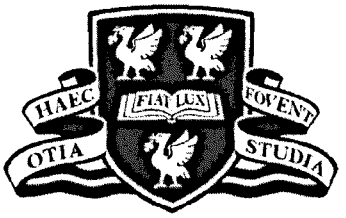

THE UNIVERSITY *of* LIVERPOOL

Estrogen receptor beta and estrogen response in breast cancer cell lines

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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ABSTRACT

Ceri Stewart: Estrogen receptor beta and estrogen response in breast cancer cell lines

Breast cancer affects 1 in 9 women in Britain and its development and treatment are greatly influenced by hormonal status, such as exposure to endogenous estrogen and expression of estrogen receptors (ERs). ERα is an established prognostic marker in breast cancer, but the role of ERβ is less certain. The ERs act to regulate gene transcription via a highly complex variety of mechanisms in response to stimuli such as estrogen, tamoxifen or fulvestrant.
In order to further define the role of ERβ isoforms in breast cancer, their role in the estrogen response must be characterised. This thesis has used a set of four breast cancer cell lines, as well as an MCF7 cell line engineered to over-express ERβ1 mRNA (MCF7β1x), to investigate the role of ERβ in estrogen response. Cells were treated with a variety of stimuli (estrogen, tamoxifen, fulvestrant, epidermal growth factor and fibroblast growth factor-2) and expression of a panel of ER isoforms, estrogen responsive genes and housekeeping genes was measured using real-time, quantitative PCR.

Estrogen response is cell line specific, both in terms of the genes affected and the level of response. These responses can be partly, but not fully, related to the levels of ERα expressed by the cell lines. Expression of individual ER isoforms varies in response to treatment in a time, stimulus and cell line specific manner. Different cell lines vary expression of different subsets of ER isoforms and MCF7β1x, which constitutively over-expresses ERβ1 mRNA, shows down-regulation of ERβ1 mRNA expression in response to estrogen. Together these data suggest that regulation may occur at the level of splicing and mRNA stability, as well as at the transcription level.
MCF7 and MCF7β1x showed remarkably similar responses to treatments. In both cell lines, similar sets of genes were .both up- and down-regulated by estrogenic and growth factor treatments. Most genes showed a similar pattern of transcriptional activation at 0 to 8 h as at 24 h, except for ERβ1 and ERβ2, indicating the importance of control of ERβ expression. It was not possible to measure the levels of ERβ1 protein in the cells, therefore the similarity in responses in MCF7 and MCF7β1x may indicate that, despite the higher levels of ERβ1 mRNA, MCF7β1x cells do not over-express ERβ1 protein.

Measurement of endogenous expression of a set of estrogen responsive genes in a panel of breast cancer cell lines in response to various stimuli has afforded new insights into the levels and variation in the response achieved in this system. Expression of ERβ mRNA was shown to be controlled in a cell line and treatment specific manner, as has previously been shown for ERα. Additionally, it was shown that this regulation was isoform specific and was maintained when the ERβ was over-expressed under the control of an exogenous promoter. This is particularly interesting, as it suggests various levels of regulation, indicating the important role of ERβ in downstream estrogen responses.

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ABBREVIATIONS

ADH	atypical ductal hyperplasia
AF1/ AF2	activating factor 1/2
ALH	atypical lobular hyperplasia
Amp	ampicillin
ANOVA	analysis of variance
beta-gal	beta-galactosidase
BHQ1	black hole quencher 1
bp	base pair
BSA	bovine serum albumin
Ca_i^{2+}	intracellular Ca^{2+}
CAT	chloramphenicol transferase (in context of reporter genes)
CDK	cyclin dependent kinase
cDNA	complementary DNA
CMV	cytomegalovirus
Ct	threshold cycle
Da	Dalton
DBD	DNA binding domain
DCCS	dextran-coated charcoal treated serum
DCIS	ductal carcinoma in situ
ddH_2O	deionised double-distilled water
ddNTPs	dideoxynucleoside triphosphates
DEPC	diethyl pyrocarbonate
DES	Diethylstilbestrol
DMBA	dimethyl benz(a)anthracene
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTPs	2'-deoxyribonucleoside triphosphates
Dox	doxycycline
dsDNA	double-stranded DNA
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EcR	ecdysone receptor
EcRE	ecdysone response element
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol bis(2-aminoethylether) tetraacetic acid
ER	estrogen receptor
ERE	estrogen response element

ERK	extracellular-signal-regulated protein kinase
ERKO	estrogen receptor knock-out mouse
EST	expressed sequence tag
estradiol	17beta-estradiol
FCS	foetal calf serum
FGF	fibroblast growth factor
gDNA	genomic DNA
HRP	horseradish peroxidase
HRT	hormone replacement therapy
ICI 182,780	Fulvestrant
Ins(1,4,5)P_3	inositol (1,4,5) trisphosphate
IPTG	isopropyl thio-beta-D-galactosidase
JNK	Jun kinase
LBD	ligand binding domain
MAPK	mitogen activated protein kinase
MCS	multiple cloning site
mER	membrane-bound ER
Mock	Cells transfected with empty vector with no insert
mRNA	messenger ribonucleic acid
MWt	molecular weight
NBCS	new-born calf serum
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PonA	ponasterone A
PVDF	polyvinylidine difluoride
Q-PCR	quantitative PCR
RE	restriction enzyme
RNA	ribonucleic acid
rpm	revolutions (or rotations) per minute
RT	reverse transcription reaction
RT-PCR	reverse trascription- polymerase chain reaction
RXR	retinoic acid receptor
SAGE	serial analysis of gene expression
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SERM	selective estrogen response modulator
SQ	starting quantity (in Q-PCR)
ssDNA	single-stranded DNA

tamoxifen	4-hydroxy-tamoxifen
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline- 0.1% Tween-20
TDLU	terminal ductal lobulo-alveolar unit
Tet	tetracycline
TetO	tetracycline operator
TetR	tetracycline repressor
TGF	Transforming growth factor
TNFα	tumour necrosis factor α
T-REx	tetracycline regulatory expression system
Tris	tris(hydroxymethyl) methylamine
UTR	untranscribed region
UV	ultra-violet
VEGF	vascular endothelial growth factor
VgEcR	modified ecdysone receptor

Chapter 1 Introduction

1.1 Introduction

Since the end of the 19th century, when it was shown that removing the major source of reproductive hormone through oophrectomy could be used to treat breast cancer [Beatson, 1896], hormone therapy has continued to be a front line treatment. The classical target of the major hormone estrogen is a transcription factor, estrogen receptor alpha (ERα), whose cDNA was first cloned in 1985 [Walter et al., 1985]. This nuclear steroid-binding receptor was shown to bind estrogen and activate gene expression via binding to a specific response element in the promoter region of target genes [Klein-Hitpass et al., 1989; Weisz et al., 1986]. Later, there was an interest in non-classical actions of the estrogen receptor [Bjornstrom and Sjoberg, 2005], such as interactions of the estrogen response with other pathways and response elements, which allow the estrogen response to be varied in a tissue, cell-type and promoter specific manner. A second estrogen receptor (ERβ) was discovered in 1996 [Mosselman et al., 1996] that was shown to be expressed in a distinct, but overlapping set of tissues to ERα and was, therefore, likely to modulate the estrogen response.

Prior to the cloning and characterisation of its cDNA, the presence or absence of the estrogen receptor in tumours was already recognised to play an important role in breast cancer development and treatment [McGuire, 1975], and drugs had been developed to inhibit the receptor for therapeutic benefit [Howell, 2005; Moseson et al., 1978]. However, treatment response is not uniform and tumours may acquire resistance to therapy.

In order to fully understand the significance of estrogen and the ERs in breast cancer, it is important to integrate the many different aspects of this field. For example, epidemiology may provide clues to the functions of estrogens and their role in both normal development and cancer, whereas studies of transcription and signal transduction may provide information about how these functions are fulfilled. An understanding of the structure and organisation of the ERs is clearly important to help understand their mechanisms of action.

1.2 Breast cancer

1.2.1 The normal breast

Development of the breast begins during embryonic growth, when the basic architecture is formed during a hormone-independent phase of growth. A single epithelial ectodermal bud develops through various stages to form a primitive structure consisting of ducts and ductules lined with one or two layers of epithelial cells and one layer of myoepithelial cells [Russo and Russo, 2004]. The breast undergoes little development between birth and puberty, with growth parallel to total body size until puberty, when hormone dependent growth and differentiation of the breast begins. Ovarian hormones induce proliferation of both the glandular tissue and the stroma. However, other signalling pathways are also involved in this phase of mammary development, for instance, growth factor signalling from the stroma is essential for the development of the ductal tree [Wiesen et al., 1999].

The mature mammary gland contains ducts ending in terminal ductal lobulo-alveolar units (TDLUs), or lobules. Lobules are categorised as types 1-4, in order of developmental phenotype. The more differentiated phenotypes express less ERα and show lower rates of proliferation. At puberty, type 1 lobules are formed. In nulliparous women, most lobules remain at this level of differentiation, with a small number of type 2 and 3 lobules developing, as each ovulatory cycle generates slightly more growth and differentiation of the ductal tree. During pregnancy, increased hormone levels induce further rapid differentiation and growth towards type 4, lactating lobules. Notch signalling has also been shown to be involved in pathway specification of cell growth during pregnancy, to regulate the balance of cell types during alveolar development [Buono et al., 2006]. By the time of birth, most of the lobules have progressed to fully active secretory lobules, although a small percentage of less differentiated lobules are still present. This is maintained throughout lactation, until after weaning a process of involution occurs, during which lobules regress to a less differentiated, resting state. However, the parous breast retains a much greater proportion of type 3 differentiated lobules than the nulliparous breast until menopause. Even after menopause, when the levels of differentiated lobules further regress, so that parous and nulliparous breast are undistinguishable histologically, the parous type 1 lobules are still less tumour prone than those of the

nulliparous breast, suggesting a subtle difference between the undifferentiated lobules of the nulliparous and parous breast [Russo and Russo, 2004].

The mammary gland consists of outer luminal epithelial and inner myoepithelial cells as well as intermediate cells with characteristics of both lineages, which are regarded as breast stem cells, and their differentiating offspring [Clarke et al., 2003; Rudland et al., 1996]. Differentiated alveolar, secretory cells are found only in pregnant or lactating breast. Cultured rat or human mammary breast tissue has been used to yield a variety of epithelial cell lines, including RAMA25 [Bennett et al., 1978], an epithelial cell line derived from a benign hyperplastic lesion of rat breast. Interestingly this cell line, and others, have been shown to generate occasional myoepithelial and alveolar-like cells in culture, and these changes can be induced by treatment with, for instance, DMSO, retinoic acid or prostaglandin [Rudland et al., 1996], indicating that these epithelial cells are multi-potent stem cells.

1.2.2 Development of breast cancer

The resting breast contains branching ducts and TDLUs, or lobules. The lobules are the site of initiation of most breast tumours [Wellings et al., 1975], which arise from the luminal epithelial, rather than myoepithelial lineage [Rudland et al., 1996]. The breast tissues contain a population of pluripotent stem cells, which are capable of regenerating an entire, differentiated mammary gland from a single cell clone [Kordon and Smith, 1998; Ormerod and Rudland, 1986; Rudland, 1991a]. These self-renewing stem cells undergo asymmetrical cell division to give rise to a population of transit amplifying and differentiated cells [Clarke, 2003] and are found throughout the epithelium, although their levels are highest in terminal end buds (TEBs) [Rudland, 1991b]. These steroid receptor-positive stem cells or their transit amplifying progeny are believed to be the source of most breast tumours [Dontu et al., 2003]. This idea is supported by the evidence that the mammary gland of rodents is most sensitive to carcinogens during the period when it contains the greatest concentrations of stem cells [Dawson, 1934]. Also, similar stem cell rich structures in the breasts of prepubertal and adolescent women are believed to account for the observation that these age groups were the most affected by breast cancers after the irradiation caused by the atomic bombing of Japan in 1945 [McGregor et al., 1977].

Tumour development is a multi-step pathway. Cells must fist escape normal control of proliferation, before developing the ability to invade other tissues and metastasize.

These changes require mutations in or loss of tumour suppressor genes or over-expression of proto-oncogenes [Bertram, 2000], however it is important to note that genes which are involved in loss of growth control are not the same as those involved in the development of the metastatic phenotype. In the breast, a series of well-characterized lesions are believed to be intermediates for invasive breast cancer [Allred et al., 2001]. Initially, atypical ductal and lobular hyperplasias (ADH and ALH) arise. These lesions show slightly higher proliferation than normal TDLUs, and in most cases the majority of cells (90%) express very high levels of ERα [Barnes and Masood, 1990; Schmitt, 1995]. These lesions may already show allelic imbalance (50% of cases at 30 loci studied), indicating a mechanism for their loss of proliferative control [Chuaqui et al., 1997; Lakhani et al., 1995; Nayar et al., 1997; O'Connell et al., 1998; Rosenberg et al., 1996]. These lesions may develop to form *in situ* carcinomas (DCIS, LCIS), which show a wide variation in phenotypes, both in terms of differentiation and other markers (proliferation, ERα expression, p53 mutations, erbB2 over-expression, allelic imbalance), reflecting the variation seen in invasive tumours [Allred et al., 2001]. In all of these markers, however, they show intermediate properties between the atypical hyperplasias and invasive tumours. Finally, further genetic changes may occur to confer the ability to invade other tissues, and invasive carcinomas are formed.

1.2.3 Risk factors in breast cancer

Breast cancer is the most common cancer in females, affecting 1 in 9 women in Britain in their lifetime [Quinn et al., 2001] and, although great advances have been made in diagnosis and treatment, the disease still kills many people every year (13200 deaths in Britain in 1999 [Quinn et al., 2001]). Many factors have been associated with breast cancer risk, including genetic factors, environmental and social factors such as breast-feeding, diet and smoking as well as physiological factors, such as menopause (reviewed in [Dumitrescu and Cotarla, 2005]).

1.2.3.1 Genetic factors

Genetics play an important role in determining breast cancer risk [Korde et al., 2004; Rebbeck, 1999]. This is supported by the observation that women who have a first-degree affected relative are more likely to develop breast cancer themselves [Collaborative Group on Hormonal Factors in Breast Cancer, 2001]. Breast cancer related genes can be divided into high and low penetrance genes. Mutations in high

penetrance genes, such as BRCA1, BRCA2 and p53 are closely related to cancer risk, and are often found in familial breast cancers [Easton et al., 1993; Oesterreich and Fuqua, 1999]. Mutations in BRCA1 or BRCA2 have been well studied as genetic factors. These are tumour suppressor genes, and mutations in these genes predispose women to breast and ovarian cancers [Warlam-Rodenhuis et al., 2005]. Various mutations in these genes can occur and the presence of a mutation, even one which is known to be deleterious, does not guarantee that the carrier will develop cancer [Ford et al., 1998]. BRCA1 protein has been shown to perform many different functions in cells, including a role in DNA damage repair and cell cycle checkpoints [Kinzler and Vogelstein, 1997]. BRCA1 co-localises with Rad51 and PCNA in the event of DNA damage, suggesting a direct role in damage repair [Scully et al., 1997]. It is also able to bind coactivators such as CBP [Pao et al., 2000] and to activate transcription of target genes [MacLachlan et al., 2000] such as p21, which is itself involved in cell cycle control and DNA damage repair [Li et al., 1999]. Mutation of the tumour suppressor gene p53 is associated with both sporadic and familial breast cancers as well as other cancers [Glebov et al., 1994; Malkin, 1994]. About 30% of breast cancers show mutations for p53, and such mutations are associated with aggressive tumours and poor clinical outcome [Chang et al., 1995; Elledge and Allred, 1994].

Low penetrance gene polymorphisms are often found at a greater frequency within the population, but their role in cancer development requires greater interactions with factors such as hormonal status and environmental exposures [Johnson-Thompson and Guthrie, 2000; Rothman et al., 2001]. One example of a low penetrance gene is CHK2, a kinase involved in cell cycle control and DNA repair [Bartek and Lukas, 2003; Lukas et al., 2003], which has been shown to be mutated in some cases of Li-Fraumeni syndrome [Bell et al., 1999]. A study of mutations in the CHK2 gene identified one mutation (T59K), which was found in a total of nine individuals with cancers, of which four were breast cancers, but in no healthy individuals [Ingvarsson et al., 2002]. A further mutation, CHK2 1100delC, expressed at a frequency of 1.1% in healthy individuals, has been shown to increase risk of breast cancer approximately 2-fold in women and 10-fold in men [Meijers-Heijboer et al., 2002]. Other low penetrance genes include BARD1, which interacts with, and is similar in structure to, BRCA1 [Karppinen et al., 2004] and the CYP19 gene, which codes for aromatase, an enzyme involved in the synthesis of estradiol [Haiman et al., 2003; Hopper et al., 2005].

1.2.3.2 Dietary factors

Various environmental and social factors can affect breast cancer risk. Not least among these are dietary factors. Generally, foods, which contain or produce carcinogens, increase cancer risks, whereas foods associated with antioxidant production decrease cancer risk. High intake of well-done meat increases risk, possibly due to the production of carcinogens in cooking [Zheng et al., 1998]. High alcohol intake increases risk [Singletary and Gapstur, 2001; Smith-Warner et al., 1998] through various mechanisms such as production of acetaldehyde and free radicals or increased levels of estrogen [Poschl and Seitz, 2004]. Low folate intake is a cancer risk factor [Duthie, 1999] and increased folate intake may be protective against breast cancer, inhibiting the effect of alcohol intake [Zhang, 2004]. Folic acid is crucial for normal DNA synthesis and repair. Also, a lack of folate can lead to reduced s-adenosylmethionine levels, which can alter DNA methylation patterns and cause inappropriate activation of proto-oncogenes. Eating fruit and vegetables is protective, which may partly reflect their content of antioxidant vitamins [Lee, 1999; Van Duyn and Pivonka, 2000]. Whilst intake of most polyunsaturated fatty acids increases cancer risk, omega-3 fatty acids may be protective, possibly involving mechanisms by which they interact with proteins of intracellular signalling pathways [Bartsch et al., 1999; Saadatian-Elahi et al., 2004]. Finally, soy intake can also decrease breast cancer risk, due to its phytoestrogen content [Bouker and Hilakivi-Clarke, 2000].

1.2.3.3 Hormonal factors

Many factors associated with hormone status play a role in breast cancer risk, demonstrating the importance of hormones to breast cancer development [Pike et al., 2004]. Early age of menarche and late age of menopause increase risk of breast cancer, whereas early pregnancy, high parity and longer duration of breast feeding all decrease the risk (reviewed in [Dumitrescu and Cotarla, 2005; Feigelson and Henderson, 1996]). Early menarche [Berkey et al., 1999; Brinton et al., 1988; Titus-Ernstoff et al., 1998] and late menopause [Collaborative Group on Hormonal Factors in Breast Cancer, 1996] both increase the number of menstrual cycles, increasing the exposure of the breast to ovarian hormones including estrogen [Bernstein, 2002]. Breast epithelium proliferates in response to ovarian hormones during the menstrual cycle, therefore, more cell divisions will occur, leading to an increased risk of mutations accumulating. Similar mechanisms underlie the observation that early

menopause, or removal of one or both of the ovaries before the menopause reduces risk of breast cancer [Kreiger et al., 1999; McPherson et al., 2000].

Various mechanisms have been proposed to explain how early and multiple pregnancies may protect against breast cancer. It has been shown that, in rats, pregnancy induces permanent differentiation of the terminal end buds of the mammary gland, and reduces the levels of breast cancer induced by DMBA [Russo et al., 1991]. Similarly, it is suggested that pregnancy may result in a reduction in the oncogenic potential of breast epithelial cells caused by reduction of growth factor expression or increase in TGFβ3 or p53 activity [D'Cruz et al., 2002; Sivaraman and Medina, 2002]. Recently, a distinct gene expression pattern in the breast has recently been identified which differentiates parous and nulliparous rats [Blakely et al., 2006]. This study confirms that parity induces changes in expression of extracellular matrix (ECM), immune system, differentiation and growth factor signalling genes, which both reinforce the mechanisms for the reduction of risk suggested above, and provide new information about the details of these mechanisms.

It is interesting to note that several of the genetic and dietary factors mentioned above may also be indirectly associated with hormonal status. Many breast cancer-related genes are associated with estrogen signalling, for instance, wild-type BRCA1 has been shown to down-regulate ER actions such as transcriptional activation [Fan et al., 1999] and membrane signalling [Razandi et al., 2004]. Several of the dietary factors mentioned also show links to estrogen signalling. Obesity is linked to postmenopausal breast cancer. It is known that obesity is often associated with higher levels of free steroid hormones, and also that aromatisation of androgens, an important source of estrogen in postmenopausal women, occurs in the adipose tissue. Therefore obesity may increase the exposure of the breast tissue to estrogen, and hence increase cancer risk [La Guardia and Giammanco, 2001].

1.3 Estrogen and breast cancer

1.3.1 Estrogen and normal mammary development.

The mammary gland is a hormonal responsive tissue, and its development and differentiation are strongly dependent on ovarian hormones. Removal of the ovaries prevents post-natal mammary development, and ER and PR knock-out mice show distinct phenotypes. The adult ERα knock-out mouse (αERKO) shows a mammary

gland structure similar to that of a new born normal female, with epithelial, stromal and connective tissues but only a small rudimentary ductal tree [Bocchinfuso and Korach, 1997]. Experiments were performed in which wild-type and αERKO stroma and epithelia were combined in a host animal to determine whether ERα expression is necessary in both of these compartments [Cunha et al., 1997]. It was shown that whilst wild-type stroma could induce the differentiation and growth of both ERα positive and negative epithelia, αERKO stroma resulted in a lack of estrogen response, even in ERα positive epithelia. However, whilst mice show ERα expression in both epithelial and stromal cells [Shyamala et al., 1997], in humans [Anderson et al., 1998], rats [Russo et al., 1999] and cows [Capuco et al., 2002] expression of ERα is restricted to the epithelia suggesting that these results may not translate directly between species. The PRKO mouse exhibits a normal pubertal ductal structure, but does not develop the necessary differentiated structures for lactation. Lobuloalveolar development in the normal mouse is stimulated by progesterone. This did not occur in the PRKO mouse [Lydon et al., 1995]. The ERβ knock-out mouse, on the other hand, shows normal breast development and lactation, suggesting that ERβ expression is not necessary for mammary development [Couse et al., 1997].

In the adult breast, epithelial proliferation is greatest during the luteal phase of the menstrual cycle, when levels of estrogen and progesterone are at their highest [Anderson et al., 1998] and this proliferative response is mainly due to the presence of high levels of estrogen [Laidlaw et al., 1995]. However, in post-menopausal breast, epithelial proliferation is greater in patients receiving combined estrogen and progesterone hormone replacement therapy (HRT) than those receiving estrogen-only HRT, indicating that progesterone has some role in stimulating proliferation, at least in the post-menopausal breast [Hofseth et al., 1999]. In the normal human breast, ERα and PR are expressed only in luminal epithelial cells [Petersen et al., 1987] and are usually coexpressed in these cells [Clarke et al., 1997]. The number of cells expressing ERα also varies throughout the menstrual cycle, being twice as high in the follicular phase as during the luteal phase [Ricketts et al., 1991]. ERβ, however, is more widely expressed, occurring in luminal epithelial and myoepithelial cells as well as stromal fibroblasts and endothelial cells [Speirs et al., 2002] and its expression does not vary with the menstrual cycle. In post-menopausal tissues, there

is a decrease in ovarian steroid levels and a corresponding decrease in proliferating cells, however, the proportion of cells expressing ERα increases [Shoker et al., 1999b] which may be related to the prevalence of hormone-dependent tumours in the post-menopausal breast.

In normal pre-menopausal breast, about 5% of epithelial cells are proliferating. These cells do not express ERα but are usually found adjacent or close to ERα/PR expressing cells [Clarke et al., 1997]. A model has been suggested in which differentiated steroid receptor-expressing cells stimulate proliferation of neighbouring cells via paracrine signalling [Anderson et al., 1998]. This model is supported by the observation that conditioned media from ERα-positive cultured cell lines treated with estrogen is able to stimulate growth of ERα-negative cell lines [Clarke et al., 1992]. Additionally, whilst PR knock-out (PRKO) mice do not undergo alveolar development of the breast, when PRKO cells are mixed with normal cells and injected into a host animal, normal alveolae are formed, suggesting that paracrine signals from the normal cells are able to rescue the PRKO cells [Brisken et al., 1998].

1.3.2 Estrogen signalling and breast cancer

Estrogen receptor signalling is key to the development of breast tumours. It has been observed that expression of the steroid receptors is perturbed during the early stages of breast cancer development. Increased expression of ERα in benign breast is a marker for breast cancer [Khan et al., 1994], and ERα is more commonly found in proliferating cells in cancers than in benign tissue [Shoker et al., 2000]. In normal tissues, ERα-positive cells rarely proliferate. However, the percentage of cells expressing both ERα and markers of proliferation is increased in hyperplasias and DCIS, where the presence of such cells correlates with the risk of developing breast cancer [Shoker et al., 1999a] . Expression of ERβ appears to decrease during cancer development [Jensen et al., 2001; Roger et al., 2001] although the details of ERβ expression during carcinogenesis remain controversial (Section 1.3.3). In normal breast, the expression levels of the two PR isoforms, PRA and PRB remain fairly constant throughout the menstrual cycle. However, as tumours develop, expression of PRA comes to predominate [Mote et al., 2002].

The change from non-proliferative to proliferative ERα-expressing cells may correspond to a change from paracrine to autocrine signalling, with the ERα-expressing cells gaining the ability to stimulate their own proliferation, or alternatively a change in intracellular signalling pathways, such that ERα becomes able to directly induce cell growth. As well as changes in expression of the steroid receptors, breast tumours also show increased expression of many coactivators such as CBP, TIF2 [Kurebayashi et al., 2000] and AIB1 [Murphy et al., 2000]. Changes in the expression of coregulatory proteins may partly mediate the change from a non-proliferative to proliferative response to estrogen in ERα-expressing cells.

1.3.3 ERβ in breast cancer

The role of ERα in breast cancer is fairly well understood (Section 1.3.2 and [Feigelson and Henderson, 1996]), however, reports vary about the role of ERβ. Expression of ERβ is found in both normal and cancerous breast tissue and cell lines using a variety of techniques, including RT-PCR and immunohistochemistry [Enmark et al., 1997; Leygue et al., 1998; Saji et al., 2000]. Using RT-PCR, ERβ mRNA was found in 35% of normal human breast samples [Speirs et al., 1999b] and using immunohistochemistry, 70% of rodent breast epithelial cells, and some stromal cells were shown to contain ERβ [Saji et al., 2000]. Unlike ERα, expression of which varies significantly during breast development, pregnancy and lactation, expression of ERβ was shown to be relatively constant. Additionally, whereas ERα is mostly confined to luminal epithelial cells, ERβ protein is also expressed in myoepithelial, stromal fibroblasts and endothelial cells in human breast [Speirs et al., 2002]. A study using semi-quantitative PCR has suggested that 90% of breast cancers express some form of ERβ mRNA (ERβ1, 2 and 5 measured separately), but that PCR results did not correlate with protein expression, as measured by immunohistochemistry [Davies et al., 2004]. This has also been observed by other groups, and is an important point, as such a discrepancy between mRNA and protein levels implies that ER expression may be regulated at post-transcriptional levels, as well as possibly explaining some of the differences between reported expression patterns of ERβ.

When comparisons are drawn between ERβ and ERα levels or between ERβ and other prognostic markers, the literature becomes even more divergent. Several

groups find no correlation between ERβ and ERα [Brouillet et al., 2001; Dotzlaw et al., 1996; Dotzlaw et al., 1999; Saunders et al., 2002b]. Others suggest a positive correlation between the two receptors [Jarvinen et al., 2000; Kurebayashi et al., 2000; Omoto et al., 2001; Omoto et al., 2002]; whereas a third set of papers suggest that ERβ and ERα show negative correlation in breast cancers [Iwao et al., 2000; Knowlden et al., 2000; Roger et al., 2001]. Each of these three sets of papers contains reports using both PCR and immunohistochemistry. However, papers reporting no correlation studied only tumour samples, whereas some of those suggesting a correlation between ERα and ERβ studied a combination of normal and tumour tissues, or used preinvasive lesions. There is no relationship between the method of measurement used, source of samples or number of samples used and whether a positive or negative correlation is observed in this series of studies.

1.3.3.1 ERβ isoforms in breast cancer

Even when looking at individual isoforms of ERβ (Section 1.4.1), there is no consensus. Some groups find no relationship between expression of ERβ isoforms and cancer progression. For instance, [Tong et al., 2002] find no correlation between mRNA expression of ERβ1-5 and clinical parameters and [Miller et al., 2006] measured levels of ERα, ERβ1 and ERβ2 protein in tumours by immunohistochemistry and found no correlation between their expression and tamoxifen sensitivity. In some reports, expression of different ERβ isoforms seems to correlate with poor prognosis. One report suggests that ERβ2 protein expression correlates with a poor response to tamoxifen in ERα-positive tumours [Saji et al., 2002]. Another group found that expression of mRNA encoding ERβ2 and ERβ5 increases compared to ERβ1 in breast cancer as tumour inflammation or grade increases [Leygue et al., 1999], whilst a further study using the same PCR assay, as well as immunohistochemistry, found that ERβ1 was higher in normal than cancer tissues, but that tumour samples were more likely to be ERβ2-positive than normal tissue [Omoto et al., 2002]. This study also showed a correlation between ERβ1 expression and positive prognostic markers (ERα-positive, PR-positive, low grade), but showed no relationship between ERβ2 expression and such markers. Similarly, [Esslimani-Sahla et al., 2005] showed an increase in ERβ2 expression during progression from normal breast to DCIS to invasive tumours by

immunohistochemistry. Finally, in complete contrast to the results of [Leygue et al., 1999], [Davies et al., 2004] found that expression of both ERβ2 and ERβ5 mRNAs were independently associated with relapse free survival in a tamoxifen treated cohort. Further work using real-time PCR and immunohistochemistry for ERβ2 confirmed this positive association [Vinayagam et al., 2006].

The two studies showing no correlation between ERβ isoform expression and outcome used the smallest cohorts of patients (30 and 36 tumours), suggesting that larger numbers of samples may be required to identify a relationship between ERβ isoform expression and clinical parameters. Similarly, although the study of Saji *et al.* evaluated the expression of ERα, ERβ2 and PR in 115 tumour samples, outcome data is not presented for the complete cohort. A subgroup of only 18 samples was used in the investigation of tamoxifen sensitivity. The therapy used was 20 mg daily for 3 months prior to surgery, similar to that studied by Miller *et al.* Saji *et al.* found that 8/9 ERβ2-negative tumours showed a response to tamoxifen, whereas only 4/9 ERβ2-positive cases responded to therapy. This is in contrast to the results of Davies *et al.* and Vinayagam *et al.* However, these latter studies used a cohort of over 100 patients who received tamoxifen as an adjuvant therapy over several years, rather than a short-term, neo-adjuvant treatment. Additionally, whilst Saji and Miller define a response to treatment in terms of tumour size reduction, Davies and Vinayagam have used measurement of time to relapse and overall survival to define response to therapy. Taken together, these data suggest that whilst ERβ2 expressing tumours may not regress on treatment with tamoxifen, they also do not progress as quickly as ERβ2-negative tumours. This may be because ERβ2 blocks both positive and negative responses to estrogenic ligands. Leygue, Omoto and Esslimani-sahla all suggest that ERβ2 (and ERβ5) are more highly expressed in tumour than normal tissues, and that they are higher in more malignant tumours. The apparent contrast between these results and the results of Davies and Vinayagam may be explained by the use of different cohorts. Whereas Davies and Vinayagam used post-menopausal, mostly ERα-positive tumours, these other studies used non-selected tumours including pre- and post-menopausal and ERα-positive and negative cases.

1.3.3.2 Prognostic significance of ERβ expression

Correlation of ERβ expression with prognostic markers such as nodal status and tumour grade and with disease outcome is also uncertain. Some groups find no

correlation between ERβ mRNA and prognostic markers [Brouillet et al., 2001; de Cremoux et al., 2002; Gustafsson and Warner, 2000; Tong et al., 2002]. In fact, whilst several studies associate ERβ expression with stages of tumour progression (normal tissue, DCIS, invasive tumour), ERα expression or survival and tamoxifen response, few papers find any association between ERβ expression and clinical markers such as nodal status, age, menopausal status and grade or size of tumour. However, [Jarvinen et al., 2000] measured ERβ protein and mRNA expression by immunohistochemistry and *in situ* hybridisation in 92 tumours and found a correlation between ERβ expression and low grade, node-negative status, pre-menopausal, low *S*-phase and ERα/PR-positive status. Conversely, [Knowlden et al., 2000] find an association between ERβ mRNA and EGFR expression, a poor prognostic marker, and [Speirs et al., 1999b] find that co-expression of ERα and ERβ mRNAs is found more often in high grade, node positive tumours. All of these groups used fairly large (60-94) cohorts of non-selected breast tumours. The differences in their conclusions may reflect the different methods used to measure ERβ expression, or simply variation between their patient cohorts.

Unlike ERβ2, expression of total ERβ or ERβ1 mRNA or protein is lower in tumours than in normal tissue [Esslimani-Sahla et al., 2005; Leygue et al., 1998; Omoto et al., 2002; Roger et al., 2001; Shaaban et al., 2003]. The relationship between ERβ1 expression and tamoxifen sensitivity is, however, less clear. One group showed that total ERβ mRNA expression was higher in tamoxifen resistant tumours than in tamoxifen sensitive tumours [Speirs et al., 1999a], however, this study only used a very small cohort (17 tumours). Using significantly more samples, [Davies et al., 2004] found no association between ERβ1 mRNA expression and survival, although [O'Neill et al., 2004] found a trend towards worse outcome in ERβ1 protein-positive samples, in a cohort of 143 patients receiving tamoxifen. Other groups have suggested that low expression of total ERβ protein may correlate with tamoxifen resistance [Esslimani-Sahla et al., 2004; Hopp et al., 2004]. It has been previously noted that ERβ mRNA and protein expression levels do not correlate well, in tumour samples. This may partly explain the discrepancies between these different studies. Additionally, whereas some studies use isoform specific assays, others have measured total protein. Therefore, the higher levels of ERβ seen by Esslimani-Sahla

and Hopp in tamoxifen sensitive tumours may correspond to the higher levels of ERβ2 and ERβ5 expression observed by Davies and Vinayagam, rather than reflecting a change in ERβ1 expression.

Overall, it is still difficult to see a clear picture of ERβ action in the breast. We know it is present, although at lower levels than ERα. Expression levels of ERβ1 decrease during tumourigenesis, but a consensus on its role in breast cancer prognosis has yet to be reached. This is partly due to the use of different methods, such as PCR or immunohistochemistry, to measure ERβ expression. Even when groups have used the same technique, different PCR primers or antibodies specific to different regions of ERβ will detect different isoforms, which may affect their results. ERβ is expressed at low levels, making it difficult to accurately measure expression, especially in small samples. Significantly, different groups have looked at different cohorts of patients, and this may lead to problems when comparing their results. It is imperative that reports give full details of the cohorts used, specificity of the ERβ assays and definitions of receptor positivity, to allow results to be compared and a full consensus on the role of ERβ in breast cancer to be reached.

1.3.4 Estrogen and breast cancer treatment

Estrogen plays an important role in both breast cancer development [Walker, 1999] and in choice of treatment [Fuqua and Cui, 2004; Miller, 2004]. From the late 19th century, it was known that removal of the ovaries was an effective treatment for some breast cancers [Beatson, 1896]. Since then, endocrine therapy has taken many steps forward, with the development of antiestrogens and selective estrogen response modulators (SERMs), which directly inhibit estrogen-dependent proliferation by binding the ligand binding domain (LBD) of the estrogen receptors [Fontana and Delmas, 2001], and also aromatase inhibitors, which block production of estrogen [Mokbel, 2002].

Tamoxifen is a SERM used as an adjuvant therapy for women, especially post-menopausal, with ERα-positive breast cancer. Five years of tamoxifen treatment is shown to decrease the risk of recurrence and mortality [Early Breast Cancer Trialists' Collaborative Group, 1998]. Tamoxifen can act as either an agonist or antagonist of estrogen action depending on the cell type, ER and promoter context [Pole et al., 2005]. For instance, whilst tamoxifen is an antagonist of estrogen in the breast

(reducing tumour growth), it acts as an agonist in the bone (protecting against osteoporosis) and the uterus (increasing the risk of endometrial cancer) [Cosman and Lindsay, 1999; Mitlak and Cohen, 1997]. It may also act through alternative mechanisms such as inhibition of protein kinase C [O'Brian et al., 1985] and other pathways [Colletta et al., 1994]. PKCδ is also implicated in tamoxifen resistance, as its over-expression can induce a tamoxifen-resistant phenotype on tamoxifen-sensitive cells and its inhibition significantly inhibits both estradiol- and tamoxifen-induced cell proliferation in antiestrogen resistant cells. Over-expression of PKCδ has been seen in breast tumours compared to normal tissue [Nabha et al., 2005]. Raloxifene is another SERM, which has been tested for use in breast cancer therapy and may be used experimentally. Raloxifene acts as an estrogen antagonist in breast and decreases the risk of breast cancer in postmenopausal women [Cummings et al., 1999; Heringa, 2003]. This drug is mainly used in osteoporosis treatment, demonstrating its estrogenicity in bone. However, unlike tamoxifen, it does not act as an estrogen in the uterus [Cosman and Lindsay, 1999; Mitlak and Cohen, 1997].

Pure antiestrogens have subsequently been developed for use in breast cancer treatment. Fulvestrant (ICI 182,780) [Wakeling and Bowler, 1992] is a pure estrogen antagonist, which binds the ER LBD, blocking ER activation and accelerating its degradation by immobilising the ER to the nuclear matrix and drawing it into close proximity to the proteasomes [Dauvois et al., 1993; Long and Nephew, 2006]. This is a particularly useful drug, as it has been shown to be effective in tamoxifen-resistant models, and is not cross-resistant with other treatments such as SERMs and aromatase inhibitors [Dodwell and Vergote, 2005]. As some patients develop resistance to tamoxifen, it is valuable to have further drugs available for their treatment [Howell et al., 2002; Steger et al., 2003].

Aromatase inhibitors can be used to reduce endogenous estrogen levels [Miller, 2006]. In post-menopausal patients, the main route of estrogen synthesis (90%) is by aromatisation of androstenedione into estrone in organs other than the ovaries [Brueggemeier, 2001]. Blocking the action of aromatase, therefore, greatly reduces the levels of circulating estrogen in the body, decreasing proliferation of ER-positive tumours [Joensuu et al., 2005]. Several third generation aromatase inhibitors such as anastrozole, exemestane, and letrozole have proved to increase survival and decrease tumour recurrence in randomised trials [Grana, 2006; Narashimamurthy et al., 2004; Thurlimann et al., 2005].

Tumours are routinely assayed for ERα status, as ERα-positive tumours are more likely to respond to endocrine therapy than ER-negative tumours [Block et al., 1975; Knight et al., 1977]. However, whilst over half of breast tumours are ERα-positive, only 70% of these tumours respond to tamoxifen therapy [Ali and Coombes, 2000] and some tumours characterised as ER-negative may respond to therapy. Obviously the estrogen response does not depend simply on expression levels of this one receptor. Many other factors are also involved in the interactions between breast cancer treatment and response, such as expression of other steroid hormone receptors and growth factor receptors [Miller et al., 2005]. ERβ may modulate the role of ERα in the breast and may show decreased expression in breast cancer compared to the normal breast [Shaaban et al., 2003]. ERβ mediates different responses to tamoxifen, raloxifene and fulvestrant than ERα at the cellular level [Fournier et al., 2001; Quaedackers et al., 2001; Wade et al., 2001], suggesting that levels of ERβ may modulate response to these drugs. Other signalling pathways and mechanisms also contribute to tamoxifen sensitivity and resistance including changes in immunology, endocrinology and pharmacokinetics [Clarke et al., 2001].

1.3.5 Estrogen and development

Estrogen and the ERs play a role in the development of breast cancer, but also in normal development of both breast (Section 1.3.1) and other tissues. Studies using knock-out mice are used to identify the developmental targets of a specific gene, without requiring detailed knowledge of the mechanism of the effects.

Aromatase catalyses the formation of estrogen from C19 steroids. In aromatase knock-out (ArKO) mice, estrogen levels are undetectable [Fisher et al., 1998]. These mice appear normal at birth, and the males show little phenotypic variation from their normal siblings. They breed normally, but show enlarged seminal vesicles and prostate, due to increased secretory content. Females, on the other hand are infertile. They show a small but apparently normal uterus, which is able to respond to exogenous estrogen, and mammary development equivalent to that of a pre-pubertal normal mouse. Their ovaries contain large follicles filled with granulosa cells, but no corpora lutea and, therefore, no ovulation. In these mice the ERs are normal, so although extremely low levels of estrogen are present, signalling through the ERs may still occur via alternative mechanisms.

Knock-out mice for the two ER genes and double knock-outs show distinct phenotypes [Curtis Hewitt et al., 2000] and will be described separately. Both male and female ERα knock-out mice are infertile, showing many changes to reproductive tissues. Female αERKO mice show hypoplastic uteri which do not respond to estrogen stimulation and no corpora lutea are seen in the ovaries, despite the presence of follicles [Lubahn et al., 1993]. Experiments using microarrays have shown that the estrogen response in the uterus is markedly reduced in αERKO mice [Hewitt et al., 2003]. These mice also show incomplete mammary development (Section 1.3.1 and [Bocchinfuso and Korach, 1997]). Male αERKO mice show low or no fertility and have small testes [Lubahn et al., 1993]. These mice also show various phenotypes in non-reproductive tissues. αERKO mice have increased white adipose tissue and exhibit glucose intolerance and insulin resistance [Heine et al., 2000]. In normal animals, stress to the bones causes the bone to grow and strengthen, a responses lost in human osteoporosis. αERKO mice also show no such response when subject to bone strains [Lee et al., 2003]. Interestingly, it has been reported that the αERKO mouse may show reduced ERβ expression in certain tissues (ovary, epididymis and prostate) indicating an interaction between the two receptors [Couse et al., 1997].

The phenotype of the ERβ knock-out (βERKO) mouse is not so clear, as different populations developed and maintained in different labs show markedly different phenotypes in terms of severity (reviewed in [Harris, 2006]). However, an indication of the roles of ERβ can be obtained by studying the available data. βERKO females show reduced fertility, with smaller, fewer litters, which may be related to reduced ovarian efficiency [Krege et al., 1998]. A distinct uterine phenotype has also been reported, showing increased amounts of glands and a very large, fluid filled lumen on estrogen stimulation [Weihua et al., 2000]. Differences in proliferative rate and PR and growth factor synthesis were also seen. Their mammary development and lactation, however, appears normal [Krege et al., 1998]. Male mice demonstrate no changes in fertility, although older mice may show prostate and bladder hyperplasia [Krege et al., 1998].

Mice lacking ERβ show changes in cardiovascular regulation. These mice show high blood pressure, ion channel dysfunction and changes in vascular constriction [Zhu et al., 2002] and also show mechanistic differences in the acute dilatory responses to

estrogen receptor agonists in isolated arteries [Cruz et al., 2005]. Several reports have identified behavioural roles for ERβ in the knock-out mice, for instance, βERKO male mice show incomplete defeminisation [Kudwa et al., 2005; Kudwa et al., 2006], females show lack of spatial learning [Rissman et al., 2002] and increased anxiety, which may be linked to an increase of 5-hydroxytryptamine 1a receptor expression in the medial amygdala [Krezel et al., 2001]. Studies have also shown morphological abnormalities in the brain of the βERKO mouse, including neuronal deficit in certain areas and degeneration of neuronal cell bodies throughout the brain as the mice age [Wang et al., 2001] and that ERβ is essential for proper embryonic development of the brain [Wang et al., 2003].

One group has created a double knock-out mouse for ERα and ERβ (αβERKO) [Couse et al., 1999]. Like the single knock-outs, these mice survive to adulthood, but show a unique reproductive phenotype, indicating that the functions of the ERs are non-redundant. Male αβERKO mice are sterile, with a grossly normal reproductive tract, but reduced sperm count and sperm motility. Females show severe hypoplasia of the uterus, similar to that seen in the αERKO mouse, indicating the requirement for ERα in post-natal, estrogen-dependent growth. In the ovaries, a distinct phenotype is observed. The prepubertal ovaries show precocious maturation, in keeping with the high levels of serum lutenising hormone (LH) observed. The mature ovaries contain primordial and growing follicles, but no corpora lutea, as well as unusual structures resembling the seminiferous tubules of the testes. The appearance of these structures in a time-dependent manner and other aspects suggests that this morphological sex-reversal is a process of redifferentiation, rather than a *de novo* formation of "male" structures.

In humans, similar roles in development for estrogen can also be deduced. The ERs show widespread distribution and distinct patterns of expression throughout the body [Enmark et al., 1997]. Both receptors are found in male [Hess et al., 1997; Saunders et al., 2002a] and female [Mowa and Iwanaga, 2000] reproductive tissues where they have roles in development and growth [Merchenthaler and Shugrue, 1999; Pelletier and El-Alfy, 2000]. Estrogen and the ERs have roles in normal bone growth and maintenance [Manolagas et al., 2002] and are known players in osteoporosis, for example, ERβ polymorphisms have been related to bone density in several studies

[Ogawa et al., 2000; Scariano et al., 2004] and studies of a man with a mutation in ERα which conferred estrogen resistance showed defects in bone maturation and mineralisation [Smith et al., 1994]. Estrogen is involved in many neurological processes via ERα and ERβ, as well as alternative ERs [Toran-Allerand, 2004a]. Estrogen and the ERs are also important in the development and treatment of many cancers, including breast (Section 1.3.2), prostate [Oh, 2002; Royuela et al., 2001] and pituitary tumours [Chaidarun et al., 1998].

These many different functions indicate that estrogen signalling is active in many different tissues. Estrogen, via the ERs, must be able to integrate different signals and activate different responses in different tissues to perform these many roles. To understand how different processes can be regulated by a single molecule, one must investigate the mechanisms by which the estrogen receptors respond to estrogen.

1.4 Structure of the estrogen receptors

The pleiotropic estrogen responses in different cells and tissues are mainly modulated by ERα and ERβ. The ERs are members of the steroid-binding nuclear receptor family. In common with other family members, they have six domains, A-F, where A/B contains the activator function 1 (AF1) region, C is the DNA binding domain (DBD) containing two zinc finger motifs, D is a hinge region, E contains the ligand binding domain (LBD) and AF2 region, as well as being involved in dimerisation and nuclear localisation and F is a less well conserved region, possibly involved in nuclear localisation (Fig. 1.1A and [Nilsson et al., 2001]). Estrogens, antiestrogens and other related molecules such as phytoestrogens bind the LBD, whereas the AF1 and AF2 regions are involved in binding of coactivator and corepressor proteins.

The two classical estrogen receptors, ERα and ERβ, are expressed from separate genes. ERα was first isolated in 1986 [Green et al., 1986; Greene et al., 1986]. The gene consists of eight exons and is located on chromosome 6q24-27 [Ponglikitmongkol et al., 1988] in humans. ERβ was first discovered in 1996 in rat prostate [Kuiper et al., 1996]. It was subsequently found to be encoded on chromosome 14q22-24 in humans and to have distinct tissue distribution and functional differences from ERα [Enmark et al., 1997]. This is also an eight-exon

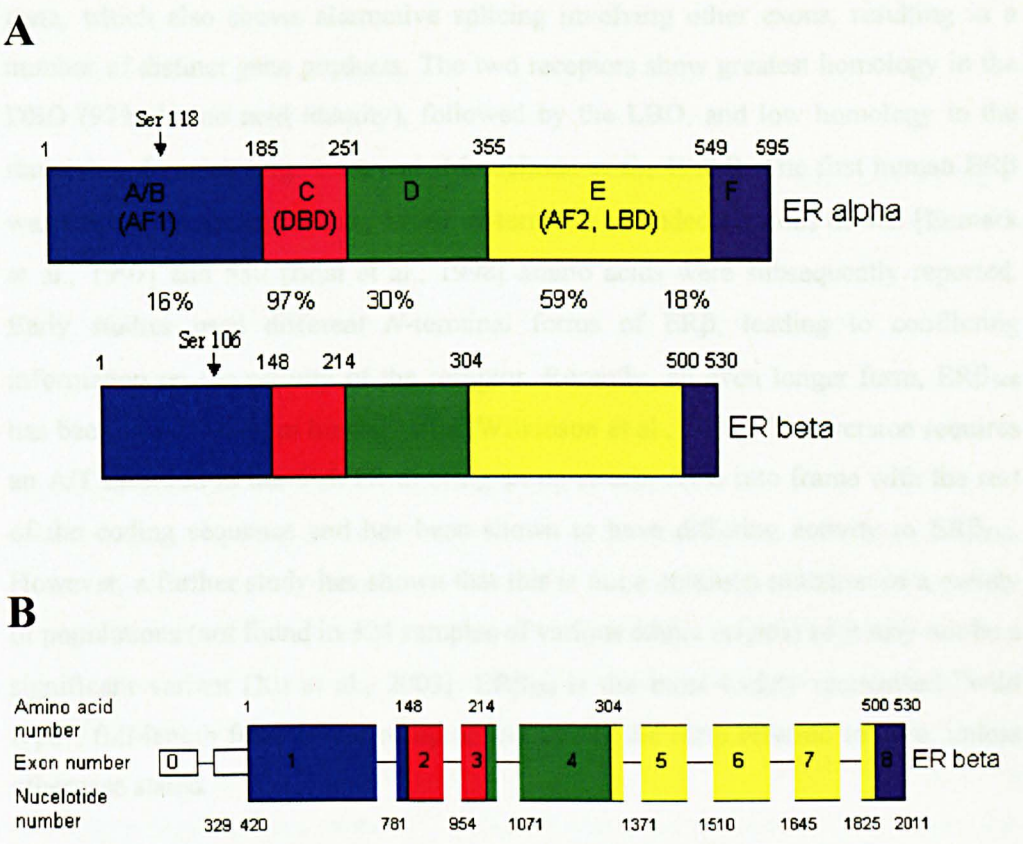


Figure 1.1 A comparison of the structural domains of the two estrogen receptors.

Both estrogen receptors are members of the nuclear steroid-binding receptor super-family, and share a common domain structure. The domains are designated A-F, as shown (Panel A). The AF1 and AF2 regions, found in domains A/B and E respectively, are responsible for coactivator and corepressor binding and other protein-protein interactions. The DBD is found in domain C and the LBD in domain E.

Panel A shows the amino acid numbers and amino acid conservation between the two receptors for each domain. A serine (Ser^{118} and Ser^{106}), which is a target of MAPK, is highlighted in each receptor. Phosphorylation of this serine by MAPK leads to ligand-independent activation of the receptor.

Panel B shows how the structural domains of ERβ relate to the exon structure. Eight exons encode the ERβ protein, with one upstream, non-coding exon, described here as exon 0.

gene, which also shows alternative splicing involving other exons, resulting in a number of distinct gene products. The two receptors show greatest homology in the DBD (97% amino acid identity), followed by the LBD, and low homology in the remaining domains (Fig. 1.1A and [Mosselman et al., 1996]). The first human ERβ was $ER\beta_{477}$ [Mosselman et al., 1996]. *N*-terminal extended versions of 485 [Enmark et al., 1997] and 530 [Bhat et al., 1998] amino acids were subsequently reported. Early studies used different *N*-terminal forms of ERβ, leading to conflicting information on the activity of the receptor. Recently, an even longer form, $ER\beta_{548}$ has been identified from human testis [Wilkinson et al., 2002]. This version requires an A/T insertion in the 5'-UTR to bring an up-stream ATG into frame with the rest of the coding sequence and has been shown to have differing activity to $ER\beta_{530}$. However, a further study has shown that this is not a common mutation in a variety of populations (not found in 324 samples of various ethnic origins) so it may not be a significant variant [Xu et al., 2003]. $ER\beta_{530}$ is the most widely recognised "wild type", full-length form of the receptor and that is the form referred to here, unless otherwise stated.

1.4.1 ER isoforms

Various isoforms of the estrogen receptors may be expressed. For ERα these usually consist of various exon deleted forms, but ERβ uses alternative last exons [Hirata et al., 2003], as well as expressing exon deleted forms. These isoforms show distinct patterns of expression and have different properties in terms of ligand binding and activation of transcription.

Different ERα exon-deleted isoforms have been shown to be expressed at the mRNA level in breast cancers [Poola, 2003a], as well as in other tumours and tissues. Poola used Q-PCR to quantitatively measure expression of wtERα, ERαΔ2, ERαΔ3, ERαΔ2/3, ERαΔ4, ERαΔ5, ERαΔ6, and ERαΔ7 in a series of breast cancer cell lines and tumours, showing that wtERα was the most highly expressed in all cases, but that the levels of expression of the other isoforms was highly heterogeneous. Initially, expression of variant isoforms was not seen to correlate with clinical parameters [Zhang et al., 1996], although [Leygue et al., 2000] found that expression of ERαΔ3 was lower and ERαΔ5 was higher in ERα-positive breast tumours than in matched normal tissue. A variant known as ERC4, which is truncated after exon 2

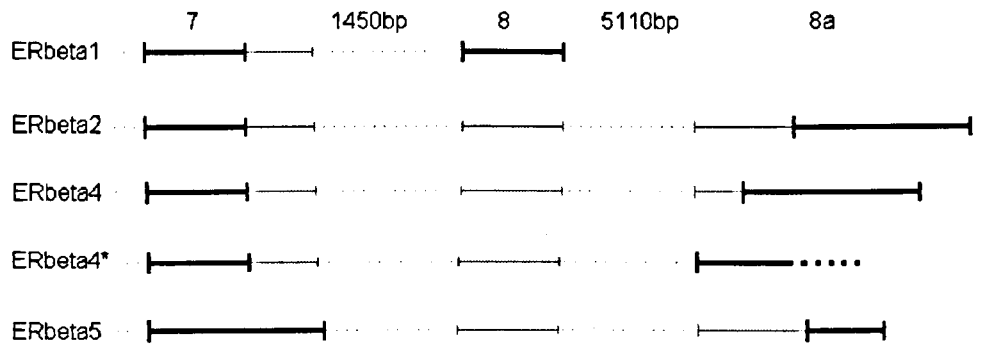


Figure 1.2 Exon usage of the *C*-terminal variants of ERβ

Several different variants of ERβ (ERβ1-5) have been reported, with different *C*-terminal exons. This figure shows how the terminal exons of several of these isoforms overlap on the genomic sequence. All isoforms share a common sequence up to exon 7. ERβ1 uses a unique exon 8, whilst the terminal exons of ERβ2, 4 and 5 all share some sequence identity, but use alternative splicing sites. ERβ3 (not shown) also uses a unique *C*-terminal exon 8, which is found approximately 142000 bp downstream of exon 8a. A novel variant, ERβ4*, which was identified when preparing standards for Q-PCR assays is included. As only an internal section of this isoform was sequenced, it is not known how far it extends in the 3' direction.

was also more highly expressed in ER/PR-positive tumours than their matched normal tissues.

ERβ has various *C*-terminal variants, of which at least five have been identified to date (ERβ1-5) [Moore et al., 1998]. These isoforms are identical to ERβ1 up to exon 7, where they diverge and use various alternatives for their last exon (Fig. 1.2), resulting in truncation of the receptor, with short isoform-specific stretches of protein at the *C*-terminal. They have distinct patterns of expression in tissues [Poola, 2003b] and tumours [Davies et al., 2004]. Exon deleted forms of ERβ have also been described, making the picture even more complicated [Lewandowski et al., 2002]. These include deletions of single (ERβΔ2, ERβΔ3, ERβΔ4) or multiple (ERβΔ2/5/6, ERβΔ5/6) exons. Deletion of exon 2 causes a frame-shift, leading to truncation within the A/B domain, whereas deletions of exon 3, 4 or 5 and 6 are in-frame deletions. Exon 3 deletion affects the DNA binding domain, exon 4 deletion affects the hinge and LBD regions, and 5/6 deletion affects the LBD (Fig. 1.1B). These exon-deleted forms also show distinct distributions, for instance, ERβΔ6 is less common in adenocarcinoma than in proliferative or secretory endometrium [Paul et al., 2004], suggesting that it may be down-regulated in carcinogenesis. Finally, combination variants with both alternative *C*-terminal domains and exon deletions are also expressed at the RNA level, for instance ERβ2Δ5, with both the exon 5 deletion and the ERβ2 *C*-terminal exon [Lewandowski et al., 2002].

Some ER isoforms have been shown to have function differently in the cell. Of the ERα single exon deletions, only ERαΔ5 and ERαΔ7 bind DNA, and then only weakly, and only ERαΔ3 binds estradiol. Whilst only ERαΔ5 showed any ability to activate transcription via an ERE, ERαΔ3 and ERαΔ5 both show dominant negative effects on wtERα [Bollig and Miksicek, 2000]. ERβ variants have also been shown to have different actions. ERβ2 (also known as ERβcx), for example, has been shown to exert a dominant negative role on ERα when heterodimers are formed [Ogawa et al., 1998] and ERβΔ5 has also been shown to exert a dominant negative effect on both ERα and ERβ [Inoue et al., 2000].

Much remains to be done to identify which of the many different variant splice forms of the mRNAs are actually expressed at the protein level, at what frequency and the actual function of these isoforms. Isoform specific antibodies are very hard to develop, as the different forms only vary in a small part of the structure. Specific

antibodies for the *C*-terminal variants ERβ1 and ERβ2 do exist, however, and have been used to show distinct distributions of the proteins in breast cancers and a poor correlation between mRNA and protein levels [O'Neill et al., 2004; Vinayagam et al., 2006].

1.5 Function of the estrogen receptors

The classical model of action for the steroid receptors is that inactive receptor is found bound to regulatory proteins, including hsp90, in the cytoplasm [Fliss et al., 2000; Pratt and Toft, 1997; Redeuilh et al., 1987; Sabbah et al., 1996]. Hormone enters the cells by diffusion and binds the LBD of the receptor, releasing the receptor from the regulatory complex and allowing dimerisation. The dimers then migrate to the nucleus, where they bind specific response elements found in the promoter regions of target genes and activate gene expression (Fig. 1.3 and [Klein-Hitpass et al., 1986]). The consensus estrogen response element (ERE) (5'-GGTCAnnnTGACC-3') occurs in the *Xenopus* vitellogenin A2 promoter, but most EREs, such as found in the pS2, vitellogenin B1, and oxytocin promoters, show some base changes, leading to different affinities for the ERs [Loven et al., 2001] and affecting coactivator binding [Hall et al., 2002]. Estrogen bound ER can also act through ERE 1/2 sites, such as is found in the progesterone receptor A (PRA) promoter [Petz and Nardulli, 2000], usually in combination with other regulatory domains such as SP1, AP1 or COUP elements.

However, the full picture is a lot more complicated than this classical model allows, involving different pathways for activation of the ERs (Section 1.5.3.1), ER acting via non-ERE sites and specific protein-protein interactions (Section 1.5.1 and Fig. 1.4) and non-genomic actions of the ERs (Section 1.5.3.2). As well as endogenous estrogen, the estrogen receptors can also bind other ligands. These can be classified as estrogens (those with similar effects to estradiol), antiestrogens (those with opposing effects to estradiol) and SERMs (Section 1.3.4), which show different responses depending on ER sub-type, target gene and cellular environment [Agnusdei and Iori, 2000; Kuiper et al., 1999; Monroe et al., 2005; Pole et al., 2005]. ERα and ERβ have been shown to respond differently to several antiestrogens and SERMs at various promoters (Section 1.5.1 and [Dutertre and Smith, 2000; Hall and

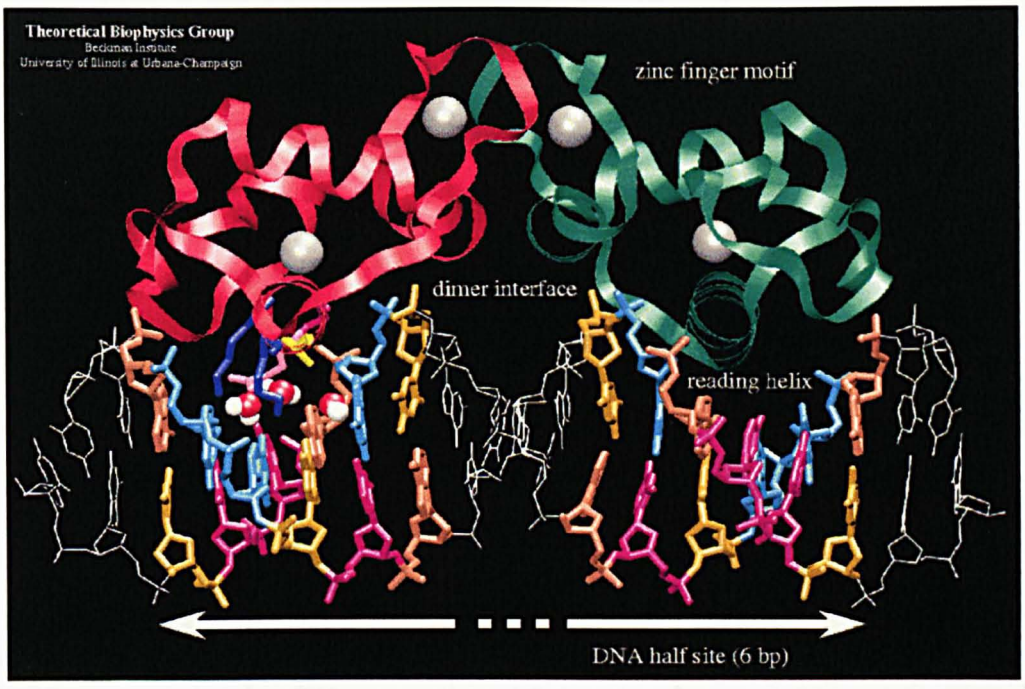


Figure 1.3 Structure of the ERα DBD bound to the ERE

ERα binds DNA as a dimer. The DNA binding domains of the two monomers are shown here as green and red ribbons. Zinc atoms involved in forming the zinc fingers of the DBD are shown in grey, and water molecules and specific amino acid side chains involved in contacting the DNA are shown in one half of the dimer. The consensus amino acids of the ERE are shown coloured.

This estrogen receptor image was made with VMD and is owned by the Theoretical and Computational Biophysics Group, NIH Resource for Macromolecular Modeling and Bioinformatics, at the Beckman Institute, University of Illinois at Urbana-Champaign [Bishop et al., 1997; Kosztin et al., 1997].

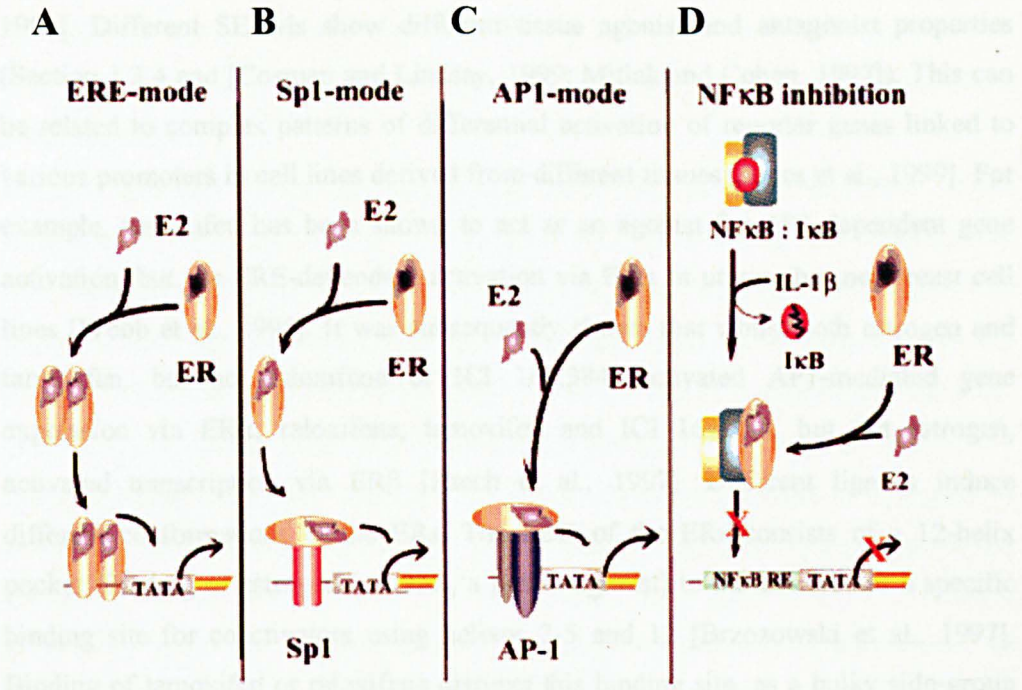


Figure 1.4 Regulation of gene transcription via the nuclear actions of the ER

Several of the ways in which estradiol-liganded ER can regulate gene transcription are shown. Classical activation of gene transcription via binding of the ERE is shown in panel A whereas panels B-D show indirect regulation of expression, via protein-protein interactions of the ER with other regulatory proteins, specifically SP1, AP1 and NFκB. Figure from [Nilsson et al., 2001].

McDonnell, 1999]) due to differences in their LBDs [Jacobs et al., 2003; Pike et al., 1999]. Different SERMs show different tissue agonist and antagonist properties (Section 1.3.4 and [Cosman and Lindsay, 1999; Mitlak and Cohen, 1997]). This can be related to complex patterns of differential activation of reporter genes linked to various promoters in cell lines derived from different tissues [Jones et al., 1999]. For example, tamoxifen has been shown to act as an agonist for AP1-dependent gene activation, but not ERE-dependent activation via ERα in uterine but not breast cell lines [Webb et al., 1995]. It was subsequently shown that whilst both estrogen and tamoxifen, but not raloxifene or ICI 164,384, activated AP1-mediated gene expression via ERα, raloxifene, tamoxifen and ICI 164,384, but not estrogen, activated transcription via ERβ [Paech et al., 1997]. Different ligands induce different conformations of the ERs. The LBD of the ERs consists of a 12-helix pocket. Binding of estrogen (or DES, a potent agonist) to the ER creates a specific binding site for coactivators using helixes 3-5 and 12 [Brzozowski et al., 1997]. Binding of tamoxifen or raloxifene disrupts this binding site, as a bulky side-group on the SERMs interferes with helix 12 positioning [Brzozowski et al., 1997; Pike et al., 1999], preventing binding of coactivators such as GRIP1 [Paige et al., 1999; Shiau et al., 1998]. The binding pockets of ERα and ERβ are slightly different, generating their ligand specificity. The pocket of ERβ is slightly smaller and there are some changes in the amino acids lining the pocket [Pike et al., 1999].

The ERs can also play a role in response to non-ligand chemicals, such as environmental contaminants, known as xenoestrogens [Ohtake et al., 2003]. Dioxins in the environment have been observed to have estrogenic effects. Dioxin binds the dioxin receptor (AhR), which forms a heterodimer with Arnt, another transcription factor. The dimerised receptor then interacts directly with either ERα or ERβ to recruit co-factors and activate transcription of estrogen responsive genes.

1.5.1 Alternative mechanisms by which the ERs may control transcription

The ERs do not act solely through binding to the ERE and activating gene transcription. They also interact with other proteins and DNA regions to affect gene transcription, often showing markedly different actions between the two receptors (Fig. 1.4). Both ERα and ERβ bind to the Fos-Jun protein dimer [Paech et al., 1997], which comprises a transcription factor that activates transcription from an AP1 site in

gene promoters. However, estradiol binding to ERα causes activation of transcription from AP1 promoters, whereas estradiol binding to ERβ inhibits such transcription. ERβ also shows much stronger activation in response to the SERM raloxifene at AP1 sites than ERα.

The ERs also bind the nuclear transcription factor SP1 [Saville et al., 2000]. In this complex, SP1 binds to GC-rich sites in promoter regions to activate transcription. Both ERs bind SP1, but only ERα-SP1 activates gene transcription in response to estradiol, although in certain cell lines some antiestrogens act as weak agonists for ERβ-SP1. Retinoic acid receptor α1 (RARα1) is activated by ERα-bound estradiol, or ERβ-bound antiestrogens via this pathway [Sun et al., 1998; Zou et al., 1999].

Many different promoters have been identified which are sensitive to estrogens or antiestrogens, but which lack an ERE [Jones et al., 1999]. These include promoters containing AP1 or SP1 sites as described above, but also other genes which do not show these motifs, for example, the human quinone reductase gene, transcription of which is activated in the presence of antiestrogens. Antiestrogens are bound by either ER, which then bind to an antioxidant response element (ARE) in the promoter region, as part of a protein complex. Activation is stronger via ERβ than via ERα, and estradiol inhibits activation of transcription of the quinone reductase gene [Montano et al., 1996; Montano et al., 1998; Montano and Katzenellenbogen, 1997]. The TGFβ3 gene is activated by SERMs and requires PKC and MAPK for activation [Lu and Giguere, 2001] and the interleukin-6 gene is down-regulated by estradiol via the ER, dependent on a region of the promoter containing a C/EBP site and a NF-κB site [Galien and Garcia, 1997; Ray et al., 1994].

1.5.2 Interactions with coregulatory proteins

When investigating the actions of steroid receptors, we must also consider the roles of transcriptional co-factors. The ERs interact with many coactivators and corepressors via the AF1 and AF2 regions, as well as by forming indirect associations [Klinge, 2000]. Many coactivators bind via helix 12 of the ligand-binding domain (AF2 region). These include members of the SRC/p160 family [McKenna et al., 1999], which act primarily by recruiting chromatin remodelling factors; the CBP/p300 coactivators [Kraus and Kadonaga, 1998], involved in histone acetylation, and the DRIP/TRAP complex, which may be involved in connecting the

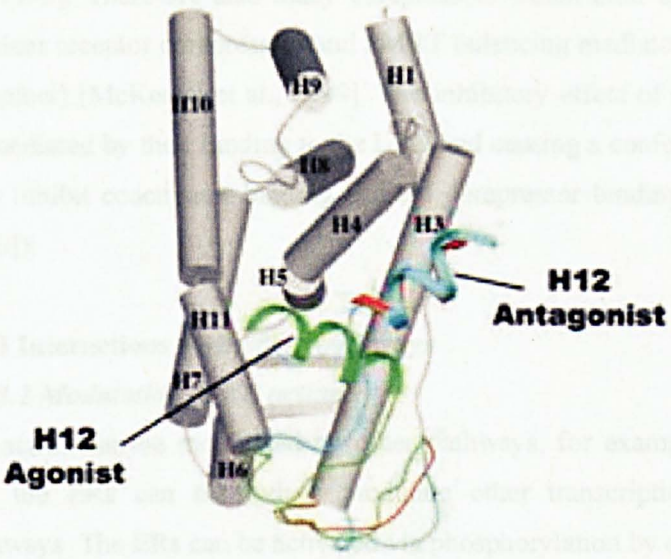


Figure 1.5 A comparison of the structures of the ERα LBD when bound to agonists and antagonists

Superposition of the three-dimensional structure of ERα LBD complexed with estradiol (green conformation), raloxifene and tamoxifen (red and blue conformations). Estradiol is the classical ligand of ERα, whereas raloxifene and tamoxifen are both SERMs. The orientation of helix 12 of the LBD (shown coloured) is important in binding coactivators. Figure from [Ruff et al., 2000].

nuclear receptors to the basal transcription machinery [Ito et al., 1999; McKenna et al., 1999]. There are also many corepressors which bind the ERs, such as N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid receptors) [McKenna et al., 1999]. The inhibitory effect of some antiestrogens may be mediated by their binding to the LBD and causing a conformational change which may inhibit coactivator binding and aid corepressor binding (Fig. 1.5 and [Klinge, 2000]).

1.5.3 Interactions with other pathways

1.5.3.1 Modulation of ER action

ER action can be modulated by other pathways, for example via phosphorylation, and the ERs can themselves modulate other transcription factors and cellular pathways. The ERs can be activated via phosphorylation by different kinases, such as mitogen-activated protein kinase (MAPK), at various sites. MAPKs are activated by a variety of growth factors and cytokines, such as IGF-1, EGF, FGFs and TNFα through the RAS-RAF pathway and have been shown to phosphorylate ser^{118} of ERα [Kato et al., 1995; Kato et al., 2000] and ser^{106} [Tremblay et al., 1999] of ERβ (Fig. 1.1) via this pathway. This phosphorylation allows the AF1 region to recruit co-factors such as p68 RNA-helicase [Endoh et al., 1999; Kato et al., 2000] and SRC-1, which aid in transcriptional activation. Phosphorylation of the AF1 region therefore mediates ligand-independent activation of the ERs as well as increasing the level of activation achieved on binding ligand.

Other pathways may also modulate ER action. ERα is reported to be dependent on Ca^{2+}-dependent binding of calmodulin for binding the ERE and activation of gene transcription [Li et al., 2005] and PKCδ can cause ERα nuclear translocation and activation, and increases transcription of an estrogen-dependent reporter gene [De Servi et al., 2005].

Another example of the interaction between different pathways is the interaction of the ER and NFκB pathways. Estrogen bound to both ERα and ERβ has been shown to inhibit the NFκB pathway at various points, by inhibiting IKK activity, inhibiting IκB degradation, competition with NFκB for coactivators and directly blocking NFκB binding to DNA (reviewed in [Kalaitzidis and Gilmore, 2005]). Conversely, there are some instances when ER and NFκB act cooperatively, for instance both

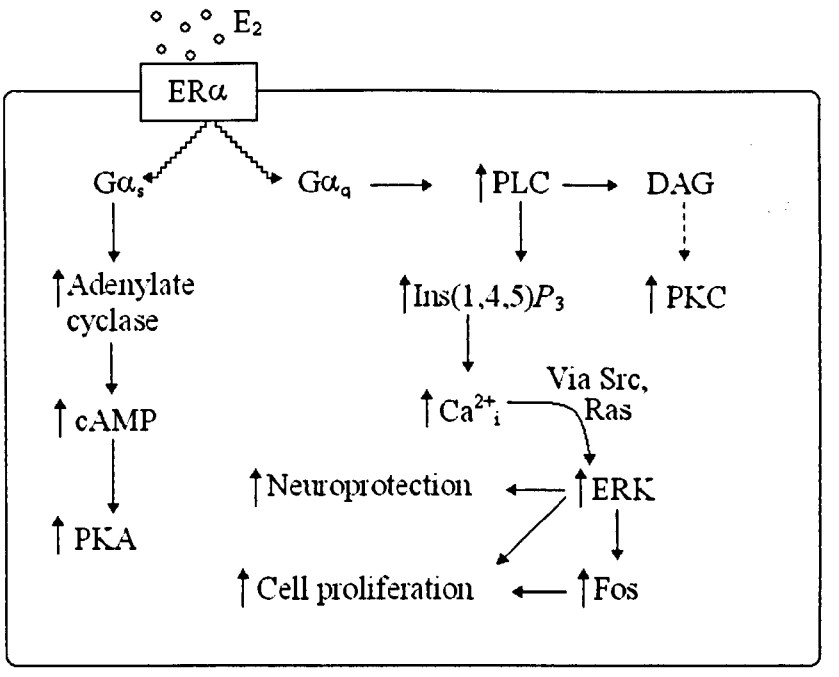


Figure 1.6 Downstream targets of mER action

Membrane bound ER (mER) may activate several signalling cascades on binding estradiol, as detailed here. Note that not all downstream targets are shown here, for instance, mER can also modulate Jun kinase. The dashed line indicates that diacyglycerol (DAG) indirectly leads to protein kinase C (PKC) activation. Figure from [Levin, 1999], with permission from Elsevier.

Additional abbreviations: Ca_i^{2+}, intracellular Ca^{2+}; ERK, extracellular signal-regulated protein kinase; Gαs/Gαq, G proteins; Ins(1,4,5)P_3, inositol (1,4,5) trisphosphate; PKA, protein kinase A; PLC, phospholipase C.

ERα and NFκB are required for the estrogen dependent up-regulation of the serotonin-1A receptor [Wissink et al., 2001].

1.5.3.2 Non-genomic roles of ER

Estrogen has a range of non-genomic actions (Fig. 1.6 and [Levin, 1999]), which may be modulated by the classical ERs, or alternative receptors, of which several have been identified [Toran-Allerand, 2004b]. The idea of a membrane receptor for estrogen is not a new one [Pietras and Szego, 1977]. Both ERα and ERβ have been shown to be localised in the membrane of cells [Pappas et al., 1995; Razandi et al., 1999], where they may initiate various signalling cascades such as activation of the MAPKs ERK1 and ERK2 via RAS-RAF [Migliaccio et al., 1996], which can lead to downstream activation of the nuclear ER, as well as stimulating cell growth, or activation of phospholipase C [Le Mellay et al., 1997], which can stimulate mobilisation of intracellular calcium stores and formation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. Estrogen, via membrane ER, has also been shown to stimulate nitric oxide synthesis [Haynes et al., 2000]. GPR30 is another membrane-bound protein associated with estrogen signalling. Initially, it was shown that there was a protein present in cell membranes, which bound estrogen with high affinity [Govind and Thampan, 2003]. This receptor was shown to be GPR30, a G-protein coupled receptor. Two different groups reported this new estrogen receptor and showed its affinity for estrogen, but reported different localisations [Revankar et al., 2005; Thomas et al., 2005].

Thomas *et al.* believe the protein is located in the cell membrane, as might be expected for such a signalling protein. They showed that GPR30 bound and was activated by both estradiol and antiestrogens, leading to G-protein activity and increased adenylyl cyclase activity. Revankar *et al.* reported GPR30 to be mostly localised to the endoplasmic reticulum, both by creation of fluorescence-tagged fusion proteins and using antibodies directed against native GPR30. Stimulation with estrogen in cells expressing GPR30 but not ERα led to mobilisation of intracellular calcium and synthesis of IP3 in the nucleus. As yet, it is unclear which of these models, or a combination of the two, is a true picture. What is clear, however, is that membrane bound ERs initiate specific and complex signalling events, which act in concert with the actions of the classical nuclear receptor [Levin, 1999], although the exact mechanisms involved may not be fully elucidated.

1.6 *In vitro* and *in silico* investigation of the role of the ERs in breast cancer

Estrogen and the estrogen response via the ERs are closely linked with breast cancer development and treatment. The action of the ERs is complex and not fully elucidated, involving interactions with many different signalling systems. Different types of study can be used to gain information about different aspects of ER signalling. Epidemiological studies can yield information about links between ER expression and breast cancer development (Section 1.2.3, 1.2.4), but cannot shed light on the mechanisms behind the links. The use of transgenic or knock-out mice suffers from a similar problem, in that an animal is a complex network of systems, and identifying the factors involved in a specific phenotypic change may be difficult. In fact, different knock-out mice for ERβ show different phenotypes [Harris, 2006], suggesting that the use of different mouse strains, different methods of generating the knock-out, or different environments may considerably affect the resulting phenotype of the knock-out (Section 1.3.5).

