ROLE OF OESTROGEN RECEPTOR PHOSPHORYLATION IN THE GROWTH OF ENDOCRINE-RESPONSIVE AND ANTI-OESTROGEN-RESISTANT BREAST CANCER CELL LINES

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Summary

EGFR/MAPK signalling has been implicated in mediating tamoxifen-resistant breast cancer cell growth in the clinic and in preclinical models. However, ERα expression and functionality has also been shown to be maintained in this condition. ERα transcriptional activity can be driven, in a ligand-independent manner, via growth factor signalling-mediated phosphorylation of ERα. The aim of this thesis was to investigate whether growth factor signalling pathways regulate phosphorylation and functionality of ERα in tamoxifen-sensitive (WT) and -resistant (TAM-R) MCF-7 breast cancer cell lines and if so whether this cross-talk mechanism plays a role in the generation and maintenance of the tamoxifen-resistant phenotype. Western blotting and immunocytochemistry assays revealed increased levels of serine 118 (S118), but not serine 167, phosphorylated ERα in TAM-R compared to WT cells. Basal S118 ERα phosphorylation was regulated by both EGFR and IGF-IR signalling pathways, via MAPK, in Tam-R cells and by IGF-1R/phosphatidylinositol 3-kinase signalling in WT cells. ERα transcriptional activity, assayed by oestrogen response element (ERE) activity and pS2 and amphiregulin (AR) mRNA levels, was similarly IGF-1R/EGFR/MAPK-regulated in TAM-R cells, whereas, ERE activity was only IGF-1R-dependent in WT cells. AP-1 and serum response element activity was EGFR/IGF-1R-independent in both cell lines. Recruitment of the co-activators p68 RNA helicase and SRC1 was EGFR/MAPK- and S118 phosphorylation-dependent in TAM-R

cells indicative of a role for S118 phosphorylation in mediating ERα transcriptional activity. The ability of ERα to regulate AR mRNA expression also suggested the existence of a self propagating autocrine growth regulatory loop in TAM-R cells. This was confirmed by the presence of ERα on the AR gene promoter, elevated basal AR mRNA expression, inhibition of EGFR, MAPK and ERα S118 phosphorylation by AR neutralising antibodies and growth promotion by AR and inhibition by the selective EGFR tyrosine kinase inhibitor gefitinib and the pure antioestrogen fulvestrant.

CONTENTS

ABBREVIATIONS

AF	Activator Function
AP-1	Activator Protein-1
AR	Amphiregulin
bp	Base Pair
BSA	Bovine Serum Albumin
BTC	Betacellulin
cAMP	cyclic Adenosine MonoPhosphate
CBP	CREB-Binding Protein
cDNA	Complementary DNA
CDK	Cyclin Dependent Kinase
C/EBP	CAAT Enhancer Binding Protein
CMF	Cyclophosphamide, Methotrexate and 5-Fluorourocil
CRE	cAMP response element
CREB	CRE Binding protein
DAB	DiAminoBenzidine tetrahydrochloride
DAPI	4'6-DiAmidino-2-Phenylindole-2HCl
DBD	DNA Binding Domain
DCCM	Defined Cell Culture Medium
DCIS	Ductal Carcinoma *In Situ*
DMSO	DiMethyl SulphOxide
DNA	DeoxyriboNucleic Acid
dNTP	DeoxyNucleotide TriPhosphate
DTT	DiThioThreital

E_2	17β-oestrogen
EBP	Enhancer Binding Protein
EDTA	EthyleneDiamine Tetracetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGTA	EthyleneGlycol Tetracetic Acid
ER	Oestrogen Receptor
EP	Epiregulin
ERE	Oestrogen Response Element
ERK	Extracellular signal-Regulated Kinases
Et-Br	Ethidium Bromide
FBS	Foetal Bovine Serum
FCS	Foetal Calf Serum
GRE	glucocorticoid response element
H	Histone
HAT	Histone acetyl transferase
HB	Heparin-binding
HBD	Hormone Binding Domain
HDAC	Histone deacetylase
HER	Heregulin Receptor
HMT	Histone methyl transferase
HRG	Heregulin
HRP	Horse Radish Peroxidase
hrs	hours

hsp	Heat Shock Protein
ICI	Imperial Chemical Industries
IGF	Insulin-like Growth Factor
IgG	ImmunoGlobulin G
JNK	c-Jun N terminal Kinases
kDa	kilo Daltons
LBD	Ligand Binding Domain
M	Molarity
mA	Milli Amps
MAPK	Mitogen Activated Protein Kinase
MCF	Mammary Carcinoma Female
MEK	MAP Kinase Extracellular Regulated Kinase
min	minutes
MMLV-RT	Moloney-Murine Leukaemia Virus – Reverse Transcriptase
NCoR	Nuclear co-repressor protein
NDF	Neu Differentiation Factor
NF-κB	Nuclear Factor-κ B
NR box	Nuclear receptor box
NRID	Nuclear Receptor Interacting Domains
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI3K	Phosphatidyl Inositol 3-Kinase
PIC	Pre-initiation complex
PKC	Protein Kinase C

RH	Random Hexamer
PMSF	Phenyl Methyl Sulforyl Fluoride
RNA	RiboNucleicAcid
rpm	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute (origin of development)
RT-PCR	Reverse-Transcription - Polymerase Chain Reaction
SDS	Sodium Docecyl Sulphate
SH	Src Homology
SMRT	Silencing mediator for retinoid and thyroid hormone receptors
SRA	Steroid receptor RNA activator
SRC	Steroid receptor co-activator
TAE	Tris Acetate-EDTA buffer
TAM	4-OH-tamoxifen
TAM-R	Tamoxifen Resistant MCF-7 cell line
TBP	TATA binding protein
TEMED	N, N, N' N, TEtraMEthyleneDiamine
TF	Transcription Factor
TGFα	Transforming Growth Factor
UWCM	University of Wales College of Medicine
UV	UltraViolet
v/v	Volume by volume
WT	Wild Type

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1.0 INTRODUCTION

1.1 Breast cancer

1.1.1 Today's breast cancer rates

Breast cancer incidence rates in Wales and England have escalated to the current day level of 114 sufferers per 100,000 women, ranking the disease as the most common cancer in these countries (Office for National Statistics).
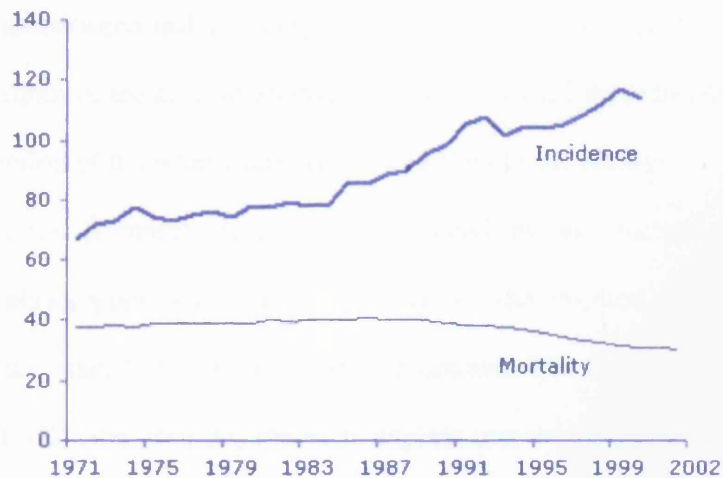
Age-standardised incidence of and mortality from female breast cancer, Wales and England, rate per 100,000.

Figure 1.1: Incidence and mortality rates of breast cancer in Wales and England between 1971 and 2002 (adapted from Office for National Statistics).

Paradoxically however, the past 16 years has seen a decrease in the mortality rate of breast cancer patients (Office for National Statistics), which can only be attributed to revolutionary advances in systemic therapy, screening and surgery. To fully understand the rationale behind these current treatment strategies, one would benefit from a historical perspective of the disease.

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1.1.2 History of breast cancer

Breast cancer has been an affliction on humanity for centuries as investigations into the disease have been recorded as far back as the ancient Egyptians. In 1862 Edwin Smith discovered a papyrus dating from the seventeenth century B.C, containing writings by an Egyptian physician who distinguished between abscesses which were considered treatable by the knife, and cancers which were deemed inoperable (Wilkins). The writings suggested that the Egyptians could distinguish between inflammatory mastitis and carcinoma of the breast and that the wisdom of the ancient anonymous author caused them to consider surgical intervention of the latter futile (Baum and Henderson 2004).

Centuries later, the Humoral theory was developed by the ancient Greek physician and philosopher Aristotle (384-322 BC), who implied that breast cancer was a local manifestation of a systemic disease. The Greek physician Galen (200-130 AD) later used this theory to explain that the systemic disorder resulted from the accumulation of black bile (melancholia). Consequently, therapy for breast cancer during the following 1600 years involved cupping, leaching, venesection, purgation and crank diets, which in fact did nothing beneficial to treat the disease, although, highlighted the danger of basing therapeutic strategies on an unproven disease model (Baum and Henderson 2004; Breast Cancer 2000).

During the mid 17th century the development of the microscope by Anthony van Leeuwenhoek allowed Muller at the beginning of the 19th century to identify the cellular nature of breast cancer. These developments, and the elegant microscopic analysis of anatomical dissections from advanced breast

cancer patients lymph nodes, allowed Rudolf Ludwig Karl Virchow (1821-1902), a leading German pathologist, in the middle of the 19th century to suggest a mechanistic model of breast cancer, with the regional nodes acting as filters to inhibit the onward spread of the cancer cells (Baum and Henderson 2004). This revolution behind the understanding of the disease made Aristotle's Humoral theory redundant and led to the assumption that cancer could be cured at an early stage with adequate surgery. Consequently, a radical surgical technique was developed which involved removal of the affected breast, total ipsilateral axillary lymph node dissection, resection of pectoral major and minor muscles and routine resection of thoracodorsal neurovascular network including the long thoracic nerve (Baum and Henderson 2004). Although Halsted takes credit for the development of this technique at the end of the 19th century, similar radical surgical techniques had been carried out in centuries previous to this, however the arrival of Virchow's theory, the timely developments throughout the 19th century in germ theory by Pasteur, antisepsis by Lister, and anaesthesia by Simpson, made Halsted's en bloc radical mastectomy a success story in controlling local breast disease (Baum and Henderson 2004; Pasteur; Lister and Antiseptic surgery; Caton 1997).

A short time after the announcement by Halsted describing his surgical procedure to treat breast cancer, a new theory was initiated. Sir George Thomas Beatson, a surgeon at the Glasgow Royal Infirmary, studied the physiology of farm animal lactation for his MD. At the time, it was assumed that lactation was controlled by nervous stimuli, yet dissections failed to demonstrate the nerves associated with lactation. Beatson made an observation that lactation

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terminates when menses restarts after a calf has been weaned and that in Australia cows are spayed to prolong lactation, which lead to his hypothesis that a link might exist between the breast and the ovary. When he observed similarities in the microscopic appearance of the lactating breast and the neoplastic breast he made the conceptual leap that if castration effects lactation then it may also affect breast cancers (Robertson, Nicholson and Hayes 2002). Beatson decided to test his hypothesis. He first reported a single case study of a young woman who had previously had a mastectomy but subsequently returned with inoperable local recurrence. He carried out bilateral oopherectomy and the patient's cancer responded. However the cancer eventually progressed and the patient died. The following week he reported a second case where a young woman presented with inoperable locally advanced breast cancer. This patient did not respond to bilateral oopherectomy. Thus in the very first two reported patients, Beatson identified the major issues that we still struggle to fully understand today. First; why do some tumours respond (endocrine sensitive), but others do not (de novo endocrine resistant)? Second; even when tumours do respond to endocrine treatment, why do they eventually progress (acquired endocrine resistance) (Robertson, Nicholson and Hayes 2002)?

Recent work by David Smith of Glasgow using the original operative records of Beatson's operating theatre showed that he had completed nine cases of oopherectomy before his report in the *Lancet*. Three out of the nine treated were a success, which is the anticipated 30% response rate for unselected cases of breast cancer to endocrine manipulation (Baum and Henderson 2004).

Continued research in the field at the beginning of the 20th century confirmed a link between the ovary and the breast but the seemingly low response rates to this first form of endocrine therapy meant that the Halstedian mastectomy, capable of removing all local disease and proving effective at reducing local recurrence, appeared to be more successful at treating breast cancer.

During the 1950s faith in the radical surgical technique began to wane firstly due to statistical analyses, which suggested that surgery alone had no effect on overall survival, and secondly due to a growing body of evidence which demonstrated that cancer cells were present in the blood of cancer patients (Ashworth 1869; Engell 1955; Fisher 1955). Fisher and Fisher confirmed these findings by demonstrating that less than 40% of ^{51}Cr labelled V_2 carcinoma cells (cells isolated from Shope virus-induced papillomas) were retained in a rabbit's popliteal node, suggesting that Virchow's mechanistic model of breast cancer progression was not the case and that cancer cells can bypass the nodes via lymphovenous channels or by direct invasion of the venous system. This hypothetical model of 'biological predeterminism' meant that the Halstedian radical mastectomy was like shutting the stable door after the horse had already bolted (Baum and Henderson 2004; Kufe et al 2003).

Following reports of favourable effects of chemotherapeutic agents on the destruction of disseminated tumour cells in experimental animals, a rationale for embarking on clinical trials of adjuvant therapy was established. The findings during the 1950's, of tumour cells in the blood of breast cancer patients prompted the NSABP in 1958 to begin the first clinical trial of adjuvant chemotherapy for breast cancer. Women entered into this study were

treated by both conventional Halsted radical mastectomy and triethylene thiophosphoramide (Thiotepa) or by radical mastectomy and placebo. Because of its effectiveness in the palliation of advanced mammary cancer, Thiotepa was administered at the time of operation and on each of the first 2 postoperative days. The results reported in 1968 indicated a significant increase in the 5-year survival of pre-menopausal women who received Thiotepa. In 1973, a second clinical trial was begun at the Istituto Nazionale Tumori in Milan to evaluate the effectiveness of a three-drug combination, cyclophosphamide, methotrexate and 5-fluorourocil (CMF) as an adjuvant to surgery. Significant reductions in treatment failure occurred in all subgroups of patients treated with CMF, however, about two-thirds of patients experienced toxicity, indicated by nausea, vomiting, anorexia, alopecia, cystitis, or amenorrhea.

During this period it was established that the ovaries mediated their effects on breast tissue, normal or cancerous, through 17β-oestradiol (oestrogen), which maintained strong interest in the possibilities of endocrine therapies despite the promising early results with chemotherapy. The understanding that treating women with oestrogenic compounds within 24 hours of coitus prevented pregnancy lead the British scientist Arthur Walpole in 1963 who was working on a morning after birth control pill at Imperial Chemical Industries (ICI), to develop the hypothesis that as his molecule ICI 46, 474 (Tamoxifen) increased fertility that it might have anti-oestrogenic properties and this might reduce breast cancer growth (Baum 1997). A publication in the British Journal of Cancer in 1971 confirmed his idea and demonstrated that 10 out of 46 patients

with late or recurrent breast cancer showed a good response to tamoxifen. Although, this was of the same order as that produced by oestrogens and androgens, which were also in use at the time, importantly tamoxifen showed very low incidence of side effects (Baum and Henderson 2004). The responsiveness of the patients to tamoxifen also meant that the drug provided a safer alternative to the invasive and disruptive surgical removal of the ovaries. These early results demonstrated tamoxifen's potential as a breast cancer agent, re-emphasising the need for effective endocrine therapy, prompting increased research in the field.

Jensen and his colleagues in the 1960's found a protein that bound oestrogen at high affinity in the cytosol of tissues responsive to the hormone. This led to the discovery that this same protein existed in approximately 80% of breast carcinomas. It was also clear that 4-hydroxytamoxifen (4-OH-tamoxifen) an active metabolite of tamoxifen, caused displacement of oestrogen from this protein and led researchers to believe that 4-OH-tamoxifen was a competitive antagonist of oestrogen. The oestrogen binding protein was named the oestrogen receptor (ER) and the application of specific antibodies to ER allowed pathologists to predict whether a patient would respond to endocrine therapy depending on the level of ER present.

1.2   Oestrogen Receptor (ER)

1.2.1   ER structure/function relationship

The discovery of the oestrogen receptor initiated decades of investigations leading us to our current understanding of the importance of the

structure/functional relationship of the receptor in the growth of breast cancer cells. There are two known subfamilies of oestrogen receptor, ERα and ERβ, each encoded by unique genes that belong to the nuclear receptor family, a large group currently totalling approximately 150 related proteins (Lewandowski et al 2002; White and Parker 1998). These family members are conserved in both primary amino acid sequence and organisation of their functional domains, A-F (Figures 1.2 and 1.3) (White and Parker 1998). The A/B domain, also referred to as the variable N-terminal region, contains the activation function (AF1) (Levenson and Jordan 1999). Activation function 2 (AF2) resides in the C terminal E domain and is ligand dependent (Levenson and Jordan 1999). In addition, the E domain contains α helical structures intricately arranged to provide the ligand-binding domain (LBD) which has high ligand specificity (Levenson and Jordan 1999). Domain C contains the DNA binding domain, the most highly conserved region throughout the whole receptor family, strongly indicating that these molecules are first and foremost transcription factors (Levenson and Jordan 1999). Domain D contains a variable hinge region allowing the protein to bend and alter conformation (Metivier 2002). It has also been shown to be involved in binding of Hsp90 whilst both the C and D domains are involved in dimerisation of the receptors (Tsai and O'Malley 1994, Levensen and Jordan 1999). ERβ is homologous to ERα at the ligand binding (58%) and DNA binding (96%) domains, whereas the A/B region, hinge domain and F region are not well conserved (Levenson and Jordan 1999).

Figure 1.2; Map of ERα domains

P 104/106   P 118

Domain A (1-38) (Metivier 2002)   Domain B (39-182) (Metivier 2002)

MTMTLHTKASGMALLHQIQGNELEPLNRPQLKIPLERPLGEVYLDSSKPAVYNYPEGAAYEFNAAAAANAQVYGQTGLPYGPGSEAAAFGSNGLGGFPPLNSVSPSPLMLLHPPPQLSPF

P 167

Domain C (183-263) (DNA binding domain) (Metivier 2002)

LQPHGQQVFYYLENEPSGYTVREAGPPAFYRPNSDNRRQGGRERLASTNDKGSMAMESAKETRYCAVCNDYASGYHYGVWSCEGCKAFFKRSIQGHNDYMCPATNQCTIDKNRRKSCQAC

               ↑ ↑   ↑ ↑ Helix 1   ↑   ↑ ↑

                 C4 Zinc finger   C4 Zinc finger

Domain D (264-302) (Hinge region) (Metivier 2002)

RLRKCYEVGMMKGGIRKDRRGGRMLKHKRQRDDGEGRGEVGS { AGDMRAANLWPSPLMIKRSKKNSLALSLTADQMVSALLDAEPPILYSEYDPTRPFSEASMMGLLTNLADRELVHMINW

Helix 2   Helix 3   Helix 4   Helix 5

Domain E (303-549) (Ligand binding domain) (Metivier 2002)

AKRVPGFVDLTLHDQVHLLECAWLEILMIGLVWRSMEHPVKLLFAPNLLLDRNQGKCVEGMVEIFDMLLATSSRFRMMNLQGEEFVCLKSIILLNSGVYTFLSSTLKSLEEKDHIHRVLD

Helix 6   Helix 7   Helix 8   Helix 9   Helix 10

P 537

Domain F (550-595) (Metivier 2002)

KITDTLIHLMAKAGLTLQQQHQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLYDLLLEMLDAHRL } HAPTSRGGASVEETDQSHLATAGSTSSHSLQKYYITGEAEGFPATV

Helix 10   Helix 11   Helix 12

_____ = AF1 (Metzger D 1997), AF2 (AF2a 282-351) (Metivier 2002, Norris JD 1997), Helix and C4 info from ExPASy Proteomics Server

Simplified map of the ERα domains

<u>**Figure 1.3; Map of ERβ domains**</u>

Domain A/B (1-148)

MDIKNSPSSLNSPSSYNCSQSILPLEHGSIYIPSSYVDSHHEYPAMTFYSPAVMNYSIPSNVTNLEGGPGRQTTSPNVLWPTPG

P 87 Human (106 Mouse)   P 105 Human (124 Mouse)

| |

Domain C (149-214) (DNA binding domain)

HLSPLVVHRQLSHLYAEPQKSPWCEARSLEHTLPVNRETLKRKVSGNRCASPVTGPGSKRDAHFCAVCSDYASGYHYGVWSCEGCKAFFKRSIQGHNDYICPATNQCTIDKNRRKSCQAC

C4 Zinc finger   C4 Zinc finger

Domain D (Hinge)

RLRKCYEVGMVKCGSRRERCGYRLVRRQRSADEQLHCAGKAKRSGGHAPRVRELLLDALSPEQLVLTLLEAEPPHVLISRPSAPFTEASMMMSLTKLADKELVHMISWAKKIPGFVELS

Helix 1   Helix 2   Helix 3

Domain E (304-500) (Ligand binding domain)

LFDQVRLLESCWMEVLMMGLMWRSIDHPGKLIFAPDLVLDRDEGKCVEGILEIFDMLLATTSRFRELKLQHKEYLCVKAMILLNSSMYPLVTATQDADSSRKLAHLLNAVTDALVWVIAKSGI

Helix 4   Helix 5   Helix 6   Helix 7   Helix 8   Helix 9

P 488

|

Domain F

SSQQQSMRLANLLMLLSHVRHASNKGMEHLLNMKCKNVVPVYDLLLEMLNAHVLRGCKSSITGSECSPAEDSKSKEGSQNPQSQ

Helix 10   Helix 11   Helix 12

Simplified map of the ERβ domains

1.2.2 Oestrogen mode of action

In the absence of ligand, ERs are predominantly distributed between the nucleus and the cytoplasm as monomers associated with chaperone proteins such as the heat shock proteins Hsp90, Hsp70 and cyclophilin 40 and p23, or as free dimers (Pratt and Toft 1997).

The classic oestrogen response involves oestrogen (E_2) diffusing through the cell plasma and/or nuclear membranes where it binds to the ligand-binding domain of the receptor (Figure 1.4) (Tsai MJ and O'Malley 1994). Oestrogen enters the narrower half of the LBD in a predominantly hydrophobic cavity composed of residues from helices 3, 6, 7, 8, 11 and 12 as well as the S1/S2 hairpin. The structure of E_2 and the hydrophobic pocket is such, that helix 12 is able to change position to pack against helices 3, 5/6, and 11, enclosing the ligand within the cavity (Shiau et al 1998).

The interaction between ER and oestrogen, and the resulting change in conformation, mainly due to the shift in position of helix 12, triggers the first key steps on the path ERα takes towards gene transcription. Namely dissociation of the chaperone molecules, dimerisation, translocation and DNA binding (Levenson and Jordan 1999).

= Chaperone proteins
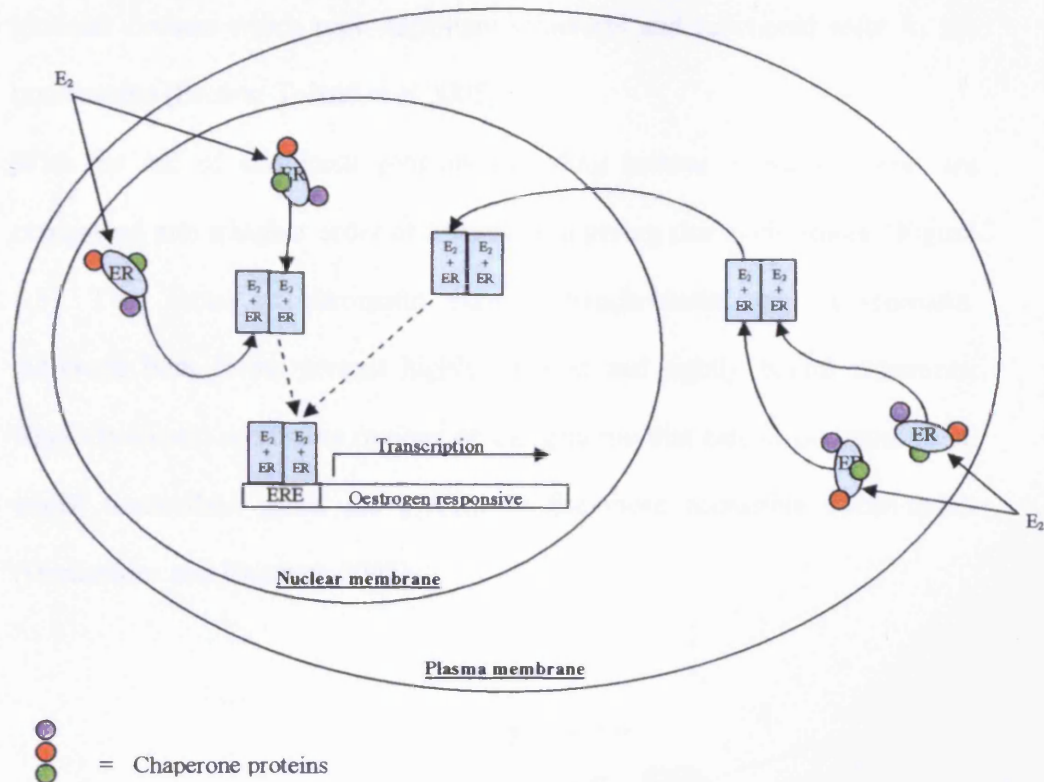
Figure 1.4: Simplified model of Oestrogen action.

1.2.3 From DNA to Chromatin; structural organisation

Two meters of DNA are tightly packed into the nucleus of a eukaryotic cell, so the DNA needs to be organised into an extremely compact and ordered structure. This is achieved by the binding of proteins such as histones that mediate successive orders of DNA folding: two copies of each histone H2A, H2B, H3 and H4 form a protein octamer core, arranged as a $(H3\text{-}H4)_2$ tetramer and two H2A-H2B dimers, around which 146 base pairs of DNA is wrapped approximately 1.7 times to form the nucleosome (McKenna 1999). Each histone consists of two domains, the N-terminal domain and the central

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globular domain which play important structural and functional roles in the nucleosome (Brower-Toland et al 2005).

With the aid of additional proteins including histone 1, nucleosomes are compacted into a higher order of organisation giving rise to chromatin (Figure 1.5). Two forms of chromatin exist; heterochromatin and euchromatin. Although both forms present highly ordered and tightly bound structures, heterochromatin represents regions of the genome that cannot be transcribed, whilst transcribed genes are present in the more accessible euchromatin (Orphanides and Reinberg 2002).
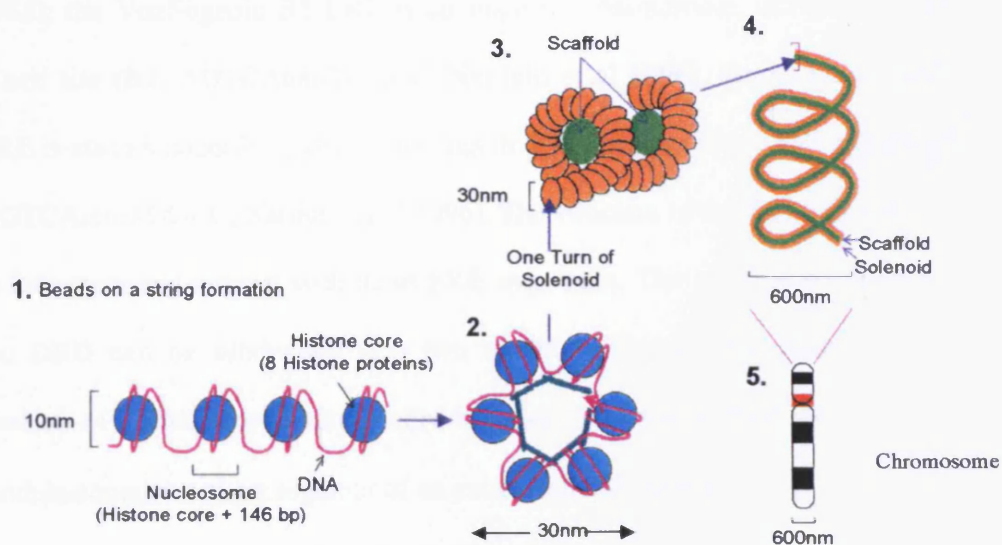
Figure 1.5: Model of DNA compaction within the cell nucleus; from the nucleosome to the chromosome (adapted from Mol biol web book). Illustrates the level of organisation required to efficiently package two meters of DNA into the nucleus of a cell.

Specific recognition sites to transcription factors exist within both forms of chromatin, however, in heterochromatin they are tightly packaged away and in

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euchromatin these recognition sequences are positioned on the outside of nucleosomes so that they are exposed, providing docking sites for transcription factors such as activated ERα.

1.2.4   Classic ER/DNA interactions

The specific sequences of DNA recognised by ER are termed oestrogen response elements (EREs) and reside in the promoter regions of genes responsive to ER transcription. EREs however are not identical within each ER responsive gene. For example, while the Vitellogenin A2 ERE (figure 1.7) is a consensus palindrome (A2, AGGTCAnnnTGACCT, Klein-Hitpass et al, 1988), the Vitellogenin B1 ERE is an imperfect palindrome, differing at the 5'half site (B1, AGTCAnnnTGACC, Nardulli et al 1996). Similarly, the pS2 ERE is also an imperfect palindrome, this time differing at the 3' half site (pS2, GGTCAnnnTGGCC, Nardulli et al 1996). The structure of the DBD allows ER to recognize and interact with these ERE sequences. The globular structure of the DBD can be subdivided into two modules (Figure 1.6). Each module consists of a Zinc coordination centre between four cystein residues. The first module contains a short segment of an antiparrallel β sheet and ends with an α-helical structure between the second pair of Zn coordinating cysteins. The β sheet helps to orientate the residues that contact the phosphate backbone of DNA. The helical structure (P-box cEGckA and down stream amino acids) provides important deoxynucleotide contacts and fits into the major groove of the DNA helix (Figure 1.7). The second module (D-box between P222 and Q226) is more important for phosphate contacts, but also has a role in

dimerisation of ER (ERα:ERα/ERβ:ERβ homodimers, ERα:ERβ heterodimer) (Tsai and O'malley 1994). Minor changes in the amino acid sequence of the DBDs of ERα and β act to change the specificity of DNA binding. Indeed, if the ER P-box is mutated from cEGckA to cGSckV, then ER recognises and binds to a glucocorticoid response element (GRE) rather than an ERE (Tsai and O'malley 1994).
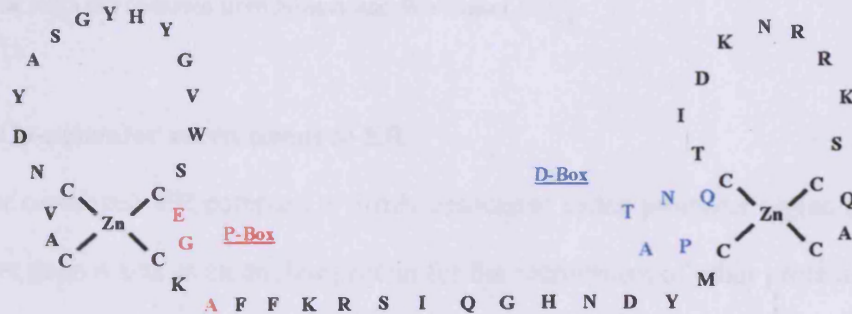
Figure 1.6: Sequence specific recognition. Amino acids of the DBD P-box (Zinc finger 1B) E=Glutamate, G=Glycine, A=Alanine, and D-box (Zinc finger 2A) P=Proline, A=Alanine, T=Threonine, N=Asparagine, Q=Glutamine, are important for the DNA recognition and dimerisation respectively (adapted from Tsai and O'Malley 1994)

AGGTCAnnnTGACCT
TCCAGTnnnACTGGA
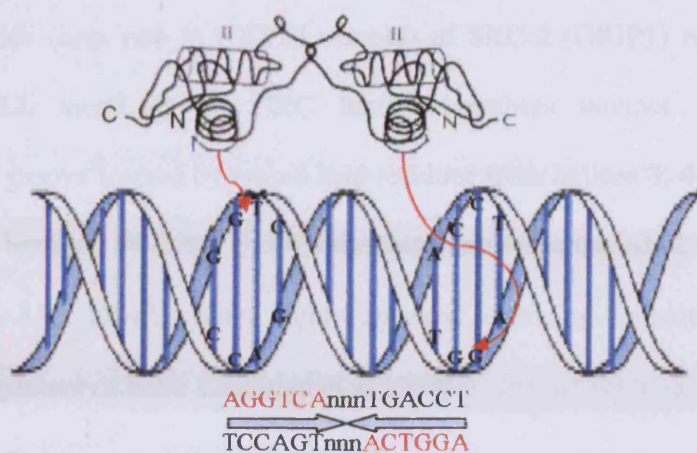
Figure 1.7: Diagram showing dimerisation of two ERs and DBD of each receptor slotting into the major grooves of DNA helix. The base sequences of the ERE are shown plus the palindrome sequence (adapted from Nussey and Whitehead 1999).

1.2.5 Co-activator recruitment to ER

Once the oestrogen–ER complex is firmly associated to the promoter region of the target gene it acts as an anchor protein for the recruitment of other proteins. These proteins are often termed co-activators since they aid the transcription of responsive genes by further protein recruitment or by modulating chromatin structure. The most well studied group of co-activators involved in ER transcription include the steroid receptor co-activator (SRC) family which contain activation domains (AD) that enable them to recruit further co-activators. In addition to their recruiting capabilities however SRC1 and SRC3 have shown enzymatic activity capable of altering the structure of chromatin (Klinge 2000). SRC proteins also contain nuclear receptor boxes (NR boxes) which consist of an LXXLL (where L is leucine and X is any amino acid) sequence shown to be important in the binding to the nuclear receptor (Shiau et

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al 1998). X-ray crystallographic analysis of ERα LBD co-crystallized with the NR box 2 peptide (area rich in LXXLL motifs) of SRC-2 (GRIP1) revealed that the LXXLL motif of the SRC family members interact with a complementary groove formed by amino acid residues from helices 3, 4, 5, and 12 and the turn between helices 3 and 4, otherwise known as the AF-2 surface of ERα (Figure 1.9). This becomes highly exposed following agonist ligand induced rearrangement of helix 12 (Shiau et al 1998).

Ligand binding to the LBD induces a conformational change that releases steric hindrance on the N-terminal of the receptor to unmask phosphorylation sites in the B domain. This leads to AF-1 activation and in some genes synergism with AF-2 (Kraus 1995; Lannigan 2003; Metivier 2001; Metivier 2002). Three conserved residues within the B domain, serine 104, 106 and 118, have been shown to be phosphorylated in response to oestrogen with residue 118 being phosphorylated to the greatest degree (Lannigan 2002). Oestradiol induced rearrangement of helix 12 and exposure of the co-activator binding sites, leads to the binding of the general transcription factor TFIIH through the nuclear receptor box (LXXLL) in its p62 subunit. TFIIH also contains a kinase subunit termed CDK7 and therefore interactions between ERα and TFIIH lead to their co-localisation and phosphorylation of serine 118 (Chen et al 2002). Significantly, these phosphorylation events also lead to recruitment of additional nuclear receptor co-activators and Endoh et al 1999, for example, demonstrated that the co-activator p68 RNA helicase is recruited to ERα AF-1 following phosphorylation of the receptor at serine 118. Studies by Metivier et al 2003 demonstrated that the recruitment of p68 rapidly occured following

oestradiol activation of ERα. Furthermore, mutants of ERα where their serine residues at 104/106/118 were substituted for alanine residues showed reduced AF-1 activity in the presence of 17β-oestradiol due to an inability to recruit the co-activator steroid receptor RNA activator (SRA) (Coleman KM et al 2004).

The enzymatic co-activators recruited to active nuclear receptors can be divided into three major groups: the ATP dependent chromatin re-modellers; the histone acetyltransferases (HATs); and the histone Methyltransferases (HMTs) (Kraus and Wong 2002).

The ATP-dependent chromatin remodelling complexes, including the SWI/SNF family, are multipolypeptide enzymes that contain an ATPase subunit and use the energy stored in ATP to mobilize or structurally alter nucleosomes (Kraus and Wong 2002). BRG1, a catalytic subunit of mammalian SWI/SNF complex, is recruited to ERα due to an interaction between the ERα LBD/DBD and the SWI/SNF BAF57 subunit. Such interactions are oestrogen dependent and are required for transcriptional activation by the estrogen receptor (Belandia et al 2002).

The link between histone acetylation and increased transcriptional activation is well established, yet the exact mechanism of how histone acetylation leads to this increase in transcription is not completely understood. Recent studies have suggested however that the acetylation of the H2A/H2B and H3/H4 N-terminal tails interrupts the charge dependent histone/DNA interaction. The reduction in electrostatic bond strength is believed to facilitate chromatin remodelling making the nucleosomal DNA more accessible to the factors essential to transcription (Brower-Toland et al 2005) (Figure 1.8). A number of different

HATs exist each having different histone targets. The most recognised of these HATs are the co-activators p300 and CBP (two closely related factors commonly referred to collectively as p300/CBP), as well as PCAF (p300/CBP-associated factor). SRC1 and SRC3 have also been shown to possess HAT activity (Klinge 2000).

Histone methylation induces a variety of responses on transcriptional activity. H3-K9 dimethylation and H3-K27 trimethylation are associated with gene silencing, whilst methylation of H3-K4, H3-K36 and H3-K79 are associated with gene activation (Kinyamu and Archer 2004). In addition, the Coactivator-associated arginine methyltransferase (CARM1) has been reported to enhance steroid dependent transcription by interacting with SRC2 resulting in methylation of the arginines 26 and more predominantly 17 of H3 N-terminal tail, whilst protein arginine methyltransferase 5 (PRMT5) methylation of H2A and H4 results in transcriptional repression (Daujat et al 2002).

1.2.6 General transcription factor (GTF) recruitment

ER/Co-activator induced re-structuring of chromatin leads to the exposure of the gene promoter TATA box; a DNA sequence which aids the recruitment of the Pre-Initiation complex (PIC) consisting of the TATA binding protein (TBP), general transcription factors (GTFs) TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH and RNA polymerase II (RNA Pol II) (Figure 1.8). Recruitment of the PIC also occurs via protein-protein interactions between co-activators and GTF or co-activators and RNA polymerase II, as well as direct interactions between ER and GTF or ER and RNA polymerase II (Metivier et

al 2003). In addition to the earlier mentioned co-activators, a family of co-activators has also been shown to be important in recruitment of the PIC called the TRAP (Thyroid hormone receptor-associated proteins)/ SMCC (SRB-MED-containing factor)/ DRIP (vitamin D receptor-interacting proteins) co-regulator family (without HAT activity) which were first identified in thyroid receptor induced transcriptional events (Ito and Roeder 2001).

The order of formation of the PIC was proposed to occur in a stepwise assembly model, initially put forward by Buratowski *et al* 1988. The PIC formation is initiated by the binding of TFIID to the TATA element via the TATA-binding protein (TBP) subunit. For some promoters the binding of TFIID is stabilized by the further association of TFIIA. This "DA" complex is recognized by TFIIB which binds and promotes the recruitment of TFIIF and RNA Pol II. The subsequent association of TFIIE and TFIIH completes PIC assembly and initiates mRNA synthesis.
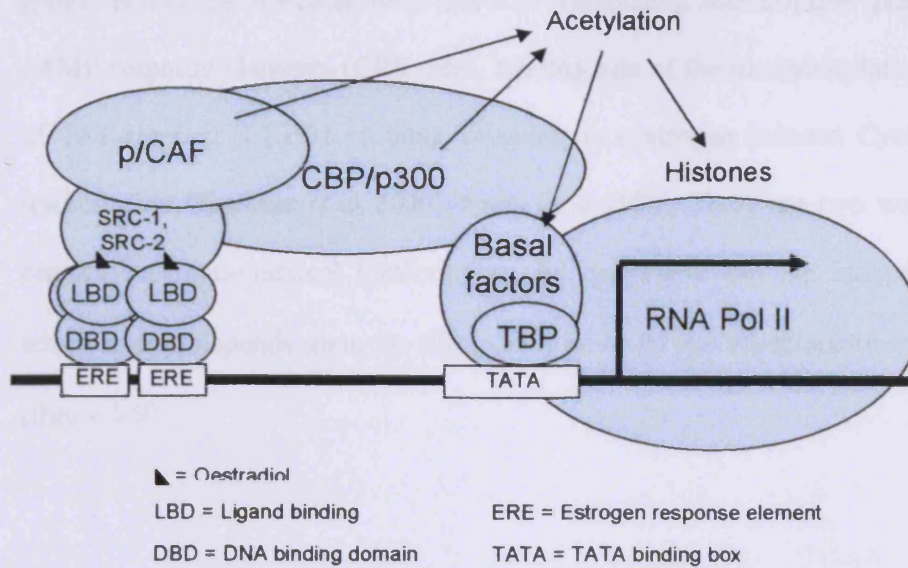
Figure 1.8: Interaction between ER dimer, co-activators and PIC on a classical Oestrogen responsive gene promoter (Modified from Moras and Gronemeyer 1998).

1.2.7 Non-classical ER transcription

Although the classic form of ER directed transcription is well-accepted, the receptor is also known to interact with an array of additional transcription factors bound to their respective response elements to initiate other transcriptional events (Figure 1.9). This is termed non-classical ER transcription and in part explains how ER can activate genes deficient of EREs in their promoter region. For example, it is well established that ER can interact with Jun at 12-O-tetradecanoylphorbol 13-acetate (TPA) response elements (AP-1 sites, the cognate binding site for the Jun/Fos complex), which underlies the oestrogen dependent transcription of the collagenase and IGF-1

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genes, whilst ER interacts with Jun/ATF (activating transcription factor) at cAMP response elements (CRE sites, binding site of the phosphorylated form of CRE-binding (CREB) protein), resulting in oestrogen induced Cyclin D1 transcription (Kushner et al 2000; Webb et al 1999). There are two ways ER can activate non-classical transcription, AF dependent and AF independent, which in turn depends upon the ER species (α or β) and the interacting ligand (figure 1.9).

ER Activation at AP-1 Sites; Two Pathways

AF Mediated

ERα with E2 or tam

AF independent

ERβ, ERαΔAF-1, with SERMs
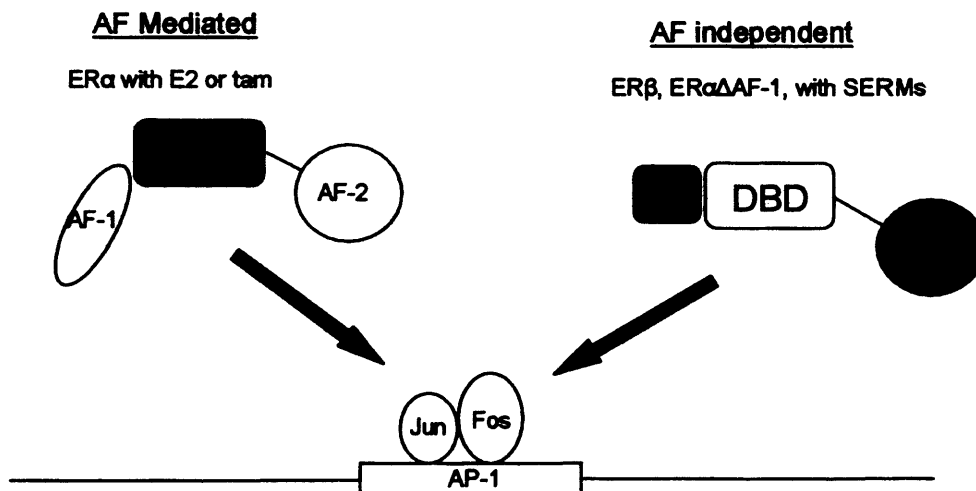
Figure 1.9: ERs use two separate pathways to AP-1. ERα with estrogen or with tamoxifen activate AP-1 through an AF mediated pathway. ERβ and AF-1 deleted ERα potently activate AP-1 through an AF independent pathway in the presence of the SERMs, raloxifene and ICI 182,780 (adapted from Kushner PJ et al (2000)).

Genetic dissections of ER have shown that the isolated LBD is a strong oestrogen-dependent activator of AP1 target genes and this activation requires the integrity of AF2. In the context of the full length ERα, mutations in AF1 also severely compromise oestrogen activation of AP1, which suggests that ERα-oestrogen complex stimulates AP-1 using both the AF-1 and AF-2 surfaces. The Fos and Jun heterodimer associated with AP1 binding sites recruit CBP/p300 and associated proteins, including the p160 co-activators. Thus the co-activator complex of CBP and p160 that is recruited by ER at an ERE is also recruited by Fos/Jun at an AP1 site. The co-activator/transcription factor contact sites differ however, as Fos/Jun contact the CBP component and the p160 co-activators associate to the pre-existing Fos/Jun/CBP complex. Regions of the p160 co-activators essential for transcription factor recruitment, such as the NR boxes, remain accessible; therefore the oestradiol-ERα complex binds to the p160 co-activators with a high affinity and in doing so triggers the AP1 transcription complex into a higher state of activity. This is known as the 'flip horizontal' model (Kushner et al, 2000). Studies by Teyssier *et al* 2001 however demonstrate that the non-classical ERα AF dependent recruitment to AP-1 sites is a result of a direct interaction between the C-terminal part of Jun and the amino acids 250-303 of ERα (DBD and hinge region), which leads to the recruitment of GRIP1. This ER/Jun interaction has been shown to occur in the presence or absence of oestradiol or 4-OH-tamoxifen, and is crucial for the stability of the ER/Jun/GRIP1 complex.

AF independent non-classical ER transcription was first shown to occur at AP-1 sites in the presence of the pure anti-oestrogen Fulvestrant, which blocks AF-

2 activity in ERα N-terminal domain-deleted mutants and ERβ, both of which are deficient of AF-1 (Kushner et al 2000). In addition, 4-OH-tamoxifen was shown to induce the ERβ/ERα N-terminal deletion mutant AF independent transcription of AP1 mediated genes but to a much lesser extent than Fulvestrant. Further studies have shown that AF independent non-classical ER transcription also occurred using full length ERα (Jakacka et al 2001; Wang et al 2004). AF independent activation, in the presence of the anti-oestrogens, was not a result of protein-protein interactions observed in the AF dependent non-classical ER transcription, nor was it dependent on sequestration of co-repressors away from the Fos/Jun complex towards the LBD of ERβ or the truncated ERα (Kushner et al 2000). Wang et al (2004) demonstrated, however, that AF independent non-classical ER transcription depended on nuclear export. They showed that transcriptional potency of classically acting and tethered ERα was unaffected by inhibition by a nuclear export inhibitor leptomycin B, yet non-classical AF independent activation of ERα by Fulvestrant was completely inhibited by the drug. The study suggests that either a critical and unique stimulatory component to this mechanism required activation or modification in the cytoplasm, or an inhibitory component of the mechanism was exported from the nucleus (Wang et al 2004).

1.3   Non-nuclear actions of Oestrogen

ER ligands initiate a myriad of responses within minutes or even seconds of contact with multiple cell types. Given the rapidity of activation, modulation of gene transcription would seem unlikely and moreover inhibitors of protein or

RNA synthesis do not block the effects. These extra-nuclear mechanisms are commonly referred to as non-nuclear or non-genomic (Nadal et al 2001). There is considerable controversy as to the nature of the non-nuclear effect of oestrogens as many studies suggest that they are mediated through membrane associated ERα, whilst others provide contradictory reports suggesting that these effects are brought about through a third subtype of ER distinct from ERα and ERβ (Nadal et al 2001). There have even been reports of non-genomic oestrogen effects independent of ER, where oestrogen binds to neurotransmitter receptor ion channels such as the 5-hydroxytryptamine 3 (5-HT$_3$) receptor and acts as an antagonist (Falkenstein et al 2000). This antagonism was not competitive as 17β-estradiol did not reduce the binding affinity of 5-HT to 5-HT$_3$ receptors (Falkenstein et al 2000). In addition, ER ligands such as 17β-estradiol, 4-OH-tamoxifen and Fulvestrant have been found to activate heptahelical G-protein-coupled receptors (GPCR), such as GPR30 via direct interactions (Filardo et al 2002; Thomas et al 2005).

The ER dependent non-nuclear actions of ER ligands have been implicated in many signalling pathways, including the second messengers Ca^{2+} and endothelial nitric oxide synthase (eNOS), receptor tyrosine kinases [e.g. epidermal growth factor receptor and insulin-like growth factor (IGF) 1 receptor (IGF-1R)], the heterotrimeric G-proteins (independent of GPCR) and protein kinases (e.g. PI 3-kinase, AKT, MAPK family members, the non-receptor tyrosine kinase Src, and protein kinases A and C) (Liao 2003). There are debates, however, as to how the oestrogen receptor associates with the membrane since it has no hydrophobic stretches that could represent

transmembrane domains. Some studies suggest that ERs may associate with the membrane through palmitoylation sequences that frequently anchor proteins to cell membranes (Acconcia et al 2005), but others suggest membrane association with ER is dependent on protein-protein interactions with membrane-associated proteins (Liao 2003).

ERα dependent release of NO involves a protein-protein interaction between ERα and the p85 subunit of PI-3-Kinase which induces the production of specific phosphatidyl inositides that promote the activation of the phosphoinositide-dependent protein kinases PDK1 and PDK2 (Simoncini et al 2000; Hisamoto et al 2001; Liao 2003). These PDKs then phosphorylate AKT/PKB which in turn activates endothelial NO synthase (eNOS). The ER dependent release of NO was found to be biphasic due to increased signalling of the ERK1/2 pathway (Nuedling et al 1999). Song et al (2002) clearly demonstrated that this increased ERK1/2 signalling was dependent on a direct interaction between ERα and Shc on the IGF-IR, which in turn leads to Shc activation, recruitment of Grb/Sos and instigation of the Ras/Raf/MAP kinase signalling. In addition to the release of NO the interactions between ERα and the anchor proteins p85 and Shc induce a plethora of cellular responses including inhibition of apoptosis and promotion of proliferation (Zhang et al 2004).

1.4   Tamoxifen

1.4.1   Classical mode of action of Tamoxifen

In most countries throughout the world tamoxifen is currently the first line endocrine agent for the treatment of ER positive early breast cancer, whilst aromatase inhibitors such as Letrozole are now the front line therapy for ER positive late stage breast cancer. Like oestrogens, tamoxifen diffuses through the plasma membrane and nuclear envelope (Clarke et al 2001), where it interacts with ER to induce a conformational change in the receptor and promotes the dissociation of chaperone proteins (such as the heat shock proteins), dimerisation and classical/non-classical DNA binding (Nicholson et al 2002). The conformation of the AF2 domain in the presence of 4-OH-tamoxifen however, shows a higher affinity for proteins that induce transcriptional repression (co-repressors), rather than transcriptional elevation (co-activators). These corepressor proteins, such as NCoR (nuclear co-repressor protein) and SMRT (silencing mediator for retinoid and thyroid hormone receptors) mediate transcriptional repression by recruiting a complex of histone deacetylases (Cohen et al 2001).

Shiau et al (1998) have revealed the crystal structure of the 4-OH-tamoxifen occupied LBD/AF-2 domain versus the full oestrogen diethylstilboestrol (DES) occupied LBD/AF-2 domain to provide answers to the mechanism by which the AF2 now recruit co-repressors rather than co-activators (Figure 1.10). As a consequence of the 4-OH-tamoxifen induced conformational changes in ER, many inter-residue van der Waals contacts present in the DES complex are

absent in the 4-OH-tamoxifen complex. This forces other residues throughout the binding pocket to adopt alternative conformations. The alternative conformations cause the helices 3, 8 and 11 of ER to shorten by one or two turns compared to those observed in the presence of oestradiol or DES. Additionally, the loop between helices 11 and 12 then becomes longer allowing for more flexibility in the positioning of helix 12. 4-OH-tamoxifen binding also effects the position of helix 12 in a more direct manner, through restricting enclosure of the LBD by helix 12. This is a result of the positioning of the flexible dimethylaminoethyl region of the 4-OH-tamoxifen side chain which protrudes out of the LBD through helices 3 and 11. The interaction between the 4-OH-tamoxifen side chain and the asparagine 351 of helix 12 is also key to the anti-oestrogenic properties of 4-OH-tamoxifen as it controls the repositioning of helix 12 (Levenson and Jordan 1999).

Helix 12 is needed to form the complete AF-2 binding surface along with additional amino acid residues from helices 3, 4, 5 and the turn between helices 3 and 4. The altered conformation of the LBD in the presence of 4-OH-tamoxifen however has surprisingly little effect on the non-helix 12 part of the binding surface (except for the shortening of helix 3). This region, named the static region, is almost identical to that seen in the DES-LBD, 4-OH-tamoxifen-LBD and E_2-LBD complexes. In the presence of 4-OH-tamoxifen helix 12 possess reduced steric hindrance and is unable to enclose the 4-OH-tamoxifen bound LBD, mimicking the hydrophobic interactions of co-activator NR boxes. It is believed that Helix 12, with a stretch of residues (residues 540 to 544) that resembles an NR box (LLEML instead of LXXLL), associate with

the static region of the AF2 thereby concealing the recognition sites for co-activator binding (Shiau 1998).
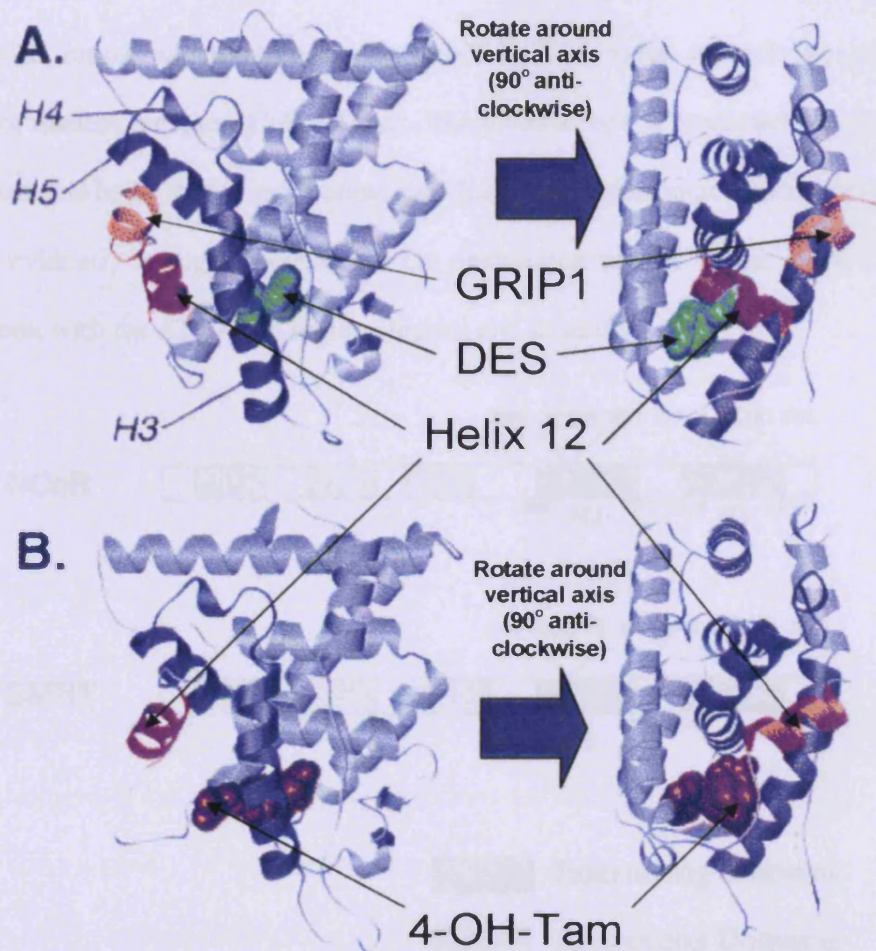
Figure 1.10: (A) Two views of the DES-ERα LBD-Nuclear receptor box II peptide complex as ribbon drawings. Helix 12 traps DES within the LDB. Conformation of helix 12 allows the GRIP1 (SRC2) peptide to bind to the AF2 domain. (B) Two views of the 4-OH-tamoxifen-ERα LBD complex as a ribbon drawing. Side chain of Tamoxifen prevents closure of LBD by helix 12. (Shiau 1998).

