INVESTIGATION OF THE MECHANISMS OF ESTROGEN RECEPTOR ACTIVATION BY PHOSPHORYLATION

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ABSTRACT

Breast cancer growth is estrogen- regulated in many cases. Estrogen actions are mediated by estrogen receptors ERα and ERβ. Whilst the involvement of ERβ in breast cancer is unresolved at present, up to 80% of breast tumours are ERα-positive and likely to respond to endocrine therapies, such as anti-estrogens. However, a substantial proportion of patients develop a resistance to such treatments. Most resistant tumours continue to express ERα and many will respond to an alternative hormonal therapy, indicating that ERα continues to be important following the emergence of resistance. One proposed mechanism for endocrine resistance is post-translational modification of the ERα, particularly phosphorylation of ERα within the transcription activation domain AF1.

This project investigates how the phosphorylation at residues within the AF1 domain is linked to ERα activation and tumour cell growth in the presence of tamoxifen and in the absence of ligand, by stable introduction of ERα phosphorylation site mutants in the MCF7 human breast cancer cell line, commonly used as a model for ERα-positive breast cancer. Characterisation of these lines suggests that phosphorylation at the sites within the AF1 domain of the ER increases the agonist activity of tamoxifen. Further studies to determine the mechanisms by which ERα activity is regulated by phosphorylation within AF1 are discussed.

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Abbreviations

ACF	ATP-utilizing chromatin assembly and remodeling factor
ACTR	Activator for thyroid and retinoid receptors
AD	Association domain
AD1	Acivation domain
AF1/AF2	Activation function 1/2
AI	Aromatase inhibitor
AIB1	Ampliphied in breast cancer 1
ANOVA	Analysis of varience
APS	Ammonium persulphate
AR	Androgen receptor
ARC	Activator-recruited cofactor
ATP	Adenosine triphosphate
BRM	Brahma
BSA	Bovine serum albumin
CAK	Cdk-activating kinase
CARM1	Coactivator-associated arginine methyltransferase 1
CBP	CREB binding protein
cdk	Cyclin dependent kinase
cDNA	Complimentary deoxyriblonucleic acid
ChIP	Chromatin immunoprecipitation
CHRAC	Chromatin accessibility complex
CML	Chronic myeloid leukemia
CoCoA	Coiled-coil coactivator A
CREB	cAMP-response element binding protein
CtBP	C-terminal binding protein
DBD	DNA binding domain
ddH2O	Distilled de-ionised water
DMEM	Dulbeco's Modified Eagles Medium
DNA	Deoxyribonucleic acid
DRIP	Vitamin D receptor interacting protein
DSS	Double Stripped Serum
DTT	Dithiothreitol
E2	Estrogen
E6-AP	E6 papillomavirus-associated protein
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERE	Estrogen receptor element
ERK	Extracellular signal regulated kinase
FCS	Fetal Calf Serum
FSH	Folicle stimulating hormone
GAC63	GRIP1-associated coactivator 63
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPS2	G-protein pathway supressor 2
GR	Glucocorticoid receptor
GREB1	Gene regulated in breast cancer 1
GRIP	Glucocorticoid receptor interacting protein
GSK3	Glycogen synthase kinase 3
GTF	General transcription factor

HA	Influenza Hemaglutinin
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HRE	hormone response element
HRG-β1	heregulin-β1
HRP	Horse radish peroxidise
HSLB	High salt lysis buffer
ICI	182, 780 ICI
IGF-1	Insulin-like growth factor-1
IGFBP5	Insulin-like growth factor binding protein 5
IKKα	I-kappa β kinase alpha
IP	Immunoaffinity purification
ISWI	Imitation switch
JNK	c-Jun N-terminal kinase
kDa	Kilo Daltons
LB	Luria Bertani
LBD	Ligand binding domain
LCoR	Ligand-dependent nuclear-receptor corepressor
LH	Luteinizing hormone
LHRH	Luteinising hormone releasing hormone
LTED	Long term estrogen deprivation
MALDI-TOF	Matrix-assisted laser desorption/ionisation time of flight
MAPK	Mitogen activated protein kinase
MCF7	Michigan Cancer Foundation -7
MEK	Mitogen-activated protein kinase kinase
MEKK1	MEK kinase 1
MMP	Matrix metalloproteinase
MNAR	Modulator of the non-genomic activities of ER
MOPS	3-(N-morpholino)propanesulphonic acid
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
NCoA	Nuclear receptor coactivator
NCoR	Nuclear receptor corepressor
NGFI-β	Nerve growth factor induced-β receptor
NL	Nuclear localization
NR	Nuclear receptor
NURF	Nucleosome-remodeling factor
OHT	4-hydroxy tamoxifen
P/CAF	p300/CBP-associated factor
p/CIP	Cointergrator-associated protein
P/S/G	Penicillin/streptomycin/L-glutamine
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PELP1	Proline-, glutamic acid-, leucine-rich protein 1
PGC1α	PPARg co-activator 1α
PH	Plekstrin homology domain
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PKC	Protein kinase C
Pol II	RNA polymerase II
PPAR	Peroxisome proliferator activated receptor
PPIL1	Peptidyl-propyl isomerise-like 1
PR	Progesterone receptor

ProTα	Prothymosin α
qRT-PCR	Quantitative (real time) RT-PCR
RAR	Retinoic acid receptor
RIP140	Receptor-interacting protein 140
RNA	Ribonucleic acid
ROR	Retinoid-related receptor
RPF-1	Retina-derived POU-domain factor 1
rpm	Revolutions per minute
RSF	Remodelling and spacing factor
RT-PCR	Reverse transcriptase polymerase chain reaction
RVR	Rev-ErbAa-related receptor
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulfate
SERM	Selective estrogen receptor modulator
SF-1	Steroidogenic factor 1
SMRT	Silencing mediator for retinoid and thyroid-hormone receptor
SR	Steroid receptor
SRC	Steroid receptor coactivator
SREBP-1α	Sterol regulatory element binding protein 1α
SUMO	Small ubiquitin-like modifier
SWI/SNF	Switch/sucrose non-fermentable
TAFII	TBP-associated factor
TBE	Tris-Borate-EDTA
TBL1	Transducin beta-like protein 1
TBLR1	Transducin beta-like protein 1 related protein
TBP	TATA binding protein
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
TF	Transcription factor
TFF1	Trefoil factor 1
TGF	Transforming growth factor
TIF2	Transcription intermediary factor 2
TR	Thyroid hormone receptor
TRAM1	Thyroid hormone receptor activator molecule 1
TRAP	TR-associated protein
U0126	1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene
VDR	Vitamin D3 receptor

I hereby declare that this submission is my own work, or if not it is clearly stated and fully acknowledged. To the best of my knowledge this report contains no material previously published or written by another person, nor material which, to a substantial extent has been accepted for the award of any degree or diploma of Imperial College or any other institution of higher leaning.

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1 INTRODUCTION

1.1 Nuclear Receptor Superfamily

Lipophilic hormones including steroids, thyroid hormones and vitamin D3 were isolated in the early 20th century but their receptors were not discovered until the early 1960's when Jensen and Jacobsen used radio-labeled estradiol to identify an estradiol-binding protein. This protein was found to translocate to the nucleus after the administration of hormones which suggested a link between hormones and gene regulation (Jensen and Jacobsen, 1962). The purification of the estrogen receptor (ER) and subsequent development of antibodies allowed the cloning of the ER gene (Green et al., 1986; Greene et al., 1986; Walter et al., 1985), now renamed ERα (Kuiper et al., 1996). The concomitant cloning of the glucocorticoid receptor (GR) gene (Hollenberg et al., 1985; Weinberger et al., 1985a) showed that ER and GR were related genes, and that they were homologous to the avian erythroblastosis virus v-erbA oncogene (Krust et al., 1986; Weinberger et al., 1985b), the cellular homologue of which (c-erbA) was subsequently shown to encode the thyroid hormone receptor (TR) gene (Sap et al., 1986). Subsequent cloning of the androgen (AR), mineralocorticoid (MR) and progesterone (PR) receptor genes further enlarged the family (Evans, 1988). Identification of other genes, largely through low stringency screening of cDNA libraries, through degenerate PCR and analysis of DNA sequence databases has expanded the number of nuclear receptor (NR) family members in man to 48, making it one of the largest families of transcription factors in higher eukaryotes. In addition to the steroid hormone receptors (AR, ER, GR, MR, PR) and TR genes, the NR superfamily now includes the vitamin D3 receptor (VDR) and retinoic acid receptors (RAR, RXR), as well as receptors that bind to a wide variety of ligands, including fatty acids, bile acids, prostaglandins, xenobiotics,

phospholipids and heme, as well as so-called 'orphan nuclear receptors' which have no known ligand or that may not require ligand binding for their activities (figure 1.1) (Chawla et al., 2001; Evans, 1988; Forman, 2005; Yin et al., 2007).

1.2 Structure of nuclear receptors

Purification of the GR led to the discovery of the three-domain structure which encompasses a DNA binding domain (DBD), ligand binding domain (LBD) and a third domain which was known to have transcriptional regulatory properties (Wrange and Gustafsson, 1978). Comparison of the amino acid sequences for ERα, GR and v-erbA with the chicken ERα identified six regions of differing amino acid sequence homology, termed A-F (N-terminal to C-terminal) (Krust et al., 1986). Of these regions, the highly conserved regions C and E, were shown to encode the DBD and LBD respectively (Kumar et al., 1986). Figure 1.1 shows a schematic diagram of the NR structure.

The NR DBD is encoded within a 66-68 amino-acid region, comprised of two zinc finger motifs in which four cysteine residues in each zinc finger coordinate one zinc ion (Hard et al., 1990; Luisi et al., 1991; Schwabe et al., 1993; Schwabe et al., 1990). Mutational analysis demonstrated that sequences in the so-called P-box at the C-terminal part of the first, or N-terminal zinc finger, determine sequence specificity of binding of NR to DNA response elements, whilst the second zinc finger is important for dimerisation (Danielsen et al., 1989; Green et al., 1988; Mader et al., 1989; Umesono and Evans, 1989). These and other studies showed that mutational switching of three amino acids in the ERα DBD P-box to the corresponding amino acids in the GR DBD was sufficient to allow ERα to bind to a GR DNA response element, in preference to an ERα response element, with the converse also being

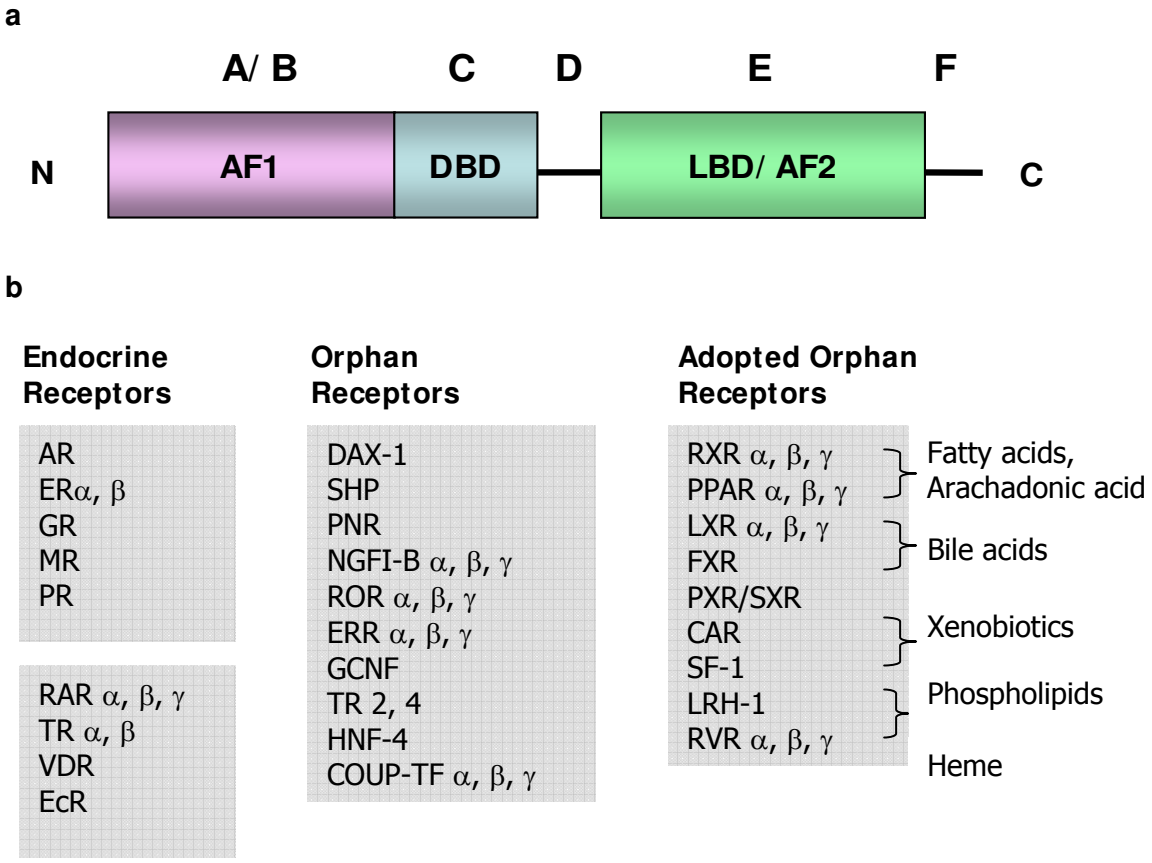


Figure 1.1 Structure and Classification of Nuclear Receptors
Nuclear receptor proteins are a structurally related group of eukaryotic transcription factors that conform to the organisation shown in (A), where AF1 corresponds to the N-terminal transcription activation domain, DBD to the DNA binding domain and LBD/AF2 to the region involved in ligand binding and the second transcription activation region. (B) The NR superfamily can be further classified according to the ligands they bind. The classic nuclear steroid receptors include the androgen (AR), estrogen (ER), glucocorticoid (GR), mineralcorticoid (MR) and progesterone receptors (PR). The adopted orphan nuclear receptors heterodimerize with the retinoid X receptor (RXR) and include peroxisome perliferator-activated receptors (PPARs), farnesoid X receptor (FXR), pregnane X receptor/ steroid xenobiotic receptor (PXR/SXR), constitutive androstane receptor (CAR), steroidogenic factor-1 (SF-1), liver receptor homologue-1 (LRH-1) and Rev-ErbAα-related receptor (RVR). Thyroid hormone (TR), retinoic acid (RAR), vitamin D3 (VDR) and ecdysone (EcR) receptors also heterodimerize with RXR yet share other characteristics with endocrine receptors. Orphan receptors include dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (DAX-1), short heterodimer partner (SHP), photoreceptor cell-specific nuclear receptor (PNR), nerve growth factor-induced gene B (NGFI-B), retinoid-related receptor (ROR), estrogen receptor-related receptors (ERR), germ cell nuclear factor (GCNF), testicular receptors (TR2, 4), hepatocyte nuclear factor 4 (HNF-4) and chicken ovalbumin upstream promoter transcription factors (COUP-TFs) (Chawla et al., 2001; Maglich et al., 2001).

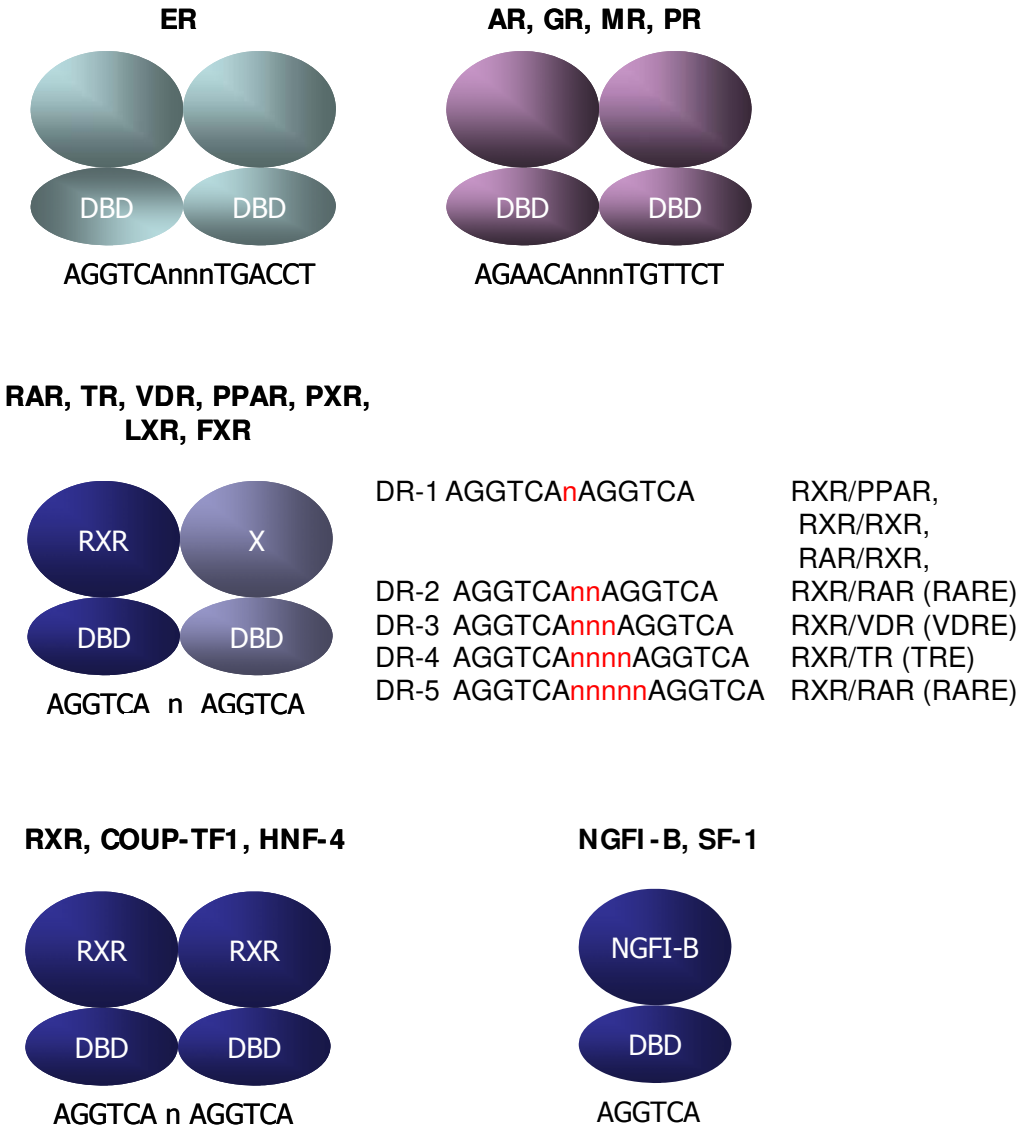


Figure 1.2 Recognition and binding of nuclear receptors to response elements

Nuclear receptors bind as homo- and heter-dimers to DNA response elements conforming to the consensus sequences shown. Receptors that form heterodimers with RXR recognise different direct repeats (DR) of the sequence separated by 1-5 base pairs. Adapted from (Mangelsdorf and Evans, 1995).

the case. Together, these studies have shown that AR, GR, MR and PR, which have the same P-box sequence, bind as homodimers to a palindromic DNA sequence conforming to the consensus sequence AGAACANNNTGTTCT (Lieberman et al., 1993; Nordeen et al., 1990; Roche et al., 1992), whilst ERα binds as a homodimer to a palindromic sequence conforming to the consensus sequence AGGTCANNNTGACCT (Klein-Hitpass et al., 1988; Walker et al., 1984), where N is any nucleotide (Figure 1.2). Although steroid receptors bind DNA as homodimers, other members of the NR superfamily form heterodimers with retinoid X receptor (RXR) and bind DNA via response elements containing direct repeats of the sequence 5'-AGGTCA-3' with between 1-5 base pairs separating the repeats, as shown in figure 1.2 (Rastinejad et al., 1995; Umesono et al., 1991).

Nuclear receptors, including the all-*trans* retinoic acid (RAR), vitamin D_3 (VDR), thyroid hormone (TR), liver X (LXR), peroxisome proliferator activated receptors (PPAR), nerve growth factor induced-B (NGFI-B) and Rev-ErbAα-related receptor (RVR) form heterodimers with RXR in this way (Bugge et al., 1992; Kliewer et al., 1992a; Kliewer et al., 1992b; Leid et al., 1992; Perlmann and Jansson, 1995; Yu et al., 1991), although VDR and RVR are also capable of forming homodimers (Harding and Lazar, 1995; Takeshita et al., 2000). A number of orphan receptors, including RVR, NGFI-B, retinoid-related receptor (ROR) and the steroidogenic factor 1 (SF-1), are capable of binding DNA as monomers. These bind sites with one repeat of the consensus sequence 5'-AAAGGTCA-3' and specific flanking 5' sequences determine response element specificity (Giguere et al., 1995; Harding and Lazar, 1995; Wilson et al., 1993; Wilson et al., 1992).

By carrying out studies using ERα, two transcriptional activation functions (AFs) were identified (Kumar and Chambon, 1988; Tora et al., 1989; Webster et al., 1988; Webster et al., 1989). The ligand-independent AF1 is located in the A/B region

and the ligand-dependent AF2 is located within the carboxyl terminus of the LBD in the E/F region. The transcriptional activation functions are capable of acting independently or synergistically in a promoter and cell-specific fashion. These observations gave rise to the hypothesis that NRs could interact with various promoter-specific transcription factors and cell-specific factors which mediated the receptor activity (Bocquel et al., 1989; Meyer et al., 1989; Tasset et al., 1990). Indeed, many such factors, labelled NR coactivator and corepressor proteins, have now been identified which facilitate gene regulation by NRs and this topic will be discussed in more detail with regard to ER later in this introduction.

The LBD within the E region is connected to the DBD by a short flexible linker and has a three-dimensional structure common to all nuclear receptors (Wurtz et al., 1996). The structure consists of eleven α-helices which come together to form a ligand-binding pocket. A twelfth α-helix (H12) forms a movable barrier to this pocket, the orientation of which is effected by the chemical structure of binding ligands and contains residues which are crucial to the function of AF2 (Bourguet et al., 1995; Henttu et al., 1997; Renaud et al., 1995). In the inactive state, corepressors of NR bind to the hydrophobic groove in the surface of the LBD formed by α-helices H3 and H4. Generally, agonists of nuclear receptor activity promote allosteric effects which cause H12 to reposition in such a way as to disrupt this hydrophobic groove, leading to dissociation of corepressors (Shiau et al., 1998). This conformational change permits the AF2 α-helix to make contact via a conserved glutamate residue with a conserved lysine residue in the H3 of the LBD (Feng et al., 1998). This provides a 'charge clamp' which binds the ends of the LXXLL motif present in coactivators (where L is Leucine and X is any other amino acid) (Heery et al., 1997; Le Douarin et al., 1996; Torchia et al., 1997). In this way, agonist binding to the NR activates the AF2 and allows interaction of the receptor with coactivator complexes

which in turn initiate transcription of the NR-regulated genes (Glass and Rosenfeld, 2000; Moras and Gronemeyer, 1998; Nettles and Greene, 2005; Shiau et al., 1998; Webster et al., 1988).

As described above, SRs homodimerise, whereas many of the non-SRs heterodimerise with RXRs. Dimerisation by NRs is mediated by the DBD and LBD (Fawell et al., 1990a; Kumar and Chambon, 1988). The dimerization domain within the LBD identified in the GR and conserved in NRs, consists of a sequence of heptad repeats of hydrophobic residues (Fawell et al., 1990a) and has been shown to act in a hormone-dependent manner, whereas the dimerization domain within the DBD is weakly constitutively active (Kumar and Chambon, 1988). The DBD dimerization domain is thought to be responsible for the selection of the spacing distance between the two halves of palindromic response elements (Hard et al., 1990; van Tilborg et al., 1995).

The molecular weight of the rat GR monomer was deduced as 87.5 kDa (Miesfeld et al., 1986). Studies around the same time showed that the nuclear envelope acted as a form of molecular sieve whereby molecules of a molecular mass less than ~70kDa could enter the nucleus by passive diffusion through nuclear pores (Lang et al., 1986; Paine et al., 1975; Peters, 1984). This gave rise to the suggestion that the GR, which is localized to the cytoplasm in the unbound state and transferred to the nucleus in the liganded state, contained nuclear localization signals; the activity of which is potentially controlled by hormone binding. Two nuclear localization (NL) signals were indeed identified in GR (Picard and Yamamoto, 1987), NL1, which is conserved in SRs, and NL2; NL1 is adjacent to the DBD and was shown to be constitutively active and NL2 is located within the LBD of GR and its activity was shown to be hormone-dependent. Not all NRs shuttle between the nucleus, however, PR and ER are constitutively present in the nucleus (King and Greene,

1984; Press and Greene, 1988; Puca et al., 1986) and do not contain the hormone-dependent NL2 nuclear localization signal (Picard and Yamamoto, 1987). The number and position of nuclear localisation signals within the NR determines the requirement for hormone-dependent nuclear localization and define which mechanism of nuclear transportation is utilized by the receptor (Hsieh et al., 1998; Savory et al., 1999; Yasmin et al., 2005).

1.3 Gene regulation by nuclear receptors

In eukaryotes, the genomic DNA is stored in the nucleus in a highly packaged state, which is termed chromatin. Chromatin is made up of units called nucleosomes; the nucleosome core is composed of 147 base pairs of DNA wrapped around histone octamers which form protein-DNA interactions (Luger et al., 1997). Nucleosomes are arranged to form chromatin domains which are very stable under physiological conditions and create a thermodynamic barrier between the DNA and transcription factors, making the chromatin structure ideal for DNA compaction. However, the nucleosome is not a fixed structure and possesses dynamic properties that are highly regulated by various protein complexes (Li et al., 2007; McKenna et al., 1999).

Covalent histone modifications, such as acetylation and phosphorylation, result in a change in the net charge of nucleosomes which studies have shown reduces the DNA-histone interactions (Hassan et al., 2006; Reinke and Horz, 2003; Zhao et al., 2005). Modification of histones has also been shown to effect the chromatin structure (Shogren-Knaak et al., 2006). The modifications are thought to act as 'tags' which are then recognised by other transcriptional regulators (Jenuwein and Allis, 2001; Strahl and Allis, 2000). Chromatin re-modelling proteins require ATP hydrolysis to alter the structure of the chromatin and work in conjunction with modifying complexes to alter the accessibility of nucleosomal DNA and allow

transcription factors, such as NRs, to access the DNA (Cosma et al., 1999; Saha et al., 2006; Smith and Peterson, 2005).

The agonist-bound NRs bind to hormone response elements (HREs) which can be several kilobase pairs away from the promoter region of the target gene. Transcription factors such as NRs allow the ordered association and dissociation of coactivator complexes which remodel chromatin and modify core histones in order to allow access to the DNA and promote gene transcription. The recruitment of the general transcription factors (GTFs) is a key factor in this process and is necessary for transcription initiation. The GTFs include RNA polymerase II (Pol II) and a variety of auxiliary components including TFII A-H. The initial rate limiting step of gene transcription involves TFIID binding to recognition sequences within the promoter region (Buratowski et al., 1989), a process which requires the direct interaction of the Mediator complex with TFIID via TATA binding protein (TBP)-associated factors (TAF_{II}s) which make up the TFIID protein complex (Baek et al., 2002). TFIID binding inititates the recruitment of the other GTFs leading to the recruitment of Pol II to the transcription start site, initiating gene transcription (Beato and Sanchez-Pacheco, 1996; McKenna et al., 1999).

These processes of chromatin modification and remodelling, recruitment of GTFs and transcription initiation are mediated by coregulators that interact with transcription factors such as NRs. The sequential exchange of coregulator complexes, which carry out a range of enzymatic activities, controls the transcriptional activity of NRs. In the absence of hormone, SR monomers are generally believed to be associated with heat shock protein complexes such as Hsp70 and Hsp90 which are thought to assist in protein maturation and maintain the SR in a stabilised, inactive form capable of binding ligand (Hernandez et al., 2002; Kosano et al., 1998).

The unliganded SR can be localized to the cytoplasm in the case of AR and GR (Picard and Yamamoto, 1987; Tyagi et al., 2000), to the nucleus in the case of ER and PR (Guiochon-Mantel et al., 1989; King and Greene, 1984) or can be distributed between the nucleus and cytoplasm as is the case with MR (Fejes-Toth et al., 1998). Upon binding hormone, the receptors dimerize, cytoplasmic receptors translocate to the nucleus and the receptors bind to sequence-specific hormone responsive elements (HRE). In the case of the non-steroid receptors that dimerise with RXR, the unliganded receptors are nuclear, are bound to promoters of regulated genes and repress gene expression through recruitment of corepressor complexes (Andersson et al., 1992; Cheskis and Freedman, 1994; Miyamoto et al., 1993). RXR itself is located in the cytoplasm in the unliganded state and forms homotetramers (Kersten et al., 1995a) which rapidly dissociate upon binding of 9-cis-retinoic acid (Kersten et al., 1995b) releasing active receptors (Kersten et al., 1998). Ligand binding to VDR has been shown to decrease the amount of DNA-bound homodimers and enhance DNA-bound monomers, which favour dimerization with RXR to form an active heterodimer (Cheskis and Freedman, 1994).

ER is also nuclear in the unliganded state (King and Greene, 1984) and recent chromatin immunoprecipitation (ChIP) studies suggest that the unliganded ER is bound to promoters of estrogen responsive genes, with coactivator proteins being recruited in transcriptionally unproductive complexes (Metivier et al., 2004). Ligand binding provides an ordered and cyclical recruitment and dissociation of a large series of diverse coactivator and corepressor complexes, resulting in cycles of chromatin remodelling, histone acetylation, methylation, deacetylation and demethylation (Metivier et al., 2003; Shang et al., 2000). Particularly important in the cyclical and ordered coregulator recruitment is the association/dissociation of ER, brought about by proteasome-mediated ER turnover (Reid et al., 2003).

1.3.1 Coregulators of nuclear receptors

Unlike NRs which are highly conserved, coregulators are incredibly diverse in their structure and enzymatic activities which can be divided into two generic classes, as mentioned previously – chromatin modifiers and chromatin remodellers. Chromatin modifiers are capable of covalently modifying histone tails and include proteins which carry out activities such as acetylation/deacetylation, methylation/demethylation, protein kinases and phosphatases, poly(ADP)ribosylases, ubiquitin and SUMO ligases. Chromatin remodellers include components of a family of ATP-dependent remodelling complexes (Rosenfeld et al., 2006). Coregulators are necessary in order to alter the chromatin structure of DNA to control initiation of transcription and are also required for recruiting GTFs and Pol II to the promoter during gene transcription. Biochemical and expression cloning approaches have been used to identify a large number of factors that interact with NRs, many of which appear to function as components of large, multiprotein complexes (Glass and Rosenfeld, 2000; Lonard and O'Malley, 2006; McKenna et al., 1999; McKenna and O'Malley, 2002). The major families of coregulators are discussed below (see figure 1.3).

1.3.1.1 Coactivators of nuclear receptors

Transcriptional coactivators have been so-termed because their co-expression with transcription factors stimulates the activity of the transcription factor of interest, at least in reporter gene studies. The notion that coactivators were involved with NR transcriptional activation first came about when it was shown that activation of multiple SRs co-expressed in a cell system caused inhibition of the SRs. Coactivators

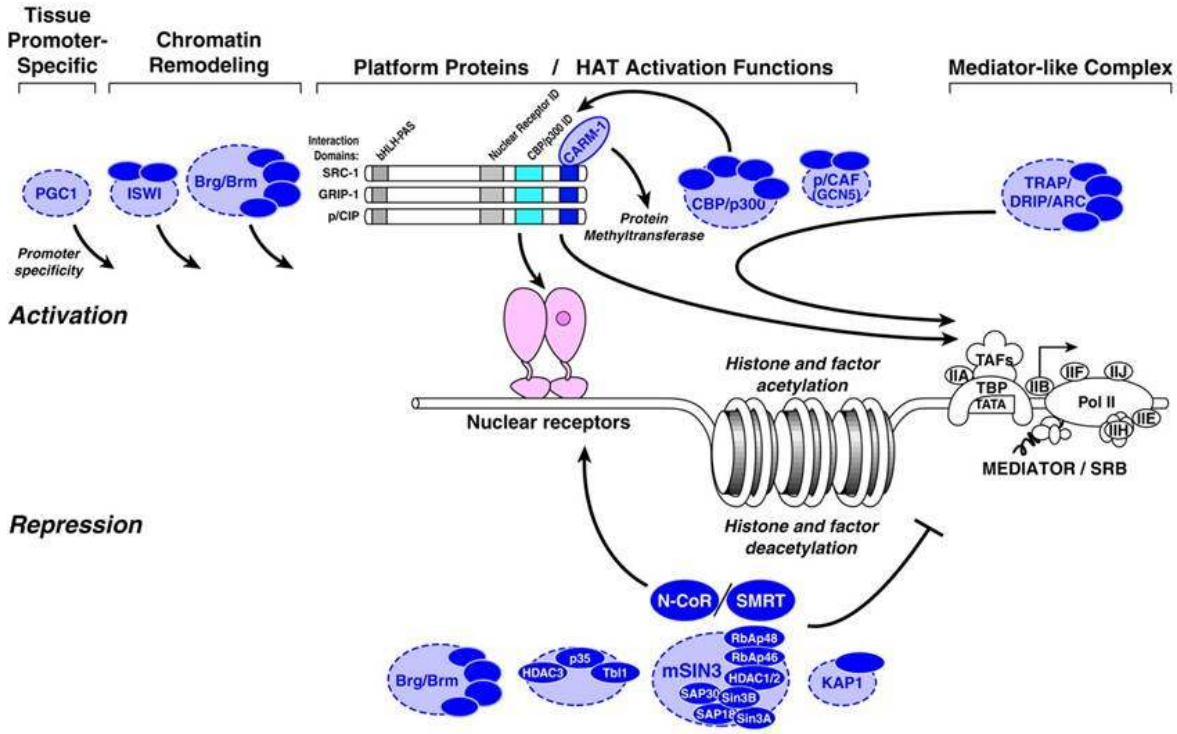


Figure 1.3 Coactivators and corepressor of nuclear receptors
Nuclear receptors bound by corepressors are transcriptionally inactive, promoting chromatin condensation. Ligand binding to the NR promotes corepressor dissociation and coactivator association, leading to chromatin remodelling and histone modifications which allow acess to DNA. Coactivators also recruit the basal transcriptional machinery to the gene promoter, initiating gene transcription. Taken from (Rosenfeld and Glass, 2001).

were identified as being limiting factors in the transcriptional process and the sequestering of coactivators leading to reduced SR activity was termed squelching. Studies have also shown that levels of coactivators vary between cell type, leading to squelching and determining the relative activity of SRs in varying cell types (Bocquel et al., 1989; Gill and Ptashne, 1988; Meyer et al., 1989; Voegel et al., 1996). It is likely that coactivators have other functions within the cell in addition to transcription initiation, such as alternative splicing, mRNA transport from the nucleus, mRNA translation and posttranslational modifications of the synthesized protein. To date, there are approximately 200 published NR coactivators, suggesting that the process of transcription intiation is a highly dynamic and orderly process involving a large number of protein complexes working together (Lonard and O'Malley, 2006). Here I shall discuss the main groups of coactivators, whose activities have been most clearly linked to gene regulation by NR.

1.3.1.1.1 ATP-dependent chromatin remodelling complexes

1.3.1.1.1.1 SWI / SNF

The SWI/SNF (switch/sucrose non-fermentable) complex was originally identified in yeast and facilitates the binding of sequence-specific transcription factors to nucleosomal DNA and has the ability to alter the chromatin structure in an ATP-dependent manner (Owen-Hughes et al., 1996). The main characteristic of the SWI/SNF family of chromatin re-modellers is the presence of a conserved ATPase subunit that belongs to the SNF2 superfamily of proteins (Eisen et al., 1995) and five other conserved core members. Homologues of SWI/SNF had been identified in *Drosophila melanogaster* (Tamkun et al., 1992) and were shown to form part of the large multi-subunit complex called Brahma (BRM) (Dingwall et al., 1995). Two

members of the SWI/SNF family have been identified in humans, BRG1 (Kwon et al., 1994) and hBRM (Wang et al., 1996) which show 70% homology to each other and are highly homologous to the Swi/Snf gene (Eisen et al., 1995). The associated factors to the BRG1 and hBRM catalytic subunits are termed BAFs, many of which share homology with factors associated with the yeast SWI/SNF ATPase (Vignali et al., 2000). Both Brg1 and hBrm1 have been shown to interact with ERα in a ligand-dependent manner (Ichinose et al., 1997), whilst BAF57, a protein present only in the mammalian SWI/SNF complex also interacts with ERα, and in addition to its role in the SWI/SNF complex, interacts with the p160 NR coactivators (Belandia et al., 2002). The ATPase activity of SWI/SNF is required for the complex to alter the orientation of the DNA on the surface of the nucleosome core, which in turn promotes transcriptional activator binding to nucleosomal DNA (Cote et al., 1994; Owen-Hughes et al., 1996).

1.3.1.1.1.2 ISWI

The imitation SWI (ISWI) family of chromatin re-modellers contains homology with the Swi/Snf gene over the region of the ATPase domain (Elfring et al., 1994) and the most well characterised are ACF (ATP-utilizing chromatin assembly and remodeling factor) (Ito et al., 1997), NURF (nucleosome-remodeling factor) (Tsukiyama and Wu, 1995), and CHRAC (chromatin accessibility complex) (Varga-Weisz et al., 1997). The remodelling and spacing factor (RSF) was identified in humans and shown to contain the ISWI protein within the complex (LeRoy et al., 1998). The ISWI complexes are smaller than SWI/SNF complexes consisting of fewer subunits but they require ATP hydrolysis in the same manner. Whereas the ATPase activity of the SWI/SNF is stimulated equally by free DNA and nucleosomal DNA

(Laurent et al., 1993), the substrate recognised by the ISWI ATPase complex is amino-terminal histone tails (Tsukiyama and Wu, 1995).

1.3.1.1.2 Histone/ protein modifying coactivators

Rates of gene transcription show a correlation with the degree of histone acetylation; hyperacetylated regions show a higher rate of transcription than hypoacetylated regions (Glass and Rosenfeld, 2000). This suggests that chromatin modifying complexes with histone acetyltransferase (HAT) activity could be required at promoter regions to overcome the repressive effects of the chromatin structure on transcription. Indeed, several HAT proteins are recruited to promoters of NR-regulated genes in a NR-dependent manner, resulting in an increase in histone acetylation within promoters of target genes (Glass and Rosenfeld, 2000; Metivier et al., 2003; Shang et al., 2000).

1.3.1.1.2.1 p160/ SRC

Proteins of approximately 160 kDa molecular mass were among the first factors found to interact with nuclear receptors in a ligand-dependent manner. Three related genes encode the p160 factors – SRC1/NCoA1, TIF2/GRIP1/SRC2/NCoA2 and p/CIP/AIB1/NCoA3/SRC3/ACTR/RAC3/TRAM1 (Chen et al., 1997; Li et al., 1997; Onate et al., 1995; Takeshita et al., 1997; Torchia et al., 1997; Voegel et al., 1996). The p160/SRC proteins are recruited by interacting with a hydrophobic groove involving α-helices 3, 4, 5 and 12 in the LBD of liganded NR, via small α-helical motifs containing the sequence leucine-X-X-leucine-leucine (LXXLL) in the p160/SRC proteins (Heery et al., 1997; Shiau et al., 1998). There are additional interactions between the C-terminal regions of p160/SRC proteins and the AF1 regions of NRs. It

is thought that the ability of p160 coactivators to interact with both AF1 and AF2 is important for the ability of these transactivation functions to synergize (Webb et al., 1998). The p160/SRC coactivators appear to act as bridging factors to mediate the recruitment of other coactivators including SWI/SNF chromatin remodelling complexes (Belandia et al., 2002), whilst the N-terminal region of p160/SRC coactivators have been shown to interact via the association domain 2 (AD2) with other cofactors, such as coactivator-associated arginine methyltransferase (CARM1) (Chen et al., 1999a), coiled-coil coactivator A (CoCoA) (Kim et al., 2003) and GAC63 (Chen et al., 2005). These interactions of p160/SRC proteins are regulated by post-translational modifications such as phosphorylation, methylation and acetylation; phosphorylation of a specific combination of residues determines which transcription factors the p160 coactivators are able to activate, allowing them to function as regulatable cofactors for a range of signalling pathways, suggesting that there maybe a "phosphorylation code" required for specific gene activation by NRs and coactivators (Wu et al., 2005). For example, the formation of a complex between SRC3/AIB1 and the ER has been shown to be dependent on the phosphorylation state of SRC3/AIB1 (Amazit et al., 2007) and ligand-dependent phosphorylation of SRC2/GRIP1 by p38 MAPK has been shown to potentiate NR activity (Frigo et al., 2006).

1.3.1.1.2.2 CBP/ p300

The CBP (cAMP-response element binding protein (CREB) binding protein) and p300 proteins are highly homologous phosphoproteins which act as histone acetyltransferases during NR-dependent transcriptional activation (Bannister and Kouzarides, 1996; Ogryzko et al., 1996). CBP/p300 contain multiple functional domains (Arany et al., 1994) including a HAT domain, a bromodomain, which is a

conserved region found in many chromatin-associated proteins capable of recognising acetylated lysines (Dhalluin et al., 1999), a KIX domain and three cysteine-histidine (CH)-rich domains which are important in mediating protein-protein interactions. These functional domains allow CBP/p300 to provide a scaffold for the assembly of multi-component transcription coactivator complexes, the association of which can be mediated by post-translational modification. Recruitment of CBP/p300 by liganded and DNA-bound NR is mediated by the p160 coactivators which interact with the KIX domain of CBP or p300 via the activation domain 1 (AD1) of the p160 coactivators (Kamei et al., 1996; Meng et al., 2004a; Yao et al., 1996). CARM1 associates with AD2 of the SRC2 p160 coactivator (Chen et al., 1999a) and has been shown to methylate SRC2 associated- CBP/p300 at residue R742 which appears to be required for SRC2 and SR-dependent transcriptional activation (Chevillard-Briet et al., 2002), whereas methylation by CARM1 within the SRC2 binding domain of CBP/p300 regulates the dissociation of SRC2 and CBP/p300 (Lee et al., 2005a). CARM1 is also involved in mediating the interaction between CBP and CREB; the cofactor CREB interacts with CBP via the KIX domain in a phosphorylation-dependent manner; this in turn is regulated by CARM1 which can methylate residues within the KIX domain, inhibiting the CREB-CBP interactions (Xu et al., 2001). There is evidence to suggest that the phosphorylation status of CBP/p300 is regulated by the cell-cycle-dependent kinases cyclin E/cdk2 with reported peaks in CBP/p300 HAT activity during the G1/S transition (Ait-Si-Ali et al., 1998), suggesting a role for CBP/p300 in promoting transcription of genes required for S-phase. In addition to their role in histone acetylation, CBP/p300 has been shown to serve a coactivator role in acetylating a range of substrates, including CREB, p53, Jun, Fos, p/CAF and $pp90^{RSK}$ (Bannister and Kouzarides, 1995; Bannister et al., 1995; Gu et al., 1999; Kwok et al., 1994; Lee et al., 1996; Nakajima et al., 1996; Yang et al., 1996). Acetylation of ERα by p300

within the hinge/ligand binding domain has also been show to occur in a ligand-dependent and coactivator-dependent manner (Kim et al., 2006; Wang et al., 2001). Acetylation of lysine residues 266 and 268 enhances DNA-binding and is thought to play a role in estrogen-dependent gene regulation (Kim et al., 2006).

1.3.1.1.2.3 P/ CAF

P/CAF (p300/CBP-associated factor) is homologous to the yeast protein GCN5 which is part of the SAGA complexes found in yeast (Grant et al., 1997; Yang et al., 1996). The SAGA complex requires the HAT activity of GCN5 in order to be transcriptionally active (Brownell et al., 1996) and in the same way P/CAF is part of a larger complex including hADAC, five TAF_{II}-like proteins and TRAP which requires the HAT activity of P/CAF in order to be transcriptionally active (Ogryzko et al., 1998; Vassilev et al., 1998). P/CAF binds to p300/CBP, contributing to the HAT activity, and also contains a bromodomain which recognises acetylated lysines within histones H3 and H4 (Kanno et al., 2004). P/CAF has been shown to be involved in gene expression not only by acetylating histones but also by acetylating a subset of transcription factors including p53 and AR (Fu et al., 2000; Gu and Roeder, 1997). Cyclin D1 is a member of the G_1 cyclin family required for eukaryotic cell cycle progression and has been shown to contribute to functions of P/CAF. Competition between cyclin D1 and P/CAF for binding to liganded AR, has been shown to result in repression of hormone-dependent AR activation (Reutens et al., 2001). Conversely, cyclin D1 has been shown to potentiate the activity of ERα by facilitating the association of ERα and P/CAF (McMahon et al., 1999).

1.3.1.1.2.4 TRAP/ DRI P/ ARC

The TR-associated protein (TRAP)/vitamin D receptor interacting protein (DRIP)/activator-recruited cofactor (ARC) complexes were originally identified separately as TR, VDR and sterol-regulated factor SREBP-1a interacting protein complexes, respectively (Fondell et al., 1996; Naar et al., 1998; Rachez et al., 1998). The three complexes have however, been shown to contain many of the same subunits and act as transcriptional coactivators to some extent for a wide range of NRs (Naar et al., 1999; Rachez et al., 1999; Yuan et al., 1998). DRIP205/TRAP220/ARC205 subunits contain an LXXLL motif that mediates recruitment to the LDB of NRs and anchors the other DRIP/TRAP/ARC subunits to the receptor (Naar et al., 1999; Rachez et al., 2000; Yuan et al., 1998). The Mediator protein complex enhances transcriptional activation by recruitment of Pol II to the gene promoter and stimulates phosphorylation of the largest subunit of Pol II by TFIIH (Jiang et al., 1998). Subunits of the TRAP/DRIP/ARC complexes have been shown to be similar, if not identical to subunits of the Mediator complex (Ito et al., 1999; Naar et al., 1999; Rachez et al., 1999) and the complexes carry out a similar role of Pol II recruitment and stimulation of TFIIH-mediated Pol II phosphorylation, enabling transcription initiation (Metivier et al., 2003).

1.3.1.1.2.5 E6-AP and RPF1

E6 papilloma virus-associated protein (E6-AP) and the closely related RPF-1, are E3 ubiquitin-protein ligases (Imhof and McDonnell, 1996; Nawaz et al., 1999b). E3 ubiquitin-protein ligases are involved in selective degradation of proteins via the 26S proteasome pathway by tagging target proteins with ubiquitin at lysine residues. ERα is subject to ligand-dependent proteolysis due to ubiquitination (Alarid et al., 1999; Horigome et al., 1988; Nirmala and Thampan, 1995) which acts to

continuously turn over ERα on responsive promoters in a cyclical manner by the cyclical association of E6-AP with the target promoter, permitting continuous responses to changes in the concentration of ligand (Reid et al., 2003). Overexpresion of E6-AP has been shown to correlate with a decrease in levels of ERα and AR via the ubiquitin-proteasome pathway (Gao et al., 2005). However, E6-AP does not appear to be involved with ubiquitin-mediated degradation of PR or TR (Nawaz et al., 1999a), with phosphorylation of the PR by MAPK being responsible for targeting the receptor for degradation (Lange et al., 2000), suggesting that E6-AP/RPF1 have specific substrates and do not act as coactivators for the whole NR superfamily. Cessation of transcription is brought about by the dissociation of RNA polymerase from the gene. As part of this process, NRs and their coactivators are degraded in an ubiquitin-proteasome-dependent manner, preventing reinitiation of transcription (Lonard and O'Malley, 2006). Ubiquitination of coactivators and NRs by E3 ligases such as E6-AP and RPF1 is therefore an important mechanism for controlling the cyclical nature of NR transcriptional activation by targeting the proteins for degradation.

1.3.1.1.2.6 PGC1α

The peroxisome-proliferator-activated receptor-γ (PPARγ) co-activator 1α (PGC1α) was originally identified as a tissue-specific coactivator of PPARγ (Puigserver et al., 1998) and has been implicated in the regulation of many important physiological processes, including adaptive thermogenesis (Puigserver et al., 1998) and hepatic gluconeogenesis (Yoon et al., 2001). PGC1α interacts with PPARγ in a ligand-independent fashion via the hinge region of the receptor and independently of a LXXLL motif within the coactivator, whereas the ligand-induced interaction with ERα (Tcherepanova et al., 2000), TR (Wu et al., 2002b), RXR (Delerive et al., 2002)

and GR (Yoon et al., 2001) depends on the AF2 region of the receptor and the LXXLL motif of the PGC1α. The tissue-specific expression of PGC-1, which is expressed at high levels in tissues such as heart, skeletal muscle, kidney, and brown fat (Esterbauer et al., 1999; Knutti et al., 2000; Puigserver et al., 1998) is induced in response to particular physiological states, such as exposure to cold or fasting (Lehman et al., 2000; Puigserver et al., 1998; Yoon et al., 2001). Induction of PGC1α leads to the activation of pathways important for energy homeostasis, such as adaptive thermogenesis, mitochondrial biogenesis, fatty acid oxidation, and gluconeogenesis by tissue-specific activation of specific enzymes, coactivators and transcription factors, including NRs (Herzig et al., 2001; Lehman et al., 2000; Puigserver et al., 1998; Vega et al., 2000; Wu et al., 1999; Yoon et al., 2001).

1.3.1.2 Nuclear Receptor Corepressors

Transcriptional activation by NRs requires the cyclical recruitment and dissociation of NRs and their coactivators which carry out chromatin remodelling and histone acetylation and methylation. Importantly, this cycling also requires chromatin remodelling and histone deacetylation and demethylation, processes performed by corepressor complexes. Additionally, many NR, such as RAR and TR, repress the expression of regulated genes when bound to gene promoters in the unliganded state (Chen and Evans, 1995; Horlein et al., 1995). Furthermore, in addition to stimulating the expression of regulated genes, agonist-bound NR repress the expression of many genes. For example, gene expression microarray analyses have shown that 70% of the estrogen-regulated genes in the MCF7 breast cancer cell line are repressed (Frasor et al., 2003). Moreover, antagonists such as the anti-estrogen Tamoxifen, prevent coactivator recruitment, but facilitate corepressor recruitment, to repress the expression of genes whose expression is stimulated by estrogen

(Brzozowski et al., 1997; Shang et al., 2000; Shiau et al., 1998). Hence, corepressors are essential for gene repression by NR, as well as being required for the expression of genes activated by NR ligands.

