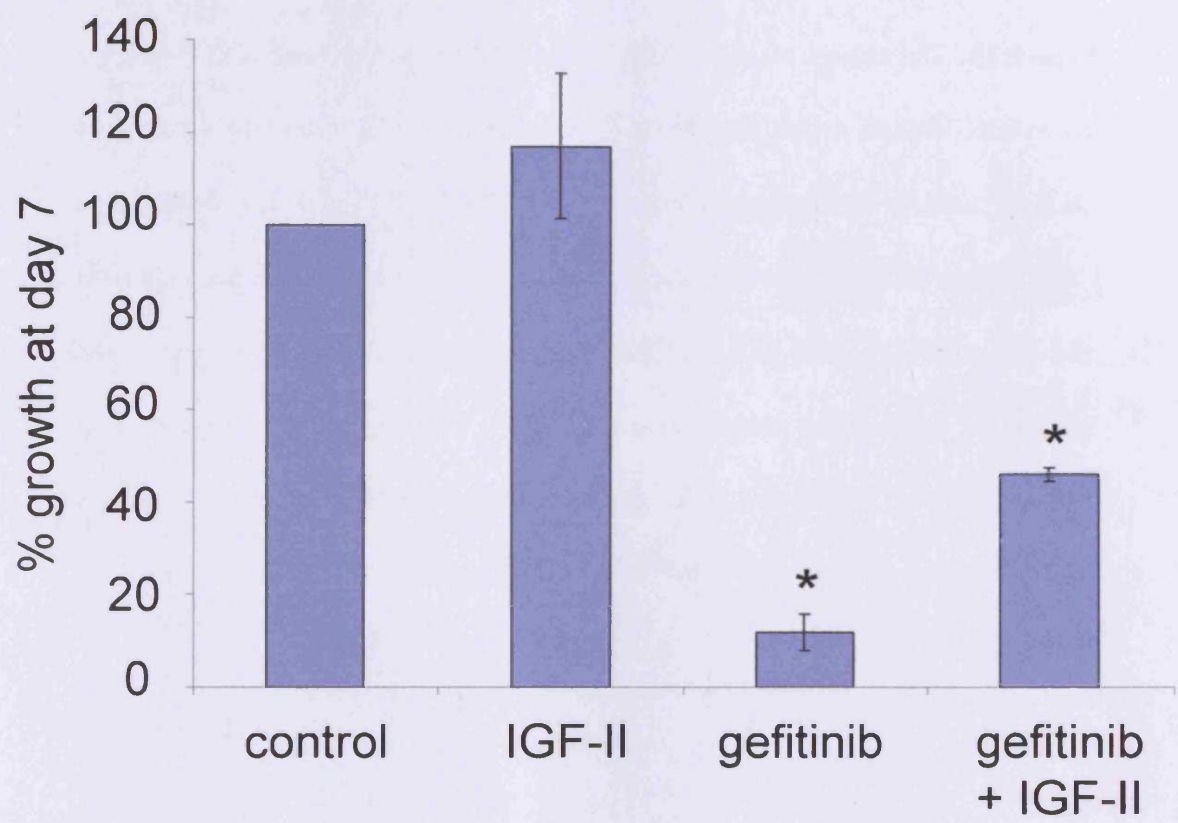
could also significantly reduce basal cell growth by approximately 35% ( $P \leq 0.001$  vs control,  $n=3$ ), unlike Tam-R cells, this moderate inhibition of growth could easily be overcome by the inclusion of IGF-II in these cells (Figure 17C).

**Figure 14.** The effects of either AG1024 or ABDP on the basal growth of Tam-R cells. Tam-R cells were grown for 7 days in phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS medium supplemented increasing concentrations of either (A) AG1024 (1-20 $\mu$ M) or (B) ABDP (0.1-10 $\mu$ M). The results are expressed as mean $\pm$ SEM of triplicate wells and are representative of three separate experiments. A p value of  $\leq 0.05^*$  was considered statistically significant.

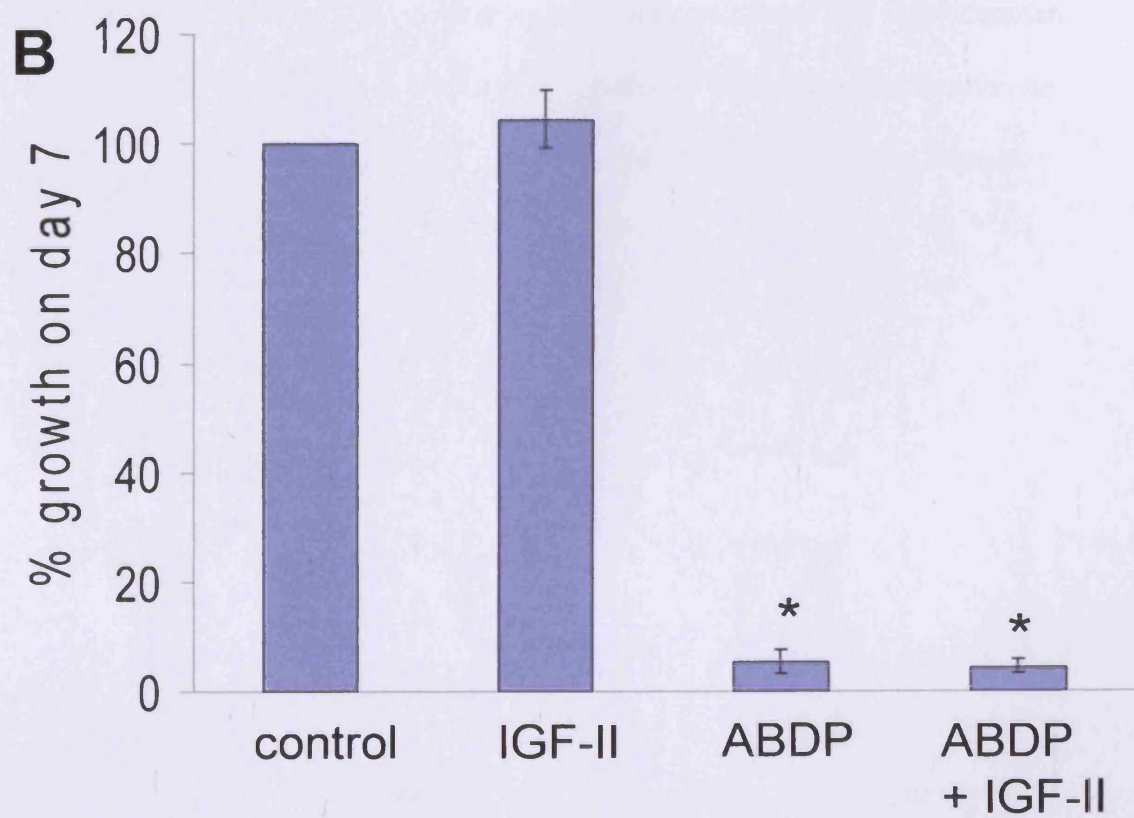
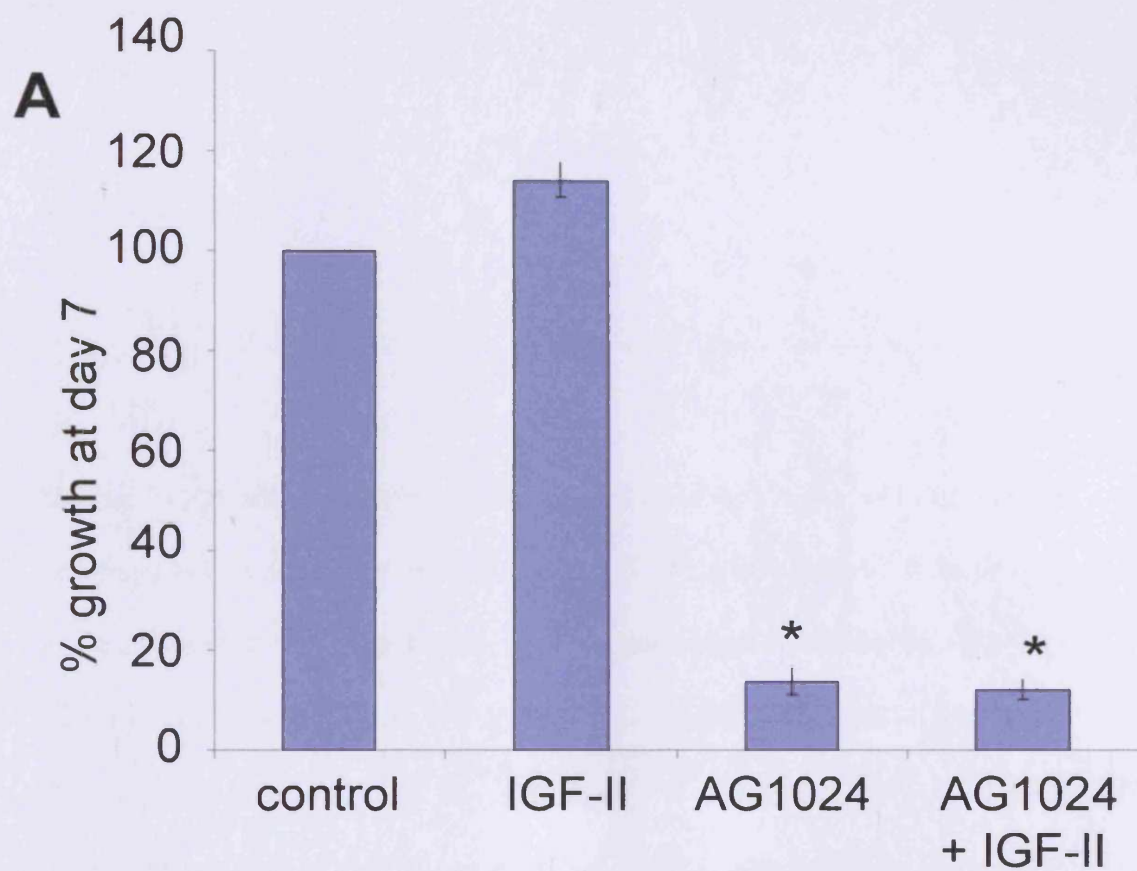


**Figure 15.** The effects of gefitinib on basal and IGF-II-primed growth of the Tam-R cells. Tam-R cells were grown for 7 days in serum-free DCCM medium supplemented with gefitinib (1 $\mu$ M), IGF-II (100ng/ml), a combination of both gefitinib and IGF-II or appropriate vehicle controls only. The results are expressed as mean $\pm$ SEM of triplicate wells and are representative of three separate experiments. A p value of  $\leq 0.05^*$  was considered statistically significant.

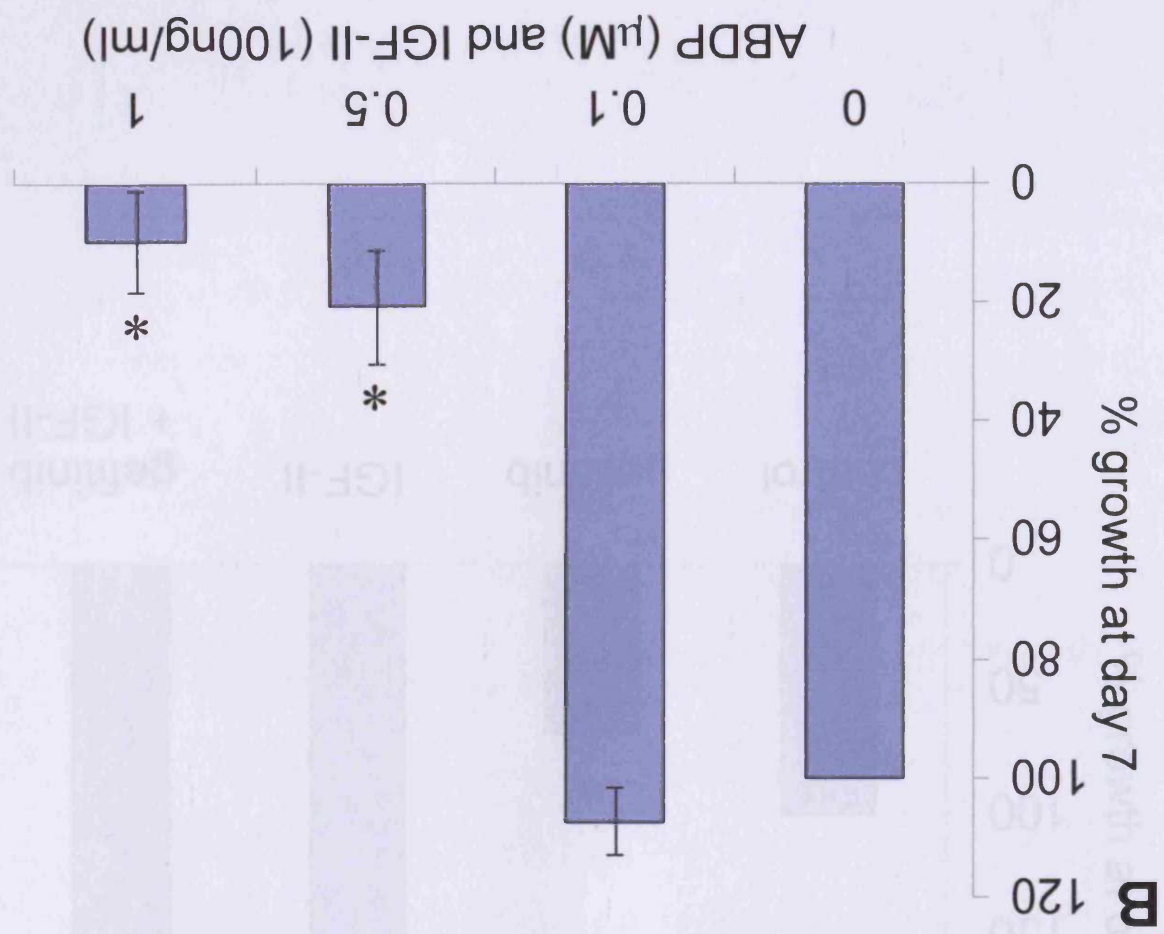
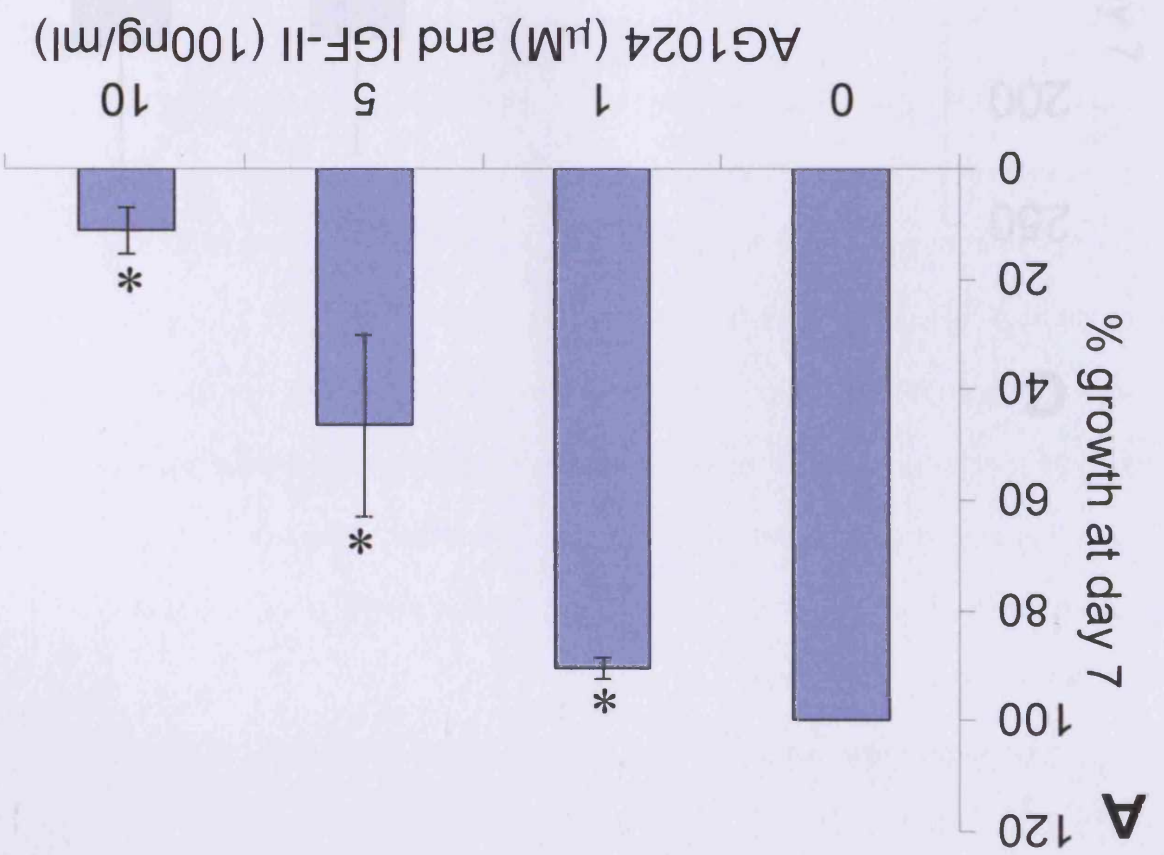




**Figure 16.** The effects of either AG1024 or ABDP on basal and IGF-II-primed growth of Tam-R cells. Tam-R cells were growth for 7 days in serum-free DCCM medium supplemented with (A) AG1024 (20 $\mu$ M), IGF-II (100ng/ml), a combination of both AG1024 and IGF-II or appropriate vehicle controls only and (B) ABDP (1 $\mu$ M), IGF-II (100ng/ml), a combination of both ABDP and IGF-II or appropriate vehicle controls only. The results are expressed as mean $\pm$ SEM of triplicate wells and are representative of three separate experiments. A p value of  $\leq 0.05^*$  was considered statistically significant.

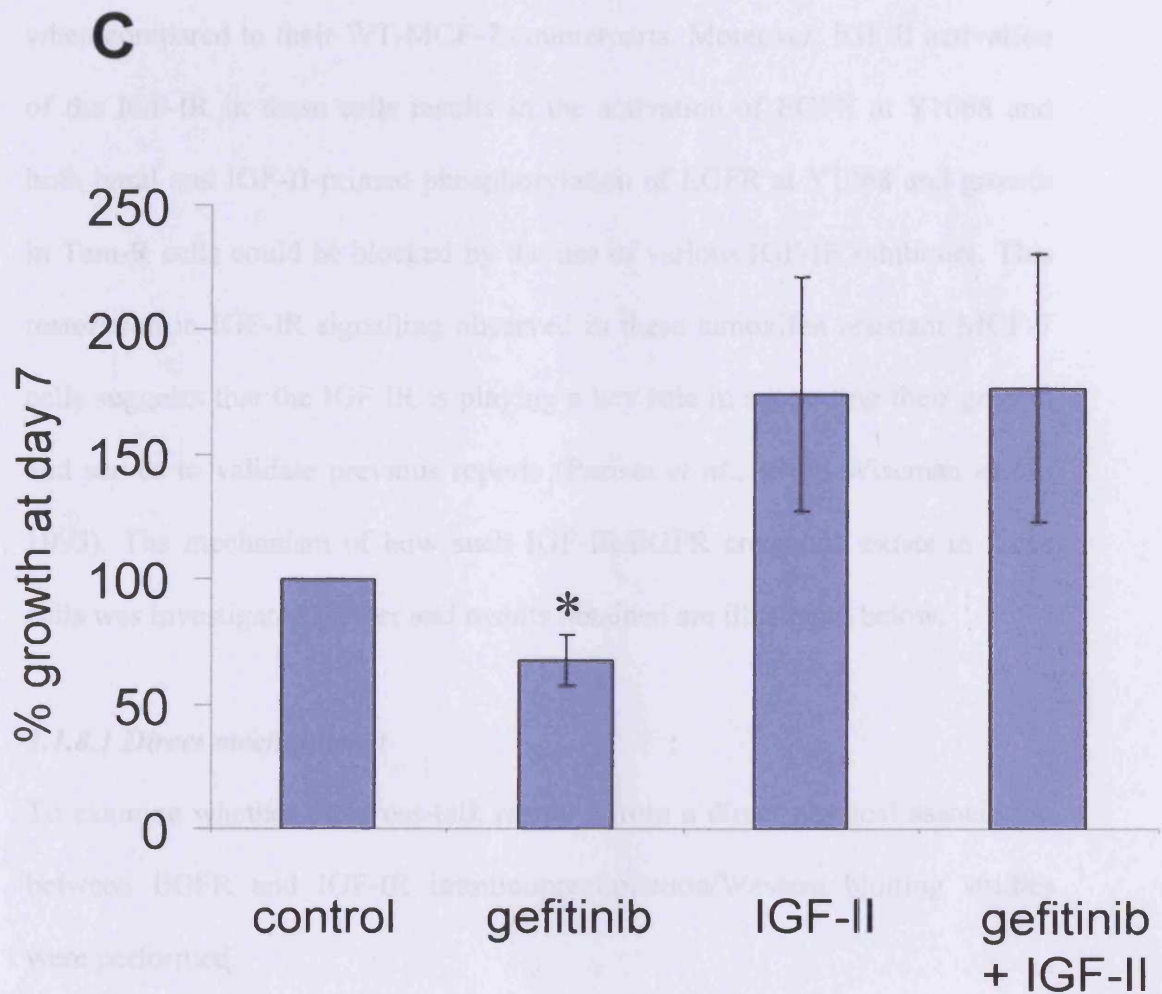


**Figure 17.** The effects of AG1024, ABDP or gefitinib on the IGF-II-primed growth of WT-MCF-7 cells. WT-MCF-7 cells were grown for 7 days in serum-free DCCM medium supplemented with increasing concentrations of (A) AG1024 (1-10 $\mu$ M) and IGF-II (100ng/ml), (B) increasing concentrations of ABDP (0.1-1 $\mu$ M) and IGF-II (100ng/ml) and (C) gefitinib (1 $\mu$ M), IGF-II (100ng/ml), a combination of both gefitinib and IGF-II or appropriate vehicle controls only. The results are expressed as mean  $\pm$  SEM of triplicate wells and are representative of three separate experiments. A p value of  $\leq 0.05^*$  was considered statistically significant.



### 3.1.5 Trans-activation mechanism of IGF-IR-induced activation of EGFR in Tam-R breast cancer cells

Results so far have shown that despite having lower total IGF-IR expression levels, the Tam-R cells nevertheless contain increased amounts of IGF-II mRNA and demonstrate an equivalent level of total phosphorylated EGFR when compared to their WT-MCF-7 counterparts. Moreover, IGF-II activation of the IGF-IR in these cells results in the activation of EGFR at Y1068 and both total and IGF-II-induced phosphorylation of EGFR at Y1068 and growth



### ***3.1.8 Trans-activation mechanisms of IGF-IR-induced activation of EGFR in Tam-R breast cancer cells:***

Results so far have shown that despite having lower total IGF-IR expression levels, the Tam-R cells nevertheless express increased amounts of IGF-II mRNA and demonstrate an equivalent level of basal phosphorylated IGF-IR when compared to their WT-MCF-7 counterparts. Moreover, IGF-II activation of the IGF-IR in these cells results in the activation of EGFR at Y1068 and both basal and IGF-II-primed phosphorylation of EGFR at Y1068 and growth in Tam-R cells could be blocked by the use of various IGF-IR inhibitors. This restoration in IGF-IR signalling observed in these tamoxifen resistant MCF-7 cells suggests that the IGF-IR is playing a key role in supporting their growth and serves to validate previous reports (Parisot *et al.*, 1999; Wiseman *et al.*, 1993). The mechanism of how such IGF-IR/EGFR cross-talk exists in these cells was investigated further and results obtained are illustrated below.

#### ***3.1.8.1 Direct mechanisms:***

To examine whether the cross-talk resulted from a direct physical association between EGFR and IGF-IR immunoprecipitation/Western blotting studies were performed.

Under both basal and IGF-II-primed conditions, no direct physical association between the two receptors could be demonstrated (Figure 18). Total levels of EGFR remained unchanged showing equivalent loading of the gel in the studies.

**Figure 18.** The effects of IGF-II on IGF-IR and EGFR heterodimer formation in Tam-R cells. Tam-R cells were incubated in serum-free DCCM medium either in the presence of IGF-II (100ng/ml) or appropriate vehicle control for 20 minutes. Following cell lysis, protein expression was assessed by Western blot analysis for (total IGF-IR and EGFR following EGFR immunoprecipitation. Data are representative of three separate experiments.



### 3.1.3.1 Indirect mechanism:

#### 3.1.3.2.1 Involvement of MMPs in IGF-II-induced EGFR activation in Tamoxifen breast cancer cells:

As no direct mechanism could be demonstrated, potential indirect mechanisms for IGF-II induced EGFR phosphorylation were next investigated. Previous studies by Rasmussen et al. (2006) have shown that IGF-induced MMP2 activation is the primary mechanism by which the plasma membrane receptor initiates the downstream signaling pathway with such a pathway being blocked by a broad-spectrum MMP inhibitor.

### Immunoprecipitation: EGFR



total IGF-IR



total EGFR

Control

IGF-II

### **3.1.8.2 Indirect mechanisms:**

#### **3.1.8.2.1 Involvement of MMPs in IGF-II-induced EGFR activation in Tam-R breast cancer cells:**

As no direct mechanism could be demonstrated, potential indirect mechanisms for IGF-II induced EGFR phosphorylation were next investigated. Previous studies by Roudabush *et al*, (2000) have shown that IGF-induced MMP activation can lead to the release of HB-EGF from the plasma membrane resulting in the transactivation of the EGFR, with such a pathway being blocked by a broad-spectrum metalloproteinase inhibitor. Pre-incubation of the Tam-R cells for 24 hours with increasing concentrations of a pan MMP-II inhibitor MMP-II (0.1-10 $\mu$ M) failed to block either basal or IGF-II-primed EGFR Y1068 and ERK1/2 phosphorylation (Figure 19). If anything, this inhibitor was shown to enhance EGFR and ERK1/2 phosphorylation in this cell line. Total levels of both EGFR and ERK1/2 proteins remained unchanged in the presence of this inhibitor.

**Figure 19.** The effects of a pan MMP-inhibitor on basal and IGF-II-primed phosphorylated and total EGFR and ERK1/2 in Tam-R cells. Tam-R cells were incubated in serum-free DCCM medium supplemented with increasing concentrations of a pan MMP-inhibitor (MMP-II; 0.01-1 $\mu$ M) or vehicle control only for 24 hours, followed by stimulation with IGF-II (100ng/ml) or appropriate vehicle control for 20 minutes. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of three separate experiments.

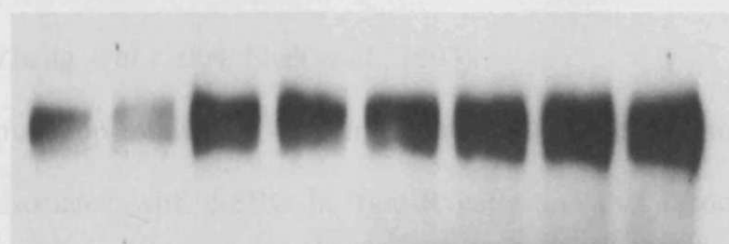
### 3.4.3.2 IGF-II promotes direct association between EGFR and $\alpha$ 5 $\beta$ 1

EGFR and  $\alpha$ 5 $\beta$ 1 in T98J brain cancer cells

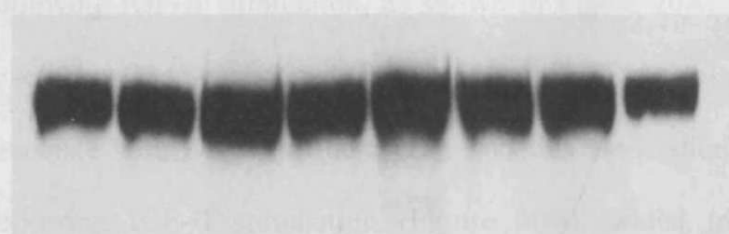
An alternative mechanism to mediate IGF-II-induced EGFR activation in T98J

B cells involves the recruitment of tyrosine kinase  $\alpha$ 5 $\beta$ 1, which has previously

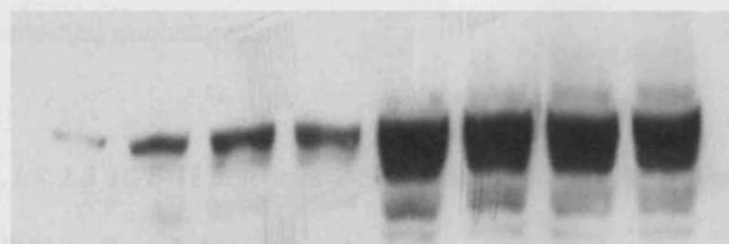
been implicated in mediating EGFR transactivation in a variety of cell lines.



phospho EGFR  
Y1068



total EGFR



phospho ERK1/2



total ERK1/2

C 0.1 1 10 C 0.1 1 10 MMP-II ( $\mu$ M)

- IGF-II

+ IGF-II

**3.1.8.2.2 IGF-II promotes direct association between IGF-IR and c-SRC and EGFR and c-SRC in Tam-R breast cancer cells:**

An alternative mechanism to mediate IGF-II-induced EGFR activation in Tam-R cells involves the non-receptor tyrosine kinase c-SRC which has previously been implicated in mediating EGFR transactivation in a variety of cell lines (Zhang *et al.*, 2004; Shah *et al.*, 2003).

Immunoprecipitation/Western blot studies revealed that total IGF-IR physically associated with c-SRC in Tam-R cells and this association was increased following IGF-II stimulation, as shown in Figure 20A, whereas total IGF-IR levels remained unchanged. Furthermore, c-SRC was also found to physically associate with EGFR in these cells and this association was again increased following IGF-II stimulation (Figure 20B), whilst total levels of EGFR remained unchanged.

**3.1.8.2.3 IGF-II activates c-SRC and promotes phosphorylation of EGFR at Y845 in Tam-R breast cancer cells:**

The ability of IGF-II to promote association of c-SRC with both EGFR and IGF-IR suggests a potential role for c-SRC in the cross-talk between the two receptors in Tam-R cells. This possible transactivation mechanism was therefore investigated further.

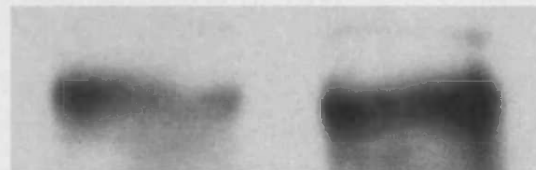
IGF-II stimulation increased phosphorylation of c-SRC at Y418 in a time-dependent manner (Figure 21A). This effect of IGF-II was, however, not demonstrated in WT-MCF-7 cells, despite these cells having similar levels of total c-SRC protein compared to Tam-R cells (Figure 21A). Such activation of c-SRC promoted phosphorylation of EGFR at Y845, a known c-SRC-

dependent phosphorylation site, in Tam-R cells also in a time-dependent and parallel manner (Figure 21B). Additionally, pre-incubation of Tam-R cells with increasing concentrations of either IGF-II neutralising antibody (1-10 $\mu$ g/ml, for 1 hour), AG1024 (5-20 $\mu$ M, for 24 hours) or ABDP (0.01-1 $\mu$ M, for 7 days) reduced basal phosphorylation levels of c-SRC at Y418 and EGFR at Y845 in these cells (Figure 22 A-C respectively). The effect of AG1024 (20 $\mu$ M) and ABDP (1 $\mu$ M) could not be overcome by IGF-II stimulation (Figure 23A and Figure 23B). Again total levels of these proteins remained unchanged for all treatment groups. Similar findings to those for AG1024, ABDP and neutralising anti- IGF-II antibodies were also observed following the silencing of the IGF-IR gene in Tam-R cells using siRNA technology. In addition to the previously described reduction in IGF-IR, EGFR and ERK1/2 phosphorylation levels (Figure 13C), siRNA also resulted in a reduction in phosphorylated c-SRC at Y418 at 4 days post transfection compared to experimental control levels (Figure 24).

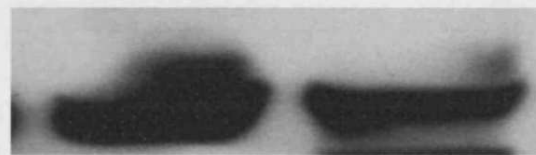
**Figure 20.** Effects of IGF-II on IGF-IR/ c-SRC and EGFR/ c-SRC heterodimer formation in Tam-R cells. Tam-R cells were incubated in serum-free DCCM medium either in presence of IGF-II (100ng/ml) or appropriate vehicle control for 20 minutes. Following cell lysis, protein expression was assessed by Western blot analysis for (A) total c-SRC and IGF-IR following IGF-IR immunoprecipitation and (B) total c-SRC and EGFR following EGFR immunoprecipitation. Data are representative of three separate experiments.