As stated above the co-repressors SMRT and NCoR, rather than the co-activators, associate to the 4-OH-tamoxifen-ER complex. This is additionally

due to subtle differences in the nuclear receptor interacting domains (NRID) of co-repressors compared to co-activator NRIDs. Significantly, the co-repressors both contain a conserved bipartite NRID, each containing a critical L-X-X-X-I-X-X-X-I/L motif, which includes the L/I-X-X-I/V-I motif termed the co-repressor nuclear receptor (CoRNR) box. The CoRNR box is predicted to form an extended α-helix that is one helical turn longer than the co-activator motif. This is evidently enough to overcome the obstruction caused by the helix 12 interaction with the AF2 static region (Jepsen and Rosenfeld 2002).
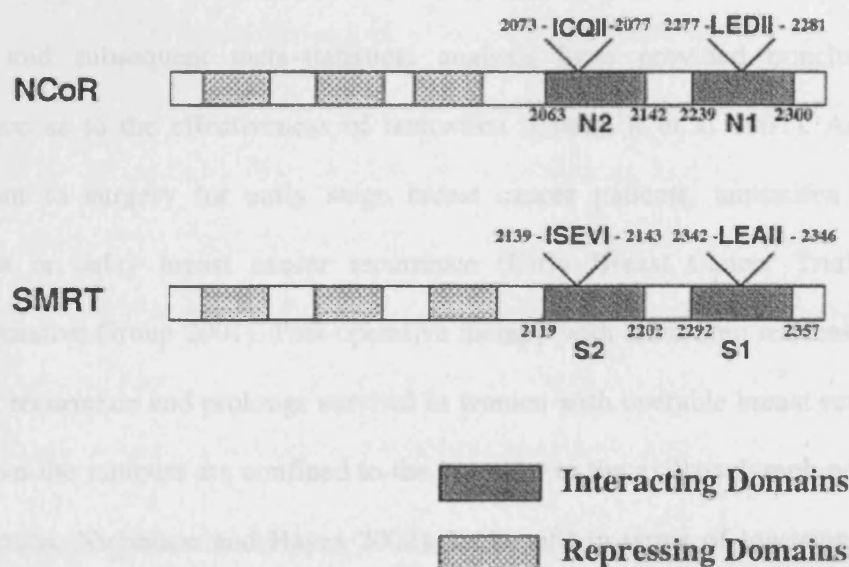
Figure 1.11: Interacting and repressing domains of the co-repressors SMRT and NCoR. Interacting domains associate with the ER whilst the repressing domains recruit histone deacetylases (Cohen et al 2001).

The independent repressor domains within NCoR and SMRT can actively recruit histone deacetylases (HDACs), which act as chromatin-remodelling factors. Currently three major classes of HDACs have been identified. Class I includes HDACs 1, 2, 3 and 8, Class II includes HDACs 4, 5, 6 and 7, and

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Class III are the NAD$^+$-dependent Sir2 family of proteins. Each HDAC is believed to reduce transcriptional activity of nuclear receptors by reducing histone acetylation and thereby the accessibility of DNA to the general transcription apparatus (Margueron et al 2004). As previously mentioned a group of HMTs, including the H3 lysine specific (H3-K9)-specific HMTs (Suv39H1 and G9a), also contribute to transcriptional repression.

1.4.2 Tamoxifen response and resistance

Clinical experience with tamoxifen now amounts to over 10 million patient years and subsequent meta-statistical analysis have provided conclusive evidence as to the effectiveness of tamoxifen (Clarke R et al 2001). As an adjuvant to surgery for early stage breast cancer patients, tamoxifen can prevent or delay breast cancer recurrence (Early Breast Cancer Trialists' Collaborative Group 2001). Post-operative therapy with tamoxifen reduces the risk of recurrence and prolongs survival in women with operable breast cancer in whom the tumours are confined to the breast or to the axillary lymph nodes (Robertson, Nicholson and Hayes 2002). Its benefit in terms of lowering the odds of recurrence and death is limited to women whose tumours express ER and/or progesterone receptor. This benefit increases with increasing receptor level and length of treatment. Benefit progressively increasing from 1 to 5 years of use. Some studies have indicated however, that more than 5 years is not additionally beneficial, and may even be detrimental, while others have not confirmed this observation (Robertson, Nicholson and Hayes 2002). Data from the Early Breast Cancer Trialists' Collaborative Group (EBCTCG 2001) indicated that tamoxifen was associated with significant reduction in

recurrence and death after a median follow up of about 10 years. The annual reductions in recurrence and death with tamoxifen as compared to placebo were 26% and 14%, respectively.

Women with advanced breast cancer have a variety of treatment options, including hormonal therapy, chemotherapy and newer biological agents. The goals of treatment are palliation of cancer related symptoms and prolongation of life. Currently available treatments probably do not cure advanced breast cancer, though some women will have sustained and long-lived disease control (Burstein HJ. 2003). Tamoxifen can be effective as palliation for women with advanced breast cancer. Once again, however, the benefits are primarily restricted to ER positive disease (Robertson, Nicholson and Hayes 2002). Overall, about 60% of patients with ER +ve tumours treated with tamoxifen have objective regression of their disease, lasting on average for 12 months. Furthermore, another 20% of patients have stabilisation of their disease for approximately 6 months (Robertson, Nicholson and Hayes 2002).

Although clearly showing the clinical value of tamoxifen, unfortunately these statistical data also highlight the inadequacies of the drug. This is particularly evident in advanced disease where ~40% of ER+ve patients derive no apparent clinical benefit from tamoxifen treatment, a figure rising to over 90% in ER − ve tumours. Such patients constitute women with de novo tamoxifen resistant disease (Robertson, Nicholson and Hayes 2002).

Significantly, as also illustrated above, even in initially responsive patients, tumour remissions are not long lasting in advanced breast cancer with patients

eventually acquiring resistance to tamoxifen. The clinical problem of de novo and acquired resistance to tamoxifen, which is also evident in primary breast cancer patients, is not fully understood and has given rise to a huge effort by researchers to identify the precise mechanisms that confer this disease state.

Several possible mechanisms have been proposed that could influence the response to tamoxifen and, when altered, contribute to resistance. These include changes in host immunity, endocrinology and tamoxifen pharmacokinetics (Clarke R et al 2001). Competition with endogenous ligands for binding to tamoxifen's primary intracellular targets (ER), and altered function of its targets (ER) may also contribute to tamoxifen resistance (Clarke et al 2001).

At the Tenovus Centre for Cancer Research our interests currently lay in the role that growth factors and their receptors play in mediating such anti-oestrogen resistance (Gee et al 2003; Hutcheson et al 2003; Knowlden et al 2003; Nicholson et al 2005). In clinical and experimental breast cancer it has been demonstrated that lack of response to endocrine therapy, together with increased metastasis and poor survival, can be associated with over expression of the epidermal growth factor receptor EGFR and c-erbB2 (Write et al 1992; Nicholson RI et al 1993; Nicholson RI et al 1994), as well as over expression of additional growth factor receptors including c-erbB3, c-erbB4 (Lupu R et al 1996; Tang CK et al 1996) and IGF-IR (Guvakova et al 1997; Stephen et al 2001). In vitro models of acquired tamoxifen resistance have further demonstrated that raised levels of EGFR may contribute to increased proliferative activity (Knowlden et al 2003; Long et al 1992; El-Zarruk et al

1999) and transfection of either EGFR or c-erbB2 into hormone-dependent breast tumour cells results in hormone-independent cell proliferation and tamoxifen resistance respectively (van Aguthen et al 1992; Benz et al 1993; Miller et al 1994; Kurokawa et al 2000).

1.4.3   Growth factor receptor signalling and tamoxifen resistance

Growth factor receptors, in particular the c-erbB family including c-erbB1 (EGFR), c-erbB2, c-erbB3 and c-erbB4 are selectively activated in various homo- and hetero-dimer combinations by a large family of peptides known as growth factors which bind to the extra-cellular domain of the receptor leading to receptor dimerisation, kinase activation and phosphorylation of specific tyrosine residues in the carboxylic-terminal domains of the receptors (Miller 2002). It must be noted however, that c-erbB2 has no known complementary ligand and c-erbB3 has no C-terminal kinase domain. The mechanism of ligand-induced dimerisation of growth factor receptors, leading to receptor activation, ultimately involves juxtaposition of the cytoplasmic domains of the receptors, allowing the kinase domains to phosphorylate each other at specific tyrosine residues. It is not completely clear how such autophosphorylation is initiated, however, one possibility is that the monomeric receptor has a low enough basal kinase activity to phosphorylate and activate the companion receptor. This then being followed by reciprocal phosphorylation. Alternatively, the interaction between the intracellular domains of the receptors in the dimer may induce conformational changes leading to an increased kinase activity (Favoni and Cupis 2000).

There are several tyrosine residues on the cytoplasmic domain of EGFR that become phosphorylated resulting in the activation of specific cell signalling pathways (Figure 1.12). Tyrosine 845 resides in the activation loop of the receptor kinase domain. Phosphorylation of tyrosine 845 may stabilize the activation loop, maintain the enzyme in an active state and provide a binding surface for substrate proteins. It has been shown that c-Src is involved in phosphorylation of the EGF receptor on tyrosine 845, which is associated with modulation of receptor function (Biscardi et al 1999). Phospho-tyrosine 992 of activated EGF receptor is a direct binding site for the phospholipase C-gamma (PLC-gamma) SH2 domain (Emlet et al 1997). This binding results in activation of PLC-gamma-mediated downstream signalling. Phosphorylation of Tyr1045 creates a major docking site for c-Cbl (Levkowitz et al 1999). Binding of c-Cbl to the activated EGF receptor leads to assembly of ubiquitination machinery to the receptor, enabling receptor ubiquitination and degradation (Ettenberg et al 1999). Phospho-tyrosine 1068 of activated EGF receptor is a direct binding site for the Grb2/SH2 domain (Rojas et al 1996). This binding results in Ras activation through a Grb2/Sos-1 signalling mechanism (Zwick et al 1999). Phospho-tyrosine 1148 provides a docking site for SHC (Zwick et al 1999). Both 1068 and 1148 are involved in activation of the MAP kinase signalling pathway.

EGF

E₂Ub — EGFR

Cbl ← Grb2 ← Shc

Gab1   Sos   PLC-γ

PI3K   Ras   IP₃   DAG

IP₃   STAT   Ca²⁺

Akt   PDK1   Raf   Targets   PKC

BAD   FKHR   Targets   MAPKK   Targets

MAPK

Targets
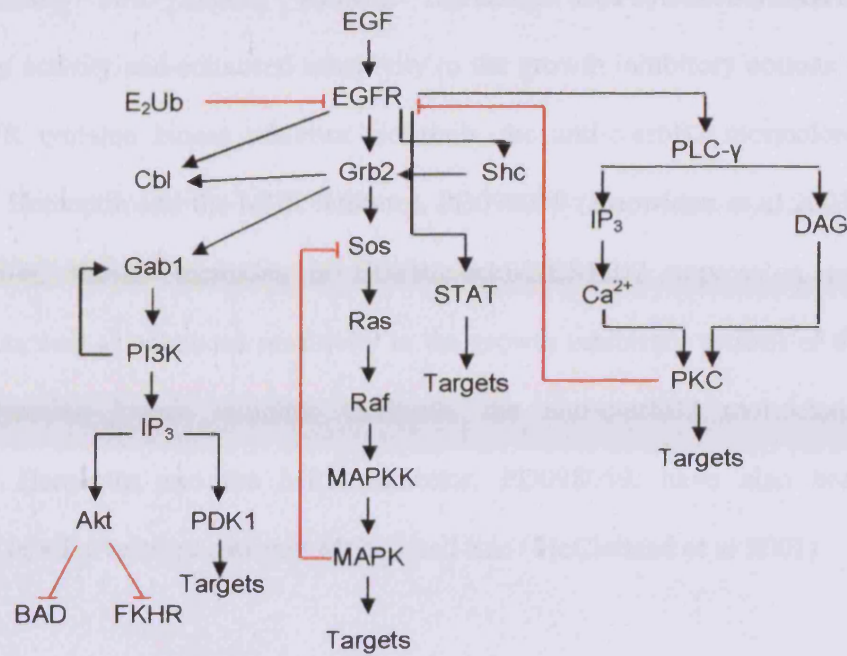
Figure 1.12: Epidermal growth factor (EGF) receptor is a 170 kDa tyrosine kinase. Ligand binding results in receptor dimerization, autophosphorylation and activation of downstream signaling pathways. Figure adapted from www.cellsignal.com

The ERK1/2 pathway is a key mediator of cell proliferation and increased activity of this signalling cascade has been associated with reduced quality and duration of response to tamoxifen and shortened disease free survival in ER-positive breast cancer patients (Mueller et al 2000; Gee et al 2001). Furthermore, constitutive activation of ERK1/2 has also been shown to contribute to anti-oestrogen resistance in MCF-7 breast cancer cell lines (Kurokawa et al 2000; El-Ashry et al 1997; Donovan et al 1997). In agreement with these findings the Tenovus group has demonstrated that an MCF-7 tamoxifen resistant (TAM-R) cell line which acquired resistance following exposure of MCF-7 cells to 100nM 4-OH-tamoxifen over a period of

approximately two months showed increased EGFR/c-erbB2/ERK1/2 signalling activity and enhanced sensitivity to the growth inhibitory actions of the EGFR tyrosine kinase inhibitor Gefitinib, the anti-c-erbB2 monoclonal antibody Herceptin and the MEK inhibitor, PD098059 (Knowlden et al 2003). In addition, similar increases in EGFR/c-erbB2/ERK1/2 expression and activity, as well as enhanced sensitivity to the growth inhibitory actions of the EGFR tyrosine kinase inhibitor Gefitinib, the anti-c-erbB2 monoclonal antibody Herceptin and the MEK inhibitor, PD098059, have also been observed in a Fulvestrant-resistant MCF-7 cell line (McClelland et al 2001).

1.4.4   ER and tamoxifen resistance

Significantly, despite TAM-R cell growth often showing a strong dependence on EGFR/c-erbB2/ERK1/2 signalling, it is clear that ERα also plays an essential role in the growth of such cells. In the study of Knowlden et al (2003), ERα was shown to be highly expressed in the TAM-R cells lines to levels equalling WT cell ERα expression, a finding mirrored in the clinical setting (Encarnacion et al 1993; Brunner et al 1993; Lykkesfeldt et al 1994; Robertson et al 1996). Furthermore, several reports have demonstrated that inhibition of the ERα signalling pathway with the pure anti-oestrogens ICI 164, 384 or Fulvestrant (Faslodex), which act in part, by degrading and down regulating ER expression (Dauvois et al 1992; Howell et al 2000; Wakeling et al 2000), significantly inhibit TAM-R cell growth both in the clinic (Howell et al 1995; Howell et al 1996; Howell et al 2002) and in cell culture models (Coopman 1994; Hu et al 1993). Interestingly, evidence is building to suggest

that the ER signalling pathway mediates its growth promoting effects in TAM-R cells through regulation of the EGFR signalling pathway (Hutcheson et al 2003). These findings were expected as the interplay with respect to ERα and growth factor signalling has been well documented both at the transcriptional regulation of the growth factors themselves (e.g. transforming growth factor-alpha (TGF-α) and IGF-II) (Bates *et al.* 1988; Lee *et al.* 1994) and their respective receptors (e.g., EGFR and IGF-IR) (Nicholson et al 2001). Hutcheson et al 2003, suggest that ER control over EGFR signalling in the TAM-R cell line may be a result of ER controlling transcription of the EGFR ligand TGFα.

Understanding the mechanism by which ERα retains its functionality in TAM-R cells, despite the continual presence of tamoxifen, and elucidating the mechanisms that TAM-R cells have adopted enabling ERα to regulate EGFR/ERK1/2 activity is of key importance to the current project.

1.5   Ligand independent activation of ER

Ligand independent activation of ERα in TAM-R cells may provide a mechanism by which ERα retains functionality. After all, this ligand independent activation has already been shown to enhance the activity of the tamoxifen-ER complex as a positive nuclear transcription factor, increasing its transcriptional and growth-promoting properties (Ali et al 1993; Kato et al 1995). It is now evident that several growth factor-induced protein kinases (e.g., ERK1/2 and Akt), in addition to their direct stimulation of proliferation and survival signals, are able to target and phosphorylate the oestrogen

regulated and other phosphorylation sites on the ER A/B domain, leading to AF-1 activation and ER transcriptional activity in a ligand independent manner (Bunone et al 1996; Kato et al 1995; Campbell et al 2001). In addition to the oestradiol effects on ERα phosphorylation at serine 118 there is overwhelming in vitro and in vivo evidence linking activation of the ERK1/2 pathway and phosphorylation of serine 118 in a ligand independent manner (Bunone 1996; Joel et al 1995; Kato et al 1995; Joel et al 1998; Lannigan 2002). Furthermore, ERK1/2 has also been implicated in phosphorylation of the ERβ (mouse) at two residues within the A/B region, namely Ser-106 and Ser-124 (Tremblay GB 1999; Tremblay A 1999). Interestingly, in cases similar to the TAM-R cells, where breast cancer cell lines express elevated growth factor signalling pathways, ERα phosphorylation at serine 118 was elevated in a growth factor/ERK1/2 dependent manner, suggesting that this event may play a role in the maintenance of ER functionality in TAM-R cells and other elevated growth factor signalling pathway conditions, such as oestrogen deprivation (Martin et al 2003; Shou et al 2004).

Although not located on the AF-1 domain, an additional conserved phosphorylation site involved in activation of the ERα AF1 domain is located at serine 167. Original studies showed that serine 167 phosphorylation was oestradiol dependent, however later studies have shown that this event is in fact ligand independent (Arnold 1995; Le Goff 1994). Several kinase pathways have been implicated in ligand independent phosphorylation of serine 167, including PI3K/AKT (Martin 2000; Campbell 2001; Sun 2001), ERK1/2/Rsk90 (Arnold 1995, Joel 1998, Frodin and Gammeltoft 1999) and

casein kinase II (Arnold 1995). Although ERK1/2 and Rsk90 belong to the same signalling pathway, the significance of their co-phosphorylation of ERα at serine 167 is not fully understood (Lannigan 2002). Phosphorylation of serine 104 and 106 however is believed to be mediated by cyclin A-CDK2 kinase in an AF2 independent mechanism because the phosphorylation event has been shown to occur in the presence and absence of ligand (Rogatsky 1999). Further phosphorylation sites exist within ER including serine 236, 305 and tyrosine 537. These sites, however appear to play roles other than activation of AF-1 (Chen et al 1999; Lannigan et al 2003; Michalides 2004).

1.6 Ligand independent recruitment of transcriptional co-activators

ER transcriptional functionality requires the recruitment of co-activators however tamoxifen is known to block such recruitment to the AF-2 domain (Levenson and Jordan 1999). Endoh et al (1999), however, suggested that co-activator recruitment can also occur in a ligand independent manner. They showed that p68 RNA helicase interacted with the A/B domain, but not with the LBD of ERα. In addition, p68 enhanced the activity of AF-1, but not AF-2, and enhanced the oestrogen and tamoxifen induced transcriptional activity of the full-length ERα in a cell-type-specific manner. The study confirmed that ERK1/2 dependent phosphorylation of ERα at serine 118 enhanced the interaction between ERα and p68 RNA helicase consequently enhancing ERα AF-1 activity. Significantly, the RNA helicase activity previously ascribed to p68 was shown to be dispensable for the ERα AF-1 co-activator activity but the

interaction region for p68 in the ERα A/B domain was essential for the full activity of ERα AF-1. Taken together, their findings show that p68 acts as a co-activator specific for the ERα AF-1 and strongly suggest that this interaction was regulated by ERK1/2-induced phosphorylation of Serine 118. Such ligand independent recruitment of co-activators to ERα AF-1 provides a mechanism which potentially maintains ERα functionality despite tamoxifen induced inhibition of AF-2.

The p68 RNA helicase belongs to a subfamily of RNA-binding DEAD-box proteins (named according to their conserved amino acid sequence motifs), which also contain the p72 RNA helicase. p72, like p68 RNA helicase, associates to the AF-1 region of ERα, but not ERβ. p72/p68 were also shown to interact directly with the activation domain 2 (AD2) of SRC family members (Watanabe et al 2001) allowing the AF-1 domain to indirectly recruit the SRC family of proteins. Interestingly, HeLa cells transfected with the full-length ERα were found to interact physically and functionally with the SRC family members and CBP in the absence of ligand and that mutation of Serine 104, 106 and 118 affects these interactions. Accordingly, ERα dephosphorylation decreases its ligand-independent interaction with SRC-1 and CBP *in vitro* (Dutertre and Smith 2003). Such interactions were also evident between ERβ and SRC-1 which was dependent on ERK2 phosphorylation of ERβ at serine 106 and 124 (Tremblay A 1999).

The co-activator Steroid Receptor RNA activator (SRA) has also been shown to interact with the ERα A/B domain in a ligand independent manner following

ERK1/2 dependent phosphorylation of serine 118 (Deblois 2003), however a later investigation by Coleman et al 2004 has suggested that this interaction between ERα A/B domain and SRA is not phosphorylation dependent and that the co-activator can also interact with ERα and ERβ AF2 domains.

1.7   Post-translational modification of co-activators

Although the phosphorylation of the ER A/B domain has long been regarded as a prerequisite to AF1 dependent activation, more recent studies suggest that phosphorylation of co-activators can also influence transcription levels.

Phosphorylation of SRC1 has been shown to occur at several residues which were found to cluster into two groups as illustrated in the figure 1.13. All of the identified phosphorylation sites have been shown to contain consensus sequences for the serine/threonine-proline-directed family of protein kinases. Two sites (serine 395 and threonine 1179) contain a perfect consensus sequence for ERK1/2, however in vitro studies by Rowan et al 2000 demonstrated that ERK-2 phosphorylated threonine 1179, serine 1185 (an adjacent site containing an imperfect ERK-1/2 motif) and to a lesser extent serine 395. Serine 372 was found to be contained within a consensus sequence for casein kinase II, although this site was not phosphorylated *in vitro* by this enzyme (Rowan 2000).
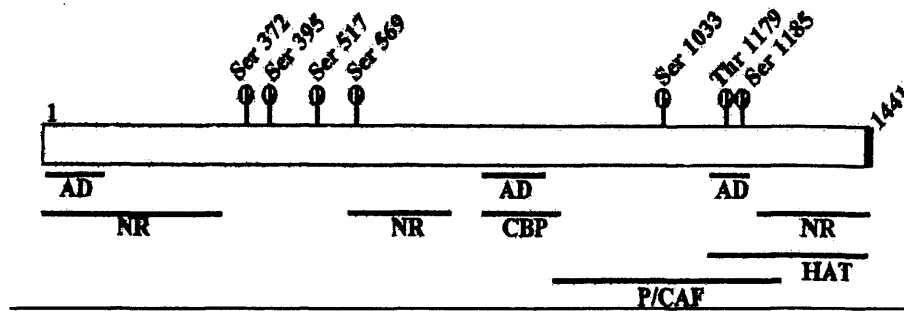
Figure 1.13: Model of SRC1. Illustrates phosphorylation residues (P), Activation domains (AD), nuclear receptor boxes (NR), CBP interacting region (CBP), P/CAF interacting region (P/CAF) and the region containing Histone Acetyl-transferase activity (HAT) (Adapted from Rowan et al 2000).

The positioning of the phosphorylation clusters in SRC1 has provided clues to their functional significance. The first group of sites involving serine 372, serine 395, serine 517 and serine 569. Serine 569 was found to be located within 64 amino acids of the first LXXLL motif of SRC-1, suggesting that these phosphorylation events influence the interaction between SRC-1 and target transcription factors (Rowan BG 2000). The second group of phosphorylation sites in the C-terminal, involving serine 1033, threonine 1179 and serine 1185, are adjacent to a major nuclear receptor interaction domain and also lie within the region of SRC-1 that interacts with the histone acetyltransferase, P/CAF. This suggests that as well as influencing the ability of SRC1 to interact with nuclear receptors these phosphorylation events may affect the ability of SRC-1 to recruit enzymatic co-activators (Rowan 2000). Threonine 1179 and serine 1185 also lie within the region of SRC-1 that was shown to possess HAT

activity and suggests a role for these phosphorylation events in the modulation of SRC-1 HAT activity (Rowan et al 2000).

Such phosphorylation events, in addition to direct phosphorylation of the receptor, may enhance transcriptional activity of ERα even in the presence of tamoxifen. After all, MCF-7 cell lines exposed to 4-OH-tamoxifen have been shown by Shou et al (2004) to recruit phosphorylated SRC3 and ERα (serine 118) to the pS2 gene promoter when over-expressing both c-erbB2 and SRC3. Interestingly ERα/SRC3 phosphorylation and their recruitment to the pS2 gene promoter were inhibited in the presence of the EGFR inhibitor gefitinib, suggesting that growth factor receptor control over both ERα and SRC3 phosphorylation dictates recruitment to gene promoters and therefore transcriptional activity in the presence of 4-OH-tamoxifen.

Aims of the study

There is now considerable evidence to indicate that increased expression and activity of the EGFR/ERK1/2 signalling pathway mediates tamoxifen-resistant growth (Knowlden et al 2003; Long et al 1992; El-Zarruk et al 1999; Mueller et al 2000; Gee et al 2003). It has also become evident that ERα expression and functionality is retained and mediates tamoxifen-resistant breast cancer growth (Robertson, 1996, Brunner et al., 1993, Lykkesfeldt et al., 1994, Encarnacion et al., 1993, Hu et al., 1993, Coopman et al., 1994, Howell et al 1995; Howell et al 1996; Howell et al 2002, Hutcheson et al., 2003). Interestingly, evidence suggests that the ER growth regulation of TAM-R cells occurs due to ER regulation of EGFR signalling pathway, perhaps through transcriptional regulation of EGFR ligands (Hutcheson et al 2003). The EGFR/C-erbB2/ERK1/2 signalling pathway, in addition to directly driving cell growth, can target and phosphorylate key serine residues within the AF-1 domain of ERα (Ali et al 1993; Bunone et al 1996; Kato et al 1995; Campbell et al 2001). Phosphorylation of these residues, in particular serine 118 promotes co-activator recruitment and activation of ERα transcriptional activity (Ali et al 1993; Endoh et al 1999; Kato et al 1995).

The aim of this thesis was, therefore, to examine growth factor-mediated phosphorylation of ERα, in tamoxifen-sensitive (WT) and –resistant (TAM-R) MCF-7 breast cancer cell lines, to elucidate whether such ligand-independent activation of ERα plays a role in the maintenance of ERα functionality in tamoxifen resistance and whether this in turn regulates EGFR signalling activity in these cells. This involved:

- Development of reproducible western blotting and ICC assays in the WT and TAM-R MCF-7 cells to evaluate ERα phosphorylation at serine 118.

- Pharmacological manipulation of EGFR and IGF-1R signalling pathways in the two cell lines to determine their role in mediating serine 118 phosphorylation of ERα.

- Determination as to whether such phosphorylation events contribute to maintaining ERα functionality in TAM-R cells by utilising immunoprecipitation/western blotting (to examine associations of ERα with transcriptional co-regulators), luciferase reporter gene assays, chromatin immunoprecipitation (ChIP) and PCR of possible ERα-dependent genes.

- Determine how EGFR signalling activity is modulated by ERα functionality in TAM-R cells and whether ERα phosphorylation at serine 118 is a contributory factor.

- Development of western blotting assays to measure additional phosphorylation sites on ERα, such as serine 167 and if possible further assessment as to their contribution to ERα functionality.

- Use of proteomic techniques such as 2D electrophoresis and MALDI/TOF mass spectrometry of immunoprecipitated ERα to identify additional phosphorylation events and interactions with novel co-regulators.

Hopefully, identification of the mechanisms responsible for the maintenance of ERα functionality in tamoxifen-resistant breast cancer cells may provide additional therapeutic targets that can be used to combat therapeutic resistance.

2.0   MATERIALS and METHODS

2.1 Materials

2.1.1 Equipment

The equipment used in this study is listed below and obtained from the following sources: BB16 function line cell incubator (Heraeus Instruments, Germany); Nikon eclipse TE200 phase contrast microscope (Nikon, UK); MDH intermed airflow vertical circulating air class II biological safety cabinet (Bioquell, UK); Beckman Coulter® Counter Multisizer II (Beckman, UK); Finn pipettes (1-10µL, 5-50µL, 20-200µL, 100-1000µL and 500µL-5mL, Thermo Labsystems, Finland); IEC micromax RF micro-centrifuge (Thermo Electron Corporation, UK); CECIL CE 2041 spectrophotometer (Cecil, UK); sample rotator (Thermo life sciences, UK); Mini-protean® 3 electrophoresis kit and powerpac 300 (BioRad laboratories Ltd, UK); Stuart scientific STR6 platform rocker (Bibby Sterilin Ltd, UK); Olympus BH-2 phase contrast microscope and Olympus DP-12 digital camera (Olympus, UK); E & G Wallac lumat LB 9507 luminometer (Jensons-PLS, UK); Labconco purifier PCR enclosure, MJ research PTC-100 thermocycler, trans-illuminator and polaroid camera (GRI, UK); 950 Watt microwave (Curries, UK); MSE soniprep 150 sonicator (Sanyo, UK); IPG phor isoelectric focusing unit with 18cm IPG strip holders, Immobiline IPG drystrips-18cm, Multiphor II electrophoresis unit (Flatbed system), EPS 350 X L power supply, Multitemp III thermostatic circulator (Amersham Pharmacia Biotech, UK).

2.1.2 Disposables and plastic ware

The disposables and plastic ware used in this study are listed below and obtained from the following sources: Sterile disposable pipettes (5mL, 10mL and 25 mL), 50mL falcon tubes, lids and cups for coulter counter (Sarstedt AG and Co, Germany); 5mL and 10mL syringes (Sherwood Medical Davis and Geck, UK); sterile needles (BD microbalance™, Becton Dickinson Ltd, UK); Nunc tissue culture plastic ware (12 well plates, universals, plates, flasks and dishes) (Fisher Scientific, UK); 5ml bijou tubes (Bibby Sterilin Ltd, UK); micro-disposable cuvettes and cell scrapers (Fisher scientific, UK); eppendorf tubes (Elkay, Ireland); polypropylene micro-capillary round tips (Sigma chemical co Ltd, UK); nitrocellulose membrane® BA 85 (0.2μMeter) (Schleicher and Schuell, Germany); filter paper grade 3 (460 X 370 mm) (Whatman; UK); luminometer tubes (Becton Dickinson, UK).

2.1.3 Media and supplements

Phenol-red RPMI, phenol-red free RPMI, foetal calf serum, PBS, trypsin, antibiotics (streptomycin/penicillin), Fungizone and L-glutamine were purchased from Invitrogen, UK.

2.1.4 Treatments

Oestrogen receptor and growth factor signalling pathway agonists and antagonists used in this study are listed below and obtained from the following sources: 4-hydroxytamoxifen (Sigma Chemical Co Ltd, UK); amphiregulin neutralising antibody (R & D systems, USA); EGFR tyrosine kinase inhibitor,

Gefitinib (Iressa, ZD1839) (gift from AstraZeneca Pharmaceuticals, UK); MEK1/2 kinase inhibitor, PD184352 (gift from Pfizer, USA); IGF-IR tyrosine kinase inhibitor AG1024 (CalBiochem, EMD Biosciences, USA); IGF-IR tyrosine kinase inhibitor ADW742 (Novartis pharmaceuticals Ltd, UK); PI3K inhibitor LY294002 and growth factors including epidermal growth factor (EGF), transforming growth factor α (TGFα), amphiregulin (Amph) and insulin like growth factor II (IGFII) (Sigma chemical Co Ltd, UK).

2.1.5 Chemicals and reagents

The chemicals and reagents used in this study are listed below and obtained from the following sources: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), acrylamide/bisacrylamide 30% v/v solution, ammonium persulphate (APS), aprotinin, bacitracin, bovine serum albumin (BSA), dimethyl sulphoxide (DMSO), dithiothrietol (DTT), ethylenediaminetetraacetic acid (EDTA), ethidium bromide, gelatine, glutaraldehyde, glycerol, glycine, HEPES, potassium chloride, leupeptin, magnesium chloride anhydrous, methyl green, mineral oil, N,N,N',N'-Tetramethylethylenediamine (TEMED), phenyl methyl sulfonyl fluoride (PMSF), pepstatin-A, phenylarsine oxide, potassium ferricyanide, potassium ferrocyanide, silver nitrate, sodium carbonate, sodium chloride, sodium dodecyl sulphate or lauryl sulphate (SDS), sodium fluoride, sodium molybdate, sodium orthovanadate, sodium thiosulphate, TRI reagent (lysis reagent), triton X-100, trizma base, trizma-HCL, tween 20 (Sigma chemical Co Ltd, UK); BioRad D$_c$ protein assay kit 2, upper buffer pH6.8/lower buffer pH

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8.8 (BioRad laboratories Ltd, UK); bromophenol blue (BDH chemicals Ltd, Poole, UK); rainbow marker 10-250 KDa, dNTPs and random hexamers, CHAPS, iodoacetamide, urea and Phast Gel^{TM} Blue R-350 coomassie blue tablets (Amersham, UK); western blocking reagent (Roche diagnostics, Germany), Chemiluminescent supersignal® west Pico, Dura and Femto (Pierce, USA); X-O-fix-fixer and X-O-dev-developer (X-O-graph Imaging System, UK); acetic acid, acetone, chloroform, dipotassium hydrogen orthophosphate anhydrous, ethanol, formaldehyde, isopropanol, methanol, hydrochloric acid, potassium dihydrogen orthophosphate and sucrose (Fisher Scientific, UK); DPX mountant (Raymond A Lamb Ltd, UK); concentrated immunostaining buffered wash solution (Euro/DPC Ltd, UK), however production of this product ceased resulting in production of in-house PBS-tween (0.02%) as a substitute; liquid DAB^{+} substrate chromogen system (K3468) and mouse/rabbit Envision system HRP DAB^{+} kits (DAKO, UK); lipofectin and MMLV reverse transcriptase (Invitrogen Life Technologies, UK); luciferase reagent kit for single luciferase assay (E4030), dual-luciferase reporter assay kit (E1910) and recombinant RNasin ribonuclease inhibitor (RNase inhibitor) (Promega, UK); Agarose and BioTaq^{TM} DNA polymerase (Bioline, UK).

2.1.6 Antibodies

The antibodies used in this study are listed below and obtained from the following sources: Monoclonal phospho-ERα serine 118 (#2511), polyclonal phospho-ERα serine 118 (#2515), phospho-ERK1/2 (#9101), total ERK1/2

(#9102), phospho-EGFR tyrosine 1068 (#2234), phospho-AKT serine 473 (#9271) antibodies (Cell Signalling Technology, New England Biolabs (NEB), UK); SRC1 (M341), SRC2 (M343), SRC3 (H270), RNA polymerase II (H-224), CBP (C-1) , NCoR (N-19), SMRT (N-20) antibodies (Santa Cruz Biotechnology Inc, USA); polyclonal phospho-ERα serine 167 (ab5701) antibody (AbCam Ltd, UK); total ERα 1D5 antibody (DAKO corporation, UK); total EGFR antibody (# 03-G, Upstate Biotechnology, UK); phospho-Y1158-IGF-IR antibody (Biosource, Belgium); total IGF-IR antibody (Insight biotechnology, UK); p68 RNA Helicase antibody (gift from Kari L Rossow and Ralf Janknecht, Department of Biochemistry and Molecular Biology, Mayo Clinic MN 55905, USA); phospho-serine Q5 antibody (Qiagen, UK); monoclonal β-actin (A1978) antibody (Sigma, UK); secondary IgG horseradish peroxidase labelled, donkey anti rabbit or sheep anti mouse (Amersham Biosciences Ltd, UK); total ERα (HC-20) antibody (Santa Cruz; USA); TFIIB and the pre-immune IgG antibodies (Active Motif; UK).

2.1.7 Plasmids

All reporter gene constructs used in this study were part of the "Mercury profiling system" purchased from Clontech (Becton and Dickinson Biosciences, Oxford, UK), with the exception of the ERE-thymidine kinase (tk)-Luc reporter plasmid DNA which was a kind gift to our group from Prof. Malcolm Parker, Imperial Cancer Research Fund (London, UK).

2.1.8 Primers

Primers used were designed manually using OLIGO (MedProbe AS, Oslo, Norway) primer pair design software. Primers were designed where possible to span intron/exon borders and specificity was checked using the European Molecular Biology Laboratory (EMBL)-GenBank database software using the BLAST program. The following primers were used together with the following assay conditions: β-actin forward 5'-GGA GCA ATG ATC TTG ATC TT and reverse 5'-CCT TCC TGG GCA TGG AGT CCT (204 bp) (24 cycles, annealing at 55°C for 1 minute); amphiregulin forward 5'-TCC TCG GGA GCC GAC TAT GAC and reverse 5'-GGA CTT TTC CCC ACA CCG (350 bp) (24 cycles, annealing at 55°C for 1 minute); β-cellulin (BTC) forward 5'-ACT GCA TCA AAG GGA GAT GC and reverse 5'-CCT GAG ACA CAT TCT GTC CA (395 bp) (33 cycles, annealing at 59°C for 30 seconds); EGF forward 5-GAG TCT GAC TCA GTC CAG AA and reverse 5-TCT ACT TGG AGC AAC AGT GG (478 bp) (33 cycles, annealing at 55°C for 1 minute); epiregulin forward 5-TCC ATC TTC TAC AGG CAG TCC and reverse 5-AGA ATC ACG GTC AAA GCC AC (304 bp) (30 cycles, annealing at 59°C for 30 seconds); HB-EGF forward 5-CGG ACC CTC CCA CTG TAT C and reverse 5-TGA CAG CAC CAC AGC CAC (300 bp) (30 cycles, annealing at 59°C for 30 seconds); TGF alpha forward 5-CCA CAC TCA GTT CTG CTT CC and reverse 5-TCT TTA TTG ATC TGC CAC AGT C (379 bp) (33 cycles, annealing at 55°C for 1 minute); GAPDH forward 5'-TAC TAG CGG TTT TAC GGG CCG and reverse 5'-TCG AAC AGG AGG AGC AGA GAG CGA, ChIP kit negative forward 5'-ATG GTT GCC ACT GGG GAT CT and

reverse 5'-TGC CAA AGC CTA GGG GAA GA (30 cycles, annealing at 55°C for 1 minute).

2.2 Methods

2.2.1 Basic cell culture conditions

Wild type (WT) MCF-7 cells, a gift from AstraZeneca Pharmaceuticals, UK, and WT T47D cells, purchased from the American tissue culture collection, were routinely maintained in phenol red-containing RPMI medium supplemented with 5% (v/v) foetal calf serum (FCS), penicillin-streptomycin (10 IU/ml–10 μg/ml), fungizone (2.5 μg/ml) and glutamine (4 mM). Cells were grown in 75cm^2 flasks and incubated at 37°C in a humidified 5% CO$_2$ atmosphere. Cultured medium was renewed every 3-4 days and cell passaging was carried out at 70-80%confluency. To avoid unwanted oestrogenic effects during experiments the WT MCF-7 and T47D cells were transferred to phenol red-free RPMI medium supplemented with 5% charcoal-stripped steroid-depleted FCS (sFCS), penicillin-streptomycin, fungizone and glutamine. This medium will be referred to as W+5% throughout the thesis.

The tamoxifen resistant (TAM-R) MCF-7 and T47D cell lines were established within Tenovus tissue culture unit by continually exposing the WT cells to 4-hydroxytamoxifen (100nM) for three months and a further six months once cells established a stable tamoxifen resistant phenotype. MCF-7 and T47D

TAM-R cells were then routinely maintained in W+5% supplemented with 4-hydroxytamoxifen (100nM).

The MCF-7 cell line doubly resistant to both tamoxifen and gefitinib (TAM/GEF-R) was established in the Tenovus tissue culture unit by exposing TAM-R MCF-7 cells to gefitinib (1μM) for a total of six months. TAM/GEF-R cells were routinely maintained in TAM-R medium supplemented with gefitinib (1μM).

The Fulvestrant resistant (FUL-R) and fulvestrant and gefitinib resistant (FUL/GEF-R) MCF-7 cell lines were established in much the same way as the TAM-R and TAM/GEF MCF-7 cells, however, fulvestrant (100nM), rather than 4-hydroxytamoxifen, was included in the culture medium.

The MCF-7 X cells, resistant to severely oestrogen and growth factor deprived conditions, were established in the Tenovus tissue culture unit by incubating WT MCF-7 cells in a severely oestrogen and growth factor deprived medium for six months, after which a stable phenotype developed. The medium consisted of phenol red-free RPMI supplemented with 5% stripped serum which was heat inactivated at 65°C for 35 minutes (XsFCS medium). MCF-7 X cells were then routinely maintained in phenol red-free RPMI + 5% XsFCS.

Routine cell culture for all cell lines involved, at 70-80% confluency, cells being detached from the flasks by incubation with trypsin-EDTA (trypsin 0.5%

v/v) for 2 minutes at 37°C. Cells were then transferred to a sterile universal container and pelleted at 1000 rpm for 5 minutes. Cell pellets were re-suspended in the appropriate cell growth media and seeded into fresh tissue culture flasks at a split ratio of 1:10.

2.2.2 Growth Studies

Cell population growth was evaluated by means of trypsin dispersion of the cell monolayers (performed in triplicate) following a 7 day incubation with either, gefitinib (1 μM), PD184352 (10 μM), AG1024 (20μM), LY2904 (10μM) fulvestrant (100 nM) or oestradiol (1nM). Controls were incubated for the same period of time with the appropriate vehicle. Cells were then measured using a coulter counter (Luton, UK). All proliferation studies were performed at least 3 times.

2.2.3 SDS-PAGE and Western blotting

2.2.3.1 Experimental cell culture

Each cell line was removed by trypsination and re-seeded at $5X10^5$ cells per 60mm dish in their respective medium. Cells were grown to 70% confluency (usually after four days) then the medium was aspirated and replaced by DCCM for 24hours. Medium was again aspirated and replaced by DCCM medium containing treatments at the stated doses and time periods: Amphiregulin neutralising antibody at 5, 10 and 20μg/mL for 1hour; gefitinib at 1, and 10μM for 10minutes; PD184352 at 1 and 10μM for 1hour; EGF at

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10ng/mL for 10 minutes; amphiregulin at 10ng/mL for 10minutes; ADW742 at 10μM for 24 hours; AG1024 at 20μM for 24 hours; LY294002 at 10μM for 1 hour; and IGFII at 30ng/mL for 10 minutes. Combination treatments involved: gefitinib (1μM) for 10 minutes followed by gefitinib (1μM) in combination with EGF (10ng/ml) for 10 mins; PD184352 (10μM) for 1hour followed by PD184352 (10μM) in combination with EGF (10n/ml) for 10minutes. All treatment groups were run along side a control arm consisting of experimental medium only. All studies involving TAM-R cells were performed with 4-OH-tamoxifen (100nM) present in the growth medium, including the TAM-R control arms.

2.2.3.2 Cell lysis

After the stated treatments, cells were washed three times with PBS (37°C) and excess PBS was aspirated off. 250μL ice cold lysis buffer (50mM Tris-HCL, 5mM EDTA, 150mM NaCl, 1% Triton X-100 (v/v) in distilled water, pH7.5) supplemented with protease and phosphatase inhibitors (2mM Na_3Vo_4, 20mM NaF, 1mM PMSF, 10 μg/mL leupeptin, 20μM phenylarsine oxide, 10μg/mL aprotinin and 10mM sodium molybdate) was then added to the 60mm dishes. The dishes were then scraped using a cell scraper. Lysate was incubated on ice for 10 minutes then the cellular contents were transferred to eppendorf tubes, centrifuged at 13,000 RPM (15 minutes at 4°C), then aliquotted and stored at -20°C until required.

Earlier assay development experiments also utilised the freeze thaw lysis technique; after the stated treatments cells were washed three times with PBS

(37°C) and excess PBS was aspirated off. 3mL of warm PBS was added to 150mm dishes and cells scraped off the surface of the plate using a cell scraper. The cell/PBS mix were then collected from the dish using a pipette and transferred to a universal which was placed on ice whilst harvesting other samples. The universals were then centrifuged at 3000RPM for 5 minutes (4°C) and placed in dry ice until being stored in a -70°C freezer. The following day, samples were removed from the freezer and 200μL of freeze thaw lysis buffer (0.477g KCL, 2.984g HEPES in 50mL distilled water (pH to 7.4), plus 20 mL glycerol) was added to each sample on ice. Volume of stock was then increased to 100mL with additional distilled water. To every 20mL of stock buffer were added, 1mM DTT, 5μg/mL Leupeptin, 5μg/mL pepstatin A, 1μg/mL Apoprotein 5μg/mL bacitracin and 1mM PMSF. Lysed cells were then placed in an eppendorf after drawing cell mixture through syringe and needle 3 times. Eppendorfs were spun down at 13000 RPM for 20 minutes (4°C), the supernatants were removed, and the protein concentrations measured, prior to storage at -70°C.

2.2.3.3 Protein concentration assay

Total protein concentrations were determined using the DC BioRad protein assay kit (Bio-Rad, UK). Absorbances were analysed at 750nm on a CECIL CE 2041 spectrophotometer.

2.2.3.4 Sample preparation for western blot

50μg of protein from each sample under investigation were mixed with 2X loading buffer (4mL 10% (w/v) SDS, 2mL of glycerol, 2.4mL of upper buffer pH6.8, 1.6mL of distilled water and 1mg of bromophenol blue, plus DTT at 15.5 mg per 1mL of loading buffer.

2.2.3.5 Sample preparation for Immunoprecipitation (IP)

Cell lysate calculated to contain 1mg protein were immunoprecipitated using 1μg of a specific antibody and incubated for 1hr gently rotating in a cold room. Twenty microlitres of protein A/G agarose, was added to the mixture and rotated gently in a cold room over night. The immune complex was centrifuged at 3500 RPM at 4°C for 5 minutes, the supernatant was removed and the complex was washed with PBS (r/t). This procedure was repeated three more times and the resultant pellet following the last centrifugation was re-suspended in 3X loading buffer (including DTT, 22mg/mL). Samples were heated to 100°C for 10 minutes to release and denature the bound proteins before gel loading.

2.2.3.6 SDS-page and western blotting

Protein samples from total cell lysates and following immunoprecipitation were subjected to electrophoresis separation on a 7.5% polyacrylamide gel then *trans*-blotted onto a nitrocellulose membrane. Afterwards, blots were blocked in a 2:20 solution of western blocking reagent and TBS-tween (10mM of trizma base, 0.1M of NaCl, 0.05% v/v Tween 20 and 0.8μM HCL in water, pH

7.5) for at least 1 hr to prevent non specific binding of antisera. Blots were then incubated in the appropriate primary antibodies; monoclonal phospho-ERα at serine 118 for 4 hrs at a 1/20000 dilution (make up fresh each time as specificity decreases), total ERα 1D5 for 4 hrs at a 1/1000 dilution, phospho-AKT and total AKT, phospho-ERK1/2 and total ERK1/2 at a 1/1000 dilution for 1hr, phospho-EGFR tyrosine 1068 for 2 nights at a 1:1000 dilution, total EGFR over night at a 1:1000 dilution made up in 1% non-fat dried milk in TBS-tween. Membranes were washed three times over 15 minutes in TBS-tween and then incubated for 1 hour with the required secondary IgG horseradish peroxidase labelled, donkey anti rabbit or sheep anti mouse, diluted 1/10000 in BM Chemiluminescence Blotting Substrate, made up in TBS-tween (1:20mLs). Membranes were then washed three times in TBS-tween for 30 minutes.

2.2.3.7 Western blotting of IP samples

Blots were incubated in monoclonal phospho-ERα at serine 118 for 4 hrs at a 1/20000 dilution (make up fresh each time as specificity decreases), total ERα 1D5 for 4 hrs at a 1/1000 dilution, phospho-tyrosine overnight at 1/1000 dilution, or total ERK1/2 at a 1/1000 dilution for a 3hr period. Membranes were washed three times over 15 minutes in TBS-tween and then incubated for 1 hour with the required secondary IgG horseradish peroxidase labelled, donkey anti rabbit or sheep anti mouse, diluted 1/10000 in BM Chemiluminescence Blotting Substrate, made up in TBS-tween (1:20mLs). Membranes were then washed three times in TBS-tween for 30 minutes.

2.2.3.8 Protein detection

Detection was performed by applying a thin film of Supersignal® WEST FEMTO/DURA/PICO chemiluminescent substrate to the membrane for 5 minutes. Hyperfilm ECL film was exposed to the membrane for between 2-30 minutes as appropriate. The film was then removed and placed in developer and fixer. Results were scanned using a BioRad model GS-700 densitometer.

2.2.3.9 Statistics

Direct comparisons between WT-MCF-7 and TAM-R cells or between control and treatment effects were assessed using a Student's t test. Differences were considered significant at the $P < 0.05$ level.

2.2.4 Immunocytochemistry (ICC)

2.2.4.1 Experimental tissue culture

Each cell line was removed by trypsination and re-suspended in W+5% and seeded onto 22-mm^2 3- aminopropyltriethoxysilane (APES)-coated glass coverslips contained in 35mm culture dishes at a density of $1X10^5$ cells/dish. After 4 days the cells were grown for 24 hrs prior to treatment in DCCM medium. Treatments included DCCM supplemented with gefitinib at 1μM (10 minutes), PD184352 at 10μM (1hr) or EGF at 10ng/mL (10 minutes). All treatment groups were run along side a control arm consisting of experimental medium only. All studies involving TAM-R cells were performed with 4-OH-

tamoxifen (100nM) present in the growth medium, including the TAM-R control arms. The treatments were removed and the cells were fixed according to the immunocytochemical assay to be performed (see below).

2.2.4.2 Cell fixing and staining

ERα phosphorylated at serine 118

For the best fixation of the immunochemical assay of ERα phosphorylated at the serine 118 residue the cell coated coverslips were immersed in 2% paraformaldehyde supplemented with sodium orthovanadate (2mM) for 20 minutes, washed in PBS for 2X5 minutes and stored in sucrose storage medium at -20^0C until required.

Coverslips were washed with PBS 2x5minutes. They were then dipped in PBS-Tween (0.02%) and blotted on a dry paper towel to remove the excess PBS-Tween (0.02%). The monoclonal ERα-phosphoserine 118 primary antibody was then added at 1/800 dilution in PBS overnight. The cover slips were washed for 2x5 minutes with PBS, then submerged in PBS-Tween (0.02%) prior to addition of the secondary antibody. One drop/coverslip of the peroxidase labelled polymer conjugated to goat anti mouse immunoglobulins from the DAKO mouse envision system HRP K4006, was added to each slip for 1 hour. The coverslips were washed (PBS (2x5minutes) and dipped in PBS-Tween (0.02%)) prior to addition of the DAKO DAB K3468 chromogen to each coverslip for 10 minutes and then removed using 2x5 minutes washes of distilled water. A counter stain of 0.5% methyl green was then applied for 10

seconds each and then rinsed 3 times with distilled water. The coverslips were then air dried and mounted onto glass slides using a DPX soluble mountant.

ERK1/2 phosphorylated at Thr202/Tyr204 assay

For the best fixation for the immunochemical assay of ERK1/2 (phospho-Thr202/Tyr204) the cell coated coverslips were immersed in 4% formal saline for 10 minutes at room temperature (r/t), followed by 2 washes in 70% ethanol over 5 minutes. The coverslips are then stored in 70% ethanol in the fridge prior to the assay.

The coverslips were washed in PBS for 6 quick washes to ensure all ethanol was removed. They were then blocked in PBS-Tween (0.02%). Excess PBS-Tween (0.02%) was removed by dabbing on a dry paper towel, then the primary phospho-ERK1/2 antibody was applied at a 1/25 dilution with PBS for 1 hour. The cover slips were washed for 2x5 minutes with PBS then dipped in PBS-Tween (0.02%) prior to addition of the secondary antibody. The Biogenex Multi Link (Biotinylated anti-various immunoglobulins (mouse, rabbit, rat, and guinea pig) secondary antibody was made up in 1% BSA/PBS and applied for 20 minutes at a 1/65 dilution. The coverslips were washed (PBS (2x5minutes) and dipped in PBS-Tween (0.02%)) prior to addition of the tertiary reagent. The Biogenex concentrated label (Streptavidin peroxidase) tertiary reagent was also made up in 1% BSA/PBS and applied for 20 minutes at a 1/65 dilution. They were washed in PBS for 2x5 minutes and dipped in PBS-Tween (0.02%). The DAKO DAB chromogen was then applied to each coverslip for 10 minutes and then removed using 3x2 minutes washes of distilled water. A counter stain

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of 0.5% methyl green was then applied for 10 seconds each and then rinsed 3 times with distilled water, allowed to dry then DPX mounted on slides.

Activated EGFR (Biosource p1068) assay

Coverslips were fixed in 2.5% phenol formal saline for 5 minutes, followed by 2 X 5 minutes washes in 70% ethanol. After 1 day storage at 4°C in ethanol, coverslips were washed in PBS (2 x 5 minutes) and again stored in the sucrose medium at -20°C. When required, coverslips were washed in PBS (2 x 5 minutes) then dipped in PBS-Tween (0.02%) prior to addition of primary antibody. The primary antibody used in this assay was the Biosource rabbit primary antibody EGFR p1068 at 1/40 dilution made up in 1%BSA in PBS. This was left to incubate overnight at room temperature then washed for 3 minutes in PBS, then PBS-Tween (0.02%) for 2 x 5 minutes prior to addition of the rabbit Envision peroxidase labelled antibody, which was left on the cells for 1 hour at room temperature. The coverslips were washed in PBS (2 X 5 minutes), then PBS-Tween (0.02%) (2 X 5 minutes) and the DAKO DAB chromogen was added for 10 minutes, followed by a distilled water wash (2 X 5 minutes). Following the counterstain of 0.5% methyl green for 10 seconds and additional washes with distilled water (2 X 5 minutes), the cover slips were dried and mounted onto a slide with the DPX mountant.

Assessment of the all slides in each assay was carried out using an Olympus BH-2 light microscope by two independent observers and estimates of percentages of cells specifically stained and, where appropriate, of staining intensity were recorded to give an H-score (range, 0–300). An H-score of 300 would describe strong staining of all (100%) tumour cells. In addition to the 3

staining intensity categories (weak, moderate, and strong) previously used, a category of very weak staining was incorporated into the H-score calculation as shown below: H-score = Σ (% very weakly stained cells x 0.5) + (% weakly stained cells x 1) + (%moderately stained cells x 2) + (% strongly stained cells x 3). Photographs were taken using the Olympus DP-12 digital camera.

2.2.4.3 Statistics

Direct comparisons of H-scores between WT-MCF-7 and TAM-R cells or between control and treatment effects were assessed using a student's t test. Differences were considered significant at the $P < 0.05$ level.

2.2.5   Transient transfection studies

2.2.5.1   *Experimental tissue culture and transfection for dual and single luciferase assays*

Each cell line was removed by trypsination and reseeded at 3 X 10^6 cells/12 well-plate in the W+5% medium for 24 hours prior to transfection. DNA-lipid-medium mixture (500μL) was prepared on day of transfection as follows. Firstly, 3μL of Lipofectin was added to 60μL of transfection medium (per well) at room temperature for 45 minutes to equilibrate. The transfection medium consisted of DCCM and L-Glutamine (200mM) used at 2% (v/v), however no antibiotics were used during the whole transfection time, because the cationic lipid reagents increase the cells permeability and allow excess delivery of antibiotics into the cells. This decreases the health of the cells and

lowers transfection efficiency. A solution of DNA (1.1µg) in transfection medium was meanwhile prepared, which comprised 400ng of an ERE-tk-luc plasmid (a modified pGL2-firefly luciferase vector possessing a thymidine kinase (tk) promoter and a single oestrogen response element (ERE) sequence. 160ng of Renilla-Luc plasmid used as an internal control for the normalisation (a modified pGL2-renilla luciferase vector with tk promoter) and 540ng of PCR-Script (a blank DNA plasmid)

The DNA mixture for the remaining luciferase assays contained: 400ng of the reporter gene construct to be studied X-p-TA/TAL-Luc (X = AP1, CRE, GRE, NFAT, NFκB and SRE). P-TA and p-TAL on their own are used as separate internal controls (i.e. in parallel wells) to assess promoter only driven transcription. 700ng of a blank DNA plasmid (here PCR-Script). The two solutions were then combined and 500µL added/well following aspiration and PBS washing of cells of overnight culture medium.