1.3.1.2.1 NCoR/ SMRT

The NR corepressors NCoR (Horlein et al., 1995) and SMRT (silencing mediator for retinoid and thyroid-hormone receptors) (Chen and Evans, 1995) are encoded by distinct genetic loci, however, they share substantial sequence homology (Ordentlich et al., 1999; Park et al., 1999) and assemble into similar or even identical complexes, with stabilizing factors such as TBL1/TBLR1 and GPS2 (Guenther et al., 2000; Li et al., 2000; Yoon et al., 2003; Zhang et al., 2002a). Although they do not harbour intrinsic enzymatic activity themselves, NCoR and SMRT facilitate the recruitment of several other corepressors, including many histone deacetylases (Huang et al., 2000; Kao et al., 2000; Li et al., 2000; Yoon et al., 2003). Regulatory events that cause dissociation of NCoR/SMRT also cause the release of the remaining corepressor complex and a loss of repression (Glass and Rosenfeld, 2000). The actions of NCoR/SMRT are thought to be regulated by multiple signal transduction pathways. Activation of MEKK1, a member of the Raf-MAPK growth factor signalling cascade, leads to the inhibition of the ability of SMRT to interact with other corepressors and NRs and causes redistribution of SMRT to the cytoplasm (Hoberg et al., 2004; Hong and Privalsky, 2000), whereas NCoR is resistant to these inhibitory effects (Jonas and Privalsky, 2004). However, different splice variants of SMRT respond differently to MEKK1 signalling with the SMRTsp18 variant showing a resistance to kinase-induced redistribution, suggesting a mechanism whereby cells can control their transcriptional response to the Raf-MAPK signalling cascade by selective expression of SMRT splice variants (Jonas et al., 2007).

NCoR and SMRT are recruited to NR LBDs through α-helical regions called the corepressor-nuclear-receptor (CoRNR) box, having a core consensus motif, LXXI/HIXXXI/L (Hu and Lazar, 1999; Nagy et al., 1999; Perissi et al., 1999). This motif is similar to the shorter α-helical LXXLL motif of coactivators and it has been proposed that the conformational change of the LBD due to ligand binding favours interaction with this shorter α-helix and so brings about the exchange of corepressors for coactivators (Perissi et al., 1999).

1.3.1.2.2 LCoR and RIP140

LCoR (ligand-dependent nuclear-receptor corepressor) and RIP140 (receptor-interacting protein 140) belong to a group of corepressors which bind agonist-bound NRs. Initial studies on RIP140 suggested the protein interacted with ER and other NRs via the LBD in such a way as to promote transcriptional activation (Cavailles et al., 1994; Cavailles et al., 1995; Joyeux et al., 1997; L'Horset et al., 1996). However, further studies showed that expression of RIP140 could not replace the function of SRC coactivators (Torchia et al., 1997) and indeed competed with SRC coactivators for binding to NRs, resulting in inhibition of NR activity (Treuter et al., 1998). However, this finding was due to the fact that similarly to coactivators, RIP140 and LCoR use a LXXLL motif to interact with the LBD of NRs (Fernandes et al., 2003; Heery et al., 1997). LCoR and RIP140-mediated repression is in fact brought about by the recruitment of HDACs and the corepressors CtBP (C-terminal binding proteins) (Carascossa et al., 2006; Castet et al., 2004; Fernandes et al., 2003; Wei et al., 2000), although the involvement of CtBP has been shown to be unnecessary for the inhibitory action of RIP140 on AR (Carascossa et al., 2006). RIP140 recruitment is not only gene-specific (Castet et al., 2006) but post-translational modifications of RIP140 control the recruitment of associated factors and nuclear export of the

corepressor (Gupta et al., 2005; Huq and Wei, 2005), suggesting that corepressors like RIP140 and LCoR provide a mechanism for NRs to down-regulate transcription of target genes upon activation of the receptor in a process that can be influenced by other signalling pathways.

1.4 Estrogen receptors

The estrogen receptor (ER) is a steroid hormone receptor which is required for the development and maintenance of the female and male reproductive systems and secondary sexual characteristics (Hewitt et al., 2005). In the hypothalamic-pituitary-gonadal axis, gonadotropin-releasing hormone is secreted in an episodic fashion from the hypothalamus to activate the production of gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) from the anterior pituitary of the brain. LH and FSH are released in a pulsatile manner to act at the gonad to control both gametogenesis (spermatogenesis or oogenesis) as well as steroidogenesis. The major sex hormones estradiol, progesterone, and testosterone are secreted in response to gonadotropins and, in turn, feedback at the level of the hypothalamus and pituitary to control normal reproductive function (Wierman, 2007). These hormones exert their effects by binding to intracellular or membrane receptors, resulting in activation of signaling pathways and transcription factors that regulate gene expression. Alterations in the regulation of these signalling molecules results in uncontrolled cell survival and proliferation, leading to tumour initiation and progression (Silva and Shupnik, 2007).

As stated above, estrogen actions are mediated by members of the NR superfamily of transcription factors, ERα and ERβ, encoded by distinct genes, with the human ERα gene being encoded on chromosome 6q and the human ERβ gene encoded on chromosome 14q (Herynk and Fuqua, 2004). The activity of ERα,

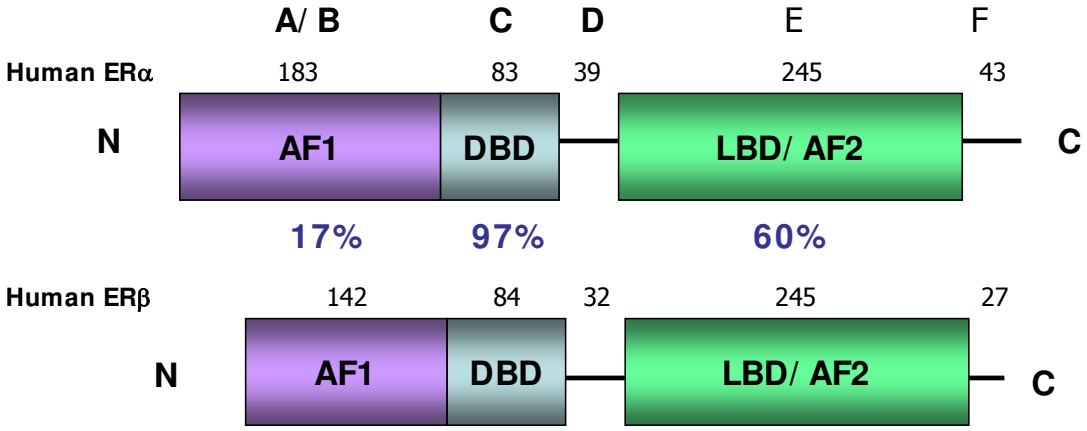


Figure 1.4 Structure of ERα and ERβ
ERα and ERβ are highly homologous, with sequence homology being greatest within the DBD which share 97% homology and the LBD/AF2 domains, which share 60% homology. The other regions are considerably less well-conserved, for example the A/B regions (AF1) share just 17% homology.

especially its involvement in breast cancer, has been studied in depth but the activities of ERβ are less well elucidated. This thesis involves work carried out using ERα; hence ERβ will be discussed briefly before a more detailed discussion of ERα.

1.4.1 Estrogen Receptor beta

The ERβ gene was identified from a prostate cDNA library in 1996 (Kuiper et al., 1996). Both ERα and ERβ bind estrogen and interact with ERE sequences, although with differing binding and transcriptional activities (Katzenellenbogen and Katzenellenbogen, 2000; Katzenellenbogen et al., 2001). Mouse knock-out studies have shown that ERβ is involved in the normal function of tissues including ovaries, uterus, lung, prostate, and for neuronal function, although in all of these tissues except the ovary, ERα is the most abundantly expressed form of ER (Couse and Korach, 1999; Couse et al., 1997; Dupont et al., 2000). In the normal breast, whereas ERα is only detected in the nuclei of luminal epithelial cells of ducts and lobules, ERβ is distributed more evenly and is the dominant form of ER (Shaw et al., 2002; Speirs et al., 2002).

Comparison of the ERα and ERβ amino acid sequences (Figure 1.4) shows that they are extremely highly conserved in the DBD, with 97% amino acid sequence homology, and are identical in the P-box, accounting for their ability to bind to ERE sequences with similar specificities and affinities (Cowley et al., 1997). There is also a high degree of homology within the LBDs of ERα and ERβ, and both receptors bind estrogen and anti-estrogens with near equal affinity (Kuiper et al., 1997), although recent studies have identified synthetic ERα and ERβ-specific ligands (De Angelis et al., 2005; Harrington et al., 2003; Harris et al., 2002; Shiau et al., 2002). These

similarities suggest that ERα and ERβ can heterodimerise, and indeed several studies have shown that the receptors can form heterodimers on DNA (Cowley et al., 1997; Pace et al., 1997). As is observed when comparing ERα from different species, the A/B region of the ER is poorly conserved between the two ER isotypes, with only 17% identity between human ERα and ERβ. Unlike ERα, the AF1 within the A/B region of ERβ has very low activity (Enmark et al., 1997).

Gene expression microarray studies in the U2OS osteosarcoma cell line, in which ERα and ERβ were stably transfected and expressed in an inducible manner, show that very few ERα regulated genes are also regulated by ERβ. Rather, these studies showed that ERα and ERβ regulate the expression of distinct sets of genes (Monroe et al., 2003; Stossi et al., 2004), suggestive of specific and distinct roles for ERα and ERβ. In reporter gene studies ERα activates gene expression much more potently than ERβ, which has led to the proposal that ERβ may act as a negative regulator of ERα activity (Lindberg et al., 2003; Pettersson et al., 2000). Additionally, five alternative splice variants of ERβ have been described; the full length ERβ (ERβ1) and the other splice variants known as ERβ2/ ERβcx, ERβ3, ERβ4 and ERβ5 (Moore et al., 1998). Of these variants, most work has concentrated on ERβ1 and ERβ2/ ERβcx. These studies have shown that whereas ERβ1 can heterodimerize with ERα at EREs, ERβ2/ ERβcx not only is unable to bind to EREs but also appears to inhibit ERα binding to EREs (Omoto et al., 2003). Hence some of these isoforms have the potential to be potent inhibitors of full-length ERβ, but also of ERα, providing evidence for the potential role of ERβ as a negative regulator of ERα. In this respect, there is evidence showing that ERβ expression is reduced in breast tumours; this decrease in ERβ mRNA levels has been suggested to be associated with breast tumorigenesis and the reduction in expression being associated with methylation of the ERβ gene (Zhao et al., 2003).

Despite these findings, the potential role of ERβ in breast cancer progression remains highly controversial. Levels of ERβ protein expression have been shown to decrease during the progression of normal breast through to invasive carcinoma (Roger et al., 2001; Skliris et al., 2003). Further, immunohistochemical studies have suggested that a high ratio of ERα:ERβ protein levels is characteristic of benign breast lesions which progress to breast cancers (Fuqua et al., 2003; Shaaban et al., 2005; Skliris et al., 2003). Antibody-based studies on clinical samples have shown that ERα-positive breast tumours which express ERβ respond better to tamoxifen treatment and therefore have a better rate of survival, although ERβ expression had little effect on the outcome of untreated patients (Hopp et al., 2004; Mann et al., 2001). This has led to suggestions that ERβ expression acts as a predictive marker for tamoxifen response in breast cancer (Hopp et al., 2004; Mann et al., 2001; Nakopoulou et al., 2004).

However, studies analysing RNA levels correlate ERβ expression with markers of poor progression (Iwao et al., 2000; Speirs et al., 1999a; Speirs et al., 1999b). Such discrepancies may be due to the fact that when RNA is measured from either breast tissue sections or chunks of tissue, the RNA represents a pool of all the different cell types present in the biopsy, including normal epithelial cells expressing ERβ and the contaminating normal epithelium may have a significant influence (Saji et al., 2005), whilst immunohistochemical detection although allowing ERß levels in breast cancer cells to be directly measured, are less quantitative, and may detect some isoforms only, depending on the antibody used (Fuqua et al., 2003; Speirs et al., 2002). Further investigation is clearly required before a clear role for ERβ in breast cancer can be established.

1.4.2 Estrogen receptor alpha

As mentioned previously, the unliganded ERα is present in the nucleus, (King and Greene, 1984) and functions primarily as a transcription factor by regulating gene expression at the mRNA level in response to binding ligand (O'Malley et al., 1968). Genomic functions of ERα describe those that involve the receptor acting as a transcription factor by interacting with DNA to either activate or repress gene expression. Classically, these genomic functions of ERα require direct interaction of ERα with DNA via ERE sequences in promoter regions of estrogen-regulated genes (Klein-Hitpass et al., 1988). Initial studies used cloning of cDNAs induced by estrogen treatment of breast cancer cells to identify a number of ERα-regulated genes, including pS2/TFF1 (trefoil factor 1) (Brown et al., 1984; Jakowlew et al., 1984) and cathepsin D (Elangovan and Moulton, 1980). Expression microarrays have allowed further identification of ERα-regulated genes by the analysis of alterations in gene expression after ligand treatment on a genome-wide scale. This technique has been used to show that 70% of estrogen-regulated genes are in fact down-regulated, including genes involved in transcriptional repression, antiproliferation and proapoptotic genes. Conversely, up-regulated genes included positive proliferation regulators, multiple growth factors, genes involved in cell cycle progression and regulatory factor-receptor loops (Frasor et al., 2003), highlighting the role of ERα in cell-cycle progression.

The identification of c-Myc (Dubik et al., 1987; Dubik and Shiu, 1988) and cyclin D1 (Altucci et al., 1996) as estrogen-regulated genes, highlighted another mechanism of ERα gene activation by indirect tethering of ERα to DNA through protein-protein interactions. These indirect protein-protein interactions of ERα at 'non-classical' response sites which do not contain ERE sequences require ERα interactions with other transcriptional elements including AP1 (c-fos/c-jun), NF-κB,

cAMP-like elements, and Sp-1 DNA binding motifs (Dubik and Shiu, 1992; Kushner et al., 2000a; Kushner et al., 2000b; Sabbah et al., 1999; Safe, 2001).

1.5 Non-classical ERα response sites

In addition to regulating gene expression by binding to ERE sequences in promoters of estrogen-regulated genes, as exemplified by the pS2 gene, ERα can also regulate gene expression by recruitment to gene promoters through interaction with other transcription factors, such as AP1, and Sp1.

Estrogen has been shown in human breast cancer cells to transiently upregulate EGFR mRNA and protein (Yarden et al., 1996); this induction appears to be due to the recruitment of ERα on the EGFR gene via protein-protein interactions with Sp1 (Salvatori et al., 2003). Many genes, including IGF-1 and c-Myc (Dubik and Shiu, 1992; Umayahara et al., 1994), are regulated by ERα recruitment to AP1-responsive elements through interaction with AP1. Estrogen-bound ERα increases the activity of AP1 by binding to the coactivators CBP and GRIP1 which are recruited by AP1 via ERα (Kushner et al., 2000b). The ERα-DBD is required for recruitment of ERα to the AP1 site and the A/B region and LBD are required for transcription activation (Cheung et al., 2005).

Sequencing of the human and mouse genomes have allowed computational analysis techniques to identify 660 genes encoding potential EREs which are conserved between the two genomes and are within 15 kb of a mRNA start site (Bourdeau et al., 2004). Recently however, chromatin immunoaffinity purification microarray (ChIP-chip) has been used to identify actual ER binding sites (Carroll et al., 2005; Carroll et al., 2006). As little as 4% of ER binding sites were mapped to the 1 kb promoter-proximal region, with the rest found to be located in distal cis-regulatory enhancer regions, some being located more than 150 kb from the target

gene transcription start site. In addition to identifying many EREs in genes for which the mechanisms by which ERα regulates their expression was unclear, these studies also demonstrated that the EREs in many estrogen-responsive genes map close to Forkhead transcription factor binding sites, and require the Forkhead transcription factor FoxA1 binding at Forkhead factor binding sites near the EREs for the estrogen-stimulated expression of estrogen-regulated target genes (Carroll et al., 2005; Laganiere et al., 2005).

The finding that ERα can regulate genes by directly binding to DNA sequences located at considerable distances from the gene promoter puts into doubt some of the ideas about indirect recruitment of ERα by other transcription factors such as AP1 and SP1. For example, the Myc gene was originally identified as being transcriptionally regulated by ERα indirectly binding the promoter region of this gene (Dubik and Shiu, 1992), however, Carroll et al have identified an ER-binding site 67 kb upstream of Myc which showed a greater increase in ERα-binding upon treatment with estrogen than the originally identified site within the promoter region (Carroll et al., 2006), suggesting that ERα interaction with the promoter region is not the primary ERα binding site. Cyclin D1 gene expression is estrogen-regulated and ERα-binding sites within the proximal promoter of cyclin D1 have been mapped to a cAMP response element (CRE), an AP1 site and a Sp1 site (Altucci et al., 1996; Castro-Rivera et al., 2001; Sabbah et al., 1999). Recent data has suggested that the ERα-binding site at the cyclin D1 gene in breast cancer cells is removed from the previously described sites and depends on a cell-type-specific enhancer, enh2, downstream of the cyclin D1 coding region (Eeckhoute et al., 2006). Estrogen stimulation of breast cancer requires cell-specific ERα-mediated stimulation of cyclin D1 expression which promotes cell cycle progression. ERα interactions are

dependent on FoxA1 which interacts directly with enh2 and other recruited transcription factors.

Further research into ERα binding sites needs to be carried out to ascertain which of the previously identified non-classical estrogen regulated genes actually bind ERα at the promoter regions indirectly, or if they are largely controlled by ERα binding directly at distal enhancer sites.

1.6 Non-genomic actions of ERα

In addition to regulating gene expression, either through direct binding to EREs or by indirect recruitment to gene promoters, ERα can regulate cellular processs through non-genomic functions for which ERα does not act as a transcription factor. These non-genomic actions of ERα tend to involve the non-reproductive actions of estrogen, such as influences on the central nervous system, cardiovascular system and the adult skeleton and include processes which control the release of calcium, secretion of prolactin, generation of phosphatidylinositol 3,4,5-triphosphate (PIP_3), nitric oxide and activation of mitogen-activated protein kinases (MAPK). These are very rapid effects which are dependent on the cell type and are not sensitive to inhibitors of transcription, although the resulting signalling cascades lead to downstream events that modulate selected gene transcription. These effects appear to require ERα and are mediated by ERα localization at the cell membrane (Marquez et al., 2006; Rai et al., 2005; Wessler et al., 2006).

Growth factor receptors are found to concentrate together with other components of the ras-MAPK signalling pathway in specific domains of the plasma membrane, termed lipid rafts (Pietras, 2003). Lipid rafts are moving platforms formed by the association of glycosphingolipids, cholesterol and specific proteins (Simons and Ikonen, 1997). ERα has been shown to associate with growth factor

receptors at the plasma membrane via caveolae-like lipid rafts which contain additional integral membrane proteins that interact with several other lipid-modified proteins involved in signal transduction, including G-proteins and protein kinase C (PKC) (Marquez et al., 2006).

The two signalling cascades which can be activated by growth factor receptor activation, Src-Shc-Ras-Raf-MAPK and phosphatidylinositol 3-kinase (PI3K)/Akt, are closely interconnected and it has been shown that the formation of a ternary complex of ERα, Src and PI3K, leads to simultaneous activation of both pathways, triggering the S-phase entry of the cell (Castoria et al., 2001). The ErbB family of growth factor receptors are receptor tyrosine kinases which possess an intrinsic protein kinase activity and include EGFR/ErbB1, ErbB2/HER2/Neu, ErbB3/HER3 and ErbB4/HER4 (Stoica et al., 2003). Ligand binding activates the growth factor receptors which recruit PI3K to the plasma membrane. PI3K is one of the key enzymes responsible for phosphoinositide metabolism and subsequent production of PIP_3 which binds Akt (Lee et al., 2005b). Akt is then activated through phosphorylation at the plasma membrane (Campbell et al., 2001) and promotes cell survival by mediating the activities of a number of transcription factors in the nucleus. Inhibitors of PI3K and dominant-negative Akt have been shown to block the effect of estrogen on ERα activity by 70-80% and 55-63% respectively and it has been suggested that Akt interacts directly with ERα via the pleckstrin homology (PH) domain of Akt. Constitutively active Akt appears to mimic the effects of estrogen in the absence of ligand and the effects of Akt are not blocked by tamoxifen (Stoica et al., 2003).

Estrogen rapidly activates the PI3K/Akt pathway by triggering the binding of ERα to the p85 regulatory subunit of PI3K (Castoria et al., 2001; Lee et al., 2005b; Simoncini et al., 2000). Estrogen-induced expression of the early response gene c-

Fos in MCF7 cells is thought to occur by activation of extracellular signal-regulated kinases (ERKs) mediated by estrogen-induced activation of the raf-MAPK signalling pathway (Almeida et al., 2006). Estrogen can also promote intracellular calcium mobilization and the generation of PIP3 in the nucleus by binding to and activating the G protein GPCR30 in the endoplasmic reticulum. Heterotrimeric G proteins are activated conventionally by members of a family of G-protein coupled receptors (GPCRs). Activated G protein subunits are competent to modulate the activity of downstream effectors (Revankar et al., 2005). The membrane-associated sub-population of ERα has been shown to interact directly with Gα-protein subunits causing activation and this has been suggested to be the mechanism behind ERα activation of endothelial nitric oxide synthase in cultured endothelium (Kumar et al., 2007; Wyckoff et al., 2001).

Activation of ERα-mediated non-genomic pathways is dependent on the structure of the ligand, with different estrogenic compounds causing activation or inhibition of kinase activity (Li et al., 2006). Wessler et al. have recently identified two substances which can cause activation of Akt and ERK through non-genomic actions of ERα whilst displaying weak activation of genomic ERα activities and suggest that these pathway-selective ERα ligands may form the basis for novel hormone replacement strategies exhibiting lesser side effects than the existing treatments which include the onset of osteoporosis and breast cancer (Wessler et al., 2006). The relative importance of the non-genomic and genomic actions of ERα remains unclear, however, and the pathway-specific ER ligands should help to clarify this position. Notwithstanding, the genomic actions of ERα are likely to be particularly important for mediating estrogen action in most cell types, particularly in breast cancer cells, since gene expression microarray and global ChIP strategies

have shown that ERα directly regulates the expression of hundreds to thousands of genes in target cells.

1.7 Regulation of ERα activity by Phosphorylation

Classically, ERα, like other NRs is activated upon binding to its cognate ligand. There is considerable evidence, however, that ERα activity can be regulated by post-translational modification, including acetylation, glycosylation and phosphorylation (Cheng and Hart, 2000; Kim et al., 2006; Lannigan, 2003). In particular, phosphorylation of ERα can result in its activation in the absence of ligand. Evidence for this mode of ERα regulation was first described *in vivo*, when it was shown that following ovariectomy, estrogen-dependent uterine proliferation could be rescued by administration of EGF and that co-administration of the anti-estrogen ICI 164, 384 could inhibit the EGF rescue, indicating that the EGF-mediated uterine proliferation in ovariectomised mice was ER-dependent. Further, EGF could not rescue uterine proliferation in ERα knockout mice. TGFα and IGF-1 can similarly stimulate uterine proliferation in an ER-dependent manner in ovariectomised mice and rats (Curtis et al., 1996; Ignar-Trowbridge et al., 1992; Nelson et al., 1991). Other studies have revealed that in addition to growth factors, dopamine and other activators of protein kinase A stimulate ERα activity in the absence of ligand (Aronica and Katzenellenbogen, 1993; Bunone et al., 1996; Chen et al., 1999b; Ignar-Trowbridge et al., 1993; Pietras et al., 1995; Power et al., 1991). Together, these findings led to a considerable effort in determining the sites of phosphorylation and mechanisms by which ERα phosphorylation can regulate its activity, and led to the proposal that ligand-independent activation of ERα by phosphorylation could contribute to resistance to anti-estrogen and estrogen withdrawal therapies in breast cancer

patients and is considered the most important post translational modification of ERα (Ali and Coombes, 2002; Lannigan, 2003; Likhite et al., 2006).

1.7.1 Serine 104/ 106/ 118

Deletional analysis and phosphopeptide mapping studies identified serines 104, 106 and 118 within the A/B region of ERα as major sites of phosphorylation (Figure 1.5) and their mutation has been shown to reduce transactivation by ERα (Ali et al., 1993; Chen et al., 2000; Chen et al., 2002; Joel et al., 1995; Kato et al., 1995; Le Goff et al., 1994).

Using mutational studies it has also been shown that S104 and S106 are phosphorylated *in vitro* by cyclin A-cdk2 in a ligand-independent manner, enhancing the transcriptional activity of ERα (Rogatsky et al., 1999). S104 and S106 can also be phosphorylated by ERK1/2 MAPK (Thomas et al., 2008), whilst their phosphorylation by GSK3α has also been described (Medunjanin et al., 2005). Studies carried out in cells, using radioactively labelled ERα transiently transfected into COS1 cells have shown that Cdk7 mediates ligand-dependent phosphorylation of S118 (Chen et al., 2000; Chen et al., 2002). Estrogen treatment stimulates ERα phosphorylation and co-transfection of Cdk7 significantly increases this. Mutation of S118 prevented Cdk7 mediated phosphorylation of ERα showing that S118 is the sole target for Cdk7. Cdk7 is part of the Cdk-activating kinase (CAK) complex of TFIIH and S118 phosphorylation occurs in a ligand-dependent manner which involves TFIIH interacting with the AF2 via the TFIIH subunits, p62 and XPD. This ligand-dependent phosphorylation of S118 by Cdk7 stimulated ERα activity in the presence of estrogen, as well as in the presence of tamoxifen. These findings were confirmed following expression of ERα in Drosophila cells (Ito et al., 2004). However, cell-based studies using Cdk7 inhibitors, DRB and roscovitine, showed that Cdk7 inhibition did not

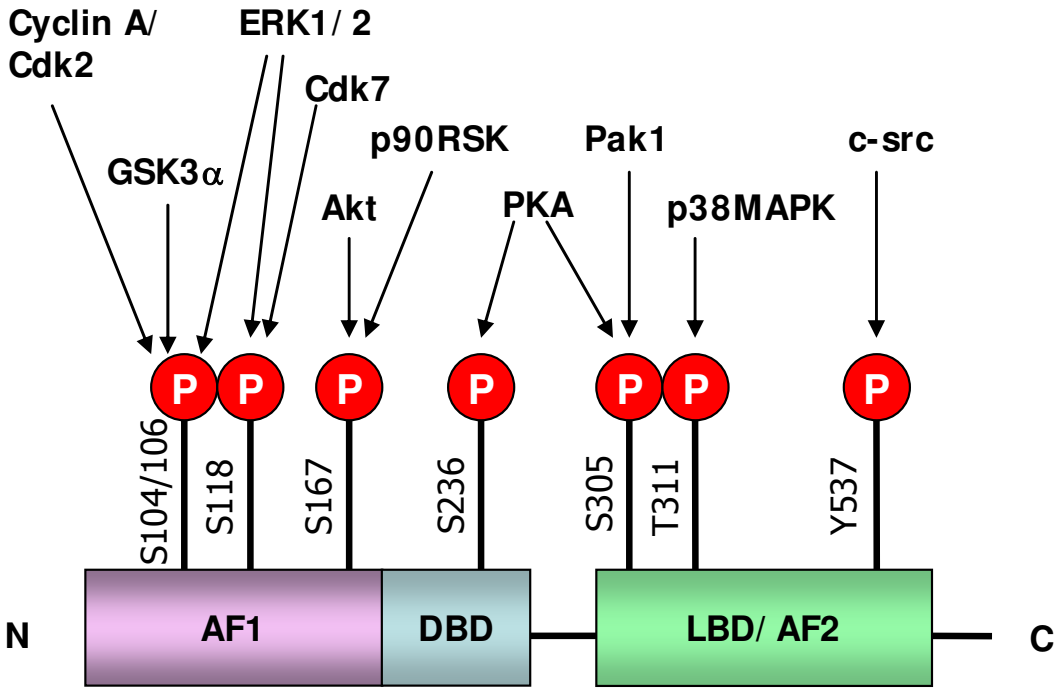


Figure 1.5 Phosphorylation sites of ERα
Phosphorylation within the AF1 domain has been shown to enhance transactivation by ERα, phosphorylation within the DBD has been shown to inhibit dimerization and phosphorylation within the LBD/AF2 region has been shown to increase estrogen sensitivity, whilst phosphorylation of Y537 appears to regulate coactivator recruitment.

inhibit estrogen-induced phosphorylation of S118 suggesting that Cdk7 does not contribute to S118 phosphorylation in the cell lines used (MCF7, ZR-75-1 and T47D) (Weitsman et al., 2006), although recent studies from our laboratory show that S118 phosphorylation is inhibited by a specific Cdk7 inhibitor (Heathcote et al., unpublished). In the study by Weitsman et al. (2006), however, inhibition of IKKα using BAY-11-7082 inhibited phosphorylation of S118, suggesting that estrogen-stimulated phophorylation of S118 may be mediated by IKKα (Weitsman et al., 2006).

In addition to its phosphorylation by Cdk7, S118 is phosphorylated in a ligand-independent manner by activation of the MAPK signalling pathway and phosphorylation by p42/p44 MAPK enables ligand-independent activation of ERα whilst enhancing ligand-dependent activation (Kato et al., 1995). Activation of p42/p44 MAPK in response to cell treatment with EGF and PMA has been used to show induction of S118 phosphorylation (Bunone et al., 1996; Chen et al., 2002; Joel et al., 1998b), whilst post-operative treatment of patients with the EGFR inhibitor Gefitnib inhibited MAPK activation and reduced S118 phosphorylation (Polychronis et al., 2005). However, immunohistochemical staining of primary breast cancer biopsies shows that high-level S118 phosphorylation is an indicator of a better prognosis and likelihood of response to tamoxifen (Murphy et al., 2004a; Murphy et al., 2004b; Sarwar et al., 2006). However, S118 phosphorylation was increased in biopsies taken from patients who relapsed following tamoxifen treatment, when compared with the pre-treatment biopsy (Sarwar et al., 2006), whilst low-level S118 phosphorylation, when coupled with high phosphorylation at S167 has been shown to be associated with improved survival (Yamashita et al., 2008), suggesting that S118 phosphorylation may indeed play a role in endocrine resistance.

1.7.2 Serine 167

Growth factors, including epidermal growth factor (EGF), insulin-like growth factor-1 (IGF1) and heregulin-β1 (HRG-β1), can modulate the genomic activities of ERα via the PI3K/Akt pathway (Stoica et al., 2003). PI3K and Akt are capable of activating ERα in the absence of estrogen by the phosphorylation of ERα at serine 167. This is thought to modulate coactivator and corepressor interactions of AF1, causing an increase in ERα activity in a promoter-specific fashion (Campbell et al., 2001). S167 is also phosphorylated in response to the activation of the MAPK pathway by p90ribosomal S6 kinase (p90RSK), a kinase downstream of MAPK (Joel et al., 1998a). However, PI3K-mediated stimulation of Akt also stimulates activation of Rsk. The relative contributions of Akt and p90RSK to S167 phosphorylation are unclear as yet but it is likely that both p90RSK and Akt regulate ERα activity with possible differences depending on cell type and extracellular signals (Lannigan, 2003). Investigation of S167 phosphorylation by immunohistochemistry showed that in patients with ERα-positive breast cancer, phospho-S167 positivity is associated with a better prognosis and likelihood of response to hormonal therapies (Jiang et al., 2007). In this study, S167 positivity was associated with phosphorylated (activated) p90RSK and with phosphorylated Akt. The association with phospho-p90RSK was considerably stronger than that with phospho-Akt and phospho-p90RSK was also associated with a better prognosis, whereas phospho-Akt was associated with worse outcome, suggesting that phosphorylation of S167 by p90RSK is more important in breast cancer cells. Evidence for S167 phosphorylation as a positive prognostic factor has also been provided by another study (as described above), which indicated that low-levl S118 phoshproylation and high-level S167 phosphorylation are predictive of a better outcome in breast cancer patients (Yamashita et al., 2008).

1.7.3 Serine 236

S236 is located within the DBD and has been shown to be phosphorylated by protein kinase A (PKA) *in vitro*, preventing receptor dimerisation and consequently DNA binding by ERα, although these effects were abrogated in the presence of ligand. However, over-expression of PKA was shown to cause phosphorylation in a ligand-independent fashion (Chen et al., 1999b). The authors showed that in the absence of ligand ERα dimerisation is mediated by the DBD and that phosphorylation of S236 by PKA inhibits DBD dimerisation, but that estrogen and tamoxifen binding by the receptor allows dimerisation through the LBD, and consequent DNA binding by ERα. Indeed, the anti-estrogen ICI 164,384, could not rescue the inhibition of DNA binding upon S236 phosphorylation, presumably since ICI 164,384 also inhibits LBD dimerisation (Metzger et al., 1995). However, Likhite et al. (2006) showed that phosphorylation of S236 increases the affinity of the receptor for estrogen without altering the affinity for the anti-estrogen tamoxifen and did not show any alterations in DNA binding or receptor dimerization (Likhite et al., 2006). They postulate that these differences may be due to receptor phosphorylation enhancing interactions with coregulators that modulate dimerization or DNA interactions.

1.7.4 Serine 305

S305 is located at the junction of the hinge region and the LBD and phosphorylation of this site by PKA *in vitro* has been linked to tamoxifen resistance (Likhite et al., 2006; Michalides et al., 2004; Zwart et al., 2007). Phosphorylation of S305 increases transcriptional activity and has been implicated in preventing acetylation of lysine 303 (Cui et al., 2004). Lys303 is often mutated to arginine in breast cancer and this mutation has been associated with hypersensitivity to estrogen (Fuqua et al., 2000).

Active p21-activated kinase 1 (Pak1), an effector of activated Rho GTPases Cdc42 and Rac1 (Manser et al., 1994), has also been reported to phosphorylate ERα at S305 in the absence of ligand, increasing the activity of ERα (Wang et al., 2002) and causing an inhibition of antiestrogenic effects of tamoxifen on ERα in tamoxifen sensitive cell lines (Rayala et al., 2006).

1.7.5 Threonine 311

Threonine 311 is located in the amino-terminus of the ERα LBD. MEKK1 activation of p38 MAPK was shown to be required for estrogen-mediated activation of ERα in endometrial and ovarian cancer cell lines (Lee et al., 2000) and subsequent studies showed that activation of p38 MAPK by estrogen resulted in the stimulation of threonine 311 phosphorylation, blocking receptor nuclear export and promoting interaction with coactivators (Lee and Bai, 2002). Mutation of T311 to alanine prevented ERα activation by estrogen by inhibiting binding of the receptor to the coactivators SRC1 and SRC2/TIF2, although the T311A mutant was still able to bind estrogen. Treatment of endometrial cancer cells with tamoxifen increased the activity of p38 MAPK (Lee and Bai, 2002), suggesting a link between p38-mediated phosphorylation of ERα and tamoxifen resistance. Indeed, patient studies have shown a link between an increase in active p38 MAPK levels, poor prognosis and tamoxifen resistance in breast cancer (Dowsett et al., 2005; Esteva et al., 2004; Gutierrez et al., 2005). However, there is also evidence to suggest that activation of p38 MAPK stimulates ERα degradation (Buck et al., 2004; Zhang and Shapiro, 2000), highlighting potential cell-specificity of these mechanisms.

1.7.6 Tyrosine 537

There is a great deal of controversy over the phosphorylation of Y537, located in the LBD. Tyrosine phosphorylation has been shown to occur in breast cancer cells over-expressing the HER2 growth factor receptor (Pietras et al., 1995) and *in vitro* studies have shown that phosphorylation of Y537 by Src (Likhite et al., 2006) blocks receptor dimerization (Arnold et al., 1997). Mutagenesis studies have been used to show that this residue is important in the modulation of ligand binding and that its mutation can lead to constitutive activation of ERα (Weis et al., 1996; White et al., 1997). Phosphorylation of Y537 has been shown to cause an increase in the affinity of ERα for estrogen, a decrease in the affinity for tamoxifen and a decrease in affinity for DNA in the presence of both estrogen and tamoxifen (Likhite et al., 2006). However, phosphorylation has not been observed by a number of other groups (Ali et al., 1993; Lahooti et al., 1994). This discrepancy may be due to the fact that tyrosine phosphorylation only occurs on a small minority of receptors, is cell type-specific or the experimental conditions used were not conducive for the detection of tyrosine phosphorylation (Lannigan, 2003; Likhite et al., 2006).

1.8 The development of the human breast

There are three phases of mammary gland growth in humans, during which important developmental events occur; embryonic, adolescent and adult phases. Firstly, during prenatal development of the mammary epithelial sprout, formation of a bilateral mammary ridge (or milk line) occurs followed by the formation of placodes at the site of each future nipple. The placodes penetrate the surrounding fat pad and a limited number of branches then sprout to form a rudimentary ductal tree before birth (Hens and Wysolmerski, 2005; Howard and Gusterson, 2000). Breast development in males is indistinguishable from females until puberty, at which point

the female breast undergoes hormone-dependent branching. During this second stage, bulbous terminal end buds form at the tips of the ducts and penetrate further into the fat pad as the ducts elongate. New primary ducts form by the division and sprouting of the terminal end buds until the entire fat pad is filled by an extensive system of branched ducts (Hinck and Silberstein, 2005). These terminal end bud structures eventually disappear from the mature gland as differentiation proceeds and the gland will stay in this form, with minor changes depending on the stage of the menstrual cycle. During the third phase, the gland undergoes a critical period of development during pregnancy where the gland prepares for functional lactation (Fenton, 2006).

The three phases of mammary branching are differentially regulated. Adolescent branching requires estrogen and ERα, adult side-branching requires progesterone and its receptor and embryonic branching is hormone independent (Bocchinfuso et al., 2000). Growth hormone (GH) is a critical pituitary hormone in mammary gland development. GH is already present before the pubertal surge in ovarian estrogens and acts via its receptor, GHR, on mammary stromal cells to elicit the expression of insulin-like growth factor 1 (IGF-1). IGF-1 then stimulates terminal end bud formation and epithelial branching in a paracrine manner and ovarian estrogens act together with GH and IGF-1 to stimulate branching (Sternlicht, 2006). It has been discovered that EGFR ligands rescue ductal development in ERα deficient mice, suggesting that EGFR promotes mammary branching downstream of ERα (Kenney et al., 2003). The EGFR-dependent activation of matrix metalloproteinase 2 (MMP-2) and MMP14 have a key role in lung branching morphogenesis (Kheradmand et al., 2002). Similarly, MMP-2 has been shown to regulate mammary ductal elongation *in vivo* and MMP-14 promotes ductal development by activating MMP-2 and collaborating with it to degrade type I collagen (Wiseman et al., 2003).

The same general processes that take place during normal mammary development, such as proliferation and invasion, also occur in malignant disease and most of the pathways that influence ductal branching have been linked with the development or progression of cancer (Sternlicht, 2006).

1.9 The involvement of ER in breast cancer

Breast cancer is the leading cancer in women in the Western World with one in nine women developing the disease at some point during their lives (Kelsey and Berkowitz, 1988). Approximately 70-80% of breast cancers express ERα and estrogen is the main stimulant in the development and growth of these tumours (Ali and Coombes, 2002; Anderson et al., 2002; EBCTCG, 1998; Johnston and Dowsett, 2003).

It is estimated that between 7-10% of normal breast epithelial cells express ERα, with levels fluctuating during the menstrual cycle (Markopoulos et al., 1988; Petersen et al., 1987; Ricketts et al., 1991). Levels of ERβ are much higher with 80-85% of normal breast cells expressing the receptor and levels do not fluctuate (Critchley et al., 2002; Palmieri et al., 2002). The ERα expressing cells do not proliferate but appear to stimulate the proliferation of surrounding ERα-negative epithelial cells (Clarke et al., 1997; Russo et al., 1999). The mechanisms which enable ERα-positive cells to be converted from non-dividing cells to proliferating under the control of estrogen is unclear (Ali and Coombes, 2002). It has been hypothesised that aberrant signalling of ER could lead to abnormal cellular proliferation and survival, potentially resulting in the development and progression of breast cancer (Herynk and Fuqua, 2004).

1.10 Treatment of breast cancer

As early as the late 1800s, it was recognised that surgical removal of the ovaries (oophorectomy) of premenopausal women with metastatic breast cancer caused tumour regression in approximately one third of patients (Beaston, 1896; Boyd, 1900). Before 1950, cancer therapy mainly concentrated on the surgical removal of tumours. Radiation therapy introduced in the 1960's was able to control local and regional disease in a non-invasive manner, however, like surgery, could not eradicate metastatic cancer (Chabner and Roberts, 2005). Chemotherapy, where cytotoxic drugs such as methotrexate are used to kill cancer cells, was introduced in the early 1950's and has the advantage over surgery and radiation therapy of being able to infiltrate the whole body and therefore come into contact with any metastatic cancer cells. Trials carried out using a combination of drugs, cyclophosphamide, methotrexate and fluorouracil, proved that adjuvant chemotherapy after complete surgical removal of breast tumours significantly extended survival (Bonadonna et al., 1976).

Targeted chemotherapeutic drugs have now been developed including imatinib mesylate (Glivec) which inhibits the kinase BCR-ABL, the tyrosine kinase KIT and the platelet derived growth factor receptor-β (PDGFRβ) and is used in the treatment of chronic myeloid leukemia (CML), gastrointestinal stromal tumours and hypereosinophilic syndrome. However, treatment leads to a rapid outgrowth of drug-resistant cells (Chabner and Roberts, 2005). Trastuzumab, more commonly known under the trade name Herceptin, is a monoclonal antibody which recognises the epidermal growth factor receptor HER2/neu and this receptor is overexpressed in between 20-30% of early-stage breast cancers (Bange et al., 2001). Trastuzumab is used to treat advanced and early-stage breast cancers which over-express HER2/neu; however, the benefit of the drug as an adjuvant treatment to prevent

recurrence is controversial with no clear consensus, partly due to the detrimental effects on cardiac function that long-term trastuzumab treatment causes (Madarnas et al., 2008; Romond et al., 2005). Lapatinib, also known as Tykerb (USA) and Tyverb (Europe), is also a monoclonal antibody which inhibits both EGFR and HER2/neu and has recently been licensed to treat advanced breast cancer which is no longer responsive to Trastuzumab, in conjunction with the chemotherapeutic drug, capecitabine (Xeloda) (Geyer et al., 2006).

1.10.1 Endocrine therapies

At present treatment of patients who present with localised disease involves surgical removal of the tumour (lumpectomy) or removal of the whole breast (mastectomy), which is usually followed by adjuvant therapy, such as endocrine treatments, in order to treat any possible micrometastases (Ali and Coombes, 2002). Endocrine therapies aim to disrupt the ER signalling pathway, either by reducing levels of circulating estrogen (such as aromatase inhibitors and LHRH agonists) or by competing with estrogen for binding to ER and thereby inhibiting the actions of the ER (such as selective estrogen receptor modulators (SERMs) and pure antiestrogens) (Howell and Dowsett, 2004). Only patients with ERα-positive disease respond to endocrine therapies (Osborne, 1998), which nevertheless makes these very important for the adjuvant treatment of breast cancer, since 70-80% of primary breast cancers are ERα positive (Ali and Coombes, 2002).

1.10.1.1 LHRH agonists

Estrogen is synthesized primarily in the ovaries of pre-menopausal women and requires the aromatase complex. Estrogen biosynthesis is subject to feedback

regulation through hormonal signals released from the pituitary gland of the brain (Ali and Coombes, 2002; Buluwela et al., 2004). Luteinising hormone (LH) and follicle-stimulating hormone (FSH) act together to control levels of estrogen and the release of both hormones is regulated by luteinising hormone releasing hormone (LHRH). LHRH agonists such as goserelin (Zoladex), buserelin, leuprolide and triptorelin cause the inhibition of LH and FSH by a negative feedback loop which causes LHRH-receptor down regulation, therefore leading to a reduction in levels of estrogen. In pre-menopausal women LHRH agonists are used as a form of medical castration (Klijn et al., 2001). However, their use is limited as estrogen synthesis in tissues other than the ovary is not affected and it has been suggested that they be used in conjunction with tamoxifen or aromatase inhibitors to treat pre-menopausal breast cancer patients (Klijn et al., 2001).

1.10.1.2 Aromatase inhibitors

Aromatase inhibitors (AIs) as a treatment for breast cancer was first investigated in the 1970s. In post-menopausal women the majority of estrogen is produced by converting androstenedione and testosterone secreted by the adrenal gland in a reaction catalysed by the aromatase complex which occurs at peripheral sites such as adipose tissue, liver and muscle (Buzdar and Howell, 2001). Two-thirds of breast carcinomas contain aromatase which is a cytochrome P450 enzyme complex (Osawa et al., 1987) and produces biologically significant amounts of estrogen local to the tumour (Santen et al., 1994). AIs block this vital enzyme therefore reducing levels of circulating estrogen. However, this is most effective only in post menopausal women due to the fact that pre-menopausal women are still capable of secreting estrogen from the ovaries (Howell and Dowsett, 2004). First generation AIs, such as the adrenotoxic antiepileptic drug aminoglutethimide, aimed

to achieve 'medical adrenalectomy' (Cash et al., 1967; Griffiths et al., 1973; Santen et al., 1974), although its clinical relevance was revealed to be due to inhibition of peripheral aromatisation (Santen et al., 1978). Second generation AIs included non-steroidal as well as steroidal inhibitors but these did not show any improved therapeutic outcome over conventional treatment (Buzdar et al., 1996b; Falkson and Falkson, 1996; Thurlimann et al., 1996). Third generation AIs including anastrazole, letrazole and exemestane however, show superior response and time to progression rates compared to first and second generation AIs (Buzdar et al., 1996a; Dombernowsky et al., 1998; Kaufmann et al., 2000) and achieved total body aromatase inhibition by 98% or more (Geisler et al., 2002; Geisler et al., 1998; Geisler et al., 1996).

1.10.1.3 Selective Estrogen Receptor Modulators (SERMs)

Tamoxifen is a non-steroidal drug which competes against estrogen for binding to the ER and inhibits receptor activation through the ligand-dependent AF2 domain. The drug was first developed in the 1960's as an antifertility drug (Harper and Walpole, 1966) and although this failed, the potential to develop the drug as a targeted breast cancer treatment was identified and by the 1980's tamoxifen had become the first line endocrine treatment for all stages of ER-positive breast cancer (Jordan, 2003). Five years of adjuvant tamoxifen treatment has been shown to provide a 50% reduction in recurrence and 25% reduction in mortality (EBCTCG, 1998).

Both estrogen and tamoxifen binding to ERα cause its dimerization and high affinity binding to EREs, however, the bulky side chain of tamoxifen prevents H12 from adopting the agonist bound position which allows coactivator interaction (Brzozowski et al., 1997). The tamoxifen-ER complex preferentially binds

corepressors that maintain the inactive state of ERα (Lavinsky et al., 1998). Although tamoxifen blocks receptor activity through the AF2, the drug has agonist activity in some tissues such as the uterus and bone, and is therefore known as a 'selective estrogen receptor modulator'. This allows other activities of ERα to proceed as normal, such as, maintenance of bone density and reduced circulating levels of cholesterol (Jordan, 2004; Jordan et al., 2003). Tamoxifen and estrogen cause conformational changes that allow interaction of ERα with a range of coactivators and corepressors and it is thought that the relative levels and/or activities of each are likely to determine whether the ligand bound has estrogenic or anti-estrogenic properties (Howell and Dowsett, 2004). Early studies indicated that tamoxifen has partial agonist activity due to its inhibition of AF2, but activation of AF1 (Berry et al., 1990; Metzger et al., 1992; Tzukerman et al., 1994). However, the picture is probably more complex, likely being dependent, as stated above, on the relative levels of coactivators and corepressors in different target tissues (Shang and Brown, 2002). Cross-signalling growth factor transduction pathways also impact on the activity of the anti-estrogen-ER complex (Johnston and Dowsett, 2003). Unfortunately, although the agonist activity of tamoxifen is desirable in some tissues, in other tissues this agonist activity is problematic, hence the intrinsic activity of the tamoxifen-ER complex results in an increase in the incidence of endometrial cancer in the lining of the uterus (Jordan et al., 2003).

1.10.1.4 Combined SERM and aromatase inhibitor treatments

Whilst tamoxifen has contributed to the significant falls in breast cancer mortality, it is clear that a significant proportion of patients receiving tamoxifen who initially present with localised disease and all of the patients who present with metastatic disease relapse (Ali and Coombes, 2002). A number of clinical trials have

therefore been set up to evaluate the use of antiestrogens and aromatase inhibitors, either to compare the treatments or to evaluate the combination or sequencing of the treatments.

The ATAC-trial (arimidex (anastrazole), tamoxifen, alone or in combination) compared anastrozole with tamoxifen for 5 years in 9366 postmenopausal women with localised breast cancer. The first report revealed improvement in relapse-free survival for anastrazole compared to tamoxifen and the two drugs taken in combination did not show any improvement compared to tamoxifen alone (Baum et al., 2002). The final report after a median follow-up of 68 months concluded that in ERα-positive post menopausal breast cancer patients, anastrozole significantly prolonged disease-free survival, time-to-recurrence and significantly reduced distant metastases and contralateral breast cancers. Anastrozole was also associated with fewer side-effects than tamoxifen, especially gynaecological problems and vascular events, but arthralgia and fractures were increased. No difference regarding overall survival was recorded (Howell et al., 2005a).

The MA-17 trial investigated the benefit of extending adjuvant therapy beyond 5 years but with use of an alternative drug after tamoxifen. This was based on the finding that five years of postoperative tamoxifen therapy prolongs disease-free and overall survival, whereas treatment with tamoxifen for a longer period does not provide any additional benefit, whilst increasing the risk of endometrial cancer and other side effects. The trial was conducted to test the effectiveness of five years of letrozole therapy in postmenopausal women with breast cancer who have completed five years of tamoxifen therapy. A total of 5187 women were enrolled and after the median follow-up at 2.4 years, the independent data and safety monitoring committee recommended termination of the trial and communication of the results to the participants as, compared with placebo, letrozole therapy after the completion of

standard tamoxifen treatment significantly improved disease-free survival (Goss et al., 2003).

The effects of exemestane treatment after 2-3 years of tamoxifen treatment were compared to continuing tamoxifen treatment for 5 years in the IES-trial (Intergroup Exemestane Study). A total of 4742 patients enrolled and the exemestane group compared with the tamoxifen group showed an absolute benefit in terms of disease-free survival of 4.7 percent at three years after randomization. Overall survival was not significantly different in the two groups, however, exemestane therapy after two to three years of tamoxifen therapy significantly improved disease-free survival compared with the standard five years of tamoxifen treatment (Coombes et al., 2004).

1.10.1.5 Pure estrogen receptor inhibitors

Fulvestrant (ICI 182,780) is a steroidal antiestrogen that inhibits the activities of both AF1 and AF2 and is therefore known as a pure antiestrogen. Fulvestrant (also known as Faslodex) competes with estrogen for binding to ERα (Wakeling et al., 1991), prevents ERα binding DNA by disrupting ERα dimerization (Fawell et al., 1990b; Parker, 1993) and promotes nuclear export of the receptor causing accumulation in the cytoplasm (Dauvois et al., 1993). The pure antiestrogen also reduces the half-life of the protein, resulting in a rapid loss of cellular ERα (Dauvois et al., 1992).