Immunoprecipitation: IGF-IR

**A**



total c-SRC



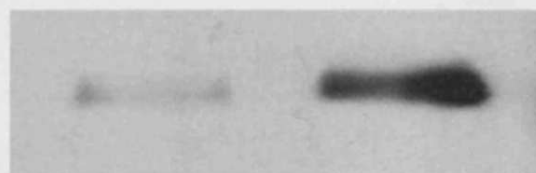
total IGF-IR

Control

IGF-II

Immunoprecipitation: EGFR

**B**



total c-SRC



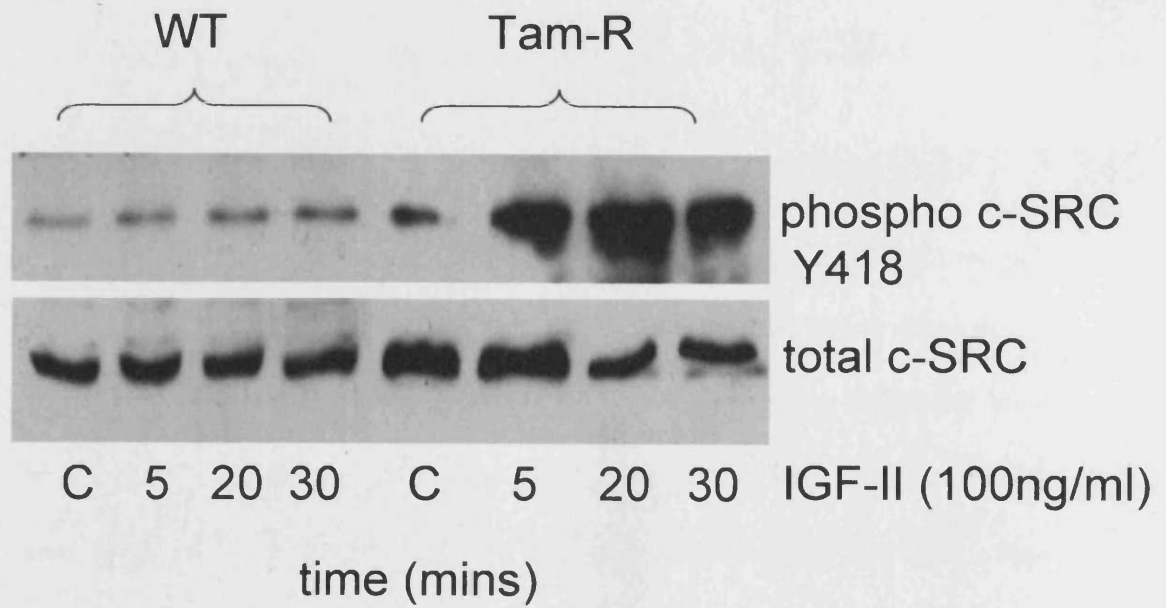
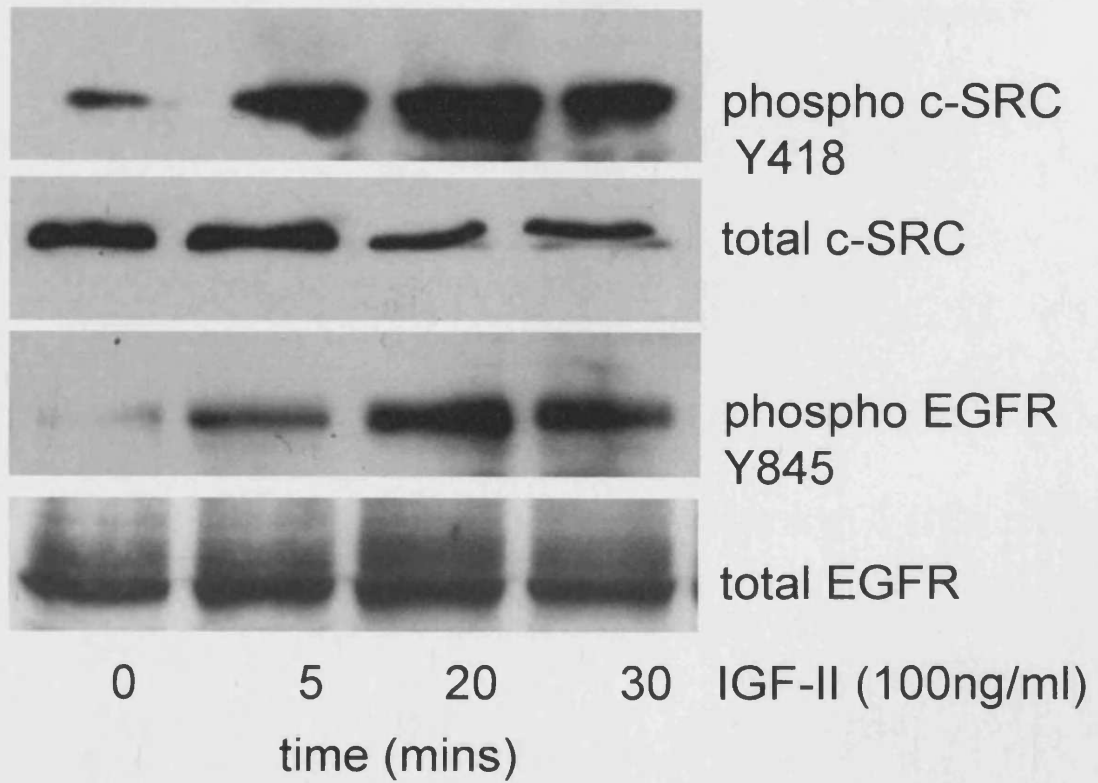
total EGFR

Control

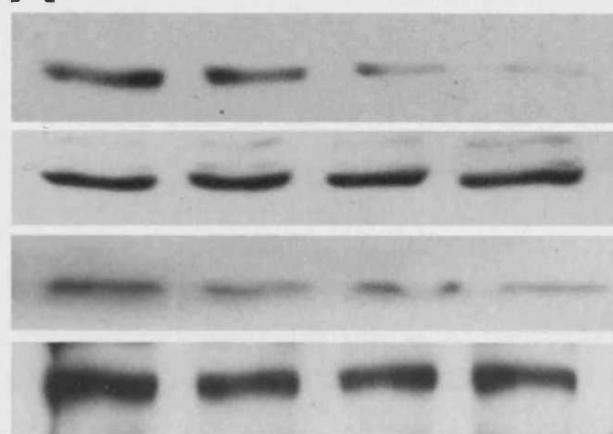
IGF-II



**Figure 21.** The effects of IGF-II on phosphorylated and total c-SRC in Tam-R and WT-MCF-7 cells and EGFR in Tam-R cells. Tam-R and WT-MCF-7 cells were incubated in serum-free DCCM medium, supplemented with IGF-II (100ng/ml) or appropriate vehicle control over 30 minutes. Following cell lysis, protein expression was assessed in (A) WT-MCF-7 and Tam-R cells and (B) Tam-R cells only by Western blot analysis. Data are representative of three separate experiments.

**A****B**

**Figure 22.** The effects of an IGF-II neutralising antibody, AG1024 and ABDP on phosphorylated and total c-SRC and EGFR in Tam-R cells. Tam-R cells were incubated in serum-free DCCM medium, supplemented with increasing concentrations of either (A) an IGF-II neutralising antibody (1-10 $\mu$ g/ml) or appropriate vehicle control for 1 hour, (B) AG1024 (5-20 $\mu$ M) or appropriate vehicle control for 24 hours or (C) ABDP (0.1-1 $\mu$ M) or appropriate vehicle control for 7 days. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of three separate experiments.

**A**

phospho c-SRC Y418

total c-SRC

phospho EGFR Y845

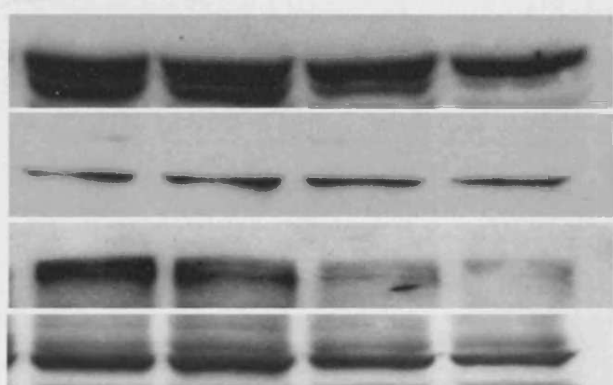
total EGFR

C

1

5

10

anti IGF-II Ab ( $\mu\text{g/ml}$ )**B**

phospho c-SRC Y418

total c-SRC

phospho EGFR Y845

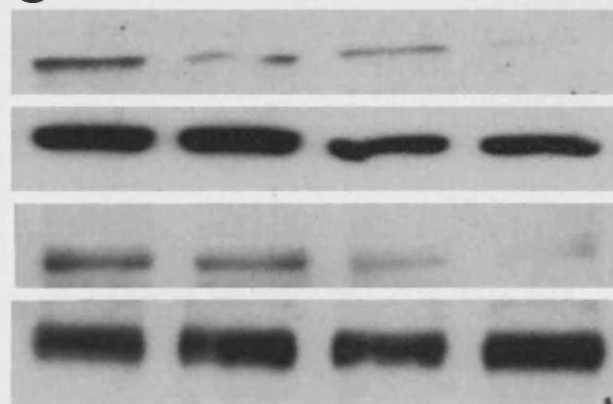
total EGFR

C

5

10

20

AG1024 ( $\mu\text{M}$ )**C**

phospho c-SRC Y418

total c-SRC

phospho EGFR Y845

total EGFR

C

0.01

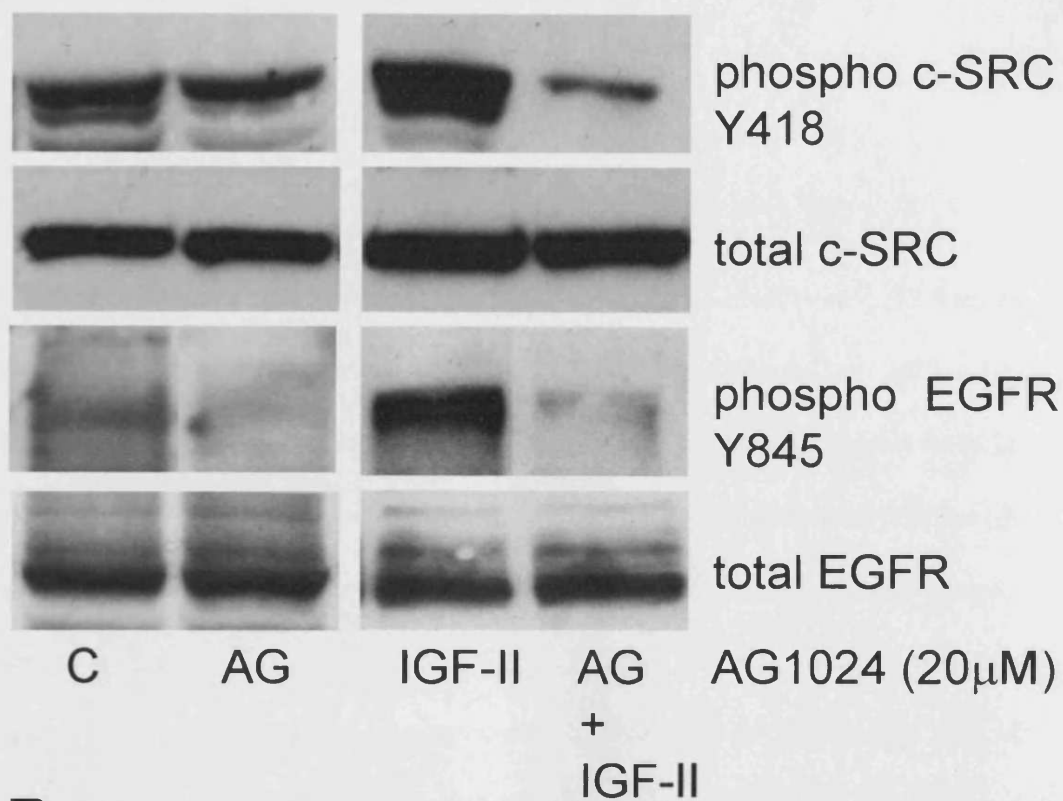
0.1

1.0

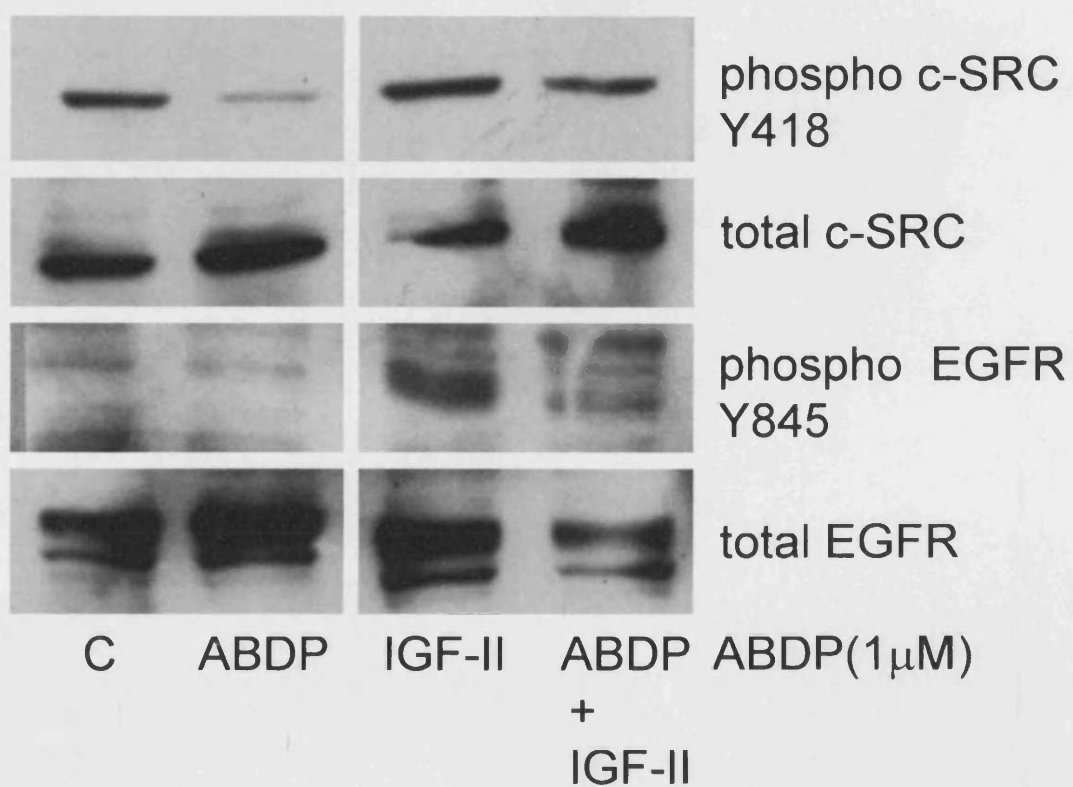
ABDP ( $\mu\text{M}$ )

**Figure 23.** The effects of AG1024 and ABDP on basal and IGF-II primed phosphorylated and total c-SRC and EGFR in Tam-R cells. Tam-R cells were incubated in serum-free DCCM medium, supplemented with (A) AG1024 (20 $\mu$ M) for 24 hours, IGF-II (100ng/ml) for 20 minutes or a combination of the two agents or appropriate vehicle controls and (B) ABDP (1 $\mu$ M) for 7 days, IGF-II (100ng/ml) for 20 minutes or a combination of the two agents or appropriate vehicle controls. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of three separate experiments.

**A**



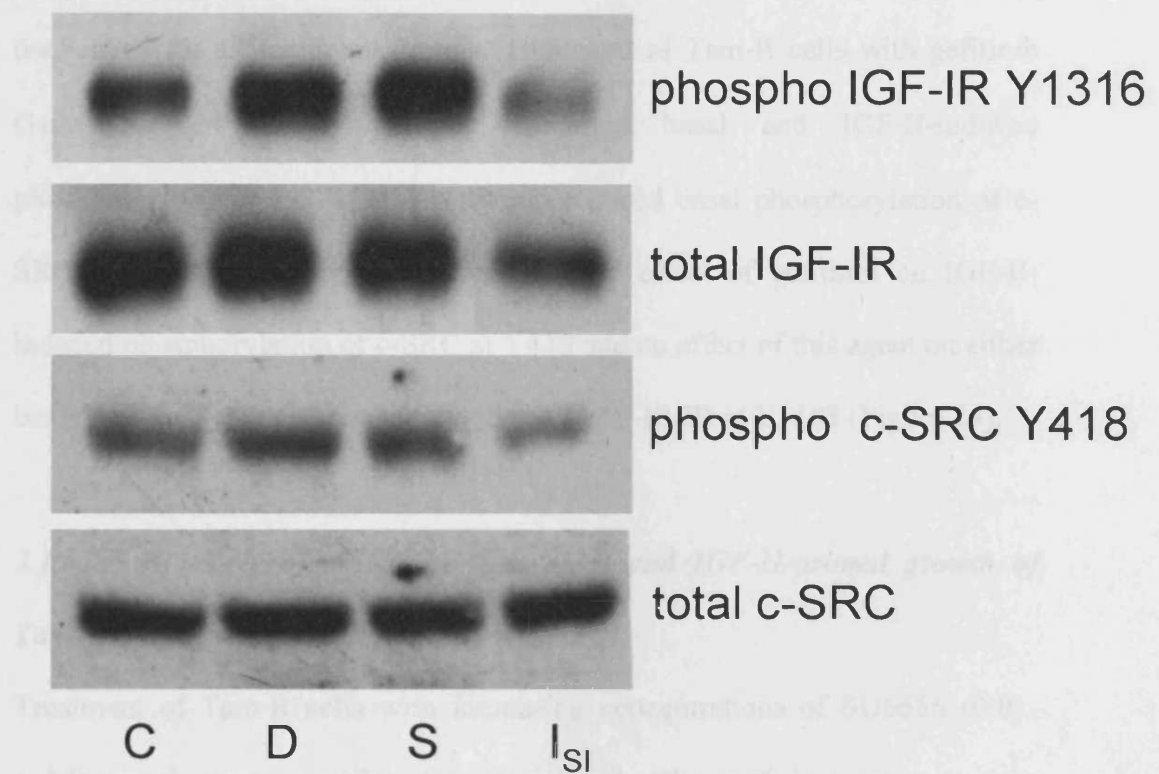
**B**



**Figure 24.** The effects of IGF-IR siRNA on phosphorylated and total IGF-IR and c-SRC in Tam-R cells. Tam-R cells were incubated in phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS for 4 days supplemented in either culture media alone (C), Dharmafect 1 transfection reagent alone (D), Dharmafect 1 transfection reagent and non-targeting scrambled siRNA mix (S; 100nM), or Dharmafect 1 transfection reagent and IGF-IR (I<sub>SI</sub>) siRNA mix (100nM). Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of two separate experiments.

### 3.3.4.4. Inhibition of c-SRC Phosphorylation and IGF-II-induced GPCR activity in T47D breast cancer cells:

Pre-treatment of T47D cells with increasing concentrations of a c-SRC specific inhibitor SU6656 (0.01-100  $\mu$ M for 24 hours) reduced basal phosphorylation of c-SRC at Y418, E477, and Y441 and IGF-II-induced phosphorylation of c-SRC at Y418 (Figure 3.3.4.4). Total protein levels were determined



Treatment of T47D cells with increasing concentrations of SU6656 (0.01-100  $\mu$ M) for 24 hours significantly reduced IGF-II-induced growth in a concentration-dependent manner with approximately a 50% reduction in cell growth observed at 10  $\mu$ M of the inhibitor (Figure 3.3.4.4). Similar to the results obtained with the c-SRC inhibitor, treatment of T47D cells with increasing concentrations of IGF-II (0.01-100 ng/ml) for 24 hours resulted in a dose-dependent increase in cell growth. However, the combination of IGF-II (100 ng/ml) and SU6656 (10  $\mu$ M) again significantly reduced cell growth by approximately 50% (Figure 3.3.4.4). These results suggest that the IGF-II-induced growth of T47D cells is dependent on c-SRC activity. To further confirm this, the effect of SU6656 on IGF-II-induced phosphorylation of IGF-IR was determined. As shown in Figure 3.3.4.4, treatment of T47D cells with increasing concentrations of SU6656 (0.01-100  $\mu$ M) for 24 hours significantly reduced IGF-II-induced phosphorylation of IGF-IR at Y1316. Total IGF-IR levels were determined by Western blot analysis and are shown in Figure 3.3.4.4. These results suggest that the IGF-II-induced phosphorylation of IGF-IR is dependent on c-SRC activity.



**3.1.8.2.4 Blockade of c-SRC inhibits basal and IGF-II -induced EGFR activity in Tam-R breast cancer cells:**

Pre-treatment of Tam-R cells with increasing concentrations of a c-SRC specific inhibitor SU6656 (0.01-1 $\mu$ M, for 24 hours) reduced basal phosphorylation of c-SRC at Y418, EGFR at Y845 and EGFR at Y1068 as shown in Figure 25A and was able to abolish the capacity of IGF-II to promote c-SRC phosphorylation (Figure 25B). Total protein levels, again, remained unchanged for all treatment groups. Treatment of Tam-R cells with gefitinib (1 $\mu$ M for 24 hours) potently inhibited basal and IGF-II-induced phosphorylation of EGFR at Y1068 and reduced basal phosphorylation of c-SRC at Y418. However, there was minimal effect of gefitinib on IGF-II-induced phosphorylation of c-SRC at Y418 and no effect of this agent on either basal or IGF-II-induced phosphorylation of IGF-IR/IR at Y1158 (Figure 26).

**3.1.8.2.5 Blockade of c-SRC inhibits basal and IGF-II-primed growth of Tam-R breast cancer cells:**

Treatment of Tam-R cells with increasing concentrations of SU6656 (0.01-1 $\mu$ M) was shown to significantly reduce basal cell growth in a concentration-dependent manner with approximately a 50% reduction in cell growth observed at 1 $\mu$ M at day 7 ( $p \leq 0.034$  vs control,  $n=3$ ) as shown in Figure 27A. Similarly, when this experiment was repeated, treatment of Tam-R cells with SU6656 (1 $\mu$ M) again significantly reduced basal cell growth by approximately 70% at day 7 ( $P \leq 0.01$  vs control,  $n=4$ ) and this inhibition could not be overcome by the inclusion of IGF-II ( $P \leq 0.05$  vs IGF-II alone,  $n=4$ , Figure 27B).

**3.1.8.2.6 Blockade of c-SRC inhibits basal and TGF $\alpha$ -induced EGFR activity and growth in Tam-R breast cancer cells:**

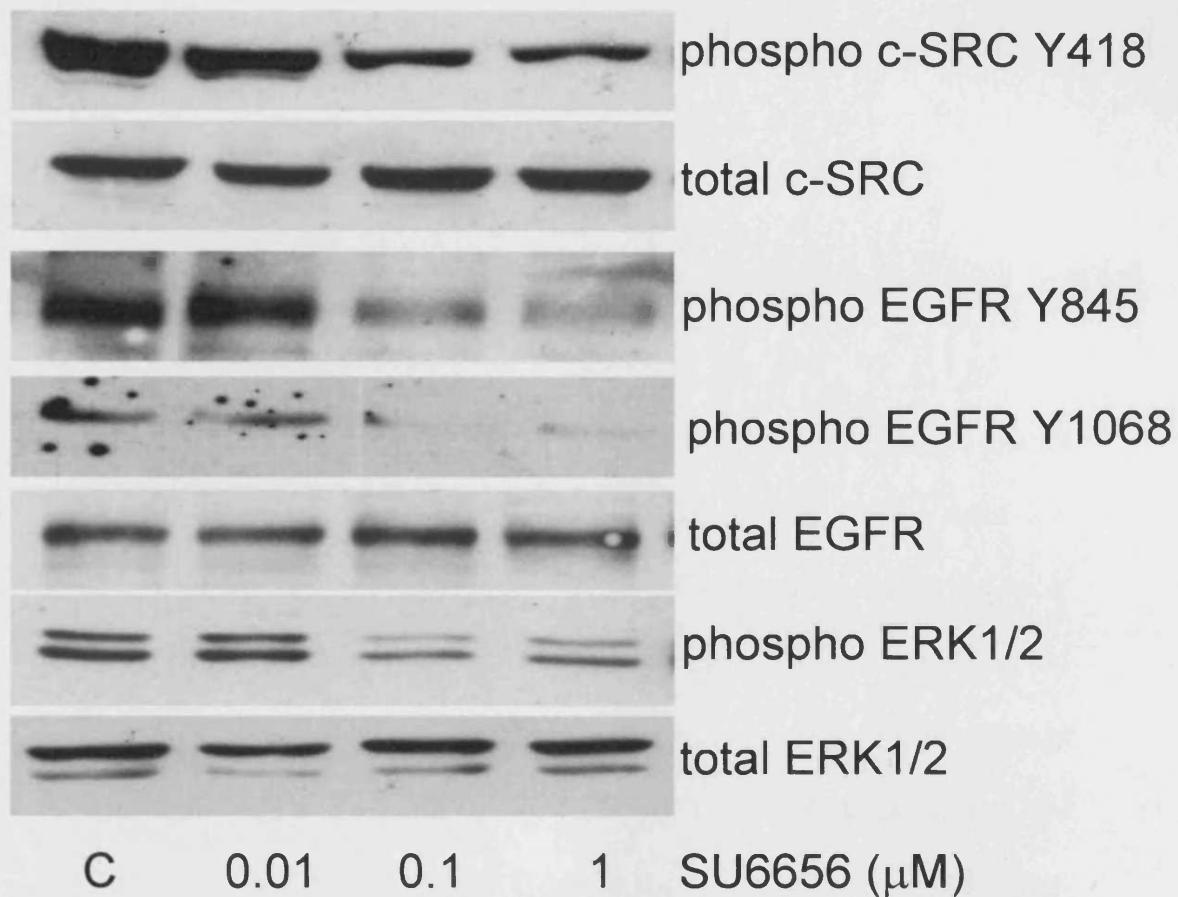
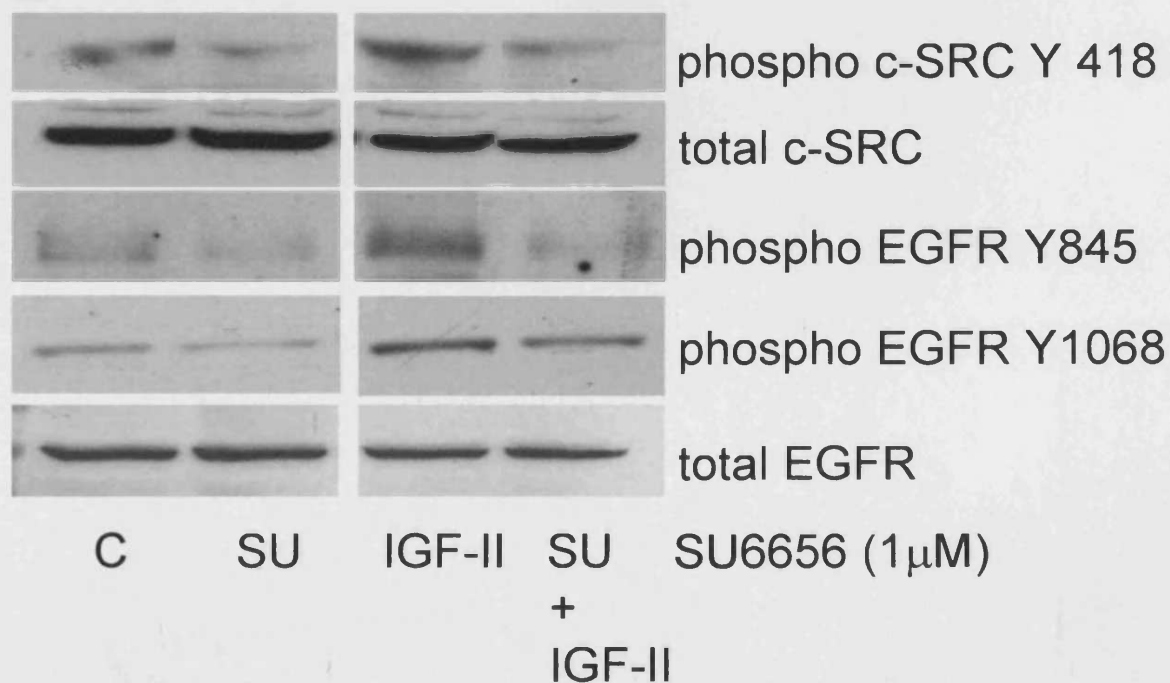
As inhibition of IGF-IR signalling via c-SRC can influence Y845 phosphorylation on EGFR and as this phosphorylation site has been postulated as playing a key role in determining ligand-dependent activation of EGFR (Biscardi *et al.*, 1999), the effects of c-SRC blockade on ligand-induced EGFR activity was assessed in Tam-R cells.

In addition to using SU6656, we also examined the inhibitory effects of another specific c-SRC inhibitor, AZM555. Inhibition of phosphorylation of c-SRC at Y418 and reduction of phosphorylation of EGFR at Y845 was again, observed, following the pre-treatment of Tam-R cells with AZM555 (1 $\mu$ M, for 24 hours) as shown in Figure 28. Furthermore, and interestingly, such inhibition of c-SRC was shown to be associated with the failure of TGF $\alpha$  (a potent activator of EGFR) to phosphorylate EGFR at Y1068 and to activate the downstream ERK1/2 MAPK pathway (over a range of concentrations 0.1-10ng/ml, Figure 28). Total levels of these proteins again remained unchanged in the presence of the inhibitor. Similar findings were observed when this experiment was repeated using TGF $\alpha$  at a single concentration of 1ng/ml for both AZM555 and SU6656 as shown in Figure 29A and Figure 29B respectively. Again total protein levels remained unchanged in these experiments.

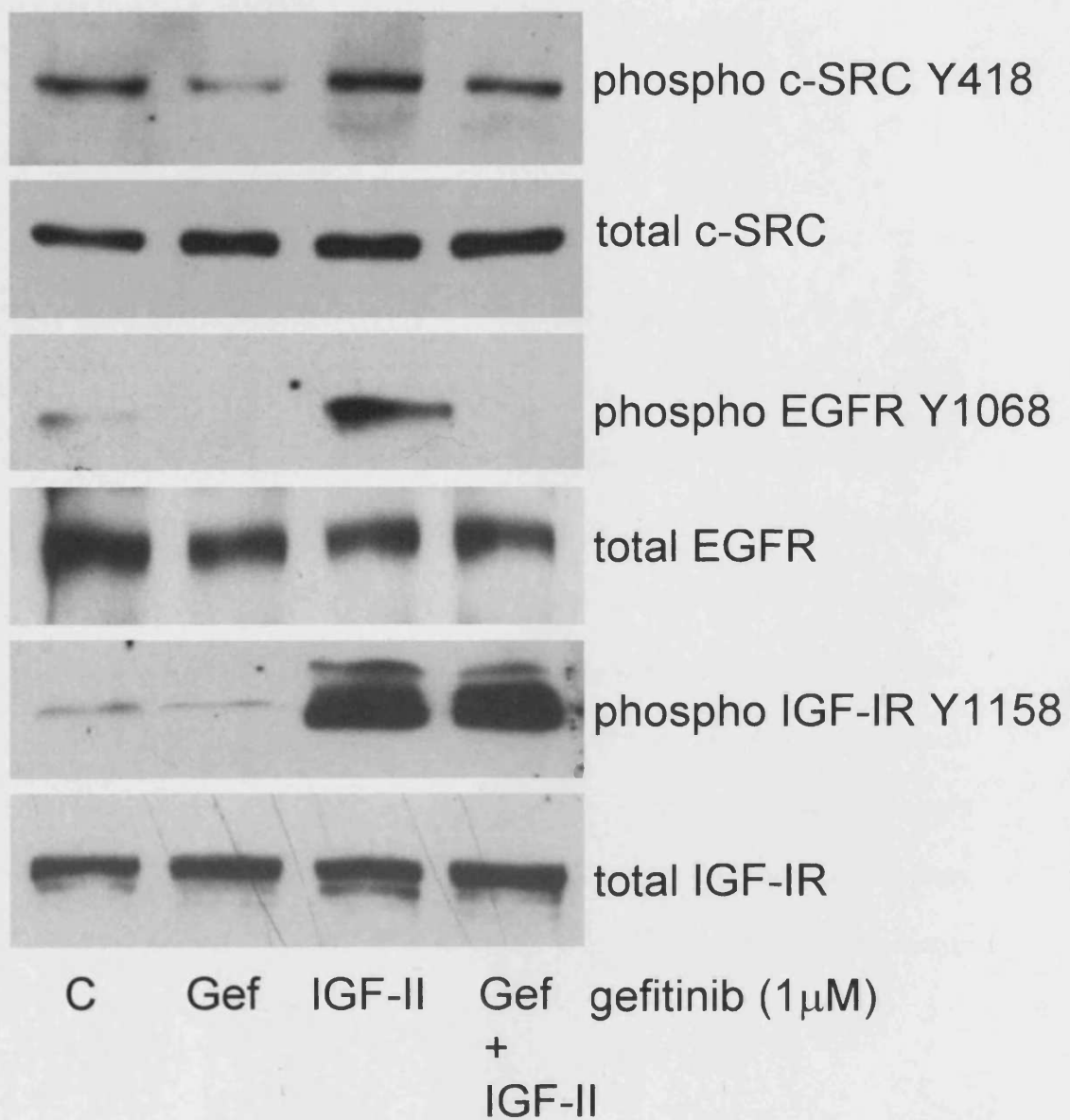
Treatment of Tam-R cells with either SU6656 (1 $\mu$ M) or AG1024 (5 $\mu$ M) was shown to significantly reduce basal cell growth by approximately 70% and 30% on day 7 ( $p \leq 0.001$  vs control,  $n=4$ ) and ( $p \leq 0.018$  vs control,  $n=3$ ) as shown in Figure 30A and Figure 30B respectively. Furthermore, this inhibition

could not be overcome by the inclusion of TGF $\alpha$  ( $P \leq 0.001$  vs TGF $\alpha$  alone,  $n=4$ ) and ( $P \leq 0.004$  vs TGF $\alpha$  alone,  $n=3$ ) as also shown in Figure 30A and Figure 30B respectively.