Individual tumours are unique in both phenotype and genotype. In order to identify factors involved in a specific aspect of cancer development, large numbers of tumours must be studied, in order to identify common factors. Studies using microarrays allow average expression in a large group of tumours with similar phenotypes to be pooled; allowing genes whose expression is associated with particular phenotypes, for instance ER status, to be identified. Such lists of genes can be used to group other tumours into phenotypic groups, or as candidate genes for investigation of tumour development pathways (Section 1.6.1). Similar experiments may be performed using cell lines, but in this case individual cell lines representing specific tumour types may be used. A study by [Liu et al., 2002a], for instance, used suppression subtraction hybridisation to identify genes differentially expressed between a benign (Huma 123) and a malignant (MCF7) human breast cancer cell line to identify genes which might be involved in the transition between these two phenotypes.

Use of cell lines can have several advantages over working with tumour samples. The first of these is that large, fresh samples may be obtained, yielding good quality

RNA, DNA and protein for analysis. Additionally, tumour samples are often heterogeneous, containing both cancerous cells, infiltrating blood cells and adjacent normal tissue, including reactive stroma, making it harder to distinguish expression patterns, without specifically isolating the tumour cells, for instance by laser capture micro-dissection. Cell lines can also be manipulated in a manner that is impossible with *in vivo* tumours (except by therapeutic treatments). Thus cell growth medium may be adjusted to vary the levels of estrogen or to mimic therapeutic treatments and cells can be treated with drugs such as actinomycin D or cycloheximide, to identify responses that are reliant on mRNA or protein synthesis, respectively. Gene expression may be controlled by transfection with expression vectors or RNAi, to investigate the effects on the cells of changing the expression of a single gene (Section 1.6.2) and reporter genes can be used to investigate the roles of particular promoter domains. The main disadvantage of using cell lines is that they are not growing in a normal environment and, therefore, may behave differently than native tumours. For instance, cell cultures are often grown as a monolayer, whereas tumours are three-dimensional.

Many different methods can be used to investigate the relationships between breast cancer and estrogen signalling. Gene expression analysis is a useful tool, as it allows genes and pathways involved in specific processes to be elucidated.

1.6.1 Gene expression analysis of tumours and cell lines

In order to identify genes related to endocrine sensitivity in tumours, and hence likely to be controlled by the ERs, several groups have used microarrays to compare gene expression in a selection of ER-positive and negative tumours. This has allowed a number of sets of genes to be identified which predict ER status or estrogen responsiveness of tumours, or even their likely response to therapy [Hayashi, 2004; Nagai et al., 2004]. Such work requires comparison of gene expression profiles across a large panel of tumours, as each tumour is unique and shows a different pattern of gene expression. Comparing different sets of tumours, such as ER-positive and negative tumours, or tumours which show different responses to therapy, allows possible target genes involved in these responses to be elucidated, but does not specifically identify genes directly controlled by the ERs, or the mechanism by which activation occurs.

Breast cancer-derived cell lines can be used as a model system to represent tumours in expression studies. This approach allows changes in gene expression to be

measured in a defined signalling background and subjected to known stimuli. Cells can be treated with or without estradiol or other treatments and changes in gene expression of known target genes measured, or new targets identified using SAGE or microarrays. One such study used microarrays to investigate changes in gene expression in MCF7 cells treated with or without estradiol for 0 to 48 h [Wang et al., 2004]. They found 55 ESTs to be up-regulated by estrogen stimulation and 38 to be down regulated, a relatively small number considering the 19000 ESTs on the array. The report lists the 25 most highly up-regulated genes, including PS2 and XBP1, but not PR.

1.6.2 Breast cancer cells transfected for ERβ expression

ERβ is believed to play a role in breast cancer development, but it is, as yet, unclear as to what that role may be (Section 1.2.4). Transfected breast cancer cell line systems can be used to investigate the role of ERβ in breast cancer by comparing estrogen responses in ERβ-negative and ERβ-positive environments.

Using transformed cell lines allows expression of a single gene to be controlled and the effects of its expression on the cells to be examined. These effects may include changes in cell proliferation and growth response, as well as changes in downstream transcriptional events. Changes in expression of individual reporter genes or endogenous gene expression can be assayed or global changes in expression examined using microarrays. Such studies are useful in the elucidation of ER action. Responses to estradiol or other treatments can be compared using similar cells expressing different levels of ER to discover the targets of the ER.

1.6.2.1 Expression of ERα or ERβ1 in MDA-MB-231

MDA-MB-231 cells have been frequently used as an "ER naïve" cell line. Their low ER expression levels make them a useful tool for studying the effects of exogenous ER expression. In one study, in order to compare the effects of ERα and ERβ, MDA-MB-231 were transfected with either ERα (previously reported in [Lazennec and Katzenellenbogen, 1999]) or full-length ERβ1 using an adenovirus-mediated system [Lazennec et al., 2001]. Both ERα and ERβ1-expressing cells activated TGFα, p21 and PS2 transcription on estradiol stimulation, but stimulation was 2-3 fold less via ERβ1 than ERα. ERα expressing cells showed an almost complete loss of c-myc expression on estradiol treatment, which was not seen in the ERβ expressing cells.

The two ERs also had different effects on cell growth. Parental cells showed no change in proliferation on addition of estradiol. ERα expressing cells showed increased proliferation in response to estradiol, whereas ERβ expressing cells showed decreased basal proliferation and no response to estradiol.

1.6.2.2 Expression of ERα or ERβ1$_{485}$ in MDA-MB-231

In another study, MDA-MB-231 cells, characterised as ERα and ERβ negative, were stably transfected to express 300 to 1600-fold higher ERβ1$_{485}$ than parental cells [Tonetti et al., 2003]. This group had also previously prepared similar cells to express ERα [Jiang and Jordan, 1992]. They found that cells showing increased ERβ$_{485}$ expression had increased basal proliferation, contrary to other reports in MDA-MB-231 and other cell lines [Lazennec et al., 2001; Paruthiyil et al., 2004; Strom et al., 2004]. The response to over-expression of ERβ might be expected to vary between cell lines, but it is harder to explain the differences between the reports of Tonetti and Lazennec. However, the two groups did use different forms of ERβ (ERβ$_{485}$ and ERβ$_{530}$), which may explain this discrepancy. Alternatively, the history of the cells used by the two groups may have been different in terms of passage number or culture conditions. In agreement with [Lazennec et al., 2001], estradiol treatment had no effect on growth rate in ERβ over-expressing MDA-MB-231 cells. Antiestrogens have previously been shown to activate AP1-dependent transcription when bound to ERβ [Paech et al., 1997]. However, in these cells, neither tamoxifen nor ICI 182,780 was able to activate a transiently transfected AP1-luciferase reporter plasmid. This may be due to the lack of other coregulatory proteins in the MDA-MB-231 environment. TGFα expression can be activated by ERα-bound estradiol or tamoxifen in MDA-MB-231 transfected with ERα [Levenson and Jordan, 1998]. Activation via tamoxifen requires helix 12 and AF1 regions to be functional [Liu et al., 2001]. TGFα was induced by estrogen but not tamoxifen in ERβ$_{485}$ expressing cells, probably reflecting the absence of an active AF1 domain in ERβ$_{485}$.

1.6.2.3 Expression of ERβ1$_{485}$ inT47D

Another study used a tetracycline-repressor based system to control inducible expression of tagged ERβ1$_{485}$ in T47D cells (an estrogen responsive, ERα expressing breast cancer cell line) [Strom et al., 2004]. They increased expression of ERβ$_{485}$ to roughly equal levels to ERα and again showed greatly decreased proliferation in

response to estrogen and corresponding changes in the estrogen response of several key cell-cycle proteins, such as a reduced response in cyclin E and cdc25A and increased levels of p27. However, it is possible that these changes in expression may be indirectly caused by arrest of the cells in G1, rather than reflecting direct transcriptional responses to ERβ. Interestingly, and in contrast to the other effects on cell cycle markers and proliferation, cyclin D1 showed earlier and greater increase in expression in response to estradiol in the presence of ERβ, supporting the notion that that ERβ, when compared to ERα, does not have a purely negative role.

1.6.2.4 Expression of ERβ1 in MCF7

Other groups have also used the approach of increasing ERβ expression in cells that already express significant levels of ERα. An adenovirus-mediated expression of full-length ERβ1 in MCF7 (which is normally ERα-positive) has been used by one group to investigate what changes this may cause [Paruthiyil et al., 2004]. Similar to the effects seen in T47D cells, ERβ1-expressing cells showed a decrease in proliferation, with 4-fold more cells in G_2-M phase than seen in control cells. Concurrently, ERβ1-expressing cells showed decreased expression of cyclin A and increased expression of p21 and p27 compared to control cells. These cells also showed reduced induction of cyclin D1 and c-myc on addition of estradiol, in contrast to the response observed in T47D [Strom et al., 2004].

1.6.2.5 Expression of ERβ1 in MCF7

Another group have used a tetracycline-based inducible system to express full-length, tagged ERβ1 in a controlled manner in MCF7 cells [Murphy et al., 2005]. Initial results using this system suggested that ERβ1 expression leads to reduced proliferation on estradiol stimulation, but that ERβ1 expression did not inhibit PR expression, when measured by PCR, showing that ERβ1 did not simply act as an ERα antagonist. Interestingly, ERβ1 over-expression led to growth stimulation by low levels of tamoxifen, but an increased sensitivity at higher levels, supporting the observation that ERβ1 expression may be associated with sensitivity to tamoxifen treatment [Hopp et al., 2004; Murphy et al., 2002; O'Neill et al., 2004]. It was noted that treatment with estradiol or ICI 182,780 decreased the half-life of both ERα and ERβ1 proteins, whereas treatment with tamoxifen stabilised the ERβ1 protein.

1.6.2.6 Expression of ERβ1 or ERβ2 in MCF7

A single study has investigated the different effects of ERβ1 and ERβ2 on ERα-positive cells [Omoto et al., 2003]. MCF7 cells were stably transfected with either ERβ1 or ERβ2 expression plasmids. In both cases transfection led to decreased growth, decreased ERE reporter activation and decreased estradiol-dependent induction of endogenous cathepsin D and IGFBP4 expression in the clones compared to mock transfected or parental cells. A custom microarray, based on genes which showed estradiol response in MCF7 cells [Inoue et al., 2002], was used to compare the estrogen responses in MCF7 and ERβ1 and ERβ2 transfected cells after 72 h with or without estradiol. MCF7 cells showed about 2/3 of the gene-set to be up-regulated by estradiol and 1/3 down regulated. ERβ1-expressing cells showed a similar pattern, but with some reduction in the levels of response. ERβ2-expressing cells showed the most marked difference in expression patterns, with 16 genes previously down-regulated showing no change or increasing and 12 genes which were previously up-regulated showing no change or decreasing. These results suggest that, in fact, ERβ2 may have the more important role in modulating ERα-dependent expression.

1.6.2.7 Discussion

These studies give various types of information about the role of ERβ in breast cancer cells. Most suggest that cells over-expressing ERβ show reduced proliferation, and were less likely to show increased proliferation in response to estradiol [Lazennec et al., 2001; Murphy et al., 2005; Omoto et al., 2003; Paruthiyil et al., 2004; Strom et al., 2004], suggesting that ERβ might be a growth inhibitor in breast cancer, and hence associated with a good prognosis [Jarvinen et al., 2000; Roger et al., 2001]. However, this response may not correspond to that of native ERβ. It has been noted that exogenous expression of ERα often leads to growth inhibition in response to estradiol, whereas in cells expressing native ERα, estradiol treatment increases proliferation [Levenson and Jordan, 1994]. A similar mechanism may occur here, although the use of different cell lines, some of which already express ERα and some ERβ, may make it more likely that this response corresponds to the normal actions of ERβ.

These studies show that ERβ does not act simply through inhibition of ERα action. ERβ up-regulates some genes in response to estrogen [Lazennec et al., 2001], usually

to a lower level than ERα. However, one group found that ERβ expression increased the estrogen response of cyclin D1 in cells already expressing ERα [Strom et al., 2004]. Using a microarray to study multiple genes at once, [Omoto et al., 2003] showed that expression of ERβ1 and ERβ2 produce distinct responses to estradiol, which differ from the response of cells expressing only ERα. Overall, these results suggest that ERβ regulates a distinct, but overlapping set of targets to ERα and has a precise role to play in the estrogen response of breast cancer cells.

It is important to note that in all these studies, expression of ERβ was up-regulated to levels similar to those of ERα. However, ERβ expression in breast cancers, as well as in other tissues and cell lines, is usually much lower than that of ERα [Knowlden et al., 2000]. It is, therefore, quite possible that the effects of ERβ expression observed in these studies, notably the impact on cell proliferation, may be the effect of abnormally high levels of ERβ, and do not reflect their roles in normal cells. Different groups have used different forms of ERβ ($ERβ1_{485}$, $ERβ1_{530}$, tagged-ERβ1), and different systems for its expression. These may give different responses, even when the same cell line is used (Section 1.6.2.2, 1.6.2.4), making it harder to define the exact role of ERβ.

1.7 Aims of the project

I have described the functions and roles of the estrogen receptors in estrogen response and breast cancer. It can be seen that gaining further understanding of the functions of ERβ is essential to understanding hormone responsive breast cancers and that there are key questions which can be answered using a cell-line based approach.

This thesis aims to investigate the effect of different ratios of the estrogen receptors on gene expression. First, the levels of estrogen receptor expression and the estrogen response in a variety of breast cancer cell lines will be investigated by Q-PCR (Chapter 3). Subsequently, an MCF7 cell line (estrogen responsive via ERα) will be engineered to express ERβ1 in an inducible manner to allow manipulation of the ERα/β ratio in these cells (Chapter 4). Using this cell line, it will be possible to observe the effects of different ERβ1 levels on gene expression (Chapter 5).

Measurements of the expression of individual genes, including the estrogen receptors and estrogen responsive genes, will be made using real-time, quantitative RT-PCR and various antibody based approaches to measure the effect of changing ERβ1 levels on the estrogen response.

Chapter 2 Materials and methods

2.1 Materials

2.1.1 General materials

General laboratory chemicals were obtained from Sigma or BDH.

4-hydroxy tamoxifen	Sigma H6278
12% precise protein gels	Pierce PIER25242
17β-estradiol	Sigma E2758
β-mercaptoethanol	Sigma M3148
Activated charcoal	Sigma C9157
Agar	Sigma (Fluka BioChemika) 05039
Ampicillin	Invitrogen A9393
Antigen unmasking solution	Vector H-3300
Autoseq plates	Amersham Biosciences 27-5340-10
Balanced Hank's salt solution	Invitrogen 14025-050
Blasticidin S HCl (50mg)	Invitrogen R210-01
Bradford micro protein assay	Bio-Rad 500-0006
BupH Tris-HEPES-SDS running buffer	Pierce 28398
Crystal mount aqueous mounting medium	Sigma C0612
Dextran	Amersham Biosciences 17-0280-08
Dimethyl sulphoxide (DMSO)	Sigma D7941
Doxycycline hyclate (1g)	Sigma D9891-1g
Dulbecco's modified Eagle's medium (high glucose)	Invitrogen 41966-029
DMEM without phenol red	Invitrogen 11880-036
DNase I (inc. 10x buffer and EDTA)	Invitrogen 18068-015
dNTPs (GeneAmp)	Applied Biosystems N-8080007
DYEnamic ET dye terminator reagent	Amersham Biosciences 9381096
Ecdysone-Inducible Mammalian Expression System	Invitrogen K1001-01
ECL advanceWestern blotting kit	Amersham Biosciences RPN 2135
Ethidium bromide	Sigma E2515
ExoSAP-IT	Amersham Biosciences US78202
External well factor solution	Bio-Rad 170-8794
Foetal bovine serum (EU approved)	Invitrogen 10270-106
G418 sulphate (geneticin) 50 mg/mL	Invitrogen 10131-019
HotStar *Taq* polymerase (inc. 10x buffer and $MgCl_2$)	Qiagen 203205
Hybond P membrane	Amersham Biosciences RPN2020F
Hyperfilm ECL	Amersham Biosciences RPN2103K
ICI 182,780	Gift from: Dr. A. E. Wakeling, Zeneca Pharmaceuticals (Macclesfield, U.K.)
Insulin (from bovine pancreas)	Sigma I5500
IPTG	Sigma I6758
iQ Supermix	Bio-Rad 170-8864
iQ SYBR green Supermix	Bio-Rad 170-8884
Kanamycin	Invitrogen 60615

Kodak LX24 developer	Axis healthcare 507 0933
Kodak AL 4 fixative	Axis healthcare 507 1071
Lab-tek 8-well permanox chamber slides	Nunc 177445
Laemmli sample buffer	Bio-Rad 161-0737
L-glutamine 200 mM	Invitrogen 25030-024
LSAB2 system inc. DAB chromogen	Dako K0675
Maxisorp plates	Nunc DIS-971-030J
NAP Blocker	GenoTech 786-190
Newborn calf serum (heat-inactivated)	Invitrogen 26010-074
Nonident P40 substitute	Sigma (Fluka BioChemika) 74385
NR Sandwich ERα ELISA	Active Motif (49296)
Nuclear extract kit	Active Motif (40010)
Oligotex mRNA Mini Kit	Qiagen 70022
Penicillin/streptomycin 100x solution	Invitrogen 15070-022
Phosphate buffered saline tablets	Sigma P4417
Ponasterone A (1 mg)	Invitrogen H101-01
Ponceau S staining solution	Sigma p7170
Primers and probes (DNA oligos)	Oswell/ Eurogentec
Prime RNase Inhibitor	Eppendorf 955154312
Protease arrest	GenoTech 786-108
QIAfilter Plasmid Midi Kit	Qiagen 12245
QIAprep 96 Turbo miniprep kit	Qiagen 27191
QIAprep spin miniprep kit	Qiagen 27104
QIAquick PCR Purification Kit	Qiagen 28104
Quik-pik electroelution capsules	Stratagene 400855
Restriction enzymes and buffers	New England Biolabs
RNase ZAP	Sigma R2020
RNeasy mini kit	Qiagen 74106
SeaKem agarose	Cambrex 50004
Sigma spin 96 well post reaction clean-up plates	Sigma S4309
SoloPack gold competent *E. coli*	Stratagene 230325
SuperScript II RT enzyme (inc. 5x first-strand buffer and 100 mM DTT)	Invitrogen 18064-014
Sybr Green (10,000x in DMSO)	Invitrogen S7563
Sypro ruby protein gel stain	Cambrex 50562
T4 DNA ligase and buffer	New England Biolabs M0202
Tet-free serum	BD Clontech 631106
TMB liquid substrate system	Sigma T8665
Top10F' *E. coli.*	Invitrogen c3030-03
TOPO-TA cloning kit inc SOC medium, pCR2.1-TOPO vector and Top 10F' *E. coli*	Invitrogen K4550-01
TransPEI transfection reagent	Eurogentec TR-0101-05
T-REx system	Invitrogen K1020-01
Triton x100	Sigma T8787
Trypsin-EDTA (0.05 % (w/v) Trypsin, 0.53 mM EDTA.4Na)	Invitrogen 25300-054
Tryptone	Sigma (Fluka BioChemika) 95039
Westran clear signal PVDF	Schleicher and Schuell 10485289
X-gal	Sigma (Fluka BioChemika) 16664

Yeast extract	Sigma (Fluka BioChemika) 70161
Zeocin	Invitrogen R250-01

2.1.2 Cell lines

MCF7, MDA-MB-231, T47D, ZR75	European Collection of Animal Cell Culture (Porton Down, U.K.)

2.1.3 Plasmids

pCR2.1-TOPO	Invitrogen from K4550-01
pIND, pVgRXR, pIND/ERβ	Invitrogen from K1001-01
pcDNA6/TR, pcDNA4/TO, pcDNA4/TO/ERβ1	Invitrogen from K1020-01

2.1.4 Size markers for DNA and protein

1kb DNA ladder	New England BioLabs N3232
100bp DNA ladder	New England BioLabs N3231
φx174 DNA- HaeIII digest	New England BioLabs N3026
lambda DNA- HindIII digest	New England BioLabs N3012
MagicMark XP Western protein standards	Invitrogen LC5602
ProSieve protein markers	Cambrex 50547
ProSieve color protein markers	Cambrex 50550

2.1.5 Recombinant proteins and antibodies

Anti-ERα mouse monoclonal (F-10)	Santa Cruz SC8002
Anti-ERβ1 rabbit polyclonal (PA1-313)	Affinity Bioreagents PA1-313
Anti-ERβ1 mouse monoclonal (PPG5/10)	Serotec MCA1974
Anti-β-actin mouse monoclonal	Biovision 3598-100
Anti-mouse IgG, HRP-linked, whole AB	Amersham Biosciences NXA931
Anti-rabbit IgG, HRP-linked, whole AB	Amersham Biosciences NA934
rERα (66kDa)	Invitrogen (Panvera) p2187
rERβ1L (59.2kDa)	Invitrogen (Panvera) p2718
rERβ1S (54kDa)	Invitrogen (Panvera) p2466

2.2 Cell culture

2.2.1 Cell culture media

2.2.1.1 Basic media

Cell culture medium was based on Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 5% (v/v) foetal calf serum (FCS), 50 units/mL penicillin, 50 μg/mL streptomycin and 2 mM L-glutamine (all Invitrogen, Paisley, U.K.). MCF7 and ZR75 were grown in this standard medium with 50 ng/mL insulin (Sigma,

Gillingham, U.K.) and 10 nM estradiol (Sigma). MDA-MB-231 and T47D were grown in standard medium with 50 ng/mL insulin.

2.2.1.2 Withdrawal medium and experimental treatments

Cells were withdrawn from estrogenic stimulation by growing in DMEM without phenol red (Invitrogen), supplemented with 5% (v/v) dextran coated charcoal treated serum (DCCS) (Section 2.2.2), 50 units/mL penicillin, 50 μg/mL streptomycin and 2 mM L-glutamine. Medium was changed daily throughout the withdrawal period. After withdrawal, cells were treated with withdrawal medium supplemented with or without 10 nM estradiol, 1 μM tamoxifen or 1 μM ICI 182,780 for 24 h as indicated in the figure legends.

2.2.1.3 Medium for MCF7 cells transfected with the ecdysone inducible system

MCF7 cells transfected with the ecdysone inducible system were grown in MCF7 medium with the addition of appropriate concentrations of zeocin (Invitrogen) and G418 (Invitrogen) (Section 4.1.1.1), the antibiotics required to maintain the transgenes. MCF7-B1 to MCF7-B13 clones used 750 μg/mL G418 and 600 μg/mL zeocin; this was subsequently reduced to 400 μg/mL of each antibiotic for later experiments. Ponasterone A (Invitrogen) was added to the medium (1-15 μM) for induction of the expression system.

2.2.1.4 Medium for MCF7 cells transfected with the T-REx system

MCF7 cells transfected with the T-REx system were grown in standard MCF7 medium, supplemented with zeocin and blasticidin (Invitrogen) to maintain the transgenes (Section 4.1.1.2). Cells were treated with at least 200 μg/mL zeocin and 1.5 μg/mL blasticidin in order to maintain selection. Tetracycline-free FCS (BD Clontech, Oxford, U.K.) was used throughout, including for the preparation of DCCS (Section 2.2.2). Induction of this system was achieved by the addition of 10-1000 ng/mL doxycycline (Dox) (Sigma).

2.2.2 Preparation of dextran-coated charcoal treated serum

This treatment removes lipophilic material, reducing the serum concentration of hormones such as estradiol, progesterone, cortisol and testosterone [Coezy et al., 1984; Seaver et al., 1984]. There are two steps to the preparation of DCCS, preparing the charcoal solution and treatment of the serum.

2.2.2.1 Preparation of DCC

To prepare dextran-coated charcoal suspension (DCC), 25 g activated charcoal (Sigma) was resuspended in 200 mL 10 mM Tris buffer (pH 8.0) and centrifuged at 1000 rpm for 10 min to remove fine particles before discarding the liquid phase. The charcoal was washed in this way twice more, resuspending the charcoal in fresh buffer each time. The charcoal was finally resuspended in 200 mL Tris buffer, transferred to a 300 mL Duran bottle, and 2.5 g Dextran 70 (Amersham Biosciences, GE Healthcare, Chalfont St Giles, U.K.) added. This was mixed gently using an electric stirrer for 15-30 min, at room temperature, before autoclaving. The DCC can be stored at 4°C for up to 1 year.

2.2.2.2 Treatment of serum

New-born calf serum (500 mL, NCS) (Invitrogen) was treated with DCC to remove steroids. The stock DCC suspension must be thoroughly resuspended before use. A 10 mL aliquot of DCC suspension was transferred to a 50 mL falcon tube, pelleted by centrifugation (1000 rpm, 4°C, 10 min) and the supernatant discarded. The pellet was resuspended into 500 mL NCS, which was incubated overnight at 4°C stirring gently with a magnetic stirrer. DCC was removed by centrifugation (3000 rpm, 20 min, 4°C). A further 10 mL aliquot of stock DCC suspension was pelleted and the supernatant discarded. The pelleted DCC was added to the serum and the serum incubated at 56°C for 40 min in a shaking water bath. This was cooled to 4°C before removal of the DCC by centrifugation (repeated centrifugation at 5000 rpm, 2-5 min, 4°C). When no further pellet was obtained on centrifugation, the serum was sterilised by filtering through a 0.45 μm filter, then a 0.20 μm filter and stored in 25 mL aliquots.

2.2.3 Maintenance of cell cultures

The cell lines used in this project were MCF7, MDA-MB-231, ZR75 and T47D (Section 3.1.2). These are all breast cancer epithelial cell lines. All cell culture used sterile, disposable plasticware, and was maintained in a humidified incubator at 37°C, 5% (v/v) CO_2. Growth medium was changed every 3-4 days, which included a PBS (Sigma) wash of the cells.

2.2.3.1 Passaging cells

Cells were passaged when ≥80% confluent, and divided 2-6 fold, depending on the cell line and requirements. Medium was removed and the cells were washed with PBS. Trypsin-EDTA (Invitrogen) (1 mL per 25 cm^2) was added, to cover the cells, and the flask was placed in the incubator for 3-5 min, by which time most of the cells had detached from the substratum. A volume of medium at least equal to the volume of trypsin-EDTA was added to the cells to inactivate the trypsin. Cells were transferred to a 25 mL universal for centrifugation (1400 rpm, 5 min). The cell pellet was resuspended in a small volume of medium and aliquots were placed into flasks containing a suitable volume of medium.

Cells were occasionally passaged directly, without centrifugation. In this case, after incubation with trypsin-EDTA, serum-containing media was added to the cells to inactivate the trypsin prior to dividing the cell suspension into flasks as above.

2.2.4 Preparing and using frozen cell stocks

2.2.4.1 Freezing cells

Frozen stocks of cells were prepared throughout the project. A 75 cm^2 tissue culture flask containing cells at 80% confluence was routinely used to prepare three cryovials of stock cells. To prepare stocks, two types of medium (I and II) were prepared as below (volumes are per two flasks or six cryovials to be frozen).

I 4 mL medium, 1 mL FCS

II 3.1 mL medium, 1 mL FCS, 0.9 mL DMSO (Sigma)

Cells in a 75 cm^2 flask were washed with PBS, trypsinised and pelleted by centrifugation. Cells were resuspended in 2.5 mL medium I. A further 2.5 mL medium II was added and mixed by pipetting. The cell suspension was pipetted into cryovials (1.5-1.8 mL per vial) and cooled gradually by freezing on ice for 1 h, freezing in a -80°C freezer overnight then transferring to liquid nitrogen for long-term storage.

2.2.4.2 Thawing frozen stocks

To thaw stock cells, 10 mL standard medium and 10 mL medium supplemented to 20% FCS were prepared. A cryovial of cells was thawed at 37°C, and cells were gently pipetted into 10 mL 20% FCS-containing medium. This was centrifuged at 1400 rpm for 5 min before the cell pellet was resuspended in 5 mL standard medium and added to a 25 cm^2 tissue culture flask containing 5 mL standard medium.

2.2.5 Transfection and clone selection

Transient and stable transfections of MCF7 cells were performed using both the Ecdysone inducible system (Section 4.1.1.1) and the T-REx system (Section 4.1.1.2). Transfections were performed using TransPEI transfection reagent (Eurogentec, Southampton, U.K.), a cationic polymer transfection reagent.

Cells were transfected in either 6 cm or 10 cm diameter dishes, as required; volumes of reagents are given for both conditions in this order. Sufficient cells were plated out to give a 50-60% confluent culture 24 h later. On the day of transfection, the medium was replaced with 5 mL (for a 6 cm plate) or 10 mL (for a 10 cm plate) fresh, serum-containing, medium. Vector DNA (5 or 7.5 μg) was diluted into 250 μL or 500 μL 150 mM NaCl solution, vortexed gently and centrifuged for 10 s at 13000 rpm. Ten μL or 15 μL TransPEI reagent was diluted into 250 μL or 500 μL 150 mM NaCl solution and also mixed and cleared by vortex and centrifugation. The TransPEI solution was added to the DNA solution by pipetting, and the mixture again mixed and cleared before incubation at room temperature for 15-30 min. The TransPEI/DNA mixture was added drop-wise onto the medium in each plate, and the plate gently mixed by swirling. After 2 h, an additional 5 mL or 10 mL serum-containing medium was added to the cells. Antibiotic selection for stable transfectants was started 24 or 48 h after transfection (Section 4.3.4, 4.4.3). At 24 h, the cells were usually passaged into 40 24-well plates, which is equivalent to a 1/10 dilution. Selection medium (0.5 mL per well) was changed at intervals of up to 7 days, until single-cell colonies began to form.

2.3 DNA Cloning

2.3.1 Preparation of media and agar plates

E. coli were grown on Luria-Bertani (LB) agar plates for individual colonies or in LB broth for plasmid preparations. Kanamycin or ampicillin selection used standard broth, but low-salt broth was required for zeocin selection. Medium recipes were as follows [Sambrook et al., 1989].

For 1 litre of LB Broth, 10 g bacto-tryptone, 5 g yeast extract and 10 g NaCl were added to 950 mL de-ionised water. This was stirred until dissolved and the pH

adjusted to 7.0 with NaOH, before making the volume up to 1 L. This was then autoclaved before the addition of antibiotics. Low salt medium used only 5 g NaCl per litre, and the pH was increased to 7.5.

Agar plates were prepared by adding 15 g agar per litre of broth before autoclaving. Antibiotics were added when the agar cooled to below 60°C, and the plates were poured aseptically.

Blue-white colour selection using the TOPO-TA cloning kit and TOP10F' cells (Invitrogen) required the addition of 40 μL X-gal (5-bromo-4-chloro-3-inodyl-beta-D-galactoside 40 mg/mL) in DMF (dimethyl formamide) and 40 μL IPTG (isopropyl thio-beta-D-galactosidase 100 mM) to each plate. These were spread onto plates immediately prior to use. Ampicillin selection was performed at 50-100 μg/mL, kanamycin was used at 50 μg/mL and zeocin was used at 25 μg/mL.

2.3.2 Bacterial transformation

Plasmids were amplified in solopack gold *E. coli* (Stratagene, Amsterdam, The Netherlands) or Top10F' *E. coli* (Invitrogen). Plasmids were transformed into the bacteria using heat shock. Briefly, dilute plasmid was added to a 25 μL aliquot of cells and incubated on ice for 30 min before a 42°C heat-shock of 60 s for solopack gold *E.coli* or 30 s for Top10F' *E.coli*. SOC recovery medium (provided with the TOPO-TA cloning kit) was added to the cells, which were incubated, shaking, at 37°C for 1 h before plating out onto warmed LB agar plates with appropriate antibiotics for colony selection, and X-gal and IPTG if blue-white screening was to be performed.

2.3.3 Preparation of plasmids

In order to produce working quantities of plasmid, *E. coli* were grown up in liquid culture (LB broth) overnight with appropriate antibiotic selection, before plasmid extraction by alkaline lysis [Birnboim and Doly, 1979] and purification of plasmid DNA by either binding to a silica gel [Vogelstein and Gillespie, 1979] or anion-exchange column.

Silica-gel binding columns were used when preparing small amounts of plasmids for initial characterisation of clones. Both spin columns (QIAprep spin miniprep kit, Qiagen, Crawley, U.K.) and a vacuum protocol which allows parallel processing of 96 clones (Qiaprep 96 Turbo miniprep kit, Qiagen) were used. In both methods,

alkaline lysis of cells is performed before neutralisation, which causes precipitation of genomic DNA and protein, and addition of salt to create the conditions required for DNA binding to the silica gel. The precipitate was collected by centrifugation and the supernatant added to the silica column and drawn through by either centrifugation or vacuum before washing to remove impurities and eluting in a suitable volume of H_2O.

Anion-exchange columns (QIAfilter midiprep kit, Qiagen) were used when preparing larger quantities of vector for further characterisation and use in downstream processes. Alkaline lysis and neutralisation was performed in a similar manner to above, but using low salt conditions and the precipitate was removed by filtration, rather than centrifugation. Samples in low-salt buffer were applied to an anion-exchange column, which was then washed with a buffer with intermediate salt levels to remove remaining RNA, protein, dye and low molecular weight impurities before elution of the samples using a high-salt buffer. Samples were desalted and concentrated by isopropanol precipitation and resuspended in H_2O.

2.3.4 Plasmid manipulation

Vectors were analysed by electrophoresis of either untreated or restriction enzyme digested samples to verify size and quality. Restriction enzymes were obtained from New England Biolabs (Hitchin, U.K) and digests were performed using their proprietary buffer system. Digests were routinely performed in 20 µL volumes at 37°C for 1 to 2 h. Restriction enzyme digest and ligation were also performed to modify vectors. Vectors were cleaved in either single or double digests using these restriction enzymes. Fragments were then separated by electrophoresis. Required bands were removed from the gel by electrophoresis using Quik-pik electroelution capsules (Stratagene), before being cleaned and concentrated by binding to a silica gel column and eluting in H_2O (QIAquick PCR Purification Kit). Fragments were ligated using T4 ligase and the appropriate buffer, in an overnight reaction at 16°C.

2.4 RNA purification, analysis and transcription

2.4.1 RNA extraction and purification from cultured cells

2.4.1.1 Extraction from cultured cells

RNA extractions were performed by lysing the cells and purifying RNA using RNA-binding silica gel columns (RNeasy Mini Kit). In initial experiments, cells were trypsinised and pelleted before lysis. Later, this protocol was replaced by direct on-dish lysis, as this gave no loss of product, and less chance of the cells developing a shock response. After addition of guanidine isothiocyanate (GITC)-containing lysis buffer to disrupt cells and inhibit RNase, cells were further homogenised by repeated aspiration through a 20-gauge needle before addition of 70% ethanol to generate appropriate conditions for binding of RNA to the column. The resulting solution was added to a silica-gel spin column which binds up to 100 µg of RNA molecules of more than 200bp. Several washes were performed with proprietary washing solutions, to remove contaminants such as proteins and DNA, before RNA was eluted in 30-60 µL H_2O.

2.4.1.2 DNase treatment

As the RNeasy RNA extraction kit does not guarantee complete DNA removal, RNA was treated with DNase, to remove any genomic DNA contamination before reverse transcription. The DNase reaction was performed in 10 µL volumes with 1 µg RNA. Reaction included 1 µL DNaseI (1 unit/µL) and 1 µL 10x DNase buffer (200 mM Tris-HCl (pH 8.4), 20 mM $MgCl_2$, 500 mM KCl). This was incubated at room temperature for 15 min before being inactivated by addition of 1 µL 25 mM EDTA and heating at 65°C for 10 min.

2.4.2 RNA analysis

Total RNA quality was periodically checked by formaldehyde-agarose gel electrophoresis and always quantified by spectrophotometry before reverse transcription was performed to yield cDNA for further analysis.

2.4.2.1 Formaldehyde-agarose electrophoresis

Samples of RNA were separated on a formaldehyde-agarose gel to assess the quality of the RNA. This method was adapted from Maniatis 7.42-7.50 [Sambrook et al., 1989].

Running buffer was prepared as a 5x stock (45 mM DEPC-treated sodium acetate, 10 mM EDTA, 100 mM MOPS). Loading buffer was prepared as 50% (v/v) glycerol, 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol FF in 1 mM EDTA (pH8.0). This was treated with 0.1 % (v/v) DEPC for 12 h at 37°C before autoclaving to destroy residual DEPC. Ethidium bromide (0.3 mg/mL) was added for visualisation

The gel tray and comb were pre-soaked in 0.1% (v/v) DEPC or RNase Zap. A 100 mL gel was prepared by melting 0.8 g agarose in 62.5 mL H_2O, and allowing the gel to cool to 65°C before adding 20 mL 5x running buffer and 18 mL 12.3 M formaldehyde (>pH 4.0). The gel was poured and allowed to set in the fume hood and pre-run at 5 V/cm for 5 min before sample loading.

Samples were prepared by combining 4.5 µL RNA sample (1-30 µg), 2 µL 5x running buffer, 3.5 µL formaldehyde and 10 µL de-ionised formamide and heating at 65°C for 15 min. Samples were chilled to 4°C and 2 µL loading buffer added before loading onto the gel. Gels were run in the fume hood, at 3-4 V/cm. Buffer must be mixed at intervals to avoid formation of a pH gradient. Gels were visualised using a FluorImager SI (Vistra Fluorescence) or a Typhoon 9400 (Amersham Biosciences).

2.4.2.2 Spectrophotometry

RNA samples were quantified by spectrophotometry. Samples were diluted between 5- and 20- fold and absorbance at 260, 280 and 320 nm measured, using a 10 µL, 0.5 cm cuvette. A_{260}/A_{280} should be 1.9-2.1, and is used to estimate the purity of the nucleic acid, since proteins absorb at 280 nm. A_{320} should be as close as possible to zero and can be used for background correction. A high A_{320} reading may indicate that air bubbles have formed in the cuvette. Concentration of RNA (ng/µL) was calculated as A_{260} x 80, as an A_{260} of one in a 1 cm cuvette is equivalent to a concentration of 40 ng/µL.

2.4.3 cDNA preparation from RNA

The reverse transcription reaction allows copying of mRNA to cDNA. Reverse transcription was performed using a known amount of total RNA, as calculated by spectrophotometry. Reverse transcription used between 100 ng and 1 µg of RNA per 20 µL reaction. Parallel controls were performed using no enzyme or no RNA and reactions were performed in duplicate, when possible. Ten µL RNA (after DNase

digestion) was combined with 1 µL 500 µg/mL Oligo dT_{17} (Oswell/Eurogentec, Southampton, UK) and 1 µL prime RNase inhibitor (Eppendorf, Fisher Scientific, Loughborough), before incubation at 65°C for 5 min. Samples were chilled on ice for 2-3 min and briefly centrifuged. Master mix was prepared as follows for each reaction: 4 µL 5x 1st strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$), 2 µL 0.1 M DTT, 1 µL 10 mM dNTPs, 1 µL SuperScript II enzyme (200 units/µL) (Invitrogen). Enzyme was replaced with H_2O for no-enzyme controls. Eight µL master mix was added to each reaction and mixed well by pipetting. Samples were incubated at 42°C for 50 min then at 70°C for 15 min and cDNA was stored at -20°C.

2.5 DNA Analysis

2.5.1 Analysis of DNA purity and integrity

2.5.1.1 Spectrophotometry

Plasmid concentration and purity was assayed by spectrophotometry. Samples were diluted as appropriate (usually 1/10) and absorbance at 260, 280 and 320 nm measured using a 0.5 cm, 10 µL cuvette. For DNA, A_{320} should be close to zero and A_{260}/A_{280} should be about 1.8. DNA concentration (µg/mL) was calculated as A_{260} x 100.

2.5.1.2 Agarose gel electrophoresis

PCR products, plasmids and restriction enzyme digest products were run on agarose gels in order to ascertain the sizes of the DNA species present and provide an indication of their amounts.

Gels of 0.7-2% (w/v) were prepared by dissolving Seakem agarose (Cambrex, Wokingham U.K.) in TAE buffer by boiling. Gels were allowed to cool to 60°C before adding 0.5 µg/mL ethidium bromide and pouring. Gels were run in TAE buffer at about 5 V/cm depending on the size of the gel. Samples were mixed with 5x gel loading buffer (0.25% (w/v) orange G, 0.025% (w/v) xylene cyanol in 40% (w/v) sucrose) before loading on the gel.

DNA was visualised using a FluorImager SI or a Typhoon 9400 and analysed using ImageQuant and ImageQuant TL (Molecular Dynamics).

2.5.2 Sequencing of DNA

2.5.2.1 Sequencing Reaction

Cleaned PCR product or plasmid underwent sequencing reactions using the DYEnamic ET dye terminator ready reaction mix (Amersham Biosciences), which includes labelled ddNTP terminators, as well as dNTPs, polymerase and a suitable buffer. Either 4 μL ExoSAP-IT cleaned PCR product (Section 2.6) or 700 ng plasmid (Section 2.3.3) were used in a 10 μL reaction with 4 μL ready reaction mix, and 0.5 μL 5 mM suitable primer, using 25 cycles of 95°C for 20 s, 50°C for 15 s, 60°C for 1 min.

2.5.2.2 Purification of sequencing reaction product

A further clean-up step was performed using either Autoseq (pre-9/05) or Sigma Spin (post-9/05) columns. Both of these products consist of 96-well format sepharose columns, which allow longer DNA molecules to pass through, whilst sequestering dNTPs and short-chain molecules such as primers. Columns were washed repeatedly with water and then 10 μL sample followed by 10 μL H_2O were applied to the columns to give sufficient volume of cleaned product for loading onto the MegaBACE. All washes were performed by centrifugation.

2.5.2.3 Sequencing

The resulting samples were sequenced using the MegaBACE 1000 (Amersham Biosciences) and results analysed using seq analyser (Amersham Biosciences) and Staden [Staden et al., 2000] programmes.

2.6 PCR

Expression levels of several genes were assayed using PCR, which was also used to amplify specific regions of DNA for sequencing. PCR and Q-PCR products were analysed by agarose gel electrophoresis to confirm product size and purity which also provided an indication of the amount of product of conventional PCR.

2.6.1 Conventional PCR

Conventional PCR was performed to amplify specific sequences and to provide an indication of the amount of product. PCR was routinely performed using a hot-lid thermal cycler in 20 μL reactions prepared as follows: 2 μL 2 mM dNTPs, 1 μL 20mM forward primer, 1 μL 20 mM reverse primer, 2 μL 10x buffer (includes 15

mM $MgCl_2$, Tris-HCl (pH 8.7), KCl, $(NH_4)_2SO_4$), 0.1 µL HotStar *Taq* polymerase (5 units/µl), 5 µL sample, 8.9 µL H_2O.

Note that primers described for use in Q-PCR (Section 2.6.3) can often also be used in conventional PCR, but that assays described here are not suitable for use in Q-PCR.

2.6.1.1 BCAS4/BCAS3 fusion gene

Primers: GAGCTCGCGCTCTTCCTGAC (upper), AGGGGCTGGCTCTCATTGGT (lower). The amplified region corresponds to bases 242-569 of the fusion gene (accession number AF361221), which spans the junction of the two parental genes. Primers were according to [Hahn et al., 2004]. Cycling conditions were 35 cycles of 94°C for 30s, 60°C for 30 s, 72°C for 30 s.

2.6.1.2 IRA1/RGS17 fusion gene

Primers: GGGAATTTCCTTGTGCCTCCA (upper), TGCTGGGGCCTTCATCATCT (lower). The amplified region runs from base 70 of the IRA1 mRNA (accession no. NM_024665) to base 435 of the RGS17 mRNA (accession no. NM_012419), giving a 367 base amplicon. Primers were according to [Hahn et al., 2004]. Cycling conditions were 35 cycles of 94°C for 30secs, 60°C for 30 s, 72°C for 30 s.

2.6.2 Colony PCR

Colony PCR was used to amplify inserts from TOPO-TA plasmids cloned into *E. coli*, for further analysis.

Colonies were picked and streaked out onto fresh agar plates for further growth, as well as being added to colony PCR reactions as follows. Fifty µL PCR mix was prepared containing 5 µL 10x PCR buffer, 5 µL 2 mM dNTPs, 0.4 µL $MgCl_2$, 1 µL 10 µM M13 (-20) forward primer, 1 µL 10 µM M13 reverse primer, 0.25 µL Hot Star *Taq* polymerase (5 units/µL), 37.35 µL ddH_2O. The colony was added to the PCR mix using a sterile pipette tip and the PCR plate centrifuged at 2000 rpm for 2 min prior to PCR. PCR cycling conditions were as follows: 95°C for 15 min (hot start) followed by 40 cycles of 94°C for 30 s, 57°C for 20 s, 72°C for 1 min before a termination step of 72°C for 10 min and a 4°C hold.

Five µL of each colony PCR was run on a 0.8% (w/v) agarose gel, with ethidium bromide, before purification of an aliquot of the PCR reaction using ExoSAP. The

PCR product was diluted 2-fold before taking a 7.5 µL aliquot and combining it with 2.5 µL ExoSAP. This was incubated at 37°C for 15 min then 80°C for 15 min for the clean-up reaction and enzyme denaturing steps.

2.6.2.1 M13 sequencing primers

M13 sequencing primers specific to regions of the TOPO-TA cloning vector were used in colony PCR reactions and in sequencing reactions using DYEnamic ET terminators (Section 2.5.2) for sequencing PCR-product inserts.

M13 (-20) forward primer: GTAAAACGACGGCCAG, M13 reverse: CAGGAAACAGCTATGAC. Primers were as recommended by the manufacturer.

2.6.3 Q-PCR

All reactions contained 1 µM primers and either Sybr-containing master mix for standard Q-PCR, or sybr-free master mix for Taqman PCR (Bio-Rad, Hemel Hempstead, U.K.). Real-time assays were usually performed with cloned standards for quantification, and used both Sybr and Taqman chemistries (Section 3.2.1). A melt-curve was generated when Sybr was used for quantification.

2.6.3.1 Cloning and preparation of Q-PCR Standards

PCR products were cloned using the TOPO-TA system (Invitrogen)(Section 2.3) and analysed by colony PCR and sequencing (Section 2.5.2) to obtain a clone with the correct insert, which was grown up and plasmid extracted (Section 2.3.3). Plasmid DNA of known concentration was diluted to give a standard curve. Standard curves were usually prepared in the range of 1 fmol/ reaction to 10^{-6} amol/ reaction, depending on the abundance of the particular cDNA.

2.6.3.2 Q-PCR primers and assays

Primers and cycling conditions for Q-PCR assays are described in Tables 2.1 and 2.2. Table 2.1 describes Sybr-based assays for ERβ used in the earlier stages of the project. Table 2.2 describes assays for the estrogen receptors, estrogen responsive genes, housekeeping genes and expression system vectors used throughout the project. Assays for expression system vectors are described further below.

2.6.3.2.1 Ecdysone inducible system

MCF7 cells transfected with the ecdysone inducible system (Invitrogen) express a modified ecdysone receptor (VgEcR) (Section 4.1.1.1). An assay was prepared to measure expression of this gene product in order to characterise expression in the

transfected cells. The amplified region corresponds to bases 3061-3143 of the vector pVgRXR which is within the coding region for the ecdysone receptor. Standard curve was prepared using dilutions of the pVgRXR vector.

2.6.3.2.2 T-REx system

MCF7 cells transfected with the T-REx system (Invitrogen) must integrate two vectors. An assay specific to a region of one of the T-REx system vectors (pcDNA6/TR) which should not be expressed was prepared (tetV). This allows measurement of vector take-up by the cells. It also allows a measurement of transcription of the tet repressor by comparing measured levels of the vector (using the tetV assay) and the repressor itself (using the tetR assay), as this second assay will measure both vector DNA and cDNA. The amplified region of TetV corresponds to bases 2314-2490 of the pcDNA6/TR vector which spans the *C*-terminal of the Tet repressor gene.

The TetR assay was used to measure expression of the repressor in cells transfected with the T-REx system. The amplified region corresponds to bases 1608-1776 of the pcDNA6/TR vector, which overlap the beginning of the repressor coding region, falling after the CMV promoter. Standard curves for both of these assays were prepared using dilutions of pcDNA6/TR.

Primer names	Primer sequences	Isoform specificity	Source
Sal Forward Sal Reverse	TCACATCTGTATGCGGAACC CGTAACACTTCCGAAGTCGG	Non-specific	Ms S. al Akilli from [Kurebayashi et al., 2000]
KIERBA KIERBB	GCTCATCTTTGCTCCAGATCTTG GATGCTTTGGTTTGGGTGATTG	Non-specific	[Iwao et al., 2000]
CSERBA CSERBB	CATCTCCTCCCAGCAGCAATC CTCCAGCAGCAGGTCATACAC	ERβ1	CES using Oligo5 and Molecular Beacon.
CSERBC CSERBD	CGTCAGGCATGCGAGTAACA GACCCCGTGATGGAGGACTT	ERβ1	CES using Oligo5 and Molecular Beacon.
CSERBE CSERBF	CTCATCTTTGCTCCAGATCTTGTT GGAGTTTTAACTCTGAAACCTTG	Non-specific	CES using primer3 software
CSERBG CSERBH	TCAGGCATGCGAGTAACAAG CTCCAGCAGCAGGTCATACA	ERβ1	CES using primer3 software

Table 2.1 (above) Primers used in non-Taqman Q-PCR assays for ERβ expression

The table lists primer pairs used to assay ERβ in Sybr-based Q-PCR assays. For each primer pair, the isoform specificity and the source of the assay is described.

Table 2.2 (over) Primers and probes used in Q-PCR assays

For each gene, primers and Taqman probe (if relevant) are listed, with their source. Primers designed in-house at CCRT were usually designed using Primer3 [Rozen and Skaletsky, 2000]. The location of the PCR amplicon is described, with respect to the sequence listed under "Target", as well as the exon location. Usual cycling conditions are also described.

Target	Primers	Taqman Probe	Location of amplicon	Cycling conditions
ERα NM_000125	CCACCAACCAGTGCACCATT GGTCTTTTCGTATCCCACCTTTG [Bieche et al., 2001]	TCCGCAAATGCTACGAAGTG 5' 6-FAM, 3' BHQ1 Mr R. Eccles (CCRT)	base 1028-1153 exons 3-4	40 cycles of 95°C for 20 s, 60°C, 20 s, 72°C 30 s.
ERβ1 AF051427	TTTGGGTGATTGCCAAGAGC AGCACGTGGGCATTCAGC [Poola, 2003b]	CCTCCCAGCAGCAATCCATGCG 5' 6-FAM, 3' TAMRA [Poola, 2003b]	base 1731-1917 exons 7-8	60 cycles of 95°C for 30 s, 64°C, 45 s.
ERβ2 AF051428	ATCCATGCGCCTGGCTAAC GAGTGTTTGAGAGGCCTTTTCTG [Critchley et al., 2002]	TCCTGATGCTCCTGTCCCTCGTCA 5' 6-FAM, 3' TAMRA [Critchley et al., 2002]	base 1771-1849 exons 7-8	60 cycles of 95°C for 20 s, 52°C, 20 s.
ERβ4 AF061054	TTTGGGTGATTGCCAAGAGC GTCTGGGTTTTATATCGTCTGCAA [Poola, 2003b]	CCTCCCAGCAGCAATCCATGCG 5' 6-FAM, 3' TAMRA [Poola, 2003b]	base 89-294 exons 7-8	60 cycles of 95°C for 30 s, 64°C, 45 s.
ERβ5 AF061055	TTTGGGTGATTGCCAAGAGC CACTTTTCCCAAATCACTTCACC [Poola, 2003b]	CCTCCCAGCAGCAATCCATGCG 5' 6-FAM, 3' TAMRA [Poola, 2003b]	base 89-265 exons 7-8	60 cycles of 95°C for 30 s, 64°C, 45 s.
Cathepsin D NM_001909	TCAGGGCGAGTACATGATCC CTCTGGGGACAGCTTGTAGC Ms J. Leslie (CCRT)	CTGCCCGCGATCACACTGAA 5' ROX, 3' BHQ1 Mr R. Eccles (CCRT)	base 1096-1189 exon 8	40 cycles of 94°C for 20 s, 64°C, 1 min.
c-myc binding protein NM_012333	ACTGGCCGAAATGAAAGAGA CCTATTCAGCACGCTTCTCC Ms J. Leslie (CCRT)	AACTGAAAGCAAAGCTTG 5' 6-FAM, 3' BHQ1 Mr R. Eccles (CCRT)	base 277-367 exons 1-2	40 cycles of 94°C for 20 s, 64°C, 1 min.
Cyclin D1 NM_053056	AACTACCTGGACCGCTTCCT CCACTTGAGCTTGTTCACCA [Paruthiyil et al., 2004].		base 456-659 exons 2-3	40 cycles of. 94°C for 20 s, 64°C, 1 min.

Target	Primers	Taqman Probe	Location of amplicon	Cycling conditions
Cytochrome C oxidase 7A subunit NM_001865	TTGGTGGTGTTCAGTTGTGG TTATCGTCCTCTGCCCAATC Ms J. Leslie (CCRT)	TGCTGGTCAGTAACAGCCAA 5' Yakima Yellow, 3' BHQ1 Mr R. Eccles (CCRT)	base 31-124 exons 1-2	40 cycles of 94°C for 20 s, 64°C, 1 min.
EFP NM_005082	GATGTGAGAAACAGGCAGCA TCCTTGTCGAGGTGGTCTCT Ms J. Leslie (CCRT)	CTTGTTGTAGCTGCTCCAC 5' 6-FAM, 3' BHQ1 Mr R. Eccles (CCRT)	base 724-843 exons 2-3	40 cycles of 94°C for 20 s, 64°C, 1 min.
Lactoferrin NM_002343	CTGTAGCGGCGGAAGTCTAC CAGACCTTGCAGTTCGTTCA Ms J. Leslie (CCRT)		base 581-690 exons 3-4	40 cycles of 94°C for 20 s, 64°C, 1 min.
Livl NM_012319	ACAAATAGCCTGGGTTGGTG AACACCCGATTCATGAGAGG Ms J. Leslie (CCRT)	CAGTTTCCTGTCTCTGCTGG 5' 6-FAM, 3' BHQ1 Mr R. Eccles (CCRT)	base 1082-1177 exons 4-5	40 cycles of 94°C for 20 s, 64°C, 1 min.
P21 NM_000389	GGCGGCAGACCAGCATGACAGATT GCAGGGGCGGCCAGGGTAT [Paruthiyil et al., 2004]		base 518-740 exons 2-3	40 cycles of 94°C for 20 s, 64°C, 1 min.
P27 NM_004064	GGGGCTCGTCTTTTCGGGGTGTTT GAGCGGGAGGGCGGAGAGGAG [Paruthiyil et al., 2004]		base 43-237 exons 1-2	40 cycles of 94°C for 20 s, 64°C, 1 min.
Progesterone receptor NM_000926	TGGGCAGATGCTGTATTTTG TGCCACATGGTAAGGCATAA Ms J. Leslie (CCRT)	CATCCGCTGTTCATTTAGTA 5' Yakima Yellow, 3' BHQ1 Mr R. Eccles (CCRT)	base 3770-3861 exons 5-6	40 cycles of 94°C for 20 s, 64°C, 1 min.
PS2 X05030	CACCATGGAGAACAAGGTGA TGACACCAGGAAAACCACAA Ms J. Leslie (CCRT)	CAGCATGGACACCAGGACCA 5' Yakima Yellow, 3' BHQ1 Mr R. Eccles (CCRT)	base 46-179 exons 1-2	40 cycles of 94°C for 20 s, 64°C, 1 min.

Target	Primers	Taqman Probe	Location of amplicon	Cycling conditions
VEGF NM_003376	TGTTCAAGCCATCCTGTGTG ATCCGCATAATCTGCATGGT Ms J. Leslie (CCRT)	CACTCCAGGCCCTCGTCATT 5' Yakima Yellow, 3' BHQ1 Mr R. Eccles (CCRT)	base 1253-1364 exons 3-4	40 cycles of 94°C for 20 s, 64°C, 1 min.
XBP1s	AAGCCAAGGGGAATGAAGT CCAGAATGCCCAACAGGATA (CCRT)	GCTGAGTCCGCAGCAGGTGCAG 5' FAM 3' BHQ1 (CCRT)		
XBP1u	AAGCCAAGGGGAATGAAGT CCAGAATGCCCAACAGGATA (CCRT)	AGCACTCAGACTACGTGCACCT 5' YakimaYellow 3'BHQ1 (CCRT)		
ARF1 NM_001024227	TCCATTTTGGTGGTTGGTTT ATCTATGCTAGGCGGGGTCT Mr R. Eccles (CCRT)	TCGAGAACACTTGAACACAC 5' 6-FAM, 3' BHQ1 Mr R. Eccles (CCRT)	base 987-1097 exon 5	50 cycles of 94°C for 20 s, 60°C for 45 s
GAPDH NM_002046	GCATCCTGGGCTACACTGAG TCCACCACCCTGTTGCTGTA Dr M. Davies (CCRT)		base 890-1052 exons 7-8	40 cycles of 95°C for 30 s, 64°C, 20 s, 72°C, 30 s.
Histone H3 NM_002107	CTATCTGGTTGGCCTTTTTGAA TCTTAAGCACGTTCTCCACGTA (CCRT)		base 412-528 exon 3	40 cycles of 95°C for 30 s, 64°C, 20 s, 72°C, 30 s.
HPRT NM_000194	GTGTTGGATATAAGCCAGACTTTGTT AACTCAACTTGAACTCTCATCTTAGGC (CCRT)		base 597-763 exons 7-9	40 cycles of 94°C for 30 s, 64°C, 1 min.
PPP1CA NM_002708	GATCTGCGGTGACATACACG CTTGCCCCTGTCCACATAGT Mr R. Eccles (CCRT)	CGACCTTCTGCGACTATTTG 5' Yakima Yellow, 3' BHQ1 Mr R. Eccles (CCRT)	base 303-398 exon 3	50 cycles of 94°C for 20 s, 60°C for 45 s.

Target	Primers	Taqman Probe	Location of amplicon	Cycling conditions
Ecdysone Receptor (VgEcR)	GGGGGACGAACTCCACTTAG CCAACATGTCCAGATCGAAA		base 3061-3143 of pVgRXR	40 cycles of 95°C for 30 s, 55°C for 30 s.
Tet vector (tetV)	GGATCCCGGGAATTCAGAT GTGATGGATTCGACCAGACA		base 2314-2490 of pcDNA6/TR	40 cycles of 94°C for 20 s, 65°C for 30 s.
Tet repressor (TetR)	GCAACGTGCTGGTTATTGTG GGCGAGTTTACGGGTTGTTA		base 1608-1776 of pcDNA6/TR	40 cycles of 94°C for 20 s, 65°C for 30 s.

2.7 Protein analysis and quantification

Expression of protein was analysed using dot and Western blotting, immunocytochemistry and an enzyme-linked immuno-absorbant assay (ELISA). For all methods other than immunostaining, protein was extracted from cells using the methods described below.

2.7.1 Protein extraction from cultured cells

Two methods were used for protein extraction, a mechanical lysis in homogenisation buffer [Hassanin, 2004] and a method specifically optimised for nuclear receptor extraction, designed in-house by Dr N. Halliwell, CCRT (unpublished).

2.7.1.1 Homogenisation buffer method

Cells were grown in 75 cm^2 flasks and treated as required. Medium was removed and the cells were washed twice with ice-cold PBS before addition of 500 µL 4°C homogenisation buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM NaCl, 1x Protease Arrest (GenoTech, Web Scientific, Crewe, U.K.)). Cells were scraped from the dish using a rubber scraper and transferred to an eppendorf tube before being passed through a 26 gauge needle 10 times. The homogenate was then centrifuged for 10 min at 4°C and the pellet discarded. The protein-containing supernatant was stored in 250 µL aliquots at -80°C.

2.7.1.2 Nuclear protein-enriching extraction method

Cell lysis buffer was prepared from stocks as required. Buffer contained 150 mM NaCl in 20 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% triton-X-100, 0.5% NP-40 substitute, 200 µM Na_3VO_4, 2.5 mM PMSF (phenylmethylsulphonyl fluoride) and 3x protease arrest (GenoTech).

Cells were grown in 75 cm^2 flasks. Medium was removed and the cells incubated in 10 mL balanced Hank's salt solution (Invitrogen) for 10 min at 37°C. The cells were then rinsed twice, with ice-cold PBS, and scraped into 1.5 mL ice-cold lysis buffer in a 6 mL bijou. Lysis was performed by incubation on ice for 30 min, gently stirring. Lysate was passed through a 26-gauge needle 10 times, avoiding frothing, before transferring to a 2 mL screw-top eppendorf. Finally, this was centrifuged at 4°C for 15 min at 13000 rpm before removing supernatant, which was stored in 60 µL aliquots, snap frozen in liquid nitrogen and stored at -80°C.

2.7.1.3 Nuclear protein extraction using the Active Motif kit

The Active Motif (Rixensart, Belgium) nuclear extract kit generates separate cytoplasmic and nuclear extracts and is recommended for use with the NR sandwich ELISA kit (Section 2.7.7.1). This kit includes a number of proprietary buffers.

Cells were cultured in a 75 cm^2 flask. Medium was removed and cells were washed with 5 ml ice-cold PBS to which phosphatase inhibitor was added and then flooded with a further 3 ml PBS/phosphatase inhibitor. Cells and PBS were scraped into a pre-chilled 15 ml tube. The cell suspension was centrifuged at 500 rpm for 5 min at 4°C to pellet the cells and the supernatant discarded.

Cells were then resuspended in 500 µl hypotonic buffer and transferred to a chilled eppendorf tube for incubation on ice for 15 min to lyse the cells. Detergent (25 µl) was added and the tube was vortex-mixed for 10 seconds then centrifuged for 30 seconds at 14000 rpm at 4°C to pellet the intact cell nuclei. The supernatant was removed at this stage and retained as the cytoplasmic fraction.

The pelleted nuclei were resuspended in 50µl complete lysis buffer and vortex-mixed for 10 sec. The resulting suspension was incubated on ice on a rocking platform for 30 min before being vortex-mixed for a further 30 sec. The solution was centrifuged at 14000 rpm at 4°C and the supernatant recovered. This supernatant comprises the nuclear protein fraction.

Both nuclear and cytoplasmic fractions were stored at -80°C for future use.

2.7.2 Bradford assay

The concentration of protein extracted from cells using the methods described above (Section 2.7.1) was measured using the Bio-Rad microprotein assay. This assay measures 2-20 µg protein in a 1 mL reaction volume. A standard curve of 2-20 µg BSA in 800 µL Tris-buffered saline (TBS, 20mM, pH 7.6) aliquots was prepared. Protein samples were also diluted for analysis, using either 4 or 16 µL of sample diluted to 800 µL using TBS. Dye reagent concentrate (200 µL) was added to each sample, as well as to buffer and water blanks and incubated for between 5 min and 1 h before measuring the absorbance at 595 nm.

2.7.3 Dot-blot assay for ERβ1

Dot-blots were performed using cell extracts and known amounts of recombinant ERβ1 (rERβ1L) as a standard. Primary antibody was a rabbit polyclonal (PA1-313,

Affinity Bioreagents, Cambridge Biosciences, Cambridge, UK) and the secondary antibody was donkey-anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Biosciences). Antibodies were diluted in a 3:1 solution of TBS-T and NAP Blocker (GenoTech). ECL Advance (Amersham Biosciences) (Section 2.7.5.3) was used to visualise peroxidase activity.

Samples of recombinant protein were spiked with 0.1 μg/μL BSA to reduce sample loss. All samples were prepared in 100 μL volumes in TBS-T (20mM TBS, pH 7.6, plus 0.1 % (v/v) Tween-20). The minifold vacuum blotting apparatus (Schleicher and Schuell, London, U.K.) was assembled with pre-wetted PVDF membrane (either Schleicher and Schuell or Amersham Biosciences). TBS-T (100 μL) was added to each well and drawn through under vacuum. Samples were added to wells and drawn onto the membrane in a 10 min vacuum step. Membranes were then cut into separate fragments, as required for antibody incubations.

Membranes were re-wetted using sequential washes in methanol (15 s), water (5 min) and TBS-T (5 min) before being blocked overnight at 4°C in ECL advance blocking reagent (Amersham Biosciences). After overnight blocking, membrane was washed in TBS-T and incubated in 1/10,000 primary antibody at room temperature for 1 h. Further washes were performed and 1/200,000 secondary antibody added for a further 1 h room temperature incubation. Blots were washed again before ECL advance was used for visualisation of spots (Section 2.7.5.3). The assay was sensitive to 100 pg recombinant ERβ1 and did not show cross reactivity with BSA.

2.7.4 Western blotting assays for ERα, ERβ1 and β-actin

Several different antibodies were used in Western blots to identify ERα, ERβ1 and β-actin in MCF7 cells. ERβ1 was detected using a 1/2000 dilution of a rabbit polyclonal antibody, PAI-313 (Affinity Bioreagents) or a 1/500 dilution of a mouse monoclonal antibody, PPG 5/10 (Serotec, Oxford, U.K.). ERα was detected using 1/1000 F-10 (Santa Cruz, Heidelberg, Germany), a mouse monoclonal and β-actin was detected using 1/2000 3398-100 (Biovision, Cambridge Biosciences, Cambridge, U.K.), also a mouse monoclonal. Specific horseradish-peroxodase conjugated secondary antibodies (Amersham Biosciences) were used at 1/500,000 (anti-rabbit) or 1/200,000 (anti-mouse) dilutions. Antibodies were diluted in 3:1 TBS-T and NAP Blocker.

Protein samples and recombinant protein standards were prepared in 33% Laemmli buffer (Bio-Rad) in a total 10-15 µL volume and heated at 70°C for 10 min before loading on a 12 % acrylamide precise protein gel (Pierce, Perbio Science U.K., Cramlington, U.K.) in a Bio-Rad electrophoresis tank with Tris-HEPES-SDS gel running buffer (Pierce) and electrophoresis performed at 100 V for 110 min. Proteins were blotted onto a pre-wetted Hybond-P PVDF membrane using the Bio-Rad tank at 100 V for 1 h.

Membrane was blocked overnight at 4°C with 2 % (w/v) ECL advance blocker and then washed with TBS-T before incubating with primary antibody for 1 h. Membrane was washed again before adding secondary antibody for a further 1 h incubation and a final series of washes. All incubations were performed with agitation. Peroxidase activity was visualised using ECL advance (Section 2.7.5.3). Blots were stripped, when necessary, by incubating in SDS-glycine (25 mM glycine, 1% (w/v) SDS) for 30 min, before re-equilibrating the membrane in TBS-T.

2.7.5 Protein visualisation and staining methods

2.7.5.1 Sypro Ruby

Gels were incubated, shaking, at 4°C overnight in the dark in Sypro ruby stain. Gels were then transferred to a 10 % (v/v) methanol, 7.5 % (v/v) glacial acetic acid solution for 30 min at room temperature for stabilisation of stain and removal of non-specific staining before visualisation of staining on the Typhoon 9100 scanner using the green laser and 610 nm bandpass filter at about 550 V. Sypro ruby staining is sensitive to < 30 ng protein per band.

2.7.5.2 Ponceau S

Ponceau S (Sigma) staining of Hybond-P membranes was used to verify protein transfer. Membrane must be kept damp throughout the procedure. Membranes were immersed in ponceau S solution, gently shaking, for 5 min. Repeated rinses with H_2O were used to reduce background staining before visualisation on the Typhoon 9100 scanner using the blue (457nm) laser and no filter, at 500 V. Staining was also observed by inspection, as it was not easily visualised by scanning. To destain, membranes were washed in 0.1 M NaOH for less than 1 min, until the bands disappeared, followed by washes in H_2O and TBS-T to re-equilibrate the membrane. Ponceau S staining states a sensitivity of 250 ng per band.

2.7.5.3 ECL advance

Immunoreactivity on Western blots and dot blots was visualised using the Amersham Biosciences ECL advance kit, following the manufacturer's protocol. ECL staining was performed in the dark or under safe light. Excess liquid was removed from membrane, and the membrane placed protein-side up on a flat surface. ECL reagents were warmed to room temperature and 3 mL of each of solutions A and B were mixed together and used to cover the membrane for 5 min. Excess liquid was again removed and the membrane wrapped in Saran-wrap and placed in an autoradiography cassette. X-ray film (Hyperfilm ECL, Amersham Biosciences) was exposed to the membrane for intervals of 1-30 min and developed using Kodak LX24 developer and AL4 fixative (Axis healthcare, Elstree, U.K.).

2.7.6 Immunocytochemical detection of ERα and ERβ1

Cells were plated onto lab-tek 8-well permanox chamber slides (Nunc, Nalge, Hereford, U.K.) and were allowed to adhere and grow for 18-24 h before fixation. Medium was aspirated and the upper structure removed from the slides which were then rinsed gently with PBS and tapped dry before being flooded with methanol for 20 min for fixation. Slides were then rinsed again with PBS and stored immersed in PBS at 4°C until staining.

In order to ascertain whether permeabilisation was required, one slide was permeabilised and one left untreated before staining for ERα. Permeabilisation was performed by incubating the slide in 0.3 % (v/v) Triton X-100 for 10 min at room temperature, before all slides were washed with TBS. Slides underwent blocking of endogenous peroxidases by immersion in 3 % (v/v) peroxide in methanol for 15 min, then rinsed in running water. Staining for estrogen receptors requires an antigen retrieval step. Antigen unmasking solution (Vector, Peterborough, U.K.) was diluted 1 in 100 before covering the slides with the solution for microwaving (1000 W, 10 min). Slides were rinsed and blocked in serum-free protein block (1% (w/v) BSA-TBS) for 10 min. All antibody incubations were performed in humid conditions. Anti-ERα primary antibody (F-10) was diluted 1 in 30 in 5 % (w/v) BSA and incubated for 40 min, ERβ1 primary (PPG5/10) was diluted 1 in 10 or 1 in 100 with 1 % (w/v) BSA and incubated overnight.

Slides were rinsed with TBS before secondary antibody (linker from DakoCytomation LSAB2 system, Dako, Ely, U.K.) was used to cover the slides for 30 min at room temperature. Again, slides were rinsed in TBS before adding streptavidin-HRP (DakoCytomation kit) for 30 min (room temperature) and rinsing again. One drop of DAB (3,3'-diaminobenzidine) chromagen per mL buffer (imidazole HCl (pH 7.5) plus H_2O_2) was prepared and incubated on slides for 5 min, before rinsing slides in running water.

Slides were counterstained with haematoxylin for 1.5 min then rinsed in running water before being dipped in acid ethanol (1 % HCl (v/v) in 70 % (v/v) EtOH) for 10 seconds then run under a warm tap for blueing. For mounting, the slides were placed on a horizontal surface and 3 drops crystal mount (Sigma) added. Slides were rotated to help the mounting medium spread to cover the entire area before drying in an oven at 40-50°C for at least 30 min. Slides were cooled to room temperature before viewing.

2.7.7 ELISA

2.7.7.1 NR sandwich ELISA for ERα

ELISA is a sensitive method for quantitatively detecting levels of a specific protein. A commercial ELISA sandwich assay (Active Motif) was used to measure ERα in the nuclear and cytoplasmic protein fractions of cultured cells. Cells were lysed and protein fractions collected using the nuclear extract kit (Section 2.7.1.3) for use in this assay. The ELISA kit comprises a 96-well plate pre-coated with a capture antibody, proprietary diluent and wash buffers, an ERα-specific antibody and a HRP-conjugated secondary antibody and substrate and stop solutions for the HRP enzyme.

Cell extracts were diluted to 50 µl using the diluent buffer and added to appropriate wells of the ELISA plate. This was incubated at room temperature on a rocking platform for 1 hour before being washed three times with the proprietary wash buffer. ERα antibody diluted 1:400 (50 µl) was then added to each well, followed by a further incubation for 1 hour and another three washes. HRP-conjugated secondary antibody diluted 1:1000 (50 µl) was then added to all wells and a further 1 hour incubation and four washes performed.

Developing solution (100 µl) (HRP substrate) was then added to each well, incubated

for 7 minutes (this time varies based on the batch of the kit, see manufacturer's instructions) until a blue colour was seen and the stop solution (100 µl) added, to turn the blue colour yellow and stop the reaction. Absorbance was then read at 450nm using a plate reader, with a reference wavelength of 655 nm.

2.7.7.2 ELISA for ERβ

An ELISA is not currently commercially available for measuring ERβ levels in cell lysates. An ELISA assay was therefore prepared to measure levels of ERβ1 in MCF7 and MCF7β1x cells. ELISA was performed using MaxiSorp flat-bottom 96-well plates (Nunc). MaxiSorp plates have a highly charged, modified polystyrene surface with high affinity for polar and hydrophobic groups. The surface can bind up to 650 ng protein per cm^2, and the base of each well has a radius of 0.35cm, giving a total binding surface, when using 100 µL, of $0.95cm^2$, giving a total binding capacity of about 600 ng protein. All incubations were performed on a rocking platform. Either recombinant ERβ1 or cell lysate was added to each well in a total volume of 100 µL carbonate coating buffer (0.15 M sodium carbonate, 0.35 M sodium bicarbonate (pH 9.6)). The plate was then sealed and incubated overnight at 4°C. The following morning the plate was emptied by inverting and banging on a hard surface, and 200 µL blocking solution (1% BSA in PBS) added to each well. This was incubated at room temperature for 2 h. The plate was dried, and rinsed with PBS-T (PBS, 0.05% (v/v) Tween-20). Primary antibody (100 µL), diluted in 3:1 PBS-T and NAP Blocker, was then added to each well, at an appropriate dilution (1/10 to 1/1000) and a further 1 h room temperature incubation performed. The plate was dried and washed with PBS-T before adding 100 µL secondary antibody (1/1000 to 1/100,000 dilution) for a further hour. Washes were performed and 200 µL TMB (3,3',5,5'-tetramethylbenzidine) substrate added, and incubated for 30 min at room temperature. The TMB reaction was stopped using 100 µL 0.5 M H_2SO_4, and the absorbance at 450 nm read.

2.8 Analysis of Q-PCR and sequencing results

Q-PCR data were analysed using the iCycler software (Bio-Rad), Excel (Microsoft), Prism 4 (GraphPad Software, Inc., San Diego, USA) and Minitab 13 (Minitab Inc., Coventry, U.K.). SPSS (Statistical Package for the Social Sciences, SPSS U.K. Inc., Woking, U.K.) and GeneSpring (Agilent Technologies, Stockport, U.K.) were also

used, to a lesser degree. The Staden package [Staden et al., 2000] was used to analyse DNA sequence data, as well as the NCBI BLAST programme [Tatusova and Madden, 1999; Wheeler et al., 2000] for sequence comparisons and identification.

2.8.1 Specific statistical tests

Several statistical tests were used to analyse Q-PCR data, using Minitab. The most commonly used tests and their uses are described here. Q-PCR data was presented graphically as mean +/- standard deviation (SD). SD is a measurement of the spread of values in a population and therefore decreases as sample range decreases or the population increases.

2.8.1.1 Establishing the difference between two data sets

In order to determine whether two data sets (such as measurements of expression of a gene in a single cell line treated with two different treatments, or two cell lines given the same treatment) were significantly different, both Student's *t*-test and Mann-Whitney were performed. Student's *t*-test considers whether the means of two independent groups of data are significantly different by making an estimate of the standard error of the difference between the two groups. Although based on an assumption of normality in the data, the *t*-test is in fact fairly robust towards non-normal data when larger data sets are involved. It becomes less robust with smaller data sets, when equal variance of the data sets also become more important [Rupert Miller, 1986]. The Mann-Whitney test is a non-parametric test of the differences between two data sets, which compares the medians of the populations. It can be more robust with small data sets as it does not assume normality, but takes no account of the overall spread of data points, as it relies on ranking the data.

2.8.1.2 Comparisons between multiple groups

ANOVA relies on similar assumptions to the *t*-test, as it is also based on the t-distribution. ANOVA allows comparisons of multiple groups, e.g., a single cell line given six different treatments (1-way ANOVA) or multiple cell lines given multiple treatments (2-way ANOVA). ANOVA establishes whether there is a significant difference between treatments, but not which treatments are outlying. A further test, e.g., Tukey's test, which generates pair-wise comparisons between the means of each data set, must be performed to determine which treatments are contributing to the variation between groups. ANOVA works best with samples of equal size (in 2-way ANOVA all samples *must* be the same size) and equal group variances. Samples

must be randomly taken and errors must be independent and normal.

ANOVA is also used to prepare plots such as the main effects plot and interactions plot which are useful in determining which factors have the largest effect, and investigating interactions between factors. Kruskal-Wallis is a nonparametric alternative to ANOVA, which tests the difference between medians of several groups. It is particularly useful for analysing normal data, but is not very robust against outliers.

2.8.1.3 Investigating the relationship between two variables

Relationships between a treatment and a gene expression response (for instance levels of Dox and ERβ1 levels, or changes in gene expression with time) were analysed by linear regression analysis. Regression analysis generates a linear relationship between the independent and dependent variables using a least-squares method. An equation for this line is generated and an ANOVA performed, allowing the significance of the slope to be determined. An R^2 value is also obtained, equivalent to the square of the correlation coefficient, which describes how well the data fit the straight line. More than one predictor can be used (multiple regression), but all predictors must be continuous. Pearson's correlation was also used in some cases, which tests how well two variables fit a linear relationship.

Chapter 3 Measuring estrogen response in breast cancer cell lines using Q-PCR

3.1 Introduction

Estrogen and the estrogen response are important in the development, prognosis and treatment of breast cancers (Section 1.3 and [Feigelson and Henderson, 1996; Iwase, 2003; Sommer and Fuqua, 2001]). Breast cancers are heterogeneous and do not show a uniform response to treatment. Response can be partly predicted using markers such as expression levels of ERα, which are associated with outcome [Knight et al., 1977], but are not fully indicative of response [Ali and Coombes, 2000].

Cell lines originally derived from mammary tumours can act as a model of breast cancer, allowing manipulation of growth conditions and signalling pathways in a way that is not possible with *in vivo* tumours (Section 1.6). Like tumours, cell lines respond differently to treatments depending on the signal transduction pathways present in the cells. Treatments can be used to mimic both hormonal status and different therapeutic treatments, such as tamoxifen [Jordan, 1997], fulvestrant [Torosian et al., 2002], and aromatase inhibitors [Kalidas and Brown, 2005]. A set of breast cancer cell lines (Section 3.1.1) was selected to be representative of different estrogen receptor levels and was treated with different stimuli. Estrogen responses were measured at a transcriptional level, allowing a comparison of responses between the cell lines. Differences in response could then be related to the different ERs expressed in each cell line to associate the response with the effectors and their known receptor interactions.