Since Fulvestrant is a complete antagonist of ER, treatment is not associated with the side effects of SERM's, such as tamoxifen which causes ERα activation via AF1, linked to an increase in cases of endometrial cancer in patients undergoing tamoxifen treatment for breast cancer (Fisher et al., 1994; Wakeling et al., 1991). Fulvestrant has been shown to inhibit agonistic effects of estrogen in endometrial

tissue in postmenopausal women (Addo et al., 2002) and shows a significant reduction in estrogenic activity in breast cancer cells compared to tamoxifen when used to treat postmenopausal women with previously untreated primary breast cancer (Robertson et al., 2001).

In vivo studies have shown that fulvestrant treatment abrogated the growth of tamoxifen-resistant MCF7 xenografts generated in nude mice (Osborne et al., 1994), suggesting a use for fulvestrant treatment of tamoxifen-resistant tumours. This was shown to be the case in two large Phase III trials (Howell et al., 2002; Osborne et al., 2002) which showed that fulvestrant treatment of advanced breast cancer in postmenopausal women whose disease had progressed on prior endocrine therapy was as effective as anastrozole. Combined analysis of the trials after a median follow-up of 15.1 months showed no statistically significant difference between fulvestrant and anastrazole treatment, although a median follow-up of 22.1 months showed that the duration of response rate of fulvestrant was 30% greater than that for anastrazole (Robertson et al., 2003). However, a median-follow up of 27 months showed that the two treatments were near-identical in terms of median time to death (Howell et al., 2005b).

These studies, and other smaller Phase II studies (Ingle et al., 2006; Mlineritsch et al., 2007; Perey et al., 2007), show the potential of fulvestrant in the treatment of postmenopausal women with advanced breast cancer who have progressed on prior endocrine therapies. Other Phase III trials are underway to evaluate the benefit of fulvestrant treatment in such settings (Evaluation of 'Faslodex' versus Exemestane Clinical Trial, Study Of 'Faslodex', Exemestane, and 'Arimidex' Trial, Southwest Oncology Group (SWOG) SO226 Trial) (Howell, 2006).

1.11 Resistance to endocrine therapies

Around 50% of patients with ERα-positive disease do not respond to tamoxifen treatment (*de novo* resistance) and a large proportion of patients who present with primary breast cancer and all those with metastatic disease who do initially respond to tamoxifen, eventually relapse (acquired resistance) (Likhite et al., 2006). ERα continues to play an important role in many tamoxifen-resistant cases as demonstrated by the finding that the aromatase inhibitor anastrazole and the complete ERα inhibitor, fulvestrant, provide tumour regression in many resistant cases (Morris and Wakeling, 2002). This suggests a mechanism of resistance whereby the activation of ERα is altered in some way and it has been hypothesised that resistance to hormonal therapies could result from growth factor-induced activation of protein kinases, resulting in phosphorylation and consequent ligand-independent activation of ERα (Stoica et al., 2003). Other possible mechanisms for endocrine resistance include loss or mutation of ERα, changes in signalling pathways, coregulator control of ERα activity or growth factor production/sensitivity, or pharmacological changes in the antiestrogen itself, including altered uptake and retention or metabolism of the antiestrogen (Katzenellenbogen et al., 1997).

1.11.1 Tamoxifen resistance due to mutations in the ERα gene

There have been very few reported mutations within the ERα gene that might suggest that mutation of the receptor is linked to resistance towards tamoxifen, with mutation being detected in only 1% of breast cancers (Roodi et al., 1995).

The somatic mutation of ERα lysine 303 to arginine has been identified in 34% of hyperplastic breast lesions (Fuqua et al., 2000). K303 is situated at the border of the hinge and LBD and the K303R mutant shows an enhanced binding to

the SRC1 coactivator at low levels of estrogen with a subsequent increased sensitivity to estrogen compared with wild-type ERα *in vitro*. This is suggestive of a potential mechanism which could promote or accelerate the development of cancer from premalignant breast lesions (Fuqua et al., 2000). Acetylation of K303 by the coactivator p300 has been suggested to reduce hormone sensitivity of ERα (Wang et al., 2001), therefore the K303N mutation may cause hormone hypersensitivity due to the inhibition of this regulatory mechanism. However, the rate of occurrence of this mutant in breast cancer is under dispute with a study of 635 newly diagnosed invasive beast tumours carried out in North America only recording a 5.7% rate of occurrence (Conway et al., 2005), a study carried out by Herynk et al (Herynk et al., 2007) recording a 50% rate of occurrence in invasive breast tumours and Zhang et al. recording no instances of the mutant in a study carried out with Japanese women (Zhang et al., 2003). This difference could be due to variability in the mutantion detection methods used, or could be due to the different ethnic make-up of the groups tested.

A mutation which substitutes tyrosine 537 in the LBD for asparagine has been shown to be equally active in the absence of ligand, presence of estrogen or the presence of tamoxifen (Zhang et al., 1997), whilst a number of other substitutions at this residue have been made experimentally. Many of these substitutions also demonstrate ligand-independent coactivator recruitment and transcriptional activity (Weiss et al; White et al). As mentioned above (section 1.7.5), Y537 was identified as being phosphorylated by members of the c-src family of tyrosine kinases, with phosphorylation causing an increase in the affinity of ERα for estrogen, a decrease in the affinity for tamoxifen and a decrease in affinity for DNA in the presence of both estrogen and tamoxifen (Likhite et al., 2006).

Deletion of a nucleotide in codon 432 that introduces a stop codon at residue 437 of ERα has been identified in a metastatic lesion of a patient but not in the primary tumour (Karnik et al., 1994), suggesting a possible role in tamoxifen resistance and metastatic spread, although there have been no other reports of this mutation occurring in a clinical situation (Herynk and Fuqua, 2004).

Expression of ERα splice variants with various deletions of the eight exons, singly and in combination, have been noted in breast cancer cells and normal breast tissue (Leygue et al., 1996a; Leygue et al., 1996b; Murphy et al., 1996; Pfeffer et al., 1995; Poola et al., 2000; Zhang et al., 1996). The ERα exon 5 deletion mutant (ERα Δ5) is probably one of the most interesting due to the lack of most of the LBD but retention of the AF1 and DBD (Fuqua et al., 1991), enabling constitutive activity of the receptor when transfected into some (Lemieux and Fuqua, 1996), but not other (Rea and Parker, 1996), breast cancer cell lines. This suggests that the variant may possess tamoxifen-resistant growth characteristics when expressed in breast cancers, although assessment of clinical samples has not shown any link between ERα Δ5 and tamoxifen resistance (Daffada et al., 1995; Madsen et al., 1997; Zhang et al., 1996). As with many mRNA splice variants, expression of wild-type ERα is greater than that of the splice variant in the majority of cases (Zhang et al., 1996) and the role of expressed splice variants is unclear at present.

1.11.2 Tamoxifen resistance due to posttranslational modifications

Activation of ERα via growth factor signalling pathways is thought to be a major factor in acquired resistance to endocrine therapies. Cooperative interactions between estrogen and growth factor receptor signalling pathways involved in ERα phosphorylation have been identified in breast tumour cells, and signalling pathways which activate MAPK, notably EGFR/HER1 and HER2/neu, appear to play critical roles

in the development of tamoxifen resistance (Marquez et al., 2006). Approximately 25-30% of breast cancers over-express HER2/neu and 50% over-express EGFR/HER1; both of these instances correlate with poor prognosis and resistance to endocrine therapy (Pietras, 2003; Stoica et al., 2003). Antiestrogen binding to ERα is thought to increase the transcription of HER2/neu gene by releasing ERα coactivators which then go on to activate the HER2/neu promoter (Jordan, 2003).

S118 phosphorylation has been linked to tamoxifen resistant growth partly due to the fact S118 phosphorylation enhances ligand-independent activity in breast cancer cell lines (Bunone et al., 1996; Joel et al., 1998b; Kato et al., 1995). MAPK has been shown to phosphorylate S118 in a ligand-independent manner in vitro (Bunone et al., 1996; Kato et al., 1995) and many in vitro studies have shown that an increase in MAPK activity due to increased expression of upstream activators leads to increased ERα activity in the presence of antiestrogens in breast cancer cell lines (Feng et al., 2001; Hutcheson et al., 2003; Knowlden et al., 2003; McClelland et al., 2001). Clinical studies have also shown an increased activity of MAPK in malignant breast tumour cells compared to normal breast tissue (Sivaraman et al., 1997). A significant relationship between elevated levels of activated MAPK and a reduced period of antiestrogen response (Gee et al., 2001), as well as relapse after antiestrogen treatment (Mueller et al., 2000), has also been reported.

However, although increased levels of active MAPK have been shown to be significantly associated with ERα phosphorylated at S118 in vitro and in breast cancer tumour samples (Britton et al., 2006; Bunone et al., 1996; Joel et al., 1998b; Murphy et al., 2004a; Sarwar et al., 2006), phosphorylated S118, which is thought to lead to tamoxifen-resistant growth and has been shown to be present in 62% of ERα-positive tumours (Murphy et al., 2004b), has also shown to be associated with lower grade tumours (Murphy et al., 2004a; Sarwar et al., 2006) and longer disease

free survival (Bergqvist et al., 2006; Murphy et al., 2004b) – characteristics suggestive of phosphorylated S118 being a good prognostic factor. This is supported by in vitro evidence that ERα phosphorylated at S118 interacts with the promoters of several estrogen regulated genes in MCF7 cells following estrogen treatment, suggesting that phospho-S118 is a marker of functional, intact ligand-dependent ERα signalling (Weitsman et al., 2006).

Studies have reported an increase in levels of both phosphorylated S118 and activated MAPK in tamoxifen-resistant cell lines (Sarwar et al., 2006; Vendrell et al., 2005). However, although an increase in phosphorylated S118 and activated MAPK was seen in tumour biopsies taken from tamoxifen-resistant breast cancer patients, comparison of pre- and post-tamoxifen resistant biopsies showed increased levels of S118 phosphorylation in post-tamoxifen resistant biopsies, this was not linked to an increase in activated MAPK compared to the pre-tamoxifen resistant biopsies (Sarwar et al., 2006). This suggests that increased activity and over-expression of growth factor receptors is not linked to an increase in phosphorylated S118 and that other signalling pathways are involved in this increase, perhaps via Cdk7 or IKKα mechanisms.

However, Gee et al. (2005) have detected phosphorylated S118 in tamoxifen-responsive as well as *de novo* and acquired tamoxifen-resistant breast tumour cells (Gee et al., 2005), whilst another study described above showed improved outcome for patients with tumours having low-level S118 phosphorylation (Yamashita et al., 2008), suggesting that S118 phosphorylation status is not linked to a better prognosis. Strong correlations between ERα, active p38 MAPK and active ERK in tamoxifen-resistant tumours compared to pre-treatment biopsies have also been reported, although this study did not assess levels of phosphorylated S118 (Gutierrez

et al., 2005). The contradictions in these findings highlight the need for more study into this potential mechanism of tamoxifen-resistance in breast cancer.

As mentioned previously (section 1.7.4), Pak1 can phosphorylate ERα at serine 305 (Rayala et al., 2006). Pak1 is a serine-threonine kinase involved in regulating the actin cytoskeleton dynamic and its expression has been shown to be deregulated in human cancers leading to increased invasivness of tumours (Adam et al., 1998; Balasenthil et al., 2004; Bokoch, 2003; Kumar et al., 2006; Stofega et al., 2004). Increased expression of Pak1 at the mRNA and protein level has been shown in up to 50% of breast cancer tumours (Balasenthil et al., 2004; Vadlamudi et al., 2000). Active Pak1 has been reported to phosphorylate ERα at S305 in the absence of ligand, increasing activity of ERα, (Wang et al., 2002) and causing an inhibition of antiestrogenic effects of tamoxifen on ERα in tamoxifen sensitive cell lines (Rayala et al., 2006). High nuclear Pak1 expression in primary breast tumours from premenopausal women has been linked with features of aggressive types of breast cancer and tamoxifen-resistance (Holm et al., 2006). Rayala et al. (2006) show that tamoxifen induces Pak1 expression and interaction with ERα in tamoxifen resistant breast cancer cell lines, but not tamoxifen sensitive lines, suggesting tamoxifen induced Pak1 expression may be linked with maintaining a tamoxifen-resistant cell phenotype. They also show evidence that phosphorylation of S305 by Pak1 leads to S118 phosphorylation.

1.11.3 Breast cancer cell growth in a reduced estrogen environment

A key step in the progression of breast cancer is the conversion of cells from an estrogen-dependent to an estrogen-independent state; however, the molecular mechanisms involved in this switch are poorly understood. Reduction of estrogen levels by surgical oophorectomy in pre-menopausal women with hormone-dependent

breast cancers causes tumour regression, however, after 12-18 months tumours begin to re-grow even in the reduced estrogen environment (Santen et al., 1990). Subsequent treatment with AIs to reduce estrogen levels further can promote tumour regression again, however, resistance to AIs eventually develops. Resistance to AIs typically develops more slowly than that to SERMs and is thought to be due to ERα overcoming the estrogen deprived conditions via mechanisms such as elevated levels of growth factor and estrogen receptors and increased transduction via the MAPK and PI3K pathways (Likhite et al., 2006; Martin et al., 2005; Santen et al., 2001).

An *in vitro* model of long term estrogen deprivation (LTED), whereby breast cancer cell lines are cultured in the absence of estrogen for 6-24 months, has been used to investigate mechanisms of ERα hypersensitivity towards estrogen. MAPK has been implicated in the development of these mechanisms, with basal levels of MAPK being increased in LTED cell lines and inhibition of the enzyme significantly reducing hypersensitivity to estrogen (Jeng et al., 2000; Yue et al., 2002; Yue et al., 2003). However, MAPK inhibition does not cause complete abrogation of estrogen hypersensitivity; LTED cell lines also show increased activity of the PI3K signalling pathway, including increased Akt activity (Santen et al., 2005a; Santen et al., 2005b). Non-genomic activities of estrogen via membrane-localised ERα and mediated by the adapter protein Shc, are thought to be in part responsible for the activation of these signalling pathways (Song et al., 2002; Zhang et al., 2002b). In support of this theory are studies showing that sub-physiological concentrations of estrogen are capable of stimulating maximal activation of MAPK in LTED cell lines (Santen et al., 2005a) and that association between Shc and ERα is markedly enhanced in these lines compared to wild-type cell lines with estrogen stimulating Shc activation (Song et al., 2002).

Growth of LTED cell lines is inhibited by treatment with the pure anti-estrogen ICI 182,780 (Martin et al., 2005) showing that the cell lines require ERα activity for growth. S118 phosphorylation of ERα has also been noted in LTED cell lines, although this was shown not to be due to MAPK-mediated phosphorylation pathways but rather stimulated by estrogen, highlighting the continued ligand-dependency of this cell line (Martin et al., 2005). Estrogen stimulates ERα transcriptional activation and also the down-regulation of ERα expression (Nawaz et al., 1999a; Saceda et al., 1988). Conversely, reduction in estrogen levels causes an increase in ERα levels which is a phenotype of many breast tumours and reported in LTED cell lines where it is thought to contribute towards estrogen hypersensitivity (Chan et al., 2002; Fowler et al., 2004; Gaskell et al., 1989; Santen et al., 2001; Thorpe et al., 1993). Induced high levels of ERα expression in breast cancer cell lines have been shown to promote gene activation in the absence of ligand in an AF1-dependent manner (Fowler et al., 2004). This concentration-dependent activation of ERα is independent of AF1 phosphorylation and p160 coactivators (Fowler et al., 2004; Fowler et al., 2006) and is thought to come about due to interaction of unliganded ERα directly with the basal transcription machinery through interactions which are usually too weak to promote transcriptional activation under normal levels of ERα expression (Fowler et al., 2006).

1.11.4 Anti-estrogen-induced gene expression

As mentioned previously, estrogen-induced activation of ERα stimulates transcriptional activities of ERα which include the activation of genes but also the repression of many genes (Frasor et al., 2003). Microarray studies have also been used to profile the effect of antiestrogens on gene expression in breast cancer cells and these studies have shown that not only do different antiestrogens have different

gene expression profiles, but they also have an antagonistic effect on the repressive actions of estrogen (Frasor et al., 2004). Many of the genes repressed by estrogen are anti-proliferative and pro-apoptotic with anti-estrogen treatment promoting transcription of these genes, expression of which would aide the antiproliferative effect of endocrine treatments. However, a number of estrogen-regulated genes which are down regulated are involved with cell growth and proliferation, including EGFR (Frasor et al., 2004; Gee et al., 2003; Wilson and Chrysogelos, 2002; Yarden et al., 2001). Tamoxifen-induced expression of EGFR has been shown to cause activation of MAPK and Akt at levels high enough to maintain S118 phosphorylation of ERα. The increased expression of EGFR over time during tamoxifen treatment is thought to contribute towards tamoxifen-resistant growth due to induced activation of ERα and subsequent expression of estrogen-regulated growth factors, such as amphiregulin, which can then activate EGFR and stimulate cell growth by this alternative signalling pathway (Britton et al., 2006; Gee et al., 2003; Knowlden et al., 2003; Yarden et al., 2001).

Tamoxifen has also been shown to regulate a set of genes not regulated by either estrogen or other SERMs (Frasor et al., 2006). Whilst microarray studies showed that a number of these genes have potential tumour suppressor roles, such as SOCS1 and PTPRG, the expression of two genes (YWHAZ and LOC441453) were found to correlate with a reduced disease-free survival in breast cancer patients (Frasor et al., 2006). Little is known about LOC441453, however, YWHAZ (also known as 14-3-3ξ) is a scaffold protein belonging to the highly conserved 14-3-3 family of proteins which regulate the cell cycle and prevent apoptosis. YWHAZ is thought to play a central role in insulin receptor and EGFR signalling and in cell cycle regulation by stabilizing and activating key signalling proteins, such as Akt and c-raf (Ogihara et al., 1997; Oksvold et al., 2004). Frasor et al. (2006) suggest that the

expression of these two genes in breast tumours could indicate a more aggressive form of cancer since increased expression of YWHAZ could lead to increased signalling of cell cycle progression pathways. In support of this is clinical evidence showing that YWHAZ expression is associated with a poor outcome following tamoxifen treatment (van 't Veer et al., 2002).

The expression of genes in breast cancer cells will obviously impact on how cell growth is affected by endocrine treatments. Analysis of any alterations in gene expression due to different treatments could provide a way of predicting the outcomes of treatment and may provide novel targets for future treatments that could be administered in conjunction with other endocrine therapies to prevent recurrence of tumour growth or even formation of resistance to endocrine therapies.

1.11.5 Transcriptional Coregulators and resistance

Denner et al. demonstrated that treatments which enhance activity of kinases or inhibit phosphatase activities cause SR activation through increasing the activity of coactivators (Denner et al., 1990). These authors showed that cells transfected with chicken PR and a PR-responsive reporter gene, when treated with 8-bromo-cyclic adenosine monophosphate (8-Br-cAMP), a stimulator of protein kinase A (PKA), showed hormone-independent, but cPR-dependent, activation of the reporter. Subsequent studies revealed that 8-Br cAMP caused activation of p42/p44 MAPK and increased phosphorylation of SRC1 which contributed to the ligand-independent activation (Rowan et al., 2000). Subsequent studies have shown that activation of ERα coactivators is capable of causing ligand-independent activation of the receptor and is a potential mechanism of endocrine-resistant growth in breast cancer cells. Many breast cancers exhibit increased expression of growth factors and their receptors, which is linked to poor prognosis and development of resistance to

endocrine therapies (Benz et al., 1992; Gutierrez et al., 2005; Klijn et al., 1992; Meng et al., 2004b; Pietras et al., 1995; Sainsbury et al., 1985; Shou et al., 2004). Indeed, tyrosine kinase inhibitors used in conjunction with tamoxifen have been shown to restore antagonistic activities of tamoxifen as they inhibit these signalling pathways (Chu et al., 2005; Kurokawa et al., 2000; Shou et al., 2004; Witters et al., 2002). Activation of growth factor signalling pathways has been shown to affect the activity of a number of ERα coactivators resulting in an increase in ERα activity in breast cancer, some instances of which will be discussed below.

The coactivator AIB1/SRC3 is overexpressed in over 50% of breast cancers and is thought to play an important role in breast cancer pathogenesis (Anzick et al., 1997; Torres-Arzayus et al., 2004; Zhou et al., 2003). It has been suggested that the ability of growth factors to modulate estrogen action may be mediated through MAPK activation of AIB1/SCR3 (Font de Mora and Brown, 2000). As mentioned above, estrogen/ERα can rapidly stimulate MAPK activity (section 1.6). One apparent target of estrogen-stimulated MAPK activity appears to be AIB1/SRC3, with MAPK phosphorylation of AIB1 stimulating its transcriptional activity (Font de Mora and Brown, 2000; Marquez et al., 2006; Zheng et al., 2005). AIB1 is capable of shuttling between the nucleus and the cytoplasm and the phosphorylation status of AIB1 has also been linked to an increased dwell-time in the nucleus (Qutob et al., 2002; Wu et al., 2002a). It has been suggested that constitutively active HER2 signalling, an activator of MAPK, would result in elevated AIB1 phosphorylation and activity, hence increasing ERα activity (Amazit et al., 2007; Shou et al., 2004). Furthermore, overexpression of AIB1 in transgenic mice causes the formation of malignant mammary tumours (Torres-Arzayus et al., 2004) and correlation between high AIB1 and HER2 expression levels and poor response to tamoxifen has been reported in breast cancer patients (Kirkegaard et al., 2007; Osborne et al., 2003).

As mentioned previously, the activity of AF1 is regulated by phosphorylation, which promotes the recruitment of coactivators to the ERα via the LBD/AF2 and interactions with AF1 (Webb et al., 1998). Moreover, the ATP-dependent RNA helicase p68 has been identified as an ERα-specific coactivator. The interaction of p68 is enhanced by MAPK-mediated phosphorylation of ERα at S118. Only the activity of AF1 and not AF2 is potentiated by p68 and the coactivator activity of p68 has been shown to be specific to ERα, even whilst ERα is bound by tamoxifen (Endoh et al., 1999). The RNA helicase activity of p68 (Hirling et al., 1989) is not required for the coactivator function of p68 and is instead due to p68 acting as a bridge between AF1 and other coactivators, including CBP (Endoh et al., 1999) and SRC1/TIF2 (Watanabe et al., 2001). Increased expression of p68 in breast cancer cell lines has been shown to cause tamoxifen-induced activation of ERα via AF1 (Fujita et al., 2003), suggesting a potential mechanism for activation of tumour cell growth in the presence of tamoxifen.

More than 80% of breast tumours exhibit an increased activity of the non-receptor Src tyrosine kinase compared with normal breast tissue (Verbeek et al., 1996). Src activation leads to the activation of signal transduction cascades including the MAP kinase subgroups ERKs via Raf/MEK/ERK kinase cascades and c-Jun N-terminal kinases (JNKs) via MEKK/JNKK/JNK. Feng et al. (Feng et al., 2001) have shown that S118 phosphorylation of ERα via Src activation of the Raf-MEK-ERK signalling cascade enhances ERα AF1 activity (Kato et al., 1995). They have also shown that Src activation of the MEKK/JNKK/JNK signalling cascade pathway enhances ERα action in a S118-independent manner due to Src-mediated enhanced activity of the CBP and GRIP1/SRC2 coactivators which can bind directly to and activate the AF1 (Feng et al., 2001; Weitsman et al., 2006). GRIP1 also contains an EGF-regulated transcriptional activation function which is activated due to

phosphorylation by p38 MAPK and promotes interaction with CBP (Frigo et al., 2006; Lopez et al., 2001). Levels of GRIP1 are seen to be increased in a proportion of breast tumours (Kurebayashi et al., 2000).

Phosphorylation of the corepressor SMRT by MEKK1, a downstream mediator of the MAPK signalling pathway, disrupts interaction of the corepressor with transcription factors such as ERα (Hong et al., 1998; Lavinsky et al., 1998; Wagner et al., 1998) and also promotes nuclear export of the corepressor (Hong and Privalsky, 2000). Activation of MEKK1 does not however affect interactions of the closely related corepressor NCoR with transcription factors (Jonas and Privalsky, 2004; Jonas et al., 2007). SMRT and NCoR are expressed as different splice variants which have different affinities for transcription factors and are expressed at varying levels in different tissues (Downes et al., 1996; Goodson et al., 2005; Malartre et al., 2004). SMRT splice variants respond differently to phosphorylation by the MAPK signalling pathway with some showing more sensitivity and a greater inhibitory response (Jonas et al., 2007). Potentially therefore, expression in breast cancer cells of SMRT splice variants with a greater response to MAPK signalling, could cause an increase in the activity of ERα.

Proline-, glutamic acid-, leucine-rich protein 1/modulator of the non-genomic activities of ER (PELP1/MNAR) has been identified as a coactivator of ERα and has similar characteristics to the p160 family of SR coactivators in that it contains an LXXLL motif and has a molecular weight of 160kDa (Vadlamudi et al., 2001; Wong et al., 2002). Although PELP1/MNAR acts as a coactivator for ERα, it has been reported to act as a corepressor for a range of other transcription factors (Choi et al., 2004). PELP1 demonstrates no intrinsic transcriptional activity, suggesting that it acts in a similar way by recruiting cofactors to the liganded ERα (Vadlamudi et al., 2001). MNAR, which was subsequently shown to be the same protein as PELP1 (Balasenthil

and Vadlamudi, 2003), was identified as a ligand-dependent ERα-interacting protein which stabilises the interaction between ERα and Src family of tyrosine kinases at the cell membrane, leading to activation of the MAPK signalling pathway (Barletta et al., 2004; Wong et al., 2002). PELP1/MNAR has also been implicated in the estrogen-regulated activation of the PI3K/Akt signalling pathway, with studies showing that PELP1/MNAR mediates an estrogen-dependent association of ERα and the p85 regulatory subunit of PI3K, which is dependent of the phosphorylation of MNAR/PELP1 by activated c-Src and leads to activation of the PI3K/Akt signalling pathway (Greger et al., 2007). Overexpression of PELP1/MNAR causes an increase in the transcriptional activity of ERα (Vadlamudi et al., 2001; Wong et al., 2002) and PELP1/MNAR expression has been reported to be increased in breast tumours compared to normal breast tissue (Vadlamudi et al., 2001), with higher levels of expression correlating to higher grade node-positive invasive tumours (Rajhans et al., 2007). The retinoblastoma protein (pRb) is involved in cell cycle progression and causes cell cycle arrest in the G1 phase by inhibiting the expression of genes required for progression into the S phase (Weinberg, 1995). Continuation of the cell cycle requires inhibition of pRb by phosphorylation of the protein, predominantly controlled by the cyclinD/CDK4 and cyclinE/CDK2 enzymes (Harbour and Dean, 2000). PELP1/MNAR has been shown to interact with pRb, increasing phosphorylation and therefore inhibition of pRb, allowing cell cycle progression (Balasenthil and Vadlamudi, 2003). A role for PELP1/MNAR in estrogen-mediated cell migration, cytoskeletal changes and anchorage-independent cell growth has been reported, suggesting that PELP1/MNAR is an oncogene, the expression of which is deregulated during cancer progression (Rajhans et al., 2007).

Alterations to different components of the cyclin/CDK/$p16^{Ink4A}$/pRb pathway through overexpression, mutation, and epigenetic gene silencing are a very common

characteristic of human cancers (Malumbres and Barbacid, 2001). The activation of CDKs by the binding of cyclins controls the progression of the cell cycle; cyclin D1 is considered a weak oncogene and its overexpression has been shown to result in estrogen-independent growth of breast cancer cells *in vitro* (Bindels et al., 2002) and development of mammary carcinoma in mouse models (Wang et al., 1994). The cyclin D1 gene is amplified in approximately 15% of breast cancers (Jirstrom et al., 2005; Ormandy et al., 2003) and the protein is overexpressed in 30–50% of breast cancers (Alle et al., 1998; Buckley et al., 1993; Gillett et al., 1994; Hui et al., 1996). Expression of cyclin D1 is regulated by estrogen (Altucci et al., 1996; Castro-Rivera et al., 2001; Eeckhoute et al., 2006; Sabbah et al., 1999) and dysregulation of PKA has been linked to an increase in cyclin D1 protein half-life (Nadella and Kirschner, 2005). Therefore, overexpression of cyclin D1 in breast cancer cells is likely due to increased activity of ERα and possibly PKA. Antiestrogen treatment has indeed been shown to cause cell cycle arrest in the G1 phase and inhibit cell proliferation due to reduced cyclin D1/CDK4 activity (Sutherland et al., 1983; Watts et al., 1995). Although cyclin D1 overexpression is associated with a better outcome for breast cancer patients (Gillett et al., 1996; Jirstrom et al., 2003; Nielsen et al., 1997; Pelosio et al., 1996), possibly due to its ability to repress the antiapoptotic transcription factor signal transducer and activator of transcription 3 (STAT3) (Bienvenu et al., 2001; Ishii et al., 2006; Quintanilla-Martinez et al., 2003), its overexpression is also linked to tamoxifen resistance due to its ability to mimic estrogenic effects on cell cycle progression (Bindels et al., 2002; Hui et al., 2002; Kilker et al., 2004; Prall et al., 1998; Wilcken et al., 1997). Cyclin D1 also acts as a coactivator for ERα independently of CDKs and potentiates the activity of ERα in the presence of estrogen whilst causing ligand-independent activation of ERα, which is not inhibited by antiestrogens, by recruiting SRC1 to the receptor (Neuman et al., 1997; Zwijsen

et al., 1998; Zwijsen et al., 1997). Treatment with tamoxifen abolishes cyclin D1-mediated repression of STAT3 and growth suppression by inducing a redistribution of cyclin D1 from STAT3 to ERα, which results in the activation of both STAT3 and ERα (Ishii et al., 2008). This may well be an important mechanism involved in the transition from tamoxifen-responsive to tamoxifen-resistant breast cancer cell growth.

Taken together, there is considerable evidence that crosstalk between estrogen signalling through ERα and cell surface receptors, other signal transduction pathways, impacting on phosphorylation of transcriptional coregulators is likely to be an important feature of breast cancer progression and response to endocrine treatments.

1.12 Aim of study

Antagonistic actions of tamoxifen in breast cancer cells occur due to inhibition of the AF2 domain of ERα. Activation of the AF1 domain, not normally involved with receptor activation in breast cells, is likely to play an important role in enabling tamoxifen-resistant breast cancer cell growth. Serine residues 104, 106 and 118 have previously been identified as phosphorylation sites within the AF1 that are phosphorylated in a ligand-independent manner by MAPK, a downstream mediator of growth factor signalling pathways. Increased expression and activity of growth factors and receptors, such as EGFR and HER2, occur in a large number of breast cancers and overexpression of HER2 in ERα positive breast cancers is linked to a poor a prognosis and tamoxifen-resistant tumour growth. During normal ERα activity, estrogen binding induces recruitment of coactivator proteins via the AF2 to the estrogen regulated gene which initiates transcription by coordinating binding of the basal transcription machinery. In breast cancer cells which are sensitive to

tamoxifen, this recruitment of coactivators and therefore activation of transcription, is inhibited by tamoxifen binding to ERα. However, over time, resistance to this mechanism of receptor inhibition occurs. We hypothesise that phosphorylation of residues S104, S106 and S118 within the AF1 cause ligand-independent activation of ERα in the presence of tamoxifen by recruiting coactivators to the AF1. However, as outlined above, phosphorylation of coactivators and corepressors may also be important for the crosstalk between cell surface receptors such as EGFR and HER2, and ERα. In order to investigate the importance of AF1 phosphorylation sites, therefore, the estrogen-responsive and ERα-positive MCF7 breast cancer cell line was stably transfected with ERα in which S104, S106 and S118 were substituted by alanine or glutamic acid residues. This would allow the importance of these phosphorylation sites for mediating estrogen responses to be determined directly. Stable lines expressing ERα and ERα AF1 phosphorylation site mutants were characterised for their growth response to estrogen withdrawal and to anti-estrogens, as was expression of estrogen-regulated genes, in order to test the importance of these phosphorylation sites in MCF7 cells. The results of these studies are presented in the following chapters.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 General chemicals and reagents

All chemicals were supplied by BDH Laboratory Supplies/Merck (Poole, UK), Sigma-Aldrich (Dorset, UK) or Fisher Scientific (Leics, UK), unless otherwise stated. Molecular biology grade reagents were used, unless otherwise stated.

2.1.2 General stock solutions

Standard solutions listed in table 2.1 were made by dissolving the appropriate quantity of reagent in distilled, de-ionised water (ddH_2O) then autoclaved and stored at room temperature, unless otherwise stated.

Table 2.1 General stock solutions

10X TBE	89mM Tris base, 89mM Boric acid, 2mM Na-EDTA, pH 8.0
TE Buffer	10mM Tris-HCl pH7.5, 1mM Na-EDTA, pH 8.0
Sodium ethylene diamine tetra-acetate (Na EDTA)	0.5M Na-EDTA, pH 8.0.
10% lauryl sodium dodecyl sulphate (SDS)	10% w/v SDS, pH 7.0. Filter sterilised. Not autoclaved.
10X phosphate buffered saline (PBS)	136mM NaCl, 3mM KCl, 8mM Na_2HPO_4, 1.5mM KH_2PO_4

2.1.3 Cell culture consumables and reagents

Tissue culture plastics were supplied by Corning/Appleton Woods (Birmingham, UK) and Triple Red Laboratory Technology (Bucks, UK). A NuAIRE DH Autoflow CO_2 Air-Jacketed Incubator (NuAIRE, Oxon, UK) maintained at 5% CO_2 was used to incubate cell cultures and cell manipulation was carried out in a NuAIRE class II, ducted laminar flow safety cabinet (NuAIRE, Oxon, UK). Dulbecco's Modified Eagle's Medium (DMEM) was supplied by Invitrogen (Paisley, UK). 0.02% Na EDTA solution, 10x trypsin solution and 5mg penicillin/streptomycin/L-glutamine (P/S/G) solution was supplied by Sigma-Aldrich (Dorset, UK). Phenol red free DMEM and fetal calf serum (FCS) were purchased from GIBCO (GibcoBRL Life Technologies, Paisley, UK). Dextran-coated charcoal-treated FCS (DSS), prepared to remove steroids, including estrogens from the FCS, was purchased from Globepharm (Surrey, UK). 17β-estradiol (E2), 4-hydroxytamoxifen (OHT), epidermal growth factor (EGF) and insulin were obtained from Sigma-Aldrich (Dorset, UK). ICI 182,780 (ICI) and U0126 were obtained from Tocris Biosciences (Avonmouth, UK). Hygromycin B was supplied by Invitrogen (Paisley, UK) and G418 was purchased from Sigma-Aldrich (Dorset, UK). Dimethyl sulphoxide (DMSO) was supplied by GIBCO (GibcoBRL Life Technologies, Paisley, UK).

2.1.4 Molecular size markers

The molecular weights of protein bands resolved using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) techniques were estimated by comparison with the Full Range Rainbow marker (MW 250, 160, 105, 75, 50, 35, 30, 25, 15, 10 kDa) supplied by Amersham Life Sicence (Bucks, UK). The sizes of DNA fragments were estimated by comparison with GeneRuler 1kb DNA ladder (250-10,000 base pairs) supplied by Fermentas Life Sciences (York, UK). The sizes of RNA were estimated by

comparison with GeneRuler Low Range DNA ladder (25-700 base pairs) purchased from Fermentas Life Sciences (York, UK).

2.1.5 Sundries

Polypropylene microfuge tubes were supplied by Corning/Appleton Woods Ltd (Birmingham, UK). Thin-walled 0.2ml PCR tubes were supplied by Advanced Biotechnologies Ltd (Surrey, UK). Whatman 3MM filter paper was obtained from Whatman International Ltd (Kent, UK). Hybond-ECL nitrocellulose blotting membrane and Hyperfilm-ECL autoradiography film was supplied by Amersham Biosciences (Bucks, UK).

2.1.6 Laboratory equipment

Centrifugation steps for volumes under 1.5mls were carried out using a Sorvall® *pico* table top centrifuge. An Innova 2000 platform shaker (New Brunswick Scientific, USA) was used for all shaking steps carried out, unless otherwise stated.

2.2 Methods

2.2.1 Routine tissue culture

The MCF-7 breast cancer cell line and derived lines were maintained at 37°C with 5% CO_2 in DMEM supplemented with 10% FCS, 100μg/ml P/S/G, 10^{-8}M estrogen and 0.1μg/ml doxycycline (DOX), which is a more stable analogue of tetracycline. Where appropriate, the medium was supplemented with the selection antibiotics Hygromycin B and G418, both at a concentration of 1μg/ml, during

routine maintenance of the cells, which were omitted one week before recruitment into and during experiments carried out using the cells.

Cultures were grown to ~80% confluencey then washed in sterile phosphate buffered saline (PBS) and treated with trypsin (1x trypsin in 0.02% Na EDTA) to release adherent cells from the plastic. Culture medium was then added to dilute the trypsin 1:3 and cells were passaged 1:5. Cultures were routinely maintained in T150 flasks.

Aliquots of cells were regularly frozen by pelletting 5 x 10^6 cells in the growth phase of the logarithmic growth curve by centrifugation for 5 minutes an 1300 rpm and re-suspending the pellet in 1ml of freezing-down medium (10% DMSO, 90% FCS). Cells were transferred to 2ml cryovials and stored at -80°C for 48 hours before being placed in liquid nitrogen for long-term storage.

2.2.2 Preparation of cell lysates using hot lysis buffer

Cell lysates for protein analysis were generally prepared using the hot lysis buffer technique. Cells were washed twice with PBS warmed to 37°C and excess PBS removed by aspiration. 0.1ml of 2x SDS-PAGE sample buffer (125mM Tris-HCl, pH6.4, 4% v/v SDS, 20% v/v glycerol, 400mM DTT, 0.002% bromophenol blue) heated to 100°C was added per 1x10^6 cells. The cells were collected by scraping off the plates using a rubber policeman and transferred to a microfuge tube and incubated at 100°C for 10 minutes before cooling on ice, aliquoting and storing at -80°C.

2.2.3 DNA Manipulations

2.2.3.1 Cell transformation using competent TG2 cells

Competent *E.coli* TG2 strain was prepared using the Hanahan method (Hanahan 1983). A starter culture was prepared by inoculating 5ml of SOB Medium (0.5% w/v yeast extract, 2% w/v tryptone, 10mM NaCl, 2.5mM KCl, 20mM $MgSO_4$) with a single bacterial colony and incubating overnight at 37°C with shaking. 3ml of the starter culture was added to 300ml of SOB medium and incubated at 37°C until the OD at 600nm reached between 0.4-0.6. The culture was placed on ice for 15 minutes then centrifuged at 2500rpm at 4°C for 15 minutes. The resulting pellet was resuspended in 90ml of RF1 buffer (100mM RbCl; 50mM $MnCl_2.4H_2O$; 30mM KOAc, pH 7.0; 10mM $CaCl_2.2H_2O$; 15% glycerol; pH 5.8) and incubated on ice for 15 minutes before centrifugation at 2500rpm at 4°C for 15 minutes. The pellet was resuspended in 21ml of RF2 buffer (10mM MOPS, pH6.8; 10mM RbCl; 75mM $CaCl_2.2H_2O$; 15% glycerol) and incubated on ice for 15 minutes. 50μl of the competent cells were transformed by incubating with 100ng of either pxpERE or pxpRL-TK DNA on ice for 30 minutes. Transformation mixtures were then incubated at 42°C for 2 minutes followed by incubation on ice for 3 minutes. 500μl of LB broth was added to the transformation mixtures and the culture incubated at 37°C with shaking (150rpm) for 60 minutes using a G2 (New Brunswick Scientific, NJ, USA) incubator. The culture was then centrifuged at 6000rpm for 3 minutes and the pellet was then resuspended in 50μl of fresh LB broth and spread on selective LB agar plates and incubated at 37°C overnight.

LB broth was prepared by suspending 25.0g LB broth (MILLER) granules (Merck, DE, USA) (10g bacto-tryptone; 5g bacto yeast; 10g NaCl, pH7.0) in 1L of ddH_2O and autoclaving the solution. Selective LB agar plates

were made by suspending 37.0g LB agar (MILLER) (Merck, DE, USA) in 1L of ddH_2O (10.0g/L peptone from casein, 5.0g/L yeast extract, 10.0g/L NaCl, 12.0g/L Agar-agar) and autoclaving the solution. After cooling, ampicillin was added to a final concentration of 50μg/ml.

2.2.3.2 Large scale plasmid preparation (maxi prep)

An individual bacterial colony from TG2 cells transformed with the plasmid of interest was used to inoculate 1ml of selective LB broth and incubated for 8 hours at 37°C with shaking (150rpm). This starter culture was then diluted 1:500 into a large conical flask containing 500ml of selective LB broth containing 50μg/ml ampicillin and incubated over night at 37°C with shaking at 150 rpm.

Plasmid DNAs were prepared from the bacterial cell culture using the Qiagen-tip500 Plasmid Maxi Purification Kit (Qiagen, DE) following the handbook instructions. For buffers used see table 2.2. The bacterial cells were harvested by centrifugation at 6000rpm for 15 minutes at 4°C using a Sorvall RC6 centrifuge. The bacterial pellet was resuspended in 10ml of resuspension buffer (Buffer P1), then lysed by adding 10ml of lysis buffer (Buffer P2) and incubated at room temperature for 5 minutes. The lysis buffer activity was neutralized by adding 10ml of Buffer P3, followed by incubation on ice for 20 minutes. The lysed cells were centrifuged at 13,000 rpm for 30 minutes at 4°C using a Sorvall RC6 to remove cell debris. The Qiagen-tip 500 was prepared by applying 10ml of equilibration buffer (Buffer QBT), allowing emptying by gravity flow. A 3-layer thick piece of muslin was placed over the Qiagen-tip 500 before adding the supernatant from the Buffer P3 step, to filter cell debris. The tip was washed twice with 30ml of wash buffer (Buffer QC) before the DNA was eluted with 15ml of Buffer QF. The DNA was precipitated by adding

10.5ml of isopropanol to the eluted DNA and centrifuged at 11,000rpm for 30 minutes at 4°C using a Sorvall RC6. The DNA pellet was further cleaned by carrying out phenol chloroform extraction and ethanol precipitation.

Table 2.2 Qiagen DNA maxi preparation buffers

Buffer P1	50 mM Tris-HCl, pH 8.0; 10mM EDTA; 100μg/ml Rnase A
Buffer P2	200 mM NaOH, 1% SDS ($^{w}/_{v}$)
Buffer P3	3.0 M potassium acetate, pH5.5
Buffer QBT	750 mM NaCl; 50 mM MOPS, pH7.0; 15% isopropanol ($^{v}/_{v}$); 0.15% Triton X-100 ($^{v}/_{v}$)
Buffer QC	1.0 M NaCl; 50 mM MOPS, pH7.0; 15% isopropanol ($^{v}/_{v}$)
Buffer QF	1.25 M NaCl; 50 mM Tris-Cl, pH8.5; 15% isopropanol ($^{v}/_{v}$)
TE Buffer	10mM Tris, pH8; 1mM EDTA

2.2.3.3 Phenol-chloroform extraction

The DNA pellet obtained from the QIAGEN column purification was resuspended in 100μl of TE buffer. The DNA concentration was then measured using a spectrophotometer (NanoDrop ND-1000, Labtech International UK) and 100μg of DNA was diluted in TE buffer to give a volume of 200μl. 100μl of phenol-chloroform was added to the DNA, vortexed and then centrifuged at 13,000rpm for 5 minutes. The top layer was transferred to a fresh microfuge tube and 1/10 volume of ice-cold 3M sodium acetate (pH 5.2) was added and then vortexed. 2-3 volumes of ice-cold 100% ethanol was added and samples were placed at -70°C for 30 minutes. Samples were then centrifuged for 10 minutes at 13000rpm. The supernatant was removed

and 500μl of 70% ethanol was added to the cell pellet before centrifugation at 13,000rpm for 10 minutes. The supernatant was then removed and the DNA pellet allowed to air dry on the bench at room temperature for 20 minutes. The DNA pellet was re-suspended in 100μl TE buffer by incubation at 4^{o}C over night and DNA concentration determined, as above.

2.2.3.4 DNA sequencing

DNA sequencing was carried out using the dideoxy chain termination method carried out by Genomics Core Laboratory, MRC Clinical Sciences, Imperial College. 3.2pmol of forward and reverse primers (Table 2.3) were added to 9μg of DNA and diluted to a final volume of 10μl with sterile water.

Table 2.3 Primer sequences for luciferase plasmid DNA sequencing

Primer	Sequence
Renilla-F1	5′ –CCA GGT CCA CTT CGC ATA TT- 3′
Renilla R1	5′ –ACC ACT GCG GAC CAG TTA TA- 3′
Renilla F2	5′ –ATG GGA TGA ATG GCC TGA TA- 3′
Renilla R2	5′ –ACC AGA TTT GCC TGA TTT GC- 3′

2.2.3.5 Restriction enzyme digestion

0.5-1 μg of DNA was digested in a volume of 20 μl, with restriction enzymes in the recommended digestion buffer (New England Biolabs, USA) and at the recommended temperature, over 1-2 hours, at which point 2μl of 6x loading dye (10 mM Tris-HCl, pH 7.6; 0.03% bromophenol blue; 0.03% xylene cyanol FF; 60%

glycerol; 60 mM EDTA) (Fermentas Life Sciences (York, UK)) was added before analysis by agarose gel electrophoresis as described in section 2.2.4.3.1.

2.2.4 MCF-7 cell transfection

MCF-7 cells were grown in DMEM without phenol red supplemented with P/S/G, 5% DSS plus or minus 0.1μg/ml DOX for 72 hours. Cells were trypsinised as described in section 2.2.1, then plated in 24-well plates, 8 $x10^4$ cells per well, in 0.5mls DMEM without phenol red supplemented with P/S/G, 5% DSS plus or minus 0.1μg/ml DOX. After 24 hrs the cells were transfected using FuGENE6 (Roche Applied Science, Indianapolis, USA)) which works by forming micelles around the DNA allowing fusion and transportation across the cell membrane. For this 0.1μg pxpERE, 0.1μg RL-TK, 0.5μg BSM were mixed and the volume made to a total of 50μl, per well. 2 μl of FuGENE6 was added to 48 μl of DMEM lacking phenol red. After a 5 minute incubation the DNA mix was added drop-wise to the FuGENE6 in DMEM lacking phenol red. They were then incubated together for 15 minutes at room temperature.

Media on the 24 well plates was replaced with fresh media containing plus or minus Dox and 30 minutes later the cells were transfected per well with 100μl of the FuGENE6/DNA mix added drop-wise. Cells were then incubated for 5 hrs at 37°C before replacing the media with fresh media containing the ligands 17β-estradiol (E2; 10^{-8}M), 4-hydroxytamoxifen (OHT; 10^{-7}M) and ICI 182, 780 (ICI; 10^{-7}M) in the presence and absence of Dox. Since the ligands were dissolved in ethanol, an equal volume of ethanol was added to the no ligand controls. The luciferase activity was then measured after 24 hours, using the Dual-Luciferase Reporter Assay system (Pomega, USA). The cells were washed with 0.5ml of ice cold PBS then lysed by adding 80μl passive lysis buffer (Promega, USA) and incubated at room temperature

with gentle shaking for 15 minutes. 50μl of the cell lysate was added to a 96 well OptiPlate (PerkinElmer, UK) and 50μl of the DualGlo firefly reagent was added to the cells in order to initiate firefly bioilluminescence which was measured using a TopCount NXT Microplate Scintillation & Luminescence Counter (Parkard Biosciences, USA). 50μl of the DualGlo Stop reagent was then added and the *Renilla* luciferase activity was measured as an internal control which was used to normalise the firefly luciferase readings.

2.2.5 Sulphorhodamine B (SRB) Growth Assay

Cells were grown with or without Dox for 96 hours, then plated at 3 $x10^3$ cells per well in a 96 well plate in 150μl of DMEM without phenol red, supplemented with 5% DSS and P/S/G in the presence or absence of Dox. Cells were plated with 6 replicates and 200μl of PBS was added to all wells around the edge of the plate to reduce evaporation of media from wells being utilised in the experiment. After cells were plated, 50μl of media containing ligand at 4x the required final concentration was added to each well, such that the final concentration of E2 was 10^{-8}M and that of OHT and ICI was 10^{-7}M. Ethanol was as added to the no ligand controls. Epidermal growth factor (EGF) (Sigma-Aldrich, Dorset, UK) was reconstituted in DMEM without phenol red containing 5% DSS and used over the range of concentrations 1ng/ml, 10ng/ml and 100ng/ml. Insulin (Sigma-Aldrich, Dorset, UK) was reconstituted in 10mM HCl and used over the range of concentrations 0.1μg/ml, 1μg/ml and 10μg/ml. An equal volume of a 10mM HCl solution was added to cells not treated with insulin. The MEK inhibitor, U0126 (Tocris Biosciences, UK), was reconstituted in DMSO and used at a final concentration of 10μM. An equal volume of DMSO was added to cells not treated with U0126 as a control.

SRB staining of cells was carried out using the Skehan method (Skehan et al., 1990). Cells were fixed by adding 100μl per well of ice-cold 40% trichloroacetic acid (TCA) gently so as not to disrupt the cells and incubating at room temperature for 1 hour. Plates were then washed 5 times in running tap water at an angle to allow the wells to fill indirectly. Cells were stained by adding 100μl per well of 0.4% ($^{w}/_{v}$) SRB (Sigma Aldrich, Dorset, UK) in 1% acetic acid and incubating at room temperature for 30 minutes. Plates were washed 5 times in 1% acetic acid and allowed to air dry overnight at room temperature. Dye was solubilised by adding 100μl per well of 10mM Tris-base and agitating the plate rapidly using a PMS-1000 shaker (Grant Instruments, UK) for 10 minutes. Absorbance was read using a spectrophotometer (Anthos 2001, Anthos Labtech Instruments, Austria) at a wavelength of 492nm.

2.2.6 RNA analysis

2.2.6.1 RNA Extraction

Cells were seeded 7.5 $x10^5$ in 10cm plates with 10mls of DMEM without phenol red, supplemented with 5% DSS and P/S/G, plus and minus Dox. Cells were harvested 96 hours later, with E2 (10^{-8} M) being added 8 or 16 hours prior to harvesting. RNA was extracted using the Qiagen RNeasy Mini Kit. Details of all buffers was withheld by the the manufacturer. Cells were washed twice with PBS and lysed in 600μl of Buffer RLT. The lysate added to a QIAshredder spin column and centrifuged for 2 minutes at full 10,000 rpm at room temperature. An equal volume of 70% ethanol was added, then after mixing was transferred 700μl at a time to an RNeasy spin column and centrifuged for 15 seconds at 10,000rpm. The flow through was discarded and the column washed with 350μl of Buffer RW1 by centrifuging for 15 seconds at 10,000rpm. DNase digestion was carried out using the RNase-free

DNase set (Qiagen, USA). The DNase I was reconstituted in ddH_2O (3 Kunitz units per μl) and 10μl diluted with 70μl of Buffer RDD. The 80μl of diluted DNase I was added directly onto the spin column membrane and incubated at room temperature for 15 minutes. The column was then washed with 350μl of Buffer RW1 and centrifuged for 15 seconds at 10,000rpm. The spin column was washed twice with 500μl of Buffer RPE and centrifuged for 15 seconds at 10,000rpm. The column was centrifuged for 15 seconds at 10,000rpm with no buffer added to remove any residual buffer before RNA was eluted by adding 50μl of RNA-free H_2O, incubating at room temperature for 5 minutes then centrifuging for 1 minute at 10,000rpm. RNA concentration was measured at 260 and 280 nm, using a spectrophotometer (Nano Drop ND-1000, Labtech International, UK), aliquoted and stored at -80°C.