Cells were left to transfect for 6 hours in the incubator with the transfecting material (500µL/well). After the incubation time, the DNA-lipid mixture was removed, the cells were washed with warm PBS and various treatments were added to the cells including: control, EGF at 30ng/mL (overnight in control then 6 hours EGF), gefitinib at 1µM for 24 hours, PD184352 at 10µM for 24 hours, fulvestrant at 100nM for 24 hours, oestradiol at 1nM for 24 hours, IGF-II at 30ng/mL (control overnight then 6hours IGF-II) AG1024 at 20 µM for 24 hours.

Following these treatments cells were washed with PBS, lysed by scraping in the presence of passive lysis buffer (200µL, Promega lysis buffer X5 diluted in

water), and lysate were stored at -80°C in eppendorf tubes, until analysis for the luciferase relative activity.

2.2.5.2 Luciferase assay

The luciferase assay was conducted in one of two ways depending on the reporter gene constructs used:

ERE-tk-Luc, dual (firefly/Renilla) luciferase assay

In this procedure the dual luciferase kit was used following the instructions given in the Promega manual .

The assay is a dual Luciferase assay, were the firefly signal, representing transactivation of ERE activity and of TK promoter, is measured following a reaction with the first reagent LAR II. The Renilla signal representing TK promoter activity only (i.e. minus the ERE), is measured following a reaction with the stop and glo reagent, which quenches the firefly luciferase signal whilst providing the substrate for the Renilla luciferase reaction.
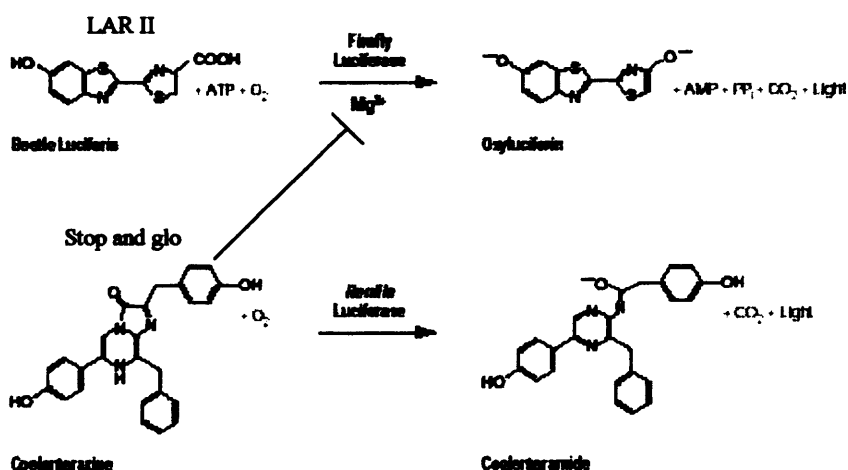
Figure 2.1; Chemical reaction involved in the firefly/renilla luciferase assay

In this assay 100μL of LARII was pre-dispensed into an appropriate number of luminometer tubes. 100μL of lysed sample was added to the tube, mixed and then placed in the luminometer where the firefly signal was measured. The tube was removed and 100μL of the stop and glo reagent added. The tube was placed back into the luminometer to give the Renilla signal.

Single (Firefly) luciferase assay.

In this procedure the single luciferase kit was used following the instructions given in the Promega manual:

First an aliquot of 100μL of luciferase reagent was pre-dispensed into an appropriate number of luminometer tubes. 100μL of lysate was added to the tube, mixed and then placed in the luminometer where the luciferase activity was measured for 10 seconds.

2.2.5.3 Transfection efficiency using β-galactosidase (β-gal)

Transfection efficiency was determined for each cell line by transfecting a set of wells with a β-gal expression vector (β-gal). The β-gal is transfected in the same way as the plasmids from the mercury profiling system using 400ng of β-gal with 700ng of PCR-script. After the incubation period of 6 hours, the transfected cells were stained for β-gal as follows: Cells were washed with r/t PBS and fixed with 0.5% (v/v) glutaraldehyde in PBS (2mLs per well) for 15 minutes at room temperature. After that time, cells were again washed with

room temperature PBS and incubated with a X-gal staining solution containing

potassium ferricyanide and potassium ferrocyanide prepared as below:-

Stocks of 300mM potassium ferricyanide/130mM MgCl2 in PBS, stored -20.

Stocks of 300mM potassium ferrocyanide/130mM $MgCl_2$ in PBS stored at -20

and

40 mg/mL X-gal dissolved in di-methyl formamide, stored in the dark at -20

were initially prepared.

The staining solution was then made by diluting 1/100 v/v each of the two

potassium cyanide solutions in PBS (final concentrations of 3mM for each

potassium cyanide) with 1/320 v/v of stock solution of the X-Gal (final

concentration 0.125 mg/mL).

Cells are left with this staining solution (2mL per well) over night at $37^{o}C$ in

the incubator. The blue colour could be seen the next day in transfected cells

only and the percentage of transfection could therefore be estimated by

counting the relative proportion of stained to non-stained cells.

2.2.5.4 Statistics

Direct comparisons between WT-MCF-7 and TAM-R cells or between control

and treatment effects were assessed using a Student's t test. Differences were

considered significant at the $P < 0.05$ level.

2.2.6 Reverse transcription-Polymerase Chain Reaction (RT-PCR)

2.2.6.1 Experimental tissue culture

Each cell line was removed by trypsination and re-plated at 1.5×10^6 cells per 100mm dish in W+5% medium. Cells were grown to 70% confluency (usually after four days), then medium was aspirated and replaced by the experimental DCCM for 24hours. To study cells under basal conditions medium was again aspirated and cells lysed. To study treatment effects medium was aspirated and replaced by experimental DCCM medium containing treatments (or control) at the stated doses and time periods: Gefitinib at 1μM for 1 hour; PD184352 at 10μM for 4hours or EGF at 10ng/mL for 1 hour; AG1024 at 20μM for 24 hours; fulvestrant at 100nM for seven days; 17β-estradiol at 1nM for 7 days. All treatment groups were run along side a control arm consisting of experimental medium only. All studies involving TAM-R cells were performed with 4-OH-tamoxifen (100nM) present in the growth medium, including the TAM-R control arms.

2.2.6.2 Cell lysis and RNA extraction

Cells were lysed by adding 1mL of TRI lysis reagent for 5 minutes at room temperature. The lysed solution was then transferred to a sterile eppendorf to which 200μL chloroform was added. The tube was gently but thoroughly mixed for 15 seconds by shaking and left to stand for up to 10 minutes at room temperature before centrifugation at 13000RPM for 30 minutes (4°C). 400μL of the top aqueous phase (containing RNA) was carefully removed to a fresh

sterile tube to which an equal volume of isopropanol was added. The tube was then mixed gently again, left to stand for 10 minutes at room temperature and centrifuged at 13000RPM for 10 minutes (4°C). The precipitated RNA (white pellet) was washed with 75% ethanol, gently vortexed, re-centrifuged (10 minutes), pellet dried (important not to let pellet dry totally as this decreases solubility) and re-suspended in sterile water (30µL).

Concentration of the RNA was measured using a spectrophotometer at 260/280 wavelength, using 1:500 dilution of RNA in water. The RNA integrity and concentration was checked by running RNA through a 2% agarose gel.

2.2.6.3 Agarose gel electrophoresis

1g of agarose was added to 50mL of Tris EDTA acetate (TEA) buffer in a 200mL glass conical flask. The agarose was dissolved in a microwave at full power for 1 minute (stopping to mix and ensure agarose does not boil over) and left to cool to approximately 40°C. Following addition of 1µL Ethidium bromide, the gel was poured into a gel tray and well comb added. When the gel set, a solution of RNA (equivalent to 1µg) was mixed to 6µL loading buffer, loaded into the lanes of the gel and run for 30 minutes at 70 volts. The gels were then visualised under UV in a dark room.

2.2.6.4 Reverse transcription

To convert the RNA molecules to complementary DNA (cDNA) of a more stable form, reverse transcription was performed. 1µg of RNA in a total of 7.5µL H_2O was added to 11µL of the RT master mix solution (comprised of

5μL dNTPs (2.5mM), 2μL PCR buffer (10X), 2μL DTT (0.1M) and 2μL of random hexamers (100μM)), denatured at 95°C for 5 minutes in a PCR machine, then cooled to 5°C in Ice for 5 minutes. Mixture was pulse spun in a micro-centrifuge to collect the mixture and placed back on ice. 1μL MMLV (reverse transcription enzyme) and 0.5μL RNase inhibitor is added to give a final volume of 20μL. The tubes are then placed in a PCR machine and reverse transcribed using the following parameters; 22°C for 10 minutes (annealing time), 42°C for 42 minutes (RT extension time) and 95°C for 5 minutes (denaturing time). The resultant cDNA was then stored at -20°C until required.

2.2.6.5 PCR

To amplify the cDNA produced in the RT step exponentially, 1μL of cDNA from each sample (equivalent to 50ng mRNA starting material assuming 100% efficiency of RT reaction) is added to a PCR master-mix solution (37.25μL of sterile distilled water, 5μL of PCR buffer (10X), 4μL of dNTPs (2.5mM), 1.25μL forward primer, 1.25μL of reverse primer, 0.3125μL of β-actin forward primer, 0.3125μL of β-actin reverse primer and 0.2μL of the Taq polymerase) to give a final volume of 50μL per sample.

Tubes were overlaid with 2 drops of sterile mineral oil and placed in the heated lid PCR machine and amplified using the following parameters:-

First cycle	95°C	2minutes (denaturing)
	55°C	1minute (annealing)
	72°C	10minutes (extension)
PCR cycles	94°C	30secs (denaturing)
	55°C	1minute (annealing)
	72°C	1minute (extension)
	repeat appropriate number of times	
Final cycle	94°C	1minute (denaturing)

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55°C   1minute (annealing)
60°C   1minute (extension)
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The PCR product was then mixed with loading buffer and run on an agarose

gel for ~30 minutes. Gels were visualised under UV light and photographed

using a Polaroid camera. The signal intensity is then measured on a

densitometer and normalised to the β-actin readings.

2.2.6.6 Statistics

Direct comparisons between WT-MCF-7 and TAM-R cells or between control

and treatment effects were assessed using a Student's t test. Differences were

considered significant at the $P < 0.05$ level.

2.2.7   Chromatin Immunoprecipitation (ChIP)

The following ChIP studies were carried out under the guidance and

supervision of Dr Martin Giles.

2.2.7.1 Experimental tissue culture

TAM-R cells cultured as monolayer in flask were removed by trypsination and

re-plated at $3X10^6$ cells per 150mm dish in W+5% medium + 100nM 4-OH-

tamoxifen (three 150mm plates required). Cells were grown to 70% confluency

(usually after four days), then medium was aspirated and replaced by the

experimental DCCM + 100nM 4-OH-tamoxifen for 24hours.

2.2.7.2 Cell fixation and chromatin isolation

When cells were ready to harvest the medium was poured off the three plates and 20mL of the fixing solution, containing 1% formaldehyde, was added to each. The cells were incubated in the fixing solution for 10 minutes at room temperature on a rocking platform. The fixing solution was removed and cells were washed by adding 15mL of ice cold PBS to each plate, rocking for 5 seconds and then pouring off the PBS. Fixation reaction was stopped by adding 15mLs of glycine (0.125M) stop solution to each of the plates, swirling to cover and then rocking at room temperature for 5 minutes. Each plate was again washed with PBS following removal of the glycine stop solution. Cell collection involved adding 2mLs of the ice cold cell scraping solution, containing PMSF/PIC, to each of the plates and scraping with rubber policemen. Cells were collected at the bottom edge of the plate by a 1mL pipette and transferred to a 15mL conical tube on ice. This was repeated in the other two plates and lysate from all three plates was pooled in the one 15mL conical tube. centrifugation at 2500RPM at 4°C for 10 minutes collected the cells at the bottom of the tube, then the supernatant was removed, and cells were re-suspended in 1.5mL ice cold lysis buffer for 30 minutes. Cells were transferred to an ice-cold Dounce homogeniser, where they were then gently dounced on ice with 10 strokes to aid nucleic release. The cell nuclei were pelleted by transferring lysate to a 15mL conical tube and centrifuging at 5000RPM for 10 minutes at 4°C. Supernatant was again carefully removed and nuclei pellet re-suspended in 1mL shearing buffer (supplemented with 5μL

PIC) and aliquot into three 1.7mL micro-centrifuge tubes. Each aliquot totalling approximately 350μL.

2.2.7.3 Chromatin shearing

Three aliquots of fixed chromatin were sheared at level 5 power using three different conditions:

a.   Five pulses of 20 seconds each, with a 30 second rest on ice between each pulse.

b.   Ten pulses of 20 seconds each, with a 30 second rest on ice between each pulse.

c.   Twenty pulses of 20 seconds each, with a 30 second rest on ice between each pulse.

The three sheared chromatin samples were centrifuged at 13,000 RPM in a 4°C micro-centrifuge for 12 minutes and the supernatants pooled by transferring each to the same fresh tube. 25μL of the sheared chromatin contained within the supernatant is removed and checked for DNA shearing efficiency and DNA concentration, whilst the remainder of the sheared chromatin is aliquot into four equal aliquots (~220μL each) and used for 4 ChIP reactions (i.e. each aliquot can be tested with four different antibodies).

2.2.7.4 Pre-clearing of chromatin

Chromatin is pre-cleared with protein G beads to reduce non-specific background. 150μL of chromatin is rotated for 2 hours at 4°C in 300μL protein G agarose beads, 177μL of ChIP buffer and 3μL of PIC, in a 1.7mL micro-

centrifuge tube. Following this, the tube is placed in a micro-centrifuge for 2 minutes at 4000RPM. After centrifugation, the tube is placed on ice for 2 minutes to let the beads settle and the supernatant is then transferred to a fresh tube. The centrifugation step was repeated several times to ensure that the agarose beads were removed from the chromatin supernatant.

2.2.7.5 Immunoprecipitation

Each chromatin preparation was used for several ChIPs (e.g. a negative control ChIP, a positive control ChIP (input DNA) and a ChIP with antibody of interest (ERα or TFIIB)). 10μL of the pre-cleared chromatin is transferred to a micro-centrifuge tube and stored at -20°C. This sample will later be used as the 'Input DNA' and stored for future analysis.

Antibody incubations were performed in 0.65 mL siliconized tubes (provided by active motif kit). Tubes were labelled and to each labelled tube, 170μL of pre-cleared chromatin was added. 7μg of total ERα antibody was also added to the ERα labelled tube, whilst 1.8μg of the negative control IgG and TFIIB to their respective labelled tubes. Antibody/chromatin mixture was left to incubate overnight on a rotator 4°C. To each of the antibody/chromatin incubations, 100 μL of fully re-suspended protein G agarose beads were added and incubated on a rotator for 2 hours at 4°C. The beads were then pelleted by centrifuging each ChIP reaction for 2 minutes at 4000RPM. Beads were allowed to settle and the supernatant removed using a pipette. To each tube, 400μL of ChIP IP buffer and PMSF/PIC was added, tubes flicked to re-suspend beads and incubate on a rotator for 1-3 minutes. Again beads were

pelleted by centrifugation and supernatant removed. 400μL of wash buffer 1 was then added, beads re-suspended and incubated on a rotator for 3 minutes, beads pelleted then supernatant removed. This step was repeated four times. 400μL of wash buffer 2 and PIC were then added, beads re-suspended, incubated on a rotator then pelleted and supernatant removed. This was repeated twice more using wash buffer 3 instead of buffer 2 and PIC.

2.2.7.6 DNA elution

In this section immunoprecipitated DNA was collected from the washed protein G beads using two elutions with 50μL ChIP elution buffer.

50μL of the ChIP elution buffer was added to each of the washed protein G bead pellets in the 0.65 mL tubes, briefly vortexed and incubated for 15 minutes at room temperature with gentle rotation. Tubes were then centrifuged for 2 minutes at 4000RPM to pellet beads and the supernatant transferred to appropriately labelled, sterile eppendorf tube. To reverse any cross links between the DNA and protein the supernatant were treated with 4μL of 5M NaCl and 1μL RNase A to each ChIP elution. The reserved Input DNA must also be taken through this procedure (90μL of water was added to Input DNA to bring the volume to 100μL). The ChIP elution and Input DNA sample were then briefly vortexed, briefly centrifuged and placed in a heating block over night at 65°C. Another brief centrifugation for 1 minute, to collect liquid from the side, then addition of 2μL 0.5M EDTA, 2μL 1M Tris-HCl pH6.5 and 2μL proteinase K solution. The mixture was briefly vortexed and centrifuged and incubated for 2 hours at 42°C to digest the remaining proteins.

2.2.7.7 DNA purification

To begin, the required number of DNA purification mini-columns are labelled on their sides and placed in their provided collection tubes in a rack.

The proteinase K-treated samples from the 42°C incubator were centrifuged briefly to collect the liquid condensed at the side of the tubes. 500μL of DNA binding buffer were then added to each DNA sample and vortexed to mix completely. Each sample was then transferred into a labelled DNA purification mini-column and centrifuged for 30 seconds at 10,000 to 15,000 RPM. The mini-column was then removed from the collection tube and placed back into the tube after discarding the flow through. 600μL of DNA wash buffer was added to each mini-column and centrifuged for 30 seconds again at 13,000. The flow through was again discarded from the mini-column and the mini-column placed back onto the collection tube. 300μL of the DNA wash buffer was added to each mini-column and centrifuged for 2 minutes at 13,000 RPM.

2.2.7.8 Primer design

The ChIP assay was used to determine the interaction of ERα with potential ERE's within the promoter region of the amphiregulin gene in TAM-R cells. A region of DNA in chromosome 4q13-q21, consisting of 1542 base pairs 5' to the start of transcription and 148 bp 3' to the start of transcription were entered into the Alibaba 2.1 transcription binding prediction software, which was set to the standard default search parameter, to locate potential ERE's (Grabe N. AliBaba2: context specific identification of transcription factor binding sites. In

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Silico Biol. 2002;2(1):S1-1). Five potential ERE's were found within a 600 bp region (GI:37953278, 671→1270) of 5' flanking DNA and primers encompassing this region were designed using the primer 3 software (Steve Rozen and Helen J. Skaletsky (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (Eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, pp 365-386

Primer 3 output for targeted area of Amphiregulin gene promoter:

Primer 1	Forward	5'-CTCCTGACCTCAGGTGATCC
		858→877 GI:37953278
	Reverse	5'-TGTTCATTTTCCTTCAACTGGA
		1012←991 GI:37953278
Primer 2	Forward	5'-GTACAGTGGCATGACCTTGG
		682→701 GI:37953278
	Reverse	5'-TGGCAAAACCCCATCTTTAC
		835←816 GI:37953278
Primer 3	Forward	5'-TTCCTGTCTCCGCTTCATTT
		1092→1111 GI:37953278
	Reverse	5'-ACTGGTGGCATACTGGCATT
		1241←1222 GI:37953278

2.2.7.9 PCR

All methods carried out for this experiment are shown in the PCR section.

Amphiregulin promoter primer 1 (33 cycles, annealing at 55°C for 1 minute)

Amphiregulin promoter primer 2 (27 cycles, annealing at 55°C for 1 minute)

Amphiregulin promoter primer 3 (33 cycles, annealing at 55°C for 1 minute)

2.2.8 Immunoprecipitation, 2D gel electrophoresis and MALDI/TOF mass spectrometry

2.2.8.1 Experimental tissue culture and Immunoprecipitation

TAM-R cells cultured as a monolayer in a flask were removed by trypsination and re-plated at 1.5×10^6 cells per 100mm dish in W+5% medium. Cells were grown to 70% confluency (usually after four days), then medium was aspirated and replaced by the experimental DCCM for 24 hours. Detergent based cell lysis was carried out as described previously. Immunoprecipitation in a 1.5mL eppendorf required 1mg of protein lysate, 1μg of ERα 1D5 antibody and 20μL of protein A/G agarose. Pellet was collected as described previously. The remaining pellet was re-suspended in 400μL of rehydration buffer [Urea (8M), CHAPS (65mM) and TRIS base (40mM)], supplemented with dithiothrietol (14mg/5mL buffer) IPG-buffer (25μL/5mL buffer) and a few grains of bromophenol blue directly prior to use.

2.2.8.2 2D gel electrophoresis (1st Dimension isoelectric focusing)

No more than 350μL of Immunoprecipitate/rehydration buffer was loaded into the centre of a clean dry IPG strip holder, taking care not to create bubbles. The IPG strip (pH3-10 NL) was gently slid face down into the IP/rehydration buffer with the pointed end directed towards the anodic end of the holder, avoiding

bubbles so that the face of the gel strip was in contact with the anode and cathode connections.

To prevent evaporation IPG cover fluid (~1mL) was pipette drop-wise into the holder thereby covering the entire length of the strip after which the lid was placed on the strip holder. The IPG strip holder was positioned on the IPG phor plate, ensuring the anode was in contact with the larger gold plate and the cathode was in contact with the smaller gold plate. The lysate was then subjected to isoelectric focussing using a programme based on guide lines by Amersham Pharmacia Biotech to deal with preparative sample loads (i.e. 100µg-2mg on the IPG-phor). The protocol used is as follows:-

Rehydration	0volts	0hrs
Step1	30volts	12hrs
Step2	200volts	1hr
Step3	500volts	1hr
Step4	1000volts	1hr
Step5	8000volts	6hrs

The current was limited to 50µA per IPG strip

Following Isoelectric focussing, strips can either go for immediate equilibration and 2nd dimension electrophoresis or they must be stored at -80^0C. Storage of strips must ideally be in long screw cap tubes but careful foil wrapping may be OK.

2.2.8.3   2nd Dimension (Horizontal electrophoresis using pre-cast gel)

Before 2nd dimension electrophoresis, the IPG strips were equilibrated in 10mLs of a buffer containing TRIS-HCL (50mM, pH 8.8), UREA (6M), glycerol (30% v/v), SDS (2% w/v) and DTT (65mM). The procedure was carried out in sealed tubes on a roller for 15 minutes at r/t. TRIS-HCL maintain an appropriate pH during electrophoresis. UREA and glycerol protect against endosmosis and improve transfer of protein from the first to the second dimension, SDS denatures proteins and forms negatively charged protein-SDS complexes whilst DTT preserves the fully reduced state of denatured proteins. The equilibration buffer was then removed and a second 15 minutes equilibration step performed using 10mLs of the above buffer containing Iodoacetamide, instead of DTT, which prevents re-oxidation of protein.

The Multiphor II horizontal electrophoresis system was used for the second dimension separation of proteins. The system has a water cooled platform which is maintained at 15^{0}C throughout electrophoresis by connections to the Multitemp III thermostatic circulator. A pre-cast polyacrylamide SDS gradient gel (ExcelGel SDS, 12-14% gel 12-14%) was removed from its packaging and orientated correctly on the cooling plate so that the polarity of the gel corresponds to that of the plate, taking care to avoid bubbles under the gel. The gel surface was left to dry briefly after removal of the coversheet before placing the colourless cathodic and yellow anodic SDS buffer strips on the respective sides of the gel, avoiding bubbles below the strip. Once drained, the IPG strip was placed carefully gel side down and 3mm in front of the clear anodic buffer strip with the pointed (acidic) end of the IPG strip directed

towards the negative side of the cooling plate. The IPG application strips were then placed under each end of the IPG strip such that they just touched the edges of the gel strip and absorbed away any water which may have leached out as electrophoresis begins. 15-20μL of Molecular weight markers (14.4-97 KDa) were applied to a separate application strip and positioned adjacent to the IPG strip. The electrode plate was positioned above the gel and each electrode aligned over the centre of each buffer strip. The electrode plate was then lowered carefully so that the electrodes were resting on the respective buffer strips. After connecting the cathode and anode electrodes to its appropriate points the lid was replaced and the gel run using the following programme:-

Step1	1000V	20mA	40W	45minutes
Step2	1000V	40mA	40W	1minute step
Step3	1000V	40mA	40W	3hr 40minutes

After the current had ran for a few minutes and the Bromophenol blue dye (thus the sample) had left the IPG strips and entered the second dimension gel by about 5mm, the power was paused. The Multiphor was opened and the IPG strip and application pieces were carefully removed. The cathode buffer gel was advanced to cover the area from which the IPG strip was removed, and the cathode electrode repositioned over the cathode buffer strip. Electrophoresis was resumed until the dye front was travelled just beneath the anode strip, at which point the procedure was terminated. Gel was removed and stained immediately as described below.

2.2.8.4 Coomassie blue (PhastGel blue R350) staining

Stock solution was prepared by dissolving one tablet of Coomassie blue R350 in 80mL of distilled water and left to stir for 5-10 minutes. 120 mL of methanol was then added and left to stir until all of the dye dissolved. Once dissolved the solution was filtered and 1 part of the filtered stock solution was mixed with 1 part of 20% acetic acid in distilled water to give a 0.1% Coomassie blue solution .

The Excel gel was briefly washed in distilled water and soaked in the 0.1% Coomassie blue stain for a maximum of 1hour. De-staining the gel was performed over 24hrs with 30% methanol, 10% Acetic acid and 60% distilled water. The de-staining fluid during this time required several changes. The gel was then wrapped in cling film and stored in the fridge at 4°C.

2.2.8.5 Silver staining

The gel was fixed with a solution containing 100mL methanol, 10mL acetic acid and 90mL of distilled water for 20minutes, followed by washing with 100mL methanol and 100mL distilled water for 10minutes. The gel was placed in distilled water over night then exposed to the sensitising reagent (0.04g Sodium thiosulphate in 200mL of distilled water) for 1minutesute followed by two 1 minute washes with distilled water. The gel was then incubated in the silver nitrate solution (0.2g silver nitrate in 200mL of distilled water) at 4°C for 20 minutes, again followed by two 1 minute washes. On the final wash the gel was transferred to another gel chamber.

2.2.8.6   *In Gel Digestion (Carried out by Dr M Morton)*

Bands were excised from the gel and cut into small pieces (~1x1mm cubes) with a clean scalpel. After several washes with water (2 or 3), the silver-stained gels were destained with a freshly prepared, 50:50 mixture of 30mM potassium ferricyanide and 100mM sodium thiosulphate. The clear gels were incubated with 200mM ammonium bicarbonate (20min), washed with water, dehydrated with acetonitrile and dried in a vacuum centrifuge. The dried gel plugs were reduced in 10mM dithiothreitol (DTT) in 100mM ammonium bicarbonate and incubated at 56°C for 45 minutes. The gel plugs were cooled to room temperature, the DTT removed and alkylated with 55mM iodoacetamide (IAA) in100mM ammonium bicarbonate at room temperature in the dark for 30 minutes. The IAA was removed; the gel plugs washed twice with 50:50 acetonitrile/100mM ammonium bicarbonate and then dried in a vacuum centrifuge. The gel plugs were rehydrated in a freshly prepared and chilled digestion buffer containing 50mM ammonium bicarbonate and 12.5ng sequencing grade trypsin at 4°C. After 45 minutes on ice any remaining digestion buffer was removed and replaced with sufficient 50mM ammonium bicarbonate (~20 to 1) to keep the gel pieces wet during overnight incubation at 37°C. Peptides were extracted from the gel by incubation with 25mM ammonium bicarbonate (20 to 1) for 10 minutes, without removing the supernatant the same volume of acetonitrile was added and incubation continued for a further 10 minutes. The supernatant was recovered and the peptides further extracted from the gels by two similar extractions with 5% formic acid and acetonitrile. All extracts were pooled, dried in a vacuum

centrifuge and redissolved in 5% formic acid (10 to 1). The peptide solutions were then purified and concentrated U-C18 ZipTips (Millipore) using the manufacturers protocol for wetting, equilibration, bonding and washing and peptides eluted directly onto the MALDI target with the matrix solution (saturated solution of -cyano-4-hydroxycinnamic acid in 50:50 acetonitrile/0.2% triflouroacetic acid (TFA), diluted 1:5 with 60:40 acetonitrile/0.2%TFA).

2.2.8.7   *MALDI-TOF mass spectrometry (Carried out by Dr M Morton)*

Peptide mass fingerprinting was carried out using a Bruker Reflex III MALDI-TOF mass spectrometer in the reflectron mode. All mass spectra were externally calibrated with a peptide mixture containing Angiotensin I and II, Substance P, Bombesin, ACTH (1-17 and 18-39) and Somatostatin. Mascot software (Matrix Science, London, UK) was used for searching the NCBInr database using monoisotopic mass values for each peptide mass spectrum. Protein identity was based a MOWSE score with a * significant value of <0.05 within the Mascot software.

3.0 RESULTS

3.1 Development of western blot assay measuring ERα phosphorylation at the serine 118 residue

Aim

The aim of this section was to develop a western blotting assay capable of detecting oestrogen receptor phosphorylation at the serine 118 residue. The cell line of choice for assay development was the breast cancer MCF-7 model. Joel et al 1998 demonstrated, using gel shift assays, that these cells showed measurable levels of phosphorylation at this residue and that oestrogen markedly increased the level of phosphorylation, providing a potentially appropriate positive control for use in subsequent experiments.

Results

Two cell lysis methods were investigated to maximize the yield of ERα extracted from WT cells, a freeze thaw lysis procedure and a detergent based lysis technique. Comparable results were obtained, as demonstrated by figure 3.1a. Subsequent experiments utilised the detergent based lysis technique as this was the more straight forward procedure.

BSA is a highly abundant protein in cell culture growth medium and has a molecular mass of ~68 KDa, which is very similar to the ERα molecular mass of 66-67KDa. As these values are so close there was a possibility that any band produced at this size by an ERα antibody may be due to non-specific binding to residual BSA, rather than specifically binding to ERα. To ensure that the 66KDa band detected by the NEB monoclonal ERα phospho-serine 118

antibody was not BSA, the cells were washed for an increasing number of times with PBS at 37°C prior to lysis and run on the gel illustrated in figure 3.1b. The NEB monoclonal antibody was used at varying concentrations as recommended by the data sheet (1:20000, 1:1000 and 1:500 dilutions). Only the 1/20000 antibody dilution was illustrated in figure 3.1b as this gave the cleanest results.

A clear strong band was present at approximately 66KDa when the cells were washed three to five times with PBS prior to lysis. In contrast, the band at 68 KDa was lost with increased washes. Where no washes were used prior to lysis it was clear that the 68 KDa protein was so abundant that it interfered with the signal from the 66KDa band. The results suggest that the band at 66KDa observed in figure 3.1b (lane 4) was in fact specific for ERα phosphorylated at serine 118 whilst the band at 68KDa was probably BSA. Subsequent experiments measuring ERα phosphorylation at serine 118 were washed three times in PBS at 37°C prior to lysis.

Further confirmation that the band detected by the ERα phospho-serine 118 antibody was in fact ERα was provided by measuring the position of the ERα band detected by the total ERα 1D5 antibody in relation to the rainbow molecular weight markers (Figure 3.2a). These measurements were then used when additional ERα phospho-serine 118 antibodies were tested to establish whether they were more efficient at detection than the NEB monoclonal ERα phospho-serine 118 antibody on WT MCF-7 cells under basal conditions, as illustrated in figure 3.2b. Each antibody was used at varying concentrations as recommended by the respective data sheets. The NEB polyclonal ERα

phospho-serine 118 antibody was used at 1:10000, 1:1000 and 1:500 dilutions, the Santa Cruz polyclonal ERα phospho-serine 118 antibody at 1:1000, 1:500 and 1:100 dilutions and the NEB monoclonal ERα phospho-serine 118 antibody at 1:20000 (previously shown to be optimum dilution). Only antibody concentrations which gave the cleanest results are illustrated in Figure 3.2b. The only ERα phosphoserine 118 antibody to reproducibly detect a clean, strong band at this position was the monoclonal NEB antibody when used at a concentration of 1:20000 (Lane 4).

Evidence that the 66KDa band was indeed ERα phosphorylated at serine 118, was provided by the study illustrated in figure 3.2c. Following protein transfer the nitrocellulose membrane was stained in Ponceau S dye and cut through the middle of the WT control lane as indicated on figure 3.2c. One half of the blot was incubated in the total ERα 1D5 antibody, whilst the other half was incubated with antibody to ERα phosphorylated at serine 118 (Figure 3.2c). Following incubation with the appropriate antibody and luminol reagents, the blot was reassembled to reveal that the band corresponding to ERα phosphorylated at serine 118 was slightly up shifted from the band corresponding to total ERα. This is consistent with studies by Joel et al (1998) demonstrating an upshift in ERα phosphorylated at serine 118 compared to the unphosphorylated form of the protein. Furthermore, the band produced by the monoclonal anti-ERα (phospho-ser 118) NEB antibody showed much greater intensity in the lane containing WT cells treated with oestradiol compared to the same band in the lane containing the WT control sample. This band was not present in the lane containing the negative control. These results were

consistent with the literature and support the view that the band was in fact

specific to ERα phosphorylated at serine 118.

Figure 3.1. Development of western blot assay measuring ERα phosphorylation at serine 118
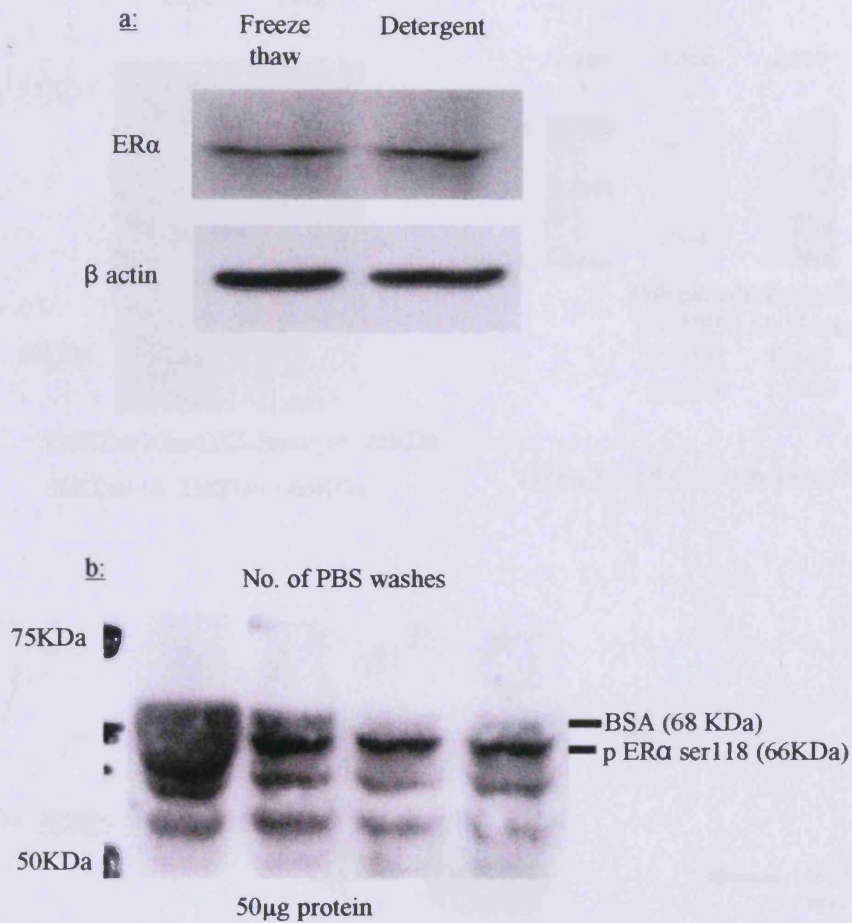
a: Level of ERα extracted from WT cells using freeze thaw cell lysis and detergent based cell lysis technique. β-actin levels show equal loading. b. Western blot of protein from WT cell samples washed with PBS 0, 1, 3 and 5 times prior to lysis, then probed for ERα phosphorylated at serine 118 with the monoclonal NEB antibody.

Figure 3.2. Development of western blot assay measuring ERα phosphorylation at serine 118

a:

Lane 1   Lane 2

75KDa

10mm

50KDa

6.5mm

ERα

(25KDa/10mm)X6.5mm=16. 25KDa

50KDa+16. 25KDa= ~66KDa

b:

Lane 1   Lane 2   Lane 3   Lane 4

75KDa

14mm

50KDa

9mm

p ERα ser118

Polyclonal Ab (NEB) 1:1000 dilution   Polyclonal Ab (Santa Cruz) 1:1000 dilution   Monoclonal Ab (NEB) 1:20000 dilution

14mm X 0.65 = ~9 mm above 50KDa marker

c:

WT C   WT C   WT E₂   -ve C

75KDa

p ERα ser118

50KDa

ERα 1D5 Ab   p ERα ser118 Ab
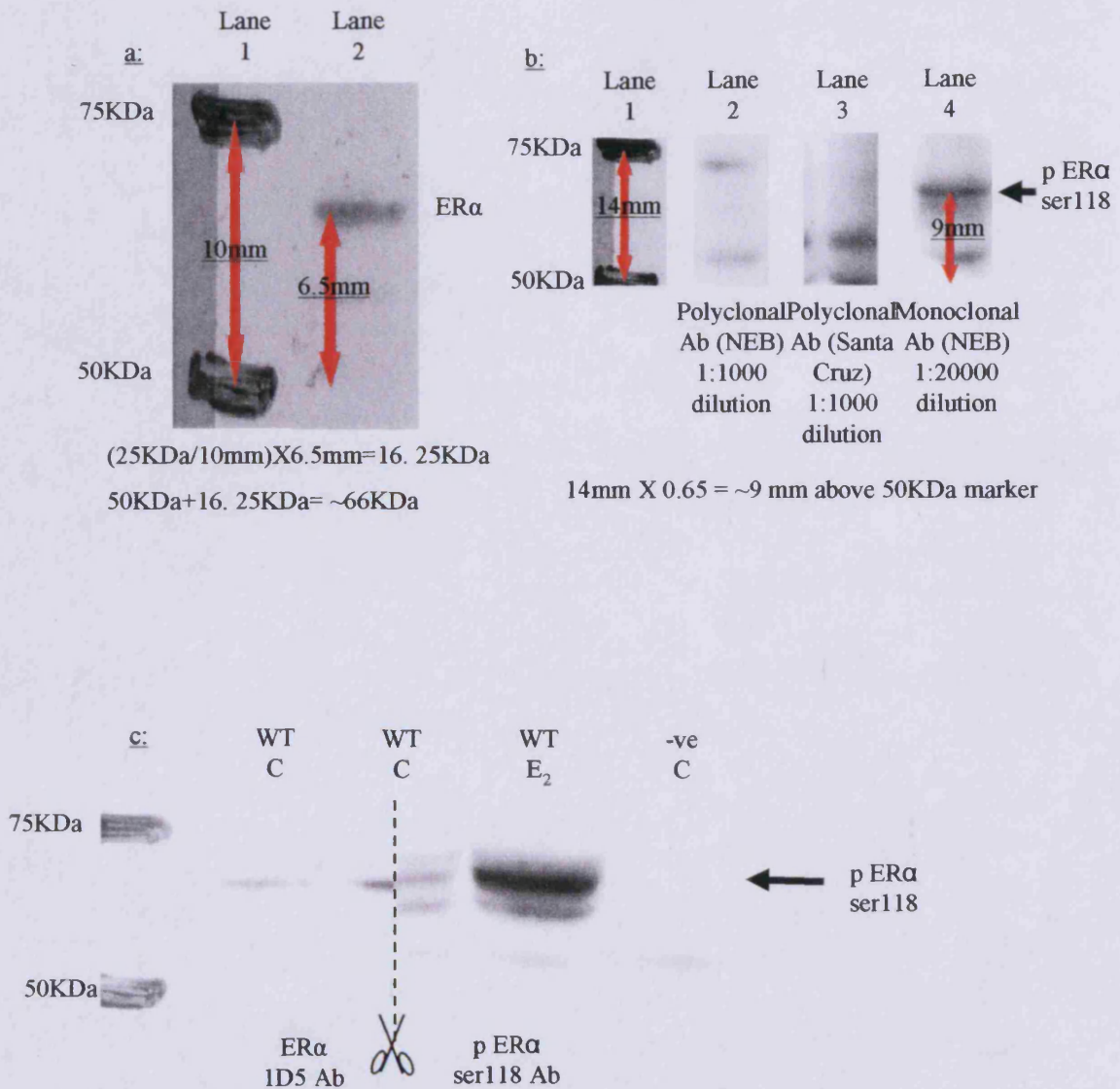
a: Lane 1 shows position of 50KDa and 75KDa rainbow markers. Lane 2 shows position of total ERα from WT cells in relation to rainbow markers. b. Lane 1 shows position of 50KDa and 75KDa rainbow markers. Lane 2 shows protein bands identified by the polyclonal NEB pERαSER118 antibody (#2515) at 1:1000 dilution (WT C). Lane 3 shows protein bands identified by the polyclonal Santa Cruz pERαSER118 antibody(#sc-12915) at 1:1000 dilution (WT C). Lane 4 shows protein bands identified by the monoclonal NEB ERαpSER118 antibody (#2511) at 1:20000 dilution. The figures are representative of two separate experiments (WT C). c. Western blot cut through lane containing WT control (WT C) sample. One half of blot, containing WT C samples was incubated in total ERα 1D5 antibody, while the other half, containing WT C, WT cells treated with E₂ (WT E₂) and Du 145 Cells (-ve C) was incubated in monoclonal NEB pERαSER118 antibody.

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3.2 Role of ERα in mediating growth of wild type (WT) and tamoxifen-resistant (TAM-R) MCF-7 breast cancer cell lines.

Aim

Tamoxifen resistant breast cancer appears to stably express ERα both in the clinical setting and in pre-clinical cell models (Robertson, 1996, Brunner et al., 1993, Lykkesfeldt et al., 1994, Encarnacion et al., 1993). Furthermore, a number of reports have indicated that down regulation of the ER with the pure anti-oestrogen, fulvestrant, inhibits tamoxifen-resistant growth in the clinic and *in vitro* suggesting that the ER has a continued role in growth regulation in this condition (Brunner et al., 1993, Lykkesfeldt et al., 1994, Coopman et al., 1994, Hu et al., 1993, Howell & Robertson, 1995, Howell et al., 1996, 2002). Similarly, the Tenovus laboratory has shown that growth of tamoxifen resistant MCF-7 breast cancer cells was sensitive to the inhibitory actions of Fulvestrant (Knowlden JM et al, 2003, Hutcheson et al 2003). This section aims to confirm these studies.

Results

At 7 days oestradiol significantly increased growth of WT (P < 0.05) and TAM-R MCF-7 cells (P < 0.05, n=3) (Figure 3.3a/b), whilst 100nM fulvestrant significantly blocked basal cell growth in both cell lines (P < 0.05, n=3 for both cell lines). Total ERα levels were comparable between the WT and TAM-R cells and 7 days exposure to 100nM fulvestrant significantly decreased ERα in each cell line (P < 0.0001, n=3 for both cell lines) (Figure 3.3c/d). The total

ERα densitometry readings were normalised to densitometry readings of β-

actin to account for any errors in protein loading.

Figure 3.3. TAM-R versus WT MCF-7 cells; role of ERα in growth.
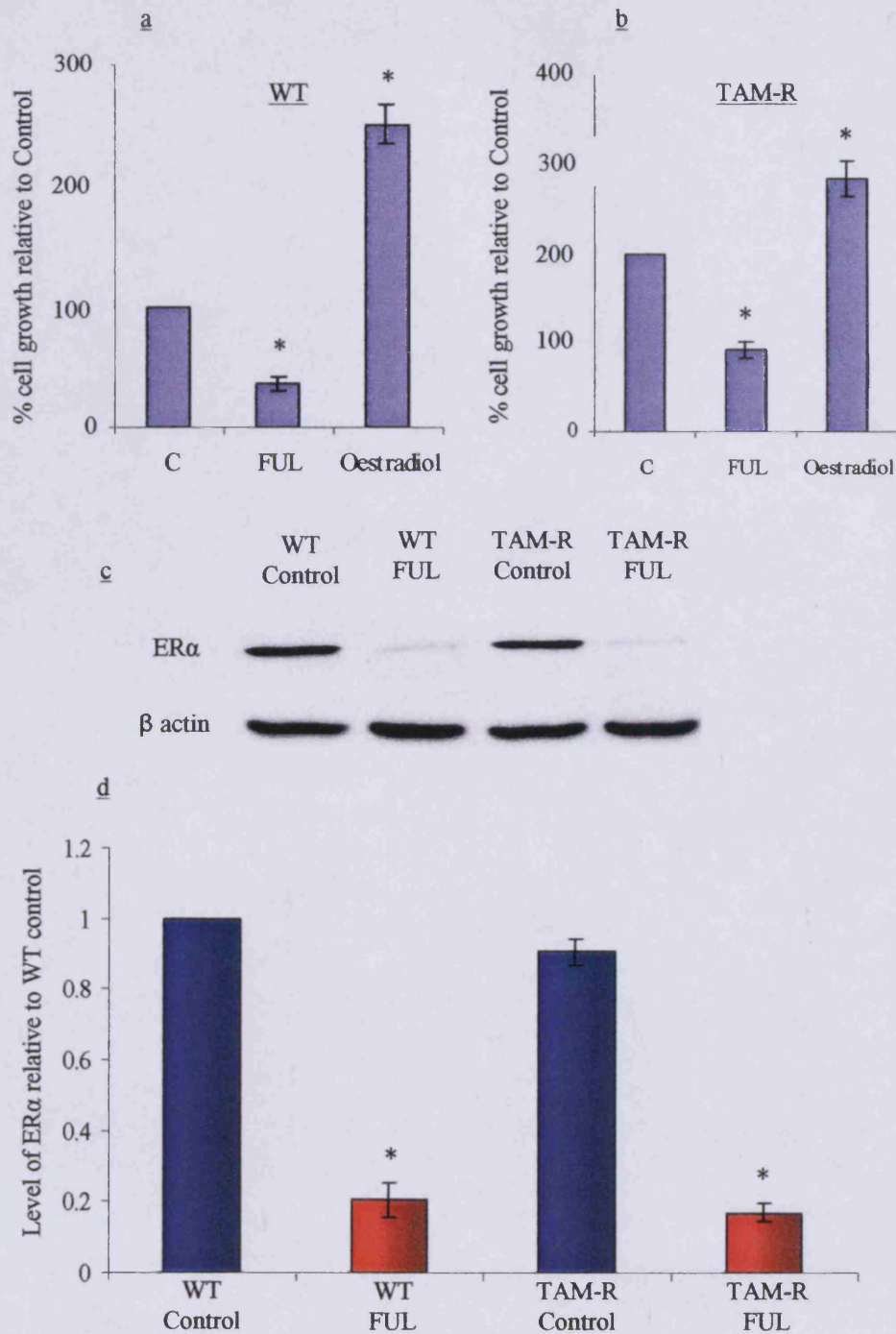
Growth responses of a) WT MCF-7 and b) TAM-R MCF-7 cells after 7 days in phenol red-free RPMI medium containg 5% charcoal-stripped serum in the absence and presence of either fulvestrant (FUL, 100 nM) or oestradiol (1 nM) (TAM-R cells are continually exposed to 100 nM TAM) (n=3). c: Western blot analysis and d:. Mean densitometry readings (± SEM) of Western blots demonstrating ERα expression in WT and TAM-R cells in either the absence or presence of fulvestrant (FUL, 100nM) for 7 days. For densitometry readings total ERα expression levels were normalised using total β-actin expression levels. * = P<0.001 compared to control.

3.3 ERα phosphorylation at serine 118 in a tamoxifen-resistant breast cancer (TAM-R) cell line.

Aim

Growth factor signalling pathways have been shown to induce phosphorylation of ERα at serine 118 via activation of ERK1/2 in multiple cell lines (Kato et al 1995; Bunone et al 1996; Lannigan et al 2003). We have previously reported that our "in-house" TAM-R cell line, under basal growth conditions, demonstrated increased EGFR/ERK1/2 signalling pathway activity when compared to the parental WT cell line. The aim of this section, therefore, was to establish whether the elevated EGFR/ERK1/2 signalling in this cell line was accompanied by increased ERα phosphorylation at serine 118.

Results

TAM-R versus WT MCF-7 cells; phosphorylation (activity) of EGFR and ERK1/2

EGFR expression and phosphorylation (tyrosine residue 1068) was significantly greater in the TAM-R cells compared to the WT cell line under basal growth conditions ($P < 0.005$, n=3 for both total and phosphorylated EGFR, figure 3.4). Phosphorylation of ERK1/2 was also significantly greater in the TAM-R cells compared to the WT cells ($P < 0.05$, n=3), whilst total ERK1/2 and β-actin expression levels remained unchanged between the sample groups (Figure 3.4).

TAM-R versus WT MCF-7 cells; ERα phosphorylation at serine 118

ERα phosphorylation at serine 118 was significantly greater in the TAM-R cells compared to the WT cells under basal growth conditions (P < 0.05, n=3), correlating with the elevated level of phosphorylated EGFR/ERK1/2 in this cell line. The level of ERα phosphorylation at serine 118 in the TAM-R cells was also significantly greater than that observed following treatment of WT cells with oestradiol (P < 0.05, n=3) (Figure 3.5). Total ERα expression levels were comparable between the two cell lines.

Figure 3.4. TAM-R versus WT MCF-7 cells; phosphorylation (activity) of EGF-R and ERK1/2.
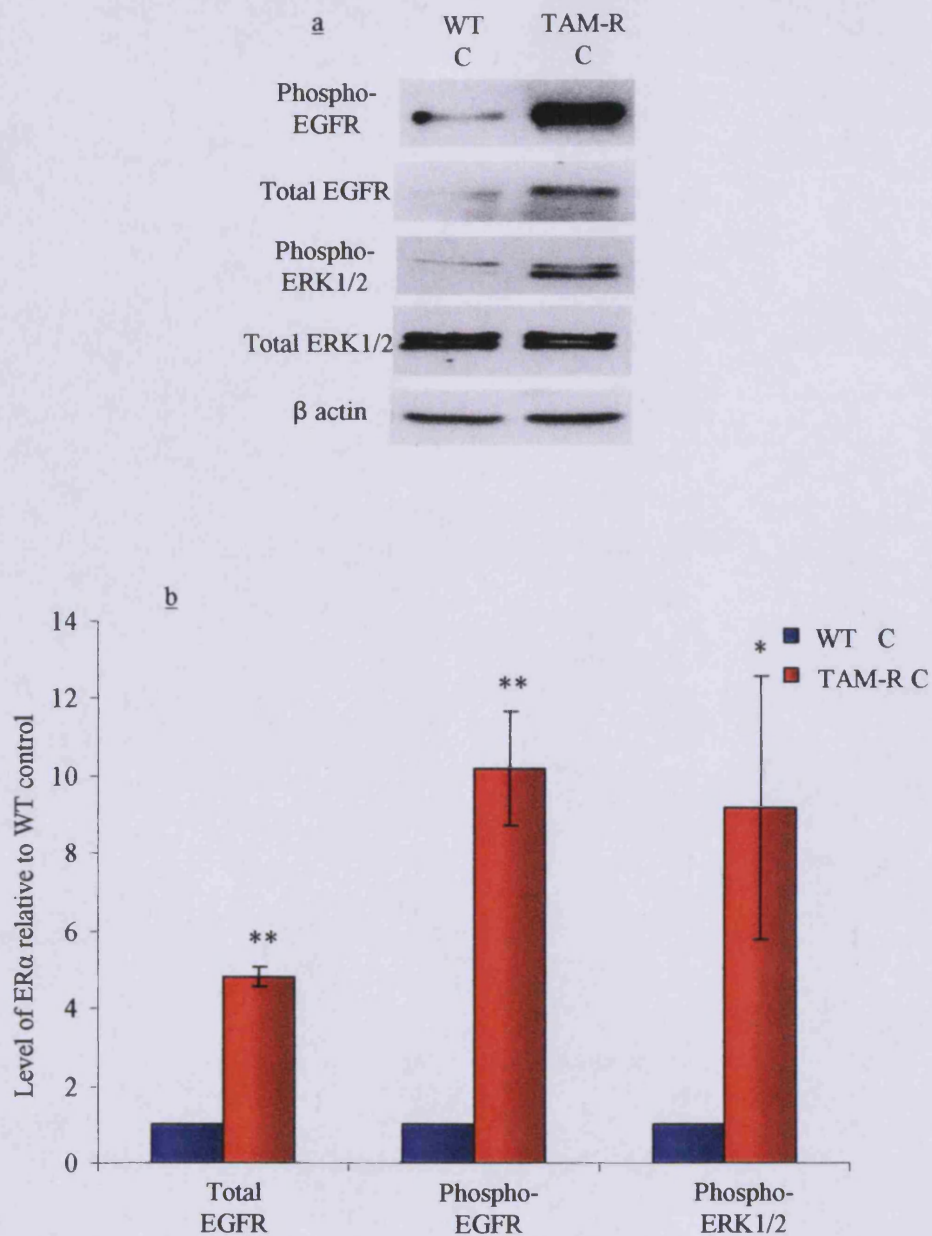
a: Expression of total and phosphorylated EGFR and ERK1/2 phosphorylation in WT and TAM-R cells under basal growth conditions (Knowlden et al 2003). b: Mean densitometry readings (± SEM) of Western blots for total and phosphorylated EGFR and phosphorylated ERK1/2 expression in WT and TAM-R cells (n=3). ERK1/2 phosphorylation levels were normalised using total ERK1/2 expression levels. Total and phospho-EGFR levels were normalised using total β-actin expression levels. * = P<0.05 compared to control. ** = P<0.01 compared to control.

Figure 3.5. TAM-R versus WT MCF-7 cells: ERα phosphorylation at serine 118
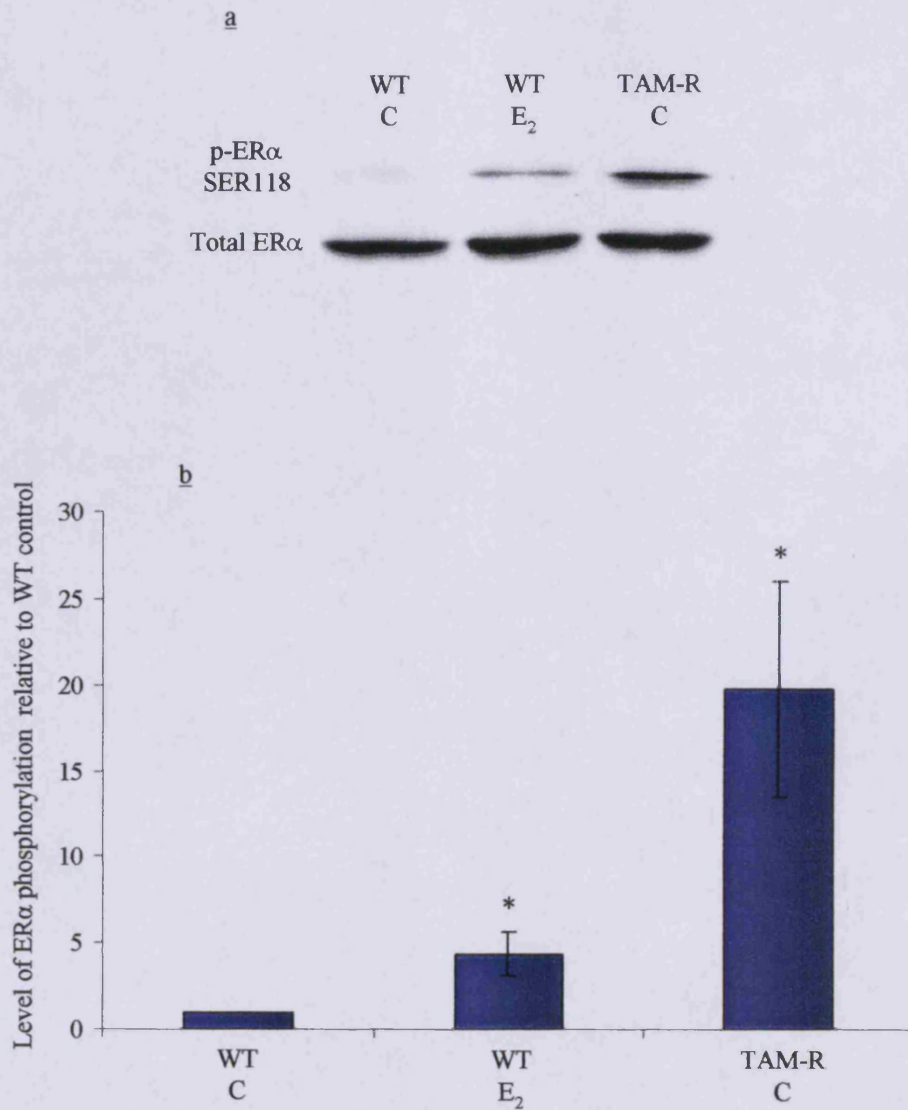
a. Western blot analysis and b. Mean densitometry readings (± SEM) of Western blots demonstrating expression of ERα phosphorylation at serine 118 in WT cells, either in the absence or presence of oestradiol (1 nM) for 5days, and in TAM-R cells under basal growth conditions. Total ERα expression demonstrates equal loading. Densitometry values for ERα phosphorylation at serine 118 were normalised using total ERα expression levels. * = P<0.05 compared to control.