**Figure 25.** The effects of SU6656 on phosphorylated and total c-SRC, EGFR and ERK1/2 and on basal and IGF-II-primed total and phosphorylated c-SRC and EGFR in Tam-R cells. Tam-R cells were incubated in serum-free DCCM medium, supplemented with (A) increasing concentrations of SU6656 (0.01-1 $\mu$ M) or appropriate vehicle control for 24 hours and (B) SU6656 (1 $\mu$ M) for 24 hours, IGF-II (100ng/ml) for 20 minutes, a combination of the two agents or appropriate vehicle controls. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of three separate experiments.

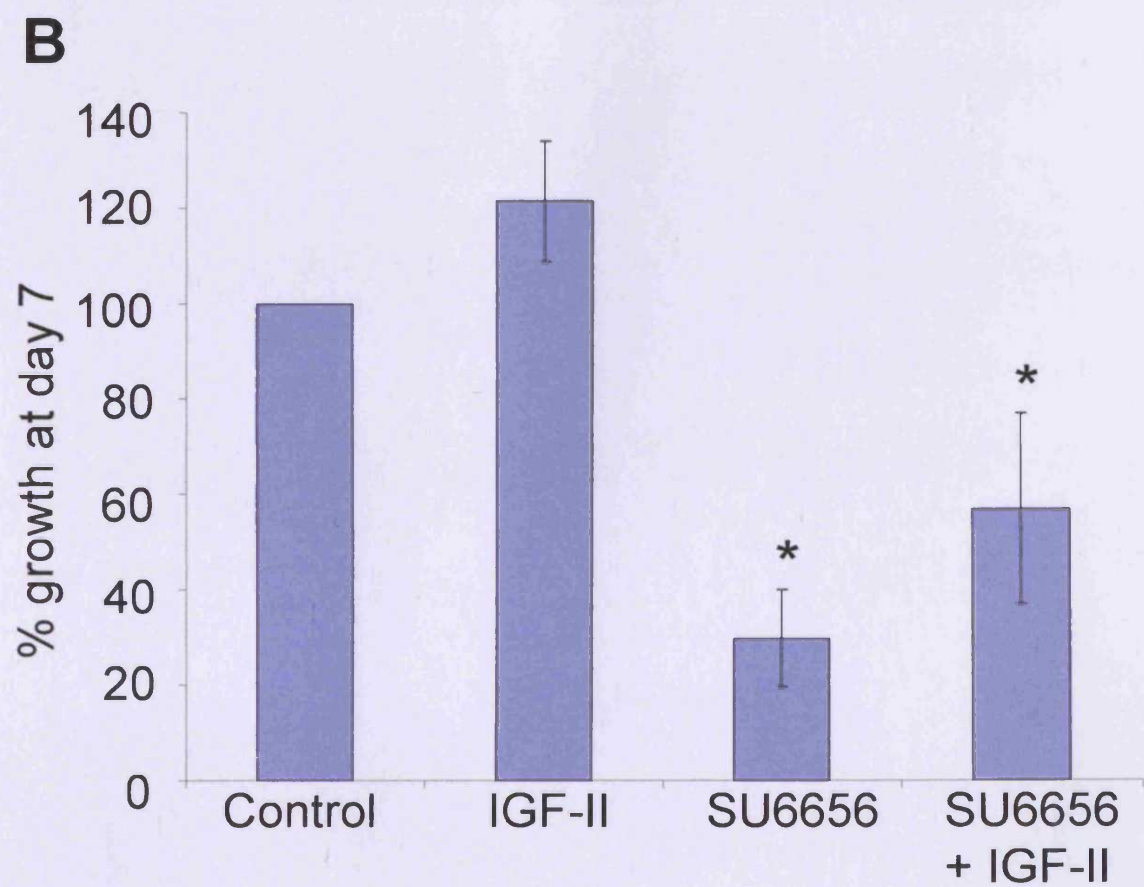
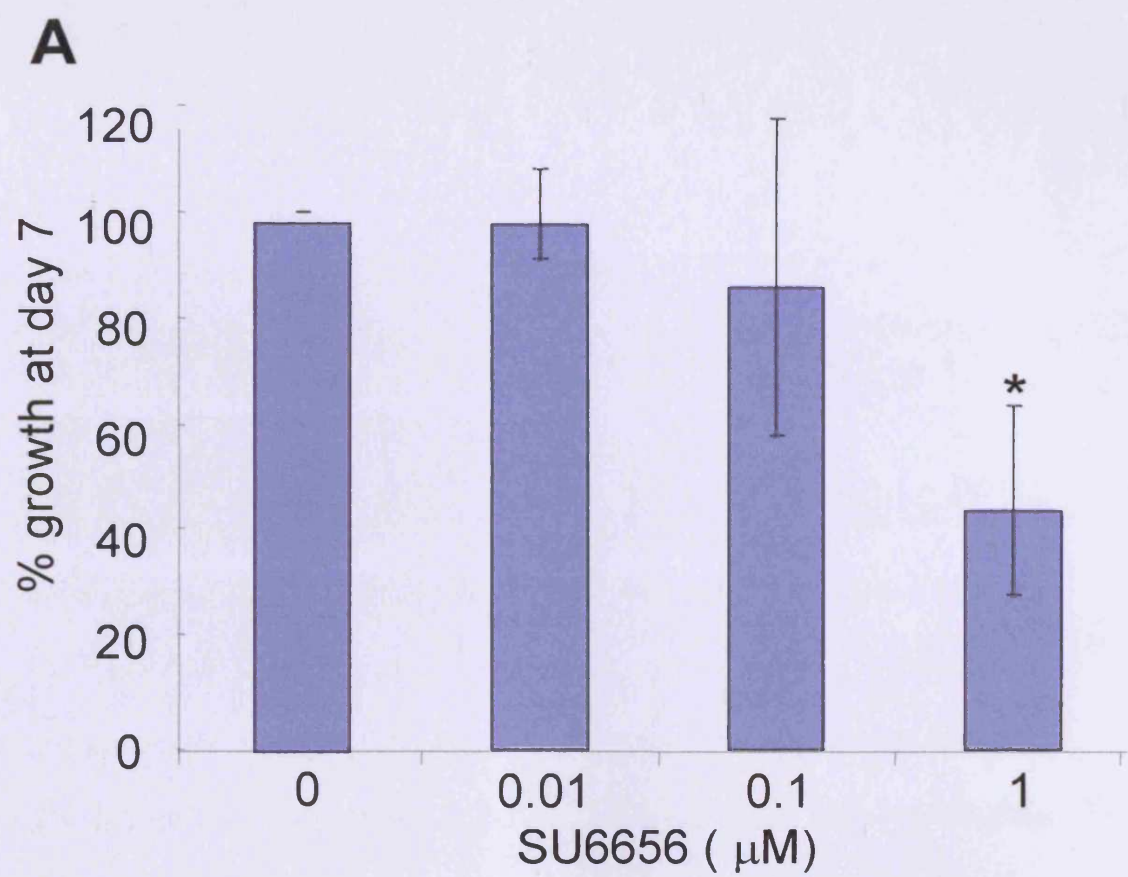
**A****B**

**Figure 26.** The effects of gefitinib on basal and IGF-II primed phosphorylated and total c-SRC, EGFR and IGF-IR in Tam-R cells. Tam-R cells were incubated in serum-free DCCM medium, supplemented with gefitinib (1 $\mu$ M) for 24 hours, IGF-II (100ng/ml) for 20 minutes, a combination of the two agents or appropriate vehicle controls. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of three separate experiments.

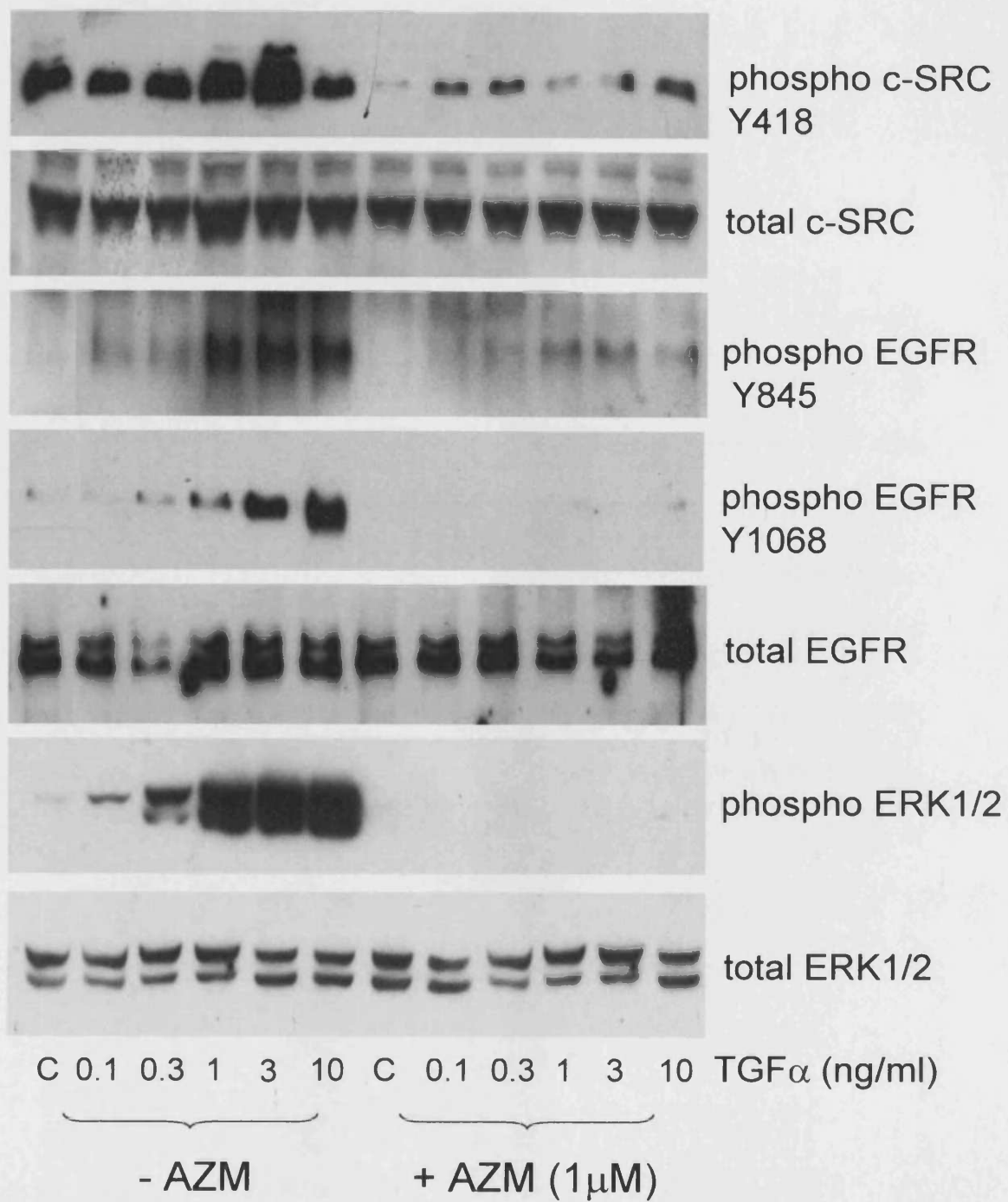


**Figure 27.** The effects of SU6656 on basal and IGF-II primed growth of Tam-R cells. Tam-R cells were grown for (A) 7 days in phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS medium supplemented with increasing concentrations of SU6656 (0.01-1  $\mu$ M) or vehicle control only and (B) Tam-R cells were grown in serum-free DCCM medium for 7 days supplemented with SU6656 (1  $\mu$ M), IGF-II (100ng/ml), a combination of the two agents or appropriate vehicle controls. The results are expressed as mean $\pm$ SEM of triplicate wells and are representative of three separate experiments. A p value of  $\leq 0.05^*$  was considered statistically significant.

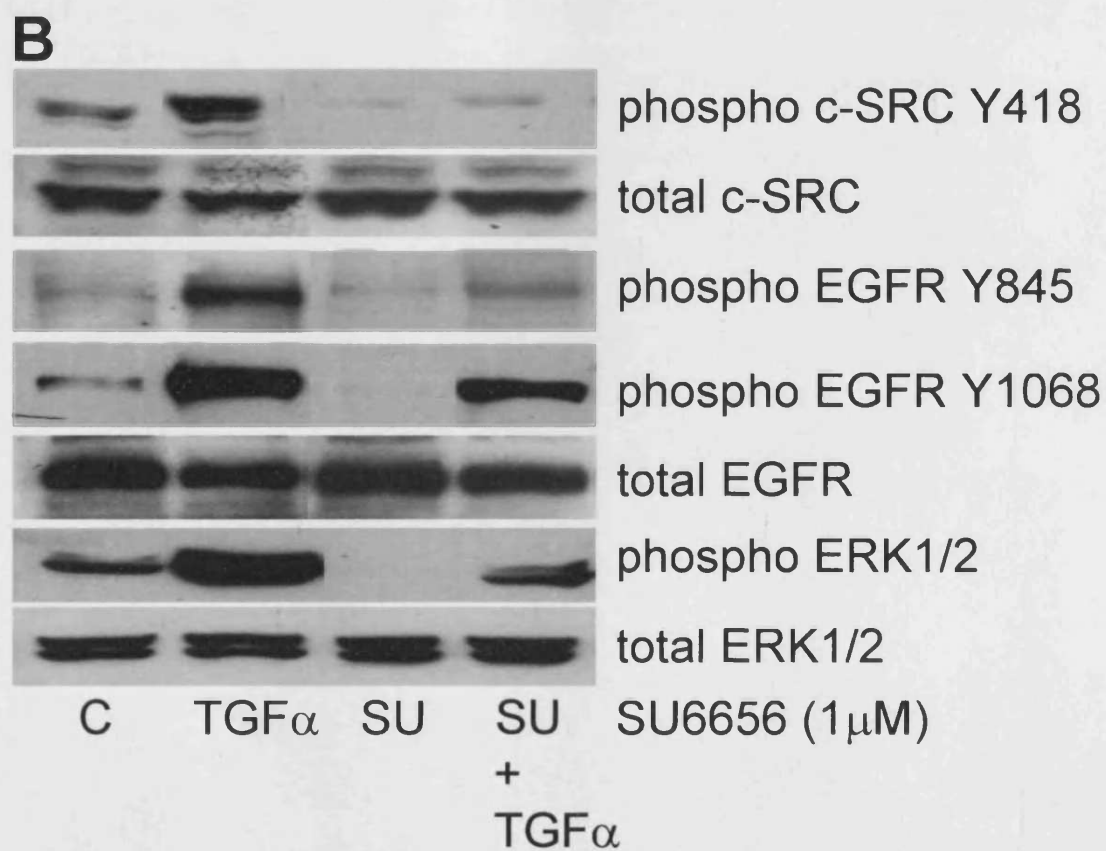
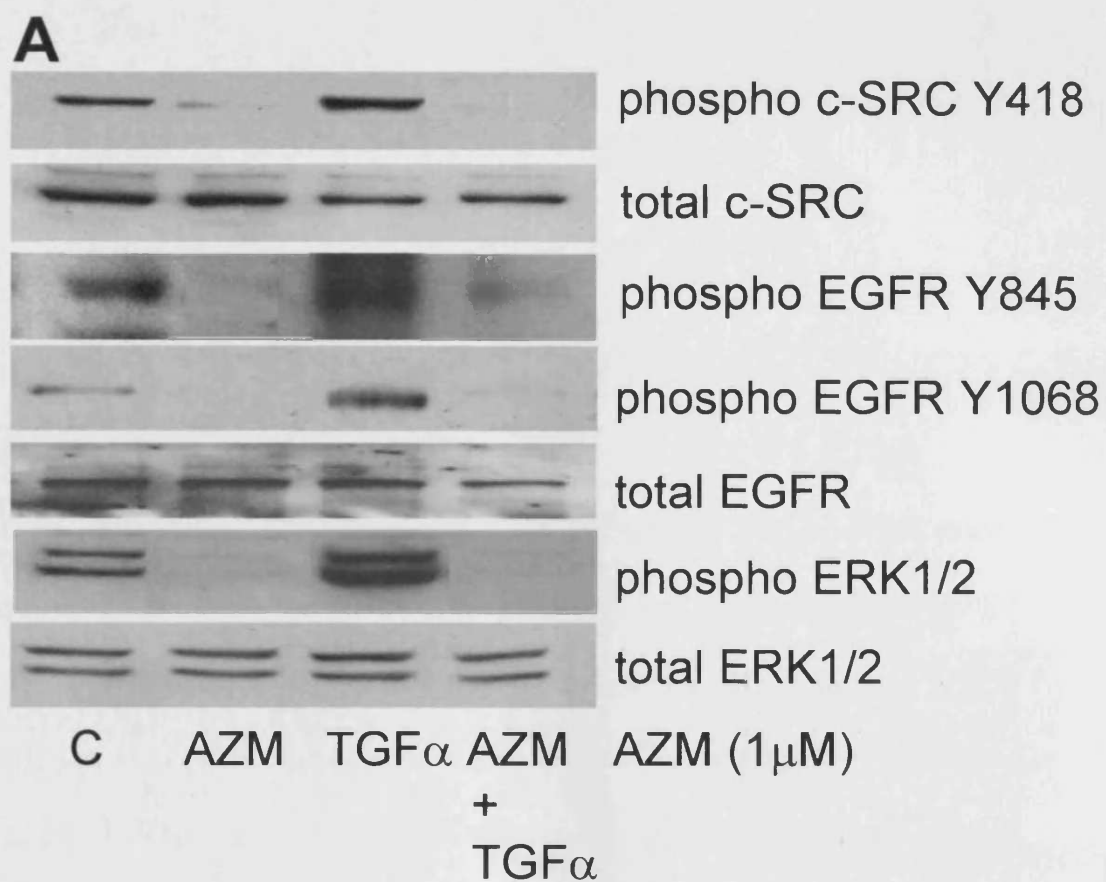




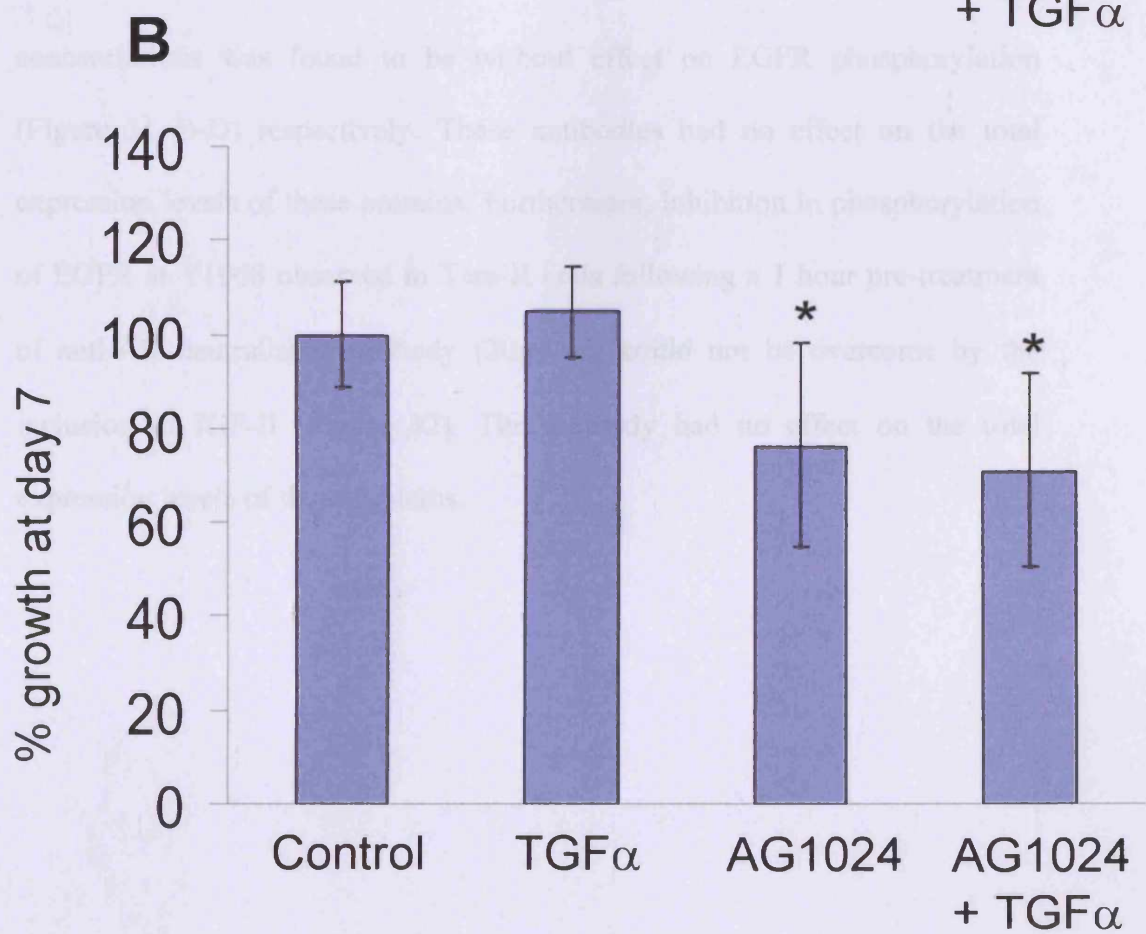
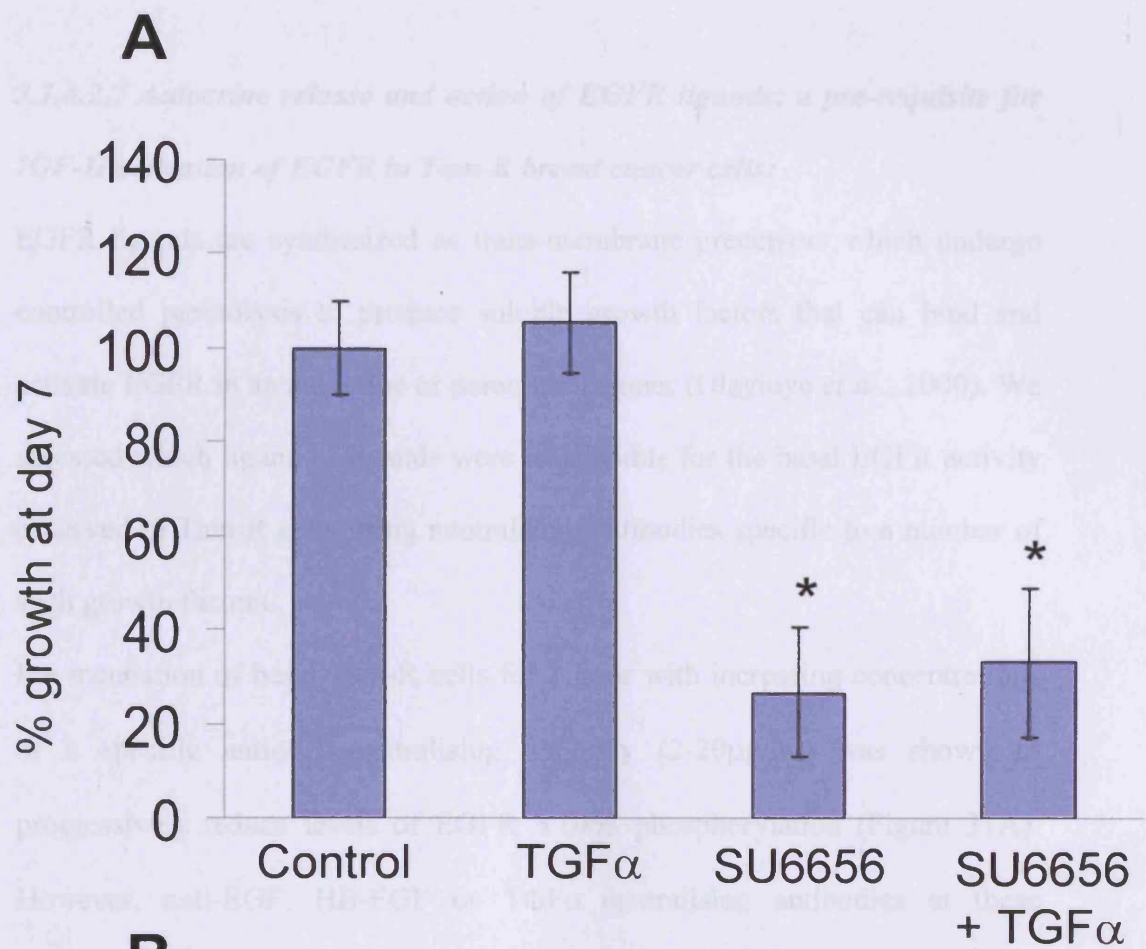
**Figure 28.** The effects of AZM555 on basal and TGF $\alpha$  primed phosphorylated and total c-SRC, EGFR and ERK1/2 in Tam-R cells. Tam-R cells were grown in serum-free DCCM medium for 24 hours, supplemented with AZM555 (1 $\mu$ M) or appropriate vehicle control, followed by stimulation with increasing concentrations of TGF $\alpha$  (0.1-10ng/ml) for 5 minutes or appropriate vehicle control. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of three separate experiments.



**Figure 29.** The effects of AZM555 or SU6656 on basal and TGF $\alpha$  primed phosphorylated and total c-SRC, EGFR and ERK1/2 in Tam-R cells. Tam-R cells were grown in serum-free DCCM medium supplemented with either (A) AZM555 (1 $\mu$ M) for 24 hours, TGF $\alpha$  (1ng/ml) for 5 minutes, a combination of the two agents or appropriate vehicle controls or (B) SU6656 (1 $\mu$ M) for 24 hours, TGF $\alpha$  (1ng/ml) for 5 minutes, a combination of the two agents or appropriate vehicle controls. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of three separate experiments.



**Figure 30.** The effects of SU6656 (1 $\mu$ M) and (B) AG1024 (5 $\mu$ M) on basal and TGF $\alpha$ -primed growth in Tam-R cells. Tam-R cells were grown in serum-free DCCM medium for 7 days supplemented with (A) SU6656 (1 $\mu$ M), TGF $\alpha$  (10ng/ml), a combination of the two agents or vehicle controls only and (B) AG1024 (5 $\mu$ M), TGF $\alpha$  (10ng/ml), a combination of the two agents or vehicle controls only. The results are expressed as mean $\pm$ SEM of triplicate wells and are representative of three separate experiments. A p value of  $\leq 0.05^*$  was considered statistically significant.



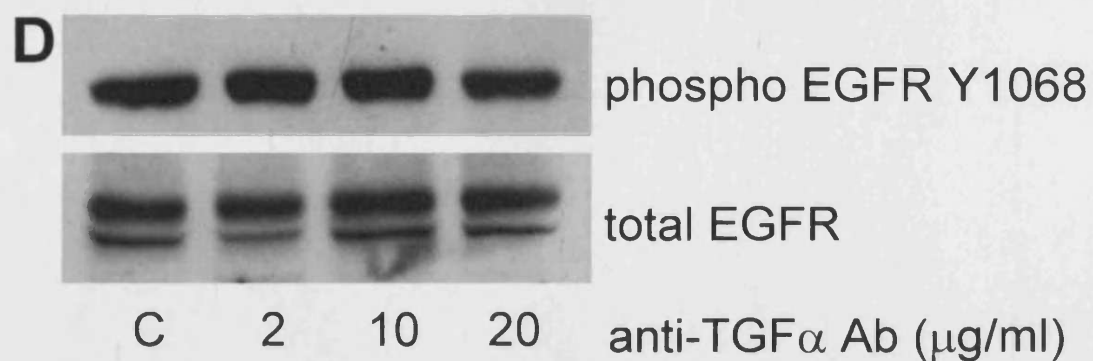
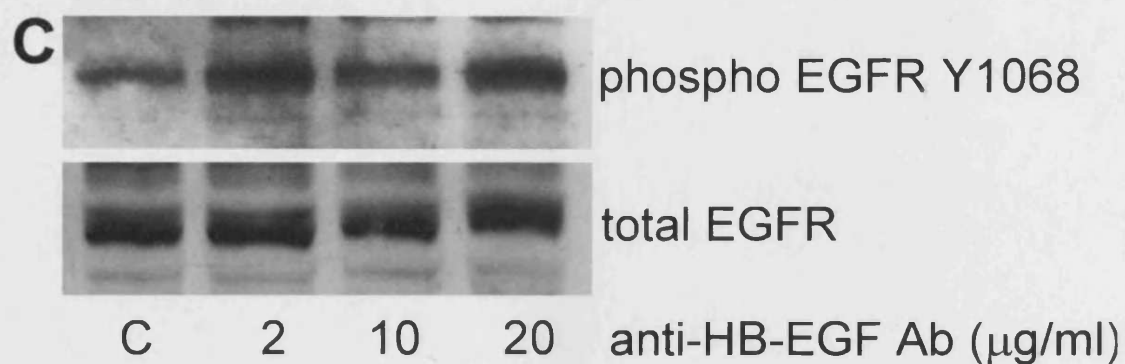
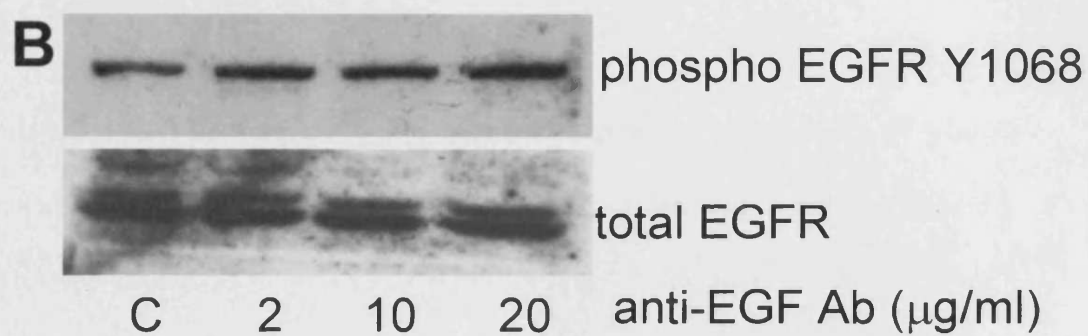
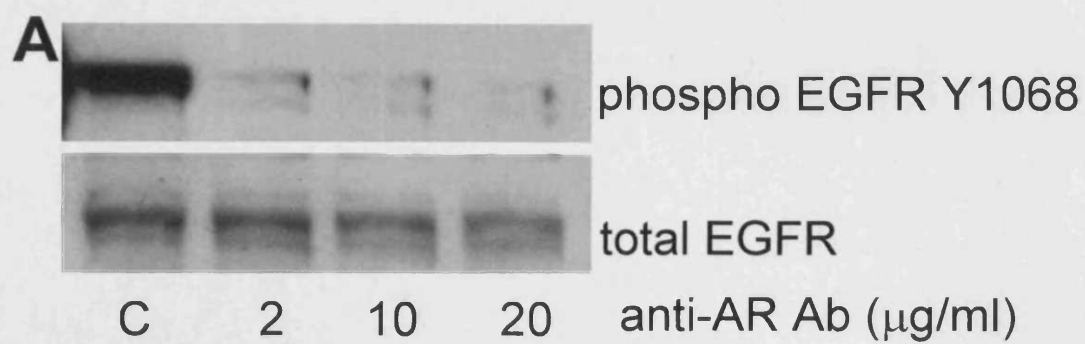
**3.1.8.2.7 Autocrine release and action of EGFR ligands: a pre-requisite for IGF-II activation of EGFR in Tam-R breast cancer cells:**

EGFR ligands are synthesized as trans-membrane precursors which undergo controlled proteolysis to produce soluble growth factors that can bind and activate EGFR in an autocrine or paracrine manner (Olayioye *et al.*, 2000). We assessed which ligand or ligands were responsible for the basal EGFR activity observed in Tam-R cells using neutralising antibodies specific to a number of such growth factors.

Pre-incubation of basal Tam-R cells for 1 hour with increasing concentrations of a specific anti-AR neutralising antibody (2-20 $\mu$ g/ml) was shown to progressively reduce levels of EGFR Y1068 phosphorylation (Figure 31A). However, anti-EGF, HB-EGF or TGF $\alpha$  neutralising antibodies at these concentrations was found to be without effect on EGFR phosphorylation (Figure 31 B-D) respectively. These antibodies had no effect on the total expression levels of these proteins. Furthermore, inhibition in phosphorylation of EGFR at Y1068 observed in Tam-R cells following a 1 hour pre-treatment of anti-AR neutralising antibody (20 $\mu$ g/ml) could not be overcome by the inclusion of IGF-II (Figure 32). The antibody had no effect on the total expression levels of these proteins.