2.2.6.2 Reverse Transcription for cDNA preparation

Complementary DNA (cDNA) was made by the reverse transcription technique using the reverse transcriptase RevertAid™ M-MuLV (Fermentas Life Sciences, York, UK). 2μg of RNA was made up to a total volume of 12μl with ddH_2O in a sterile tube. The master mixture was prepared on ice as follows for each sample of RNA; 2μl of 10mM dNTPs (Fermentas Life Sciences, York, UK), 1μl (200ng) of random primers (pd(N)6, Pharmacia, USA) , 4μl of 5x RT Buffer (Fermentas Life Sciences, York, UK) (250mM Tris-HCl, pH8.3; 250mM KCl; 20nM $MgCl_2$; 50mM DTT) and 1μl (200u) of RevertAid™ M-MuLV. 8μl of the master mixture was added to the 12μl of diluted RNA to give a total volume of 20μl. The reaction mixture was incubated at 42°C for 60 minutes then the reaction stopped by heating at 95°C for 5 minutes using a GeneAmp® PCRSystem 9700 (PE Applied Biosystems, UK). The cDNA was diluted 1:5 with ddH_2O and stored at -20°C.

2.2.6.3 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was carried out using Pre-Aliquoted ReddyMix™ PCR Master Mix, 25μl reaction (ABgene, UK). The primers used are shown in table 2.6. A master mix of primers was prepared using 2.5μl of each of the forward and reverse PCR primers and 5.5μl of ddH_2O per reaction. 2μl of cDNA, prepared as in the last section, was added per reaction to the ReddyMix™ PCR Master Mix tube and 10.5μl of the primer master mix was then added per reaction. The reaction mix was incubated at 95°C for 5 minutes to denature the cDNA. A cycle of heating at 94°C for denaturing to take place, cooling to 55°C to allow the oligonucleotide primers to anneal to the target sequences and then heating to 72°C for DNA synthesis to take place was carried out. The reaction was held at each temperature for 1 minute and the number of cycles carried out was dependent on the gene of interest and stored a 4°C.

Table 2.4 RT-PCR primers

Primer	Sequence	Accession number
GAPDH-F	TCCCATCACCATCTTCCA	NM_002046
GAPDH-R	CATCACGCCACAGTTTCC	NM_002046
ER-alpha-F	CAGATGGTCAGTGCCTTGTTGG	X03635
ER-alpha-R	CCAAGAGCAAGTTAGGAGCAAACAG	X03635
pS2-F	TTGGAGCAGAGAGGAGGCAATG	X00474
pS2-R	TTAGGATAGAAGCACCAGGGGACC	X00474
PR-F	CCAGAGCCCACAAATACAGCTTC	AF016381
PR-R	GCCAGCCTGACAGCACTTTCTAAG	AF016381
Cathepsin D-F	CATTCCCGAGGTGCTCAAGAAC	NM 001909
Cathepsin D-R	TCTTCACGTAGGTGCTGGACTTG	NM 001909
GREB1-F	AAGGAGGGCTGGAAACAAAT	BC054502
GREB1-R	CATTGTGGCCATTGTCATCT	BC054502

2.2.6.3.1 Agarose Gel Electrophoresis

PCR products were analysed by agarose gel electrophoresis. Samples were resolved on a 1% ($^{w}/_{v}$) agarose gel prepared in TBE and containing 0.25μl/ml ethidium bromide, run at 100V for 1 hour and electrophoresis was carried out in horizontal submersion gel tanks (Thistle Scientific, UK) in 1x TBE buffer at 150 volts for 30 minutes. Agarose gels were visualized using an UV transilluminator.

2.2.6.4 Real-time (Quantitative) RT-PCR

Real-time or quantitative RT-PCR (qRT-PCR) was carried out using the TaqMan® Gene Expression Assays (Applied Biosystems, USA). This uses TaqMan® Fast Universal PCR Master Mix (2x) No AmpErase UNG (Applied Biosystems, USA) which contains a hot-start DNA polymerase. The probes used are shown in table 2.8. A master mix of probes was prepared on ice using 10μl of the TaqMan® Fast Universal PCR Master Mix (2x) No AmpErase UNG, 1μl of the probe and 7μl of ddH_2O per reaction. 18μl of the probe master mix was then added per well into a MicroAmp Fast Optical 96-well Reaction Plate (0.1ml) (Applied Biosystems, USA) and 2μl of cDNA was added per reaction. The plate was sealed with a MicroAmp Optical Adhesive Film and centrifuged at 1200rpm for 1 minute at 4°C. The assay was carried out using an Applied Biosystems 7900HT Fast Real-Time PCR machine. The reaction was heated at 95°C for 20 seconds and then a thermal cycle of 95°C for 1 second followed by 60°C for 20 seconds was carried out for 40 cycles.

Table 2.5 qRT-PCR primers
Shown are the ABI Taqman assay numbers. These were purchased from ABI (CA, USA). Primer sequences are withheld by the manufacturer.

ERα	Hs00174860_m1 ESR1
pS2	Hs00170216_m1 TFF1
Cathepsin D	Hs00157201_m1 CTSD
GREB1	Hs00536409_m1 GREB1
PR	Hs00172183_m1 PGR
GAPDH	Hs99999905_m1 GAPDH

2.2.7 Microarray

The HumanWG-6 v2 Illumina Expression BeadChip (Illumina, USA) microarray platform was used to carry out whole-genome expression analysis comparing the cell lines. This technology probes for 48,804 transcripts using 79-mer oligonucleotides, combining a 50-mer gene-specific probe and a unique 29-mer "address" sequence, that are covalently attached to silica beads (Figure 2.1). These 3 micron beads are randomly assembled and bound within microwells on planar silica slides. The 29-mer address sequences present on each bead are the targets for a hybridization-based decoding procedure to map the array, identifying the location of each bead. Subsequent hybridization of the chip with labelled cRNA, derived by cDNA synthesis and single round in vitro transcription amplification of cellular total RNA using the Illumina RNA amplification Kit, can be quantified and correlated with the location of each gene-specific bead, using the Illumina Bead Array reader. Subsequent analysis of the generated data was performed using GeneSpring 7 software (Agilent Technologies, Cheshire, UK).

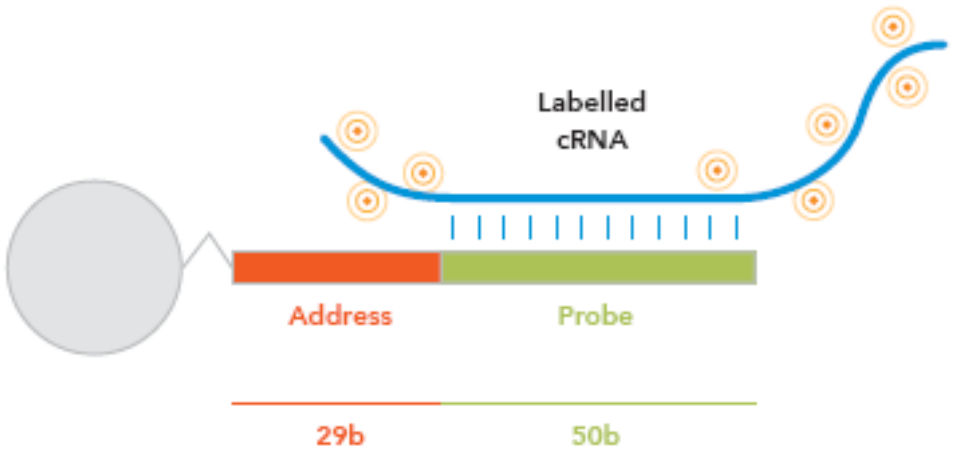


Figure 2.1 Illumina gene expression probe design
The oligonucleotides attached to beads in Illumina gene expression arrays contain a 29-base address used in the manufacturing/decoding process and a 50-base gene-specific probe. Each address and probe sequence combination has been selected bioinformatically, followed by a functional screen in the laboratory to ensure that no cross-hybridization is observed. Gene-specific probe design is accomplished through a multi-step bioinformatics algorithm that scores potential probes by considering the uniqueness compared with other genes, the sequence complexity, self-complementarity for hairpin structure prediction, melting temperature for hybridization uniformity and the distance from the 3' end of the transcript. Probe design also takes into account exon structure, enabling design of probes that target specific splice isoforms. For simplicity, this bead only shows one oligomer attached; actual beads have thousands of copies of the same sequence attached. (Taken from Illumina Gene Expression Profiling Technical Bulletin)

2.2.8 Immunoaffinity Purification

FLAG- and HA- double-tagged ERα was purified using FLAG and HA immunoprecipitation kits from Sigma Aldrich (Dorset, UK), as described below.

2.2.8.1 Harvesting cells

Cells were grown in DMEM with phenol red, supplemented with 10% FCS and P/S/G in the presence of E2 (10^{-8}M) with or without Dox for 72 hours. Cells were trypsinised as described in section 2.2.7 then plated at 7.5 x10^5 cells per 10cm plate in DMEM without phenol red, supplemented with 5% DSS and P/S/G in the presence or absence of E2 (10^{-8}M) with or without Dox and cultured for a further 72 hours at 37°C, 5% CO_2.

Cells were harvested at 4°C by one of three methods; using Sigma lysis buffer supplied with the kit, using high salt lysis buffer (HSLB) with 500mM NaCl or using modified HSLB with 150mM NaCl (Table 2.9). Protein concentration was ascertained by Bradford assay as described in section 2.2.5.2. An input sample was made by adding an equal volume of 2x Laemmli buffer (Table 2.9) to the lysate regardless of lysis buffer used to harvest cells.

2.2.8.1.1 Harvesting cells using Sigma lysis buffer

Cells were washed twice in ice cold PBS and 125μl of Sigma lysis buffer was added per 1 x10^6 cells, at 4°C with gentle rocking for 15 minutes, according to the Sigma instruction manual. Cells were scraped from the plate using a rubber policeman and lysates were collected in 1.5ml microfuge tubes. Samples were spun at 13,000rpm for 10 minutes at 4°C to remove any

remaining debris and the supernatant was placed in a fresh microfuge tube before storing at -80°C.

Table 2.6 Immunoaffinitiy purification buffers

Sigma lysis buffer	50 mM Tris, pH 7.4; 150mM NaCl; 1mM EDTA; 1% Triton® X-100; supplemented with 1x proteinase inhibitors (Roche)
HSLB (500 mM NaCl)	10mM Tris, pH 7.4; 500mM NaCl; 1mM EDTA; 0.1% NP40; 1x proteinase inhibitors (Roche); 1mM PMSF
Modified HSLB (no NaCl)	10mM Tris pH 7.4, 1mM EDTA, 0.1% NP40; 1x proteinase inhibitors (Roche); 1mM PMSF
2x Laemmli Buffer	1M Tris, pH6.8; 15% glycerol; 2% $^{v}/_{v}$ SDS; 50% $^{v}/_{v}$ ddH_2O; 0.1% $^{w}/_{v}$ bromophenol blue; 400mM DTT
Elution buffer	0.1M Glycine, pH3.5
Wash buffer	50mM Tris-HCl, pH7.4; 150mM NaCl

2.2.8.1.2 Harvesting cells using HSLB

Cells were washed twice in ice cold PBS then harvested by scraping with a rubber policeman in 500µl ice cold HSLB containing 500mM NaCl. Lysates were incubated on ice for 10 minutes then sonication was carried out for 5 seconds at 25% using a Soniprep 150 (MSE, UK). Samples were spun at 13,000rpm for 10 minutes at 4°C to remove any remaining debris and the supernatant was placed in a fresh microfuge tube before storing at -80°C.

2.2.8.1.3 Harvesting cells using modified HSLB

Cells were washed twice in ice-cold PBS then harvested by scrapping with a rubber policeman in 1ml ice cold PBS and spinning at 6,000rpm, 4°C for 10 minutes. The pellet was resuspended in 150µl HSLB with 500mM NaCl and sonication was

carried out for 5 seconds at 25% using a Soniprep 150 (MSE, UK). The samples were then spun at 13,000 rpm for 10 minutes at 4°C to remove any remaining debris. The supernatant was placed in a fresh microfuge tube and 350μl modified HSLB (Table 2.9) was added to dilute the NaCl to a final concentration of 150mM. Lysates were then stored at -80°C.

2.2.8.2 Determining protein concentration by Bradford assay

Protein concentrations were determined using the Bradford assay. Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, DE) was diluted 1:5 with ddH_2O and 200μl of the diluted reagent added per well of a 96 well plate. Known concentrations of BSA were used to produce a standard curve and known volumes of lysates were compared to this to determine protein concentration of the lysates. All measurements were performed in triplicate and absorbance was read using a spectrophotometer (Anthos 2001, Anthos Labtech Instruments, Austria) at wavelength 620nm.

2.2.8.3 Anti-FLAG Immunoaffinity Purification

The Sigma FLAG Tagged Protein Immunoprecipitation Kit was used to purify FLAG-ERα-HA from whole cell lysates. 20μl of anti-FLAG M2-agarose affinity gel was used per mg of protein. The gel was washed 5 times with 1x wash buffer (Table 2.9) at a volume equal to 20 times the volume of gel and centrifuged at 13,000rpm for 1 minute at 4°C and then the supernatant was removed. Between 1-10mg of whole cell lysate was then added to the gel and incubated overnight at 4°C with gentle rocking. The gel was centrifuged at 13,000rpm for 1 minute at 4°C and supernatant removed before washing 3 times in 1x wash buffer as before. The bound proteins

were eluted using peptide elution, acid elution or by the addition of Laemmli buffer, as described below.

2.2.8.3.1 Elution of FLAG-tagged protein with 3x FLAG peptide

A 5μg/μl solution of 3x FLAG peptide (N-Met-Asp-Tyr-Lys-Asp-His-Asp-Gly-Asp-Tyr-Lys-Asp-His-Asp-Ile-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C) was prepared as stated in the manufacturers manual by dissolving in 1x wash buffer (Table 2.9). A working concentration of 150ng/μl was produced by diluting 3μl of this stock peptide with 100μl of 1x wash buffer. The FLAG-tagged protein was eluted from the beads by adding 50μl of the working stock 3x FLAG peptide solution and incubating for 30 minutes at 4°C with gentle rocking. The gel was then centrifuged at 13,000rpm for 2 minutes at 4°C. The supernatant was transferred to a fresh microfuge tube and stored at -20°C.

2.2.8.3.2 Elution of FLAG-tagged protein by acid elution

FLAG-tagged proteins were eluted from the gel by adding 100μl of elution buffer (Table 2.9) and incubating at room temperature for 5 minutes with gentle rocking. The sample was then centrifuged at 13,000rpm for 5 minutes and the supernatant transferred to a fresh microfuge tube containing 10μl of 10x wash buffer then stored at -20°C.

2.2.8.3.3 Elution of FLAG-tagged protein with Laemmli Buffer

FLAG-tagged proteins were eluted from the gel by adding 50μl of 2x Laemmli Buffer to each sample and boiling for 3 minutes. The samples were then centrifuged

at 13,000rpm for 1 minute at room temperature and the supernatants transferred to a fresh microfuge tube then stored at -20°C.

2.2.8.4 Anti-HA Immunoaffinity Purification

The Sigma Anti-HA Immunoprecipitation Kit was used to purify FLAG-ERα-HA from whole cell lysates by the HA tag. 20μl of anti-HA-agarose affinity gel was used per mg of protein. Between 1-10mg of whole cell lysate was added to the gel and incubated over night at 4°C with gentle rocking. A minimum total volume of 600μl was achieved by adding 1x wash buffer if required. The gel was then washed 5 times in 700μl of 1x wash buffer by centrifugation at 13,000rpm for 1 minute at 4°C. The HA-tagged protein was eluted by the addition of Laemmli buffer as above, or with peptide elution (below).

2.2.8.4.1 Elution of HA-tagged protein with HA peptide

The HA peptide (Sigma, US) was prepared in ddH_2O at a stock concentration of 1mg/ml. A working concentration of 150ng/μl was produced by diluting 15μl of this stock peptide with 85μl of ddH_2O. The HA-tagged protein was eluted from the beads by adding 50μl of the working concentration HA peptide solution and incubating for 30 minutes at 4°C with gentle rocking. The gel was then centrifuged at 13,000rpm for 2 minutes at 4°C. The supernatant was transferred to a fresh microfuge tube and stored at -20°C.

2.2.8.5 Double Immunoaffinity Purification

Proteins eluted using the 3xFLAG peptide elution method were further purified by loading the eluate onto anti-HA-agarose affinity gel. Proteins were eluted from anti-FLAG M2-Agarose affinity gel as described in section 2.2.5.3.1; however, 100μl of 150ng/μl 3x FLAG peptide was used instead of 50μl. After the initial elution was carried out and the supernatant removed, the process was repeated again with 100μl of 150ng/μl 3x FLAG peptide and the total volume from both elutions (200μl) was loaded onto 40μl of anti-HA-agarose affinity gel. A final volume of 600μl was achieved by adding 400μl of 1x wash buffer. Incubation and wash steps were then carried out as described in section 2.2.5.4. Proteins were eluted by the HA peptide elution method as detailed in section 2.2.5.4.1.

2.2.9 Protein Analysis

2.2.9.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were denatured in sample buffer containing SDS according to the method of protein extraction required. After boiling the samples at 95°C for 5 minutes, they were loaded onto 8x7cm SDS-polyacrylamide gels, the percentage of which was determined by the size of the protein of interest (see Table 2.10 for gel composition). A discontinuous buffer system was used which consists of a non-restrictive large pore gel (stacking gel) which is layered on top of a separating gel (resolving gel). Electrophoresis was performed with running buffer (Table 2.11) at 60 volts for 45 minutes and then 100 volts for approximately 150 minutes using Mighty Small II gel apparatus (Amersham Biosciences, UK).

Table 2.7 Components of SDS-PAGE Gels

	7% Resolving Gel	10% Resolving Gel	15% resolving gel	Stacking Gel
Lower Buffer (4x)	5ml	5ml	5ml	-
Upper Buffer (10x)	-	-	-	0.5ml
Bis-acrylamide (30% stock solution)	4.7ml	6.7ml	10.7ml	0.5ml
ddH_2O	10.3ml	8.3ml	5.3ml	4ml
10% (w/v) APS	200µl	200µl	200µl	50µl
TEMED	20µl	20µl	20µl	5µl

Table 2.8 SDS-PAGE and Western Buffers

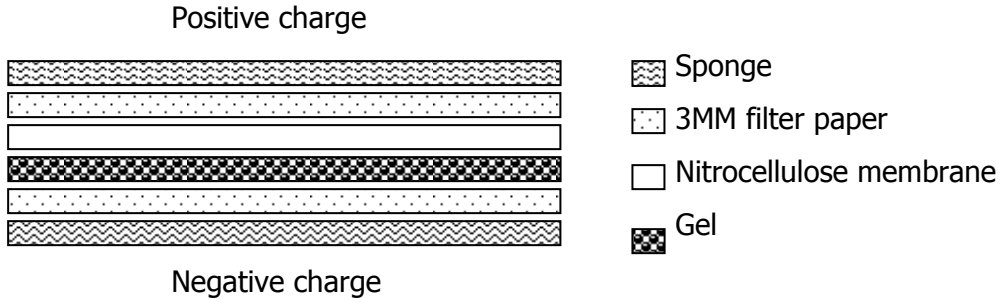
10x Running buffer	0.25M Tris base, 1.0M glycine, 35mM SDS
50x Electrotransfer buffer	0.25M Tris base, 1.0M glycine
1x Electrotransfer buffer	1x Electrotransfer buffer, 20% $^{v}/_{v}$ methanol, diluted in ddH_2O
Washing buffer	0.1% $^{v}/_{v}$ Tween 20 in PBS
Blocking buffer	5% $^{w}/_{v}$ Marvel dried skimmed milk dissolved in washing buffer

2.2.9.2 Western Blotting

Proteins were resolved by SDS-PAGE and then transferred from the gel to Hybond-ECL nitrocellulose membrane (Amersham Biosciences, UK) by electrophoresis. The resolving gel and membrane were set up as in figure 2.2. The proteins are drawn from the gel onto the membrane by the negative to positive flow of current. Gel electrotransfer was carried out using the Bio-Rad Mini Trans-Blot Cell at 100 volts for 90 minutes with electrotransfer buffer (Table 2.11). The nitrocellulose membrane was then incubated in 10ml of blocking buffer (Table 2.11) for 30 minutes at room temperature with gentle rocking before the primary antibody was added. The membrane was incubated overnight at 4°C with gentle rocking in 10ml of primary antibody which was diluted in blocking buffer as shown in table

2.12. Membranes were then washed four times, over a period of one hour, using washing buffer (Table 2.11) at room temperature with gentle rocking. The membrane was then incubated with horse radish peroxidise (HRP)-conjugated goat

Figure 2.2 Preparing membrane and gel for electrophoresis



anti-rabbit or anti-mouse immunoglubulins, as appropriate. The membrane was incubated for 1 hour at room temperature with gentle shaking in 10ml of secondary antibody diluted in blocking buffer (see table 2.13 for dilutions). Membranes were then washed as before over a period of one hour. Following washes, the membranes were incubated with SuperSignal® West Pico Enhanced Chemiluminescent (ECL) Substrate (Pierce, US). The HRP enzyme complex catalyzes the conversion of the ECL substrate into a sensitized reagent which on further oxidation by the addition of hydrogen peroxide produces an excited carbonyl which emits light as it decays from a triplet to a singlet carbonyl. Signals were detected by exposing high performance autoradiography film (Amersham, UK) to the membrane. The film was developed using a Konica SRX-101A X Ray Developer.

Table 2.9 Primary antibody information

Antibody	Antigen	Dilution	Antigen raised in	Company
ER6F11	Estrogen Receptor α	1:2000	Mouse monoclonal	Nova Castra
HA (3F10)	HA-peptide	1:500	HRP-conjugated	Roche
AC-15	β-Actin	1:20,000	Mouse monoclonal	AbCam
CTD-19	Cathepsin D	1:1000	Mouse monoclonal	AbCam
FL-84	pS2	1:400	Rabbit polyclonal	Santa Cruz
C-19	Progesterone receptor	1: 200	Rabbit polyclonal	Santa Cruz

Table 2.10 Secondary antibody information

Antibody	Dilution	Antigen raised in	Company
α mouse HRP conjugated	1:2000	Goat polyclonal	DAKO
α rabbit HRP conjugated	1:2000	Goat polyclonal	DAKO

2.2.9.3 SYPRO Ruby Gel Stain

After SDS-PAGE, the resolving gel was placed in a clean plastic dish (Sterelin, Barloworld Scientific UK) where it was washed in 25ml of fixing solution (10% methanol, 7% acetic acid) for 30 minutes with rocking at room temperature. After this time, the fixing solution was removed and 50ml SYPRO Ruby protein gel stain (Bio-Rad, DE) added. After gentle agitation for 120 minutes, the gel stain was removed and the gel washed in 25ml of fixing solution for 30 minutes. Gel imaging was then carried out using a Typhoon 8600 Variable Mode Imager (Amersham Pharmacia Biotech, UK).

2.2.9.4 Silver Stain

Silver staining of the resolving gel was carried out using the ProteoSilver™ Plus Silver Stain kit (Sigma, USA) following the manufacturers instructions. All steps were carried out at room temperature with shaking at 60rpm. The resolving gel was

placed in a clean 10cm square Petri dish (Sterelin, Barloworld Scientific UK) and fixed by incubating in 25ml of fixing solution (50% ethanol, 10% acetic acid) for 40 minutes. The gel was then washed in 25ml of a 30% ethanol solution for 10 minutes followed by a 10 minute wash with 25ml of ddH_2O. The gel was then treated with 25ml of the Sensitizer Solution for 10 minutes then washed twice for 10 minutes each with 25ml of ddH_2O. The gel was equilibrated with 25ml of the Silver Solution for 10 minutes then washed for 1 minute with 25ml of ddH_2O before being developed by treating the gel with 25ml of the Developer Solution for up to 7 minutes until the desired staining intensity was achieved. The developing reaction was stopped by adding 2.5ml of ProteoSilver Stop Solution to the Developer Solution and incubating for 5 minutes.

3 RESULTS

3.1 Characterising MCF7-TO cell lines

The MCF7 human breast cancer-derived cell line is the most commonly used model system for estrogen-responsive breast cancer. MCF7 cells grow in an estrogen-dependent manner, with their growth being inhibited by anti-estrogens or by estrogen withdrawal. They express ERα and demonstrate estrogen and anti-estrogen regulation of the expression of estrogen-responsive genes. Previous studies from the laboratory have used a MCF7-derived line (MCF7-TO) (BD Biosciences, UK), which stably expresses TetR-VP16, allowing tetracycline regulation of the expression of genes of interest (Buluwela et al., 2005). The MCF7-TO cells continue to express ERα, express estrogen-responsive genes in an estrogen and anti-estrogen-regulated manner, grow in a ligand-dependent manner and form estrogen-dependent tumours in xenografts (Buluwela et al., 2005), and in this context they are indistinguishable from the parental MCF7 cells. MCF7-TO cells were stably transduced with pRevTRE-ERα, (MCF7-TO-ERα) encoding HA and FLAG epitope tag sequences at the 5' and 3' ends respectively, to facilitate immunoaffinity purification of ERα and associated proteins. For this, HA-ERα-FLAG was cloned into the retroviral plasmid pRevTRE, which encodes a tetracycline responsive promoter to provide tetracycline regulation of the gene of interest and the hygromycin B resistance gene, to allow for selection of stable transformants. Stable MCF7-TO lines were also generated to express HA- and FLAG-tagged ERα in which serines 104, 106 and 118 have been substituted by alanine residues (MCF7-TO-ERα(AAA)) to prevent phosphorylation at these sites. Finally, stable lines were generated, in which serines 104, 106 and 118 have been substituted by glutamic acid (MCF7-TO-ERα(EEE)). Serine residues are often replaced by glutamic or aspartic acids, to "mimic" phosphorylation, and have

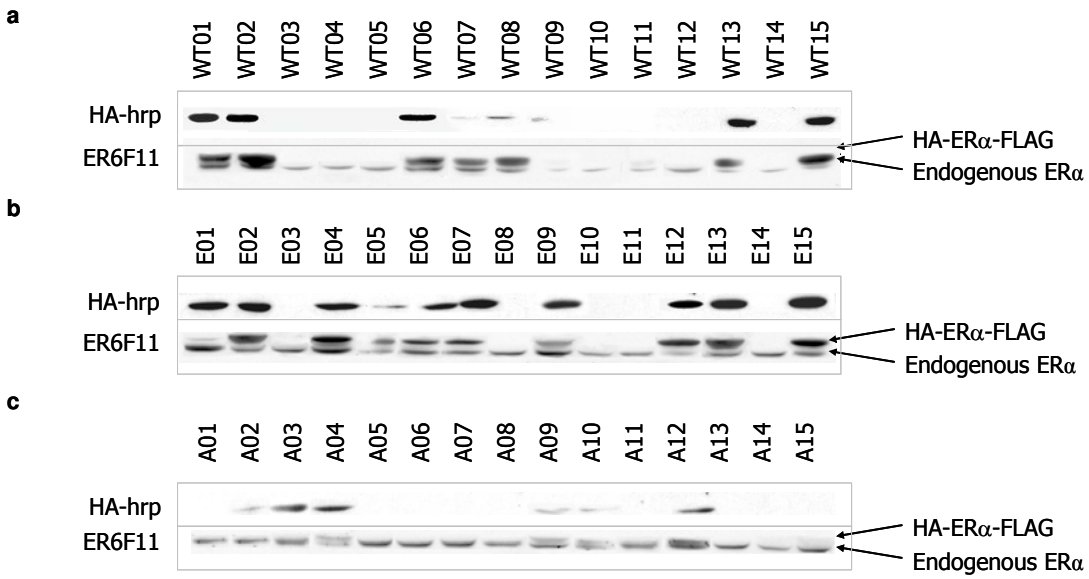


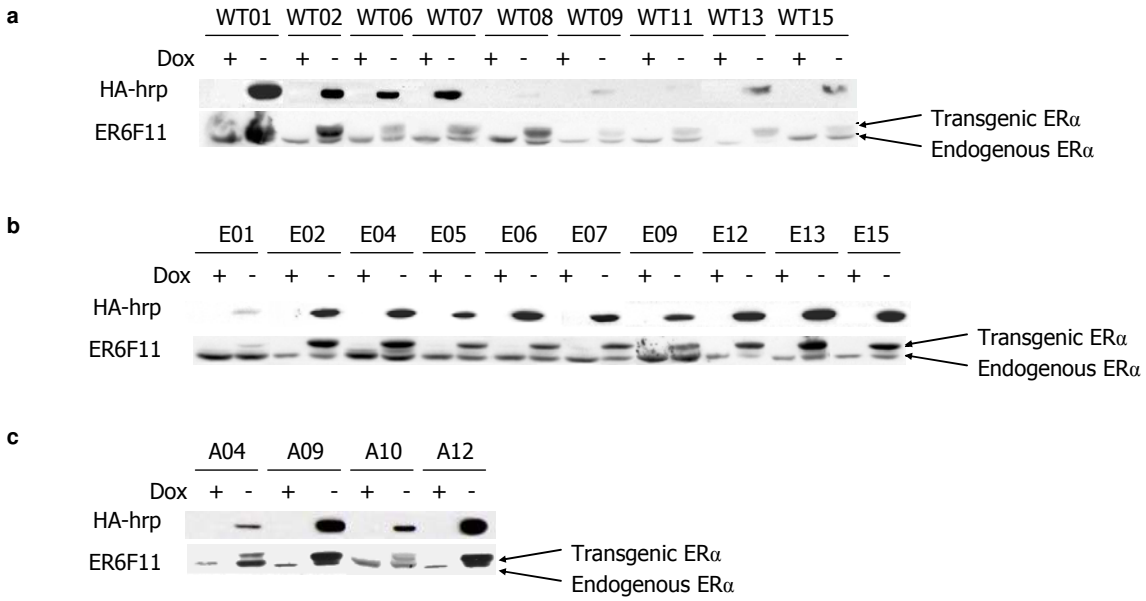
Figure 3.1 Expression levels of ERα in MCF7-TO-ERα clones
Whole cell lysates were made for the (a) MCF7-TO-ERα cell line (clones WT01-WT15), (b) MCF7-TO-ERα(EEE) cell line (clones E01-E15) and (c) MCF7-TO-ERα(AAA) cell line (clones A01-A15) using the hot lysis method 96hr after Dox removal. Lysates were immunoblotted using HA-hrp (top blot) which identifies the HA tag of the HA-ERα-FLAG construct, and ER6F11 (bottom blot) which identifies both endogenous and exogenous ERα.

previously been described for ERα (Ali et al., 1993; Chen et al., 2002; Thomas et al., 2008). As these lines express the gene of interest following removal of tetracycline, routine culturing was performed in the presence of doxycycline (Dox), a more stable form of tetracycline, to prevent expression of HA-ERα-FLAG, HA-ERα(EEE)-FLAG and HA-ERα(AAA)-FLAG and any possible long-term changes that high levels of ERα expression might cause. These lines were generated prior to my arrival (R. Thomas et al., unpublished) but had not been characterised beyond preliminary confirmation of the expression of the transgene in a few of the cloned lines. For further experiments, it was therefore necessary to characterise the clones for expression of the epitope-tagged ERα.

Lysates prepared from fifteen hygromycin B-resistant clones arising from each of the retroviral transductions with pRevTRE-ERα, pRevTRE-ERα(EEE) and pRevTRE-ERα(AAA) 120 hours after removal of Dox from the medium, were immunoblotted for ERα and HA-ERα-FLAG. Immunoblotting using an antibody for the HA tag, showed that nine of the clones selected following transduction for HA-ERα-FLAG (named WT01-WT15), expressed a polypeptide of the expected size, which was confirmed by immunoblotting using the ERα-specific antibody (ER6F11) (Fig. 3.1a). As seen in the ERα immunoblot, HA-ERα-FLAG migrates slightly more slowly than endogenous ERα, presumably due to the HA and FLAG tags. Ten of the MCF7-TO-ERα(EEE) clones (named E01-E15) expressed HA-ERα(EEE)-FLAG, the polypeptide migrating more slowly than endogenous ERα (Fig. 3.1b). Four of the the MCF7-TO-ERα(AAA) clones (named A01-A15) expressed HA-ERα(AAA)-FLAG (Fig. 3.1c). Clones that expressed the "transgene" were next tested for Dox regulation of HA-ERα-FLAG, HA-ERα(EEE)-FLAG and HA-ERα(AAA)-FLAG expression, by immunoblotting of lysates prepared in the absence or presence of Dox (Fig. 3.2). All of the positive clones demonstrated Dox regulation with little or no transgene

expression being detected in the presence of Dox (Fig. 3.2). Based on these data, three clones were selected for further investigation based on the level of HA- and FLAG-tagged ERα expression compared to the expression level of endogenous ERα; one clone from each cell line that demonstrated high, one medium and one demonstrating low levels of HA-ERα-FLAG expression compared to endogenous ERα, were selected for further study, in order to determine if the level of HA-ERα-FLAG expression influenced the ligand response of the cells. For the MCF7-TO-ERα cell line (Fig. 3.2a) clone WT02 was chosen as expressing high, clone WT06 (intermediate expression) and clone WT11 (low level expression) of HA-ERα-FLAG. For the MCF7-TO-ERα(EEE) cell line (Fig. 3.2b) clone E04 was chosen as expressing high, clone E06 as expressing medium and clone E01 as expressing low levels of HA-ERα(EEE)-FLAG. For the MCF7-TO-ERα(AAA) cell line (Fig. 3.2c) clone A12 was chosen as expressing high, clone A10 as expressing medium and clone A04 as expressing low levels of HA-ERα(AAA)- FLAG. All subsequent studies were performed using these nine lines, together with MCF7-TO cells as controls.

A time course experiment was next carried out to assess transgene expression over time and to identify when maximal levels of transgene expression are observed (Fig. 3.3). This was carried out in order to determine how long clones should be grown in the absence of Dox to achieve stable levels of HA-ERα-FLAG protein. From the time course, expression generally reached near maximal 96 hours following removal of Dox, although higher levels were achieved at 144 hours (Fig 3.3 a-c). A longer time course showed levelling out of expression at 168 hours (Fig. 3.3 d). However, as there was substantial expression at 96 hours, this time-point was used for subsequent studies as it provided the most appropriate staging for the ligand experiments.



d

	Low expression	Medium expression	High expression
MCF7-TO-ERα	WT11	WT06	WT02
MCF7-TO-ERα(EEE)	E01	E06	E04
MCF7-TO-ERα(AAA)	A04	A10	A12

Figure 3.2. Western blotting of MCF7-TO-ERα clones demonstrates Dox regulation of transgene expression

Whole cell lysates of (a) MCF7-TO-ERα, (b) MCF7-TO-ERα(EEE) and (c) MCF7-TO-ERα(AAA) clones were made using the hot lysis method 120 hours after Dox removal. Lysates were probed using antibodies for HA and ERα (ER6F11). Clones displaying low, medium and high levels of HA-ERα-FLAG expression compared to endogenous ERα were chosen as listed in (d).

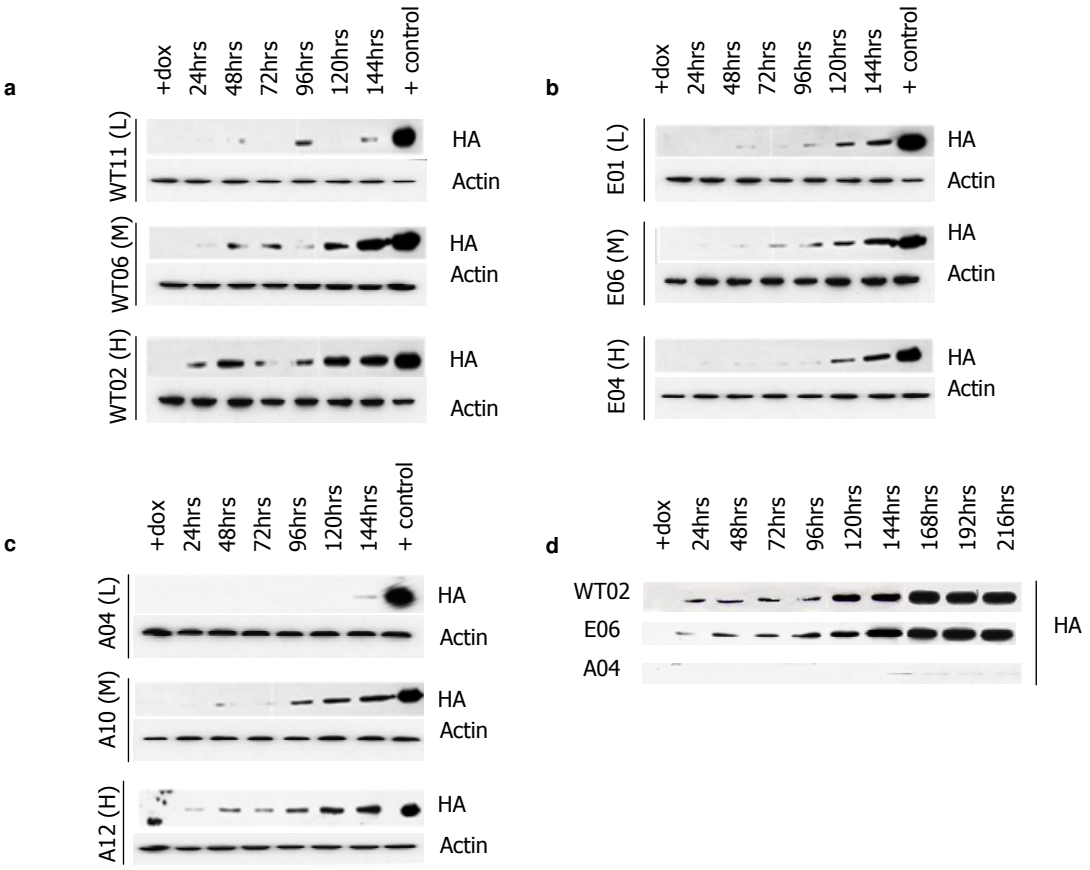


Figure 3.3 Time course of HA-ERα-FLAG induction after removal of Dox
The expression of HA and FLAG tagged ERα in the presence of estrogen for three clones from each cell line was determined at 24, 48, 72, 96, 120 and 144 hours after Dox removal. (a) MCF7-TO-ERα clone WT11 represented low, WT06 medium and WT02 high levels of HA-ERα-FLAG expression. (b) MCF7-TO-ERα(EEE) clone E01 represented low, E06 medium and E04 high levels of HA-ERα(EEE)-FLAG expression. (c) MCF7-TO-ERα(AAA) clone A01 represented low, A10 medium and A12 high levels of HA-ERα(AAA)-FLAG expression. Low, medium and high clones are denoted by L, M and H respectively. (d) The time course was extended up to 216 hours for MCF7-TO-ERα(WT02), MCF7-TO-ERα(EEE)(E06) and MCF7-TO-ERα(AAA)(A04).

3.2 Reporter Gene Assay Shows Dox-Regulated Increased Stimulation of ERα Activity in Stable Lines

In order to determine how over-expression of ERα, or the expression of ERα(EEE) and ERα(AAA) alters the estrogen-responses in the MCF7-TO lines, reporter gene assays were carried out following transient transfection with an estrogen-responsive firefly luciferase reporter gene encoding three EREs (pXpERE3) and the RLTK renilla luciferase reporter gene DNA (Promega, UK) as a control for transfection efficiency. Receptor activity was measured after treatment with vehicle (ethanol) (Fig.3.4a), 17β-estradiol (E2) (10^{-8}M) (Fig3.4b), 4-hydroxytamoxifen (OHT) (10^{-7}M) (Fig.3.4c) and ICI 182, 780 (ICI) (10^{-7}M) (Fig3.4d) for 24 hours (section 2.1.1.2). These concentrations were chosen to mimic physiological concentrations of the individual drug. The reporter gene activities for each of the three chosen clones for MCF7-TO-ERα (WT11, WT06, WT02), MCF7-TO-ERα(EEE) (E01, E06, E04) and MCF7-TO-ERα(AAA) (A04, A10, A12) were measured in triplicate and the average shown in figure 3.4. In the parental (MCF7-TO) cells, reporter gene activity was stimulated by E2, and to a lower extent by the partial ERα inhibitor, OHT. There was no significant difference in reporter gene activity in the presence or absence of Dox for the MCF7-TO cell line under any of the conditions tested, indicating that Dox does not influence ERα activity in MCF7-TO cells.

In the presence of Dox, each cell line displayed a similar profile to MCF7-TO parental cells, displaying an estrogen-stimulated increase in activity (Fig.3.4b) which was reduced in the presence of OHT (Fig.3.4c) and reduced further in the presence of ICI (Fig.3.4d), to levels similar as that in the absence of ligand (Fig.3.4a). In the presence of Dox, each clone would be expected to express similar levels of ERα to the MCF7-TO parental cell line and as such show similar levels of ERα activity. However, all the clones showed slightly varying levels of ERα activity under all

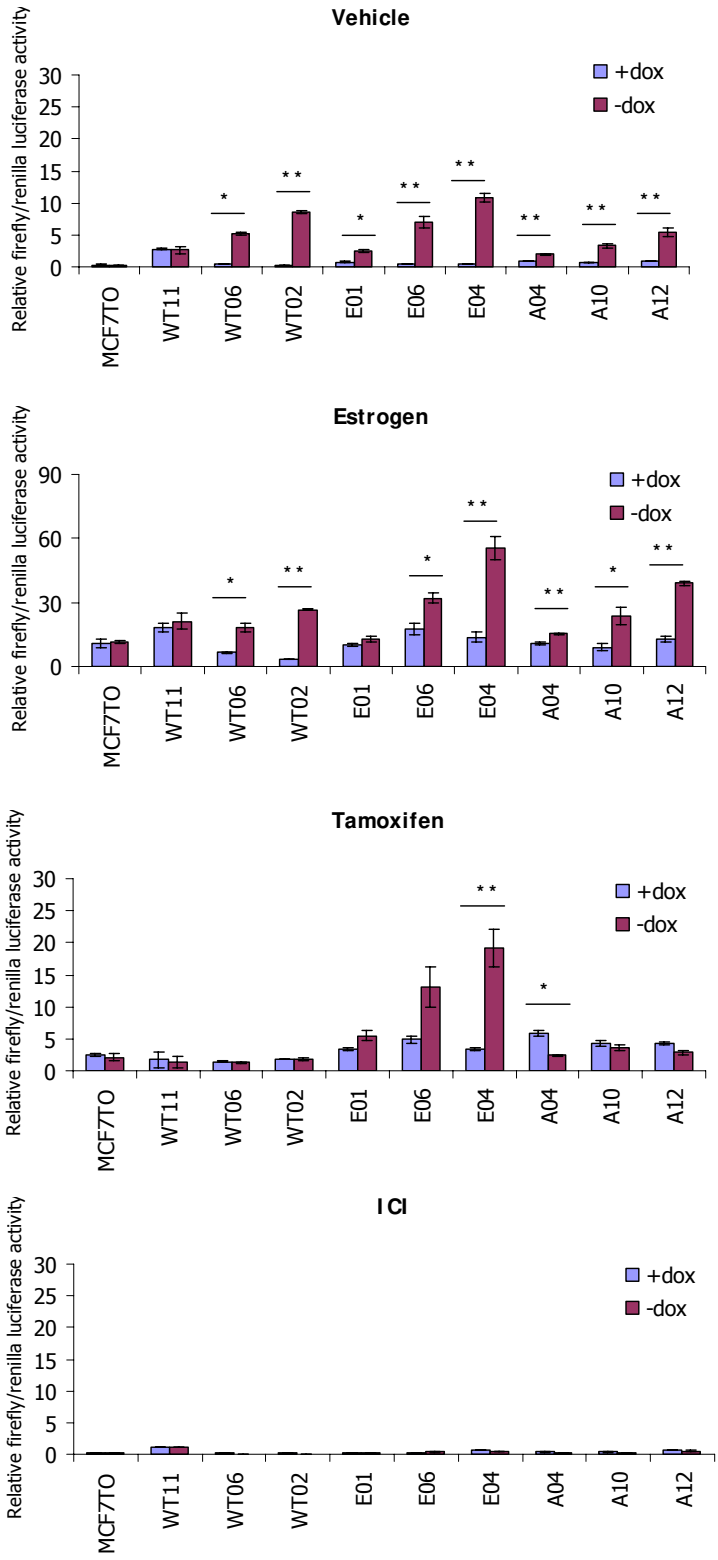


Figure 3.4 Transactivation activity of HA-ERα-FLAG constructs
Luciferase reporter gene assays were used to measure ERα activity in the presence of vehicle (EtOH) (a), E2 (10^{-8} M) (b), OHT (10^{-7} M) (c) and ICI (10^{-7} M) (d). 0.1μg pxpERE, 0.1μg RL-TK and 0.5μg BSM was used per well to transfect $8x10^4$ cells per well using FuGENE6. The data represent the average activities of 3 replicates for each clone; (in order of low to high expression) MCF7-TO-ERα clones WT11, WT06, WT02, MCF7-TO-ERα(EEE) clones E02, E06, E04 and MCF7-TO-ERα(AAA) clones A04, A10, A12. The error bars represent the standard errors of the mean. Statistical differences with p values ≤ 0.01 denoted by * and p values ≤ 0.001 denoted by **

conditions in the presence of Dox compared with each other and MCF7-TO. WT11, E06, E04 and A12 consistently showed higher levels of activity compared to MCF7-TO under all conditions suggesting a higher level of endogenous ERα expression in these clones and may be indicative of some transgene expression in the presence of Dox.

In the absence of ligand (fig. 3.4a), removal of Dox and subsequent expression of ERα, ERα(EEE) and ERα(AAA) caused a significant increase in reporter gene activity compared to in the presence of Dox, except for MCF7-TO-ERα clone WT11. Interestingly, clones expressing the lowest levels of HA and FLAG tagged ERα (WT11, E01 and A04) showed the smallest increase in reporter gene activity in the absence of Dox compared to in the presence of Dox whereas those expressing the highest levels of HA and FLAG tagged ERα (WT02, E04 and A12) showed the largest increase. In a similar way, E2 -stimulated reporter gene activity was increased in the absence of Dox compared to in the presence of Dox – the level of increase correlating to the level of ERα expression in each clone with WT11, E01 and A04 showing the smallest increase and WT02, W04 and A12 showing the largest increase (fig. 3.4b). Since this occurred for all cell lines regardless of the ERα mutant expressed, this suggests that ERα over-expression is sufficient for increased levels of reporter gene activity in the presence and absence of E2, with ERα phosphorylation not contributing greatly to this effect.

OHT treatment (Fig.3.4c) caused an inhibition of ERα activity for all the cell lines compared to the acitivty measured in the presence of E2 in the presence and absence of Dox. In the absence of Dox, MCF7-TO-ERα clones showed no significant difference in activity compared to in the presence of Dox, MCF7-TO-ERα(EEE) clones showed a significant increase in activity and MCF7-TO-ERα(AAA) clones showed a significant decrease in activity compared to in the presence of Dox during OHT

treatment. This suggests that whereas the phosphorylation status of these AF1 sites did not have an effect on ERα activity in the presence or absence of E2, phosphorylation may be significant in the presence of OHT as an increase in ERα acitivity shown by clones expressing glutamic acid residues at these sites appears to be inhibited due to the expression of alanine residues.

3.3 Expression analysis of Estrogen-Regulated genes by immunoblotting

Cells were treated with estrogen in the presence and absence of Dox to investigate how over-expression of ERα and the phosphorylation site mutants affects the expression of endogenous estrogen-regulated genes. The cells were grown in the presence or absence of Dox for 120 hours before the addition of E2 (10^{-7}M) and cell lysates were prepared a further 24 hours later (Fig. 3.5). Immunoblotting for ß-actin was used as a control for protein loading.

The MCF7-TO cell line (Fig. 3.5a) shows endogenous ERα expression which is not altered by Dox treatment, and no expression of HA-ERα-FLAG, as expected. The cell lines expressing epitope-tagged forms of ERα (Fig. 3.5b-d) show Dox regulation of the tagged protein as seen by the HA blots and the upper band of the ERα blots where tagged ERα, ERα(EEE) and ERα(AAA) are not present in the presence of Dox. There appears to be low level expression of HA-ERα-FLAG in the MCF7-TO-ERα clone WT06 (Fig. 3.5b) in the presence of Dox, indicating 'leaky' expression of HA-ERα-FLAG in this clone.