Section 3.4 Development of Immunocytochemical (ICC) assay measuring ERα phosphorylation at the serine 118 residue in TAM-R cells

Aim

ERα phosphorylation at serine 118 in the TAM-R cells had not previously been researched at the time of this investigation and therefore immunocytochemical (ICC) evidence in addition to western blotting was sought. The aim was to develop an ICC assay capable of measuring the effects of pharmacological agents on ERα phosphorylation at serine 118 in the TAM-R cell line. ICC has the advantage of revealing the cellular localisation of investigated proteins and is also of emerging importance in monitoring signal transduction using phospho-specific antibodies.

Results

When developing a new ICC assay it is essential to determine the most appropriate fixative. Based on previous laboratory experience the ability of 2% Paraformaldehyde with sodium orthovanadate (2mM) (20 minutes) to fix cells and preserve phosphorylated epitopes was compared to a range of other fixatives including 2% paraformaldehyde (10mins), 4% formaldehyde in PBS (10mins) or formal saline (10mins). These reagents retained adequate morphology and comparable levels of the antigen directed to the antibody for ERα phosphorylated at serine 118 at a 1/800 dilution as revealed by figure 3.6a, b, c and d. The decision to use a 1/800 dilution of primary antibody was also based on previous laboratory experience. Closer inspection of the cells

revealed that the highest level of nuclear staining in the TAM-R cells was obtained using the monoclonal antibody to ERα phosphorylated at serine 118, when cells were fixed with either 2% paraformaldehyde vanadate for 20 mins or 4% formaldehyde for 10 minutes or (figure 3.6 a and c respectively). TAM-R cells fixed with 2% paraformaldehyde or formal saline for 10 minutes did not produce slides with sharp enough staining (Figure 3.6b and d respectively). The polyclonal NEB antibody at a range of dilutions was also tested with the two most successful fixatives, 2% paraformaldehyde vanadate or 4%formaldehyde, in the WT versus TAM-R cells (Figure 3.7). An increased antibody dilution of 1 in 20 produced immunostaining in both WT and TAM-R cells following fixation with either fixative (Figure 3.7). However, the polyclonal ERα phosphoserine 118 antibody was not used for further studies for several reasons. Many non-specific bands were observed in western blotting experiments when the polyclonal antibody was used at such high concentrations. This therefore suggests that the staining in the polyclonal stained cells may refer to non-specific interactions with the antibody. The images did not appear to produce such sharp and clear signals as the monoclonal antibody. Furthermore the price of using this antibody at such concentrations was far greater than using the monoclonal.

WT cells compared to TAM-R cells fixed with 4% formaldehyde in PBS for 10 minutes, showed lower levels of staining using the monoclonal antibody to ERα phosphorylated at serine 118 (figure 3.8a). However, the difference between the level of ERα phosphorylation at serine 118 in WT and TAM-R

cells was more evident using 2% paraformaldehyde orthovanadate (Figure 3.8b). Furthermore, across the several coverslips examined, paraformaldehyde orthovanadate produced the lowest level of background staining out of all the fixatives tested.

In agreement with the western blotting results and correlating with the elevated levels of phosphorylated EGFR/ERK1/2, TAM-R cells fixed with 2% paraformaldehyde orthovanadate, immunostained with monoclonal antibody to ERα phosphorylated at serine 118 have significantly greater nuclear H-score values than the WT cells (P < 0.001, n=3) (Figure 3.8c). The increase in staining between the two cell lines appears to be predominantly nuclear, although cytoplasmic staining also increased slightly. Interestingly, intensity of immunostaining of the cells for ERα phosphorylated at serine 118 was not uniform from one cell to another. There was considerable heterogeneity even within the intensely stained TAM-R cells. This correlates with the heterogeneity observed in TAM-R cells immunostained for EGFR and ERK1/2 (Knowlden et al 2003).

The ER negative MDA 231 cells were also fixed with 2% paraformaldehyde vanadate then immunostained for ERα phosphorylated at serine 118 and used as a negative control (Figure 3.8d). Although the slides produced extremely weak signals, immunostaining was detected in the nucleus of approximately 5% of cells. There was also evidence of weak membrane staining. Additional studies are needed to determine whether such staining was unspecific or whether low levels of ERα exist in the MDA 231 cells, a finding not observed with several total ERα antibodies. Determination of the origin of the MDA 231

cytoplasmic staining could provide further clues as to the origin of the

cytoplasmic staining observed in the WT and TAM-R cells.

Figure 3.6. Development of Immunocytochemical assay to measure the level of ERα phosphorylation at serine 118
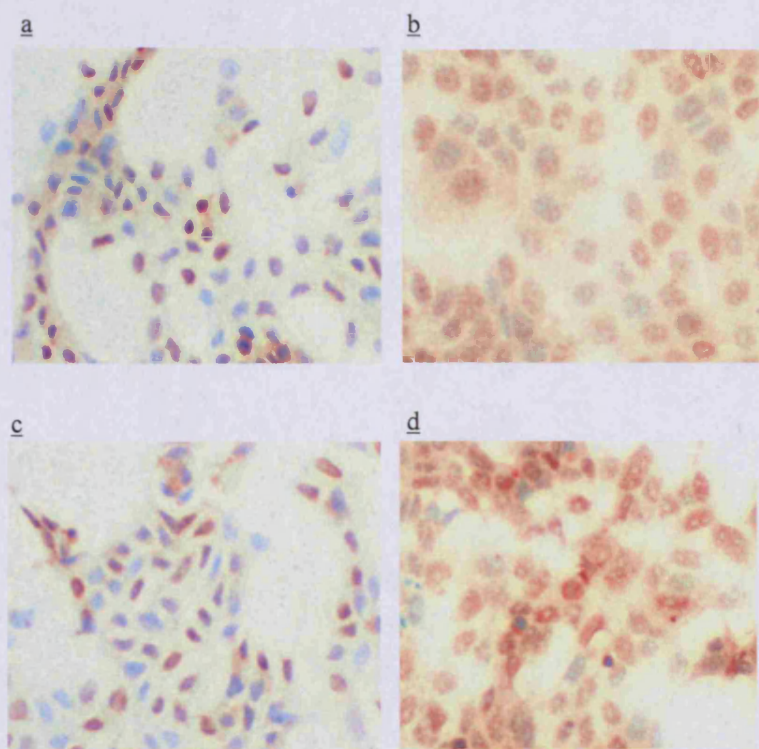
Immunocytochemistry of TAM-R cells under basal growth conditions fixed with; a) 2% Paraformaldehyde Vanadate 20 mins, b) 2% Paraformaldehyde 10 mins, c) 4% Formaldehyde in PBS 10 mins, d) Formal Saline 10 mins and immunostained using the monoclonal ERα ser118 NEB Ab (1/800). Magnification X20.

Figure 3.7. Development of Immunocytochemical assay to measure the level of ERα phosphorylation at serine 118

WT   TAM-R

a

b
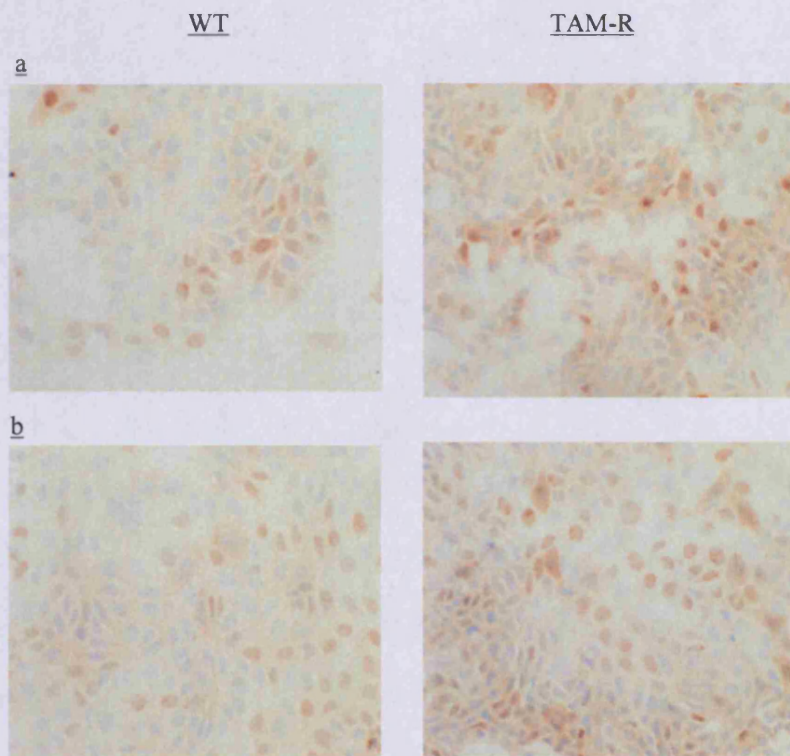
Immunocytochemistry of WT and TAM-R cells fixed with either a. 2% Paraformaldehyde Vanadate for 20 mins or b. 4% Formaldehyde in PBS for 10 mins and incubated with polyclonal ERα ser 118 NEB Ab (1/20). Magnification X20.

Figure 3.8. Development of Immunocytochemical assay to measure the level of ERα phosphorylation at serine 118
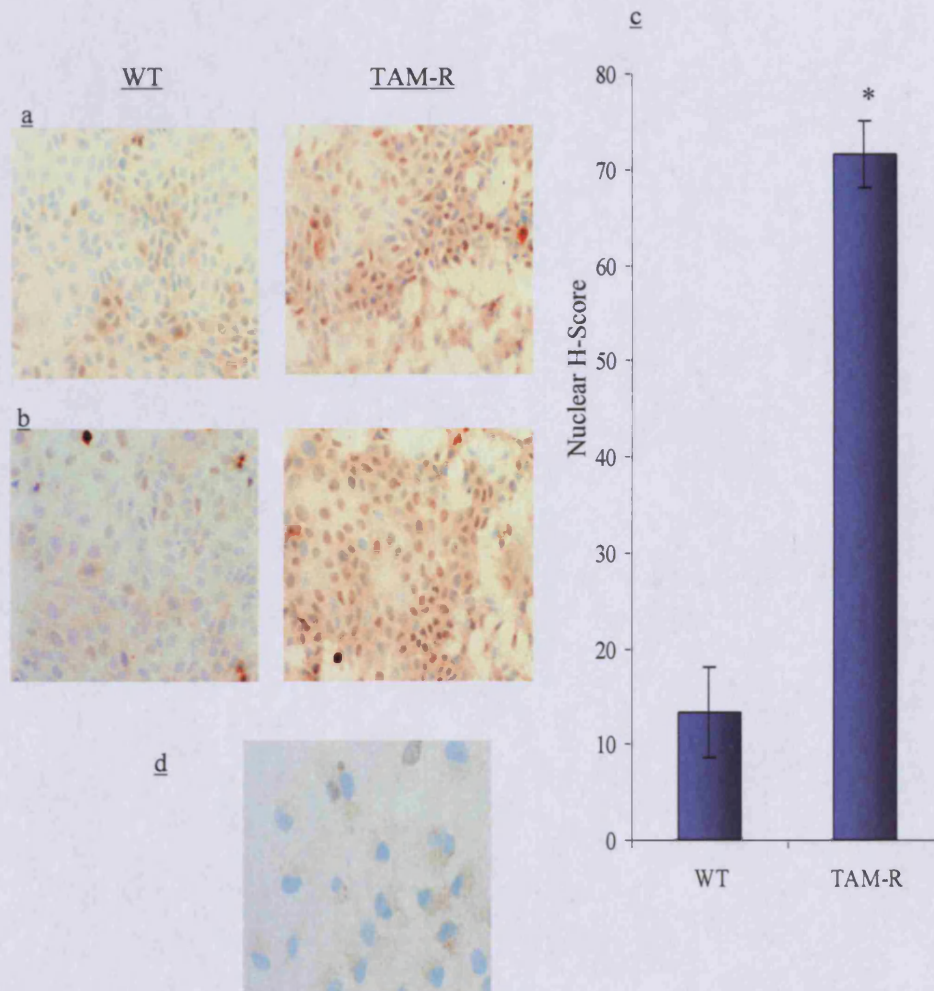
Immunocytochemistry of WT cells and TAM-R cells under basal growth conditions fixed with either a. 4% Formaldehyde in PBS (10 mins) or b. 2% Paraformaldehyde orthovanadate (PFV) (20mins), All slides at X20 magnification. Following fixation cells were immunostained with monoclonal ERα ser118 antibody (1/800). c: Mean H-score values (± SEM) from three separate experiments measuring nuclear ERα phosphorylation at serine 118 in TAM-R versus WT cells under basal growth conditions (n=3). * = P < 0.001 compared to control. d. Negative control consisting of ERα negative MDA231 cells fixed with PFV and immunostained for ERα (phospho-ser 118).

3.5 Modulation of the EGFR/ERK1/2 signalling pathway and its effects on ERα phosphorylation at serine 118 in TAM-R MCF-7 cells

Aim

Section 3 demonstrated increased levels of ERα phosphorylation at serine 118 in association with enhanced phosphorylation (activity) of EGFR/ERK1/2 in TAM-R cells. The influence of EGFR/ERK1/2 signalling on ERα phosphorylation at serine 118 was therefore investigated further in this cell line.

Results

Effects of the EGFR ligand EGF on TAM-R MCF-7 cells

Treatment of TAM-R cells with EGF significantly increased levels of phosphorylated EGFR ($P < 0.01$, n=3) and ERK1/2 ($P < 0.05$, n=3) (Figure 3.9). These increases in EGFR/ERK1/2 phosphorylation were accompanied by significant increases in ERα phosphorylation at serine 118 ($P < 0.05$, n=3), as shown by the western blots in Figure 3.9. There was no effect of this ligand on total EGFR, ERK1/2 and ERα protein expression. ICC also demonstrated that EGF significantly increased levels of phosphorylated EGFR ($P < 0.0001$, n=3), ERK1/2 ($P < 0.0001$, n=3) and ERα at serine 118 ($P < 0.0001$, n=3) (Figure 3.10b/c). Closer inspection of the slides in figure 3.10a revealed that EGF induced a substantial increase in the frequency and intensity of nuclei positively immunostained for ERα phosphorylated at serine 118. Cytoplasmic

and membrane staining for ERα phosphorylated at serine 118 in these samples, however, remained unchanged. Phosphorylated ERK1/2 showed obvious increases in cytoplasmic and nuclear staining when treated with EGF, whilst phosphorylated EGFR displayed an increased frequency of strong membrane immunostaining. The low levels of cytoplasmic phosphorylated EGFR staining appeared to be unchanged by this treatment.

Effects of EGFR and MEK inhibition on the EGF-induced increases in ERα and ERK1/2 phosphorylation in TAM-R MCF-7 cells

Western blotting demonstrated that the selective EGFR tyrosine kinase inhibitor gefitinib prevented increased phosphorylation of both ERK1/2 and ERα in response to EGF in TAM-R cells. The MEK1/2 inhibitor PD184352 also clearly inhibited the EGF-induced increases in ERα and ERK1/2 phosphorylation although its effectiveness on EGF-induced ERα phosphorylation was less pronounced than that of gefitinib. There was no effect of these treatments on total ERα and ERK1/2 levels (Figure 3.11, n=2).

3.6: Modulation of the IGF-IR signalling pathway and the effects on ERα phosphorylation at serine 118 in TAM-R MCF-7 cells

Aim

Studies by Kato at al 1995 have demonstrated that the IGF-IR signalling pathway, in addition to EGFR signalling, can regulate the level of ERα phosphorylation at serine 118 in breast cancer cells. As TAM-R cells also express IGF-IR the current section investigated whether the IGF-IR signalling pathway contributed to ERα phosphorylation at serine 118 in the TAM-R cell line.

Results

Insulin like growth factor II (IGF-II) effects on TAM-R MCF-7 cells

IGF-II treatment significantly increased phosphorylation of IGF-IR ($P < 0.05$, n=3), ERK1/2 ($P < 0.05$, n=3) and ERα (serine 118) ($P < 0.05$, n=3) in TAM-R cells, whilst having no effect on total expression levels of these proteins (Figure 3.12a/b).

Figure 3.9. Epidermal growth factor (EGF) effects on TAM-R MCF-7 cells
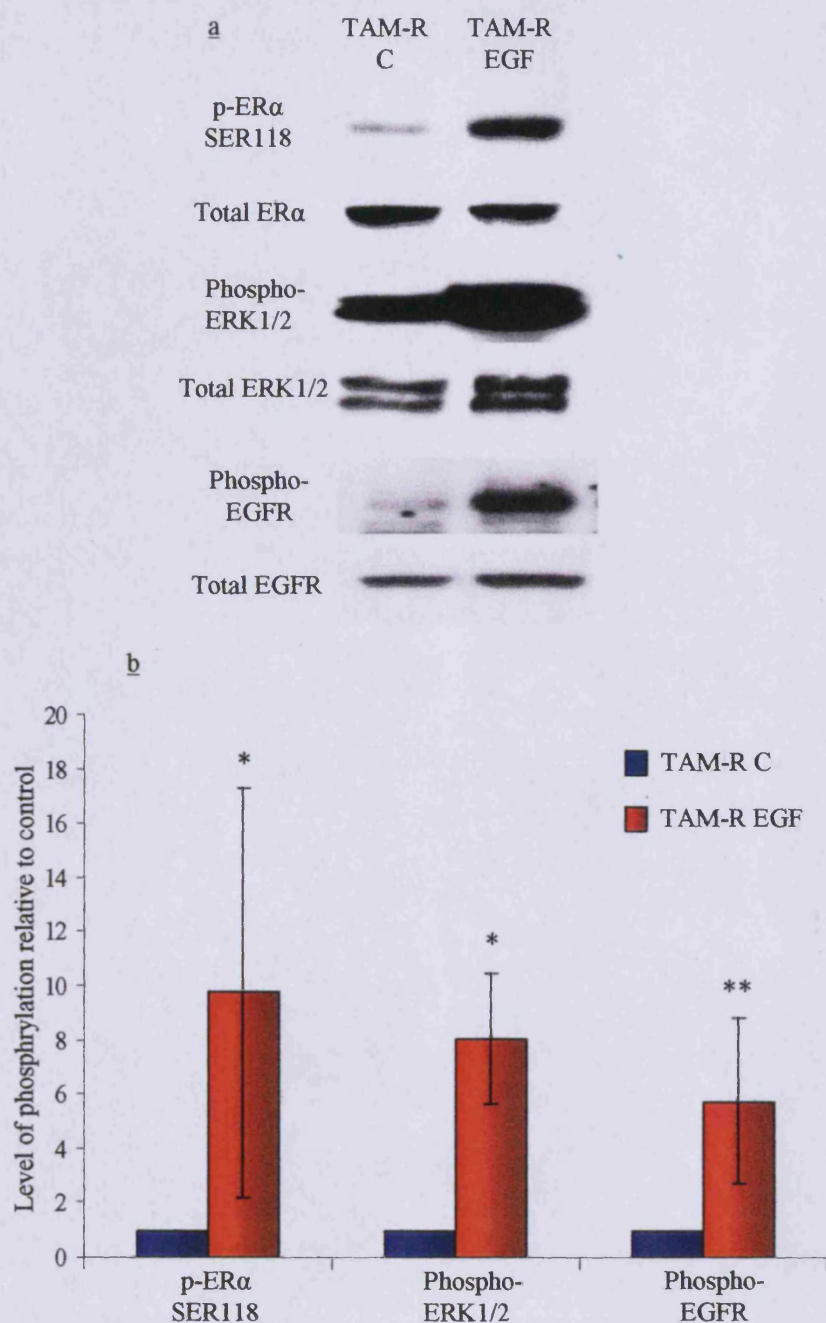
a: Western blot analysis of ERα (SER 118), ERK1/2 and EGFR phosphorylation in TAM-R MCF-7 cells in either the absence or presence of EGF (10ng/ml) for 10 minutes. Equal loading demonstrated by no change in total protein expression levels. b: Mean densitometry readings (+/-SEM) from Western blots for ERα (SER 118), ERK1/2 and EGFR phosphorylation in TAM-R cells in either the absence or presence of EGF (10ng/ml) for 10 minutes (n=3). Phosphorylation levels were normalised using total protein expression levels. * = P<0.05 compared to control. ** = P<0.01 compared to control.

Figure 3.10. Epidermal growth factor (EGF) effects on TAM-R MCF-7 cells
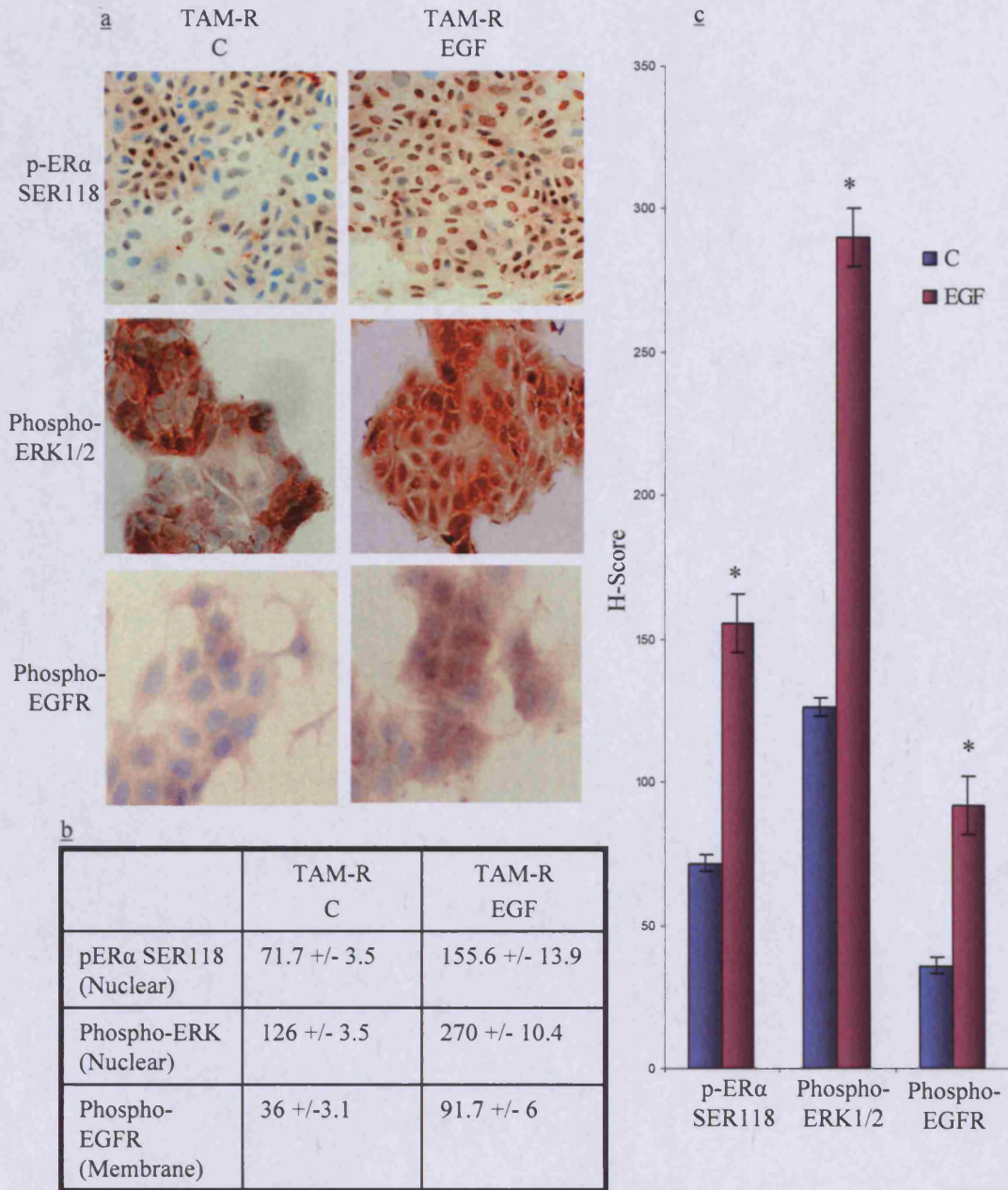
	TAM-R C	TAM-R EGF
pERα SER118 (Nuclear)	71.7 +/- 3.5	155.6 +/- 13.9
Phospho-ERK (Nuclear)	126 +/- 3.5	270 +/- 10.4
Phospho-EGFR (Membrane)	36 +/-3.1	91.7 +/- 6

a: Immunocytochemistry and b/c. Mean H-scores values (±SEM) of ERα (SER 118), ERK1/2 and EGFR phosphorylation in TAM-R cells in either the absence or presence of EGF (10ng/ml) for 10 minutes (n=3) (X20 magnification for ICC). * = P<0.001 compared to TAM-R control.

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Figure 3.11. Gefitinib/PD184352 +/- EGF effects on TAM-R MCF-7 cells
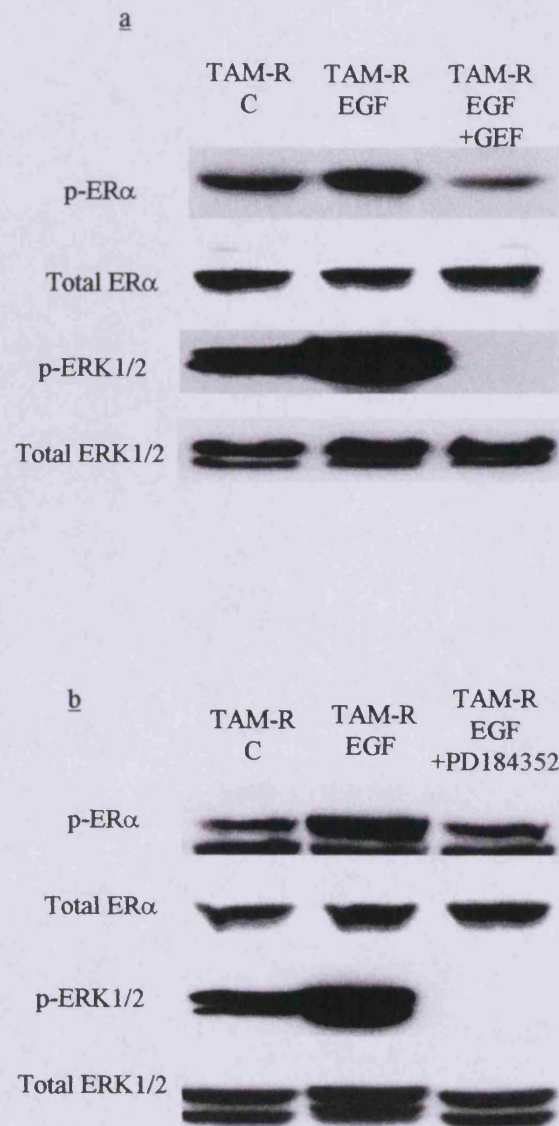
Western blot analysis of ERα (SER 118) and ERK1/2 phosphorylation in TAM-R MCF-7 cells a. in the absence and following treatment of cells with either EGF or a combination of EGF and gefitinib (1 μM, 10mins). b. in the absence and following treatment of cells with either EGF (10 ng/ml, 10mins) or a combination of EGF and PD184352 (10 μM, 1hr). Each blot is representative of two separate experiments (n=2).

Figure 3.12. Insulin like growth factor II (IGFII) effects on TAM-R MCF-7 cells
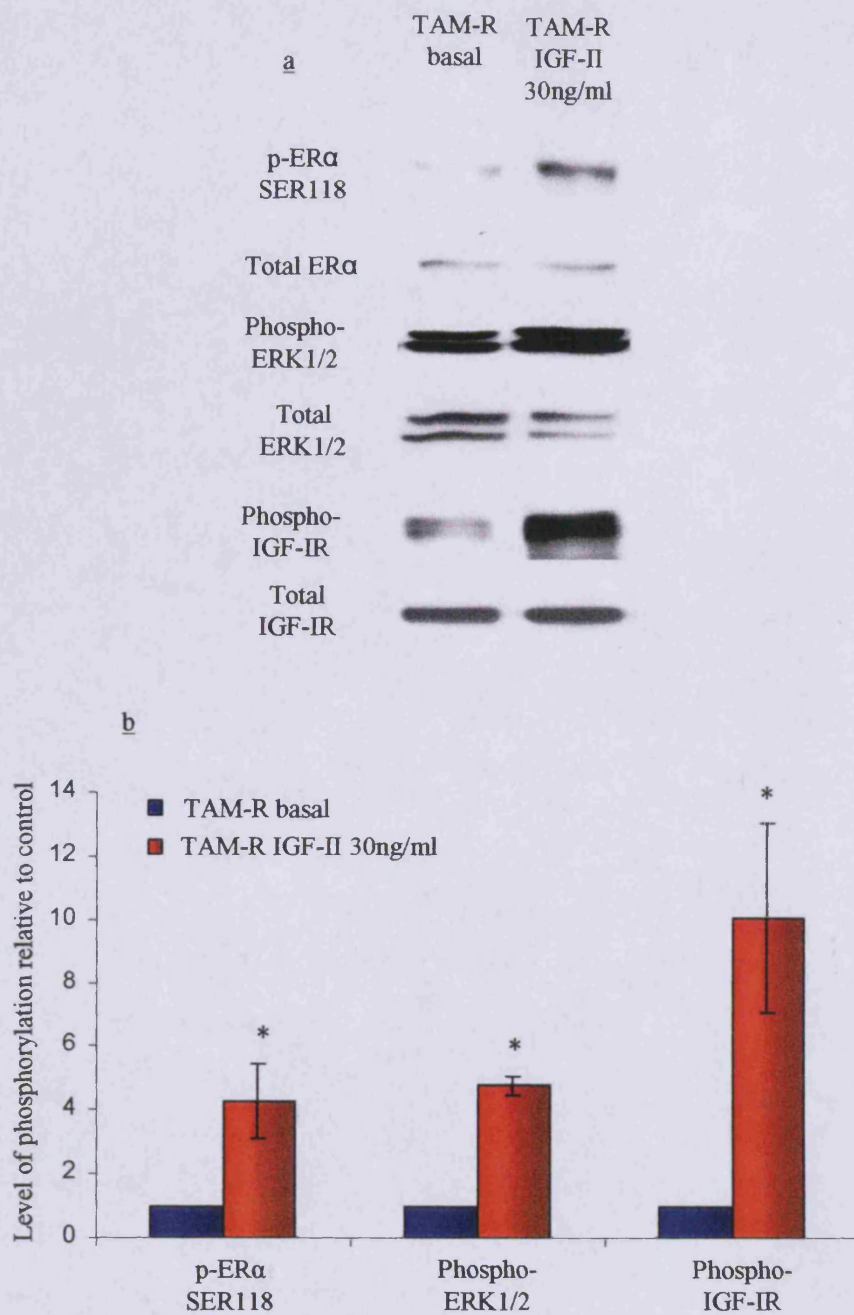
a: Western blot analysis of total and phosphorylated ERα (SER 118), ERK1/2 and IGF-IR expression levels in TAM-R MCF-7 cells in the absence and presence of IGF-II (10ng/ml) for 10 minutes. Equal loading is demonstrated by equal levels of the respective total proteins. b: Mean densitometry values (±SEM) of ERα (SER 118), ERK1/2 and IGF-1R phosphorylation in TAM-R cells in the absence and presence of IGF-II (10ng/ml) for 10 minutes (n=3). Phosphorylation levels were normalised using total protein expression levels. * = P<0.05 compared to control.

3.7 Regulation of ERα phosphorylation at serine 118 in TAM-R cells under basal growth conditions

Aims

The previous sections demonstrated that stimulating the EGFR and IGF-IR signalling pathways induced elevated ERα phosphorylation at serine 118 in the TAM-R cells. The role of the current section is to understand whether these pathways are important in regulating phosphorylation at serine 118 under basal growth conditions and whether, as a consequence, they influence basal TAM-R cell growth.

Results

Effects of gefitinib (GEF) on TAM-R MCF-7 cells under basal growth conditions

Growth of TAM-R MCF-7 cells at 7 days was significantly reduced in the presence of gefitinib at concentrations of $1\mu M$ ($P < 0.05$, n=3) and $10\mu M$ ($P < 0.01$, n=3; Figure 3.13a). Furthermore, gefitinib treatment significantly inhibited phosphorylation of EGFR ($P < 0.05$, n=3) and ERK1/2 ($P < 0.01$, n=3) in TAM-R cells in a concentration dependent manner. Such decreases in EGFR/ERK1/2 phosphorylation in response to gefitinib were accompanied by a significant reduction in the levels of phosphorylated ERα at serine 118 ($P < 0.05$, n=3) (Figure 3.13b/c). There was no effect of either concentration of gefitinib on total EGFR, ERK1/2 and ERα expression levels.

In agreement with western blotting the immunocytochemical studies revealed that exposure of TAM-R cells to gefitinib significantly reduced H-score values for phosphorylated EGFR (P < 0.0001, n=3), ERK1/2 (P < 0.0001, n=3) and ERα at serine 118 (P < 0.0001, n=3, figure 3.14b/c). Gefitinib significantly decreased the frequency and intensity of nuclei positively immunostained for ERα phosphorylated at serine 118 with cytoplasmic and membrane staining in these samples remaining predominantly unchanged. Phosphorylated ERK1/2 showed dramatic reductions in cytoplasmic and nuclear staining, whilst EGFR showed dramatic reductions in membrane staining following exposure to gefitinib.

Effects of PD184352 (PD) on TAM-R MCF-7 cells under basal growth conditions

Growth of TAM-R MCF-7 cells at 7 days was significantly reduced in the presence of PD184352 at concentrations of 1µM (P < 0.05, n=3) and 10µM (P < 0.005, n=3; Figure 3.15a). Western blotting further revealed that PD184352, like gefitinib, significantly reduced phosphorylation of ERK1/2 (P < 0.005, n=3) and ERα at serine 118 (P < 0.05, n=3) in a concentration dependent manner whilst having no effect on total ERK1/2 and ERα protein levels (Figure 3.15b/c). H-Score results from 3 separate experiments confirmed the western blotting findings with PD184352 significantly reducing ERK1/2 (P < 0.0001, n=3) and ERα phosphorylation (P < 0.0001, n=3) in the TAM-R cells (Figure 3.16b/c). TAM-R cells immunostained for ERα phosphorylated at serine 118 showed a dramatic reduction in the intensity and frequency of positively

stained cell nuclei following exposure to 10μM PD184352 (Figure 3.15a), however, cytoplasmic and membrane staining remained unchanged in these samples. TAM-R cells immunostained for phosphorylated ERK1/2 showed a parallel reduction in nuclear staining following exposure to PD184352. The phospho-ERK1/2 cytoplasmic staining was also reduced considerably.

Effects of the IGF-IR inhibitor AG1024 on TAM-R MCF-7 cells under basal growth conditions

TAM-R cells treated with AG1024 for 7 days showed a significant decrease in growth compared to control ($P < 0.05$, n=3, Figure 3.17a). Furthermore, AG1024 significantly reduced phosphorylated IGF-IR ($P < 0.05$, n=3), ERK1/2 ($P < 0.05$, n=3) and ERα at serine 118 ($P < 0.05$, n=3). Total ERα, ERK1/2 and IGF-IR protein levels remained unchanged following this treatment (Figure 3.17b/c).

Effect of AG1024 and Gefitinib in combination on TAM-R MCF-7 cells under basal growth conditions

TAM-R cells treated with either AG1024 or gefitinib alone, exhibited significant reductions in ERα and ERK1/2 phosphorylation. However, a combination of these two agents had no significant further effect on ERα and ERK1/2 phosphorylation when compared to the effects observed following treatment with gefitinib alone (Figure 3.18). There was again no effect of these treatments on total ERα and ERK1/2 expression levels.

Figure 3.13. Gefitinib (GEF) effects on TAM-R MCF-7 cells

a: Growth responses of TAM-R MCF-7 cells after 7 days in phenol red-free RPMI medium containg 5% charcoal-stripped serum in the absence and presence of gefitinib (GEF, 1 and 10 μM) (n=3). b: Western blot analysis of total and phosphorylated ERα (SER 118), ERK1/2 and EGFR expression levels in TAM-R MCF-7 cells in the absence and presence of gefitinib (1 and 10 μM) for 10 minutes. Equal loading demonstrated by equal levels of total proteins. c: Mean densitometry readings (±SEM) of ERα (SER 118), ERK1/2 and EGFR phosphorylation in TAM-R cells in the absence and presence of gefitinib (1 and 10 μM) (10minutes). Phosphorylation levels were normalised using the respective total protein expression levels (n=3). * = P<0.05 compared to control. ** = P<0.01 compared to control

Figure 3.14. Gefitinib (GEF) effects on TAM-R MCF-7 cells
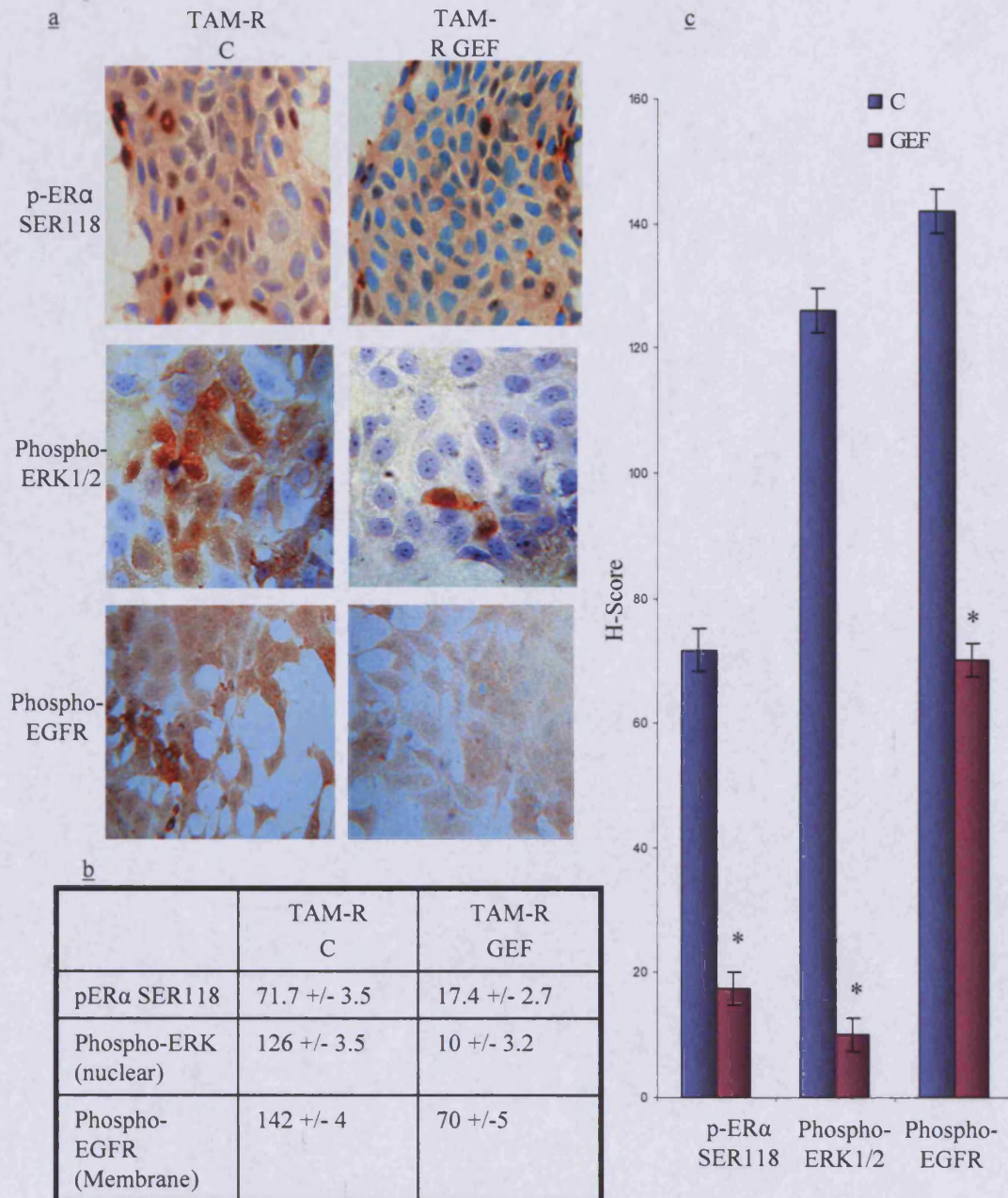
	TAM-R C	TAM-R GEF
pERα SER118	71.7 +/- 3.5	17.4 +/- 2.7
Phospho-ERK (nuclear)	126 +/- 3.5	10 +/- 3.2
Phospho-EGFR (Membrane)	142 +/- 4	70 +/-5

a: Immunocytochemistry and b/c. Mean H-scores values (±SEM) of ERα (SER 118), ERK1/2 and EGFR phosphorylation in TAM-R cells in either the absence or presence of gefitinib (1 μM) for 10 minutes (n=3) (X20 magnification for ICC). * = P<0.001 compared to TAM-R control.

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Figure 3.15. PD184352 (PD) effects on TAM-R MCF-7 cells

a: Growth responses of TAM-R MCF-7 cells after 7 days in phenol red-free RPMI medium containing 5% charcoal-stripped serum in the absence and presence of PD184352 (PD184, 1 and 10 μM) (n=3). b: Western blot analysis of total and phosphorylated ERα (SER 118), and ERK1/2 expression levels in TAM-R MCF-7 cells in the absence and presence of PD184352 (1 and 10 μM) for 1 hour. Equal loading demonstrated by equal levels of total proteins. c: Mean densitometry readings (±SEM) of ERα (SER 118), and ERK1/2 phosphorylation in TAM-R cells in the absence and presence of PD184352 (1 and 10 μM) (1 hour). Phosphorylation levels were normalised using the respective total protein expression levels (n=3). * = P<0.05 compared to control. ** = P<0.01 compared to control

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Figure 3.16. PD184352 (PD) effects on TAM-R MCF-7 cells
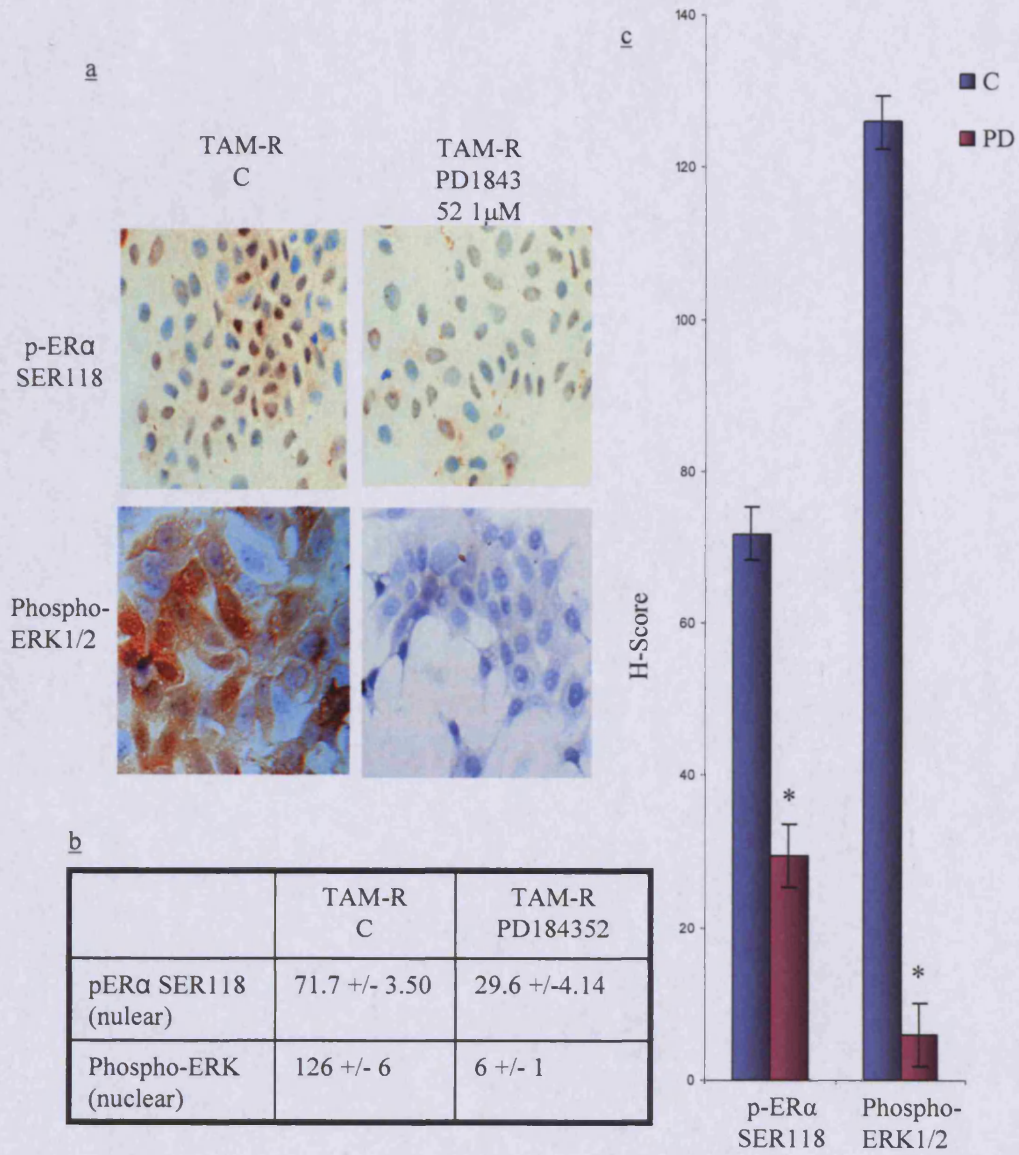
a

c

b

	TAM-R C	TAM-R PD184352
pERα SER118 (nulear)	71.7 +/- 3.50	29.6 +/-4.14
Phospho-ERK (nuclear)	126 +/- 6	6 +/- 1

a: Immunocytochemistry and b/c. Mean H-scores values (±SEM) of ERα (SER 118) and ERK1/2 phosphorylation in TAM-R cells in either the absence or presence of PD184352 (10 μM) for 1 hour (n=3) (X20 magnification for ICC). * = P<0.001 compared to TAM-R control.

Figure 3.17. Effects of the IGF-IR inhibitor AG1024 on TAM-R MCF-7 cells
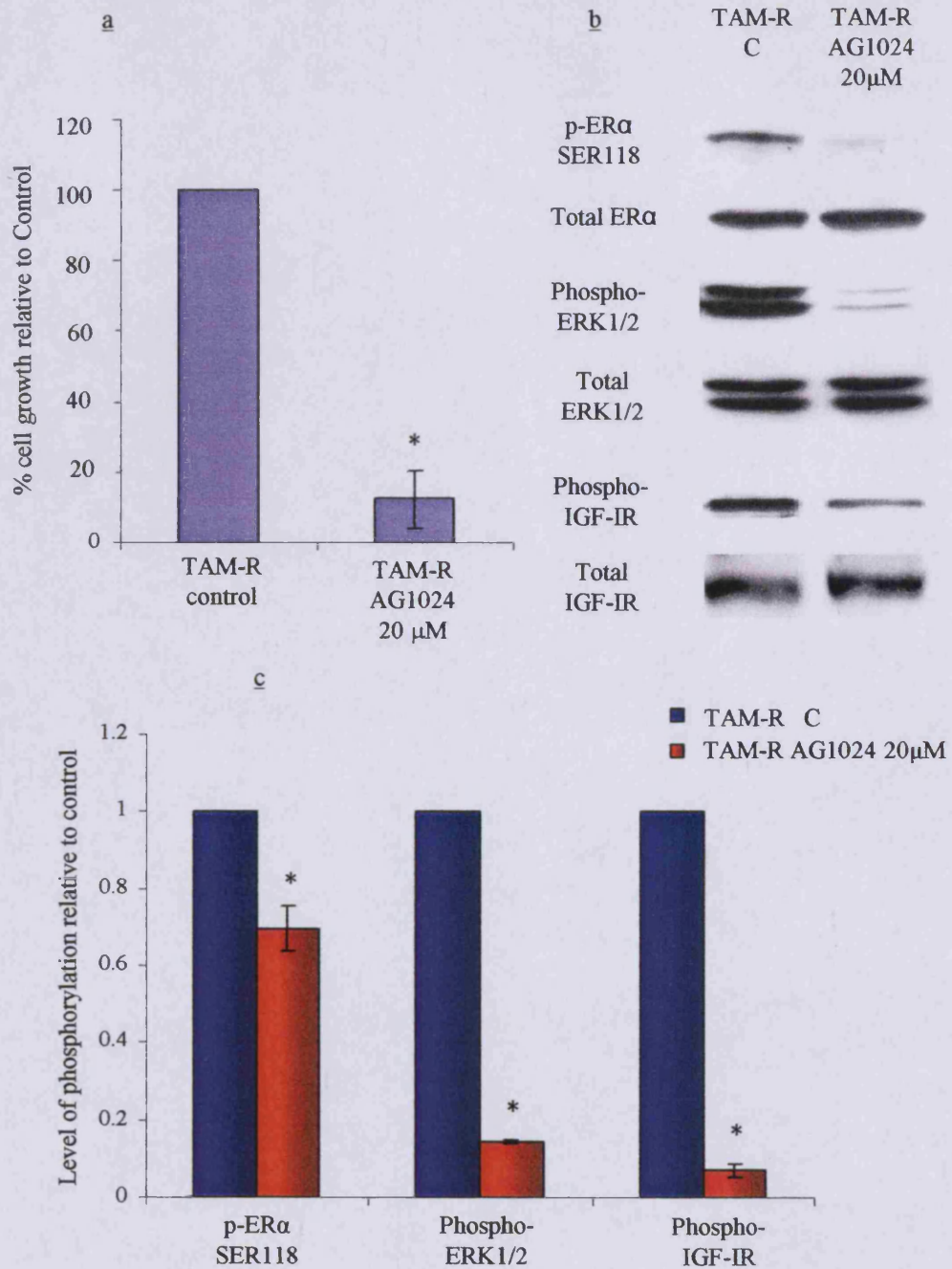
a: Growth responses of TAM-R MCF-7 cells after 7 days in phenol red-free RPMI medium containing 5% charcoal-stripped serum in the absence and presence of AG1024 (20 μM) (n=3). b: Western blot analysis of total and phosphorylated ERα (SER 118), ERK1/2 and IGF-1R expression levels in TAM-R MCF-7 cells in the absence and presence of AG1024 (20 μM) for 24 hours. Equal loading demonstrated by equal levels of total proteins. c: Mean densitometry readings (±SEM) of ERα (SER 118), ERK1/2 and IGF-1R phosphorylation in TAM-R cells in the absence and presence of AG1024 (20 μM) (24hours). Phosphorylation levels were normalised using the respective total protein expression levels (n=3). * = P<0.05 compared to control.
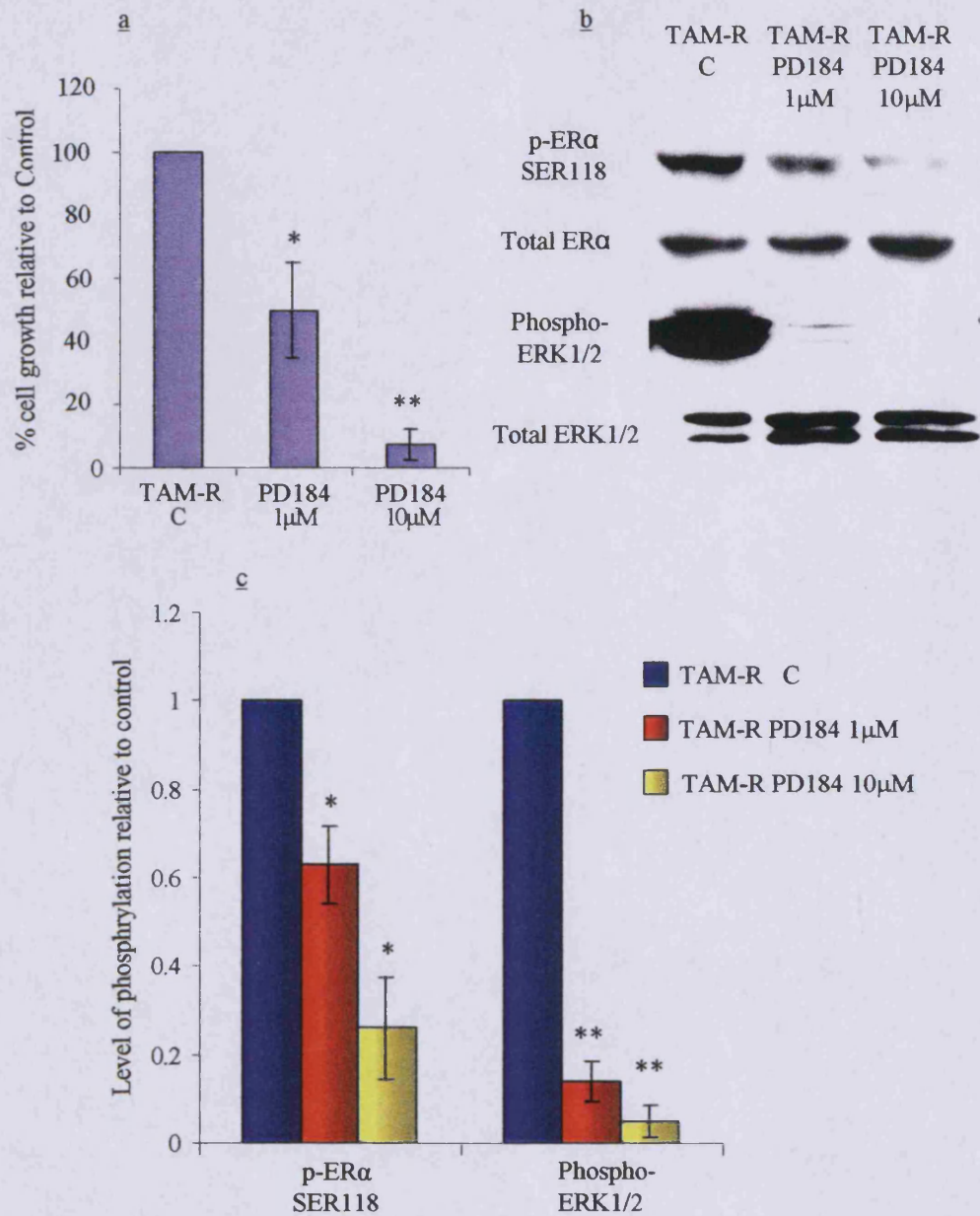
Figure 3.18. Effect of AG1024 and Gefitinib in combination on TAM-R MCF-7 cells
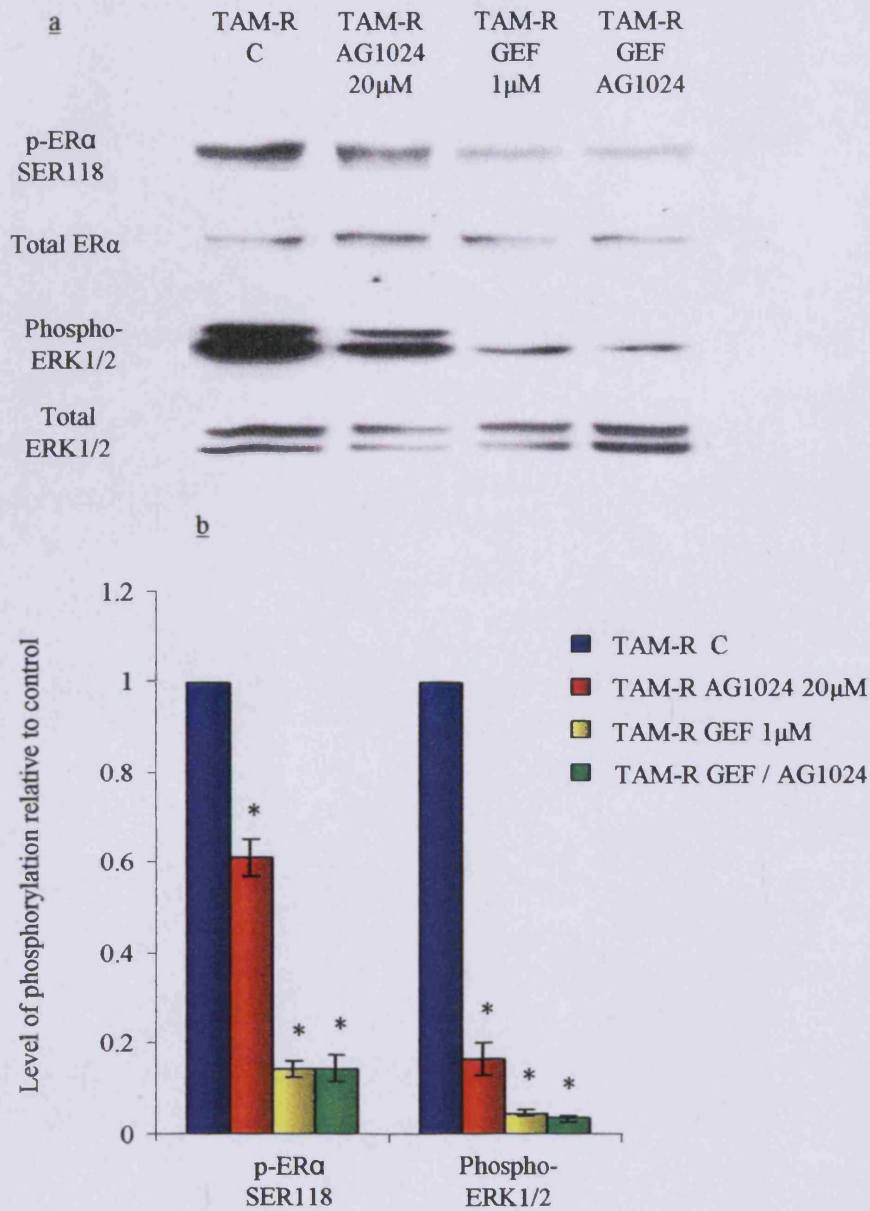
a: Western blot analysis of total and phosphorylated ERα (SER 118) and ERK1/2 expression levels in TAM-R MCF-7 cells in the absence and following treatment of cells with either gefitinib alone (1 μM), AG1024 alone (20 μM) or the two agents in combination. Equal loading is demonstrated by equal levels of the respective total proteins. b: Mean densitometry values (±SEM) of ERα (SER 118) and ERK1/2 phosphorylation in TAM-R cells in the absence and following treatment of cells with either gefitinib alone (1 μM), AG1024 alone (20 μM) or the two agents in combination. (n=3). Phosphorylation levels were normalised using total protein expression levels. * = P<0.05 compared to control.

3.8 Modulation of the EGFR/ERK1/2 signalling pathway and its effects on ERα phosphorylation at serine 118 in WT MCF-7 cells

Aim

EGFR/ERK1/2 signal transduction pathways clearly play a role in the phosphorylation of ERα in TAM-R cells. The WT cell line also showed measurable levels of ERα phosphorylation at this residue, yet expressed significantly less phosphorylated EGFR and ERK1/2, therefore this section investigated whether EGFR/ERK1/2 signalling is involved in the phosphorylation of ERα at serine 118 in the WT cell line.

Effects of EGF on WT MCF-7 cells

EGF significantly increased levels of phosphorylated EGFR ($P < 0.01$, n=3) and ERK1/2 ($P < 0.05$, n=3) in the WT cells (Figure 3.19). These EGF-induced increases in EGFR/ERK1/2 phosphorylation were accompanied by significant increases in ERα phosphorylation at serine 118 ($P < 0.05$, n=3), as shown by the western blots in Figure 3.19. There was again no effect of this ligand on total protein expression in these cells.

Effects of EGFR and MEK inhibition on EGF-induced actions in WT MCF-7 cells

EGF-induced increases in ERα and ERK1/2 phosphorylation were completely inhibited by both gefitinib and PD184352 in the WT cell line (Figure 3.20).

There was again no effect of these inhibitors on total ERα and ERK1/2 expression levels in these cells.

3.9 Modulation of the IGF-IR signalling pathway and the effects on ERα phosphorylation at serine 118 in WT MCF-7 cells

Aims

IGF-IR signal transduction pathway has been shown to induce phosphorylation of ERα in the TAM-R MCF-7 cell line. As WT cells also express phosphorylated IGF-IR this section investigated whether the IGF-IR is involved in the phosphorylation of ERα at serine 118 in WT cells.

Results

Insulin like growth factor-II (IGF-II) effects on WT MCF-7 cells

IGF-II significantly increased phosphorylation levels of IGF-IR (P < 0.01, n=3), ERK1/2 (P < 0.01, n=3) and ERα at serine 118 (P < 0.01, n=3) in WT cells but had no effect on total expression levels of these proteins in this cell line (Figure 3.21).

Figure 3.19. Epidermal growth factor (EGF) effects on WT MCF-7 cells
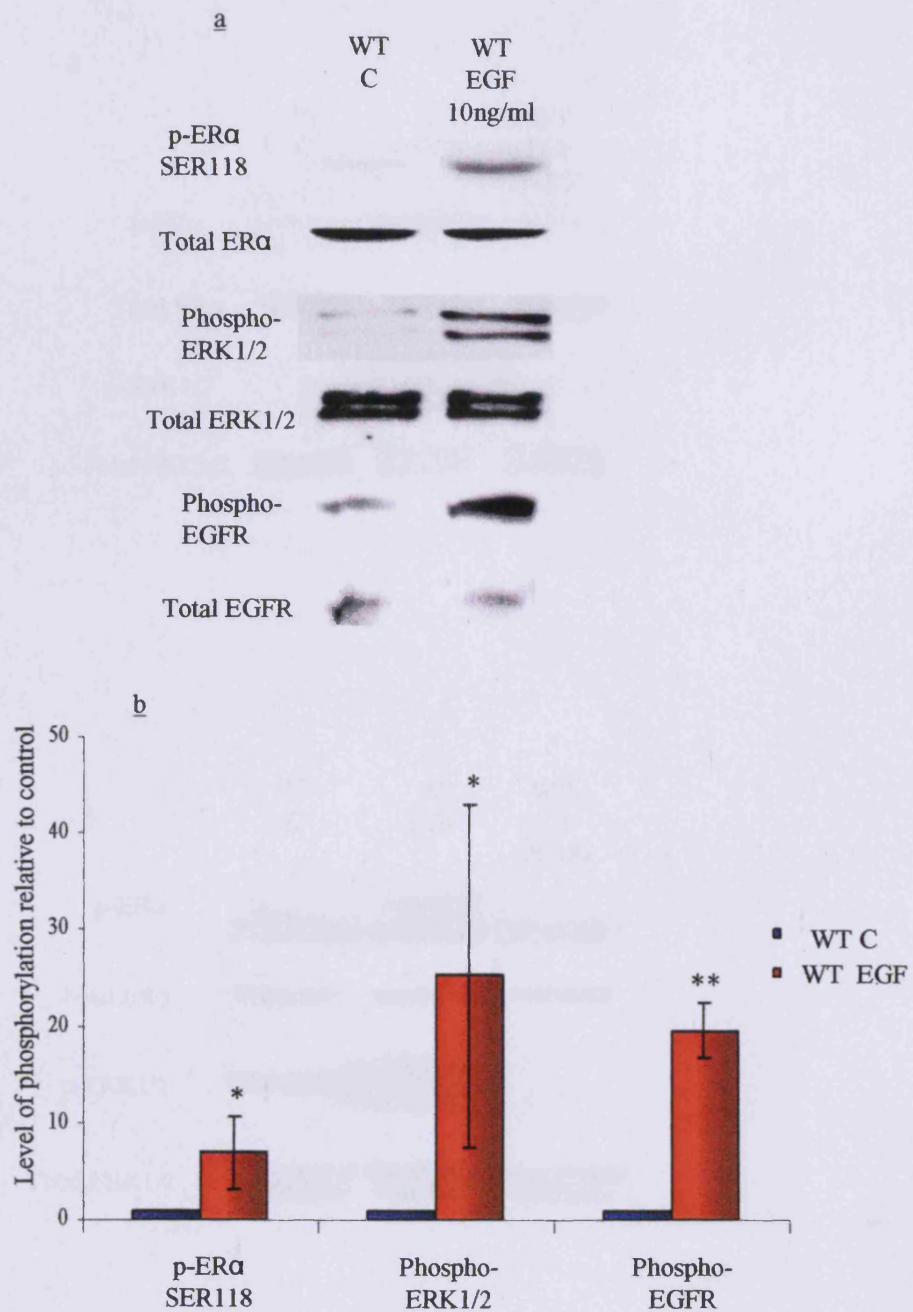
a: Western blot analysis of ERα (SER 118), ERK1/2 and EGFR phosphorylation in WT MCF-7 cells in either the absence or presence of EGF (10ng/ml) for 10 minutes. Equal loading demonstrated by no change in total protein expression levels. b: Mean densitometry readings (±SEM) from Western blots for ERα (SER 118), ERK1/2 and EGFR phosphorylation in WT cells in either the absence or presence of EGF (10ng/ml) for 10 minutes (n=3). Phosphorylation levels were normalised using total protein expression levels. * = P<0.05 compared to control. ** = P<0.01 compared to control.

Figure 3.20. PD184352/Gefitinib +/- EGF effects on WT MCF-7 cells

a

b
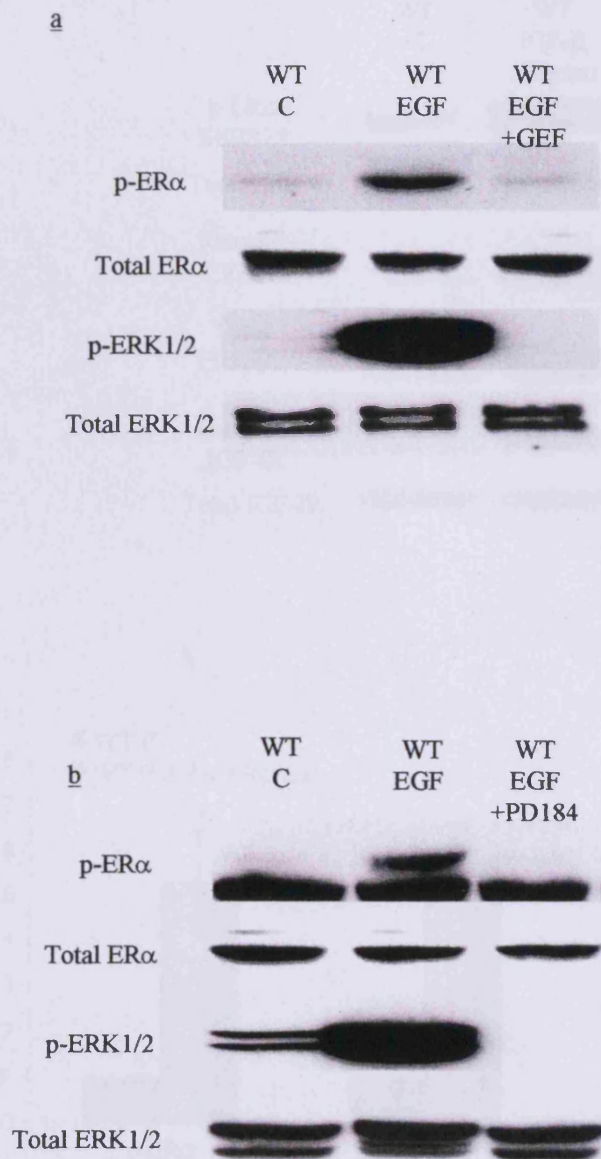
Western blot analysis of ERα (SER 118) and ERK1/2 phosphorylation in WT MCF-7 cells a. in the absence and following treatment of cells with either EGF (10 ng/ml) or a combination of EGF and gefitinib (1 μM). b. in the absence and following treatment of cells with either EGF (10 ng/ml) or a combination of EGF and PD184352 (10 μM). Each blot is representative of two separate experiments (n=2).

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Figure 3.21. Insulin like growth factor II (IGFII) effects on WT MCF-7 cells
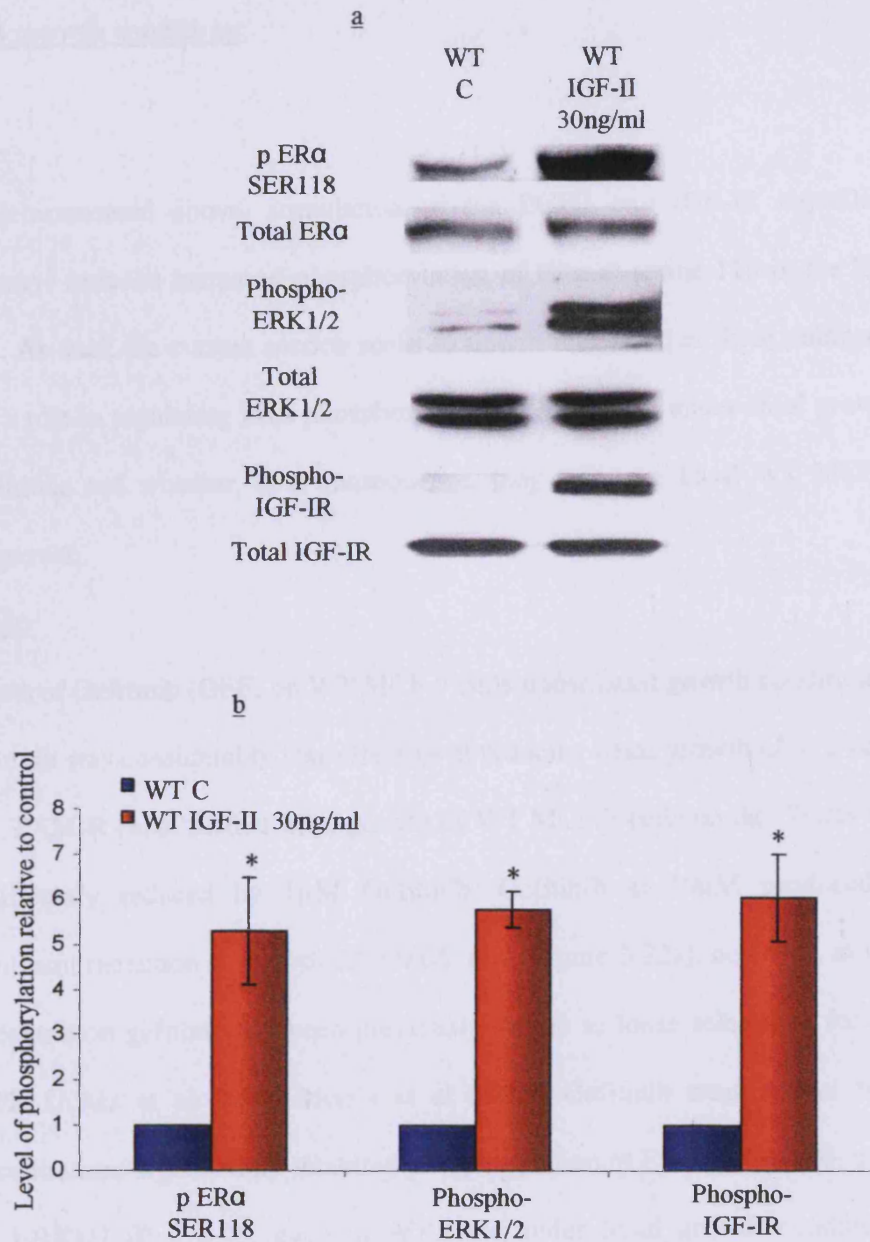
a: Western blot analysis of total and phosphorylated ERα (SER 118), ERK1/2 and IGF-IR expression levels in WT MCF-7 cells in the absence and presence of IGF-II (10ng/ml) for 10 minutes. Equal loading is demonstrated by equal levels of the respective total proteins. b: Mean densitometry values (±SEM) of ERα (SER 118), ERK1/2 and IGF-1R phosphorylation in WT cells in the absence and presence of IGF-II (10ng/ml) for 10 minutes (n=3). Phosphorylation levels were normalised using total protein expression levels. * = P<0.05 compared to control.

3.10 Regulation of ERα phosphorylation at serine 118 in WT cells under basal growth conditions

Aims

As demonstrated above, stimulation of the EGFR and IGF-IR signalling pathways induced increased phosphorylation of ERα at serine 118 in the WT cells. As such the current section seeks to understand whether these pathways play a role in regulating ERα phosphorylation at serine 118 under basal growth conditions and whether, as a consequence, they influence basal WT MCF-7 cell growth.

Results

Effects of Gefitinib (GEF) on WT MCF-7 cells under basal growth conditions

Gefitinib was considerably less effective at reducing basal growth of WT cells than TAM-R cells. Statistically, growth of WT MCF-7 cells on day 7 was not significantly reduced by 1μM Gefitinib. Gefitinib at 10μM produced a significant reduction in growth ($P < 0.05$, n=3, Figure 3.22a), however, at this concentration gefitinib has been previously shown to loose selectivity for the EGFR (Jones et al 2004; Morris et al 2002). Gefitinib treatment at both concentrations significantly inhibited phosphorylation of EGFR ($P < 0.05$, n=3) and ERK1/2 ($P < 0.05$, n=3) in WT cells under basal growth conditions. Despite such EGFR/ERK1/2 inhibition, gefitinib was ineffective at reducing basal ERα phosphorylation at serine 118. Total EGFR, ERK1/2 and ERα levels remained unchanged throughout the study (Figure 3.22b/c).

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Effects of PD184352 (PD) on WT MCF-7 cells under basal growth conditions

As with gefitinib, PD184352 was less effective at reducing growth of WT cells than TAM-R cells. Growth of WT MCF-7 cells on day 7 was not significantly reduced by 1 µM PD184352. Increasing the PD184352 concentration to 10µM however, produced a significant reduction in growth ($P < 0.05$, n=3, Figure 3.23a).

Both concentrations of PD184352 induced significant reductions in ERK1/2 phosphorylation ($P < 0.001$, n=3), yet unlike in the TAM-R cells, this reduction in ERK1/2 phosphorylation was not accompanied by a reduction in ERα phosphorylation at serine 118 with levels of this phosphorylated protein remaining mainly unchanged even following treatment with 10 µM PD184352 (Figure 3.23b/c). There was no effect of PD184352 on total ERα and ERK1/2 levels in these cells.

Effects of the IGF-IR inhibitors AG1024 and ADW742 on WT MCF-7 cells

Growth of WT MCF-7 cells after 7 days was significantly reduced by both AG1024 ($P < 0.05$, n=3) and ADW742 ($P < 0.05$, n=3) (Figure 3.24a). Western blots demonstrate that treatment with either AG1024 or ADW742 significantly decreased IGF-IR phosphorylation ($P < 0.05$, n=3) and ERα phosphorylation at serine 118 ($P < 0.05$, n=3) in this cell line whilst having no effect on total ERα and IGF-IR expression (Figure 3.24 b/c).

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Effects of a PI3 kinase inhibitor LY294002 on WT MCF-7 cells

PI3K is an important down stream kinase of the IGF-IR signalling pathway therefore its role was investigated in WT cell growth and ERα phosphorylation at serine 118.

7 day exposure to LY294002 significantly reduced WT cell growth by 85 +/-5% (P < 0.05, n=3; Figure 3.25a). Westerns demonstrate that LY294002 significantly reduced levels of phosphorylated AKT (P < 0.05, n=3) and ERα at serine 118 (P < 0.05, n=3) in WT cells (Figure 3.25 b/c). Interestingly, ERα was also significantly reduced in theses cells following treatment with LY294002 (P < 0.05, n=3).

Figure 3.22. Gefitinib (GEF) effects on WT MCF-7 cells

a: Growth responses of WT MCF-7 cells after 7 days in phenol red-free RPMI medium containing 5% charcoal-stripped serum in the absence and presence of gefitinib (GEF, 1 and 10 μM) (n=3). b: Western blot analysis of total and phosphorylated ERα (SER 118), ERK1/2 and EGFR expression levels in WT MCF-7 cells in the absence and presence of gefitinib (1 and 10 μM) for 10 minutes. Equal loading demonstrated by equal levels of total proteins. c: Mean densitometry readings (±SEM) of ERα (SER 118), ERK1/2 and EGFR phosphorylation in WT cells in the absence and presence of gefitinib (1 and 10 μM) (10 minutes). Phosphorylation levels were normalised using the respective total protein expression levels (n=3). * = $P<0.05$ compared to control. ** = $P<0.01$ compared to control

Figure 3.23. PD184352 (PD) effects on WT MCF-7 cells

a: Growth responses of WT MCF-7 cells after 7 days in phenol red-free RPMI medium containing 5% charcoal-stripped serum in the absence and presence of PD184352 (PD184, 1 and 10 μM) (n=3). b: Western blot analysis of total and phosphorylated ERα (SER 118), and ERK1/2 expression levels in WT MCF-7 cells in the absence and presence of PD184352 (1 and 10 μM) for 1hour. Equal loading demonstrated by equal levels of total proteins. c: Mean densitometry readings (±SEM) of ERα (SER 118), and ERK1/2 phosphorylation in WT cells in the absence and presence of PD184352 (1 and 10 μM) (1 hour). Phosphorylation levels were normalised using the respective total protein expression levels (n=3). * = P<0.05 compared to control. ** = P<0.01 compared to control

Figure 3.24. Effects of the IGF-IR inhibitors AG1024 and ADW on WT MCF-7 cells
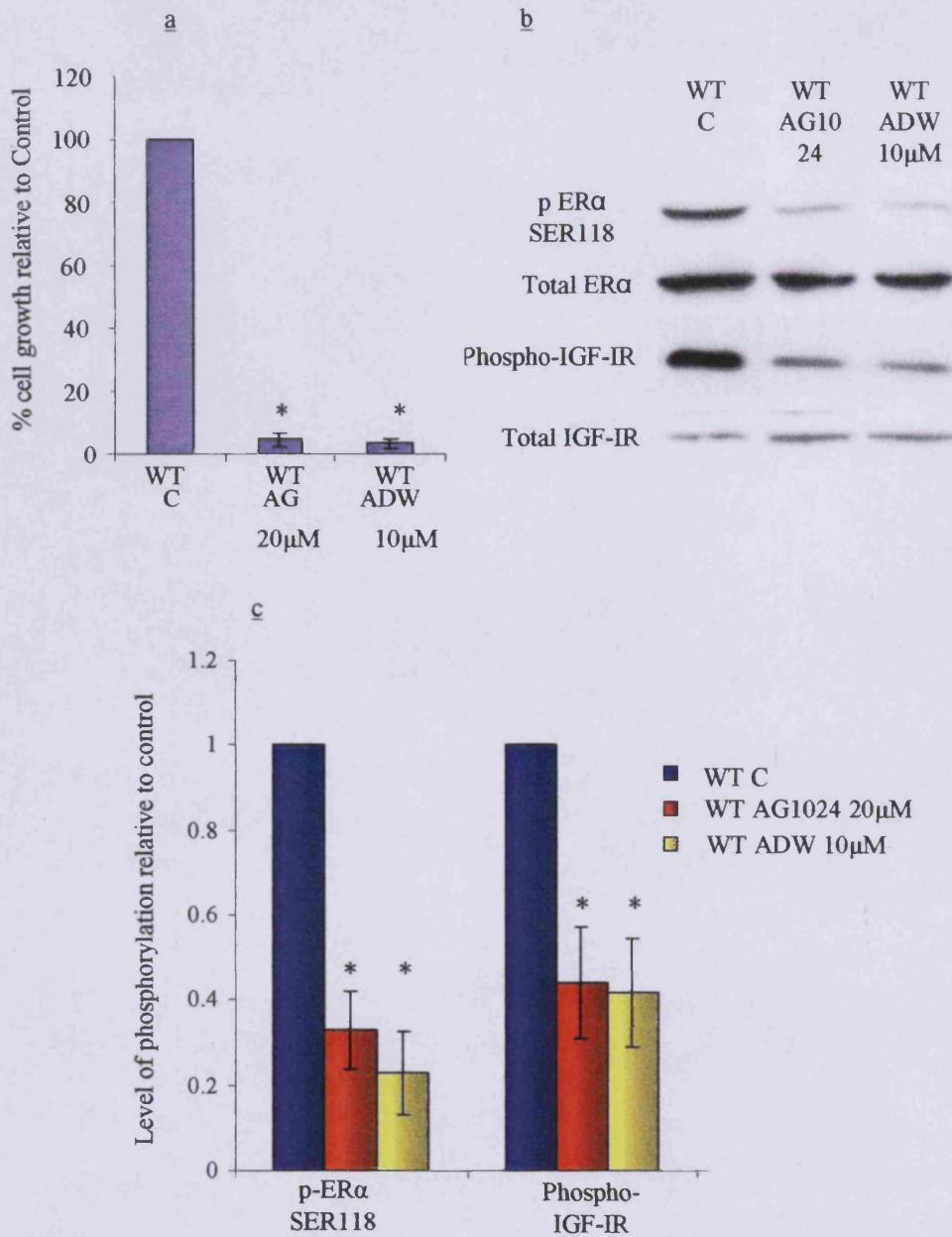
a: Growth responses of WT MCF-7 cells after 7 days in phenol red-free RPMI medium containing 5% charcoal-stripped serum in the absence and presence of AG1024 (20 µM) or ADW (10µM) (n=3). b: Western blot analysis of total and phosphorylated ERα (SER 118) and IGF-IR expression levels in WT MCF-7 cells in the absence and presence of AG1024 (20 µM) or ADW (10µM) for 24 hours. Equal loading demonstrated by equal levels of total protein. c: Mean densitometry readings (+/-SEM) of ERα (SER 118) and IGF-IR phosphorylation in WT cells in the absence and presence of AG1024 (20 µM) or ADW (10µM). Phosphorylation levels normalised using respective total protein expression levels. * = P<0.05 (significant) compared to control.

Figure 3.25. Effects of the PI3 kinase inhibitor LY294002 on WT MCF-7 cells
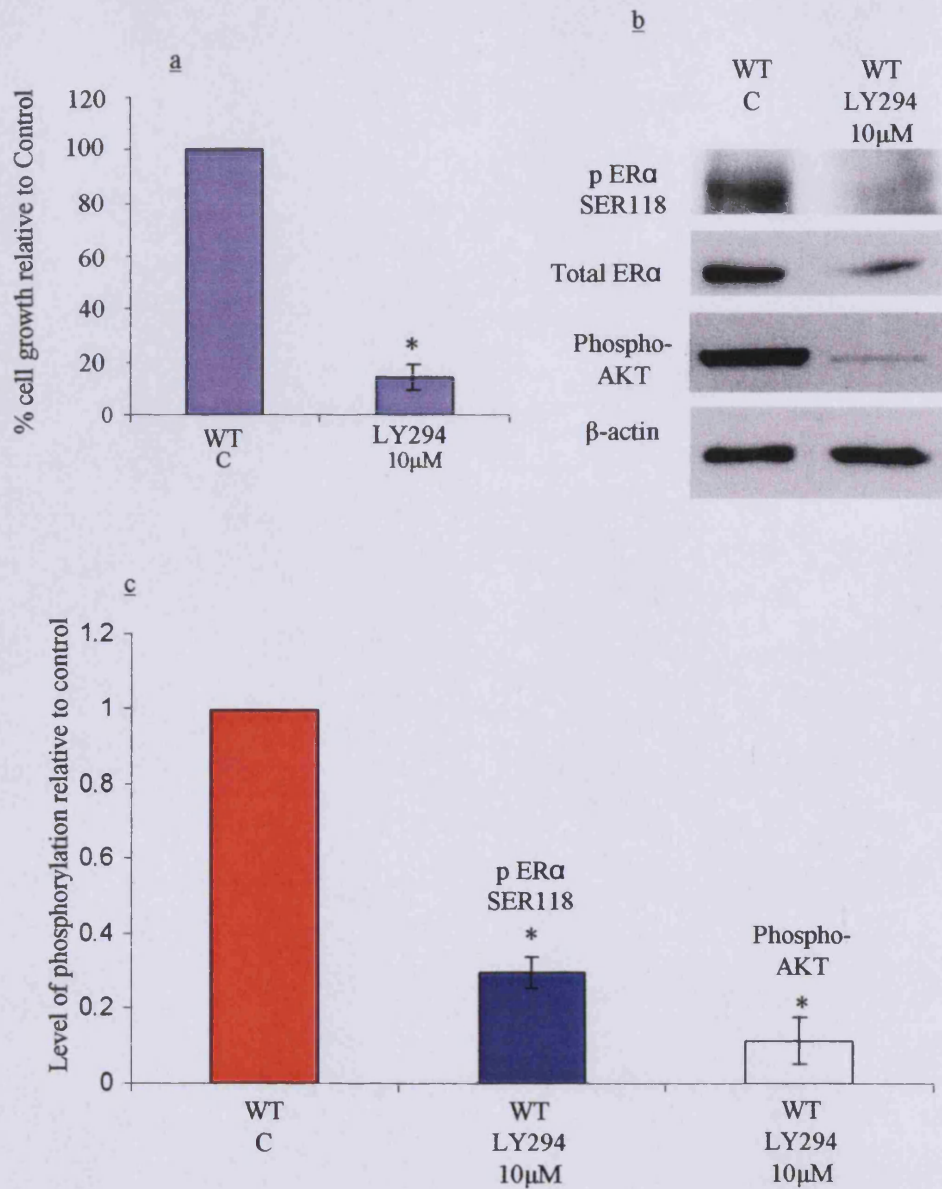
a: Growth responses of WT MCF-7 cells after 7 days in phenol red-free RPMI medium containing 5% charcoal-stripped serum in the absence and presence of LY294002 (10 μM) (n=3). b: Western blot analysis of total and phosphorylated ERα (serine 118) and AKT expression levels in WT MCF-7 cells in the absence and presence of LY294002 (10 μM) for 1 hour. Equal loading demonstrated by equal levels of total proteins c: Mean densitometry readings (+/- SEM) of ERα (SER 118) and AKT phosphorylation in WT cells in the absence and presence of LY294002 (10 μM) for 1 hour. ERα phosphorylation levels were normalised using total ERα protein levels. AKT phosphorylation levels normalised to actin. * = P<0.05 (significant) compared to control.

3.11 Cross talk between growth factor signal transduction pathways and ERα at serine 118; effect on transcription at ERE containing reporter genes in TAM-R and WT MCF-7 cell lines

Aims

Several pharmacological and mutational studies have demonstrated that phosphorylation of ERα at serine 118 can contribute to the transcriptional activity of ERα at reporter gene constructs containing EREs within their promoter regions (Bunone et al 1996; Joel et al 1995; Kato et al 1995). The previous sections have shown that EGFR and IGF-IR signal transduction pathways, via ERK1/2, regulate the phosphorylation of ERα at serine 118 in TAM-R cells under basal conditions. IGF-R and not EGFR however, was shown to regulate ERα phosphorylation at serine 118 in the WT cells under basal conditions. The aim of this section, therefore, is to elucidate whether the cross talk between growth factor receptor signalling pathways and ERα at serine 118 effects ER function at ERE dependent reporter genes in TAM-R and WT MCF-7 cells.

Results

Transfection efficiency of reporter gene plasmids in WT and TAM-R cells

An estimate of the transfection efficiency of WT and TAM-R cells was required in order to normalise any results from subsequent comparative investigations between the two cell lines. A higher proportion of the WT cells were successfully transfected with the plasmid containing the β-galactosidase

gene (blue stained cells), in comparison with transfected TAM-R cells (Figure 3.26a). A quantitative difference in transfection efficiency was shown to exists between the two cell lines by counting the percentage of transfected (blue stained) cells compared to unstained cells. The β-Galactosidase assay shows that the transfection efficiency of TAM-R cells is approximately 30% that of WT cells (Figure 3.26b).

Level of transcriptional activity at ERE containing reporter genes transiently transfected into WT and TAM-R cells.

Basal ERE-dependent luciferase activity was approximately 85% lower in TAM-R cells than in the WT cells ($P < 0.05$, n=7; Figure 3.27a). However, following normalisation according to the relative transfection efficiency of the cells, basal ERE dependent luciferase activity in TAM-R cells was seen to be approximately 50% of basal WT ERE activity ($P < 0.05$, n=7) (Figure 3.27b).

Pharmacological modulation of ERE activity in TAM-R cells

Significantly, while ERE activity in TAM-R cells is strongly enhanced by oestradiol ($P > 0.05$, n =3) and reduced by fulvestrant ($P < 0.05$, n =3; Figure 3.28) it is also influenced by modulators of EGFR and ERK1/2, correlating with their effects on ERα phosphorylation at serine 118 (Figure 3.29). Thus EGF significantly increased ERE activity in TAM-R cells ($P < 0.05$, n=3), while basal ERE activity was inhibited by both gefitinib ($P < 0.05$, n=3) and PD184352 ($P < 0.05$, n=3) in this cell line. Similarly, ERE activity in TAM-R cells was also shown to be under the regulation of the IGF-IR signalling

pathway (Figure 3.30), with IGF-II, but not IGF-I, significantly increasing ERE activity in the TAM-R cells (P < 0.05, n=3). Interestingly, TAM-R cells treated with AG1024 for 24 hours at 20μM demonstrated an approximate 25 % reduction in basal ERE activity although this did not reach statistical significance.

Pharmacological modulation of ERE activity in WT cells.

As previously observed by Hutcheson et al 2003 ERE activity in WT cells was regulated by ERα, with oestradiol significantly increasing ERE activity (P < 0.05, n=3) and basal ERE activity being reduced by approximately 30% following treatment of this cell line with fulvestrant. Correlating with ERα phosphorylation at serine 118, inhibition of the EGFR/ERK1/2 signalling pathway had no effect on ERE activity, whilst EGF significantly enhanced ERE activity in the WT cell line (P < 0.05, n=3; Figure 3.32). Further correlations between ERα phosphorylation at serine 118 and ERE activity were also observed following regulation of IGF-IR signalling in WT cells. IGF-I and IGF-II significantly increased ERE activity (P < 0.001, n=3 for both ligands), whilst AG1024 significantly reduced basal ERE activity in WT cells (P < 0.001, n=3; Figure 3.33).

Figure 3.26. β-Galactosidase cell staining assay to measure transfection efficiency in WT and TAM-R cells

a.

b.
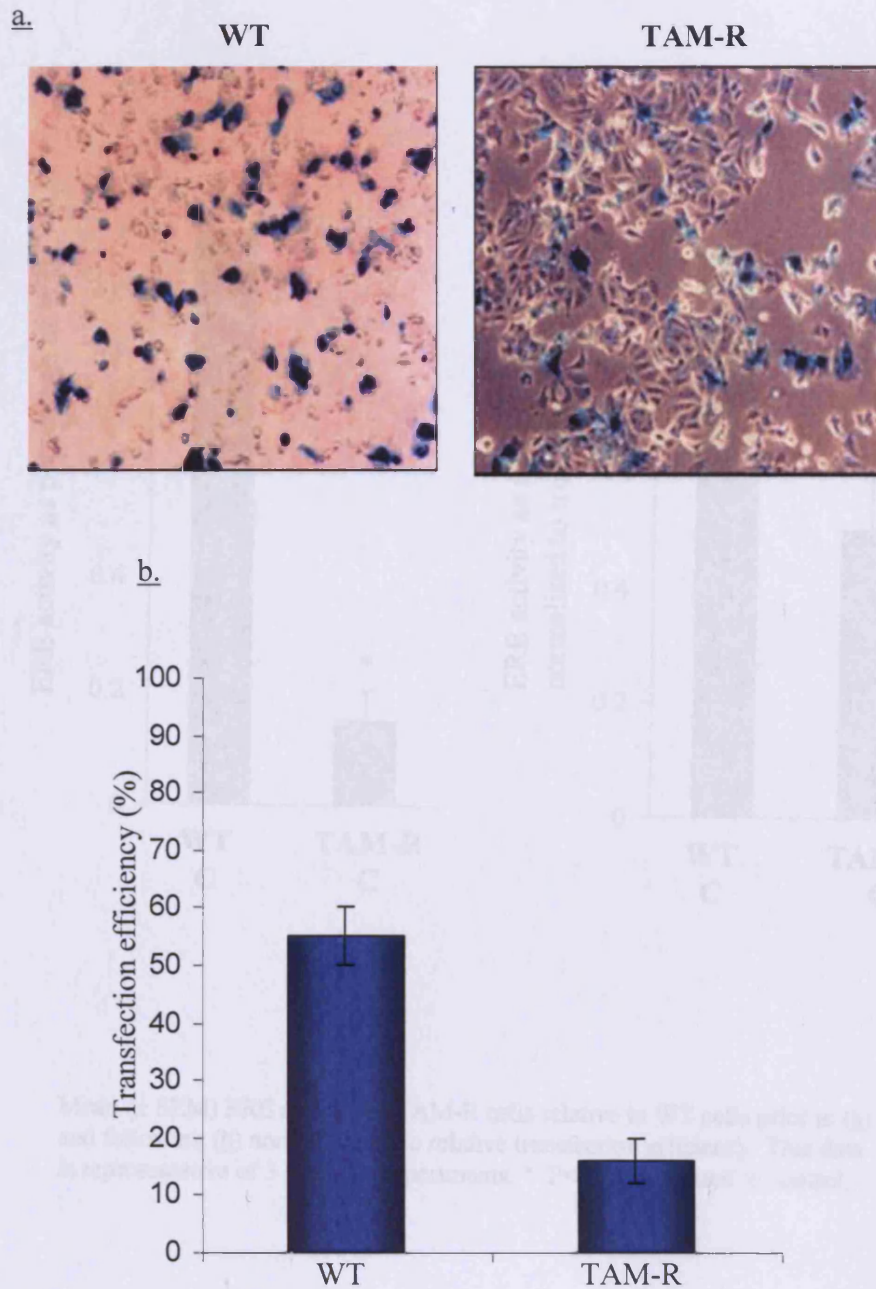
a. Staining of WT and TAM-R cells transfected with β-galactosidase. Blue stained nuclei have been successfully transfected. b. Mean (± SEM) β-galactosidase transfection efficiency in WT and TAM-R cells being representative of seven separate experiments.

Figure 3.27. Luciferase reporter gene assay to measure
ERE activity in WT and TAM-R cells.
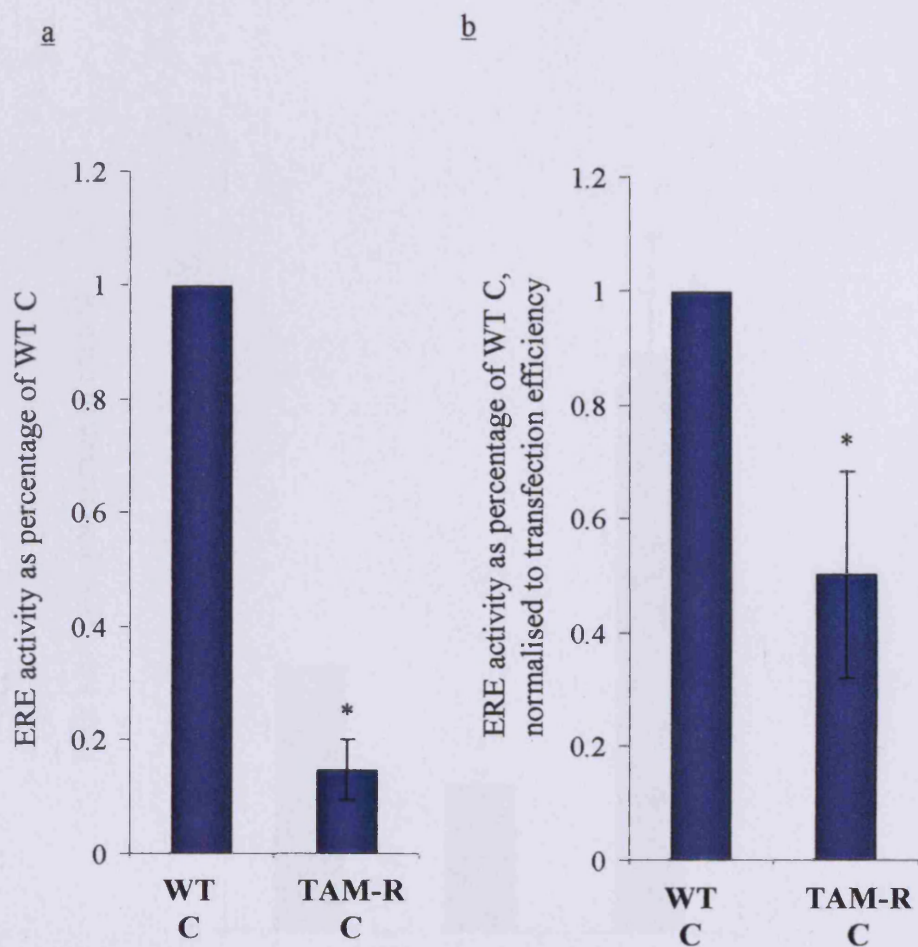
Mean (± SEM) ERE activity in TAM-R cells relative to WT cells prior to (a)
and following (b) normalisation to relative transfection efficiency. This data
is representative of 3 separate experiments. * P<0.05 compared to control.

Figure 3.28. Effects of ER ligands on ER transcriptional activity in TAM-R cells.
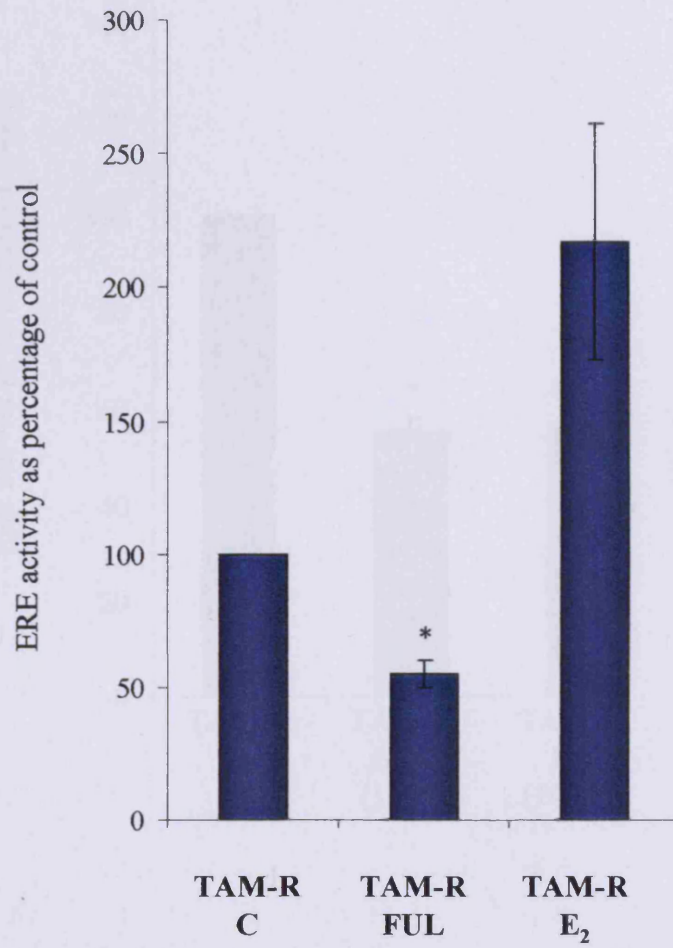
Mean (± SEM) ERE activity in TAM-R cells in the absence and presence of either 100 nM Fulvestrant or 1 nM oestradiol for 24 hours (n=3). The data was normalised to the control ERE activity. * P<0.05 compared to control.

Figure 3.29. Effect of EGFR ligands on ER transcriptional activity in TAM-R cells
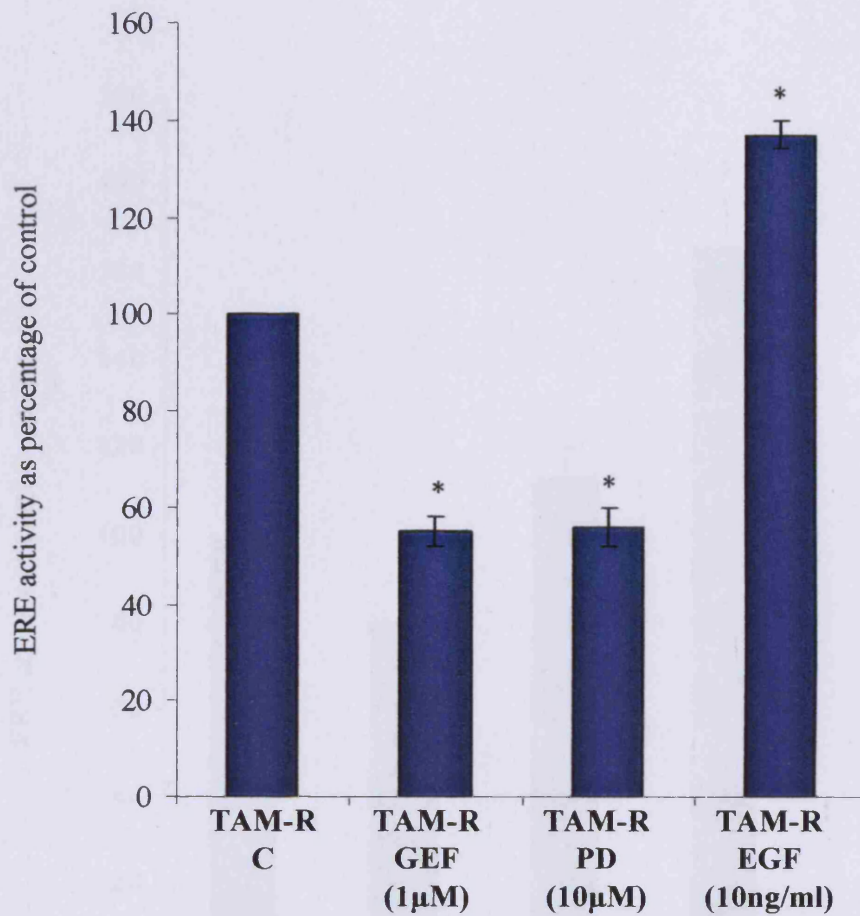
Mean (± SEM) ERE activity in TAM-R cells in the absence and presence of either 1μM gefitinib 24hrs (TAM-R GEF), 10μM PD184352 24hrs (TAM-R PD) or 10ng/ml EGF for 6hrs (TAM-R EGF) (n=3). The data was normalised to the control ERE activity. * P<0.05 compared to control.

Figure 3.30. Effect of IGF-IR ligands on ER transcriptional activity in TAM-R cells
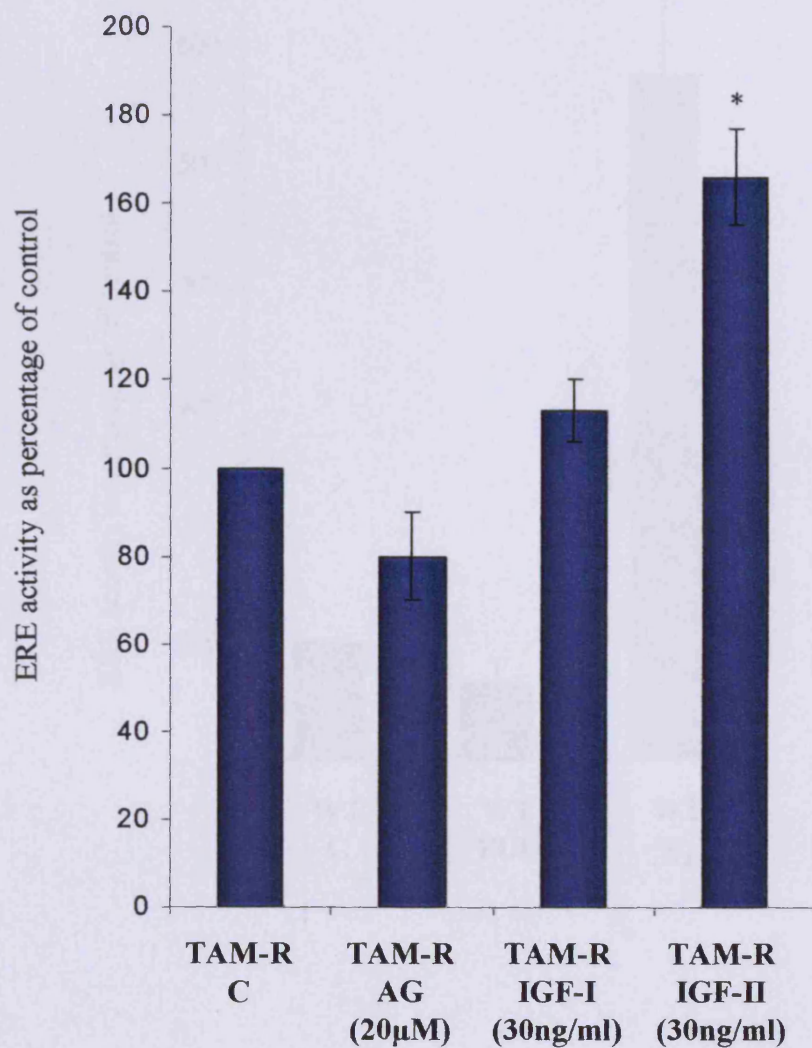
Mean (± SEM) ERE activity in the absence and presence of either 20μM AG1024 for 24 hrs or 30ng/ml IGF-I/IGF-II for 6hrs. The data was normalised to the control ERE activity. * P<0.05 compared to control.

Figure 3.31. Effects of ER ligands on ER transcriptional activity in WT cells
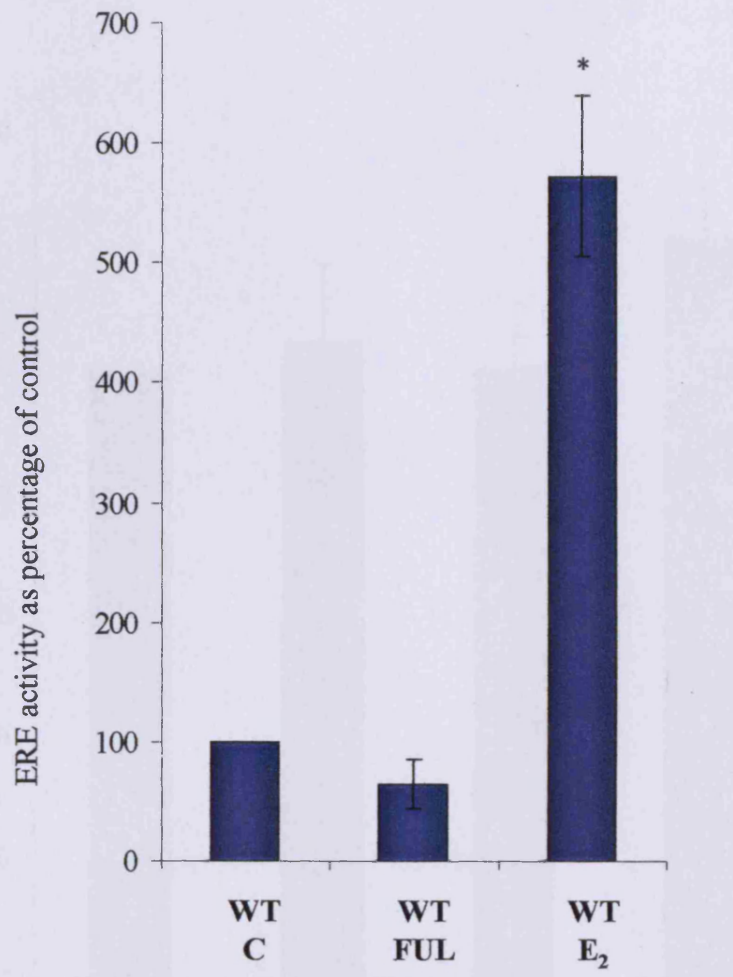
Mean (± SEM) ERE activity in WT cells in the absence and presence of either 100 nM Fulvestrant (WT FUL) or 1 nM oestradiol for 24 hrs (TAM-R E_2) (n=3). The data was normalised to the control ERE activity. * P<0.05 compared to control.

Figure 3.32. Effects of EGFR ligands on ER transcriptional activity in WT cells
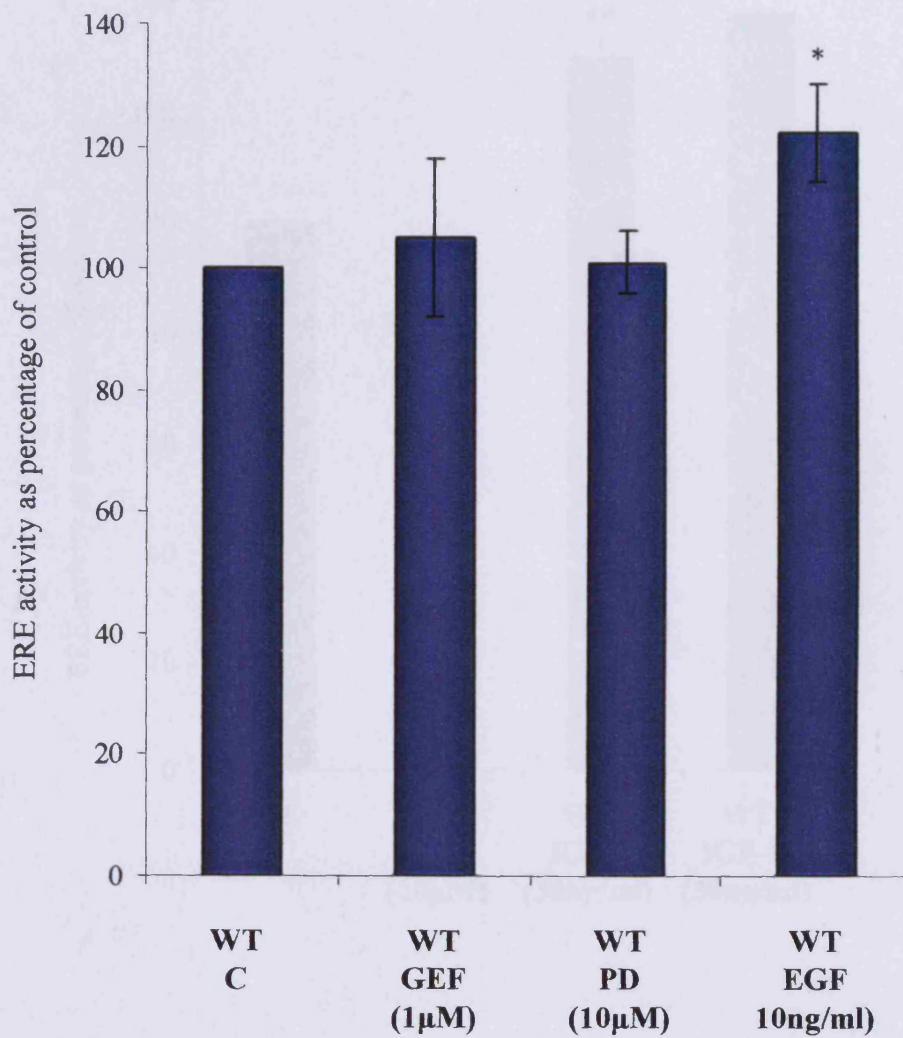
Mean (± SEM) ERE activity in WT cells in the absence and presence of either 1μM gefitinib for 24hrs, 10μM PD184352 for 24 hrs or 10ng/ml EGF for 6hrs (n=3). The data was normalised to the control ERE activity. * P < 0.05 compared to control.

Figure 3.33. Effects of the IGF-IR ligands on ER transcriptional activity in WT cells
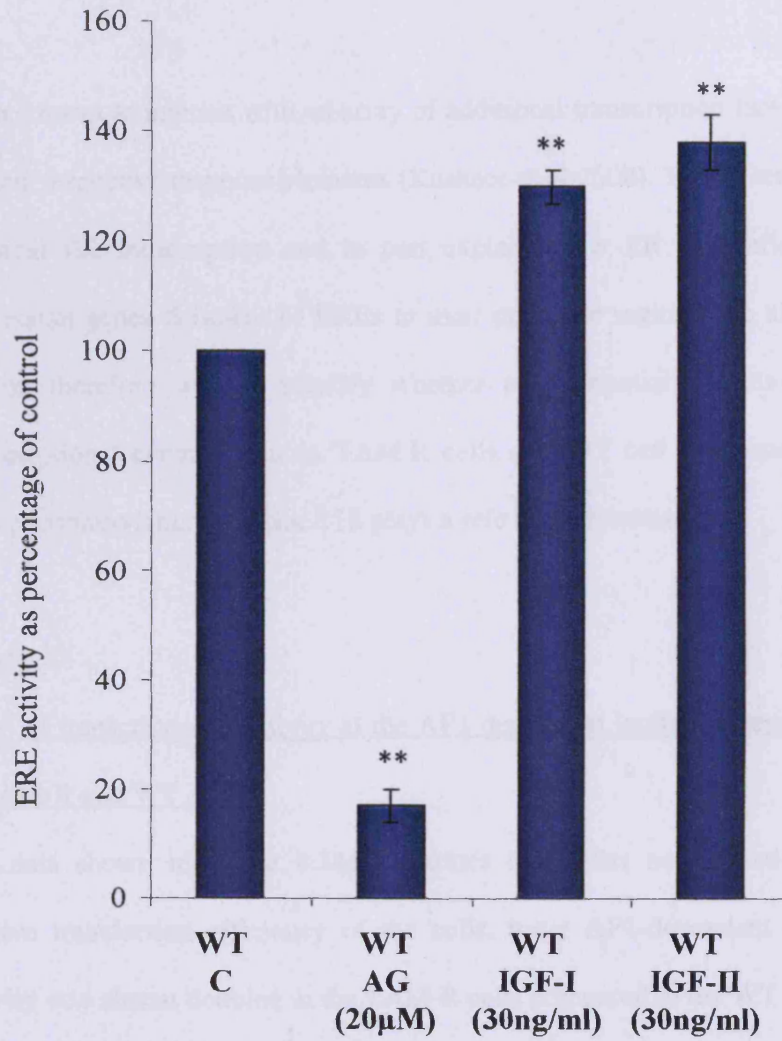
Mean (± SEM) ERE activity in WT cells in the absence and presence of either 20µM AG1024 for 24 hrs, or 30ng/ml IGF-I/IGF-II for 6hrs (n=3). The data was normalised to the control ERE activity. ** P<0.001 compared to control.

3.12 Pharmacological modulation of response elements other than ERE in transfected TAM-R and WT cells

Aims

ER is known to interact with an array of additional transcription factors bound to their respective response elements (Kushner et al 2000). This is termed non-classical ER transcription and in part explains how ER can influence the expression genes deficient of EREs in their promoter region. The aim of this section therefore was to identify whether any potential non-classical ER transcriptional events occur in TAM-R cells and WT cell lines and whether ERα phosphorylation at serine 118 plays a role in such events.

Results

Level of transcriptional activity at the AP1 dependent luciferase reporter gene in TAM-R and WT cells

The data shown in figure 3.34a illustrates that, after normalisation to the relative transfection efficiency of the cells, basal AP1-dependent luciferase activity was almost doubled in the TAM-R cells compared to the WT cell line.

Effect of EGFR activity modulators on AP1 activity in TAM-R and WT cells

AP-1 activity in TAM-R and WT cells showed some correlations with ERα phosphorylation at serine 118. Figure 3.34b and c shows that EGF was a potent stimulator of AP-1 activity in TAM-R and WT cells and while gefitinib and PD184352 lacked inhibitory effects in WT cells, gefitinib showed some inhibitory activity in TAM-R cells consistent with the high levels of EGFR

signalling in this cell type. The 12-O-tetradecanoyl-phorbol 13-acetate (TPA) positive control was less effective than EGF at increasing AP1 dependent transcription. Interestingly however, EGFR modulation of AP-1 activity appears independent of non-classical ER events and hence ERα phosphorylation, as fulvestrant showed no inhibitory effects on AP-1 activity in TAM-R or WT cells in the presence or absence of EGF (Figure 3.34 b/c).

Level of transcriptional activity at the SRE dependent luciferase reporter gene in TAM-R and WT cells

Figure 3.35a revealed that, after normalisation to the relative transfection efficiency of the cells, basal SRE-dependent luciferase activity was approximately 6 times greater in TAM-R cells than it was in the WT cell line.

Effect of EGFR activity modulators on SRE activity in TAM-R and WT cells

Figure 3.35 shows that EGFR signalling is a modulator of SRE activity in TAM-R cells. Thus EGF stimulates, while gefitinib and PD184352 inhibits SRE activity in this cell type. The results contrast with those obtained in WT cells where EGF, gefitinib and PD184352 are largely without effect. Importantly, as with the AP-1 response element, fulvestrant was unable to reduce SRE activity in TAM-R cells in the presence or absence of EGF (Figure 3.35).

The importance of non-classical ERα transcriptional activity in TAM-R cells on a variety of other reporter gene constructs containing response elements to heat shock (HSE), NFκB, Glucocorticoid (GRE) and cAMP (CRE) were also

investigated. Again however, fulvestrant had no effect on the basal activity of these response elements (not illustrated). Clearly ERα and hence ERα phosphorylation at serine 118 appears less significant to the transcriptional activity of these additional response elements. The remaining studies therefore focussed on ERE containing genes.

Figure 3.34. Effects of EGFR activity modulators on AP-1 activity in TAM-R and WT cells
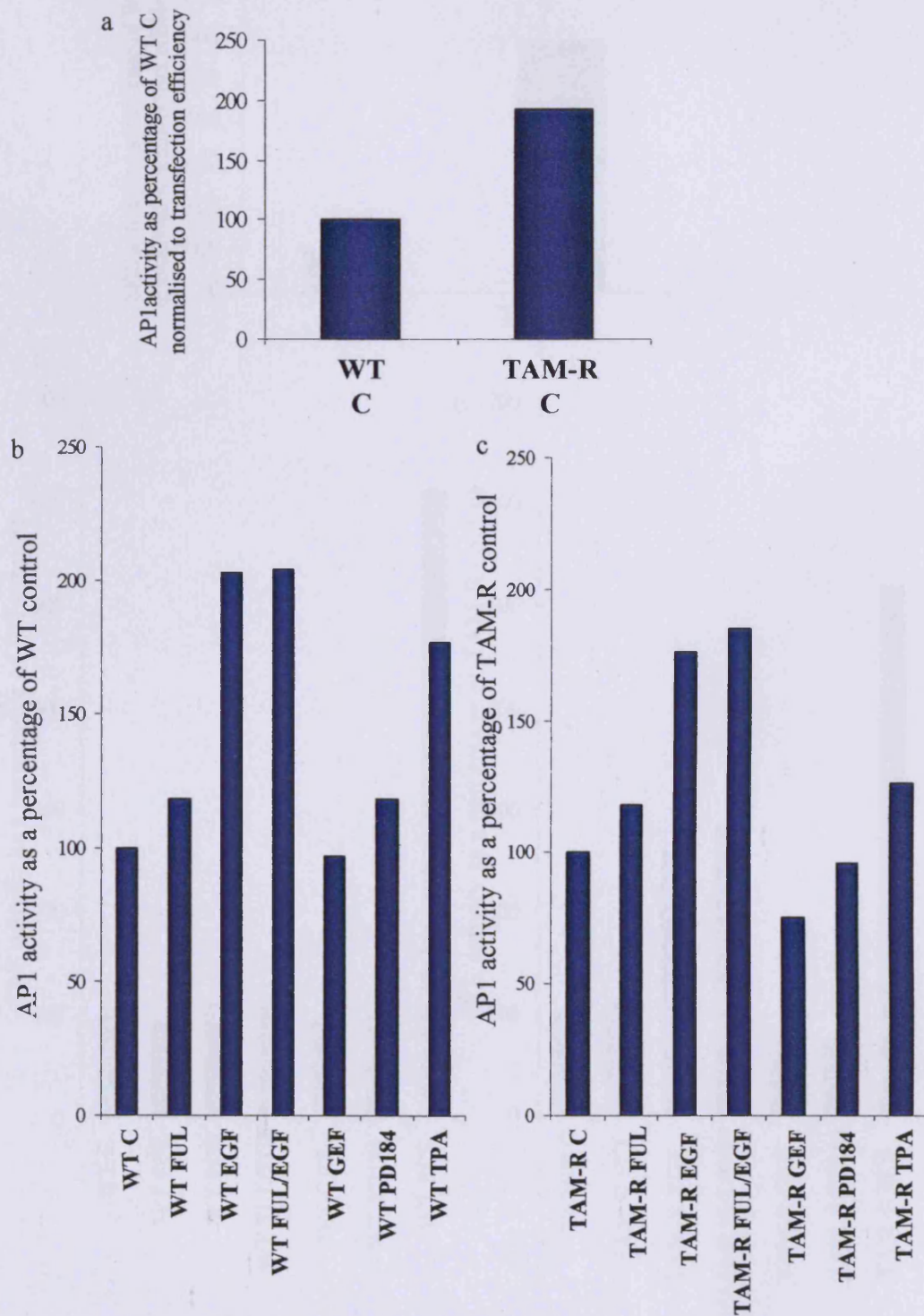
a. AP1 activity in TAM-R cells relative to WT cells following their normalisation with transfection efficiency. Level of AP1 activity in b) WT and c) TAM-R cells following treatment with either 100 nM Fulvestrant, 10 ng/ml EGF, 1 μM Gefitinib, 10 μM PD184352 or 1 μM TPA. The data was normalised to the control ERE activity (n=1).

Figure 3.35. Effects of EGFR activity modulators on SRE activity in TAM-R and WT cells
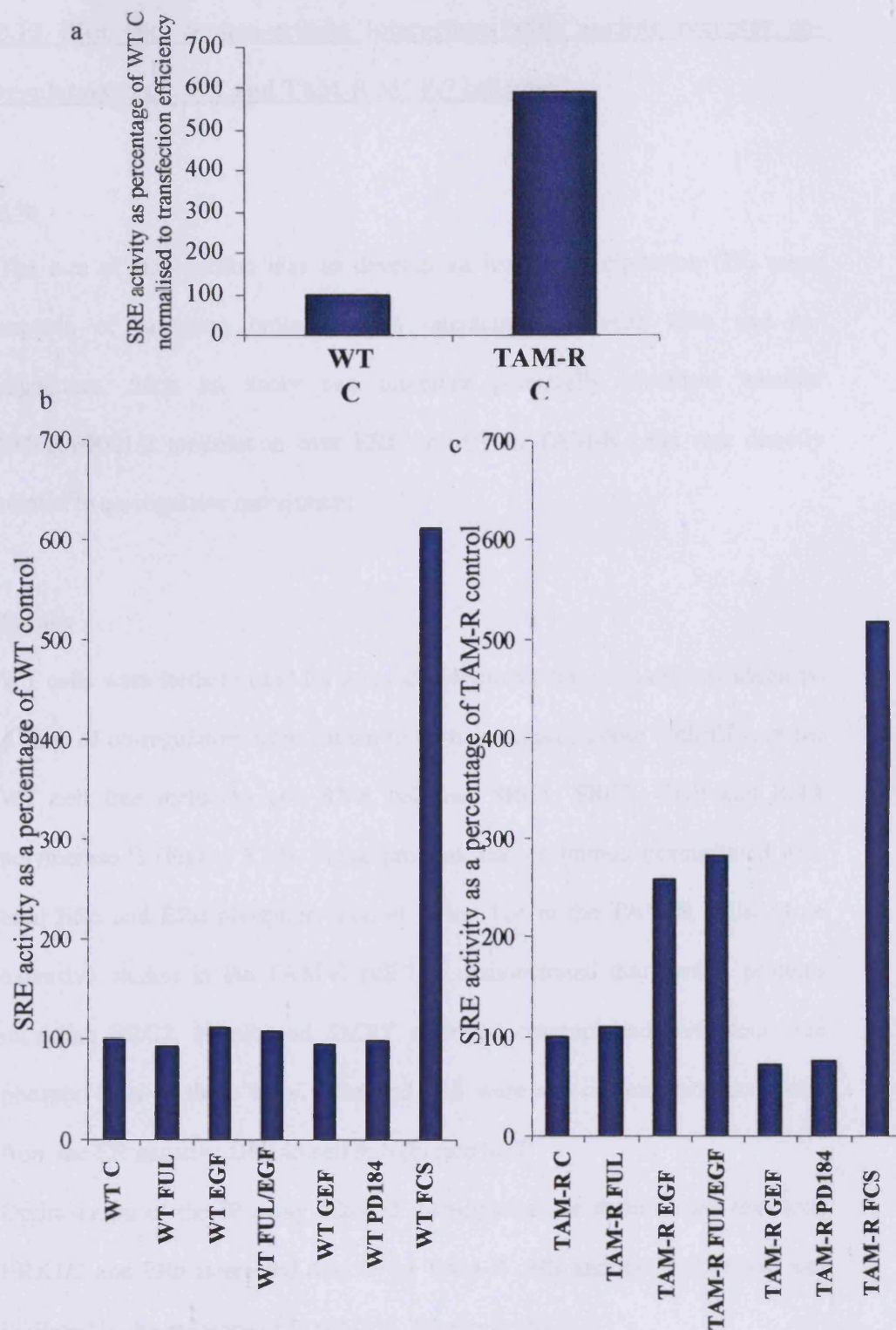
a. SRE activity in TAM-R cells relative to WT cells following their normalisation with transfection efficiency. Level of SRE activity in b) WT and c) TAM-R cells following treatment with either 100 nM Fulvestrant, 10 ng/ml EGF, 1 μM Gefitinib, 10 μM PD184352 or 5% FCS. The data was normalised to the control ERE activity (n=1).

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3.13 ERα and protein-protein interactions with nuclear receptor co-regulators in the WT and TAM-R MCF-7 cell lines.

Aim

The aim of this section was to develop an immunoprecipitation (IP) assay capable of detecting protein-protein interactions between ERα and co-regulators. Such an assay can therefore potentially elucidate whether EGFR/ERK1/2 modulation over ERE activity in TAM-R cells was directly related to co-regulator recruitment.

Results

WT cells were initially used for assay development due to sample availability. A host of co-regulators were shown to co-immunoprecipitate with ERα in the WT cell line including p68 RNA helicase, SRC1, SRC3, CBP and RNA polymerase II (Figure 3.37). These proteins also co-immunoprecipitated with total ERα and ERα phosphorylated at serine 118 in the TAM-R cells. More extensive studies in the TAM-R cell line demonstrated that further proteins including SRC2, NCoR and SMRT co-immunoprecipitated with total and phospho-ERα in these cells. ERα and p68 were not co-immunoprecipitated from the ER negative DU145 cell line (Figure 3.37).

Optimisation of the IP assay allowed investigations to demonstrate that total ERK1/2 and ERα interacted directly in TAM-R cells and this interaction was inhibited in the presence of PD184352 (Figure 3.38).

Figure 3.36. Immunoprecipitation studies demonstrate that ERα interacts with several co-regulator proteins in the WT cell line.
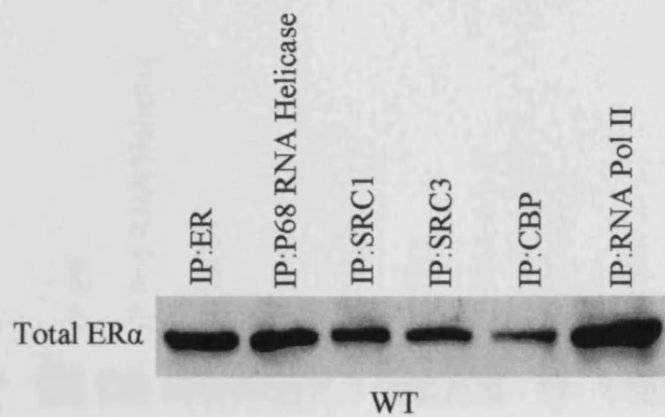
WT cell lysate immunoprecipitated for; ERα, p68 RNA helicase, SRC1, SRC3, CBP, RNA pol II. Each immunoprecipitate run on gel in separate lanes and blot incubated in total ERα primary antibody.

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Figure 3.37. Immunoprecipitation studies demonstrate that ERα interacts with several co-regulator proteins in the TAM-R cell line.
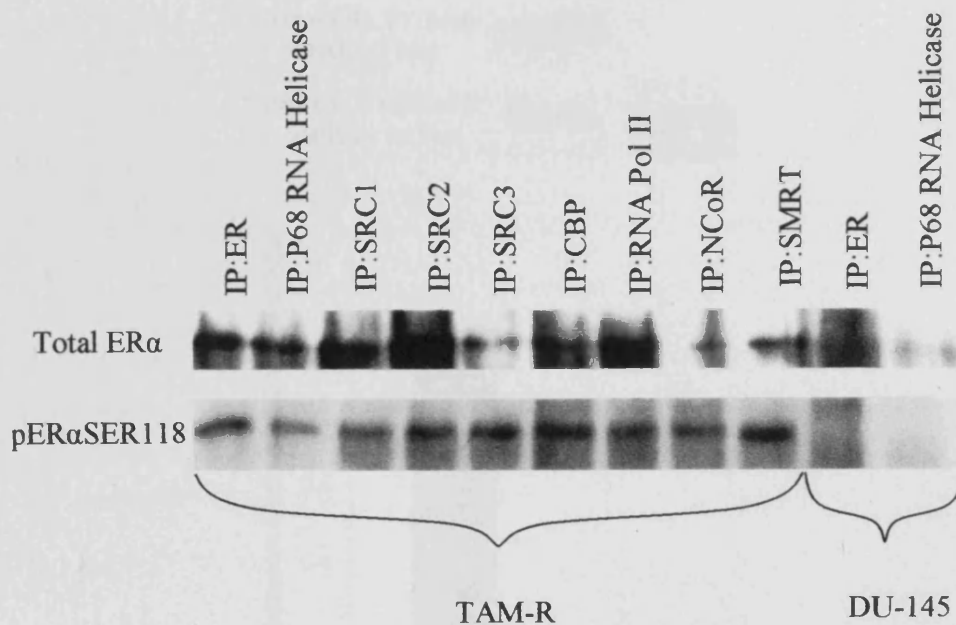
TAM-R cell lysate immunopreciptated for; ERα, p68 RNA helicase, SRC1, SRC2, SRC3, CBP, RNA pol II, NCoR or SMRT. DU-145 cell lysate immunoprecipitated for ERα or p68 RNA helicase. Each immunoprecipitate run on gel in separate lanes. Western blots incubated in either total ERα or pERαSER118 primary antibody.

Figure 3.38. Immunoprecipitation studies demonstrate that the interaction between ERK1/2 and ERα is dependent on ERK1/2 activity in the TAM-R cell line.
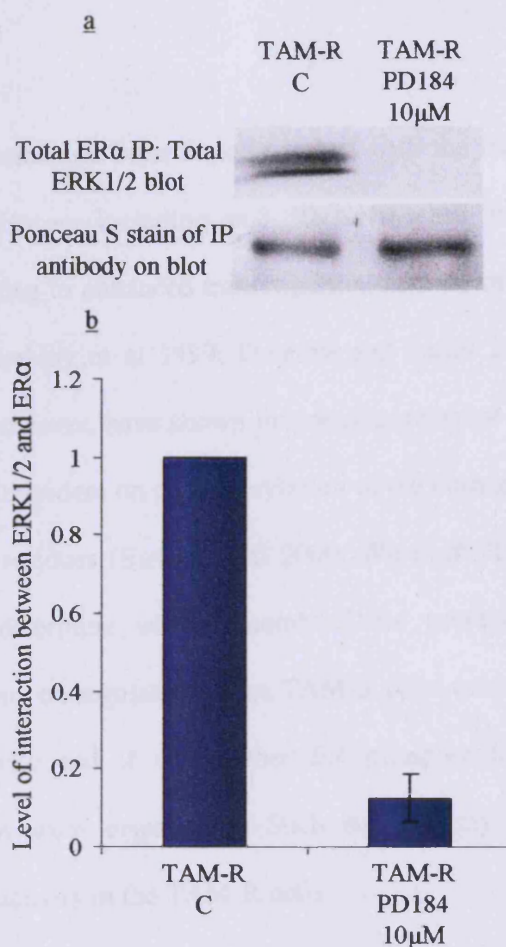
a: Western blot analysis and b: Densitometry values (expressed as mean ± SEM) of total ERK1/2 expression following immunoprecipitation with total ERα in TAM-R cells treated with either PD184352 (10µM) or appropriate vehicle control for 1 hour (n=2). Densitometry values were normalised to Ponceau S staining of IP antibody to account for possible errors in sample loading.

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3.14 ERα phosphorylation at serine 118 and recruitment of nuclear receptor co-activators in TAM-R MCF-7 cell lines

Aims

ER phosphorylation has been directly linked with the recruitment of nuclear receptor co-activators including p68 RNA helicase and the SRC family members, resulting in enhanced transcriptional activity of the receptor (Endoh et al 1999; Tremblay et al 1999; Dutertre and Smith 2003; Watanabe et al 2003). Others however, have shown that nuclear receptor interactions with co-regulators are dependent on phosphorylation of the co-regulators themselves at specific serine residues (Rowan et al 2000; Wu et al 2004). The aim of this section is to determine whether some of the protein-protein interactions between ERα and co-regulators in the TAM-R cells were dependent on EGFR signalling activity and if so whether ER phosphorylation or co-regulator phosphorylation were responsible. Such studies may explain how EGFR mediates ERE activity in the TAM-R cells.

Results

The nuclear receptor co-activator p68 RNA helicase associates with ERα in an AF-1 dependent/AF-2 independent manner (Endoh et al 1999). In support of these findings and correlating with ERα phosphorylation at serine 118, modulation of EGFR in the TAM-R cells directly influenced recruitment of p68 to ERα, as gefitinib strongly decreased, while EGF strongly increased the ERα-p68 RNA helicase interaction (Figure 3.39). Interestingly the level of

phosphorylation at the serine residues of p68 RNA helicase were unchanged between treatment arms. Gel loading was equal as shown by the level of ponceau S stained antibody (Figure 3.39).

The interaction between ERα and SRC1 was also dependent on EGFR activity in TAM-R cells as gefitinib strongly decreased, while EGF strongly increased the ERα-SRC1 interaction (Figure 3.40). The level of phosphorylation at the serine residues of SRC1, however, again remained unchanged by EGFR signalling activity modulation. Ponceau S staining of the blot demonstrated equal sample loading (Figure 3.40)

The interaction between ERα and SRC3 was not as dependent on the EGFR activity in the TAM-R cell line as the interaction between ERα and p68 or SRC1. Gefitinib decreased the interaction between these proteins in TAM-R cells by approximately 20%, whilst EGF showed no effect. Interestingly the level of phosphorylation on the serine residues of SRC3 decreased dramatically in TAM-R cells treated with gefitinib, while EGF clearly increased SRC3 serine phosphorylation. Gel loading was equal as shown by the level of ponceau S staining of the blot (Figure 3.41).

Growth factor signalling pathways are also reported to mediate the interaction between co-repressors and ERα in tamoxifen treated breast cancer cells (Carroll et al 2003), therefore, regulation of SMRT/ERα interactions by EGFR signalling activity was also investigated in TAM-R cells. Gefitinib and EGF had no effect on SMRT association with ERα (Figure 3.42), furthermore, the

level of phosphorylation on the serine residues of SMRT were unchanged

following treatment with these agents. Gel loading was equal as shown by the

level of ponceau S staining of the blot (Figure 3.42).

Figure 3.39. EGF-R signalling regulates the level of interaction between ERα and the transcriptional coregulator p68 RNA Helicase
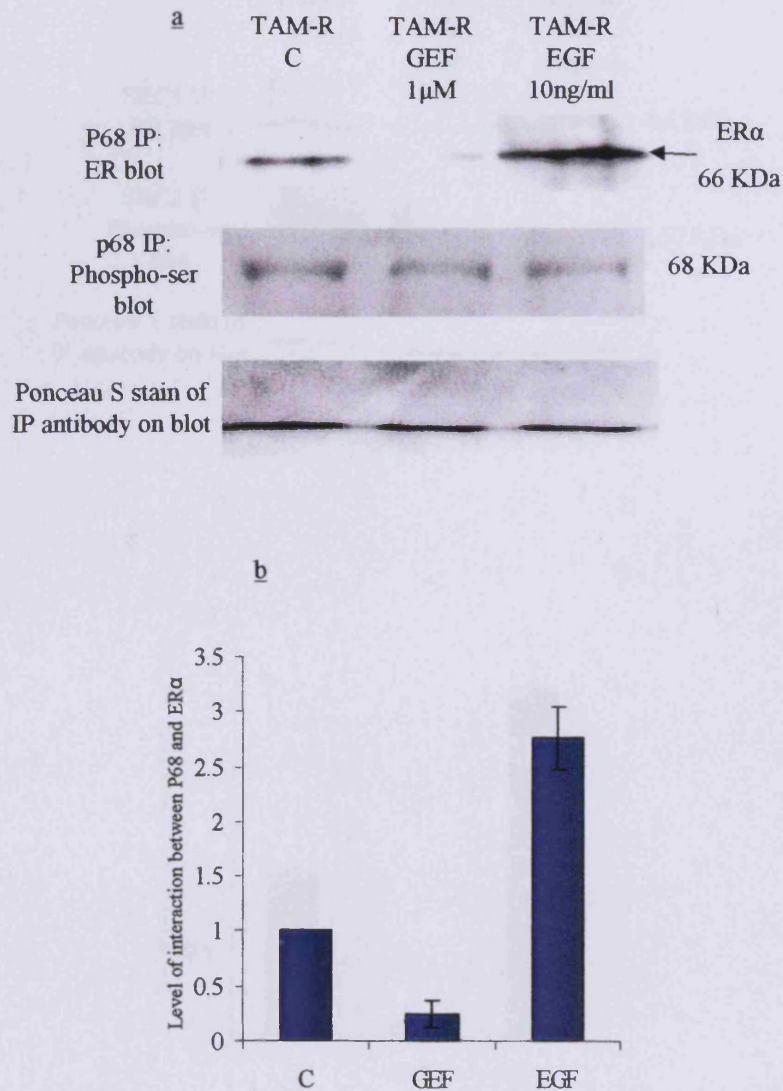
a: Western blot analysis and of total ERα and phospho-serine expression following immunoprecipitation with p68 RNA helicase in TAM-R cells treated with either gefitinb (1 μM), EGF (10 ng/ml) or ethanol control. b: Densitometry values (expressed as mean ± SEM) of total ERα expression following immunoprecipitation with p68 RNA helicase in TAM-R cells treated with either gefitinb, EGF or ethanol control (n=2). Values were normalised to Ponceau S staining of IP antibody to account for possible errors in sample loading.

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Figure 3.40. EGF-R signalling regulates the level of interaction between ER and the transcriptional coregulator SRC1
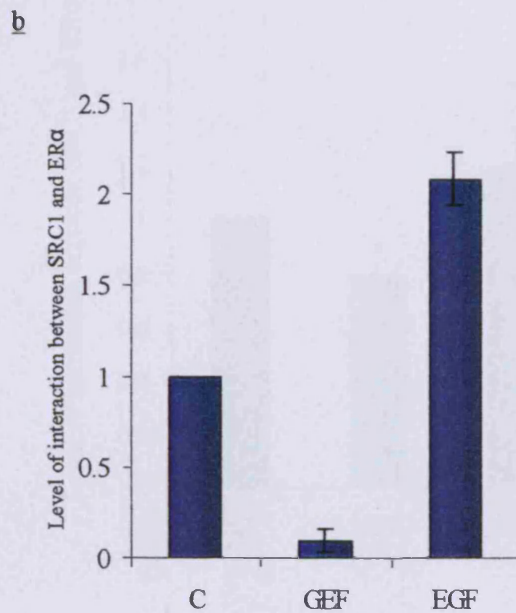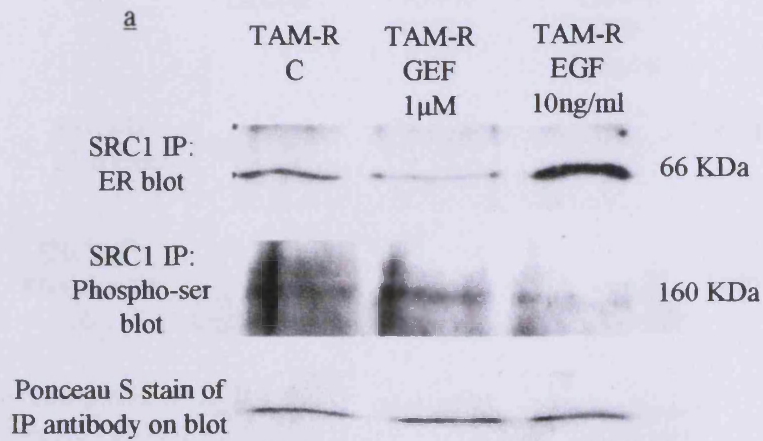
a: Western blot analysis and of total ERα and phospho-serine expression following immunoprecipitation with SRC1 in TAM-R cells treated with either gefitinb (1 μM), EGF (10 ng/ml) or ethanol control. b: Densitometry values (expressed as mean ± SEM) of total ERα expression following immunoprecipitation with SRC1 in TAM-R cells treated with either gefitinb, EGF or ethanol control (n=2). Values were normalised to Ponceau S staining of IP antibody to account for possible errors in sample loading.

Figure 3.41. Effect of EGF-R signalling on the interaction between ERα and the transcriptional co-regulator SRC3
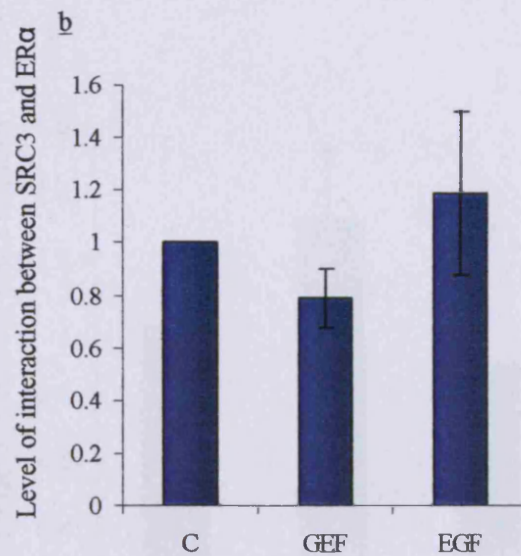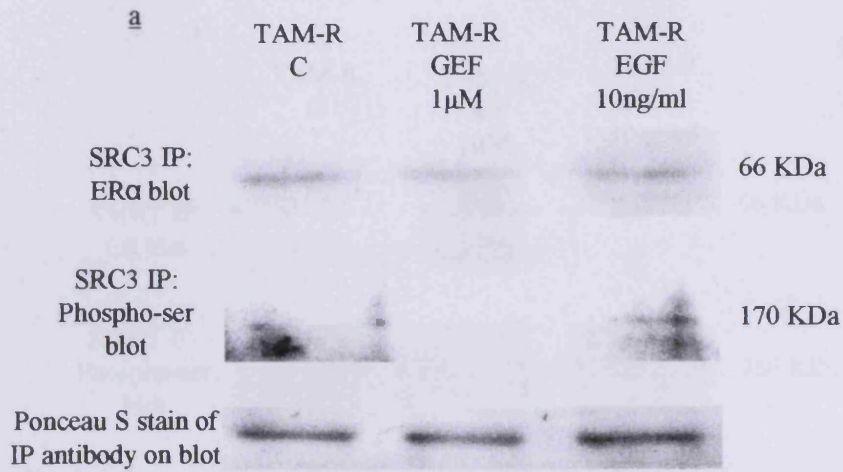
a: Western blot analysis and of total ERα and phospho-serine expression following immunoprecipitation with SRC3 in TAM-R cells treated with either gefitinb (1 μM), EGF (10 ng/ml) or ethanol control. b: Densitometry values (expressed as mean ± SEM) of total ERα expression following immunoprecipitation with SRC3 in TAM-R cells treated with either gefitinb, EGF or ethanol control (n=2). Values were normalised to Ponceau S staining of IP antibody to account for possible errors in sample loading.

Figure 3.42. EGF-R signalling does not regulate the level of interaction between ER and the transcriptional coregulator SMRT
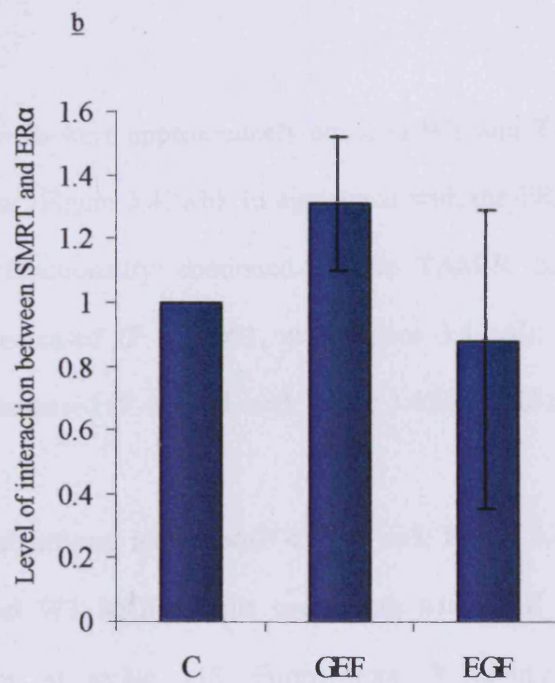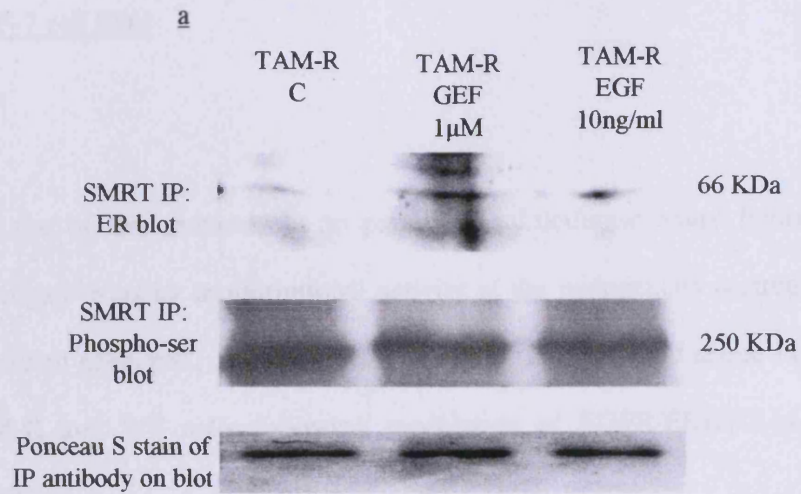
a: Western blot analysis and of total ERα and phospho-serine expression following immunoprecipitation with SMRT in TAM-R cells treated with either gefitinb (1 μM), EGF (10 ng/ml) or ethanol control. b: Densitometry values (expressed as mean ± SEM) of total ERα expression following immunoprecipitation with SMRT in TAM-R cells treated with either gefitinb, EGF or ethanol control (n=2). Values were normalised to Ponceau S staining of IP antibody to account for possible errors in sample loading.

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3.15: ERα phosphorylation at serine 118 and effects on transcriptional regulation of endogenous oestrogen dependent genes in TAM-R and WT MCF-7 cell lines

Aim

The aim of this section was to parallel the Luciferase assay findings and investigate whether transcriptional activity at the endogenous oestrogen/ERE dependent gene, pS2, correlated with ERα phosphorylation at serine 118 in the TAM-R and WT cells following modulation of EGFR/ERK1/2 signalling activity.

Results

pS2 mRNA levels were approximately equal in WT and TAM-R cells under basal conditions (Figure 3.43a/b). In agreement with the ERE luciferase assay findings ER functionality continued in the TAM-R cells as oestradiol significantly increased (P < 0.001, n=3; figure 3.43c/d), whilst fulvestrant significantly decreased (P < 0.005, n=3; figure 3.43c/d) pS2 mRNA in both cell lines.

EGF induced significant increases (P < 0.05, n=3; Figure 3.44) in pS2 mRNA in TAM-R and WT MCF-7 cells correlating with ERE activity and ERα phosphorylation at serine 118. Furthermore, Fulvestrant was shown to significantly inhibit these EGF-induced increases in pS2 mRNA (P < 0.05, n=3; figure 3.44). Gefitinib and PD184352 significantly reduced pS2 mRNA levels in the TAM-R cells (P < 0.05, n=3 for both; figure 3.45c) whilst having

no significant effects in the WT cells (figure 3.45b), again correlating with

ERα phosphorylation at serine 118 and ERE luciferase transcriptional activity

in these cell lines.

Figure 3.43. Modulation of pS2 mRNA levels in WT and TAM-R MCF-7 cells

a
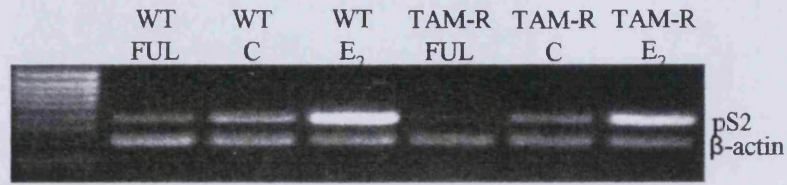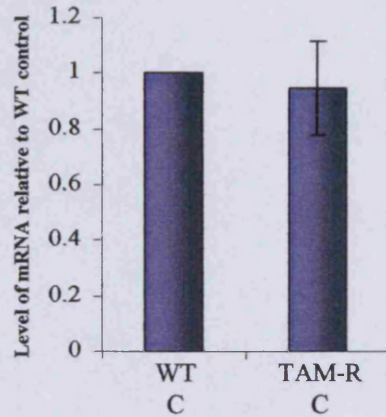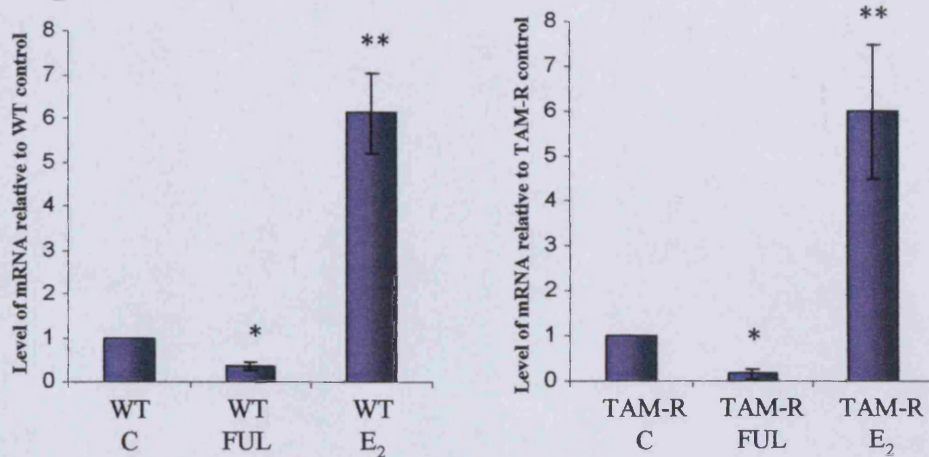
a. pS2 mRNA expression levels in WT and TAM-R cells in the absence and presence of either 100 nM Fulvestrant or 1 nM oestradiol. β-actin was used as an internal control. Densitimetry values for pS2 mRNA levels in WT vs TAM-R cells either b. under basal growth conditions or c. in the absence and presence of either 100 nM fulvestrant or 1 nM oestradiol following normalisation to β-actin levels (n=3). * P<0.05 compared to control. ** P<0.01 compared to control.

Figure 3.44. Modulation of pS2 mRNA levels in WT and TAM-R MCF-7 cells
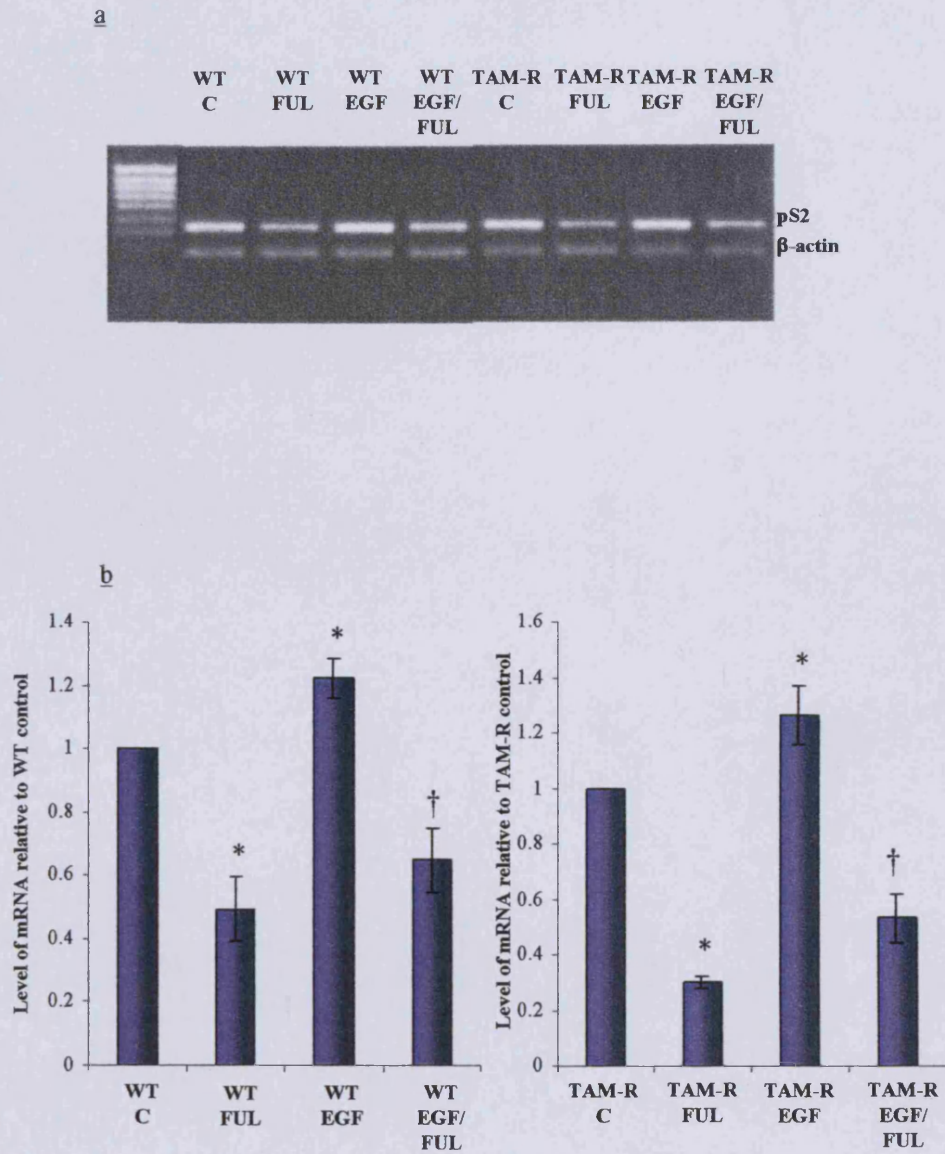
a. RT-PCR analysis and b. Densitometry values of pS2 mRNA expression levels in WT and TAM-R cells in the absence and presence of either 100 nM fulvestrant (FUL), 10 ng/ml EGF or a combination of the two agents. β-actin was used as an internal control. b. Densitimetry values were normalised to β-actin levels (n=3). * P<0.05 compared to control. † P<0.05 compared to EGF.

Figure 3.45. Modulation of PS2 mRNA levels in WT and TAM-R MCF-7 cells
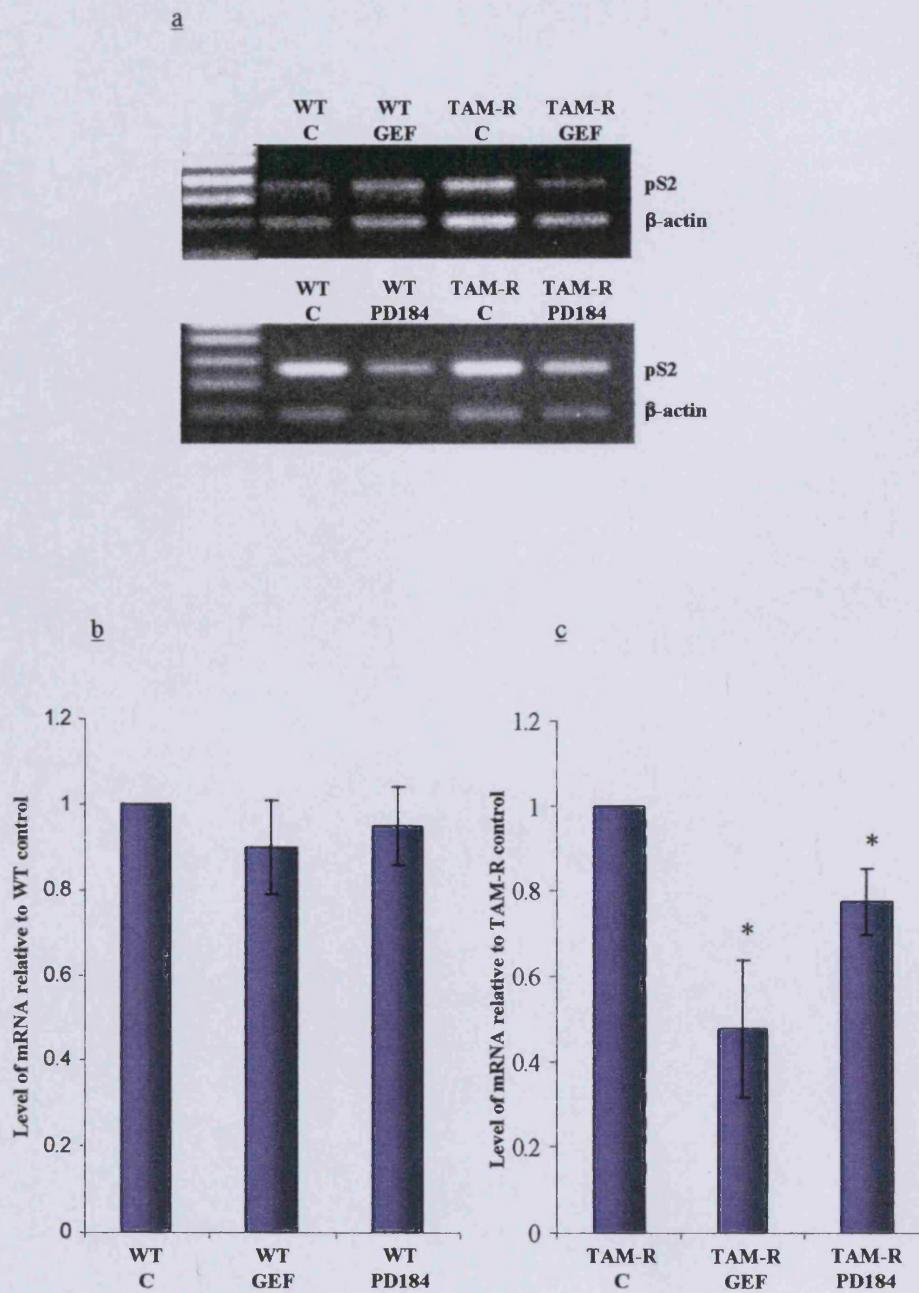
a. RT-PCR analysis and b. Densitometry values of pS2 mRNA expression levels in WT and TAM-R cells in the absence and presence of either 1 μM gefitinib for 1 hour (GEF) or 10μM PD184352 (PD184) . β-actin was used as an internal control. b. Densitimetry values were normalised to β-actin levels (n=3). * P<0.05 compared to control.

3.16 ER regulation of EGFR ligands in TAM-R and WT cell lines

Aim

EGFR signalling activity is dependent on a functional ER in TAM-R cells under basal conditions as fulvestrant significantly inhibits EGFR/ERK1/2 phosphorylation (Hutcheson et al 2003). Early findings showed that TGFα expression levels were also decreased by fulvestrant, whilst total EGFR/ERK1/2 expression levels remained unchanged, suggesting that ER regulation of EGFR activity was mediated through transcriptional regulation of EGFR ligands (Hutcheson et al 2003). The aim of this section was to investigate the effects of fulvestrant on EGFR ligands in TAM-R cells more extensively and study whether EGFR/ERK1/2 dependent phosphorylation at serine 118 influences ER-mediated transcription of these ligands.

Results

Of the EGFR ligands examined only epiregulin and amphiregulin (AR) appeared to be under obvious ER regulation. Epiregulin mRNA levels were significantly reduced by fulvestrant in TAM-R ($P < 0.05$, n=3) and WT cells ($P < 0.05$, n=3; Figure 3.46). Fulvestrant also significantly reduced AR mRNA levels in the two cell lines ($P < 0.05$, n=3 for both cell lines; Figure 3.47). TGFα levels appeared to be modulated in WT and TAM-R cells, albeity weakly, by fulvestrant however this was not found to be significant. Significance may be reached with further repeat experiments to increase the n value that used in the Hutcheson et al study (n=7). Similarly there was no

significant effect of fulvestrant on either EGF, HB-EGF or β-cellulin mRNA expression in the two cell lines (Figure 3.46). Only amphiregulin showed significantly raised levels of mRNA in the TAM-R compared to WT cells ($P <$ 0.05, n=3; Figure 3.47), correlating with the elevated EGFR/ERK1/2 signalling activity observed previously in this cell line. Oestradiol also significantly increased amphiregulin transcription in the TAM-R and WT cell lines ($P <$ 0.05, n=3 for both cell lines; figure 3.47) confirming ER-dependent transcription of this gene.

EGF induced significant increases in amphiregulin mRNA in the TAM-R and WT cell lines ($P < 0.05$, n=3 for both cell lines; Figure 3.48) correlating with ERE activity and ERα phosphorylation at serine 118. Furthermore fulvestrant significantly inhibited the EGF induced increases in both cell lines ($P < 0.05$, n=3; Figure 3.48).

Gefitinib ($P < 0.05$, n=3; figure 3.49) and PD184352 ($P < 0.05$, n=3; figure 3.49) significantly reduced basal amphiregulin mRNA levels in the TAM-R cells, whilst showing no effect in the WT cells, again correlating with ERE activity and ERα phosphorylation at serine 118 in these two cell lines (Britton et al 2005). AG1024 also induced a reduction in basal amphiregulin mRNA expression in TAM-R cells, however, this was not found to be statistically significant (Figure 3.49). Interestingly, AG1024 showed no significant effects on amphiregulin mRNA levels in the WT cells (figure 3.49).

Figure 3.46. ERα regulation of EGFR ligand mRNA levels in WT and TAM-R MCF-7 cells
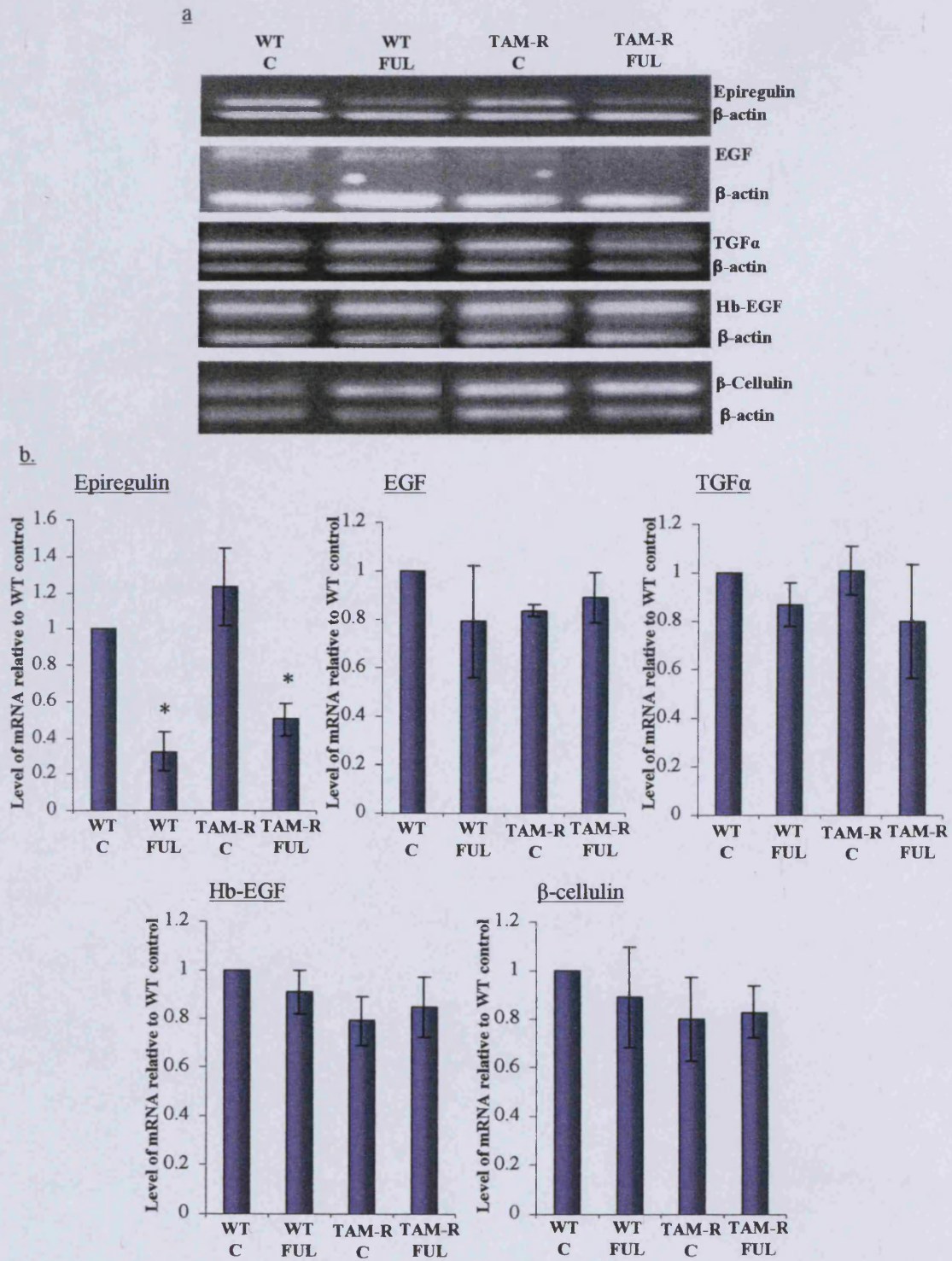
a. RT-PCR analysis and b. Densitometry values for epiregulin, EGF, TGFα, HB-EGF and β-cellulin mRNA expression in WT and TAM-R cells either in the absence or presence of 100 nM fulvestrant for 7 days (n=3). β-actin was used as an internal control in RT-PCR studies and densitometry values were normalised to β-actin levels to take account of any errors in sample loading. * $P < 0.05$ compared to control.

Figure 3.47. Modulation of Amphiregulin mRNA levels in WT and TAM-R MCF-7 cells
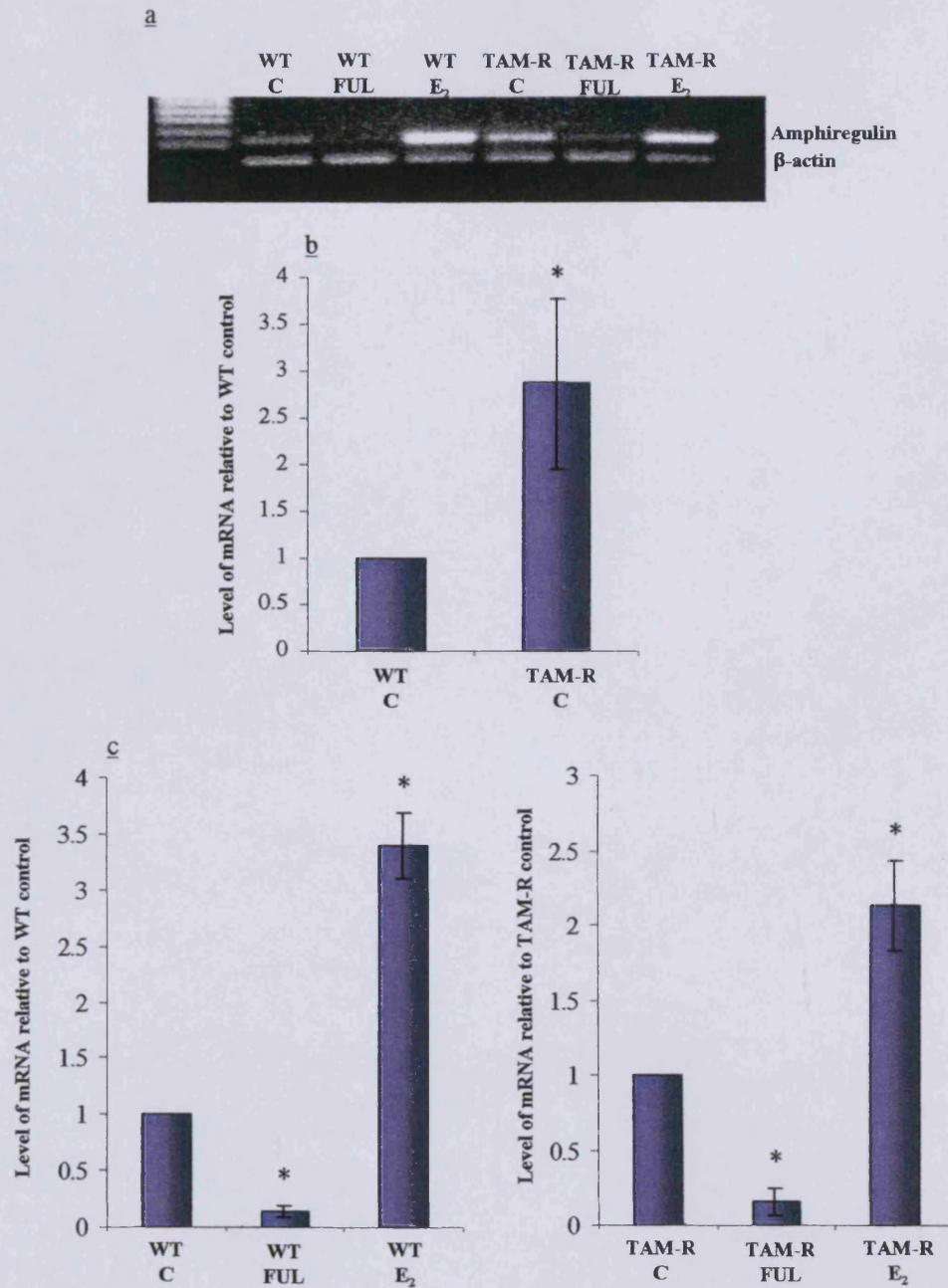
a. RT-PCR analysis of amphiregulin mRNA expression in WT and TAM-R cells in the absence and presence of either 100 nM fulvestrant or 1 nM oestradiol for 7 days. β-actin was used as an internal control. b. Densitometry values for amphiregulin mRNA expression under basal growth conditions in WT and TAM-R cells. c. Densitometry values for amphiregulin mRNA expression in WT and TAM-R cells in the absence and presence of either 100 nM fulvestrant or 1 nM oestradiol for 7 days (n=3). All densitometry values were normalised to β-actin levels to take account of any errors in sample loading. * P<0.05 compared to control.

Figure 3.48. Modulation of Amphiregulin mRNA levels in WT and TAM-R MCF-7 cells
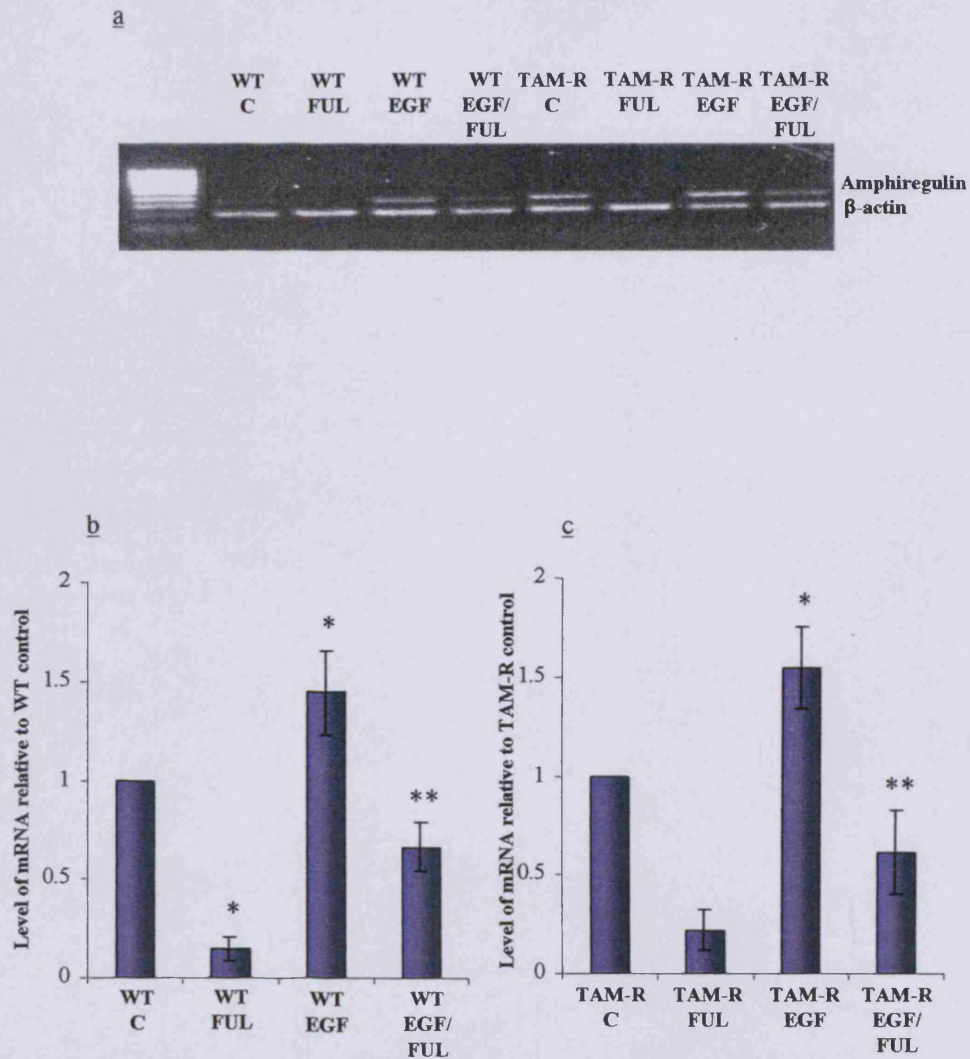
a. RT-PCR analysis and b. Densitometry values for amphiregulin mRNA expression in WT and TAM-R cells in the absence and presence of either 100 nM fulvestrant (FUL), 10 ng/ml EGF or a combination of the two agents for 7 days (n=3). β-actin was used as an internal control in RT-PCR studies and densitometry values were normalised to β-actin levels to take account of any errors in sample loading. * P<0.05 compared to control.

Figure 3.49. Modulation of Amphiregulin mRNA levels in WT and TAM-R MCF-7 cells
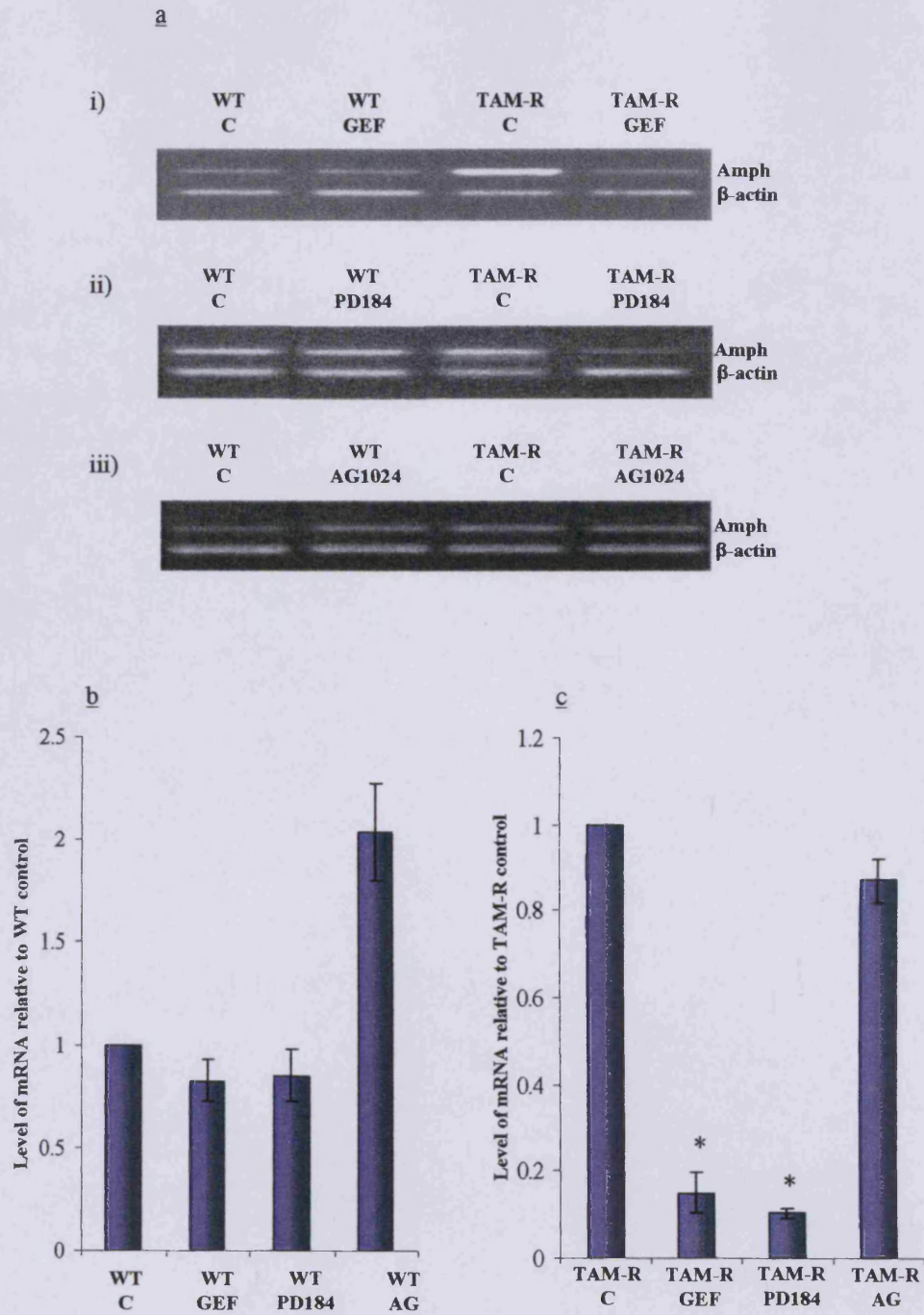
a. RT-PCR analysis and b. Densitometry values for amphiregulin mRNA expression in WT and TAM-R cells in the absence and presence of either (i) 1μM gefitinib (GEF) for 1 hour, (ii) 10 μM PD184352 (PD184) for 4 hours or (iii) 20 μM AG1024 for 24 hours (n=3). β-actin was used as an internal control in RT-PCR studies and densitometry values were normalised to β-actin levels to take account of any errors in sample loading. * P<0.05 compared to control.

3.17 Interaction between ERα and the Amphiregulin promoter in TAM-R cells

Aims

The data suggests that EGFR/ERK1/2 signal transduction pathway may regulate amphiregulin transcription in TAM-R cells by modulating ERα phosphorylation at serine 118 and therefore transcriptional activity at ERE dependent genes. The aim of this section was to use the Chromatin Immunoprecipitation (ChIP) technique to examine whether ERα interacts with potential EREs identified within the putative amphiregulin promoter, thereby providing supporting evidence that amphiregulin transcription in the TAM-R cells may depend on this cross talk between the EGFR and ERα signalling pathways.

ChIP assay development

The Active Motif ChIP-IT kit used in this procedure contained an internal control test ensuring low background staining and high specificity. Low background was demonstrated following ChIP using antibodies to ERα and TFIIB with signals of similar intensity to the negative control being generated following PCR with the negative control primer (Figure 3.50a). As expected the negative control primer produced a strong signal in the presence of total DNA following PCR (Figure 3.50a). High specificity of the assay was demonstrated as ChIP using the TFIIB antibody produced a signal of greater intensity than ChIP with ERα or negative control antibodies following PCR in

the presence of a GAPDH primer (Figure 3.50b). Once again, as expected, the GAPDH primer produced a strong signal in the presence of total DNA following PCR.

The Alibaba 2.1 transcription factor binding software identified 5 potential ERE's within a 600bp region (Accession number: AY442340 or Gene identifier: gi|37953278, nucleotides 671 to 1270) of the amphiregulin gene promoter. Three primer pairs were designed by the Primer 3 design software to encompass this ERE rich region then run on the NCBI nucleotide-nucleotide blast programme to confirm their specificity to the amphiregulin gene promoter region. Initially the ability of each primer pair to bind efficiency to total DNA was assessed. Only primer pairs 2 and 3 produced signals, therefore primer 1 was excluded from further studies (Figure 3.50c).

Efficiency of the primers to associate to DNA ChIP'ed with the total ERα antibody was then tested. Primer 3 was found to be incapable of producing a band with ER ChIP'ed DNA in both WT and TAM-R cells and so was also excluded from future studies (Figure 3.50d).

Results

Primer 2 produced a signal, following a PCR reaction, in the presence of DNA ChIP'ed using the ERα antibody in both oestradiol treated WT cells and control TAM-R cells, suggesting that ERα may associate to this region of the amphiregulin gene promoter in these two cell lines under these conditions (Figure 3.51a and b). The data in control (untreated) WT cells suggests that ER association with this putative ERE within the AR promoter is non-specific in

untreated WT cells but specific in the WT cells treated with oestradiol as there is a clear difference in the intensity of the signal between ER ChIP and −ve control in the oestradiol treated WT cells, which was not so evident in the untreated WT cells. The high signal from the negative control in the untreated WT cells suggests high non-specific binding between the primer and ChIP'ed DNA from the ER ChIP sample.

TAM-R cells showed a significant difference in intensity of the primer 2 band between the −ve control sample and the ER ChIP sample, suggesting that ERα has a highly specific affinity for this ERE rich region of the amphiregulin promoter in TAM-R cells (P < 0.001, n=6; figure 3.51a/b).

Figure 3.50. ChIP assay development
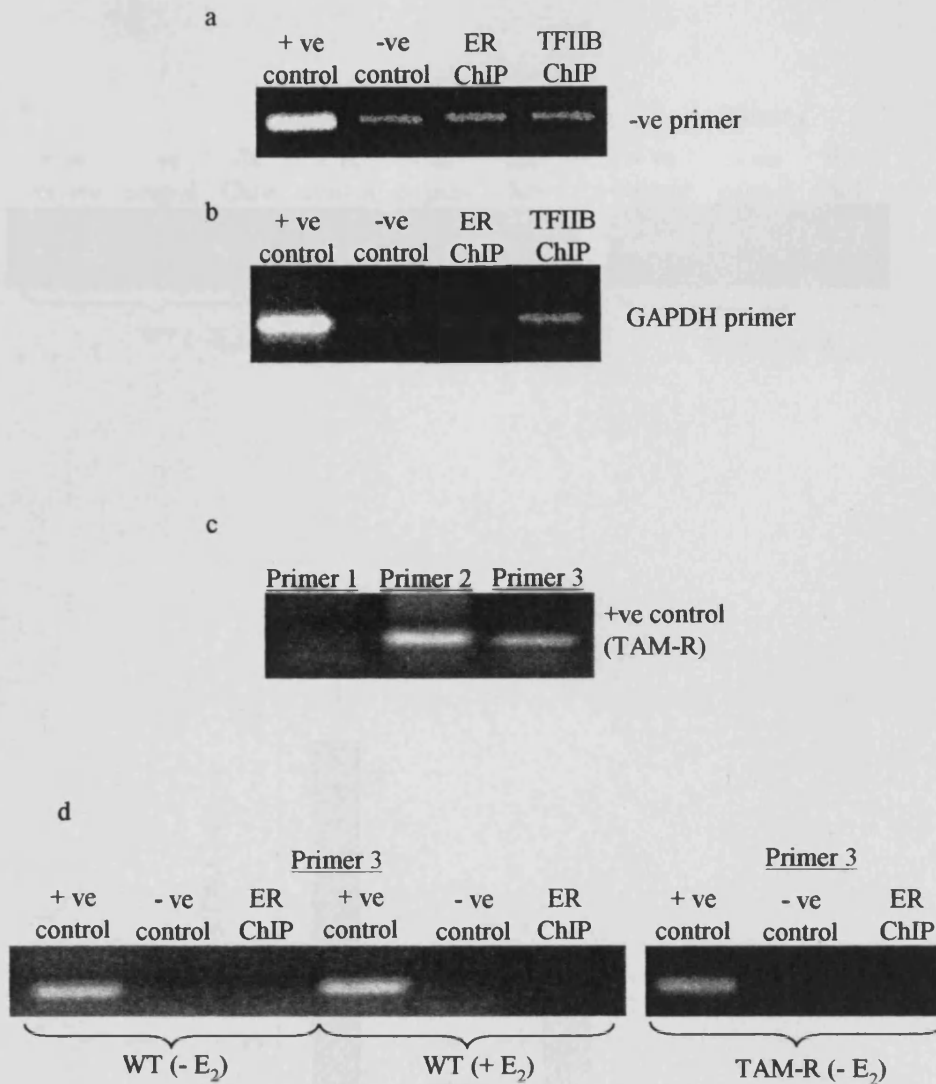
a. Negative control primers flank region of genomic DNA containing no binding sites for transcription factors. PCR product is 174 bp long. b. GAPDH primers flank the TFIIB site (positive control) of the constitutively active GAPDH promoter. Samples in a. and b. include total DNA (+ve control), IgG antibody ChIP (-ve control), ERα antibody ChIP and the TFIIB antibody ChIP. c. Binding efficiencies of primers 1, 2 and 3 to total DNA (+ve control). d. Binding efficiency of primer 3; in +ve control, -ve control and DNA ChIP'ed using ER Ab (ER ChIP). Samples were obtained from WT and TAM-R control cells in the absence of oestradiol (–E2), or WT cells in the presence of oestradiol (+E2).

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Figure 3.51. ERα binds to the promoter region of the Amphiregulin gene in TAM-R and WT cells
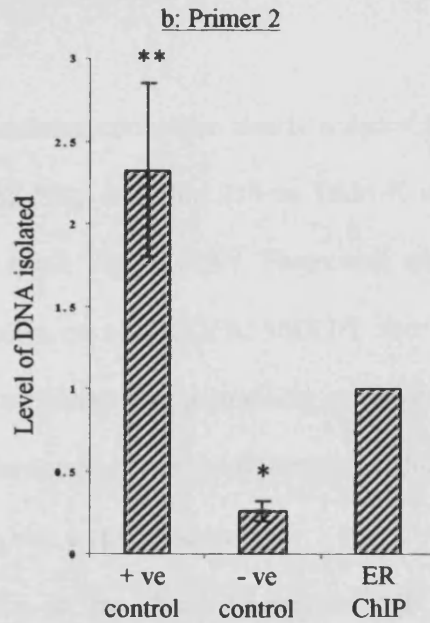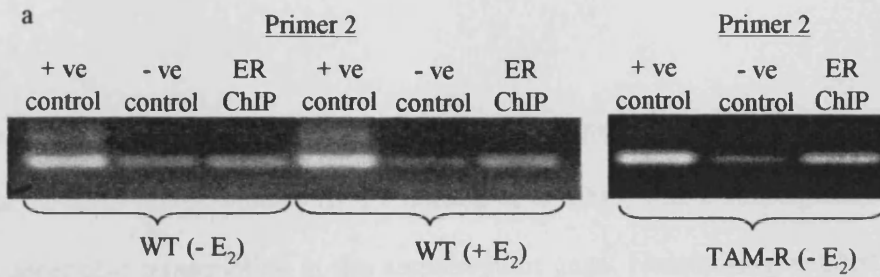
a. Binding efficiency of primer 2; in +ve control, -ve control and ER ChIP.
Samples were obtained from WT and TAM-R cells in the absence of
oestradiol (–E2) or WT cells in the presence of oestradiol (+E2). b. Level of
interaction between primer 2 and the +ve control, -ve control and DNA
ChIP'ed with ER Ab from TAM-R cells. * = P<0.005 compared to ER chip
sample. ** = P<0.001 compared to ER chip sample

3.18 Effect of Amphiregulin on EGFR/ERK1/2 activity and ERα phosphorylation at serine 118 in TAM-R cells.

Aim

The data suggests that ER regulation over EGFR signalling activity in TAM-R cells, as observed previously by Hutcheson et al 2003, was a consequence of ER-dependent transcription at the amphiregulin gene. Neutralising antibodies to several EGFR ligands which sequester the target growth factor were used to confirm the role of amphiregulin.

Results

Amphiregulin neutralising antibodies clearly reduced levels of phosphorylated EGFR, ERK1/2 and ERα at serine 118 in TAM-R cells in a concentration-dependent manner (n=2; Figure 3.52). There was no effect of amphiregulin neutralising antibodies on total EGFR, ERK1/2 and ERα protein expression levels. Unlike the amphiregulin neutralising antibody, TGFα, EGF and HB-EGF neutralising antibodies were ineffective at reducing the level of EGFR phosphorylation on two separate occasions.

Further confirmation of the effects of amphiregulin on TAM-R cells were demonstrated in figure 3.53 as amphiregulin greatly increased the level of phosphorylated EGFR, ERK1/2 and ERα at serine 118 in a concentration-dependent manner. There was again no effect of amphiregulin treatment on total EGFR, ERK1/2 and ERα expression.

Figure 3.52. Amphiregulin neutralising antibody reduces ERα phosphorylation at serine 118 in the TAM-R cell line
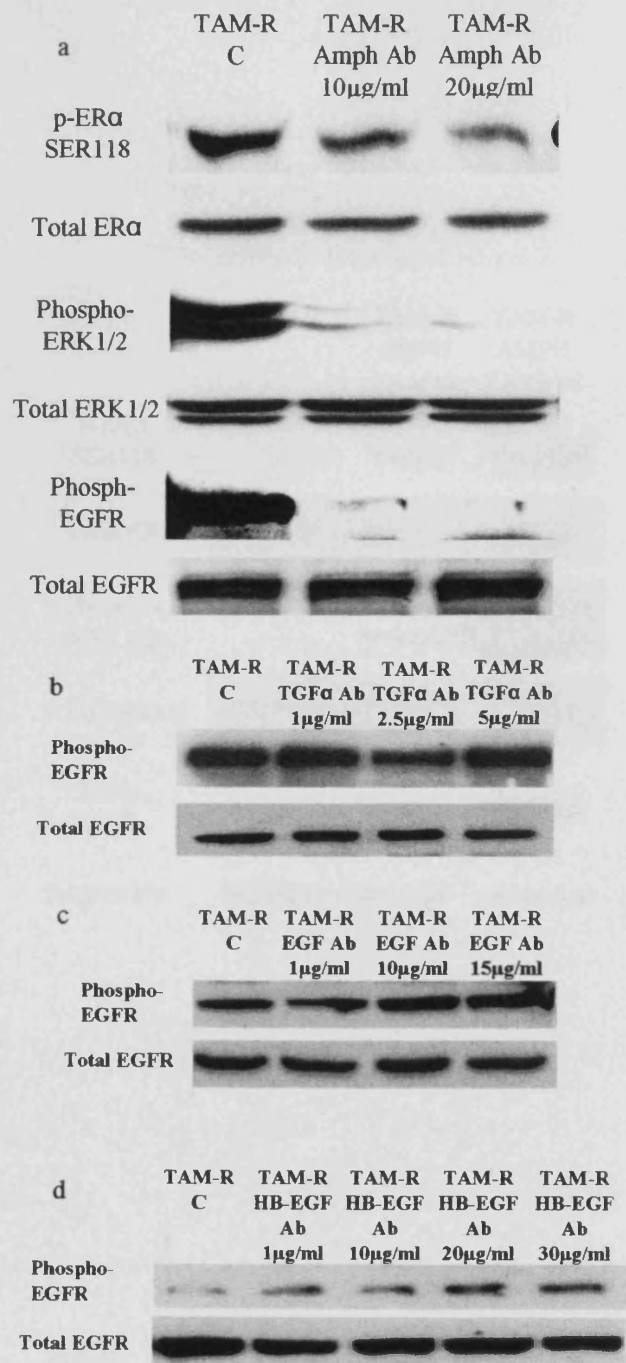
Western analysis of **a.** EGFR/ERK1/2 phosphorylation and ERα phosphorylation at serine 118 in TAM-R cells either in the absence or presence of increasing concentrations of amphiregulin neutralising antibody for 1 hour (n=2), and **b. c. d.** EGFR phosphorylation in TAM-R cells in the absence or presence of increasing concentrations of either TGFα, EGF or HB-EGF neutralising antibodies for 1 hour (n=2).

Figure 3.53. Amphiregulin increases the level of ER phosphorylation at serine 118 in TAM-R cells
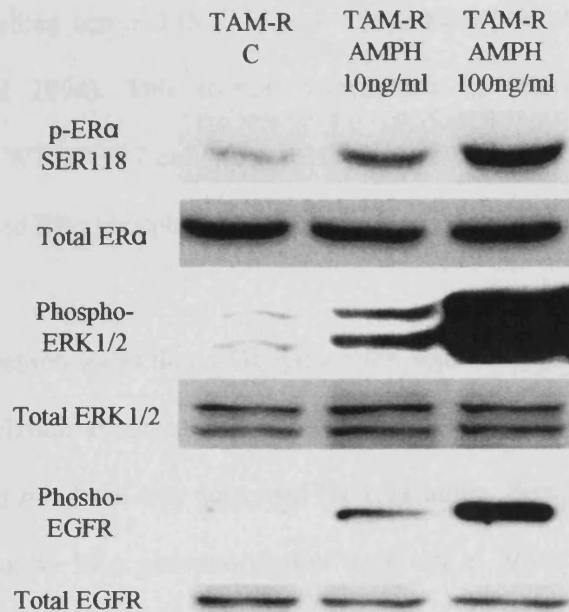
Western analysis of EGFR/ERK1/2 phosphorylation and ERα phosphorylation at serine 118 in TAM-R cells in the absence and presence of either 10ng/ml or 100ng/ml amphiregulin for 10 minutes. Figure is representative of two separate experiments.

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3.19 Short term 4-OH-tamoxifen treatment in WT MCF-7 cells and the effects on ERα phosphorylation at serine 118

Aims

In addition to oestrogen, protein kinase activators and growth factors; anti-oestrogens are also known to increase phosphorylation of ERα at a number of residues including serine 118 (Ali et al 1993; Katzenellenbogen et al 1995; Lahooti et al 1994). This section investigated the short term effects of tamoxifen in WT MCF-7 cells to assess the contribution of this antioestrogen to the increased ERα phosphorylation at serine 118 observed in TAM-R cells.

Results

Figure 3.54 demonstrates that 4-OH-tamoxifen significantly increased the level of phosphorylation at serine 118 in WT MCF-7 cells after only 10 minutes treatment and this level was sustained for four hours. Significantly, however, these increases in ERα phosphorylation were not as great as those observed earlier between the WT and the TAM-R MCF-7 cell lines under basal growth conditions.

ERK1/2 phosphorylation increased but not significantly, whilst AKT phosphorylation was unchanged, in WT cells treated with 4-OH-tamoxifen (Figure 3.55). Furthermore, neither gefitinib nor PD184352, had any effect on the 4-OH-tamoxifen induced increase in ERα phosphorylation at serine 118 despite significantly inhibiting ERK1/2 phosphorylation ($P < 0.05$, n=3, Figure 3.55).

Interestingly, the pure anti-oestrogen Fulvestrant, after one hour treatment of WT cells, also induced an increase in the level of ERα phosphorylation

compared to the control. This increase in serine 118 ERa phosphorylation was significantly greater than that observed with 4-OH-tamoxifen treatment (P < 0.001, n=3) despite fulvestrant substantially reducing total ERα levels (Figure 3.55).

Figure 3.54. Short term Tamoxifen (TAM) effects in WT MCF-7 cells
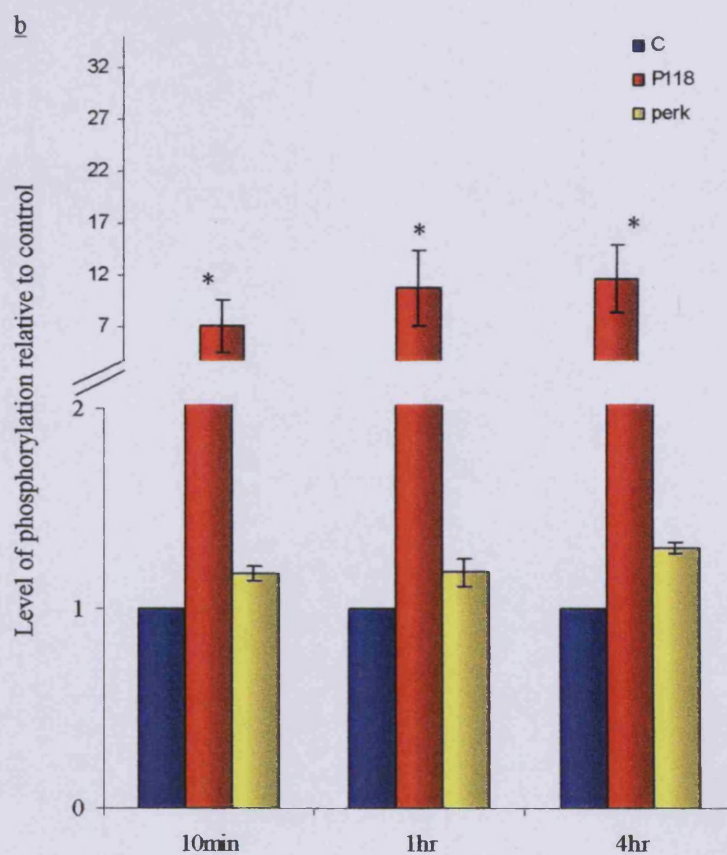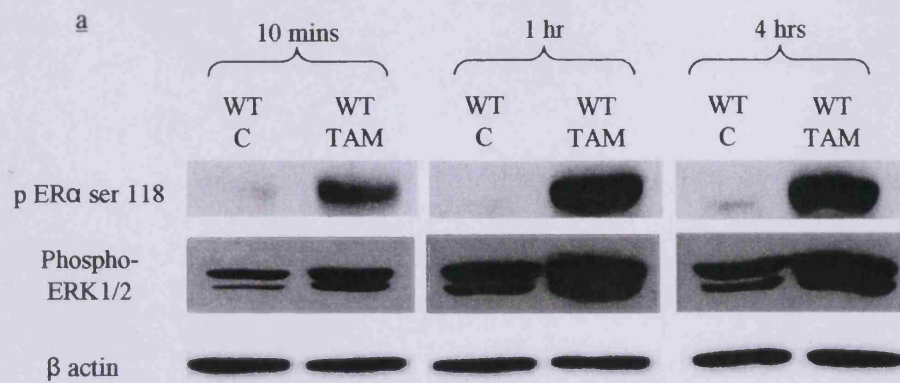
a: Western blot analysis and b. Mean densitometry readings ($\pm$ SEM) of western blots demonstrating expression of phosphorylated ERK1/2 and ERα at serine 118 in WT MCF-7 cells either in the absence or presence of tamoxifen (100nM) for 10 mins, 1hr and 4hrs (n=3). Equal loading was confirmed by consistent expression levels of β-actin. Densitometry values were normalised using the β-actin expression levels. * P<0.05 compared to control.

Figure 3.55. Short term Tamoxifen (TAM) and Fulvestrant (FUL) effects in WT MCF-7 cells
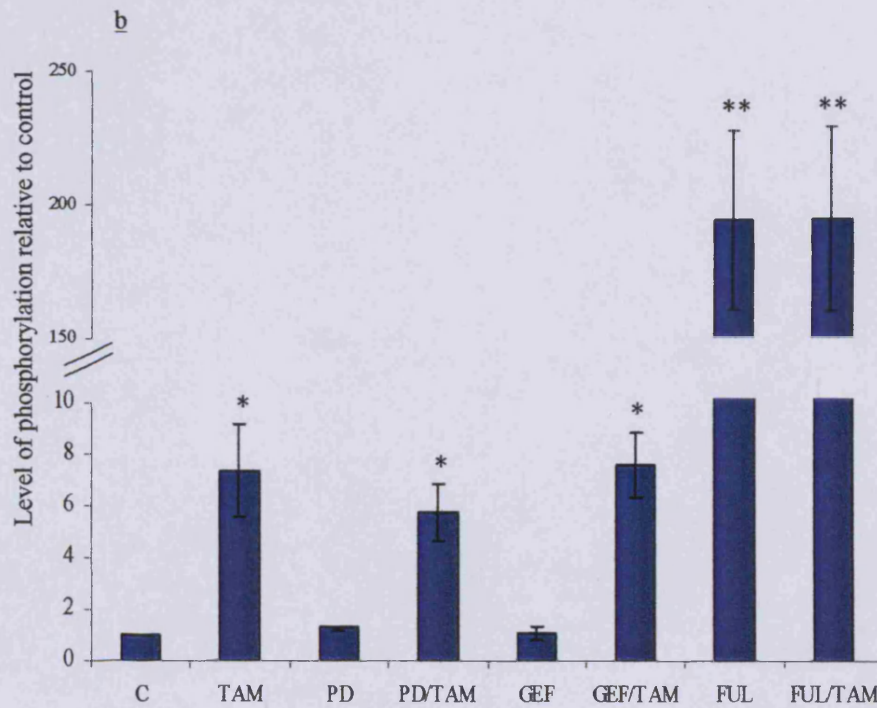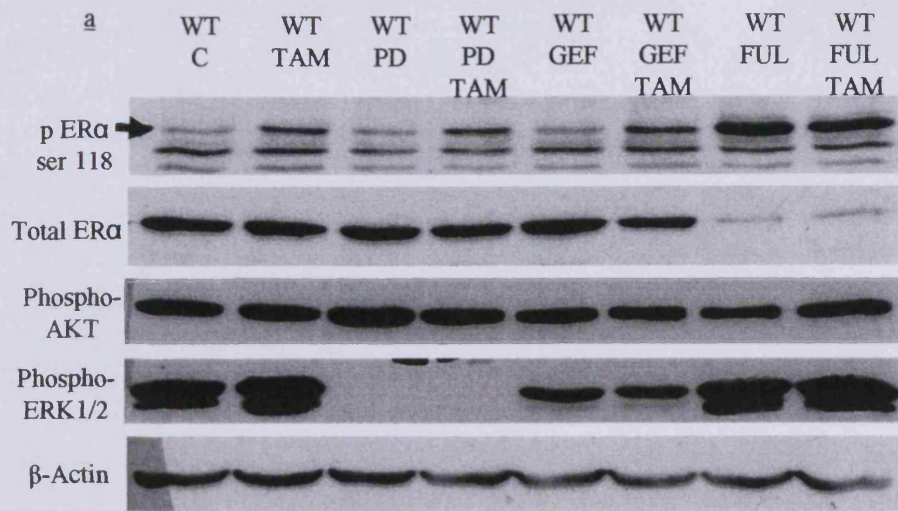
a

| | WT C | WT TAM | WT PD | WT PD TAM | WT GEF | WT GEF TAM | WT FUL | WT FUL TAM |

p ERα ser 118

Total ERα

Phospho-AKT

Phospho-ERK1/2

β-Actin

b

a: Western blot analysis and b. Mean densitometry readings (± SEM) of western blots demonstrating expression of phosphorylated ERK1/2, AKT and ERα at serine 118 in WT MCF-7 cells in the absence and presence of either 4-OH-tamoxifen (100nM, 10minutes, TAM), fulvestrant (100nM, 1 hour, FUL), gefitinib (I μM, 10 minutes, GEF), PD184352 (10 mM, 1 hour, PD184), a combination of either gefitinib or PD184352 and 4-OH-tamoxifen or a combination of fulvestrant and tamoxifen (10minutes) (n=3). Equal loading was confirmed by consistent expression levels of β-actin. Densitometry values were normalised using the β-actin expression levels. * P<0.05 compared to control. ** P<0.001 compared to control

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3.20 Long term 4-OH-tamoxifen treatment in WT MCF-7 cells and the effects on ERα phosphorylation at serine 118

Aim

As shown above, 4-OH-tamoxifen induced increases in ERα phosphorylation within minutes of treatment and this increase was sustained for up to 4 hours. Gee et al (2003) demonstrated that no significant decreases in the rate of growth of the WT MCF-7 cells were observed in the presence of 4-OH-tamoxifen until day seven of treatment and at day 15 the growth curve begins to decline. The aim of this section was to study WT cells treated with 4-OH-tamoxifen for these longer durations to elucidate the significance of ERα phosphorylation at serine 118 in response to 4-OH-tamoxifen on cell growth.

Results

At five days 4-OH-tamoxifen treated MCF-7 cells continued to grow steadily, although at a slower rate than the untreated MCF-7 cells (Figure 3.56a). During this time the level of ERα phosphorylation at serine 118 remained elevated over the untreated cells, whilst ERK1/2 increased only slightly (Figure 3.56b). After 14 days there was no further net growth of the 4-OH-tamoxifen-treated cells resulting in a significant difference in cell number compared to the control cells (P < 0.001, n=3). The decrease in growth rate was accompanied by a dramatic drop in ERα and ERK1/2 phosphorylation to almost undetectable levels, compared to the 14 day control (n=1).

Figure 3.56. Long-term Tamoxifen treatments in WT cells
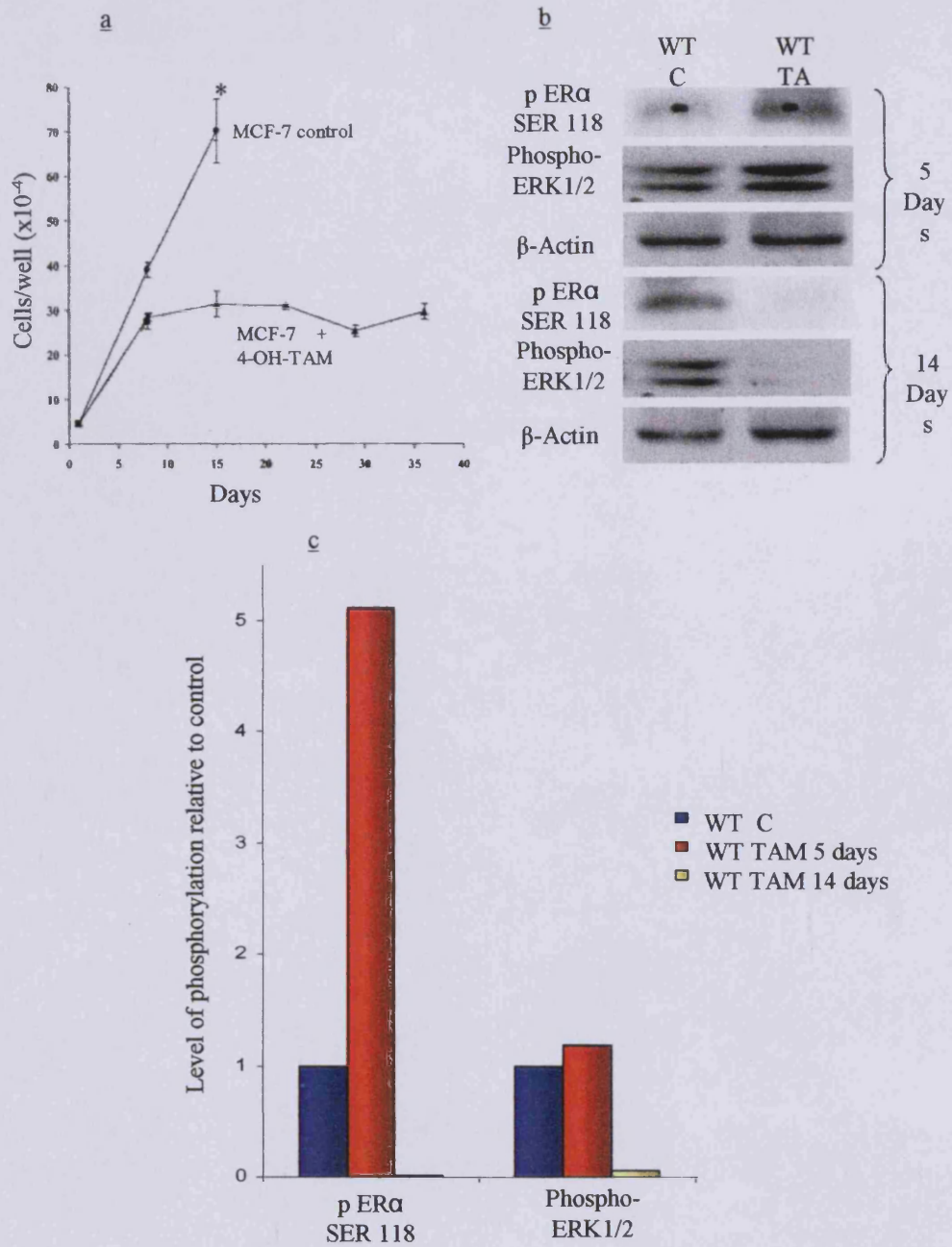
a: Growth responses of WT MCF-7 cells after 7 days in phenol red-free RPMI medium containing 5% charcoal-stripped serum in the absence (CON;●) and presence of 4-OH-tamoxifen (0.1 μM, ▲). Results are expressed as mean (± SD) of triplicate cell counts (Adapted from Gee et al 2003). b: Western blot analysis and c. Mean densitometry readings (± SEM) of phosphorylated ERK1/2 and ERα at serine 118 in WT MCF-7 cells in the absence and presence of 4-OH-tamoxifen (100nM) after 5 or 14 days (n=1). Equal loading was confirmed by consistent expression levels of β-actin. Densitometry values were normalised to β-actin expression levels. * P<0.001 compared to control

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3.21 Development of Western blot assay measuring ERα phosphorylation at serine 167

Aim

EGF-induced activation of ERK1/2 reportedly increased ERα phosphorylation at serine 167 via ERK1/2 and p90 ribosomal S6 kinase (Lannigan 2003). The aim of this section was to investigate whether elevated EGFR/ERK1/2 signalling in TAM-R cells increases ERα phosphorylation at serine 167 as well as at serine 118.

Results

Figure 3.57a demonstrates that the Abcam antibody to ERα phosphorylated at serine 167 produced many bands at approximately 67 KDa. The band which was positioned closest to 67 KDa was not the strongest of these bands casting doubt as to whether the 67KDa band was specific. The remaining experiments were used to confirm which band corresponded to ERα. To aid identification of the specific band a sample containing protein from TAM-R cells immunoprecipitated with the total ERα 1D5 antibody was run along side the other samples. The immunoprecipitated sample contained only one band around the 67 KDa region but this was slightly up shifted from the band in the non-immunoprecipitated samples positioned at exactly 67KDa (Figure 3.57b). The same upshift with immunoprecipitated proteins was observed in blots probed for ERα phosphorylated at serine 118 and total ERα (Figure 3.58a). These blots were used to demonstrate that proteins from immunoprecipitated

samples are prone to up shift suggesting that the band labelled 67KDa in figure 3.57b is in fact ERα, despite the immunoprecipitated/non-immunoprecipitated bands not being aligned precisely.

Figure 3.58b provides additional evidence that the band labelled 67KDa in figure 3.57b is ERα. The samples were all run along side each other in a gel and transferred to a nitrocellulose membrane, stained with ponceau S and cut down the side of the well containing TAM-R T47D cells. The two membrane sections were then probed in antibody to either ERα phosphorylated at serine 167 or ERα phosphorylated at serine 118. Once reassembled in exactly the same position as prior to cutting it became evident that a band was located on the ERα serine 167 blot at exactly the same position as the band for ERα phosphorylated at serine 118. With closer inspection of the serine 167 blot one can see that this band corresponds to the 67KDa band identified earlier on figure 3.57b.

Once the band for ERα phosphorylated at serine 167 had been identified it was clear that there was no clear difference in the expression of this phosphorylated form of the protein between the WT and TAM-R cell line (adding to the reason for continuing the more extensive investigations on ERα phosphorylation at serine 118 rather than serine 167). The same level of expression of ERα phosphorylated at serine 167 was also observed in the MCF-7 cells resistant to 4-OH-tamoxifen and Gefitinib (TAM/GEF-R MCF-7), yet expression was reduced dramatically in the MCF-7 cells resistant to Tamoxifen and Herceptin (TAM/HER-R MCF-7). Interestingly, serine 167 phosphorylation was detectable in the Fulvestrant resistant MCF-7 cell line (FUL-R MCF-7), but

was less apparent in the MCF-7 cells resistant to both Fulvestrant and Gefitinib (FUL/GEF-R MCF-7). The cells resistant to oestrogen deprivation MCF-7 X) showed the greatest level of serine 167 phosphorylation of ERα and high levels of this phosphorylated form of ERα was also observed in the WT T47D breast cancer cell line. The TAM-R T47D cells showed detectable levels of phosphorylation at serine 167 but to a much lesser extent than that observed in the WT T47D cells.

Expression levels of ERα phosphorylation at serine 118 across these cell lines followed a different pattern to that observed with serine 167 phosphorylation levels. Firstly, TAM-R cells expressed considerably greater levels of ERα phosphorylated at serine 118 compared to the WT cell line, confirming earlier findings. MCF-7 cells resistant to both Tamoxifen and Gefitinib (TAM/GEF-R MCF-7) or Tamoxifen and Herceptin (TAM/HER-R MCF-7) or MCF-7 cells that have become resistant to oestrogen deprivation (MCF-7 X), showed similar levels as the TAM-R cells. The fulvestrant-resistant cell line did not express ERα phosphorylated at serine 118, yet interestingly, the MCF-7 cells resistant to Fulvestrant and Gefitinib (FUL/GEF-R MCF-7) expressed weak levels of phosphorylated serine 118. Expression of ERα phosphorylated at serine 118 in the WT and TAM-R T47D cell lines followed the same pattern as the WT and TAM-R MCF-7 cells yet this may be attributed to the expression level of total ERα as the TAM-R T47 D cells express greater levels of total ERα than the WT T47D cell line.

Figure 3.57 Development of western blot assay measuring ERα phosphorylation at serine 167
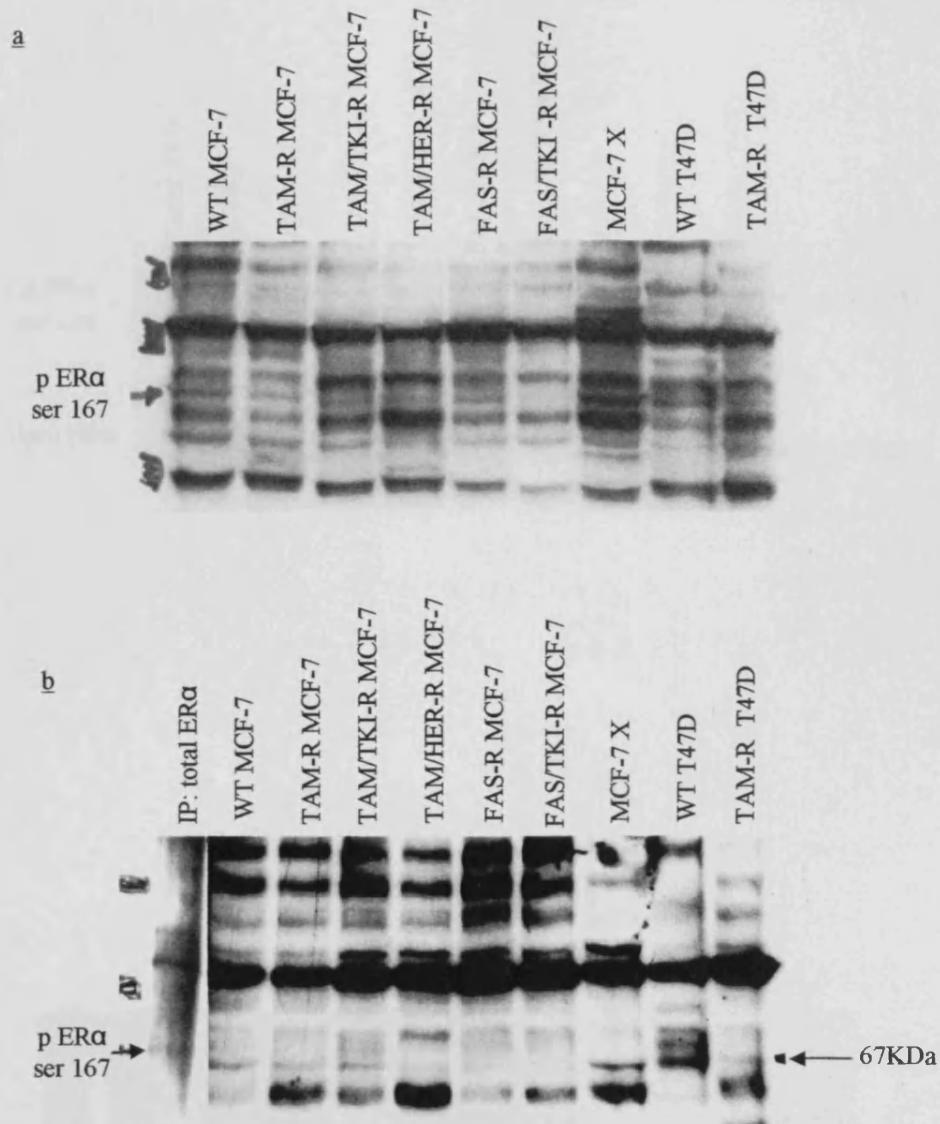
a: ERα phospho-SER 118 and total ERα blots containing protein from EGF treated TAM-R cells that were Immunoprecipitated for total ERα. Also contains protein samples from several different cell lines (as labelled) under basal conditions, that were not immuno-precipitated. b. ERα phospho-SER 167 blot containing protein from EGF treated TAM-R cells that were Immunoprecipitated for total ERα. Also contains protein samples from several different cell lines (as labelled) under basal conditions, that were not immuno-precipitated

3.58 Development of western blot assay measuring ERα phosphorylation at serine 167
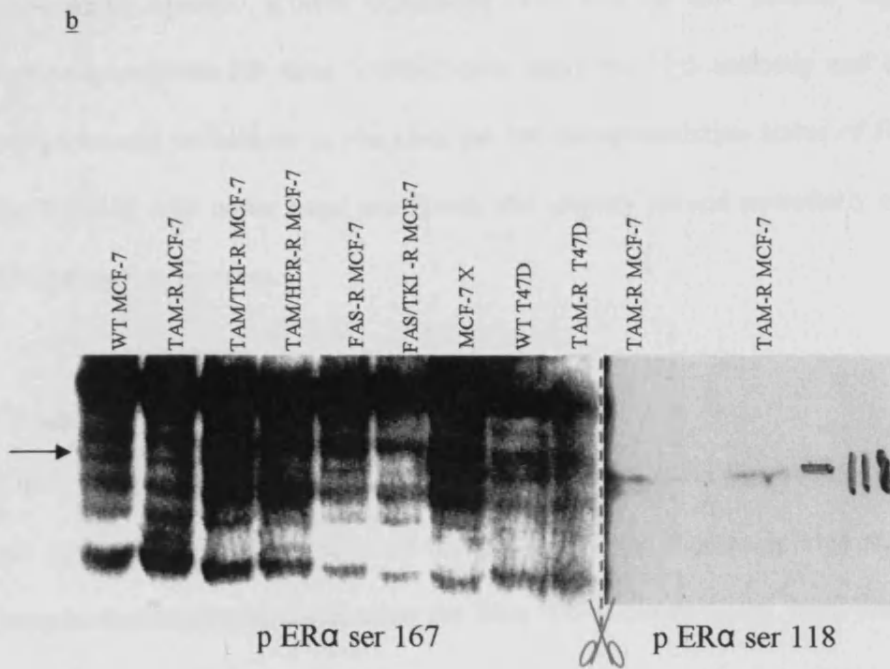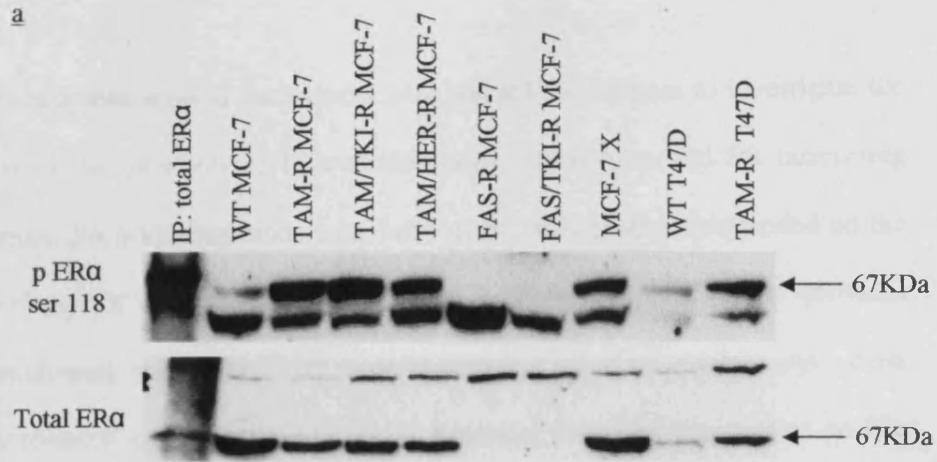
a: ERα phospho-SER 167 blot containing protein from several different cell lines (as labelled) under basal conditions (repeat experiment of figure 28b). b. Western blot cut as indicated. Left side of blot contains samples from several different cell lines (as labelled) and incubated in ERα phospho-SER 167 antibody. Right side of blot contained TAM-R C sample and was incubated in pERαSER118 antibody.

3.22 Two-dimensional gel electrophoresis and MALDI/TOF MS

Aim

This study has utilised the western blot and ICC techniques to investigate the phosphorylation status of ERα at the serine 118 residue and ER interacting proteins. Such investigations have been time consuming and depended on the specificity of all the antibodies used. This post-genomic age provides technologies, such as 2D electrophoresis, chromatography and mass spectrometry, capable of analysing all potential phosphorylation sites on ER, identifying the array of different proteins associated with ER, and the means to identify the many splice variants of ER within clinical samples and various cell lines under specific growth conditions. The aim of this section was to immunoprecipitate ER from TAM-R cells using the 1D5 antibody and carry out proteomic techniques to elucidate the full phosphorylation status of ER in the TAM-R cells under basal conditions and identify all and potentially novel ER interacting proteins.

Results

The coomassie blue stained gel in figure 3.59 illustrates the first attempt of 2D gel electrophoresis. Each spot on the gel represents coomassie blue stained proteins immunoprecipitated, using the ERα 1D5 antibody (1μg), from TAM-R cell lysate (total protein content = 1mg). Each numbered spot was excised using a sterile scalpel, subjected to in gel digestion, then analysed using a Bruker Reflex III MALDI-TOF mass spectrometer in the reflection mode. The

Mass spectrometer data were entered into the Mascot search to produce a probability based mowse score, which predicts the likelyhood that the analysed peptides belong to a specific protein. Results are displayed in Table 1a and 1b. The coomassie blue stained gel in figure 3.60 illustrated the second attempt of 2D gel electrophoresis. Each spot on the gel represented coomassie blue stained proteins immunoprecipitated, using the ERα 1D5 antibody (10μg), from TAM-R cell lysate (total protein content = 10mg). The gel was re-stained with silver stain (figure 3.61) to reveal protein spots lower than 1ng. Each numbered spot was analysed as above. The results are displayed in table 2a and 2b.

Figure 3.59. Coomasie blue stained 2 Dimensional gel containing proteins immunoprecipitated, using ERα specific antibody, from TAM-R cells.
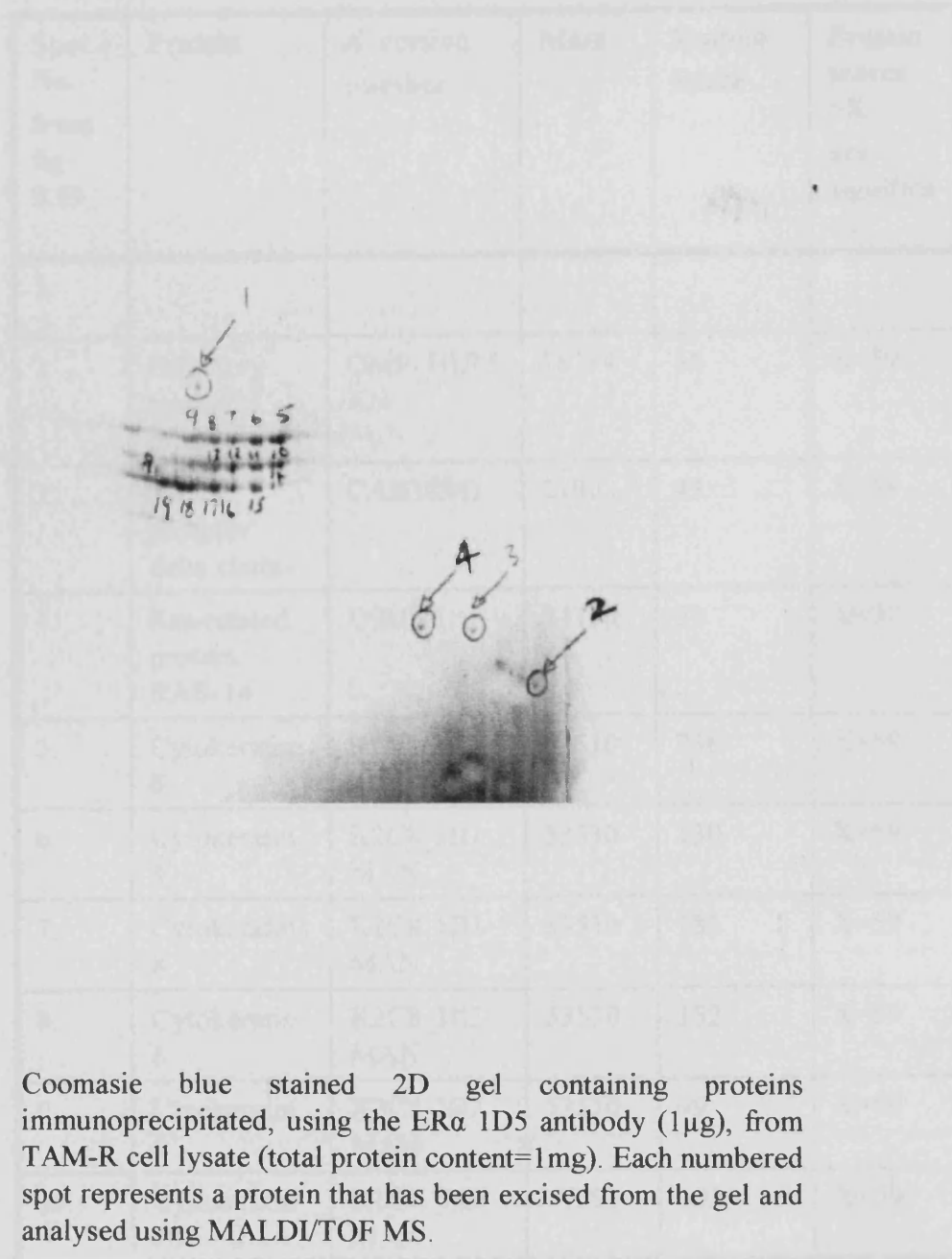
Coomasie blue stained 2D gel containing proteins immunoprecipitated, using the ERα 1D5 antibody (1μg), from TAM-R cell lysate (total protein content=1mg). Each numbered spot represents a protein that has been excised from the gel and analysed using MALDI/TOF MS.

Table 1a. Mascot search results from MALDI/TOF mass spectrometry data, of samples cut from the coomasie stained gel in figure 3.59

Spot No. from fig 3.59	Protein	Accession number	Mass	Protein Score	Protein scores >X are significant
1.					
2.	Olfactory marker protein	OMP_HUMAN	18794	56	X=59
3.	T-Cell receptor delta chain	CAB38241	2190	43	X=59
4.	Ras-related protein RAB-14	Q9UI11	24110	59	X=59
5.	Cytokeratin 8	K2C8_HUMAN	53510	236	X=59
6.	Cytokeratin 8	K2C8_HUMAN	53510	130	X=59
7.	Cytokeratin 8	K2C8_HUMAN	53510	183	X=59
8.	Cytokeratin 8	K2C8_HUMAN	53510	152	X=59
9.	Cytokeratin 8	K2C8_HUMAN	53510	99	X=59
10.	Cytokeratin 18	K1CR_HUMAN	47897	192	X=59

Identity of proteins excised from the gel (numbered spots 1-10, Figure 3.59) and analysed by MALDI/TOF mass spectrometry. The protein score is - 10*Log(P), where P is the probability that the observed match is a random event.

Table 1b. Mascot search results from MALDI/TOF mass spectrometry data, of samples cut from the coomasie stained gel in figure 3.59

Spot No. from fig 3.59	Protein	Accession number	Mass	Protein Score	Protein scores >X are significant
11.	Cytokeratin 18	K1CR_HUMAN	47897	165	X=59
12.	Keratin 10	KRHUO	59720	110	X=59
13.	Cytokeratin 18	K1CR_HUMAN	47897	126	X=59
14.	Keratin 19	KRHU9	44079	150	X=59
15.	Keratin 19	KRHU9	44079	188	X=59
16.	Keratin 19	KRHU9	44079	156	X=59
17.	Keratin 19	KRHU9	44079	163	X=59
18.	Keratin 19	KRHU9	44079	112	X=59
19.	Keratin 10	KRHUO	59720	78	X=59
20.	Cytokeratin 8	K2C8_HUMAN	53510	173	X=59

Identity of proteins excised from the gel (numbered spots 11-20, Figure 3.59) and analysed by MALDI/TOF mass spectrometry. The protein score is - 10*Log(P), where P is the probability that the observed match is a random event.

Figure 3.60. Repeat coomasie blue stained 2 Dimensional gel containing proteins immunoprecipitated, using ERα specific antibody, from TAM-R cells.
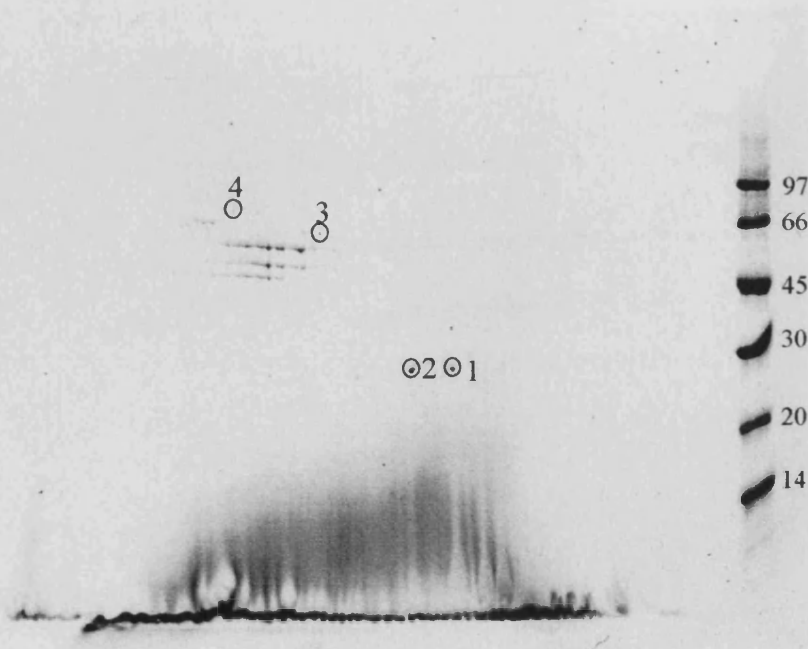
Repeat coomasie blue stained 2D gel containing proteins immunoprecipitated, using the ERα 1D5 antibody (10µg), from TAM-R cell lysate (total protein content=10mg). Each numbered spot represents a protein that has been excised from the gel and analysed using MALDI/TOF MS.
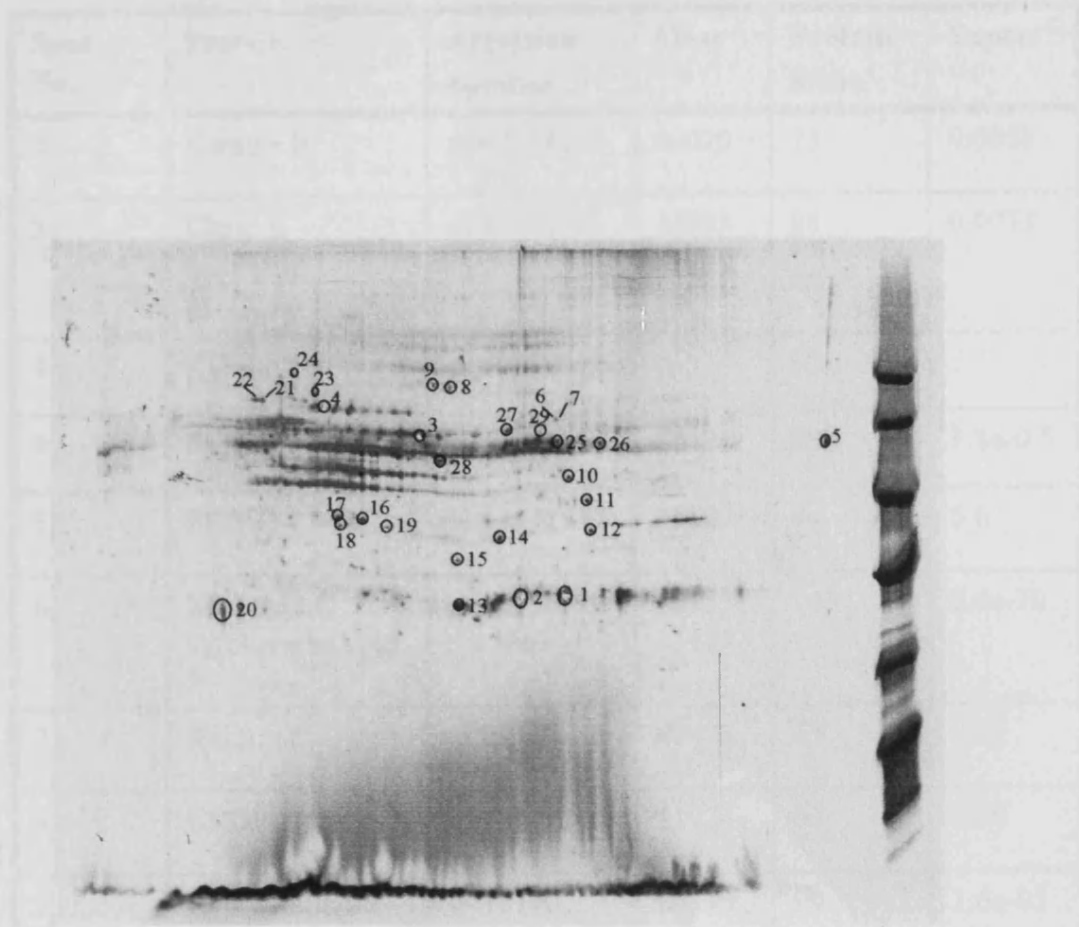
Same gel as in figure 3.60, but re-stained using the silver
staining technique. Each numbered spot represents a protein
that has been excised from the gel and analysed using
MALDI/TOF MS.

Table 2a. Mascot search results from MALDI/TOF mass spectrometry data, of samples cut from the silver stained gel in figure 3.61

Spot No.	Protein	Accession number	Mass	Protein Score	Expect
1.	Keratin 10	gi:40354192	59020	73	0.0058
2.	Chain L, Igg1 Fab Fragment	gi:4558340	23693	88	0.0033
3.	Keratin 10	gi:40354192		92	
4.	Keratin 9	gi:4557705	62178	98	1.8e-0.5
5.	FKSG14	gi:11141855	31677	44	5.6
6.	Mixture of cytokeratin 1 and 9	gi:55956899 + 1346343	62255+ 66149	143	6.4e-10
7.	Keratin 6	gi:27465517	60472	63	0.06
8.	CRDP 12	gi:13899173	117341	57	0.26
9.	Cytokeratin 2	gi:181402	66110	99	1.6e-05
10.	IgG heavy chain	gi:49523848	13533	36	
11.	Zinc finger protein	gi:7019591	84893	52	0.89
12.	PPIL3b	gi:14043400	18385	51	0.93
13.	Unnamed protein	gi:16552024	41878	47	2.6
14.	Pleckstrin and sec7	gi:14150035	85292	54	0.47
15.	Unnamed protein	gi:10438010	24432	48	1.9

Identity of proteins excised from the gel (numbered spots 1-15, Figure 3.61) and analysed by MALDI/TOF mass spectrometry. The protein score is -10*Log(P), where P is the probability that the observed match is a random event. Expect is the probability that the match is a random event.

Table 2b. Mascot search results from MALDI/TOF mass spectrometry data, of samples cut from the silver stained gel in figure 3.61

Spot No.	Protein	Accession number	Mass	Score	Protein score >X significant
16.	Protein XP	gi:51466965	28654	64	0.054
17.	Keratin 10	gi:40354192	59020	69	0.015
18.	Mix of Keratin 10 and Cytokeratin 9	gi:307086 gi:435476	46473 62320	159	1.5e-11
19.	Keratin 2a	gi:4713260	65678	77	0.0026
20.	Keratin 1	gi:17318569	66198	51	0.99
21.	Keratin 10	gi:40354192	59020	95	3.7e-5
22.	Mix of Keratin 10 and Keratin 2a	gi:40354192 gi:47132620	59020 65678	194	4.9e-15
23.	Keratin 6a	gi:46812692	60323	78	0.002
24.	Mix of Keratin 10 and keratin 2a	gi:40354192 gi:47132620	59020 65678	187	2.4e-14
25.	Cytokeratin 1	gi:1346343	66149	74	0.0055
26.	Keratin 6a	gi:46812692	60323	76	0.0034
27.	Mixture of Cytokeratin 1 and 9	gi:435476 gi:1346343	62320 66149	174	4.9e-13

Identity of proteins excised from the gel (numbered spots 16-27, Figure 3.61) and analysed by MALDI/TOF mass spectrometry. The protein score is -10*Log(P), where P is the probability that the observed match is a random event. Expect is the probability that the match is a random event.

4.0 DISCUSSION

Tamoxifen resistant breast cancers appear to stably express ERα both in the clinical setting and in preclinical cell models (Robertson, 1996, Brunner et al., 1993, Lykkesfeldt et al., 1994, Encarnacion et al., 1993; Hutcheson et al 2003). Furthermore, a number of reports have indicated that down regulation of the ER with the pure anti-oestrogen, fulvestrant, inhibits tamoxifen-resistant growth in the clinic and *in vitro* suggesting that the ER has a continued role in growth regulation in this condition (Brunner et al., 1993, Lykkesfeldt et al., 1994, Coopman et al., 1994, Hu et al., 1993, Howell & Robertson, 1995, Howell et al., 1996, 2002). Similarly, the Tenovus laboratory has shown that growth of a tamoxifen resistant MCF-7 breast cancer cell line is sensitive to the growth inhibitory actions of fulvestrant (Knowlden JM et al, 2003, Hutcheson et al 2003). In support of these findings the current study has confirmed that ERα expression levels are comparable in the tamoxifen-resistant (TAM-R) and WT MCF-7 cell lines and that fulvestrant induced down regulation of ERα and consequently reduced growth in these two cell lines. The primary aim of the current study, therefore, was to elucidate a potential mechanism, utilised by the tamoxifen resistant MCF-7 breast cancer cell line, to maintain ER functionality despite the continued presence of tamoxifen. Identification of such a mechanism may lead to the development of a more effective therapy for the treatment of acquired tamoxifen resistant breast cancer.

In addition to ER playing a continued role in the growth of tamoxifen resistant breast cancer, this condition has also been associated with a dependence on growth factors, their respective receptors and constituents of the growth factor receptor signalling pathway (Clarke et al 2001; Nicholson et al 2005). In

clinical breast cancer it has been demonstrated that lack of response to endocrine therapy, together with increased metastasis and poor survival, can be associated with over expression of the epidermal growth factor receptor EGFR and c-erbB2 (Write et al 1992; Nicholson et al 1993; Nicholson et al 1994). In vitro models of acquired tamoxifen resistance have further demonstrated that raised levels of EGFR may contribute to increased proliferative activity (Long et al 1992; El-Zarruk et al 1999, Knowlden et al 2003; Hutcheson et al 2003) and transfection of either EGFR or c-erbB2 into hormone-dependent breast tumour cells resulted in hormone-independent cell proliferation and tamoxifen resistance respectively (van Aguthen et al 1992; Benz et al 1992; Miller et al 1994; Kurokawa et al 2000; Hutcheson et al 2003, Osborne K 2005). Experimental evidence has suggested that IGF-IR signalling may also play an important role in the development and growth of tamoxifen resistant cell lines (Knowlden et al 2005; Parisot et al 1999; Wiseman et al 1993). Over-expression of IGF-II, IGF-IR and IRS-1 has been shown to reduce sensitivity of MCF-7 and T47-D breast cancer cell lines to tamoxifen treatment (Daly et al., 1991, Surmacz & Burgaud, 1995, Abdul-Wahab et al., 1999) and tamoxifen-resistant MCF-7 cells demonstrate increased sensitivity to both the proliferative effects of IGF-I/II and the growth inhibitory actions of the selective IGF-1R tyrosine kinase inhibitor AG1024 and the IGF-1R monoclonal antibody αIR-3 (Knowlden et al 2005; Parisot et al 1999; Wiseman et al 1993). Interestingly, the increased reliance on IGF-1R signalling is observed in tamoxifen-resistant cell lines despite expression levels of IGF-1R being lower in these cells compared to their parental, tamoxifen-sensitive, cell

lines (Brockdorff et al 2003; van den Berg et al 1996; McCotter et al 1996; Knowlden et al 2003).

Both EGFR and IGF-1R regulate cell proliferation through activation of downstream signal transduction cascades, in particular the mitogen-activated protein kinase (MAPK) pathway. Increased activity of MAPK (or extracellular signal-regulated kinase [ERK]) has been associated with reduced quality and duration of response to tamoxifen and shortened disease free survival in ER-positive breast cancer patients (Mueller et al 2000; Gee et al 2001). Furthermore, constitutive activation of ERK1/2 has also been shown to contribute to anti-oestrogen resistance in MCF-7 breast cancer cell lines (Kurokawa et al 2000; El-Ashry et al 1997; Donovan et al 1997). The current study supports the above findings as blockade of EGFR, IGF-1R and ERK1/2 signalling activity using gefitinib (selective EGFR tyrosine kinase inhibitor), AG1024 (selective IGF-1R tyrosine kinase inhibitor) and PD184352 (selective MEK1/2 inhibitor), respectively, induced significant reductions in TAM-R cell growth, in agreement with previous Tenovus findings (Knowlden et al 2003, Jones et al., 2005, Knowlden et al., 2005).

Interestingly, evidence exists to suggest that in addition to direct stimulatory effects on cell growth, EGFR and IGF-1R signalling can also target and phosphorylate key serine residues within the AF-1 domain of ERα in endocrine responsive breast cancer cells (Bunone et al 1996; Kato at al 1995, Joel et al., 1998, Lannigan, 2003). Phosphorylation of these residues, in particular serine 118 which has been suggested as a target for ERK1/2 MAPK, promotes co-activator recruitment and activation of ERα transcriptional activity (Bunone et

al 1996; Kato at al 1995; Joel et al 1998; Endoh et al 1999; Deblois et al 2003).

It is possible that such ligand-independent activation of ERα may play a role in endocrine resistance as increased levels of serine 118 and serine 167 phosphorylated ERα have been reported in breast cancer cell lines resistant to both tamoxifen and long-term oestrogen deprivation (Campbell et al 2001; Chan et al 2002; Martin et al 2003; Shou et al 2004; Vendrell et al 2005). Based on these findings and the knowledge that the EGFR and IGF-1R signalling pathways are active in the TAM-R cell line, we hypothesised that cross talk between these signalling pathways and ERα, via ERK1/2-mediated phosphorylation of ERα at serine 118, may provide a mechanism to maintain ER transcriptional functionality in these cells despite the presence of tamoxifen.

Early evidence to suggest that this may in fact be the case was provided by the western blotting assay developed in this study which revealed that there was a significant increase in the level of basal ERα phosphorylation at serine 118 in the TAM-R compared to the WT MCF-7 cell line. Furthermore, this increased level of serine 118 phosphorylated ERα in TAM-R cells correlated with the elevated EGFR/ERK1/2 signalling activity observed in this cell line. These findings were confirmed by a developed ICC assay, which indicated that serine 118 phosphorylated ERα was localised predominantly within the nuclei of the TAM-R cells. This is consistent with reports from other groups who have shown enhanced ERK1/2 activity in association with increased ERα phosphorylation at serine 118 in other MCF-7 breast cancer cell lines resistant to tamoxifen (Vendrell et al 2005; Shou et al 2004). The higher levels of ERα

phosphorylation was not a result of changes in total ERα expression as levels of this receptor were equivalent in the two cell lines as previously reported and confirmed above (Hutcheson et al 2003).

Conformational changes in ERα induced by ligand interactions have also been reported to induce phosphorylation of serine 118 (Chen et al 2000; Joel et al 1998; Shou et al 2004). However, this study shows that, although tamoxifen clearly induced significant increases in ERα phosphorylation at serine 118 in the WT MCF-7 cells, as early as 10 minutes post treatment and lasting up to five days, after 14 days treatment ERα phosphorylation levels had reduced to well below basal WT values. Interestingly, ERα phosphorylation levels closely correlated with cell growth following tamoxifen treatment in WT cells, with cells continuing to proliferate whilst levels of ERα phosphorylation were evident during the first week of tamoxifen treatment but being growth inhibited at day 14 when levels of ERα phosphorylation were substantially reduced. It should also be noted that phosphorylation of ERα at serine 118 in TAM-R cells was significantly greater than the tamoxifen-induced increases in ERα phosphorylation observed in WT cells suggesting that additional factors alongside tamoxifen binding, such as EGFR/MAPK signalling, were required to generate the levels observed in TAM-R cells.

Confirmation that the EGFR/ERK1/2 signalling pathway was indeed responsible for much of the ERα phosphorylation at serine 118 in TAM-R cells was provided by treating the cells with EGF, which significantly up-regulated activation of EGFR/ERK1/2 signalling and ERα phosphorylation, whilst the selective inhibitors of EGFR and MEK1/2, gefitinib and PD184352,

significantly reduced ERα phosphorylation. This regulation of ERα phosphorylation at serine 118 by EGFR driven ERK1/2 activity was emphasised by the finding that ERK1/2 was physically associated with ERα in the TAM-R cells and this association could be blocked using PD184352. These findings are again consistent with the Shou et al (2005) report which also demonstrated EGFR dependent regulation of ERα phosphorylation at serine 118 in their HER2 over-expressing MCF-7 breast cancer cell line in the presence of tamoxifen.

Modulation of IGF-1R signalling was also found to influence ERα phosphorylation at serine 118 in the TAM-R cell line. Stimulation of IGF-1R signalling with IGF-II promoted ERK1/2 activation and increased serine 118 phosphorylation in the tamoxifen-resistant cells. Furthermore, inhibition of IGF-1R activity using AG1024 reduced both ERK1/2 and ERα phosphorylation levels in this cell line. Recently, it has been reported that, in addition to direct activation of ERK1/2 via the Shc/Ras/Raf/MEK1/2 pathway, IGF-IR signalling can promote ERK1/2 activation indirectly through transactivation of EGFR via a c-src-dependent mechanism in the TAM-R cell line (Knowlden et al 2005). It is likely that IGF-1R regulates serine 118 phosphorylation of ERα via EGFR in TAM-R cells as blockade of IGF-1R signalling with AG1024 provided no additive inhibitory effect on ERα phosphorylation at serine 118 when given in combination with gefitinib in these cells.

Growth factor signal transduction pathways clearly played a role in regulating the phosphorylation of ERα in TAM-R cells, therefore, investigations were

carried out to elucidate whether the same pathways still played a role in maintaining the low but measurable levels of ERα phosphorylation at serine 118 in the parent WT MCF-7 cell line or whether this phosphorylation was dependent on other factors such as background oestrogen levels found in the charcoal stripped serum (Chen et al 2002; Joel et al 1998). Early results showed that EGF and IGF-II both significantly increased ERα phosphorylation at serine 118 in the WT cells, as previously reported by Joel et al (1998). Under basal conditions, however, ERα phosphorylation was not reduced by either EGFR or ERK1/2 inhibition using gefitinib and PD184352 respectively, but was significantly reduced by AG1024 inhibition of IGF-IR. These results compliment growth studies in WT MCF-7 cells where gefitinib and PD184352 were less effective than AG1024 at reducing growth, probably due to the relatively low expression and phosphorylation levels of EGFR/ERK1/2. Thus, although priming of the EGFR signalling pathway with EGF demonstrated that EGFR dependent phosphorylation of ERα at serine 118 via ERK1/2 can take place, under basal growth conditions IGF-1R appears the dominant mediator of ERα phosphorylation at serine 118 through an ERK1/2-independent pathway.

To further examine the regulatory role of IGF-IR in the WT cells the PI3K/AKT signalling pathway, another down stream IGF-IR signalling pathway independent of the Ras/Raf/ERK1/2, was inhibited using the PI3K inhibitor LY294002. Although LY294002 significantly reduced ERα phosphorylation at serine 118 in these cells, total ERα levels were also reduced in the presence of this agent. This observation may relate to the findings by Pasapera Limón et al (2003) who demonstrated that LY294002 could act as a

direct competitive antagonist of ERα and as a consequence accelerate protease degradation of the receptor, possibly in a similar fashion to fulvestrant (Carlson et al 2005; McClelland et al 1995). Interestingly, in the current study fulvestrant treatment of WT cells induced an enormous increase in ERα phosphorylation at serine 118 after one hour despite significantly down regulating total ERα levels. These data suggest that differences exist between the LY294002- and fulvestrant-induced degradation of ERα in the WT cells.

Overall, the findings suggest that a growth factor signalling pathway independent of that found to regulate ERα phosphorylation at serine 118 in TAM-R cells exists in WT cells. In support of the findings that IGF-IR possibly via PI3K/AKT regulated ERα phosphorylation at serine 118 in the WT cells, Martin et al (2000) have previously demonstrated that AKT dependent phosphorylation of serine 118 and other N-terminal serine residues such as 104, 106 and 167 appear to play a role in the activation of ERα in COS1 cells. They also demonstrated that in MCF-7 cells AKT activation was blocked by inhibitors of PI3K such as wortmannin which, unlike LY294002, have no affinity for ERα. Moreover, stable transfection of the MCF-7 cells with a dominant negative AKT mutant blocked the effects of EGF and IGF-I on ER-alpha expression and activity, whereas stable transfection of cells with a constitutively active AKT mutant mimicked the effects of EGF and IGF-I (Martin et al 2000).

Prior to discovering that IGF-1R provides a level of regulation over ERα phosphorylation at serine 118 in WT cells under basal conditions, probably through a PI3K/AKT dependent mechanism, this study demonstrated that

tamoxifen induced increases in ERα phosphorylation at serine 118 were independent of the EGFR/ERK1/2 signalling pathway as gefitinib and PD184352 were ineffective at inhibiting such increases. Based on these observations it appears necessary to carryout a study to investigate whether the IGF-1R/PI3K/AKT pathway mediates the tamoxifen induced increase in ERα phosphorylation at serine 118 in the WT cells.

Interestingly, within both the TAM-R and WT cell lines the growth factor inhibitors used in this study to reduce ERα phosphorylation at serine 118, were not fully effective. For example, although gefitinib, AG1024 and PD184352 lowered ERα phosphorylation at serine 118 in TAM-R cells, either alone or in combination, it is clear that a measurable level of ERα phosphorylation remained under conditions where EGFR, IGF-1R and ERK1/2 appeared completely blocked by the inhibitors. Similarly, when gefitinib or PD184352 were used in combination with EGF in the TAM-R cells it is clear that while the inhibitors prevented the EGF induced increase in ERα phosphorylation at serine 118 and in the case of gefitinib even reduced ERα phosphorylation below the control, once again residual ERα phosphorylation at serine 118 remained despite relatively complete inhibition of EGFR and ERK1/2. There are several possible explanations for these observations. Firstly, the western blotting assay may not be sensitive enough to detect any residual low levels of phosphorylated EGFR, IGF-IR or ERK1/2 following exposure of cells to the inhibitors, where such residual signalling may be sufficient to induce some ERα phosphorylation. Alternatively, other kinases may come into play, including CDK-7, which has been previously implicated in ERα

phosphorylation at serine 118 (Chen et al 2000; Chen et al 2002; Ito et al 2004). Importantly however, CDK-7-induced ERα phosphorylation has been suggested to be dependent on oestradiol induced recruitment of TFIIH to the AF-2 domain, an event unlikely to occur in the presence of tamoxifen due to the altered position of helix 12 (Chen et al 2000). The findings that ERK1/2, AKT and CDK7 have all been implicated in phosphorylation of ERα at serine 118 does in fact highlight that a level of redundancy exists within the mechanisms phosphorylating ERα at serine 118 and such pathways may either function alongside one another or take over from the dominant phosphorylating pathway immediately after inhibition, due to their increased access to the phosphorylation domain.

A further example suggesting redundancy in the kinases responsible for phosphorylating ERα at serine 118 has been provided by examining ERα phosphorylation status across a number of our MCF-7 resistant cells lines. These studies have clearly shown that cells doubly resistant to tamoxifen and inhibitors of two of the principle proteins implicated in phosphorylation of ERα at serine 118, namely EGFR and HER2 (TAM/TKI-R and TAM/HER-R MCF-7 respectively), have very high levels of phosphorylated ERα at serine 118. Indeed, their ERα phosphorylation levels are actually greater than the parent TAM-R cells. Thus, after an initial inhibition of ERα phosphorylation at serine 118 and blockade of cell growth promoted by gefitinib and herceptin, the cells eventually find mechanisms to re-establish elevated ERα phosphorylation at serine 118. Early studies by Dr Helen Jones (personal communication) within the Tenovus laboratories have shown that TAM/TKI-R cells are dependent on

IGF-IR and PKC signalling to mediate growth and ERα phosphorylation at serine 118, implicating an additional kinase capable of phosphorylating ERα. These suggestions of redundancy within the system regulating ERα phosphorylation at serine 118 potentially highlight the need to carry out large scale in vitro kinase assays and kinase library screening assays within a range of breast cancer cell lines to evaluate other potential kinases responsible for serine 118 phosphorylation. Alternatively it may be possible to design antagonistic compounds or peptides to inhibit AF-1 phosphorylation by direct interaction with ERα rather than targeting single kinases.

It should be noted at this point that although ERα phosphorylation at serine 118 may play a role in the growth of acquired resistant breast cancer cell models clinical studies by Murphy LC et al 2004 have strongly suggested that such a phosphorylation event appears to play no role in de novo tamoxifen resistance. In fact their studies show that along with ERα and progesterone positive status, ERα phosphorylation at serine 118 corresponds to longer disease free survival in node-negative patients who were subsequently treated with adjuvant tamoxifen. Such discrepancies between the role of ERα phosphorylation at serine 118 in de novo and acquired resistance may relate to the observations that the ligand dependent mechanism may be prominent in the clinical tumour samples, whereas the current study suggests that ligand independent phosphorylation of ERα plays a much stronger role in the acquired tamoxifen resistant MCF-7 cell line.

To better understand the biological significance of the EGFR/ERK1/2 dependent phosphorylation of ERα at serine 118 in TAM-R cells, the recruitment of nuclear receptor co-regulators to ERα was next investigated. Binding of tamoxifen to ERα is believed to cause a conformational change in the receptor allowing recruitment of co-repressors to the AF-2 domain whilst preventing association with co-activators (Lavinsky et al 1998; Levenson and Jordan 1999; Shiau et al 1998; Shang et al 2000; Shang & Brown 2002; Smith & O'Malley 2004). However, phosphorylation of serine 118 within the AF-1 domain has been shown to promote co-activator recruitment to ERα in MCF-7 cells and thus activate transcriptional activity of the receptor in the presence of tamoxifen, hence emphasising the potential importance of the AF-1 domain and it's phosphorylation dependent activation in the TAM-R cells (Dutertre and Smith 2003; Endoh et al 1999; Lavinsky et al 1998). Immunoprecipitation and Western blotting studies were used in the current study to examine whether serine 118 phosphorylation of ERα influenced co-regulator recruitment. Significantly, in TAM-R cells both co-repressors (nuclear receptor corepressor (NCoR) and SMRT) and co-activators (SRC1, SRC3, CBP and p68 RNA Helicase) were found to be associated with ERα phosphorylated at serine 118. Similarly, Shou et al., 2004 have reported that tamoxifen treatment promotes recruitment of ERα and the co-activators SRC3, CBP and p300 to the ER-regulated pS2 gene promotor in a *de novo* tamoxifen-resistant MCF-7 cell line engineered to over-express HER2. However, in this model, association of co-repressors to ERα was only observed following inhibition of EGFR signalling and thus reduction in serine 118 phosphorylated ERα levels.

Interestingly in our TAM-R cell line recruitment of the co-activators p68 RNA helicase and SRC1 by ERα was regulated by EGFR/ERK1/2 signalling activity. In support of this, it has previously been reported that p68 RNA helicase preferentially binds ERα when phosphorylated at serine 118 by ERK1/2 (Endoh et al., 1999). It should be noted that direct, ERK1/2-mediated, serine phosphorylation of both co-activator and co-repressor proteins has also been shown to regulate their ability to associate with ERα (Font de Mora & Brown, 2000, Rowan et al., 2000, Lopez et al., 2001, Jonas & Privalsky, 2004) and thus ER-driven transcriptional activation may be a function of each of these serine phosphorylation steps. Interestingly, over expression of the co-activator SRC3 has also been shown to correlate with resistance to tamoxifen in breast cancer patients and EGFR-dependent phosphorylation of this co-activator has also been proposed to mediate tamoxifen resistance in HER2 over expressing MCF-7 cells (Shou et al., 2004). Although SRC3 was phosphorylated on serine residues in our TAM-R cells, and this phosphorylation was under EGFR/ERK1/2 regulation, no evidence was obtained that recruitment of the co-activator SRC3 to ERα was similarly under the regulation of this signalling pathway. Immunoprecipitation studies also revealed no effect of either gefitinib or EGF treatment on serine phosphorylation of SRC1, p68 RNA helicase and SMRT, suggesting that their recruitment to ERα is sufficient to promote transcriptional activity in TAM-R cells. As would be expected, immunoprecipitation studies carried out in the WT cell line showed strong associations between ERα, the co-activators p68 RNA helicase, SRC1, SRC3, CBP and RNA polymerase II.

The fact that both co-activators and co-repressors were found to associate with phosphorylated ERα in the TAM-R cells is counterintuitive. In the most simplistic of terms the. primary role of co-activators is to increase the accessibility of DNA to the pre-initiation complex, by principally weakening the interaction between DNA and histones either through ATP dependent mechanisms or through acetylation of the N-terminal tails of the histones (Klinge CM 2000). Conversely, the primary role of co-repressors is to promote chromatin condensation and inhibition of transcription predominantly through recruitment of histone deacetylases (Klinge CM 2000). Acetylation and deacetylation of nucleosomal histones by these coactivator and corepressor complexes, operating together with other covalent histone modifications such as methylation, phosphorylation and ubiquitination creates a 'histone code' and the prevalence or activity of either the coactivators or corepressors determines transcriptional activation or transcriptional repression respectively (Kraus and Wong 2002). However, in the present study both co-activators and co-repressors associate with ERα in TAM-R cells, therefore it is possible that the positive effects of co-activators, associated to the AF-1 region of tamoxifen bound-ERα phosphorylated at serine 118, on transcriptional activity may be cancelled out by the inhibitory effects of co-repressors associated to the AF-2 region of the same ERα. There are several possible scenarios that may occur to prevent this paradox. Firstly the balance of coactivators versus co-repressors bound to ERα may be un-equal, therefore, the co-regulator group with greater numbers will predominate. Secondly, it has been shown that the response elements of gene promoters themselves can influence the binding of co-

regulators to a nuclear receptor (Klinge et al 2004). Natural and synthetic EREs with different nucleotide sequences were found to influence ER binding affinity, conformation, and transcriptional activity, through effects on ER interactions with coactivators and corepressors. For example, CHO-K1 cells transfected with ERα or ERβ showed ERE sequence-dependent differences in the functional interaction of ERα and ERβ with coactivators such as SRC-1, SRC-2, SRC-3, CBP and SRA, and corepressors such as NCoR and SMRT, as well as secondary coactivators such as CARM1 and PRMT1. These differences in co-regulator recruitment were observed in both the absence and presence of ligands such as oestradiol and 4-hydroxytamoxifen. This ability of ERE's to influence co-regulator recruitment by ERα could be assessed in TAM-R cells by chromatin immunoprecipitation assays allowing comparison of co-regulator recruitment by ERα at promoters of ER-regulated genes that are either transcribed or not transcribed in TAM-R cells. Thirdly, the balance of co-activator versus co-repressor may be equal but the activity of the co-regulator may potentially be regulated by post-translational modification. Phosphorylation and acetylation of the nuclear receptor co-regulators has been reported to influence the level of co-regulator activity (Klinge et al 2000). The phosphorylation events are regulated by signalling kinase pathways originating from the plasma membrane, however, acetylation and even methylation events have been reported to be regulated by other co-regulators (Rowen et al 2000; Wu et al 2004; Sterner and Berger 2000). For example co-Factor AcetylTransferases (FATs) such as p300/CBP and PCAF regulate the co-activator SRC3 (ACTR) activity by acetylating a region adjacent to the

LXXLL motif. The two other members of the p160 family of nuclear receptor coactivators, SRC-1 and SRC-2 (TIF2), can also be efficiently acetylated by p300/CBP in vitro (Sterner and Berger 2000). It is therefore possible that, based on these reports, co-activators associated with AF-1 may repress the co-repressors associated to the AF-2 domain of the tamoxifen bound-ERα allowing transcription of the gene in TAM-R cells. These discoveries indicate that further investigations are needed to elucidate all the possible posttranslational modifications that occur on co-regulator proteins and elucidate whether such modifications can be carried out by coactivators on corepressors and visa versa.

Significantly, the immunoprecipitation studies also revealed that RNA polymerase II was recruited to ERα phosphorylated at serine 118 in the TAM-R cells adding further weight to the concept that ERα was transcriptionally active. To examine this possibility in more detail we measured ERE-mediated gene transcription using a transiently-transfected reporter gene assay system. Initial evaluation revealed that TAM-R ERE levels were approximately 50% of those observed in WT cells, indicating a high level of basal ERα transcriptional activity despite the presence of tamoxifen. Further evaluation of basal ERE activity in the two cell lines revealed that pretreatment with fulvestrant, and thus downregulation of ERα, resulted in suppression of ERE activity in both cell lines. It should be noted that as well as activating gene transcription at classical ERE's, ERα can also influence transcription at alternative response elements. For example, protein-protein interactions between the ERα and

members of the AP-1 complex have been shown to enhance transcriptional activity of AP-1 responsive genes (Kushner et al 2000). Furthermore, it has been suggested that the partial oestrogenic activity of tamoxifen can be mediated by ER:AP-1 interactions (Webb et al 1995) and increased AP-1 activity has been reported in tamoxifen-resistant clinical samples and breast cancer cells (Dumont et al 1996; Johnston et al 1999). This does not, however, appear significant in either our WT or TAM-R cells since fulvestrant had no inhibitory effect on AP-1 reporter gene activity in either cell line. Similarly, fulvestrant showed no inhibitory effect on the basal activity of reporter gene constructs containing serum response elements (SRE) in either WT or TAM-R cells and reporter gene constructs containing response elements to heat shock (HSE), NFκB, Glucocorticoid (GRE) and cAMP (CRE) were also insensitive to the inhibitory action of fulvestrant in TAM-R cells. These findings confirmed our previous investigations with respect to basal ERE activity in the TAM-R and WT cells (Hutcheson et al 2003) and would suggest that ERα exerts its action primarily through ERE's in these cell lines. Interestingly a slight increase was observed in AP-1 and SRE activity in the WT and TAM-R cells in the presence of fulvestrant, which has also been reported by Wang et al (2004) in 293A cells. Wang et al (2004) did not believe that this was due to protein-protein interactions or sequestration of co-repressors away from the AP-1 complex but involved a nuclear shuttling event since Leptomycin B, which inhibits nuclear export shuttling of proteins from the nucleus to the cytoplasm, completely inhibited the fulvestrant induced activation of AP-1.

The ERE luciferase assay also revealed that ERα transcriptional activity was regulated by the EGFR/IGF-1R/ERK1/2 signalling pathway, being increased by EGF and IGF-II and inhibited by gefitinib, AG1024 and PD184352, providing further indirect support for the involvement of EGFR/IGF-1R/ERK1/2-mediated serine 118 ERα phosphorylation in the regulation of ERα activity in TAM-R cells. Although the involvement of ERα serine 118 phosphorylation in the regulation of ERα activity was further emphasised by the ability of the EGFR/IGF-1R/ERK1/2 signalling pathway to regulate ERE activity in these cells, it is important to note that the EGFR/IGF-1R signalling pathways can also mediate TAM-R cell growth directly through AP-1 and SREs (Whitmarsh and Davis 1996), as shown by the stimulatory effects of EGF, and inhibitory effects of gefitinib on AP-1 and SRE in the TAM-R cells. Thus, it is likely that ERα transcriptional activity is only one aspect of EGFR/IGF-1R signalling activity contributing to the proliferative response in these cells. Interestingly, in our studies PD184352 was inhibitory on SRE activity but not AP-1 activity in the TAM-R cells, suggesting that while EGFR/ERK1/2 may modulate SRE activity, the EGFR may mediate its effects on AP-1 activity through another signalling cascade, possibly Jun Kinase, JNK (Whitmarsh and Davis 1996).

Based on the findings that ER exerts its action primarily through ERE's in WT cells, the ERE luciferase assay was used to elucidate whether ligand independent activation of ERα, possibly through phosphorylation of serine 118, has an effect on transcriptional activity. Interestingly there were strong

correlations between ERE luciferase activity and ERα phosphorylation as EGF, IGF-I and IGF-II significantly increased, whilst AG1024 significantly inhibited ER transcriptional activity. Again correlating with ERα phosphorylation at serine 118 in WT cells, gefitinib and PD184352 were ineffective at reducing ER transcriptional activity at the ERE reporter gene. The ERE luciferase assay proved to be a convenient and reproducible technique to measure ER transcriptional activity, however the technique only assesses acute effects (within 24 hrs) and does not necessarily fully reflect ER dependent transcriptional events within the genome of the investigated cell lines. Reverse transcription (rt)-PCR, therefore, was used to measure the level of transcription at an endogenous oestrogen responsive gene. The pS2 gene was selected for investigation as its promoter contains a single imperfect ERE which confers oestrogen-responsiveness to this gene (Kim et al 2000), contrasting with the progesterone receptor which contains no classical full length ERE in its promoters (Flototto et al 2004; Petz et al 2004). The present study demonstrated that pS2 was still ERα-regulated in both WT and TAM-R cell lines being increased by oestradiol and reduced by fulvestrant treatment. In the present study, pS2 mRNA levels in WT and TAM-R MCF-7 cells were found to be comparable, whereas, a previous report by Hutcheson et al (2003) had demonstrated a 50% reduction in TAM-R pS2 mRNA levels compared to the WT cells. The reasons for this discrepancy are unclear but may simply reflect differences in experimental conditions used in the two studies.

Consistent with the previous findings concerning ER/ERE transcriptional activity and phosphorylation at serine 118, pS2 mRNA levels in TAM-R cells

were increased significantly in the presence of EGF and significantly decreased in the presence of gefitinib and PD184352. In the WT cell line, however, only EGFR stimulation had any measurable effect on pS2 mRNA levels. Significantly, EGF induced increase in transcription of the pS2 gene in the TAM-R and WT cells was also found to be significantly inhibited by fulvestrant confirming the importance of the ER to the EGF-induced events. Interestingly, an ability of EGF to regulate pS2 gene transcription through ER–independent mechanisms has also been reported by other groups. In the ER negative HepG2 cell line ER independent activation of the pS2 gene via an ERK1/2-mediated activation of Fos and Jun at the AP-1 site has been demonstrated. However, in that study transcription of the pS2 gene was shown to be substantially greater in HepG2 cells transfected with wild type ERα (Barkhem et al 2002). Furthermore Shou et al (2004) demonstrated that in the presence of tamoxifen their de novo tamoxifen resistant MCF-7 cell line engineered to over-express HER2, maintained strong recruitment of ERα and the co-activators SRC3, CBP, p300 to the pS2 gene promoter as well as promoting increased acetylation of this promoter. The addition of gefitinib, however, inhibited acetylation of the pS2 promoter due to inhibition of the co-activator recruitment and enhanced co-repressor recruitment to ERα.

Having established that EGFR/ERK1/2 signalling activity regulated ERα function in TAM-R cells, attention was focused on a potential bidirectional form of cross talk between ERα-EGFR/ERK1/2 signalling pathways described by Hutcheson et al (2003), who showed that fulvestrant induced reduction in

TAM-R cell growth was a consequence of reduced EGFR/ERK1/2 signalling in these cells (Knowlden et al 2003; Hutcheson et al 2003). In this study, ERα was believed to modulate these effects on EGFR signalling through regulating the availability of EGFR ligands such as TGFα (Hutcheson et al 2003). However, the present findings indicate that a role for TGFα is unlikely as fulvestrant only weakly reduced mRNA levels of this ligand in the TAM-R cell line and an anti-TGFα neutralizing antibody had no obvious effect on basal EGFR activity in these cells. In addition there were no inhibitory effects on TAM-R EGFR activity observed in the presence of neutralising antibodies to EGF and Hb-EGF. This study did, however, find that neutralizing antibodies to amphiregulin, another EGFR ligand which has also been shown to be ERα-regulated in a range of breast cancer cell lines, including MCF-7 (Martinez-Lacaci et al., 1995), reduced basal phosphorylation levels of EGFR/ERK1/2 and ERα phosphorylation at serine 118 in TAM-R cells. In support of these findings, PCR studies showed that fulvestrant significantly inhibited the mRNA levels of amphiregulin in TAM-R cells, yet EGF, Hb-EGF and β-cellulin mRNA levels were unaffected by fulvestrant. Interestingly another EGFR ligand in addition to amphiregulin was found to be ER dependent in the TAM-R cells as fulvestrant significantly inhibited the mRNA levels of Epiregulin. Unfortunately a neutralising antibody to Epiregulin was not available at the time of the study.

Consistent with ERα phosphorylation at serine 118, expression of amphiregulin mRNA was significantly higher in TAM-R compared to the parental WT cells and was also under the regulation of EGFR/ERK1/2 signalling being increased

by EGF and reduced by gefitinib and PD184352 in the TAM-R cell line. Amphiregulin was also reduced by AG1024 however this was not found to be statistically significant, perhaps due to the inhibitors reduced ability to inhibit ERα phosphorylation at serine 118. Regulation of amphiregulin expression by both ERα and ERK1/2 signalling has also been reported in MCF-7 cells engineered to over-express constitutively active raf-1 (Weinstein-Oppenheimer et al., 2002). Additional support for ERα regulation of amphiregulin in TAM-R cells was provided by ChIP assays which demonstrated association of ERα with a putative ERE site within the region of 1531-1378bp from the start site of the amphiregulin promoter. Thus, although it is possible that EGFR/ERK1/2 control over amphiregulin transcription is direct, the ChIP data and the ability of fulvestrant to reduce EGF-mediated amphiregulin mRNA expression implies ERα regulation, again implicating a role for serine 118 phosphorylation of the AF-1 domain in mediating this process.

Similar studies in the WT cells demonstrated that EGF also induced increased amphiregulin transcription, which appeared to depend on ER due to the significant reduction in the EGF-induced response following treatment with fulvestrant. Again consistent with ERα phosphorylation levels at serine 118, EGFR/ERK1/2 inhibition with gefitinib and PD184352 was ineffective at reducing amphiregulin mRNA levels, however, a discrepancy was observed in the WT cells following IGF-IR inhibition with AG1024. In fact, AG1024 almost doubled the level of amphiregulin transcription, despite previously showing clear reductions in the levels of ERE transcriptional activity and ERα phosphorylation at serine 118 in the WT cells. A possible explanation for this

discrepancy may be linked with the observation from the ChIP studies that in WT cells ERα does not appear to interact strongly with the region of the amphiregulin promoter complementary to primer 2 unless oestradiol is present. The elevated ERα phosphorylation at serine 118 and the presence of tamoxifen in the ERα LBD may be required to allow this interaction to occur in TAM-R cells. As these factors are not present in WT cells a completely different mechanism may exist in the regulation of transcriptional activity at the amphiregulin promoter in WT cells compared to the TAM-R cells, hence the lack of dependence on ERα phosphorylation.

The EGFR/ERK1/2 signalling pathway clearly plays an important role in the phosphorylation of ERα at serine 118 in TAM-R cells however EGF-induced activation of EGFR is also reported to increase ERα phosphorylation at serine 167 via ERK1/2 and p90 ribosomal S6 kinase (Lannigan 2003). Furthermore, Joel et al, Smith et al, and Clarke et al have all observed ERK1/2 dependent phosphorylation of ERα at serine 167, therefore this study investigated whether the elevated EGFR/ERK1/2 signalling in TAM-R cells also increases ERα phosphorylation at serine 167. Importantly, no real change in the level of ERα phosphorylation at serine 167 between the WT and TAM-R cell lines was observed. Interestingly, MCF-7 cells which have acquired resistance to growth conditions in the absence of oestrogen and serum growth factors displayed very high levels of serine 167 phosphorylation compared to both WT and TAM-R cells, an observation that correlates with increased ERE activity in transient transfection assays (Staka et al 2005). The WT T47D cells also displayed

elevated serine 167 phosphorylation, despite expressing considerably lower levels of total ERα, however further studies are required to identify the relevance of such phosphorylation events in this cell line.

- The current project has focused separately on individual ERα phosphorylation events and ER-coregulator interactions in order to gain an insight into the mechanisms that govern ERα functionality in the TAM-R cells continually exposed to tamoxifen. Although successful in answering many questions, other clear post-translational events and co-regulator interactions, may impact on ERα activation. In an attempt to answer some of these questions; such as the complete phosphorylation status of the ER in our MCF-7 cell lines and the identity of the many different ERα interacting proteins, proteomic technologies such as 2D gel electrophoresis and MALDI/TOF Mass spectrometry were used. These techniques have been used to study protein phosphorylation and the profile of protein-protein interactions in different pathological conditions and signalling pathways (Schilling et al 2005; Gibson BW 2005).

Although the MS analysis was possible through collaboration with Dr M Morton, University hospital of Wales; only a limited number of samples could be processed due to time restrictions. For this reason excision of the protein spots from the 2D gel was selective and only spots around the 66-68 KDa size range and 6.5-7.1 pI value were selected as the full length ERα has a molecular weight of approximately 67KDa and a pI of approximately 6.8, although other isoforms of ERα are detected at different KDa and pI (Puddefoot JR et al 1993). Importantly however, full length ERα was not identified on the gels

produced. An interesting theme throughout the results obtained from the proteomic experiments was the number of cytokeratins (numbers 1, 2, 6, 8, 9, 10, 18 and 19), that co-immunoprecipitated with ERα. There are three possible reasons for the presence of cytokeratins in the immunoprecipitates. Firstly, the cytokeratins may arise from contaminants, possibly hair and skin. This, however, is unlikely because the cytokeratins identified are commonly expressed in epithelial cells, rather than those from dermal origins. Secondly, the cytokeratins associated with the 1D5 antibody in a non-specific manner. Further studies are needed to examine this and would require preclearing with a non-specific monoclonal mouse antibody or washing the beads used in the immunoprecipitation under more stringent conditions. Thirdly, it is possible that ERα associates with cytokeratins within the cell in a biologically relevant manner, perhaps relating to the trafficking of the receptor from cytoplasm to nucleus. Indeed, cytokeratins 8 and 18, which were strongly co-immunoprecipitated with ERα, have previously been implicated in cell signalling events (Davezac et al 2004). If such an interaction is real, this study is the first to reveal a direct binding between ER and cytokeratins. In support of this Spencer et al 1998 showed that the interaction between nuclear DNA and the cytokeratins 8, 18 and 19 in hormone dependent ER positive T47D breast cancer cells was increased in the presence of oestrogens and decreased following oestrogen depletion and treatment with the pure antioestrogen ICI164,384. Further studies are obviously required to elucidate whether these ER dependent interactions between DNA and cytokeratins are real and aid the

transcriptional control of specific genes downstream of ER, or alternatively whether ER itself is acting as a linker between the DNA and cytokeratin. Potentially, once the ERα is isolated from the breast cancer cell in high enough quantities, then tandem mass spectrometry of the intact protein, peptide mass finger printing following proteolysis, or a combination of these two techniques can be used to identify all possible posttranslational modifications of the receptor. Such approaches have already proved successful in identifying all posttranslational modifications of the chromatin histones (Espino et al 2005). In combination with pharmacological studies a posttranslational finger print system may be developed to inform the investigator which signalling pathways are modulating ERα posttranslational modifications and therefore functionality. Similar analysis of ERα isolated from breast cancer patients may then be compared against the various post-translational fingerprints and inform the oncologist of what pathways maintain ERα functionality in that particular patient, therefore providing more accurate therapeutic targets.

Conclusion

This study suggests that a self propagating autocrine growth regulatory loop exists in our TAM-R cell line due to bidirectional cross talk between EGFR/ERK1/2 and ERα signal transduction pathways. Furthermore, the EGFR/ERK1/2 dependent phosphorylation of ERα appears to be supported by the IGF-IR signalling pathway. IGF-IR also played a role in phosphorylation at serine 118 in the WT cell line however this involved the PI3K/AKT signalling pathway and was totally independent of EGFR/ERK1/2 under basal growth conditions. EGFR/ERK1/2 dependent phosphorylation of ERα at serine 118 within the TAM-R cell line results in recruitment of the coactivators p68 RNA helicase and SRC1, activation of ERα in a ligand-independent manner resulting in enhanced ERE dependent gene transcription. Similarly in the WT cells, IGF-IR signalling contributed to ERE dependent transcriptional activity. A consequence of the increased ERα transcriptional activity in TAM-R cells was the generation of amphiregulin which when released by the cells can act in an autocrine manner to regulate EGFR/ERK1/2 signalling activity and thus, cell growth.

It should be noted that this data is only correlative and perhaps more detailed studies are required to demonstrate that the EGFR/ERK1/2 mediated regulation over ERα transcriptional activity is conclusively dependent on this phosphorylation event. Previous studies have used serine to alanine point mutated forms of ERα to demonstrate the functional significance of ERα phosphorylation at serine 118 (Ali et al 1993; Bunone et al 1996; Joel et al 1998; Le Goff et al 1994; Rogatsky et al 1999), therefore similar experiments

involving mutational analysis of ERα in the TAM-R cells are required to conclusively confirm the role of this phosphorylation event in the TAM-R cells. Unfortunately, however, all of these mutational studies were carried out in ER negative cell lines stably transfected with point mutant ERα and developing an experimental system where TAM-R cells express such serine to alanine point mutations as replacements of the endogenous receptor poses many practical problems, therefore the strategy used in this investigation appears the best possible.

Despite these considerations the present findings clearly demonstrate that cross-talk between growth factor signalling pathways and ERα plays a central role in regulating growth of breast cancer cells lines, in particular the MCF-7 model of acquired tamoxifen resistance, and indicate that targeting these phosphorylation events on ERα whilst maintaining tamoxifen induced inhibition of AF-2 may prove beneficial in the therapy of this disease.

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