**Figure 31.** The effects of AR, EGF, HB-EGF and TGF $\alpha$  neutralising antibodies on basal phosphorylated and total EGFR protein expression in Tam-R cells. Tam-R cells were incubated in serum-free DCCM medium supplemented with increasing concentrations of (A) AR, (B) EGF, (C) HB-EGF and (D) TGF $\alpha$  neutralising antibodies (2-20 $\mu$ g/ml) or appropriate vehicle control for 1 hour. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of two separate experiments, except for amphiregulin which was performed three times.



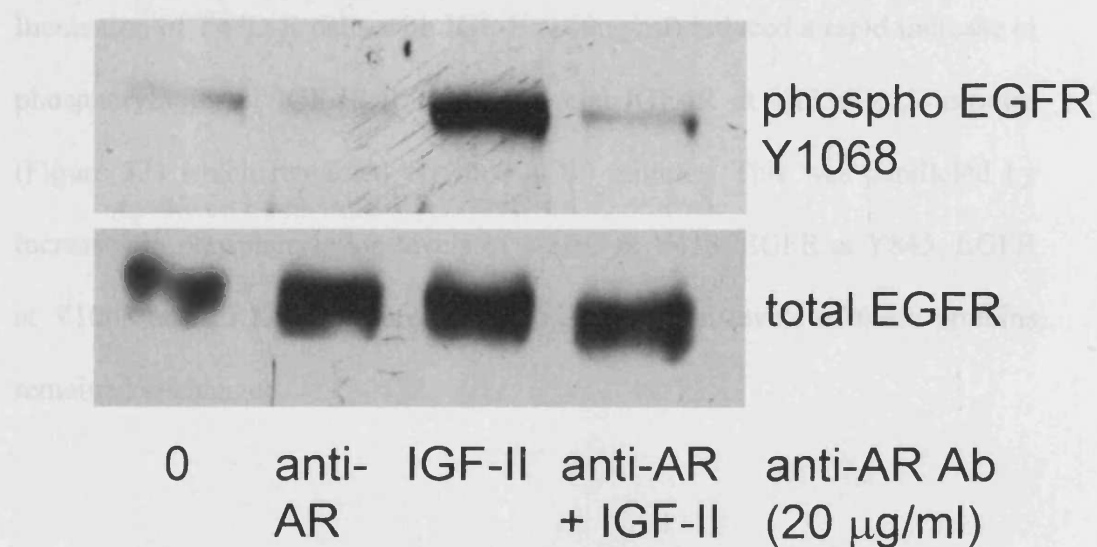
**Figure 32.** The effects of an AR neutralising antibody on basal and IGF-II-primed phosphorylated and total EGFR protein expression in Tam-R cells. Tam-R cells were incubated in serum-free DCCM medium supplemented with AR neutralising antibody (20µg/ml) for 1 hour, IGF-II for 20 minutes or a combination of these two agents or appropriate vehicle controls. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of two separate experiments.

### 3.1.1 IGF-IR/EGFR cross-talk in T47D-R breast cancer cells

To assess whether this novel  $\alpha$ -SRC-dependent IGF-II-induced activation of EGFR phosphorylation was unique to breast cells, we examined the induction of similar responses in a variety of human cancer cell lines. In all cases, the induction of EGFR phosphorylation was observed in T47D-R cells, but not in T47D-R cells.

### 3.1.2 IGF-II promotes IGF-IR/EGFR activity in T47D-R breast cancer cells

cells



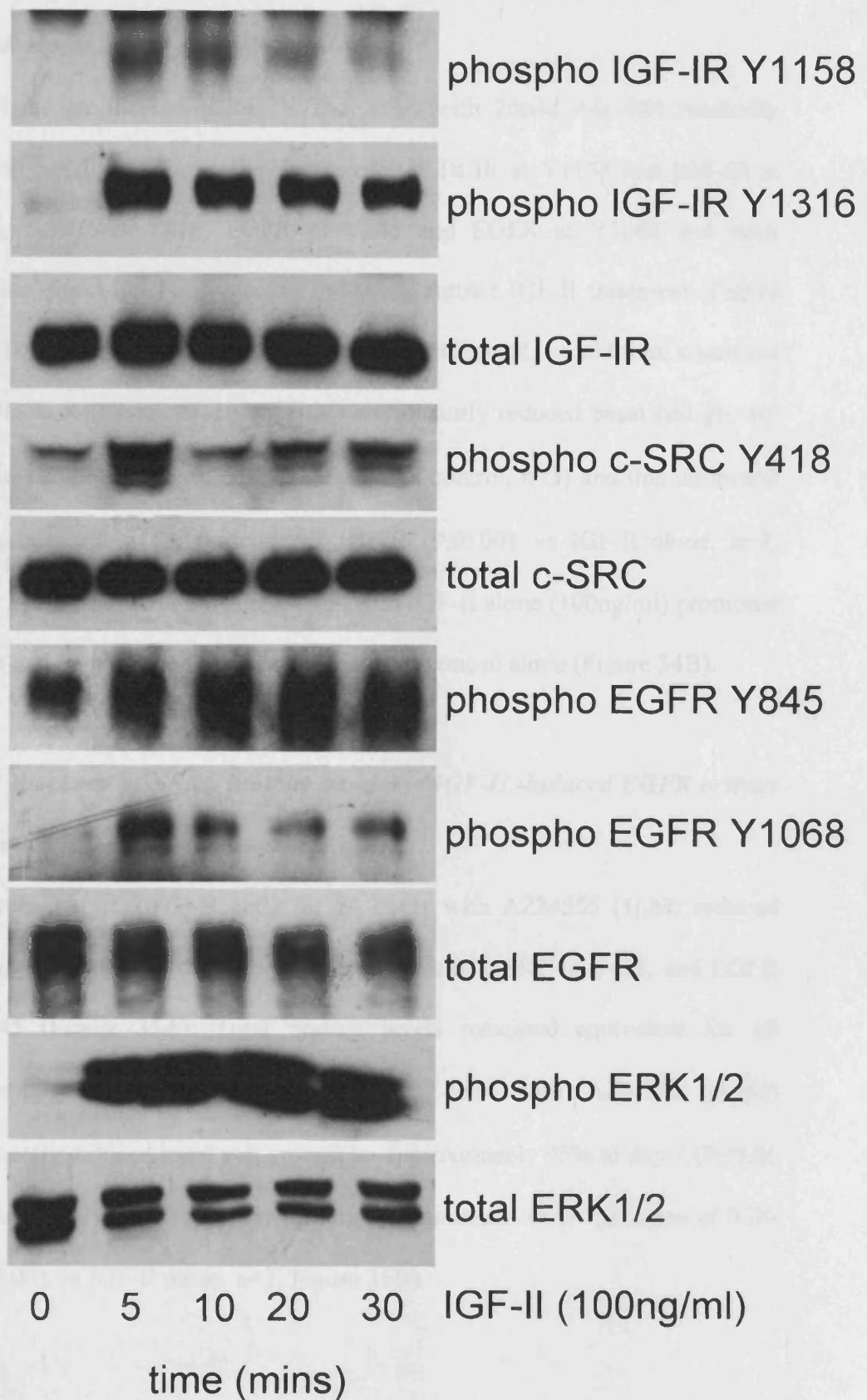
### ***3.1.9 IGF-IR/EGFR cross-talk in T47D-R breast cancer cells:***

To assess whether this novel c-SRC-dependent IGF-II-induced activation of EGFR phosphorylation was unique to Tam-R cells, we examined this mechanism in another tamoxifen resistant breast cancer cell line derived from wild-type T47D cells, termed T47D-R.

#### ***3.1.9.1 IGF-II promotes IGF-IR/EGFR activity in T47D-R breast cancer cells:***

Incubation of T47D-R cells with IGF-II (100ng/ml) induced a rapid increase in phosphorylation of IGF-IR/IR at Y1158 and IGF-IR at Y1316 at 5 minutes (Figure 33) which remained elevated at 30 minutes. This was paralleled by increases in phosphorylation levels of c-SRC at Y418, EGFR at Y845, EGFR at Y1068 and ERK1/2 (Figure 33). Total expression levels of these proteins remained unchanged.

**Figure 33.** The effects of IGF-II on phosphorylated and total IGF-IR, c-SRC, EGFR and ERK1/2 protein expression in T47D-R cells. T47D-R cells were incubated in serum-free DCCM medium supplemented with IGF-II (100ng/ml) or appropriate vehicle control over a 30 minute time period. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of three separate experiments.



***3.1.9.2 IGF-IR blockade inhibits IGF-IR/EGFR activity and growth in T47D-R breast cancer cells:***

A 24 hour pre-incubation of T47D-R cells with 20 $\mu$ M AG1024 markedly inhibited basal phosphorylation levels of IGF-IR/IR at Y1158 and IGF-IR at Y1316, c-SRC at Y418, EGFR at Y845 and EGFR at Y1068 and such inhibition could not be overcome following further IGF-II treatment (Figure 34A). Total levels of each protein remained unchanged. In addition, treatment of T47D-R cells with AG1024 (5 $\mu$ M) significantly reduced basal cell growth by approximately 30% on day 7 ( $P \leq 0.001$  vs control,  $n=3$ ) and this inhibition was maintained in the presence of IGF-II ( $P \leq 0.001$  vs IGF-II alone,  $n=3$ , Figure 34B). Stimulation of these cells with IGF-II alone (100ng/ml) promoted growth by approximately 20% at day 7 versus control alone (Figure 34B).

***3.1.9.3 Blockade of c-SRC inhibits basal and IGF-II-induced EGFR activity and growth in T47D-R breast cancer cells:***

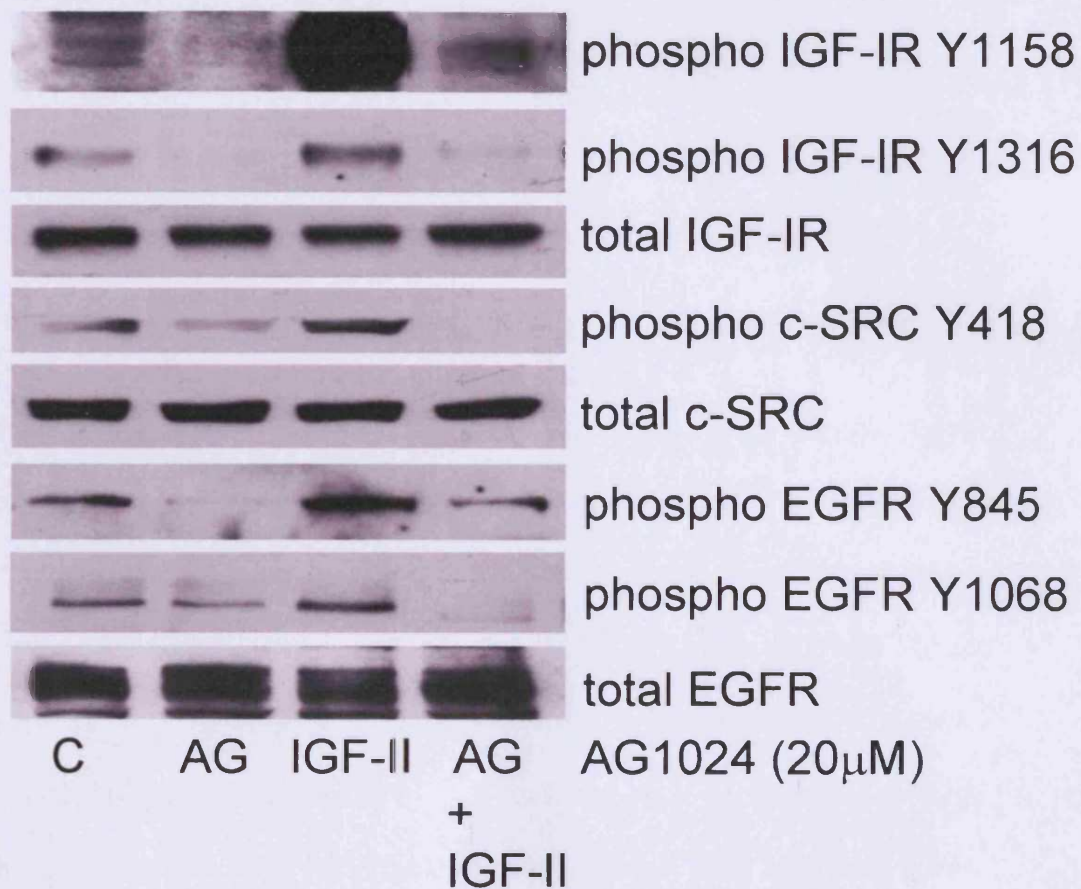
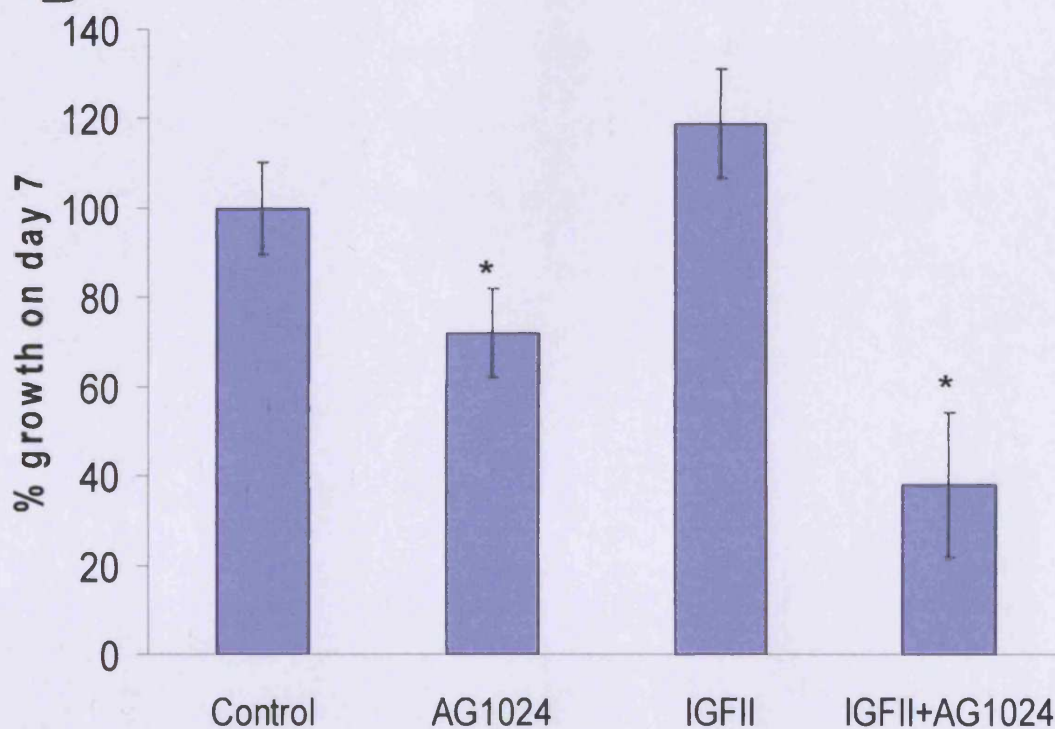
Pre-incubation of T47D-R cells for 24 hours with AZM555 (1 $\mu$ M) reduced basal and IGF-II-primed phosphorylation levels of c-SRC at Y418, and EGFR at Y845 (Figure 35A). Total protein levels remained equivalent for all treatment groups. Treatment of T47D-R cells with AZM555 (1 $\mu$ M) significantly reduced basal cell growth by approximately 95% at day 7 ( $P \leq 0.01$  vs control,  $n=3$ ) and this effect was largely maintained in the presence of IGF-II ( $P \leq 0.001$  vs IGF-II alone,  $n=3$ , Figure 35B).



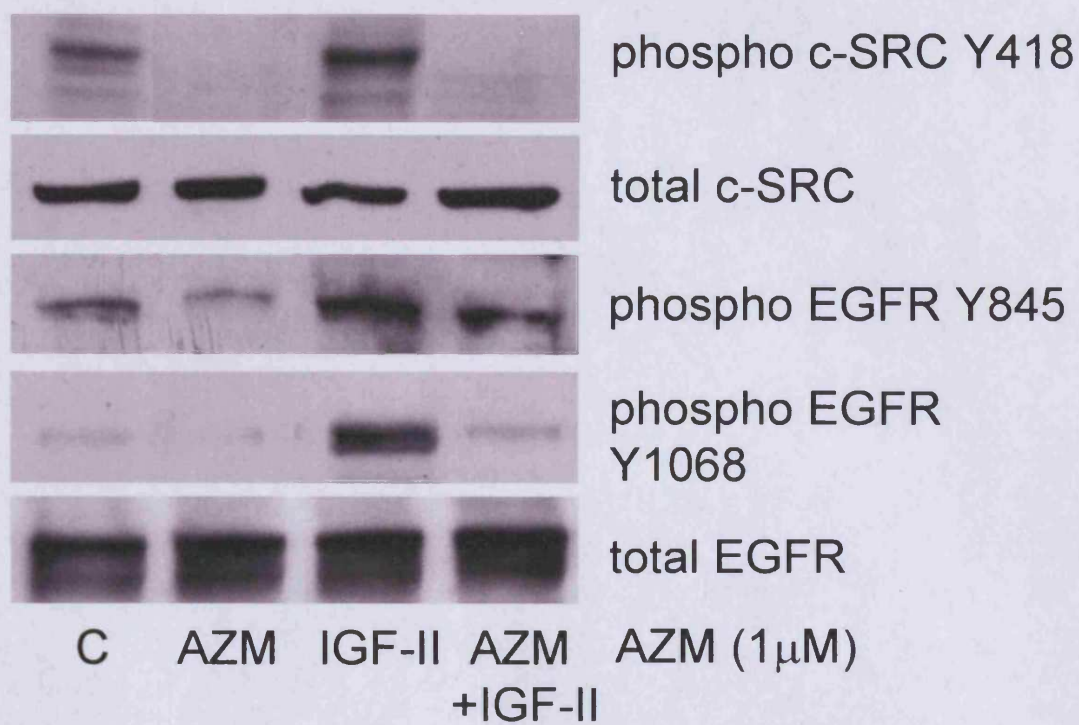
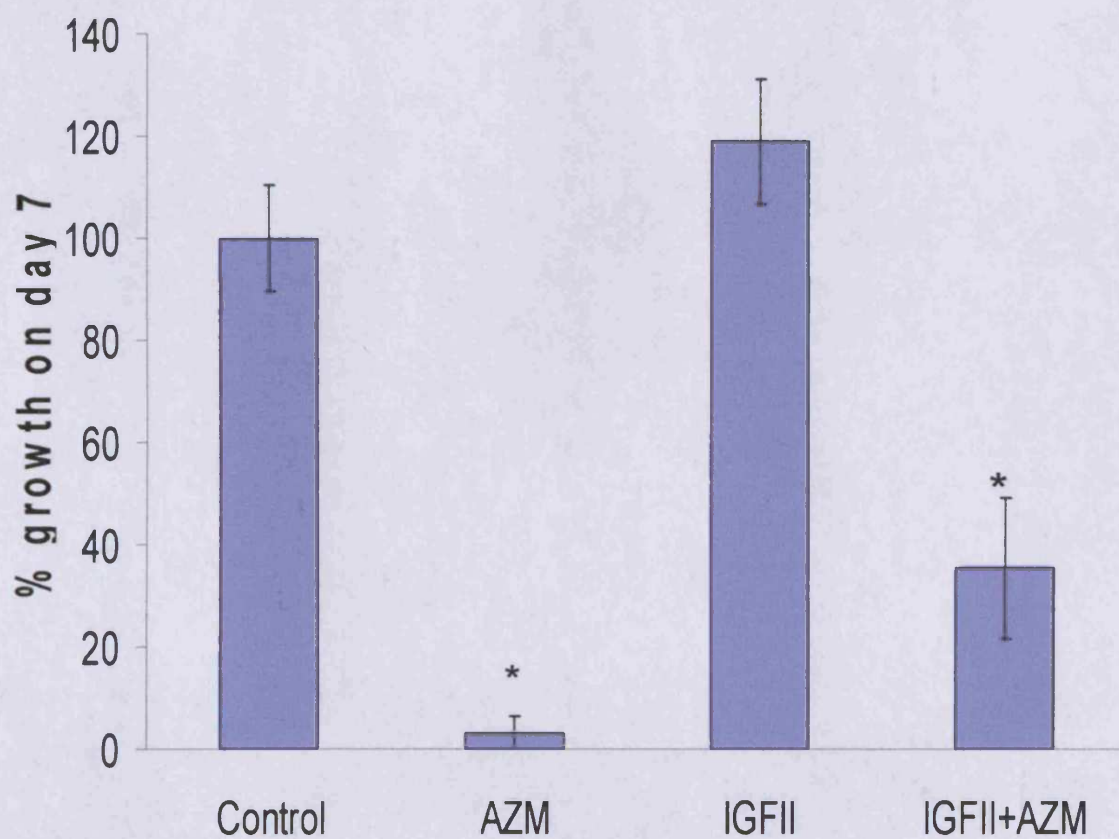
***3.1.9.4 Blockade of c-SRC inhibits basal and TGF $\alpha$ -induced EGFR activity in T47D-R breast cancer cells:***

Interestingly, as already shown for the MCF-7 derived Tam-R cells, inhibition of c-SRC at Y418 phosphorylation and reduction in phosphorylation of EGFR at Y845 by AZM555 (1 $\mu$ M) in the T47D-R cells was associated with a reduced ability of TGF $\alpha$  (1ng/ml) to phosphorylate EGFR at Y1068 (Figure 36). Total protein levels remained equivalent for all treatment groups.

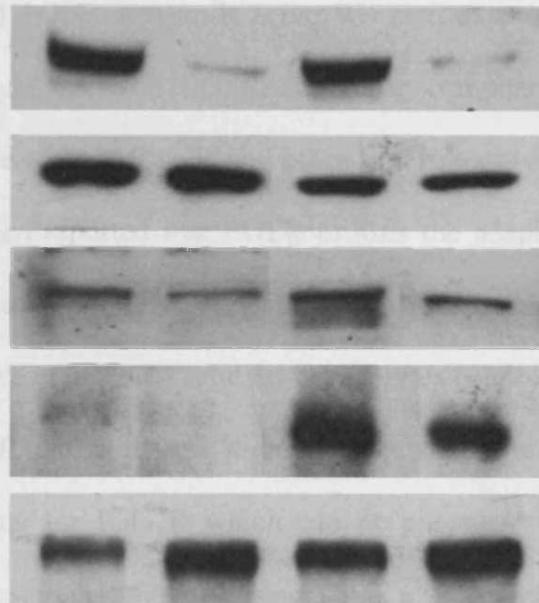
**Figure 34.** The effects of AG1024 on basal and IGF-II-primed phosphorylated and total IGF-IR, c-SRC, EGFR protein expression and growth in T47D-R cells. T47D-R cells were incubated in serum-free DCCM medium supplemented with either (A) AG1024 (20 $\mu$ M) for 24 hours, IGF-II (100ng/ml) for 20 minutes, a combination of the two agents or appropriate vehicle control. Protein expression was assessed by Western analysis and data are representative of three separate experiments. (B) T47D-R cells were incubated in serum-free DCCM medium for 7 days supplemented with either AG1024 (5 $\mu$ M), IGF-II (100ng/ml), a combination of the two agents or appropriate vehicle control. The results are expressed as mean $\pm$ SEM of triplicate wells and are representative of three separate experiments. A p value of  $\leq 0.05^*$  was considered statistically significant.

**A****B**

**Figure 35.** The effects of AZM555 on basal and IGF-II-primed phosphorylated and total c-SRC and EGFR protein expression and growth in T47D-R cells. T47D-R cells were incubated in serum-free DCCM medium supplemented with either (A) AZM555 (1 $\mu$ M) for 24 hours, IGF-II (100ng/ml) for 20 minutes, a combination of the two agents or appropriate vehicle control. Protein expression was assessed by Western analysis and data are representative of three separate experiments. (B) T47D-R cells were incubated in serum-free DCCM medium for 7 days supplemented AZM555 (1 $\mu$ M), IGF-II (100ng/ml), a combination of the two agents or appropriate vehicle control. The results are expressed as mean $\pm$ SEM of triplicate wells and are representative of three separate experiments. A p value of  $\leq 0.05^*$  was considered statistically significant.

**A****B**

**Figure 36.** The effects of AZM555 on basal and TGF $\alpha$  primed phosphorylated and total c-SRC and EGFR protein expression in T47D-R cells. T47D-R cells were incubated in serum-free DCCM medium, supplemented with either AZM555 (1 $\mu$ M) for 24 hours, TGF $\alpha$  (1ng/ml) for 5 minutes, or a combination of the two agents or appropriate vehicle controls. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of three separate experiments.



phospho c-SRC Y418

total c-SRC

phospho EGFR Y845

phospho EGFR Y1068

total EGFR

C AZM TGF $\alpha$  AZM AZM (1 $\mu$ M)  
+  
TGF $\alpha$

### **3.2 Section 2:**

#### **3.2.1 *IRS-1 involvement in EGFR and IGF-IR signalling in EGFR positive cancer cells:***

We have identified a novel uni-directional cross-talk mechanism between IGF-IR and EGFR in Tam-R cells. We next examined whether there was further cross-talk between EGFR and other components of the IGF-IR signalling cascade in Tam-R cells and other EGFR positive cancer cell lines. Recently, it has been reported that EGFR utilises the adaptor protein IRS-1 in recruiting and activating the PI3-K pathway in human epidermoid A431 carcinoma cells (Fujioka *et al.*, 2001) and rat hepatocytes (Fujioka and Ui, 2001). In the second part of this study we therefore, focussed our attention on IRS-1, a major substrate for IGF-IR which can be phosphorylated at a number of tyrosine residues, in particular tyrosine Y896, a Grb2 binding site leading to recruitment of the MAPK signalling pathway, and Y612 which is a binding site for the p85 subunit of PI3-K, key pathways contributing to the oncogenic potential of IGF-IR (White, 1997; Hers *et al.*, 2002; Esposito *et al.*, 2001).

#### **3.2.2 *Basal expression and activity of IGF-IR downstream components in WT-MCF-7 and Tam-R breast cancer cells:***

Although examination of the basal levels of total IRS-1 expression by Western analysis only revealed a moderate reduction in IRS-1 levels in the Tam-R compared to the WT-MCF-7 breast cancer cells, assessment of the basal IRS-1 phosphorylation profile, interestingly, revealed distinct differences between the cells. In WT-MCF-7 cells the dominant phosphorylation site of IRS-1 was Y612 while in Tam-R cells phosphorylation of IRS-1 at Y896 predominated

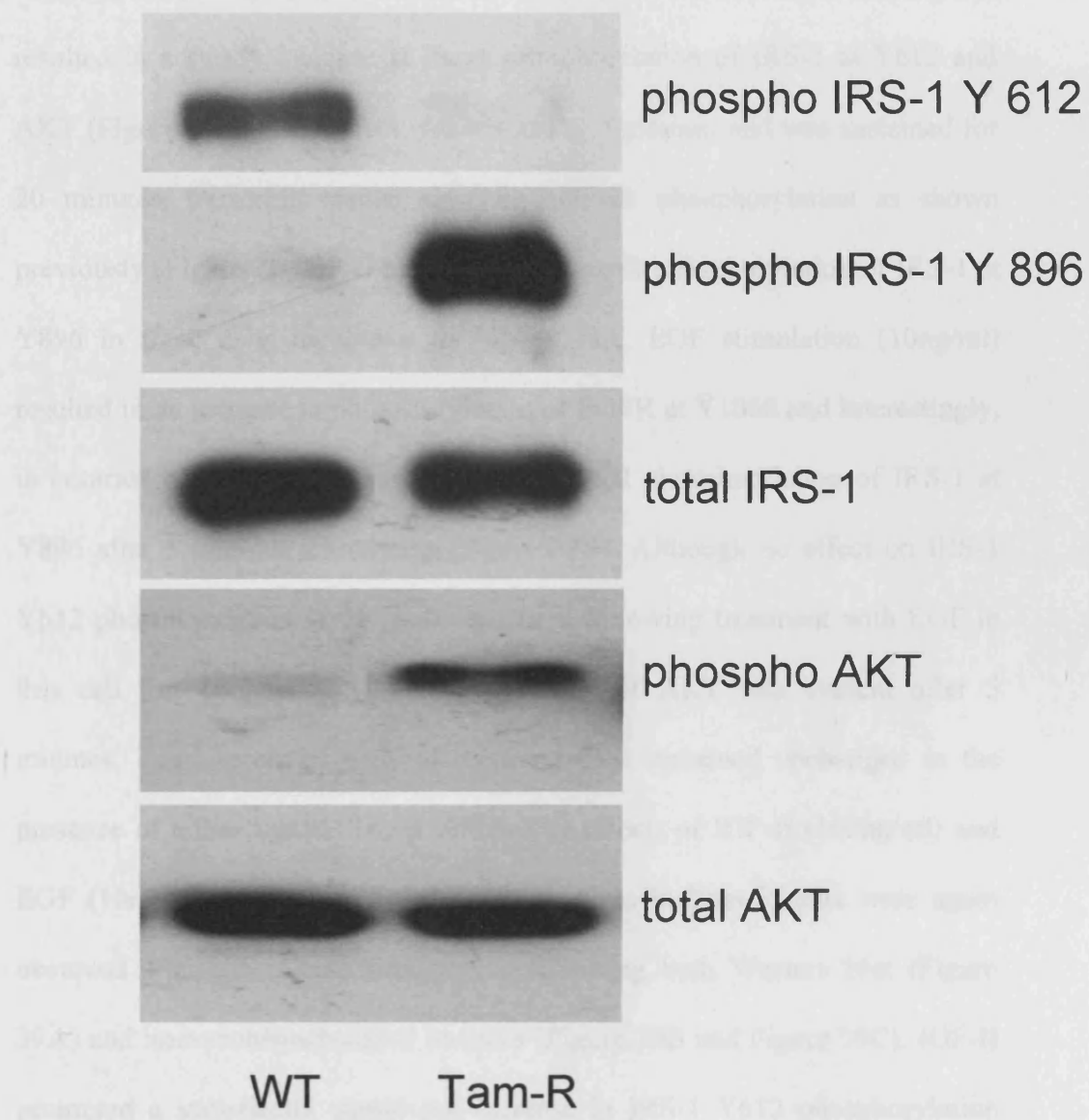


(Figure 37). Parallel Western blots also revealed that Tam-R cells express increased levels of phosphorylated AKT compared to WT-MCF-7 cells, corroborating previous findings (Knowlden *et al.*, 2003; Jordan *et al.*, 2003).

**Figure 37.** Basal levels of both phosphorylated and total IRS-1 and AKT protein expression in WT-MCF-7 and TAM-R cells. WT-MCF-7 and TAM-R cells were incubated in serum-free DCCM medium for 24 hours. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of three separate experiments.

3.2.3 Effects of ligand-stimulation of IRS-1 phosphorylation in Tam-R breast cancer cells

The membrane was incubated with the IRS-1 phosphorylation antibody in Tam-R cells, and the results of IRS-1 and AKT levels were analyzed by Western blot using the indicated antibodies.

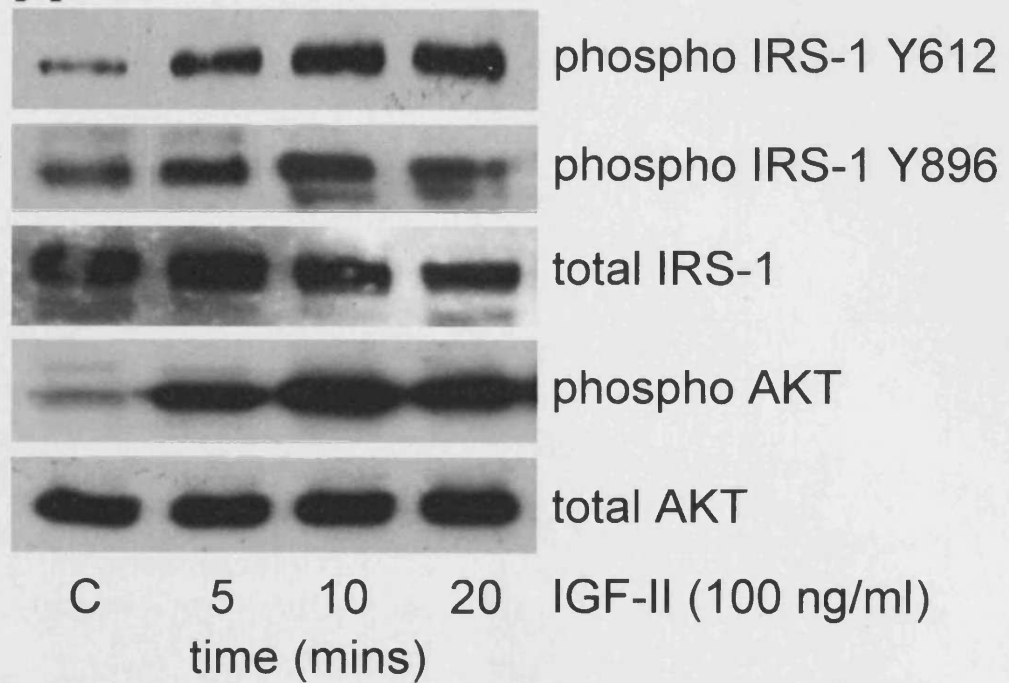
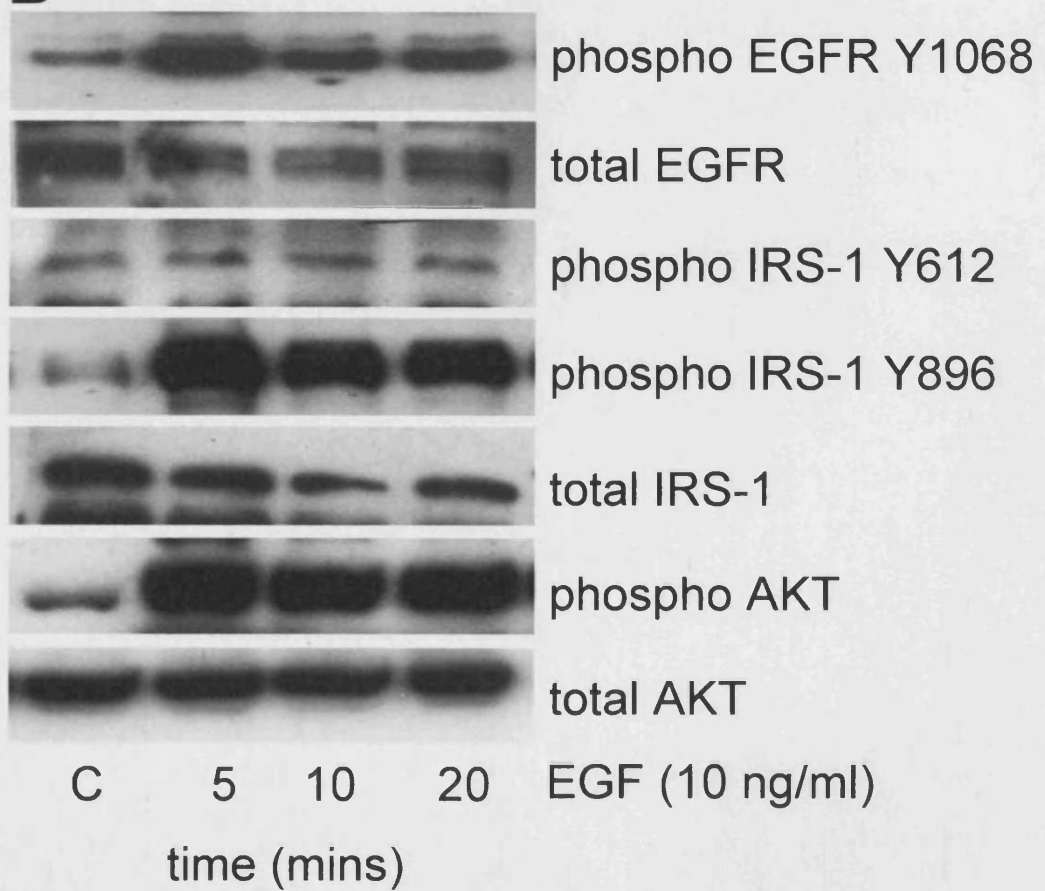


### ***3.2.3 Effects of ligand stimulation of IRS-1 phosphorylation in Tam-R breast cancer cells:***

To examine more extensively the IRS-1 phosphorylation profile in Tam-R cells, the effects of IGF-II and EGF ligand stimulation were investigated by Western blot analysis. Incubation of Tam-R cells with IGF-II (100ng/ml) resulted in a steady increase in basal phosphorylation of IRS-1 at Y612 and AKT (Figure 38A). This effect was evident at 5 minutes and was sustained for 20 minutes, mirroring results seen for IGF-IR phosphorylation as shown previously (Figure 7). IGF-II induced only a small phosphorylation of IRS-1 at Y896 in these cells as shown in Figure 38A. EGF stimulation (10ng/ml) resulted in an increase in phosphorylation of EGFR at Y1068 and interestingly, in contrast to IGF-II, EGF promoted substantial phosphorylation of IRS-1 at Y896 after 5 minutes stimulation (figure 38B). Although no effect on IRS-1 Y612 phosphorylation levels were observed following treatment with EGF in this cell line (Figure 38B), phosphorylation of AKT was evident after 5 minutes. Total levels of each of these proteins remained unchanged in the presence of either ligand. These differential effects of IGF-II (100ng/ml) and EGF (10ng/ml) on IRS-1 phosphorylation states in Tam-R cells were again observed after a 5 minute time period following both Western blot (Figure 39A) and immunohistochemical analysis (Figure 39B and Figure 39C). IGF-II promoted a statistically significant increase in IRS-1 Y612 phosphorylation compared to control ( $P<0.05$ ,  $n=4$ ; Table 1), whereas EGF promoted significant phosphorylation of IRS-1 at Y896 compared to control ( $P<0.05$ ,  $n=4$ ; Figure 39A, 39B and 39C, Table 1) as determined by HScore analysis of immunocytochemical staining. Neither ligand influenced total expression

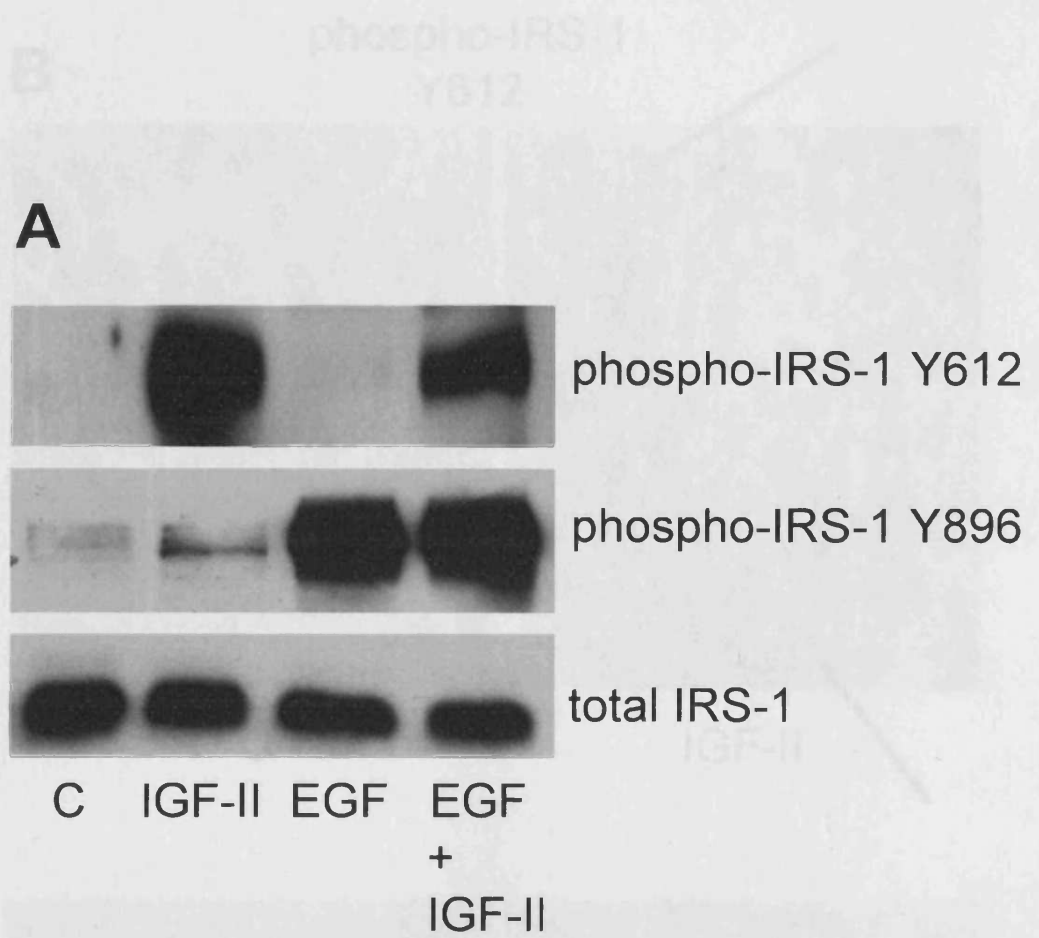
levels of these proteins. Phosphorylated IRS-1 Y612 basal expression was shown to be situated at both the plasma membrane and in the cytoplasm, albeit at low levels following immunocytochemical staining. However, after IGF-II treatment there was clearly more membrane staining which was of a punctated nature, as depicted by arrows (Figure 39B). Phosphorylated IRS-1 Y896 expression was predominantly localised to the plasma membrane, although there was some cytoplasmic staining (Figure 39C).

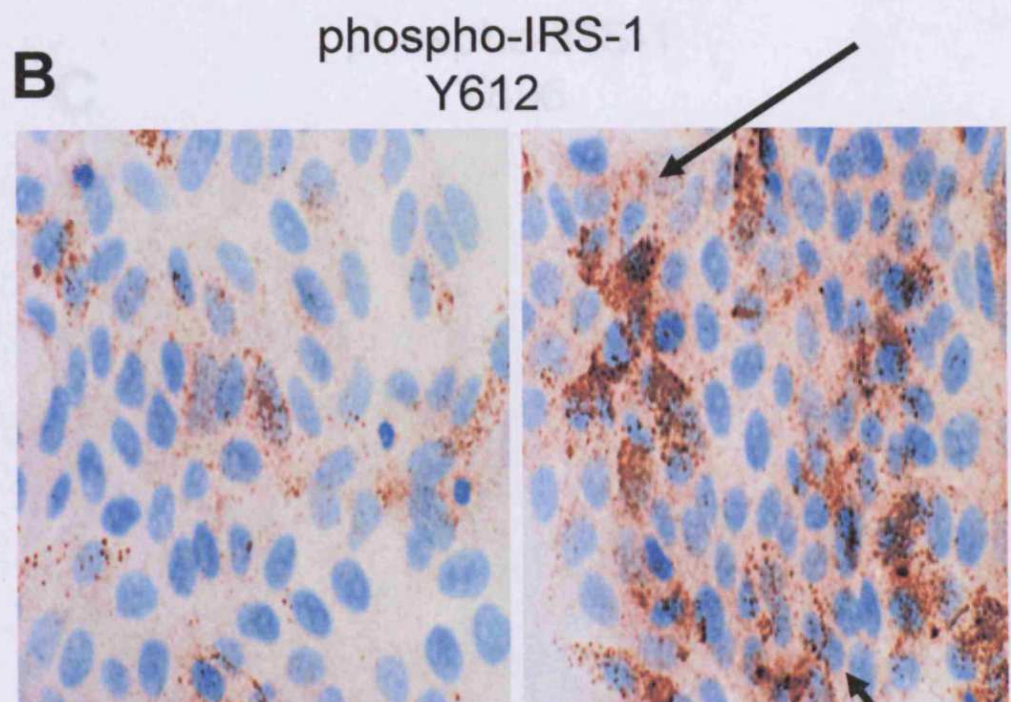
**Figure 38.** The effects of IGF-II on phosphorylated and total IRS-1 and AKT and EGF on phosphorylated and total EGFR, IRS-1 and AKT protein expression in Tam-R cells. Tam-R cells were incubated in serum-free DCCM medium supplemented with (A) IGF-II (100ng/ml) or appropriate vehicle control and (B) EGF (10ng/ml) or appropriate vehicle control over a 20 minute time period. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of three separate experiments.

**A****B**

**Figure 39.** The effects of IGF-II, EGF or a combination of the two agents on phosphorylated and total IRS-1 in Tam-R cells. Tam-R cells were incubated in serum-free DCCM medium supplemented with IGF-II (100ng/ml), EGF (10ng/ml), a combination of both IGF-II and EGF or appropriate vehicle controls for 5 minutes respectively. Cells were assessed by (A) Western blot and (B and C) immunocytochemical analysis. Data are representative of three separate experiments for each methodology. Original magnification (x 40) for immunocytochemical analysis. Black arrows depict areas of punctated plasma membrane staining.

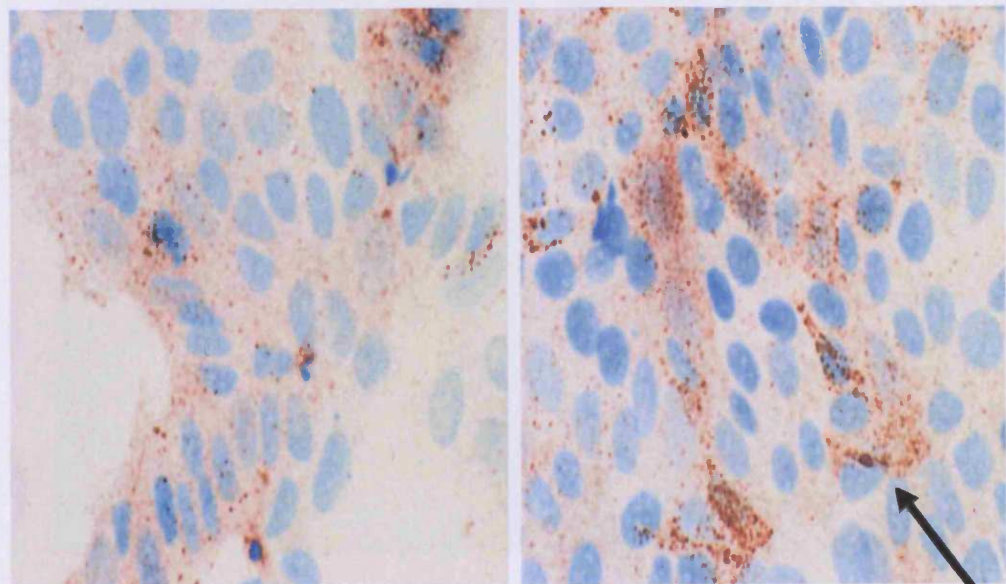






**C**

IGF-II



EGF

EGF + IGF-II

### 3.2.4 IRS-1 associates with EGFR in T47D breast cancer cells

The novel finding that EGF could result in the phosphorylation of IRS-1 at

Y896 would suggest that this phosphorylation is a key event in the

leading to IRS-1 recruitment and physical association with EGFR.

physical association between IRS-1 and EGFR.

Figure 17 shows that

likely due to IRS-1 phosphorylation.

blotting with anti-phosphotyrosine antibody.

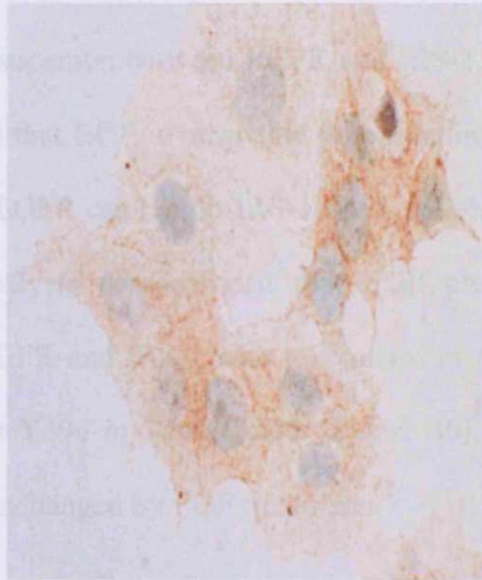
between EGF and

of IRS-1 at Y896 in

remained unchanged by

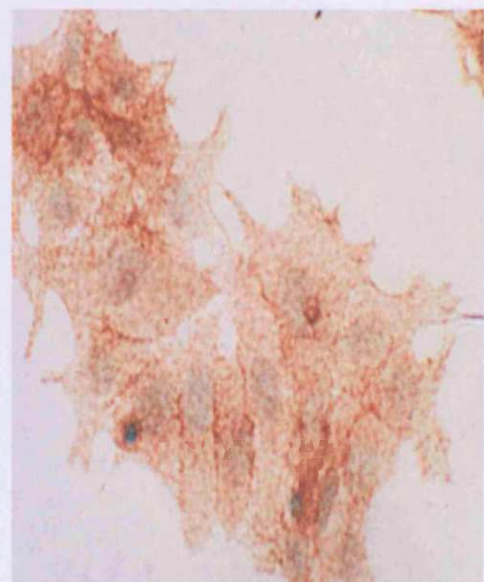
phospho-IRS-1  
Y896

C



C

IGF-II



EGF

EGF + IGF-II

#### ***3.2.4 IRS-1 associates with EGFR in Tam-R breast cancer cells:***

The novel finding that EGF could result in the phosphorylation of IRS-1 at Y896 would suggest that either EGF alone can directly activate IGF-IR, leading to IRS-1 recruitment and phosphorylation or alternatively, promote the physical association between EGFR and IRS-1. As we have previously shown (Figure 12) that EGF is incapable of activating the IGF-IR in these cells it is likely that EGFR can recruit IRS-1 itself. Indeed immunoprecipitation/Western blotting analysis demonstrated that EGF promoted a physical association between EGFR and IRS-1 with a resultant increased level of phosphorylation of IRS-1 at Y896 in Tam-R cells (Figure 40). Total EGFR and IRS-1 levels remained unchanged by EGF treatment.

**Figure 40.** Effects of EGF on EGFR and IRS-1 heterodimer formation in Tam-R cells. Tam-R cells were incubated in serum-free DCCM medium either in the presence of EGF (10ng/ml) or appropriate vehicle control for 5 minutes. Following cell lysis, protein expression was assessed by Western blot analysis of phosphorylated IRS-1, total EGFR and IRS-1 following EGFR immunoprecipitation. Data are representative of three separate experiments.



### 3.2.3 EGFR is the dominant regulator of IRS-1 in T47D breast cancer cells

Western blotting and immunoprecipitation analysis revealed that co-treatment of T47D cells with EGF and IGF-1 resulted in phosphorylation of IRS-1 at Sub Y612 and at Y896. However, levels of Y612 phosphorylation were significantly lower following co-treatment with the two ligands.

#### Immunoprecipitation: total EGFR antibody

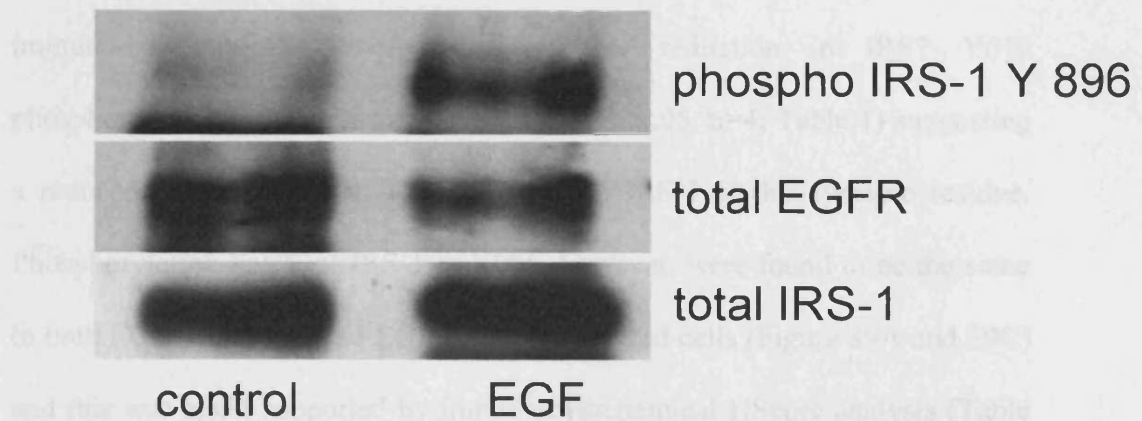


Fig. 3. Total levels of IRS-1 remained unchanged for all treatments studied.

### 3.2.6 EGFR blockade promotes association of IRS-1 with IGF-1R in T47D breast cancer cells

Treatment of T47D cells with increasing concentrations of gefitinib (100 nM to 1  $\mu$ M) for 7 days was shown to inhibit phosphorylation of EGFR in a concentration dependent manner. Such inhibition was mirrored by the subsequent inhibition of phosphorylation of IRS-1 at Y612 (Figure 4A). Interestingly, IRS-1 Y612 phosphorylation, as assessed by IRS-1 Y612, was shown to increase as the concentration of gefitinib increased (Figure 4A). Total protein levels remained unchanged following treatment. A time course study performed on T47D cells revealed that the observed increase in IRS-1 Y612 phosphorylation occurred as early as day 1 post gefitinib treatment.

### ***3.2.5 EGFR is the dominant recruiter of IRS-1 in Tam-R breast cancer cells:***

Western blotting and immunocytochemical analysis revealed that co-treatment of Tam-R cells with EGF and IGF-II resulted in phosphorylation of IRS-1 at both Y612 and at Y896, however, levels of Y612 phosphorylation were considerably lower following combined treatment with the two ligands compared with IGF-II stimulation alone (Figure 39A, 39B and 39C). Following immunocytochemical HScore analysis, this reduction in IRS1 Y612 phosphorylation was found to be significant ( $P < 0.05$ ,  $n = 4$ ; Table 1) suggesting a reduced ability of IGF-II to phosphorylate IRS-1 at this tyrosine residue. Phosphorylation levels of IRS-1 at Y896, however, were found to be the same in both EGF stimulated and EGF/IGF-II stimulated cells (Figure 39A and 39C) and this was again supported by immunocytochemical HScore analysis (Table 1). Total levels of IRS-1 remained unchanged for all treatments studied.

### ***3.2.6 EGFR blockade promotes association of IRS-1 with IGF-IR in Tam-R breast cancer cells:***

Treatment of Tam-R cells with increasing concentrations of gefitinib (0.01-1  $\mu$ M) for 7 days was shown to inhibit phosphorylation of EGFR at Y1068 in a concentration dependent manner. Such inhibition was mirrored by the subsequent inhibition in phosphorylation of IRS-1 at Y896 (Figure 41A). Interestingly, IRS-1 Y612 phosphorylation, in contrast to IRS-1 Y896, was shown to increase as the concentration of gefitinib increased (Figure 41A). Total protein levels remained unchanged following treatment. A time course study performed on Tam-R cells revealed that this observed increase in IRS-1 Y612 phosphorylation occurred as early as day 1 post gefitinib treatment

(Figure 41B). In addition and interestingly, this unexpected increase in IRS-1 Y612 phosphorylation was associated with an increase in AKT activity, although this was only observed following 7 days gefitinib treatment, with AKT phosphorylation levels inhibited up to the 4 day treatment point (Figure 41B). Total protein levels remained unchanged at all treatment times. A 7 day gefitinib only treatment generated identical findings (Figure 42A) with a reduction in phosphorylation of IRS-1 at Y896 and an increase in phosphorylation of IRS-1 at Y612 and AKT. Similar results were observed by immunocytochemistry (Figure 42B) where immunocytochemical staining of IRS-1 Y612 phosphorylation levels were again shown to be localised to both the cytoplasm and at the plasma membrane regions in a punctated form, whereas IRS-1 Y896 phosphorylated levels were more membrane associated. These changes were shown to be significant following immunocytochemical HScore analysis ( $P < 0.05$ ,  $n = 4$  for both phosphorylated forms; Table 1). Moreover, in the presence of IGF-II, phosphorylation of IRS-1 at Y612 and AKT could be significantly enhanced following 7 day gefitinib treatment of Tam-R cells (Figure 43). Again total protein levels remained unchanged for all treatment groups. Immunoprecipitation/Western blotting analysis revealed that a 7 day gefitinib ( $1\mu\text{M}$ ) challenge of Tam-R cells resulted in a reduced association of IRS-1 with EGFR and an obvious increased association of IRS-1 with IGF-IR (Figure 44). Total levels of IRS-1 remained the same both in the presence and absence of gefitinib.



**Table 1**

**Upper:** Tam-R cells were grown on coverslips in phenol-red/steroid-and serum free DCCM for 24 hours then challenged with either IGF-II (100 ng/ml), EGF (10 ng/ml), a combination of the two ligands, or the appropriate vehicle control for 5 minutes prior to fixation.

**Lower:** Tam-R cells were grown on coverslips in phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS supplemented with either gefitinib (1  $\mu$ M) or vehicle control for 7 days prior to fixation. Combined cytoplasmic and plasma membrane staining intensity and percentage positivity for phosphorylated IRS-1 Y612 and Y896 were assessed by HScore analysis. HScore values are representative of the assessment of four fields of view per coverslip in triplicate experiments.\*  $P < 0.05$  vs Control, †  $P < 0.05$  vs IGF-II.

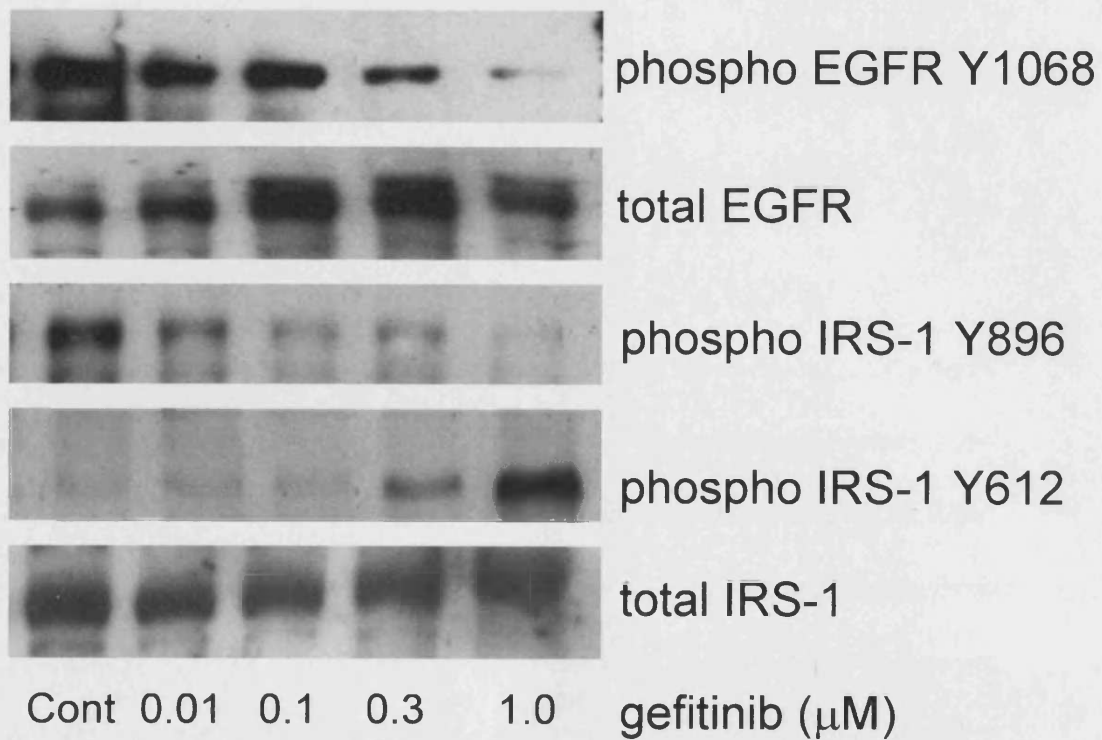
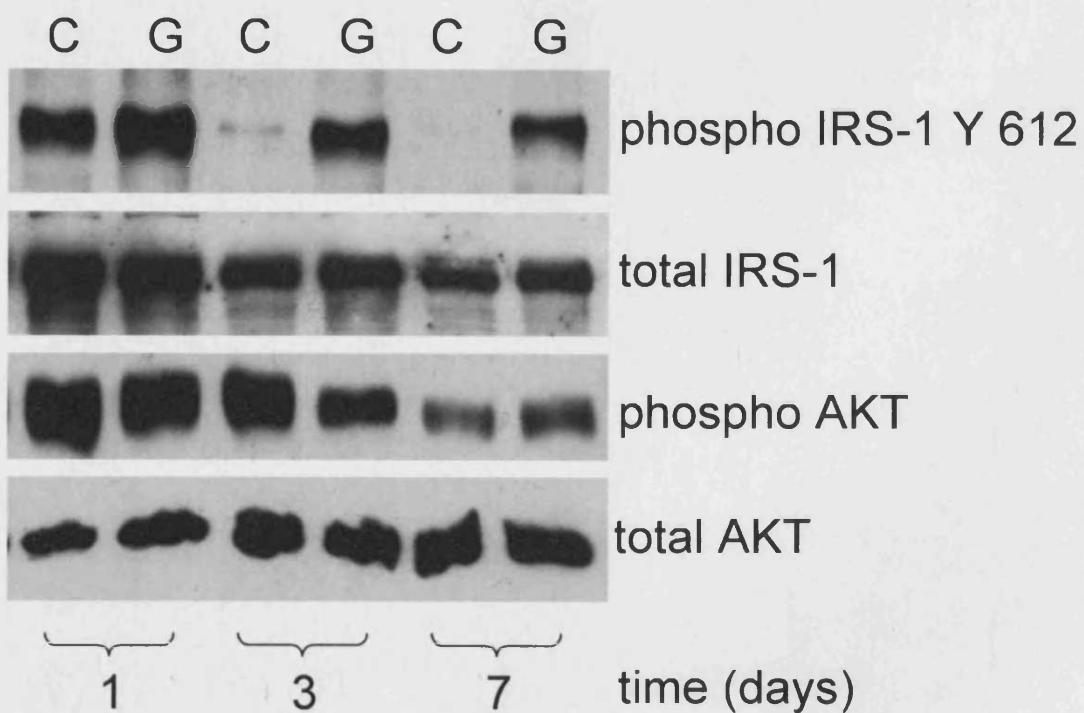
**Table 1.** Effects of EGFR and IGF-IR activation status on immunocytochemically-determined phosphorylated IRS-1 expression in TAM-R cells.

	<b>IRS-1 Y612</b>	<b>IRS-1 Y896</b>
	Median HScore	Median HScore
<b>Control</b>	30	70
<b>IGF-II</b>	120 *	47.5
<b>EGF</b>	12.5	170 *
<b>IGF-II + EGF</b>	42.5 †	165

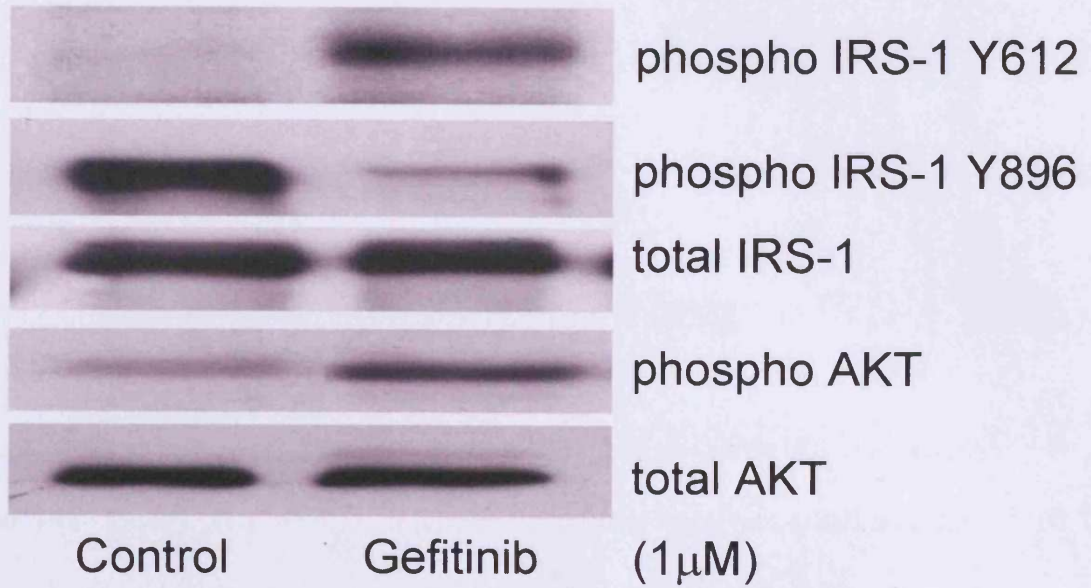
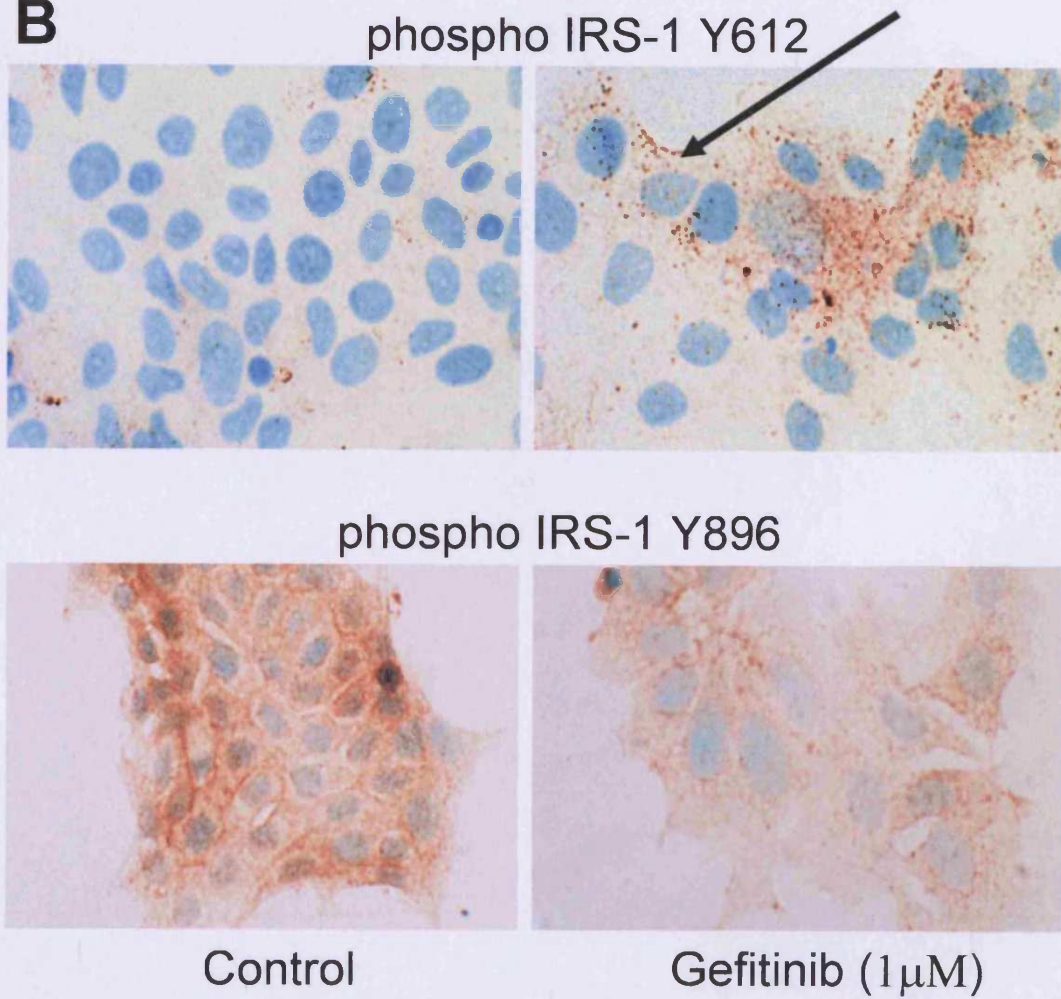
  

<b>Control</b>	7.5	137.5
<b>Gefitinib</b>	70 *	45 *

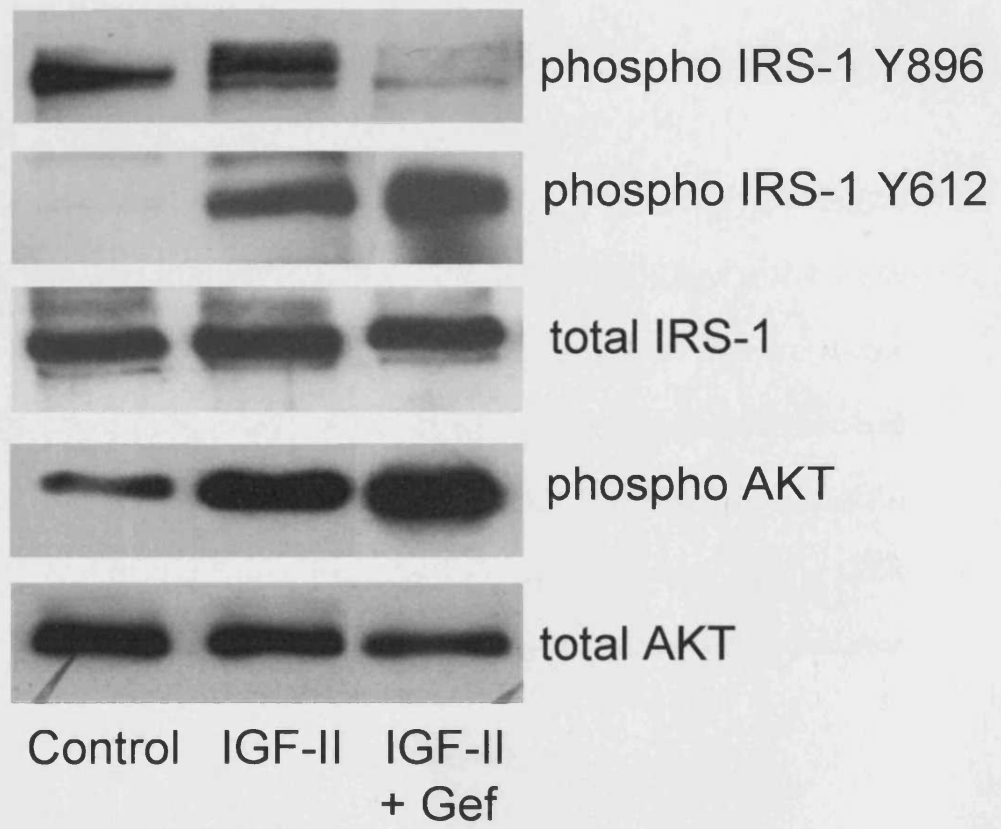
**Figure 41.** The effects of gefitinib on phosphorylated and total EGFR, IRS-1 and AKT protein expression in Tam-R cells. Tam-R cells were grown in phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS for 7 days supplemented with increasing concentrations of (A) gefitinib (0.01-1 $\mu$ M) or appropriate vehicle control (Cont) and (B) gefitinib (G; 1 $\mu$ M) or appropriate vehicle control (C) for between 1 and 7 days. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of three separate experiments.

**A****B**

**Figure 42.** The effects of gefitinib on basal phosphorylated and total IRS-1 and AKT protein expression in Tam-R cells. Tam-R cells were grown in phenol red-free RPMI medium containing 5% charcoal steroid-depleted FCS for 7 days supplemented with gefitinib (1 $\mu$ M) or appropriate vehicle control. Protein expression was assessed by (A) Western blot or (B) immunocytochemical analysis. Data are representative of three separate experiments for each methodology. Original magnification (x 40) for immunocytochemical analysis. Black arrows depict areas of punctated plasma membrane staining.

**A****B**

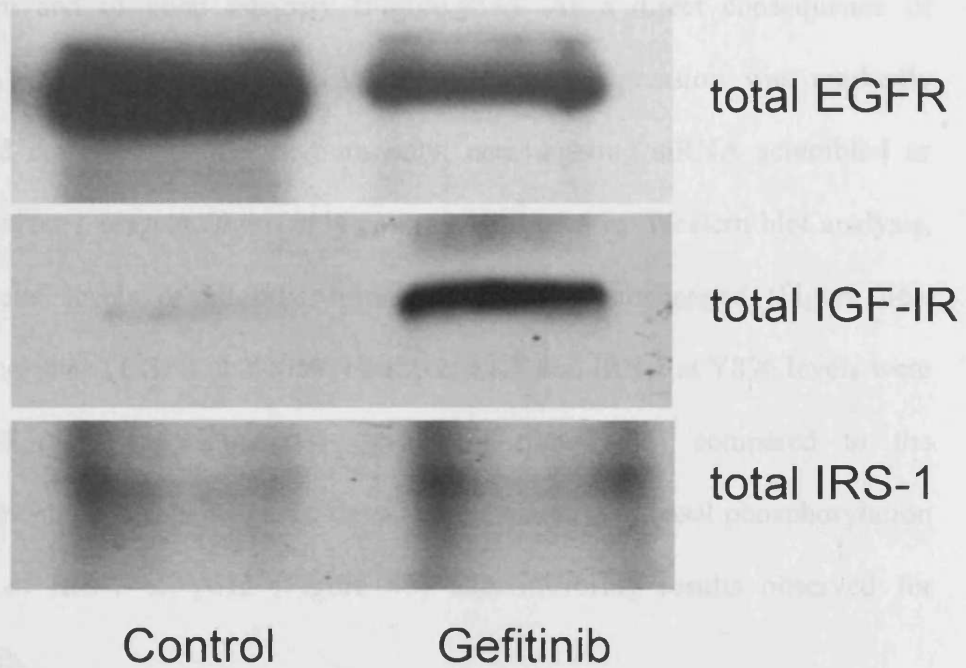
**Figure 43.** The effects of gefitinib on basal and IGF-II primed phosphorylated and total IRS-1 and AKT protein expression in Tam-R cells. Tam-R cells were grown in serum-free DCCM medium, supplemented with gefitinib (1 $\mu$ M) for 7 days, IGF-II (100ng/ml) for 5 minutes or a combination of both gefitinib and IGF-II or appropriate vehicle controls. Phosphorylated and total IRS-1 and AKT protein expression was assessed by Western blot analysis. Data are representative of at least three separate experiments.





**Figure 44.** The effects of gefitinib on basal heterodimer formation between IRS-1/EGFR and IRS-1/IGF-IR in Tam-R cells. Tam-R cells were grown in phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS supplemented with gefitinib (1 $\mu$ M) or appropriate vehicle control for 7 days. Following cell lysis, protein expression of total EGFR, IGF-IR and IRS-1 was assessed by Western blot analysis following IRS-1 immunoprecipitation. Data are representative of three separate experiments.

### Immunoprecipitation: total IRS-1 antibody

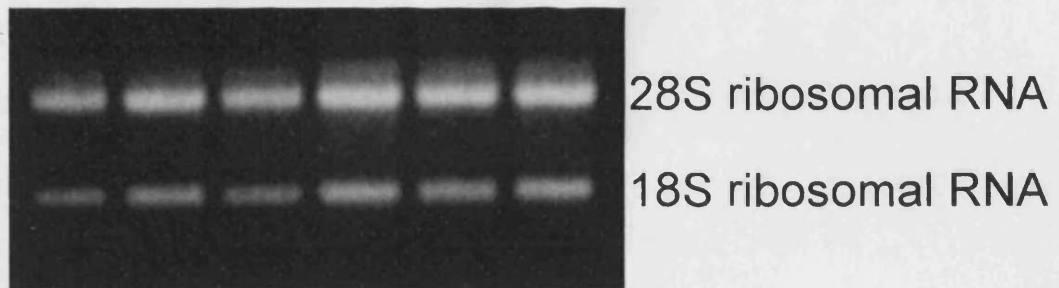


Similar findings to those for gefitinib were also observed following the silencing of the EGFR gene using targeted siRNA technology. The siRNA was firstly shown to specifically down-regulate total EGFR mRNA expression following semi-quantitative RT-PCR analysis (Figure 45B) with the integrity and constancy of the RNA prior to RT-PCR analysis found to be relatively constant and of good integrity (Figure 45A). As a direct consequence of mRNA knockdown with siRNA, EGFR protein expression was markedly reduced compared to the medium only, non-targeting siRNA scrambled or Dharmafect 1 reagent (lipid) only controls as shown by Western blot analysis, with total levels of all other proteins remaining unchanged (Figure 46). Phosphorylated EGFR at Y1068, ERK1/2, AKT and IRS-1 at Y896 levels were reduced quite substantially 4 days post transfection compared to the experimental controls, whereas, there was an increase in basal phosphorylation levels of IRS-1 at Y612 (Figure 46) thus mirroring results observed for gefitinib.

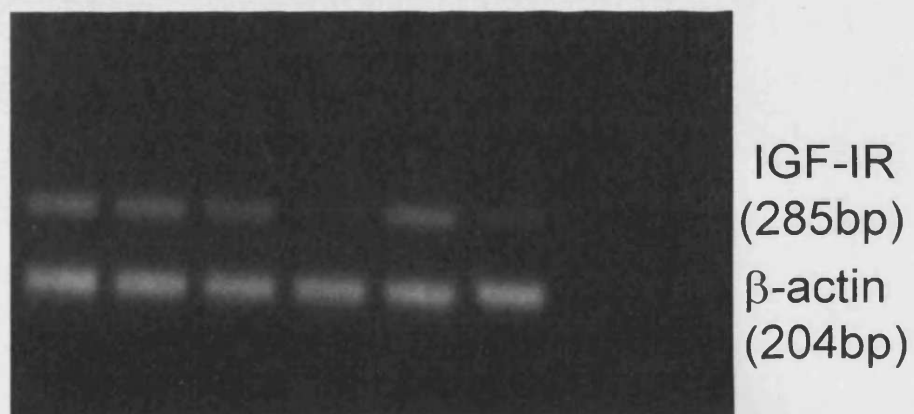
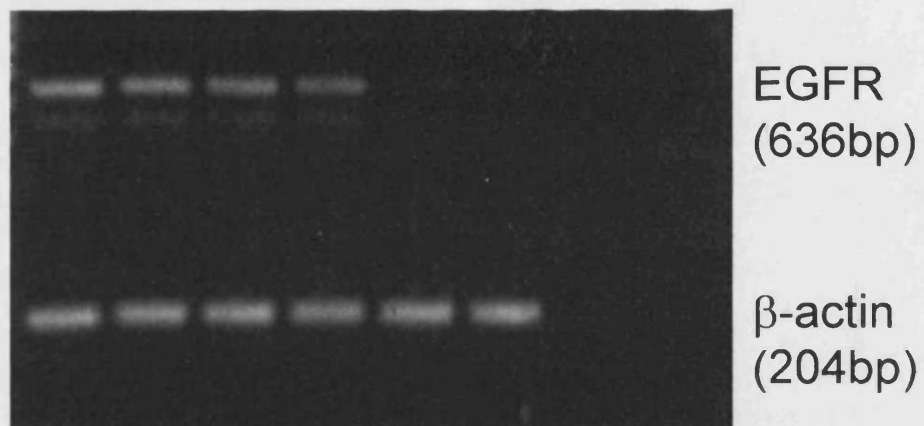
The increase in IGF-IR signalling component activity seen in Tam-R cells in response to gefitinib was maintained in our departmental 'in house' gefitinib resistant Tam-R cells (Tam/TKI-R), with both IRS-1 Y612 and AKT phosphorylation levels increased compared to Tam-R cells (Figure 47). There was no difference in total AKT and IRS-1 expression in the Tam/TKI-R compared to the Tam-R cell line (Figure 47).

**Figure 45.** The effects of IGF-IR, EGFR or a combination of the two siRNAs on total RNA and expression levels of EGFR, IGF-IR and  $\beta$ -actin mRNA in Tam-R cells. Tam-R cells were incubated in phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS supplemented in either culture media alone (C), Dharmafect 1 transfection reagent alone (D), Dharmafect 1 transfection reagent and non-targeting scrambled siRNA mix (S; 100nM), Dharmafect 1 transfection reagent and either IGF-IR (I<sub>SI</sub>) or EGFR (E<sub>SI</sub>) siRNA mix (100nM) or Dharmafect 1 transfection reagent and both EGFR (E<sub>SI</sub>) and IGF-IR (I<sub>SI</sub>) siRNAs in combination (100nM of each) for 2 days for RNA and RT-PCR analysis. Following cell lysis, total RNA and mRNA expression was assessed by (A) ethidium bromide stained agarose gel, (B) semi-quantitative RT-PCR analysis. Data are representative of two separate experiments.

**A**

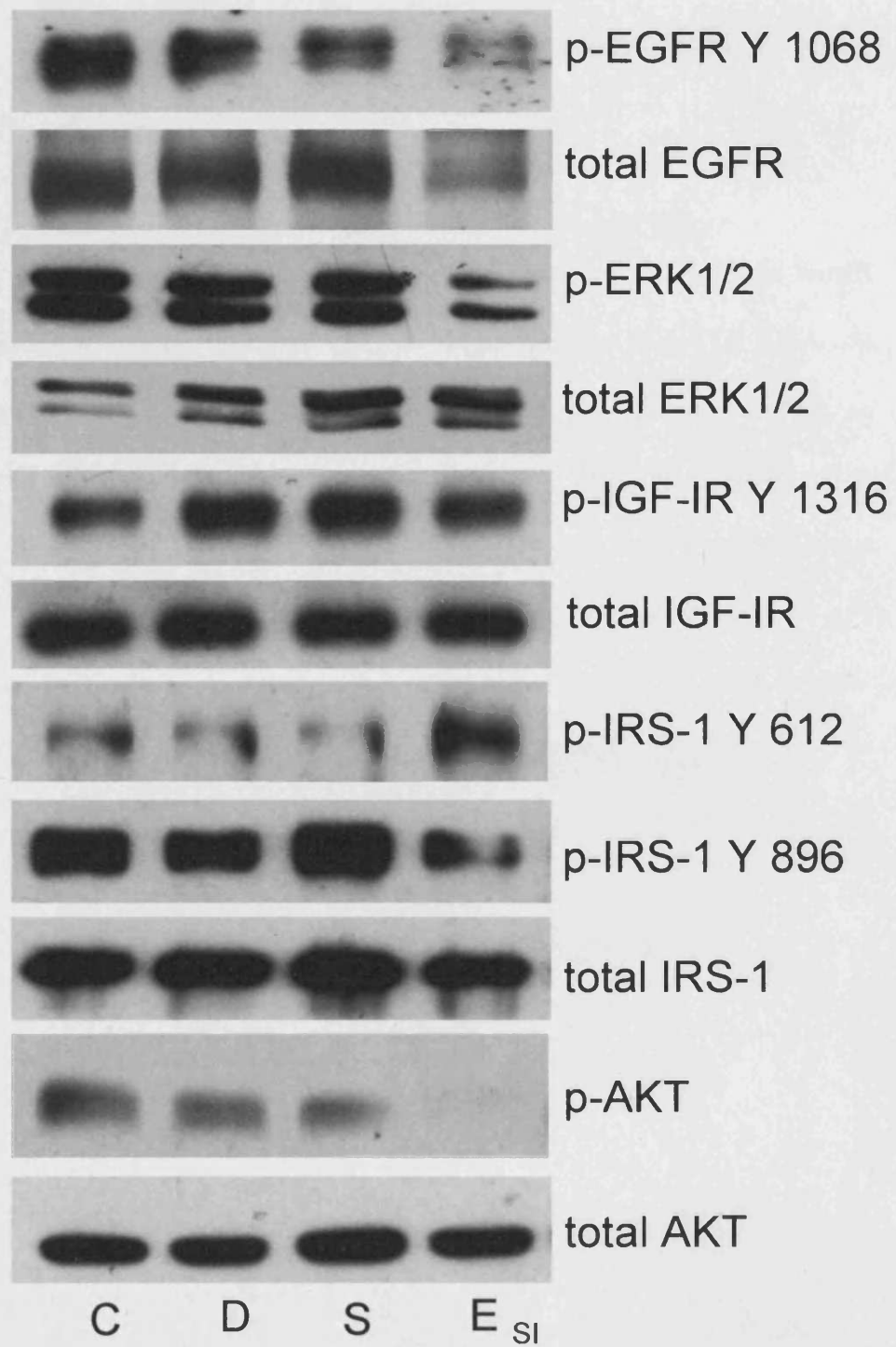


**B**



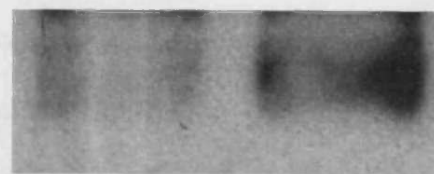
C D S I<sub>SI</sub> E<sub>SI</sub> EI<sub>SI</sub> -ve -ve  
RT PCR

**Figure 46.** The effects of EGFR siRNA on phosphorylated and total EGFR, ERK1/2, IGF-IR, IRS-1 and AKT protein expression in Tam-R cells. Tam-R cells were incubated in phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS for 4 days supplemented in either culture media alone (C), Dharmafect 1 transfection reagent alone (D), Dharmafect 1 transfection reagent and non-targeting scrambled siRNA mix (S; 100nM), or Dharmafect 1 transfection reagent and EGFR (E<sub>SI</sub>) siRNA mix (100nM). Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of two separate experiments.

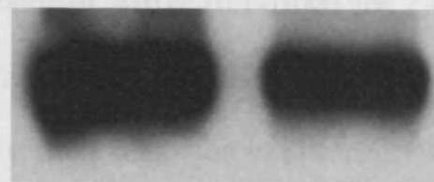


**Figure 47.** Basal expression levels of phosphorylated and total IGF-IR, IRS-1 and AKT in Tam-R and Tam-R/gef-R cells. Tam-R and Tam-R/gef-R cells were incubated in serum-free DCCM medium for 24 hours. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of three separate experiments.

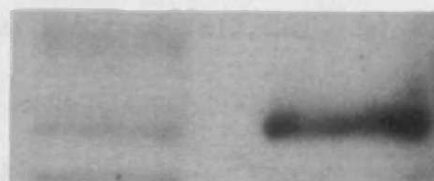




phospho -IGF-1R Y1316



total IGF-IR



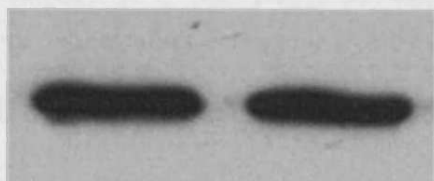
phospho -IRS-1 Y612



total IRS-1



phospho AKT



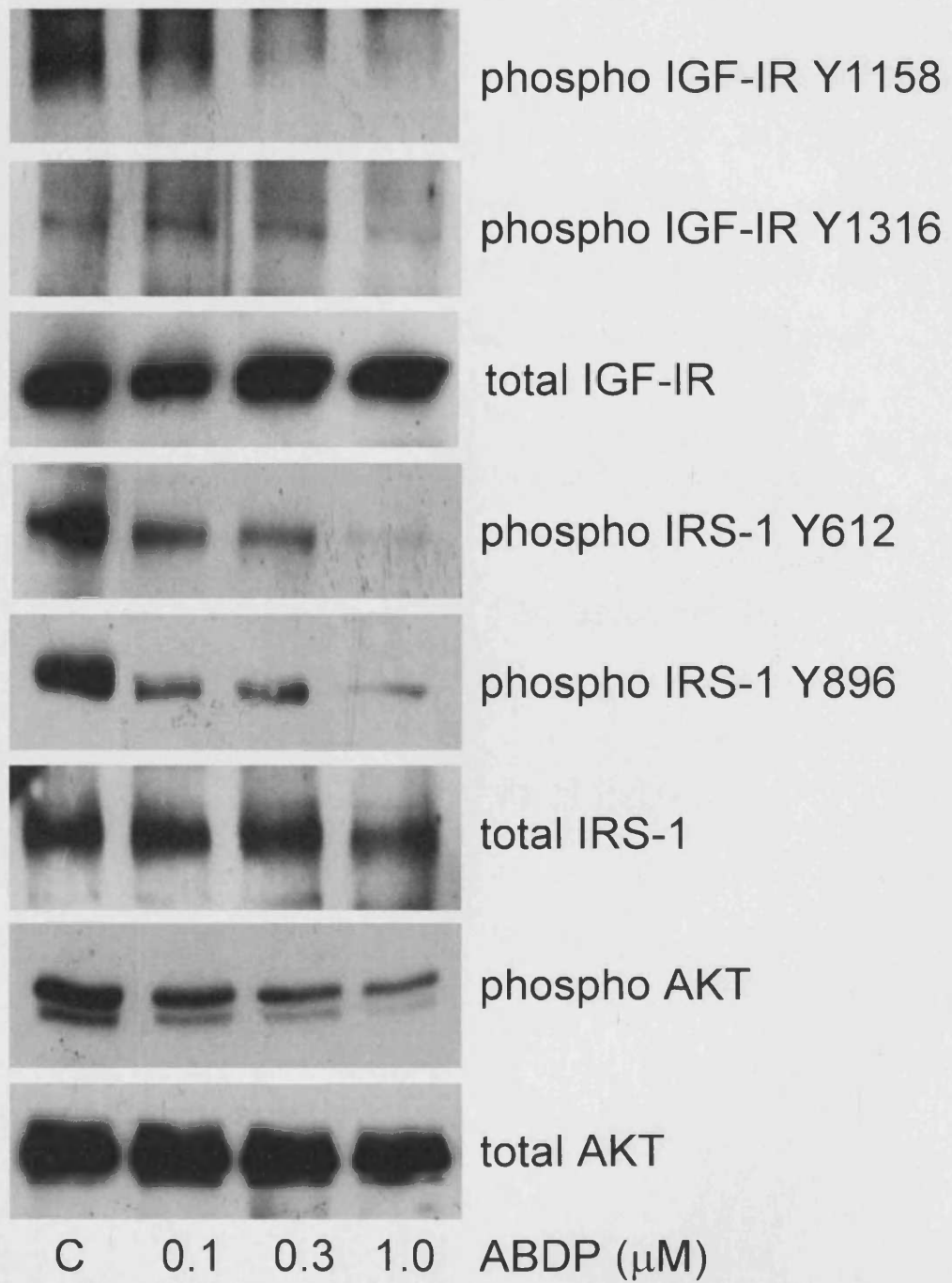
total AKT

Tam-R Tam/ gef-R

### ***3.2.7 IGF-IR blockade inhibits basal IRS-1 signalling activity:***

The effects of an IGF-IR inhibitor on IRS-1 phosphorylation in these cells was also examined. Incubation of Tam-R cells in the presence of increasing concentrations of ABDP (0.1-1 $\mu$ M) for 7 days was shown to inhibit IGF-IR activity (Figure 48) and as shown previously (Figure 10) and this was reflected by the subsequent inhibition in phosphorylation of the down-stream signalling components IRS-1 at Y612, IRS-1 at Y896 and AKT in a concentration dependent manner (Figure 48), with maximum inhibitory effect again observed at 1 $\mu$ M). Moreover, similar findings to those for ABDP were also observed following the silencing of the IGF-IR gene in Tam-R cells using siRNA technology. SiRNA down-regulation of IGF-IR mRNA (Figure 13B and Figure 45C) and protein expression (Figure 13C and Figure 49) also resulted in a reduction in phosphorylation of IRS-1 at Y612, IRS-1 at Y896 and AKT levels 4 days post transfection compared to the medium only, non-targeting siRNA scrambled or Dharmafect 1 reagent (lipid) only controls (Figure 49) with total levels of all other proteins remaining unchanged.

**Figure 48.** The effects of ABDP on basal phosphorylated and total IGF-IR, IRS-1 and AKT protein expression in Tam-R cells. Tam-R cells were grown in phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS for 7 days supplemented with increasing concentrations of ABDP (0.1-1 $\mu$ M) or appropriate vehicle control. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of three separate experiments.



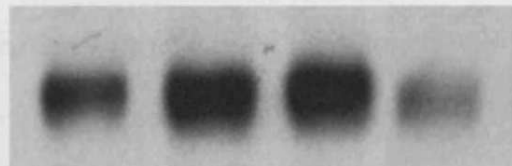
**Figure 49.** The effects of IGFR siRNA on phosphorylated and total IGF-IR, IRS-1 and AKT protein expression in Tam-R cells. Tam-R cells were incubated in phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS for 4 days supplemented in either culture media alone (C), Dharmafect 1 transfection reagent alone (D), Dharmafect 1 transfection reagent and non-targeting scrambled siRNA mix (S; 100nM) or Dharmafect 1 transfection reagent and IGF-IR (I<sub>SI</sub>) siRNA mix (100nM). Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of two separate experiments.

### 3.1.3 Co-localization of IGF-1R and IGF-1R primary antibody with IGF-1R

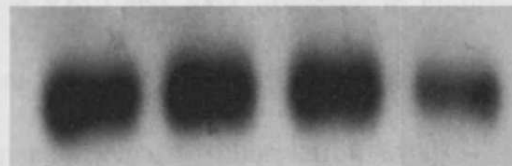
#### IGF-1R signaling and growth of T47D breast cancer cells

In an attempt to further the potential relevance of IGF-1R signaling to our

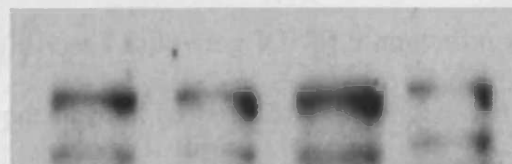
T47D cells, we have used a panel of antibodies to determine the effect of growth on



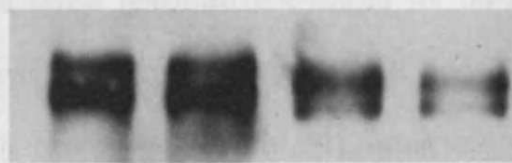
phospho IGF-IR Y 1316



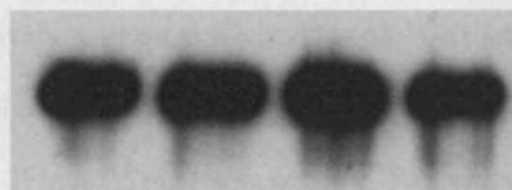
total IGF-IR



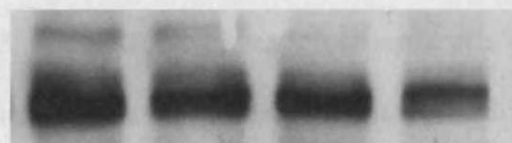
phospho IRS-1 Y 612



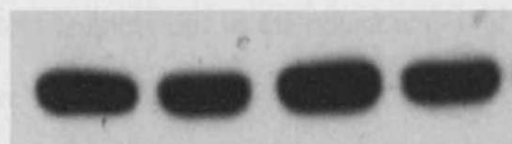
phospho IRS-1 Y 896



total IRS-1



phospho AKT



total AKT

C D S I<sub>Sl</sub>

### ***3.2.8 Co-targeting of EGFR and IGF-IR potentially inhibits both EGFR/IGF-IR signalling and growth of Tam-R breast cancer cells:***

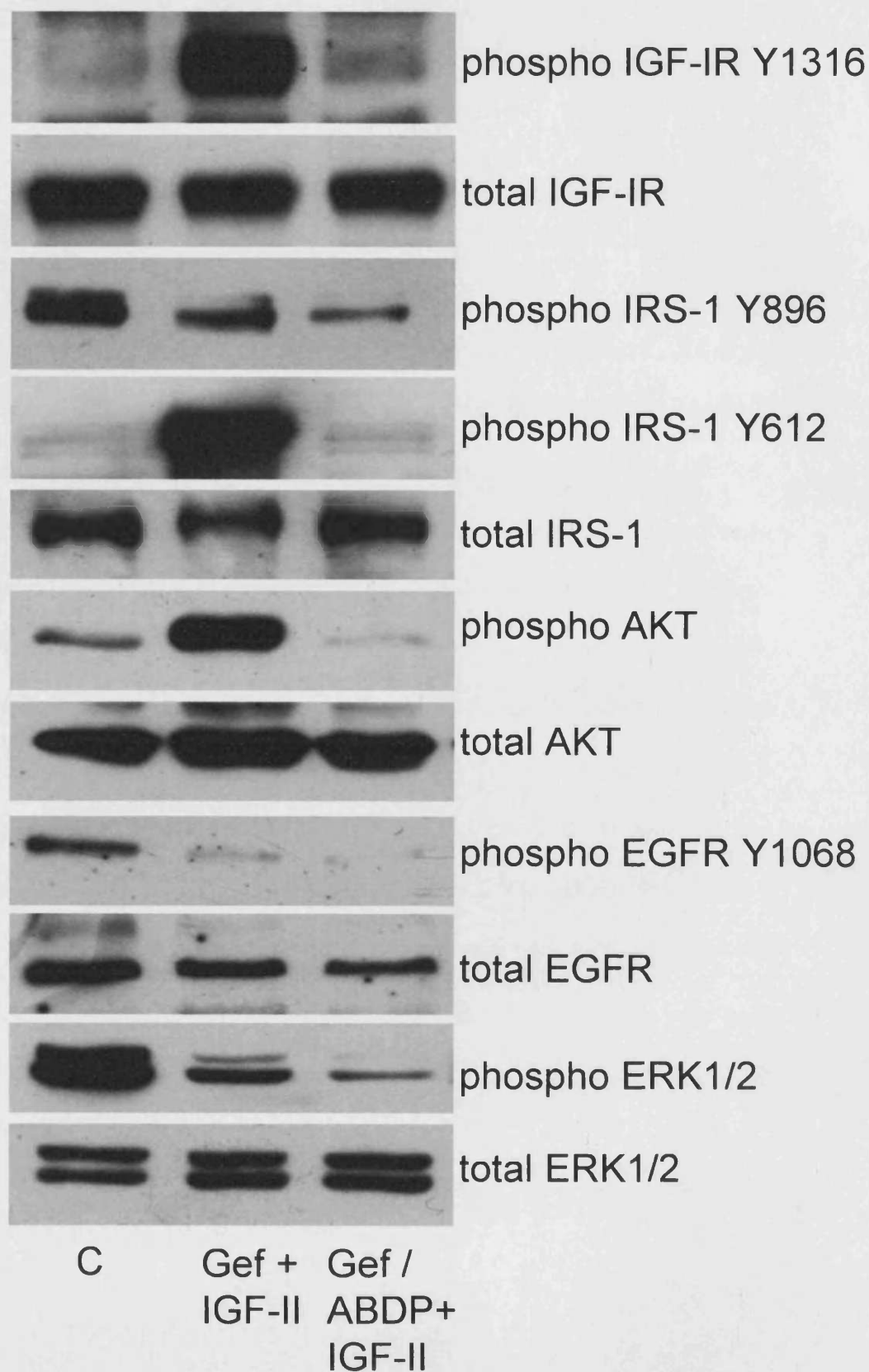
In an attempt to prevent the gefitinib induction of IGF-IR signalling in our Tam-R cells, studies were carried out to determine the effect of gefitinib in combination with an IGF-IR inhibitor on IGF-IR signalling.

The gefitinib-dependent enhancement of IGF-II-mediated phosphorylation of IRS-1 at Y612 and AKT observed in Figure 43 was reversed by treatment of the Tam-R cells with ABDP (1 $\mu$ M) in combination with gefitinib (1 $\mu$ M; Figure 50). In addition, phosphorylation of IRS-1 at Y896, EGFR at Y1068, and ERK1/2 levels following IGF-II stimulation were inhibited to a greater extent in the presence of both inhibitors compared to gefitinib alone (Figure 50). Total protein levels remained unchanged for all treatment groups. Similarly, the selective knockdown of both EGFR and IGF-IR mRNA (Figure 45B) and protein expression (Figure 51) using siRNA technology in Tam-R cells effectively prevented the enhanced IGF-II-mediated IRS-1 Y612 and AKT phosphorylation observed following transfection with siRNA targeting EGFR alone (Figure 51). However, the further reduction in IRS-1 Y896, EGFR Y1068, and ERK1/2 phosphorylation levels seen with gefitinib and ABDP in combination was not so apparent following siRNA co-targeting of these receptors possibly due to the potent action of EGFR siRNA alone in this study (Figure 51).

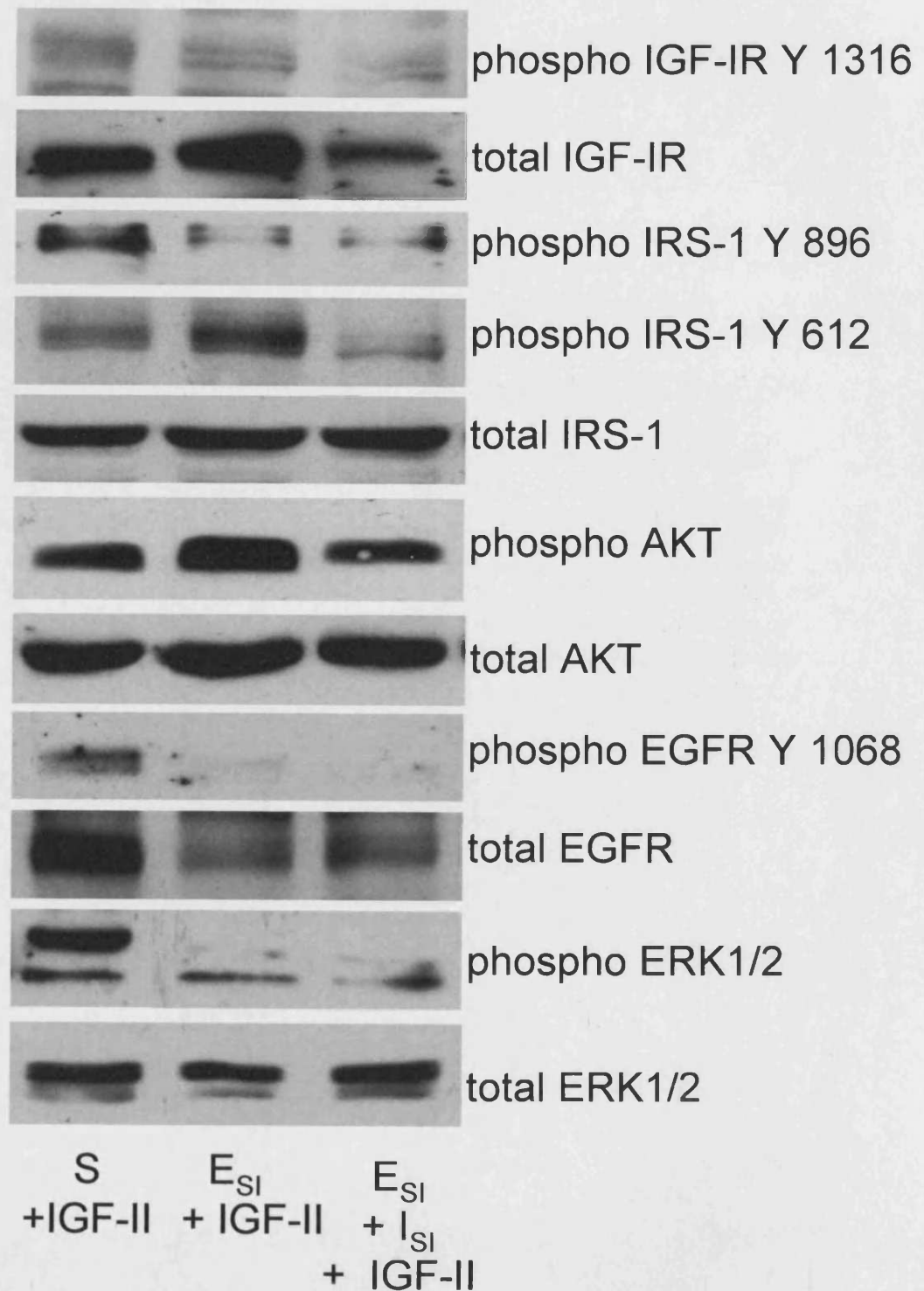
Growth of Tam-R cells was significantly reduced by treatment with gefitinib (1  $\mu$ M) for 7 days ( $P < 0.001$ ,  $n = 3$ ), however, a further significant inhibition of growth was observed following treatment with gefitinib in combination with the IGF-IR tyrosine kinase inhibitor ABDP ( $P < 0.001$ ,  $n = 3$ ; Figure 52).

**Figure 50.** The effects of gefitinib or a combination of gefitinib with ABDP on IGF-II-primed phosphorylation of total IGF-IR, IRS-1, AKT, EGFR and ERK1/2 protein expression in Tam-R cells. Tam-R cells were grown in serum-free DCCM medium, supplemented with either gefitinib (1 $\mu$ M) for 4 days or appropriate vehicle control, gefitinib (1 $\mu$ M) in combination with ABDP (1 $\mu$ M) for 4 days or appropriate vehicle control and subsequently challenged with either IGF-II (100 ng/ml) or vehicle control for 5 minutes. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of three separate experiments.





**Figure 51.** The effects of EGFR siRNA and EGFR in combination with IGF-IR siRNA on IGF-II-primed phosphorylated and total IGF-IR, IRS-1, AKT, EGFR and ERK1/2 protein expression in Tam-R cells. Tam-R cells were incubated in phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS for 4 days supplemented with either Dharmafect 1 transfection reagent and non-targeting scrambled siRNA mix (100nM), Dharmafect 1 transfection reagent and EGFR siRNA mix (100nM) or Dharmafect 1 transfection reagent, EGFR siRNA (100nM and IGF-IR siRNA mix (100nM) and subsequently challenged with IGF-II (100ng/ml) for 5 minutes. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of at least three separate experiments.

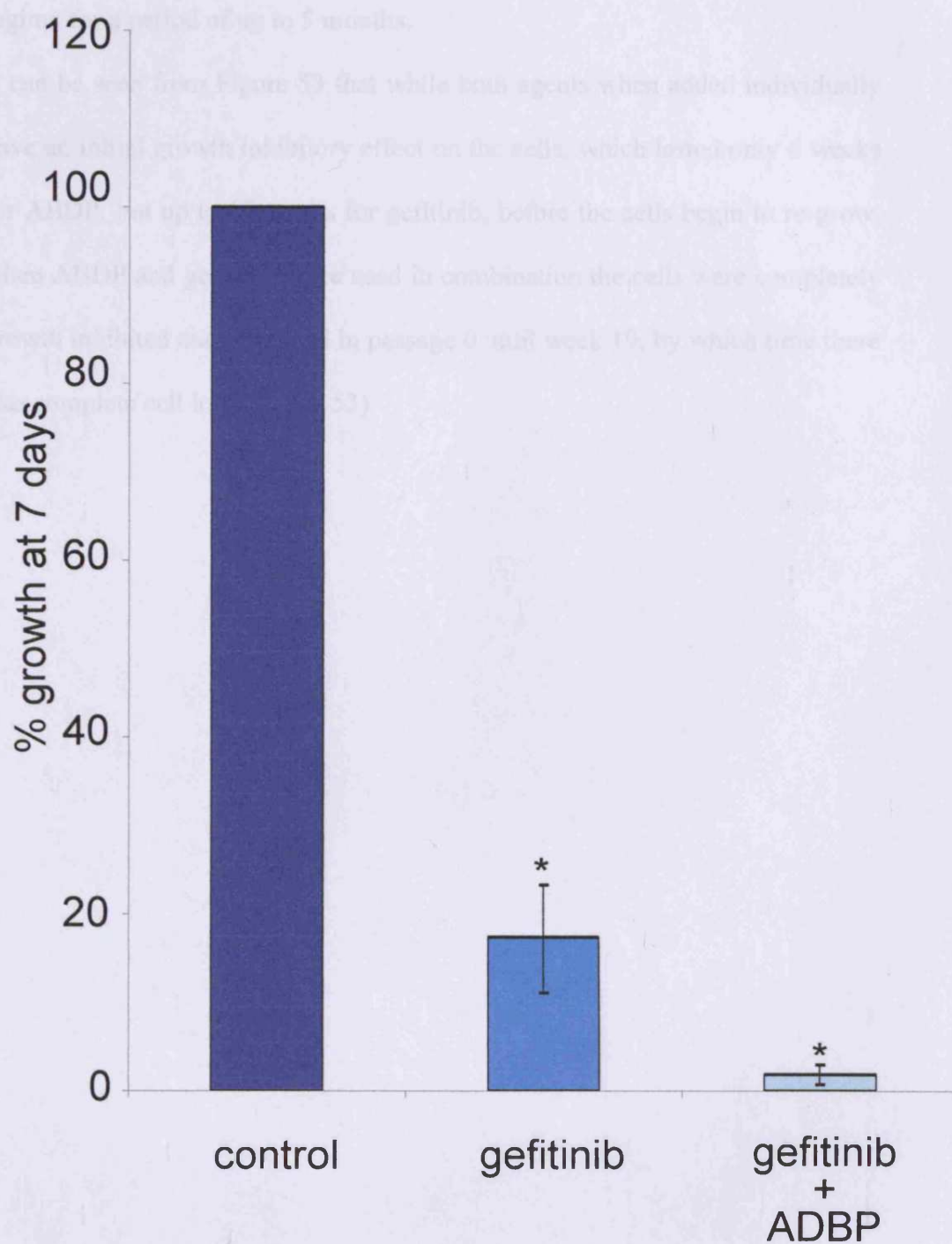


**Figure 52.** The effects of gefitinib or gefitinib and ABDP in combination on the basal growth of Tam-R cells. Tam-R cells were grown in phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS for 7 days supplemented with gefitinib (1 $\mu$ M) or gefitinib and ABDP in combination (1 $\mu$ M of each). The results are expressed as mean $\pm$ SEM of triplicate wells and are representative of three separate experiments. A p value of  $\leq 0.05^*$  was considered statistically significant.

### 4.2.9 Long-term response of EGFR and IGF-1R blockade inhibits growth of Tumor breast cancer cells

To determine whether the success of this novel system combined EGFR and IGF-1R blockade was significant, Tumor-R cells were exposed to this treatment regimen for up to 5 months.

It can be seen from Figure 5D that while both agents when added individually have an inhibitory growth inhibitory effect on the cells, which lasted only 3 weeks for gefitinib and up to 5 weeks for ADBP, before the cells begin to regrow, when ADBP and gefitinib were used in combination the cells were completely growth inhibited for up to 5 months (passage 0 until week 19, by which time there was a slight regrowth of the cells).



***3.2.9 Long-term exposure of EGFR and IGF-IR blockade inhibits growth of Tam-R breast cancer cells:***

To determine whether the success of this more potent combined EGFR and IGF-IR blockade was maintained, Tam-R cells were exposed to this treatment regime for a period of up to 5 months.

It can be seen from Figure 53 that while both agents when added individually have an initial growth inhibitory effect on the cells, which lasted only 6 weeks for ABDP, but up to 15 weeks for gefitinib, before the cells begin to re-grow, when ABDP and gefitinib were used in combination the cells were completely growth inhibited and remained in passage 0 until week 19, by which time there was complete cell loss (Figure 53).

**Figure 53.** The effects of gefitinib, ABDP, or a combination of gefitinib and ABDP on long-term growth of Tam-R cells. Tam-R cells were grown for up to 23 weeks in phenol red-free RPMI medium containing 5% charcoal-stripped steroid-depleted FCS supplemented with gefitinib (1 $\mu$ M), ABDP (0.25 $\mu$ M), a combination of gefitinib and ABDP or appropriate vehicle control. The effects of growth were assessed by defining passage number.





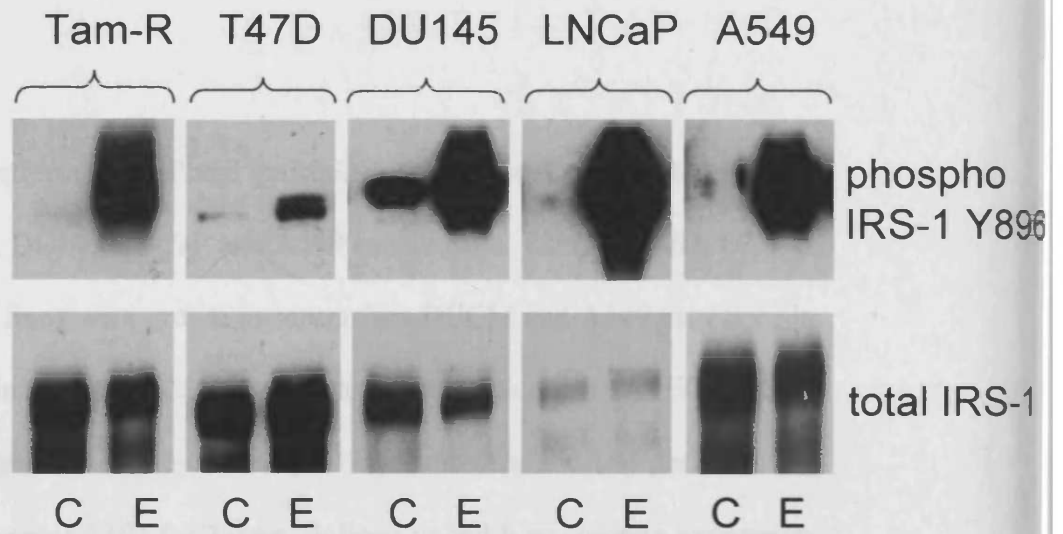
### ***3.2.10 EGFR recruits IRS-1 in a range of EGFR-positive cancer cell lines:***

Further immunoprecipitation/Western blot studies were performed in a range of cancer cell types to investigate whether this ability of EGFR to recruit and phosphorylate IRS-1 was specific to Tam-R MCF-7 cells or a more general cancer cell phenomenon.

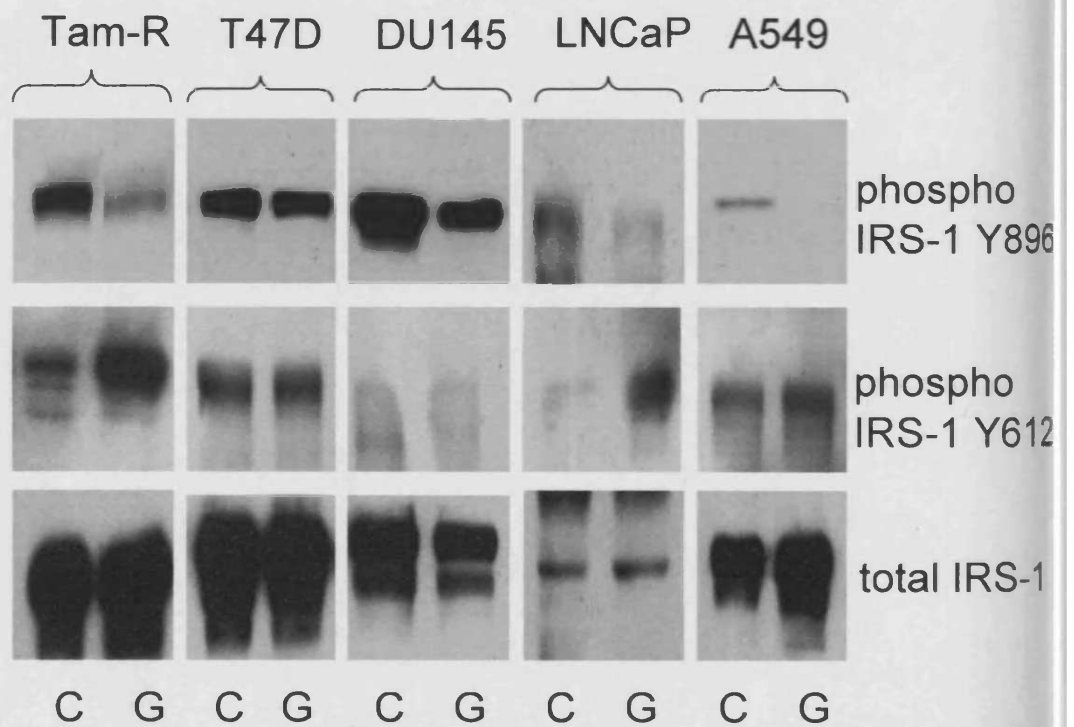
Treatment of T47D breast, LNCaP and DU145 prostate and A549 NSCLC cancer cells with EGF (10ng/ml) dramatically increased the phosphorylation levels of IRS-1 at Y896 compared to the controls, as previously seen for Tam-R cells, as shown by Western analysis (Figure 54A). In addition, treatment of these cell lines with gefitinib (1 $\mu$ M) for 7 days, revealed that basal phosphorylation levels of IRS-1 at Y896 were inhibited in all cell lines to varying degrees, again, mirroring results seen for Tam-R cells (Figure 54B). Moreover, such treatment resulted in an increase in phosphorylation of IRS-1 at Y612 in the LNCaP cells (Figure 54B) as seen previously for Tam-R cells. However, there was no significant effect of gefitinib on IRS-1 Y612 phosphorylation levels in the other three cancer cell lines studied (Figure 54B). Total protein IRS-1 levels were found to be differentially expressed among the cancer cell types with LNCaPs expressing the least amount of IRS-1. There was no effect of any of the treatments on total protein expression in any of the cell lines studied.

**Figure 54.** The effects of EGF and gefitinib on phosphorylated and total IRS-1 in Tam-R T47D, DU145 LNCaP and A549 cancer cells. Tam-R, T47D, DU145 and LNCaP cell lines were grown in serum-free DCCM and A549 cancer cells in DCCM containing 0.5% FCS each supplemented with either (A) EGF (E; 10 ng/ml) or appropriate vehicle control (C) for 5 minutes and (B) gefitinib (G; 1 $\mu$ M) or vehicle control (C) for 7 days. Following cell lysis, protein expression was assessed by Western blot analysis. Data are representative of at least three separate experiments.

**A**



**B**



### ***3.2.11 IRS-1 associates with EGFR in LNCaP prostate cancer cells:***

Further analysis of LNCaP cells demonstrated that EGF promoted a direct physical association between EGFR and IRS-1 resulting in increased phosphorylation of IRS-1 at Y896 (Figure 55A) following immunoprecipitation / Western blotting analysis, mirroring results seen for Tam-R cells. Total EGFR protein levels remained unchanged following EGF treatment.

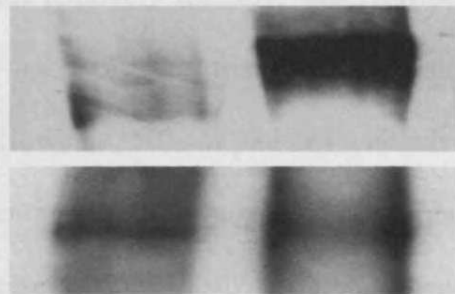
### ***3.2.12 EGFR blockade enhances association of IRS-1 with IGF-IR in LNCaP prostate cancer cells:***

Treatment of LNCaP prostate cancer cells with gefitinib (1 $\mu$ M) resulted in a loss in association between EGFR and IRS-1 and a reduction in IRS-1 Y896 phosphorylation (Figure 55B), whilst association of IRS-1 with IGF-IR and IRS-1 phosphorylation at Y612 were enhanced (Figure 55C), paralleling the results observed in Tam-R cells. Once again total protein levels remained unchanged following gefitinib treatment.

**Figure 55.** The effects of EGF on heterodimer formation between EGFR and IRS-1 and the effects of gefitinib on heterodimer formation between EGFR and IRS-1 and IGF-IR and IRS-1 in LNCaP cells. LNCaP cells were incubated in serum-free DCCM medium either in the presence of EGF (10ng/ml) or appropriate vehicle control for 5 minutes. Following cell lysis, protein expression was assessed by Western blot analysis for (A) phosphorylated IRS-1 and total EGFR following EGFR immunoprecipitation. LNCaP cells were grown for 7 days in serum-free DCCM medium supplemented with gefitinib (1 $\mu$ M) or vehicle control only. Following cell lysis, protein expression was assessed by Western blot analysis for (B) phosphorylated IRS-1 and total EGFR and (C) phosphorylated IRS-1 and total IGF-IR following EGFR and IGF-IR immunoprecipitation respectively. Data are representative of three separate experiments.

Immunoprecipitation: total EGFR

**A**



phospho IRS-1 Y896

total EGFR

control

EGF

Immunoprecipitation: total EGFR

**B**



phospho IRS-1 Y896

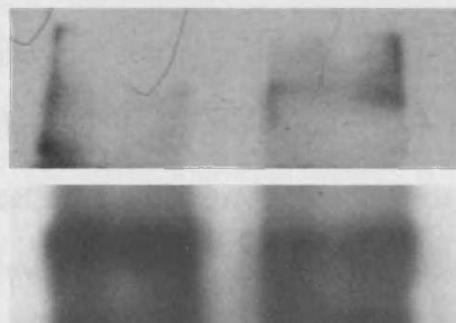
total EGFR

control

gefitinib

Immunoprecipitation: total IGF-IR

**C**



phospho IRS-1 Y612

total IGF-IR

control

gefitinib

#### **4.0 Discussion:**

Tamoxifen, until relatively recently, was considered by many to be the first-line endocrine agent for the treatment of oestrogen receptor (ER)-positive breast cancer. However, 50% of patients with advanced disease do not respond to first-line treatment with tamoxifen. Furthermore, almost all patients with metastatic disease and up to 40% of the patients that receive tamoxifen as adjuvant therapy will eventually relapse due to the development of tamoxifen resistance (Howell *et al.*, 1993; Osborne and Fuqua, 1994; Normanno *et al.*, 2005; Ring and Dowsett, 2004). Acquired resistance to tamoxifen is thus a serious therapeutic problem and major efforts are now being made to understand the underlying mechanisms responsible for this condition (Howell *et al.*, 1993; Osborne and Fuqua, 1994; Clarke *et al.*, 2001; Normanno *et al.*, 2005; Nicholson *et al.*, 2004; Nicholson *et al.*, 2005). Although a small proportion of patients show loss of ER on tamoxifen relapse (Dowsett *et al.*, 2003; Ring and Dowsett, 2004), it is unlikely to be the major mechanism contributing to the resistant state as the majority of tamoxifen-resistant tumours and breast cancer cell lines demonstrate sustained ER content and remain responsive to pure anti-oestrogen therapy (Encarnacion *et al.*, 1993; Brunner *et al.*, 1993; Lykkesfeldt *et al.*, 1994; Robertson, 1996). Therefore, it has been proposed that cells in the presence of anti-oestrogens use an alternative growth regulatory pathway (Nicholson *et al.*, 2004; Nicholson *et al.*, 2005). Indeed, there is now an increasing body of evidence that suggests that alterations in growth factor signalling networks, that are also highly interactive with the ER, can readily promote anti-hormone resistance in breast cancer. Such signalling networks including members of the type 1 super family of RTKs such as EGFR

and c-erbB2 (Nicholson *et al.*, 2004; Nicholson *et al.*, 2005). Studies from our group have demonstrated that WT-MCF-7 breast cancer cells are able to establish an alternative basal growth regulatory pathway following long-term treatment in the presence of tamoxifen (Knowlden *et al.*, 2003). Upon acquisition of resistance to this anti-oestrogen these cells, designated Tam-R, utilise a mechanism of growth which involves the autocrine release and action of the EGFR-specific ligand AR inducing marked increases in EGFR/c-erbB2 dimerisation /phosphorylation and subsequent downstream activation of ERK1/2 MAPK and Akt (Knowlden *et al.*, 2003; Britton *et al.*, 2006; Jordan *et al.*, 2004).

More recently, another growth factor signalling pathway has been shown to be functional in breast cancer, the type II receptor tyrosine kinase IGF-IR signalling cascade (Surmacz, 2000). Increased expression and activation of IGF-IR and its associated downstream signalling components have been reported in clinical breast cancer specimens and linked to disease progression and recurrence (Rocha *et al.*, 1997; Turner *et al.*, 1997). Furthermore, expression of IGF-IR has been found in most breast cancer cell lines including endocrine-responsive MCF-7 cells (Surmacz, 2000; Lee *et al.*, 1999; Stewart *et al.*, 1990) where a significant degree of productive cross-talk has now been shown to exist between the IGF-IR and the ER (Hamelers and Steenbergh, 2003; Yee and Lee, 2000; Lee *et al.*, 1997). In addition to tamoxifen possessing anti-oestrogenic activity, through its ability to block ER/ERE signalling, it also has anti-growth factor actions and can perturb cross-talk between oestrogens and the IGF-IR. Indeed, it has been shown to inhibit IGF-I-



mediated proliferation in ER-positive breast cancer cells (Yee and Lee, 2000; Lee *et al.*, 1997; Guvakova *et al.*, 1997).

Although there is extensive evidence supporting a role for IGF-IR signalling in endocrine responsive breast cancer, there is also some limited experimental and clinical data suggesting an IGF-IR involvement in endocrine resistance. Indeed, it has been reported that tamoxifen-resistant MCF-7 cell lines demonstrate increased sensitivity to the proliferative effects of IGF-I/II following treatment with either oestradiol or tamoxifen (Parisot *et al.*, 1999; Wiseman *et al.*, 1993) and the selective anti-IGF-1R monoclonal antibody  $\alpha$ IR-3 has been shown to block growth of tamoxifen-resistant MCF-7 cell variants (Parisot *et al.*, 1999). Significantly, there is now growing evidence to suggest that a degree of cross-talk exists between members of the type I and type II RTK families and their ligands in several cell models. Studies have highlighted the presence of a direct association between IGF-IR and EGFR in breast cancer cells (Shou *et al.*, 2003), in NSCLC cells (Morgillo *et al.*, 2006), in normal mammary epithelial cells (Ahmad *et al.*, 2004) and between IGF-IR and c-erbB2 in mammary epithelial cancer cells, including MCF-7 cells (Balana *et al.*, 2001). Support for the presence of indirect mechanisms of cross-talk between IGF-IR and EGFR and their ligands have also been reported in a range of cell lines (Coppola *et al.*, 1994; Vardy *et al.*, 1995; Burgaud and Baserga; 1996, Roudabush *et al.*, 2000; Hurbin *et al.*, 2002; Wang *et al.*, 2002; Morgillo *et al.*, 2006; Desbois-Mouthon *et al.*, 2006), although, evidence for this in breast cancer cell lines remains very limited (Gilmore *et al.*, 2002).

Since high levels of cross-talk have been shown to exist between EGFR and

IGF-IR in breast cancer (Shou *et al.*, 2003; Gilmore *et al.*, 2002) and as both receptors have been implicated in endocrine resistant breast cancer, in the present study, we sought to investigate the role played by the IGF-II/IGF-IR axis in a MCF-7 breast cancer cell model of acquired resistance to anti-hormones by examining potential areas of cross-talk between IGF-IR and EGFR. Three major observations were made:

1. IGF-IR signalling contributes to EGFR responses in tamoxifen-resistant breast cancer by a c-SRC-dependent mechanism.
2. Activated EGFR sequesters IRS-1 as part of its growth mechanism in Tam-R cells and EGFR inhibition facilitates IRS-1 interactions with IGF-IR as a survival pathway.
3. Co-targeting of EGFR/IGF-IR is more effective in the treatment of Tam-R cells, than single agent therapy.

These observations are discussed below.

#### ***4.1 EGFR and IGF-IR cross-talk in Tam-R WT-MCF-7 breast cancer cells:***

##### ***A c-SRC-mediated event:***

Initial studies demonstrated that Tam-R cells have lower levels of IGF-IR than their WT-MCF-7 counterparts, consistent with previous studies in tamoxifen-resistant MCF-7 and ZR-75-1 cells (Brockdorf *et al.*, 2003; McCotter *et al.*, 1996). The reduction in IGF-1R levels in the Tam-R cells was not altogether surprising as this gene has been shown to be oestrogen-regulated in MCF-7 cells (Surmacz, and Burgaud, 1995), indeed, a reduction in IGF-1R protein expression was observed 6 weeks post tamoxifen treatment in WT-MCF-7 cells in the present study. However, it should also be noted that there was no parallel

reduction in IGF-1R mRNA expression at the same 6 weeks time point in this cell line. Such discrepancies in IGF-IR levels observed may simply be due to differing inherent procedural sensitivities or differing protein or mRNA stability times. Despite having lower IGF-IR expression levels, the Tam-R cells nevertheless expressed an equivalent level of phosphorylated IGF-IR to that observed in the WT-MCF-7 cells. Phosphorylation of IGF-1R, and not insulin receptor, was confirmed by both Western blotting and immunocytochemistry using an antibody targeting IGF-1R phosphorylated at Y1316. This antibody, unlike those targeting Y1158 and Y1131 which were also used in this study, does not cross-react with insulin receptor. This maintenance of IGF-1R activity through to acquisition of tamoxifen-resistance was also associated with increased levels of IGF-II mRNA in the Tam-R cell line, a result in accordance with Lee & Yee, (1995). IGF-I mRNA, however, could not be detected in either cell line, in agreement with Quinn and colleagues (1996). IGF-II, like IGF-1R, has been shown to be oestrogen-regulated in MCF-7 cells (Osborne *et al.*, 1989; Lee *et al.*, 1994) and there was a suggestion of a reduction in IGF-II mRNA levels 6 weeks post tamoxifen treatment of WT MCF-7 cells in the present study. A possible explanation for the subsequent increase in IGF-II mRNA expression observed in the Tam-R cell may reside in the fact that Tam-R cells also express elevated levels of EGFR/MAPK-mediated serine 118 phosphorylated ER $\alpha$ , compared to the parental WT-MCF-7 cells (Britton *et al.*, 2006). It is thought that such phosphorylation of ER $\alpha$  results in re-activation of this protein as a nuclear transcription factor leading to re-expression of previously suppressed oestrogen-regulated genes, such as AR (Britton *et al.*, 2006). Thus, it is possible that the re-activation of ER $\alpha$  function in Tam-R cells

may also mediate re-expression of IGF-II mRNA in these cells. Indeed, in support of this suggestion it has been shown that inhibition of ER $\alpha$  function using the pure anti-oestrogen fulvestrant results in reduction of IGF-II mRNA in the Tam-R cell line (unpublished personal observation), although it is noteworthy that no equivalent studies at a protein level have as yet, been undertaken. Significantly, the lack of increased expression of IGF-IR versus IGF-II in Tam-R cells, may reflect their differential regulation by AF-1 and AF-2 regulatory elements in ERs. Thus, the phosphorylation of ER $\alpha$  at serine 118 results in the re-activation of the AF-1 domain of ER $\alpha$  and hence enhancement of IGF-II levels, whilst the AF-2 domain and IGF-IR expression remains inhibited due to binding of tamoxifen and recruitment of co-repressor proteins to this domain (Britton *et al.*, 2006). However, further studies are required to examine this possibility.

Importantly, the maintenance of IGF-1R phosphorylation in Tam-R cells suggests that the IGF-1R plays a role in tamoxifen resistance, supporting the previous findings of Parisot *et al.*, (1999) and Wiseman *et al.*, (1993). Indeed, in the current study, exogenous IGF-II promoted an increase in cell proliferation as well as an increase in IGF-IR phosphorylation in both a concentration and time-dependent manner in Tam-R cells, as well as in their WT-MCF7 counterparts. Significantly, however, IGF-II also increased EGFR phosphorylation in the Tam-R cells, a finding not evident in WT-MCF-7 cells. Interestingly, treatment of Tam-R cells with the IGF-IR tyrosine kinase inhibitors AG1024 and ABDP inhibited both basal and IGF-II-primed IGF-IR and EGFR/ERK1/2 signalling and significantly inhibited cell growth. Equivalent findings were also observed following treatment of these cells with

an IGF-II neutralizing antibody and following knockdown of IGF-IR mRNA expression using siRNA technology. Similarly, Parisot *et al*, (1999), have demonstrated that growth of a tamoxifen-resistant MCF-7/2-23 cell line can be enhanced by IGF-I and inhibited by the IGF-IR monoclonal antibody,  $\alpha$ IR-3.

These present findings in Tam-R cells confirm a role for IGF-IR, through its ability to cross-talk and activate EGFR, in mediating tamoxifen-resistant cell growth and such cross-talk is initiated, under basal growth conditions, by the autocrine release and action of IGF-II. Furthermore, since the activation of EGFR with the exogenous growth factor EGF failed to promote IGF-IR phosphorylation in Tam-R cells, this IGF-IR/EGFR cross-talk mechanism is apparently unidirectional in this cell line. A role for IGF-IR/EGFR cross-talk in mediating tamoxifen-resistant growth is further supported by the finding that treatment of Tam-R cells with the selective EGFR tyrosine kinase inhibitor gefitinib, potently inhibited IGF-II-induced EGFR activity and proliferation whilst having no effect on either basal or ligand-induced activation of IGF-IR. Although, to date, there appears to be no conclusive clinical data reported in the literature regarding the relevance of IGF-IR expression to tamoxifen resistance, our group has recently developed immunocytochemical assays for the detection of both total and phospho-specific IGF-IR Y1316 expression in clinical breast cancer specimens. When these assays were applied to a small cohort of tumours, taken from patients who have ER-positive/EGFR-positive acquired tamoxifen resistance, IGF-IR expression and activity were readily detectable (Gee *et al.*, 2005). This indicates that such IGF-IR signalling observed *in vivo* may indeed be functional and play a role in supporting the growth of such

tumours in acquired tamoxifen resistance, complementing our findings in Tam-R cells.

It should be noted that, a similar reduction in growth was also observed in WT-MCF-7 cells with both AG1024 and ABDP inhibiting the growth stimulatory effects of IGF-II, this growth inhibitory effect being associated with blockade of IGF-IR activation in these cells. Interestingly, AG1024 was also shown to enhance, rather than inhibit, ERK1/2 phosphorylation following treatment of WT-MCF-7 cells. This may reflect the fact that AG1024 is not as selective for IGF-IR as ABDP and may have some off-target effects at the concentration used in this study. Furthermore, IGF-II was also shown to have no effect on EGFR phosphorylation in WT-MCF7 cells. The inability to detect IGF-II-induced increases in EGFR phosphorylation in the parental cells would indicate that such a cross-talk mechanism is not functional in these cells and may simply be due to their very low EGFR expression levels (Knowlden *et al.*, 2003).

As already mentioned, there already exists a wealth of evidence that cross-talk occurs between the IGF-IR and EGFR and their ligands, where both direct and indirect interactions have been observed in several different cell types including the breast (Shou *et al.*, 2003; Ahmad *et al.*, 2004; Gilmore *et al.*, 2002; Coppola *et al.*, 1994; Vardy *et al.*, 1995; Burgaud and Baserga; 1996, Roudabush *et al.*, 2000; Hurbin *et al.*, 2002; Wang *et al.*, 2002; Morgillo *et al.*, 2006; Desbois-Mouthon *et al.*, 2006). However, mechanistically, we found no evidence to suggest that interactions between the IGF-IR and EGFR in the Tam-R cells occurs through a direct physical association of the receptors. Indeed, our own studies revealed that the stimulation of Tam-R cells with IGF-

II resulted in a time-delayed phosphorylation of EGFR. A recent report successfully identified active heterodimers between these two receptors that could be disrupted by gefitinib in both MCF-7 and T47D breast cancer cell lines (Shou *et al.*, 2003). This latter data being supported by an investigation in mammary epithelial cells (Ahmad *et al.*, 2004). Contrastingly, Gilmore *et al.*, (2002) has demonstrated that IGF-I indirectly trans-activated EGFR and its downstream MAPK signalling pathway in mammary epithelial cells, although they failed to identify the mechanism involved

In an attempt to further dissect how this cross-talk is occurring in our Tam-R cells, we performed a series of experiments examining other possible indirect mechanisms. Firstly, we investigated the potential role of MMPs. A report by (Roudabush *et al.*, (2000) demonstrated that trans-activation of EGFR can occur via an autocrine/paracrine mechanism involving MMP-dependent release of HB-EGF in response to IGF stimulation in Cos-7 cells, with such a pathway being blocked by a broad-spectrum MMP inhibitor 1,10-phenanthroline. Similarly, a more recent report by Desbois-Mouthon *et al.*, (2006), also showed that trans-activation of EGFR can occur in hepatocellular carcinoma cells by a mechanism involving TACE-dependent release of AR, which results in downstream activation of ERK1/2 and ultimately leading to cell proliferation. We examined the effects of a broad spectrum MMP inhibitor, MMP-II, to determine whether it would block EGFR activation in our Tam-R cells. However, treatment of the cells with a range of concentrations of this inhibitor appeared to have no inhibitory effect on basal or IGF-II-primed EGFR phosphorylation and if anything, MMP-II was shown to enhance levels of EGFR phosphorylation, thus suggesting that this was not the mechanism used

by these cells. This was in agreement with another report who showed that trans-activation of EGFR could not be blocked with another broad spectrum MMP inhibitor GM6001, Ilomastat (Olayioye *et al.*, 2000). It is possible, that the increased EGFR activity observed in these cells following MMP-II treatment is simply due to some off-target effects promoted by this broad-spectrum MMP inhibitor.

Having established that MMPs are unlikely to be involved in EGFR/IGF-IR cross-talk in our tamoxifen-resistant MCF-7 cells, we next examined the potential role of c-SRC. c-SRC is one member of a closely related family of non-receptor tyrosine kinases that share important regulatory mechanisms. Other members of this group also include: Lck; Hyk; Blk; Fyn; Lyn; Fgr; Yes and Yrk (Sicheri and Kuriyan, 1997). The domain structure of c-SRC consists of a myristylated N-terminal “unique” domain, important for cell membrane interactions, which differs among family members. This region is followed by an SH3 domain, an SH2 domain, a tyrosine kinase domain, and a short C-terminal tail (Superti-Furga, 1995). The activity of c-SRC is regulated by tyrosine phosphorylation at two sites with opposing effects. In humans, phosphorylation of tyrosine Y418 in the activation loop of the kinase domain up-regulates the enzyme and is required for full catalytic activity, whereas phosphorylation of Y527 in the C-terminal tail renders the enzyme less active. The enzyme is phosphorylated ‘*in vivo*’ at either Y418 or Y527, but not both at the same time (Superti-Furga and Courtneidge, 1995; Superti-Furga, 1995). Extensive biochemical and genetic characterizations have led to a model for c-SRC regulation in which the interaction of the phosphorylated C-terminal tail



with the SH2 domain locks the molecule in an inhibited and closed conformation (Matsuda *et al.*, 1990).

c-SRC functions as a transducer of trans-membrane signals arising from a variety of polypeptide growth factor receptors, including the EGFR in a range of cell lines including breast cancer (Luttrell *et al.*, 1994; Roche *et al.*, 1995; Abram and Courtneidge, 2000; Zhang *et al.*, 2004; Shah *et al.*, 2003). It has been reported to be over expressed in a variety of cancers and in breast cancer over expression of this protein was found to occur in a majority of patients (Biscardi *et al.*, 2000). Moreover, it has been shown to be a critical component of EGF-induced mitogenesis and may cooperate with the EGFR synergistically in promoting breast cancer progression (Maa *et al.*, 1995; Luttrell *et al.*, 1988). With respect to the current study c-SRC has also been shown to be activated by IGF-I in 3T3-L1 pre-adipocytes (Boney *et al.*, 2001) and in neuroblastoma cells (Bence-Hanulec *et al.*, 2000) and shown to mediate EGFR trans-activation through direct association with EGFR and phosphorylation of tyrosine residues Y845 and Y1101.

The effect of IGF-II on c-SRC activation in Tam-R cells was first examined. c-SRC Y418 phosphorylation levels observed under basal conditions were enhanced following the treatment of these cells with IGF-II in a time-dependent manner, although IGF-II was shown to have no effect on c-SRC phosphorylation levels in WT-MCF-7 cells despite total c-SRC levels being similar in the two cell lines. This may be due to the ability of IGF-II to potentially promote a strong interaction between IGF-IR and IRS-1 in these cells, thus limiting the amount of IGF-IR to link up with other molecules such as c-SRC (as discussed later in section 4.2). A similar inhibition of basal and

also IGF-II-induced c-SRC activity was observed following a block on IGF-IR activation using the IGF-IR inhibitors AG1024 and ABDP. Interestingly, such basal c-SRC activity in Tam-R cells could be reduced following a block on IGF-II autocrine action using an IGF-II neutralising antibody, in addition to knocking down of IGF-IR using IGF-IR siRNA technology. This indicated that basal c-SRC activity in Tam-R cells was mediated in part through phosphorylated IGF-IR as a result of the autocrine release and action of IGF-II. Activation of c-SRC by EGFR was also apparent in Tam-R cells because TGF $\alpha$ -promoted c-SRC phosphorylation and basal c-SRC activity was sensitive to inhibition by gefitinib. However, gefitinib only partially reduced phosphorylation of c-SRC in response to IGF-II, indicating that a proportion of IGF-II-induced c-SRC activity occurred upstream of the EGFR. This was further supported by the finding that IGF-IR and c-SRC physically interact in Tam-R cells, and this association was increased after IGF-II stimulation.

There are five known auto phosphorylation sites on EGFR, including the major Y1068 site, studied in this report. The recent appearance of two new unique non- auto-phosphorylation sites Y845 and Y1101 have now also been identified on EGFR which are c-SRC-dependent and have been shown to form complexes with c-SRC, in several cell lines including MCF-7 cells (Sato *et al.*, 1995; Maa *et al.*, 1995; Luttrell *et al.*, 1994; Biscardi *et al.*, 1999; Stover *et al.*, 1995). In support of these findings, we also demonstrated a physical association between c-SRC and EGFR under basal conditions of growth in Tam-R cells and levels were enhanced following IGF-II treatment. c-SRC has been shown to mediate EGFR transactivation through direct association with EGFR and phosphorylation of these tyrosine residues Y845 and Y1101

(Biscardi *et al.*, 1999; Stover *et al.*, 1995). We observed an increase in phosphorylation of EGFR at Y845 following IGF-II stimulation and this effect could again be blocked by pre-treatment with AG1024 or ABDP confirming the involvement of the IGF-1R in Tam-R cells.

We have further demonstrated that this c-SRC-dependent EGFR transactivation mechanism plays a key role in regulating EGFR activity and consequently Tam-R cell growth because the c-SRC tyrosine kinase inhibitor SU6656 reduced both basal and IGF-II-primed c-SRC and EGFR signalling and inhibited proliferative activity in this cell line. Indeed, SU6656 inhibited phosphorylation of EGFR at both Y845 and Y1068, suggesting that c-SRC can also regulate EGFR auto-phosphorylation in these cells. A simple explanation for this is that activated c-SRC when bound to EGFR is capable of phosphorylating EGFR on both auto- and non auto-phosphorylation sites (Stover *et al.*, 1995). However, studies have also shown that cells that express a mutant form of Y845 display a decrease in their ability to respond mitogenically to EGF, suggesting that this c-SRC-mediated phosphorylation site is important for ligand-dependent receptor activation (Biscardi *et al.*, 1999). Furthermore, crystallographic studies have shown that phosphorylation of Y845 on EGFR homologs helps stabilize the enzyme, thus maintaining it in an active state (Biscardi *et al.*, 1999). It has therefore been proposed that ligand activation of EGFR may be under Y845 regulation. Our findings support this proposition because TGF $\alpha$ -induced activation of EGFR/ERK1/2 phosphorylation was considerably abrogated in Tam-R cells pre-treated with two c-SRC inhibitors SU6656 and AZM555. Moreover, the presence of TGF $\alpha$  failed to overcome the growth inhibitory effects of either SU6656 or AG1024

in Tam-R cells, thus further indicating the importance of the c-SRC-dependent phosphorylation of EGFR at Y845.

In order to determine whether the presence of an EGF ligand is a pre-requisite for EGFR activation in Tam-R cells following IGF-II-mediated c-SRC activation of EGFR at Y845, additional studies were performed. Interestingly, with the use of neutralising antibodies surprisingly, the EGF ligand AR, rather than EGF, HB-EGF or TGF $\alpha$ , three well-known EGF ligands, was largely responsible for the basal activation of EGFR observed in Tam-R cells (Britton *et al.*, 2006). In support of these findings is a similar study investigating cross-talk between IGF-IR and EGFR, undertaken very recently by Desbois-Mouthon *et al.*, (2006) in hepatocellular carcinoma cells. They also provided clear evidence with the use of neutralising antibodies that AR rather EGF, HB-EGF or TGF $\alpha$ , was mainly responsible for the trans-activation of EGFR following IGF-II stimulation, although the mechanism involved was shown to be TACE-dependent. Moreover, a recent RT-PCR study within our group also demonstrated increased AR mRNA expression levels in Tam-R cells compared to their WT counterparts, whereas the expression levels of other EGF ligands examined remained the same in both cell lines (Britton *et al.*, 2006). In light of these findings, an additional experimental study showed that the stimulatory effects of IGF-II could not overcome the inhibitory action of an AR neutralising antibody on EGFR activation in Tam-R cells, thus clearly indicating that in order for IGF-II to promote EGFR activation in Tam-R cells, the presence of an EGF ligand such as AR is an essential requirement.

In agreement with our Tam-R data, we also found that under basal growth conditions both AG1024 and SU6656 inhibited phosphorylation of c-SRC and

EGFR at both Y845 and Y1068 residues in a tamoxifen-resistant T47D breast cancer cell line. Furthermore, phosphorylation of IGF-IR, c-SRC, and EGFR at Y845 and at Y1068 could be enhanced by IGF-II, and this was again sensitive to the inhibitory actions of both AG1024 and SU6656 in this cell line. The importance of c-SRC-dependent phosphorylation of EGFR at Y845 was also demonstrated in T47D-R cells because inhibition of c-SRC activity with SU6656 reduced the ability of TGF $\alpha$  to promote auto-phosphorylation of EGFR at Y1068. These findings from T47D-R cells would indicate that this cross-talk mechanism is not unique to Tam-R cells but may be a more general tamoxifen-resistant phenomenon.

***4.2 IRS-1 involvement in EGFR and IGF-IR signalling in Tam-R WT-MCF-7 breast cancer cells in addition to and other EGFR positive cancer cell types:***

We have recently demonstrated (Knowlden *et al.*, 2003) that the EGFR-positive Tam-R MCF-7 breast cancer cell line is growth inhibited by the selective EGFR tyrosine kinase inhibitor gefitinib (Iressa, ZD1839), a quinazoline derivative which competitively blocks the binding of adenosine tri-phosphate to the receptors tyrosine kinase domain (Baselga and Arteaga, 2005). However further studies have demonstrated that following prolonged exposure to this agent (4-6 months) the cells start to re-grow due to acquisition of resistance (Jones *et al.*, 2004). Such experimental data may have important clinical implications. Indeed, several phase II clinical trials are now in place examining gefitinib monotherapy in patients with tamoxifen-resistant ER

positive breast cancer, in addition to other cancer types (Robertson *et al.*, 2003, Gutteridge *et al.*, 2004, Barton *et al.*, 2001 and Cohen *et al.*, 2003). Preliminary data would indicate that gefitinib was well tolerated and provided tumour remissions and disease stabilization in such patients. However, despite this clear therapeutic promise clinical trials have disappointingly revealed evidence of primary/*de novo* and acquired resistance to EGFR inhibitors such as gefitinib (Barton *et al.*, 2001; Cohen *et al.*, 2003). A range of possible resistance mechanisms have been identified in both preclinical and clinical studies and include receptor mutation (Lynch *et al.*, 2004; Paez *et al.*, 2004; Riedel and Febbo, 2005), loss of downstream effector components (Bianco *et al.*, 2003) and activation of alternative oncogenic signalling pathways (Liu *et al.*, 2001; Chakravarti *et al.*, 2002; Camirand *et al.*, 2005; Jones *et al.*, 2004). A candidate resistance mechanism to anti-c-erbB receptor therapy in a number of cancer types is the IGF-IR signalling pathway and this receptor has been linked to disease progression and recurrence in clinical breast cancer (Rocha *et al.*, 1997; Turner *et al.*, 1997). In this light, we have gone on to show that growth of our dual tamoxifen/gefitinib-resistant breast cancer cells is indeed mediated by signalling via the IGF-IR and PI3K/Akt and protein kinase C delta (PKC $\delta$ ) pathways and these cells display an increased sensitivity to growth inhibition by the IGF-IR inhibitor AG1024 (Jones *et al.*, 2004). Similarly, we have reported that growth of a gefitinib-resistant DU145 prostate cancer cell line is also dependent on IGF-IR/AKT/PKC $\delta$  signalling activity (Jones *et al.*, 2004). Increased IGF-IR signalling activity has also been implicated in the development of resistance to the selective EGFR tyrosine kinase inhibitor AG1478 in glioblastoma cells (Chakravarti *et al.*, 2002) and also to the anti-

EGFR monoclonal antibody 225 in the DiFi human colorectal cancer cell line (Liu *et al.*, 2001).

These findings would suggest another novel cross-talk mechanism exists between EGFR and IGF-1R whereby EGFR is capable of suppressing IGF-1R signalling activity. Thus, if EGFR activity is inhibited with an agent such as gefitinib this would remove the suppressive action of EGFR and allow IGF-1R signalling to then drive resistant cell growth. The aim of this second part of the thesis was to determine whether such a cross-talk mechanism exists in the EGFR-positive Tam-R cell line and if so whether such a mechanism was unique to Tam-R cells or was potentially active in other EGFR-positive cancer cell lines. We have previously demonstrated that EGF stimulation of Tam-R cells had no effect on IGF-1R activity, therefore, any potential suppressive effect of EGFR on IGF-1R signalling must be on components downstream of IGF-1R. Initial studies focused on the key adaptor protein for IGF-1R, IRS-1 and revealed that IRS-1 was expressed in Tam-R cells at levels slightly lower than those observed in WT cells. This small reduction is again a likely consequence of the fact that IRS-1 is an oestrogen-regulated gene in MCF-7 cells (Molloy *et al.*, 2000) and therefore would be down-regulated by tamoxifen treatment. Indeed, a 2 and 6 week treatment of WT MCF-7 cells with tamoxifen reduced IRS-1 mRNA and protein expression in the present study. Interestingly, examination of the phosphorylation profiles of IRS-1 in this cell line revealed differential expression of two tyrosine phosphorylated forms of this adaptor protein. IRS-1 can be phosphorylated at multiple tyrosine residues following association with IR/IGF-IR; two key residues which when phosphorylated play a central role in recruitment of important downstream

signal transduction cascades being Y612 and Y896. Phosphorylation of IRS-1 at Y612 has been shown to act as a docking site for the p85 regulatory subunit of PI3-K which when activated serves to drive Akt activity, whereas, Y896 phosphorylation of this adaptor protein acts as a recruitment site for the adaptor protein Grb2 which is involved in triggering the MAPK signalling pathway (Esposito *et al.*, 2001; Hers *et al.*, 2002). Treatment of Tam-R cells with IGF-II promoted predominantly Y612 phosphorylation of IRS-1, with only a small increase in IRS-1 Y896 phosphorylation being observed in response to this ligand. In contrast, EGF caused a sharp increase in Y896 phosphorylated IRS-1 levels whilst having no effect on IRS Y612 levels in this cell line. These findings suggest that firstly, IRS-1 Y612 phosphorylation appears to be solely under the regulation of the IGF-IR in Tam-R cells, thus, IGF-IR signals primarily through PI3K/Akt pathway in these cells. Secondly, EGFR appears to utilise IRS-1 as part of its mechanism to engage the MAPK signalling cascade in this same cell line which was further supported by immunoprecipitation/Western blotting studies which revealed that EGF-induced phosphorylation of IRS-1 at Y896 resulted from a direct association of EGFR with IRS-1. The small increase in IRS-1 Y896 phosphorylation in response to IGF-II may reflect the ability of IGF-II to activate EGFR, the cross-talk mechanism demonstrated to be active in this cell line as described earlier in this study.

The ability of EGFR to recruit IRS-1 is a novel signalling phenomenon that has not previously been described to date in breast cancer cells. However, we have found that such a phenomenon is not unique to tamoxifen-resistant MCF-7 breast cancer cells as EGF can also promote phosphorylation of IRS-1 at Y896



in a range of EGFR-positive cancer cell lines, T47D breast cancer cells, DU145 and LNCaP prostate cancer cells and A549 non small cell lung carcinoma cells. Furthermore, in LNCaP cells this effect of EGF is again a result of direct association of EGFR with IRS-1. EGF dependent IRS-1 phosphorylation has also been reported in human epidermoid carcinoma A431 cells and in primary cultures of rat hepatocytes (Fujioka *et al.*, 2001; Fujioka and Ui, 2001). However, it should also be noted that EGF is without effect on tyrosine phosphorylation of IRS-1 in 3T3-L1 adipocytes transfected with EGFR (Hardy *et al.*, 1995). It is not entirely surprising that EGFR can bind IRS-1. A potential interaction between EGFR and IRS-1 has been predicted from the binding of peptides, representing the physical sites of EGFR tyrosine phosphorylation, to protein microarrays comprising all SH2 and PTB domains encoded in the human genome (Jones *et al.*, 2006). Furthermore, the phosphorylated NPXY motifs in activated insulin and IGF-IR receptors to which the phosphotyrosine binding domains of IRS molecules bind are also present in the C-terminus region of EGFR (Songyang *et al.*, 1995). Indeed, the presence of all three of these NPXY motifs found in EGFR were found to be indispensable for IRS-1 to be tyrosine phosphorylated in response to EGF in EGFR-transfected Chinese hamster ovary cells (Fujioka *et al.*, 2001).

The EGFR appears to be the dominant recruiter of IRS-1 in Tam-R cells as IRS-1 Y896 is the principal phosphorylated form of this adaptor protein under basal growth conditions. This dominance of EGFR over IGF-IR to recruit IRS-1 was further emphasised by the finding that co-treatment of Tam-R cells with EGF and IGF-II resulted in a reduced ability of IGF-II to promote Y612 phosphorylation of IRS-1 whilst there was no effect of this co-treatment regime

on EGF-induced increases in IRS-1 Y896. This indicates that the EGFR/IRS-1 association in Tam-R cells prevents recruitment of IRS-1 by IGF-IR thus potentially serving to actively limit signalling via this receptor while further promoting the EGFR/MAPK pathway that is central to Tam-R cell growth. Similar findings have been reported in a prostate epithelial cell line, CPTX 1532, where EGF was shown to inhibit IGF-I-dependent degradation of IRS-1 (Zhang *et al.*, 2000).

We next examined the effect of EGFR blockade on this novel interplay between EGFR, IGF-IR and IRS-1, using gefitinib. As expected, a 7 day gefitinib treatment potently inhibited EGFR and ERK1/2 MAPK activity in Tam-R cells confirming our previous findings (Knowlden *et al.*, 2003), however, an increase in Akt phosphorylation was also observed in response to this treatment regime. Interestingly, a time course study assessing the effects of gefitinib on Akt activity revealed that gefitinib was inhibitory up to day 4, corroborating previous findings in this cell line (Jordan *et al.*, 2004), but by day 7 was stimulatory. A possible explanation for this gefitinib-induced stimulation of Akt activity at this later time point was provided by the finding that gefitinib treatment also resulted in the loss of EGFR and IRS-1 association, a reduction in Y896 IRS-1, enhanced association of IRS-1 with IGF-IR and increased Y612 IRS-1 phosphorylation levels at day 7. These additional novel findings indicate that gefitinib may alter the dynamics of the EGFR/IGF-IR/IRS-1 cross-talk system, promoting IGF-IR signalling by allowing IRS-1 to re-associate with IGF-IR an event that can then serve to stimulate downstream PI3-K/Akt signalling. It should be noted that this re-establishment of IGF-IR /PI3K /Akt signalling following gefitinib treatment was not associated with any

increase in phosphorylated or total IGF-IR expression. This ability of gefitinib to promote IGF-IR signalling in Tam-R cells was further evidenced by the ability of gefitinib to further enhance IGF-II-induced phosphorylation of IRS-1 Y612 and Akt when compared to IGF-II treatment alone. Activation of such a pathway may facilitate the ability of the cells to survive gefitinib challenge in the short-term and in the long-term provide a mechanism to drive resistant growth. We confirmed that these effects resulted from a selective inhibition of EGFR by gefitinib as similar findings were observed following knockdown of EGFR expression using siRNA technology.

The ability of gefitinib to promote IGF-IR signalling was not unique to the Tam-R cell line as similar findings were also observed in the LNCaP prostate cancer cell line. Indeed, a 7 day treatment of LNCaP cells with gefitinib enhanced IRS-1 Y612 phosphorylation with subsequent immunoprecipitation/Western blot analysis again revealing a loss of association of EGFR with IRS-1 and increased association of IRS-1 with IGF-IR in these cells as observed in Tam-R cells. It has previously been reported that LNCaP cells do not express IRS-1 protein (Reiss *et al.*, 2000), however, we were able to detect very low levels of this protein in the present study possibly due to the use of more sensitive detection reagents in our Western blotting studies. That such a cross-talk mechanism between EGFR and IGF-IR was evident in LNCaPs would suggest that EGFR was the dominant recruiter of IRS-1 in these cells. It is also likely that low expression levels of IRS-1 in LNCaP compared to T47D, DU145 and A549 cells would serve to greatly limit the availability of this adaptor protein and so further emphasise any alterations in IRS-1 recruitment and phosphorylation patterns observed under basal

conditions and following gefitinib exposure. It is likely that this novel cross-talk mechanism is not functional in T47D cells as gefitinib was fairly ineffective at reducing Y896 IRS-1 phosphorylation levels, whilst in A549 cells expression levels of Y612 IRS-1 were higher than Y896 IRS-1 suggesting that EGFR was not the dominant recruiter of IRS-1 in these cells. DU145 cells do not appear to express Y612 phosphorylated IRS-1 suggesting that basal IGF-IR signalling is compromised in these cells possibly as a consequence of the serum-free conditions used in this study which is believed to suppress IGF-IR expression and down-regulate activity (an unpublished personal observation).

The re-establishment of the IGF-IR signalling pathway has been shown to be the dominant growth mechanism utilised by two of our 'in house' Tam-R/gefitinib resistant breast cancer and Du145/gefitinib resistant prostate cancer cell models (Jones *et al*; 2004). We have also shown that these Tam-R/gefitinib resistant cells have increased IRS-1 Y612 and Akt activity compared to Tam-R cells alone, thus suggesting that signalling through this pathway has indeed been maintained throughout the development of gefitinib resistance. This is supported by an *in vitro* study that observed an association between increased Akt activity and *de novo* gefitinib resistance in MDA-468 cells results from a lack of PTEN (Bianco *et al.*, 2003). Also, there is an early indication that elevated AKT may similarly be associated with *de novo* resistance in clinical breast cancer via the re-establishment of the IGF-IR signalling pathway as the dominant growth mechanism in gefitinib resistance (Agrawal *et al.*, 2005). Moreover, a more recent study by Camirand *et al*, (2005) also demonstrated that over expression of IGF-IR in SK-BR-3 breast cancer cells was sufficient to

cause a marked enhancement in gefitinib resistance.

#### ***4.3. Co-targeting of EGFR and IGF-IR potentially inhibits IGF-IR/EGFR activity and growth of Tam-R cells.***

As gefitinib treatment appeared to rapidly enhance IGF-IR signalling in Tam-R cells we next assessed the effect of targeting IGF-IR in combination with gefitinib in an attempt to abrogate this potential resistance mechanism. Treatment of Tam-R cells with a combination of gefitinib and the selective IGF-IR tyrosine kinase inhibitor ABDP prevented the activation of IGF-IR by IGF-II and blocked the gefitinib-dependent enhancement of IRS-1 Y612 and phosphorylation in response to this ligand. Again these results were shown to be a consequence of selective inhibition of EGFR and IGF-IR as similar results were observed following combined knockdown of EGFR and IGF-IR with siRNA technology. Greater inhibition of phosphorylated levels of IRS-1 Y896, EGFR Y1068 and ERK1/2 were also observed in those cells treated with the combination of ABDP and gefitinib compared with gefitinib alone, reflecting the important role played by IGF-IR in facilitating EGFR signalling activity in Tam-R cells as described earlier in this thesis. This more effective inhibition of IGF-IR and EGFR signalling pathways by combination treatment also translated out into a greater inhibition of cell growth compared to either agent alone supporting the findings of Camirand *et al*, (2005) who reported additive or synergistic inhibitory effects on breast cancer cell growth in cells treated with a combination of the selective IGF-IR tyrosine kinase inhibitor AG1024 and gefitinib compared to either agent alone.

Previous studies within our group have attempted to delay or prevent the development of these resistant states by combining drugs that anticipate and therefore, abrogate the resistance mechanism. Indeed, gefitinib has been used in combination with tamoxifen in treating MCF-7 cells, where gefitinib is used in anticipation of these cells adopting EGFR signalling (Gee *et al.*, 2003). Such treatment resulted in a dramatic loss in cell number, with no cells viable after 12 weeks. In the present study we examined the effects of long-term combination therapy targeting EGFR and IGF-IR in Tam-R cells. At 4-5 months, we found that Tam-R cells treated with gefitinib alone show evidence of re-growth, with cell numbers doubling over a 2 week time frame, as previously reported (Jones *et al.*, 2005). However, Tam-R cells treated with a combination of gefitinib and ABDP were completely growth inhibited, which resulted in total cell loss by week 19. Similarly, another study from our group examining the effectiveness of the co-treatment of gefitinib and AG1024 in Tam-R cells also showed a complete loss of cells after only 6 weeks in culture (Nicholson *et al.*, 2004).

Clearly, further work is required to fully establish the long-term effects of combination compared to monotherapy in these cells. However, these data indicate that targeting the gefitinib-induced IGF-IR signalling in Tam-R cells may provide a mechanism to prevent cells surviving the initial challenge with this anti-EGFR agent and ultimately block the development of a resistant phenotype. Evidence from such cell models, therefore, strongly suggests that combination therapies, as opposed to mono-therapies will result in a far better outcome for patients with both primary and acquired resistant disease. There are currently several clinical trials on going, assessing the efficacy of

combinations of tyrosine kinase inhibitors, such as gefitinib with various endocrine agents in the tamoxifen-resistant/second line setting, together with several randomized phase II/III trials in the first-line setting (Johnston, 2005). The primary endpoint for these trials is to investigate whether time to disease progression can be significantly prolonged by the addition of a TKI to endocrine therapy, thus delaying or preventing the emergence of resistance demonstrated in various preclinical models such as those described above

#### ***4.4 Summary and conclusion:***

In conclusion, the first half of this thesis demonstrates that following inhibition of IGF-IR signalling by tamoxifen in endocrine-responsive MCF-7 breast cancer cells, IGF-IR signalling is re-instigated upon acquisition of tamoxifen resistance. Moreover, these studies have further identified a novel unidirectional cross-talk mechanism between EGFR and IGF-IR, whereby IGF-IR promotes EGFR signalling via a c-SRC dependent pathway, which is active not only in MCF-7 but also T47D breast cancer cells resistant to the growth inhibitory effects of tamoxifen. This ability of IGF-IR to support EGFR signalling, the dominant growth regulatory pathway in these two cell lines, identifies the IGF-IR as an important and potent mediator of tamoxifen-resistant breast cancer cell growth and would suggest that targeting the IGF-IR may prove to be highly effective, not only in endocrine responsive breast cancer (Nicholson and Gee, 2000), but also in those patients who have gone on to acquire tamoxifen resistance. The identification of c-SRC as a key player in this cross-talk mechanism would also indicate that targeting this signalling element may also prove valuable in the management of this disease. Indeed, c-

SRC has been shown to play a central role in mediating the enhanced invasive capacity of tamoxifen-resistant MCF-7 cells (Hiscox *et al.*, 2004), thus inhibiting this non-receptor tyrosine kinase may prove an effective means to target both the proliferative and aggressive features of this condition. The second half of this thesis identifies a second highly novel mechanism of cross-talk between IGF-IR and EGFR signalling pathways in tamoxifen-resistant MCF-7 breast cancer cells whereby EGFR, through its ability to recruit and bind IRS-1, can limit the availability of this adaptor protein to associate with IGF-IR and so suppress down-stream signalling via this receptor. Once again this mechanism is not specific to tamoxifen-resistant MCF-7 cells as it was also found to be active in LNCaP prostate cancer cells in these studies. An important consequence of this cross-talk was demonstrated when these cell lines were treated with the anti-EGFR agent gefitinib. Blockade of EGFR with this agent disrupted EGFR/IRS-1 association permitting association of IRS-1 with IGF-IR and re-establishment of IGF-IR signalling. In tamoxifen-resistant MCF-7 cells IGF-IR signalling is the dominant mediator of gefitinib-resistant cell growth (Jones *et al.*, 2004), thus gefitinib appears to play an active role in limiting its own efficacy by promoting activation of a key resistance pathway in these cells. These findings clearly demonstrate that as a consequence of the high degree of cross-talk that exists between growth factor signalling pathways in cancer cells we must take into consideration that targeting a single protein in this complex signalling array may adversely influence the quality and duration of response. Deciphering both the inhibitory and inductive effects of these targeted agents provides us with the opportunity to design effective strategies to combat such resistance mechanisms and improve response to initial therapy.



Indeed as proof of principle, in the final part of this thesis studies targeting IGF-IR signalling in combination with gefitinib, to anticipate the inductive action of gefitinib on this pathway, generated a more effective inhibition of tamoxifen-resistant MCF-7 breast cancer cell signalling activity and growth compared to gefitinib alone. Furthermore, re-establishment of cell growth following long-term treatment with these agents was not observed. Thus, targeting the IGF-IR may also provide both a potent therapy for gefitinib resistance and, if given in combination with gefitinib, may prevent the development of this resistant state.

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