Cathepsin D is a well-characterised estrogen-regulated gene. In agreement with previous findings, cathepsin D expression was stimulated by E2 both in the presence and absence of Dox in all cell lines (Fig. 3.5a-d). In cell lines expressing tagged forms of ERα (Fig. 3.5b-d) an increase in the levels of cathepsin D can be

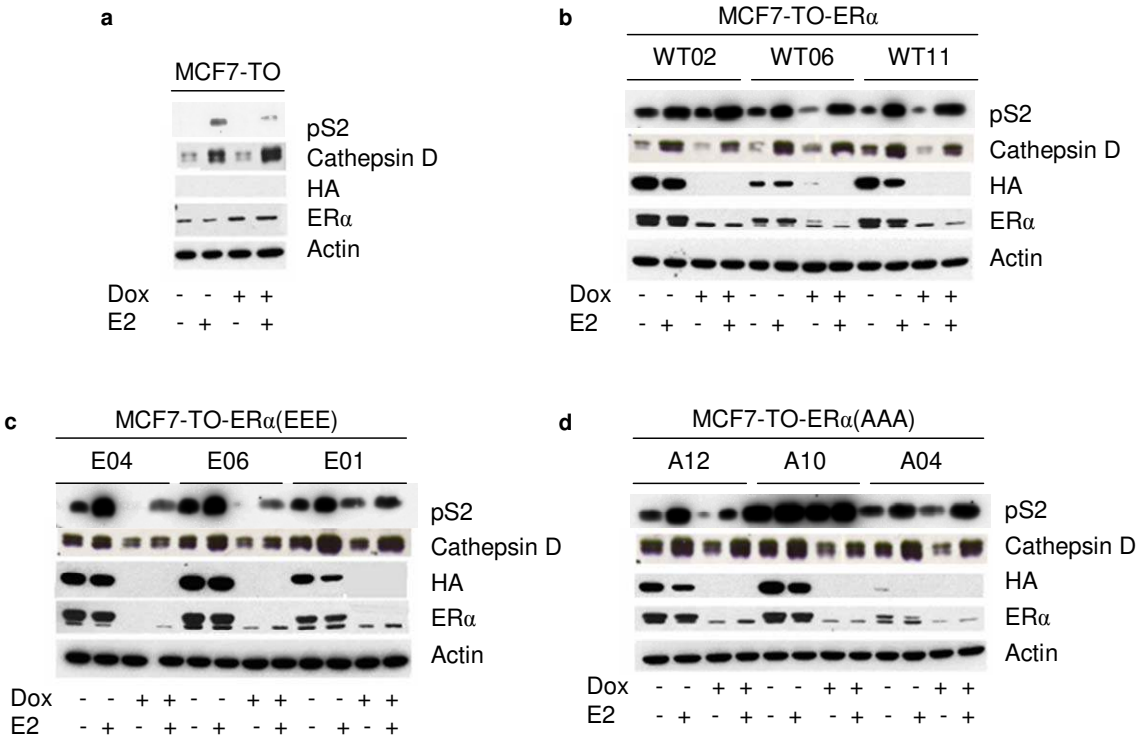


Figure 3.5 Expression of estrogen-regulated genes in the presence and absence of HA-ERα-FLAG constructs and estrogen

Cells were grown in the presence or absence of Dox for 96 hrs before treatment with vehicle (ethanol) or E2 (10^{-7}M) for 24 hrs. Cells were then harvested and western blot analysis carried out for estrogen regulated genes pS2 and cathepsin D, the HA tag, ERα and β-Actin as a loading control. The three clones from each cell line are in the order from left to right of high to low levels of phospho-mutant ERα expression.

seen in the absence of Dox compared to the presence of Dox with and without E2 treatment.

Levels of the estrogen-regulated gene, pS2, were also stimulated by E2 in all the cell lines in the presence and absence of Dox (Fig. 3.5a-d). MCF7-TO showed no expression of pS2 in the absence of ligand in either the presence or absence of Dox, although lower levels of pS2 overall were found in this line (Fig.3.5a). All MCF7-TO-ERα, MCF7-TO-ERα(EEE) and MCF7-TO-ERα(AAA) clones showed ligand-independent pS2 expression which was increased in response to E2 in the presence and absence of Dox (Fig. 3.5b-d) and both ligand-independent and estrogen-dependent pS2 expression was increased in the absence of Dox compared to in the presence of Dox.

Levels of pS2 expression remained similar in the presence and absence of Dox for the MCF7-TO cell line. However, MCF7-TO-ERα(EEE) clones (Fig. 3.5c) displayed a larger increase in ligand-independent pS2 expression compared to MCF7-TO-ERα (Fig. 3.5b) and MCF7-TO-ERα(AAA) (Fig. 3.5d) clones in the absence of Dox compared to in the presence of Dox. MCF7-TO-ERα(AAA) clone A10 showed abnormally high levels of pS2 expression compared to the other clones within the MCF7-TO-ERα(AAA) cell line.

These data show that over-expression of ERα leads to an increase in expression of cathepsin D and pS2, both in the presence and absence of E2. However, expression of ERα(EEE) (Fig. 3.5c) appears to cause an increase in ligand-independent expression of pS2 greater than that seen by cell lines expressing WT ERα or ERα(AAA). This suggests that phosphorylated AF1 residues may cause an increase in ligand-independent expression of some E2-regulated genes.

3.4 Determination of the expression of estrogen responsive genes using RT-PCR and real-time PCR

In order to determine if the altered expression of pS2 and cathepsin D was also observed at the transcriptional level, total RNA was isolated from cells after 8 hours and 16 hours following estrogen treatment. Reverse transcriptase PCR (RT-PCR) and real-time (quantitative) RT-PCR (qRT-PCR) were used to examine expression of a number of estrogen-regulated genes; pS2, cathepsin D, PR and GREB1, as well as ERα, with GAPDH serving as control. For these experiments, the cell line that appeared to express HA-ERα-FLAG (line WT06) at levels similar to those of endogenous ERα, as determined by immunoblotting, was used. On this basis, the E06 and A10 lines were used to represent MCF7-TO-ERα(EEE) and MCF7-TO-ERα(AAA), respectively.

qRT-PCR was carried out using validated primer sets from ABI on a Taqman 7900HT machine. Expression for each gene is depicted relative to expression levels of GAPDH and is shown as fold expression relative to expression level in the absence of E2 and the presence of Dox for MCF7-TO cells (Figure 3.6). Both real-time RT-PCR (Fig. 3.6e) and RT-PCR (Fig. 3.7) showed that, as expected, ERα mRNA levels were elevated in the MCF7-TO-ERα, MCF7-TO-ERα(EEE) and MCF7-TO-ERα(AAA) lines, compared to MCF7-TO cells in the absence, but not in the presence of Dox, as the PCR primers were designed against the ERα coding sequences and hence would detect both the endogenous and "transgene" ERα. This increase in ERα expression in the absence of Dox for the MCF7-TO-ERα, MCF7-TO-ERα(EEE) and MCF7-TO-ERα(AAA) lines was also seen at the protein level (Fig. 3.5). However, whilst levels of ERα expression were similar in the MCF7-TO-ERα and MCF7-TO-ERα(EEE) lines, levels of ERα expression in the MCF7-TO-ERα(AAA) line were considerably higher in the absence of Dox, compared to the other lines as analysed by real-time RT-PCR

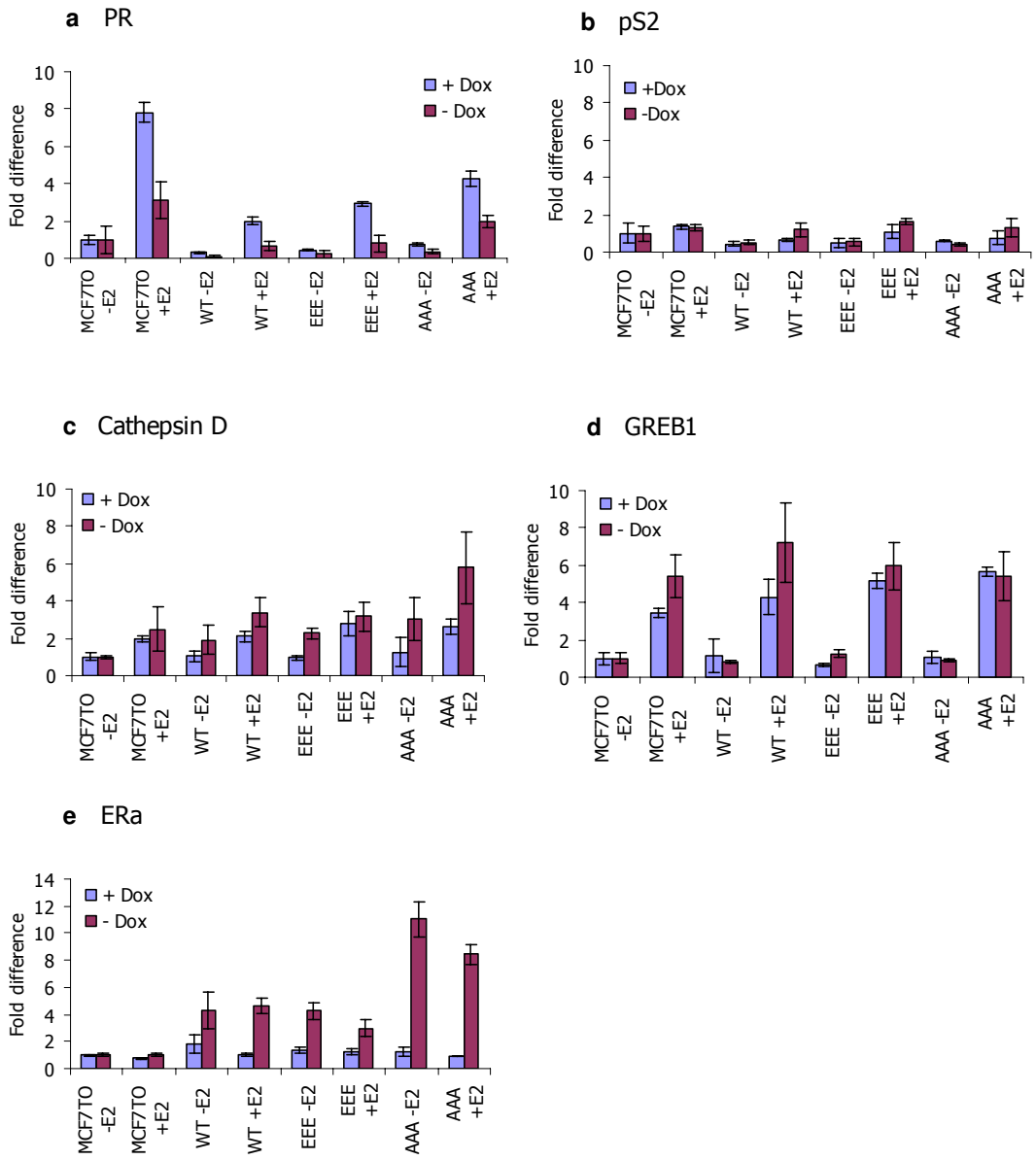


Figure 3.6 Expression of estrogen-regulated genes in response to estrogen and HA-ERα-FLAG constructs

Cells were treated with ethanol or E2 (10^{-8}M) in the presence and absence of DOX. Cells were harvested after 8 hours of estrogen treatment and total RNA extracted. Real-time PCR was performed in triplicate for three clones of each cell line MCF7-TO-ERα (WT), MCF7-TO-ERα(EEE) (EEE) and MCF7-TO-ERα(AAA) (AAA) using Taqman gene expression primers for the estrogen-regulated genes PR (a), pS2 (b), cathepsin D (c), GREB1 (d) and ERα (e). Averages are shown for each cell line plus MCF7-TO as a control. Readings were corrected against GAPDH and fold difference is shown relative to MCF7-TO +Dox -E2.

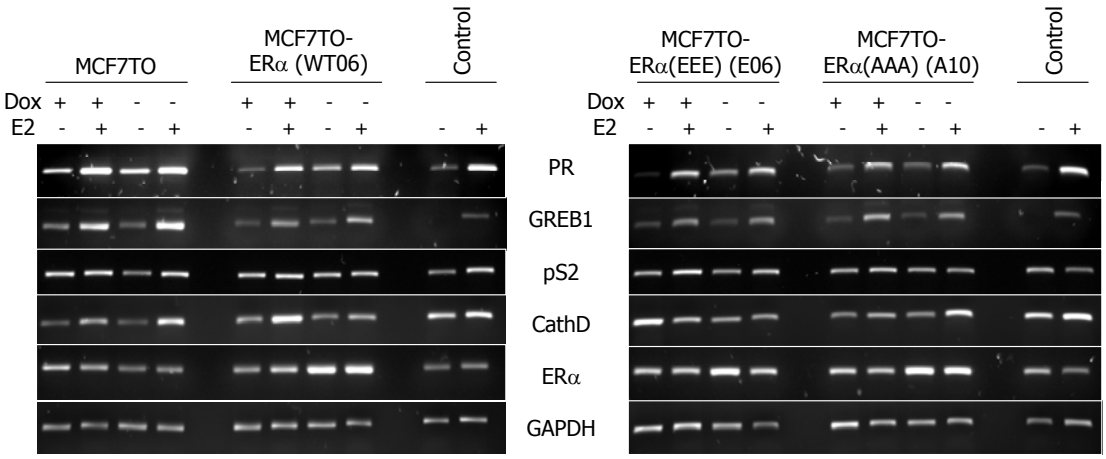


Figure 3.7 Expression of estrogen-regulated genes in response to estrogen in individual HA-ERα-FLAG construct cell lines
Cell lines expressing similar levels of exogenous ERα compared to endogenoue ERα were treated with ethanol or E2 (10^{-8}M) in the presence and absence of Dox. Cells were harvested after 16 hours of E2 treatment and total RNA extracted. RT-PCR was performed using primers for the estrogen-regulated genes progesterone receptor (PR), GREB1, pS2, cathepsin D (CathD), ERα and GAPDH as a loading control. RNA extracted from COS cells transfected with ERα grown in the presence and absence of E2 was used as a positive control.

(Fig. 3.6e). This high-level expression at the RNA level was not observed at the protein level (Fig. 3.5) or by RT-PCR (Fig. 3.7).

Expression of all four estrogen-regulated genes was stimulated by E2 in the MCF7-TO cell line at 8 and 16 hours after addition of E2 as shown by real-time RT-PCR and RT-PCR (Fig. 3.6 and Fig. 3.7). In MCF7-TO cells, the expression of pS2, cathepsin D and GREB1 were stimulated similarly in the presence or absence of Dox, in agreement with the immunoblotting data. Further, despite the apparent increase in levels of pS2 and cathepsin D protein seen in the absence of Dox in the MCF7-TO-ERα, MCF7-TO-ERα(EEE) and MCF7-TO-ERα(AAA) cell lines, there was only a small, and statistically insignifcant increase in expression of these genes, as analysed by semi-quantitative RT-PCR (Fig. 3.6) and real-time RT-PCR (Fig. 3.7), with 8 or 16 hours E2 treatment.

Although PR gene expression was stimulated by estrogen in all of the lines in the absence or presence of Dox, a greater level of PR expression was observed in the MCF7-TO cells than in the transgene lines as shown by both qRT-PCR (Fig. 3.6a) and RT-PCR (Fig. 3.7). Moreover, qRT-PCR analysis of PR expression showed an 8-fold induction in response to estrogen in the presence of Dox for the MCF7-TO cell line, however, this was reduced to a 3-fold induction in the absence of Dox (Fig. 3.6a). This is surprising as the MCF7-TO cell line is not expected to show any reaction to Dox. However, this effect of Dox was not seen by RT-PCR analysis of PR (Fig. 3.7) or for any of the other genes examined.

Together, these data suggest that overexpression of ERα leads to only a small effect on expression of the pS2, cathepsin D and GREB1 genes over 8 or 16 hours treatment with E2, although immunoblotting for pS2 and cathepsin D proteins suggests that the increased expression is sufficient for the increase in levels of these proteins, as judged by immunoblotting.

3.5 Whole-genome analysis of MCF7-TO cell lines by microarray

In order to investigate if expression of WT ERα, ERα(EEE) or ERα(AAA) affected the expression of specific genes, whole-genome expression analysis was carried out in triplicate for one representative clone from each of the ERα variants, WT06, E01 and A10, and the parental MCF7-TO was used as a control. WT06 and A10 were used as these clones expressed moderate levels of exogenous ERα. E06 should have been used for the MCF7-TO-ERα(EEE) cell line for this purpose but results in figure 3.5 showed the clone had infact the highest level of ERα(EEE) expression compared to E04 (high expresser) and E01 (low expresser) and it was decided that clone E01 expressed more suitable levels. Total RNA was extracted from cells after 8 hours of E2 (10^{-8}M) treatment in the presence and absence of Dox, using Qiagen RNA prep kit, as described in section 2.2.4.1.

Raw microarray data was subjected to quality control and normalisation criteria. Firstly, data from probes with very low signal were removed from the analysis as background noise could potentially give rise to false positives. As part of the default normalisation procedures, probes which had a normalised value of less than 0.1 were set to 0.1, further offsetting false-positive detection in this low expression area. Standard normalisation to the 50th percentile, as recommended for Illumina data by Agilent (Genespring), was applied to all data sets, aiding the comparison of data from slide to slide and sample to sample. Normalised data from all samples were subjected to cluster analysis to generate a dendogram relating treatment groups (Figure 3.8). Firstly the cluster analysis divided the four cell lines into two groups, namely: (i) MCF7-TO and MCF7-TO-ERα (WT06) and (ii) MCF7-TO-ERα(AAA) (A10) and MCF7-TO-ERα(EEE) (E01). With the exception of one of the three MCF7-TO samples treated with Dox and lacking E2, cluster (i) separates the two lines, MCF7-TO and WT06. Similarly, cluster (ii) divides cleanly into in the A10

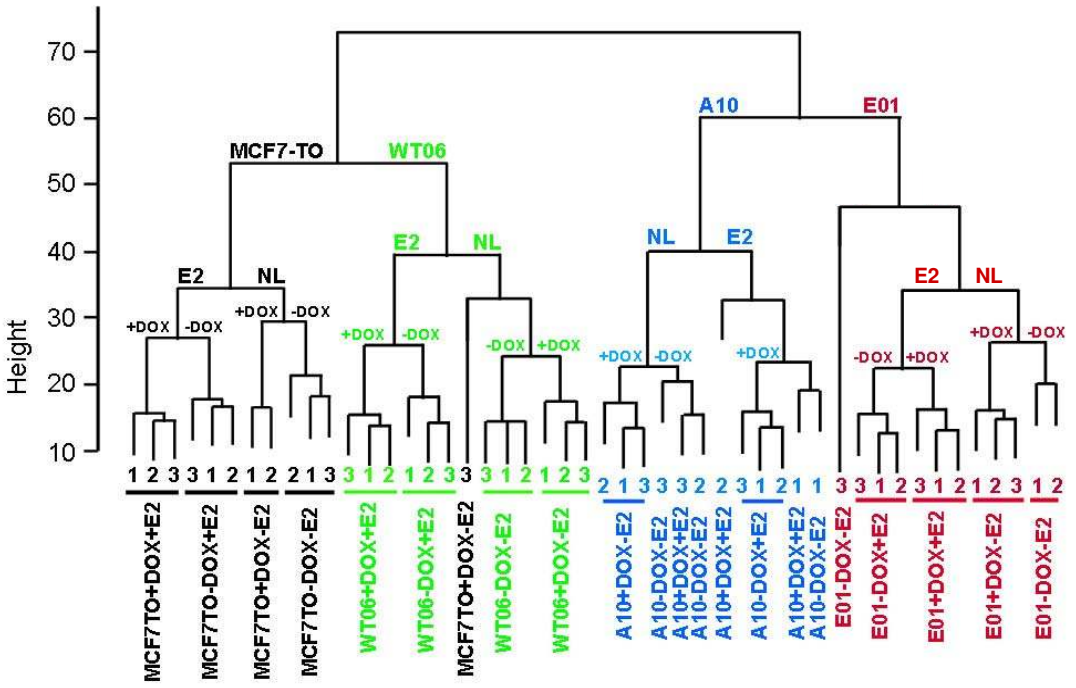


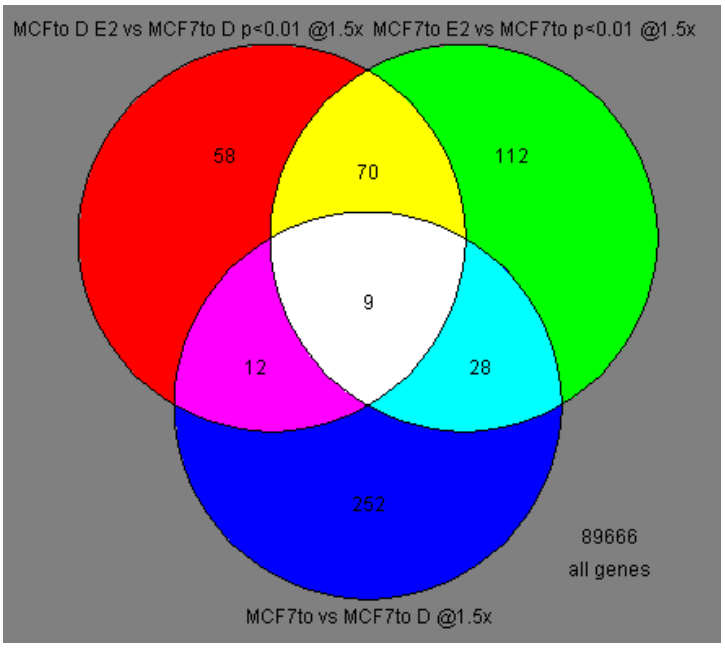
Figure 3.8 Global clustering of normalised data
Shown is the tree cluster analysis of the normalised microarray data for MCF7-TO-ERα (WT06) (green), MCF7-TO-ERα(EEE) (E06) (red), MCF7-TO-ERα(AAA) (A10) (blue) and the control cell line MCF7-TO (black). The cells were cultured in the presence or absence of Dox for 96 hours. RNA was prepared 8 hours following the addition of E2 (10^{-8}M). Microarrays analysis was carried out using 3 replicate RNA preparations for each condition.

and E01 lines. With the exception of the A10 line, clustering could distinguish E2 treated samples from the no ligand controls. In the case of the A10 line, the no ligand and Dox-treated replicate samples cluster together, as do the E2 treated samples in the absence of Dox. These are the samples that would express ERα(AAA). However, the samples treated with Dox and E2 fail to form a tight cluster and show relationship with the other treatments. Overall, this suggests the possibility that E2 regulation in this line is distinct from that seen in the other lines examined. It is also noted that in the case of the E01 line, one of the no ligand samples does not cluster with the bioreplicates for this treatment and is as closely related to any other treatment. Taken together, the cluster analysis shows that there is a high degree of correlation between related samples, apart from clone A10 where correlation was less defined.

For analysis of differentially expressed genes, replicates were combined to give a mean and standard error for the expression of each gene within a particular experimental condition. One-way ANOVA analysis was performed on the log scale data of a pair of experimental conditions (eg. MCF7-TO in the absence of ligand (NL) versus MCF7-TO treated with E2) using a parametric test, assuming variances were equal (student's t-test). Variances were assumed to be equal as all data are based on the same parental cell line (MCF7-TO), and therefore the number of genes whose expression is changed in different clones and treatments are expected to be only a small proportion of all genes. Differentially expressed genes were considered statistically significant at $p <= 0.01$. These genes were then filtered according to fold change between the two experimental conditions and lists of genes that were up or down regulated by 1.5- or 2- fold were generated.

The cluster analysis showed that the MCF7-TO and WT06 lines were most similar to each other, whilst the E2 regulation is unclear for the ERα(AAA) line A10.

a



b

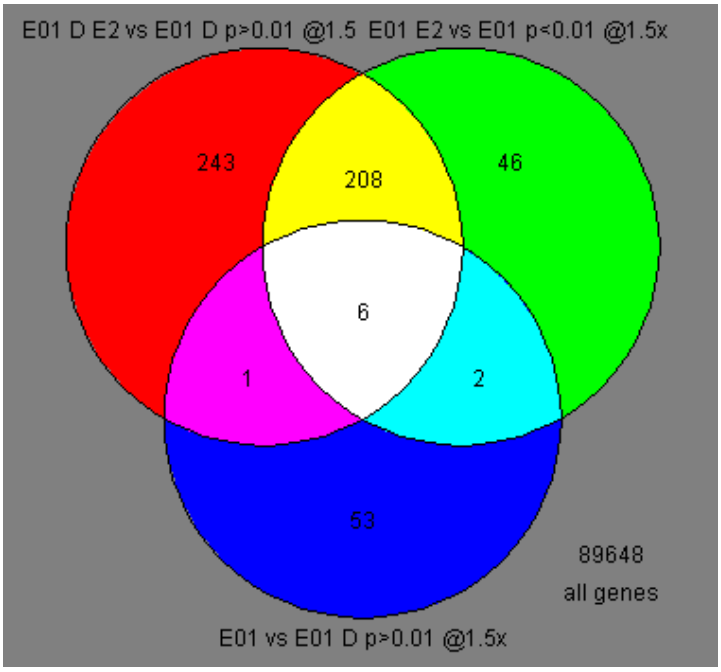


Figure 3.9 Venn diagrams showing the selection of estrogen-regulated genes
Normalised microarray data was analysed to identify the number of estrogen-regulated genes in the presence and absence of Dox for the MCF7-TO cell line (a) and MCF7-TO-ERα(EEE) E01 cell line (b). Genes up or down regulated by E2 greater than 1.5-fold (p>0.01) in the presence of Dox are shown in the top left section, those regulated in the absence of Dox (i.e. during transgene expression) are shown in the top right section. Genes up or down regulated by Dox greater than 1.5-fold (p>0.01) are shown in the bottom section

Therefore, the first analysis carried out compared expression profiles of the MCF7-TO line with the ERα(EEE) line (E01). Comparison of genes in the presence and absence of E2 identified 128 genes which showed up or down estrogen-regulation greater than 1.5-fold in the MCF7-TO cell line in the presence of Dox and 182 in the absence of Dox (Figure 3.9a); 70 genes were estrogen-regulated in the presence and absence of Dox. By comparing estrogen-regulated genes with those regulated by at least 1.5-fold in the presence and absence of Dox alone, a further 49 genes which displayed estrogen-regulation were also shown to be Dox regulated. The same analysis was

carried out for the E01 cell line (Figure 3.9b) which identified 451 genes that were up or down regulated by at least 1.5-fold in response to E2 in the presence of Dox and 254 genes in the absence of Dox; 208 genes showed estrogen-regulation in the presence and absence of Dox.

Full lists of estrogen regulated genes for the MCF7-TO and MCF7-TO-ERα(EEE) cell line are shown in appendix 1 and 2. Classical estrogen-regulated genes were shown to be up-regulated in these cell lines. Cathepsin D, pS2 and GREB1 expression were up-regulated 1.6, 1.2 and 1.8 -fold in the parental cell line in the presence of Dox and 2.4, 1.3 and 1.6 -fold in the absence of Dox, respectively. E01 showed a 1.9, 1.2 and 1.5 –fold induction of these genes in the presence of Dox and 1.8, 1.2 and 1.7 –fold induction in the absence of Dox, respectively. MCF7-TO-ERα(EEE) showed similar control of these estrogen regulated genes to the parental cell line and expression of ERα(EEE) did not alter the estrogen-induction of these genes.

Further analysis was carried out to identify genes which were regulated due to the expression of ERα(EEE) in the absence of E2 (Fig. 3.10). This would theortically identify any genes specifically regulated by ERα in the absence of E2 due

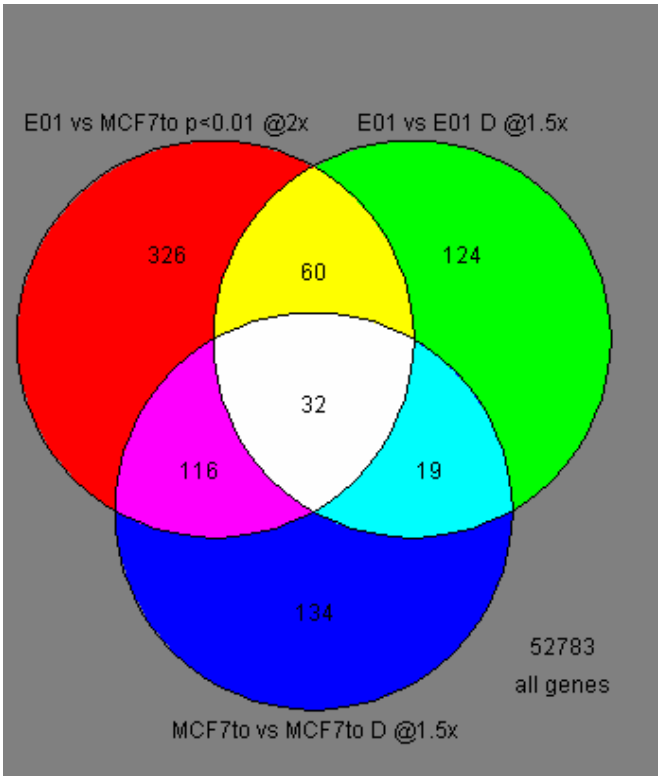


Figure 3.10 Venn Diagram identifying genes regulated due to expression of ERα(EEE) in an E2-independent manner
Genes which are up or down regulated 1.5-fold ($p<0.01$) in the absence of Dox (i.e. when ERα(EEE) is expressed) in the E01 clone, but not in MCF7-TO, are shown in the top left circle. Genes which are up- or down-regulated 1.5-fold ($p<0.01$) due to the expression of ERα(EEE) are shown in the top right circle. Genes which are up- or down-regulated 1.5-fold ($p<0.01$) by Dox treatment in the MCF7-TO cell are shown in the bottom circle. Genes which are specifically regulated due to expression of ERα(EEE) in the absence of estrogen and shown in the yellow segment.

to phosphorylation of ERα. Firstly, all genes regulated by Dox treatment were identified by comparing MCF7-TO gene expression in the presence and absence of Dox. Genes which were regulated by ERα(EEE) expression were identified by comparing MCF7-TO-ERα(EEE) gene regulation in the presence and absence of Dox. This identified 235 genes which were up or down regulated by at least 1.5-fold due to the expression of ERα(EEE), of which, 51 were also seen to be Dox regulated in the parental cell line. These 51 genes that were Dox regulated in the parental cell line were of no interest and discarded from further analysis. In order to identify which of the remaining 184 genes regulated by ERα(EEE) expression were specific to the MCF7-TO-ERα(EEE) cell line, genes regulated in the absence of Dox for E01 and MCF7-TO were compared. This identified 534 genes that were up or down regulated by at least 2-fold in the MCF7-TO-ERα(EEE) cell line and not the parental cell line. Of these, 148 also showed Dox regulation in the parental cell line of at least 1.5-fold and were discarded from further analysis. Only 60 of the genes regulated in the absence of Dox specifically in the MCF7-TO-ERα(EEE) cell line were also up or down regulated 1.5-fold in response to Dox – the remaining 326 genes showed regulation in the E01 and not the MCF7-TO cell line in the absence of Dox, but were not regulated specifically by the expression of ERα(EEE). The 60 genes regulated in response to ERα(EEE) expression alone are detailed in table 3.2. Many of the genes identified are involved with the control of cell growth, gene transcription or protein degradation; however, four genes were highlighted as being of interest – LOC643287, peptidyl-propyl isomerise-like 1 (PPIL1/CYPL1), pp32r1 and insulin-like growth factor binding protein 5 (IGFBP5).

Levels of LOC643287 were increased 2-fold due to expression of ERα(EEE). Very little is known about LOC643287, however, LOC643287 has a similar sequence to prothymosin α (ProTα). ProTα is a protein involved in mammalian cellular

proliferation and has been shown to be increased in breast cancer (Dominguez et al., 1993; Tsitsiloni et al., 1993). These data suggest that expression of ERα(EEE) causes an increase in expression of a gene which is similar to ProTα. Levels of LOC643287 induction in WT06 and A10 in the absence of Dox compared to the presence of Dox were 1.0 and 1.3 –fold, respectively.

PPIL1 is a member of the cyclophin family of peptidyl-prolyl isomerases which carry out cis-trans isomerization of the peptide bond on the N-terminal side of proline residues in proteins (Hunter, 1998), and increased expression of PPIL1 has been shown to promote growth of colon cancer cells (Obama et al., 2006). Microarray studies have also shown an elevation of PPIL1 expression in cervical, pancreatic and gastric cancers (Obama et al., 2006). Levels of PPIL1 expression were increased 1.6-fold in response to ERα(EEE) expression in contrast to WT06 and A10 cell lines which showed a 0.9 and 1.2 -fold change in the absence of Dox compared to the presence of Dox, respectively.

The pp32r1 has been shown to have oncogenic properties in prostatic adencarcinoma cells (Kochevar et al., 2004) and over-expression of the gene has been linked to transformation and tumourigenesis (Bai et al., 2001). Levels of pp32r1 expression were increased 1.6-fold in response to ERα(EEE) expression in contrast to WT06 and A10 cell lines which showed a 0.9 and 1.0 – fold change in the absence of Dox compared to the presence of Dox, respectively.

This suggests that the increase in expression of these three genes, LOC643287, PPIL1 and pp32r1, expression of which could potentially be linked to an increase in cancerous cell growth, is not due to the general increase in levels of ERα expression in the absence of Dox compared to in the presence of Dox for these cell lines, but that ERα(EEE) expression specifically causes an increase in the levels of expression.

	Fold	Common name	Genebank	Description
ILMN_102158	2.546	ENSG00000210082	CR612552	full-length cDNA clone XCL0BB001ZD04 of Neuroblastoma of Homo sapiens (human).
ILMN_37869	2.073	LOC643287	XM_928075	similar to prothymosin, alpha (gene sequence 28)
ILMN_45269	2.054	LOC645968	XM_928934	similar to 40S ribosomal protein S3a (V-fos transformation effector protein)
ILMN_483	1.923	TAF9	NM_001015892	Transcription initiation factor TFIID subunit 9
ILMN_43617	1.901	PSMC1	NM_002802	26S protease regulatory subunit 4
ILMN_32918	1.898	GNG10	NM_001015882	DnaJ homolog Guanine nucleotide-binding protein gamma-10 subunit precursor
ILMN_44829	1.878	LOC647436	XM_937113	similar to ribosomal protein L5
ILMN_36846	1.867	ENS G00000172895	XM_941189	Novel Ras related
ILMN_10494	1.86	LPC2A; ANX2L1; ANX2P1	NR_001562	annexin A2 pseudogene 1
ILMN_29952	1.858	HRP1; TRE2; TRE17; Tre-2	NM_004505	ubiquitin specific peptidase 6 (Tre-2 oncogene)
ILMN_14666	1.845	FKSG17	NR_002182	nascent-polypeptide-associated complex alpha polypeptide pseudogene 1
ILMN_30365	1.844	CSN2; SGN2; ALIEN; TRIP15	NM_004236	COP9 constitutive photomorphogenic homolog subunit 2 (Arabidopsis)
ILMN_36491	1.837			
ILMN_36919	1.837	RPL37	XM_294473	60S ribosomal protein L37
ILMN_137601	1.801	SMN2	NM_022875	Survival motor neuron protein (Component of gems 1) (Gemin-1)
ILMN_25663	1.785	CEN3; MGC12502; MGC138245	NM_004365	centrin, EF-hand protein, 3 (CDC31 homolog, yeast)
ILMN_17969	1.724			
ILMN_40674	1.712		XR_017481	Similar to chaperonin containing TCP1, subunit 8
ILMN_25676	1.693	SRB7; MED21	NM_004264	SRB7 suppressor of RNA polymerase B homolog (yeast)
ILMN_14333	1.691	GAL3; MAC2; CBP35; GALBP; GALIG; LGALS2	NM_002306	lectin, galactoside-binding, soluble, 3
ILMN_23425	1.672	ANX4; PIG28; MGC75105; DKFZp686H02120	NM_001153	annexin A4
ILMN_31870	1.666	cdc42	XM_935770	Cell division control protein 42 homolog (G25K GTP-binding protein)
ILMN_3174	1.665	H1; huH1; p64H1; CLIC4L; FLJ38640; DKFZP566G223	NM_013943	chloride intracellular channel 4
ILMN_39721	1.663	LOC643997	XM_292963	similar to peptidylprolyl isomerase A isoform 1
ILMN_45053	1.647	LOC644131	XM_929729	similar to chaperonin containing TCP1, subunit 8
ILMN_7321	1.64	Txl; TXNL; TRP32; TXL-1	NM_004786	thioredoxin-like 1
ILMN_40173	1.611	PSMC1	XM_931995	26S protease regulatory subunit 4
ILMN_32246	1.602	LOC653631	XM_930476	hypothetical LOC653631
ILMN_31803	1.598			
ILMN_11992	1.593	PP32R1	NM_012403	acidic (leucine-rich) nuclear phosphoprotein 32 family, member C
ILMN_39811	1.592	ROCK1	NM_005406	Rho-associated protein kinase 1
ILMN_9395	1.59	FLJ42355; KIAA0276	NM_015115	DCN1, defective in cullin neddylation 1, domain containing 4 (S. cerevisiae)
ILMN_35777	1.589	LOC654121	XM_942442	similar to c.elegans C49H3.3
ILMN_83371	1.583			
ILMN_34205	1.581			
ILMN_19855	1.568	PABP3; PABPL3	NM_030979	poly(A) binding protein, cytoplasmic 3
ILMN_31701	1.565	eIF4E	XM_945579	eukaryotic translation initiation factor 4E
ILMN_20257	1.562	C1GALT; T-synthase	NM_020156	core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase, 1
ILMN_30246	1.556	CYPL1; hCyPX; MGC678; PPIase; CGI-124	NM_016059	peptidylprolyl isomerase (cyclophilin)-like 1
ILMN_6208	1.551	MDS011; MDS025; MGC104239	NM_021825	coiled-coil domain containing 90B

ILMN_137289	1.549	C7orf28B	NM_198097	Uncharacterized protein C7orf28
ILMN_15777	1.548	ACHAP; C6orf82; MGC111154	NM_015921	cutA divalent cation tolerance homolog (E. coli)
ILMN_31759	1.548	LOC642502	XM_926594	hCG1776980
ILMN_5108	1.547	caveolin 2 (CAV2),	NM_001233	scaffolding protein within caveolar membranes
ILMN_4441	1.546	MMTRA1B	NM_021105	phospholipid scramblase 1
ILMN_40627	1.543	RPS26P10	XM_376787	ribosomal protein S26 pseudogene 10
ILMN_23856	1.542	MATN2	NM_002380	matrilin 2
ILMN_25705	1.542	MGC11102	NM_032325	hypothetical protein MGC11102
ILMN_34041	1.542	LOC645351	XM_928393	similar to SMT3 suppressor of mif two 3 homolog 2
ILMN_37790	1.537			
ILMN_42347	1.535	TPM3		Tropomyosin alpha-3 chain
ILMN_22810	1.53	MGC-24; MUC-24; endolyn	NM_006016	CD164 molecule, sialomucin
ILMN_2393	1.528	FKSG30; ACT	NM_001017421	kappa-actin
ILMN_31523	1.519	LOC347376	XM_937928	hCG1749005
ILMN_11438	1.517	CASP6	NP_001217	caspase 6 alpha form preproprotein
ILMN_19744	1.514	ACAD-8; FLJ22590	NM_014384	acyl-Coenzyme A dehydrogenase family, member 8
ILMN_30283	1.511	NKAP; FLJ22626	NM_024528	NF-kappaB activating protein
ILMN_13483	1.51	myr1	NM_012223	myosin IB
ILMN_137901	1.505	MRPL30	NM_145212	39S ribosomal protein L30
ILMN_3066	0.629	IGFBP5	NM_000599	Insulin-like growth factor-binding protein 5 precursor

Table 3.2 Genes regulated due to expression of ERα(EEE)
Genes were identified by comparing those up or down regulated in the E01 cell line in the absence of Dox (i.e. during transgene expression) 1.5-fold or more compared to those regulatred in the presence of Dox ($p<0.01$) when ERα(EEE) was not expressed. Genes that were regulated by Dox in the parental cell line were exclude from this list.

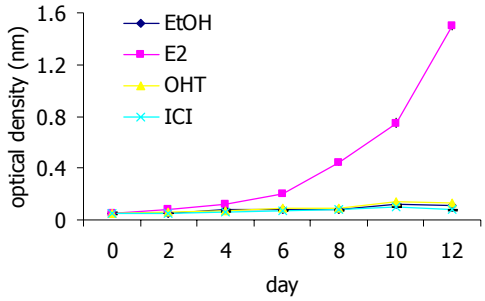
IGFBP5 has been shown to act as a tumour suppressor by inhibiting angiogenesis (Chun et al., 2008), however, levels have been shown to be increased in breast carcinoma (Wang et al., 2008). Levels of IGFBP5 expression were decreased 1.6-fold in response to ERα(EEE) expression. However, expression levels were also decreased in WT06 and A10 cell lines in the absence of Dox compared to the presence of Dox by 1.8 and 1.3 –fold, respectively. This suggests that overexpression of ERα in the absence of Dox causes a decrease in levels of IGFBP5 expression.

3.6 Effects of HA-ERα-FLAG and ERα mutant expression on MCF7 cell growth in the presence of estrogen and anti-estrogens

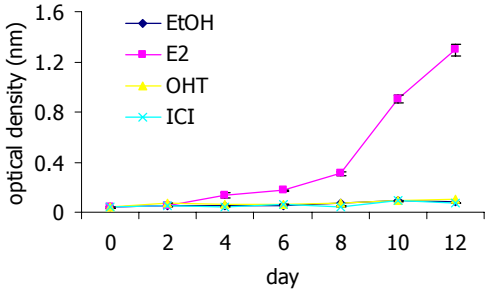
MCF7 cell growth requires estrogen and is inhibited by anti-estrogens, such as OHT and ICI. Cell growth was determined using the SRB growth assay, in which cells were cultured in the absence of ligand, or in the presence of E2 (10^{-7}M), OHT (10^{-8}M) and ICI (10^{-8}M), plus and minus Dox, over a period of 12 days (Fig. 3.11). As expected, in both the presence and absence of Dox there was no growth of the parental (MCF7-TO) cells in the absence of ligand, with growth being stimulated by E2, but not stimulated in response to OHT or ICI (Fig. 3.11a). In the presence of Dox, the growth curve shows that the cells are in a state of exponential growth up to 12 days. In the absence of Dox the graph appears to suggest cell growth rate is slowing down and beginning to plateau within the 12 day time period although a similar optical density, and therefore cell density, is reached.

The growth of MCF7-TO-ERα (Fig. 3.11b-d), MCF7-TO-ERα(EEE) (Fig. 3.11e-g) and MCF7-TO-ERα(AAA) (Fig. 3.11h-j) lines in response to ligands were similar to that of MCF7-TO cells in the presence of Dox, with the exception of E06 (the medium ERα(EEE) expresser, Fig. 3.11g), where the estrogen-stimulated growth was considerably lower than that seen in other lines.

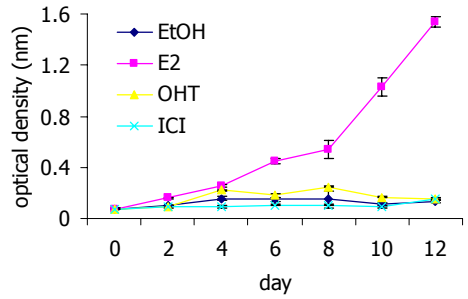
a **MCF7TO + Dox**



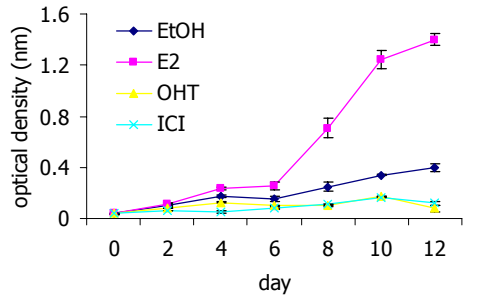
MCF7TO -Dox



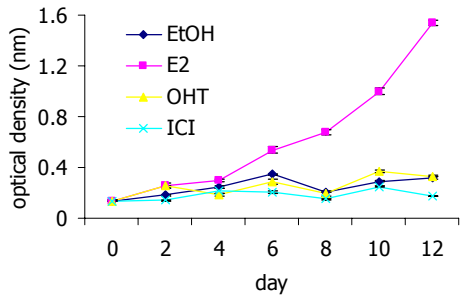
b **WT11 + Dox**



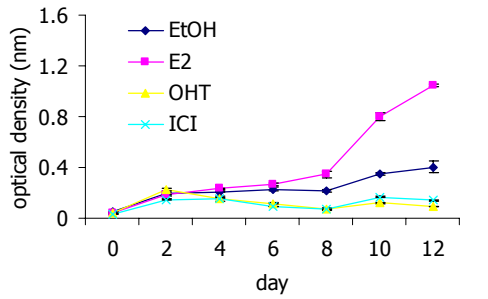
WT11 -Dox



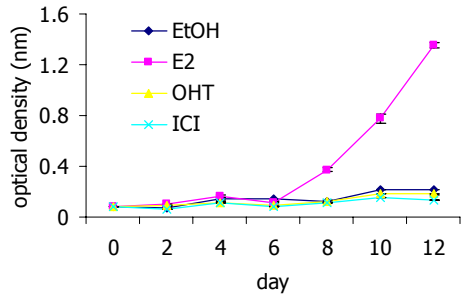
c **WT06 + Dox**



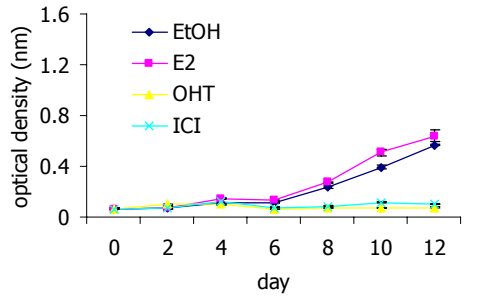
WT06 -Dox



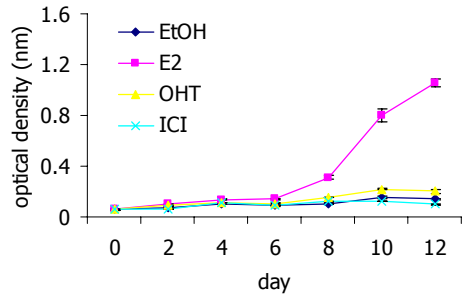
d **WT02 + Dox**



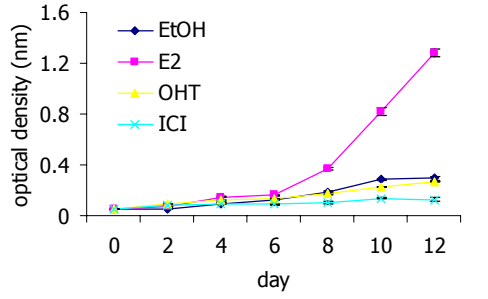
WT02 -Dox



e **E01 + Dox**



E01 -Dox



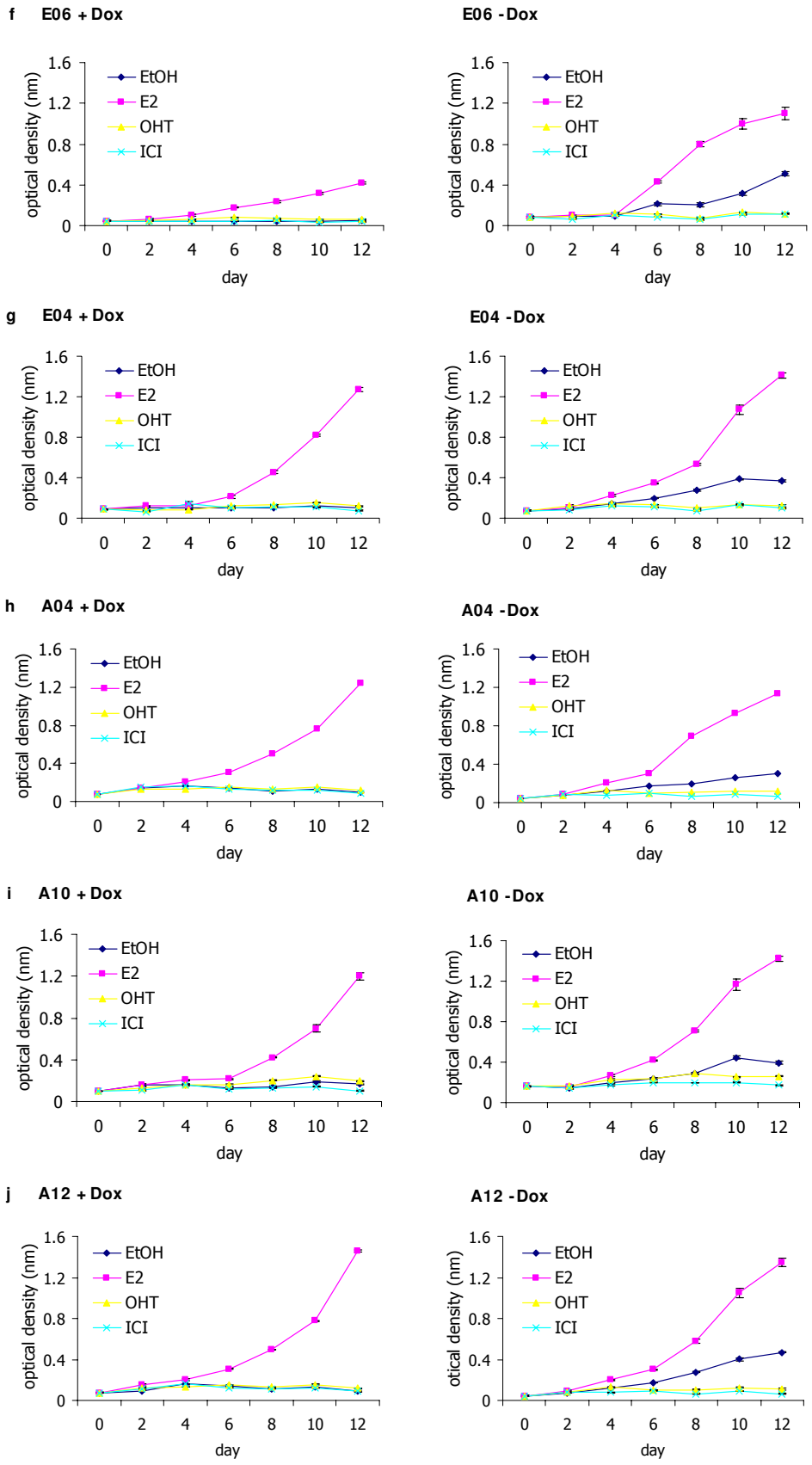


Figure 3.11 SRB growth assays in the presence of estrogen and anti-estrogens
Sulphorhodamine B assays were carried out to assess cell growth, in the presence of vehicle (EtOH), E2 (10^{-8}M), OHT (10^{-7}M) and ICI (10^{-7}M) in the presence and absence of Dox for all MCF7-TO-ERα clones (b, c, d), MCF7-TO-ERα(EEE) clones (e, f, g), MCF7-TO-ERα(AAA) (h, i, j) and the parental cell line MCF7TO as a control (a). The order of clones is low, medium and high expression of transgenic ERα in each case.

Similar to the parental cell line, although E2-stimulated growth is still exponential by day 12 in the presence of Dox, in the absence of Dox (i.e. when the transgenes are expressed) growth appears to plateau for all the MCF7-TO-ERα, MCF7-TO-ERα(EEE) and MCF7-TO-ERα(AAA) clones (except E01). E2-stimulated cell growth in clones WT11, E04, A04 and A12 reached a similar cell density by day 12 in the presence and absence of Dox whereas E01, E06 and A10 showed slightly higher cell density when the transgenes were expressed in the absence of Dox compared to in the presence of Dox. WT02 and WT06 showed a reduction in cell density by day 12 n the absence of Dox compared to the presence of Dox. How the clones responded to E2 and Dox was not dependent on either the type or level of phosphorylation mutant expressed. Since this plateauing of cell growth also occured for the parental cell line, this suggests these differences are due to Dox treatment and not the mutant ERα.