3.1.1 Cell lines and treatments

A panel of four human malignant mammary epithelial cell lines, MCF7, T47D, ZR75 and MDA-MB-231 (Table 3.1 A), was chosen to represent a selection of both estrogen responsive and non-responsive breast cancer cell types. MCF7 was derived from a pleural effusion of a mammary tumour in 1970 [Soule et al., 1973] and is a differentiated epithelial cell line, expressing estrogen receptor (ERα) and showing strong growth responses to estrogen and insulin [Engel and Young, 1978]. ZR75 was also derived from a pleural effusion and its growth can be stimulated by estrogen and

A

Cell line	karyotype	tumour status	ERα status	source
MCF7	aneuploid	malignant	positive	Soule *et al.*, 1973
T47D	aneuploid	malignant	positive	Engel and Young, 1978
ZR75	aneuploid	malignant	positive	Engel *et al.*, 1978
MDA-MB-231	diploid	malignant	negative	Cailleau *et al.*, 1974

B

Treatment	Estradiol	Tamoxifen	ICI 182,780
None	none	none	none
E2	10 nM	none	none
Tam	none	1 μM	none
Tam+E2	10 nM	1 μM	none
ICI	none	none	1 μM
ICI + E2	10 nM	none	1 μM

C

Estrogen Receptors	Housekeeping genes	Estrogen responsive genes
ERα	HPRT	Cathepsin D
ERβ1	GAPDH	Cyclin D1
ERβ2	ARF1	EFP
ERβ5	PPP1CA	Liv1
		p21 (KIP1)
		PR
		PS2 (TFF1)
		VEGF
		XBP1S
		XBP1U

Table 3.1 Cell lines, treatments and Q-PCR assays used to investigate estrogen response in breast cancer cell lines

Experiments were performed using a set of breast cancer cell lines to investigate the estrogen response in these cells. Cells of each type were treated with a variety of stimuli and changes in expression of a set of genes measured.

(A) Details of the set of breast cancer cell lines, (B) The different experimental treatments used, (C) The set of genes assayed by Q-PCR.

insulin, whilst being inhibited by glucocorticoid and androgen [Engel et al., 1978]. T47D expresses estrogen, progesterone, glucocorticoid and androgen receptors [Keydar et al., 1979]. Finally, MDA-MB-231 was established in 1973 from a pleural effusion of a tumour showing a poorly differentiated phenotype [Cailleau et al., 1974]. This cell line is regarded as non-hormone responsive and does not express large amounts of the hormone receptors [Engel and Young, 1978]. To investigate a range of responses, cultures were withdrawn from estrogen stimulation (Section 2.2.1.2) before treatment with combinations of estradiol, ICI 182,780 and tamoxifen in fresh withdrawal medium for 24 h (Table 3.1 B), after which mRNA levels were measured.

Although the ERα status of these cell lines has been previously determined, it has also been shown that cells of the same cell line maintained under different conditions and in different laboratories may express different levels of ERα. In order to determine the levels of ERα protein expressed by the various cell lines used in this thesis, an ELISA (Section 2.7.7.1) was performed. For each cell line, cells were grown either in standard medium or in withdrawal medium (Section 2.2.1.2). Protein was extracted using the nuclear extract kit (Section 2.7.1.3) in order to separate the nuclear and cytoplasmic fractions. The assay was performed using both the nuclear and cytoplasmic fractions to determine ERα expression and localisation. ERα was shown to be mainly located in the nucleus in these cells, although cytoplasmic levels were proportional to nuclear levels (data not shown). In both the nuclear and cytoplasmic fractions, ERα levels were higher in the cells grown in complete medium than withdrawal medium, indicating that the presence of estradiol may increase the expression of ERα protein (Figure 3.1). The rank order level of expression of ERα in the cells is: MCF7>T47D>ZR75>MDA-MB-231, although in compete medium, ZR75 shows a similar level of expression to T47D. Expression of ERα in MDA-MB-231 cells was at the limit of detection, in accordance with their reported ER-naïve status.

Estradiol is the classic activating ligand for the ERs and was used to establish the cells' estrogen response. ICI 182,780 (Fulvestrant) is a pure antiestrogen (Section 1.3.4, [Wakeling and Bowler, 1992]) and tamoxifen is a SERM (Section 1.3.4). The combination treatments, estradiol and ICI 182,780, as well as estradiol and tamoxifen, were also included, as when ICI 182,780 or

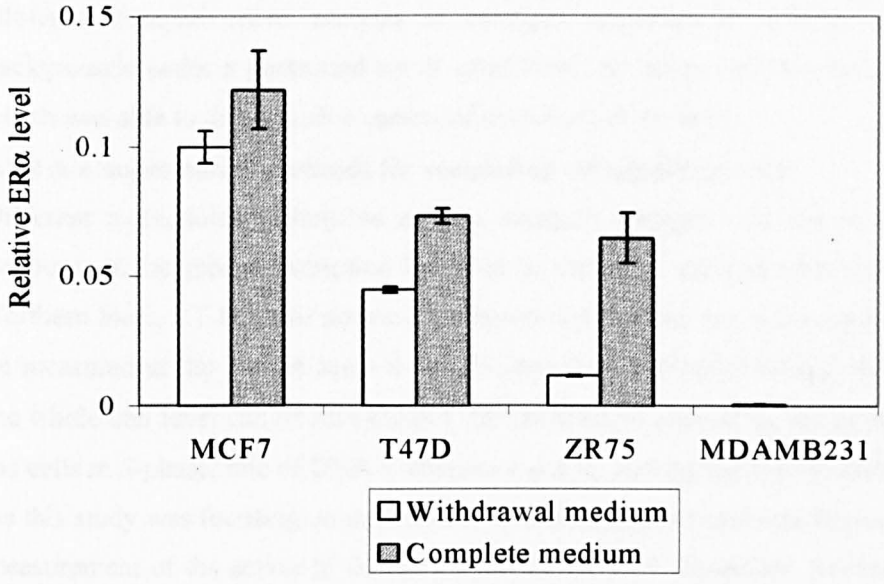


Figure 3.1: Expression of ERα protein in a set of breast cancer cell lines

Levels of ERα were measured in the nuclear protein fractions for the set of breast cancer cell lines used in this study using an ELISA assay (Section 2.7.7.1). Relative levels of expression are shown here for cells grown either in standard medium or after withdrawal for six days (mean +/- SEM 3 ELISA replicates from a single biological experiment) (Section 2.2.1.2).

tamoxifen are used as treatments for breast cancer, this is usually in the context of an estrogen-positive environment. Investigation of responses to the combined treatments is, therefore, appropriate, as it allows inhibition of estrogenic effects by the antiestrogens to be observed. Thus, the use of this set of cell lines and effectors allows a comprehensive analysis of estrogen responses in different signalling backgrounds under a controlled set of conditions. An assay was therefore required, which was able to distinguish a variety of responses in the cells.

3.1.2 A comparison of methods for measuring estrogen response

Different methodologies may be used to measure estrogen response in cell lines. Response at the gene transcription level can be measured using reporter gene assays, Northern blots, RT-PCR, or arrays. Expression of estrogen responsive genes can also be measured at the protein level using, for example, Western blotting. Response at the whole cell level can be investigated, for instance by measuring the percentage of the cells in *S*-phase, rate of DNA synthesis or simply comparing growth rates.

As this study was focusing on the role of the ERs in controlling transcription, a direct measurement of the action of the receptors was required. Therefore, transcription of estrogen responsive genes was measured, rather than protein or cell-growth response, as this gives a more direct measurement of ER function. Protein expression is a poor marker of transcriptional rates, with poor correlation between mRNA and protein levels being observed in many different systems [Chen et al., 2002; Washburn et al., 2003], as protein levels rely on mRNA stability, rates of translation and rates of protein degradation, as well as rates of transcription. Investigation of cell growth responses is useful when comparing the behaviour of cells expressing different receptors [Omoto et al., 2003]. However, it does not give a direct insight into the mechanisms of the estrogen response, particularly when comparing different cell lines, in which many signalling pathways may be dissimilar. As the major function of the ERs is to regulate gene transcription rates, the more direct measurement of mRNA was chosen.

Reporter gene assays allow measurement of transcriptional stimulation via a specific promoter, as used in, for example, [Singh et al., 2003]. Although these assays can give interesting results, they are not fully indicative of the transcriptional response in native chromatin, when other DNA regulatory elements [Paech et al., 1997], chromatin structure [Cheung et al., 2003] and coactivating complexes [Klinge, 2000] are likely to be involved. Reporter gene assays also generally allow only a single or a

small number of promoters to be investigated at a time, whereas measuring native gene expression can allow analysis of several different response elements in parallel.

3.1.2.1 Arrays: general considerations

An obvious method to measure changes in expression in a large number of target genes would be the use of arrays [van Berkum and Holstege, 2001]. However, arrays do have disadvantages, which may, in our case, outweigh the advantage of being able to measure the expression levels of many genes at a time. cDNA array experiments allow gene expression in two labelled samples to be compared. This allows measurement of the ratio of expression of a target in the two samples, but does not quantify the actual levels of expression. Single channel oligonucleotide arrays, e.g. Affymetrix, give a supposedly quantitative measurement of gene expression, but this is highly dependent on probe design, with different probes for overlapping regions giving different measurements of expression [Draghici, 2003]. This means that it would be difficult to use arrays to measure gene expression changes over the large set of cell lines and treatments proposed (a total of 20 samples). The sensitivity of array-based assays has also been questioned. An experiment comparing Q-PCR results with Affymetrix array results showed that genes with low levels of expression such as transcription factors could not be reliably quantified using the array [Czechowski et al., 2004]. Also, expression ratios measured using arrays are consistently underestimated, especially when using cDNA microarrays [Yuen et al., 2002].

When using arrays, replicate experiments should be performed to give reliable results, which can be expensive and slow [Lee et al., 2000]. Expression analysis and validation of the large data sets obtained from array-based experiments is also complicated and time consuming [Simon et al., 2003]. Finally, expression changes identified using arrays must be validated using another method such as Q-PCR, adding an extra stage to the process [Larkin et al., 2005].

3.1.2.2 Q-PCR: general considerations

A different method of examining gene transcription levels is to use a PCR based assay. Real-time, or quantitative PCR (Q-PCR) is a rapid and robust method of measuring changes in gene expression levels [Bustin, 2000]. Q-PCR is highly sensitive and has a wide dynamic range. Assays for many different genes can be performed from a single RNA sample, and expression of genes can be accurately measured in multiple samples in parallel. Primers and probes can be designed to

ensure only specific genes of interest are measured, and can even be designed to analyse expression of specific isoforms of a gene. This means that assays can be performed for several different ER isoforms, allowing quantification of their relative levels, as well as analysing expression of different estrogen responsive genes, which are influenced by the ERs in different ways (Section 3.1.3).

Thus, Q-PCR was chosen over an array-based assay, since the aim is to investigate the response to estradiol of a selected set of known estrogen responsive genes rather than to discover new estradiol responsive genes where the mechanism of the response was unknown. Q-PCR is also less time consuming and easier to validate, and more suitable for use when comparing multiple samples.

3.1.3 Selection of genes to be assayed

In order to investigate the levels of ERs in the cell lines and the response to estrogenic treatments, Q-PCR assays were prepared for the ERs, a panel of genes reported to be estrogen responsive and a set of housekeeping genes, the latter for use as controls. The genes used in the experiments are listed in Table 3.1 C, primers and PCR design are described in Section 2.6.3, and the selection criteria for the genes used are described here.

3.1.3.1 Estrogen receptors

ERα is a key player in estrogen response (Chapter 1) and different isoforms of ERβ have been shown to have varying roles in signal transduction [Omoto et al., 2003]. Assays for mRNA expression of ERα and various isoforms of ERβ were prepared. The Q-PCR assay for ERα expression uses primers spanning exons 3-4 (Table 2.2). This means it will detect full-length transcripts and some exon deleted forms, but not transcripts that exclude these exons, or are truncated before exon 3.

Several different assays for ERβ mRNA were tested (Table 2.1), but final experiments were performed using a set of Taqman assays with primers and probes spanning exons 7-8 (Table 2.2). These were specific to different isoforms of ERβ. Assays for ERβ1, 4 and 5 were adapted from [Poola, 2003b], but the ERβ2 assay from that paper was shown to have poor specificity as both primers and probe were located in regions shared by ERβ2, 4 and 5. An assay for ERβ2 was, therefore, prepared using the primers and probe from [Critchley et al., 2002]. These assays are

able to reveal the relative levels of the *C*-terminal variants of ERβ, but cannot detect any internal isoform deletions such as ERβΔ5 [Inoue et al., 2000].

3.1.3.2 Estrogen responsive genes

A subset of the many genes shown to be regulated by estrogen was selected for investigation in this study, based on their different modes of regulation.

Cathepsin D

Cathepsin D is a lysosomal aspartyl protease composed of a dimer of disulfide-linked heavy and light chains, both produced from a single protein precursor. Transcription of this gene is initiated from several sites, including one which is a start site for an estrogen-regulated transcript [Cavailles et al., 1993]. Mutations in this gene are involved in the pathogenesis of several diseases, including breast cancer and possibly Alzheimer's disease.

The cathepsin D promoter contains an imperfect ERE as well as an SP1/ half-ERE site [Wang et al., 1997a], which binds ERα with greater affinity than ERβ1 in a gel-shift assay [Hyder et al., 1999]. Binding of both ERα and SP1 to the promoter is required for a full estrogen response. This was determined by use of wild-type and mutant promoters coupled to a CAT reporter gene in transient transfection assays using MCF7 cells [Krishnan et al., 1994].

C-myc binding protein

The c-myc binding protein gene (*amy-1/mycbp*) encodes a protein that binds to the *N*-terminal region of MYC and stimulates the activation of E box-dependent transcription by MYC. Expression of another c-myc binding protein (MM-1) was found to be regulated by tamoxifen [Pole et al., 2005].

An assay was prepared to analyse expression of this gene. Early studies showed no significant changes in expression of this gene in response to treatment with estrogen, tamoxifen or ICI 182,780, so it was excluded from the final set of responsive genes.

Cyclin D1

Cyclin D1 is a cell cycle regulatory protein, which varies in its expression and degradation throughout the cell cycle and participates in the regulation of the G1/S transition. The cyclin D1 gene has no known ERE, but is rapidly expressed in response to estradiol treatment in MCF7 cells [Altucci et al., 1996; Foster and Wimalasena, 1996; Planas-Silva and Weinberg, 1997; Prall et al., 1997]. The

mechanism of the estrogen response may vary in a cell-type specific manner, as several different response elements have been identified in different studies.

In MCF7 cells, induction of cyclin D1 has been mapped to a cAMP response element [Sabbah et al., 1999]. The estrogen response was not, however, dependent on cAMP in MCF7 or HeLa cells, but involved binding of a c-Jun/ATF-2 heterodimer to the response element, which then interacts with estradiol-bound ERα to activate gene transcription. The core response element (-96 to -29) was not sufficient to activate gene expression when removed from the promoter context, suggesting other regions might also be involved in stabilising the protein complex. Another group investigated estrogen response in ZR75 cells [Castro-Rivera et al., 2001]. They found multiple SP1-binding sites, as well as the cAMP response element, were involved in the estradiol response in these cells. A reporter gene construct under the control of the cyclin D1 promoter was stimulated when co-transfected with an ERα expression plasmid, but not when ERβ was used. In this study, activation at the cAMP response element was shown to be protein kinase A (PKA) dependent.

The roles of ERα and ERβ1 in the control of cyclin D1 expression were further investigated by transiently transfecting HeLa cells with expression vectors for either ERα, ERβ1 or an ERα mutant which is superactive at AP1 sites [Liu et al., 2002b]. By measuring both expression from a luciferase reporter gene linked to the cyclin D1 promoter and endogenous cyclin D1 protein, they showed that both the cAMP response element and an upstream AP1 site were necessary for full induction of the reporter gene. They also found that ERα activated cyclin D1 expression in response to estrogens, whereas ERβ1 activated expression in response to antiestrogens, and repressed expression to below basal levels in the presence of estrogens. When ERα and ERβ were co-expressed, induction with estrogen was completely silenced, whereas the antiestrogen response was intermediate between the responses shown with either receptor alone.

Cytochrome C oxidase VIIa

Cytochrome C oxidase (COX) protein is found in the mitochondria, where it catalyses electron transfer from reduced cytochrome C to oxygen. It exists as a heteromeric complex consisting of three catalytic subunits encoded by mitochondrial genes and multiple structural subunits encoded by nuclear genes. This nuclear gene (COX7A) encodes polypeptide 1 (muscle isoform) of subunit VIIa. Other

polypeptides of subunit VIIa are present in both muscle and non-muscle tissues, and are encoded by different genes.

A related gene, *cox7rp/cox7a2l* (NCBI GeneID: 9167), was shown to contain a consensus ERE in the promoter region and to be up-regulated by estradiol in MCF7 cells [Watanabe et al., 1998]. The assay for COX7A mRNA showed no estrogen response in early studies, so was excluded from subsequent experiments.

Estrogen-responsive finger protein

Estrogen-responsive finger protein (EFP, ZNF147) is a member of the tripartite motif (TRIM) family and is found in the cytoplasm. The presence of potential DNA-binding and dimerisation-transactivation domains suggests that this protein may act as a transcription factor, similar to several other members of the TRIM family. EFP is thought to mediate estrogen actions in breast cancer as a primary response gene, and its expression has been observed in tumours [Ikeda et al., 2000]. In MCF7 xenografts, inhibition of EFP expression by anti-sense oligonucleotide reduces tumour growth, whereas MCF7 cells over-expressing EFP formed tumours in xenografts even in an estrogen deprived environment [Horie et al., 2003]. The EFP promoter contains a consensus ERE which can activate transcription of a reporter gene in response to estradiol. Expression of the gene is up-regulated in breast epithelial cells (HBL-100) in response to estrogen at both the mRNA and protein levels. mRNA expression was increased by 2 h, peaked at 10 h, and returned to basal levels by 20 h after stimulation [Inoue et al., 1993]. In MCF7 cells, EFP mRNA expression is also rapidly increased in response to estradiol treatment (within 0.5 h), and expression returns to basal levels within 8 hours [Ikeda et al., 2000].

Lactoferrin

Lactoferrin (LTF) belongs to a family of iron-binding proteins that modulate iron metabolism, haemopoiesis, and immunologic reactions. The lactoferrin promoter contains an imperfect ERE and two nearby half-EREs, among other elements. The imperfect ERE and overlapping COUP binding site were sufficient to generate estrogen response when tested in a reporter gene assay in a human endometrial carcinoma cell line [Teng et al., 1992].

Although an assay for LTF mRNA was prepared, it showed poor precision, giving very large errors when repeated measurements were performed. This is probably due to the very low levels of expression of this gene in the cell lines used. The assay was therefore excluded from the set of genes analysed in later experiments.

Liv1

Liv1 (SLC39A6) belongs to a subfamily of zinc-transporting proteins [Taylor and Nicholson, 2003]. Its expression is strongly correlated with ERα status in breast cancers [Manning et al., 1995] and its expression is stimulated by estradiol in ZR75 breast cancer cells [Manning et al., 1988]. Promoter analysis has not been reported for this gene, so the exact mechanism of the estrogen response is unknown. However, analysis of the region between –2000 and +100 using the TFSEARCH programme (http://www.cbrc.jp/research/db/TFSEARCH.html), which searches sequences for putative binding sites and transcription activation domains based on data from the TRANSFAC databases [Heinemeyer et al., 1998], identified several putative AP1, SP1 and CREB binding sites, but no ERE. The Dragon ERE finder version 2.0 (http://sdmc.lit.org.sg/ERE-V2/index) [Bajic et al., 2003] also found no ERE at the default sensitivity (83%) but found possible matches at –123 and –1951 when the sensitivity was raised to 94%. Despite the lack of promoter information, liv1 was included in the set of estrogen responsive genes, due to its strong response in previous studies.

P21

$P21^{cip1}$ is a cyclin-dependent kinase inhibitor. It binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and so helps regulate cell cycle progression through G1. P21 can also interact with proliferating cell nuclear antigen (PCNA), a DNA polymerase accessory factor, and play a regulatory role in *S*-phase DNA replication and DNA damage repair. P21 is not a classical estrogen responsive gene, but [Paruthiyil et al., 2004] suggest that over-expression of ERβ1 may lead to ligand independent up-regulation of this gene.

P27

$p27^{kip1}$ is also a cyclin-dependent kinase inhibitor, which acts on cyclin E-CDK2 or cyclin D-CDK4 complexes and, thus, controls cell cycle progression at G1. Expression of p27 has been implicated as a prognostic factor in breast cancer [Barnes et al., 2003] and may be regulated by estrogen [Foster et al., 2003]. It may also play a part in ERβ response, as transfected MCF7 cells, which over-express ERβ1, express increased levels of p27. These cells are also G2 arrested, showing a 4-fold increase in the percentage of cells in G2/M phase 96 hours after transfection [Paruthiyil et al., 2004]. Although a Q-PCR assay for p27 mRNA was designed, it was not sufficiently

sensitive to accurately measure levels of p27 mRNA in the cell lines. This may be due to the quality of the assay, or may be indicative of extremely low levels of expression of this gene in the cells studied.

Progesterone receptor

Progesterone receptor (PR) is a member of the steroid receptor super-family and mediates the physiological effects of progesterone. Progesterone plays a central role in reproduction. The PR gene uses separate promoters and translational start sites to produce two isoforms, PRA and PRB, which possesses an additional 164 amino acids at its *N*-terminus [Kastner et al., 1990]. Although PRA and PRB share several structural domains, they are distinct transcription factors that mediate their own response genes and downstream effects with little overlap [Giangrande et al., 2000; Vegeto et al., 1993]. The primers used in this study span exons 5-6 and therefore amplify both forms of PR mRNA.

Both forms of PR show increased expression in response to estrogens in breast cancer cell lines such as MCF7 [Nardulli et al., 1988], although their expression is decreased by estrogen treatment in ovarian cells [Mukherjee et al., 2005]. Multiple PR mRNAs are found in PR positive cell lines (MCF7 and T47D), but not in PR negative cell lines (MDA-MB-231 and LY2) [Read et al., 1988]. The lack of PR expression seen in MDA-MB-231 cells may be related to the low ERα expression levels in these cells.

The PRA promoter contains an ERE half-site upstream of two SP1 sites, a region shown to be important in estrogen responsiveness [Petz and Nardulli, 2000]. The PRB promoter contains two SP1 sites (-80/-34 region) [Schultz et al., 2003], which confer estrogen responsiveness on a heterologous promoter in a reporter gene assay, when co-transfected with an ERα-expressing plasmid. PRB expression has been shown to be regulated in a cell line, ER sub-type and treatment specific manner [Flototto et al., 2004]. In a series of co-transfection assays, expression from the PRB promoter was shown to be activated via ERα in response to estradiol in Ishikawa (endometrium) and HeLa (cervix) cells, and via either ERα or ERβ1 in response to SERMs. However, the levels of response to different treatments varied between these cell lines. Surprisingly, SK-BR-3 (breast cancer) cells showed a different response, in that expression was inhibited by estradiol and up-regulated by SERMs in the presence of either ER subtype.

PS2

PS2 or trefoil factor 1 (TFF1) is a secreted protein, expressed in the gastrointestinal mucosa, as well as in human tumours. PS2 is expressed by MCF7 cells in response to estradiol treatment [Masiakowski et al., 1982]. This has been shown to be a rapid, primary response [Brown et al., 1984]. PS2 expression also correlates with ERα in breast cancers [Tong et al., 1999]. The PS2 promoter contains an imperfect ERE, which is essential to the estrogen response, permitting estradiol-dependent activation of a reporter gene in HeLa cells [Berry et al., 1989] and which binds both ERα and ERβ1 *in vitro* [Hyder et al., 1999].

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is involved in normal development of the vasculature and in cancer development. It exists as a glycosylated homodimer and exerts several effects on endothelial cells including promoting cell growth and migration, inhibiting apoptosis and increasing vascular permeability. The VEGF gene is expressed as multiple splice variants via alternate splicing of exons 6 and 7 [Tischer et al., 1991]. The primers used span exons 3-4, and therefore will detect any of these forms.

The VEGF promoter contains multiple regulatory domains, involved in estrogen-dependent activation. In the endometrium it can be up-regulated by ERα via an SP1 binding domain and also via estrogen induced HIF-1 binding to the promoter, suggesting that this factor may be involved in estradiol stimulated VEGF expression [Koos et al., 2005]. A further study using endometrial cell lines showed that expression from the VEGF promoter can be up-regulated by ERα (3.2-fold) and ERβ (2.3-fold) in an estradiol-dependent manner via a variant ERE located 1.5 kb up-stream from the transcription start site [Mueller et al., 2000].

In MCF7 cells, VEGF mRNA and protein are up-regulated by estradiol or tamoxifen, but this induction was inhibited by ICI 182,780 [Ruohola et al., 1999]. A series of experiments to investigate the regulation of VEGF by ERα and ERβ in breast cancer cell lines showed that VEGF mRNA was induced to maximum levels in MCF7 cells 6 hours after estradiol treatment, but could not be induced in MDA-MB-231 cells [Buteau-Lozano et al., 2002]. Transient transfection of a reporter gene construct, in addition to expression constructs for either ERα or ERβ, showed that the response to different ligands varied in a cell type and receptor subtype dependent manner.

Activation of a reporter gene under the control of a reduced fragment of the VEGF promoter containing a variant ERE (-1520) could be achieved in response to ERα-bound estradiol in MCF7 but not in MDA-MB-231 cells, confirming that multiple different response mechanisms may be involved in the estrogen-dependent activation of VEGF.

X-box binding protein 1

X-box binding protein 1 (XBP1) is a transcription factor originally identified by its ability to bind a specific promoter motif via a leucine zipper motif [Liou et al., 1990] and which is involved in the stress response. It has more recently been found to interact with the ERs, activating ERα in a ligand-independent manner [Ding et al., 2003]. XBP1 mRNA undergoes unconventional splicing, with 26 nucleotides being specifically removed from within an exon by IRE1α. This splicing results in the expression of two different forms, XBP1U and XBP1S, which are 261 and 376 amino acids long, respectively [Yoshida et al., 2001]. XBP1S is the more potent activator of ERα regulated expression. It has been suggested that XBP1 may be involved in chromatin unfolding to allow ER controlled expression [Fang et al., 2004].

Interestingly, as well as being involved in regulation of ER-dependent expression, XBP1 has been shown to be up-regulated in response to estradiol in several microarray experiments [Bouras et al., 2002; Finlin et al., 2001; Wang et al., 2004]. This led to its inclusion as an estrogen responsive gene in the target gene set, using primers, which differentiate the two forms of the mRNA. This allows investigation of the expression of both forms of the mRNA and, therefore, permits analysis of whether the production of XBP1 is controlled by ER at the transcriptional or splicing level.

3.1.3.3 Housekeeping genes

A set of "housekeeping genes" were chosen and used for normalising Q-PCR results. Normalising using housekeeping genes allows correction for RNA quality and reverse transcription efficiency and is described more fully in Section 3.2.5. For larger experiments a "housekeeping ratio" was calculated by averaging expression levels of several housekeeping genes.

ADP-ribosylation factor 1

ADP-ribosylation factor 1 (ARF1) is a member of the human ARF gene family. The family members encode small guanine nucleotide-binding proteins that play a role in vesicular trafficking as activators of phospholipase D. The ARF1 protein is localized to the Golgi apparatus and has a central role in intra-Golgi transport. Multiple alternatively spliced transcript variants encoding the same protein have been found for this gene.

ARF1 was investigated as a housekeeping gene based on its use in a set of control genes produced by Stratagene (#720130). It is expressed at low levels, making it an ideal control when measuring genes, which are also expressed at low levels, such as the ERs. Experiments showed that ARF1 expression was conserved across the set of cell lines and treatments, confirming its suitability as a housekeeping gene.

Glyceraldehyde-3-phosphate dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is involved in carbohydrate metabolism, specifically the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate. The enzyme consists of a tetramer of identical chains. A GAPDH pseudogene has been mapped to Xp21-p11 and 15 GAPDH-like loci have been identified. Primers were selected to cross an intron-exon boundary to produce a cDNA specific assay (Table 2.2).

GAPDH is identified as a likely housekeeping gene in [Eisenberg and Levanon, 2003] and is used as such in many studies. Although its levels have been shown to vary in different tissues, it is still suitable for use in controlled situations [Barber et al., 2005] and its expression has been shown to correlate well with a variety of other housekeeping genes [de Kok et al., 2005; Vandesompele et al., 2002]. GAPDH is expressed at relatively high levels.

Histone H3

Histone H3 has been widely used as a housekeeping gene, and is listed in [Eisenberg and Levanon, 2003] and other studies on housekeeping genes. It is a major protein in chromatin, being part of the nucleosome structure. Although initially selected as a possible housekeeping gene, it was shown that its mRNA levels varied strongly between treatments in the cell lines, making it unsuitable for use in this study.

Hypoxanthine phosphoribosyltransferase 1

Hypoxanthine phosphoribosyltransferase 1 (HPRT) is highly expressed and is involved in metabolic salvage of purines. It is widely used as a housekeeping gene,

giving good results in several studies, e.g., [Vandesompele et al., 2002]. It has been shown to correlate well with a set of ten other housekeeping genes [de Kok et al., 2005], being the gene which was most suited to replacing using these multiple genes as a single reference.

PPP1CA

Protein phosphatase 1 (PPP1) is a serine-threonine phosphatase, involved in the regulation of many cellular processes. PPP1CA has been shown to be ubiquitously expressed in a variety of cancer cell lines [Takakura et al., 2001], suggesting its relevance as a housekeeping gene. It was also listed as a low expression control in a set of controls produced by Stratagene (product #720130). Experiments confirmed that it was expressed constitutively in the cell lines chosen for the study and was, therefore, suitable for use as a low-abundance housekeeping gene.

3.1.4 Measuring estrogen response in breast cancer cell lines

Genes were chosen for expression analysis by Q-PCR (Section 3.1.3) and assays designed and optimised for these genes (Section 2.6, 3.2). Experiments were also performed to validate the Q-PCR method and to test the variability and precision of the assay (Section 3.2).

Once the assays were fully validated, cells were treated with a variety of different estrogenic stimuli for 24 hours before RNA extraction, reverse transcription and Q-PCR analysis of gene expression (Chapter 2, Section 3.1.2). Gene expression was normalised using the expression patterns of a set of housekeeping genes (Section 3.1.3.3, 3.2.5) and estrogen responses were analysed (Section 3.3.3). This allowed changes of expression levels in a single cell type under different treatments as well as the effect of different signal transduction environments on the estrogen response to be investigated.

Experiments were performed to establish the unique pattern of estrogen receptor isoform expression in each cell line. Expression of the ER isoforms, estrogen responsive genes and housekeeping genes were measured after different treatments and estrogen responses related to the ERα and ERβ status of the cell lines.

3.2 Development of Q-PCR assays

To investigate estrogen responses in cell lines, a set of Q-PCR assays to analyse expression of ERα, three ERβ isoforms, a set of four housekeeping genes and ten estrogen responsive genes were prepared (Section 2.6, 3.1.3, Table 3.1 C). Q-PCR primers for each gene were chosen either from the literature or designed in-house using Oligo5 (NBI, Plymouth, MN, USA), Primer3 [Rozen and Skaletsky, 2000] or Beacon Designer (PREMIER Biosoft international). Primers were selected based on their internal sequence and location within the gene. Q-PCR works optimally with a short PCR amplicon of 75 to 150 bp, allowing melting and annealing/extension steps to be quite short (Table 3.2 A), but also limiting the possible locations of primers. Assays were preferentially selected which crossed an exon boundary, in order to prevent amplification of any contaminating gDNA and primers were BLAST searched [Wheeler et al., 2000] to confirm their specificity. Once primers were chosen, PCR cycling conditions were individually optimised for each assay (Section 3.2.2, Fig. 3.3). Q-PCR assays can be performed using either a DNA binding dye such as Sybr green [Giglio et al., 2003; Zipper et al., 2004] or a fluorescent probe, such as is used in the Taqman (Applied Biosystems) and scorpion systems [Bustin, 2000]. These systems have different advantages and disadvantages and so were compared to investigate which would be more appropriate.

3.2.1 A comparison of Sybr and Taqman assays

Fluorescence in a Q-PCR assay can be generated either using a non-specific dsDNA binding dye such as Sybr green, or using a probe specific to the PCR amplicon, as used in the Taqman system [Bustin, 2000]. The Taqman assay shows increased specificity over conventional real-time PCR by use of a third gene-specific oligonucleotide [Bustin, 2000]. Taqman assays also have the benefit over Sybr dye of being able to perform multiplex PCR, in which more than one set of primers and probes are included in a single assay, allowing simultaneous analysis of more than one gene product. The probe, specific to an area within the PCR amplicon, incorporates a fluorescent reporter dye at its 5' end and a quencher at the 3' end. The probe binds the single stranded DNA during the annealing step and is displaced and cleaved by the *Taq* polymerase during the extension step. This cleavage releases the

dye from the quencher and the resulting fluorescence can then be measured.

Using the Taqman assay, primer dimerisation or non-specific amplification do not lead to erroneously increased fluorescence, although these should still be minimised, as they will interfere with the efficiency of the PCR reaction. The level of fluorescence generated using the Taqman system is lower than seen using Sybr, as only one probe with a single fluorophore molecule attached binds per DNA fragment, compared to many Sybr molecules binding each dsDNA. Use of a DNA-binding dye allows generation of a melt curve after PCR to test whether a single or multiple products have been formed (Section 2.6). This is not possible with a Taqman probe, so electrophoresis should be performed after Taqman PCR to confirm the product size and purity.

3.2.1.1 Experimental comparison of Sybr and Taqman assays

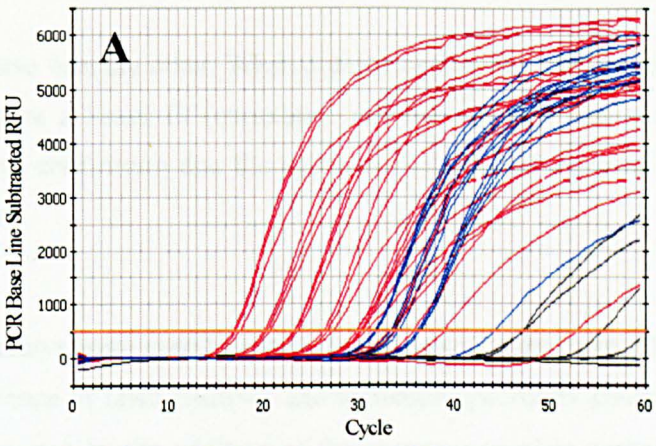
In order to investigate the relative merits of the Sybr and Taqman methods, Q-PCR for ERβ was performed using the same samples and primers, with either Sybr dye or a Taqman probe (Fig. 3.2). PCR using Sybr gave much higher levels of fluorescence; up to 6000 rfu (Fig. 3.2 A) compared to 1200 rfu for the Taqman assay (Fig. 3.2 C), as well as earlier threshold cycle values (mean 1.9 cycles earlier), as fluorescence rose above baseline levels much sooner. However, the Sybr method will detect any double stranded DNA present in the reaction, as well as showing weak fluorescence when bound to single stranded DNA [Zipper et al., 2004]. This can lead to false positive results in negative controls, as primer dimers or non-specific amplification can occur, and the resulting dsDNA is bound by the Sybr dye. This is likely to explain results with nominal starting quantity (SQ) much smaller than 10^{-7} amol/reaction, which corresponds to less than one molecule per reaction (Fig. 3.2 B).

The Taqman assay showed no non-specific fluorescence, with no negative controls rising above base line fluorescence (Fig. 3.2 C-D). However, the total levels of fluorescence are much lower. Taqman assays showed a much "noisier" amplification curve (compare Fig. 3.2 A and C). This may simply reflect the larger signal with the Sybr assay rendering the noise in the amplification curve smaller. This "noise" could cause a reduction in precision of the assay, however, in this experiment, of the four samples included as unknowns, two of these showed greater standard deviation in the Sybr assay, and two in Taqman assay, suggesting that neither assay is significantly

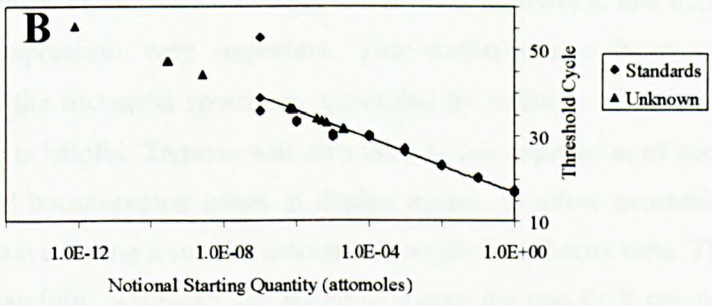
Figure 3.2 Comparison of Sybr and Taqman assays for ERβ1

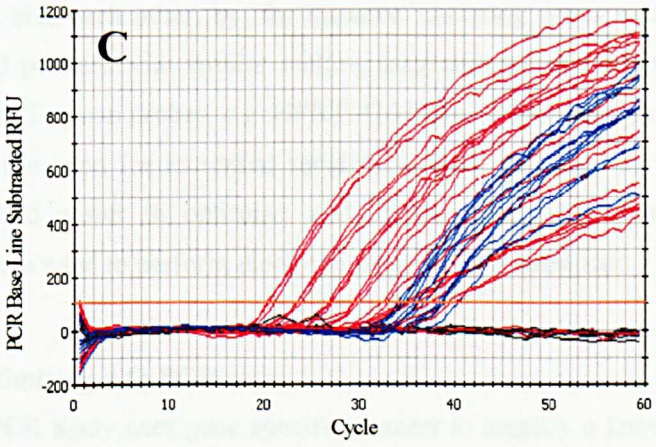
PCR was performed using ERβ1 specific primers, with and without a Taqman probe (Section 3.2.1) to assess the relative merits of the two systems. For each method, PCR was performed using a standard curve of diluted plasmid (red traces on PCR amplification graphs), 4 different cDNA samples (blue) and negative controls (black). Standard curves were prepared using a dilution series of plasmid. Dilutions from 1 amol/reaction to a nominal 10^{-7} amol/reaction were used in triplicate. The equations of the standard curves were used to calculate nominal starting quantities for the unknown samples for each PCR method.

(A) Fluorescence generated during PCR for the Sybr assay, (B) standard curve for the Sybr assay, (C) Fluorescence generated during PCR for the Taqman assay, (D) standard curve for the Taqman assay.

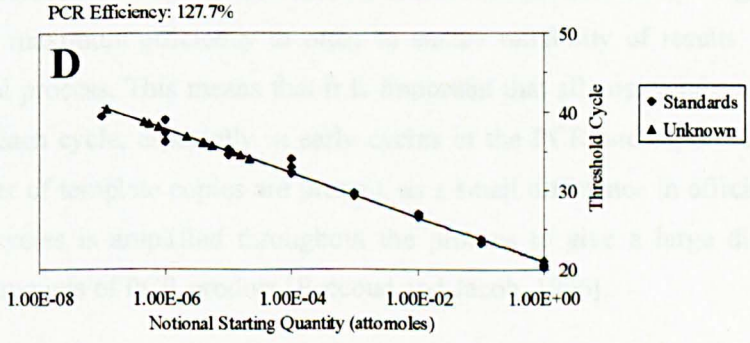


Correlation Coefficient: 0.919 Y=-3.190X + 16.802
PCR efficiency: 105.8%





Correlation Coefficient: 0.993 Y = -2.799 X + 20.959
PCR Efficiency: 127.7%



more precise than the other. When comparing the standard curves, the Taqman assay actually gave a better fit (correlation coefficient 0.993 compared to 0.919 for the Sybr assay) confirming that the apparent noisiness of the assay does not affect the consistency.

Taqman assays were used to measure expression of different ERβ isoforms because of the absence of false-positives and increased specificity generated by the Taqman assay, imparted by the addition of the sequence-specific probe. The ERβ isoforms are only expressed at low levels, making the distinction between low expression and absence of expression very important. The isoforms also have very similar sequences, so the increased specificity generated by addition of an extra isoform-specific probe is helpful. Taqman was also used to test expression of some estrogen responsive and housekeeping genes in duplex assays, to allow expression of more genes to be assayed using a smaller amount of sample in a shorter time. These duplex assays were carefully optimised and tested to ensure the two PCR reactions did not inhibit or bias each other, by, for instance, choosing genes with similar expression levels and primers with similar melting temperatures in these assays. Some assays did not use Taqman probes, e.g. HPRT, GAPDH. Where gene expression was higher, reducing the need for increased sensitivity, Sybr was used as this methodology is cheaper and easier to optimise, and allows generation of a melt-curve graph, reducing the need to perform electrophoresis of PCR products.

3.2.2 Optimising a Q-PCR assay

Each Q-PCR assay uses gene-specific primers to amplify a known region of cDNA. PCR is optimised by varying the contents of the reaction and the cycling conditions to achieve maximum efficiency in order to ensure reliability of results. PCR is an exponential process. This means that it is important that all copies of a template are copied at each cycle, especially in early cycles of the PCR and especially if only a low number of template copies are present, as a small difference in efficiency in the first few cycles is amplified throughout the process to give a large difference in finishing amounts of PCR product [Peccoud and Jacob, 1996].

PCR efficiency can vary due to the amount of template in the starting mixture, the contents of the reaction mix such as Mg^{2+} or KCl levels [Henegariu et al., 1997], carry-over from upstream processes, such as EDTA, NaCl, or GITC, and the use of non-optimum temperatures during PCR cycling. Of course, the starting amount of template cannot be selected, so each assay was optimised so that the reaction mixture and PCR cycling conditions were optimal for the particular primers. As all Q-PCR was performed using the Bio-Rad iCyler, assays were prepared using Bio-Rad Q-PCR master mix (Section 2.6). This reduced the amount of variation of the PCR reaction contents possible during optimisation, so that only levels of primers or cycling conditions could be changed.

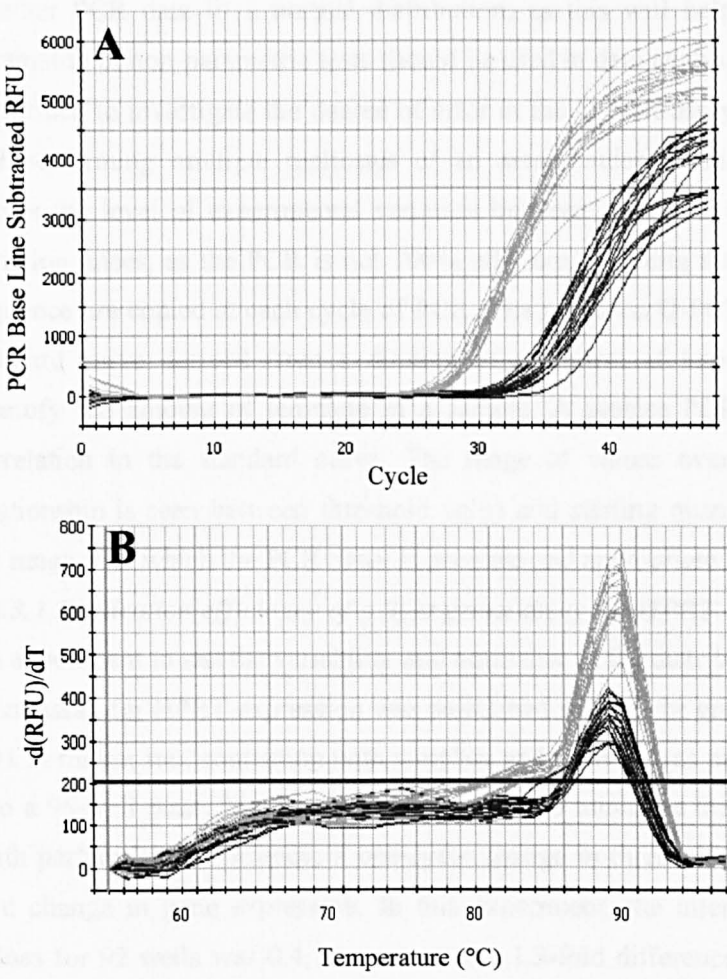
An example of Q-PCR optimisation is shown (Fig. 3.3), using primers for ERβ1 taken from [Poola, 2003b] (Table 2.2), Sybr detection and two different primer concentrations (0.1 μM and 1 μM). A two-step PCR assay was performed, with a gradient of annealing/extension temperatures from 55 to 65°C. This PCR assay was shown to have good specificity, generating a single PCR product with no significant primer dimer formation (Fig. 3.3 B). The assay was significantly less efficient when less primer was used, since the threshold cycle was an average of six cycles higher with the lower primer concentration (Student's *t*-test $p<0.0005$, Fig. 3.3 A). When 0.1 μM primers were used, the greatest efficiency was seen at an annealing temperature of 61.2°C, with a range of threshold cycle (Ct) values of 4.4. Ct was significantly associated with temperature (1-way ANOVA $p=0.002$) at this primer concentration. At 1 μM primers, efficiency was less strongly affected by annealing temperature. The range of Ct values was 1.9, and the relationship using 1-way ANOVA was weaker ($p=0.033$), showing that the assay is more robust at the higher concentration of primers (1 μM). In this case, a higher annealing temperature, e.g., 64°C, would be chosen to help minimise non-specific binding of the primers and, therefore, increase PCR efficiency.

Optimising the PCR assay can minimise the amount of variability seen, but a certain amount of variation is unavoidable, due to the nature of the PCR reaction. Quantifying this variation allows the degree of confidence in the assay to be established.

Figure 3.3 Optimising a Q-PCR assay for ERβ1 using Poola's primer set

Q-PCR (Section 2.6.3 and [Poola, 2003b]) was performed using either 1 μM or 0.1 μM primers (shown in light and dark grey, respectively, in panels A and B). Different cycling conditions were also tested; here a 2-step PCR was performed with melting at 94°C for 20 s and combined annealing/amplification at 55 to 65°C for 60 s. All replicates used a diluted sample of testis cDNA as template and Sybr green was used for quantification.

(A) The fluorescence increase during PCR, which is proportional to the amount of PCR product obtained; *(B) Melt curve*, which is generated by gradually increasing the temperature from 55°C to 98°C, whilst monitoring the level of fluorescence. dsDNA molecules of a particular size and composition will denature at a specific temperature, giving a peak in the melt curve graph. If high levels of primer dimers are present, a second peak is seen with a lower melting temperature than that of the PCR product; *(C) PCR efficiencies* using different primer concentrations and different annealing/extension temperatures. Note that higher threshold cycle is equivalent to lower PCR efficiency.


A
PCR Base Line Subtracted RFU
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
Cycle
B
-d(RFU)/dT
800
700
600
500
400
300
200
100
0
60
70
80
90
Temperature (°C)
C
Threshold Cycle
40
35
30
25
55
60
65
Melting temperature (°C)
* 0.1 µM primer
x 1 µM primer

3.2.3 Investigating variability in the Q-PCR assay

It was important to know the precision of the Q-PCR assay. It is also useful to know whether PCR data fit a normal distribution, as this will help determine whether parametric or non-parametric tests should be used in data analysis. Experiments were performed to investigate the degree of error in the Q-PCR assay using master mixes and performing multiple replicates of an assay under identical conditions. This allows the level of experimental variation between results to be established. Such variation arises, as the PCR is not 100% efficient, meaning that not all copies of a sequence are copied at each cycle of PCR. The real-time Q-PCR method relies on a standard curve derived from a dilution of a control of known concentration to quantify the amount of template in a sample. A precise PCR assay shows good correlation in the standard curve. The range of values over which a log-linear relationship is seen between threshold value and starting quantity of target defines the range over which the PCR assay is accurate and appropriate.

3.2.3.1 Replication efficiency of a Sybr green assay for HPRT

An experiment to test the variability and normality of the distribution of 92 replicates of an assay for HPRT expression was performed using Sybr green (Section 2.6, Fig. 3.4). A master mix containing both template and primers was prepared and aliquoted into a 96-well plate. The master mix was used to minimise the levels of user error. With perfect PCR efficiency, a one-cycle change in threshold is equivalent to a 2-fold change in gene expression. In this experiment, the interquartile range of Ct values for 92 wells was 0.4, equivalent to a 1.3-fold difference in gene expression, and the 95% confidence interval (calculated as mean +/- 1.96 SD) was 1.2, equivalent to a 2.3-fold difference in expression.

There are many different tests for normality. Minitab, for instance, gives a choice of three tests, the Anderson-Darling, the Ryan-Joiner and the Kolmogorov-Smirnov.

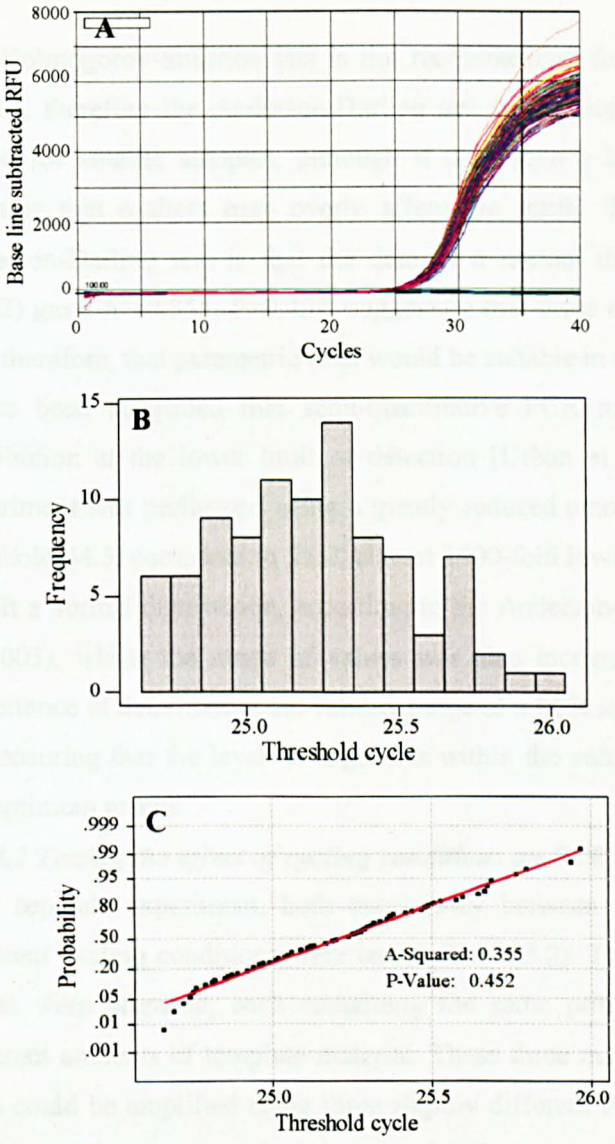


Figure 3.4 Variability and normality of 92 replicates of a Q-PCR assay for HPRT.

Ninety-two replicate Q-PCR assays for HPRT were performed using a single master mix to test for between-well variability. Master mix contained primers, iCycler Sybr green mastermix and a standard sample and was aliquoted into a 96-well plate for analysis.

(A) PCR amplification for 92 replicates, (B) Frequency histogram of threshold values obtained from (A), (C) Anderson-Darling normality plot for Ct values from (A).

The Kolmogorov-Smirnov test is not recommended for small samples (<100 data points), therefore the Anderson-Darling test was chosen as it is regarded as more robust for smaller samples, although it does give a large weighting to the tails, meaning that outliers may overly affect the result. The null hypothesis for the Anderson-Darling test is that the data fit a normal distribution. This experiment (n=92) gave A^2=0.355, P=0.452, suggesting that these data fit a normal distribution and, therefore, that parametric tests would be suitable in their analysis.

It has been suggested that semi-quantitative PCR may deviate from a normal distribution at the lower limit of detection [Urban et al., 2003]. When a similar experiment was performed using a greatly reduced amount of target (n=96, average threshold 34.5, compared to 25.2, almost 1000-fold lower expression) the results did not fit a normal distribution, according to the Anderson-Darling statistic (A2=1.268, P=0.003), whilst the range of values was also increased. These data confirm the importance of determining the validity range of a PCR assay using the standard curve and ensuring that the level of target lies within the valid range of the PCR assay to get optimum results.

3.2.3.2 Testing the effect of cycling conditions on PCR variability

In a separate experiment, both consistency between replicates and the effect of different cycling conditions were tested (Table 3.2). Three different 96-well master mixes were prepared, each containing the same primers and template, but with different amounts of template material. These three master mixes were split so that each could be amplified using three slightly different PCR protocols (Table 3.2 A). Again, threshold cycle values were analysed. Standard deviation (SD) of Ct values ranged from 0.4 to 1.8 across these experiments. This is equivalent to a 95% confidence interval (mean +/- 1.96 SD) of between 1.6 and 7.1 Ct, or between 3.0 and 130-fold difference in expression. Regardless of PCR conditions, SD was always greatest for the middle dilution of sample (template amount = 1, Ct $\approx$ 19). The 3-step PCR assay gave more precise results than either of the 2-step assays, as it gave the smallest SD for each of the three sample dilutions. The "2-step long" protocol, with extended melting and annealing/extension times, seems to have the lowest PCR efficiency, as it gave the highest Ct value for all samples. The 3-step PCR may be more precise because the optimal extension temperature for the polymerase can be

A

2 step short	2 step long	3 step
94.0°C for 20 s 65.0°C for 30 s x 40 cycles	94.0°C for 45 s 65.0°C for 45 s x 40 cycles	94.0°C for 20 s 60.0°C for 30 s 72.0°C for 30 s x 40 cycles

B

PCR[a]	Template amount[b]	N[c]	N missing[d]	Mean[e]	Median[f]	StDev[g]	SEM[h]	A^{2i}	P-value[j]
2 step short	0.01	31	1	27.3	27.2	0.6	0.10	0.45	0.256
	1	30	2	18.8	19.5	1.6	0.30	2.14	<0.0005
	100	32	0	11.2	11.2	1.1	0.19	0.25	0.723
2 step long	0.01	32	0	28.1	28.3	0.9	0.15	2.62	<0.0005
	1	32	0	19.8	20.4	1.8	0.32	4.19	<0.0005
	100	32	0	12.2	12.1	0.9	0.16	0.27	0.656
3 step	0.01	32	0	27.4	27.4	0.4	0.07	0.48	0.213
	1	32	0	19.3	19.7	1.2	0.21	4.60	<0.0005
	100	31	1	11.4	11.6	0.9	0.17	0.55	0.144

Cycling conditions as described in Table 3.1 A. [b] Amount of PCR template used, arbitrary units. [c] Number of samples (of a possible 32) reaching threshold fluorescence. [d] Number of samples not reaching threshold, and therefore excluded from analysis. [e] Mean threshold cycle (Ct). [f] Median Ct. [g] Standard deviation of Ct values. [h] Standard error of the mean of Ct values. [i] A^2 Anderson Darling normality test statistic: the larger the value, the greater the deviation from the normal distribution. [J] P-value for the Anderson-Darling normality test (H_0 is that the data fit a normal distribution).

Table 3.2 Variability in Q-PCR under different PCR cycling conditions.

To investigate the effect of template concentration and PCR cycling conditions on PCR efficiency and error, three 96-well PCR master-mixes were prepared using the same primers and template in each, but using 100, 1 and 0.01 units of template per well (Section 2.6.3). The template used was the pcDNA6/TR vector and primers were specific to the Tet Repressor region of the vector. These three master-mixes were each split between three PCR plates, which were run with slightly different PCR protocols. Statistical analysis was performed on each set of 32 Q-PCR results.

(A) Different cycling conditions used, (B) Statistical information from analysis of threshold cycle values.

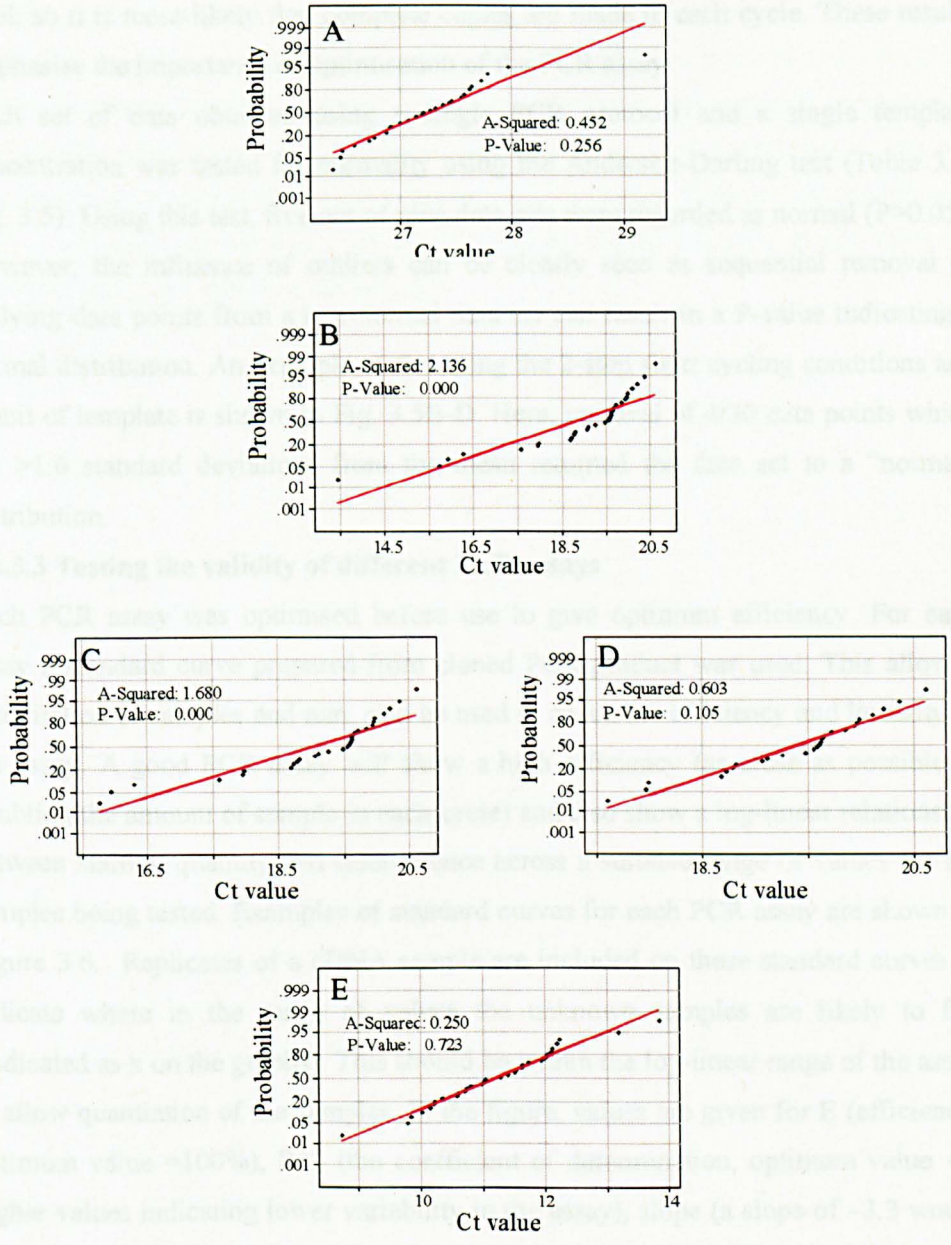


Figure 3.5 Anderson-Darling normality plots for PCR replicates

Normality plots are shown for the threshold values obtained using the 2 step short protocol (Table 3.2A). Threshold values for different amounts of starting material are graphed together.

Normality plots for threshold values using (A) 0.01 unit template, (B) 1 unit template (C) 1 unit template with one outlier removed, (D) 1 unit template with four outliers removed, (E) 100 units template.

used, so it is more likely that complete copies are made in each cycle. These results emphasise the importance of optimisation of the PCR assay.

Each set of data obtained using a single PCR protocol and a single template concentration was tested for normality using the Anderson-Darling test (Table 3.2, Fig. 3.5). Using this test, five out of nine data sets were regarded as normal (P>0.05). However, the influence of outliers can be clearly seen as sequential removal of outlying data points from a non-normal data set can result in a P-value indicating a normal distribution. An example of this using the 2-step short cycling conditions and 1 unit of template is shown in Fig. 3.5B-D. Here, removal of 4/30 data points which lay >1.6 standard deviations from the mean returned the data set to a "normal" distribution.

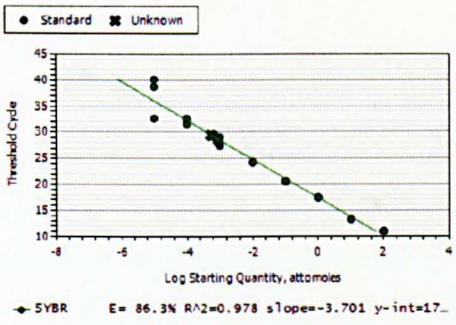
3.2.3.3 Testing the validity of different PCR assays

Each PCR assay was optimised before use to give optimum efficiency. For each assay a standard curve prepared from cloned PCR product was used. This allowed quantitation of samples and may also be used to check the efficiency and linearity of the assay. A good PCR assay will show a high efficiency (as close as possible to doubling the amount of sample in each cycle) and also show a log-linear relationship between starting quantity and fluorescence across a suitable range of values for the samples being tested. Examples of standard curves for each PCR assay are shown in Figure 3.6. Replicates of a cDNA sample are included on these standard curves to indicate where in the range of values the unknown samples are likely to fall (indicated as x on the graphs). This should be within the log-linear range of the assay to allow quantiation of the samples. In the figure, values are given for E (efficiency, optimum value =100%), R^2 (the coefficient of determination, optimum value =1, higher values indicating lower variability in the assay), slope (a slope of –3.3 would be expected, if E=100%, variation from this indicates lower efficiency) and the y-intercept of the graph. The concentration of the standard is known and is measured in attomoles in these experiments. Note that as one mole represents about $6x10^{23}$ molecules, 10^{-6} attomoles is equivalent to only six molecules and has therefore been defined as the lower limit for any standard curve.

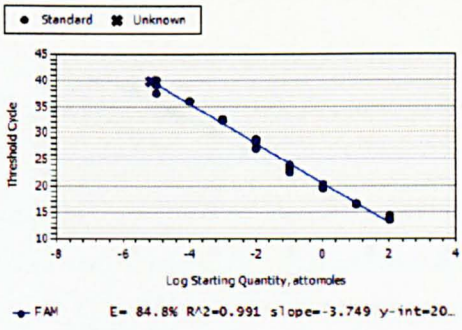
In some cases, an increased variation between samples is seen at the lower limit of the assay. This is to be expected, as an error in replicating a single copy will have a greater effect when starting from a smaller number of copies [Peccoud and Jacob,

Figure 3.6 Standard curve graphs for Q-PCR gene expression assays

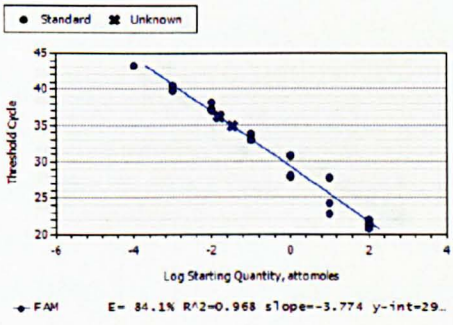
Standard curves were prepared using a dilution series of cloned PCR product for each assay. These may be used to test the efficiency, range of log-linearity and precision of the assays as well as allowing quantitative measurements of gene expression. Here, standard curves for the housekeeping, ER and estrogen responsive genes that were used in the present series of experiments are shown, detailing the efficiency (E), variability (R^2) and the approximate level of expression in cell lines ("unknown" samples). Graphs are taken directly from the Bio-Rad iQ5 iCycler software (version 2.0).



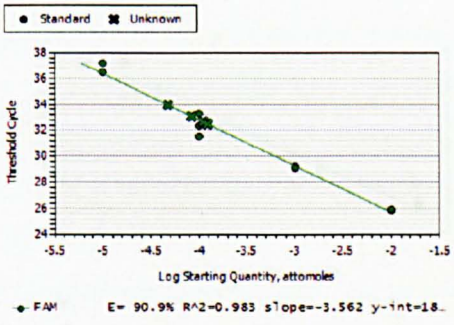
PCR Standard Curve : ERa standard curve.opd



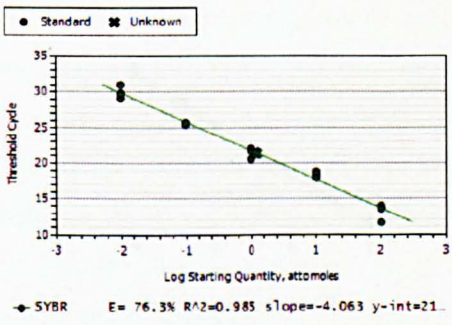
PCR Standard Curve : ERbeta1 standard curve.opd



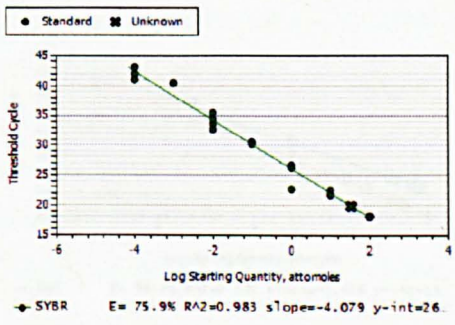
PCR Standard Curve : ERbeta2 standard curve.opd



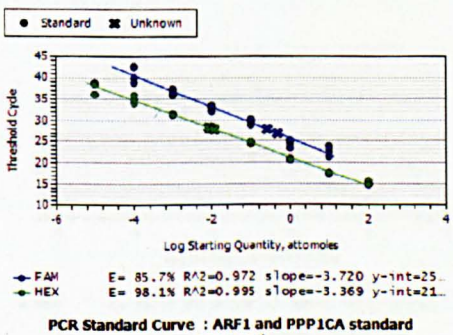
PCR Standard Curve : ERbeta5 standard curve.opd



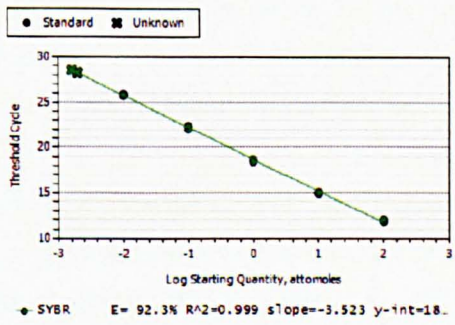
PCR Standard Curve : HPRT standard curve.opd



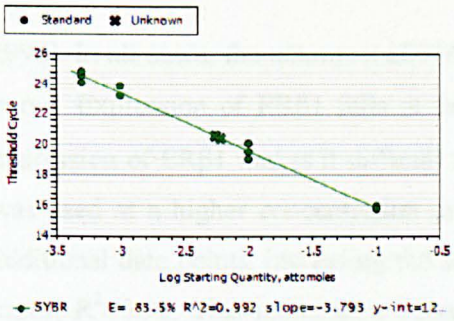
PCR Standard Curve : GAPDH standard curve.opd



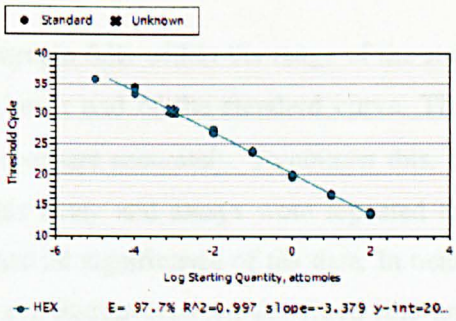
PCR Standard Curve : ARF1 and PPP1CA standard curve.opd



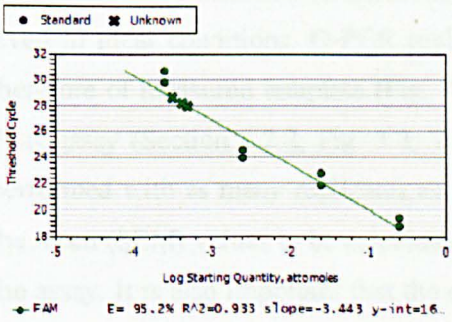
PCR Standard Curve : CathepsinD standard curve.opd



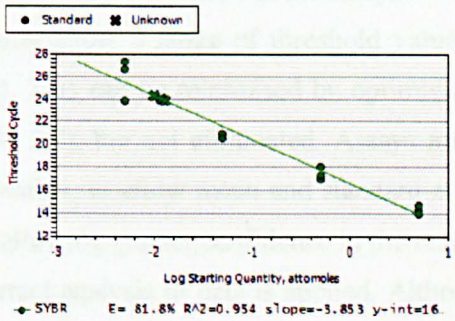
PCR Standard Curve : CyclinD1 standard curve.opd



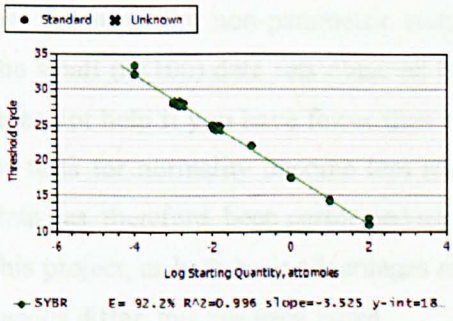
PCR Standard Curve : VEGF standard curve.opd



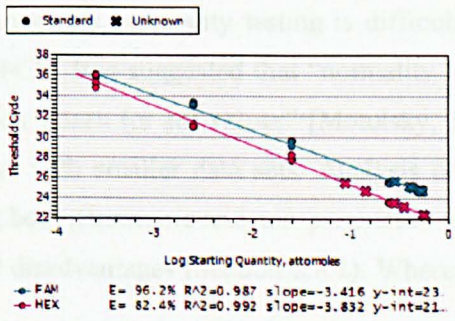
PCR Standard Curve : EFP standard curve.opd



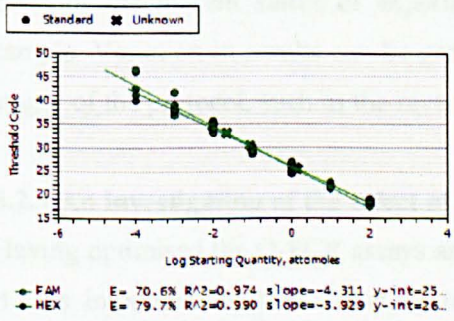
PCR Standard Curve : p21 standard curve.opd



PCR Standard Curve : PR standard curve.opd



PCR Standard Curve : Liv1 and PS2 standard curve.opd



PCR Standard Curve : XBP1 standard curve.opd

1996]. In all cases, the unknown cDNA sample falls within the range of the standard curve. Expression of ERβ1 falls at the lower end of the standard curve. The low expression of ERβ1 makes it difficult to measure accurately. To counter this, cDNA was used at a higher concentration in this assay and assays were repeated to give additional data points, increasing the statistical significance of the data. In nearly all cases, R^2>0.95. This indicates a strong correlation between the fitted line and the data points of the standard cure and reflects the high precision of the assays.

Even in ideal conditions, Q-PCR replicates show a range of threshold values and therefore of measured template (Fig. 3.4). This can be minimised by optimising the PCR assay (Section 3.2.2, Fig. 3.3, Table 3.2), but not eliminated. Assays must be performed with as many replicates as possible, to allow mean and standard error of the mean (SEM) values to be calculated, allowing greater confidence in the results of the assay. It is also important that the correct analysis of data is applied. Although it is useful to have an indication of the normality of a data set in deciding whether to use parametric or non-parametric statistical tests, normality testing is difficult with the small (n<100) data sets obtained by PCR. It is suggested that "normality testing does not help if you have fewer than a few dozen (or so) values" [Motulsky, 1999], as tests for normality become less robust with smaller data sets. Analysis of PCR data has, therefore, been performed using both parametric and non-parametric tests in this project, as both have advantages and disadvantages (Section 2.8.1). Where these results differ, this has been noted.

Each of the present series of experiments was performed using a single cDNA sample. Variation in results can be generated at the Q-PCR stage, but also at earlier stages of the protocol, such as the reverse transcription reaction.

3.2.4 An investigation of the effect of sample preparation on Q-PCR results

Having optimised the Q-PCR assays and established their level of intrinsic variation, it was important to ensure that upstream processes did not adversely affect the accuracy and precision of the PCR assay. Cells were grown and treated before their RNA was extracted for expression analysis (Section 2.4.1). The RNA was treated with DNase and cDNA prepared using a reverse transcription (RT) reaction (Section 2.4.3). cDNA from the RT was then diluted and used in Q-PCR analysis (Section 2.6). Variability introduced at any of these steps could affect the measured levels of

gene expression.

3.2.4.1 Determining the effect of mRNA purification

Total RNA was extracted from cells (Section 2.4.1). An experiment was performed to test whether use of mRNA would give more accurate and sensitive results than using total RNA. In this case, mRNA was obtained by affinity purification of polyA RNA from total RNA using polystyrene-latex beads with covalently attached $dC_{10}T_{20}$ (Qiagen oligotex system). Eight different total RNA extracts were obtained from cultured cells (Section 2.4) and an aliquot of each was purified using the oligotex system to obtain mRNA. Total RNA was reverse transcribed using 1μg RNA per reaction, whilst 10μl (the maximum amount) mRNA was used to give the best chance of increasing sensitivity. Expression of eight genes was measured in the cDNAs (Fig. 3.7). Each cDNA was used in triplicate in each assay (Fig. 3.7A). The mean value for each cDNA was taken, and paired *t*-tests used to assess whether a significant difference was seen between samples transcribed from total or mRNA (Fig. 3.7 B-I). No improvement in sensitivity or precision was obtained by treating the RNA in this way, presumably due to the use of oligo dT during reverse transcription to select for polyA+ mRNA, so further experiments were performed using total RNA.

3.2.4.2 Investigating DNase treatment

DNase treatment was included, as the RNA extraction method is not totally efficient in removing DNA (Section 2.4.1.2, [Bustin, 2002]). This treatment should prevent any carry-over of gDNA into the cDNA, ideally without affecting downstream processes. Removal of gDNA is important, as some Q-PCR assays did not cross an exon boundary, due to sequence limitations, and were therefore not cDNA specific. Additionally, in cells transfected with inducible expression systems (Chapters 4 and 5) the gDNA contained multiple copies of the cDNA sequence for ERβ1 and the induction system proteins encoded by the vectors, so complete gDNA removal would be essential for accurate analysis of any of these gene products in transfected cells.

DNase treatment might have unwanted side effects. Any DNase that was not inactivated could break down the cDNA, whereas the DNase buffer or EDTA might inhibit the RT reaction. If these problems occurred, DNase addition would reduce measured gene expression in subsequent Q-PCR steps.

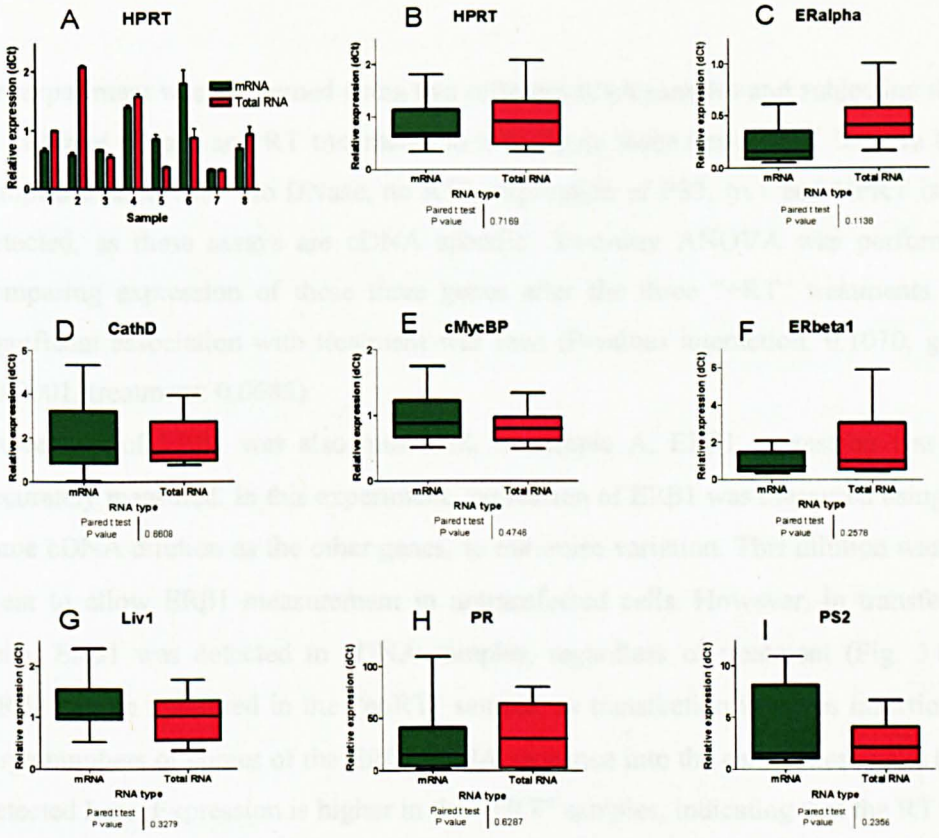


Figure 3.7: measured gene expression in samples with and without mRNA purification

Eight different total RNA extracts were obtained from cultured cells (Section 2.4) and an aliquot of each was further purified using the oligotex system to obtain mRNA. Total RNA was reverse transcribed using 1μg RNA per reaction, whilst 10μl (the maximum amount) mRNA was used to give the best chance of increasing sensitivity. Reverse transcriptions were diluted 1 in 20 for PCR and expression of eight genes was measured in the cDNAs, using the delta-Ct method to give relative expression. Each cDNA was used in triplicate in each assay. The mean value for each cDNA was taken, and paired *t*-tests used to assess whether a significant difference was seen between samples transcribed from total or mRNA.

(A) Expression of HPRT in eight RNA samples measured in cDNA prepared from mRNA (green) or total RNA (red).

(B-I) Box-plots show range of expression of each gene in eight different RNAs, measured in cDNA prepared from mRNA (green) or total RNA (red).

An experiment was performed using two different RNA samples and subjecting them to different DNase and RT treatments to investigate these issues (Fig. 3.8). In both samples treated with "no DNase, no RT", expression of PS2, liv1 and HPRT is not detected, as these assays are cDNA specific. Two-way ANOVA was performed, comparing expression of these three genes after the three "+RT" treatments. No significant association with treatment was seen (P-values interaction: 0.1070, gene: <0.0001, treatment: 0.0685).

Expression of ERβ1 was also measured. In sample A, ERβ1 expression was not accurately measured. In this experiment, expression of ERβ1 was measured using the same cDNA dilution as the other genes, to minimise variation. This dilution was too great to allow ERβ1 measurement in untransfected cells. However, in transfected cells, ERβ1 was detected in cDNA samples, regardless of treatment (Fig. 3.8B). ERβ1 can be measured in the "noRT" sample, as transfection involves insertion of large numbers of copies of the ERβ1 cDNA sequence into the cells. These have been detected here. Expression is higher in the "+RT" samples, indicating that the RT was successful, however, in the absence of a "+DNase –RT" control, we cannot tell whether all contaminating sequences were removed during the DNase step. When ERβ1 data was analysed using ANOVA, no significant difference was seen between treatments (P=0.9144).

These experiments showed that DNase treatment has no adverse effects on the RT reaction or cDNA quality. DNase treatment is essential when using non-cDNA specific PCR assays, or when working with transfected cells. DNase treatment will therefore be routinely used to treat RNA samples prior to RT.

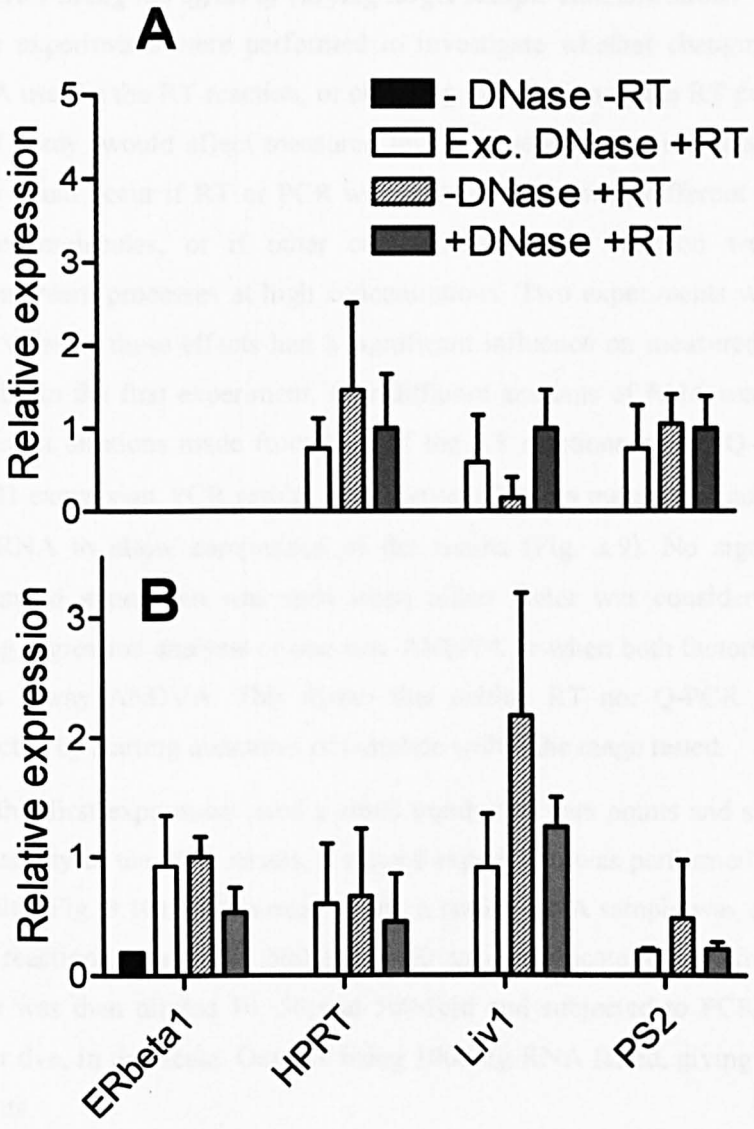
Figure 3.8 The effect of DNase treatment on RT efficiency.

Two different RNA samples each underwent four different DNase and RT treatments before being used for Q-PCR analysis of gene expression in order to test whether different DNase and RT treatments affected gene expression measurements. One sample (A) was extracted from untreated MCF7 cells, and the other (B) from MCF7 cells transiently transfected using the ecdysone inducible system for ERβ1 expression (Section 4.3.3).

Samples were treated using the DNase protocol (Section 2.4.1.2) with or without enzyme, as appropriate, except the second group of samples, which were excluded entirely, in order to test whether the DNase treatment step affected results. All samples were then subjected to the RT protocol (Section 2.4.3), although one group of samples had reverse transcriptase enzyme excluded (+DNase, -RT).

Each different DNase and RT treatment was performed in duplicate on each sample, giving eight RT reactions per sample and Q-PCR was performed in duplicate on each RT, giving a total of 16 Q-PCR results for each gene per sample or four Q-PCR results for each DNase and RT treatment. Expression levels of several genes were analysed for each sample. Relative expression of ERβ1, HPRT, liv1 and PS2 (calculated using the delta-Ct method) is shown here as mean +/- SD for each sample. Note that no bar is seen for "-DNase, -RT" as no PCR reached threshold.

(A) Relative expression in sample A, MCF7 cells, (B) Relative expression in sample B, MCF7 cells transfected to over-express ERβ1.


A
- DNase -RT
Exc. DNase +RT
-DNase +RT
+DNase +RT
Relative expression
5
4
3
2
1
0
B
Relative expression
3
2
1
0
ERbeta1
HPRT
Liv1
PS2

3.2.4.3 Testing the effect of varying target sample concentrations

Two experiments were performed to investigate whether changing the amount of RNA used in the RT reaction, or changing the dilution of the RT product into the Q-PCR assay, would affect measured levels of gene expression (Figs. 3.9 and 3.10). This could occur if RT or PCR was more efficient with different concentrations of target molecules, or if other components of the reaction were inhibitory to downstream processes at high concentrations. Two experiments were performed to test whether these effects had a significant influence on measured gene expression levels. In the first experiment, four different amounts of RNA were used and three different dilutions made from each of the RT reactions before Q-PCR analysis for ERβ1 expression. PCR results were converted into a measure of gene expression per ng RNA to allow comparison of the results (Fig. 3.9). No significant effect on measured expression was seen when either factor was considered independently using regression analysis or one-way ANOVA or when both factors were considered in a 2-way ANOVA. This shows that neither RT nor Q-PCR efficiencies were affected by starting quantities of template within the range tested.

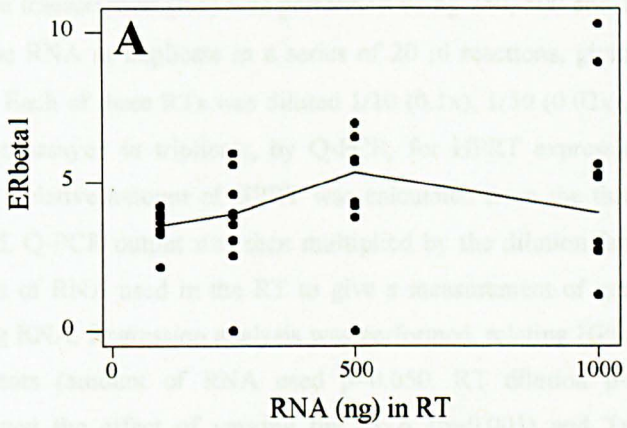
As this first experiment used a small number of data points and showed quite high variability in the PCR results, a second experiment was performed, to confirm these results (Fig. 3.10). In this experiment, a pooled RNA sample was used in a series of RT reactions, using 250, 500 and 1000 ng in duplicate. cDNA from each of these RTs was then diluted 10, 50 and 500-fold and subjected to PCR for HPRT using Sybr dye, in duplicate. One RT using 1000 ng RNA failed, giving a total n=30 data points.

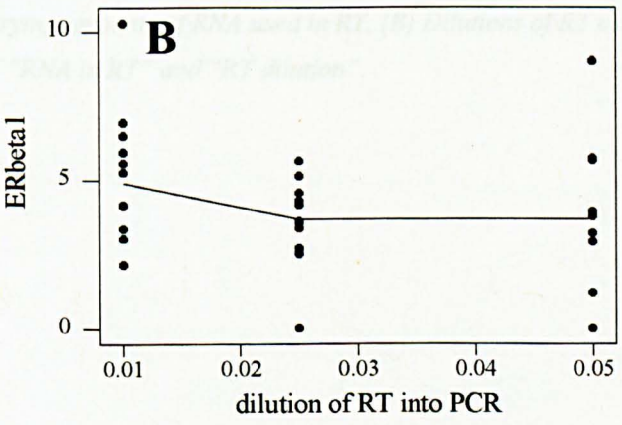
Threshold values were converted to a measurement of relative expression using the ΔCt method and normalised to the amount of RNA used by multiplying by the dilution factor used and dividing by the amount of RNA used in the RT. The effect of different treatments on measured expression was measured using regression analysis and 1-way ANOVA. It was not possible to perform 2-way ANOVA, as excluding the failed RT led to an imbalance in the number of data points in each data set. In this experiment, dilution of the RT into the PCR reaction had no significant effect on the result. However, using 250ng RNA in the RT led to a significantly lower measurement of gene expression (Fig. 3.10).

Figure 3.9 Analysis of variation in the Q-PCR assay for ERβ1 on varying the amount of RNA used in the RT reaction and the dilution of RT used in the PCR reaction.

Reverse transcription (RT) was performed using 100, 250, 500 and 1000 ng testis RNA in a series of 20 µl reactions (Section 2.4.3). Each of these RTs was diluted 1/20 (0.05x), 1/40 (0.025x), and 1/100 (0.001x) and 5µl aliquots assayed in triplicate, by Q-PCR, for ERβ1 expression using Sybr dye (Section 2.6.3). Q-PCR output was multiplied by the dilution factor used and divided by the amount of RNA used in the RT to give a measurement of gene expression relative to the starting RNA. Analysis was performed, relating ERβ1 expression to different RT treatments. The amount of RNA used and the RT dilution had no significant effect on measured ERβ1 (regression analysis p=0.096 and p=0.188, respectively).

(A) Varying amounts of RNA used in RT, (B) Dilutions of RT used in Q-PCR, (C) Interaction plot of "RNA in RT" and "RT dilution".


A
10
5
0
ERbeta1
0
500
1000
RNA (ng) in RT


B
10
5
0
ERbeta1
0.01
0.02
0.03
0.04
0.05
dilution of RT into PCR

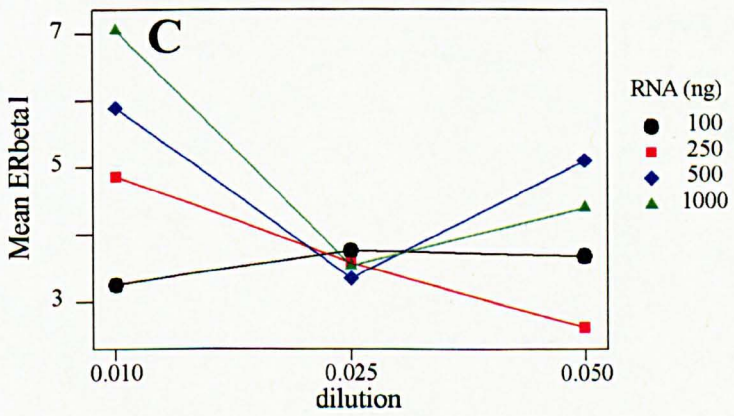
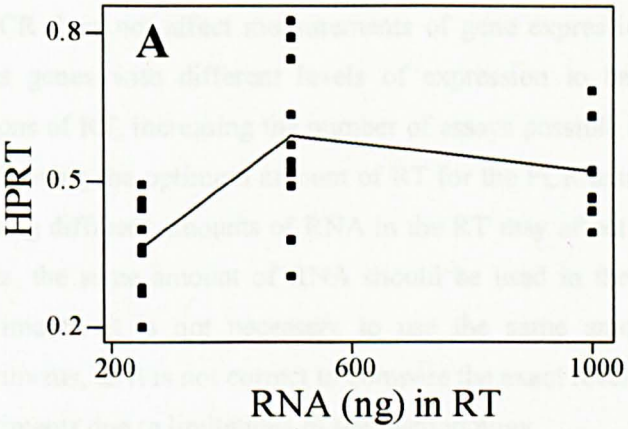
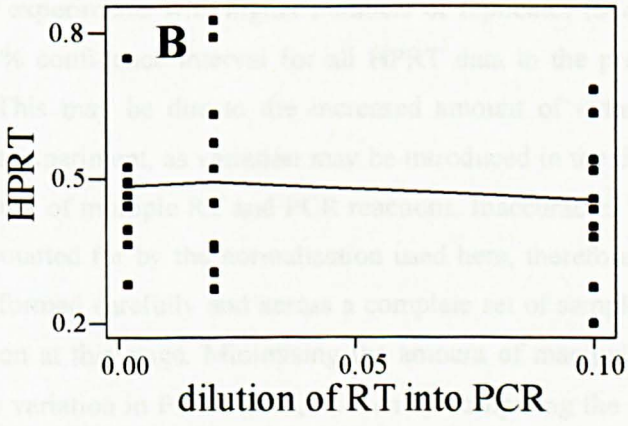
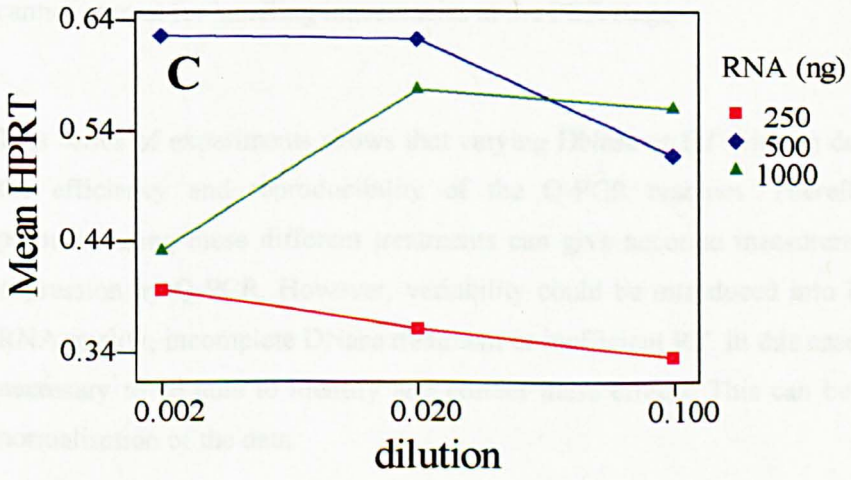

C
7
5
3
Mean ERbeta1
0.010
0.025
0.050
dilution
RNA (ng)
100
250
500
1000

Figure 3.10 Analysis of variation in the Q-PCR assay for HPRT on varying the amount of RNA used in the RT reaction and the dilution of RT used in the PCR reaction.
Reverse transcription (RT) was performed using 250, 500 and 1000 ng mixed breast cancer cell line RNA in duplicate in a series of 20 µl reactions, giving six RT reactions (Section 2.4.3). Each of these RTs was diluted 1/10 (0.1x), 1/50 (0.02x), and 1/500 (0.002x) and 5µl aliquots assayed in triplicate, by Q-PCR, for HPRT expression using Sybr dye (Section 2.6.3). Relative amount of HPRT was calculated from the threshold values using the ΔCt method. Q-PCR output was then multiplied by the dilution factor used and divided by the amount of RNA used in the RT to give a measurement of gene expression relative to the starting RNA. Regression analysis was performed, relating HPRT expression to different RT treatments (amount of RNA used p=0.050, RT dilution p=0.448). One-way ANOVA confirmed the effect of varying the RNA (p=0.001) and Tukey's pairwise comparison confirmed that the outlying value was expression using 250ng RNA.

(A) Varying amounts of RNA used in RT, (B) Dilutions of RT used in Q-PCR, (C) Interaction plot of "RNA in RT" and "RT dilution".


A
0.8
0.5
0.2
HPRT
200
600
1000
RNA (ng) in RT


B
0.8
0.5
0.2
HPRT
0.00
0.05
0.10
dilution of RT into PCR


C
0.64
0.54
0.44
0.34
Mean HPRT
0.002
0.020
0.100
dilution
RNA (ng)
250
500
1000

Together, these two experiments suggest that using different dilutions of the RT in the PCR does not affect measurements of gene expression. This is important as it allows genes with different levels of expression to be analysed using different dilutions of RT, increasing the number of assays possible from a single RNA sample and allowing the optimum amount of RT for the PCR assay to be used in each case. As using different amounts of RNA in the RT may affect measured gene expression results, the same amount of RNA should be used in the RT within each series of experiments. It is not necessary to use the same amount of RNA in different experiments, as it is not correct to compare the exact levels of expression in different experiments due to limitations of the methodology.

The variability within the data in these experiments is much higher than observed in earlier experiments with higher numbers of replicates (Section 3.2.3). For instance, the 95% confidence interval for all HPRT data in the present experiment is 0.18-0.80. This may be due to the increased amount of manipulation involved in the current experiment, as variation may be introduced in the different dilution steps and in the use of multiple RT and PCR reactions. Inaccuracies in the dilutions would not be accounted for by the normalisation used here, therefore dilutions of cDNA must be performed carefully and across a complete set of samples in parallel to minimise variation at this stage. Minimising the amount of manipulation of samples can hep reduce variation in PCR results, as seen by comparing the present results to those in section 3.2.3. Normalisation using a housekeeping gene may help reduce variation caused in dilutions and set-up prior and during the RT stage (Section 3.2.5) but this cannot correct for handling inaccuracies at the PCR stage.

This series of experiments shows that varying DNase or RT dilution does not affect the efficiency and reproducibility of the Q-PCR reaction. Therefore, samples prepared using these different treatments can give accurate measurements of gene expression by Q-PCR. However, variability could be introduced into PCR by poor RNA quality, incomplete DNase treatment or inefficient RT. In this case, it would be necessary to be able to identify and correct these effects. This can be achieved by normalisation of the data.

3.2.5 Normalisation of Q-PCR data

Variation in RNA quality and RT efficiency between samples could lead to differences in gene expression being missed or exaggerated. Samples of RNA extracted from cultured cells were assayed by formaldehyde-agarose gel electrophoresis to confirm integrity of the samples, and showed well-defined bands for the ribosomal RNAs, confirming that the RNA was not degraded (Section 2.4.2.1, data not shown). To minimise differences between samples, RNA samples were analysed by spectrophotometry to check their concentration and identify any protein contamination (Section 2.4.2.2) and an equal amount of each RNA was used per RT in each experiment. The actual amount of RNA used in a particular experiment was chosen based on the amount of RNA available and the number of RT and PCR experiments to be performed. Generally, 1μg RNA was used in a 20μl reverse transcription reaction, but occasionally smaller amounts were used to conserve RNA. As far as possible, two RTs were performed using each RNA sample and multiple PCRs were performed using each cDNA sample, so that any variation due to differences in the RT or PCR efficiency could be identified.

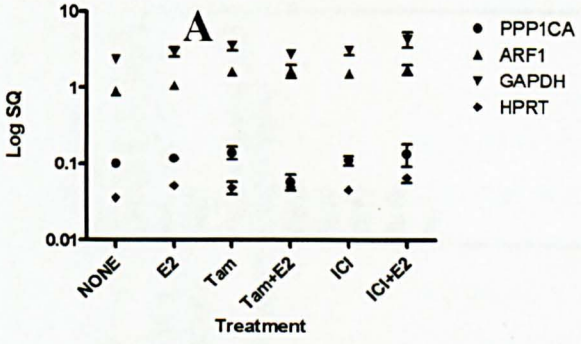
To identify variation in expression caused by sample preparation rather than biological differences, it was necessary to use a set of genes whose expression would not be expected to vary biologically, the housekeeping genes. Five housekeeping genes were initially selected, HPRT, GAPDH, ARF1, PPP1CA and histone H3 (Section 3.1.3.3). Histone H3 expression was poorly conserved in cells given different treatments (data not shown), so was not used routinely. Use of a panel of housekeeping genes, rather than a single gene, gives a more robust normalisation as any changes in expression in a single housekeeping gene are averaged with the levels of the other genes [Vandesompele et al., 2002].

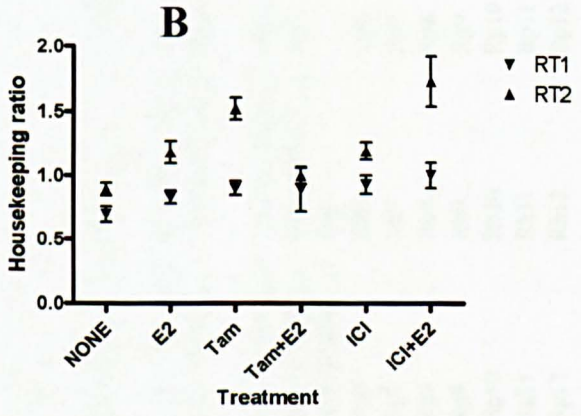
3.2.5.1 The normalisation protocol

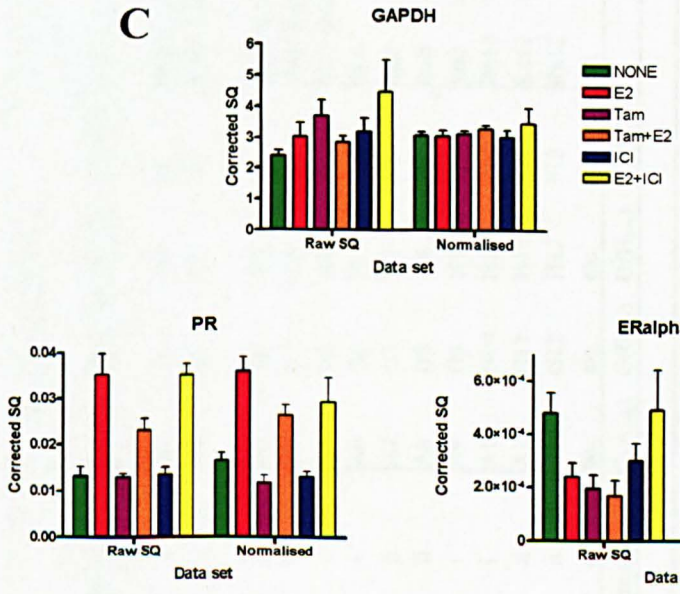
Expression levels of four housekeeping genes (HPRT, GAPDH, ARF1 and PPP1CA) were assayed for each RNA sample and gene expression in each cell line was normalised using a housekeeping ratio calculated using the combined relative expression of this set of genes. Q-PCR data obtained from each RT reaction were normalised separately, to allow any variation caused by the upstream processes to be taken into account. Each Q-PCR data point was divided by a housekeeping ratio to give normalised expression values used for further analysis (Figure 3.11, Table 3.3).

Figure 3.11 Normalisation of Q-PCR data in T47D cells under a variety of treatments. T47D cells were withdrawn from estrogenic stimulation before being treated with six different treatments (Section 2.2.1.2). The treatments were unmodified medium, 10 nM estradiol (E2), 1 μM ICI 182,780 (ICI), 10 nM estradiol and 1 μM ICI 182,780 (ICI+E2), 1 μM tamoxifen (Tam) or 10 nM estradiol and 1 μM tamoxifen (Tam+E2). After 24 hours, RNA was extracted for RT and Q-PCR analysis of a set of 18 genes.
Expression data for each gene were normalised using a housekeeping ratio (Section 3.2.5.1). To measure expression of each gene, each RNA sample was used in duplicate RT reactions, which then underwent duplicate Q-PCR reactions, giving 4 values for each treatment. A set of four housekeeping genes (A) was used to obtain a normalisation ratio for Q-PCR data (B, Section 3.2.5). Raw Q-PCR data for all genes, e.g. panel C, were normalised using the housekeeping ratio calculated using the average expression of four housekeeping genes under different treatments and in different replicate RT reactions. These normalised data were then analysed to investigate estrogen response.

(A) Q-PCR data, shown as log nominal starting quantity (SQ) for expression of housekeeping genes in T47D cells after different treatments, (B) Housekeeping ratios calculated from the set of housekeeping genes for each RT reaction. (C) Expression of GAPDH, ERα and PR shown before and after normalisation using the housekeeping ratio.


A
Log SQ
10
1
0.1
0.01
NONE
E2
Tam
Tam+E2
ICI
ICI+E2
Treatment
PPP1CA
ARF1
GAPDH
HPRT


B
Housekeeping ratio
2.0
1.5
1.0
0.5
0.0
NONE
E2
Tam
Tam+E2
ICI
ICI+E2
Treatment
RT1
RT2


C
GAPDH
Corrected SQ
Raw SQ
Normalised
Data set
NONE
E2
Tam
Tam+E2
ICI
E2+ICI
PR
Corrected SQ
0.04
0.03
0.02
0.01
0.00
Raw SQ
Normalised
Data set
ERalpha
Corrected SQ
6.0×10⁻⁴
4.0×10⁻⁴
2.0×10⁻⁴
0
Raw SQ
Normalised
Data set

A		SQ values (raw PCR data)				Relative expression (R)				
Treatment	RT	ARF1	GAPDH	HPRT	PPP1CA	ARF1	GAPDH	HPRT	PPP1CA	Housekeeping ratio (Hk)
Z	i	A1	G1	H1	P1	Ra1=A1/G(A_{1-n})	Rg1=G1/G(G_{1-n})	Rh1=H1/G(H_{1-n})	Rp1=P1/G(P_{1-n})	HkZi=G(Ra1-2, Rg1-2, Rh1-2, Gp1-2)
	i	A2	G2	H2	P2	Ra2=A2/G(A_{1-n})	Rg2=G2/G(G_{1-n})	Rh2=H2/G(H_{1-n})	Rp2=P2/G(P_{1-n})	HkZi
	ii	A3	G3	H3	P3	Ra3=A3/G(A_{1-n})	Rg3=G3/G(G_{1-n})	Rh3=H3/G(H_{1-n})	Rp3=P3/G(P_{1-n})	HkZii=G(Ra3-4, Rg3-4, Rh3-4, Gp3-4
	ii	A4	G4	H4	P4	Ra4=A4/G(A_{1-n})	Rg4=G4/G(G_{1-n})	Rh4=H4/G(H_{1-n})	Rp4	HkZii
Y	i	A5	G5	H5	P5	Ra5=A5/G(A_{1-n})	Rg5=G5/G(G_{1-n})	Rh5		HkYi
	i	A6	G6	H6	P6	Ra6	Rg6	Rh6	Rp6	HkYi
	ii	A7	G7	H7	P7	Ra7	Rg7	Rh7	Rp7	HkYii
	ii	A8	G8	H8	P8	Ra8	Rg8	Rh8	Rp8	etc.
X	i	A9	G9	H9	P9	Ra9	Rg9	Rh9	Rp9	
	i	A10	G10	H10	P10	Ra10	Rg10	Rh10	Rp10	
	ii	A11	G11	H11	P11	Ra11	Rg11	Rh11	Rp11	
	ii	A12	G12	H12	P12	Ra12	Rg12	Rh12	Rp12	
etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.	
Geometric mean (G)		G(A_{1-n})	G(G_{1-n})	G(H_{1-n})	G(P_{1-n})					

B		Normalised values					
Treatment	RT	ARF1	GAPDH	HPRT	PPP1CA	Gene B	Gene C
Z	i	A1/HkZi	G1/HkZi	H1/HkZi	P1/HkZi	B1/HkZi	C1/HkZi
	i	A2/HkZi	G2/HkZi	H2/HkZi	P2/HkZi	B2/HkZi	C2/HkZi
	ii	A3/HkZii	G3/HkZii	H3/HkZii	P3/HkZii	B3/HkZii	C3/HkZii
	ii	A4/HkZii	G4/HkZii	H4/HkZii	P4/HkZii	B4/HkZii	C4/HkZii
Y	i	A5/HkYi	G5/HkYi	H5/HkYi	P5/HkYi	B5/HkYi	C5/HkYi
	i	A6/HkYi	G6/HkYi	H6/HkYi	P6/HkYi	B6/HkYi	C6/HkYi
etc.	etc.	etc.	etc.	etc.	etc.	etc.	etc.

Table 3.3 Calculation and application of the housekeeping ratio

Housekeeping ratios were calculated per RT *(A)*. Relative expression of the housekeeping genes was calculated by dividing by the geometric mean. The mean of these values was calculated for each RT to give the housekeeping ratio. For normalisation *(B)*, all PCR values were divided by the appropriate housekeeping ratio.

For normalisation, PCR results for each cell line after all treatments were considered as a set. This could include analysis of up to 18 genes under six different conditions, with e.g. four PCR data points per condition. Each cell line was considered separately as the level of expression of the housekeeping genes was not always constant across cell lines. For each housekeeping gene, the geometric mean in the cell line was calculated. Each individual PCR result for that gene was then divided by the mean value to give relative measured expression. The geometric mean of all PCR data points for all housekeeping genes was then obtained for each RT reaction (e.g. the geometric mean of two PCR values for each of four housekeeping genes). This mean value was used as the housekeeping ratio for that reverse transcription reaction. The raw starting quantity (SQ) for every PCR data point for all genes was then divided by this housekeeping ratio to give normalised expression, thus taking into account variation in efficiency between RT reactions and any differences in the quality of the RNA.

3.3 Investigating estrogen receptor expression and estrogen response in a set of breast cancer cell lines

To investigate estrogen responses in breast cancer cell lines, experiments were performed using four different types of human malignant mammary epithelial cells; MCF7, MDA-MB-231, T47D and ZR75 (Section 3.1.1). These cells express different levels of the estrogen receptors and were, therefore, expected to show different estrogen responses. The cells were treated with a variety of different stimuli to investigate the estrogen response at the gene transcription level (Section 3.1.1). Cells were treated with combinations of estrogen, ICI 182,780 and tamoxifen and expression levels of the estrogen receptors, estrogen responsive genes and housekeeping genes were measured by Q-PCR (Section 3.1.3 and Table 3.1 C). Experiments were performed to compare expression between cells of the same type given different treatments, as well as between different cell lines given the same treatment. Several repeat experiments were performed using each cell line to establish the reproducibility of the estrogen responses.

3.3.1 Experiment design and data normalisation

Two sets of experiments were performed. In the initial series of experiments, cells from different cell lines were treated, in parallel, with various stimuli in single biological replicates. For each treatment, two RTs were performed and two PCRs performed from each RT to give four data points for each RNA sample or cell line/treatment combination. These experiments allow changes in expression within a cell line in response to treatment to be examined, as well as allowing a comparison of gene expression between cell lines, as PCR for different cell lines was performed on the same PCR plate. In total, this series of experiments included three replicates for each of MCF7, MDA-MB-231, T47D and two replicates for ZR75, as these cells were slower growing. An MCF7 cell line transfected to over-express ERβ1 (MCF7β1x) was also included in this series of experiments, but those results are discussed elsewhere (Section 5.2.1). Normalised expression values +/- SEM for this series of experiments are shown in Appendix A.

A second series of experiments was then performed in which cells from each cell line (MCF7, MDA-MB-231, T47D and ZR75) were treated in duplicate with each stimulus, to give biological replication. Each RNA sample from a single dish of cells then underwent two RTs, before two PCRs were performed from each RT, giving a total of eight data points per cell line/treatment combination (see Appendix B for normalised results).

Data from each experiment were normalised using a housekeeping ratio calculated from a set of four housekeeping genes to allow for variation in RT efficiency and any differences in RNA quality between samples (Section 3.2.5, Fig. 3.11, Table 3.3). The housekeeping ratio was calculated from the expression patterns of HPRT, GAPDH, ARF1 and PPP1CA. In most cases, comparison of raw expression data and normalised expression data showed no gross changes in expression patterns, only variation in the significance of expression changes. This is to be expected, as the experiments were designed to minimise errors, which would cause large amounts of normalisation to be necessary. For example, in one experiment using four cell lines and five treatments, giving a total of 20 samples and 40 RTs, the mean +/- SEM of the housekeeping ratios was 0.9-1.1.

Normalised gene expression was analysed to investigate estrogen response. PCR results under different treatments and in different cell lines were compared using a variety of statistical tests. ANOVA and Kruskal-Wallis tests were used initially to

identify those cell lines and experiments where varying treatments had an effect on gene expression levels. Student's *t*-test and Mann-Whitney were then used to identify statistically significant changes in expression. Experiments using a large number of replicates suggest that Q-PCR data often follow a normal distribution (Section 3.2.3, Figs. 3.4 and 3.5). However, as this distribution cannot be confirmed in smaller data sets, both parametric and non-parametric tests were used, as non-parametric testing may be more suitable in the absence of proof of normality, but parametric tests are more powerful. It should be noted that in the present analysis, these tests usually gave similar results. In order to combine multiple experiments performed on different days, expression data for each gene were further normalised relative to expression in untreated cells. This allows expression in the same cell line in multiple separate experiments to be analysed using 2-way ANOVA. The results of this test will show whether changes in expression in response to treatment are more significant that the variation in response between experiments.

Use of multiple RT and PCR reactions per sample allows any errors caused by inaccuracies in reaction set-up to be easily identified and excluded, repeated or corrected by normalisation. Occasionally, spurious data points were excluded when a single replicate from a set (of four or eight points) was outlying by a large margin (e.g. 100 or 1000-fold) and where the remaining data points were well clustered. Additionally, in some assays, samples may not have reached threshold fluorescence and were therefore below the level of detection and regarded as zero expression. PCR experiments were occasionally repeated where a poor standard curve was obtained or replicates showed large variation, as this might suggest correctable errors had occurred during PCR set-up. Where replicate RTs gave different values, this could be corrected by normalisation using the housekeeping genes (Section 3.2.5). As experiments were repeated several times, estrogen responses seen in multiple experiments were regarded as more robust, as these differences are less likely to have been caused by either experimental variation or random variation in expression within the cells.

3.3.2 Estrogen receptor expression in breast cancer cell lines

In order to relate estrogen response to the expression of the estrogen receptors in the cell lines, levels of ERα, ERβ1, ERβ2 and ERβ5 mRNAs were measured in each sample. This allows a comparison of ER levels in untreated cell lines, and also

observation of any changes in ER levels with treatments.

To compare the levels of gene expression between cell lines, PCR for multiple cell lines must be performed in the same 96-well PCR assay. Three experiments from the first series (Appendix A) satisfy this requirement, allowing comparison of four, three and two cell lines, respectively. Figure 3.12 shows expression levels of ERα and three ERβ isoforms in all cell lines in the absence of any treatment in these experiments. To facilitate comparison of results between different experiments, expression levels of each receptor isoform are shown relative to expression in MDA-MB-231 cells, which is given an expression level of one.

For all of the ER isoforms, the magnitude of differences in expression between cell lines varies between experiments. However, the rank values of expression are conserved across experiments. This suggests that we can confidently determine which cell lines express higher levels of the isoforms, but that the magnitude of the differences is affected by experimental variation and differences in culture conditions.

ERα mRNA expression (Fig. 3.12 A) is highest in T47D cells, followed by MCF7, ZR75 and MDA-MB-231 (where it is much lower than the other three cell lines). This does not fully correspond to the protein levels, where MCF7>T47D>ZR75>>MDA-MB-231, in untreated cells (Fig. 3.1), however, it does reinforce the low level of expression in MDA-MB-231 cells. The differences between protein and mRNA may reflect an imbalance caused by differences in translational rates or protein degradation in the different cell lines, or differences between the growth conditions and passage number of the cells used in the two assays.

ERβ1 expression (Fig. 3.12 B) is very low in all cell lines, barely reaching the limit of detection of the PCR assay (Section 3.2.3.3). In these experiments, expression is highest in MDA-MB-231 cells > ZR75 > T47D = MCF7, which appear to express similar levels. In one of three experiments, ERβ1 expression in T47D cells was below the limit of detection.

ERβ2 expression (Fig. 3.12 C) is also highest in MDA-MB-231 cells > MCF7 > T47D > ZR75. In one of two experiments, expression in ZR75 did not reach the limit of detection.

Relative levels of ERβ5 in different cell lines vary quite strongly between

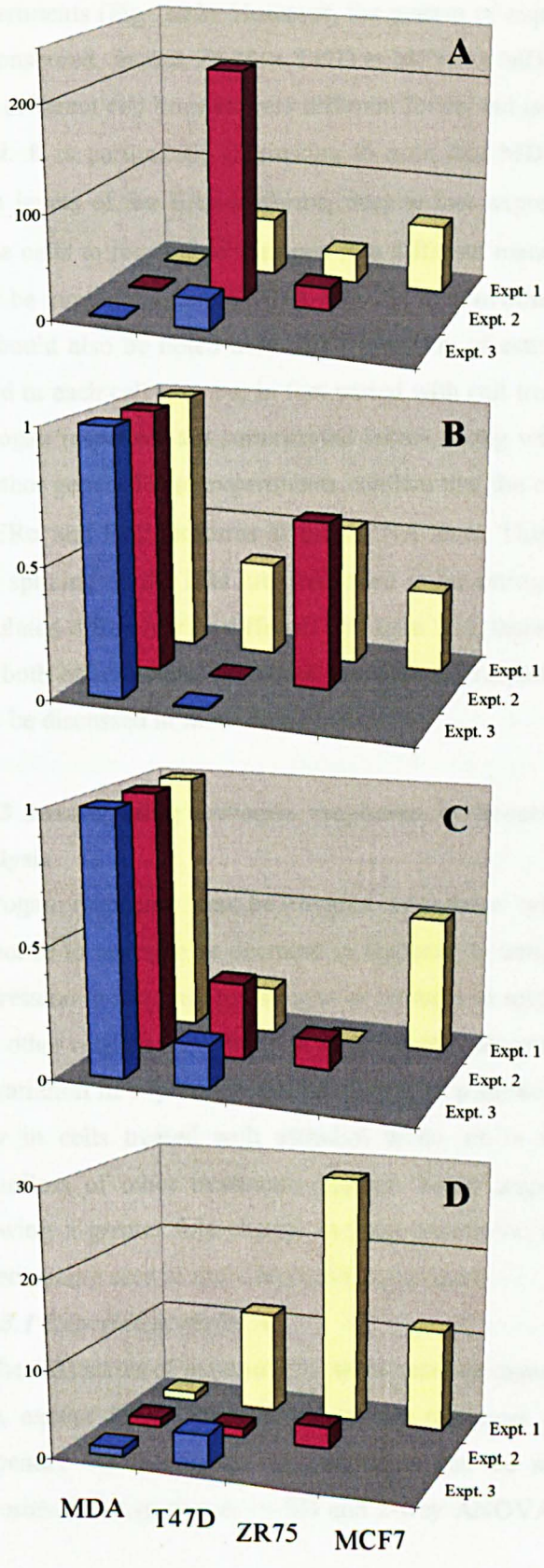
Figure 3.12 Relative estrogen receptor expression in unstimulated cell lines.

During the first series of experiments (Appendix A), three experiments were performed using MCF7, MDA-MB-231, T47D and ZR75 cells in parallel to investigate changes in gene expression between different treatments and across cell lines. Experiment 1 used all four cell lines, Experiment 2 included MDA-MB-231, T47D and ZR75, and Experiment 3 used only MDA-MB-231 and T47D cells.

For each experiment, cells were withdrawn from stimulation before being treated with different treatments (Section 2.2.1.2). After 24 hours, RNA was extracted for RT and Q-PCR. Gene expression was normalised using a housekeeping ratio calculated from expression of four different housekeeping genes for each cell line (Section 3.2.5).

These data show normalised ER expression in unstimulated cells over these three replicate experiments. Expression of each gene in each experiment has been normalised so that expression in MDA-MB-231 = 1 to allow easier comparison of replicate experiments. Each value shown is a mean of four Q–PCR replicates.

(A) *Relative expression of ERα*, (B) *Relative expression of ERβ1*, (C) *Relative expression of ERβ2*, (D) *Relative expression of ERβ5*.


A
200
100
0
Expt. 1
Expt. 2
Expt. 3
B
1
0.5
0
Expt. 1
Expt. 2
Expt. 3
C
1
0.5
0
Expt. 1
Expt. 2
Expt. 3
D
30
20
10
0
Expt. 1
Expt. 2
Expt. 3
MDA
T47D
ZR75
MCF7

experiments (Fig. 3.12). However, the pattern of expression in different experiments is conserved, in that ZR75 > T47D = MCF7 > MDA-MB-231. These results show that different cell lines express different forms and isoforms of the ERs at the mRNA level. It is particularly interesting to note that MDA-MB-231 expresses relatively high levels of the ERβ isoforms, despite low expression of ERα. This may allow these cells to respond to estrogen in a different manner than the other cells, as ERβ may be more dominant in MDA-MB-231 than in other cells.

It should also be noted here that expression of estrogen receptor isoforms was not fixed in each cell line, but in fact varied with cell treatment (see Appendices). These estrogen responses are summarised below, along with results for estrogen response in other genes. These experiments confirm that the cell lines express different levels of ERα and ERβ isoforms at the mRNA level. They also show that the expression and splicing of the ERs are controlled in an estrogen dependent manner, which is regulated differently in different cell lines and, therefore, that the estrogen receptors are both effectors and targets of the estrogen response. The details of these effects will be discussed in more detail below.

3.3.3 Investigating estrogen responses in breast cancer cell lines: statistical analysis

Estrogen responses can be divided into three types: increase in expression in response to estrogen or decrease in response to antiestrogen treatments; decrease in expression in response to estrogen or increase in response to antiestrogen treatments; and other responses. Within the three broad categories of response, different patterns of variation in expression can be shown. A positive estrogen response may be seen only in cells treated with estradiol alone, or in all cells treated with estradiol, regardless of other treatments applied. Some responses are stronger than others, showing a greater fold-change in gene expression and some are more robust than others, being seen in more replicate experiments.

3.3.3.1 Experiment series A

In the first series of experiments, three separate experiments were performed per cell line, except ZR75, for which only two replicates were performed (Section 3.3.1, Appendix A). Responses to treatments can be seen in each of these separate experiments (Expression +/- SD and 2-way ANOVA (cell line vs. treatment) values

are shown in Appendix A). However, in order to focus on the most robust responses, data were further normalised with respect to expression in untreated cells to allow comparison of responses in a given cell line across different experiments. These relative expression levels were subjected to 2-way ANOVA where the variables were "treatment" (does adding different treatments affect expression?) and "experiment" (do you see the same levels of expression in different experiments?) (Table 3.4). ANOVA also generates a third factor "interaction", which can be interpreted as "is the same response to treatment seen in different experiments?". This can be used as a marker of the reliability of a response. ANOVA is a parametric test; therefore the data should be relatively normally distributed, with similar variances in the data sets, although deviation from normality is tolerated, as long as the variances are similar. 2-way ANOVA is useful as it allows investigation of whether responses to treatment are well conserved across replicate experiments. The treatment Tam+E2 was excluded from analysis, as it was not included in all experiments and 2-way ANOVA requires a balanced design. In a few cases (ERβ1, ERβ2, PS2, XBP1u and XBP1s) data sets were further excluded. This was either due to an absence of expression in untreated cells, preventing calculation of the relative expression (ERβ1 in T47D, ERβ2 in ZR75 and PR in MDA-MB-231) or to a gene only being included in a single experiment, in the case of the XBP1 variants in ZR75 cells.

The 2-way ANOVA results show significant variation in gene expression between replicate experiments in a number of genes, including housekeeping genes, notably PPP1CA. For this gene, differences between experiments generate around half the total variation in expression, with a highly significant p-value in all cell lines. A further 14-25% of the variation is contributed by "interaction", suggesting that changes in expression in differently treated cells are not conserved between experiments. These results suggest that there may be more variation in expression than is ideal in a housekeeping gene, although this is the rationale behind using multiple housekeeping genes (Section 3.2.5). However, they do suggest that variation between experiments, which cannot be resolved using the housekeeping normalisation, is the major factor in changes in PPP1CA expression. Variation between experiments may be caused by biological or experimental factors (Section 3.3.4.1)

Of the cell lines, all except MDA-MB-231 show responses to treatment in a similar

Table 3.4 A summary of results of 2-way ANOVA analysis of relative gene expression in each cell line in replicate experiments (Series A)

In experiment series A, up to three experiments per cell line were performed using MCF7, MDA-MB-231, T47D and ZR75 cells to investigate changes in gene expression between different treatments and across cell lines (Section 3.3.3.1 and Appendix A). For each experiment, cells were withdrawn from estrogenic stimulation before being treated basic medium (None), 10 nM estradiol (E2), 1 μM ICI 182,780 (ICI), 10 nM estradiol and 1 μM ICI (ICI+E2), 1 μM tamoxifen (Tam) or 10 nM estradiol and 1 μM tamoxifen (Tam+E2). After 24 hours, RNA was extracted for RT and Q-PCR.

Gene expression was normalised using a housekeeping ratio (Section 3.2.5) before being further normalised to give expression relative to untreated cells for each cell line. This normalisation allows comparison of changes in expression in the same cell line in different experiments. For each cell line, 2-way ANOVA was performed comparing expression in a given gene across multiple treatments and experiments.

This table summarises the % variation and P-values obtained for "experiment" "treatment" and "interaction" for each cell line/gene product combination.

	Cell line	MDAMB231		MCF7		T47D		ZR75	
Gene product	Source of variation	% total variation	P value	% total variation	P value	% total variation	P value	% total variation	P value
ARF1	Interaction	7.87	0.7664	8.41	0.7215	8.28	0.6651	13.57	0.0579
	Treatment	6.07	0.4511	2.16	0.8499	13.77	0.0618	3.27	0.6512
	Experiment	13.26	0.0232	18.02	0.0063	14.08	0.0113	43.6	<0.0001
PPP1CA	Interaction	17.63	0.0242	14.48	0.028	25.15	<0.0001	14.36	0.0052
	Treatment	0.73	0.9334	2.98	0.4164	9.96	0.0009	6.15	0.1256
	Experiment	42.05	<0.0001	49.07	<0.0001	45.13	<0.0001	55.99	<0.0001
GAPDH	Interaction	18.71	0.1065	8.86	0.7749	11.68	0.4567	11.89	0.3071
	Treatment	0.71	0.9455	1.7	0.9208	7.6	0.1126	11.24	0.3347
	Experiment	23.3	0.0001	6	0.2097	22.53	0.0002	6.14	0.1169
HPRT	Interaction	9.87	0.6517	17.49	0.0088	8.61	0.7895	13.57	0.1535
	Treatment	0.93	0.9663	9.01	0.025	1.23	0.9545	24.25	0.0256
	Experiment	14.66	0.0176	40.66	<0.0001	6.56	0.1829	5.87	0.0873
ERalpha	Interaction	15.17	0.2168	26.22	0.001	14.26	0.0956	10.54	0.2249
	Treatment	21.61	0.0071	21.51	0.0034	1.39	0.8373	22.95	0.024
	Experiment	2.88	0.3507	16.6	0.0002	40.58	<0.0001	14.07	0.0081
ERbeta1	Interaction	8.14	0.8954	16.84	0.1455	12.05	0.4468	13.2	0.2943
	Treatment	2.48	0.7554	15.97	0.0253	14.59	0.0612	10.31	0.4171
	Experiment	10.73	0.0517	8.77	0.043	5.84	0.1546	0.04	0.9058
ERbeta2	Interaction	19.53	0.0064	9.26	0.7288	18.16	<0.0001		
	Treatment	3.77	0.3161	2.4	0.8501	61.69	<0.0001	Could not calculate*	
	Experiment	41.9	<0.0001	8.65	0.0983	13.75	<0.0001		
ERbeta5	Interaction	13.89	0.4254	10.81	0.6439	12.43	0.4274	11.75	0.1791
	Treatment	6.87	0.4058	3.28	0.7661	13.73	0.0023	12.69	0.1506
	Experiment	3.64	0.3468	5.3	0.2383	17.75	0.0003	23.33	0.001

	Cell line	MDA-MB-231		MCF7		T47D		ZR75	
Gene product	Source of variation	% total variation	P value	% total variation	P value	% total variation	P value	% total variation	P value
cathepsinD	Interaction	9.77	0.5755	18.66	0.0204	14.99	0.0353	4.22	0.6867
	Treatment	5.74	0.4267	13.28	0.0004	23.23	0.0001	33.77	0.0054
	Experiment	18.78	0.0035	25.28	<0.0001	25.47	<0.0001	6.44	0.0719
cyclinD1	Interaction	11.36	0.4966	8.14	0.7547	8.85	0.7296	8.33	0.5484
	Treatment	7.32	0.3211	15.91	0.061	9.3	0.259	5	0.7594
	Experiment	13.11	0.0192	2.39	0.4863	5.57	0.2046	6.4	0.1325
EFP	Interaction	10.95	0.5146	23.2	0.0001	9.8	0.6257	22.46	0.0519
	Treatment	21.77	0.0122	3.83	0.1595	15.37	0.0609	11.78	0.2596
	Experiment	1.33	0.6452	48.07	<0.0001	3.82	0.3079	2.43	0.2919
liv1	Interaction	20.67	0.1066	22.58	0.0052	9.23	0.7699	3.66	0.7598
	Treatment	5.72	0.2599	26.9	<0.0001	14.92	0.0172	26.77	0.0205
	Experiment	10.33	0.0274	9.09	0.0074	7.33	0.1046	10.73	0.0262
PR	Interaction	16.26	0.0527	25.82	<0.0001	11.76	0.0547	18.88	<0.0001
	Treatment	9.47	0.0575	56.77	<0.0001	46.58	<0.0001	55.2	<0.0001
	Experiment	31.26	<0.0001	6.33	<0.0001	10.28	0.0017	20.17	<0.0001
PS2	Interaction	Could not calculate*		14.55	0.038	16.69	0.0649	5.35	0.2932
	Treatment			41.29	<0.0001	12.63	0.0258	61.7	<0.0001
	Experiment			8.36	0.0089	24.29	<0.0001	2.03	0.1706
p21	Interaction	26.14	0.0137	9.32	0.3981	10.81	0.6006	6.91	0.5924
	Treatment	3.11	0.6211	22.47	0.0016	13.06	0.119	14.18	0.2406
	Experiment	17.92	0.0014	19.37	0.0005	0.68	0.8174	5.81	0.1331
VEGF	Interaction	37.64	0.0009	13.51	0.1932	17.75	0.0535	19.02	0.0231
	Treatment	5.12	0.3558	11.18	0.0604	4.5	0.38	14.52	0.061
	Experiment	6.08	0.0799	23.79	0.0002	30.63	<0.0001	23.42	0.0003
XBP1s	Interaction	5.28	0.8286	5.25	0.7838	11.24	0.4006	Could not calculate^	
	Treatment	5.18	0.8342	15.12	0.2467	3.97	0.8637		
	Experiment	0.01	0.9571	2.03	0.3382	8.19	0.0575		
XBP1u	Interaction	10.59	0.1758	15.05	0.1882	20.6	0.0001	Could not calculate^	
	Treatment	18.33	0.0296	8.59	0.4875	21.9	<0.0001		
	Experiment	24.43	0.0001	8.12	0.0457	36.15	<0.0001		

* 1 or more data sets were excluded, as untreated cells showed measured expression of zero, preventing calculation of relative expression. This resulted in only a single experiment being available so that 2-way ANOVA could not be performed.

^ Expression of these genes was only measured in a single experiment in this cell line.

numbers of genes. The reduced number of responses shown by MDA-MB-231 cells is consistent with their ERα negative status. The fact that some response to treatment is seen in these cells probably reflects a response via ERβ, or through alternative mechanisms for the estrogenic compounds such as estradiol binding GPR30 [Revankar et al., 2005; Thomas et al., 2005] and inhibition of PKC [O'Brian et al., 1985] and other pathways [Colletta et al., 1994] by tamoxifen.

The majority of the genes, including the housekeeping genes, show a significant response in at least one cell line. Exceptions to this are ARF1, GAPDH, cyclin D1, EFP, VEGF and XBP1s. Whilst ANOVA shows whether there is a response to changes in treatment, post-testing using Bonferroni's test can identify exactly which treatment causes this response. This test is quite conservative, to allow for multiple testing, and identifies those treatments and experiments where the strongest difference in expression is seen. In some cases, ANOVA will show a significant response to treatment overall, but no specific changes are identified by the Bonferroni's post-test, suggesting that ANOVA may be detecting background variation in expression, rather than a more robust response to treatment. A robust response would be one where Bonferroni's identifies the response in multiple replicate experiments.

Specific estrogen responses observed in these experiments as well as the second series of experiments will be discussed below on a gene-by-gene basis (Section 3.3.3.3).

3.3.3.2 Experiment B

To validate the responses seen in the first series of experiments, a further large-scale experiment was performed. In this experiment, cells from each cell line were treated in duplicate with each of the six treatments, giving a total of twelve cell samples per cell line. Each cell sample underwent duplicate RTs, which were then used in duplicate PCRs, generating a total of eight PCR data points per cell line/ treatment combination. This design generates more PCR points per treatment, giving more weight to any statistical analysis and also allows an investigation of whether the variation between experiments seen in the first experiment would also be seen in cells of the same passage and confluence treated at exactly the same time. Due to the increased number of samples per cell line, PCR for each gene was performed on each cell line independently, thus direct comparisons cannot be made between the levels of expression of each gene in different cell lines. Results for this experiment are

shown in Appendix B, as mean expression +/- SD, with 1-way ANOVA results (comparing treatments within each cell line).

As PCR was performed for each cell line separately in this experiment, one-way ANOVA was used to investigate changes in expression in each cell line independently. As well as ANOVA, a similar non-parametric test, Kruksal-Wallis, was also performed. If the data are not correctly distributed for ANOVA, the non-parametric test may be more accurate, however, non-parametric tests are less powerful with small samples, thus may miss significant responses detected by ANOVA. As mentioned previously, ANOVA is based on normality of the data and equal variances, although the requirement for normality is less strong. A test for equality of variances was therefore performed for each data set. Bartlett's test for equal variances gives a small p-value when the samples have different variances, i.e. if $p<0.05$, ANOVA may not be valid. However, Bartlett's test has a much stronger requirement for normality that ANOVA does. Small p-values may therefore reflect non-normality rather than difference in variances, which may lead to rejecting the ANOVA result unnecessarily [Motulsky, 1999].

A summary of p-values for Bartlett's test for equal variances, 1-way ANOVA and the Kruskal-Wallis test for each cell line/gene combination is shown in Table 3.5. ANOVA assumes that all groups have equal variances, hence a $p>0.05$ should be obtained from Bartlett's test to use ANOVA. However, this requirement is less strict when the sample sizes are the same (as in this case) [Motulsky, 1999], hence ANOVA has always been calculated, as well as the non-parametric statistic. Although Bartlett's statistic has $P<0.05$ in many cases, the ANOVA and non-parametric results are often still similar, indicating that these results are fairly robust. These results suggest that different treatments affect expression of virtually every gene, including housekeeping genes, in virtually every cell line. Post-testing is important to identify where the differences in expression lie and their size. In this case, Tukey's post-test was used on the ANOVA data to identify the source of variation. This test is conservative to allow for multiple testing and generates a mean difference and significance value between each pair of treatments. Although all data for post-testing is not shown, examples from MCF7 are shown below.

Both ANOVA and Kruskal-Wallis suggest ARF1 shows a significant response to treatment, although this is a housekeeping gene (ANOVA $p=0.003$). Tukey's post-test finds that values for "no treatment" and "E2+Tam" are significantly different

Table 3.5 A summary of results of 1-way ANOVA and a non-parametric analysis of relative gene expression in each cell line in experiment B

In experiment B, MCF7, MDA-MB-231, T47D and ZR75 cells were used to investigate changes in gene expression between different treatments and across cell lines (Section 3.3.1 and Appendix A). For each experiment, cells were withdrawn from estrogenic stimulation before being treated in duplicate with basic medium (None), 10 nM estradiol (E2), 1 μM ICI 182,780 (ICI), 10 nM estradiol and 1 μM ICI (ICI+E2), 1 μM tamoxifen (Tam) or 10 nM estradiol and 1 μM tamoxifen (Tam+E2). After 24 hours, RNA was extracted for RT and Q-PCR.

Gene expression was normalised using a housekeeping ratio to correct for experimental variation (Section 3.2.5). For each cell line, analysis performed comparing expression in a given gene across multiple treatments and experiments. This table summarises the P-values obtained for Bartlett's test for equal variances, 1-way ANOVA and the Kruskal-Wallis test for each cell line/gene product combination. Post testing was also used to determine the source of variation between groups (data not shown).

Gene product	Test	MDA-MB-231	MCF7	T47D	ZR75
ARF1	Bartlett's test	0.0003	0.0017	0.0143	<0.0001
	ANOVA	0.3594	0.0028	0.2794	0.0238
	K-W*	0.238	0.004	0.091	0.008
GAPDH	Bartlett's test	<0.0001	<0.0001	0.0033	0.3001
	ANOVA	0.0432	0.0005	0.0011	0.0193
	K-W	0.003	0.002	0.001	0.077
HPRT	Bartlett's test	<0.0001	<0.0001	0.0003	0.0193
	ANOVA	<0.0001	0.0465	0.0616	0.0039
	K-W	<0.0001	0.003	0.011	0.03
PPP1CA	Bartlett's test	0.6191	0.0456	0.0028	0.0808
	ANOVA	0.424	0.1005	0.0098	0.3267
	K-W	0.576	0.151	0.23	0.451
ERalpha	Bartlett's test	0.0002	<0.0001	0.0058	0.0157
	ANOVA	0.4944	<0.0001	0.1069	0.2174
	K-W	0.918	<0.0001	0.327	0.5
ERbeta2	Bartlett's test	<0.0001	0.8013	<0.0001	0.0008
	ANOVA	0.0325	0.0159	<0.0001	0.7148
	K-W	0.184	0.02	<0.0001	0.273
ERbeta5	Bartlett's test	<0.0001	<0.0001	<0.0001	<0.0001
	ANOVA	0.0073	0.0644	0.0019	0.1391
	K-W	0.033	0.366	0.001	0.16
Liv1	Bartlett's test	<0.0001	0.0009	0.0132	0.0005
	ANOVA	0.0014	<0.0001	0.2194	0.0024
	K-W	<0.0001	<0.0001	0.164	0.018
PR	Bartlett's test	n/a	<0.0001	0.0006	0.0001
	ANOVA	0.673	<0.0001	0.0003	<0.0001
	K-W	0.829	<0.0001	<0.0001	<0.0001
PS2	Bartlett's test	<0.0001	<0.0001	<0.0001	<0.0001
	ANOVA	0.0749	0.0007	<0.0001	<0.0001
	K-W	0.013	<0.0001	<0.0001	0.001
XBP1s	Bartlett's test	0.0031	<0.0001	0.0695	0.0094
	ANOVA	0.7294	0.001	0.0274	<0.0001
	K-W	0.665	<0.0001	0.005	0.001
XBP1u	Bartlett's test	<0.0001	0.0025	0.0101	0.0022
	ANOVA	0.4752	<0.0001	0.0067	0.0018
	K-W	0.169	<0.0001	0.03	0.008

*K-W: Kruksal-Wallis

from each other and "E2" and "E2+Tam" are different ($p<0.05$) The mean value for both untreated and estradiol treated cells is 0.34 (the two lowest values), whilst the value for "E2+Tam" is 0.54 (the highest value), equivalent to a 1.6x difference. This post-testing suggests that the first anlaysis is detecting the background variation in expression, rather than a specific response to treatment, as the differing treatments are not supported by a biological hypothesis and the fold change is small.

Again, both ANOVA and Kruskal-Wallis show a significant response to treatment for PS2 (ANOVA $p=0.0007$). In this case, Tukey's post test shows significant differences between estradiol treated cells and untreated ($p<0.01$), tamoxifen treated ($p<0.01$), "E2+Tam" treated ($p<0.05$), "ICI" treated ($p<0.01$) and "E2+ICI" treated ($p<0.01$), but not between any other treatments. A 5.5-fold difference is seen between untreated and estradiol treated cells. Here, the post-test supports the hypothesis that estradiol leads to an increase in PS2 expression and that this increase is inhibited by anti-estrogen treatment.

This analysis will be further discussed in the gene-by-gene analysis below (Section 3.3.4.2).

3.3.3.3 Combined analysis of both experimental data sets

Analysis of the two separate experiments shows some similarities and some differences in response (Tables 3.6 and 3.7). In order to investigate responses to treatment throughout the whole data set, the two experiments must be combined into a single analysis. Both data sets were normalised using the housekeeping ratio and further adjusted to give expression relative to untreated cells. In order to generate a balanced design for 2-way ANOVA, experiment B was split into two sub-experiments, each containing one plate of cells given each of the six treatments. ANOVA was performed using the resulting four (ZR75) or five (MCF7, MDA-MB-231 and T47D) dta sets for each cell line/ gene combination. Initially, the treatment Tam+E2 was excluded (Table 3.6) as this was not included in all experiments and resulted in an unbalanced design. In cases where post-testing indicated a response to tamoxifen, ANOVA was repeated excluding those experiments where Tam+E2 was not included, allowing this treatment to be included in the investigation (data not shown).

2-way ANOVA generates % variation and p-values for treatment, experiment and interaction. These have been described previously (Section 3.3.3.1). As with the analysis of individual experiments, large numbers of significant p-values were

Table 3.6 A summary of the combined results of experiments A and B using 2-way ANOVA analysis of relative gene expression in each cell line

In the two series of experiments, MCF7, MDA-MB-231, T47D and ZR75 cells were used to investigate changes in gene expression between different treatments and across cell lines (Sections 3.3.1 and Appendices A and B). For each experiment, cells were withdrawn from estrogenic stimulation before being treated in duplicate with basic medium (None), 10 nM estradiol (E2), 1 μM ICI 182,780 (ICI), 10 nM estradiol and 1 μM ICI (ICI+E2), 1 μM tamoxifen (Tam) or 10 nM estradiol and 1 μM tamoxifen (Tam+E2). After 24 hours, RNA was extracted for RT and Q-PCR.

Gene expression was normalised using a housekeeping ratio to correct for experimental variation (Section 3.2.5). In order to give equal numbers of data points per treatment in experiments A and B, experiment B was split into two sub-groups, each including one set of cells given each of the six treatments. Expression was then further normalised to give expression relative to untreated cells in each experiment. This gave up to five experiments per cell line per gene.

For each cell line, 2-way ANOVA was performed comparing expression in a given gene across multiple treatments and experiments. The treatment Tam+E2 was excluded from initial analyses, as it was not included in all experiments, resulting in an unbalanced design. This table summarises the % variation and P-values obtained for "experiment" "treatment" and "interaction" for each cell line/gene product combination.

	Source of Variation	MDA-MB-231 % total variation	MDA-MB-231 P value	MCF7 % total variation	MCF7 P value	T47D % total variation	T47D P value	ZR75 % total variation	ZR75 P value
ARF1	Interaction	18.7	0.1562	17.85	0.1263	6.71	0.9755	24.82	0.0003
	Treatment	5.77	0.1478	8.4	0.0316	5.39	0.2702	9.42	0.0043
	Experiment	20.27	0.0003	17.56	0.0004	12.28	0.0234	32.54	<0.0001
GAPDH	Interaction	18.78	0.0411	11.07	0.7535	27.32	<0.0001	18.29	0.2423
	Treatment	1.47	0.6074	0.59	0.9596	8.44	<0.0001	6.94	0.2194
	Experiment	31.16	<0.0001	17.38	0.0023	39.11	<0.0001	4.44	0.2951
HPRT	Interaction	14.91	0.0901	20.35	0.0008	19.51	0.015	17.69	0.1877
	Treatment	5.07	0.0798	6.13	0.01	5.23	0.068	15.14	0.0105
	Experiment	36.31	<0.0001	41.42	<0.0001	32.38	<0.0001	4.37	0.2542
PPP1CA	Interaction	14.19	0.1329	14.52	0.0135	24.14	<0.0001	10.98	0.3455
	Treatment	1.66	0.6018	2.98	0.1429	5.92	0.0024	4.96	0.2
	Experiment	38.98	<0.0001	51.06	<0.0001	45.55	<0.0001	35.97	<0.0001
ERalpha	Interaction	23.95	0.0315	30.79	<0.0001	22.33	0.0009	16.25	0.1525
	Treatment	11.44	0.0088	29.92	<0.0001	5.4	0.0301	11.54	0.0196
	Experiment	6.09	0.1108	13.73	<0.0001	36.54	<0.0001	17.74	0.0007
ERbeta1	Interaction	8.14	0.8954	16.84	0.1455	12.05	0.4468	13.2	0.2943
	Treatment	2.48	0.7554	15.97	0.0253	14.59	0.0612	10.31	0.4171
	Experiment	10.73	0.0517	8.77	0.043	5.84	0.1546	0.04	0.9058
ERbeta2	Interaction	18.32	0.0145	9.26	0.9026	16.12	<0.0001	23.52	0.0377
	Treatment	3.6	0.1621	3.85	0.4487	69.43	<0.0001	11.8	0.0751
	Experiment	38.02	<0.0001	9.56	0.0649	8.35	<0.0001	4.98	0.1151
ERbeta5	Interaction	23.08	0.003	14.93	0.5905	19.85	0.0254	13.51	0.4103
	Treatment	3.18	0.2323	2.4	0.6871	18.73	<0.0001	10.62	0.0521
	Experiment	36.47	<0.0001	5.45	0.282	14.45	0.0004	12.05	0.015

	Source of Variation	MDA-MB-231 % total variation	MDA-MB-231 P value	MCF7 % total variation	MCF7 P value	T47D % total variation	T47D P value	ZR75 % total variation	ZR75 P value
CathepsinD	Interaction	9.77	0.5755	18.66	0.0204	14.99	0.0353	4.22	0.6867
	Treatment	5.74	0.4267	13.28	0.0004	23.23	0.0001	33.77	0.0054
	Experiment	18.78	0.0035	25.28	<0.0001	25.47	<0.0001	6.44	0.0719
CyclinD1	Interaction	11.36	0.4966	8.14	0.7547	8.85	0.7296	8.33	0.5484
	Treatment	7.32	0.3211	15.91	0.061	9.3	0.259	5	0.7594
	Experiment	13.11	0.0192	2.39	0.4863	5.57	0.2046	6.4	0.1325
EFP	Interaction	10.95	0.5146	23.2	0.0001	9.8	0.6257	22.46	0.0519
	Treatment	21.77	0.0122	3.83	0.1595	15.37	0.0609	11.78	0.2596
	Experiment	1.33	0.6452	48.07	<0.0001	3.82	0.3079	2.43	0.2919
Liv1	Interaction	21.23	0.034	20	0.0035	21.64	0.0291	8.15	0.6885
	Treatment	4.96	0.0889	29.64	<0.0001	1.91	0.5244	23.04	0.0002
	Experiment	20.37	<0.0001	11.91	0.0002	23.13	<0.0001	15.17	0.0018
P21	Interaction	26.14	0.0137	9.32	0.3981	10.81	0.6006	6.91	0.5924
	Treatment	3.11	0.6211	22.47	0.0016	13.06	0.119	14.18	0.2406
	Experiment	17.92	0.0014	19.37	0.0005	0.68	0.8174	5.81	0.1331
PR	Interaction	16.26	0.0527	22.31	<0.0001	19.54	0.0007	18.61	<0.0001
	Treatment	9.47	0.0575	61.14	<0.0001	35.38	<0.0001	65.12	<0.0001
	Experiment	31.26	<0.0001	7.58	<0.0001	14.32	<0.0001	8.44	<0.0001
PS2	Interaction	26.39	0.0049	19.4	0.0458	47.96	<0.0001	16.2	0.0027
	Treatment	5.9	0.0778	28.03	<0.0001	34.29	<0.0001	46.43	<0.0001
	Experiment	17.24	0.0002	2.23	0.5105	11.39	<0.0001	10.05	0.0003
VEGF	Interaction	37.64	0.0009	13.51	0.1932	17.75	0.0535	19.02	0.0231
	Treatment	5.12	0.3558	11.18	0.0604	4.5	0.38	14.52	0.061
	Experiment	6.08	0.0799	23.79	0.0002	30.63	<0.0001	23.42	0.0003
XBP1s	Interaction	5.8	0.9916	11.63	0.4813	19.2	0.1325	16.87	0.0142
	Treatment	5.1	0.5231	4.27	0.3776	13.31	0.0138	23.72	<0.0001
	Experiment	2.13	0.6255	27.33	<0.0001	5.59	0.0991	23.35	<0.0001
XBP1u	Interaction	15.5	0.1954	17.35	0.1874	29.67	0.0002	35.63	<0.0001
	Treatment	5.37	0.2327	5.61	0.2624	15.62	0.0002	21.92	<0.0001
	Experiment	24.09	<0.0001	16.12	0.0007	14.05	<0.0001	27.1	<0.0001

obtained. Post-testing using Bonferroni's test was used to identify the source of variation, reliability of response and magnitude of response. Data for the combined series of experiments was also analysed using stand-alone tests to investigate the hypothesis that treating cells with estradiol, tamoxifen or ICI 182,780 would affect expression. This analysis was performed on each cell line and gene data set. For each set of cells (A1 - A5 and B1 - B2), the mean values of the normalised data were generated, so that untreated cells gave expression "1" and a value for cells treated with each of the three drugs was obtained. Up to five such pairs of data were obtained per cell line and gene. This paired data was analysed using the Student's paired *t*-test and Wilcoxon's signed ranks test to compare untreated expression to each of the treatments (Table 3.7). The paired *t*-test was used as the data represent paired observations at a single time, however the *t*-test has assumptions of normality and equality of variances that cannot be adequately tested with small samples. Wilcoxon's test was used to compare the median values for each treatment to a hypothetical median of "1". This test does not make assumptions about the distribution of the data points, but is less powerful for small samples. Brief observations of the data suggest that these analyses generate significant responses to treatment in fewer cases than the 2-way ANOVA. This type of analysis may be less powerful as it only includes the mean values for each experiment, whilst the ANOVA includes the full data set, taking the range of the values into account. However, responses detected by both the ANOVA and these stand-alone tests may be regarded as more robust.

Results of both of these analyses will be discussed on a gene-by-gene basis below (Section 3.3.4).

3.3.4 Specific estrogen responses

3.3.4.1 Identifying sources of variation in the data

The first point to note is that the actual measured levels of expression of a specific gene are not always consistent between experiments, despite the use of a quantified standard curve. This can be seen by comparing corrected SQ values for a particular gene across different experiments (Appendix A) and at least partly explains the difference between experiments seen in 2-way ANOVA analysis (Table 3.4 and 3.6). Measured expression may vary 100-fold, although the pattern of expression is similar in each case. This is not simply due to using more RNA, or more efficient RT, in

Table 3.7: Analysis of responses to treatment in multiple experiments using the Student's paired *t*-test and Wilcoxon signed ranks test

For all experiments (sets A and B) data were normalised using a housekeeping ratio (Section 3.2.5) and further normalised to give expression relative to the average "untreated" value for each cell line and each gene (section 3.1.3). These normalisations allow the data from multiple experiments to be easily compared. Expression in untreated cells and those treated with estradiol, tamoxifen and ICI 182,780 was then compared. As mean expression in untreated cells was always "1", due to the normalisation procedure, the Student's paired *t*-test and Wilcoxon signed ranks test were used to analyse the data. The mean expression in each experiment was entered into these tests; generating a maximum of five data points for untreated and treated cells. This analysis will identify significant responses conserved across experiments.

The table shows those responses reaching significance in each cell line, giving the P-value and fold change in expression relative to untreated for each significant response. The ZR75 cell line was only used in 2 of 3 experiments in experiment set A. Where genes were not tested in experiment set B, this means there are only two total data sets for ZR75, insufficient for the paired *t*-test. This is shown as "No T-test data" in the table.

Abbreviations: T-test: Student's *t*-test, W: Wilcoxon signed ranks test. Empty cells indicate that no comparisons reach significance using either test.

	MDA-MB-231	MCF7	T47D	ZR75
ARF1	W Tam: p=0.0313, 1.1x		W E2: p=0.0313, 0.8x	
GAPDH				
HPRT				T test E2: p= 0.0416, 1.6x
PPP1CA				
ERα		W E2: p=0.0313, 0.7x T test E2: p= 0.0199, 0.6x		
ERβ1				
ERβ2			W E2: p=0.0313, 22.5x T test E2: p=0.0066, 22x	
ERβ5			W ICI: p= 0.0313 0.7x T test ICI: p= 0.0580	
Cathepsin D				No T-test data
Cyclin D1				No T-test data
EFP	T test E2: p=0.0038, 0.7x		T test Tam: p=0.0229, 0.5x	No T-test data
Liv1	W E2: p=0.0313, 0.7x, ICI: p=0.0156, 0.6x	W E2: p=0.0469, 1.4x Tam: p=0.0313, 0.5x ICI: p=0.0156, 0.5x T test: Tam: 0.0362, 0.6x, ICI p=0.0006, 0.5x		
p21				No T-test data
PR		W E2: p=0.0313, 4.7x ICI: p=0.0313, 0.4x T test E2: p=0.0346, 4.9x ICI: p=0.0243, 0.5x	W E2: p=0.0313, 3.7x T test E2: p=0.0373, 3.5x	W E2: p=0.0625, 7.4x T test E2: p=0.0495, 6.5x
PS2		W E2: p=0.0625, 2.9x Tam: p=0.0313, 0.4x ICI: p=0.0313, 0.4x	W E2: p=0.0313, 2000x	W E2: p=0.0625, 33x ICI: p=0.0625, 2x

	MDA-MB-231	MCF7	T47D	ZR75
VEGF	T test E2: p=0.0030, 0.8x			No T-test data
XBP1u				T test E2: p=0.0334, 0.6x
XBP1s	T test Tam: p=0.0002, 1.2x			

some experiments than others, as different genes are not elevated in the same experiment. Nor can it be related to normalisation, as this takes into account only relative not actual levels of gene expression of the housekeeping genes and, therefore, only adjusts measured expression levels by less than 2-fold (Section 3.2.5). Hence, these differences must be due to actual differences in expression levels, or variation between PCR experiments.

Variation in PCR could involve a difference in threshold cycle, or a difference in quantification caused by differences in the standard curve. Both standard curve samples and cDNA samples could suffer degradation during storage [Wilkening and Bader, 2004], although these effects might only be expected to cause a 2-fold not 100-fold difference. One group [Bustin, 2002] have conducted an experiment in which three workers used the same samples and reagents to prepare replicate PCRs. After quantification, the average values obtained by the three workers ranged from $8.7x10^5$ to $2.7x10^3$ copies/μg RNA, a 300-fold difference. They also showed a difference of almost 10-fold between values obtained with two batches of probe in a Taqman assay. These differences suggest that, although quantification within a 96-well plate Q-PCR assay is valid, comparing actual levels of expression of different genes or the same gene in different assays may be less valid.

As well as basic experimental variation, there are several other sources of variation in these experiments. The first is the use of different cells, either in parallel or at different times. It is, of course, impossible to use exactly the same cells for different treatments and experiments. In order to minimise variation, cells given different treatments within the same experiment were split from a single flask only at the start of the estrogen withdrawal period and cells were used at similar passages and confluence in separate experiments.

Results from similar cells in totally separate experiments (Experiment set A), show that there is a strong variation between similar cells treated on different days (Table 3.4). This may relate to small differences in passage number, confluence, age of media and so on. Results for experiment B were examined using regression analysis. Expression data for a single gene in a single cell line were input as response, with "treatment" (1-6) "cells" (1-2) and "RT" (1-2) as predictors. Data from different cell lines, both before and after normalisation using the housekeeping ratio were examined in this way. Regardless of whether a response to treatment was seen, "cells" and "RT" did not play a reproducible role in the regression equation across

multiples genes in a single cell type. This indicates that cells which have gone through withdrawal and treatment in parallel show a much lower degree of variation than those treated independently.

3.3.4.2 Gene by gene analysis

Expression of each gene was analysed individually using both ANOVA to identify trends in expression (Tables 3.4 to 3.6) and the Student's paired *t*-test and Wilcoxon to identify specific responses to treatment (Table 3.7). Here, statistical analysis of all experiments will be discussed in detail on a gene-by-gene basis. Responses will be related to the literature in section 3.4.3.

ARF1

ARF1 was used as a housekeeping gene. In experiment set A, 2-way ANOVA results comparing both different cell lines in the same experiment (Appendix A) and different experiments using the same cell line (Table 3.4) show variation in expression between experiments and between cell lines, but not between different treatments in a single cell line. Analysis of Experiment B and the combined data from both experiments shows responses to treatment in both MCF7 and ZR75 cells using ANOVA (Tables 3.5 and 3.6). Post-testing using Bonferroni's post-test indicates that these differences are only seen in a single replicate experiment and probably indicate background variation in expression, rather than a specific responses to treatment, as discussed in Section 3.3.3.2.

Analysis of specific responses in the combined data showed a small decrease in expression in response to estradiol in T47D cells and a small increase in response to tamoxifen in MDA-MB-231 cells, which reach significance using the Wilcoxon test only (Table 3.7). As these responses are small and not detected by other tests (*t*-test, ANOVA), they are not regarded as significant.

GAPDH

Similarly to ARF1, GAPDH showed no response to treatment in the ANOVA analysis of experiment set A, but did show responses in experiment B and the combined analysis (Tables 3.4 to 3.6). Again, the responses are only seen in a single experiment on post-testing (Bonferroni's post-test). Expression also varies between experiments and between cell lines, suggesting that the responses seen are due to background variation in expression, as might be expected. Analysis of response to treatment across all experiments using stand-alone tests also shows no significant responses (Table 3.7).

HPRT

ANOVA investigation of HPRT expression in experiment set A shows a significant response to treatment in both MCF7 and ZR75 cells (Table 3.4). Bonferroni's post testing showed that significant changes in expression were observed in only a single experiment for each of these cell types. Experiment B and the combined data also showed significant responses (Tables 3.5 and 3.6). Post-testing of the combined data again showed that specific responses were only observed in a single replicate experiment in each case.

Analysis of the combined data using the Student's paired *t*-test and Wilcoxon shows a response to treatment for ZR75 only, where a 1.6-fold increase is seen in response to estradiol using the *t*-test (Table 3.7). Whilst this corresponds to the result of the ANOVA analysis, the lack of support from the non-parametric test or repeatability shown by Bonferroni's test, suggest that his is probably not a true response.

PPP1CA

As with the other housekeeping genes, some variation in expression is seen for PPP1CA. When results of experiment set A were analysed by 2-way ANOVA (Table 3.4), significant values for interaction and experiment were observed in all cell lines, indicating that levels of expression varied between experiments and that there was some variation in expression after different treatments, but this response was not usually conserved between experiments (Section 3.3.3.1). The major source of variation was "experiment", contributing about 50% of the variation in each cell line. In one cell line (T47D), a significant response to treatment was observed, however post-testing using Bonferroni's test showed that no specific response was conserved across two or more experiments. In experiment B, T47D cells again show a significant response to treatment (Table 3.5) Tukey's post-testing suggests that untreated cells show significantly higher expression than all other treatments except tamoxifen alone. As this does not support a biological hypothesis, it may simply reflect variation in expression.

Both series of experiments were combined and analysed in a 2-way ANOVA (Table 3.6). These results show a strong association between treatment and expression in T47D cells only. Post-testing showed a significant difference between no treatment and estradiol treated cells in two experiments in T47D cells. However, one experiment showed a reduction and one an increase in expression in response to estradiol, indicating that this probably does not represent a true response to

treatment.

To further test the combined experimental data, the Student's paired *t*-test and Wilcoxon were used to compare expression with and without drug addition (Table 3.7). No response was seen in any cell line in this analysis, reinforcing the suggestion that PPP1CA shows no true response to treatment.

ERα

Expression of ERα shows a significant response to treatment in different sub-sets of cell lines in experiments A and B (Tables 3.4 and 3.5). When the two sets of experiments combined, all cell lines show a significant response (Table 3.6). Use of Bonferroni post-tests shows that no specific response (e.g. none vs. E2) reaches significance in more than two of five experiments in this ANOVA.

Analysis of the combined data sets using stand-alone tests may help identify the key responses in each cell line (Table 3.7). In this analysis, only MCF7 cells showed a response, showing decreased expression in response to estradiol using either the parametric or non-parametric test. These data suggest that there may be a small yet significant response to estradiol in MCF7 cells. This response is not fully reproducible, which may be due to its small magnitude.

ERβ1

Expression of ERβ1 is generally low, making it difficult to define changes in expression due to the decreased precision of Q-PCR using very small starting amounts of template. However, combining multiple experiments can help to overcome this problem to generate meaningful statistics. In experiment series A, some experiments gave good data with little PCR dropout (e.g. Appendix A1), whilst in other experiments ERβ1 levels were too low to be measured under certain conditions. For instance, in T47D cells, expression after estradiol treatment is always measurable, but expression in untreated cells or those treated with antiestrogens is often below measurable levels. This causes problems with respect to normalisation, as other genes have been normalised to untreated cells to allow multiple experiments to be combined. If expression is zero in untreated cells, this is not possible. Variation between experiments in a single cell line is indicative of the fact that expression of this gene is low and that small changes such as different RT efficiencies or storage of the cDNA may affect the ability to measure ERβ1. In experiment series B, it was generally not possible to measure ERβ1 in the cDNAs. This could be due to

problems with the cells or with the assay itself, although a standard curve could still be amplified (discussed further in Section 3.4.2). As a large amount of cDNA was required to perform the assay, it was not possible to repeat the assay further and no additional data was generated for ERβ1.

Analysis of experiment set A by 2-way ANOVA shows a significant response to treatment in MCF7 cells only. Bonferroni's post-test suggests this may be an up-regulation in response to ICI 182,780 treatment. Analysis of the combined data for series A using stand-alone tests shows no significant response to treatment (Table 3.7). These results suggest that insufficient data is available to determine whether ERβ1 is affected by treatment in these cell lines.

ERβ2

Expression of ERβ2 is generally higher than that of ERβ1, making it easier to measure. ANOVA analysis shows a conserved response to treatment in T47D cells across both series of experiments (Tables 3.4 to 3.6), with responses to treatment also seen in MCF7 and MDA-MB-231, in experiment B only. Bonferroni's post-testing of the combined data for T47D showed an increase in expression in response to estradiol in all replicate experiments. Analysis of the combined dataset using stand-alone tests shows a significant response in T47D cells only where estradiol shows strong up-regulation (22-fold) using either statistical test (Table 3.7).

ERβ5

ERβ5 is the third isoform of ERβ that was measured in this study. ANOVA results of experiment series A suggest a response to treatment in T47D cells only, similar to other ERβ isoforms (Table 3.4). Analysis of these data (Experiment series A only) using the Student's *t*-test and Mann-Whitney shows a significant decrease in expression in response to tamoxifen treatment in both T47D and ZR75 cells (not shown). ANOVA analysis of experiment series B shows a significant response to treatment in MDA-MB-231 and T47D cells (Table 3.5). When the data from the two sets of experiments are combined, ANOVA suggests a significant response to treatment in T47D cells alone (Table 3.6). Post-testing suggest this response is an increase in expression in cells treated with estradiol alone versus those treated with ICI 182,780 ($p<0.05$) or estradiol and ICI 182,780 ($p<0.01$). These results probably reflect an increase in expression in response to estradiol, which is too small to be detected by post-testing in these experiments. The strong anti-estrogen effect of ICI

182,780 is sufficient to reduce expression to lower than basal (untreated) levels, thereby generating a significant difference between estradiol and ICI 182,780 treated cells. This result is reinforced by the results of the stand alone tests, where ICI 182,780 reduces expression 0.7-fold in T47D cells, reaching significance in the Wilcoxon test ($p=0.03$) and nearing significance in the paired *t*-test ($p=0.06$).

PR

Progesterone receptor is a classic estrogen-responsive gene and up-regulation of PR in response to estradiol has long been used as a marker of estrogen response. As expected, expression of PR was significantly increased by estradiol treatment in all cell lines except MDA-MB-231 in both sets of experiments. ANOVA shows a response to treatment (Tables 3.4 to 3.6), which is confirmed as a conserved response to estradiol addition using Bonferroni's post-testing.

Stand-alone tests using the combined data support the results of ANOVA, showing up-regulation in response to estradiol in MCF7 (5-fold), T47D (4-fold) and ZR75 (7-fold) cells (Table 3.7). MCF7 cells also show a significant down-regulation in response to ICI 182,780 in this analysis, suggesting a basal level of expression that can be further inhibited by anti-estrogens.

PS2

This gene is used as a marker of estradiol response in both cell lines and in breast cancers, where its expression correlates well with ERα expression and may correlate with other prognostic markers [Gillesby and Zacharewski, 1999; Looi et al., 2001; Surowiak et al., 2001]. Analysis of data from the two experiments, either alone or combined, shows a significant response to treatment in all cell lines except MDA-MB-231 (Tables 3.4 to 3.6). Post-testing confirms this as a response to estradiol, as expected, although this response does not reach significance in all replicate experiments, suggesting this response may be less robust that the PR response or the ERβ2 response in T47D cells.

Stand-alone tests showed significant responses to estradiol in MCF7 (3-fold), T47D (2000-fold) and ZR75 (30-fold) cells, although these were only detected using the non-parametric Wilcoxon test, again possibly reflecting the lower reliability of this response (Table 3.7). Note that the response seen in T47D cells is particularly large. This is because expression in untreated cells was virtually undetectable, suffering some PCR drop-out, thus lowering the average value. MCF7 cells also showed decreased expression in response to tamoxifen or ICI 182,780 in this analysis,

reflecting the results seen for PR.

Cathepsin D

ANOVA analysis of cathepsin D expression in Experiment set A shows significant response to treatment in all cell lines, except MDA-MB-231 (Table 3.4). However, post-testing using Bonferroni's test shows that these significant responses (increased expression in response to estradiol) are only significant in a single experiment in each case. This gene was not measured in experiment B. Stand-alone tests show no significant response to treatment. These results suggest that whilst the "estrogen responsive" cell lines may up-regulate cathepsin D in response to estradiol, the response may be small or not very robust, making them difficult to measure reproducibly.

Liv1

Using 2-way ANOVA to analyse the results of experiment set A, response to treatment reaches significance in all cell lines except MDA-MB-231. Post-testing, however, only identifies a response to estrogen treatment in MCF7 cells in a single experiment. In experiment B, significant responses are seen in all cell lines except T47D. Combining the results of experiments A and B shows significant response to treatment in 2-way ANOVA in MCF7 and ZR75 cells, but not MDA-MB-231 or T47D, although the source of these responses cannot be identified by post-testing (Table 3.6).

Stand-alone testing showed no response in T47D or ZR75 cells. The Wilcoxon test suggested that expression was decreased by estradiol or ICI 182,780 in MDA-MB-231 cells. This may reflect background variation in expression, as it does not support a biological hypothesis. In MCF7 cells, expression is slightly increased by estradiol (1.4-fold, Wilcoxon only) and decreased by tamoxifen or ICI 182,780 (2-fold, both *t*-test and Wilcoxon). These results suggest that MCF7 cells do show a small response to treatment in this gene, but that its size makes it difficult to reproducibly detect.

XBP1

ANOVA analysis of experiment A alone shows no response to treatment in XBP1s, and a response to treatment in MDA-MB-231 and T47D cells for XBP1u (Table 3.4). Conversely, experiment B alone shows a response to treatment in all cell lines except MDA-MB-231 for both splice variants (Table 3.5). ANOVA analysis of the two experiments combined shows a response in T47D and ZR75 cells for both splice variants (Table 3.6). Using stand-alone tests on the combined data set, MDA-MB-

231 cells show a decrease in expression in response to tamoxifen and ZR75 cells show a decrease in response to estradiol, both using the *t*-test alone (Table 3.7).

Post-testing for XBP1s expression in ZR75 cells reveals a confusing pattern of significant differences. No response is conserved across the three data sets available. The strongest response (compared to untreated) is seen for the combined treatment estradiol and tamoxifen, which shows a highly significant increase in two of three data sets. Neither estradiol nor tamoxifen alone generates a similar response. No response is seen using the stand-alone tests (Table 3.7) suggesting that any response to treatment may be small or insufficiently robust to be detected with the present series of experiments. XBP1u shows similar results. Bonferroni's test shows that XBP1u is strongly up-regulated by tamoxifen or tamoxifen and estradiol in a single experiment. However in the other two data sets, expression appears to be slightly reduced in the presence of tamoxifen, suggesting that expression of this variant may vary strongly between experiments. Using the stand-alone tests, a significant decrease in expression is seen in response to estradiol (*t*-test), however this is not supported by the Wilcoxon's test or ANOVA data (Table 3.7).

In the case of T47D cells, post-testing again suggests that the response to treatment in both splice variants is seen in only a single replicate experiment (out of a total of four). The data suggest a possible trend towards increased expression in response to estradiol or tamoxifen, but this cannot be confirmed.

None of the responses show a large fold-change in expression, and responses do not reach significance in multiple replicate experiments, or using multiple statistical tests. These results suggest that expression of both variants of XBP1 may be affected by treatment, particularly in T47D and ZR75 cells, however these changes are small or easily perturbed and cannot be confirmed by the present series of experiments.

Cyclin D1

In the present study, expression of cyclin D1 was not significantly affected by treatment in experiment set A, when analysed using 2-way ANOVA (Table 3.4) or stand-alone tests (Table 3.7). This gene was not included in experiment B.

EFP

EFP showed a significant response to treatment in only MDA-MB-231 cells in experiment set A, using 2-way ANOVA (Table 3.4). Post-testing shows this as a significant decrease in expression in response to tamoxifen in one of three replicates. This gene was not included in experiment B. Stand-alone tests show a decrease in

expression in MDA-MB-231 cells in response to estradiol (*t*-test) and in T47D cells in response to tamoxifen (*t*-test) (Table 3.7). These results suggest that no change in EFP at 24 hours can be reliably measured in these cell lines.

p21

Analysis of experiment set A using 2-way ANOVA shows a response to treatment in MCF7 cells only for p21 (Table 3.4). Bonferroni's post-test shows up-regulation by ICI 182,780 in a single replicate experiment. This gene was not included in experiment B. Stand-alone tests showed no significant response to treatment (Table 3.7). These results suggest that p21 expression may be affected by treatment, but that this response is not robust or large enough to be measured in this series of experiments.

VEGF

Analysis of the first series of experiments using ANOVA did not detect a significant response to treatment for this gene (Table 3.4). This gene was not included in experiment B. Stand-alone tests showed a decrease in expression in response to estradiol in MDA-MB-231 cells, using the *t*-test alone (Table 3.7). These results suggest that VEGF may show a negative response to estradiol; however, the power of these experiments was too low to reliably detect this response.

3.4 Discussion

3.4.1 Validation of the Q-PCR assay

Before using Q-PCR assays as the basis of an investigation of estrogen response, it was necessary to assess the levels of variability in the Q-PCR assay, in order to define the level of precision. A series of experiments was, therefore, performed to identify sources of variability in the methods used and to minimise and define the amount of variation (Section 3.2).

Changes in upstream processes such as mRNA purification and DNase treatment were shown to have no effect on Q-PCR variability or measured expression levels (Section 2.3.4). Varying the amount of cDNA used (RT dilution) in each PCR reaction had no significant effect, whereas varying the amount of RNA in the RT did affect measured expression. The amount of RNA used per RT was, therefore, measured and kept constant within each experiment. Q-PCR experiments using large numbers of replicates were performed to show the spread of values obtained from a

single sample. The precision of the assay was affected by the cycling conditions used (Section 3.2.3), emphasising the importance of optimising the assay. Q-PCR values show a normal distribution (n=92) (Fig. 3.4), with 95% confidence interval for threshold cycle of 1.2 (approx. 2.3-fold). This figure will vary with the precision of the assay. Each assay was therefore optimised to improve precision, indicated by a coefficient of determination, (R^2) value of >0.95 for the standard curve (Section 3.2.3.3).

3.4.2 Basal estrogen receptor levels in the cell lines

A set of four cell lines was used to investigate the estrogen response (Section 3.1.1, Table 3.1 C). Expression of ERα and three ERβ isoforms was measured at the mRNA level, to allow responses to treatment to be related to the different patterns of expression of the estrogen receptors. The level of expression of all ERβ isoforms was much lower than that of ERα, in accordance with published results [Murphy et al., 2005; Strom et al., 2004], and different cell lines express different patterns of ERβ isoform mRNAs (Fig. 3.12). In this study, the three cell lines usually regarded as estrogen responsive; MCF7, T47D and ZR75; showed significantly higher levels of expression of ERα mRNA than did MDA-MB-231, which is regarded as non-hormone responsive [Engel and Young, 1978] and is reported to be ER-naïve by some groups [Lazennec et al., 2001]. In this study, MDA-MB-231 cells expressed detectable levels of ERα mRNA (Section 3.3.3.1, Fig. 3.12), in accordance with other reports, which suggest that these cells express low levels of the ERs, rather than being completely ER negative [Tonetti et al., 2003; Vladusic et al., 2000]. However, expression of ERα protein in MDA-MB-231 cells was at or below the limit of detection of the ELISA used here (Section 3.1.1, Fig. 3.1). Expression in the other cell lines was in order of highest to lowest MCF7 > T47D > ZR75, whilst serum starving reduced expression in all cell lines.

Few papers have compared expression of ERβ between cell lines. The present data suggest that all of the studied cell lines express similar levels of ERβ, showing variation of approximately 10-fold between cell lines, compared to 100-fold for ERα (Fig. 3.12). In contrast to the low level of ERα expression in MDA-MB-231 cells, these cells express relatively high levels of ERβ1 and ERβ2, but little ERβ5. Other reports using non-isoform specific assays support this high overall level of

expression. For instance, [Dotzlaw et al., 1997] measure higher levels of total ERβ mRNA in MDA-MB-231 cells than in T47D cells and [Fuqua et al., 1999] show that MDA-MB-231 cells contain more total ERβ protein than MCF7 and also express predominantly different isoforms of the receptor, although the isoforms are not identified. Another report, however, suggests that expression of ERβ1 mRNA is higher in T47D cells than in either MDA-MB-231 or MCF7 [Vladusic et al., 2000]. The work in this thesis suggests the ranked expression level of ERβ is MDA-MB-231 > ZR75 > MCF7 = T47D in unstimulated cells. This discrepancy may be caused by differences in withdrawal protocol, as ERβ1 expression is strongly stimulated by estradiol in T47D cells.

In this study, it was particularly difficult to measure expression of ERβ1 in these cell lines. The ability to measure ERβ1 varied between experiments, rather than between cell lines, suggesting that ERβ1 expression was generally on the limit of detection for the assay used and that small changes in experimental conditions such as differences in RT efficiency or cDNA storage time may be enough to push the samples below the limit of detection. In experiment series B, it was not possible to detect ERβ1 in the cell lines. Although it seems unlikely that all four cell lines had lost ERβ1 expression, it has been suggested that ERβ might be a growth inhibitor in breast cancer [Jarvinen et al., 2000; Roger et al., 2001] whilst cells over-expressing ERβ show reduced proliferation, and were less likely to show increased proliferation in response to estradiol (Section 1.6.2 and [Lazennec et al., 2001; Murphy et al., 2005; Omoto et al., 2003; Paruthiyil et al., 2004; Strom et al., 2004]). This could result in a down-regulation of ERβ expression in long-term culture.

Several studies have over-expressed ERβ1 in the same breast cancer cell lines as used here. In many cases, these groups were unable to detect ERβ1 mRNA or protein in non-transfected MDA-MB-231 or MCF7 cells [Lazennec et al., 2001; Omoto et al., 2003; Paruthiyil et al., 2004]. Other groups detect low levels of ERβ1 protein and mRNA in MDA-MB-231 [Tonetti et al., 2003], whilst [Strom et al., 2005] measure ERβ1 mRNA but not protein in T47D. This last study, however does not note whether or not the cells were grown in the presence of estradiol for this assay, which would affect ERβ1 mRNA levels in these cells significantly. These results confirm that ERβ1 expression in these cell lines is low and may be difficult to detect.

Information in the literature regarding the relative levels of ER expression in different cell lines is fairly heterogeneous, reflecting different methods of measurement, the difficulty in measuring ERβ due to its low level of expression and different isoform specificity of different studies. Additionally, cells cultured by different groups show differences in cell behaviour. For instance, the T47D cell line has been described as possessing properties ranging from completely estrogen independent to classically estrogen dependent and responsive [Pink and Jordan, 1996] and, similarly, MCF7 cells lacking ERα expression or showing no growth response to estradiol have been described [Shaw et al., 2006; Sonnenschein et al., 1994]. Finally, ER expression may be modulated by treatment (Section 3.3.4.2). Thus, studies performed using different growth medium and treatment regimes may elicit different levels of ER expression. These factors are likely to explain much of the differences between reported results as well as discrepancies between this study and reports from the literature.

3.4.3 Variation in expression of housekeeping genes

Four housekeeping genes were chosen and expression of all genes (including the housekeeping genes themselves) was normalised to this set of genes (Section 3.2.5). In both sets of experiments, as well as in the combined results, significant responses to treatment were seen in the housekeeping genes (Section 3.3.4, Tables 3.4 to 3.7). Ideally, the housekeeping genes should not vary at all in treated cells; however, this is unlikely to be achieved as differences in the cell cultures (e.g. confluence, rate of growth) and experimental variation (during RNA preparation and cDNA synthesis) may all contribute to generate changes in expression, even disregarding any genuine responses to treatment. These effects are the reason for using a panel of housekeeping genes, as this allows more accurate normalisation for experimental variation. Although responses to treatment are observed in all housekeeping genes, these are mostly not highly significant (Table 3.7) or reflect a response in a single experimental replicate (Section 3.3.4.2 and Appendices). Thus, these changes may reflect changes in the cells other than direct response to treatment.

3.4.4 Estrogen responsiveness in breast cancer cell lines

These experiments set out to relate the estrogen response, in terms of transcription of estrogen responsive genes, to the levels of estrogen receptors expressed by the cell

lines. Some of the selected estrogen responsive genes (Section 3.1.3.2) did not show a significant response to treatment in cell lines, which might be expected from the literature. This may partly reflect the 24 hour time selected for the experiment, which may miss some more transient, early responses, such as induction of transcription of EFP [Ikeda et al., 2000] or VEGF [Buteau-Lozano et al., 2002]. Note that experiments were later performed using MCF7 cells to address this question (Section 5.2.2). Additionally, some estrogen stimulation experiments have used cells synchronised prior to stimulation [Altucci et al., 1996]. Although the cells used in this project were withdrawn from estrogenic stimulation before treatment, no additional treatment to synchronise the cells was used. Thus, responses such as induction of expression of cyclin D1 may be masked by the heterogeneity of the population.

Responses to treatment in some genes are seen in multiple, but not all, replicate experiments (Section 3.3.4, Tables 3.4 to 3.7 and Appendices). This could suggest that these responses are not genuine responses to treatment, but simply fluctuations in expression of the gene product. Conversely, the responses may be either relatively small or transient and therefore do not always reach significance, or may only occur under certain conditions, related to the confluence and passage number of the cell cultures. Several different statistical tests were used to determine whether responses to treatment could be regarded as significant. Different tests have different strengths and weaknesses, such that in some cases a response would reach significance in one test (e.g. Wilcoxon) but not another (e.g. Student's *t*-test). Tests based on the *t* distribution such as the Student's *t*-test and ANOVA are sensitive, but require a certain degree of normality and equality of variances. Post-tests such as Tukey's and Bonferroni's are robust against false positives observed due to multiple testing, but are therefore conservative and may generate false negatives. Non-parametric tests such as Wilcoxon's signed ranks test and Kruksal-Wallis are robust against non-normality and un-equal variances, but have low power with smaller samples. In this study, several different tests were used for each data set. Responses were regarded as more robust if they were detected using multiple different tests.

It has proved difficult to accurately measure expression of some genes, especially the ERβ isoforms, due to their low levels of expression. ERβ1, above all, often shows high levels of "PCR dropout" in assays, leading to high levels of error (Section 3.3.4.2). PCR reactions with a low target copy-number show inherently higher errors

because of particle distribution statistics, which predict that it will require a much greater number of replicates to differentiate five from ten copies of RNA than for the differentiation of 5000 from 10000 copies [Peccoud and Jacob, 1996]. Additionally, any differences in efficiency of the RT and PCR reactions, or small errors in set-up will reflect more strongly on low copy templates, possibly even moving them over the threshold of detection. In such cases, maximising the amount of cDNA used in each PCR reaction and increasing the number of PCR replicates and replicate experiments can help to generate more reliable and significant data sets.

3.4.4.1 Expression of estrogen receptor isoforms is cell line and treatment specific

The present study shows that different cell lines show distinct estrogen responses (Section 3.3, Tables 3.4 to 3.7 and Appendices). Interestingly, expression of estrogen receptor isoforms, as well as other genes, is regulated in a cell line and treatment dependent manner. This may provide a mechanism by which downstream estrogen responses can be differently controlled in the cell lines.

In this study, expression of ERα may be decreased by estradiol treatment in MCF7 cells (Section 3.3.4.2). Previous studies of ERα expression in MCF7 and T47D cells [Pink and Jordan, 1996; Read et al., 1989] suggested that ERα mRNA was up-regulated by estradiol in T47D and down-regulated in MCF7 at 24 hours, corresponding to the responses seen here in MCF7. T47D cells did not show a significant response in the current study.

It has been shown previously that culturing ZR75 cells in tamoxifen for up to a year resulted in an almost complete loss of ERα protein expression [van den Berg et al., 1989] and that treatment with tamoxifen rapidly reduced uterine ERα expression in ovariectomised rats [Reed et al., 2005]. In the current study, ZR75 cells do not show a conserved response to tamoxifen at the mRNA level. These differences may be due to the difference in timescale from the experiments described in the literature and to differences between mRNA and protein expression. It is notable, however that growth in withdrawal medium (removal of estrogen) for one week reduced ERα protein expression in all cell lines (This reaches significance in T47D and ZR75 cells by Student's *t*-test) (Fig. 3.1).

Expression of ERβ1 has been previously reported to be estradiol dependent in T47D cells, where mRNA expression was shown to be up-regulated 30 to 40-fold in response to estradiol [Vladusic et al., 2000]. This induction was inhibited by

tamoxifen, ICI 182,780, actinomycin D or cyclohexymide treatments, indicating that mRNA and protein synthesis are required for induction. In the present study, no significant responses to treatment were observed in ERβ1, probably due to measurement difficulties. However, ERβ2 is strongly up-regulated by estradiol in T47D cells (Section 3.3.4.2). Interestingly, only a very weak increase in ERβ5 mRNA is seen in response to estradiol (although expression may be reduced by ICI 182,780), suggesting that regulation of splicing events may be involved, rather than only an up-regulation of transcription. The estrogen receptor has recently been shown to be involved in regulation of gene splicing [Auboeuf et al., 2004; Auboeuf et al., 2002; Masuhiro et al., 2005]. One study using various mini-genes coupled to different promoters showed that estradiol could mediate changes in splicing patterns in a cell-type, promoter and ER subtype dependent manner [Auboeuf et al., 2002]. This may represent a mechanism by which auto-regulation of the ERs may occur.

3.4.4.2 estrogen responses in other genes

As well as the estrogen receptors, other genes also show changes in expression in response to treatment. As might be expected, these responses vary in a cell line dependent manner, which suggests that the mechanisms of the estrogen response may vary in different cell lines, as has been suggested by previous studies. Estrogen-responsive cyclin D1 expression, for instance, was shown to be PKA-independent in MCF7 cells [Sabbah et al., 1999] but PKA-dependent in ZR75 cells [Castro-Rivera et al., 2001]. The rate of induction of EFP expression appears to differ between cell lines [Ikeda et al., 2000; Inoue et al., 1993] and both PR [Flototto et al., 2004] and VEGF [Buteau-Lozano et al., 2002] show different responses in different breast cancer cell lines (Section 3.1.3.2). It is particularly noteworthy that even when both the ER and the responsive gene are expressed from transiently transfected expression vectors, the response may still differ between cell lines [Buteau-Lozano et al., 2002]. Similarly, exogenous expression of ERα in MDA-MB-231 cells was insufficient to generate estradiol dependent cyclin D1 expression [Planas-Silva et al., 1999], confirming that other endogenous factors play a role in the estrogen response.

PR

Progesterone receptor is a classic estrogen-responsive gene and up-regulation of PR in response to estradiol has long been used as a marker of estrogen response. It is expected to show increase mRNA expression response to estradiol in all cell lines

except MDA-MB-231. Studies have shown that MCF7 cells show an increase in expression of PR mRNA and protein in response to estradiol [Nardulli et al., 1988]. One study investigating differential regulation of PRA and PRB in MCF7, T47D and ZR75 cells over 48 h found overall PR mRNA expression was up-regulated by estradiol in all three cell lines (note that the present assay will detect both forms of PR) [Vienonen et al., 2002]. A different study showed that T47D cells increase PRB expression preferentially on estradiol stimulation [Graham et al., 1995], whilst a non estrogen-responsive T47D cell line shows no increase in expression [Horwitz et al., 1982; Nardulli and Katzenellenbogen, 1988].

ANOVA and two-sample tests show a significant increase with estradiol treatment in all cell lines except MDA-MB-231 in both sets of experiments, as would be expected (Section 3.3.4.2, Tables 3.4 to 3.7). These responses are also inhibited by the addition of anti-estrogens. In MCF7 cells, anti-estrogens may even reduce expression to below baseline levels. This robust induction and inhibition of over-expression confirms that these cells have a functional mechanism for estrogen response.

PS2

This gene is often used as a marker of estrogen response in both cell lines and in breast cancers, where its expression correlates well with ERα expression and may correlate with other prognostic markers [Gillesby and Zacharewski, 1999; Looi et al., 2001; Surowiak et al., 2001]. PS2 was originally shown to be estrogen responsive in MCF7 cells [Masiakowski et al., 1982], showing a 10-fold increase in mRNA expression in response to estrogen and no response to tamoxifen [Westley et al., 1984]. Many other studies using SAGE, PCR, Northern blot and microarrays confirm this response, giving between 5- and 16-fold increases in expression in response to estrogen [Bouras et al., 2002; El-Tanani and Green, 1997a; Inadera et al., 2000; Kim et al., 2000; Seth et al., 2002]. PS2 also shows an estrogen response of more than 16-fold in ZR75 cells [Cappelletti et al., 1996; Manning et al., 1988], but not in MDA-MB-231 cells [Cappelletti et al., 1996].

Here, statistical analysis shows a response to estradiol, which is inhibited by anti-estrogens, in all cell lines except MDA-MB-231 (Section 3.3.4.2). The magnitude of response in ZR75 also appears to be greater than in MCF7 cells (Table 3.7), as suggested by the literature.

Cathepsin D

Cathepsin D mRNA and protein expression have been previously shown to be

induced by estradiol in ERα-positive cell lines (including MCF7 and ZR75 but excluding MDA-MB-231, HeLa and BT20) [Cappelletti et al., 1996; Cavailles et al., 1993; Couissi et al., 1997; Rochefort, 1990; Touitou et al., 1991]. A SAGE study investigating estrogen and tamoxifen responses in MCF7 and ZR75 cells showed that mRNA expression was increased in response to estradiol in both cell lines [Seth et al., 2002], whilst another SAGE study also showed a 3.8-fold increase in expression in MCF7 cells over 24 hours [Inadera et al., 2000]. A study in which MDA-MB-231 cells were stably transfected to express ERα suggested that the native cells did not show estrogen-regulated cathepsin D expression, but that introduction of the ER permitted estrogen induced expression [Touitou et al., 1991].

Analysis of cathepsin D expression in the present series of experiments shows that the response to estrogen does not reach significance across the series of experiments. This may be due to limitation of the assay to detect changes, or suggest that this response is less robust to changes in cell culture than other responses such as increase in PR and PS2 levels.

Liv1

Liv1 was first identified in a screen of estrogen responsive ESTs in ZR75 cells, where expression was increased 4-fold in response to estrogen [Manning et al., 1988]. Although later papers have investigated the relationship between liv1 expression and prognostic markers in breast cancer [Kasper et al., 2005; McClelland et al., 1998], few groups have investigated its behaviour in cell lines. El-Tanani and Green used liv1 extensively as a marker of estrogen-related response in MCF7 cells [el-Tanani and Green, 1996a; el-Tanani and Green, 1996b; el-Tanani and Green, 1996c; El-Tanani and Green, 1997a; El-Tanani and Green, 1997b] and suggest a 10-fold induction can be obtained by estradiol treatment of these cells.

In this study, a statistically significant response was not seen in any cell line (Section 3.3.4.2). However, the data do suggest that expression may be up-regulated by estradiol in MCF7 cells, but that the power of these experiments was too low to give this significance. These results do not support or deny the reports in the literature due to the low power of the assay.

XBP1

Expression of XBP1 was shown to be up-regulated by estrogen treatment in various microarray-based experiments using MCF7 cells [Bouras et al., 2002; Finlin et al., 2001; Wang et al., 2004]. XBP1 mRNA levels are rapidly up-regulated, and remain

elevated up to 24 hours. Array [Finlin et al., 2001] and PCR [Wang et al., 2004] data suggest that XBP1 expression may be up-regulated up to 4-fold.

The present series of experiments showed that expression varied between treatments, but that no clear pattern of response to treatments could be confirmed (Section 3.3.4.2). The small size and lack of reproducibility of these changes is surprising, but may reflect the different method of measurement (Q-PCR rather than array or standard PCR) used in the current study, and the more specific measurement of the differently spliced forms. Additionally, different studies have used different growth and withdrawal conditions, which will affect basal levels of expression. XBP1 is also known to be a stress response gene. Therefore, its levels may change in response to stresses to the cells, such as the addition of ethanol carrier or antiestrogen to the medium, as well as to estradiol addition. This may make it difficult to identify estrogen-specific responses.

Cyclin D1

In the present study, expression of cyclin D1 is not significantly affected by treatment (Section 3.3.4.2). The literature includes many reports of up-regulation of cyclin D1 expression in MCF7 [Altucci et al., 1996; Foster and Wimalasena, 1996; Planas-Silva and Weinberg, 1997; Prall et al., 1997; Sabbah et al., 1999; Wang et al., 1998] and ZR75 [Castro-Rivera et al., 2001] cells. However, many of these groups report early induction of cyclin D1 expression, with mRNA levels peaking at 6 to 8 hours, and returning to close to base line levels by 24 hours, as one might expect for a cell cycle regulated gene. In addition, most studies used cells that had been synchronised prior to treatment, making changes in expression of cell cycle related proteins such as cyclin D1 easier to detect. This was not performed in the present study.

EFP

EFP showed no conserved response to treatment in these experiments (Section 3.3.4.2). This lack of response to estradiol may be due to the time-course of the experiment. Previous studies showed that expression of EFP was rapidly induced and returned to basal levels by 8 hours [Ikeda et al., 2000] or 20 hours [Inoue et al., 1993], depending on the cell line studied, whereas the present study measured expression at 24 hours. The possible response to tamoxifen is interesting, as it suggests that the inhibition of expression caused by tamoxifen may be more persistent that the positive response to estradiol.

p21

This gene was included in the set of estrogen responsive genes, because it was reported to show an increase in expression in a cell line transfected to over-express ERβ1 [Paruthiyil et al., 2004]. The present study suggests no robust response to treatment, although it may show a small increase in response to anti-estrogen in MCF7 (Section 3.3.4.2). p21 is a cyclin-dependent kinase inhibitor, which helps regulate cell cycle progression. It is, therefore, likely that any changes in expression of p21, both in this study and in the report of Paruthiyil *et al.*, are simply a reflection of changes in the rate of cell proliferation.

VEGF

VEGF was chosen as an estrogen responsive gene on the basis of reports in the literature that both ERα and ERβ could mediate estrogen-stimulated expression of a reporter construct containing the VEGF promoter (Section 3.1.3.2, [Buteau-Lozano et al., 2002]). In fact, the response of endogenous VEGF to estradiol stimulation in MCF7 cells was also measured [Buteau-Lozano et al., 2002], showing a maximum 1.6-fold increase in mRNA expression at 6 h, with levels then falling to close to base line by 24 h. Another study measured endogenous VEGF expression in MCF7 and MDA-MB-231 cells in response to estrogen treatment [Ruohola et al., 1999]. They show no response to estradiol in MDA-MB-231 cells, and a biphasic increase in mRNA levels in MCF7 cells. An initial, early peak of expression occurs, before expression returns to basal levels at 8 h. At 24 h expression is approximately 2-fold higher than basal levels, and levels then continue to rise until 72 h.

The present results show no significant response to treatment at 24 h (Section 3.3.4.2). It is noteworthy that the two reports of endogenous VEGF induction in MCF7 cells suggest markedly different time courses for this induction. This may be explained by the use of different medium and withdrawal conditions in the reports, or reflect variation in the behaviour of different populations of MCF7 cells, but may explain why no response is seen in this study.

3.4.5 A discussion of sources of variation in reported estrogen responses

In some cases, changes in gene expression do not correspond entirely to that reported in the literature, nor are the responses reported in the literature entirely consistent. In some cases, genes shown to be up-regulated by estrogen in the literature have not shown a significant response in this study. This may reflect the variation in

experimental systems used. The estrogen responsiveness of many genes has been investigated using either reporter-coupled promoter constructs, or cell lines transfected to over-express the ERs. Reporter genes may show stronger activation than endogenous genes, due to the accessibility of the construct, compared to the gene in native chromatin. However, native genes may also be able to recruit coactivator proteins, which may not be recruited by the reporter construct. In many cases, ER-expressing cells lines do not generate activation of these reporters without additional exogenous ER expression suggesting that only in the presence of excess ER do these responses occur.

Different cell lines show different estrogen responses. Additionally, in some cases, a single cell line may show different results in different studies. One example of this is PR induction in T47D cells. T47D cells have been reported to be both estrogen responsive and non-responsive in terms of PR expression by different groups, due to use of different sub-lines [Graham et al., 1995; Horwitz et al., 1982; Nardulli and Katzenellenbogen, 1988; Vienonen et al., 2002]. Different results may also be obtained due to different experimental procedures. A single cell line may be grown in different medium, different withdrawal protocols may be used, with or without additional cell cycle synchronisation, induction will use different levels of estradiol with a variety of carriers and measurements may be made over different time courses. Methods of measurement of mRNA expression of estrogen-responsive genes also differ, e.g. dot blots, Northern blots, PCR and Q-PCR, as do the different regions of the mRNA employed as target.

Finally, estrogen response was identified in this study if expression was significantly changed by estradiol treatment using multiple statistical tests such as ANOVA, Student's *t*-test or Wilcoxon's signed ranks test. Other studies use different measures of response, which may affect the significance of the response.

Despite the variation in estrogen responses in both the literature and the current study, some commonalities are seen. ERα expression varies differently with treatment in each cell line, in accordance with the literature, and the estrogen response of ERβ isoform mRNA expression corresponds with the preliminary results in the literature (Section 3.4.4.1). Key estrogen responsive genes such as PR and PS2 showed a response to estradiol as expected, however other responses (VEGF, EFP, cyclin D1) did not correspond to those in the literature (Section 3.4.5). These differences may be partly explained by variation in methodology, particularly to the

use of techniques such as reporter genes, transfected ERs or synchronised cells in the literature. They may also reflect the different growth conditions or genetic drift between cells used by different groups.

3.4.6 Conclusion

In this study, different cell lines show different types and magnitudes of responses when given the same set of estrogenic treatments (Section 3.3.4, Tables 3.4 to 3.7). These varying responses can be partly, but not fully, associated with the expressed levels of ERα (Section 3.3.4). For instance, MDA-MB-231 shows the lowest level of ERα expression, and shows no reproducible estrogen responses. However, although the other cell lines express similar levels of ERα protein and mRNA (Figs. 3.1 and 3.12), their estrogen responses are markedly different (Tables 3.4 to 3.7).

Despite its low levels of endogenous expression, the structure and function of ERβ make it an obvious candidate as a modulator of the estrogen response. Over-expression of ERβ1 has been shown to affect expression of some of the target genes used here [Liu et al., 2002b; Paruthiyil et al., 2004]. However, the effects of the different isoforms of ER are complex, making associations between ERβ expression and response difficult to identify. For example, when either ERβ1 or ERβ2 were constitutively over-expressed in MCF7 cells [Omoto et al., 2003], the estrogen response of cathepsin D was reduced, whereas the PS2 response to estrogen was decreased by ERβ1 expression but not ERβ2 expression, and ERα expression was down-regulated in response to estradiol in native cells and those over-expressing ERβ1, but up-regulated in cells over-expressing ERβ2, confirming that ER isoforms behave differently at different promoters.

It cannot be stated that differences in estrogen responsiveness in the cell lines are directly due to expression of different ER isoforms, as different cell lines may also express different levels of cofactors and use different growth factor signalling pathways, all of which will affect the estrogen response [Atanaskova et al., 2002; Dobrzycka et al., 2003; Lee et al., 2001; McKenna et al., 1999]. This explains why many groups resort to transfection experiments to unravel the roles of specific factors, despite the disadvantages of this technique.

Although the responses to treatments seen in the set of cell lines are difficult to relate to ER levels, they may give a more accurate representation of responses seen in real

breast cancers than given by transfection experiments. Transfection experiments often rely on insertion of multiple copies of an expression vector, or choosing clones which express high levels of a transgene [Omoto et al., 2003; Tonetti et al., 2003], thereby generating expression levels of the ERs which are much higher than usually seen. Responses to treatment may, therefore, not be comparable to those seen *in vivo*, where expression levels may be much lower [Chen et al., 2005].

In order to determine whether expression levels of individual ER isoforms are directly affecting expression of other genes it would be valuable to be able to control their expression without changing other variables within the cell. One method to achieve this is through transfection of a cell line to over-express the isoform of interest. It is particularly valuable to be able to control the expression of the inserted gene in an inducible manner, allowing gene expression to be measured in the same clone of cells with expression either "on" or "off", and allowing the level of expression of the inserted gene to be controlled, allowing expression levels closer to those seen endogenously to be achieved. This strategy is pursued in Chapter 4.

Chapter 4 Preparation of an inducible system for ERβ1 expression in MCF7 cells

4.1 Introduction

Analysis of the estrogen response in a set of breast cancer cell lines showed that this varied at the transcriptional level in a cell line dependent manner (Chapter 3). This is supported by the findings of [Saville et al., 2000] and [Watanabe et al., 1997] among others, who showed that aspects of estrogen and tamoxifen responses were dependent on cell type. Although the variation in estrogen responses could be partly attributed to ERα expression, other factors, such as ERβ or coregulators were also likely be involved in modulating estrogen response, as the responses did not simply correlate with ERα expression. For instance, T47D and MCF7 express similar levels of ERα, but showed different estrogen responses (Section 3.3.3), including different changes in expression levels of the ERβ isoforms. It is likely that ERβ plays a modulating role in the estrogen response, as ERβ isoforms have been shown to differentially regulate the estrogen response (Section 1.4.1, 1.5). However, using a set of different cell lines does not allow this hypothesis to be tested conclusively, as there are many other differences between the cell lines, such as growth factor receptor expression levels and autocrine loops, which also interact to affect transcription of estrogen responsive genes.

To examine the role of ERβ more stringently in breast cancer cell lines, a cell line was required which could express an ERβ isoform at different levels against a uniform signalling background. ERβ1 was chosen as the isoform to investigate, as this is the so-called full length form. It has a structure most similar to that of ERα (Fig. 1.1) and although it is not the most highly expressed of the ERβ is
expression has been associated with regulation of gene expression (Section 1.5) and aspects of breast cancer development (Section 1.3.3).

Of the cell lines, one was to be selected with relatively high expression of ERα, in order to investigate the effect of ERβ1 expression in the presence of ERα. This would allow both direct gene expression changes in response to ERβ1 and modulation of the ERα response to be investigated. T47D and MCF7 were suitable candidates based on this criterion (Figs. 3.1, 3.12). MCF7 and T47D express similar

levels of ERβ5 mRNA, but MCF7 express higher levels of ERβ2 mRNA and T47D express higher levels of ERβ1 mRNA (Fig. 3.12). T47D also showed much greater variation in expression of ERβ mRNAs following treatment with estradiol and antiestrogens (Table 3.7). MCF7 was selected, as it would show a greater difference in expression between endogenous and induced ERβ1 levels. Also, the greater stability of the endogenous ERβ1 expression levels should allow better quantification of the response with respect to expressed ERβ1 levels. Finally, it is valuable to be able to confirm the lineage of the transformed cells. MCF7 cells are reported to express certain fusion genes due to chromosomal rearrangements which are not seen in other cell lines (Section 4.4.6, [Barlund et al., 2002; Hahn et al., 2004]). This gives a simple method to confirm the lineage of MCF7 cells, which is not necessarily available with other cell lines.

4.1.1 A comparison of methods for exogenous expression of a gene in cell lines

A method of increasing expression of ERβ1 in the cells was required. Cells can be transfected to over-express a gene of interest in a variety of ways. Constitutive over-expression can be achieved most simply by insertion of the gene of interest into a vector under the control of a strong viral promoter such as the CMV promoter and transfection of the vector into the cells. However, this approach may lead to extremely high levels of expression, compared to physiological levels. Over-expression of the gene of interest may also inhibit cell growth, or change the behaviour of the cells.

Alternatively, the gene may be transfected into cells using an inducible system, where its expression is under the control of a second protein expressed from an inserted vector as part of the control system. Examples of this type of system include the ecdysone inducible mammalian expression system (Invitrogen, Section 4.1.1.1), the T-REx system (Invitrogen, Section 4.1.1.2), the Tet-On and Tet-Off systems (BD Clontech) and the RheoSwitch system (New England BioLabs), which allow expression of the gene of interest to be switched on or off within a transfected cell line and may also allow different levels of expression to be achieved, so that different levels of the gene product may be investigated.

Cells may be transfected transiently or stable clones may be isolated by antibiotic selection. The resulting stable integration of the expression system into the cells'

DNA is preferable, as this allows multiple experiments to be performed using the same cells. It also allows several clones to be screened and the most suitable selected for further experiments.

4.1.1.1 Introducing the ecdysone inducible system

Initially an ecdysone inducible system (Invitrogen) was chosen for the controlled expression of ERβ1 (Fig. 4.1). This system uses a modified receptor for an insect hormone to regulate expression of the target gene, resulting in a highly specific system. It was reported to show strong and controllable inducibility and to have little or no "leakiness" [No et al., 1996]. It has been reported by other groups to generate inducible expression of a target gene in cell culture (40 references listed in [Lafont and Dinan, 2003] and reviewed in [Fussenegger, 2001]).

The ecdysone inducible system is based on the *Drosophila melanogaster* moulting hormone system. *In vivo*, ecdysone binds to the ecdysone receptor (EcR), a nuclear steroid receptor, which then dimerises with *ultraspiracle* (USP). The dimerised receptor binds specific response elements, activating gene transcription. In the transfection system, both RXR (the mammalian homologue of USP), and a modified ecdysone receptor (VgEcR) are expressed constitutively from the vector pVgRXR. Expression of the gene of interest is activated when ponasterone A (PonA) is added to the cells and binds the ecdysone receptor, allowing the dimerised receptor to bind to a specific response element in the promoter of the downstream gene and to activate transcription. The ecdysone receptor has been modified to contain the VP16 transactivation domain, as well as having a modified DBD, which allows it to recognise a half-site of the glucocorticoid response element (5'-AGAACA-3') instead of its natural response element. RXR recognises an EcR half-site (5'-AGTGCA-3'), allowing the dimerised receptor to only bind the specific hybrid response elements found on the vector pIND, reducing the chance of endogenous receptors activating the system, or of the introduced ecdysone receptor activating endogenous genes [No et al., 1996]. This is important as other receptors such as the farsenoid X receptor have been shown to bind to normal EcRE and activate it at high concentrations of ligand [Lee et al., 1992].

Stable transfection is maintained by the presence of an antibiotic resistance gene in each vector. pIND contains a neomycin resistance gene which confers resistance to G418. This is an aminoglycoside which interferes with ribosomal function to block protein synthesis [Colbere-Garapin et al., 1981; Jimenez and Davies, 1980].

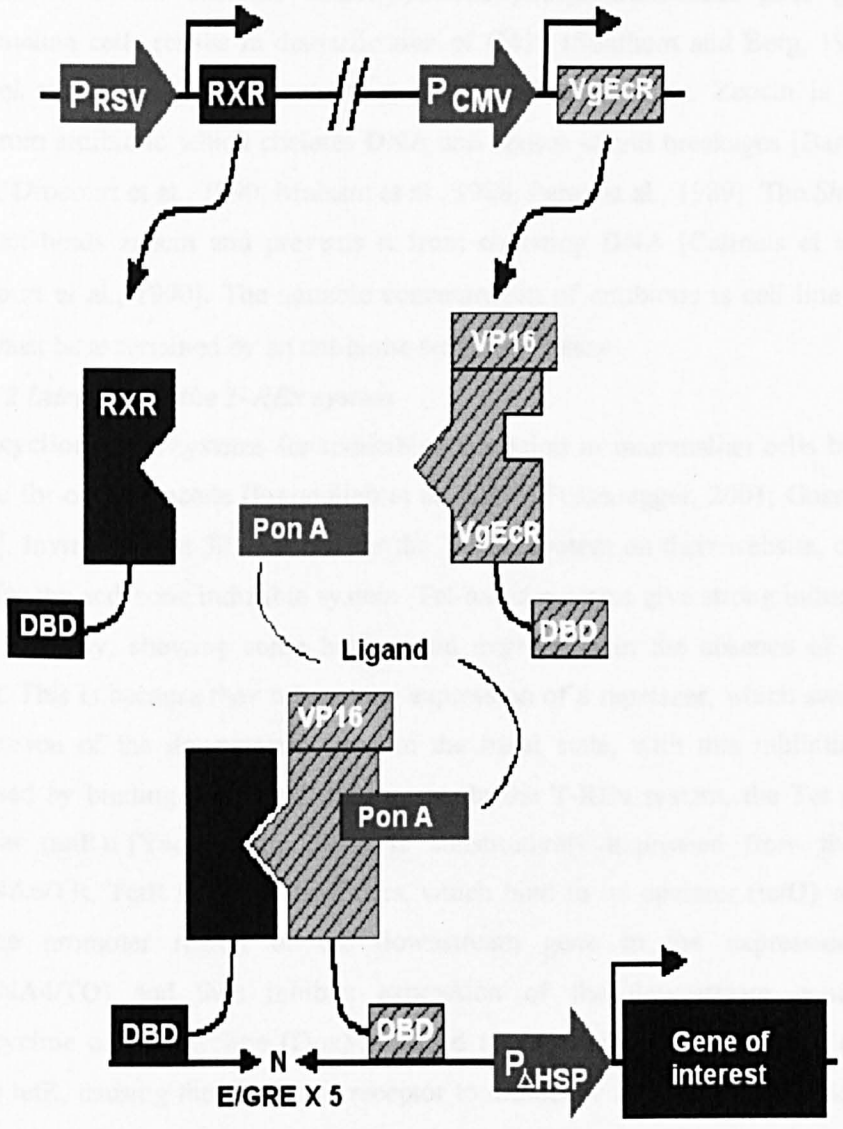


Figure 4.1 Mechanism of the ecdysone inducible system

Cells are stably transfected with two vectors, pVgRXR and pIND/gene of interest. RXR, the retinoic acid receptor, and VgEcR, a modified ecdysone receptor, are constitutively expressed from the pVgRXR plasmid. On addition of Ponasterone A, an ecdysone homologue, the two receptors dimerise and bind to repeats of the hybrid response element in the promoter for the gene of interest in the vector pIND. This activates transcription of the gene of interest from a minimal heat-shock promoter. Different levels of induction are achieved by varying the levels of Ponasterone A.

Reproduced from "Ecdysone-Inducible Mammalian Expression System" with permission of Invitrogen Ltd., Paisley, UK.

Expression of the bacterial aminoglycoside phosphotransferase gene (APH) in mammalian cells results in detoxification of G418 [Southern and Berg, 1982]. The control vector, pVgRXR, contains a zeocin resistance gene. Zeocin is a broad-spectrum antibiotic which chelates DNA and causes strand breakages [Baron et al., 1992; Drocourt et al., 1990; Mulsant et al., 1988; Perez et al., 1989]. The *Sh ble* gene product binds zeocin and prevents it from chelating DNA [Calmels et al., 1991; Drocourt et al., 1990]. The suitable concentration of antibiotic is cell line specific, and must be ascertained by an antibiotic sensitivity assay.

4.1.1.2 Introducing the T-REx system

Tetracycline-based systems for inducible expression in mammalian cells have been in use for over a decade [Freundlieb et al., 1997; Fussenegger, 2001; Gossen et al., 1993]. Invitrogen list 32 citations for the T-REx system on their website, compared to 8 for the ecdysone inducible system. Tet-based systems give strong induction, but may be leaky, showing some background expression in the absence of inducing agent. This is because they rely on the expression of a repressor, which switches off expression of the downstream gene in the basal state, with this inhibition being released by binding of the inducing agent. In the T-REx system, the Tet repressor protein (tetR) [Yao et al., 1998] is constitutively expressed from the vector pcDNA6/TR. TetR forms homodimers, which bind to tet operator (tetO) sequences in the promoter region of the downstream gene in the expression vector (pcDNA4/TO) and thus inhibits expression of the downstream gene. When tetracycline or doxycycline (Dox) is added to the medium, it enters the cells and binds tetR, causing the dimerised receptor to dissociate from the DNA binding site. Expression of the downstream gene is no longer inhibited and transcription can occur (Fig. 4.2).

Presence of the vectors is maintained in the cells by antibiotic selection, in this case with zeocin (Section 4.1.1.1) and blasticidin. This is a nucleoside antibiotic, which inhibits protein synthesis in both prokaryotic and eukaryotic cells [Takeuchi et al., 1958; Yamaguchi et al., 1965]. Resistance is conferred by expression of a deaminase which converts blasticidin S to a non-toxic deaminohydroxy derivative [Izumi et al., 1991]. Expression of the gene of interest in this system is from a strong promoter, allowing high levels of induction. However, it is essential that sufficient tetR is expressed to completely inhibit expression in the basal state. On transfection, more copies of the vector encoding tetR are inserted than the vector encoding the gene of

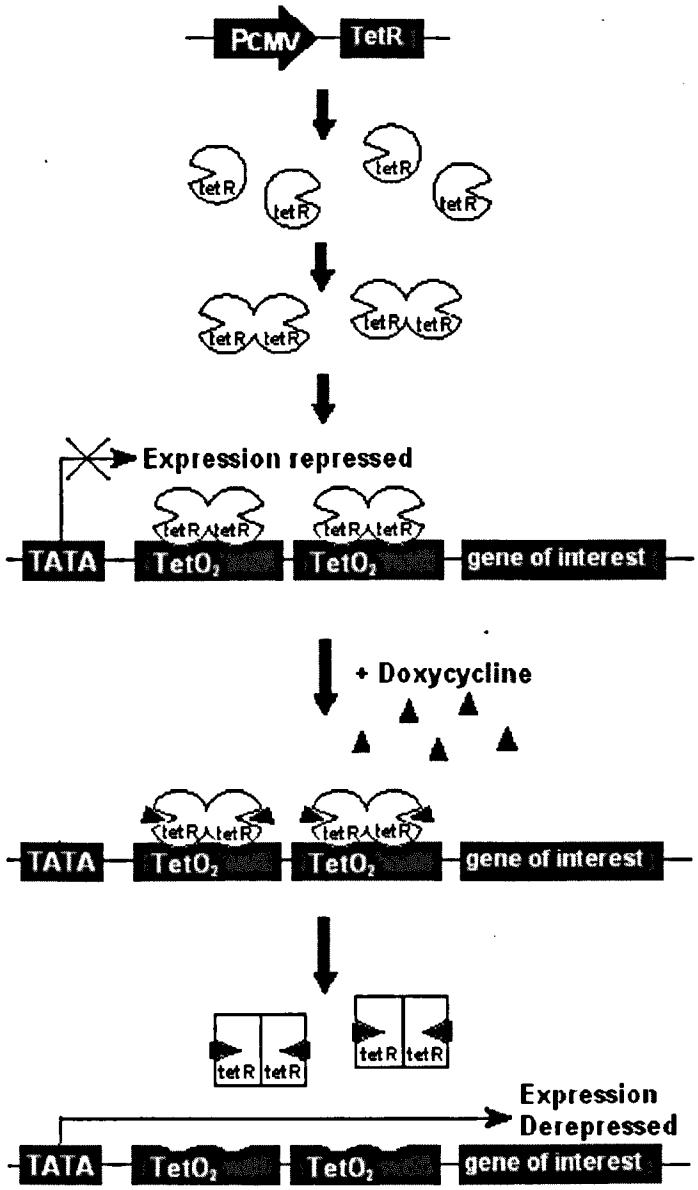


Figure 4.2 Mechanism of the T-REx system

Cells are stably transfected with the two vectors pcDNA6/TR and pcDNA4/TO/inserted gene. Tet repressor is constitutively expressed from the pcDNA6/TR vector. This dimerises and binds to the Tet operator sequences in the promoter region associated with the gene of interest in the pcDNA4/TO vector, repressing transcription. Addition of doxycycline, a tetracycline analogue, to the growth medium causes de-repression. The antibiotic binds to the TetR homodimers, causing a conformational change and a loss of repression, allowing expression of the gene of interest.

Taken from "T-REx system", with permission of Invitrogen Ltd., Paisley, UK.

interest, to ensure a high ratio of tetR molecules to tetO sequences. Certified Tet-free serum must also be used, as some sources of serum contain the inducing antibiotic which is routinely used to treat cattle.

4.2 Investigating antibiotic sensitivity of MCF7 cells

Before attempting transfection, it is important to know the sensitivity of the parental cells to the antibiotics in use. Three different antibiotics were used in this project; zeocin, G418 and blasticidin.

Before MCF7 cells were transfected with the ecdysone system, the insensitivity to zeocin and G418 was determined (Fig. 4.3). Cells were plated in the presence of different levels of either G418 or zeocin and their growth monitored. G418 (Fig. 4.3 A) was cytostatic at 250-375 μg/mL, and cytotoxic at 625 μg/mL and above. In contrast, zeocin (Fig. 4.3 B) was cytostatic at 125 μg/mL, but showed inefficient cytotoxicity, even at high concentrations (1000 μg/mL).

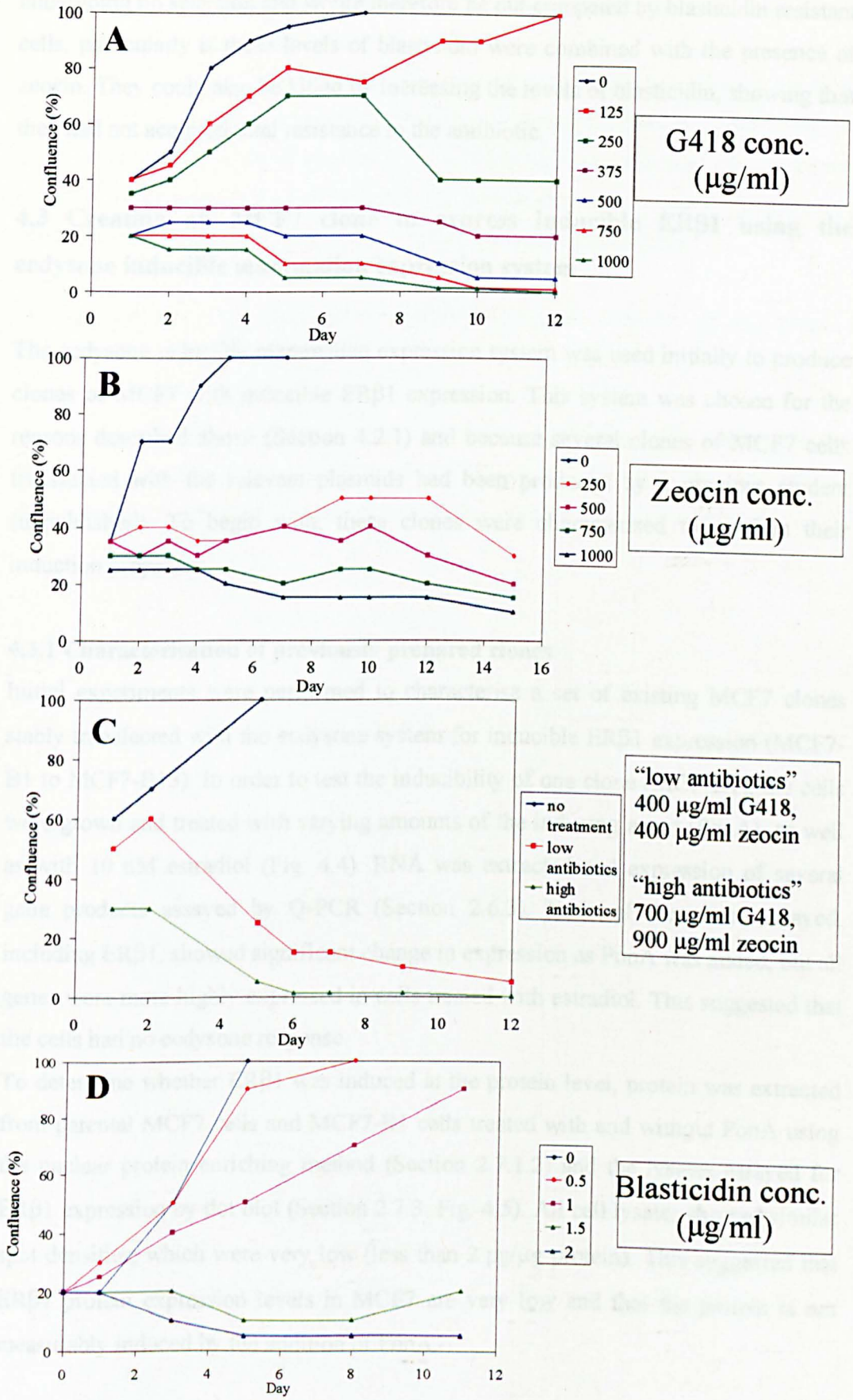
The cumulative effects of the two antibiotics were also investigated by treating cells with either a cytostatic dose of both antibiotics (400 μg/mL of each) or a cytotoxic dose of both antibiotics (900 μg/mL zeocin + 700 μg/mL G418) (Fig. 4.3 C). Both of these combinations of antibiotics led to cell death, although more slowly at the lower dose. It is important that the dose of antibiotic used for selection is high enough to prevent the growth of non-transfected cells, whilst not damaging or inhibiting the growth of transfected cells, containing the antibiotic resistance gene. This experiment showed that a lower dose of each antibiotic could be used when the two were given together.

The T-REx system uses zeocin and blasticidin for selection. In an initial experiment with six blasticidin concentrations from 0 to 10 μg/mL, as recommended by the manufacturer, 2.5 μg/mL was sufficient to produce cytotoxicity (confluence reduced from 50 to 10% in 4 days). A subsequent experiment (Fig. 4.3 D) showed that below 1.25 μg/mL blasticidin had little effect on cell growth, whereas from 1.5 to 2 μg/mL blasticidin showed a cytostatic effect. At these concentrations, some cells were killed quickly (0 to 5 days), but there appeared to be a subset of cells, which were resistant to these levels of antibiotic and were able to survive, even showing some growth over the 11 day period of the experiment. These cells did not grow as quickly as cells

Figure 4.3 Antibiotic sensitivity of MCF7 cells

To maintain the transfected vectors in the cell lines, antibiotic selection is required. In order to determine the sensitivity of MCF7, cells were treated with varying levels of different antibiotics and their growth assessed by visual examination. Cells were passaged on day 0, and antibiotic selection was begun immediately. Medium was changed every 3-4 days throughout the experiments. For clarity, a sub-set of the treatments used are shown on each graph.

(A) MCF7 sensitivity to G418, (B) Sensitivity to zeocin, (C) Sensitivity to combined G418 and zeocin, (D) Sensitivity to blasticidin.


A
Confluence (%)
Day
0
125
250
375
500
750
1000
G418 conc. (µg/ml)
B
Confluence (%)
Day
0
250
500
750
1000
Zeocin conc. (µg/ml)
C
Confluence (%)
Day
no treatment
low antibiotics
high antibiotics
"low antibiotics"
400 µg/ml G418,
400 µg/ml zeocin
"high antibiotics"
700 µg/ml G418,
900 µg/ml zeocin
D
Confluence (%)
Day
0
0.5
1
1.5
2
Blasticidin conc. (µg/ml)

undergoing no selection and would therefore be out-competed by blasticidin resistant cells, particularly if these levels of blasticidin were combined with the presence of zeocin. They could also be killed by increasing the levels of blasticidin, showing that they had not acquired total resistance to the antibiotic.

4.3 Creating an MCF7 clone to express inducible ERβ1 using the ecdysone inducible mammalian expression system

The ecdysone inducible mammalian expression system was used initially to produce clones of MCF7 with inducible ERβ1 expression. This system was chosen for the reasons described above (Section 4.2.1) and because several clones of MCF7 cells transfected with the relevant plasmids had been produced by a previous student (unpublished). To begin with, these clones were characterised to confirm their induction properties.

4.3.1 Characterisation of previously prepared clones

Initial experiments were performed to characterise a set of existing MCF7 clones stably transfected with the ecdysone system for inducible ERβ1 expression (MCF7-B1 to MCF7-B13). In order to test the inducibility of one clone (MCF-B1), the cells were grown and treated with varying amounts of the inducing agent (PonA), as well as with 10 nM estradiol (Fig. 4.4). RNA was extracted and expression of several gene products assayed by Q-PCR (Section 2.6.3). None of the genes assayed, including ERβ1, showed significant change in expression as PonA was added, but all genes were more highly expressed in cells treated with estradiol. This suggested that the cells had no ecdysone response.

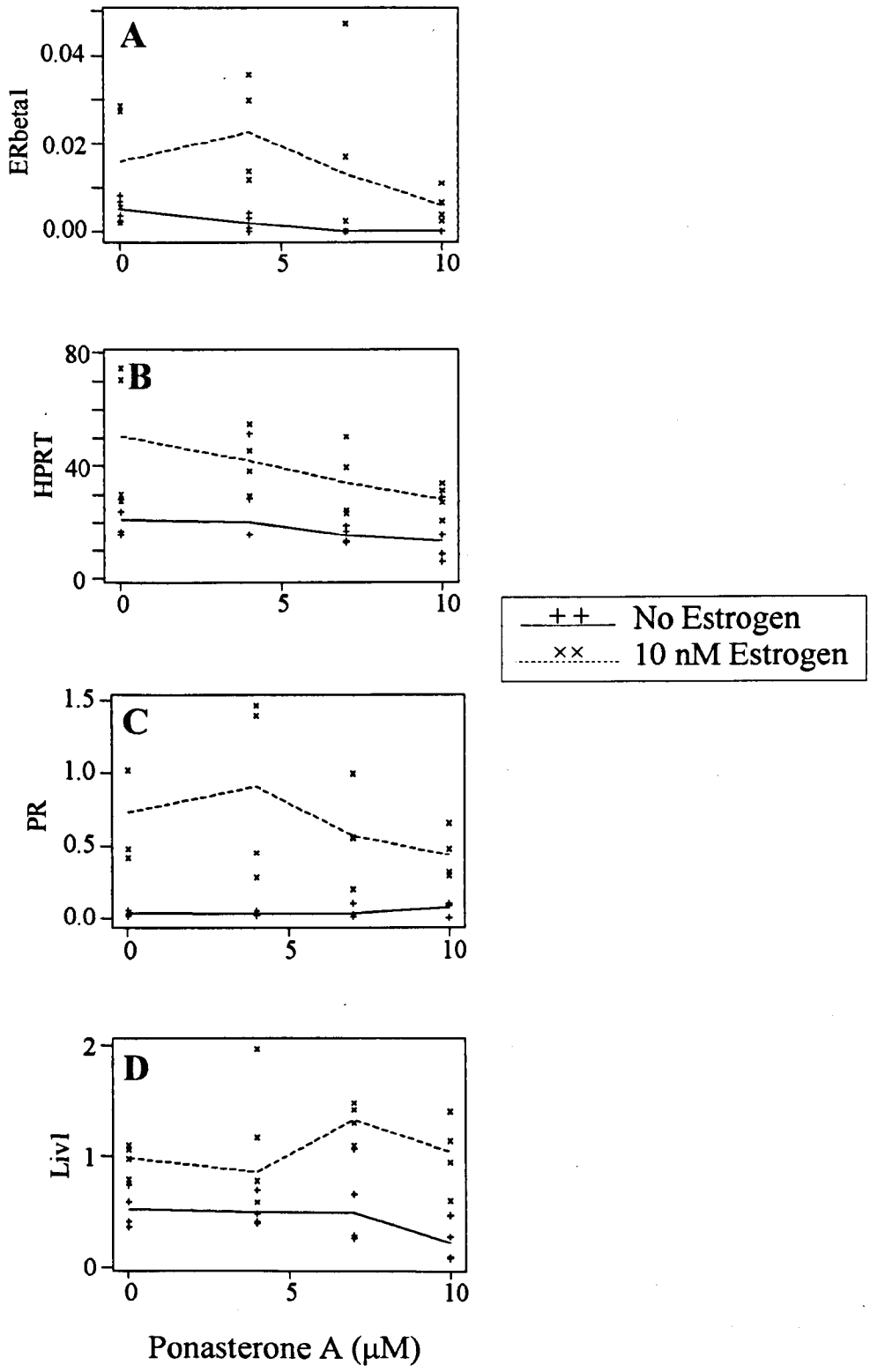
To determine whether ERβ1 was induced at the protein level, protein was extracted from parental MCF7 cells and MCF7-B1 cells treated with and without PonA using the nuclear protein enriching method (Section 2.7.1.2) and the lysates assayed for ERβ1 expression by dot blot (Section 2.7.3, Fig. 4.5). All cell lysates showed similar spot densities, which were very low (less than 2 pg/μg protein). This suggested that ERβ1 protein expression levels in MCF7 are very low and that the protein is not measurably induced by the addition of PonA.

Figure 4.4 Expression of ERβ1, HPRT, PR and liv1 in MCF7 cells stably transfected with the ecdysone expression system

"MCF7-B1", a clone of cells prepared using the ecdysone inducible system by Ms S. al Akilli (Section 4.2.1), were withdrawn from estrogen stimulation then treated with different levels (0 to 10 μM) of PonA (Section 2.2.1.3, 3.2.1, Fig. 4.1), with and without 10 nM estradiol, for 24 hours. RNA was extracted, RT performed and expression of several genes assayed by Q-PCR (Section 2.6.3). Gene expression uses an arbitrary scale.

(A) Expression of ERβ1, (B) Expression of HPRT, (C) Expression of PR, (D) Expression of liv1.

Cells treated with estradiol showed higher expression of all genes than without estradiol (Student's *t*-test ERβ1 $p=0.002$, HPRT $p=0.001$, PR and liv1 $p<0.001$). However, none of the genes showed significant association between PonA addition and expression.


A
ERbeta1
0.04
0.02
0.00
0
5
10
B
HPRT
80
40
0
C
PR
1.5
1.0
0.5
0.0
D
Liv1
2
1
0
++ No Estrogen
×× 10 nM Estrogen
Ponasterone A (μM)

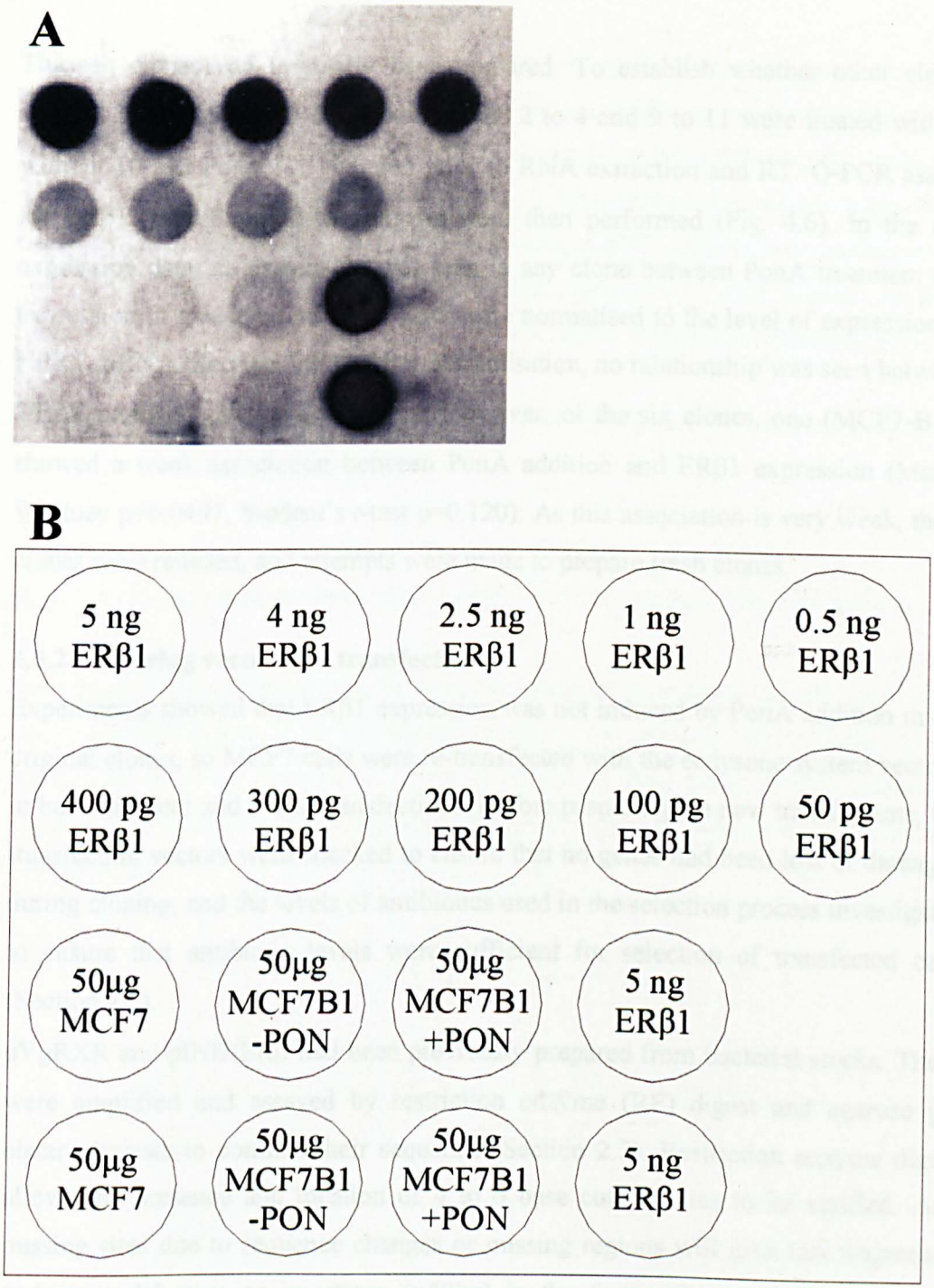


Figure 4.5 Dot blot analysis of ERβ1 expression in MCF7 and MCF7-B1 cells

A dot blot was performed using 50 µg of protein from MCF7 and MCF7-B1 cells treated with and without 10 µM PonA. A standard curve of 5000 to 50 pg recombinant ERβ1 is also shown.

Thirteen clones had originally been prepared. To establish whether other clones showed ERβ1 induction, cells from clones 2 to 4 and 9 to 11 were treated with or without 10 μM PonA for 24 hours prior to RNA extraction and RT. Q-PCR assays for ERβ1, HPRT and ERα mRNAs were then performed (Fig. 4.6). In the raw expression data, no correlation was seen in any clone between PonA treatment and expression of measured mRNAs. Data were normalised to the level of expression of HPRT mRNA (Section 3.2.5). After normalisation, no relationship was seen between HPRT or ERα and PonA treatment. However, of the six clones, one (MCF7-B11) showed a weak association between PonA addition and ERβ1 expression (Mann-Whitney p=0.0497, Student's *t*-test p=0.120). As this association is very weak, these clones were rejected, and attempts were made to prepare fresh clones.

4.3.2 Preparing vectors for transfection

Experiments showed that ERβ1 expression was not induced by PonA addition in the original clones, so MCF7 cells were re-transfected with the ecdysone system vectors, in both transient and stable transfections. Before preparing the new transfectants, the transfection vectors were checked to ensure that no genes had been lost or damaged during cloning, and the levels of antibiotics used in the selection process investigated to ensure that antibiotic levels were sufficient for selection of transfected cells (Section 4.2).

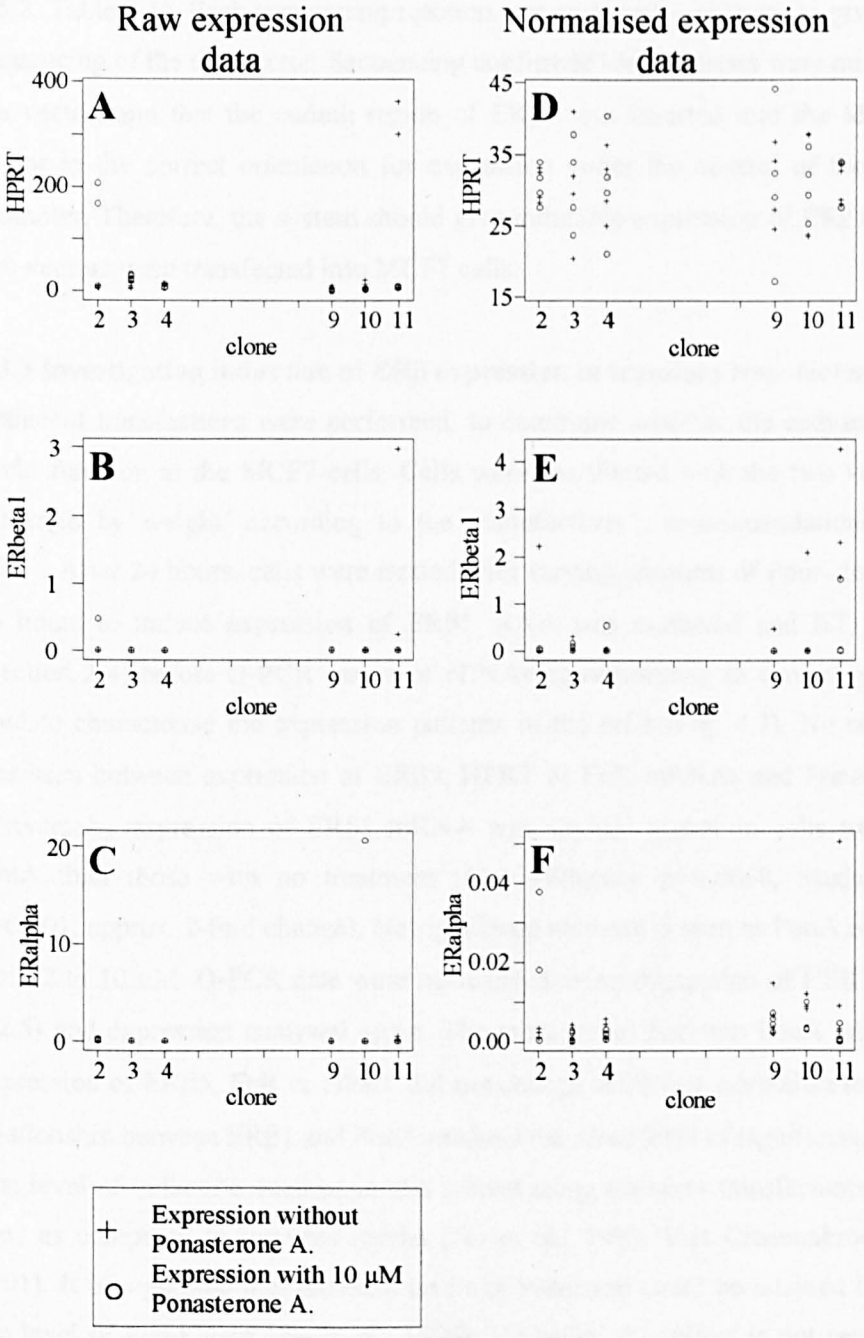
pVgRXR and pIND/ERβ had been previously prepared from bacterial stocks. These were amplified and assayed by restriction enzyme (RE) digest and agarose gel electrophoresis to confirm their sequence (Section 2.3). Restriction enzyme digest allows the presence and location of 4 to 6 base cutting sites to be verified. Any missing sites due to sequence changes or missing regions will give less fragments, and gross deletions or insertions (>50bp) in the fragments can be identified by changes in the sizes of the fragments. pVgRXR preps showed correct digest patterns, but pIND/ERβ stocks were shown to consist of a mixed population, as different RE digest patterns were obtained from different clones.

pIND/ERβ was transformed into *E. coli* and 96 clones prepared (Section 2.3). All of the clones were analysed by RE digest, and a suitable clone was picked, which showed the correct digest pattern. This clone was further amplified and completely

Figure 4.6 Expression of ERβ1, ERα and HPRT in several "ecdysone inducible" clones. Thirteen different clones were prepared by a previous worker to express ERβ1 using the ecdysone inducible expression system. Six of these (2-4 and 9-11) were treated with and without 10 μM ponasterone A for 24 hours prior to RNA extraction in order to assess levels and induction of expression of ERβ1. For each clone, two RT reactions were performed and each of these was used in duplicated PCR reactions, giving four Q-PCR readings per RNA sample in total. Results for three genes are shown, before and after normalisation using the housekeeping gene HPRT.
In each panel, expression in untreated cells (+) and in cells treated with 10 μM PonA (o) are shown. Gene expression is shown on an arbitrary scale.

(A) Expression of HPRT, (B) Expression of ERβ1, (C) Expression of ERα, (D) Normalised HPRT expression, (E) Normalised ERβ1 expression, (F) Normalised ERα expression.

No significant relationship is seen between expression of these genes and treatment in the raw or normalised data except for normalised ERβ1. For this data set, two outlying data points were removed, from clones 2 and 11. Clone 11 then shows a weak association between PonA addition and increased ERβ1 expression (Mann-Whitney p=0.0497, Student's t-test p=0.120).


Raw expression data
Normalised expression data
A
B
C
D
E
F
HPRT
ERbeta1
ERalpha
clone
2 3 4 9 10 11
Expression without Ponasterone A.
Expression with 10 µM Ponasterone A.

sequenced using 48 primers positioned throughout the vector sequence (Section 2.5.2, Table 4.1). Each sequencing reaction was analysed four times to give accurate sequencing of the full vector. Sequencing confirmed that no genes were missing from the vector, and that the coding region of ERβ1 was inserted into the MCS of the vector in the correct orientation for expression under the control of the ecdysone promoter. Therefore, the system should give inducible expression of ERβ1 when the two vectors were transfected into MCF7 cells.

4.3.3 Investigating induction of ERβ expression in transient transfectants

Transient transfections were performed, to determine whether the ecdysone system could function in the MCF7 cells. Cells were transfected with the two vectors in a 1:1 ratio by weight, according to the manufacturer's recommendations (Section 2.2.5). After 24 hours, cells were treated with varying amounts of PonA for a further 24 hours to induce expression of ERβ1. RNA was extracted and RT performed (Section 2.4) before Q-PCR assays of cDNAs corresponding to several genes were used to characterise the expression patterns in the cells (Fig. 4.7). No relationship was seen between expression of ERβ5, HPRT or EcR mRNAs and PonA addition. Conversely, expression of ERβ1 mRNA was slightly higher in cells treated with PonA than those with no treatment (Mann-Whitney $p=0.0668$, Student's *t*-test $p=0.001$, approx. 2-fold change). No significant increase is seen as PonA is increased from 2 to 10 μM. Q-PCR data were normalised using expression of HPRT (Section 3.2.5) and expression analysed again. The relationship between PonA addition and expression of ERβ5, EcR or HPRT did not change following normalisation, and the relationship between ERβ1 and PonA retained the same level of significance.

The level of induction attained in this system using transient transfectants was very low, as compared to reported results [No et al., 1996; Van Craenenbroeck et al., 2001]. It is suggested that different levels of induction could be attained by varying the level of PonA used [No et al., 1996]. However, this effect is not seen in these transient transfectants.

Forward primers

Primer name	primer sequence	position
pINDa	TACCCTCGACCGCCGGAGT	183
pINDb	CCGAGAAAGAAGAACTCACA	452
SALFWD	TCACATCTGTATGCGGAACC	947
1144F	TGGAGGTCTGGTCGTGTGAAG	1144
1345F	TGTGCGGAGACAGAGAAGTG	1345
CSERBE	CTCATCTTTGCTCCAGATCTTGTT	1721
CSERBA	CATCTCCTCCCAGCAGCAATC	1996
CSERBC	CGTCAGGCATGCGAGTAACA	2053
2395F	ATTGCATCGCATTGTCTGAG	2395
2692F	TTTCCCCGTCAAGCTCTAAA	2692
2933F	CGATTTCGGCCTATTGGTTA	2933
3378F	ACAGGATGAGGATCGTTTCG	3378
3719F	TGCTCCTGCCGAGAAAGTAT	3719
4198F	GACTCTGGGGTTCGAAATGA	4198
4565F	TATCCGCTCACAATTCCACA	4565
4958F	CCTGACGAGCATCACAAAAA	4958
5310F	GCAGAGCGAGGTATGTAGGC	5310
5791F	GATACGGGAGGGCTTACCAT	5791
5950F	TTGCCGGGAAGCTAGAGTAA	5950
6321F	ATAATACCGCGCCACATAGC	6321
6490F	AGCAAAAACAGGAAGGCAAA	6490

Reverse primers

Primer name	primer sequence	position
42R	AGCGGTATCGAGAACAATGC	42
183R	ACTCCCGGGTACTGAGCTTT	183
484R	AACGCTAGCTGTGTGTGAGTTC	484
pINDg	GGGGTAAGATGGATTGACTG	715
pINDh	GCAGGGCTATAGAATGTCAT	797
pINDi	GATAACTGGCGATGGACC	929
pINDf	GGCGCAACGGTTCCCACTA	1041
SALREV	CGTAACACTTCCGAAGTCGG	1273
1369R	GTCGGCACTTCTCTGTCTCC	1369
1773R	TCTACGCATTTCCCCTCATC	1773
CSERBF	GGAGTTTTAACTCTCGAAACCTTG	1819
CSERBB	CTCCAGCAGCAGGTCATACAC	2121
CSERBD	GACCCCGTGATGGAGGACTT	2171
pINDd	GGGCAAACAACAGATGGCTG	2308
pINDc	ACGGGGGAGGGGCAAACAA	2317
pINDe	CCTACTCAGACAATGCGATG	2399
2830R	AAGGGCGAAAAACCGTCTAT	2830
3167R	CGGGACTATGGTTGCTGACT	3167
3404R	AATCATGCGAAACGATCCTC	3404
3738R	ATACTTTCTCGGCAGGAGCA	3738
4217R	TCATTTCGAACCCCAGAGTC	4217
4584R	TGTGGAATTGTGAGCGGATA	4584
4977R	TTTTTGTGATGCTCGTCAGG	4977
5329R	GCCTACATACCTCGCTCTGC	5329
5804R	AGCCCTCCCGTATCGTAGTT	5804
6165R	GCGGCCAACTTACTTCTGAC	6165
6340R	GCTATGTGGCGCGGTATTAT	6340

Table 4.1 Sequencing primers used to confirm the sequence of the vector pIND/ERβ

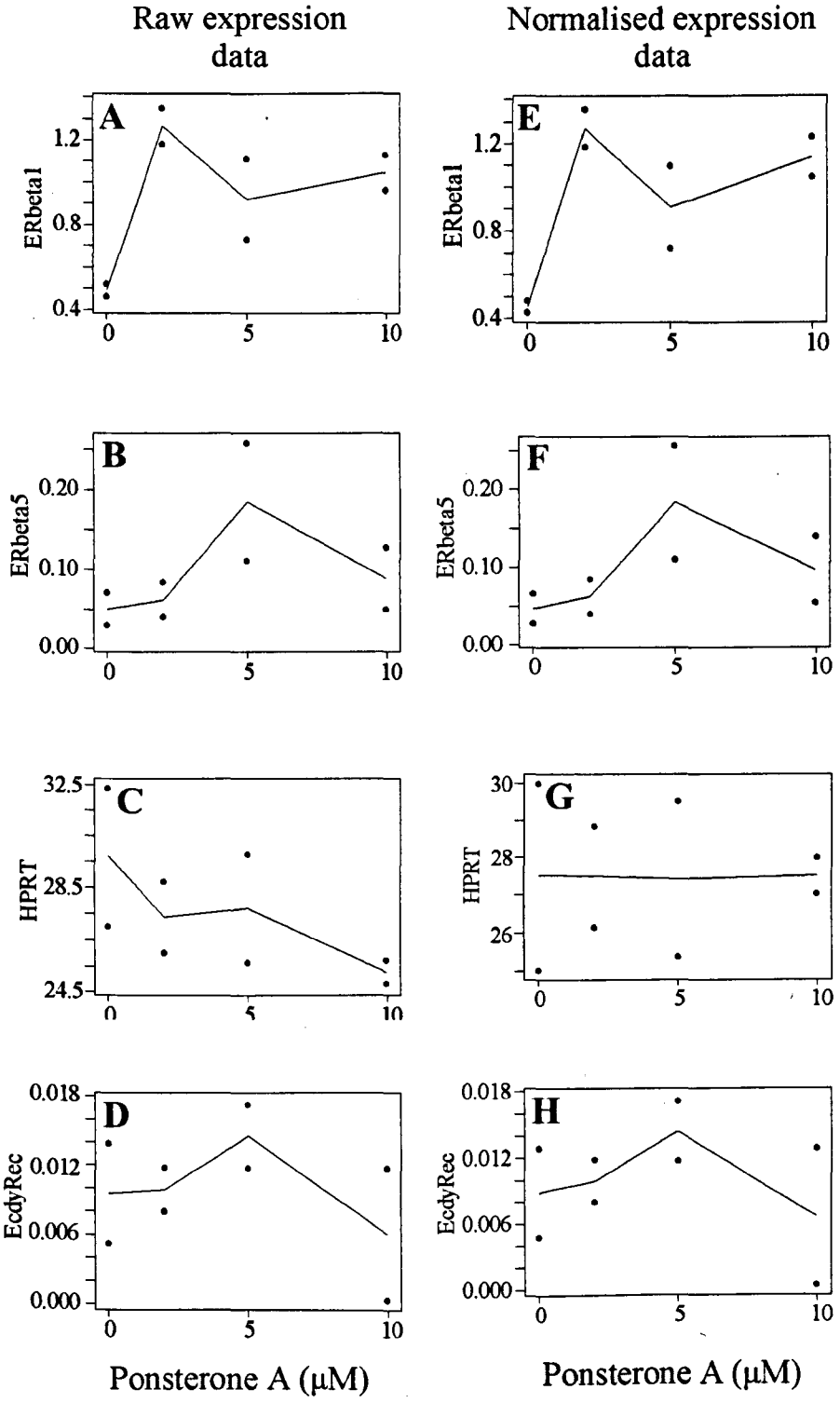
Stock of the vector pIND/ERβ was cloned into *E. coli* and analysed by RE digest (Section 2.3). A clone showing the correct RE digest pattern was chosen and completely sequenced. The primers used to sequence the vector are shown here, with their positions in the vector sequence. Two sequencing reactions were performed using each primer, and each sequencing reaction was run on the MegaBase sequencer more than once to confirm the sequence.

Figure 4.7 Expression of several genes in MCF7 cells transiently transfected with the ecdysone inducible expression system for the expression of ERβ1

Cells were transiently transfected with the ecdysone system, and after 24 hours were treated with different levels of PonA for a further 24 hours before RNA extraction, RT and expression analysis by Q-PCR. Each RNA sample underwent a single RT reaction, which was then used in duplicate Q-PCR assays for each gene product. Data are shown before and after normalisation using HPRT expression.

(A) ERβ1 expression, (B) ERβ5 expression, (C) HPRT expression (D) EcR expression, (E) Normalised ERβ1 expression, (F) Normalised ERβ5 expression, (G) Normalised HPRT expression (H) Normalised EcR expression

No relationship was seen between expression of ERβ5, HPRT or EcR and PonA addition in raw or normalised data. ERβ1 expression was higher when PonA was added (Student's *t*-test p=0.001, Mann-Whitney p=0.0668 using either raw or normalised data) but no increase in ERβ1 is seen as ponasterone levels are increased from 2 to 10 μM.


Raw expression data
Normalised expression data
A
E
ERbeta1
1.2
0.8
0.4
0
5
10
B
F
ERbeta5
0.20
0.10
0.00
C
G
HPRT
32.5
28.5
24.5
30
28
26
D
H
EcdyRec
0.018
0.012
0.006
0.000
Ponsterone A (μM)
Ponsterone A (μM)

4.3.4 Stable transfection of MCF7 with the ecdysone system to give inducible ERβ1 expression.

In order to obtain MCF7 stably transfected with the ecdysone system for ERβ1 expression, cells were transfected (Section 2.2.5) and after 24 hours were split into forty 24-well plates and selection with antibiotics begun using 400 μg/mL of each of the two antibiotics. No clones were obtained following selection with antibiotics. Whether this was due to using overly high levels of antibiotic, or whether the cells did not take up the vectors or express the antibiotic resistance genes from the vectors is unknown. However, the combination of poor results from the existing stable cones and the transient transfectants, as well as the difficulties in obtaining stable clones led to the investigation of an alternative system.

4.4 Creating an MCF7 clone to express inducible ERβ1 using the T-REx system

As the ecdysone system had failed to produce stably transfected cells, which expressed ERβ1 in an inducible manner, experiments were begun using the T-REx system (Section 4.1.1.2). This allows expression of a gene of interest under the control of a repressor, which is constitutively expressed. The system comprises two vectors, one encoding the repressor and one encoding the gene of interest. Both vectors also include antibiotic resistance genes to maintain them in the cells.

4.4.1 Preparing vectors for the T-REx system

The control vector, pcDNA6/TR, was simply amplified from purchased stock (Section 2.3.3) and assayed by RE digest and electrophoresis (Section 2.3.4) to confirm the gross structure before use. Preparation of pcDNA4/TO/ERβ required insertion of the ERβ1 cDNA sequence into the pcDNA4/TO vector. This was achieved by removing the ERβ1 fragment from the MCS of the pIND/ERβ vector, which had been previously analysed to confirm the exact sequence (Section 4.3.2), and inserting it into the MCS of the pcDNA4/TO vector.

The ERβ1 fragment was obtained by digestion of the pIND/ERβ vector with BamHI and XhoI in a double digest since the pcDNA4/TO vector possesses sites for these enzymes in its MCS. The large pcDNA4/TO fragment and the small ERβ1 fragments

were separated from the remaining fragments by electrophoresis and removed from the gel by electroelution. The DNA was then cleaned and concentrated before ligation to create the pcDNA4/ERβ1 vector (Section 2.3.4). The ligation mixture was cloned into *E. coli* and sixteen colonies were picked and grown on (Section 2.3.2). Glycerol stocks of these were prepared and vector was extracted from each clone. Each vector was analysed by running a sample on a gel, with and without prior digestion with a selection of RE enzymes. Of the 16 clones, one was lost during processing, and 14 out of the remaining 15 showed the correct RE digest pattern, and were, therefore, suitable for use in transfection reactions.

4.4.2 Induction of ERβ in transient transfectants

Transient transfections of the two vectors into MCF7 cells were performed in order to confirm that the system was functional. The vectors of the T-REx system were transfected into MCF7 cells at a ratio of 6:1 (control: expression vectors) according to the manufacturer's recommendations (Section 2.2.5). Cells either underwent mock transfection, with no vector, or were transfected with the two vectors. After 24 hours the transfected cells were treated with increasing levels (0, 10, 100, 1000 ng/mL) of doxycycline (Dox), and after a further 24 hours, RNA was extracted. RT and Q-PCR analysis of gene expression were performed to investigate induction (Fig. 4.8).

Assays were performed for a number of gene products, including ERβ1, HPRT, GAPDH and TetV- a pcDNA6/TR vector specific assay (Section 2.6.3). Tet vector (Fig. 4.8 B) was absent from the mock-transfected cells and showed much higher levels when RNA was not treated with DNase, as expected. However, measured levels of all genes were higher when no DNase was used. This suggests the DNase treatment had some inhibitory effect on the RT, or damaged the RNA or cDNA in this assay. ERβ1 (Fig. 4.8 A) mRNA is induced with Dox treatment. Comparing mock transfected to transfected cells, expression of ERβ1 mRNA is about 85,000-fold higher (Student's *t*-test, $p<0.0005$). In the transfectants, expression of ERβ1 shows a 2-fold increase, as Dox is increased from 0 ng/mL to 1000 ng/mL (*t*-test, $p=0.019$), and regression analysis shows a significant relationship between log Dox and ERβ1 expression ($R^2=42\%$, $p=0.001$).

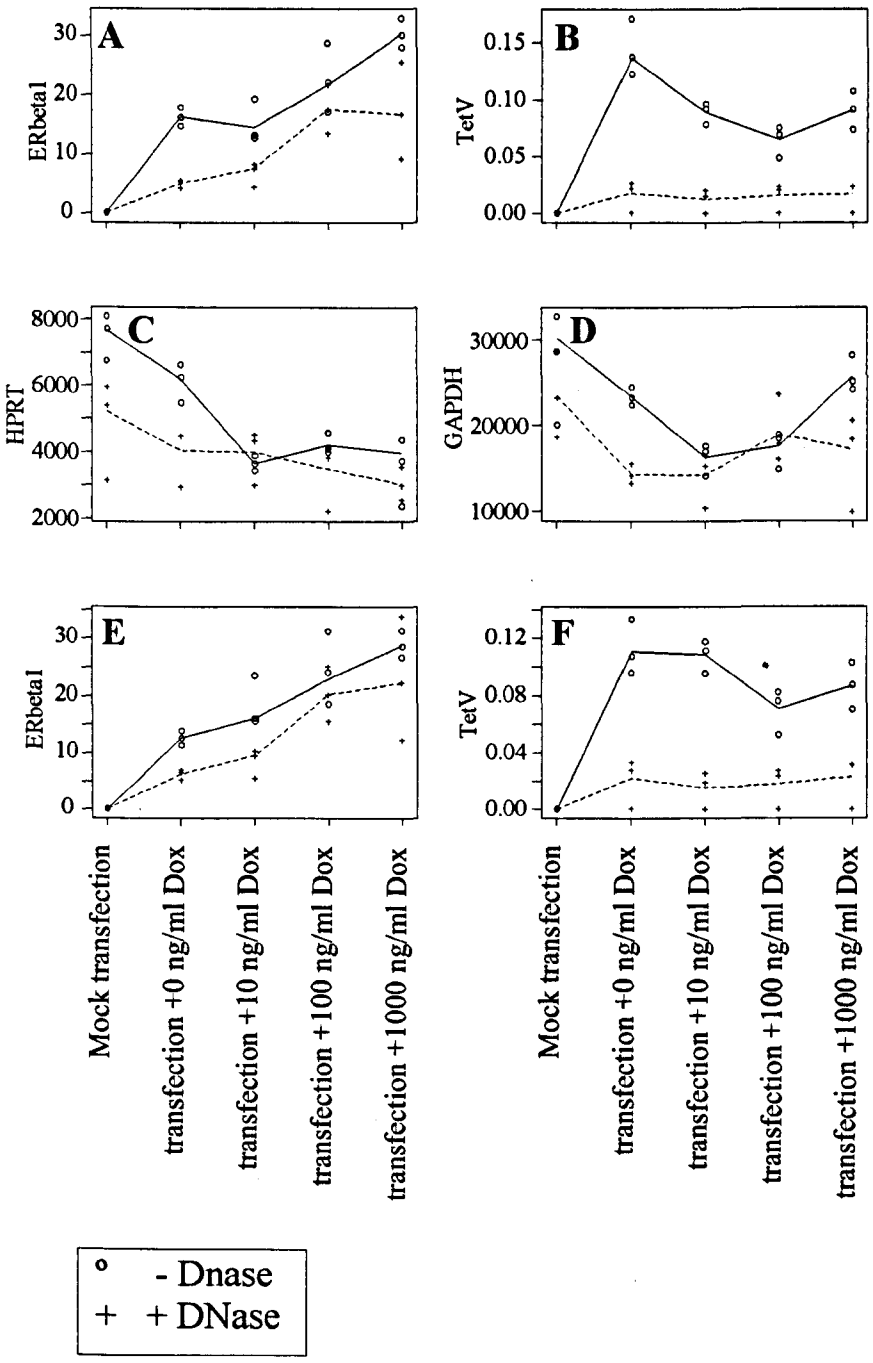
Data were normalised using an average of HPRT and GAPDH expression (Section 3.2.5). No major changes in expression pattern are seen with this normalisation, e.g.

Figure 4.8 Gene expression in MCF7 transiently transfected with the T-REx system to express ERβ1

Cells were transfected with the T-REx system for inducible expression of ERβ1. A mock transfection was also included. After 24 hours cells were treated with different amounts of Dox, the inducing agent, and after a further 24 hours cells were killed and RNA extracted for Q-RT-PCR analysis. Mock-transfected cells were not treated with Dox. Transfected cells were treated with 0, 10, 100 or 1000 ng Dox per ml media. Each RNA sample was used in two RT reactions, with and without DNase treatment. Each RT was then diluted into triplicate Q-PCR reactions for expression analysis. Raw data is shown for expression of five Q-PCR assays, and data normalised using GAPDH and HPRT expression for two of these.

(A) ERβ1 expression, (B) Measured pcDNA4/TR vector, (C) HPRT expression, (D) GAPDH expression (E) Normalised ERβ1 expression, (F) Normalised measured pcDNA4/TR.

Transfected cells show significantly higher ERβ1 expression than mock-transfected cells (85000-fold increase, Student's *t*-test $p<0.0005$). ERβ1 expression in transfected cells increases with increased Dox addition (Student's *t*-test, cells treated with 1000 ng Dox and without Dox, 2.2-fold increase, $p=0.019$). This response is augmented in the normalised data (Student's *t*-test, cells treated with 1000 ng Dox and without Dox 2.8-fold increase, $p=0.002$). HPRT shows a decrease in expression in response to Dox treatment (regression analysis $R^2=38\%$, $p<0.0005$). All assays show lower values when DNase treatment was used (Student's *t*-test ERβ1 $p=0.039$, pcDNA4/TR $p<0.0005$, HPRT $p<0.0005$, GAPDH $p=0.024$). Normalisation does not strongly affect the results (Student's *t*-test for pcDNA4/TR raw data vs. normalised data $p=0.916$), although the significance of the Dox response in ERβ1 is increased (see above).


A
B
C
D
E
F
ERbeta1
TetV
HPRT
GAPDH
Mock transfection
transfection +0 ng/ml Dox
transfection +10 ng/ml Dox
transfection +100 ng/ml Dox
transfection +1000 ng/ml Dox
- Dnase
+ DNase

TetV (Fig. 4.8 F). ERβ1 (Fig. 4.8 E), however, shows a slightly stronger association between Dox and increased expression after data normalisation. As Dox increases from 0 ng/mL to 1000 ng/mL measured ERβ1 increases 2.7-fold (*t*-test comparing 0 and 1000 ng/mL, p=0.002; regression analysis R^2=57%, p<0.0005).

The large difference in expression between mock transfected and transfected cells was an encouraging result. It was hypothesised that the inducibility of the cells might increase in stably transfected cells. The T-REx system relies on expression of a repressor to inhibit expression of the gene of interest. In the transient system, the cells may not have had sufficient time to synthesise enough Tet repressor to fully repress expression of ERβ1. Thus, in stably transfected cells, the difference between induced and non-induced expression of ERβ1 could be increased.

4.4.3 Preparation of MCF7 cells stably transfected with the T-REx system to inducibly express ERβ1

MCF7 cells were transfected as described above (Section 2.2.5, 4.4.2) and after 24 hours the cells were passaged into 24-well plates for clonal selection. Several different approaches to antibiotic selection were tested. Initially, cells were passaged 1 in 10 at 24 hours into 24-well plates, and antibiotics added immediately. This resulted in no surviving clones, so the transfection was repeated, this time passaging the cells after 24 hours and beginning antibiotic selection after a further 24 hours, to allow the cells more time to express the antibiotic resistance genes and to become established. Cells were also treated with both antibiotics from the outset or with blasticidin alone or zeocin alone for five days, before addition of the second antibiotic. Plates not immediately treated with zeocin showed a greater number of surviving colonies. This may be due to the mode of action of zeocin, which is to cause DNA strand breakages [Baron et al., 1992]. The zeocin resistance gene product binds and sequesters zeocin, preventing its action. However, a recent report [Oliva-Trastoy et al., 2005] suggests that in cells expressing the *Sh ble* resistance gene, some DNA damage still occurs. Possibly, addition of zeocin too soon after transfection could lead to irreparable damage to the cells' genome, due to insufficient expression of the resistance protein.

A pooled population of stably transfected cells (MCF7β1x) was obtained by maintaining transfected cells in large dishes and pooling all surviving cells. Several

clones of cells were also obtained by limit dilution of the transfected cells into 24-well plates and selection of clones derived from a single cell. Although selection was performed using relatively low levels of the two antibiotics, (200 μg/mL zeocin, 1.5 μg/mL blasticindin), tests performed using pooled stably transfected cells showed they could survive much higher levels of antibiotics; up to 1000 μg/mL zeocin and 10 μg/mL blasticidin (data not shown), which would kill parental MCF7 cells.

4.4.4 Investigating inducibility and gene expression in a pooled population of stably transfected cells

4.4.4.1 Induction of ERβ1 expression over 24 hours

Low passage number MCF7β1x cells (passage 4) were treated with 0, 0.5 or 5 μg/mL Dox for 24 hours before RNA extraction, RT and Q-PCR analysis. Expression analysis was performed for several genes (Fig. 4.9). Expression of ERβ1 mRNA is increased approximately 10-fold in Dox treated compared to non-treated cells (*t*-test $p<0.0005$), but no change is seen between cells treated with 0.5 or 5 μg/mL Dox (*t*-test $p=0.242$) (Fig. 4.9 A). The other gene products assayed (ERβ2, ERα, TetR and GAPDH) do not show significant changes in expression with respect to Dox addition (Figs 4.9 B-E). One RNA sample, taken from cells treated with 0.5 μg/mL Dox and not treated with DNase, showed unusually low levels of expression of GAPDH, Tet Repressor and ERα compared to other samples. Due to this, data were normalised using expression of GAPDH (Section 3.2.5). This normalisation by a housekeeping gene was performed because the reduced expression across several genes suggested that this sample had less cDNA than the other samples, either due to poor quality or insufficient amounts of RNA being used in the RT, or a poor RT reaction. In the normalised data (Figs 4.9 F-G as examples), similar patterns of expression were seen, but without the drop in expression in the one sample. ERβ1 expression showed similar levels of significance for induction with Dox addition (Student's *t*-test comparing treated to untreated cells $p=0.000$, approx. 12-fold difference). Other genes do not show any association with Dox treatment after normalisation.

4.4.4.2 Induction of ERβ1 over a 96 hour time course

The effect of Dox addition on gene expression over a longer time course was also investigated. MCF7β1x cells at passage 6 were treated with 1 μg/mL Dox and RNA

Figure 4.9 Gene expression in a MCF7 cell line stably transfected with the T-REx system for ERβ1 expression.

MCF7 were transfected with the T-REx system and stable transfectants generated by antibiotic selection. A pooled population of surviving cells at low passage number was tested for response to Dox. Cells were treated with 0, 0.5 or 5 μg/ml Dox for 24 hours before RNA extraction. Each RNA was used in two RT reactions, with and without prior DNase treatment and each RT was used in triplicate Q-PCR reactions to measure expression of various genes. Raw data are shown for five genes, and data normalised using GAPDH expression are shown for two of these.

(A) ERβ1 expression, (B) ERβ2 expression, (C) GAPDH expression, (D) TetR expression, (E) ERα expression, (F) Normalised ERβ1 expression, (G) Normalised GAPDH expression.

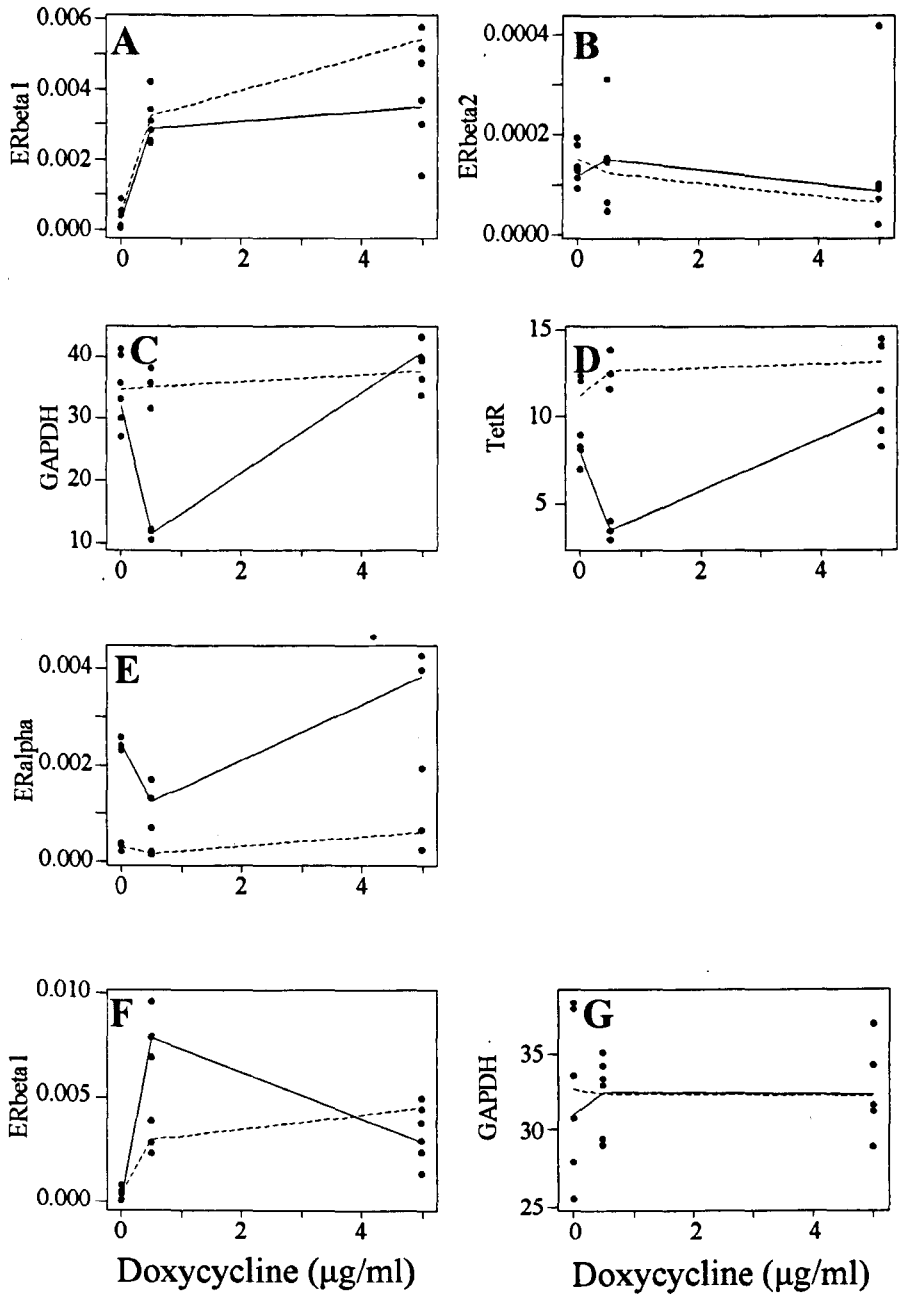
In the raw data, ERβ1 expression is significantly increased when cells are treated with 0.5 μg/ml Dox compared to untreated cells (Student's *t*-test $p<0.0005$, approx 10-fold increase), but no change is seen between 0.5 μg/ml Dox and 5 μg/ml Dox (Student's *t*-test $p=0.242$). Similar results were seen in the normalised data.

ERβ2 expression is not significantly affected by either DNase treatment or Dox addition.

Expression of GAPDH shows no overall trends with Dox addition or DNase treatment. However, expression is significantly reduced in cells treated with 0.5 μg/ml Dox and treated without DNase prior to RT compared to all other data sets. After normalisation, this outlier is lost.

TetR expression is lower in samples not treated with DNase (Student's *t*-test $p=0.001$). It is also significantly decreased in cells treated with 0.5 μg/ml Dox and treated without DNase prior to RT compared to other data sets.

Measured expression of ERα is significantly higher in samples not treated with DNase (*t*-test $p=0.001$). Regression analysis suggests a slight increase in ERα expression as Dox increases, but a Student's *t*-test comparing cells treated with 0 μg/ml Dox and 5 μg/ml Dox shows no significant difference ($p=0.522$).


A
ERbeta1
0.006
0.004
0.002
0.000
B
ERbeta2
0.0004
0.0002
0.0000
C
GAPDH
40
30
20
10
D
TetR
15
10
5
E
ERalpha
0.004
0.002
0.000
F
ERbeta1
0.010
0.005
0.000
G
GAPDH
35
30
25
0
2
4
Doxycycline (µg/ml)
Doxycycline (µg/ml)
+ DNase treatment
- DNase treatment

extracted after 0, 12, 24, 36, 48, 72 and 96 hours. DNase treatment, RT and Q-PCR analysis were performed to investigate expression of mRNAs corresponding to a set of genes including ERβ1, ERβ2, Tet repressor, liv1, PPP1CA and ARF1 (Fig. 4.10). Both raw data (Figs 4.10 A-F) and data normalised using ARF1 and PPP1CA (e.g., Figs 4.10 G-H) were analysed. Several genes show changes in expression over time. However, is it impossible to tell whether this is due to the addition of Dox or to other factors involved in cell growth, such as the increase in confluence in the dish, or the cells using up some component of the growth medium. Focusing on expression of ERβ1, the raw data shows no increase in expression after Dox addition. In fact, expression is significantly reduced by 96 hours, compared to that at 0 hours (*t*-test, p=0.021). After normalisation, some increase in expression is seen between 0 and 36 hours (*t*-test p=0.043), but the overall trend is still towards a decrease in expression with time (regression analysis p=0.027).

4.4.4.3 Further verification of induction over 24 hours

In order to investigate the cells' response to Dox in a non-estrogen stimulated environment, and to confirm whether cells retained their Dox response, fifteen dishes of MCF7β1x cells were grown in estradiol-free medium (Section 2.2.1.2) for a week before being treated with different levels of Dox for 24 hours. Cells were treated with 0, 0.5, 1, 2 or 5 μg/mL Dox in triplicate. RNA was extracted from each dish of cells, treated with DNase and RT performed in duplicate. Q-PCR analysis was performed in duplicate on each RT, giving a total of four Q-PCR replicates for each of three biological replicates (Fig. 4.11). In the raw data, no relationship is seen between expression of ERβ1 mRNA and Dox treatment. All other gene-products assayed show a slight decrease in expression as Dox is increased. After normalisation using ARF1 and PPP1CA (Section 3.2.5), an increase in expression of ERβ1 was observed when comparing cells with no Dox to all cells treated with Dox (1.7-fold, *t*-test p<0.0005). However, regression analysis of the relationship between ERβ1 and Dox (R^2= 14%, p< 0.0005) suggests that Dox levels only explain a small fraction of the variability in ERβ1 mRNA levels. The R^2 value is much lower than that seen in the transient transfectants (Section 4.4.2). Other gene-products (ARF1, PPP1CA and TetR) show no relationship with Dox treatment. These results confirm the results of the 96 hour experiment, that the cells had lost their original 10-fold response to Dox (Section 4.4.4.1). However, these cells do still express significantly higher levels of

Figure 4.10 Gene expression over 96 hours post-induction in a pooled, stably transfected MCF7 cell line.

Cells from a pooled population of MCF7 cells stably transfected with the T-REx system were treated with 1 μg/ml Dox in standard medium and RNA extracted for Q-RT-PCR analysis at 0, 12, 24, 36, 48, 72 and 96 hours post-treatment. All samples were treated with DNase prior to RT, which was performed in duplicate on each sample, and Q-PCR in duplicate from each RT, giving four data points per RNA sample. Raw data are shown for a set of five genes, and data normalised using PPP1CA and ARF1 are shown for two of these genes.

(A) Expression of ERβ1, (B) TetR expression, (C) ERβ2 expression, (D) Liv1 expression, (E) PPP1CA expression, (F) ARF1 expression, (G) Normalised ERβ1 expression, (H) Normalised TetR expression.

After 96 hours, expression of ERβ1 is significantly lower than initial levels (Student's *t*-test p=0.021). After normalisation, expression shows a significant increase between 0 and 36 hours (*t*-test p=0.043), but the overall trend still shows a decrease in expression over time (regression analysis p=0.027).

TetR and liv1 both show increased expression with time over the 96 hours following Dox addition (regression analysis for both genes p<0.0005). Normalisation of TetR data does not affect the general trend (regression analysis, p=0.010).

ERβ2 expression shows no significant change with time (regression analysis p=0.087).

The housekeeping genes (PPP1CA and ARF1) both show a decrease in expression for the first 36 hours of the experiment, before increasing again between 36 and 96 hours, giving an overall significant increase in expression between 0 and 96 hours.

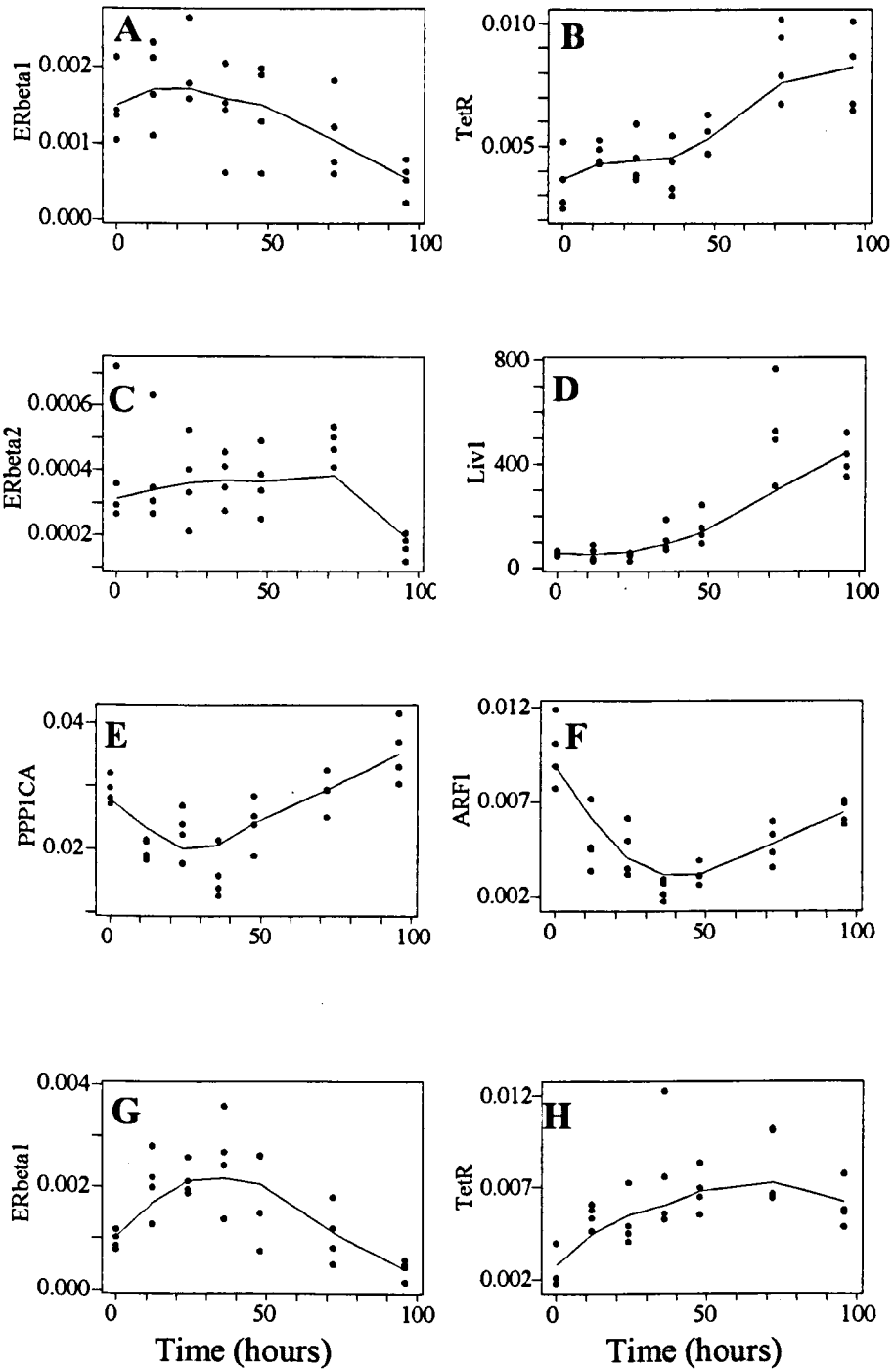

A
ERbeta1
0.002
0.001
0.000
0
50
100
B
TetR
0.010
0.005
C
ERbeta2
0.0006
0.0004
0.0002
D
Liv1
800
400
0
E
PPP1CA
0.04
0.02
F
ARF1
0.012
0.007
0.002
G
ERbeta1
0.004
H
TetR
Time (hours)
Time (hours)

Figure 4.11 Gene expression in MCF7β1x cells treated with increasing amounts of Dox for 24 hours

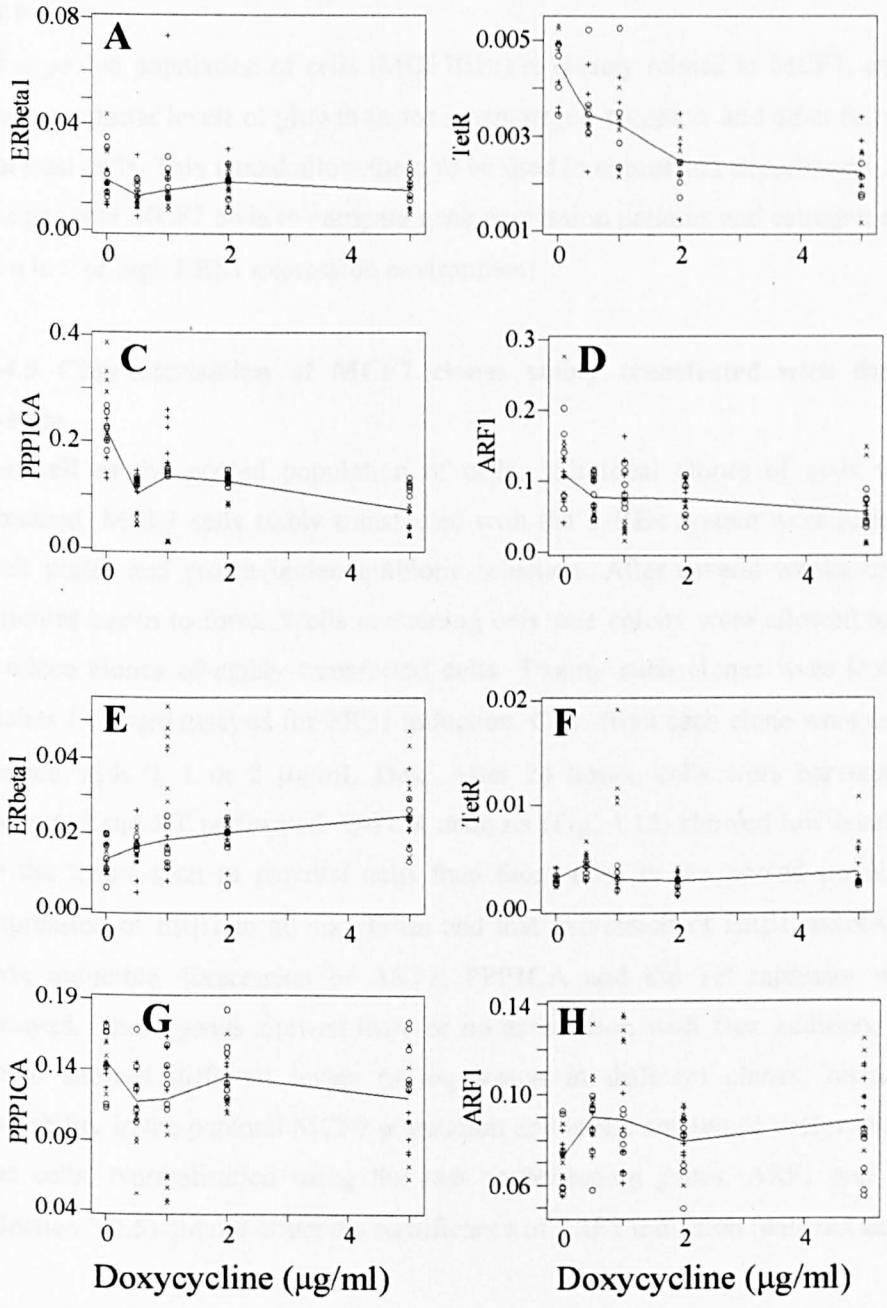
Multiple dishes of MCF7 cells transfected with the T-REx system (Section 2.2.5, Fig. 4.6) (MCF7β1x) were treated with increasing levels of Dox (0 to 5 μg/ml) for 24 hours. Before Dox treatment, these cells had been withdrawn from estrogen, and the experiment was performed in phenol red-free medium (Section 2.2.1.2). Fifteen dishes of cells were used in total. These were treated, in triplicate, with 0, 0.5, 1, 2 and 5 μg/ml Dox. For each RNA sample, RTs were performed in duplicate (with DNase treatment) and Q-PCR was performed in triplicate on each RT sample, giving six PCR data points per RNA sample. Expression of four genes is shown before and after normalisation using ARF1 and PPP1CA expression. The three replicate RNA samples for each treatment were arbitrarily assigned the symbols x, + and o.

(A) ERβ1 expression, (B) TetR expression, (C) PPP1CA expression, (D) ARF1 expression, (E) Normalised ERβ1 expression, (F) Normalised TetR expression, (G) Normalised PPP1CA expression, (H) Normalised ARF1 expression.

ERβ1 is not induced by Dox addition in the raw data (regression analysis p=0.245). After normalisation, a 1.7-fold increase is seen, comparing data for all cells with added Dox to all cells without Dox (Student's *t*-test, p<0.0005).

TetR expression decreases with Dox addition in the raw data (regression analysis p<0.0005). This response is lost in the normalised data, where some samples have become outlying (notably group "x" at 1 μg/ml Dox).

PPP1CA and ARF1 show a slight decrease in expression as Dox increases (regression analysis p<0.0005). This relationship is lost on normalisation.


A
ERbeta1
0.08
0.04
0.00
0
2
4
B
TetR
0.005
0.003
0.001
C
PPP1CA
0.4
0.2
0.0
D
ARF1
0.3
0.2
0.1
0.0
E
ERbeta1
0.04
0.02
0.00
F
TetR
0.02
0.01
0.00
G
PPP1CA
0.19
0.14
0.09
0.04
H
ARF1
0.14
0.10
0.06
Doxycycline (µg/ml)
Doxycycline (µg/ml)

ERβ1 than the parental cells, making then valuable as an experimental tool in their own right.

This pooled population of cells (MCF7β1x) is closely related to MCF7, and should express similar levels of growth factor receptors, co-receptors and other factors as the parental cells. This would allow them to be used in expression experiments alongside the parental MCF7 cells to compare gene expression patterns and estrogen responses in a low or high ERβ1 expression environment.

4.4.5 Characterisation of MCF7 clones stably transfected with the T-REx system

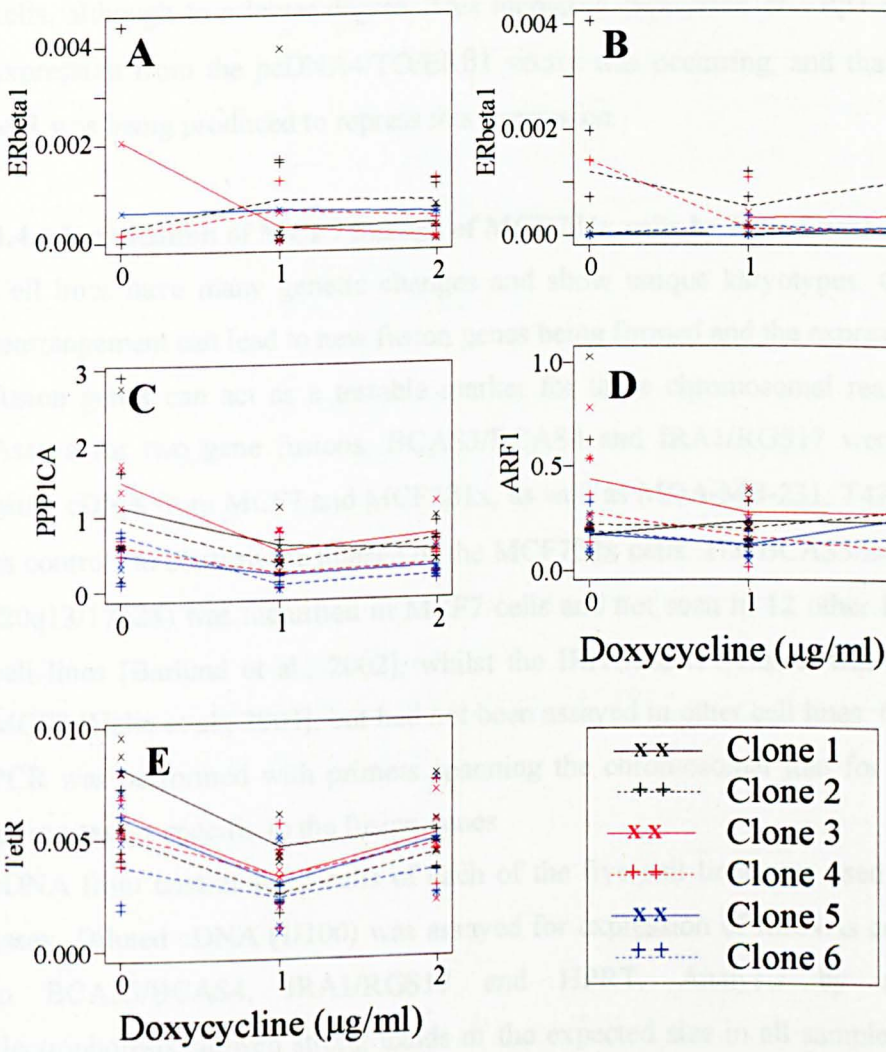
As well as the pooled population of cells, individual clones of cells were also obtained. MCF7 cells stably transfected with the T-REx system were split into 24-well plates and grown under antibiotic selection. After several weeks, single cell colonies began to form. Wells containing only one colony were allowed to grow to produce clones of stably transfected cells. Twenty such clones were isolated and clones 1-6 were assayed for ERβ1 induction. Cells from each clone were grown and treated with 0, 1 or 2 μg/mL Dox. After 24 hours, cells were harvested, RNA extracted and RT performed. Q-PCR analysis (Fig. 4.12) showed low levels (closer to the levels seen in parental cells than those seen in the pooled population) of expression of ERβ1 in all six clones and that expression of ERβ1 mRNA was not Dox inducible. Expression of ARF1, PPP1CA and the Tet repressor were also assayed. These genes showed little or no association with Dox addition, although some showed different levels of expression in different clones, reflecting the variability in the parental MCF7 population and in the amount of vector taken up by the cells. Normalisation using the two housekeeping genes, ARF1 and PPP1CA (Section 3.2.5) did not affect the significance of ERβ1 induction (data not shown).

The lack of inducibility in both these clones and the mixed population of cells was disappointing. Both vectors used in the transfection reactions showed no gross sequence errors before transfection, and indeed, the system showed some inducibility in transiently transfected MCF7. The cells survived selection with antibiotics and must, therefore, have retained both vectors (or portions of the vectors) in their genomes. Expression of ERβ1 mRNA was greatly increased in the mixed population

Figure 4.12 Gene expression in six clones transformed with the T-REx system.
Several clones of cells stably transfected with the T-REx system for inducible ERβ1 were obtained. To investigate inducibility in the clones 1 to 6, cells were treated with 0, 1 or 2 μg/ml Dox for 24 hours before RNA extraction. RT was performed and expression of a set of genes was measured by Q-PCR. Note that two separate Q-PCR assays for ERβ1 were performed.

(A) ERβ1 expression (Assay 1), (B) ERβ1 expression (Assay 2), (C) PPP1CA expression, (D) ARF1 expression, (E) TetR expression.

No relationship was seen between ERβ1 expression and Dox treatment in either assay. The results of the two assays show poor correlation (Pearson correlation = 0.132, p = 0.528). Expression of ARF1, PPP1CA and TetR vary between clones (1-way ANOVA p=0.097, 0.003, 0.043 respectively). PPP1CA and TetR also show a small but significant decrease in expression with Dox addition (regression analysis R^2= 14%, 7%, p=0.001, 0.022).


A
B
C
D
E
ERbeta1
ERbeta1
PPP1CA
ARF1
TetR
0.004
0.002
0.000
0.004
0.002
0.000
3
2
1
0
1.0
0.5
0.0
0.010
0.005
0.000
0
1
2
Doxycycline (µg/ml)
Doxycycline (µg/ml)
Clone 1
Clone 2
Clone 3
Clone 4
Clone 5
Clone 6

of cells compared to the parental cells. Expression was also increased in the clones of cells, although to a lesser degree. This increased expression of ERβ1 suggests that expression from the pcDNA4/TO/ERβ1 vector was occurring, and that insufficient tetR was being produced to repress this expression.

4.4.6 Verification of MCF7 lineage of MCF7β1x cells by fusion gene analysis

Cell lines have many genetic changes and show unique karyotypes. Chromosome rearrangement can lead to new fusion genes being formed and the expression of these fusion genes can act as a testable marker for these chromosomal rearrangements. Assays for two gene fusions, BCAS3/BCAS4 and IRA1/RGS17 were performed using cDNA from MCF7 and MCF7β1x, as well as MDA-MB-231, T47D and ZR75 as controls to confirm the lineage of the MCF7β1x cells. The BCAS3/BCAS4 fusion (20q13/17q23) was identified in MCF7 cells and not seen in 12 other breast cancer cell lines [Barlund et al., 2002], whilst the IRA1/RGS17 fusion was identified in MCF7 [Hahn et al., 2004], but had not been assayed in other cell lines. Conventional PCR was performed with primers spanning the chromosomal join for each fusion, giving assays specific to the fusion genes.

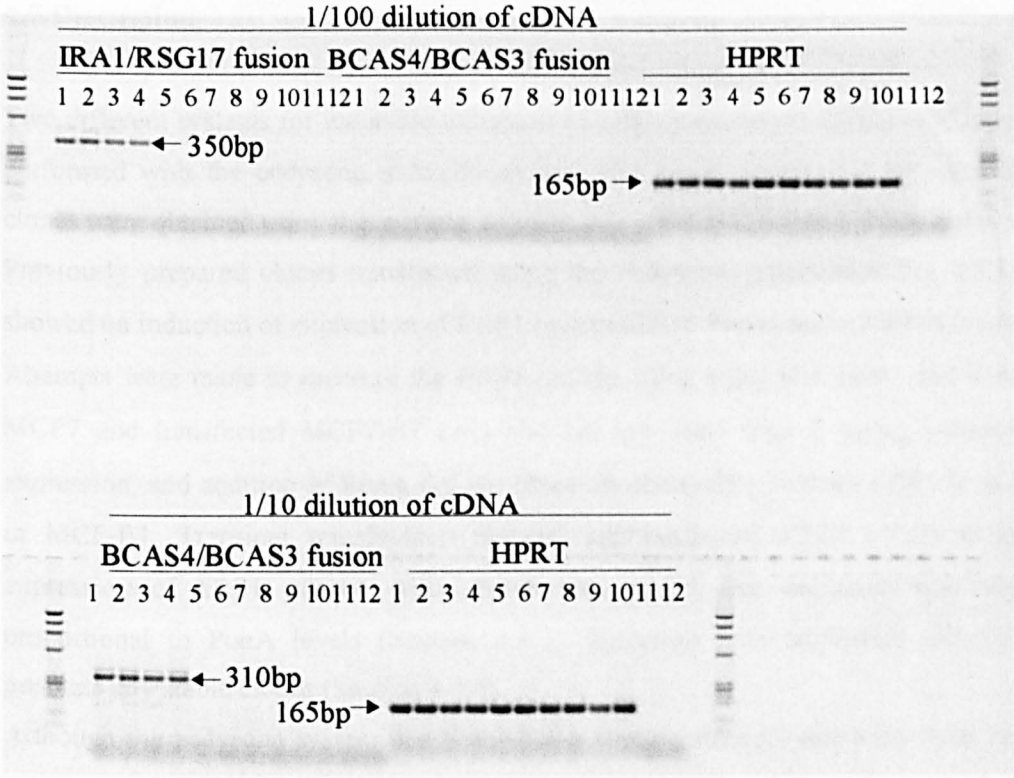
cDNA from unstimulated cells of each of the five cell lines was used in this PCR assay. Diluted cDNA (1/100) was assayed for expression of mRNAs corresponding to BCAS3/BCAS4, IRA1/RGS17 and HPRT. Analysis by agarose gel electrophoresis showed strong bands of the expected size in all samples for HPRT, whilst only MCF7 and MCF7β1x cells showed bands for the fusion genes (Fig. 4.13). Both cell lines showed strong bands for IRA1/RGS17 and very weak bands for BCAS3/BCAS4. PCR for BCAS3/BCAS4 and HPRT was repeated using 1/10 diluted cDNA in an attempt to generate stronger bands on electrophoresis. Expression of BCAS3/BCAS4 was seen only in MCF7 and MCF7β1x cells, whereas expression of HPRT was high in all samples. These data confirm that these fusion genes are expressed in a cell line specific manner, related to the specific chromosomal rearrangements found in different cell lines. The presence of the fusion gene products in MCF7β1x cDNA, therefore, confirms that these cells are of the MCF7 lineage.

Figure 4.13 Expression of fusion genes in MCF7, MCF7β1x and three other cell lines

PCR was performed on cDNA taken from MCF7β1x, MCF7, MDA-MB-231, T47D and ZR75 cells in order to confirm the lineage of the MCF7β1x cells. Expression of two fusion genes, BCAS4/BCAS3 and IRA1/RGS17 as well as a housekeeping gene, HPRT, was analysed in the five cell lines. The fusion genes are reported to be expressed in a cell line specific manner as they are formed only after specific chromosomal rearrangements. Expression of the fusion genes can therefore be used as a marker of MCF7 lineage (Section 4.4.6).

PCR was performed using 1/10 and 1/100 dilutions of the cDNA, and 5μl aliquots were run on a gel, alongside a ϕx174 DNA marker (Section 2.5.1.2). The gel contained ethidium bromide and was visualised using the Typhoon scanner. Band sizes were calculated using ImageQuant software.

All cell lines show strong expression of HPRT. Only MCF7 and MCF7β1x show expression of the two fusion genes. IRA1/RGS17 expression is significantly higher than BCAS4/BCAS3 expression, as bands for BCAS4/BCAS3 are only visible when the higher concentration of cDNA was used in the PCR.



1,2	MCF7 cDNA
3,4	MCF7β1x cDNA
5,6	MDAMB231 cDNA
7,8	T47D cDNA
9,10	ZR75 cDNA
11,12	H_2O control

4.5 Discussion

Two different systems for inducible induction of ERβ1 were tested. Initial work was performed with the ecdysone inducible system [No et al., 1996], but no suitable clones were obtained using this system, so work was refocused on the T-REx system. Previously prepared clones transfected using the ecdysone system (Section 4.3.1) showed no induction of expression of ERβ1 in response to PonA, at the mRNA level. Attempts were made to measure the ERβ1 protein using a dot blot assay, but both MCF7 and transfected MCF7-B1 cells showed low (less than 2 pg/μg protein) expression, and addition of PonA did not cause an observable increase ERβ1 levels in MCF-B1. Transient transfectants showed approximately 2-fold induction of expression of ERβ1 mRNA when PonA was added, but induction was not proportional to PonA levels (Section 4.3.3). Selection with antibiotics did not generate any stable clones (Section 4.3.4).

Although the ecdysone system has been shown to give strong inducibility with no leakiness in some systems, there are reports of it being less than perfect in other situations. Although No *et al.* report stronger induction using the ecdysone system than using the tetracycline repressor (tTA) or activator (rtTA) systems [No et al., 1996], others have found the opposite to be the case when comparing the ecdysone system and Tet-On systems [Van Craenenbroeck et al., 2001]. A group using this system to express IGFBP-3 in MCF7 cells produced 16 clones, of which two showed constitutive over-expression of IGFBP-3, three showed inducible expression and the remaining 11 showed no increased expression, suggesting that the system may be difficult to establish in MCF7 [Kim et al., 2004]. Other groups have found it impossible to generate stable clones in certain cell lines including L929sA (a mouse fibrosarcoma cell line) [Van Craenenbroeck et al., 2001], CHO cells [Zhang et al., 2000] and HeLa cells (personal communication from Invitrogen reported in [Van Craenenbroeck et al., 2001]). A possible explanation for this problem is given in [Yeh and Shatkin, 1994], which describes identification of a p21 protein from HeLa cells which is able to down-regulate expression from a RSV LTR promoter. This is the constitutive promoter used to direct expression of RXR from the pVgRXR vector. The presence of this p21 protein in cells might prevent sufficient RXR being expressed to allow the cells to respond to PonA addition.

The T-REx system (Section 4.1.2) was chosen as a replacement method for producing ERβ1 inducible cells (Section 4.4). Promising results were obtained at the transient transfection stage (Section 4.4.2). Transient transfectants showed constitutive expression of ERβ1, which was 85000-fold higher than in mock transfectants and a 2-fold increase in ERβ1 was seen on addition of Dox. Stable transfectants showed variable induction of ERβ1. Initially, a 10-fold induction of expression was observed at low passage number in the mixed population (MCF7β1x) (Section 4.4.4.1). However, in subsequent experiments the induction was reduced to less than 2-fold (Section 4.4.4.3). Six clones of transfected cells were obtained and characterised (Section 4.4.5), which did not show inducible ERβ1 expression.

A previous study suggested that levels of induction with the Tet-On system were significantly different in different cell lines (2 to 16-fold induction in HEK293 clones, compared to 52 to 225-fold induction in L929sA clones) [Van Craenenbroeck et al., 2001]. The lack of inducibility in these clones may, therefore, be either due to poor functioning of this system in MCF7 cells, or to integration of insufficient amounts of pcDNA6/TR vector. Alternatively, since the level of transfected cDNA in a cell clone is effectively random, more than six clones may need to be isolated and analysed to isolate one in which ERβ1 expression was inducible with Dox. The mixed population of cells (MCF7β1x) and, to a lesser extent, the stable clones, express higher levels of ERβ1 mRNA than MCF7 cells, suggesting that the expression vectors are integrated, but that insufficient TetR is expressed, possibly due to integration of insufficient copies of the pcDNA6/TR vector (Section 4.4.5).

Fusion gene analysis was used to confirm the lineage of the MCF7β1x cell population. Fusion gene expression is caused by specific chromosomal rearrangements. Two fusion genes were shown to be expressed in both MCF7 and MCF7β1x cells, but not in MDA-MB-231, T47D or ZR75, confirming that these cell lines share a common lineage, not shared with the other cell lines used in the project.

To investigate the levels of ERα and ERβ1 protein in the MCF7 and MCF7β1x cells, a series of experiments were performed to determine whether the increased levels of ERβ1 mRNA corresponded to an increase in ERβ1 protein (Chapter 5).

Despite the lack of inducibility in the cloned cells, the mixed population of transfected MCF7 cells was shown to have constitutively higher expression of ERβ1 mRNA than parental MCF7 cells, allowing it to be used as a new cell line in parallel

experiments with MCF7 cells to investigate gene expression patterns in high and low ERβ1 environments (Chapter 5). An inducible system would allow a single cell line to be used in expression experiments using different stimuli in combination with different levels of inducing agent, to compare the gene expression response at different levels of expression of ERβ1. Nonetheless, comparing gene expression responses in MCF7 and MCF7β1x cells, a closely related pair of cell lines with differing levels of expression of ERβ1, may yield valuable information. In previous experiments, changes in expression of target genes in response to treatment were often hard to identify (Section 3.3.3). It may be that the relatively large difference in expression of ERβ1 between these two cell lines may facilitate observation of any ERβ1-modulated response.

CHAPTER 5 Estrogen responses in MCF7 and in MCF7β1x cells

5.1 Introduction

MCF7 cells were stably transfected using the T-REx system (Invitrogen) with the intention of producing a cell line, which would express ERβ1 in an inducible manner (Chapter 4). A population of cells (MCF7β1x) was isolated, which showed an induction of ERβ1 mRNA upon addition of Dox (about 2-fold) (Section 4.4.4) and possessed a much higher basal level of expression of ERβ1 than the parental MCF7 cells. As the increase in expression on induction was much smaller than the difference between basal expression in MCF7β1x and MCF7 cells, experiments were performed to compare gene expression in these two cell lines to investigate the effects of over-expression of ERβ1 on downstream gene expression (Sections 5.2-5.4).

The two types of cells were treated in parallel with a variety of estrogenic treatments (Section 3.1.1) and their estrogen responses compared using Q-PCR (Sections 3.1.2 and 3.1.3). Transcriptional responses were measured at 24 hours (Section 5.2.1), as well as over an 8 hour time course to investigate temporal effects on gene expression (Section 5.2.2). The cells were also treated with estradiol in combination with either EGF or FGF-2, to investigate whether growth factor treatment had any effect on downstream gene expression in the cell lines, and whether this effect was different in the two cell lines (Section 5.3).

Experiments were carried out to investigate the levels of protein expression in the two cell lines. ERα, ERβ1 and β-actin (a housekeeping gene) were assayed by Western blotting (Section 5.4), and an ELISA for ERβ1 was developed and used on lysates from the two cell lines (Section 5.5). Immunocytochemistry was also used to visualise the pattern of immunoreactive ERα and ERβ1 in the two cell lines to explore whether all cells were expressing similar levels of the ERs and to identify the intracellular location of the proteins (Section 5.6).

5.2 Analysis of estrogen response in low and high ERβ1 environments

5.2.1 Estrogen response after 24 hours

MCF7β1x is a transfected variant of MCF7, which over-expresses ERβ1 mRNA (Section 4.4.4). In order to investigate whether over-expression of ERβ1 had any effect on downstream gene expression, MCF7 and MCF7β1x cells were subjected, in parallel, to various estrogenic treatments. Duplicate experiments were performed using both cell lines, in which cells were withdrawn from estradiol stimulation and treated with the same set of six treatments, as used previously (Table 3.1 B). Expression of the ERs, estrogen responsive genes and housekeeping genes (Table 3.1 C) were measured in each cell line after each treatment, and estrogen responses were compared between treatments in each cell line (Fig. 5.1). Expression could also be compared between cell lines, when Q-PCR was performed at the same time. Estrogen responses in the two cell lines are summarised in table 5.1 A, whilst full results are shown in Appendix A (experiments A4 and A5).

A further experiment was performed in which the two types of cells were given no 10 nM estradiol alone or 1 μM ICI 182,780 alone, in order to confirm the responses seen in the previous experiment. Three replicate samples of each cell line were used for each treatment and the results pooled. Genes, which showed changes in expression in response to estrogen in this experiment, are shown in table 5.1 B. No genes showed a consistent change in expression in response to ICI 182,780 compared to untreated cells in this experiment. In Table 5.1, genes that are consistently regulated by estradiol in a cell line in both experiments are shown in bold.

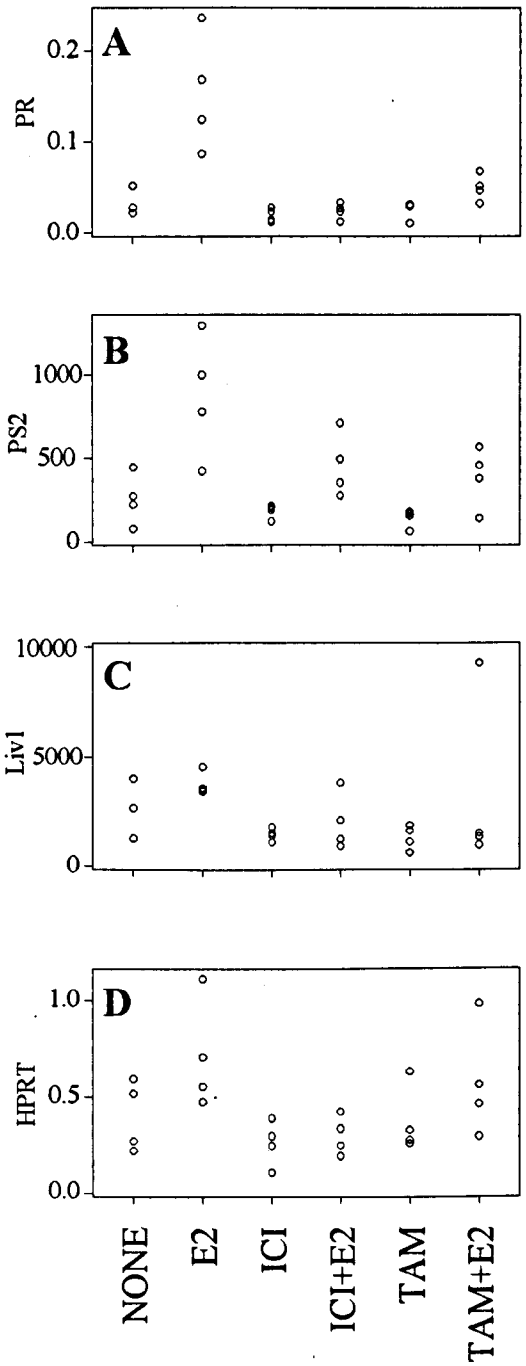
5.2.1.1 Gene expression responses

Many genes are up-regulated by estradiol in the two cell lines. As seen previously (Section 3.3.3), liv1 expression was increased with estradiol treatment in MCF7 cells in all experiments. PS2 and PR also show significant induction in all experiments, with several other genes being up-regulated in a subset of experiments. MCF7β1x cells show responses in the same set of genes as MCF7 cells, both in terms of the genes consistently up-regulated and those which only sometimes reached significance. Some genes were also down-regulated by estrogen treatment. Only MCF7β1x cells show consistent down regulation of any genes; ERβ1 and EFP.

Figure 5.1 Examples of genes showing an estrogen response in MCF7β1x cells.
Several replicate experiments were performed treating MCF7 and MCF7β1x cells with and without estradiol either alone or in combination with ICI 182,780 or tamoxifen. RNA was extracted and RT performed before Q-PCR analysis of a set of 18 mRNAs. Data were normalised using a housekeeping ratio calculated using changes in expression of the four housekeeping genes HPRT, GAPDH, ARF1 and PPP1CA. Shown here are the normalised PCR results for a set of four mRNAs which illustrate different levels of significance in their estrogen responses in MCF7β1x cells.

(A) PR, (B) PS2, (C) Liv1, (D) HPRT.

PR expression is significantly higher in both cells treated with estradiol only (Student's *t*-test, approx 5-fold higher, p=0.031) and in all cells treated with estradiol compared to those with no estradiol (approx 3-fold higher, p=0.029)
PS2 expression is not significantly higher in cells treated with estradiol only, compared to other treatments (p=0.052). However, comparing all cells treated with estradiol to those with no estradiol a significant change is seen (approx 3-fold higher, p=0.002).
Liv1 expression is significantly higher in cells treated with estradiol only, compared to other treatments (approx 3.6-fold higher, p=0.003). No increase in expression is seen when comparing all cells whose treatments included estradiol to those with no estradiol (p=0.087).
Although by visual inspection HPRT expression appears increased in cells treated with estradiol, this increase is not significant (p=0.065).


A
PR
0.2
0.1
0.0
B
PS2
1000
500
0
C
Liv1
10000
5000
0
D
HPRT
1.0
0.5
0.0
NONE
E2
ICI
ICI+E2
TAM
TAM+E2

	MCF7β1x	MCF7
Increased with E2	**Liv1, PS2**, XBP1U, **PR**	**Liv1, PS2**, XBP1U, XBP1S, **PR**, HPRT
	Liv1, PS2, XBP1U, **PR**	
	Liv1, PS2, PR, HPRT, ERβ2, CathD	**Liv1, PS2, PR**, CathD
Decreased with E2	**ERβ1, EFP**, VEGF	VEGF
	ERβ1, EFP, VEGF	
	ERβ1, EFP	None
Other changes	None	None
	ERβ5 increased with Tam	
	ERα decreased with Tam or ICI	ERα increased with ICI

	MCF7β1x	MCF7
Increased with E2	**Liv1, PS2**, XBP1U, XBP1S, **PR**, CathD, ERβ2	**Liv1, PS2**, XBP1U, XBP1S, **PR**, HPRT, CathD
Decreased with E2	VEGF	ERβ5, VEGF, EFP

Table 5.1 Summary of estrogen responses in MCF7 and MCF7β1x cells treated with estrogen, ICI 182,780 and tamoxifen.

Replicate experiments were performed treating estradiol-withdrawn MCF7 and MCF7β1x cells with and without estradiol either alone or in combination with ICI 182,780 or tamoxifen, giving a total of six different treatments. A further experiment was performed using the same cell lines, but only three treatments (no treatment, estradiol and ICI 182,780). These treatments were performed in triplicate for each cell line. In each experiment, RNA was extracted and RT performed before Q-PCR analysis of a set of 18 mRNAs. Data were normalised using a housekeeping ratio calculated using changes in expression of the four housekeeping genes HPRT, GAPDH, ARF1 and PPP1CA.

Panels *(A)* and *(B)* show summaries of the estrogen response in these two experiments. In panel *(A)*, a gene is included if its expression shows a significant increase or decrease either in estradiol treated cells alone, all estradiol treatments compared to all estradiol-free treatments or both. Genes which show an estrogen response in all replicate experiments are shown in bold. In panel *(B)*, mRNAs are listed which are increased in estradiol treated cells when compared to all other treatments. In most cases, expression in non-treated and ICI 182,780 treated cells are not significantly different, although ERα expression is slightly lower in ICI 182,780 treated MCF7 than non-treated MCF7 (*t*-test $p<0.0005$ and $p=0.002$ in 2 replicate PCRs), but higher in ICI 182,780 treated MCF7β1x than untreated cells (*t*-test only significant in 1/2 replicate PCRs, $p=0.036$). Genes shown in bold are also increased in all replicates of the experiment shown in panel A.

Overall, VEGF is also down-regulated in both cell lines in a large subset (5/7) of experiments.

The down-regulation of EFP is surprising, as EFP is regarded as an estrogen responsive gene. However, EFP is reported to increase in response to estradiol and return to basal levels within 8 hours of stimulation in MCF7 cells [Ikeda et al., 2000]. As these experiments were conducted over 24 hours it is possible that an initial stimulation of EFP expression could be followed by a breakdown of excess EFP mRNA in the cells, leading to lower than base line levels after 24 hours.

The down-regulation of VEGF mRNA levels by estradiol is also in contrast to reports in the literature (Section 3.1.3.2). However, although both ERα and ERβ have been shown to mediate estrogen-stimulated expression of a reporter construct containing the VEGF promoter, this may not reflect the effect on the endogenous gene. One study [Buteau-Lozano et al., 2002] did measure the response of endogenous VEGF to estradiol stimulation in MCF7 cells, and noted only a maximum 1.6-fold increase in mRNA expression at 6 hours, with levels then falling towards base line by 24 hours. However, they make no mention of serum withdrawal prior to treatment, which may significantly affect results.

The reduced expression of ERβ1 after estradiol stimulation seen in MCF7β1x is in contrast to the increase in expression seen in T47D cells (Section 3.3.4.2). In MCF7β1x cells, most of the ERβ1 mRNA is likely to be transcribed from the vector cDNA rather than the endogenous ERβ1 gene, and is therefore independent of any control sequences found in the promoter region of the ERβ gene or of promoter-specific splicing mechanisms. This apparent down-regulation is, therefore, unlikely to be caused by inhibition of transcription of the ERβ gene, and other possible mechanisms must be hypothesised such as increased rate of mRNA breakdown (Section 5.7.1).

The response to treatment after 24 hours was shown to be similar in MCF7 and MCF7β1x cells, both in terms of the genes affected and the magnitude of response. Another aspect of response, which could be affected by different levels of ERβ1, is the time-dependence of the response and an experiment to investigate this in the two cell lines was, therefore, performed.

5.2.2 Response to estradiol over an 8 hour time course

The estrogen response of genes may occur over less than 24 hours. One example of this is EFP, which has been shown to increase expression and return to baseline levels in the first 8 hours after estrogen stimulation [Ikeda et al., 2000]. This would suggest that induction of the mRNA encoded by the EFP gene might not be detected after 24 hours treatment of cells with estrogen. Cyclin D1 is another gene that may show an early, transient response to estrogen. A luciferase reporter assay coupled to the cyclin D1 promoter showed estradiol induced expression in MCF7 peaking at 7 h, and returning to near baseline levels by 24 h [Sabbah et al., 1999]. However, an experiment using ZR75 cells showed cyclin D1 mRNA levels to peak at 0.5 h, but then to be maintained a somewhat elevated level for 24 h [Castro-Rivera et al., 2001], suggesting that either cell type or the different assays used may affect the period of the response. In any event, one would expect cell cycle-dependent genes such as cyclin D to have responses that are tied to their biological function. Many experiments investigating estrogen responsive genes have only used fixed time periods such as 8, 24 or 48 h, so an investigation of a shorter time course may yield valuable information about the early induction of the set of estradiol controlled genes.

In order to investigate whether ERβ1 over-expression affected the rate of gene expression changes, an experiment was performed over an 8 hour time course. MCF7 and MCF7β1x cells were treated with 10 nM estradiol or 1 μM ICI 182,780 and RNA extracted 0, 1, 2, 4 and 8 hours after treatment to investigate early estrogen responses. Q-PCR analysis was carried out and the results normalised as usual. Two samples (MCF7 treated with estradiol and with ICI 182,780 at 2 hours) were lost during processing, leaving a total of 28 samples. Because of the large number of samples, only one RT reaction was performed per RNA that was used in duplicate Q-PCR assays for each gene. A subset of normalised expression data are shown in figure 5.2.

5.2.2.1 Gene expression responses

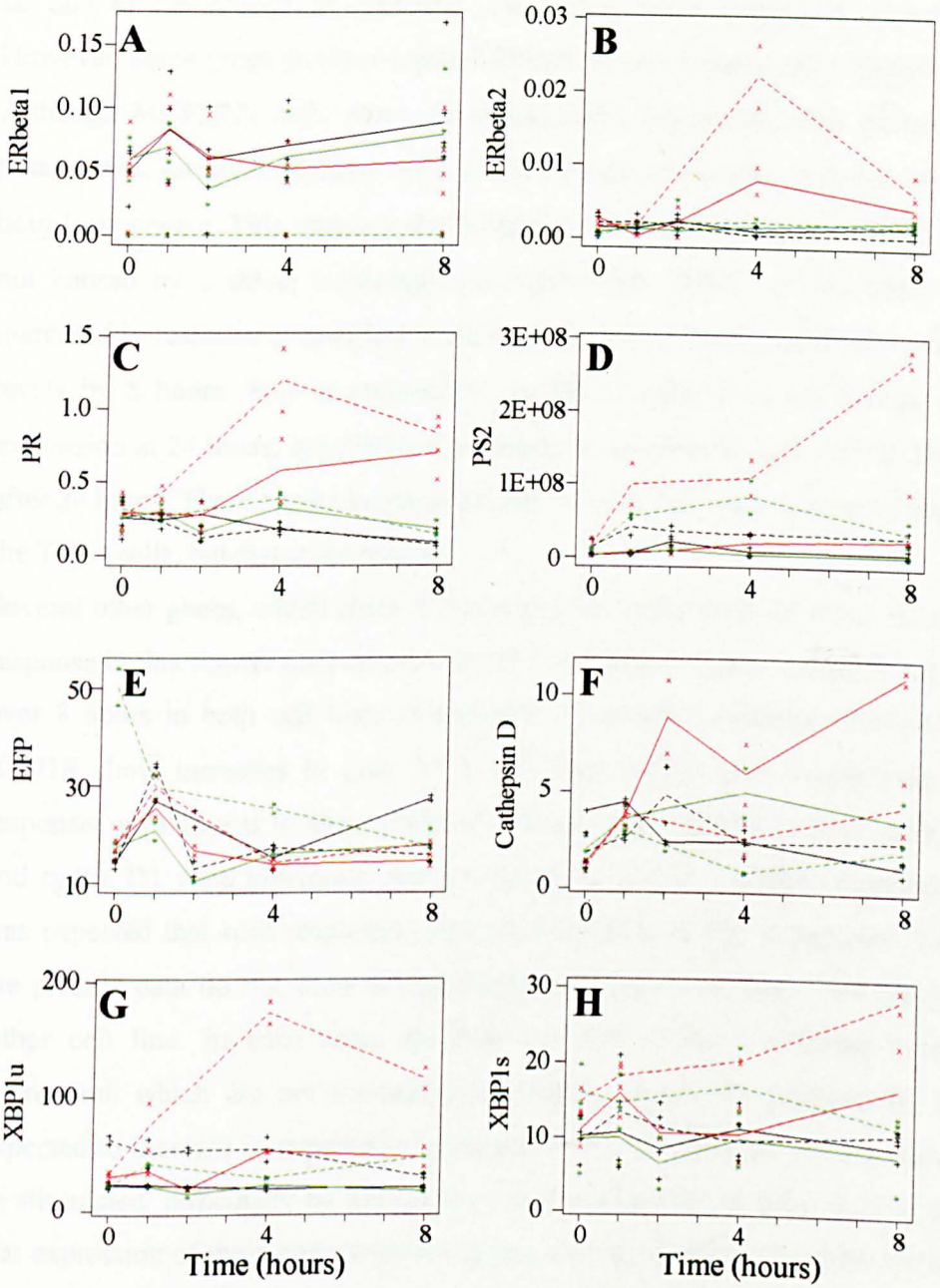
Most genes showed no significant change in expression over the 8 h time period, or showed a slight peak in expression at 1 hour before returning to a constant expression level, e.g., EFP (Fig. 5.2 E). Cells were treated with fresh medium when treatments were added at the start of the time course, so it is possible that the initial peak, as it is seen in many different genes regardless of treatment and cell type, may

Figure 5.2 Examples of estrogen responses in MCF7 and MCF7β1x cells over an 8 hour time course

MCF7 and MCF7β1x cells were withdrawn from stimulation before treatment with no treatment, 10 nM estradiol or 1 μM ICI 182,780. RNA was extracted 0, 1, 2, 4 and 8 hours after treatment, and Q-PCR assays of expression of a set of ERs, housekeeping genes and estradiol sensitive genes performed. Data were normalised using the set of housekeeping genes. Two samples were lost during processing- MCF7 cells treated with estradiol or ICI 182,780 for 2 h.

Normalised expression of several mRNAs in both cell lines after both treatments are shown (amol/μg RNA).

(A) ERβ1, (B) ERβ2 (C) PR, (D) PS2, (E) EFP, (F) Cathepsin D, (G) XBP1U, (H) XBP1S.


A
B
C
D
E
F
G
H
ERbeta1
ERbeta2
PR
PS2
EFP
Cathepsin D
XBP1u
XBP1s
Time (hours)
Time (hours)
MCFβ1x no treat.
MCFβ1x + E2
MCFβ1x + ICI
MCF no treat.
MCF + E2
MCF + ICI

be due to the change of medium, rather than being specific to the treatment. However, some genes do show a more distinct, treatment-dependent response.

Although MCF7β1x cells show decreased ERβ1 expression after 24 hours when treated with estradiol (Section 5.2.1.1), no change in expression is seen over the 8 hour time course. This supports the hypothesis that the reduction in ERβ1 levels in not caused by a direct transcriptional mechanism. ERβ2, on the other hand, is increased in response to estradiol in both cell lines at 4 hours, returning to near basal levels by 8 hours. This is noteworthy, as MCF7 cells show no change in ERβ2 expression at 24 hours, but ERβ2 is increased in response to estradiol in T47D cells after 24 hours. These results suggest that MCF7 cells may show the same response as the T47D cells, but that it is transient.

Several other genes, which show a response to estradiol after 24 hours, also show a response in this shorter time course. PR, PS2 and XBP1U show a significant increase over 8 hours in both cell lines in response to estradiol, whereas cathepsin D and XBP1S show increases in only MCF7β1x and MCF7 cells, respectively. These responses also appear to show different patterns with respect to time. EFP, VEGF and cyclin D1 were previously reported to show an early estrogen response, and it was expected that such responses might be identified in this experiment. However, the present data do not show a significant response to estrogen in these genes in either cell line. In each case, the data are very "noisy", showing changes in expression, which are not treatment dependent. Cyclin D1, particularly, may be expected to increase in expression in response to fresh medium, as cell growth may be stimulated, principally by the increase in nutrients such as glucose. It is possible that expression of these early response genes may be regulated by other components of the growth medium, or by the cell confluence or growth rate to such an extent that any estrogen response is masked.

5.3 Analysis of growth factor response in MCF7 and MCF7β1x cells

Measured estrogen response may be affected by time and by levels of estrogen receptors, but the ERs do not act alone. Growth factors and growth factor receptors, for instance, are intimately involved in the estrogen response (Section 1.5.3 and, e.g.

[Kato, 2001; Lee et al., 2001; Levin, 2003]). An experiment was performed to investigate the effect of two growth factors, EGF and FGF-2 (basic FGF), on estrogen response in MCF and MCF7β1x cells. EGF can act via MAPK to phosphorylate both ERα [Kato et al., 1995] and ERβ [Tremblay et al., 1999] in the AF1 region, causing activation. Estradiol and EGF have previously been shown to have an additive effect on estradiol controlled genes [El-Tanani and Green, 1997a] and a synergistic effect in breast cancer [Gullick, 1990; Murphy et al., 1990].

The FGFs have a less defined role in relation to estrogen response and breast cancer. FGF-2 protein was detected in all breast tumours in one study [Relf et al., 1997], although it is suggested that it is expressed at lower levels in breast tumours than in normal or non-malignant breast [Anandappa et al., 1994]. FGF-2 is not expressed by MCF7 cells, but all four FGF receptors are expressed in these cells [Johnson et al., 1998]. FGF-2 has been reported to act as both a mitogen [Briozzo et al., 1991; Delehedde et al., 1996; Peyrat et al., 1991; Rahmoune et al., 1998; Vercoutter-Edouart et al., 2000] and a growth inhibitor [Fenig et al., 1997; Johnson et al., 1998; Wang et al., 1997b] of MCF7 cells. The confusion may arise due to the conflicting actions of FGF-2 on the cells. This growth factor stimulates MAPK action and cyclin D1 expression, but also stimulates p21 expression, therefore modulating both growth activating and inhibitory signals [Johnson et al., 1998; Wang et al., 1997b]. An experiment using an MCF7 cell line which was selected to grow in serum-free media suggested that FGF-2 up-regulates PS2 expression synergistically with estradiol, but inhibited both PR and ERα expression, suggesting that FGF-2 may modulate the estradiol response in a more complex manner than EGF [Garnier et al., 2003].

To investigate the effects of EGF and FGF-2 on estrogen responses in MCF7 and MCF7β1x cells, duplicate experiments were performed in which MCF7 and MCF7β1x cells were each withdrawn from estradiol stimulation before treatment with 10 nM estradiol, either alone or in combination with 3 ng/mL EGF or 1 ng/mL FGF-2. Each experiment, therefore, involved a total of six treatments for each cell line. Expression of the set of estrogen responsive genes, housekeeping genes and estrogen receptors was analysed and normalised using the average expression of the four housekeeping genes. Normalised data from each experiment was analysed using 2-way ANOVA to compare the effects of estrogen and growth factor treatments (Table 5.2). Two-way ANOVA gives a significance value for the influence of

gene name	MCF7β1		MCF7	
	exp 1	exp 2	exp 1	exp 2
HPRT		E		E
GAPDH	E		I	
ARF1			I	
PPP1CA	GF	E		I
ERα	E			E
ERβ1	**E, GF**	**E, GF, I**	I	
ERβ2				E
ERβ5		E	I	E
CathD		E	GF	
P21				
PR	E, I		**E**	**E**
Liv1	E, GF, I		E	
PS2	E, **GF**	**GF**	**E**, GF	**E**
VEGF	**E**	**E**	E, GF, I	
EFP		E	E	
CyclinD1				E
XBP1S		GF	E	
XBP1U	**E**	**E**, GF	E	GF

Table 5.2 Summary of genes showing response to estrogen, EGF and FGF-2 treatments in MCF7 and MCF7β1x cells

For each cell line, cells were treated with six different treatments; with either no added growth factor, added EGF or added FGF-2 in combination with estradiol. This experiment was performed in duplicate. Q-PCR analysis of a set of gene products was performed for each experiment, and the data normalised using the four housekeeping genes before analysis. 2-way ANOVA was performed on each gene's data set, comparing expression with the different estradiol and growth factor treatments. The table shows the complete set of gene products analysed and shows which gave significant ($p<0.05$) values for either response to estrogen (E), response to growth factor (GF) or the interaction between estradiol and GF (I) in each experiment. Gene names in bold highlight those mRNAs which showed some response to growth factor treatment and responses are shown in bold where the significant response is conserved across the 2 experiments. Note this table does not show whether mRNAs are up- or down-regulated in response to any individual treatment, only whether the response is significant.

response to estradiol, growth factor or interaction, are listed, and where the same result was obtained in each experiment it is shown in bold. Genes which show any response to growth factor have been highlighted in bold. Note that no distinction is made between positive and negative responses.

5.3.1 Gene expression responses

The duplicate experiments show slightly different patterns of responses. Experiment 1 shows a greater number of responses to estradiol and to growth factor treatments, whereas experiment 2 shows a greater number of significant values for interaction between the two types of treatment. The first experiment also shows a greater number of genes down-regulated by either growth factor treatment, whereas the second experiment shows more up-regulated genes. These differences may reflect variation in factors such as the confluence of the cell cultures on the two occasions. MCF7 cells show contact inhibition of growth; therefore, in more confluent conditions, changes in the signalling network within the cells may prevent a positive response to growth factors.

Responses to estradiol correspond to results seen in previous experiments (Tables 3.2 and 5.1). ERβ1 was down-regulated by estradiol in MCF7β1x cells in both replicate experiments, and VEGF and EFP show reduced expression in at least 1/2 experiments in each cell line. Similarly, PS2, liv1, PR and XBP1U are up-regulated by estradiol in at least 1/2 experiments in each cell line, in accordance with previous results. Several gene products show significant changes in expression in response to changes in growth factor treatment in at least one experiment. PPP1CA, ERβ1, cathepsin D and liv1 all show decreased expression on addition of growth factor and PS2, VEGF, XBP1U and XBP1S show increased expression (Table 5.2). Genes show different responses in terms of magnitude, reproducibility and which growth factor generates the largest response. ERβ1 and PS2 are the only genes in which a significant growth factor response is conserved in both experiments.

In MCF7β1x cells expression of ERβ1 is down-regulated by estradiol and by growth factor addition, in both replicate experiments. In the first experiment, expression is decreased in cells treated with EGF, compared to all others, whereas in the second experiment, both EGF and FGF-2 addition reduce expression by a similar amount. This down-regulation of ERβ1 is unlikely to be caused by reduction in the rate of

estrogen treatment, growth factor treatment (none, EGF or FGF-2) and interaction between these two treatments. Gene products, which showed a significant ($p<0.05$)expression, as most expression is expected to be from the transfected cDNA, therefore treatment with growth factors may lead to increased ERβ1 mRNA degradation. Interestingly, although ERβ1 expression is not significantly affected by estradiol or growth factor addition in MCF7 cells, non-significant changes in expression in response to growth factors mirror those seen in MCF7β1x cells, suggesting that this response may be an endogenous response, the magnitude of which is increased in the presence of excess ERβ1 mRNA.

PS2 expression is significantly increased in response to estradiol treatment in 3/4 experiments, with a trend towards higher expression with estradiol in the fourth. Expression is also affected by growth factor addition. In both cell lines, expression is lower in cells with no growth factor added, than if either growth factor has been added to the medium, with both growth factors showing similar levels of induction. This effect is significant in the 2-way ANOVA in 3/4 experiments (Table 5.2). The effect can also be analysed using a Student's *t*-test, comparing measured expression with and without growth factor addition. In these tests, significance is reached in 1/2 experiments for each cell line (*t*-tests exp. 1: MCF7 $p=0.016$, MCF7β1x $p=0.068$; exp. 2: MCF7 $p=0.174$, MCF7β1x $p<0.0005$). These results agree with those of [Garnier et al., 2003] who showed PS2 up-regulation by FGF-2 and [Barkhem et al., 2002] who showed that PS2 induction could be reduced by use of MAPK inhibitors, i.e., by inhibiting the downstream effectors of growth factors.

XBP1 is an estrogen responsive gene, which has two expressed forms (Section 3.1.3.2). In these experiments, both forms show some response to estradiol and to growth factors, although these responses are not well conserved between the two replicate experiments. XBP1U is up-regulated by estradiol in MCFβ1x cells in both replicate experiments, and in MCF7 cells in 1/2 experiments. In the first experiment, no response to growth factor is seen in either cell line, but in the second experiment, expression is significantly increased in response to either growth factor to similar levels compared to untreated cells in both cell lines, when using 2-way ANOVA. The Student's *t*-test confirms an increase in expression in treated cells compared to untreated cells in MCFβ1x ($p=0.037$) although the increase in MCF7 cells does not reach significance ($p=0.060$). XBP1S shows a less robust response to estradiol, being

significantly up-regulated in only one experiment using MCF7 cells. It also shows an increase in expression in response to growth factor treatment in MCF7β1x. In the first experiment, both growth factors generate a non-significant trend towards increased expression, whereas in the second experiment expression is significantly higher in MCF7β1x cells treated with either growth factor than those treated with no growth factor (*t*-test p=0.012).

Both PPP1CA and liv1 show decreased expression in response to growth factor treatment in MCF7β1x cells in the first experiment, and showed no trend towards changed expression in other experiments. This is in contrast to a report which showed that liv1 was up-regulated by EGF, similarly to PS2 [El-Tanani and Green, 1997a]. MCF7β1x cells in the first experiment showed a greater number of genes down-regulated than other experiments, suggesting that these changes in expression may only occur in certain conditions. Both VEGF and cathepsin D showed significant responses to growth factor in 1/4 experiments. VEGF mRNA has been shown to be up-regulated by FGF-2 in bone cells [Saadeh et al., 2000] and peritoneal mesothelial cells [Sako et al., 2003] and FGF-2 stimulation has been shown to increase VEGF release in T47D cells via a mechanism involving both the PI3K/Akt and the MEK1/ERK pathways [Shi et al., 2005], indicating that VEGF may be regarded as a FGF-2, as well as estrogen-dependent gene. However, in the present study this response was not consistently observed. High VEGF expression and secretion is associated with over-expression of many EGF related proteins such as EGFR, *ras* and ERb-B2 [Petit et al., 1997; Rak et al., 1995], however it is not known whether EGF directly up-regulates VEGF expression. Previously, expression of cathepsin D mRNA has been shown to be up-regulated by EGF in MCF7 cells [Cavailles et al., 1989], but although cathepsin D has been shown to potentiate FGF-2 action [Briozzo et al., 1991], it is not known whether FGF-2 regulates cathepsin D expression in breast cancer cells. Neither VEGF nor cathepsin D showed consistent responses to growth factor treatments, showing trends towards up-regulation by growth factors in some experiments, and down-regulation in others. It is interesting to note that in 3/4 experiments these two genes show opposite trends in response to growth factor treatments. For example, one gene will show highest expression in untreated cells, followed by those treated with EGF, then FGF-2, whereas the other gene will show the reverse pattern. A stronger response to FGF-2 than to EGF is

always seen. These contrasting patterns of response may suggest that VEGF and cathepsin D are controlled by similar pathways, but in an opposing manner, for instance activation of a specific intracellular signalling pathway may lead to the up-regulation of expression of one of these genes and the down regulation of the other.
As well as comparing responses to estradiol and growth factor individually, the 2-way ANOVA assay also gives a significance value to the interaction between estrogen response and growth factor response. This interaction value is related to either a synergistic or inhibitory effect of one treatment upon the response to the other. In these experiments, interaction may be significant when estradiol and growth factor responses are not significant, or there can be a response to estradiol or growth factor with no interaction between the two. No interactions are conserved at a significant level between replicate experiments suggesting that no synergistic effects of estradiol and growth factors are occurring in these cells. This is in accordance with [El-Tanani and Green, 1997a] who suggested that estradiol and EGF could up-regulate estrogen responsive genes in an additive manner in MCF7 cells, but in contrast to various other studies [Gullick, 1990; Murphy et al., 1990] who suggest that these effects should be synergistic. Similarly for FGF-2, [Garnier et al., 2003] showed a synergistic up-regulation of PS2 expression by estradiol and growth factor, whereas we see only an additive effect.

Although MCF7β1x cells express increased levels of ERβ1 mRNA, it is at the protein level that ERβ1 acts on gene transcription. In order to identify whether ERβ1 protein was increased in MCF7β1x cells, a series of assays were performed to measure protein expression in MCF7 and MCF7β1x cells.

5.4 Western blot analysis of ERα, ERβ1 and β-actin in MCF7 and MCF7β1x cells

MCF7β1x cells over-express ERβ1 mRNA. In order to investigate whether this over-expression was seen at the protein level, Western blot analysis was undertaken. Lysates were obtained from both MCF7 and MCF7β1 cells using two different methods and levels of ERα, ERβ1 and β-actin compared in the two cell lines.

5.4.1 Analysis of lysates extracted using the homogenisation buffer method

Initially, protein was extracted from untreated MCF7 and MCF7β1x cells using the homogenisation buffer method (Section 2.7.1.1). Aliquots of each protein sample (5-20 μg) were loaded onto a gel alongside known amounts of recombinant ERβ1 of both the long (rERβ1L) (530aa) and short (rERβ1S) (485aa) forms and recombinant ERα (rERα). After electrotransfer the blot was probed with PA1-313 polyclonal antibody (Fig. 5.3 A, Section 2.7.4), to measure ERβ1 immunoreactivity. The antibody bound both forms of rERβ1, but did not bind rERα. Both MCF7 and MCF7β1x lysates showed strong bands (greater than 6 ng rERβ1). However, this band was at a slightly higher molecular weight than either form of recombinant ERβ1 raising suspicions that the band could be non-specific. This was addressed by using a different antibody in subsequent assays (Section 5.4.2). The blot was subsequently stripped and re-probed with an anti-β-actin antibody to determine whether the lanes contained similar levels of protein (Fig. 5.3 B, Section 2.7.4). Strong bands of similar strength for MCF7 and MCF7β1x cells were observed, between the 40 and 50 kDa bands of the molecular weight marker, as expected (β-actin has MWt 44 kDa).

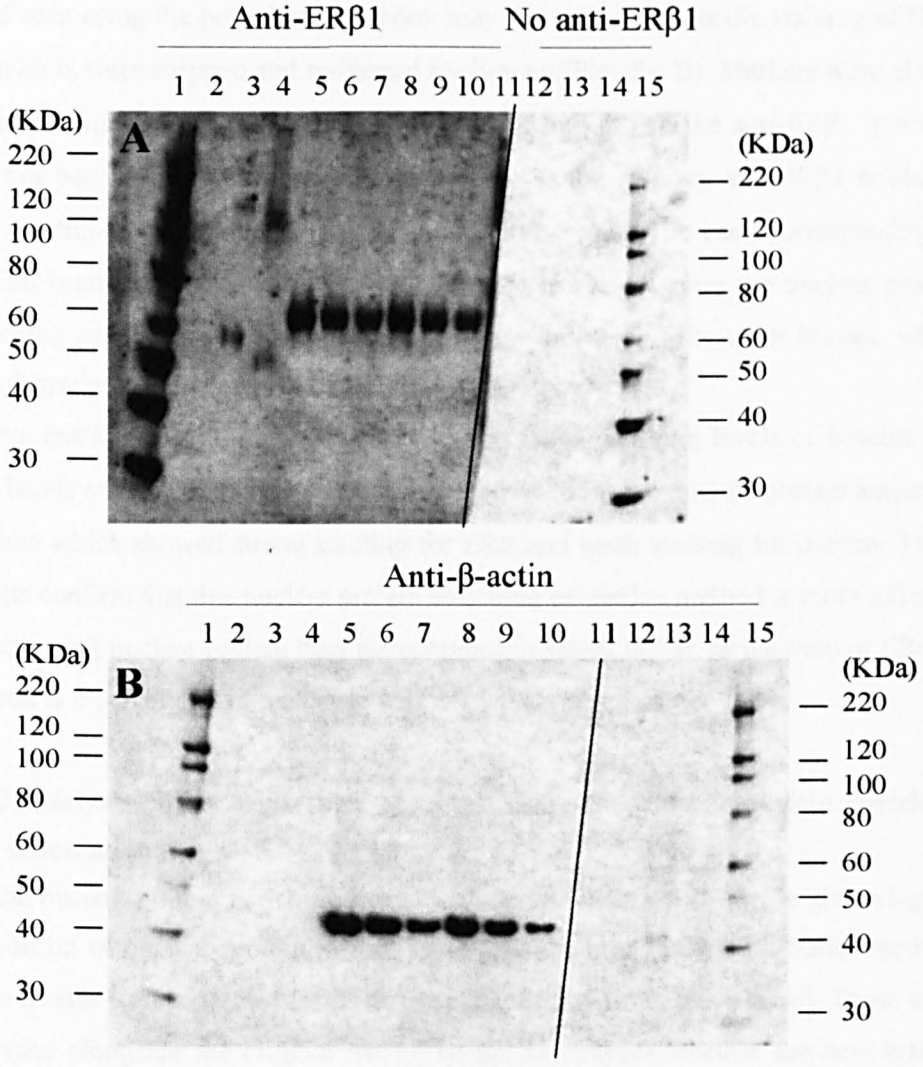
5.4.2 A comparison of the two protein extraction methods

To compare the homogenisation buffer protein extraction method (Section 2.7.1.1) and the nuclear protein enriching extraction method (Section 2.7.1.2), both lysates extracted using the homogenisation method and an MCF7 extract previously prepared using the nuclear protein enriching extraction method (Section 4.3.1) were analysed by SDS-PAGE. The membrane corresponding to a single gel was cut in half, to allow staining with two different mouse monoclonal antibodies. The anti-ERα antibody (F10) detected a band in the MCF7 lysate extracted using the nuclear protein enriching extraction method, but only very faint bands in the samples extracted using homogenisation buffer (Fig. 5.4 A). The band was of the same apparent molecular weight, but of lower intensity than the band for 2 ng recombinant ERα. The anti-ERβ1 antibody (PPG5/10) blot showed strong bands for both recombinant ERβ1 samples, and no band for ERα (Fig. 5.4 A). No bands were observed for any cell lysate sample, in contrast to the strong bands seen when using the PA1-313 antibody (Section 5.4.1, Fig. 5.3 B). This confirms the concerns that the

Figure 5.3 Western blot analysis of ERβ1 and β-actin in MCF7 and MCFβ1x lysates extracted using homogenisation buffer.

Protein was extracted from untreated MCF7 and MCF7β1x cells using homogenisation buffer (Section 2.7.1.1). Protein was quantified using a Bradford assay before being used for Western blot analysis (Sections 2.7.2, 2.7.4, 2.7.5.3). Samples were run on a 15-lane gel and blotted onto a PVDF membrane. This was then cut into 2 sections along lane 11 containing coloured markers. Lanes 1-11 were probed with primary and secondary antibodies in each blot, and lanes 11-15 were proed with secondary antibody only. Membrane was assayed for ERβ1, before being stripped and re-probed for ERα, then being stripped a final time and probed for β-actin.

(A) ERβ1 Western blot (PAI313 antibody), (B) β-actin western blot (Biovision antibody).



Lane loading
1 Invitrogen XP protein marker
2 10 ng rERα
3 6 ng rERβ1(long form)
4 6 ng rERβ1(short form)
5 20 µg MCF7 protein
6 10 µg MCF7 protein
7 5 µg MCF7 protein
8 20 µg MCF7β1x protein
9 10 µg MCF7β1x protein
10 5 µg MCF7β1x protein
11 ProSieve coloured marker
12 Mixed rERs
13 20 µg MCF7 protein
14 20 µg MCF7β1x protein
15 Invitrogen XP protein marker

band seen using the polyclonal antibody may not represent specific staining of ERβ. Both blots were stripped and re-blotted for β-actin (Fig. 5.4 B). Markers were almost entirely stripped from the blot. A faint band is seen where the anti-ERβ1 antibody has not been entirely stripped (shown boxed), as the primary anti-ERβ1 antibody, like the anti-β-actin antibody, was raised in mouse cells. The band corresponding to β-actin immunoreactivity for the MCF7 lysate extracted using the nuclear protein enriching extraction method is much weaker than those in the other lysates, which are of similar densities.

Lysate extracted using homogenisation buffer contained high levels of β-actin, but low levels of ERα, in contrast to lysate extracted using the nuclear protein enriching method which showed strong staining for ERα and weak staining for β-actin. These results confirm that this nuclear protein enriching extraction method is more efficient in extracting nuclear protein than using homogenisation buffer, as the ratio of ERα to β-actin is much higher in the lysate extracted using this method.

5.4.3 Analysis of further lysates prepared using the nuclear protein enriching extraction method

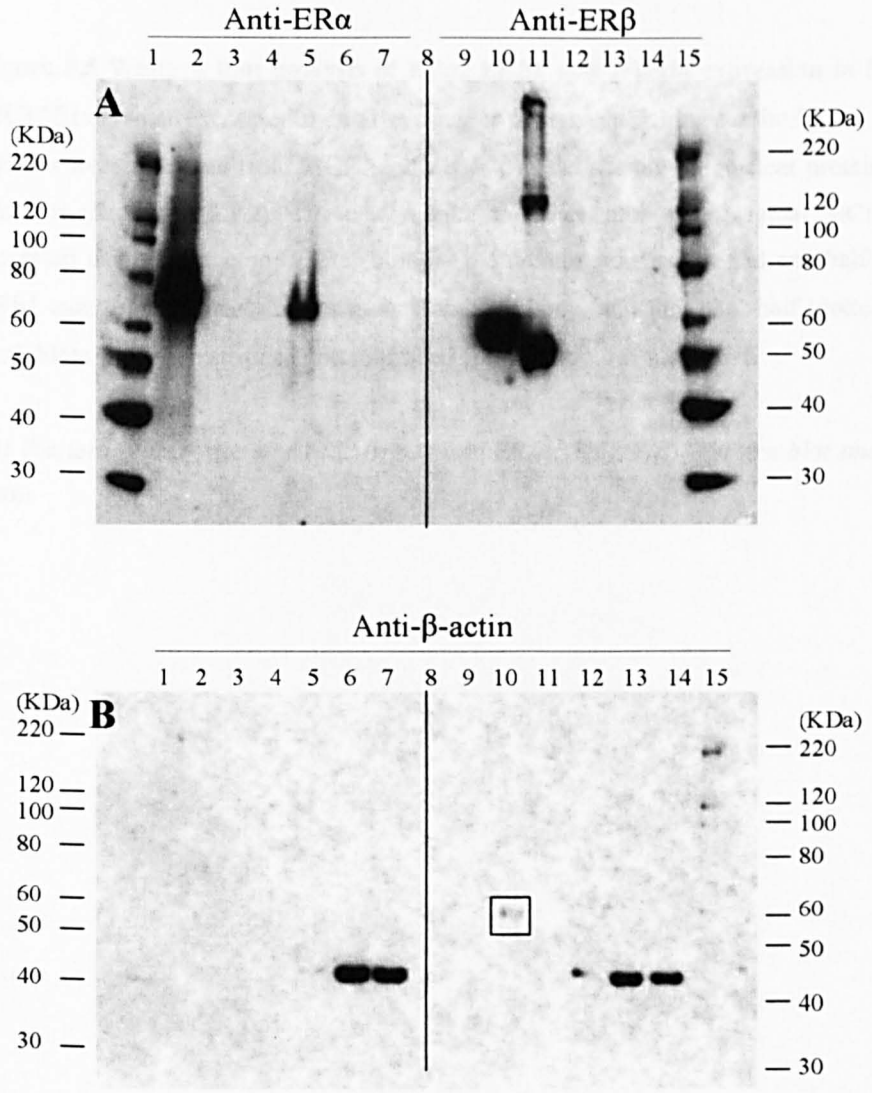
As the nuclear protein enriching extraction method had been shown to give a higher proportion of nuclear protein in the lysate than the homogenisation buffer method, fresh lysates were extracted from MCF7 and MCF7β1 using this method. These were analysed alongside the original MCF7 lysate, to confirm whether the new lysates were of similar quality and composition to the original lysate.

Less ERα immunoreactivity was seen in the new lysates (Fig. 5.5 A lanes 2-3) than in the original lysate (Fig. 5.5 A lane 4). All bands were of the same molecular weight, but less intense than the band for 2 ng rERα. Although bands were seen for both rERβ1 samples (30 ng of each form in lanes 12-13), no specific bands were obtained in any lysate using the anti-ERβ1 antibody, although some non-specific immunoreactivity is seen (Fig. 5.5 A). After staining for the ERs, both membranes were stripped and re-probed for β-actin expression (Fig. 5.5 B). Bands are seen for all three lysates at 44 kDa and less β-actin immunoreactivity is seen in the new lysates than the old. As the levels of both ERα and β-actin immunoreactivity are lower in the new lysates than in the original lysate, it suggests that in fact less total

Figure 5.4 Western blot analysis of protein expression in MCF7 and MCF7β1x cells, comparing lysates extracted using different methods.

MCF7 and MCF7β1x lysates prepared using homogenisation buffer (Section 2.7.1.1) were run on a gel alongside an MCF7 lysate extracted at an earlier date using the enriched nuclear protein extraction method (Section 2.7.1.2) to ascertain whether any differences were seen between these extraction methods. The 15-lane gel was cut into two similarly loaded pieces. One was blotted for ERβ1 using PPPG5/10, whilst the other was blotted for ERα using a fresh, verified, batch of antibody. Both blots were then stripped and re-probed for β-actin.

(A) Western blot analysis of ERα (left) and ERβ1 (right), (B) Western blot analysis of β-actin.

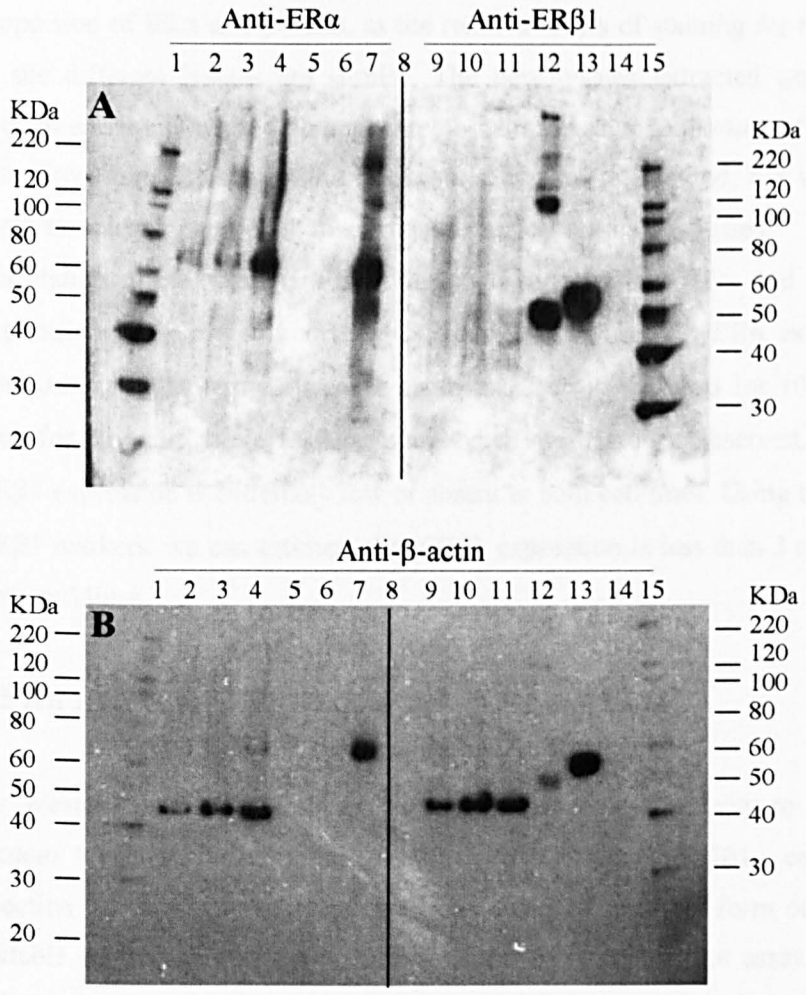


Lane loading
1 Invitrogen XP marker
2 2 ng rERα
3 30 ng rERβ1 (long form)
4 30 ng rERβ1 (short form)
5 10 µg MCF7 lysate (nuclear-enriching method)
6 10 µg MCF7 lysate (homogenisation method)
7 10 µg MCF7β1x lysate (homogenisation method)
8 ProSieve coloured marker
9 2 ng rERα
10 30 ng rERβ1 (long form)
11 30 ng rERβ1 (short form)
12 10 µg MCF7 lysate (nuclear-enriching method)
13 10 µg MCF7 lysate (homogenisation method)
14 10 µg MCF7β1x lysate (homogenisation method)
15 Invitrogen XP marker

Figure 5.5 Western blot analysis of ERα, ERβ1 and β-actin expression in MCF7 and MCF7β1x lysates extracted using the nuclear protein enriching method.

Lysates were extracted from MCF7 and MCF7β1x cells using the nuclear protein enriching protocol (Section 2.7.1.2). These were run on a gel, alongside original MCF7 samples extracted using this method. After blotting the membrane was cut and one half blotted for ERβ1 using the Serotech mouse monoclonal antibody, and the other half blotted for ERα. Both blots were then stripped and re-probed for β-actin.

(A) Western blot analysis of ERα (right) and ERβ1 (left). (B) Western blot analysis of β-actin



Lane loading
1 Invitrogen XP marker
2 20 µg new MCF7β1x lysate
3 20 µg new MCF7 lysate
4 20 µg original MCF7 lysate
5 30 ng rERβ1 (short form)
6 30 ng rERβ1 (long form)
7 2 ng rERα
8 ProSieve coloured marker
9 20 µg new MCF7β1x lysate
10 20 µg new MCF7 lysate
11 20 µg original MCF7 lysate
12 30 ng rERβ1 (short form)
13 30 ng rERβ1 (long form)
14 2 ng rERα
15 Invitrogen XP marker

Note: All lysates extracted using the nuclear protein enriching method.

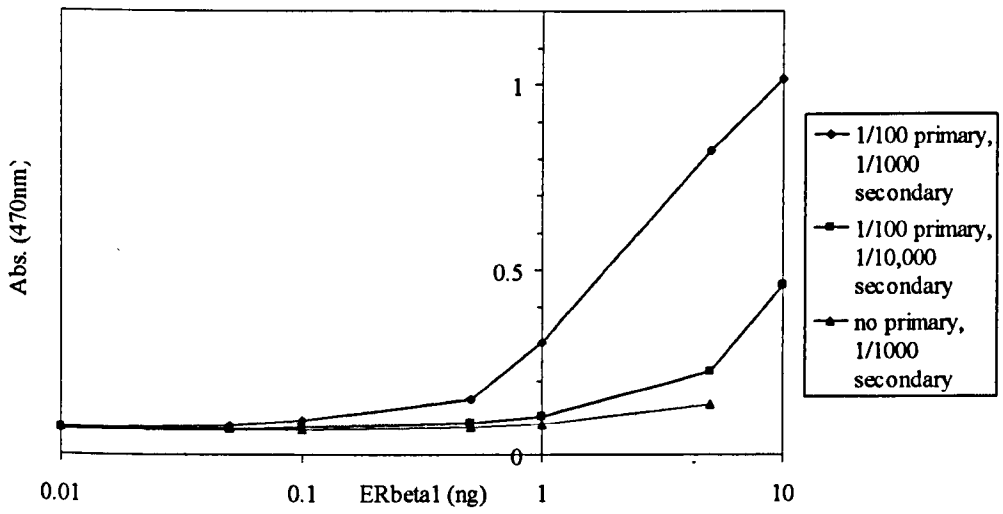
protein was loaded on the gel for these samples. All lysates seem to contain a similar proportion of ERα and β-actin, as the relative levels of staining for the two proteins in the different lysates are similar. The new lysates extracted using the nuclear protein enriching method do appear to contain a greater proportion of nuclear protein than those extracted using the homogenisation buffer method, but without running these samples in parallel on the same gel, this cannot be quantified.

Throughout these western blot assays, we can see that ERα and β-actin protein expression levels are similar in MCF7 and MCF7β1x cells. ERα expression is less than 0.2 ng/μg protein, as weaker bands are seen in the blots for 10 μg lysate than seen for 2 ng rERα. No ERβ1 immunoreactivity has been observed, indicating that ERβ1 expression is extremely low or absent in both cell lines. Using the recombinant ERβ1 markers, we can estimate that ERβ1 expression is less than 3 ng/μg protein in both cell lines.

5.5 An ELISA for ERβ1 expression in the cell lines

As Western blotting was shown to have insufficient sensitivity to measure ERβ1 protein levels in lysates extracted from MCF7 and MCF7β1x cells, an ELISA (Section 2.7.7) was developed. ELISA is a highly sensitive form of protein assay, suitable for measuring low abundance proteins. Initially, the assay was validated using recombinant protein, before testing it using the cell lysates. In the first assay, 10 ng aliquots of rERβ1L were bound to Maxisorp plates in coating buffer. Primary antibody (PPG5/10) and secondary antibody (peroxidase linked anti-mouse) were applied at 1/10 to 1/1000 and 1/100 to 1/100000 dilutions, respectively. Primary antibody concentration was shown to have little effect on absorbance, but absorbance increased as secondary antibody concentration increases. This increase was strongest between 1/100000 and 1/10000, and gradually decreased as the concentration increased. 1/1000 and 1/100 secondary antibody dilutions gave very similar results, suggesting that saturation had been reached. This validation experiment showed that ELISA was a suitable method for measuring rERβ1.

A further assay was performed to investigate the sensitivity of the assay using rERβ1 (Fig. 5.6). Wells were coated with between 10 and 0.005 ng rERβ1L in coating buffer, or filled with coating buffer alone as a negative control. Primary antibody was



5.6 Standard curves for ELISA detection of ERβ1, using different antibody concentrations

ELISA (Section 2.7.7) was tested using standard dilutions of rERβ1L. Changing the dilution of primary antibody made little difference to the sensitivity of the assay, but changing the dilution of the secondary antibody had a much greater effect.

Detection of the product is achieved by measuring the absorbance at 470 nm. No primary antibody controls show a background absorbance of about 0.075.

Using a higher concentration of secondary antibody (1/1000) generates a standard curve with a detection sensitivity of between 0.1 and 0.5 ng ERβ1. The sensitivity using 1/10,000 secondary antibody is only between 1 and 5 ng ERβ1.

used at a 1/100 dilution and secondary antibody applied at both 1/1000 and 1/10000 dilutions. A "no primary antibody" control series was also prepared. These assays showed that the higher secondary antibody concentration gave greater sensitivity. Using 1/1000 secondary antibody, absorbance is proportional to the log of rERβ1. The assay is sensitive to between 0.5 and 0.1 ng per well, before readings reach baseline values. Both "no ERβ1" and "no primary antibody" controls show an absorbance of around 0.075 in this assay.

The maxisorp plates bind up to 650 ng protein per cm^2, and each well has a binding surface area of 0.95 cm^2, when using 100 μL solution, allowing a total maximum binding of 600 ng protein per well. In order to achieve the maximum possible protein binding, 1, 5 and 10 μg aliquots of MCF7 and MCF7β1x lysates were loaded onto the plate alongside a set of rERβ1 standards, and treated with 1/100 primary and 1/1000 secondary antibody. Each lysate (5 ng) was also spiked with 1ng rERβ1 to investigate whether protein overload would affect ER binding. Wells coated with lysate alone did not show absorbance values above background. This suggests that less than 0.5 ng ERβ1 was bound to these wells, and therefore that the lysates contained less than 2 ng/μg protein. Wells coated with a mixture of lysate and 1 ng rERβ1 gave lower absorbance values than those containing 1 ng rERβ1 alone (equivalent to between 0.2 and 0.5 ng), suggesting competition by the multiple proteins in a complex mixture is leading to an under-estimation of total ERβ1 using this method.

This assay was not significantly more sensitive than the Western blot. Western blotting can easily detect 6 ng rERβ1, and 10 μg protein can be loaded, giving a sensitivity of < 0.6 ng/μg. The ELISA detected 0.5 ng ERβ1, but not 0.1 ng. As each well can bind only 0.6 μg total protein, this gives a sensitivity of between 0.2 and 0.8 ng/μg. This did not allow detection of ERβ1 protein in the cell lines, suggesting that ERβ1 protein is either expressed at levels < 0.6 ng/μg total protein, or is not expressed in either cell line.

5.5.1 Competitive ELISA

Competitive ELISA may be more sensitive than a direct ELISA method. An assay was performed to test this method. Wells were coated with either 5 or 10 ng rERβ1L, primary antibody was used at a 1/500 or 1/5000 dilution and secondary antibody was

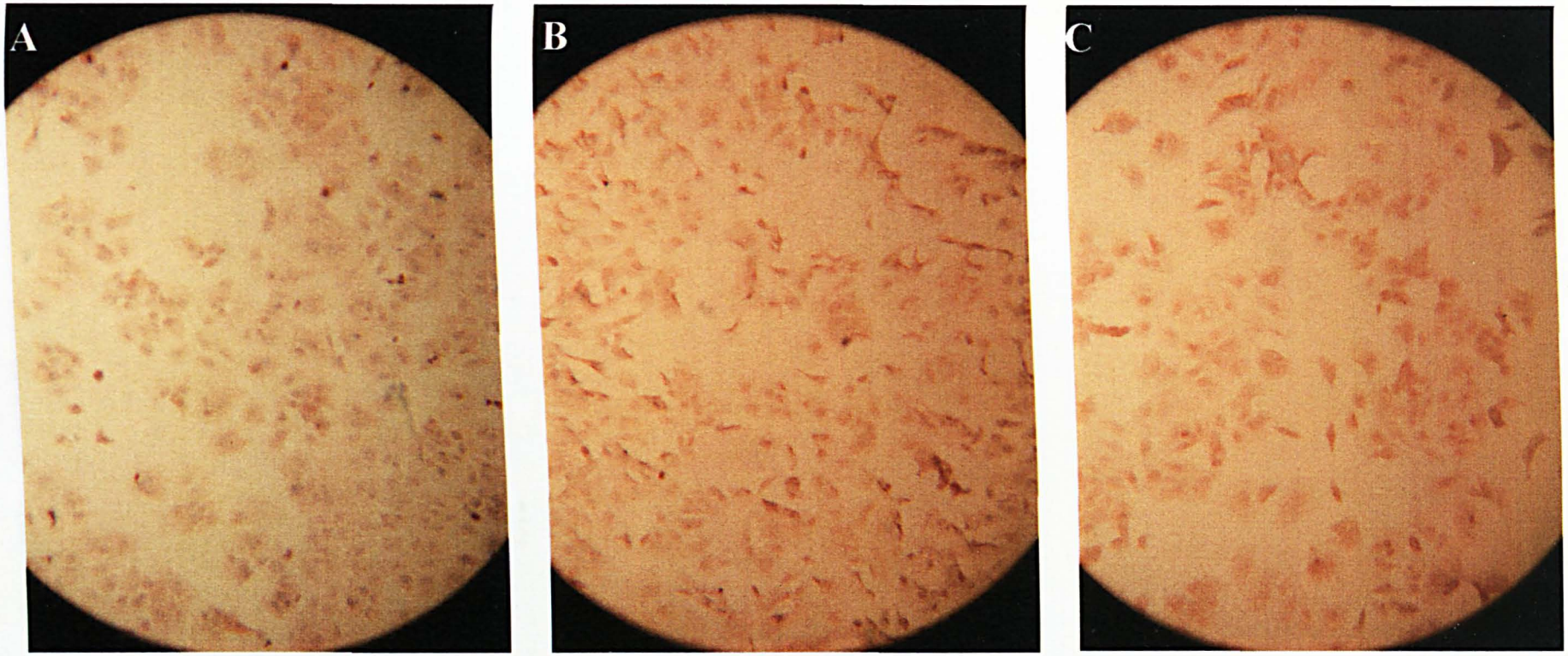
used at either 1/200 or 1/1000 dilution. Before adding to the plate, primary antibody was mixed with 0, 0.01, 0.1 or 1 ng rERβ1 as a competitor and either added directly to the plate, or left for 1 h prior to addition. Secondary antibody and enzyme substrate were used as previously (Sections 2.7.7, 5.5). As expected, absorbance was significantly higher when greater amounts of coating protein, primary or secondary antibodies were used. The change in absorbance in response to different levels of competitor were, however, very small. In some cases, absorbance was, in fact, greater when 0.01 ng competitor was used that in the absence of competitor and a typical difference between absorbance with 0 and 1 ng competitor was only 0.03, compared to up to 0.20 with the standard assay. This small change in absorbance suggests that this method would not be suitable for measuring small amounts of ERβ1 with these antibodies.

5.6 Immunostaining of MCF7 and MCF7β1x cells for ERα and ERβ1

Immunostaining allows the location of proteins within the cell, as well as the levels in the cells to be visualised. Established methods for staining paraffin-embedded tissue for ERα and ERβ1 [O'Neill et al., 2004; Shaaban et al., 2003; Shoker et al., 1999a] were adapted for use on cultured cells (Section 2.7.6). MCF7 and MCF7β1x cells were grown in parallel on multi-well slides, allowing them to be stained simultaneously, reducing the chance of variation in staining being caused by experimental handling. Cells were stained for ERα using three slightly different protocols in order to define the optimum treatment for the slides (Section 5.6.1, Figs 5.7, 5.8). Based on these results, cells stained for ERβ1 always underwent permeabilisation and unmasking. To test for any non-specific secondary antibody binding, and to optimise the anti-ERβ1 levels, a "no primary antibody" control and two different primary antibody concentrations (1/10 and 1/100) were used (Section 5.6.2, Figs 5.9, 5.10).

5.6.1 Immunostaining for ERα

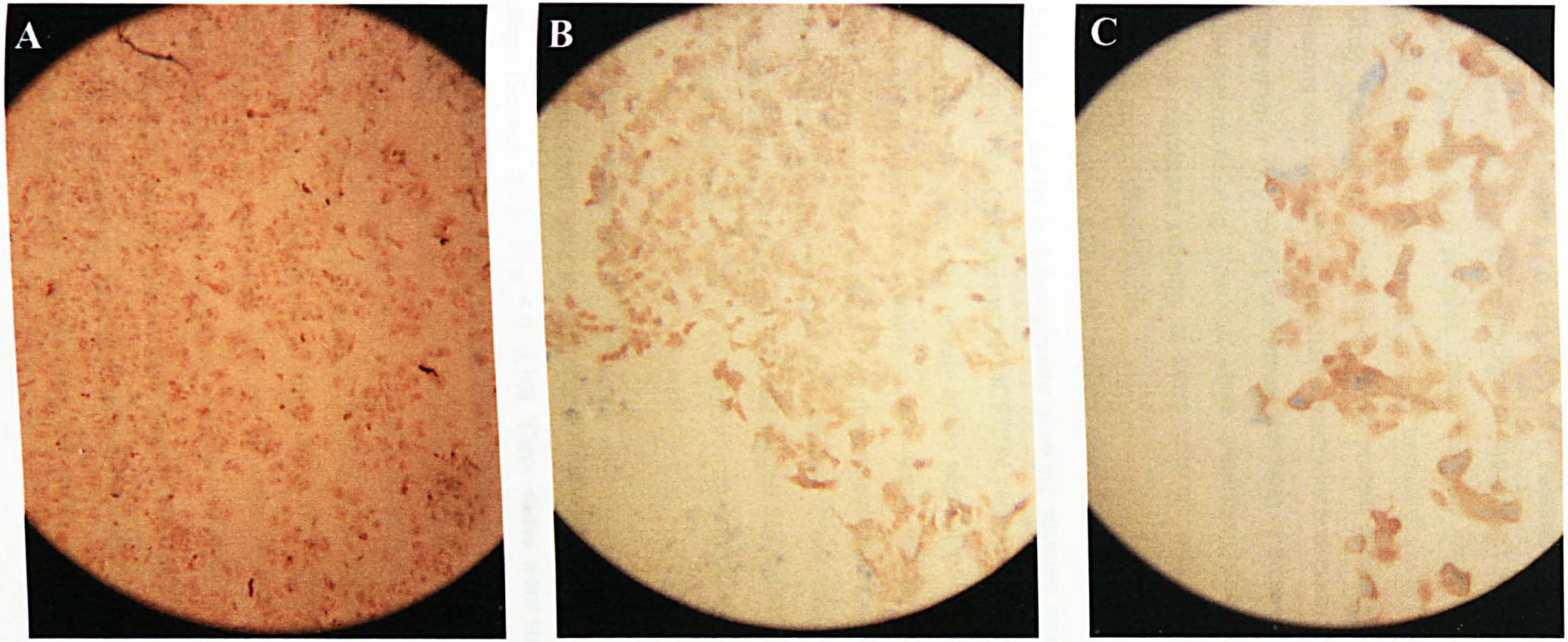
MCF7 and MCF7β1 cells were stained for ERα using three different protocols, to determine the optimum protocol for staining the directly fixed cells. The treatments were "including permeabilisation and unmasking" and excluding one or the other of



5.7 Immunostaining of MCF7 cells for ERα

MCF7 cells were immunostained for ERα (Section 2.7.6). During processing, slides were treated with both permeabilisation and unmasking, or excluding one of these treatments.

(A) MCF7 cells, permeabilised, (B) MCF7 cells, unmasked, (C) MCF7 cells, both permeabilised and unmasked.



5.8 Immunostaining of MCF7β1x cells for ERα

MCF7β1x cells were immunostained for ERα (Section 2.7.6). During processing, slides were treated with both permeabilisation and unmasking, or excluding one of these treatments.

(A) MCF7β1x cells, permeabilised, (B) MCF7β1x cells, unmasked, (C) MCF7β1x cells, both permeabilised and unmasked.

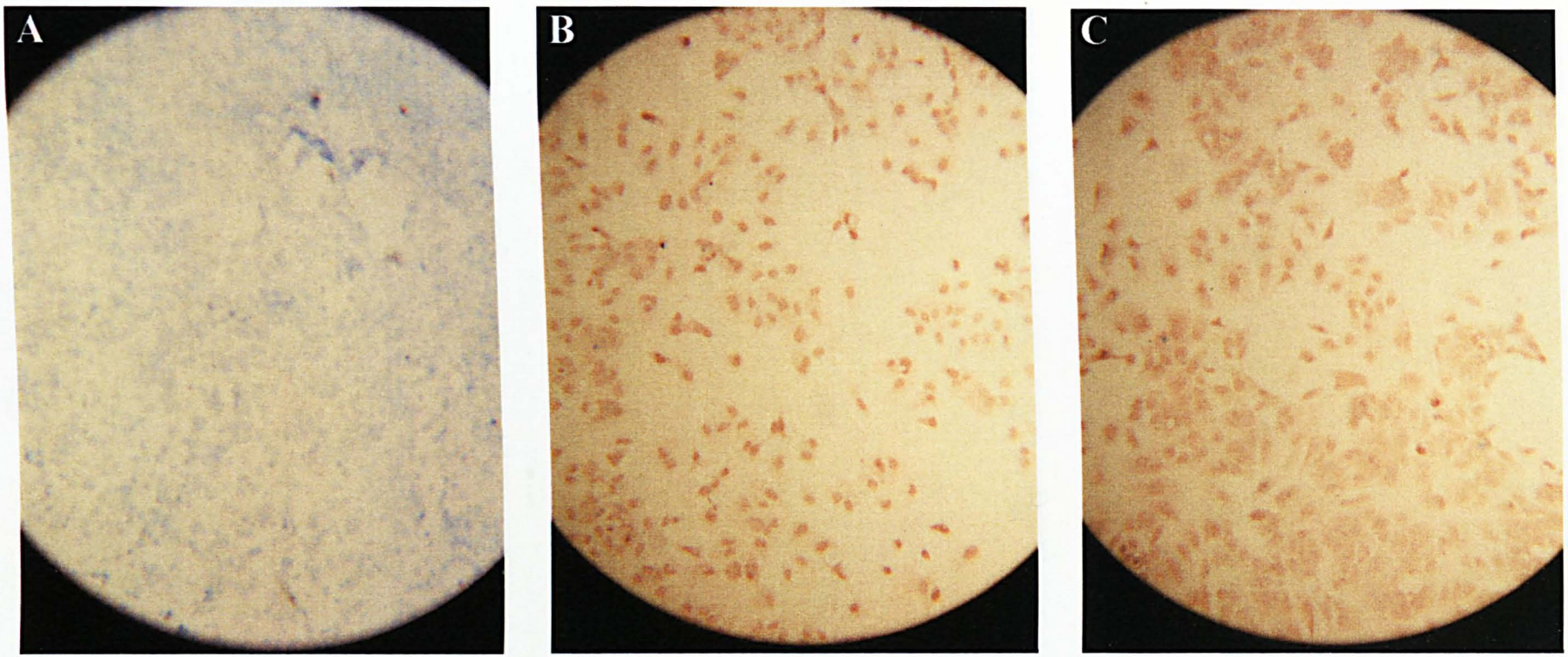
these steps (Section 2.7.6, Figs 5.7, 5.8). Permeabilisation permits easier access of antibodies and stains to the nucleus of the cells. It was used here as, unlike when using formalin-fixed samples, intact cells are fixed to the slide. Unmasking is necessary to detect certain antigens by immunostaining [Cattoretti et al., 1993], yhe likely mechanism for its action being protein denaturation exposing the epitope. Staining was strongest in the slide treated with both permeabilisation and unmasking steps (Figs 5.7 C, 5.8 C), confirming that both of these treatments were required to fully permit staining. Note that where cytoplasmic staining is described, this could also be membrane staining, as intact cells are used, meaning these two types of staining are indistinguishable.

MCF7 cells (Fig. 5.7) show nuclear staining, with weaker cytoplasmic staining. Staining of nuclei is heterogeneous showing stronger staining in some cells than others (Fig. 5.7 C). Staining of MCF7β1x cells was rather less homogenous (Fig. 5.8). Although some cells, usually at the edge of cell groups, show strong nuclear staining, in other cells staining is very weak, and in some cases the stain even appears to be totally excluded from the nucleus. Both cell types were grown on the same slides, so it is unlikely that staining issues could explain the differences between the two cell lines. This would suggest that the MCF7β1x population is more heterogeneous than the MCF7 population with respect to ERα distribution.

5.6.2 Immunostaining for ERβ1

Staining for ERβ1 used the full protocol including permeabilisation and unmasking steps (Section 2.7.6, Figs 5.9, 5.10). Three slides were stained using "no primary antibody" or PPG5/10 at 1/10 and 1/100 dilutions. These dilutions were greater than described in the literature, as the batch in use is a 10x concentrated stock. Primary antibody controls (Fig. 5.9 A, 5.10 A) showed no (brown) DAB staining, confirming that non-specific secondary antibody binding is not occurring. However, these results do not confirm the specificity of the primary antibodies to the correct ER epitopes. Nevertheless, the antibodies used are the same as those used in Western blotting procedures, where good specificity was observed (Section 5.4).

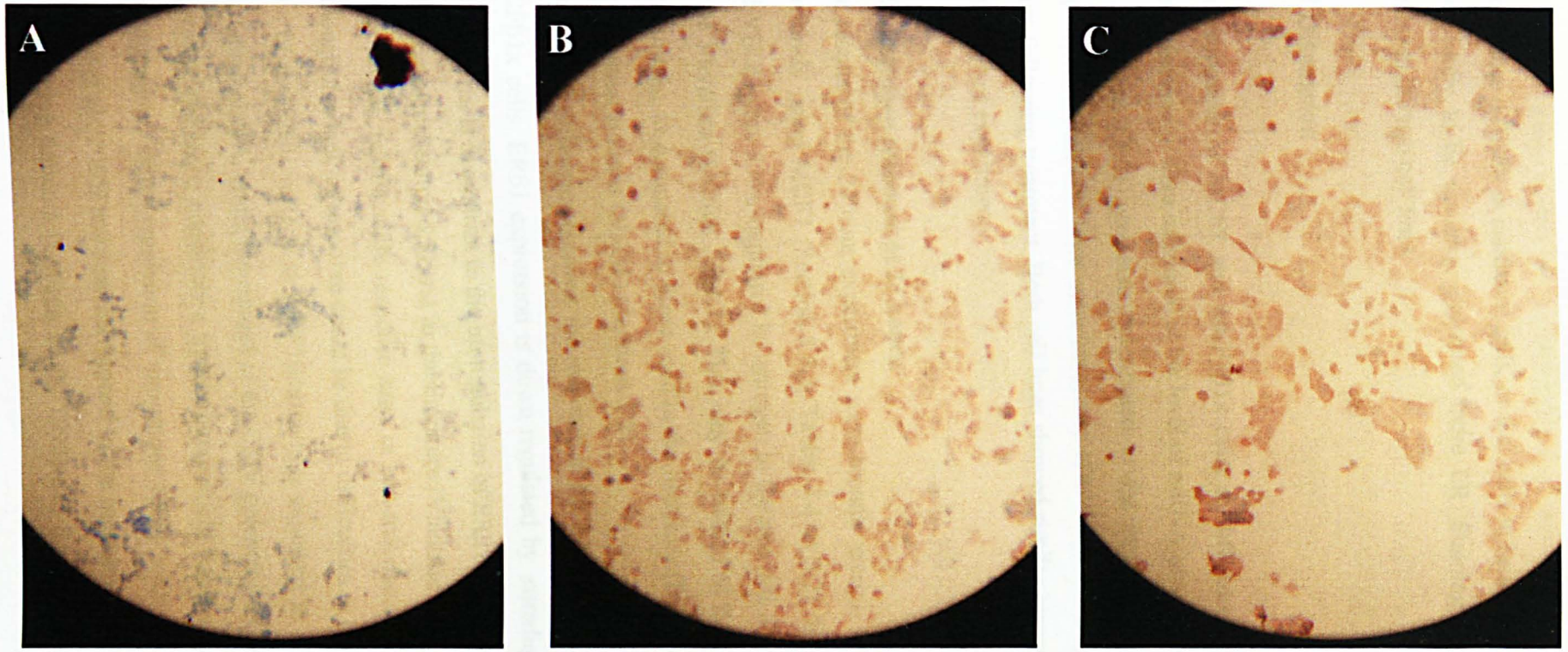
Nuclear ERβ1 staining was stronger in both cell lines than cytoplasmic staining. As seen with ERα staining, individual cells showed stronger staining than those in more confluent areas, suggesting that this was either an effect of antibody penetration



5.9 Immunostaining of MCF7 cells for ERβ1

MCF7 cells were immunostained for ERβ1 (Section 2.7.6). During processing, slides were both permeabilised and unmasked. ERβ1 antibody was used as either no antibody, 1/10 or 1/100 antibody.

(A) MCF7 cells, no primary antibody, (B) MCF7 cells, 1/100 primary antibody, (C) MCF7 cells, 1/10 primary antibody.



5.10 Immunostaining of MCF7β1x cells for ERβ1

MCF7β1x cells were immunostained for ERβ1 (Section 2.7.6). During processing, slides were both permeabilised and unmasked. ERβ1 antibody was used as either no antibody, 1/10 or 1/100 antibody.

(A) MCF7β1x cells, no primary antibody, (B) MCF7β1x cells, 1/100 primary antibody, (C) MCF7β1x cells, 1/10 primary antibody.

being more difficult in confluent cells, or that cells in less confluent areas, which are more likely to be dividing, expressed more ER. Staining patterns for ERβ were similar in the two cell lines.

5.7 Discussion

The responses of MCF7 and MCF7β1x cells to different estrogenic and growth factor treatments were measured at the level of mRNA expression and the expression of ERα and ERβ1 was also determined at the protein level. Gene expression responses in both cell lines were measured after 24 hours and over the first eight hours following treatment. Both cell lines showed similar responses to treatments in this series of experiments.

5.7.1 Estrogen response at 24 hours

The results of the 24 hour experiment were broadly similar to the results obtained previously using MCF7 (Section 3.3.3, Tables 3.4 to 3.7). Interestingly, both EFP and VEGF were down-regulated by estrogen in the two cell lines, contrary to reports in the literature (Section 5.2.1.1). This may be related to the time course of these experiments, as compared to those used in the literature (EFP), or to use of different experimental systems (VEGF) (Sections 5.2).

The striking difference between the response in MCF7 and MCF7β1x cells is that in MCFβ1x cells, ERβ1 expression is down regulated by estradiol treatment (Section 5.2.1.1). This is in contrast to the up-regulation of ERβ1 obtained in T47D cells, both in this project (Section 3.3) and in the literature [Vladusic et al., 2000], and the lack of significant, reproducible response seen in non-transfected, parental MCF7 cells (although this lack of response could be due to difficulties in detecting the response). Most of the ERβ1 mRNA in MCF7β1x cells is expected to be expressed from the transfected cDNA, under the control of the CMV promoter and Tet repressor-binding sequences, therefore, constitutive transcription would be expected in the absence of inducing agent. Down-regulation of exogenous ERβ1 mRNA was also observed by [Murphy et al., 2005] and may correspond to the increased ERα:ERβ ratio observed in ER-positive tumours [Leygue et al., 1998] and the down-regulation of ERβ by

estrogen in mouse uterine stroma [Weihua et al., 2000]. Most likely, down-regulation of mRNA levels is via an increased rate of mRNA degradation. Alternatively, it is possible that the levels of ERβ1 mRNA remain unchanged, whilst an overall increase in transcription occurs, a phenomena observed in sheep endothelial cells after estradiol treatment [Ing et al., 1996]. In this case, the fraction of transcripts which represent ERβ1 would be reduced. If the levels of expression of the housekeeping genes, but not ERβ1, was increased in parallel with the overall increase in expression rates, this effect would not be offset by normalisation. This argument may be supported by the observation that in 4/6 experiments in which MCF7β1x cells were treated with and without estradiol in parallel, RNA concentration was higher in the estradiol treated cells. However, the increased RNA concentration may also be due to the presence of a greater number of cells after estradiol stimulation, rather than an increase in the amount of RNA per cell.

As previously described (Section 3.3.4.2, [Pink and Jordan, 1996]), ERα mRNA is down-regulated by estradiol in MCF7 cells. This is regarded as being due to both transcriptional and post-transcriptional mechanisms [Saceda et al., 1988], and is in contrast to the stabilisation of ERα mRNA seen in the endometrium [Ing and Ott, 1999]. Estradiol and other steroid hormones have also been shown to have both stabilising and destabilising effects on other mRNAs in various tissues (reviewed in [Ing, 2005]) via, for instance, activation of a sequence specific endonuclease [Cunningham et al., 2001]. It is, therefore, possible that the excess, exogenous ERβ1 mRNA in MCF7β1x cells is targeted by the same destabilisation as affects endogenous ERα mRNA.

5.7.2 Estrogen response over 8 hours

In order to test the hypothesis that different genes may respond to estradiol over different time course, both cell lines were treated with estradiol or ICI 182,780 and RNA was extracted between 0 and 8 h after treatment. Many genes, such as PR, PS2, XBP1S, XBP1U and cathepsin D, which show increased expression after 24 hours (Table 5.1), also show increased expression by 4 or 8 hours after treatment, although the time courses of response in different genes do appear to be different (Section 5.2.2.1 and Fig. 5.2). However, given that changes may not be replicated over multiple experiments (e.g. Sections 5.2.1.1 5.3.1), further replicates, followed by

measurements of the cells' biological responses, e.g., cell proliferation, are needed to ascertain fully this conclusion. Although both cell lines show a peak in expression of several genes at one hour with estradiol treatment, the same peak is often seen in either untreated or ICI 182,780 treated cells. Possibly repeating the experiment without the change of medium, or changing the medium some hours before the experiment, might help isolate the effects of estradiol treatment over the shorter time course.

Some genes appear to behave differently over 8 hours than after 24 hours. ERβ1 expression, for instance, is consistently decreased in MCF7β1x cells after 24 hours of estradiol treatment, but showed no change in expression over 8 h. This reinforces the suggestion that this decrease is not a primary transcriptional response. Conversely, ERβ2 is not consistently up-regulated after 24 hours in MCF7 or MCF7β1x cells, but did show increased expression after 4 hours in both of these cell lines. Although other responses were observed over 8 hours, most genes showed the same response in both MCF7 and MCF7β1x cells. The exceptions to this are cathepsin D and XBP1U, but as these are genes which have previously shown sporadic responses over 24 hours (Appendices and Tables 5.1, 5.2), it is possible that repeating this experiment might show similar responses in the two cell lines.

The different patterns of ERβ expression in different cell lines with respect to time are particularly important, as differences in the expression of the estrogen receptors will affect down-stream estrogen responses. ERβ2, for instance, has been shown to inhibit ERα-dependent expression [Ogawa et al., 1998]. In these experiments ERβ2 shows only transient, early induction in MCF7 cells, returning to basal levels by 8 hours, but strong induction at 24 hours in T47D cells (Sections 5.2.2.1, 3.3.4.2). Assuming that these differences in expression patterns are reflected in different levels of the ERβ2 protein, this is a mechanism by which these two cell lines may be able to differently modulate their downstream responses to estrogen.

5.7.3 Response to growth factor treatment

MCF7 cells and MCF7β1x cells were treated with estrogen and growth factors to investigate whether EGF or FGF-2 had an effect on the estrogen response, and whether increased ERβ1 mRNA expression had an effect on the cellular response to the growth factors. Several responses to growth factor treatment were seen, although

these were not always preserved in repeated experiments (Section 5.3.1). Different genes showed both increases and decreases in expression in response to growth factors, as they do to estradiol. Most responses were similar in both cell lines, and with both growth factors, although expression of ERβ1 was significantly decreased in response to growth factor treatment in MCF7β1x cells, but not in MCF7, similarly to the effects of estradiol.

Growth factor signalling involves many different intracellular pathways. For instance, both EGF and FGF-2 signalling may use both the MAPK and PKC pathways, but may be expected to stimulate these pathways to a different extent, since their links to these pathways via adaptor proteins are different [Schlessinger, 2004]. EGF is generally regarded as a mitogen for MCF7 cells [Karey and Sirbasku, 1988; van der Burg et al., 1988], whereas FGF-2 has been reported to act as both a mitogen [Briozzo et al., 1991; Karey and Sirbasku, 1988] and a growth inhibitor [Fenig et al., 1997; Johnson et al., 1998; Wang et al., 1997b], reflecting the complexity of the signalling network as well as the differences between the assays used in these papers. Whilst most of these studies used MCF7 cells grown in DMEM with foetal calf serum, [Karey and Sirbasku, 1988] used a serum-free system. [Briozzo et al., 1991] report that FGF-2 addition increases the rate of DNA synthesis in MCF7 cells, whilst [Karey and Sirbasku, 1988] report an increased rate of doubling, and [Fenig et al., 1997; Johnson et al., 1998; Wang et al., 1997b] use a measurement of the percentage of cells in each phase of the cell cycle as their main method of measurement.

There are few reports describing the modulation of gene expression in response to growth factor treatment in breast cancer cell lines. Most studies focus on the effect on proliferation or the mechanisms of growth factor signalling. Those studies which specifically investigate cross-talk between the growth factor and estrogen pathways often use reporter genes to measure any response [Kato et al., 1995; Tremblay et al., 1999]. However, some data is available regarding the effect on estrogen-responsive gene expression of growth factors in MCF7 cells.

One study involved treatment of MCF7 cells with combinations of estradiol and 10 ng/ml EGF [El-Tanani and Green, 1997a]. This study showed an additive effect of these two stimuli on the expression of both PS2 and liv1. In the present study, however, only PS2 responds to EGF in this way. This may reflect differences in experimental treatments. El-Tanani and Green used a serum-free medium for their

study, and used a higher concentration of EGF than used in the present study, both of which may alter the signalling background against with changes in gene expression are measured. However, induction of liv1 by estradiol was also much lower in the present study than in this previous study (Section 3.4.4.2), so the absence of response to growth factor may reflect this same poorer activation in our cells.

Another study investigated the effect of FGF-2 treatment on MCF7 cells, both in terms of proliferation and of expression of ERα, PR and PS2 [Garnier et al., 2003]. This study used 1 ng/ml FGF-2, as in the present study, but also used a serum-free, defined medium. They found that FGF-2 treatment inhibited estradiol dependent PR expression and down-regulated ERα protein, but also acted synergistically with estradiol to stimulate expression of PS2. In contrast to this, the present study shows no effect of FGF-2 on ERα or PR mRNA levels. However, Garnier *et al.* measured the receptor protein levels after 4 days of treatment, whereas the present study measured mRNA levels after 24 h, which may explain this discrepancy. Garnier *et al.* also showed a synergistic up-regulation of PS2 mRNA after 18 h of treatment with estradiol and FGF-2. In the present study, FGF-2 up-regulates PS2 expression at 24 hours, but this effect is additive, rather than synergistic. Possibly this disparity may reflect the use of serum-free media in the previous study, or be a reflection of the slightly different time points used in the two studies. Although the treated serum (Section 2.2.2) used in the present study is steroid-depleted, other components of the serum remain, which may have a strong effect on cell growth and signalling. These include platelet-derived growth factor (PDGF), which stimulates the growth of breast epithelial cells [Taverna et al., 1991], and transferrin, which is necessary for the cells to utilise the low levels of iron in the media [Barnes, 1984; Riss and Sirbasku, 1987; Rudland et al., 1977].

The present study shows the expression of a small subset of estrogen responsive genes to be modulated by either EGF or FGF-2. In most cases, responses to the two growth factors were indistinguishable, suggesting that, in this context, they may be acting through similar pathways. Several responses seen in previous studies were not observed here, possibly use of a serum-free medium might allow responses to growth factors to be more easily distinguished.

5.7.4 Protein expression

To investigate levels of protein expression, Western blotting was performed for ERα, ERβ1 and β-actin (Section 5.4). ERα and β-actin were both expressed at similar levels in the two cell lines, but the assay was not sufficiently sensitive to detect ERβ1 in either cell line. An ELISA was then designed, as this technique is reported to have high sensitivity (Section 5.5). The assay was sensitive to between 0.1 and 0.5 ng recombinant ERβ1, but did not detect expression in either cell lysate, suggesting that ERβ1 expression is less than 1 ng/μg total protein in both cell lines. These results confirm that ERβ1 protein is expressed at very low levels or is absent in the cell lines.

It was suggested that the sensitivity of the ELISA might be increased by using either a sandwich assay, using two different antibodies specific to different regions of the ERβ1 molecule or a competitive assay. This method involves binding a known amount of rERβ1 to the plate, and pre-incubating a limiting amount of the primary antibody with either a known amount of rERβ1 or lysate before adding it to the plate. A competitive assay was designed and tested using rERβ1L (Section 5.5.1), but was shown to be less sensitive than the standard ELISA. A sandwich ELISA is available commercially for ERα, which claims to detect ERα in 0.6 μg nuclear MCF7 extract, and allows up to 10 μg extract to be loaded (Active Motif). As the present study suggests that ERα protein expression in MCF7 is < 0.2 ng/μg (Section 5.4), this suggests the commercial assay is sensitive to about 0.1 ng ERα. The ELISA for ERβ1 used in the present study was able to detect 0.5 ng ERβ1, but only 0.6 μg cell extract could be loaded. This suggests that an assay similar to the commercially available ERα assay may show increased sensitivity over the method used here.

Fixed MCF7 and MCF7β1x cells were immunostained for expression of ERα and ERβ1 (Section 5.6). It was noted that MCF7β1x cells are generally smaller than the parental cell line. Staining using anti-ERβ1 showed mostly nuclear staining and similar levels of staining in both cell lines (Fig. 5.9, 5.10) Staining was stronger in cells in less confluent areas, which may be either an artefact of the staining procedure, or suggest that dividing cells express higher levels of ERβ1. Staining using anti-ERα (Fig. 5.7, 5.8) was dependent on staining procedure. MCF7 cells showed variable nuclear staining as well as a background level of cytoplasmic

staining. MCF7β1x cells showed a more unexpected pattern of staining. As noted for ERβ1, staining was stronger in cells on the edges of groups. However, although some cells showed fairly strong nuclear staining, in other cells stain was excluded from the nucleus, leaving the blue counter-stain visible.

A further control, which could have been used with any of the protein-analysis methods to ensure the specificity of the assays, would involve addition of excess, unbound recombinant protein or blocking peptide to the primary antibody. This should inhibit specific binding, allowing any non-specific binding to be identified.

5.7.5 Conclusion

Results from the series of PCR experiments (Sections 5.2-5.3) reinforced the pattern of estrogen response seen in standard MCF7 cells previously (Chapter 3), showing similar responses over 24 h, and adding more information about early responses and interactions with growth factors. However, few significant differences were seen between responses in the parental MCF7 and the ERβ1 over-expressing cells. This lack of variation could be attributed to a variety of factors. Firstly, no quantitative measurement of ERβ1 protein in the cells was achieved (Section 5.4, 5.5), so it is unknown whether the up-regulation of ERβ1 at the mRNA level results in a corresponding increase in the level of protein. It has been shown that ERβ1 mRNA does not correlate with protein levels in breast cancers [O'Neill et al., 2004; Shaw et al., 2002]. The mRNA may not be translated, or, if the MCF7β1x cells are over-expressing ERβ1 protein, it could be rapidly destroyed or inactivated. Alternatively, if ERβ1 protein levels were increased in the MCF7β1x cells, this would suggest that ERβ1 plays a relatively small role in modulating the estrogen response of the series of genes in this study, the cells instead relying on the levels of ERα and other co-factors to control the estrogen response.

The estrogen response of breast cancer cell lines has been shown to vary in a cell line and treatment dependent manner (Chapter 3). The experiments in this chapter have shown that expression of different genes and isoforms are regulated in a time-dependent manner in MCF7 cells and that growth factor treatment can affect the estrogen response. Despite the lack of a quantitative measure of ERβ1 protein in the MCF7 and MCF7β1x cell lines, these data nevertheless give interesting information about the estrogen response in MCF7 cells.

Chapter 6 Discussion

This thesis has investigated the role of ERβ in the estrogen responses of breast cancer cell lines. Understanding the mechanisms by which cells respond to estrogenic stimuli may help further our understanding of their responses to endogenous estrogen and to hormone therapy-type treatments. ERβ is expected to play a role in these responses, as it has been shown to directly mediate estrogen response and to modulate response via ERα in a variety of systems (Section 1.5 and e.g. [Monroe et al., 2005; Murphy et al., 2005; Omoto et al., 2003]). However, the role of ERβ in breast cancer is not yet fully elucidated with conflicting reports as to its prognostic value (Section 1.3.3). It is hoped that developing an understanding of the role of ERβ in signalling may help to predict its role in breast cancer.

To investigate the relationship between endogenous ERβ expression and estrogen responses, a panel of breast cancer cell lines with differing levels of expression of ER subtypes and isoforms was used (Chapter 3), which allowed patterns of estrogen response, measured under defined growth conditions, to be related to the endogenous expression of ERs in the cell lines. However, the pattern of estrogen receptor expression in these cell lines was shown to be highly complex, making it difficult to directly relate the responses to treatment to expression of specific ERs (Section 3.4.6). Further experiments were subsequently performed using MCF7 cells and a population of cells over-expressing ERβ1 mRNA (MCF7β1x), in order to further define the estrogen response in MCF7 cells and to investigate the effect of ERβ1 over-expression on these responses (Chapter 5).

6.1 Approach

6.1.1 Defining an estrogen response

The estrogen receptors are known to act through many different mechanisms, and different ERs mediate different levels and types of response at different promoters (Section 1.5, 3.1.3.2). In order to observe a variety of estrogen responses, expression of a set of ERs, estrogen responsive genes and housekeeping genes was measured by Q-PCR in each experiment (Section 3.1.4). This type of assay was chosen for reasons

discussed previously (Section 3.1.2), briefly, that such a system would allow efficient and sensitive measurement of a variety of different estrogen responses in terms of endogenous gene expression. A stringent limit was set on how estrogen responses were determined by using multiple statistical tests including the Student's *t*-test and ANOVA, whilsy replicate experiments were also performed and combined for analysis to offset variation. Each Q-PCR assay was individually optimised (Section 3.2) and results of preparatory experiments suggested that changes in expression of less than 2-fold could be distinguished by calculating the mean +/- SEM. In practice, the level of sensitivity varied between assays and samples. The fold-response which could be detected was defined using a cut-off of P=0.05 in a set of statistical tests (Tables 3.4 to 3.7) and sensitivity could be improved by including increased numbers of replicates in each test. These assays were able to detect and quantify estrogen responses and to differentiate between different patterns of responses in the various cell lines in a statistically relevant manner.

6.1.2 Establishment of the MCF7β1x cell line

Experiments were performed with the aim of preparing an MCF7 cell line to express ERβ1 in an inducible fashion (Chapter 4). Such a cell line would facilitate investigation of the effects of different levels of ERβ1 expression in a defined signalling background. A cell line showing constitutive over-expression of ERβ1 was generated using the T-REx system, which was then further characterised and used in downstream experiments (Chapter 5).

The genotype of the MCF7β1x cells was shown to be of the same lineage as that of the MCF7 cells by measuring mRNA expression of fusion genes generated by specific chromosomal rearrangements (Section 4.4.6). This assay provides a quick and easy method of detecting specific genotypic lesions without having to extract genomic DNA or prepare chromosome spreads. Both fusion genes were shown to be expressed in MCF7 and MCF7β1x cells, but not in MDA-MB-231, T47D or ZR75 cells. BCAS3/BCAS4 had previously been shown to be expressed only in MCF7, in a set of 12 cell lines, but its expression had not previously been assessed in MDA-MB-231, T47D or ZR75 [Barlund et al., 2002]. IRA1/RGS17 had been identified in MCF7, and its cell line-specific expression hypothesised, but not tested [Hahn et al.,

2004]. The present data support this hypothesis, as well as further reinforcing the MCF7 specificity of BCAS3/BCAS4 expression.

6.2 Estrogen response in breast cancer cell lines

Different cell lines show diverse responses to treatment, both in terms of the genes affected and the levels of response (Section 3.3.3). The results of this study correspond partly, but not fully, to reports in the literature in this respect (Section 3.4.4.2). Discrepancies between studies may be due to variation in phenotype of cell lines, different experimental conditions (growth medium, withdrawal regime, concentration of effectors) and variation in the method used to identify changes in expression (northern blot, PCR, Western blot) (Section 3.4.5). A previous report showed that the estrogen response of cells varied depending on previous growth conditions [Read et al., 1989], and many variations in estrogen responsiveness of, for instance, MCF7 and T47D cells, have been reported. Interestingly, a microarray based study measuring estrogen response in MCF7 cells failed to observe a response in PR expression, but did observe up-regulation of PS2 and XBP1 mRNAs [Wang et al., 2004], illustrating that not even all classic responses are observed in all studies. It has been shown that expression patterns in breast cancer cell lines (MCF7 and ZR75) undergoing different treatments (estrogen and tamoxifen) cluster by cell line rather than by treatment [Seth et al., 2002], indicating that the signalling environment of the cell plays an important role in determining the response to these effectors. Differences in estrogen response between cell lines may be due to their different levels of ER, or to the presence of other factors in the cells.

Duplicate experiments in this study did not always show significant changes in expression of the same genes (Tables 3.4 to 3.6, 5.2). This is likely to be due to slight variations in cell growth conditions, such as differences in passage number, cell confluence or cell growth rate. Cultured cells are very sensitive to small perturbations in growth conditions, for instance, changes in temperature or CO_2 levels caused by opening the incubator, so slight changes in cell confluence may well have a profound effect on gene transcription. For example cultured cells undergo contact inhibition at high densities, which may affect the gene expression response to a growth-stimulatory treatment such as estrogen. Cell confluence may also have an

indirect effect on the cellular environment as, at higher confluence, cells will use up nutrients more rapidly and produce more waste products. High densities may only be local, due to variations in cell distribution over the culture surface. To guard against variation caused by differences in growth conditions, medium was prepared in the same way on each occasion, the same amount of medium used routinely, and cells were used within a narrow passage range (Section 2.2). However, there was still considerable variation between experiments and a clear priority for the future would be to identify the source of this variation with a view to eliminating it.

6.2.1 The estrogen receptors are estrogen responsive genes

Basal expression of ER isoforms is different in each cell line. ERα expression was shown to vary in response to treatment in accordance with the literature (Section 3.4.3.1). Expression of ERβ might also be expected to be regulated by estrogenic treatment, as was demonstrated in Section 3.3.3. Interestingly, this regulation was shown to be isoform-specific, with a different subset of isoforms being affected in each cell line. The present study shows both ERβ1 and ERβ2 mRNAs to be up-regulated by estradiol in T47D cells, with ERβ2 showing the stronger response, but that ERβ5 is not affected (Table 3.7). In accordance with this, expression of ERβ1 has been previously shown to be up-regulated by estradiol in T47D cells, although expression of other isoforms was not measured [Vladusic et al., 2000]. Similarly, ZR75 cells show down-regulation of ERβ5 only in response to tamoxifen or estradiol (Table 3.7). It has previously been observed that ERα protein expression is lost in ZR75 cells after long term culture in tamoxifen [van den Berg et al., 1989], but the observation of ERβ isoform-specific down-regulation is novel. The isoform-specificity of these responses to both estradiol and tamoxifen suggests that regulation of splicing may be involved. Estradiol, acting via the ERs, has been shown to regulate splicing patterns in a cell-type dependent manner [Auboeuf et al., 2002], indicating a mechanism by which this may occur (Section 3.4.3.1). These data suggest that tamoxifen may be able to modulate a similar response.

In the first series of experiments, MCF7 cells showed sporadic down-regulation of ERβ1 mRNA in response to estradiol (Table 3.4 and appendix A), and this response was enhanced in the ERβ1 over-expressing cells (Table 5.1). Expression of ERβ1 mRNA was also down-regulated by EGF and FGF-2 in MCF7β1x cells (Section

5.3.1). The observation that regulation is conserved and enhanced in the transfected cells is significant, as most of the ERβ1 mRNA in these cells is expressed from the inserted cDNA, and is, therefore, independent of the control of the endogenous ERβ promoter. This suggests that down-regulation of ERβ1 mRNA in MCF7 cells may occur primarily through an increased rate of degradation, rather than a reduction in the rate of transcription. Down-regulation of ERα mRNA in MCF7 cells is regarded as being partially mediated by such post-translational mechanisms [Saceda et al., 1988], so it is feasible that a similar mechanism may affect ERβ1 mRNA.

MCF7 and MCF7β1x cells were also used to investigate estrogen response between 0 and 8 h (Section 5.2.2). Most genes showed a similar response over this time as at 24 hours (Section 5.2.2.2, Fig. 5.2). Interestingly, the exceptions were ERβ1 and ERβ2. ERβ1 is down-regulated by estradiol in MCF7β1x cells after 24 hours, but showed no response over 8 hours, whereas ERβ2 shows no response to treatment after 24 hours, but did show a peak in expression in response to estradiol after 4 hours, in both cell lines. Expression of ERβ5 is not affected by treatment at either time point. These data indicate that expression of different ER isoforms is regulated in a time-dependent manner in these cells.

6.2.1.1 Measurement of ERs in breast cancer

Expression of ERα and, increasingly, ERβ isoforms may be used as a prognostic marker in breast cancer. Over-expression of ERα in benign breast is associated with an increased risk of breast cancer [Khan et al., 1994], and ERα expressing tumours are more likely to respond to endocrine therapy [Ali and Coombes, 2000]. ERα expression is usually measured at the protein level, however, the level of expression of ERα protein and of its mRNA has been shown to correlate well in a series of tumour samples [O'Neill et al., 2004], suggesting that variation of mRNA expression in response to treatment may reflect a similar variation at the protein level. However, a study using MCF7 and T47D cells shows that although ERα protein and mRNA show similar responses to estrogen, this correlation may not be conserved in response to tamoxifen or ICI 182,780 [Pink and Jordan, 1996]. Thus, although expression of ERα mRNA and protein are coupled in cells stimulated by natural or endogenous estrogens, such as tumour samples and untreated and estradiol treated cell lines, this coupling may be lost on treatment with artificial ER ligands which may change the rate of ER protein degradation or mRNA stability. Levels of ERβ isoforms show

weaker correlation between mRNA and protein in clinical samples, e.g. ERβ1, [O'Neill et al., 2004], which may partly explain the discrepancies between different reports of the prognostic value of ERβ (Section 1.2.3) and also indicate that ERβ protein and mRNA levels and not so closely coupled as is seen for ERα. However, the present results suggest that despite the lack of correlation between mRNA and protein levels, regulation of ERβ mRNAs may play an important role in estrogen response.

6.2.1.2 Clinical implications of estrogen responsive ER expression

The present results, as well as those of studies such as [Pink and Jordan, 1996], suggest that different breast cancer-derived cell lines respond differently to estrogen or therapeutic treatments, with respect to expression of ERs. It follows that different breast tumours will also show completely different responses to therapies. For example, two tumours may initially express similar levels of estrogen receptors, as do the cell lines MCF7 and T47D. As these tumours would be ERα-positive, they might be treated with aromatase inhibitors, thus reducing the levels of circulating estrogen. The "MCF7-like" tumour will respond by decreasing expression of ERα and possibly ERβ1, whereas the "T47D-like" tumour will respond by decreasing levels of ERα and increasing ERβ1 and ERβ2 expression. These differences might be expected to have a profound effect on the downstream response to treatment. These data suggest that the single time-point measurements of ER expression used in prognostic investigations may not give as much information about phenotype, with respect to estrogen responsiveness, as previously believed. This may help to explain the 30% of ERα positive tumours which do not respond to therapy in the predicted manner [Ali and Coombes, 2000].

6.3 Measurement of ER protein expression in MCF7 cells

MCF7β1x cells over-express ERβ1 mRNA, but unfortunately it proved impossible to show whether this over-expression was conserved at the protein level (Sections 5.4-5.5, 5.7.4). Immunostaining of MCF7 and MCF7β1x cells (Section 5.6) showed that MCF7β1x cells were generally smaller than the parental MCF7 cells, indicating some phenotypic change between the two cell lines. Staining for ERβ1 was similar in the two cell lines, with stronger nuclear staining in cells in less confluent areas (Figs

5.9, 5.10). Anti-ERα staining also showed variable nuclear staining. Some MCF7β1x cells apparently showed ERα staining excluded from the nucleus, which was not seen in MCF7 cells.

These slight differences in phenotype between the two cell lines do not apparently result in significantly different patterns of gene expression at the mRNA level, except with regard to ERβ1 expression (Section 5.2, 5.3). This indicates that either these cells do not over-express ERβ1 protein, or that any over-expression has no down-stream effect on expression of the target genes. This, however, would seem surprising, given the degree of control of expression of individual ERβ isoforms observed in this study (Sections 3.3.3, 5.2.1.1, 6.3). The most likely role of ERβ1 is in modulating the expression of estrogen responsive genes. If changing the levels of ERβ1 in cells has no effect on downstream estrogen responsive gene expression, regulation of ERβ expression would serve no purpose. It appears more likely, therefore, that the MCF7β1x cells are somehow preventing ERβ1 protein from being accumulated from the mRNA expressed from the transfected cDNA, rather than the ERβ1 protein having no downstream effects. This might be related to the down-regulation of ERβ1 mRNA in the presence of estrogen or indicate an extra level of control of ER expression at the level of translation, which might help explain the lack of correlation between ERβ mRNA and protein levels.

6.4 Perspectives

This project has investigated various aspects of the response to estrogenic treatments at the level of gene expression in breast cancer cell lines. Several questions have been raised, which could be addressed by further experiments.

Both the 8 h time course and the growth factor experiments performed with the MCF7 and MCF7β1x cells would benefit from further replication to validate the responses observed. In addition, it would be interesting to perform these experiments using the other cell lines in order to determine whether these responses varied in different cell lines. Experiments could also be performed using, for instance, actinomycin D or cyclohexymide in order to determine whether the gene expression responses observed are dependent on mRNA or protein synthesis. This would indicate whether expression changes were affected by, for instance, estrogen-

dependent changes in estrogen receptor levels and also indicate whether some of the estrogen responses seen are secondary responses to changes in levels of transcription-related proteins such as XBP1 and EFP.

A more sensitive assay for ERβ1 protein, and possibly also other isoforms, would allow the question of whether the transfected cells over-express ERβ1 at the protein level to be addressed and also allow investigation of whether the regulation of expression of ERβ mRNA is conserved at the protein level.

The original aim of the transfection experiments was to create a cell line in which ERβ1 expression was inducible. This remains an important milestone for the field and, given the complexity of isoform expression, further experiments should be performed to create such a cell line, in parallel with cell lines expressing other ERβ isoforms in an inducible manner.

A key outcome of this work has been the overview of the complexity of estrogen signalling. The time-dependent regulation of specific ER isoforms points to a constantly changing signalling environment. Acquiring dynamic, multi-parametric data could shed light on the complex regulation of ER expression and the downstream effects of this regulation. Results of the experiment using EGF and FGF-2 to modulate the estrogen response of MCF7 and MCF7β1x cells give a hint of the interactions between the estrogen response and other signalling pathways. *In vivo*, tumours exist in a complex environment, under the influence of many signals. These include the endogenous and exogenous estrogens studied in this project, as well as endocrine, paracrine and autocrine growth factors, of which EGF and FGF-2 are examples, as is pleiotrophin [Riegel and Wellstein, 1994], which has been shown to be endogenously expressed by, and required for growth of, MDA-MB-231 cells [Hamma-Kourbali et al., 2006; Wellstein et al., 1992]. Additionally, tumours are affected by "matrix signals" that result from the pathological breakdown of matrix in invasive tumours, e.g., the release of small leucine rich repeat proteoglycans including decorin and biglycan, which bind to receptor tyrosine kinases (RTKs), such as EGFR, as alternative ligands [Moscatello et al., 1998]. Given the known interactions between estrogen response and other signalling pathways, it seems likely that many of these signals may modulate the estrogen response of tumour cells. Further work using combinations of treatments, or measurement of levels of

endogenously expressed growth factors, is likely to yield important information about these interactions and provide much needed insights into the application of treatments with multiple receptor antagonists.

Appendix A

Expression of a panel of genes in up to four different cell lines in experiment series A (Section 3.3.1 and 5.2)

Experiment series A comprised five separate experiments. Each of these used a different combination of cell lines, as listed below. In each experiment, cells were treated, with various stimuli in single biological replicates. Expression of a panel of genes (Section 3.1.3) was then measured by Q-PCR. Expression of each gene was assayed using a single PCR plate, allowing comparison of expression between, as well as within cell lines.

Here, normalised (Section 3.2.5) expression of each gene in each cell line after different treatments is shown as mean + SD. The unit of measurement is SQ, or starting quantity. This was calculated using a cloned standard and in the raw data is equivalent to attomoles of target per PCR reaction and as such it can be assumed that genes with higher SQ show higher expression. Beneath each graph, results of a 2-way ANOVA are summarised. These results indicate whether expression differs in each cell line, whether a significant response to changes in treatment is observed and whether this response is conserved in different cell lines (interaction).

Cell lines assayed in each experiment

Experiment A1: MCF7, MDA-MB-231, T47D and ZR75.

Experiment A2: MDA-MB-231, T47D and ZR75.

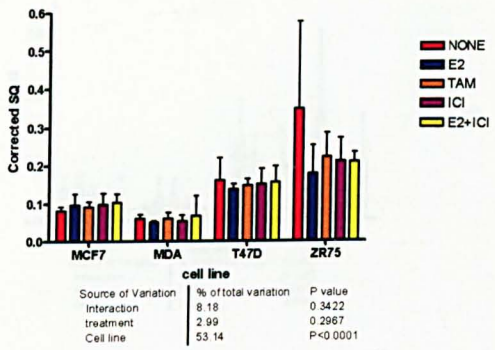
Experiment A3: MDA-MB-231 and T47D.

Experiment A4: MCF7 and MCF7β1x.

Experiment A5: MCF7 and MCF7β1x.

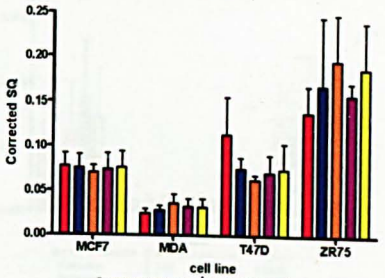
7.1 Experiment A1

ARF1



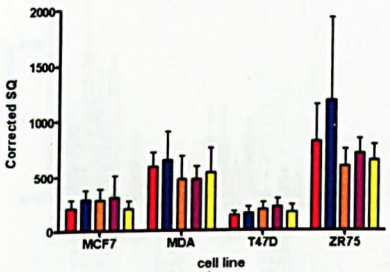
Source of Variation	% of total variation	P value
Interaction	8.18	0.3422
treatment	2.99	0.2967
Cell line	53.14	P<0.0001

PPP



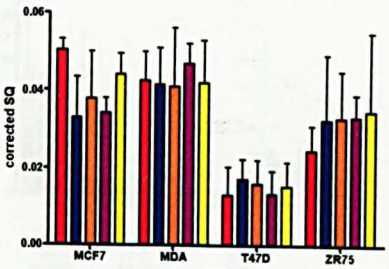
Source of Variation	% of total variation	P value
Interaction	5.33	0.1800
treatment	0.28	0.9220
cell line	75.68	P<0.0001

GAPDH



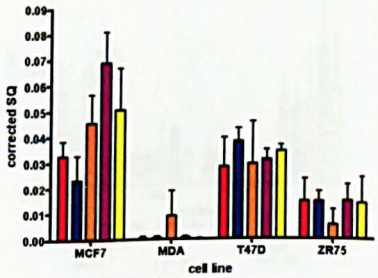
Source of Variation	% of total variation	P value
Interaction	7.59	0.3781
treatment	4.12	0.1429
cell line	53.73	P<0.0001

HPRT



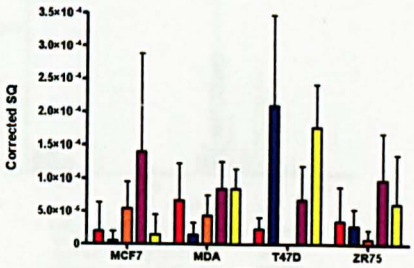
Source of Variation	% of total variation	P value
Interaction	7.14	0.4595
treatment	0.52	0.9264
cell line	56.68	P<0.0001

ER alpha



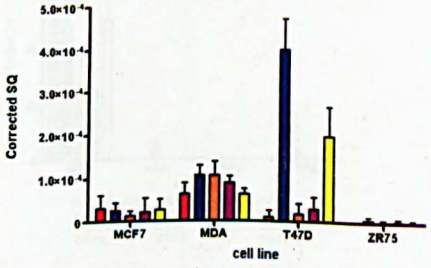
Source of Variation	% of total variation	P value
Interaction	14.34	P<0.0001
treatment	3.46	0.0086
cell line	68.40	P<0.0001

ERbeta1



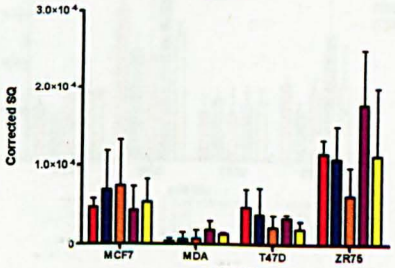
Source of Variation	% of total variation	P value
Interaction	34.57	0.0003
treatment	12.53	0.0055
cell line	6.79	0.0401

ERbeta2



Source of Variation	% of total variation	P value
Interaction	46.42	P<0.0001
treatment	17.87	P<0.0001
cell line	28.61	P<0.0001

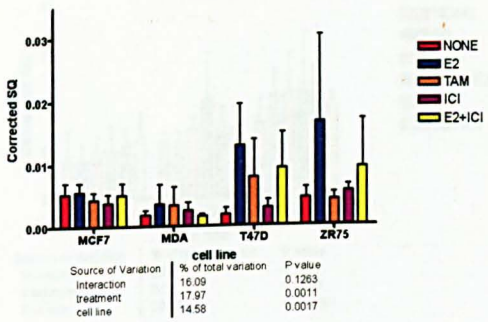
ERbeta5



Source of Variation	% of total variation	P value
Interaction	11.34	0.0948
treatment	2.43	0.3734
cell line	52.41	P<0.0001

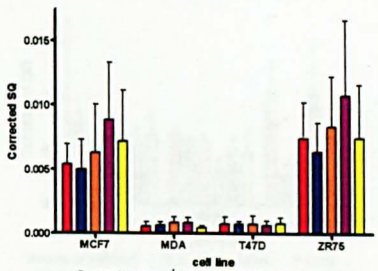
Experiment A1

CathD



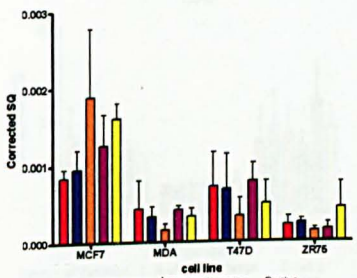
Source of Variation	% of total variation	P value
Interaction	16.09	0.1263
treatment	17.97	0.0011
cell line	14.58	0.0017

cyclinD1



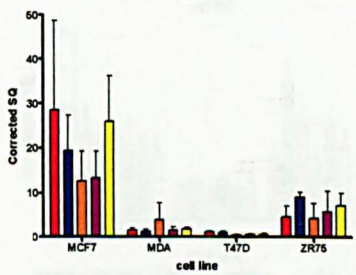
Source of Variation	% of total variation	P value
Interaction	2.98	0.9035
treatment	2.91	0.2198
cell line	84.58	P<0.0001

EFP



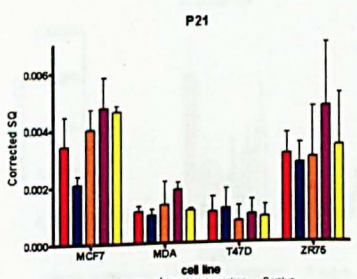
Source of Variation	% of total variation	P value
Interaction	15.55	0.0019
treatment	1.32	0.5380
cell line	58.35	P<0.0001

liv1



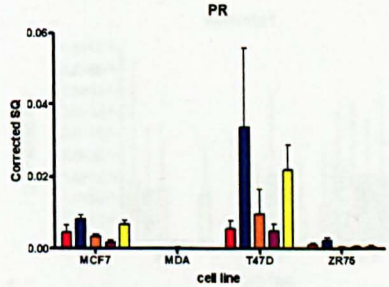
Source of Variation	% of total variation	P value
Interaction	9.27	0.0995
treatment	2.75	0.2203
cell line	80.06	P<0.0001

P21



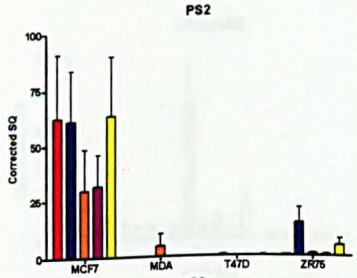
Source of Variation	% of total variation	P value
Interaction	7.73	0.1469
treatment	7.43	0.0037
cell line	59.19	P<0.0001

PR



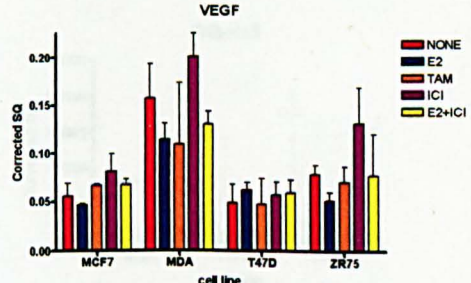
Source of Variation	% of total variation	P value
Interaction	22.49	P<0.0001
treatment	12.96	P<0.0001
cell line	39.63	P<0.0001

PS2



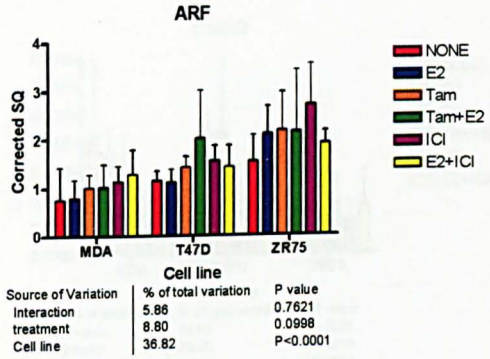
Source of Variation	% of total variation	P value
Interaction	8.14	0.0132
treatment	3.30	0.0265
cell line	71.60	P<0.0001

VEGF

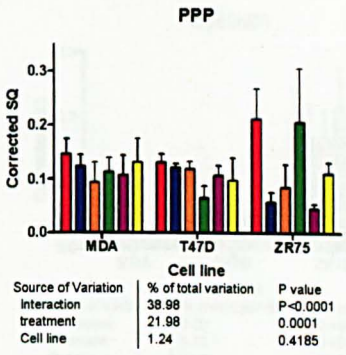


Source of Variation	% of total variation	P value
Interaction	9.73	0.0179
treatment	13.90	P<0.0001
cell line	55.12	P<0.0001

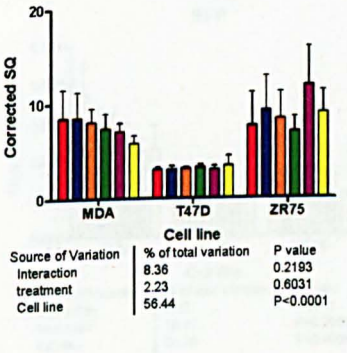
7.2 Experiment A2



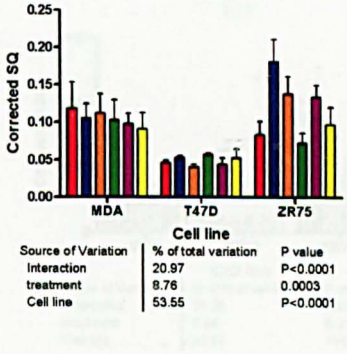
Source of Variation	% of total variation	P value
Interaction	5.86	0.7621
treatment	8.80	0.0998
Cell line	36.82	P<0.0001



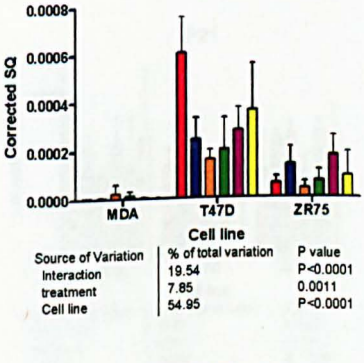
Source of Variation	% of total variation	P value
Interaction	38.98	P<0.0001
treatment	21.98	0.0001
Cell line	1.24	0.4185



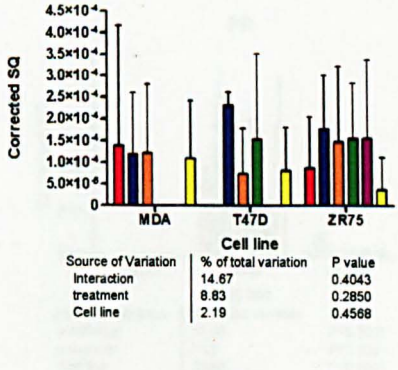
Source of Variation	% of total variation	P value
Interaction	8.36	0.2193
treatment	2.23	0.6031
Cell line	56.44	P<0.0001



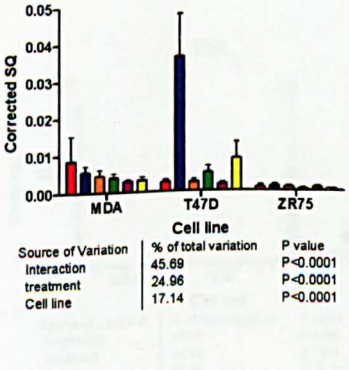
Source of Variation	% of total variation	P value
Interaction	20.97	P<0.0001
treatment	8.76	0.0003
Cell line	53.55	P<0.0001



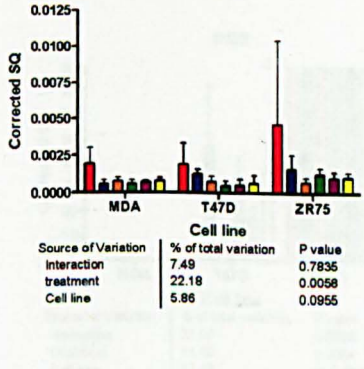
Source of Variation	% of total variation	P value
Interaction	19.54	P<0.0001
treatment	7.85	0.0011
Cell line	54.95	P<0.0001



Source of Variation	% of total variation	P value
Interaction	14.67	0.4043
treatment	8.83	0.2850
Cell line	2.19	0.4568

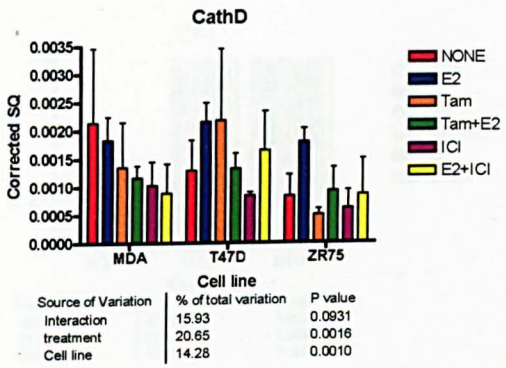


Source of Variation	% of total variation	P value
Interaction	45.69	P<0.0001
treatment	24.96	P<0.0001
Cell line	17.14	P<0.0001

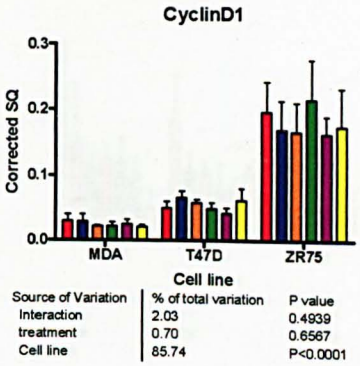


Source of Variation	% of total variation	P value
Interaction	7.49	0.7835
treatment	22.18	0.0058
Cell line	5.86	0.0955

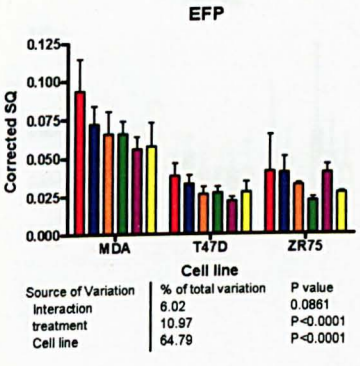
Experiment A2



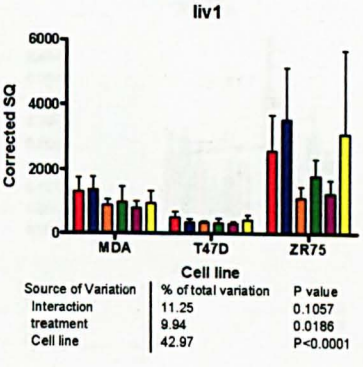
Source of Variation	% of total variation	P value
Interaction	15.93	0.0931
treatment	20.65	0.0016
Cell line	14.28	0.0010



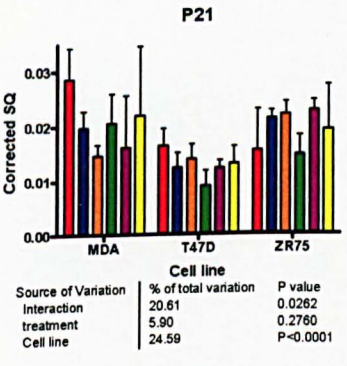
Source of Variation	% of total variation	P value
Interaction	2.03	0.4939
treatment	0.70	0.6567
Cell line	85.74	P<0.0001



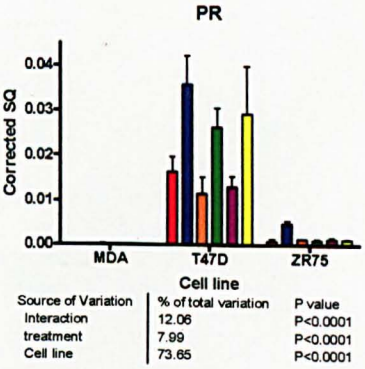
Source of Variation	% of total variation	P value
Interaction	6.02	0.0861
treatment	10.97	P<0.0001
Cell line	64.79	P<0.0001



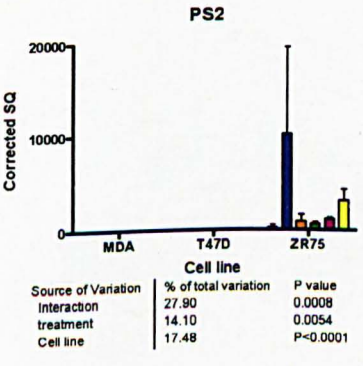
Source of Variation	% of total variation	P value
Interaction	11.25	0.1057
treatment	9.94	0.0186
Cell line	42.97	P<0.0001



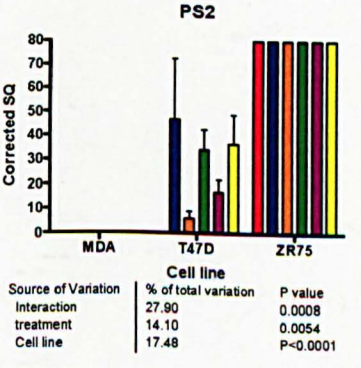
Source of Variation	% of total variation	P value
Interaction	20.61	0.0262
treatment	5.90	0.2760
Cell line	24.59	P<0.0001



Source of Variation	% of total variation	P value
Interaction	12.06	P<0.0001
treatment	7.99	P<0.0001
Cell line	73.65	P<0.0001

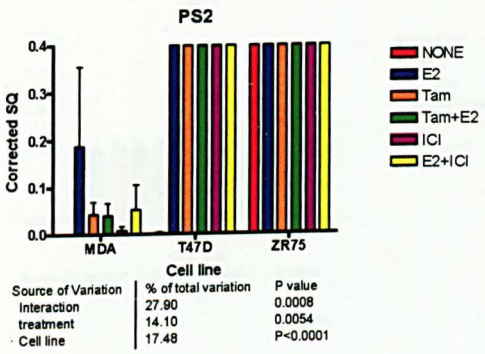


Source of Variation	% of total variation	P value
Interaction	27.90	0.0008
treatment	14.10	0.0054
Cell line	17.48	P<0.0001

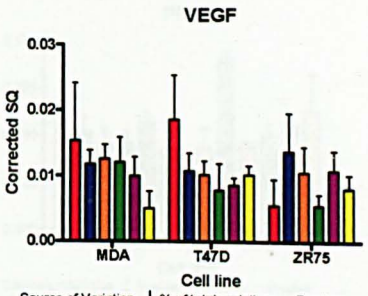


Source of Variation	% of total variation	P value
Interaction	27.90	0.0008
treatment	14.10	0.0054
Cell line	17.48	P<0.0001

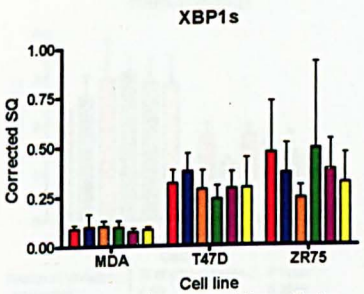
Experiment A2



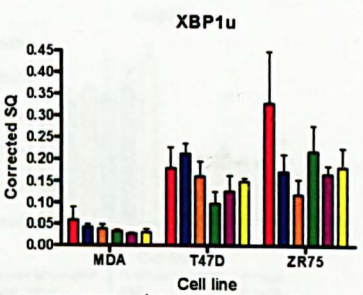
Source of Variation	% of total variation	P value
Interaction	27.90	0.0008
treatment	14.10	0.0054
Cell line	17.48	P<0.0001



Source of Variation	% of total variation	P value
Interaction	29.71	0.0027
treatment	16.25	0.0081
Cell line	3.97	0.1273

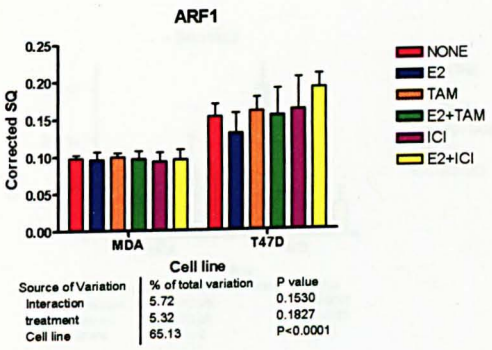


Source of Variation	% of total variation	P value
Interaction	6.48	0.6899
treatment	2.40	0.7422
Cell line	43.40	P<0.0001

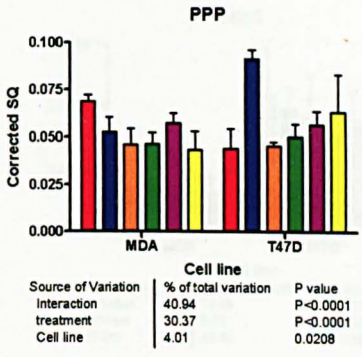


Source of Variation	% of total variation	P value
Interaction	13.89	P<0.0001
treatment	10.78	P<0.0001
Cell line	59.31	P<0.0001

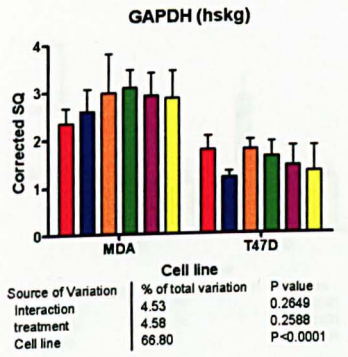
7.3 Experiment A3



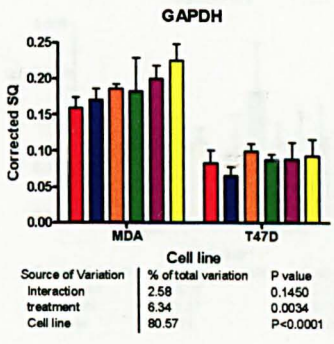
Source of Variation	% of total variation	P value
Interaction	5.72	0.1530
treatment	5.32	0.1827
Cell line	65.13	P<0.0001



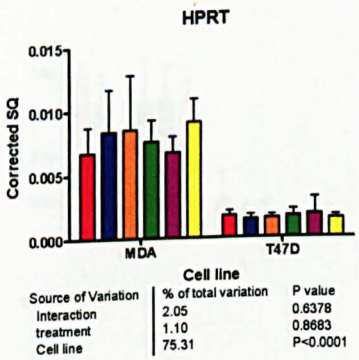
Source of Variation	% of total variation	P value
Interaction	40.94	P<0.0001
treatment	30.37	P<0.0001
Cell line	4.01	0.0208



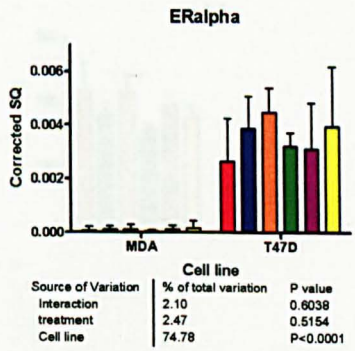
Source of Variation	% of total variation	P value
Interaction	4.53	0.2649
treatment	4.58	0.2588
Cell line	66.80	P<0.0001



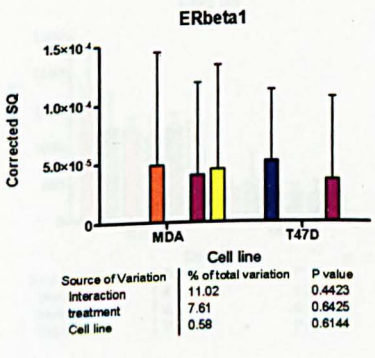
Source of Variation	% of total variation	P value
Interaction	2.58	0.1450
treatment	6.34	0.0034
Cell line	80.57	P<0.0001



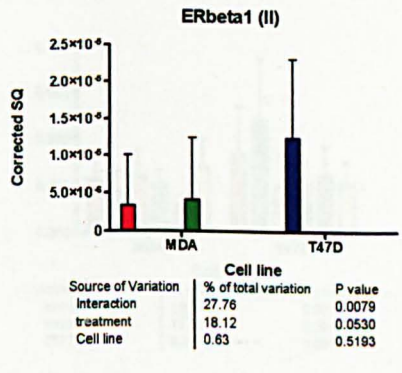
Source of Variation	% of total variation	P value
Interaction	2.05	0.6378
treatment	1.10	0.8683
Cell line	75.31	P<0.0001



Source of Variation	% of total variation	P value
Interaction	2.10	0.6038
treatment	2.47	0.5154
Cell line	74.78	P<0.0001

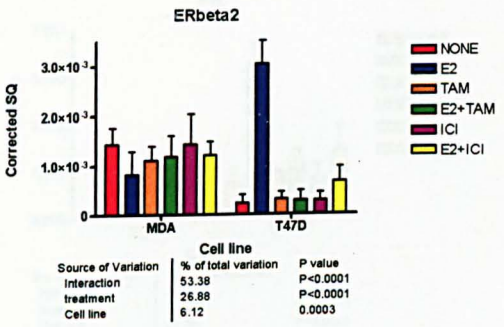


Source of Variation	% of total variation	P value
Interaction	11.02	0.4423
treatment	7.61	0.6425
Cell line	0.58	0.6144

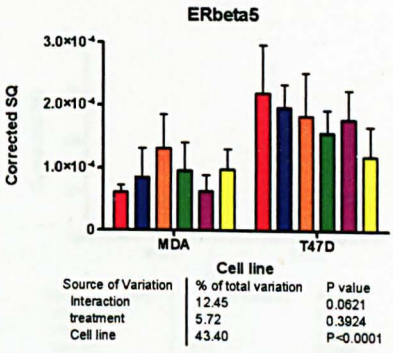


Source of Variation	% of total variation	P value
Interaction	27.76	0.0079
treatment	18.12	0.0530
Cell line	0.63	0.5193

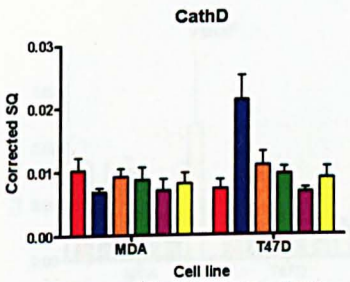
Experiment A3



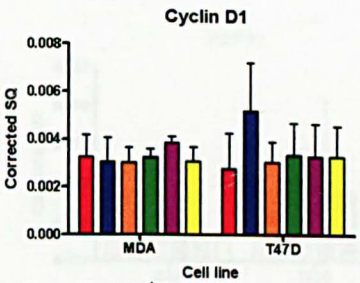
Source of Variation	% of total variation	P value
Interaction	53.38	P<0.0001
treatment	26.88	P<0.0001
Cell line	6.12	0.0003



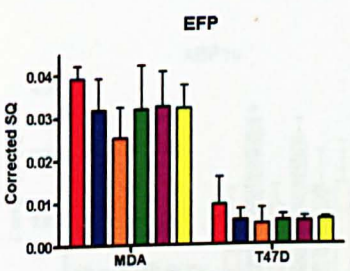
Source of Variation	% of total variation	P value
Interaction	12.45	0.0621
treatment	5.72	0.3924
Cell line	43.40	P<0.0001



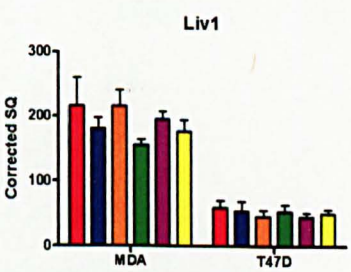
Source of Variation	% of total variation	P value
Interaction	46.00	P<0.0001
treatment	29.74	P<0.0001
Cell line	8.52	P<0.0001



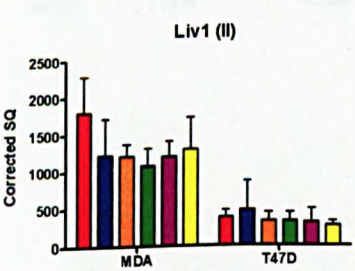
Source of Variation	% of total variation	P value
Interaction	15.04	0.2177
treatment	11.11	0.3776
Cell line	1.11	0.4636



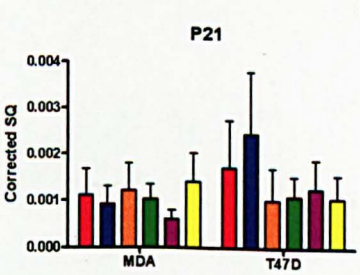
Source of Variation	% of total variation	P value
Interaction	1.01	0.6933
treatment	3.67	0.0741
Cell line	83.39	P<0.0001



Source of Variation	% of total variation	P value
Interaction	2.34	0.0106
treatment	2.25	0.0126
Cell line	90.65	P<0.0001

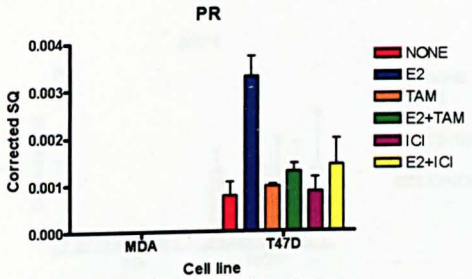


Source of Variation	% of total variation	P value
Interaction	4.14	0.2077
treatment	4.96	0.1326
Cell line	71.32	P<0.0001

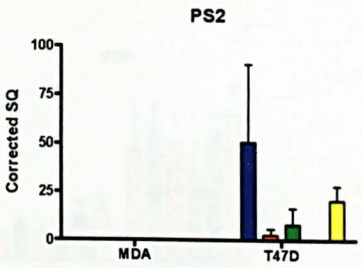


Source of Variation	% of total variation	P value
Interaction	18.85	0.0817
treatment	11.49	0.2814
Cell line	6.53	0.0615

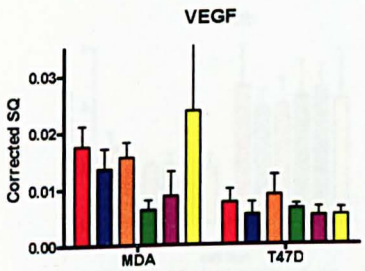
Experiment A3



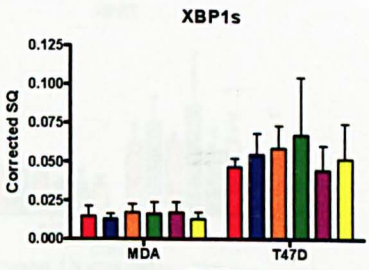
Source of Variation	% of total variation	P value
Interaction	20.23	P<0.0001
treatment	20.28	P<0.0001
Cell line	54.79	P<0.0001



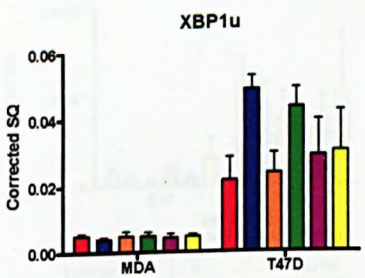
Source of Variation	% of total variation	P value
Interaction	25.62	0.0008
treatment	25.62	0.0008
Cell line	14.96	0.0003



Source of Variation	% of total variation	P value
Interaction	16.54	0.0105
treatment	18.07	0.0066
Cell line	31.09	P<0.0001

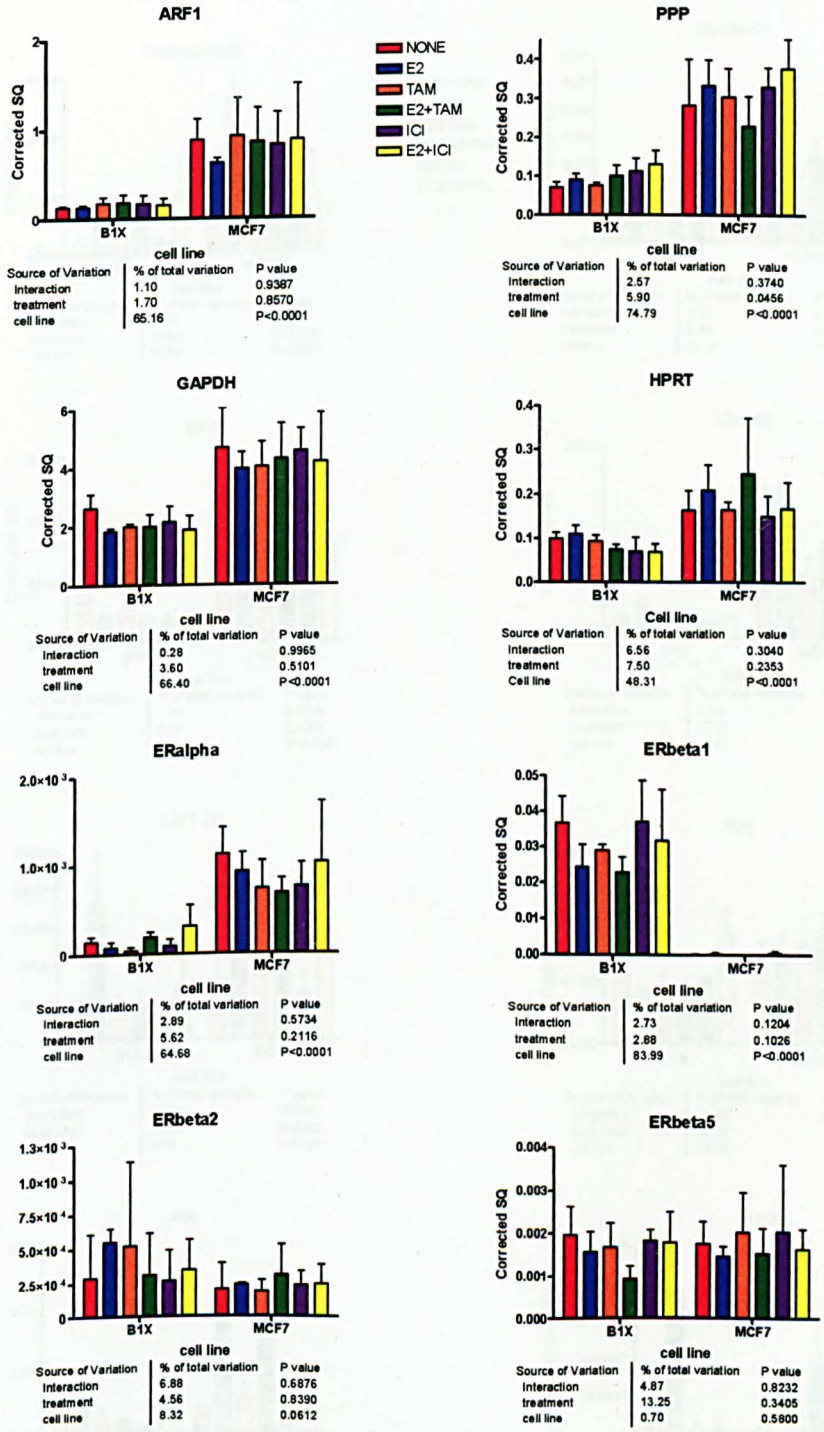


Source of Variation	% of total variation	P value
Interaction	2.25	0.7394
treatment	2.78	0.6444
Cell line	65.33	P<0.0001

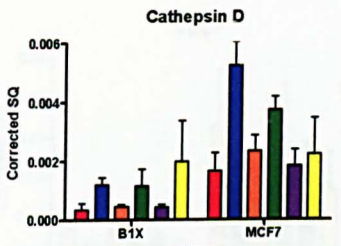


Source of Variation	% of total variation	P value
Interaction	9.68	P<0.0001
treatmnet	8.71	0.0001
Cell line	72.44	P<0.0001

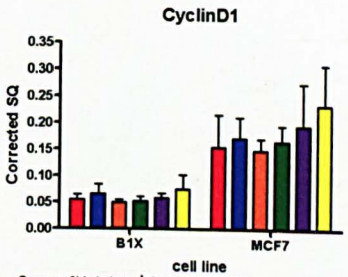
7.4 Experiment A4



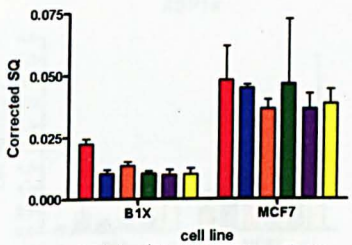
7.4 Experiment A4



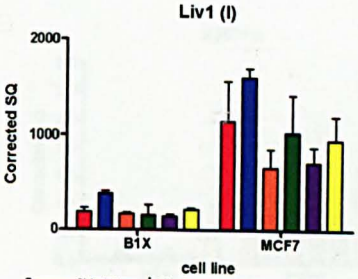
Source of Variation	% of total variation	P value
Interaction	15.57	0.0001
treatment	27.82	P<0.0001
cell line	40.86	P<0.0001



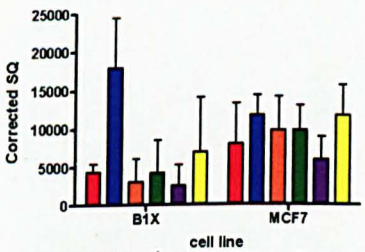
Source of Variation	% of total variation	P value
Interaction	2.34	0.6199
treatment	6.48	0.1070
cell line	67.47	P<0.0001



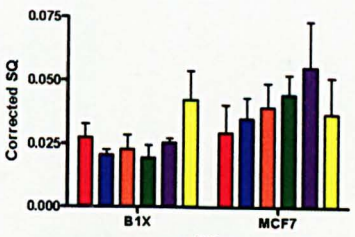
Source of Variation	% of total variation	P value
Interaction	1.88	0.6569
treatment	5.39	0.1213
cell line	72.14	P<0.0001



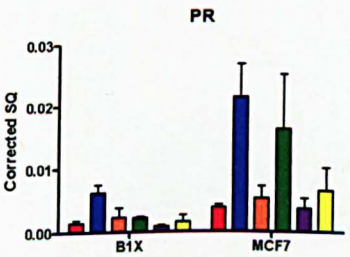
Source of Variation	% of total variation	P value
Interaction	6.12	0.0091
treatment	15.15	P<0.0001
cell line	66.62	P<0.0001



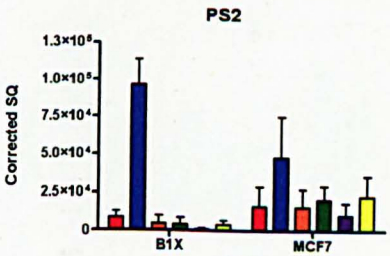
Source of Variation	% of total variation	P value
Interaction	14.45	0.0526
treatment	36.25	0.0003
cell line	6.70	0.0227



Source of Variation	% of total variation	P value
Interaction	21.40	0.0049
treatment	14.36	0.0337
cell line	26.53	P<0.0001

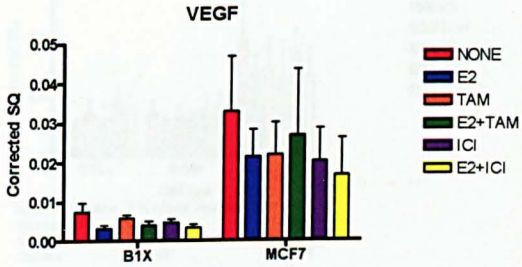


Source of Variation	% of total variation	P value
Interaction	16.11	0.0002
treatment	38.54	P<0.0001
cell line	27.22	P<0.0001

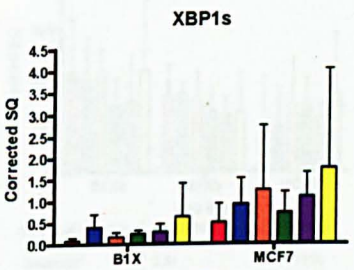


Source of Variation	% of total variation	P value
Interaction	17.29	P<0.0001
treatment	68.65	P<0.0001
cell line	0.18	0.5043

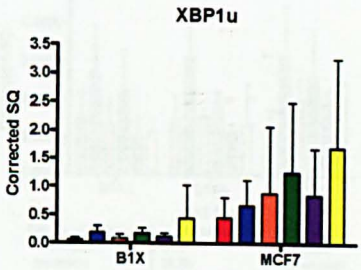
Experiment A4



Source of Variation	% of total variation	P value
Interaction	3.07	0.6304
treatment	6.93	0.1940
cell line	58.20	P<0.0001

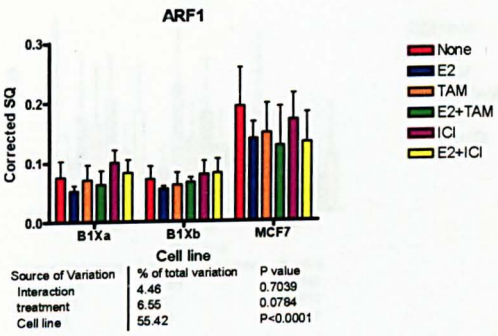


Source of Variation	% of total variation	P value
Interaction	2.53	0.9345
treatment	9.41	0.4609
cell line	16.78	0.0061

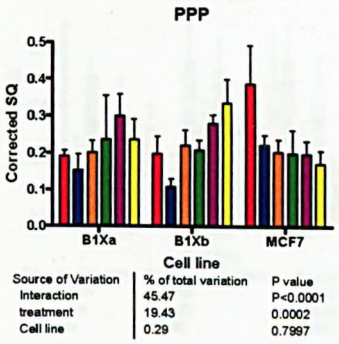


Source of Variation	% of total variation	P value
Interaction	3.46	0.8476
treatment	10.11	0.3484
cell line	23.58	0.0008

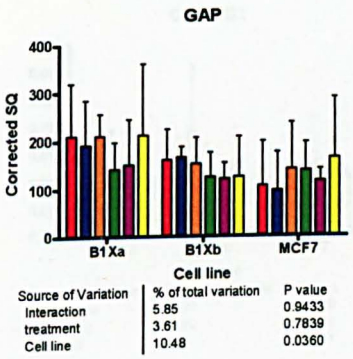
7.5 Experiment A5



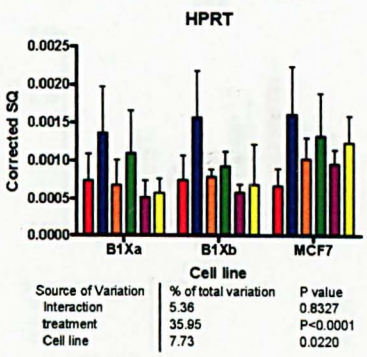
Source of Variation	% of total variation	P value
Interaction	4.46	0.7039
treatment	6.55	0.0784
Cell line	55.42	P<0.0001



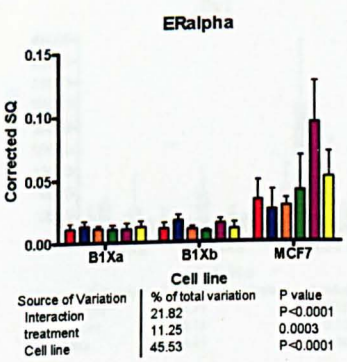
Source of Variation	% of total variation	P value
Interaction	45.47	P<0.0001
treatment	19.43	0.0002
Cell line	0.29	0.7997



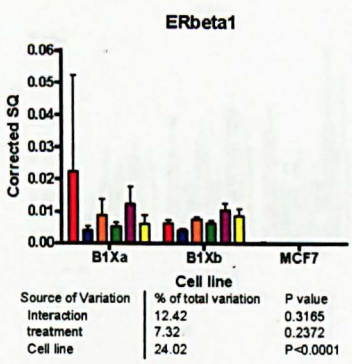
Source of Variation	% of total variation	P value
Interaction	5.85	0.9433
treatment	3.61	0.7839
Cell line	10.48	0.0360



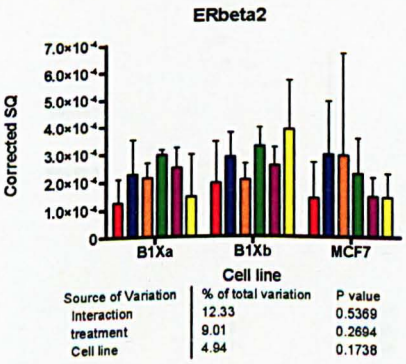
Source of Variation	% of total variation	P value
Interaction	5.36	0.8327
treatment	35.95	P<0.0001
Cell line	7.73	0.0220



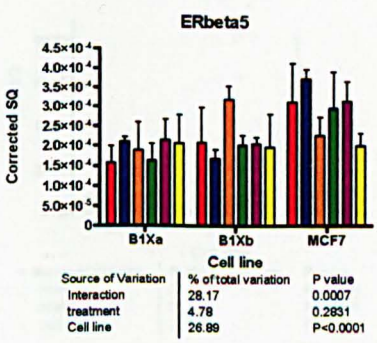
Source of Variation	% of total variation	P value
Interaction	21.82	P<0.0001
treatment	11.25	0.0003
Cell line	45.53	P<0.0001



Source of Variation	% of total variation	P value
Interaction	12.42	0.3165
treatment	7.32	0.2372
Cell line	24.02	P<0.0001

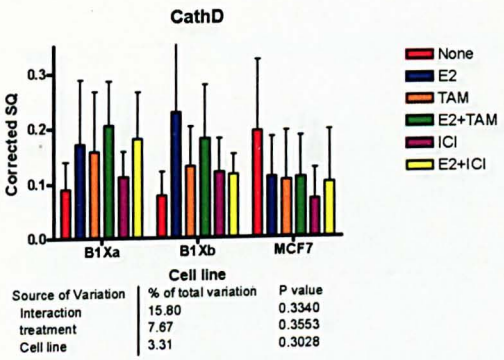


Source of Variation	% of total variation	P value
Interaction	12.33	0.5369
treatment	9.01	0.2694
Cell line	4.94	0.1738

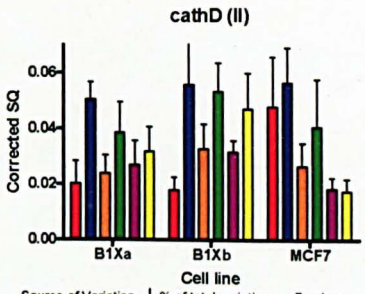


Source of Variation	% of total variation	P value
Interaction	28.17	0.0007
treatment	4.78	0.2831
Cell line	26.89	P<0.0001

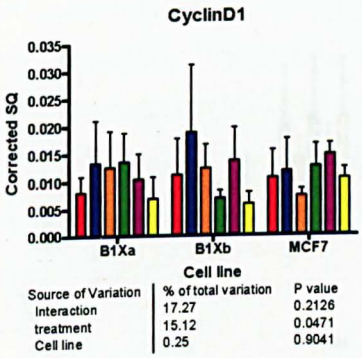
Experiment A5



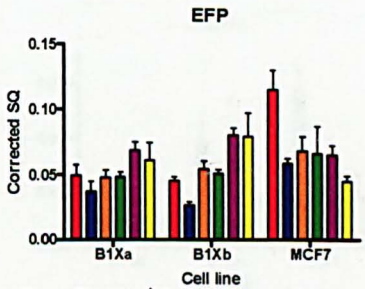
Source of Variation	% of total variation	P value
Interaction	15.80	0.3340
treatment	7.67	0.3553
Cell line	3.31	0.3028



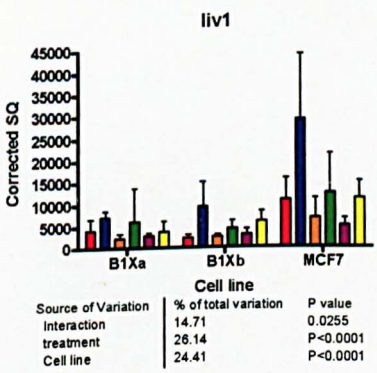
Source of Variation	% of total variation	P value
Interaction	23.05	0.0005
treatment	40.78	P<0.0001
Cell line	3.98	0.0431



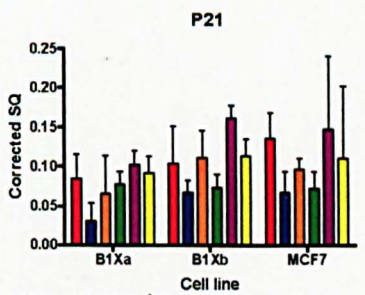
Source of Variation	% of total variation	P value
Interaction	17.27	0.2126
treatment	15.12	0.0471
Cell line	0.25	0.9041



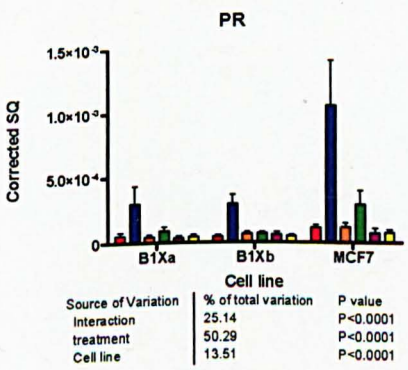
Source of Variation	% of total variation	P value
Interaction	46.99	P<0.0001
treatment	23.90	P<0.0001
Cell line	13.35	P<0.0001



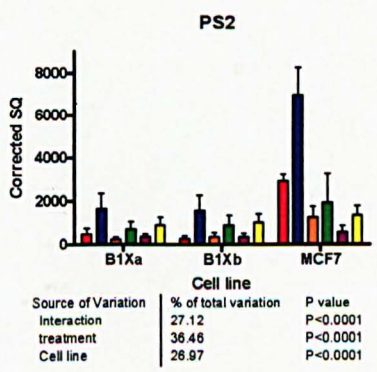
Source of Variation	% of total variation	P value
Interaction	14.71	0.0255
treatment	26.14	P<0.0001
Cell line	24.41	P<0.0001



Source of Variation	% of total variation	P value
Interaction	5.15	0.8787
treatment	30.81	0.0002
Cell line	9.04	0.0164

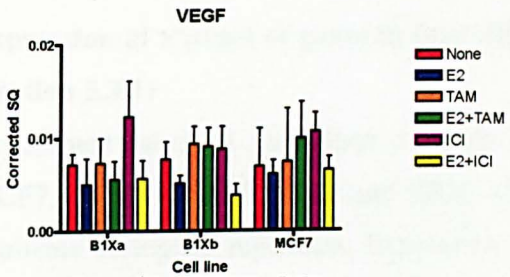


Source of Variation	% of total variation	P value
Interaction	25.14	P<0.0001
treatment	50.29	P<0.0001
Cell line	13.51	P<0.0001

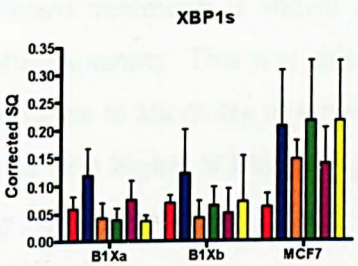


Source of Variation	% of total variation	P value
Interaction	27.12	P<0.0001
treatment	36.46	P<0.0001
Cell line	26.97	P<0.0001

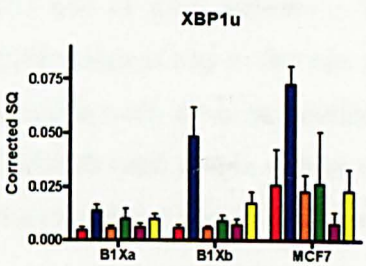
Experiment A5



Source of Variation	% of total variation	P value
Interaction	12.42	0.2835
treatment	32.62	P<0.0001
Cell line	1.16	0.5621



Source of Variation	% of total variation	P value
Interaction	12.40	0.1167
treatment	12.09	0.0129
Cell line	34.98	P<0.0001



Source of Variation	% of total variation	P value
Interaction	16.96	P<0.0001
treatment	43.98	P<0.0001
Cell line	22.84	P<0.0001

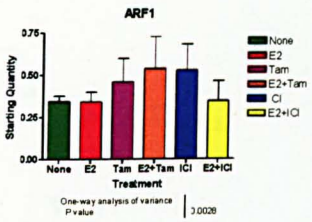
Appendix B

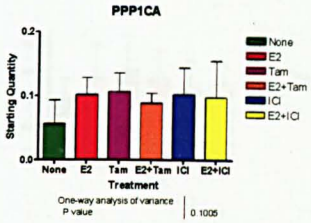
Expression of a panel of genes in four different cell lines in experiment series B (Section 3.3.1)

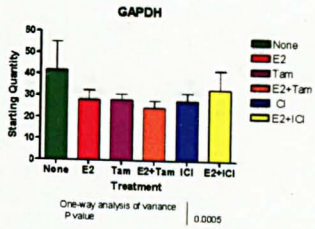
Experiment series B comprised a single set of experiments using the cell lines MCF7, MDA-MB-231, T47D and ZR75. Cells were treated with various stimuli in duplicate biological replicates. Expression of a panel of genes (Section 3.1.3) was then measured by Q-PCR.

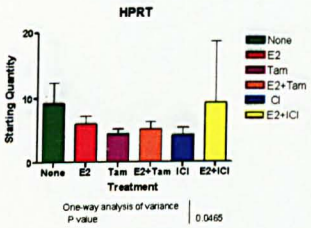
Here, normalised (Section 3.2.5) expression of each gene in each cell line after different treatments is shown as mean + SD. The unit of measurement is SQ, or starting quantity. This was calculated using a cloned standard and in the raw data is equivalent to attomoles of target per PCR reaction and as such it can be assumed that genes with higher SQ show higher expression. Beneath each graph, results of a 1-way ANOVA are summarised, indicating whether a significant response to treatment is seen.

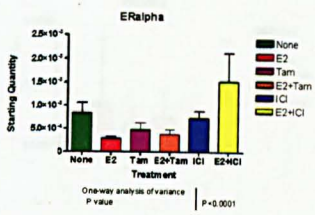
Normalised data for MCF7

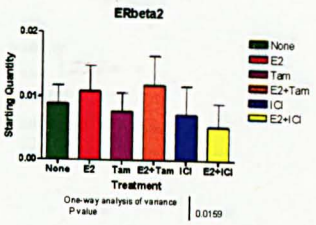


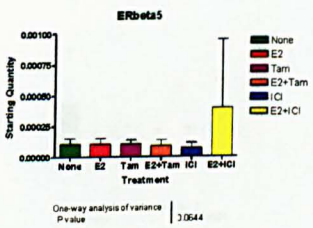


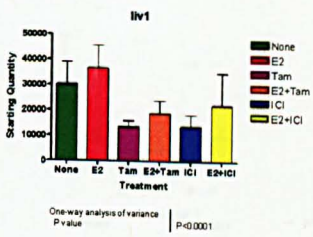


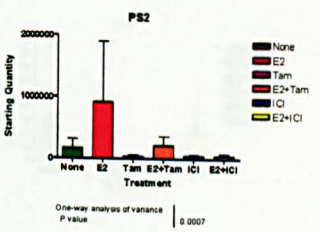


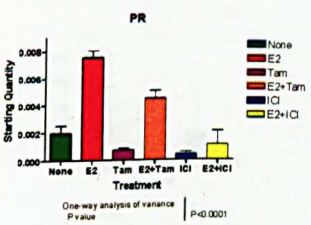


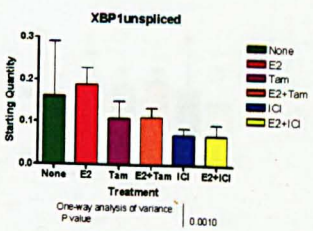


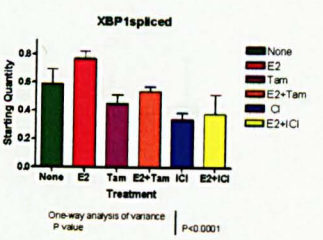




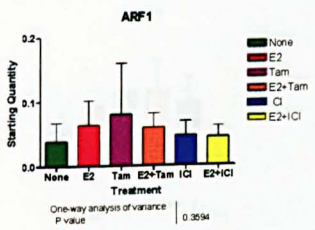


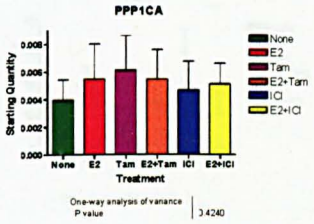


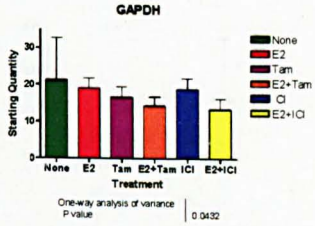


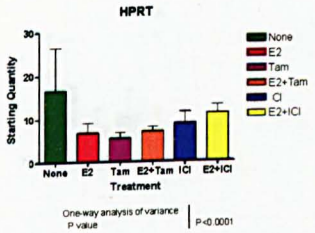


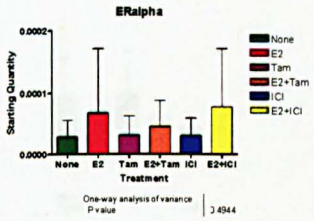
Normalised data for MDAMB231

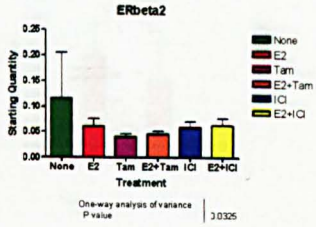


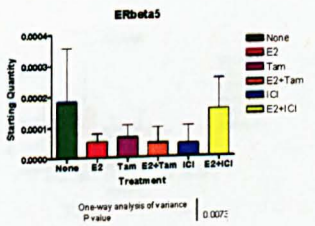


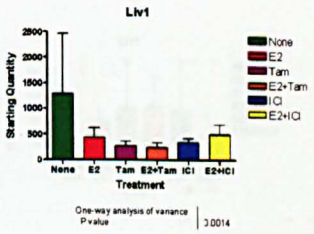


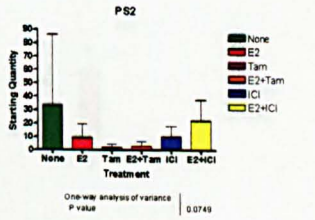


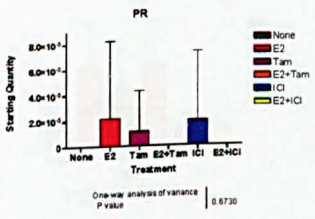


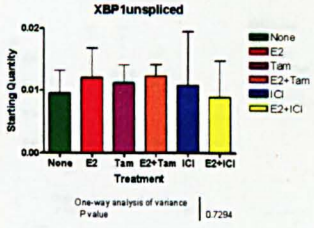


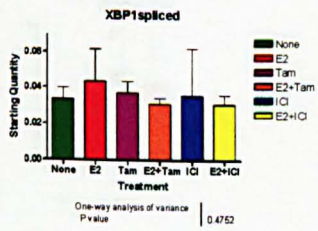












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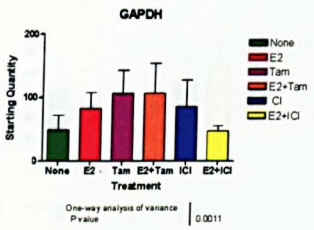

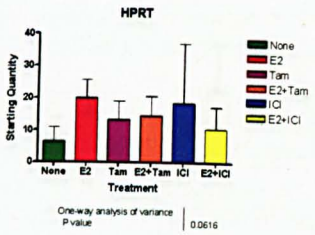

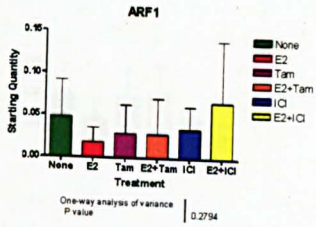

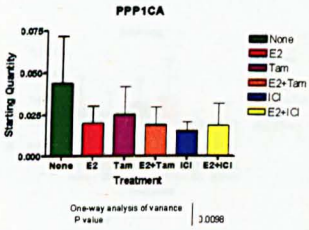

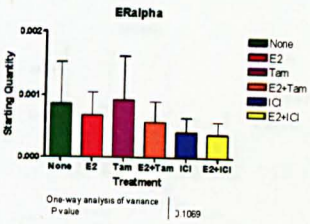

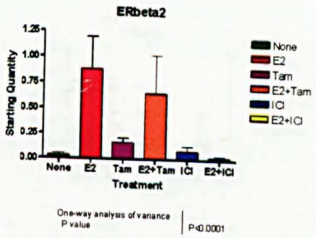

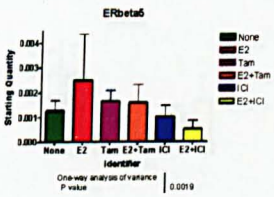

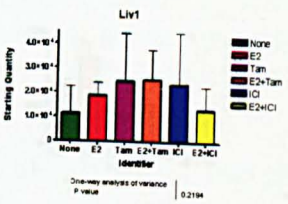

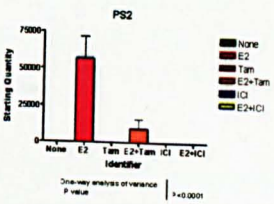

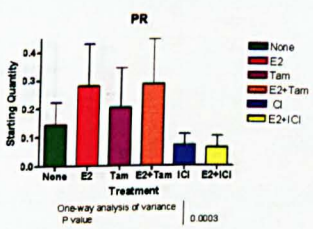

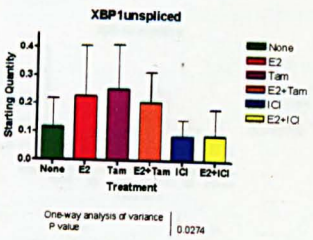

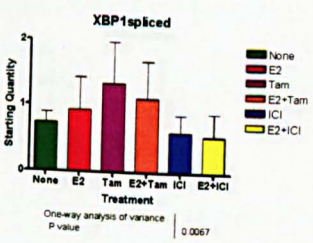

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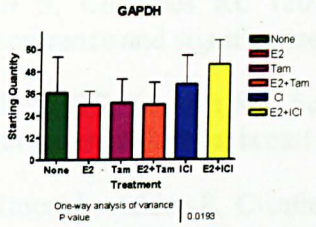

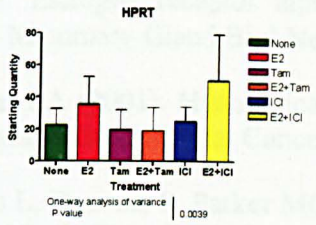

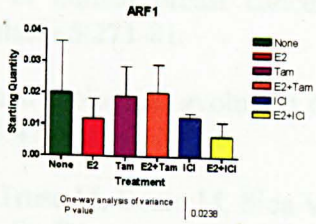

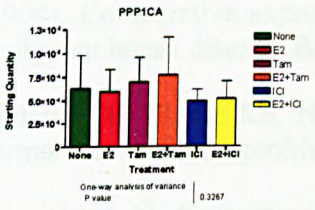

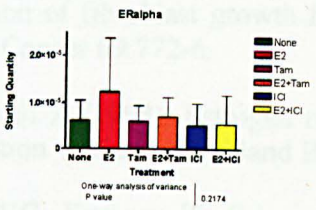

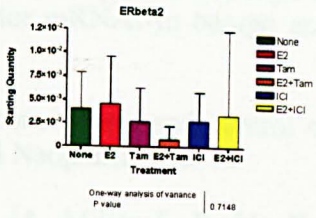

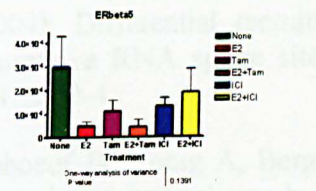

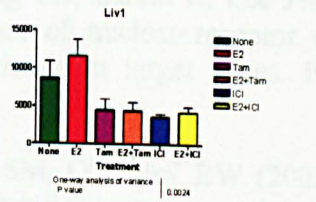

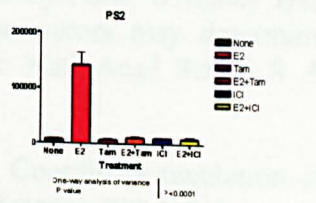

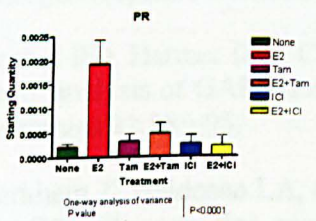

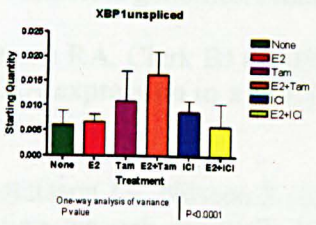

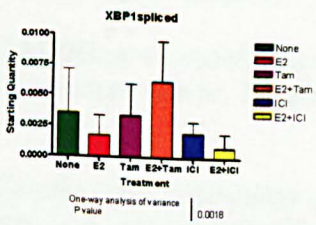

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