Similar to the parental cell line, cell growth was not stimulated by either OHT or ICI for any of the MCF7-TO-ERα, MCF7-TO-ERα(EEE) or MCF7-TO-ERα(AAA) clones in the absence of Dox. This suggests that neither over-expression of ERα or expression of ERα phospho-mutants is sufficient to stimulate growth in the presence of OHT. Ligand-independent cell growth however, was consistently increased for all of the lines expressing tagged forms of ERα (Fig. 3.11 b-j) in the absence of Dox compared to in the presence of Dox. The lack of an obvious difference in ligand-independent growth between cell lines expressing ERα, ERα(EEE) or ERα(AAA) suggests that this increase in growth is due to overexpression of ERα induced by the expression of WT ERα, ERα(EEE) and ERα(AAA) in the absence of Dox, with the phosphorylation site mutantions not contributing to cell growth.

3.6.1 Growth of ERα, ERα(EEE) and ERα(AAA) expressing MCF7-TO cells in the presence of epidermal growth factor

Previous studies have shown that S104, S106 and S118 are phosphorylated in a ligand-independent manner by p42/p44 MAPK (Chen et al., 2002; Kato et al., 1995), a downstream kinase of the Raf-MAPK signalling cascade. Activation of p42/p44 MAPK in response to cell treatment with EGF and PMA has been used to show induction of S118 phosphorylation (Bunone et al. 1996; Joel et al. 1998; Chen et al. 2002; Sarwar et al. 2006). In order to determine if mutation of S104, S106 and S118 alters the response of MCF7 cells to activation of the MAPK pathway by EGF, growth assays were carried out in the presence and absence of EGF using the conditions of the previous experiment; in the presence of vehicle (EtOH), E2, OHT and ICI, all in the presence and absence of Dox. The growth was measured after 8 days as this time point provided sufficient ligand-induced growth, with differences between the ligands to be evident, without the cells reaching confluence (Figure 3.12). Graphs show the growth at day eight relative to the cell numbers 24 hours after plating.

In the MCF7-TO parental cell line, Dox treatment did not have a significant affect on ligand-independent or estrogen-dependent cell growth, either in the presence or absence of EGF. EGF treatment did however cause a significant increase in ligand-independent cell growth in the presence and absence of Dox. Estrogen-dependent cell growth was not significantly affected by EGF treatment (Fig. 3.12a). Dox treatment caused a significant decrease in cell growth in the presence of OHT and ICI, which was not affected by EGF treatment. This suggests that the cell growth of the parental line is increased due to the presence of Dox under these conditions, which is unexpected since the parental cell line does not express any Dox-regulated proteins.

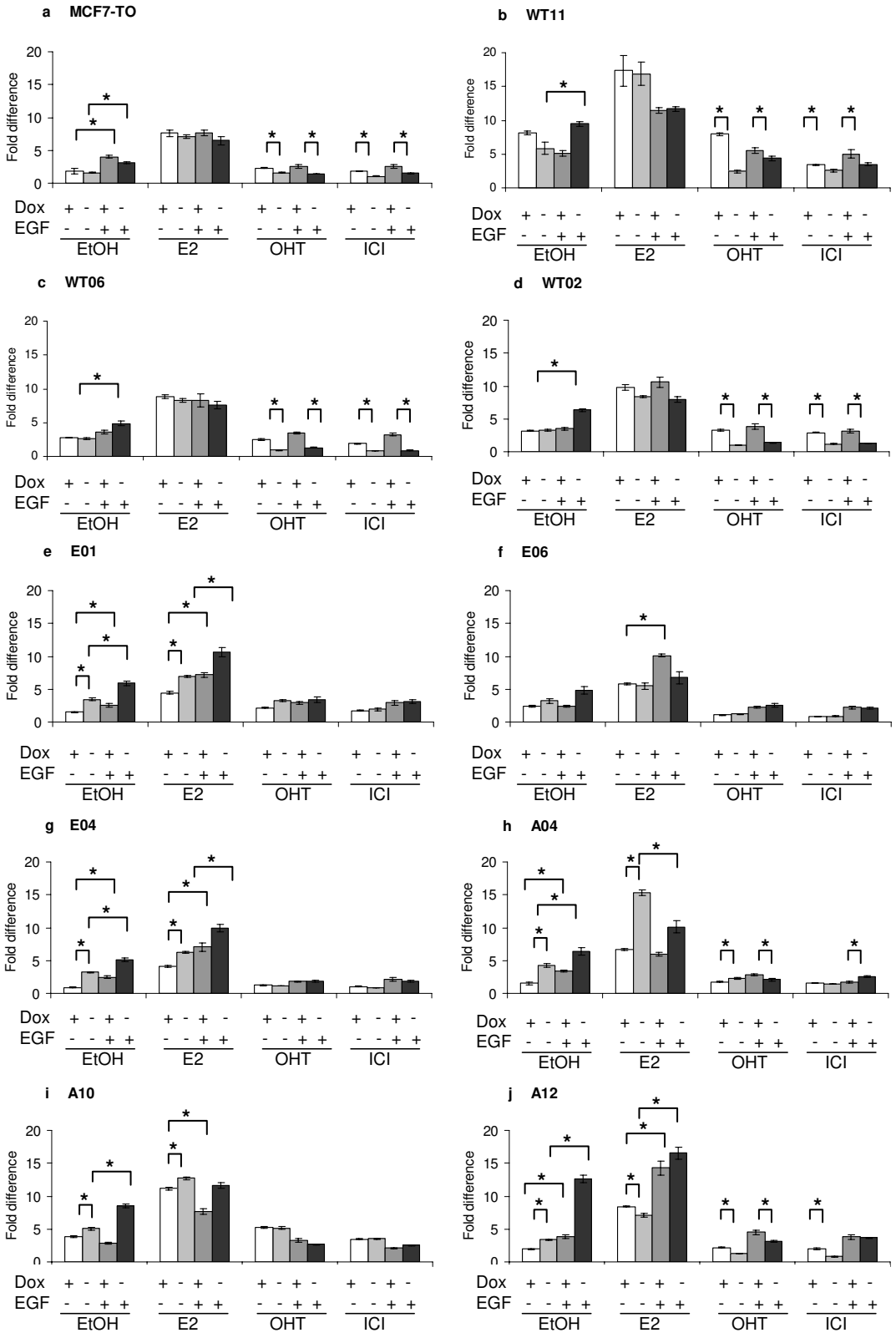


Figure 3.12 Cell growth in the presence of epidermal growth factor
Sulphorhodamine B assays were carried out to assess cell growth, in the presence of vehicle (EtOH), E2 (10^{-8}M), OHT (10^{-7}M) and ICI (10^{-7}M) in the presence and absence of Dox, with and without EGF treatment (10ng/ml) for all MCF7-TO-ERα clones (b, c, d), MCF7-TO-ERα(EEE) clones (e, f, g), MCF7-TO-ERα(AAA) (h, i, j) and the parental cell line MCF7TO as a control (a). Cell growth is shown at day 8 relative to the plating efficiency measured 24 hours after plating cells. Standard error is shown based on 6 replicates. Clones are in order of lowest to highest mutant ERα expression. Statistical differences between treatments with a p value <0.01 are denoted by an *.

There was no significant difference in ligand-independent cell growth with or without Dox treatment in the MCF7-TO-ERα clones (Fig. 3.12b-c). MCF7-TO-ERα(EEE) clones E01 (Fig. 3.12e) and E04 (Fig. 3.12g) showed a significant increase in ligand-independent cell growth when the transgene was expressed in the absence of Dox compared to the presence of Dox; however E06 (Fig. 3.12f) did not show this but more importantly, all three MCF7-TO-ERα(AAA) clones (Fig. 3.12h-j) showed a significant increase similar to clones E01 and E04. Since two clones from the MCF7-TO-ERα(EEE) cell line and all three MCF7-TO-ERα(AAA) cell lines showed an increase in ligand-independent cell growth due to Dox removal, this suggests that this is due to the over expression of ERα in these cell lines and not the phosphorylation status of the phospho-mutant.

Treatment with EGF caused a significant increase in ligand-independent cell growth in the absence of Dox for all the clones except clone E06, which showed a slight, but not significant increase. The degree of increase did not appear to correlate with the levels of Dox-regulated ERα, ERα(EEE) or ERα(AAA) expression as shown by Western blotting in figure 3.2. E01, E04, A04 and A12 also showed a significant increase in ligand-independent cell growth in the presence of Dox as well, however, in all four cases the increase was more pronounced when the transgenes were expressed (-Dox). This suggests that EGF treatment causes an increase in ligand-independent cell growth in some, but not all, cell lines. Overexpression of ERα increases this effect since ligand-independent cell growth was increased due to the expression of both ERα(EEE) and ERα(AAA) as well as WT ERα so cannot be said to be phospho-specific.

E2-dependent cell growth was increased significantly due to the expression of Dox-regulated ERα in the absence of Dox compared to in the presence of Dox for clones E01 (Fig. 3.12e), E04 (Fig. 3.12g), A04 (Fig. 3.12h) and A10 (Fig. 3.12i). A12

(Fig. 3.12j) showed a significant decrease in E2-stimulated cell growth after the removal of Dox and expression of the transgene. E2-stimulated cell growth was not significantly affected by Dox treatment for any of the MCF7-TO-ERα clones (Fig. 3.12b-d) or E06. Similar to the increase seen in ligand-independent cell growth in the absence of Dox, these data suggest the increase in E2-stimulated cell growth is due to the over-expression of ERα in these lines and not due to the phosphorylation status of ERα.

EGF treatment caused a significant increase in E2-stimulated cell growth in the presence and absence of Dox for clones E01, E04 and A12; however, A04 showed a significant decrease in cell growth in the absence of Dox due to EGF treatment, as did A10 in the presence of Dox where as E06 showed a significant increase in the presence of Dox but no significant change in cell growth in the absence of Dox due to EGF treatment. The MCF7-TO-ERα clones showed no difference in cell growth due to EGF treatment. Overall, these data show that EGF treatment had no consistent affect on cell growth and the increases or decreases appear to be clone-specific and not related to over-expression of ERα or the phosphorylation status of ERα.

Similar to the control cell line, all MCF7-TO-ERα clones and A12 showed a significant decrease in OHT and ICI stimulated cell growth in the absence of Dox compared to in the presence of Dox. In general, EGF treatment caused an increase in OHT and ICI-stimulated cell growth, but since the changes observed with Dox or EGF treatment were consistently seen in the presence of both OHT and ICI, this suggests they were not due to the involvement of ERα since ERα activity is totally inhibited by ICI treatment (fig. 3.4).

Similar to the previous findings in figure 3.11, these results show that over-expression of ERα can cause an increase in ligand-independent cell growth, and for

some clones estrogen-dependent cell growth, but this appears to be unrelated to the phosphorylation status of the three residues within the AF1 of ERα or to overall levels of ERα expression. These results suggest that treatment with EGF causes a general increase in ligand-independent cell growth which appears to be enhanced by over-expression of ERα, but not phosphorylation, as overexpression of ERα in the absence of Dox caused further increases compared to growth in the presence of Dox across the clones from MCF7-TO-ERα, MCF7-TO-ERα(EEE) and MCF7-TO-ERα(AAA) cell lines. Treatment with EGF also appears to increase E2-stimulated cell growth, again, regardless of the phosphorylation status of the AF1 residues, although over-expression of ERα caused a further increase in this effect in some, but not all cases.

3.6.2 Growth of ERα, ERα(EEE) and ERα(AAA) expressing MCF7-TO cells in the presence of insulin

Insulin is a growth factor which, similar to EGF, causes activation of the Ras-MAPK signalling pathway (Porras et al., 1998). Insulin-like growth factor signalling has been implicated in resistance to endocrine treatments (Gee et al., 2005; Knowlden et al., 2005). In order to determine if insulin stimulates the ligand-independent growth of MCF7 and ERα lines, growth assays were carried out as performed for EGF above (Fig. 3.13). Since the insulin was prepared in 10mM HCl, the cells not treated with insulin were treated with an equal volume of 10mM HCl. Graphs depict the growth measured at day 8 relative to that measured 24 hours after plating of cells (Fig. 3.13).

Cell growth of the MCF7-TO cell line was stimulated by treatment with E2 and less so with OHT treatment whereas cell growth was not stimulated in the absence of ligand and severely inhibited by ICI treatment (Fig. 3.13a). Dox treatment in

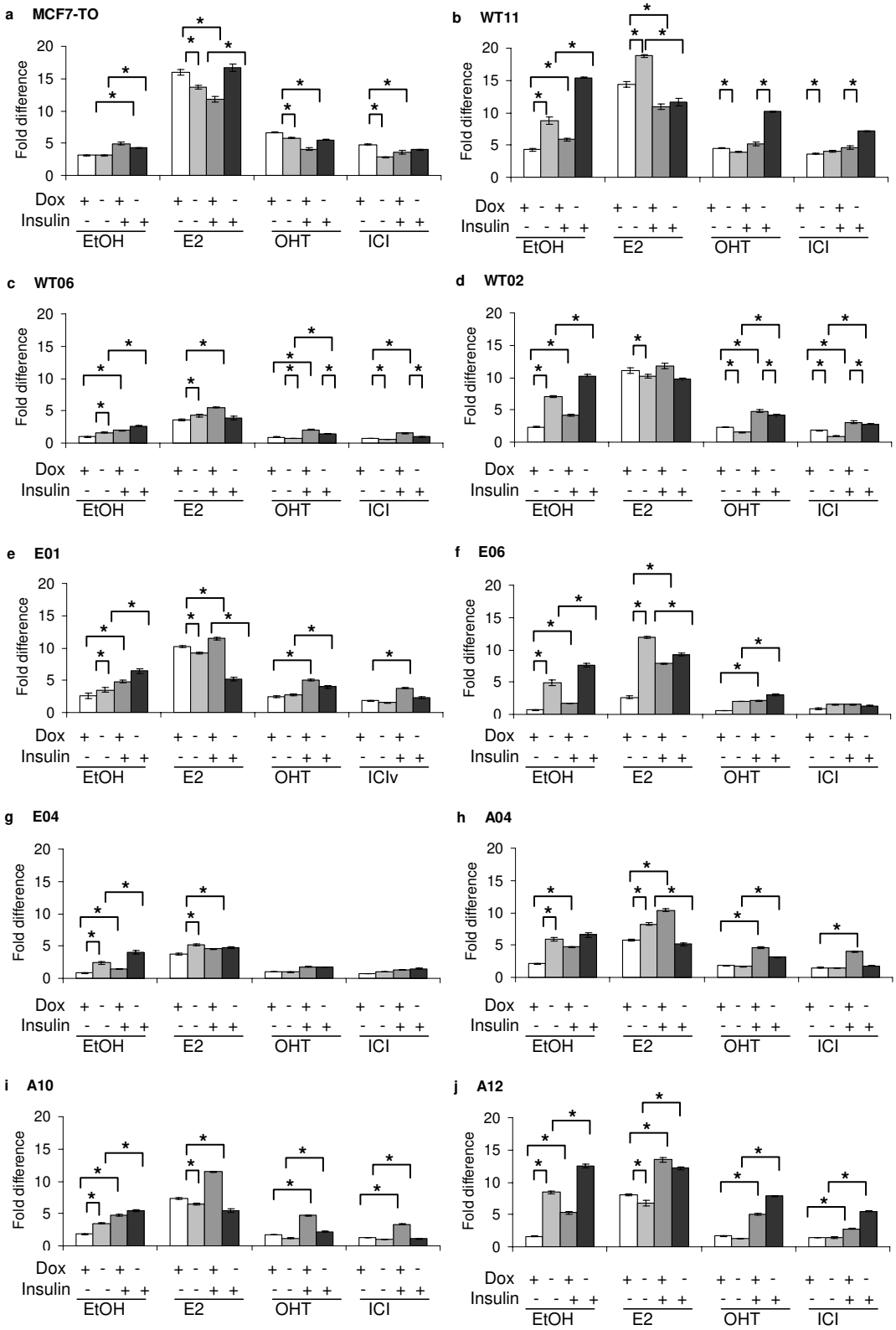


Figure 3.13 Cell growth in the presence of insulin

Sulphorhodamine B assays were carried out to assess cell growth, in the presence of vehicle (EtOH), E2 (10^{-8}M), OHT (10^{-7}M) and ICI (10^{-7}M) in the presence and absence of Dox, with and without insulin treatment (1μg/ml) for all MCF7-TO-ERα clones (b, c, d), MCF7-TO-ERα(EEE) clones (e, f, g), MCF7-TO-ERα(AAA) (h, i, j) and the parental cell line MCF7TO as a control (a). Cell growth is shown at day 8 relative to the plating efficiency measured 24 hours after plating cells. Clones are in order of lowest to highest mutant ERα expression. Standard error is shown based on the readings of 6 replicates. Statistical differences between treatments with a p value <0.01 are denoted by an *.

conjunctin with insulin treatment causd a significant reduction in cell growth in the presence of E2, OHT and ICI compared to Dox treatment in the absence of insulin. Treatment with insulin caused a significant increase in ligand-independent cell growth in the presence and absence of Dox. E2-dependent cell growth was decreased significantly by insulin in the presence of Dox from 16- to 12- fold, although increased significantly in the absence of Dox from 13.5- to 16.5- fold. Cell growth in the presence of OHT and ICI was decreased significantly due to insulin in the presence of Dox yet remained similar in the absence of Dox.

All the MCF7-TO-ERα, MCF7-TO-ERα(EEE) and MCF7-TO-ERα(AAA) clones (Fig. 3.13b-j) displayed a significant increase in ligand-independent cell growth due to expression of the Dox-regulated ERα in the absence of Dox compared to in the presence of Dox. Similar to the control cell line but to a greater extent, ligand-independent cell growth increased in response to insulin treatment in the presence and absence of Dox for all MCF7-TO-ERα, MCF7-TO-ERα(EEE) and MCF7-TO-ERα(AAA) clones, except A04 which did not show a significant increase in growth in the absence of Dox. The level of increase in ligand-independent cell growth induced by insulin treatment was similar in the presence and absence of Dox and was not influenced by the levels of Dox-regulated ERα expression, although ligand-independent cell growth was higher in the absence of Dox. This suggests that the expression of Dox-regulated ERα causes an increase in ligand-independent cell growth which is independent of the phosphorylation status of ERα and this affect is increased due to insulin treatment.

E2-stimulated cell growth was significantly increased due to expression of ERα, ERα(EEE)or ERα(AAA) in clones WT11, WT06, E06, E04 and A04, yet significantly decreased in clones WT02, E01, A10 and A12. Treatment with insulin in the presence of Dox caused a significant increase in E2-stimulated cell growth in all

clones, except WT02 which showed a slight but not significant increase, and WT11 which showed a significant decrease in cell growth similar to the control cell line. Treatment with insulin in the absence of Dox caused a significant decrease in E2-stimulated cell growth for clones WT11, E01, E06 and A04, whereas a significant increase in cell growth was shown for clone A12, similar to the control cell line. All other clones showed no significant change in E2-stimulated cell growth due to insulin treatment in the absence of Dox. These data suggest that insulin has no specific effect on E2-induced cell growth.

Slight increases in cell growth in the presence of OHT and ICI in response to insulin were seen in the presence and absence of Dox for all the clones, although cell growth remained the lowest under these conditions compared to in the presence or absence of E2.

3.6.3 Cell growth in the presence of the MEK inhibitor U0126

Tyrosine kinase receptors such as EGFR stimulate the activity of c-raf, which in turn activates MAPK kinase (MAPKK, also known as MEK), which is an upstream activator of p42/p44 MAPK (Lange-Carter et al., 1993). The U0126 compound inhibits the MEK family members MEK1 and MEK2 (Favata et al., 1998). If phosphorylation of S104, S106 and S118 by MAPK is important for the growth of MCF7 cells, then treatment with U0126 would be expected to inhibit the growth of MCF7-TO (parental cells) as well as the growth of lines expressing HA-ERα-FLAG, but should not influence the growth of the MCF7-TO-ERα(EEE) or MCF7-TO-ERα(AAA) lines. In order to test this possibility, the lines were treated with U0126 (10μM) (Thomas et al., 2008) in the presence of vehicle (EtOH), E2, OHT and ICI, plus and minus Dox, in order to investigate how inhibition of p42/p44 MAPK activation affected cell growth. Cells which were not treated with U0126 were treated with an equal volume

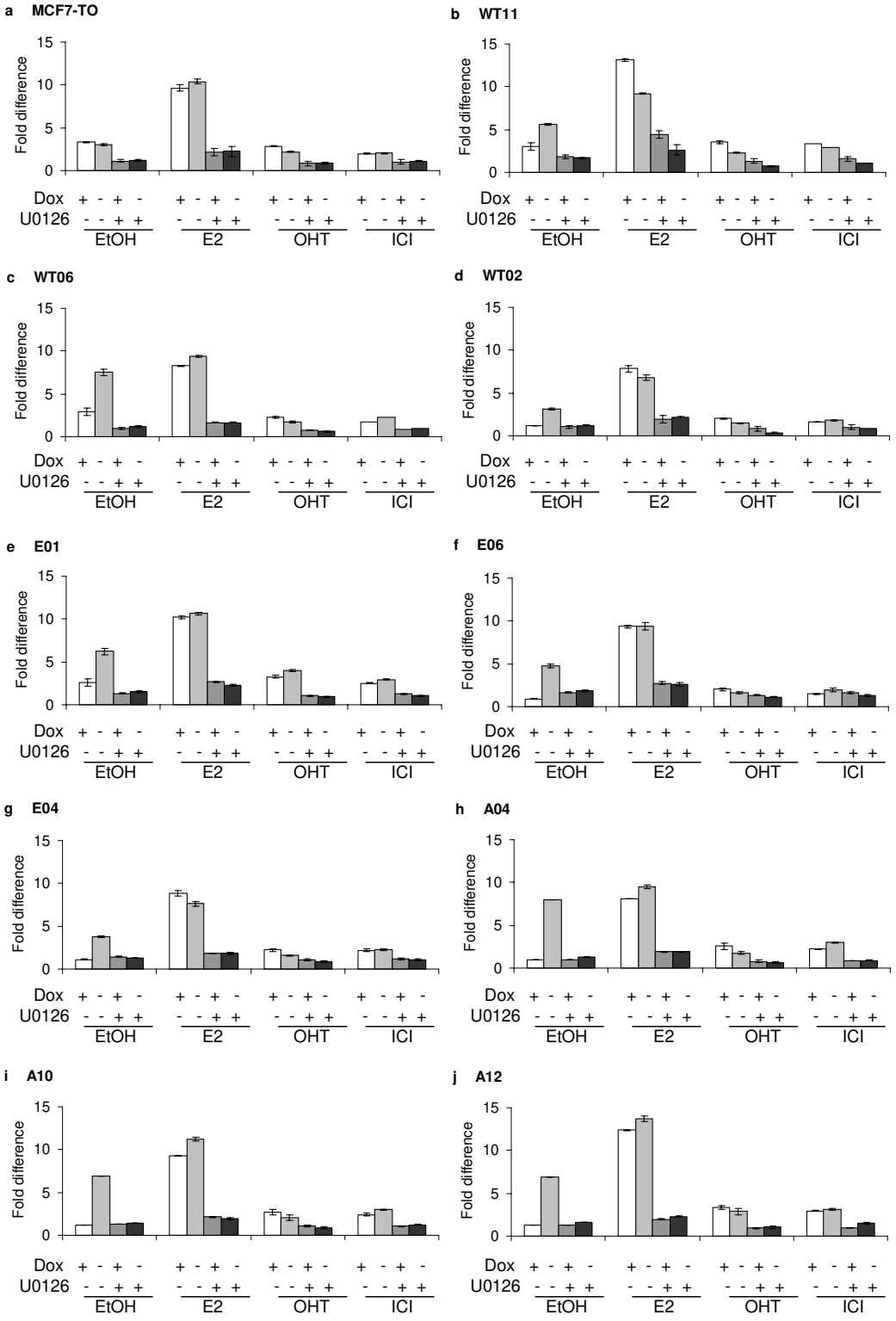


Figure 3.14 Cell growth in the presence of MEK inhibitor U0126
Sulphorhodamine B assays were carried out to assess cell growth, in the presence of vehicle (EtOH), E2 (10^{-8}M), OHT (10^{-7}M) and ICI (10^{-7}M) in the presence and absence of Dox, with and without U0126 treatment (10μM) for all MCF7-TO-ERα clones (b, c, d), MCF7-TO-ERα(EEE) clones (e, f, g), MCF7-TO-ERα(AAA) (h, i, j) and the parental cell line MCF7TO as a control (a). Cell growth is shown at day 8 relative to the plating efficiency measured 24 hours after plating cells. Standard error is shown based on the readings of 6 replicates. Clones are in order of lowest to highest mutant ERα expression. Statistical differences between treatments with a p value <0.01 are denoted by an *.

of the vehicle DMSO. Graphs depict the growth measured at day 8 relative to that measured 24 hours after plating of cells (Fig. 3.14).

U0126 inhibited the ligand-stimulated growth of MCF7-TO cells (Fig. 3.14a), indicating that MAPK activity is important for the growth of MCF7 cells. Growth of the ERα lines was similarly inhibited by U0126, regardless of the nature of the residues at S104, S106 and S118, suggesting that the action of U0126 is independent of ERα phosphorylation status at these sites (Fig. 3.14b-j). Further, the ligand-independent growth in the absence of Dox in the MCF7-TO-ERα, MCF7-TO-ERα(EEE) and MCF7-TO-ERα(AAA) lines was also inhibited by U0126. Together, these data show that MAPK activity is important for the growth of MCF7 cells, and that phosphorylation of ERα at these residues is not able to overcome the affects of total MAPK inhibition.

3.7 Identification of proteins associated with ERα using immunoaffinity purification

Immunoaffinity purification was used to isolate HA and FLAG tagged ERα protein and associated proteins from whole cell lysates. Isolated proteins were analysed by SDS-PAGE followed by silver stain and mass spectrometory was then carried out on any bands which occurred on the gel. Firstly, the method of producing lysates was explored in order to ascertain which lysis buffer produced whole cell lysates with intact ERα in the largest quantities.

3.7.1 Testing lysis buffers

Three lysis buffers were tested to produce cell lysates using the high salt lysis method as SDS-containing lysis buffers used for the studies in previous sections

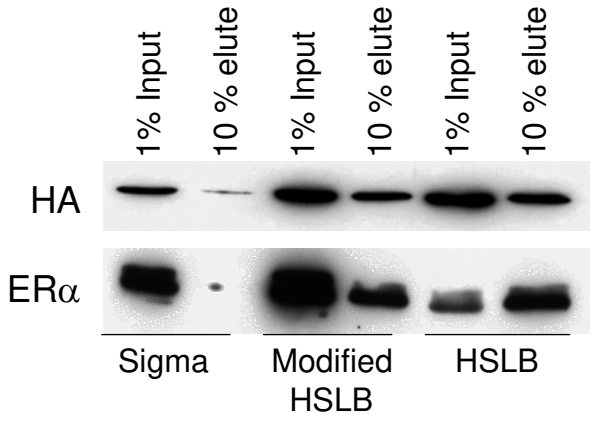


Figure 3.15 Western blot showing different cell lysis techniques used for immunoaffinity purification
Lysates of clone WT02 were made using Sigma lysis buffer (lanes 1 and 2), modified HSLB (lanes 3 and 4) and HSLB (lanes 5 and 6). For each of the three sets of lysates, immunoaffinity purification was carried out using the Sigma FLAG Tagged Protein Immunoprecipitation Kit, proteins eluted by sample buffer elution and 10% of elute loaded (lanes 2, 4 and 6). Whole cell extracts were generated by adding Laemmli buffer and the equivalent of 1% of the volume of whole cell lysate added to the immunoaffinity purification column was loaded (lanes 1, 3 and 5). Levels of HA-ERα-FLAG protein (HA) were determined by immunoblotting.

would interfere with the immunoaffinity purification. All tests were done using clone WT02 as an example of a clone expressing HA-ERα-FLAG. Preliminary experiments showed very poor ERα yield using the Sigma lysis buffer with a salt concentration of 150mM NaCl provided in the immunoaffinity purification kit used, so this lysis buffer was compared to the HSLB routinely used in the lab, which has a salt concentration of 500mM NaCl. A modified HSLB technique was also developed which lysed the cells at a higher salt concentration of 500mM NaCl, then the lysates were diluted to give a final salt concentration of 150mM NaCl, same as the Sigma lysis buffer, before loading onto the column. Immnoaffinity purification was carried out using the Sigma FLAG Tagged Protein Immunoprecipitation Kit for 1mg of protein from each type of cell lysis technique used, as measured by Bradford assay. Figure 3.15 shows the results of each cell lysis technique used with the levels of ERα measured in 1% of whole cell lysate input compared to the ERα isolated using immunoaffinity purification. Lanes A, C and E show the levels of HA-ERα-FLAG measured in the whole cell extract from Sigma lysis buffer, modified HSLB and HSLB, respectively. Levels of HA-ERα-FLAG measured from whole cell extract inputs produced using Sigma lysis buffer (lane A), as shown by immunoblot HA, clearly show a lower level of HA-ERα-FLAG protein compared to that measured using the modified HSLB (lane C) and HSLB (lane E). All lysates showed lower levels of immunopurified HA-ERα-FLAG in 10% of eluate compared to that in 1% of input, however, the Sigma lysis technique again showed the lowest level of protein. The modified HSLB technique and HSLB technique showed very similar levels of HA-ERα-FLAG in the input and after immunoaffinity purification, suggesting that the different concentrations of NaCl do not adversely affect the immunoaffinity purification. Based on these findings, the HSLB technique was used for further experiments due to the simpler technique compared to the modified HSLB technique.

3.7.2 Testing methods of elution of immunoprecipitated ERα

Three methods of eluting proteins bound to the immunoaffinity purification column are available; elution using Laemmli buffer, peptide elution in which the immunoprecipitates are incubated with a molar excess of the peptide corresponding to the epitope recognised by the primary antibody and acid elution, in which a low pH solution is used to dissociate the antibody-antigen complex. These three methods were compared in order to ascertain which provided the best elution method for HA-ERα-FLAG. Figure 3.16a shows Laemmli buffer elution following immunoaffinity purification using the HA tag (lane 2) and using the FLAG tag (lane 3). This shows that more HA-ERα-FLAG is removed from the column using the FLAG tag as both the upper blot identifying the HA tag and the lower blot identifying total ERα show considerably higher levels of HA-ERα-FLAG. Lane 4 shows peptide elution following immunoaffinity purification using the FLAG tag and lane 5 shows the same procedure using the HA tag. For both the HA and FLAG tags, peptide elution yields considerably lower levels of HA-ERα-FLAG compared to that produced using sample buffer elution, indicating inefficient competition. Figure 3.13b shows acid elution (lane B) compared to elution by the addition of Laemmli buffer (lane D) following immunoaffinity purification using the FLAG tag; this method of immunoaffinity purification was used over HA tag as it yielded higher levels of HA-ERα-FLAG in figure 3.16a. Laemmli buffer elution was carried out on the same immunoaffinity purification column after acid elution to remove any remaining HA-ERα-FLAG protein still bound to the column (lane C) The results in figure 3.16b show that acid elution method yielded very little HA-ERα-FLAG from the column (lane B). Indeed, addition of Laemmli buffer following acid elution (lane C) shows that a similar level of HA-ERα-FLAG remained bound to the column as was eluted by the addition of Laemmli buffer alone (lane D).

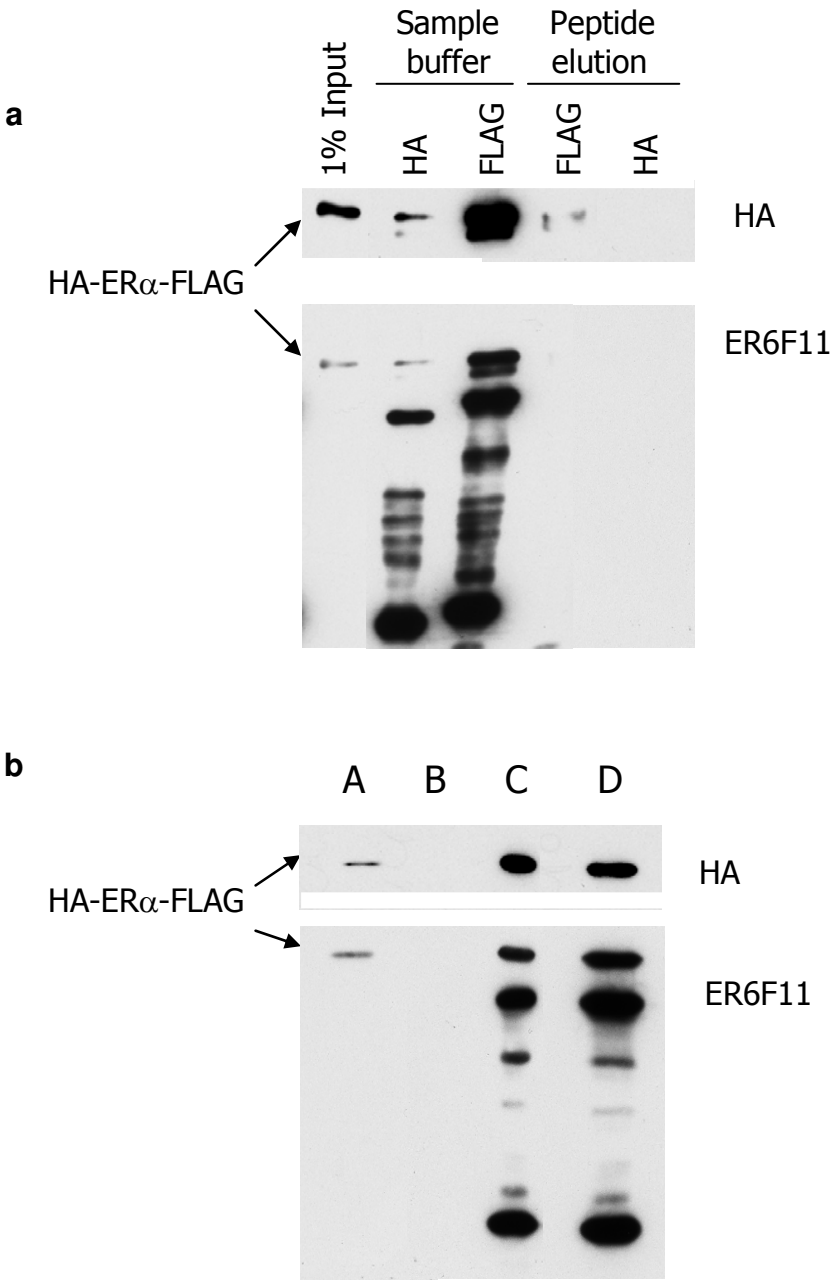


Figure 3.16 Testing methods of elution of bound proteins after immunoaffinity purification

(a) Sample buffer elution (lane 2 and 3) and peptide elution techniques (lane 4 and 5) were compared after immunoaffinity purification of whole cell lysate from clone WT02 using the FLAG-tag (lane 3 and 4) and HA-tag (lane 2 and 5). 10% of the eluate was loaded in each case. The equivalent of 1% of the volume of whole cell lysate added to the immunoaffinity purification column was loaded in lane 1 (Input). (b) Acid elution (B) was compared to the sample buffer elution technique (D) after immunoaffinity purification of whole cell lysate from clone WT02 using the FLAG-tag. Sample buffer elution was carried out on the column after acid elution (C). 10% of the eluate was loaded in each case. The equivalent of 1% of the volume of whole cell lysate added to the immunoaffinity purification column was loaded in lane A. HA-ERα-FLAG was identified using HA-hrp (HA) and ER6F11.

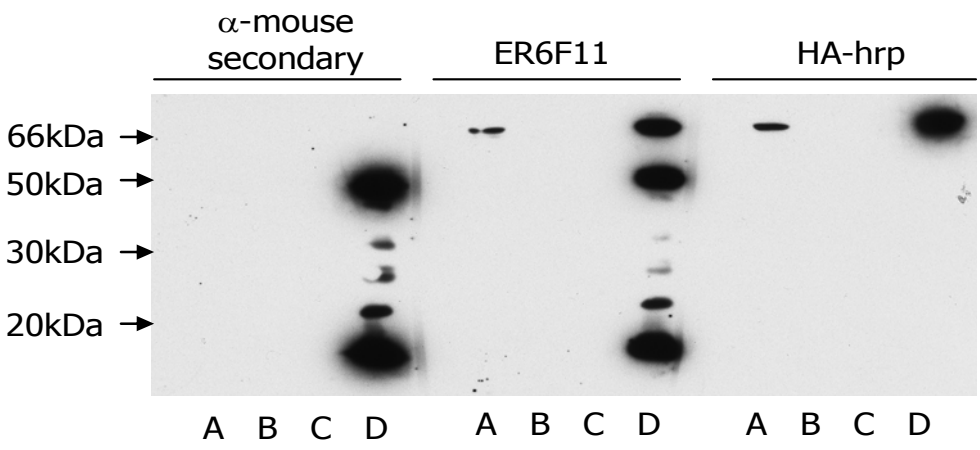


Figure 3.17 Western blot showing artefacts detected during sample buffer elution of immunopurified proteins
(A) 1% of the volume of whole cell extract added to agarose gel for IP. B) 1% of the volume of whole cell extract after IP using the FLAG tag. C) 1% of the volume of the first wash of the agarose gel after IP. D) 10% of the immunopurified protein. Samples probed with α-mouse-hrp secondary antibody, ERα followed by α-mouse-hrp secondary antibody and HA-hrp conjugated antibody.

These results would suggest that elution with Laemmli buffer was the preferred method as it yielded higher levels of HA-ERα-FLAG from the column compared to acid elution and peptide elution. However, as seen following immunoblotting for ERα (ER6F11 antibody) in figures 3.16a and 3.16b, a number of polypeptides not related to ERα were also identified. These were investigated further, and as shown in figure 3.17, they were also seen when a secondary anti- mouse hrp-conjugated antibody was used on its own of in order to identify the ER6F11 primary antibody, suggesting that the additional bands are immunoglobulins and are not related to ERα.

3.7.3 Testing methods of band identification

Eluted proteins were separated using SDS-PAGE, followed by staining of the gel to highlight separate bands. Two methods of gel staining were investigated for this use; SYPRO Ruby staining and silver staining. Identification of a range between 1-1000ng of protein marker, recombinant ERα and BSA were tested using the two methods, as shown in figure 3.18. Silver staining of the gel produced clearer protein bands and also allowed identification of lower amounts of protein compared to SYPRO Ruby staining. Silver staining was therefore used to identify HA-ERα-FLAG and associated proteins from the immunoaffinity purification eluates.

3.7.4 Identification of ERα and associated proteins

Immunoaffinity purification was carried out using the FLAG tag and HA tag separately and also using FLAG tag followed by immunnoaffinity purification of the eluate using the HA tag. Clone WT02 was again used and compared to the control MCF7-TO which contains no HA-ERα-FLAG and therefore no protein should bind to or be eluted from the column. Peptide elution was used to minimise IgG elution and

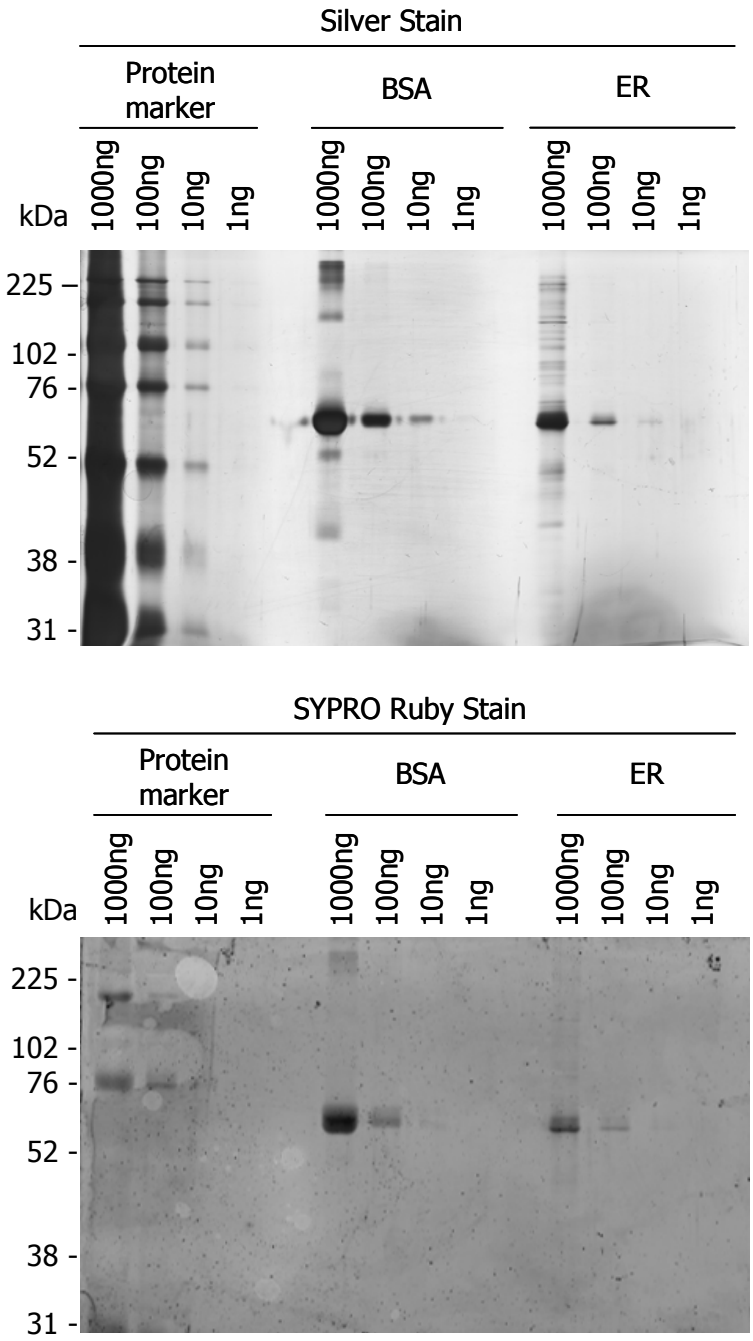


Figure 3.18 Silver stain and SYPRO Ruby staining of resolving gels
1000ng, 100ng, 10ng and 1ng of protein marker, BSA and recombinant ERα were separated using SDS-PAGE and either SYPRO Ruby staining or silver staining was carried out on the resolving gel

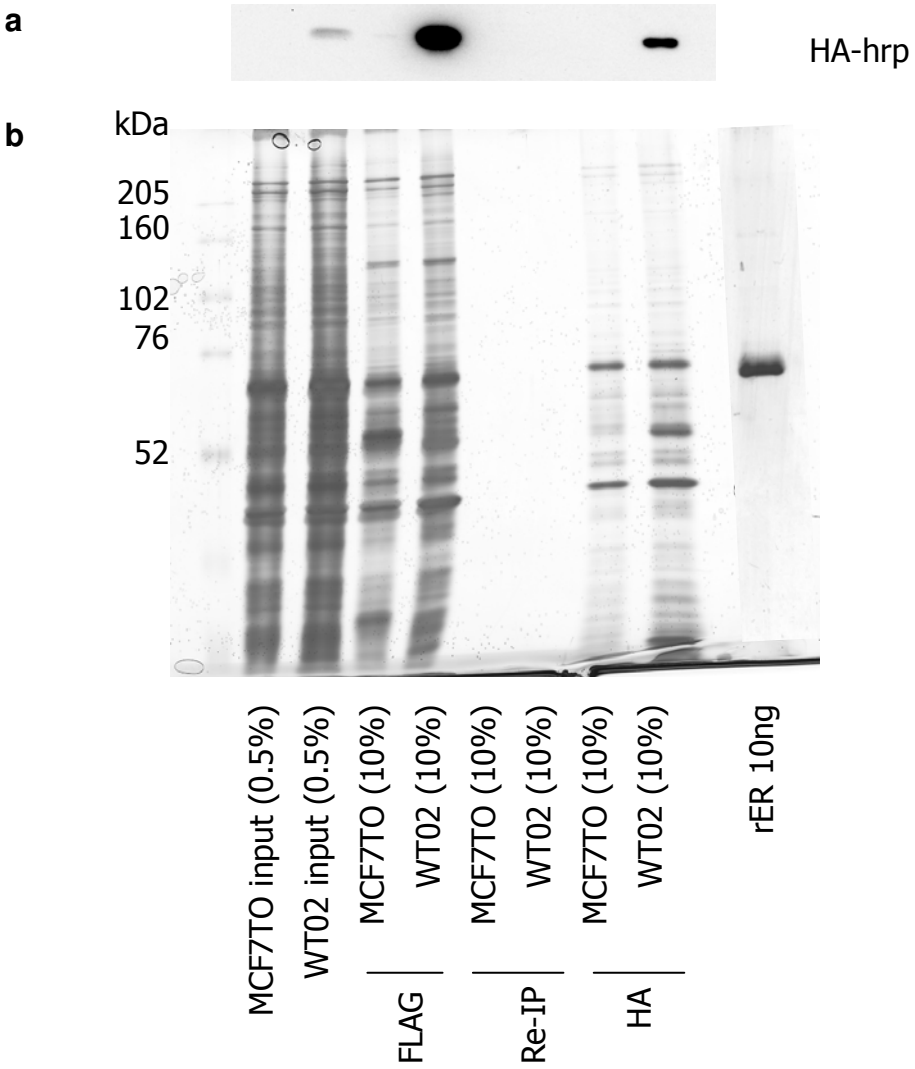


Figure 3.19 Silver stain of immunoaffinity purified proteins
FLAG-tag (FLAG) and HA-tag (HA) immunoaffinity purifications were carried out using WT02 whole cell lysate and MCF7-TO as a control. HA-tag immunoaffinity purification was carried out on elute from FLAG-tag immunoaffinity purification (Re-IP). Recombinant ERα was loaded as an indicator. HA-ERα-FLAG was identified by Western blot using HA-hrp antibody (a) and silver stain was carried out on the samples to identify total protein (b). The equivilant of 0.5% of the total volume loaded onto the column was loaded as input. 10% of the eluate from the column was loaded for each sample.

silver staining was carried out on the resolving gel after SDS-PAGE. The result displayed in figure 3.19b shows that although peptide elution does not appear to elute IgG compared to Laemmli buffer elution when analysed by Western blotting, the silver stain clearly shows non-specific binding of proteins which have been eluted as the majority of the bands present in the WT02 lanes are also present for MCF7-TO. The results in figure 3.19a and 3.19b also show, as seen in figure 3.16, that immunoaffinity purification using the FLAG tag produces a higher yield of HA-ERα-FLAG than when using the HA tag. This may explain why FLAG tag followed by HA tag immunoaffinity purification (Re-IP) yielded such low levels of protein.

FLAG tag immunoaffinity purification was carried out for clone MCF7-TO-ERα WT02 with increased wash steps to try to reduce non-specific binding. MALDI-TOF mass spectrometory was then used to identify protein bands from the gel and to see if ERα could be identified from the gel using this method. As seen in figure 3.20, the majority of bands were similar to non-specific bands seen in figure 3.19b. Mass spectrometory carried out on these bands (table 3.2) did not identify ERα and the majority of the bands tested were abundant ribosomal proteins involved with translation, tubulin or keratin. These are unlikely to represent ERα interacting proteins, so should therefore not have been present on the column after washing. Due to time constraints this was not pursued any further.

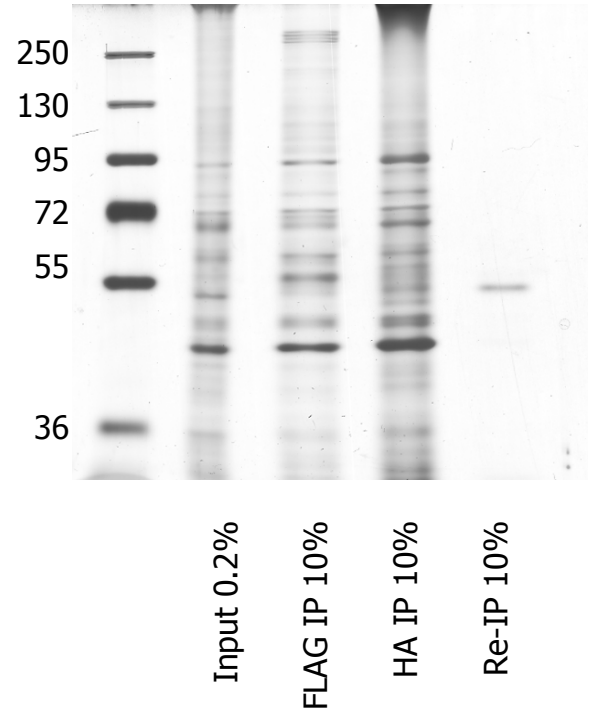


Figure 3.20 Silver stain of immunoaffinity purified proteins which were carried forward for mass spectrometory
FLAG-tag (FLAG) and HA-tag (HA) immunoaffinity purifications were carried out using WT02 whole cell lysate. HA-tag immunoaffinity purification was carried out on elute from FLAG-tag immunoaffinity purification (Re-IP). The equivilant of 0.2% of the total volume loaded onto the column was loaded as input. 10% of the eluate from the column was loaded for each sample.

Protein	Molecular weight (Da)
60S ribosomal protein L18	21485
40S ribosomal protein S7	22108
40S ribosomal protein S9	22442
40S ribosomal unit S8	24056
Ribosomal protein L13	24247
60S ribosomal protein L10a (CSA-19)	24682
Ribosomal protein LP0	27280
DNA-binding protein TAXREB107	32707
Ribosomal protein L6	32723
60S acidic ribosomal protein P0 (L10E)	34255
Keratin 19	44074
Ribosomal protein L3	46101
Keratin 18	47908
Tubulin alpha-1	49906
Tubulin alpha-6	49877
Tubulin alpha-8	50075
Tubulin alpha-3	50117
Keratin 8	53525
Keratin 9	62111
Heat shock-related 70kDa protein 2	70003
Heat shock 70kDa protein 1A	70020
Polyadenylate-binding protein 1	70653
Heat shock protein CA	73581
Nucleolin (protein C23)	76194
Heat shock protein 75 kDa	79993
TRAP1 (fragment)	79339

Table 3.2 Proteins identified using MALDI-TOF mass spectrometory
Carried out by Robin Wait at Imperial College Charing Cross Hospital

4 DISCUSSION

ERα is a steroid hormone receptor which is required for the growth and development of the male and female reproductive systems (Hewitt et al., 2005). Ligand binding results in the recruitment of coactivator complexes to the ERα which promotes its activation, allowing stimulation or inhibition of the expression of estrogen-regulated genes (Ali and Coombes, 2002; Doisneau-Sixou et al., 2003; Glass and Rosenfeld, 2000). Under normal circumstances, ERα expressing cells in breast tissue do not proliferate but stimulate the proliferation of surrounding ERα-negative epithelial cells (Clarke et al., 1997; Russo et al., 1999). In breast tumours, ERα expressing cells are converted from non-dividing cells into proliferating cells, the growth of which is stimulated by estrogen. However, how cells are converted from non-proliferating to proliferating, is unclear (Ali and Coombes, 2002). It has been hypothesised that aberrant signalling of ERα could lead to abnormal cellular proliferation and survival, potentially resulting in the development and progression of breast cancer (Herynk and Fuqua, 2004).

The SERM, tamoxifen, is used to treat ERα-positive breast cancer and works by binding to and blocking the activity of the AF2 domain, which is required for estrogen-regulated activation of ERα. A large proportion of patients who present with primary breast cancer and all those with metastatic disease, who initially respond to tamoxifen, eventually acquire resistance to tamoxifen and relapse (Likhite et al. 2006). However, ERα continues to play an important role in many tamoxifen-resistant cases (Morris and Wakeling 2002), suggesting a mechanism of resistance whereby the activity of ERα is altered in some way to overcome the inhibition of AF2 activity by tamoxifen. Although tamoxifen blocks ERα activity through the AF2, it has partial agonist properties through the activation of the second transactivation domain, AF1 (Berry, Metzger et al. 1990; Metzger, Losson et al. 1992; Tzukerman,

Esty et al. 1994), and it has been suggested that activation of the AF1 domain is involved in tamoxifen-resistant cell growth.

There is considerable evidence that ERα activity can be regulated by post-translational modification, including acetylation, glycosylation and phosphorylation (Cheng and Hart 2000; Lannigan 2003; Kim, Woo et al. 2006). Serines 104, 106 and 118 within the AF1 region of ERα have been identified as major sites of phosphorylation and their mutation has been shown to reduce the transcriptional activity of ERα (Ali et al. 1993; Le Goff et al. 1994; Joel et al. 1995; Kato et al. 1995; Chen et al. 2000; Chen et al. 2002). These three residues have all been shown to be phosphorylated by MAPK and are required for the agonist functions of tamoxifen (Bunone et al., 1996; Chen et al., 2002; Joel et al., 1998; Kato et al., 1995; Thomas et al., 2008). These and other studies have suggested that elevated S118 phosphorylation may be indicative of non-response to endocrine therapies. However, immunohistochemical studies reported since the initiation of the current project, have shown that ERα is phosphorylated at S118 in 62% of ERα-positive tumours (Murphy et al., 2004b), but that S118 phosphorylation is associated with better prognosis (Bergqvist et al., 2006; Britton et al., 2006; Bunone et al., 1996; Joel et al., 1998; Murphy et al., 2004a; Murphy et al., 2004b; Sarwar et al., 2006), although another study showed that low phosphorylation of S118, at least when accompanied by high-level S167 phosphorylation is associated with a better outcome (Yamashita et al., 2008). Furthermore, S118 phosphorylation has been reported to be higher in biopsies taken from recurrent tumours in patients who had received tamoxifen, compared to the pre-tamoxifen treatment biopsies from the same patients (Sarwar et al., 2006). Hence, the picture remains far from clear, with respect to S118 phosphorylation in vivo. No immunohistochemical studies for ERα phosphorylated at S104 and/or S106 have been reported to date. This thesis attempts to ascertain how

phosphorylation of S104, S106 and S118, affects the function of ERα in the presence of estrogen and anti-estrogens and what impact this has on cell growth using the MCF7 breast cancer cell line as a model system.

4.1 Characterising the MCF7-TO cell lines

MCF7-TO lines were generated in order to assess the effects of phosphorylation at the three residues S104, S106 and S118 within the AF1 domain. Generated were lines expressing ERα, or mutant forms of ERα in which S104, S106 and S118 were substituted by glutamic acid or alanines. The MCF7 human breast cancer cell line was used as it is a commonly used model of breast cancer, in that it expresses ERα, requires estrogen for its growth, and its growth is inhibited by anti-estrogens (Buluwela et al., 2005). The Tet-Off system was used as it enabled controlled expression of the mutant and wild type forms of ERα by removal of Dox from the growth medium which prevented any possible long-term changes that high levels of ERα expression might cause. The mutant and wild type forms of ERα introduced into the cells were N- and C-terminally tagged with HA and FLAG epitope tags, respectively. This was carried out so that it would be possible to identify these forms of ERα from the endogenous ERα present in the cells.

Immunoblotting of whole cell lysates in the absence of Dox demonstrated successful generation of lines expressing each introduced form of ERα, at varying levels, in a Dox-regulated manner (Figure 3.1). Clones expressing HA-ERα-FLAG, HA-ERα(EEE)-FLAG and HA-ERα(AAA)-FLAG were then taken forward to test for Dox control of the tagged ERα (Fig. 3.2) and based on these results, three clones from each cell line were chosen according to the level of introduced ERα expression compared to endogenous ERα; one with high, one medium and one with low levels

of tagged ERα compared to endogenous ERα. This was done so that any effects due to the level of tagged ERα expression in subsequent experiments would be identified.

4.2 The effect of phosphorylation on the activity of ERα

Luciferase reporter gene assays were carried out to assess how the expression of ERα, ERα(EEE) and ERα(AAA) affected the activity of the ERα relative to endogenous ERα expressed in MCF7-TO (Fig. 3.4). Expression of WT ERα, ERα(EEE) and ERα(AAA) caused an increase in the overall levels of ERα expression, and a consequent increase in the levels of ERα activity in the absence of ligand and presence of estrogen. Although all clones expressing WT ERα, ERα(EEE) and ERα(AAA) caused an increase in ligand-independent activity, expression of ERα(EEE) displayed the greatest increase in activity, suggesting that phosphorylation at S104, S106 and S118 within the AF1 may promote the activity of ERα in the absence of ligand.

Tamoxifen stimulated ERα activity to a lesser extent than estrogen. Interestingly, there was increased ERα activity in the presence of OHT in the case of the MCF7-TO- ERα and ERα(EEE) lines and activity in the presence of OHT was reduced in MCF7-TO-ERα(AAA) compared to the parental cells. These results suggest that the ability to phosphorylate these three residues within AF1 stimulates the activity of ERα in the presence of OHT as expression of ERα(AAA), which cannot be phosphorylated, was less active, whilst ERα(EEE) was more active, than WT ERα. The results also suggest that there is moderate stimulation of phosphorylation of these residues under the culture conditions used, as expression of WT ERα increases activity more than expression of ERα(AAA), possibly because WT ERα is being phosphorylated by endogenous signalling mechanisms. It is of course possible that

the reduction in ERα activity for the ERα(AAA) mutant is due to the replacement of the serines, rather than due to phosphorylation of the serines residues, although the higher activity of the ERα(EEE) mutant suggests that phosphorylation is an important feature of ERα activity.

ICI inhibited ERα activity, regardless of the phosphorylation status of the residues at positions 104, 106 and 118, which is in agreement with the previous findings showing that ICI inhibits the activity of AF1, as well as AF2 (Wakeling et al., 1991).

4.3 The effect of ERα phosphorylation on the expression of endogenous estrogen-regulated genes

The effect of the phosphorylation status of S104, S106 and S118 on the expression of a number of estrogen-regulated genes in response to estrogen was investigated at the protein level and at the mRNA level. Analysis of all the genes studied showed a level of estrogen-stimulation within all the cell lines at either the protein level or the mRNA level or both. Overall, these data show that over-expression of ERα causes an increase in expression of estrogen-responsive genes and that protein expression of some genes are also affected by the phosphorylation status of S104, S106 and S118 within the ERα.

Data shown in figure 3.5 suggests that over-expression of ERα causes an increase in ligand-independent and estrogen-dependent expression of pS2 and cathepsin D at the protein level. Expression of ERα(EEE) appears to promote ligand-independent pS2 protein expression, however, levels of cathepsin D, pS2 and also GREB1 mRNA were not seen to increase significantly due to overexpression of ERα or due to expression of phosphorylation mutants (Fig. 3.6b and c, Fig. 3.7). This suggests that phosphorylation of S104, S106 and S118 causes an increase protein

expression of at least some estrogen-regulated genes but also that this effect may be gene-specific and that not all estrogen-regulated genes respond in the same way. Microarray analysis of the whole genome allowed for further analysis of how phosphorylation of the AF1 may affect other genes, both estrogen-regulated and not. By comparing whole-genome gene analysis of MCF7-TO-ERα(EEE) with MCF7-TO, 60 genes were identified as being up-regulated due to expression of ERα(EEE) in the absence of E2. The majority of these genes were seen to be involved in gene transcription and protein degradation; however, LOC643287, PPIL1, p32r1 and IGFBP5 appeared to be of particular interest.

LOC643287 has a similar sequence to the more widely studied protein, ProTα. ProTα is a highly conserved nuclear protein which is ubiquitously expressed in mammalian tissues and is involved with chromatin remodelling (Diaz-Jullien et al., 1996; Papamarcaki and Tsolas, 1994) and cellular proliferation (Clinton et al., 1991; Eschenfeldt and Berger, 1986; Gomez-Marquez et al., 1989; Sburlati et al., 1991). Levels of ProTα are increased in proliferating cells (Tsitsiloni et al., 1993) whilst repressed in quiescent cells (Dominguez et al., 1993); in accordance with this involvement in cell growth, ProTα has also been shown to be expressed in various tumours of different origins. Overexpression of ProTα in a rat fibroblast cell line has been shown to result in loss of contact inhibition, anchorage-independent growth, and decreased serum dependence (Orre et al., 2001) whereas inhibition of ProTα using antisense oligomers causes an inhibition of myeloma cell growth (Sburlati et al., 1991). Most interestingly in respect to this project, not only has ERα been shown to regulate the transcriptional activity of the ProTα gene in response to E2 (Bianco and Montano, 2002), but levels of ProTα have been shown to be higher in breast tumours compared to normal breast tissue (Magdalena et al., 2000), indicating a possible role in breast cancer progression. A number of studies raise possible reasons

as to why an increase in ProTα may be involved in breast cancer progression, including reported interactions with the transcriptional co-activator CBP, both in vivo and in vitro, acting as a co-factor stimulating the transcriptional activity of CBP (Karetsou et al., 2002). Expression of ProTα has also been shown to interact with the co-repressor REA (repressor of estrogen receptor activity) causing an increase in ERα activity by sequestering the co-repressor (Martini et al., 2000).

The results of this study appear to suggest that ERα phosphorylated within the AF1 causes an increase in expression of LOC643287, a gene similar to ProTα. Phosphorylation of the AF1 has been shown to cause an increase in activity of ERα (Ali et al., 1993; Chen et al., 2000; Kato et al., 1995) and levels of phosphorylated AF1 have been shown to be increased in breast cancer (Murphy et al., 2004a; Sarwar et al., 2006). These results could indicate a possible mechanism whereby phosphorylation of the AF1 causes an increase in cell proliferation by increasing the expression of LOC643287 which may act in a similar manner to ProTα in promoting cellular proliferation. Unfortunately no probe for ProTα is included in the microarray carried out and it would be interesting in the future to identify how ProTα regulation is affected by ERα phosphorylation.

PPIL1 is a cyclophilin-related protein, sharing 41.6% identity of the amino acid sequence with human cyclophilin A (Ozaki et al., 1996). Cyclophilins are one of three families of peptidyl-prolyl isomerases; the cyclophilin family which form complexes with the immunosuppressant cyclosporin A, the immunosuppresant FK506 binding protein (FKBP) family and the parvulin family (Hunter, 1998). Peptidyl prolyl isomerases carry out cis-trans isomerization of the peptide bond on the N-terminal side of proline residues in proteins – a process which plays a role in protein folding, the assembly and disassembly of protein complexes, protein trafficking and regulation of protein activity (Schmid, 1995; Shaw, 2002). Microarray studies carried

out using colon cancer tissues identified PPIL1 as being specifically up-regulated in tumours compared to normal colon tissue (Lin et al., 2002). Further studies showed that expression of PPIL1 promotes growth of colon cancer cells and that the protein interacts with SNW1/SKIP and stathmin (Obama et al., 2006). SNW1/SKIP is a transcriptional coactivator which plays an important role in transcriptional regulation, pre-mRNA splicing and cell cycle regulation (Folk et al., 2004). Stathmin, also known as oncoprotein 18, is over-expressed in luekaemia (Hanash et al., 1988), highly proliferative breast cancers (Curmi et al., 2000) and malignant ovarian cancers (Price et al., 2000) causing microtubule destabalization by stimulating microtubule catastrophes and sequestering tubulin dimers - a process involved with transformation allowing cancer cells to grow independently of substrate anchorage (Cassimeris, 2002). The authors suggest that elevated expression of PPIL1 may play an important role in the proliferation of cancer cells through the control of SNW1/SKIP modulated genes associated with proliferation and cell cycle progression through association with stathmin (Obama et al., 2006). Microarray studies also showed that expression of PPIL1 was elevated in cervical, pancreatic and gastric cancers (Obama et al., 2006) and the data from the microarray data here suggest that the expression could also be elevated in breast cancers, possibly due to phosphorylation of ERα.

The pp32r1 gene (or ANP32C) is a member of the acidic leucine-rich nuclear phosphoprotein 32 family (ANP32). Whereas the closely-related family member pp32 has anti-oncogenic properties and is involved with controlling gene expression, signalling pathways and apoptotic pathways, pp32r1 has been shown to have oncogenic properties (Brody et al., 1999; Kadkol et al., 1999). Transfection of NIH-3T3 cells over-expressing pp32r1 into nude mice has been shown to lead to the formation of tumours, suggesting that over-expression of pp32r1 stimulates

transformation and tumourigenesis (Bai et al., 2001). Studies have identified pp32r1 as being highly expressed in prostatic adenocarcinoma cell lines with little expression shown in breast cancer cell lines (Kochevar et al., 2004). The data presented here suggests that pp32r1 expression is increased in response to ERα phosphorylation, possibly stimulating tumourigenesis.

IGFBP5 is one of six members of the IGFBP family and binds IGF, enhancing or suppressing its activity in a tissue and cell-specific manner (Beattie et al., 2006; Schneider et al., 2002). Studies have shown expression of IGFBP5 causes a decrease in tumour growth and tumour vascularity in a xenograft model of human ovarian cancer (Chun et al., 2008). Although very low levels of IGFBP5 were detected in normal breast epithelium, moderate to high levels of IGFBP5 were detected in 50% of invasive breast carcinomas tested in a recent study carried out by Wang et al. (Wang et al., 2008). Although expression of IGFBP5 correlated with the expression of PR and Her2, no correlation was shown with tumour size or the expression of ERα (Wang et al., 2008). The data here suggests that over-expression of ERα causes a decrease in the levels of IGFBP5 expression and presumably the anti-tumuorigenic affect of IGFBP5.

Taken together, initial analysis of the microarray studies carried out suggests that phosphorylation within the AF1 of ERα promotes tumour cell growth by causing an increase in expression of at least two genes involved in tumourigenesis, PPIL1 and pp32r1, and potentially a third also, LOC643287. Over-expression of ERα appears to causes a decrease in at least one gene, IGFBP5, which has anti-tumourigenic properties, therefore also promoting tumourigenic activity.

4.4 The effect of ERα phosphorylation on cell growth

The above-described data indicate that ERα over-expression results in an increased expression of estrogen-regulated genes. Moreover, the activity of ERα(EEE) was higher in the presence of estrogen and OHT, as well as in the absence of ligand, when compared with WT ERα, suggesting that the glutamic acid residues at these positions stimulate ERα activity. In growth assays, however, the picture was clearly more complex. In the presence of Dox, all of the lines showed a similar growth profile to MCF7-TO, as expected, with little or no growth in the presence of OHT, ICI or in the absence of ligand. In the MCF7-TO-ERα, ERα(EEE) and ERα(AAA) lines, there was significant growth in the absence of ligand in all cases, when Dox was removed, indicating that the growth stimulation was due to the over-expression of ERα and not the phosphorylation status of the S104, S106 and S118 residues within the AF1. Growth in the presence of estrogen, tamoxifen and ICI was not stimulated in the ERα, ERα(EEE) or ERα(AAA) lines, even though the levels of ERα activity as assessed by gene expression analysis and reporter gene assays suggested that cell growth might be stimulated in the presence of tamoxifen. These growth assays suggest that phosphorylation of the residues within the AF1 does not play a significant part in regulating MCF7 cell growth. A number of studies have shown that re-introducing ERα expression into ERα-negative breast cancer cell lines such as MDA-MB 231 cells, re-sensitizes the cells to hormone treatment; however, subsequent E2 treatment suppresses cell proliferation (Jiang and Jordan, 1992; Lazennec and Katzenellenbogen, 1999; Zajchowski et al., 1993). These results described here however, are similar to a number of studies carried out using MCF7 cell lines which have shown that over-expression of ERα causes an increase in ligand-independent cell proliferation, but does not affect estrogen-stimulated cell growth (Fowler et al., 2004; Fowler et al., 2006). It has also been shown that

increased activity due to over-expression of ERα requires the AF1, but is independent of AF1 phosphorylation (Fowler et al., 2004) which is supported by the results presented here.

Since S104, S106 and S118 phosphorylation is stimulated by the MAPK pathway, stimulators of cell surface receptors that activate this pathway were carried out. However, neither EGF nor insulin stimulated the growth of these lines in a differential manner, indicating that mutation of S104, S106 and S118 is insufficient to prevent the growth stimulation through the MAPK pathway, at least in this context, where ERα is over-expressed.

There is relatively little EGF receptor expression in MCF7 cells, although IGF receptors are expressed (Pacher et al., 2007). Additionally, other tyrosine kinase receptors might provide sufficient MAPK activation for additional EGF or insulin to make an insufficient difference in this study. Indeed, MAPK activation by EGF and insulin was not determined. In order to address this issue, growth assays were carried out in the presence of the MEK inhibitor, U0126, caused inhibition of cell growth for all cell lines under all conditions in the presence and absence of Dox. Growth inhibition by U0126 was observed, with the inhibition not being rescued by ERα over-expression, or indeed by the ERα(EEE) mutant.

Together, these findings indicate that MAPK activation is important for the growth of MCF7 cells, but that the growth stimulation is independent of a requirement for phosphorylation at S104, S106 or S118. There are a number of potential reasons for these findings. The simplest is that over-expression of ERα is sufficient to provide the growth benefit that phosphorylation might provide. There are other protein kinases that have been shown to phosphorylate these residues, namely CDK2 and GSK3 in the case of S104, S106, CDK7 in the case of S118. These kinases may provide sufficient phosphorylation at these sites to prevent evident

differences between ERα and ERα(EEE) lines to be observed. However, differences might have been expected for the ERα(AAA), which also did not show any differences. It is also possible, as discussed in Chapter 1, that MAPK-mediated phosphorylation of coactivators, in particular AIB1 and SRC1, and/or the corepressors NCoR and SMRT, is rather more important for ERα function than the phosphorylation of the residues in AF1, at least when ERα is over-expressed.

4.5 Identification of ERα co-immunoprecipitating proteins

The MCF7-TO lines described here were generated by stable integration of ERα HA and FLAG epitope tagged at the N- and C-terminus, respectively. This allowed the immunoaffinity purification of HA-ERα-FLAG, HA-ERα(EEE)-FLAG and HA-ERα(AAA)-FLAG polypeptides from cell extracts, with the aim of co-purifying proteins that interact with ERα and specifically, by comparison of proteins that interact with ERα and ERα phosphorylation site mutants, of proteins that interact preferentially with phosphorylated ERα. However, a number of problems occurred using this method, firstly, the difficulties associated with generation of sufficiently large amounts of cell extracts to allow the purification of interacting proteins. Further, although the immunoprecipitation of ERα was extremely easy, the process was inefficient, with much of the ERα remaining bound to the antibody agarose beads, at least using the gentle methods of elution required to maintain ERα interacting proteins. Finally, despite the fact that HA and FLAG antibodies coupled directly to agarose beads were used, considerable amounts of antibody were eluted from the gels using the Laemmli buffer method of elution, but also using the rather more gentle peptide elution method, whilst acid elution proved to be inefficient. When immunoprecipitates were subjected to mass spectrometric sequencing, the major proteins were immunoglobulin's and tubulin. Indeed, it was not possible even

to identify ERα in the immunoprecipitates following silver staining, although there was some ERα in the immunoprecipitates as demonstrated by Western blotting. This could be due to other more abundant protein bands at a similar molecular mass masking the presence of ERα, or it could be due to the fact that samples were not concentrated enough for silver staining of gels to highlight the protein. Silver staining of gels is considerably less sensitive than Western blotting so a larger amount of protein would be required in order for the protein to be visualised on a gel by silver stain compared to that required by Western blotting.

This work shows that in order for silver staining to be used as a method of visualising co-immunoprecipitating proteins of ERα, the method of washing the column of un-bound proteins requires improving and the elute needs to be concentrated in order to reach high enough levels of protein to be visualised on the gel by silver stain. Unfortunately, there was not enough time to explore this further during this project. The problems encountered using silver stain and mass spectrometry to identify novel ERα-interacting proteins could possibly be overcome by using the tandem affinity purification (TAP)-tag method. This method involves fusion of the TAP tag to the target protein and then introduction of the construct into the host. The TAP tag consists of two protein tags separated by a cleavage site. For example, the yeast TAP tag consists of two IgG binding units of protein A from Staphylococcus aureus, a cleavage site for the tobacco etch virus (TEV) protease and a calmodulin binding peptide (CBP). The target protein is first purified by protein A binding to IgG sepharose, then separated using TEV-protesase cleavage. The eluate is then incubated with calmodulin sepharose and released with ethylene glycol tetraacetic acid. This method has been shown to increase the amount of target protein recovered and the increased efficiency compared to immunoaffinity purification with other tags, including HA and FLAG, reduces the amount of cellular

starting material required (Burckstummer et al., 2006; Collins and Choudhary, 2008; Tsai and Carstens, 2006).

4.6 Final conclusions and future work

These results show, in agreement with other studies carried out, that over-expression of ERα causes an increase in levels of receptor activity and cell proliferation. Although an increase in ERα activity was observed in the presence and absence of E2 due to over-expression of ERα, as seen by reporter gene assays and an increase in pS2 and cathepsin D protein expression, cell growth was only increased in the absence of ligand due to ERα over-expression, with no additional effect when S104, S106 and S118 were substituted by glutamic acid residues, despite the fact that these substitutions increase ERα activity in reporter gene assays. However, microarray analysis does suggest that phosphorylation within the AF1 leads to a ligand-independent increase in certain genes – most interestingly with respect to this project was the upregulation of at least two genes previously shown to be involved with cancer cell progression (PPIL1 and pp32r1) and the downregulation of a tumour suppressor gene (IGFBP5). This does suggest that AF1 phosphorylation could promote cancer cell growth progression.

However, data shown here indicates that phosphorylation at these sites does not contribute greatly to cell growth, at least when ERα is over-expressed. A study carried out using MCF7 cells over-expressing ERα in a tetracycline-inducible manner suggested that increased levels of ERα protein results in an increased occupancy of EREs by ERα at promoter sites of estrogen-regulated genes, independent of estrogen binding (Fowler et al., 2004). Although this was seen to result in increased pS2 mRNA levels in the presence and absence of E2 this study showed that, in agreement with the results presented here, over-expression of ERα was not capable

of increasing cell growth in the presence of E2 and the authors suggested that increased levels of ERα have a squelching effect on co-repressors in the absence of ligand (Fowler et al., 2004). This means that the cells effectively 'run-out' of co-repressors that are thought to normally bind ERα in the unliganded state due to the abnormally high levels of ERα protein, allowing for un-bound ERα protein to take on an active role resulting in an increase in ligand-independent cellular proliferation. In a similar way, in the presence of estrogen the increased levels of ERα protein may potentially put such a demand on available co-activators that levels of activity, although increased for some genes, do not increase sufficiently to cause an increase in cell proliferation.

Due to the method used in this study of expressing ERα, ERα(EEE) and ERα(AAA) in the ERα-positive MCF7 cell, the effect of ERα phosphorylation was difficult to elucidate as results were complicated by the effects of ERα overexpression. The MCF7 cell line was chosen due to the fact that it represents an anti-estrogen-sensitive cell line which expresses estrogen-regulated genes and these characteristics were important for these studies. To avoid the added affect of ERα over-expression, gene knock-in techniques could be used in future. This has been widely used to introduce small changes in mice through homologous recombination (Christian et al., 2008; McDevitt et al., 2008; Sinkevicius et al., 2008). Short interfering RNA (siRNA) is a method of 'silencing' gene expression *in vitro* and as such could be used to prevent endogenous ERα expression, although the knock-down effect on the gene of interest is only transient, the level of reduction in gene expression may be incomplete, and whilst such a method could be used to knockdown expression of endogenous ERα, the over-expression provided by the introduced gene may still be problematic. Further, expression of the introduced gene is mediated by a CMV promoter and TetR-VP16, whose regulation in the cell cycle

will be distinct from that of the endogenous ERα gene, which may also influence estrogen responses in MCF-7 cells. Recent reports have utilised a homologous recombination system that has been used to generate knock-in mutations in cell lines. The method uses the adenovirus associated virus (AAV) based vectors to allow infection for high-level cell take up of the vector, thereby maximising the chances of homologous recombination. However, most genes in cell lines are present as two copies (and in many cases, due to chromosomal instability, there may be additional copies of some genes), so two homologous recombination events are required, making this a difficult task to achieve. Nevertheless, several reports of gene knockouts and knockins have used this methodology (Arena et al., 2007; Kim et al., 2008) and it may provide a method for introduction of the substitutions in ERα phosphorylation sites.

Increased expression of ERα is a common phenotype in many post-menopausal breast cancer cases and is linked to an increase in cell growth and poor patient survival (Black et al., 1983; Fabris et al., 1987; Holst et al., 2007; Hupperets et al., 1997; Masters et al., 1978; Sancho-Garnier et al., 1995; Thorpe et al., 1993). The work described here supports the hypothesis that this increased ERα expression leads to an increase in estrogen-independent cell growth. However, phosphorylation of ERα at S104, S106 and S118 within the AF1 does not appear to further regulate breast cancer cell growth, despite previous findings using reporter gene studies showing that phosphorylation at these sites stimulates ERα activity. Although a number of studies have been carried out which show that MAPK signalling pathways increase phosphorylation of these residues, resulting in increased ERα activity, this study shows that the stimulation of this pathway increases cell growth independently of the phosphorylation status of ERα. This is interesting given recent

immunohsitochemical staining studies showing that increased levels of S118 phosphorylation are indicative of a better prognosis in breast cancer patients.

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Appendix 1a

Genes up or down regulated 1.5-fold in the MFC7-TO cell line by estrogen in the presence and absence of Dox

Ilumina probe	Fold-induction	Gene name	Gene bank code	Gene description
ILMN_20319	3.892			
ILMN_24929	2.964	CAP70; CLAMP; PDZD1; NHERF3	NM_002614	PDZ domain containing 1
ILMN_5543	2.896	D53; hD53; MGC8556; TPD52L2	NM_001003396	tumor protein D52-like 1
ILMN_20958	2.873	MGC134798; MGC134799; Link-GEFII	NM_016339	Rap guanine nucleotide exchange factor (GEF)-like 1
ILMN_24346	2.749			
ILMN_25919	2.711	CTRP6; ZACRP6	NM_031910	C1q and tumor necrosis factor related protein 6
ILMN_25992	2.699	OGR1; MGC111379	NM_003485	G protein-coupled receptor 68
ILMN_6412	2.675	RNPC6; FLJ26355; FLJ30829; FLJ37697; dJ259A10.1	NM_153020	RNA binding motif protein 24
ILMN_14912	2.536	B1F; CPF; FTF; B1F2; hB1F; LRH-1; FTZ-F1; hB1F-2; FTZ-F1beta	NM_205860	nuclear receptor subfamily 5, group A, member 2
ILMN_24376	2.522	DEP.6; FLJ12428; FLJ13854; DKFZp564B1778	NM_022783	DEP domain containing 6
ILMN_7163	2.469			
ILMN_9309	2.391	BP-4; IBP4; IGFBP-4; HT29-IGFBP	NM_001552	insulin-like growth factor binding protein 4
ILMN_3259	2.369	PTMP; PP2Cm; UG0882E07; DKFZp667B084; DKFZp761G058	NM_152542	protein phosphatase 1K (PP2C domain containing)
ILMN_11008	2.347	MGC26847	NM_145006	sushi domain containing 3
ILMN_6004	2.289			
ILMN_16132	2.274	EGFL2; MEGF3; CDHF10; FLJ34118; FLJ42737; FLJ45143; FLJ45845; KIAA0279; Flamingo1	NM_001408	cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila)
ILMN_2451	2.172	SGK1	NM_005627	serum/glucocorticoid regulated kinase
ILMN_2778	2.092	PGES; PIG12; PP102; PP1294; MGST-IV; MGST1L1; TP53I12; MGC10317; MGST1-L1	NM_004878	prostaglandin E synthase
ILMN_27781	2.035	ALP; DKFZp686L0362	NM_014476	PDZ and LIM domain 3
ILMN_115591	2.005		AW364673	PM3-DT0037-231299-001-e06 DT0037 Homo sapiens cDNA, mRNA sequence.
ILMN_24745	1.963			
ILMN_21479	1.93			
ILMN_9429	1.929	HBI; p52; Hsp56; FKBP52; FKBP59; PPIase	NM_002014	FK506 binding protein 4, 59kDa
ILMN_26335	1.906	AMZ1; KIAA1950	NM_133463	archaemetzincin-1
ILMN_25446	1.893	E16; CD98; LAT1; 4F2LC; MPE16; hLAT1; D16S469E	NM_003486	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5
ILMN_10499	1.885	GRL; CHF1; HRT2; HERP1; HESR2; MGC10720	NM_012259	hairy/enhancer-of-split related with YRPW motif 2
ILMN_24807	1.853	HSU79274	NM_013300	chromosome 12 open reading frame 24
ILMN_21535	1.853			
ILMN_29405	1.851	CRBP4; CRBPIV; MGC70641	NM_052960	retinol binding protein 7, cellular
ILMN_3806	1.819		NM_153344	
ILMN_15343	1.795	FLJ32104	NM_153034	zinc finger protein 488
ILMN_25666	1.785	SemA; SEMA5; SEMAA; semaV; LUCA-1; FLJ34863	NM_001005914	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B
ILMN_19956	1.769	GREB1; KIAA0575	NM_148903	GREB1 protein
ILMN_20988	1.767	FLJ21145; FTHFSDC1; dJ292B18.2; RP1-292B18.2; DKFZp586G1517	NM_015440	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like
ILMN_12660	1.757	RDGBB; RDGBB1; RDGB-BETA	NM_181671	phosphatidylinositol transfer protein, cytoplasmic 1
ILMN_7086	1.745			
ILMN_20377	1.728	SPG31; C2orf23; FLJ13110	NM_022912	receptor accessory protein 1
ILMN_23624	1.715			
ILMN_74292	1.693			
ILMN_18253	1.69	C12orf7; DKFZp686O1689	NM_182608	ankyrin repeat domain 33
ILMN_12566	1.683	JAK2	NM_004972	Janus kinase 2 (a protein tyrosine kinase)
ILMN_11540	1.678	NEH2; MGC2832; MGC4505; FLJ31644	NM_145043	nei like 2 (E. coli)
ILMN_14782	1.665	PCPE2	NM_013363	procollagen C-endopeptidase enhancer 2
ILMN_7220	1.658	MGC33510	NM_152765	chromosome 8 open reading frame 46
ILMN_29566	1.655	FLJ22416; FLJ25593	NM_014344	four jointed box 1 (Drosophila)
ILMN_25424	1.651	P1; PK; A14GALT; A4GALT1	NM_017436	alpha 1,4-galactosyltransferase (globotriaosylceramide synthase)

Genes up or down regulated 1.5-fold in the MFC7-TO cell line by estrogen in the presence and absence of Dox contd.

Illumina probe	Fold-induction	Gene name	Gene bank code	Gene description
ILMN_17796	1.65	K13; CK13; MGC3781; MGC161462	NM_002274	keratin 13
ILMN_3555	1.646	KIAA1026; RP1-21O18.1	NM_001018000	kazrin
ILMN_29636	1.635	LOC89944; MST114; MSTP114	NM_138342	hypothetical protein BC008326
ILMN_20677	1.615	FLJ11266; TU12B1-TY	NM_016575	5'-nucleotidase domain containing 3
ILMN_119940	1.604	DKFZp762C1112	AK095472	transmembrane protein 64
ILMN_15639	1.6	CPSD; CLN10; MGC2311	NM_001909	cathepsin D
ILMN_26070	1.596	TOSS; KCNK8; TWIK2; K2p6.1; TWIK-2; FLJ12282	NM_004823	potassium channel, subfamily K, member 6
ILMN_16561	1.591			
ILMN_4623	1.56	OCIM	NM_138768	myeloma overexpressed gene (in a subset of t(11;14) positive multiple myelomas)
ILMN_1421	1.538	EBP50; NHERF; NHERF1	NM_004252	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1
ILMN_13740	1.53			
ILMN_29702	1.528	CDT2; RAMP; DCAF2; L2DTL	NM_016448	denticleless homolog (Drosophila)
ILMN_34249	1.511		XM_372111	
ILMN_28089	1.506	FLJ43600; dJ310O13.5	NM_080625	chromosome 20 open reading frame 160
ILMN_26680	0.596	REPRIMO; FLJ90327	NM_019845	reprimo, TP53 dependent G2 arrest mediator candidate
ILMN_27027	0.575	BRPK; PARK6; FLJ27236	NM_032409	PTEN induced putative kinase 1
ILMN_7361	0.561			
ILMN_23267	0.534	C4.4A	NM_014400	LY6/PLAUR domain containing 3
ILMN_26701	0.522	MAD4; MST149; MSTP149	NM_006454	MAX dimerization protein 4
ILMN_11864	0.519	FLJ25974; dJ382I10.1	NM_001031743	chromosome 6 open reading frame 165
ILMN_14320	0.489			
ILMN_71954	0.475	RAB27B	AF131784	RAB27B, member RAS oncogene family
ILMN_14524	0.418	BJ-TSA-9; MGC14128	NM_032899	family with sequence similarity 83, member A
ILMN_3777	0.414	BP4; NBK; BIP1	NM_001197	BCL2-interacting killer (apoptosis-inducing)

Appendix 1b

Genes up or down regulated 1.5-fold in the MFC7-TO cell line by estrogen in the presence of Dox

Ilumina probe	Fold-induction	Gene name	Gene bank code	Gene description
ILMN_104092	3.623		BE041196	Transcribed locus
ILMN_122276	1.957		DB005376	Transcribed locus
ILMN_17836	1.85	NPH3	NM_007225	neurexophilin 3
ILMN_2445	1.683	PAPT; SPS1; SPDSY; SRML1	NM_003132	spermidine synthase
ILMN_14815	1.644	EAAC1; EAAT3	NM_004170	solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag)
ILMN_2720	1.612	RP3; TCTE1L; TCTEX1L	NM_006520	dynein, light chain, Tctex-type 3
ILMN_92257	1.602		CF887340	Transcribed locus
ILMN_16684	1.592	KSP37	NM_031950	fibroblast growth factor binding protein 2
ILMN_19350	1.59	FLJ25330; DKFZp434A119	NM_178452	leucine rich repeat containing 50
ILMN_6931	1.589	FLJ20811	NM_019007	armadillo repeat containing, X-linked 6
ILMN_1521	1.573			
ILMN_6757	1.552	MGC18079; PRO19651	NM_144675	GSG1-like
ILMN_15268	1.524	PR52; PR55G; IMYPNO; IMYPNO1; MGC33570	NM_181876	protein phosphatase 2 (formerly 2A), regulatory subunit B, gamma isoform
ILMN_102475	1.52		BF676681	Transcribed locus, strongly similar to XP_530653.1 hypothetical protein XP_530653
ILMN_13769	1.519	PEC-60; MGC133107	NM_014471	serine peptidase inhibitor, Kazal type 4
ILMN_6685	1.509	C23; FLJ45706	NM_005381	nucleolin
ILMN_12916	1.506	H731; MGC33046; MGC33047	NM_145341	programmed cell death 4 (neoplastic transformation inhibitor)
ILMN_11739	0.666	MAR; IRF-1	NM_002198	interferon regulatory factor 1
ILMN_16812	0.665	PBGS; ALADH; MGC5057	NM_001003945	aminolevulinate, delta-, dehydratase
ILMN_3455	0.664			
ILMN_17461	0.663		NM_145748	
ILMN_693	0.659	ATP-BL	NM_004913	chromosome 16 open reading frame 7
ILMN_17641	0.657	FLJ13725; KIAA1930	NM_024519	family with sequence similarity 65, member A
ILMN_2236	0.653	C10orf45; FLJ45505; MGC11034; MGC90261	NM_031453	family with sequence similarity 107, member B
ILMN_16682	0.653			
ILMN_20707	0.652	ZABC1	NM_006526	zinc finger protein 217
ILMN_21728	0.643	MGC119418; MGC119419; MGC119420	NM_033131	wingless-type MMTV integration site family, member 3A
ILMN_5460	0.637	DMP4	NM_020223	family with sequence similarity 20, member C
ILMN_3465	0.633	DKFZp686I1536	NM_005920	myocyte enhancer factor 2D
ILMN_138674	0.633			
ILMN_138640	0.632			
ILMN_84360	0.631		XM_168073	
ILMN_18161	0.624	ARHS; RHO6; FLJ42294	NM_014470	Rho family GTPase 1
ILMN_8157	0.623			
ILMN_20870	0.623	CBX6	NM_014292	chromobox homolog 6
ILMN_9568	0.613	MGC4954; FLJ20636; DKFZp564L0862	NM_024087	ankyrin repeat and SOCS box-containing 9
ILMN_2275	0.612			
ILMN_20024	0.612	DSAEC	NM_006111	acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)
ILMN_18339	0.611			
ILMN_13834	0.611	STAG1; PMEPA1	NM_199169	transmembrane, prostate androgen induced RNA
ILMN_4627	0.609	KYNU	NM_003937	kynureninase (L-kynurenine hydrolase)
ILMN_6524	0.604			
ILMN_8828	0.6	CGI-127	NM_016061	yippee-like 5 (Drosophila)
ILMN_20617	0.594	CA-VB; MGC39962	NM_007220	carbonic anhydrase VB, mitochondrial
ILMN_12475	0.583	NSP2; SH2D3B; KIAA0554	NM_003567	breast cancer anti-estrogen resistance 3
ILMN_9754	0.579			
ILMN_25787	0.577	APPD; MGC4090; PHAFIN1; ZFYVE15	NM_024310	pleckstrin homology domain containing, family F (with FYVE domain) member 1
ILMN_16215	0.575			

Genes up or down regulated 1.5-fold in the MFC7-TO cell line by estrogen in the presence of Dox contd.

Illumina probe	Fold-induction	Gene name	Gene bank code	Gene description
ILMN_16215	0.575			
ILMN_16203	0.567	SIP; Teap; FLJ22139; p53DINP1; TP53DINP1; TP53INP1A; TP53INP1B; DKFZp434M1317	NM_033285	tumor protein p53 inducible nuclear protein 1
ILMN_136977	0.554			
ILMN_20440	0.552	NL2; ARP4; FIAF; PGAR; HFARP; pp1158; ANGPTL2	NM_139314	angiopoietin-like 4
ILMN_20678	0.552	Ga55; KIAA1103; DKFZp686K18126	NM_006283	transforming, acidic coiled-coil containing protein 1
ILMN_84654	0.533		BM728728	Transcribed locus
ILMN_20048	0.513	DRAM; FLJ11259	NM_018370	damage-regulated autophagy modulator
ILMN_11058	0.513	mGlu4; GPRC1D; MGLUR4	NM_000841	glutamate receptor, metabotropic 4
ILMN_22926	0.506	FLJ39281; KIAA1319; DKFZp779N1112	NM_020770	cingulin
ILMN_6009	0.495			
ILMN_13405	0.479	RAP1GA1; KIAA0474; rap1GAPII	NM_002885	RAP1 GTPase activating protein

Appendix 1c

Genes up or down regulated 1.5-fold in the MFC7-TO cell line by estrogen in the absence of Dox

Ilumina probe	Fold-induction	Gene name	Gene bank code	Gene description
ILMN_20831	2.673			
ILMN_3680	2.548			
ILMN_27477	2.366	Ssc2; FLJ20334	NM_017770	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 2
ILMN_20145	2.334			
ILMN_10483	2.259			
ILMN_15875	2.138	efg; Cmyb; c-myb; c-myb_CDS	NM_005375	v-myb myeloblastosis viral oncogene homolog (avian)
ILMN_29523	2.061	TSK; E2IG4; LRRC54	NM_015516	tsukushin
ILMN_9856	2.056	239FB; FAM1B; C11orf8; D11S302E; Hs.46638; dJ873F21.1; dJ1024C24.1	NM_001584	metallophosphoesterase domain containing 2
ILMN_4419	2.022	DDF1; PARP7; PARP-1; PARP-7; FLJ40466; DKFZP434J214; DKFZp686N0351; DKFZp686P1838	NM_015508	TCDD-inducible poly(ADP-ribose) polymerase
ILMN_138827	1.998			
ILMN_29306	1.982	GTK; RAK; PTK5	NM_002031	fyn-related kinase
ILMN_28725	1.97	STC-2; STCRP	NM_003714	stanniocalcin 2
ILMN_12229	1.944	COL12A1L; BA209D8.1; DJ234P15.1	NM_080645	collagen, type XII, alpha 1
ILMN_11880	1.94			
ILMN_18562	1.9			
ILMN_16823	1.875	MRGH; SOXB	NM_005634	SRY (sex determining region Y)-box 3
ILMN_3319	1.856	LIG1; LIG-1; DKFZP586O1624	NM_015541	leucine-rich repeats and immunoglobulin-like domains 1
ILMN_28130	1.849	c-Myc	NM_002467	v-myc myelocytomatosis viral oncogene homolog (avian)
ILMN_27341	1.801	AMY; NOE1; OlfA; NOELIN1	NM_014279	olfactomedin 1
ILMN_21069	1.792	30ST3A1; 3OST3A1	NM_006042	heparan sulfate (glucosamine) 3-O-sulfotransferase 3A1
ILMN_421	1.779	GLUT; GLUT1; MGC141895; MGC141896	NM_006516	solute carrier family 2 (facilitated glucose transporter), member 1
ILMN_38793	1.772		XM_378883	
ILMN_6528	1.752			
ILMN_122489	1.736		BX099724	Transcribed locus
ILMN_78063	1.736		BX109404	Transcribed locus
ILMN_22427	1.724			
ILMN_796	1.721	CAXII; FLJ20151; HsT18816	NM_001218	carbonic anhydrase XII
ILMN_17460	1.712	PSA; EPIP; PSAT; MGC1460	NM_021154	phosphoserine aminotransferase 1
ILMN_4339	1.696	RIP140	NM_003489	nuclear receptor interacting protein 1
ILMN_44323	1.695			
ILMN_15791	1.689			
ILMN_3868	1.686			
ILMN_4560	1.682	RAI13; NORPEG; KIAA1334; DKFZp564G013	NM_015577	retinoic acid induced 14
ILMN_28485	1.682			
ILMN_25543	1.681	ITRPK1	NM_014216	inositol 1,3,4-triphosphate 5/6 kinase
ILMN_23075	1.677	CP47; CP49; LIFL-L; MGC142078; MGC142080	NM_003571	beaded filament structural protein 2, phakinin
ILMN_12640	1.675	KIAA0124	NM_015201	block of proliferation 1
ILMN_7737	1.675			
ILMN_11251	1.671	EP3; EP3e; EP3-I; EP3-II; EP3-IV; EP3-III; MGC27302; MGC141828; MGC141829	NM_198719	prostaglandin E receptor 3 (subtype EP3)
ILMN_17132	1.67			
ILMN_137293	1.665			
ILMN_27101	1.646	DKFZp686D143; DKFZp686M031; DKFZp686H1748	NM_198859	prickle homolog 2 (Drosophila)
ILMN_1793	1.628	CAD	NM_004341	carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase
ILMN_8761	1.623	P5C; P5CR; PYCR; PIG45; PP222	NM_153824	pyrroline-5-carboxylate reductase 1
ILMN_12826	1.621	4F2; CD98; MDU1; 4F2HC; 4T2HC; NACAE; CD98HC	NM_001013251	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2
ILMN_23424	1.62	PKCE; MGC125656; MGC125657; nPKC-epsilon	NM_005400	protein kinase C, epsilon
ILMN_6255	1.617	DKFZp686A17191	NM_003174	supervillin
ILMN_22619	1.616			

Genes up or down regulated 1.5-fold in the MFC7-TO cell line by estrogen in the absence of Dox contd.

Illumina probe	Fold-induction	Gene name	Gene bank code	Gene description
ILMN_107396	1.616		CD640673	Transcribed locus
ILMN_12273	1.608	DKFZp547M2010	NM_173078	SLIT and NTRK-like family, member 4
ILMN_35248	1.604			
ILMN_6687	1.594		NM_172234	
ILMN_19723	1.586	MCT9; C10orf36; FLJ43803	NM_194298	solute carrier family 16, member 9 (monocarboxylic acid transporter 9)
ILMN_3768	1.585		NM_152488	
ILMN_14354	1.567	MDG1; ERdj4; MST049; MSTP049; DKFZP564F1862	NM_012328	DnaJ (Hsp40) homolog, subfamily B, member 9
ILMN_21252	1.564	FLJ40080; PRTD-NY2; MGC102908; DKFZP434I092	NM_015668	regulator of G-protein signalling 22
ILMN_3578	1.559			
ILMN_29880	1.556	RAR; NR1B1	NM_001024809	retinoic acid receptor, alpha
ILMN_20212	1.555			
ILMN_21497	1.549	CDSP; OCTN2; FLJ46769	NM_003060	solute carrier family 22 (organic cation transporter), member 5
ILMN_23434	1.545	DFNB8; DFNB10; ECHOS1; TADG12	NM_032405	transmembrane protease, serine 3
ILMN_27552	1.544	POPX1; PP2CH; KIAA1072; DKFZp781F1422	NM_014906	protein phosphatase 1E (PP2C domain containing)
ILMN_5233	1.542			
ILMN_19730	1.541	E2F-2	NM_004091	E2F transcription factor 2
ILMN_12108	1.541	HSPG; HSPG1; SYND2	NM_002998	syndecan 2
ILMN_1920	1.538			
ILMN_17000	1.535	NFBP; ALG-4; KIAA0185	NM_014976	programmed cell death 11
ILMN_14410	1.534	YTM1; FLJ10881; FLJ12719; FLJ12720	NM_018256	WD repeat domain 12
ILMN_139066	1.534	HHH; ORC1; ORNT1; D13S327	NM_014252	solute carrier family 25 (mitochondrial carrier; ornithine transporter) member 15
ILMN_8158	1.531			
ILMN_3189	1.529	K15; CK15; K1CO	NM_002275	keratin 15
ILMN_31247	1.528			
ILMN_4970	1.527			
ILMN_12235	1.522			
ILMN_26499	1.521	FLJ20374	NM_017793	ribonuclease P 25kDa subunit
ILMN_3286	1.517	ACF7; MACF; OFC4; ABP620; FLJ45612; FLJ46776; KIAA0465; KIAA1251	NM_012090	microtubule-actin crosslinking factor 1
ILMN_12236	1.516			
ILMN_16225	1.513	STC	NM_003155	stanniocalcin 1
ILMN_16240	1.513	Ga19; NATH; TBDN100	NM_057175	NMDA receptor regulated 1
ILMN_1930	1.512	MRT4; C1orf33; dJ657E11.4	NM_016183	mRNA turnover 4 homolog (S. cerevisiae)
ILMN_29619	1.511			
ILMN_27367	1.511	CAP2	NM_006366	CAP, adenylate cyclase-associated protein, 2 (yeast)
ILMN_21352	1.51			
ILMN_29425	1.509	Gsh-4	NM_033343	LIM homeobox 4
ILMN_14503	1.508	IRS2	NM_003749	insulin receptor substrate 2
ILMN_14119	1.508	FLJ12439	NM_023077	chromosome 1 open reading frame 163
ILMN_137692	1.507			
ILMN_15415	1.507	GUA; GURDB; RH-II/GU; RH-II/GuA; DKFZp686F21172	NM_004728	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21
ILMN_22397	1.506	CGI-77	NM_016023	OTU domain containing 6B
ILMN_8524	1.506	SBBI31; PRO1284	NM_014035	sorting nexing 24
ILMN_4322	1.505	KANK; KIAA0172; MGC43128; DKFZp451G231	NM_153186	ankyrin repeat domain 15
ILMN_18906	1.505	CTPS	NM_001905	CTP synthase
ILMN_19769	1.503			
ILMN_8276	1.503	MGC4308	NM_032359	chromosome 3 open reading frame 26
ILMN_137784	1.502			
ILMN_18208	1.502			
ILMN_92748	1.502		BX101722	Transcribed locus, weakly similar to NP_001074253.1

Genes up or down regulated 1.5-fold in the MFC7-TO cell line by estrogen in the absence of Dox contd.

Illumina probe	Fold-induction	Gene name	Gene bank code	Gene description
ILMN_11360	1.502	AD24; FAD24; FLJ12820; C10orf117	NM_022451	nucleolar complex associated 3 homolog (S. cerevisiae)
ILMN_27879	1.501	GLOXD1; 4-HPPD-L; MGC15668; RP4-534D1.1	NM_032756	4-hydroxyphenylpyruvate dioxygenase-like
ILMN_137537	0.661			
ILMN_16249	0.657			
ILMN_17925	0.637	MGC10500	NM_031477	yippee-like 3 (Drosophila)
ILMN_21443	0.633			
ILMN_13120	0.633	MGC59937	NM_199001	Similar to RIKEN cDNA 2310002J15 gene
ILMN_22606	0.623	GRX; GRX1; MGC117407	NM_002064	glutaredoxin (thioltransferase)
ILMN_29440	0.601	B7X; B7H4; B7S1; B7-H4; B7h.5; VCTN1; PRO1291; FLJ22418; RP11-229A19.4	NM_024626	V-set domain containing T cell activation inhibitor 1
ILMN_89196	0.59	FAM84A	AK025063	Family with sequence similarity 84, member A
ILMN_13811	0.585	ARVD; FLJ16571; TGF-beta3	NM_003239	transforming growth factor, beta 3
ILMN_2714	0.577	FLJ45464; ANKYRIN-G	NM_001149	ankyrin 3, node of Ranvier (ankyrin G)
ILMN_13175	0.576	OP-1	NM_001719	bone morphogenetic protein 7 (osteogenic protein 1)
ILMN_18672	0.481			
ILMN_6834	0.306	L6; H-L6; M3S1; TAAL6	NM_014220	transmembrane 4 L six family member 1

Appendix 2a

Genes up or down regulated 1.5-fold in the MFC7-TO-ERα(EEE) cell line by estrogen in the presence and absence of Dox

Ilumina probe	Fold-induction	Gene name	Gene bank code	Gene description
ILMN_20319	4.912			
ILMN_4631	4.297	PBSF; SDF1; SDF1A; SDF1B; TPAR1; SCYB12; SDF-1a; SDF-1b; TLSF-a; TLSF-b	NM_199168	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
ILMN_16782	3.966	PBSF; SDF1; SDF1A; SDF1B; TPAR1; SCYB12; SDF-1a; SDF-1b; TLSF-a; TLSF-b	NM_000609	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
ILMN_25919	3.748	CTRP6; ZACRP6	NM_031910	C1q and tumor necrosis factor related protein 6
ILMN_15875	3.668	efg; Cmyb; c-myb; c-myb_CDS	NM_005375	v-myb myeloblastosis viral oncogene homolog (avian)
ILMN_25992	3.649	OGR1; MGC111379	NM_003485	G protein-coupled receptor 68
ILMN_20958	3.569	MGC134798; MGC134799; Link-GEFII	NM_016339	Rap guanine nucleotide exchange factor (GEF)-like 1
ILMN_9309	3.508	BP-4; IBP4; IGFBP-4; HT29-IGFBP	NM_001552	insulin-like growth factor binding protein 4
ILMN_28190	3.342	TOSO	NM_005449	Fas apoptotic inhibitory molecule 3
ILMN_23624	3.31			
ILMN_5543	3.206	D53; hD53; MGC8556; TPD52L2	NM_001003396	tumor protein D52-like 1
ILMN_28725	3.126	STC-2; STCRP	NM_003714	stanniocalcin 2
ILMN_25446	2.838	E16; CD98; LAT1; 4F2LC; MPE16; hLAT1; D16S469E	NM_003486	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5
ILMN_4109	2.775			
ILMN_27517	2.69	BCOX; BRCOX; THCCox; BRCACOX	NM_003500	acyl-Coenzyme A oxidase 2, branched chain
ILMN_28750	2.636	CAII; Car2; CA II; CA-II	NM_000067	carbonic anhydrase II
ILMN_24346	2.623			
ILMN_12478	2.612	MSP; PSP; IGBF; MSPB; PN44; PRPS; PSP57; PSP94; PSP-94	NM_002443	microseminoprotein, beta-
ILMN_12434	2.596	MGC15754	NM_032918	RAS-like, estrogen-regulated, growth inhibitor
ILMN_16132	2.564	EGFL2; MEGF3; CDHF10; FLJ34118; FLJ42737; FLJ45143; FLJ45845; KIAA0279; Flamingo1	NM_001408	cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila)
ILMN_25666	2.558	SemA; SEMA5; SEMAA; semaV; LUCA-1; FLJ34863	NM_001005914	sema domain, immunoglobulin domain (Ig), short basic domain, secreted
ILMN_6528	2.534			
ILMN_77290	2.505		AK125960	CDNA FLJ43972 fis, clone TESTI4017961
ILMN_10483	2.496			
ILMN_13950	2.472			
ILMN_5293	2.464	XBP2; TREB5	NM_005080	X-box binding protein 1
ILMN_421	2.426	GLUT; GLUT1; MGC141895; MGC141896	NM_006516	solute carrier family 2 (facilitated glucose transporter), member 1
ILMN_7086	2.425			
ILMN_138827	2.417			
ILMN_19350	2.391	FLJ25330; DKFZp434A119	NM_178452	leucine rich repeat containing 50
ILMN_22479	2.368			
ILMN_6004	2.347			
ILMN_115591	2.344		AW364673	PM3-DT0037-231299-001-e06 DT0037 Homo sapiens cDNA, mRNA sequence.
ILMN_29523	2.287	TSK; E2IG4; LRRC54	NM_015516	tsukushin
ILMN_24376	2.264	DEP.6; FLJ12428; FLJ13854; DKFZp564B1778	NM_022783	DEP domain containing 6
ILMN_7163	2.24			
ILMN_9856	2.234	239FB; FAM1B; C11orf8; D11S302E; Hs.46638; dJ873F21.1; dJ1024C24.1	NM_001584	metallophosphoesterase domain containing 2
ILMN_7737	2.213			
ILMN_2380	2.154	PTC; MTC1; HSCR1; MEN2A; MEN2B; RET51; CDHF12; RET-ELE1	NM_020630	ret proto-oncogene
ILMN_4348	2.147	KIAA0442; MGC13140	NM_015570	autism susceptibility candidate 2
ILMN_4623	2.143	OCIM	NM_138768	myeloma overexpressed gene (in a subset of t(11;14) positive multiple myelomas)
ILMN_6304	2.101	ZFYVE5; FLJ00004; MGC117260	NM_033086	FYVE, RhoGEF and PH domain containing 3
ILMN_13740	2.097			
ILMN_11008	2.095	MGC26847	NM_145006	sushi domain containing 3
ILMN_15836	2.094	FLJ40960; DKFZp451P0116	NM_080737	synaptotagmin-like 4 (granuphilin-a)
ILMN_1421	2.085	EBP50; NHERF; NHERF1	NM_004252	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1
ILMN_27341	2.084	AMY; NOE1; OlfA; NOELIN1	NM_014279	olfactomedin 1
ILMN_29702	2.068	CDT2; RAMP; DCAF2; L2DTL	NM_016448	denticleless homolog (Drosophila)

Genes up or down regulated 1.5-fold in the MFC7-TO-ERα(EEE) cell line by estrogen in the presence and absence of Dox contd.

Ilumina probe	Fold-induction	Gene name	Gene bank code	Gene description
ILMN_1421	2.315	EBP50; NHERF; NHERF1	NM_004252	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1
ILMN_111275	2.308		AK127526	CDNA FLJ45619 fis, clone BRTHA3027318
ILMN_3259	2.292	PTMP; PP2Cm; UG0882E07; DKFZp667B084; DKFZp761G058	NM_152542	protein phosphatase 1K (PP2C domain containing)
ILMN_4348	2.279	KIAA0442; MGC13140	NM_015570	autism susceptibility candidate 2
ILMN_6004	2.265			
ILMN_6304	2.247	ZFYVE5; FLJ00004; MGC117260	NM_033086	FYVE, RhoGEF and PH domain containing 3
ILMN_29566	2.212	FLJ22416; FLJ25593	NM_014344	four jointed box 1 (Drosophila)
ILMN_27341	2.207	AMY; NOE1; OlfA; NOELIN1	NM_014279	olfactomedin 1
ILMN_17240	2.206	BDGI; OKL38	NM_013370	oxidative stress induced growth inhibitor 1
ILMN_7033	2.205	PSF3; FLJ13912	NM_022770	GINS complex subunit 3 (Psf3 homolog)
ILMN_6412	2.196	RNPC6; FLJ26355; FLJ30829; FLJ37697; dJ259A10.1	NM_153020	RNA binding motif protein 24
ILMN_71996	2.188	FLJ38860	AK096179	chromosome 7 open reading frame 40
ILMN_14815	2.177	EAAC1; EAAT3	NM_004170	solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), m
ILMN_38793	2.173		XM_378883	
ILMN_25424	2.167	P1; PK; A14GALT; A4GALT1	NM_017436	alpha 1,4-galactosyltransferase (globotriaosylceramide synthase)
ILMN_14410	2.163	YTM1; FLJ10881; FLJ12719; FLJ12720	NM_018256	WD repeat domain 12
ILMN_11049	2.149	p7; RRP4; Rrp4p; hRrp4p	NM_014285	exosome component 2
ILMN_2483	2.147	CNA43; PRO2249; MGC126776	NM_018518	minichromosome maintenance complex component 10
ILMN_4339	2.147	RIP140	NM_003489	nuclear receptor interacting protein 1
ILMN_29523	2.145	TSK; E2IG4; LRRC54	NM_015516	tsukushin
ILMN_18063	2.142	PNP; PUNP; PRO1837; MGC117396; MGC125915; MGC125916	NM_000270	nucleoside phosphorylase
ILMN_18253	2.133	C12orf7; DKFZp686O1689	NM_182608	ankyrin repeat domain 33
ILMN_20135	2.127	AMD; ADOMETDC; FLJ26964; DKFZp313L1234	NM_001033059	adenosylmethionine decarboxylase 1
ILMN_7086	2.12			
ILMN_16107	2.119	ACT; AACT; GIG24; GIG25; MGC88254	NM_001085	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3
ILMN_23200	2.117	ZLYAR; FLJ20425	NM_017816	Ly1 antibody reactive homolog (mouse)
ILMN_14912	2.098	B1F; CPF; FTF; B1F2; hB1F; LRH-1; FTZ-F1; hB1F-2; FTZ-F1beta	NM_205860	nuclear receptor subfamily 5, group A, member 2
ILMN_89110	2.097		BC038580	CDNA clone IMAGE:5418341
ILMN_3189	2.072	K15; CK15; K1CO	NM_002275	keratin 15
ILMN_686	2.072	DGU; UDG; UNG1; HIGM4; UNG15; DKFZp781L1143	NM_080911	uracil-DNA glycosylase
ILMN_13861	2.065			
ILMN_6750	2.065	NPYR	NM_000909	neuropeptide Y receptor Y1
ILMN_9429	2.047	HBI; p52; Hsp56; FKBP52; FKBP59; PPIase	NM_002014	FK506 binding protein 4, 59kDa
ILMN_28089	2.027	FLJ43600; dJ310O13.5	NM_080625	chromosome 20 open reading frame 160
ILMN_139385	2.018			
ILMN_9627	2.014			
ILMN_24929	2.003	CAP70; CLAMP; PDZD1; NHERF3	NM_002614	PDZ domain containing 1
ILMN_17647	1.99	H2; RLXH2; bA12D24.1.1; bA12D24.1.2	NM_005059	relaxin 2
ILMN_21371	1.988	AOI; FH1; SCT; TAP; LRS1; TABP; FLN1L; ABP-278; filamin B; DKFZp686O033; DKFZp686A1668	NM_001457	filamin B, beta (actin binding protein 278)
ILMN_4419	1.984	DDF1; PARP7; PARP-1; PARP-7; FLJ40466; DKFZP434J214; DKFZp686N0351; DKFZp686P1838	NM_015508	TCDD-inducible poly(ADP-ribose) polymerase
ILMN_3319	1.977	LIG1; LIG-1; DKFZP586O1624	NM_015541	leucine-rich repeats and immunoglobulin-like domains 1
ILMN_15662	1.968	FLJ20516	NM_017858	TIMELESS interacting protein
ILMN_15791	1.958			
ILMN_6687	1.957		NM_172234	
ILMN_20779	1.954	HRH-J8	NM_004398	DEAD (Asp-Glu-Ala-Asp) box polypeptide 10
ILMN_12005	1.948			
ILMN_18562	1.936			
ILMN_20145	1.933			
ILMN_20498	1.925	EPS15R	NM_021235	epidermal growth factor receptor pathway substrate 15-like 1

Genes up or down regulated 1.5-fold in the MFC7-TO-ERα(EEE) cell line by estrogen in the presence and absence of Dox contd.

Illumina probe	Fold-induction	Gene name	Gene bank code	Gene description
ILMN_15694	1.923	HSPC111; HSPC185	NM_016391	hypothetical protein HSPC111
ILMN_11038	1.918	FLJ25416; FLJ13936	NM_145018	hypothetical protein FLJ25416
ILMN_129106	1.918			
ILMN_12273	1.916	DKFZp547M2010	NM_173078	SLIT and NTRK-like family, member 4
ILMN_3286	1.915	ACF7; MACF; OFC4; ABP620; FLJ45612; FLJ46776; KIAA0465; KIAA1251	NM_012090	microtubule-actin crosslinking factor 1
ILMN_19730	1.914	E2F-2	NM_004091	E2F transcription factor 2
ILMN_29306	1.911	GTK; RAK; PTK5	NM_002031	fyn-related kinase
ILMN_21836	1.903	ORC1; PARC1; HSORC1	NM_004153	origin recognition complex, subunit 1-like (yeast)
ILMN_15639	1.9	CPSD; CLN10; MGC2311	NM_001909	cathepsin D
ILMN_2775	1.894	DKC; NAP57; NOLA4; XAP101; dyskerin	NM_001363	dyskeratosis congenita 1, dyskerin
ILMN_20677	1.889	FLJ11266; TU12B1-TY	NM_016575	5'-nucleotidase domain containing 3
ILMN_5835	1.867			
ILMN_18906	1.864	CTPS	NM_001905	CTP synthase
ILMN_13740	1.861			
ILMN_19508	1.86	PEN; PUF6; XTP5; HLA-HA8; MGC8749; RP11-526D20.2	NM_014878	KIAA0020
ILMN_2451	1.85	SGK1	NM_005627	serum/glucocorticoid regulated kinase
ILMN_16562	1.847	P54; FKBP51; FKBP54; PPIase; Ptg-10; MGC111006	NM_004117	FK506 binding protein 5
ILMN_1930	1.832	MRT4; C1orf33; dJ657E11.4	NM_016183	mRNA turnover 4 homolog (S. cerevisiae)
ILMN_119940	1.827	DKFZp762C1112	AK095472	transmembrane protein 64
ILMN_10386	1.825			
ILMN_29425	1.824	Gsh-4	NM_033343	LIM homeobox 4
ILMN_137484	1.823			
ILMN_26335	1.814	AMZ1; KIAA1950	NM_133463	archaemetzincin-1
ILMN_2469	1.813	PERB11.2	NM_005931	MHC class I polypeptide-related sequence B
ILMN_12640	1.811	KIAA0124	NM_015201	block of proliferation 1
ILMN_25234	1.81	GRAB	NM_013401	RAB3A interacting protein (rabin3)-like 1
ILMN_4671	1.807			
ILMN_11360	1.807	AD24; FAD24; FLJ12820; C10orf117	NM_022451	nucleolar complex associated 3 homolog (S. cerevisiae)
ILMN_23283	1.806	UK; UMPK; TSA903	NM_012474	uridine-cytidine kinase 2
ILMN_9848	1.805			
ILMN_11616	1.802	RFC38; MGC5276	NM_002915	replication factor C (activator 1) 3, 38kDa
ILMN_25241	1.798	FLJ34389	NM_152649	mixed lineage kinase domain-like
ILMN_10445	1.797	PRC; KIAA0595; MGC74642; RP11-302K17.6	NM_015062	peroxisome proliferator-activated receptor gamma, coactivator-related 1
ILMN_25553	1.794	HCC5; P1.h; RLFB; MGC1157; P1-MCM3	NM_002388	minichromosome maintenance complex component 3
ILMN_20959	1.793	KIAA1313; DKFZp686P1843	NM_020766	protocadherin 19
ILMN_16823	1.793	MRGH; SOXB	NM_005634	SRY (sex determining region Y)-box 3
ILMN_25134	1.787			
ILMN_20988	1.787	FLJ21145; FTHFSDC1; dJ292B18.2; RP1-292B18.2; DKFZp586G1517	NM_015440	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like
ILMN_12401	1.785	AEN; pp12744; FLJ12484; FLJ12562	NM_022767	interferon stimulated exonuclease gene 20kDa-like 1
ILMN_23434	1.782	DFNB8; DFNB10; ECHOS1; TADG12	NM_032405	transmembrane protease, serine 3
ILMN_26070	1.78	TOSS; KCNK8; TWIK2; K2p6.1; TWIK-2; FLJ12282	NM_004823	potassium channel, subfamily K, member 6
ILMN_3628	1.778	DCC1; MGC5528	NM_024094	defective in sister chromatid cohesion homolog 1 (S. cerevisiae)
ILMN_13630	1.778	FBL1; FLB1; FBXL1; MGC1366	NM_032637	S-phase kinase-associated protein 2 (p45)
ILMN_138917	1.773			
ILMN_73496	1.769		AJ420536	MRNA full length insert cDNA clone EUROIMAGE 994183
ILMN_3617	1.766			
ILMN_4560	1.757	RAI13; NORPEG; KIAA1334; DKFZp564G013	NM_015577	retinoic acid induced 14
ILMN_137293	1.756			
ILMN_28734	1.755	FLJ20485; KIAA1897; MGC17720	NM_019042	pseudouridylate synthase 7 homolog (S. cerevisiae)

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Ilumina probe	Fold-induction	Gene name	Gene bank code	Gene description
ILMN_3578	1.755			
ILMN_1663	1.747			
ILMN_14675	1.745	LH2; TLH	NM_182943	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2
ILMN_7178	1.744	EVN; NBN	NM_003976	artemin
ILMN_7092	1.743			
ILMN_1920	1.739			
ILMN_15226	1.737	VDR; CP2B; CYP1; PDDR; VDD1; VDDR; VDDRI; CYP27B; P450c1; CYP1alpha	NM_000785	cytochrome P450, family 27, subfamily B, polypeptide 1
ILMN_26929	1.735	AC3; KIAA0511	NM_004036	adenylate cyclase 3
ILMN_20377	1.73	SPG31; C2orf23; FLJ13110	NM_022912	receptor accessory protein 1
ILMN_18997	1.73	PEO; PEOA3; SANDO; TWINL; C10orf2; FLJ21832	NM_021830	progressive external ophthalmoplegia 1
ILMN_13393	1.728	MTP18; HSPC242	NM_016498	mitochondrial protein 18 kDa
ILMN_12235	1.725			
ILMN_4065	1.722	SEP3; MGC133218; bK250D10.3	NM_019106	septin 3
ILMN_25232	1.717	MGC2603; FLJ14264	NM_024037	chromosome 1 open reading frame 135
ILMN_21964	1.714	PIM	NM_002648	pim-1 oncogene
ILMN_823	1.71	IK8; kH1; KCNF; KV5.1; MGC33316	NM_002236	potassium voltage-gated channel, subfamily F, member 1
ILMN_22619	1.71			
ILMN_1940	1.709		NM_178867	
ILMN_27367	1.705	CAP2	NM_006366	CAP, adenylate cyclase-associated protein, 2 (yeast)
ILMN_31247	1.705			
ILMN_19008	1.705	FLJ32549	NM_152440	hypothetical protein FLJ32549
ILMN_29509	1.703	RP13-15M17.2	NM_001010866	hypothetical protein LOC199953
ILMN_6032	1.699	ADE2; AIRC; PAIS; ADE2H1; MGC1343; MGC5024; DKFZp781N1372	NM_006452	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxa
ILMN_15343	1.698	FLJ32104	NM_153034	zinc finger protein 488
ILMN_20557	1.697	ABH2; hABH2; MGC90512	NM_001001655	alkB, alkylation repair homolog 2 (E. coli)
ILMN_20831	1.697			
ILMN_5197	1.697	CRT; CTR; CTR1	NM_001742	calcitonin receptor
ILMN_19962	1.695	RhoBP; p76RBE	NM_033103	rhophilin, Rho GTPase binding protein 2
ILMN_137856	1.695			
ILMN_139066	1.695	HHH; ORC1; ORNT1; D13S327	NM_014252	solute carrier family 25 (mitochondrial carrier; ornithine transporter) member 15
ILMN_6255	1.694	DKFZp686A17191	NM_003174	supervillin
ILMN_14119	1.676	FLJ12439	NM_023077	chromosome 1 open reading frame 163
ILMN_137728	1.646			
ILMN_8159	1.646	RCN; RCAL; PIG20; FLJ37041	NM_002901	reticulocalbin 1, EF-hand calcium binding domain
ILMN_8355	1.637	KHK	NM_000221	ketohexokinase (fructokinase)
ILMN_26079	1.623	MGC3265	NM_024028	prenylcysteine oxidase 1 like
ILMN_3335	1.605	CEP2; BORG1	NM_006779	CDC42 effector protein (Rho GTPase binding) 2
ILMN_28485	1.6			
ILMN_7614	1.588	BAP28; FLJ10359; MGC72083; RP11-385F5.3	NM_018072	HEAT repeat containing 1
ILMN_23424	1.583	PKCE; MGC125656; MGC125657; nPKC-epsilon	NM_005400	protein kinase C, epsilon
ILMN_21352	1.578			
ILMN_5233	1.575			
ILMN_19769	1.562			
ILMN_27952	1.561	KIAA1594; MGC117261	NM_020935	ubiquitin specific peptidase 37
ILMN_2574	1.55	NAIC; CIRHIN; TEX292; FLJ14728; KIAA1988	NM_032830	cirrhosis, autosomal recessive 1A (cirhin)
ILMN_10925	1.545	NOP14; C4orf9; RES4-25	NM_003703	nucleolar protein 14
ILMN_13970	1.54	V; RASGRP; hRasGRP1; MGC129998; MGC129999; CALDAG-GEFI; CALDAG-GEFII	NM_005739	RAS guanyl releasing protein 1 (calcium and DAG-regulated)
ILMN_4401	1.534			

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Illumina probe	Fold-induction	Gene name	Gene bank code	Gene description
ILMN_34249	1.532		XM_372111	
ILMN_139141	1.528			
ILMN_16548	1.515			
ILMN_12229	1.511	COL12A1L; BA209D8.1; DJ234P15.1	NM_080645	collagen, type XII, alpha 1
ILMN_19956	1.508	GREB1; KIAA0575	NM_148903	GREB1 protein
ILMN_15035	0.666	P80; IL1R; IL1RA; CD121A; D2S1473; IL-1R-alpha	NM_000877	interleukin 1 receptor, type I
ILMN_13811	0.64	ARVD; FLJ16571; TGF-beta3	NM_003239	transforming growth factor, beta 3
ILMN_18672	0.602			
ILMN_2714	0.581	FLJ45464; ANKYRIN-G	NM_001149	ankyrin 3, node of Ranvier (ankyrin G)
ILMN_27092	0.575	SOM; TFCP2L4; MGC46624	NM_021180	grainyhead-like 3 (Drosophila)
ILMN_3374	0.57	CCN2; NOV2; HCS24; IGFBP8; MGC102839	NM_001901	connective tissue growth factor
ILMN_138502	0.558	PGM5	XM_936702	phosphoglucomutase 5; synonym: PGMRP; phosphoglucomutase-related protein; Homo sap
ILMN_10700	0.539	PGMRP	NM_021965	phosphoglucomutase 5
ILMN_6834	0.443	L6; H-L6; M3S1; TAAL6	NM_014220	transmembrane 4 L six family member 1

Appendix 2b

Genes up or down regulated 1.5-fold in the MFC7-TO-ERa(EEE) cell line by estrogen in the presence of Dox

Ilumina probe	Fold-induction	Gene name	Gene bank code	Gene description
ILMN_27879	2.189	GLOXD1; 4-HPPD-L; MGC15668; RP4-534D1.1	NM_032756	4-hydroxyphenylpyruvate dioxygenase-like
ILMN_11882	2.147	DNC; TPC; MUP1; MCPHA	NM_021734	solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19
ILMN_3806	2.137		NM_153344	
ILMN_17288	2.114	CG1; F18	NM_005491	chromosome X open reading frame 6
ILMN_9407	1.986			
ILMN_33181	1.974			
ILMN_6169	1.959	MLASA; MGC11268	NM_025215	pseudouridylate synthase 1
ILMN_122276	1.954		DB005376	Transcribed locus
ILMN_25327	1.953	CHK1	NM_001274	CHK1 checkpoint homolog (S. pombe)
ILMN_16146	1.949			
ILMN_28432	1.926			
ILMN_19931	1.906	CD122; P70-75	NM_000878	interleukin 2 receptor, beta
ILMN_11540	1.9	NEH2; MGC2832; MGC4505; FLJ31644	NM_145043	nei like 2 (E. coli)
ILMN_11282	1.898	VIA; SEMA; HT018; SEMAQ; SEMA6A1; KIAA1368; sema VIa	NM_020796	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A
ILMN_22839	1.895	HUFI-2; FLJ20248; FLJ22683; DKFZp434H2035	NM_017724	leucine rich repeat (in FLII) interacting protein 2
ILMN_20212	1.891			
ILMN_8352	1.887	DDP; MTS; DDP1; DFN1; MGC12262	NM_004085	translocase of inner mitochondrial membrane 8 homolog A (yeast)
ILMN_18208	1.884			
ILMN_24423	1.882	PIP1; MAK11; WDR84; hPIP1; FLJ20624; bA421M1.5; RP11-421M1.5	NM_017906	PAK1 interacting protein 1
ILMN_11880	1.881			
ILMN_3555	1.868	KIAA1026; RP1-21O18.1	NM_001018000	kazrin
ILMN_6222	1.853			
ILMN_839	1.846			
ILMN_7438	1.84	VLCS; FATP2; VLACS; ACSVL1; FACVL1; hFACVL1; HsT17226	NM_003645	solute carrier family 27 (fatty acid transporter), member 2
ILMN_10275	1.838	RPS7	NM_001011	ribosomal protein S7
ILMN_29292	1.835	TERE1; RP4-796F18.1	NM_013319	UbiA prenyltransferase domain containing 1
ILMN_19682	1.831	MGC35274; DKFZp686I2243	NM_153374	LysM, putative peptidoglycan-binding, domain containing 2
ILMN_20584	1.821	Hsk1; CDC7L1; HsCDC7; huCDC7; MGC117361; MGC126237; MGC126238	NM_003503	cell division cycle 7 homolog (S. cerevisiae)
ILMN_4787	1.816	PCDH13; FLJ22218	NM_022843	protocadherin 20
ILMN_18538	1.806	ASM; BWS; ASM1; MGC4485; PRO2605; D11S813E	NR_002196	H19, imprinted maternally expressed untranslated mRNA
ILMN_22207	1.804	FLJ20231; KIAA0690; DKFZp762P1116	NM_015179	ribosomal RNA processing 12 homolog (S. cerevisiae)
ILMN_28300	1.796	KD93; CGI-37; HSPC031; FLJ10296	NM_016101	nuclear import 7 homolog (S. cerevisiae)
ILMN_2445	1.795	PAPT; SPS1; SPDSY; SRML1	NM_003132	spermidine synthase
ILMN_4970	1.791			
ILMN_16870	1.787			
ILMN_14503	1.785	IRS2	NM_003749	insulin receptor substrate 2
ILMN_26359	1.783	TPT; COQ1; TPRT; hDPS1; MGC70953; RP13-16H11.3	NM_014317	prenyl (decaprenyl) diphosphate synthase, subunit 1
ILMN_6934	1.783	p12B; RRP46; RRP41B; Rrp46p; hRrp46p; MGC12901; MGC111224	NM_020158	exosome component 5
ILMN_6438	1.778	FLJ20244	NM_017722	TRM1 tRNA methyltransferase 1 homolog (S. cerevisiae)
ILMN_15415	1.778	GUA; GURDB; RH-II/GU; RH-II/GuA; DKFZp686F21172	NM_004728	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21
ILMN_29880	1.777	RAR; NR1B1	NM_001024809	retinoic acid receptor, alpha
ILMN_26854	1.774	PSF2; Pfs2; HSPC037	NM_016095	GINS complex subunit 2 (Psf2 homolog)
ILMN_36850	1.773		XM_943393	
ILMN_8276	1.771	MGC4308	NM_032359	chromosome 3 open reading frame 26
ILMN_3481	1.771	TRM82	NM_033661	WD repeat domain 4
ILMN_26865	1.77	P15; MTR2	NM_013248	NTF2-like export factor 1
ILMN_7620	1.76	9G8; AAG3; HSSG1; RBM37; ZCRB2; ZCCHC20	NM_001031684	splicing factor, arginine/serine-rich 7, 35kDa

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Illumina probe	Fold-induction	Gene name	Gene bank code	Gene description
ILMN_6778	1.749	GPAT; ATASE	NM_002703	phosphoribosyl pyrophosphate amidotransferase
ILMN_4503	1.747	BRIX; FLJ11100	NM_018321	brix domain containing 2
ILMN_10469	1.745	PEP-19	NM_006198	Purkinje cell protein 4
ILMN_14354	1.74	MDG1; ERdj4; MST049; MSTP049; DKFZP564F1862	NM_012328	DnaJ (Hsp40) homolog, subfamily B, member 9
ILMN_22410	1.738			
ILMN_17191	1.737	ZNF485	NM_145312	zinc finger protein 485
ILMN_11044	1.731	DNTNP	NM_001013622	family with sequence similarity 53, member A
ILMN_10472	1.731	RBP3; E2F-1; RBBP3	NM_005225	E2F transcription factor 1
ILMN_11783	1.728	DSU; WDT2; FLJ10116; MGC90296	NM_018000	melanoregulin
ILMN_2452	1.726	APMCF1	NM_021203	signal recognition particle receptor, B subunit
ILMN_796	1.722	CAXII; FLJ20151; HsT18816	NM_001218	carbonic anhydrase XII
ILMN_18980	1.722	HMGCS; MGC90332	NM_002130	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)
ILMN_16867	1.719	SIP; COAA; SYTIP1; DKFZp779J0927	NM_006328	RNA binding motif protein 14
ILMN_28266	1.719			
ILMN_25628	1.717	MAGI1; FLJ31349; RP11-486M3.1	NM_173515	CNKSR family member 3
ILMN_12826	1.706	4F2; CD98; MDU1; 4F2HC; 4T2HC; NACAE; CD98HC	NM_001013251	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2
ILMN_23659	1.705			
ILMN_28415	1.703			
ILMN_20587	1.702	TRA16	NM_176880	TR4 orphan receptor associated protein TRA16
ILMN_3124	1.699			
ILMN_25046	1.696	PIR51	NM_006479	RAD51 associated protein 1
ILMN_26499	1.692	FLJ20374	NM_017793	ribonuclease P 25kDa subunit
ILMN_14509	1.685	MCT6; MCT7	NM_004694	solute carrier family 16, member 6 (monocarboxylic acid transporter 7)
ILMN_13841	1.684	NOP56	NM_006392	nucleolar protein 5A (56kDa with KKE/D repeat)
ILMN_26228	1.683	GCS	NM_003358	UDP-glucose ceramide glucosyltransferase
ILMN_23782	1.683	NMDMC	NM_006636	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate c
ILMN_16202	1.682			
ILMN_24576	1.678			
ILMN_17058	1.678	BinCARD; MGC11115; MGC110898; bA370F5.1	NM_032310	chromosome 9 open reading frame 89
ILMN_26871	1.675	FAM44B	NM_138369	family with sequence similarity 44, member B
ILMN_11416	1.674	MGC52057	NM_194317	LY6/PLAUR domain containing 6
ILMN_21529	1.674	AMD; ADOMETDC; FLJ26964; DKFZp313L1234	NM_001634	adenosylmethionine decarboxylase 1
ILMN_25107	1.672			
ILMN_1277	1.671			
ILMN_7282	1.671	CTHRC1	NM_138455	collagen triple helix repeat containing 1
ILMN_9565	1.67			
ILMN_21654	1.666			
ILMN_14185	1.666			
ILMN_25543	1.662	ITRPK1	NM_014216	inositol 1,3,4-triphosphate 5/6 kinase
ILMN_19136	1.662	TC4; Gsp1; ARA24	NM_006325	RAN, member RAS oncogene family
ILMN_24076	1.661	CPT; CPT1	NM_020244	choline phosphotransferase 1
ILMN_15773	1.66	PAC1; C21LRP; LRPC21	NM_003720	Down syndrome critical region gene 2
ILMN_11384	1.659	FLJ38663	NM_152269	chromosome 12 open reading frame 65
ILMN_17691	1.657	SM15; IFNRP; SKMc15; FLJ40446	NM_006764	interferon-related developmental regulator 2
ILMN_1600	1.656			
ILMN_28398	1.646			
ILMN_5923	1.643			
ILMN_27470	1.642	MIA40; FLJ31709	NM_144636	coiled-coil-helix-coiled-coil-helix domain containing 4

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Illumina probe	Fold-induction	Gene name	Gene bank code	Gene description
ILMN_1129	1.639	CHRNA5	NM_000745	cholinergic receptor, nicotinic, alpha 5
ILMN_139061	1.637			
ILMN_8864	1.637			
ILMN_5998	1.636			
ILMN_30048	1.635	FLJ10110; FLJ25795	NM_017998	chromosome 9 open reading frame 40
ILMN_136949	1.634			
ILMN_24738	1.634			
ILMN_28887	1.633	HPE5	NM_007129	Zic family member 2 (odd-paired homolog, Drosophila)
ILMN_29346	1.633	MOS; HMCS; FLJ20733	NM_017947	molybdenum cofactor sulfurase
ILMN_4090	1.633	ASB-18; DKFZp313I1130	NM_212556	ankyrin repeat and SOCS box-containing 18
ILMN_25512	1.628	C2orf8; MGC2454	NM_025264	THUMP domain containing 2
ILMN_5592	1.627	HUFI-2; FLJ20248; FLJ22683; DKFZp434H2035	NM_006309	leucine rich repeat (in FLII) interacting protein 2
ILMN_26243	1.626	PNO1	NM_020143	partner of NOB1 homolog (S. cerevisiae)
ILMN_17929	1.625	KIAA0990	NM_014918	carbohydrate (chondroitin) synthase 1
ILMN_20191	1.625			
ILMN_19458	1.624	FLJ11184	NM_018352	hypothetical protein FLJ11184
ILMN_3651	1.624	TPA1; FLJ10826; KIAA1612	NM_018233	2-oxoglutarate and iron-dependent oxygenase domain containing 1
ILMN_137400	1.623			
ILMN_19705	1.621	DPE2	NM_002692	polymerase (DNA directed), epsilon 2 (p59 subunit)
ILMN_13264	1.621	MSS1; MTGP1; THDF1; GTPBG3; FLJ14700	NM_032620	GTP binding protein 3 (mitochondrial)
ILMN_137663	1.62			
ILMN_21330	1.617			
ILMN_20107	1.616	CDC46; MGC5315; P1-CDC46	NM_006739	minichromosome maintenance complex component 5
ILMN_25365	1.616	MGC13096	NM_032346	programmed cell death 2-like
ILMN_1191	1.614	DDX33; FLJ21972; DKFZp762F2011	NM_020162	DEAH (Asp-Glu-Ala-His) box polypeptide 33
ILMN_10377	1.613	HS11; HCP15; bA513I15.3	NR_001561	cytochrome c, somatic-like 1
ILMN_138712	1.612			
ILMN_2731	1.612			
ILMN_7824	1.611	FLJ44259; MGC11386; MGC138577	NM_032933	chromosome 18 open reading frame 45
ILMN_137123	1.606			
ILMN_19282	1.604	AIRS; GARS; PAIS; PGFT; PRGS; GARTF; MGC47764	NM_175085	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosp
ILMN_21009	1.603	NRF3	NM_004289	nuclear factor (erythroid-derived 2)-like 3
ILMN_44093	1.601			
ILMN_21387	1.601	GAC1; LRRN5; LRANK1	NM_006338	leucine rich repeat neuronal 2
ILMN_138283	1.6	CDV3	XM_945271	CDV3 homolog (mouse); synonym: H41; expression increases in HER-2/neu overexpressing
ILMN_28822	1.6	ARP; MGC142148; MGC142150	NM_006010	arginine-rich, mutated in early stage tumors
ILMN_25948	1.6	SSF; SSF1; SSF2; BXDC3; MGC14226; MGC45852	NM_020230	peter pan homolog (Drosophila)
ILMN_5297	1.598			
ILMN_5520	1.598	KF; DKFZp686F03259	NM_001010982	arylformamidase
ILMN_7455	1.597	SL21; MAPO6D1; FLJ12748	NM_024871	MAP6 domain containing 1
ILMN_2614	1.595	C2F; NEP1; Grcc2f	NM_006331	EMG1 nucleolar protein homolog (S. cerevisiae)
ILMN_23187	1.593	FLJ23459	NM_024775	gem (nuclear organelle) associated protein 6
ILMN_19233	1.59	BL2; ST17; IGSF4; NECL2; RA175; TSLC1; IGSF4A; Necl-2; SYNCAM; MGC51880; DKFZp686F1789	NM_014333	cell adhesion molecule 1
ILMN_20725	1.588	C6orf93; FLJ14909; dJ468K18.4	NM_032860	LTV1 homolog (S. cerevisiae)
ILMN_9227	1.588	My013; HRPAP20; HSPC125; bA22L21.1	NM_014165	chromosome 6 open reading frame 66
ILMN_13904	1.587	FLJ20739; DKFZp547L163	NM_017949	CUE domain containing 1
ILMN_9443	1.586	RPML7; MRP-L7; HSPC145	NM_014175	mitochondrial ribosomal protein L15
ILMN_28644	1.584	CT120; FLJ22282	NM_024792	family with sequence similarity 57, member A

Genes up or down regulated 1.5-fold in the MFC7-TO-ERa(EEE) cell line by estrogen in the presence of Dox contd.

Illumina probe	Fold-induction	Gene name	Gene bank code	Gene description
ILMN_17000	1.583	NFBP; ALG-4; KIAA0185	NM_014976	programmed cell death 11
ILMN_5016	1.583			
ILMN_6271	1.582			
ILMN_12933	1.581			
ILMN_9763	1.58	EMI1; FBX5; Fbxo31	NM_012177	F-box protein 5
ILMN_5885	1.58			
ILMN_5465	1.58	HEPP; FLJ20764; MGC19517	NM_017955	cell division cycle associated 4
ILMN_20713	1.579			
ILMN_22486	1.577	XTP3TPA; CDA03; RS21C6; MGC5627	NM_024096	XTP3-transactivated protein A
ILMN_14448	1.577			
ILMN_3366	1.576	MMTN; B17.2L; mimitin; FLJ22398	NM_174889	NDUFA12-like
ILMN_17736	1.576	WDR50; CGI-48	NM_016001	UTP18, small subunit (SSU) processome component, homolog (yeast)
ILMN_20839	1.576	FLJ20729; FLJ20760; NY-BR-75; MGC131963	NM_017953	chromosome 1 open reading frame 181
ILMN_27786	1.575	E1; MASA; MST145; FLJ12594; DKFZp586M0524	NM_021204	enolase-phosphatase 1
ILMN_6600	1.573	FLJ35155	NM_152531	chromosome 3 open reading frame 21
ILMN_29359	1.573	FLJ30322	NM_006753	surfeit 6
ILMN_1157	1.572	HSP105; HSP105A; HSP105B; KIAA0201; NY-CO-25; DKFZp686M05240	NM_006644	heat shock 105kDa/110kDa protein 1
ILMN_2102	1.572			
ILMN_1435	1.571	AMY; NOE1; OlfA; NOELIN1	NM_006334	olfactomedin 1
ILMN_9573	1.57	PIG50; HSPC150	NM_014176	ubiquitin-conjugating enzyme E2T (putative)
ILMN_14505	1.569	HSNOV1; FLJ13018; DKFZp667H1615	NM_017515	solute carrier family 35, member F2
ILMN_137480	1.569			
ILMN_41369	1.568			
ILMN_8861	1.568	ILRS; IARS1; PRO0785; FLJ20736	NM_002161	isoleucyl-tRNA synthetase
ILMN_12579	1.567		NM_002097	
ILMN_2645	1.566	L15; RAMA3; NY-REN-41	NM_030771	coiled-coil domain containing 34
ILMN_13686	1.564	FLJ16540; FLJ33039; KIAA0303; DKFZp686N1467; DKFZp686E18148	NM_198828	microtubule associated serine/threonine kinase family member 4
ILMN_29459	1.564	TOM40; PEREC1; C19orf1; PER-EC1; D19S1177E	NM_006114	translocase of outer mitochondrial membrane 40 homolog (yeast)
ILMN_13640	1.563	D15Wsu75e; MGC138384; DJ347H13.4	NM_015704	DNA segment, Chr 15, Wayne State University 75, expressed
ILMN_29128	1.563			
ILMN_9187	1.562			
ILMN_138567	1.562			
ILMN_7574	1.562	KIAA0286; DKFZp686N1768	NM_015257	KIAA0286 protein
ILMN_24243	1.561	RBP4; HSRBP4; HSRPB4	NM_004805	polymerase (RNA) II (DNA directed) polypeptide D
ILMN_14714	1.557			
ILMN_11989	1.554	H41	NM_017548	CDV3 homolog (mouse)
ILMN_22997	1.554	L1mt; BM022	NM_020236	mitochondrial ribosomal protein L1
ILMN_15799	1.554	KG19; BNAS1; HCCR-2	NM_080626	BRI3 binding protein
ILMN_138954	1.554			
ILMN_16596	1.553	RVB1; ECP54; TIP49; NMP238; TIP49A	NM_003707	RuvB-like 1 (E. coli)
ILMN_5375	1.553			
ILMN_7413	1.553	HuF2	NM_003594	transcription termination factor, RNA polymerase II
ILMN_22427	1.552			
ILMN_45032	1.551			
ILMN_31467	1.551	LOC389895	XM_372255	similar to CG4768-PA
ILMN_32712	1.55			
ILMN_3686	1.55	CCT6; Cctz; HTR3; TCPZ; TCP20; MoDP-2; TTCP20; CCT-zeta; MGC126214; MGC126215; CCT-zeta-1; T	NM_001009186	chaperonin containing TCP1, subunit 6A (zeta 1)
ILMN_521	1.549	SQLE	NM_003129	squalene epoxidase

Genes up or down regulated 1.5-fold in the MFC7-TO-ERa(EEE) cell line by estrogen in the presence of Dox contd.

Ilumina probe	Fold-induction	Gene name	Gene bank code	Gene description
ILMN_8524	1.548	SBBI31; PRO1284	NM_014035	sorting nexing 24
ILMN_138281	1.548			
ILMN_21022	1.546	CAT56; MGC161733; MGC161735	NM_025263	proline rich 3
ILMN_24080	1.543	FLJ20272	NM_017735	tetratricopeptide repeat domain 27
ILMN_15060	1.543	MCSC; PCSCL; SCAMC-2; KIAA1896; MGC105138; MGC119514; MGC119515	NM_052901	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 25
ILMN_26547	1.543			
ILMN_13298	1.542	ALP; hALP; FLJ10774; FLJ12179; FLJ23850; KIAA1709; DKFZp434C116	NM_024662	N-acetyltransferase 10
ILMN_1133	1.541	MCM2; CDC47; P85MCM; P1CDC47; PNAS-146; CDABP0042; P1.1-MCM3	NM_182776	minichromosome maintenance complex component 7
ILMN_22164	1.539	EIF1A; EIF4C; eIF-1A; eIF-4C	NM_001412	eukaryotic translation initiation factor 1A, X-linked
ILMN_137045	1.538			
ILMN_21470	1.535			
ILMN_7176	1.534	ZIP14; cig19; LZT-Hs4; KIAA0062	NM_015359	solute carrier family 39 (zinc transporter), member 14
ILMN_13265	1.534	RaLP; MGC34023	NM_203349	SHC (Src homology 2 domain containing) family, member 4
ILMN_28730	1.534			
ILMN_15311	1.534			
ILMN_8178	1.532			
ILMN_1163	1.531			
ILMN_18895	1.53	DUP; RIS2	NM_030928	chromatin licensing and DNA replication factor 1
ILMN_9607	1.527	SPF; TAP; TAP1; C22orf6; KIAA1186; KIAA1658; MGC65053	NM_012429	SEC14-like 2 (S. cerevisiae)
ILMN_1346	1.525	PSO2; SNM1; KIAA0086	NM_014881	DNA cross-link repair 1A (PSO2 homolog, S. cerevisiae)
ILMN_15061	1.525	16E1BP	NM_004237	thyroid hormone receptor interactor 13
ILMN_4860	1.524	GPE1BP; IG/EBP-1	NM_001806	CCAAT/enhancer binding protein (C/EBP), gamma
ILMN_14687	1.523	GNIP; RNF90	NM_033342	tripartite motif-containing 7
ILMN_1278	1.522			
ILMN_2138	1.521	SLOS	NM_001360	7-dehydrocholesterol reductase
ILMN_1073	1.519	MST; LST005; FLJ10504; RP11-29H23.3; DKFZp686B1757; DKFZp686I01261	NM_018116	misato homolog 1 (Drosophila)
ILMN_4868	1.518	p23; p25; p35; CDK5R; NCK5A; CDK5P35; MGC33831; p35nck5a	NM_003885	cyclin-dependent kinase 5, regulatory subunit 1 (p35)
ILMN_27477	1.517	Ssc2; FLJ20334	NM_017770	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 2
ILMN_23394	1.514	HPRT; HGPRT	NM_000194	hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)
ILMN_17555	1.514	ZPC; ZP3A; ZP3B; HUMZP3; ZP3-372; ZP3-424; ZP3-474	NM_007155	zona pellucida glycoprotein 3 (sperm receptor)
ILMN_24467	1.514	FLJ23384	NM_024758	agmatine ureohydrolase (agmatinase)
ILMN_137078	1.513			
ILMN_10462	1.51	CIS; G18; SOCS; CIS-1	NM_145071	cytokine inducible SH2-containing protein
ILMN_3370	1.509	UCH37; CGI-70	NM_015984	ubiquitin carboxyl-terminal hydrolase L5
ILMN_13820	1.508			
ILMN_23513	1.508	RDC7	NM_000674	adenosine A1 receptor
ILMN_72262	1.507		AA305151	EST176292 Colon carcinoma (Caco-2) cell line II Homo sapiens cDNA 5' end, mRNA sequer
ILMN_995	1.506			
ILMN_4548	1.504	FLJ12981	NM_203394	E2F transcription factor 7
ILMN_1150	1.504	FA2; FAB; FACB; FAAP90; FAAP95	NM_001018113	Fanconi anemia, complementation group B
ILMN_11087	1.503	GNA14	NM_004297	guanine nucleotide binding protein (G protein), alpha 14
ILMN_24438	1.503	PLAC1	NM_021796	placenta-specific 1
ILMN_6685	1.503	C23; FLJ45706	NM_005381	nucleolin
ILMN_78063	1.501		BX109404	Transcribed locus
ILMN_24968	1.501	ERP46; UNQ364; EndoPDI; MGC3178	NM_030810	thioredoxin domain containing 5
ILMN_16245	1.501	RAMP1	NM_005855	receptor (G protein-coupled) activity modifying protein 1
ILMN_22150	1.5	APG-1; Osp94	NM_014278	heat shock 70kDa protein 4-like
ILMN_29440	0.666	B7X; B7H4; B7S1; B7-H4; B7h.5; VCTN1; PRO1291; FLJ22418; RP11-229A19.4	NM_024626	V-set domain containing T cell activation inhibitor 1
ILMN_3294	0.666			
ILMN_13176	0.649	Dig2; REDD1; REDD-1; RTP801; FLJ20500; RP11-442H21.1	NM_019058	DNA-damage-inducible transcript 4
ILMN_97949	0.582		AK124402	CDNA FLJ42411 fis, clone BLADE2001133
ILMN_8269	0.505	AIBC1; NABC1	NM_003657	breast carcinoma amplified sequence 1

Appendix 2c

Genes up or down regulated 1.5-fold in the MFC7-TO-ERa(EEE) cell line by estrogen in the absence of Dox

Illumina probe	Fold-induction	Gene name	Gene bank code	Gene description
ILMN_138010	1.848			
ILMN_71180	1.797	AK3; AK4	AK026966	adenylate kinase 3-like 1
ILMN_17460	1.788	PSA; EPIP; PSAT; MGC1460	NM_021154	phosphoserine aminotransferase 1
ILMN_19358	1.782	WTX; FLJ39827; RP11-403E24.2	NM_152424	family with sequence similarity 123B
ILMN_22974	1.723	AIRS; GARS; PAIS; PGFT; PRGS; GARTF; MGC47764	NM_000819	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosp
ILMN_27722	1.693	FLJ13265	NM_024877	cyclin N-terminal domain containing 2
ILMN_22093	1.684	CEB1; CEBP1	NM_016323	hect domain and RLD 5
ILMN_22590	1.68	P130; NOPP130; NOPP140; KIAA0035; NS5ATP13	NM_004741	nucleolar and coiled-body phosphoprotein 1
ILMN_122489	1.678		BX099724	Transcribed locus
ILMN_27030	1.672	c-fos	NM_005252	v-fos FBJ murine osteosarcoma viral oncogene homolog
ILMN_15022	1.668	FLJ22329; MGC117270	NM_024656	glycosyltransferase 25 domain containing 1
ILMN_20313	1.664	FLJ39501	NM_173483	cytochrome P450, family 4, subfamily F, polypeptide 22
ILMN_4380	1.614			
ILMN_2720	1.608	RP3; TCTE1L; TCTEX1L	NM_006520	dynein, light chain, Tctex-type 3
ILMN_138245	1.605			
ILMN_28955	1.593			
ILMN_17615	1.589	PURL; FGAMS; FGARAT; KIAA0361	NM_012393	phosphoribosylformylglycinamidine synthase (FGAR amidotransferase)
ILMN_19052	1.586	GMP; NT5B; PNT5; cN-II	NM_012229	5'-nucleotidase, cytosolic II
ILMN_13503	1.579	YRS; YTS; TYRRS; CMTDIC	NM_003680	tyrosyl-tRNA synthetase
ILMN_7098	1.574	CCN5; CT58; CTGF-L	NM_003881	WNT1 inducible signaling pathway protein 2
ILMN_21069	1.573	30ST3A1; 3OST3A1	NM_006042	heparan sulfate (glucosamine) 3-O-sulfotransferase 3A1
ILMN_2707	1.56	SEN2; SEN2L; MGC2776; MGC4440	NM_025265	tRNA splicing endonuclease 2 homolog (S. cerevisiae)
ILMN_14614	1.548			
ILMN_41356	1.548		XM_939432	
ILMN_2030	1.547			
ILMN_20013	1.547	NRF; ITBA4	NM_017544	NF-kappaB repressing factor
ILMN_35248	1.533			
ILMN_21497	1.531	CDSP; OCTN2; FLJ46769	NM_003060	solute carrier family 22 (organic cation transporter), member 5
ILMN_19670	1.53	RNF192; FLJ45273; MGC126711; MGC126713	NM_198461	LON peptidase N-terminal domain and ring finger 2
ILMN_12909	1.513	TFR; CD71; TFR1; TRFR	NM_003234	transferrin receptor (p90, CD71)
ILMN_16938	1.509	FLJ20105; MGC131695	NM_017669	FLJ20105 protein
ILMN_9737	1.507	LPAAT-e; 1-AGPAT5; LPAAT-epsilon	NM_018361	1-acylglycerol-3-phosphate O-acyltransferase 5 (lysophosphatidic acid acyltransferase, epsil
ILMN_137162	1.504			
ILMN_24745	1.504			
ILMN_10290	0.658			
ILMN_11864	0.654	FLJ25974; dJ382I10.1	NM_001031743	chromosome 6 open reading frame 165
ILMN_13561	0.653	AGS1; DEXRAS1; MGC:26290	NM_016084	RAS, dexamethasone-induced 1
ILMN_18931	0.652	CTR2; COPT2; hCTR2	NM_001860	solute carrier family 31 (copper transporters), member 2
ILMN_22862	0.652	SK; DKK-1	NM_012242	dickkopf homolog 1 (Xenopus laevis)
ILMN_2905	0.624	RAB27B	NM_004163	RAB27B, member RAS oncogene family
ILMN_35465	0.62			
ILMN_16203	0.611	SIP; Teap; FLJ22139; p53DINP1; TP53DINP1; TP53INP1A; TP53INP1B; DKFZp434M1317	NM_033285	tumor protein p53 inducible nuclear protein 1
ILMN_22593	0.603		NM_207035	
ILMN_22606	0.588	GRX; GRX1; MGC117407	NM_002064	glutaredoxin (thioltransferase)
ILMN_5938	0.581			
ILMN_28481	0.576	GIG8; ID2A; ID2H; MGC26389	NM_002166	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein