Effects of Long-term Tamoxifen Treatment on ERα Expression in Breast Cancer Cells

by

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Doctor of Philosophy

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In loving memory of

my grand mother Fatma Bensmail (25 Sept 1922-5 Apr 2008)
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Cancer: facts and figures</td>
<td>1</td>
</tr>
<tr>
<td>1.2</td>
<td>Breast cancer</td>
<td>2</td>
</tr>
<tr>
<td>1.2.1</td>
<td>Incidence and risk factors</td>
<td>2</td>
</tr>
<tr>
<td>1.2.2</td>
<td>Breast cancer development and subtypes</td>
<td>4</td>
</tr>
<tr>
<td>1.3</td>
<td>Oestrogen and Breast cancer</td>
<td>6</td>
</tr>
<tr>
<td>1.3.1</td>
<td>Oestrogen synthesis</td>
<td>6</td>
</tr>
<tr>
<td>1.3.2</td>
<td>Oestrogen Receptors: gene structure</td>
<td>7</td>
</tr>
<tr>
<td>1.3.2.1</td>
<td>Alternative promoter usage</td>
<td>9</td>
</tr>
<tr>
<td>1.3.2.2</td>
<td>Alternative splicing (exon skipping)</td>
<td>10</td>
</tr>
<tr>
<td>1.3.3</td>
<td>Oestrogen Receptors: protein structure and function</td>
<td>11</td>
</tr>
<tr>
<td>1.3.4</td>
<td>Oestrogen Receptor: cofactors</td>
<td>15</td>
</tr>
<tr>
<td>1.3.5</td>
<td>ER signalling</td>
<td>18</td>
</tr>
<tr>
<td>1.3.5.1</td>
<td>Classical genomic action of ERs</td>
<td>18</td>
</tr>
<tr>
<td>1.3.5.2</td>
<td>Non-Classical genomic action of ERs</td>
<td>21</td>
</tr>
<tr>
<td>1.3.5.3</td>
<td>Non-genomic action of ERs</td>
<td>21</td>
</tr>
<tr>
<td>1.3.5.4</td>
<td>Convergence of genomic and non-genomic actions of ERs</td>
<td>23</td>
</tr>
<tr>
<td>1.4</td>
<td>Regulation of ERα expression in breast cancer</td>
<td>24</td>
</tr>
<tr>
<td>1.4.1</td>
<td>Transcriptional regulation</td>
<td>24</td>
</tr>
<tr>
<td>1.4.2</td>
<td>Post-transcriptional regulation</td>
<td>28</td>
</tr>
<tr>
<td>1.4.3</td>
<td>Post-translational regulation</td>
<td>29</td>
</tr>
<tr>
<td>1.5</td>
<td>Anti-hormone therapy: response and resistance</td>
<td>31</td>
</tr>
<tr>
<td>1.5.1</td>
<td>Selective Oestrogen Receptor Modulators (SERMs)</td>
<td>31</td>
</tr>
<tr>
<td>1.5.2</td>
<td>Selective Oestrogen Receptor Downregulators (SERDs) and Aromatase inhibitors</td>
<td>34</td>
</tr>
<tr>
<td>1.5.3</td>
<td>Anti-hormone resistance</td>
<td>35</td>
</tr>
<tr>
<td>1.5.3.1</td>
<td>Growth factor signalling</td>
<td>36</td>
</tr>
<tr>
<td>1.5.3.2</td>
<td>Loss of ERα expression</td>
<td>40</td>
</tr>
</tbody>
</table>
CHAPTER 2. MATERIAL & METHODS

2.1 Routine cell culture maintenance ........................................48
  2.1.1 Cell lines .................................................................48
    2.1.1.1 MCF-7 cells .........................................................48
    2.1.1.2 TamR cells .........................................................49
    2.1.1.3 FasR cells ..........................................................49
    2.1.1.4 Tam/TK1-R cells ..................................................50
  2.1.2 Routine technical procedures for cell culture ......................50
    2.1.2.1 Cell passaging .....................................................50
    2.1.2.2 Cryo-preservation of cell lines ...............................50
    2.1.2.3 Charcoal stripping procedure for 100 ml FCS ...............51
    2.1.2.4 Cell counting .......................................................51
  2.1.3 Treatments ......................................................................52

2.2 Gene expression analysis ....................................................52
  2.2.1 Total RNA extraction ..................................................52
  2.2.2 Nucleic acid quantification by spectrophotometry ................53
  2.2.3 cDNA synthesis by reverse-transcription PCR (RT-PCR) .......53
  2.2.4 Real time quantitative PCR (qPCR) analysis of total ERα and ERα mRNA variants ......54
    2.2.4.1 Primers ...............................................................55
    2.2.4.2 Preparation of cDNA standards ...............................58
    2.2.4.3 Real-time qPCR reaction set ups ...............................58
    2.2.4.4 Data analysis .......................................................59
  2.2.5 Semi-quantitative PCR ..................................................61
  2.2.6 Agarose gel electrophoresis ............................................62

2.3 Protein expression analysis ..................................................62
  2.3.1 Protein cell lysis .........................................................62
  2.3.2 Determination of protein concentration .............................63
  2.3.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) ............64
  2.3.4 Western blotting .........................................................65
  2.3.5 Immunoblotting ..........................................................66
CHAPTER 3. RESULTS

3.1 Validation of primers used for qPCR analysis of variant-ERα mRNA expression ......74
  3.1.1 Verification of primer-specific PCR products by semi-qPCR and melting curve analysis 74
  3.1.2 E2-induced downregulation of ERα mRNA expression in MCF-7 cells .......................77

3.2 Loss of ERα expression in response to anti-hormone treatments: Proof of principle with fulvestrant in vitro ..................................................................................................................79

3.3 Analysis of ERα expression in TamR cells ...................................................................81
  3.3.1 TamR-18 cells ..................................................................................................................81
  3.3.2 Effects of short-term tamoxifen treatment on ERα expression levels ..................................85
  3.3.3 Effects of long-term tamoxifen treatment on ERα expression levels ...................................88
  3.3.4 Sensitivity to fulvestrant and basal growth rate of TamR-30 cells .................................91

3.4 Molecular mechanisms for ERα loss in TamR cells .......................................................94
  3.4.1 Role of EGFR signalling ..................................................................................................94
  3.4.2 Role of epigenetic mechanisms .....................................................................................111
  3.4.3 Role of alternative growth factor signalling pathways ...................................................115

3.5 Analysis of cell motility and invasiveness in TamR-30 cells ...........................................128
3.6 Potential Transcription factors regulating ERα expression in TamR cells: preliminary studies
3.6.1 AliBaba2.1 and Affymetrix database
3.6.2 AP2γ
3.6.3 FoxO3a

CHAPTER 4. DISCUSSION
4.1 Rationale and aims of this study
4.2 In vitro models of anti-hormone-resistance with depleted ERα levels
4.3 ERα promoter usage in TamR cells
4.4 Mechanisms of ERα loss in TamR cells
4.4.1 A reversible and “adaptive” mechanisms
4.4.2 An EGFR- independent mechanism
4.4.3 A Src-dependent mechanism
4.5 Therapeutic implications of the AZD0530-induced restoration of ERα expression
4.6 Potential upstream signalling elements for the Src-mediated loss of ERα expression
4.7 Potential transcription factors regulating ERα expression in TamR cells
4.8 Summary and conclusion

CHAPTER 5. REFERENCES
References

APPENDICES
Appendix A
Appendix B
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~ Summary ~

Tamoxifen has long been the drug of choice in endocrine therapy of oestrogen receptor α (ERα)-positive breast cancer and still remains widely used due to its well-documented beneficial effect. However, its efficacy is often limited by the onset of acquired resistance and several clinical studies have suggested that a proportion of tumours, which are initially ERα-positive, lack the receptor at the time of tamoxifen relapse in the adjuvant or metastatic setting. The mechanisms underlying acquired ERα-negativity remain largely unknown and their elucidation is of therapeutic importance since breast cancer lacking ERα is associated with endocrine resistance, aggressive tumour biology and poor prognosis. This study addressed the issue of acquired ERα-negativity during tamoxifen therapy by assessing ERα expression in tamoxifen-resistant MCF-7-derived cells, which were cultured in the presence of the anti-hormone over a 30-month period. Results showed a progressive and significant reduction of total ERα mRNA and protein expression in response to tamoxifen therapy and this was accompanied with greatly increased aggressive tumour cell behaviour. The tamoxifen-treatment regimen also resulted in reduced expression of all ERα mRNA variants, which are generated through alternative promoter usage of the ERα gene. Such reduction was most apparent for ERα-mRNA variants C. Importantly, pharmacological modulation of cell signalling pathways identified the epidermal growth factor receptor (EGFR) signalling maintaining ERα levels, whilst c-Src kinase activity appeared to be the key underlying cause of ERα loss during tamoxifen therapy. Encouragingly, even after a 30-month treatment regime with tamoxifen, ERα loss was reversible with a c-Src inhibitor. The data presented in this thesis suggest that combinations of anti-hormones with c-Src inhibitors could retain ERα functions during tamoxifen therapy and prevent a drift towards more aggressive cancer cell behaviour.
~ Publications and presentations ~


~ Abbreviations ~

ADP  adenosine di-phosphate
AIB1  amplified in breast cancer 1
APS  ammonium persulfate
ATP  adenosine tri-phosphate
5-AZA  5-azadeoxycytidine
bp  base pairs
BSA  bovine serum albumin
CARM1  coactivator-associated arginine methyltransferase 1
cDNA  complementary DNA
CoAs  coactivators
C-terminal  carboxyl-terminal
DAB  3-3' -diaminobenzidine
DAPI  4'6-diamidino-2-phenylindole-2-hydrochloride
DAX-1  dosage-sensitive sex-reversal, adrenal hypoplasia congenital, X chromosome
DBD  DNA binding domain
DMSO  dimethyl sulfoxide
DNA  deoxyribonucleic acid
dNTP  deoxynucleotide tri-phosphate
DPX  di-butylphthalatexylene
DTT  di-thiothreitol
E2  17β-oestradiol
ECM  extracellular matrix
EDTA  ethylene diamine tetraacetic acid
EGF  epidermal growth factor
EGFR  epidermal growth factor receptor
EGTA  ethylene glycol tetraacetic acid
EMT  epithelial-mesenchymal transition
ER  oestrogen receptor
ERE  oestrogen response element
ER-ICA  oestrogen receptor immunocytochemical assay
Erk  extracellular-signal regulated kinase
FasR  faslodex-resistant MCF-7 cells
FCS  foetal calf serum
GF  growth factor
Grb2  growth factor receptor-bound protein 2
GTFs  general transcription factors
HCl  hydrochloric acid
HER  human epidermal growth factor receptor
HRG  heregulin
HRP  horse-radish peroxidase
HSP  heat shock protein
ICC  immunocytochemistry
IgG  immunoglobulin G
IGF  Insulin-like growth factor
IGF-IR  Insulin-like growth factor-I receptor
K$_2$HPO$_4$  di-potassium hydrogen orthophosphate anhydrous
kDa  kilo Daltons
KH$_2$PO$_4$  potassium di-hydrogen orthophosphate
LBD  ligand binding domain
M  molar
MAPK  mitogen-activated protein kinase
MCF  Michigan Cancer Foundation
MEK  MAPK extra-cellular signal-regulated kinase
MgCl$_2$  magnesium chloride
mRNA  messenger RNA
NaCl  sodium chloride
NaOH  sodium hydroxide
Na$_3$VO$_4$  sodium orthovanadate
NEDD8  neural precursor cell expressed developmentally down-regulated 8
<table>
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</tr>
</thead>
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<tr>
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<td>optical density</td>
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<td>4-hydroxytamoxifen</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>PI3K</td>
<td>phosphatidylinositol-3-kinase</td>
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<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
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<td>qPCR</td>
<td>quantitative (real-time) PCR</td>
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<td>REA</td>
<td>repressor of oestrogen action</td>
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<td>RH</td>
<td>random hexamers</td>
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<td>receptor-interacting protein 140</td>
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<td>ribonucleic acid</td>
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<td>rpm</td>
<td>revolution per minute</td>
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<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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<td>RT-PCR</td>
<td>reverse transcription PCR</td>
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<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SDS</td>
<td>sodium-dodecyl-sulphate</td>
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<tr>
<td>Ser</td>
<td>serine</td>
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<tr>
<td>SERD</td>
<td>selective oestrogen receptor down-regulator</td>
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<td>SERM</td>
<td>selective oestrogen receptor modulator</td>
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<tr>
<td>SFCS</td>
<td>charcoal-stripped foetal calf serum</td>
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<tr>
<td>SHC</td>
<td>Src and collagen-homology protein</td>
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<td>SMRT</td>
<td>silencing mediator of retinoid and thyroid hormone receptors</td>
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<td>son of sevenless protein</td>
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<td>TamR</td>
<td>tamoxifen-resistant MCF-7 cell line</td>
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<td>Full Form</td>
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<td>TBS</td>
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<td>N,N,N',N'-tetramethylene-diamine</td>
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<td>TESPA</td>
<td>3-aminopropytriethoxysilane</td>
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<td>transforming growth factor</td>
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<td>tyrosine</td>
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<td>U</td>
<td>Unit of enzyme activity</td>
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<td>phenol-red free RPMI</td>
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Chapter 1

~ Introduction ~
1.1 Cancer: facts and figures

Cancer can be viewed as a set of chronic diseases characterised by an uncontrolled tissue growth, in which cells divide in defiance of normal restraints and are capable of invading and colonizing other tissues, ultimately forming fatal metastasis. There are over 200 types of cancer but the most commonly diagnosed are lung, colorectal, prostate and breast cancers, which accounted for over half of all new cases diagnosed in 2005 (Office for National Statistics, 2005; Cancer Research UK, 2007). Cancer development is complex and is now well acknowledged to be an evolutionary process at the cell level in which genomic instability plays a significant role (Schneider & Kulesz-Martin, 2004). A good example of genomic instability is that normal cells do not display is the mutation of the p53 tumour suppressor protein, famously known as the “guardian of the genome” for its protective role against DNA damage. Over 50% of human cancers lack the pro-apoptotic function of p53 due to mutations occurring in the TP53 gene (Vogelstein et al., 2000). As reviewed by Hanahan & Weinberg (2000), most if not all human cancers share six common physiological properties which define the pathological characteristic of cell malignancy, known as the “hallmark of cancer”. These are (i) self-sufficiency in growth signals (activation of many dominant oncogenes), (ii) insensitivity to growth-inhibitory signals (loss of key tumour suppressor genes), which lead to (iii) evasion from the programmed cell-death (apoptosis), (iv) un-limited replicative potential (telomere maintenance), (v) sustained angiogenesis, and (vi) metastasis.

It is estimated that 1 in 3 people will develop some form of cancer over the course of a lifetime with more than half of all cancer cases diagnosed in persons aged 65 and older. In the UK there were just over 250,000 diagnosed cases of cancer each year in 2002-2004 with an average of 154,000 cancer-related deaths each year during the same period (Office for National Statistics, 2005). In 2007, cancer killed 7.9 million people worldwide and this figure could rise to over 10 million by 2030 with poor countries shouldering the heaviest burden from the disease (World Health Organization, 2007). Yet about 50% of malignant diseases could be prevented if significant widespread changes are made on population’s behaviour towards tobacco,
alcohol consumption, physical activity, and sun exposure to mention just a few (Stein & Colditz, 2004).

1.2 Breast cancer

1.2.1 Incidence and risk factors

With an estimated 1 million new cases each year in the world, breast cancer represents by far the most common form of cancer in women (Ferlay et al., 2004). The incidence and mortality rates can vary up to five fold across the globe but are the highest in developed countries due to lifestyle-related causes. In the UK, breast cancer accounts for just over 30% of all new female cancers with more than 44,000 new cases diagnosed in 2004. The overall lifetime risk (all ages) of developing breast cancer is 1 in 9 but this increases considerably with age particularly after 50 (Office for National Statistics, 2005; Cancer Research UK, 2007). Although it is rare, breast cancer in men can also be diagnosed with around 300 new cases each year in the UK (Cancer Research UK, 2007). The introduction of national mammographic screening across the UK in 1988, together with improved treatments has led to a considerable decrease in mortality from breast cancer while the incidence has increased (Figure 1.1).
Figure 1.1  Age standardised (European) incidence and mortality rates of female breast cancer between 1975 and 2005 in the UK (adapted from the Office for National Statistics, 2007).
One of the strongest epidemiological risk factors for breast cancer is age but many other non-genetic predisposing factors have been identified, most of which are related to the Western lifestyle (McPherson et al., 2000). They include early menarche (start of the menstrual periods), late menopause, delayed age at first pregnancy, lack of breast feeding, prolonged hormone replacement therapy, and post-menopausal obesity. While the vast majority of breast cancer cases are thought to be sporadic with no apparent hereditary traits, a smaller proportion (5-10%) is known to be linked to the inheritance of one or several mutated genes. The most established ones are the tumour suppressor genes BRCA1/BRCA2, whose mutations account for almost half of familial breast cancers and increase the overall lifetime risk of getting the disease by up to 80% by the age of 70 (Ford et al., 1998). Other mutated genes like TP53 and PTEN can also contribute to hereditary breast cancer although to a much lesser extent. It is believed that additional genes could be involved (McPherson et al., 2000). Clearly, breast cancer is a complex disease involving numerous genetic and non-genetic predisposing factors. However, the etiology of the disease can be assigned to two main categories: failure to maintain genomic stability and excessive exposure to oestrogens, which is the focus of this thesis (Imyanitov & Hanson, 2004).

1.2.2 Breast cancer development and subtypes

The human breast is a complex organ that begins development early in gestation and subsequently undergoes dramatic changes in size, shape and function during the transition from puberty to menopause. Breast tissue comprises of lobules, a collection of milk-producing glands, and ducts, which carry the milk from the lobules to the nipple surrounded by the breast stroma consisting of fatty connective tissue and numerous vessels of the blood and lymphatic system. The ducts are formed by a basal layer of contractile, myo-epithelial cells and a luminal layer of specialized epithelial cells (Russo & Russo, 2004). Breast cancers can arise from the epithelial cells that line the ducts (ductal carcinoma in situ, DCIS), or from the lobules (lobular carcinoma in situ, LCIS). DCIS is more common and accounts for 20-30% of all newly diagnosed cases of breast cancers while LCIS accounts for
approximately 10% (Olivotto et al., 2006). Both DCIS and LCIS represent pre-invasive malignant lesions in which the abnormal breast epithelial cells are contained within the mammary epithelial structures, and where no invasion of the basement membrane and no infiltration of the breast stroma is apparent (Howard & Gusterson, 2000). Whilst nearly all premalignant epithelial cells (from the DCIS and LCIS lesions) express high levels of the oestrogen receptor (ER, section 1.3), normal breast epithelial cells are for the most part ER-negative (Allred et al., 2004). The origins of ER-positive breast cancer cells still remain controversial but it is believed that they may arise from either ER-positive or adjacent ER-negative progenitor cells, which are located at the growing, terminal end of the duct (Dontu et al., 2004).

It is now well acknowledged that breast cancer is a complex heterogeneous disease with diverse histo-pathologies, genetic variations, and clinical outcomes. The pioneering works from Perou et al. (2000) and Sorlie et al. (2001), using global gene expression profiling in invasive breast carcinoma, have demonstrated that breast cancer could be classified into 5 categories: luminal A, luminal B, HER-2 overexpressing, basal-like and normal breast-like. The luminal subtypes (or non-basal subtypes) largely corresponded to phenotypically ER-positive tumours, with the luminal subgroup A characterized by a higher ER expression and superior clinical outcome than the luminal subgroup B. In contrast, the remaining subtypes (or basal subtypes) corresponded to ER-negative breast tumours, which are associated with poor clinical outcomes. Basal-like breast cancers show considerable cross-over with the clinically aggressive “triple-negative” (i.e.: ER/PR/HER-2 negative) breast tumours, a group of cancers with a particularly poor prognosis and for which no effective tailored therapies currently exist. Due to the complexity and heterogeneity of human breast cancer, no single breast cancer cell model is truly representative of the disease. However, studies by Neve et al. (2006) using a panel of 51 widely-used breast cancer cell lines demonstrated that these cell models share many of the genetic and genomic aberrations found in human breast cancer and closely represent a number of clinical breast cancer subtypes. For example, the widely used MCF-7 cell line (originally derived from human pleural effusion of breast carcinoma metastasis) corresponds to the most prevalent luminal A breast cancer subtype, thus making this cell line clinically relevant (Neve et al., 2006).


1.3 Oestrogen and Breast cancer

The first evidence implicating oestrogen and breast cancer growth was provided in 1896 by the British physician Beatson who discovered that ovariectomy resulted in tumour regression in premenopausal women with metastatic breast cancer (MacGregor & Jordan, 1998). Based on the specific binding of radioactive oestrogen in the uterus of rats, Jensen & Jacobsen first came to the conclusion that the biological effects of oestrogen were most likely to be mediated by oestrogen receptors, ERs (Jensen & Jacobson, 1962). This led to the development of the first ER assays in the 1960s to predict whether breast cancer patients would respond to oestrogen depletion by ovariectomy (premenopausal women) or adrenalectomy (postmenopausal women).

1.3.1 Oestrogen synthesis

The physiological effects of oestrogen are numerous and include the development and maintenance of the female reproductive organs, the reproductive cycle, the cardiovascular and central nervous system and bone metabolism (Nilsson & Gustafsson, 2000). Importantly, growth and development of breast tumours are dependent on oestradiol-17β (E2), which is the most potent and main circulating oestrogen. In premenopausal women, E2 is mainly produced in the ovaries through aromatization of androgens by the cytochrome P450 aromatase enzyme complex, all under the stimulatory effects of the pituitary hormones LH (Luteinizing hormone) and FSH (follicle-stimulating hormone). In postmenopausal women, aromatization from androgens occurs in peripheral tissues such as brain, liver or fat tissues and provides the main source of circulating oestrogen (Dowsett et al., 2005). Importantly, postmenopausal breast tissues are able to locally produce E2 at levels which can be much higher than the corresponding plasma levels. Such local oestrogen production is now known to be mainly due to high aromatase activity exhibited by both the stromal and epithelial cell component of breast tissues (Blankenstein et al., 1999).
1.3.2 Oestrogen Receptors: gene structure

More than two decades after the first discovery of the ER by Jensen & Jacobson (1962), the gene encoding the receptor was cloned from the breast cancer cell line MCF-7 and was named ERα or ESR1 (Green et al., 1986; Greene et al., 1986). A second and functionally distinct receptor, named ERβ, was later found (Kuiper et al., 1996). While the specific functions of ERβ in normal and neoplastic tissues remain largely unknown, the role of ERα in breast carcinogenesis is relatively well understood. The complex genomic organization of the human ERα gene has been extensively analyzed and a widely acknowledged nomenclature that describes the promoter region of the gene has been published (Koš et al., 2001). With the exception of the promoter region, the sequence of the human ERα cDNA shows a high degree of conservation with that of other species including rat, mouse, and chicken (Koš et al., 2001). The ERα gene is a large genetic unit spanning over 300 kb on the long arm of chromosome 6 (Gosden et al., 1986). This includes a large promoter region stretching over 150kb and a protein coding region of ~140 kb containing 8 exons and 7 introns. As illustrated in Figure 1.2, transcription from the ERα gene is complex and can occur from at least 7 promoters (or upstream exons: A→F, and T), which can be located more than 150 kb upstream of the originally described transcription start site “+1” (Green et al., 1986). All upstream exons can be spliced into a common acceptor splice site that is highly conserved amongst species and located at position “+163” in exon 1 of the protein coding region. Alternative usage and splicing of these promoters during transcription generate multiple mRNA variants, which differ in their 5’-UTRs (untranslated regions) but encode a common ERα protein that is 66 kDa in size (ERα-66; Gannon et al., 2002).
Figure 1.2  Genomic organization of the promoter region of the ERα gene. Grey boxes represent upstream exons with broken arrows depicting start of transcription. Numbers below upstream exons correspond to the distance from the originally described transcription start site (+1) in base pairs. Numbers between exons show the size of major introns in kilobase pairs. Broke lines below exons symbolize observed alternative splicing of the different promoters to the common acceptor splice site located at (+163) and represented by an open triangle (adapted from Koš et al., 2001).
1.3.2.1 Alternative promoter usage

It is now well recognised that one of the implications of multiple promoters in the ERα gene is the sensitive tuning of ERα expression according to cell requirement in a tissue- and developmental stage-specific manner (Koš et al., 2001). ERα transcripts originating from the proximal promoters A and B as well as the distal promoter C have been shown to be the predominant ones in tissues expressing relatively high levels of ERα including the mammary gland, the ovary and endometrium (Flouriot et al., 1998). In contrast, the more distal promoters E and F contribute most to the pool of ERα mRNA in liver and osteoblasts, respectively, where ERα expression is less abundant (Flouriot et al., 1998; Lambertini et al., 2003). In addition, the role of E2 in the development of the male reproductive tract has been further demonstrated by the identification of the promoter T (T1+T2), which has been shown to be used almost exclusively in testis and in the epididymis (Brand et al, 2002).

The considerable increase of ERα levels, characteristically observed in breast tumours in comparison with normal breast tissues, has been associated with the increased expression of the ERα transcripts A, B, and C. The latter have been shown to be up-regulated by up to 30-fold in cancerous breast tissues compared with normal breast tissues (Donaghue et al., 1999; Flouriot et al., 1998; Grandien et al., 1995). The individual contribution of each transcript to the total pool of ERα mRNA can be variable. While some studies have shown that transcript A is the most predominately expressed and account for ~50% of all ERα transcripts in the breast cancer cell line MCF-7 (Flouriot et al., 1998), others have reported that transcripts originating from promoter C are the most abundant and may largely be responsible for the increased expression of ERα (Tanimoto et al., 1999; ). In contrast, transcripts originating from the promoters E and F are less abundant but are still contributing towards the total level of ERα mRNA in ERα-positive cells (Donaghue et al., 1999). Overall, it has been suggested that the high levels of ERα expression is determined by the number of the contributing promoters rather than their selective use, as illustrated by the observations that non-breast cancer cells use fewer promoters than their cancerous counterparts (Donaghue et al., 1999).
1.3.2.2 Alternative splicing (exon skipping)

In addition to alternative promoter usage, single or multiple exons from the protein coding region of the ERα gene can be depleted during the processing of the primary transcript (exon skipping). This ultimately results in the production of ERα protein variants that are structurally and functionally different (Ferro et al., 2003). The so-called alternative splicing occurs in 60% of all human genes and its mechanisms can be greatly affected by promoters (i.e.: promoter identity and occupancy) through recruitment of factors that have dual functions in transcription and splicing (Kornblihtt, 2005). In MCF-7 cells for example, Flouriot & colleagues (2000) have reported the existence of an ERα mRNA isoform depleted of the entire exon 1 containing the A/B domain of the ERα gene, and therefore resulting in an AF1-truncated ERα protein that is 46 kDa in size (ERα-46). The same authors have shown that ERα-46 is encoded by distinct mRNA variants originating from the alternative splicing of promoters E and F directly into exon 2 of the ERα gene. It was shown that ERα-46 can heterodimerize with wild-type ERα-66 and greatly inhibits the overall transcriptional activity of the receptor in cells where ERα signalling is mainly mediated through the AF1 activation domain. Importantly, it has been reported that the overall prevalence of the exon-skipped-ERα mRNA variants is increased in breast tumours. This is illustrated, for example, by the exon 7 deletion form of ERα which has been shown to contribute ~20% of the total ERα mRNA pool in MCF-7 cells (Fasco, 1998). The increased expression of alternatively spliced ERα mRNAs can ultimately result in the synthesis of ERα protein isoforms that may exhibit variations in oestrogen/anti-oestrogen binding properties (Poola & Speirs, 2001).
1.3.3 Oestrogen Receptors: protein structure and function

ERs belong to the nuclear receptor superfamily (NR), which consists of approximately 150 different members spanning across a wide array of animal species including vertebrate and invertebrates (Mangelsdorf et al., 1995). Other members include progesterone, thyroid hormone, retinoid and vitamin-D receptors as well as the so-called orphan receptors for which no ligands have been identified. Like most nuclear receptors, ERα and ERβ function as ligand-dependent transcription factors that regulate the expression of a large array of specific target genes (McDonnell & Norris, 2002). ERα protein is thought to be the predominant receptor for the regulation of oestrogen responsive genes in breast cancer (Fuqua et al., 2003). The two receptors are functionally distinct, have different tissue distribution, different ligand activation and so play different roles in gene activation (Nilsson & Gustafsson, 2000). However, ERα and ERβ share a common modular structure, which includes 6 functional domains (A-F) that characterises all members of the NR family (Figure 1.3).

The N-terminal region contains the poorly conserved activation function domain AF1 (A/B domain). The poor conservation within the A/B domain of ERα and ERβ is believed to explain, at least in part, the functional differences between the two receptors by interacting with different transcription factors (Nilsson & Gustafsson, 2000). Probably the best example is the fact that ERα and ERβ have opposite transcriptional effects on API promoter sites when bound to oestrogen (Paech et al., 1997). Oestrogen-bound ERα activates while ERβ inhibits gene transcription from an API site. The DNA binding domain (DBD, C domain) represents the most highly conserved region consisting of two zinc-fingers that are crucial for the binding of the receptor to target gene. The hinge region (D domain) contains nuclear localisation sequences and also several motifs for post-translational modifications of the receptors such as acetylation and sumoylation, which can regulate the transcriptional activity of ERα (Sentis et al., 2005; Wang et al., 2001).
Figure 1.3  **Structural organization of ERα and ERβ proteins.** Both receptors consist of six functional domains (A-F) including the transactivation domains AF-1 and AF-2, the DNA binding domain (DBD) and the ligand binding domain (LBD). The percentage represents the degree of homology between the two receptors (adapted from Shao & Brown, 2004).
Most of the C-terminal region (E/F domain) encompasses the ligand-binding domain (LBD) harbouring a dimerisation surface and most importantly the activation function domain AF2. The latter is involved in the ligand-dependant interactions of the receptors with numerous cofactors (McDonnell & Norris, 2002). Crystallographic analysis of the ERα LBD in the presence of either an agonist or an antagonist has revealed the presence of a highly mobile hydrophobic segment, α-helix 12 (H12), whose position is altered by the binding of ligands (Figure 1.4, Brzozowski et al., 1997). Upon binding of oestrogen, H12 is positioned over the ligand-binding pocket and, together with segments H3-H5, results in the formation of a hydrophobic groove that exposes “docking sites” for the binding of co-activator proteins. In contrast, the bulky side chain of anti-oestrogens displaces H12 thus preventing interaction of the AF2 domain with co-activators. Overall, one major function for ligands is to alter the conformation of the LBD, which ultimately dictates the type of cofactors interacting with ERs (White & Parker, 1998).
Figure 1.4 Structural model of the ligand binding domain of ERα and the alteration in the positioning of helix H12 in the presence of oestrogen or anti-oestrogen. The yellow spheres schematically represents the area of ligand binding (adapted from White & Parker, 1998).
1.3.4 Oestrogen Receptor: cofactors

In order to regulate the expression of their target genes, ligand-activated ERs recruit a multitude of co-regulatory proteins, which can essentially be viewed as proteins that complement the cellular effects of ERs by either enhancing (coactivators, CoAs) or attenuating (corepressors, CoRs) ER mediated-gene transcription (Table 1.1). The degree of complexity to which nuclear receptors can interact with cofactors is illustrated by the ever-growing number of identified NR coactivators (~200, Lonard & O'Malley, 2006). The overall multiprotein complex formed by ERs and coactivators at the promoter regions of target genes ultimately results in the remodeling of the local chromatin structure and recruitment of the basal transcriptional machinery. Coactivators have been shown to contain a conserved “LXXLL” motif (also known as “NR box”), which binds specific sites within the AF2 domain of ERs (McKenna & O'Malley, 2002). A common functional property of most (but not all) coactivators is histone acetyltransferase (HAT) activity. HAT enzymes catalyze the acetylation of lysine residues of the N-terminal tails of histones which, together with other DNA modifications, results in local decondensation of the chromatin necessary for gene activation (Umov & Wolffe, 2001). Probably the most established AF2-interacting coactivators are the p160 family, which contain intrinsic HAT activity but whose primary functions are thought to be the recruitment of other coactivators and HATs like CBP (CREB binding protein)/p300, and pCAF (CBP-associated factor). For example, members of the p160 family include SRC-3 (better known as amplified in breast cancer, AIB1), which is amplified and/or overexpressed in over half of breast cancers (Massarweh & Schiff, 2006).

Corepressors are equally important in the regulation of ER-mediated gene expression and repress transcription when interacting with apo-ERs (unliganded) or antagonist-bound receptors. As reviewed by Dobrzycka et al. (2003), corepressors can exert their transcriptional repression through a wide variety of mechanisms including chromatin remodeling, binding competition with coactivators, sequestration of ERs in the cytoplasm, and interference with DNA binding. For example, some corepressors negatively regulate transcription due to their ability to recruit histone deacetylases (HDACs), which participate in the condensation of chromatin therefore preventing the transcriptional apparatus from accessing DNA. The most established
corepressors for ERs include NCoRs (nuclear corepressors) and SMRT (silencing mediator of retinoid and thyroid hormone receptor). It is now increasingly believed that, although having opposite functions, both coactivators and corepressors may coexist in the same multiprotein complex. Ligand binding on the receptors would result in a dynamic and rapid reorientation of the coactivators/corepressors, whose balance and activity sensitively tune the expression of the nuclear receptor target genes (Kumar et al., 2005).
Table 1.1  Oestrogen receptor cofactors

<table>
<thead>
<tr>
<th>Name</th>
<th>ER binding site</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCoR</td>
<td>LBD</td>
<td>HDACs</td>
<td>Lavinsky et al., 1998</td>
</tr>
<tr>
<td>SMRT</td>
<td>LBD</td>
<td>HDACs</td>
<td>Smith et al., 1997</td>
</tr>
<tr>
<td>RIP140</td>
<td>LBD</td>
<td>HDACs, competition with coactivators</td>
<td>Cavailles et al., 1995</td>
</tr>
<tr>
<td>DAX-1</td>
<td>AF-2</td>
<td>Competition with coactivators, Inhibition of ERα dimerization</td>
<td>Zhang et al., 2000</td>
</tr>
<tr>
<td>REA</td>
<td>LBD</td>
<td>Competition with coactivators</td>
<td>Klinge et al., 1997</td>
</tr>
<tr>
<td>NEDD8</td>
<td>LBD</td>
<td>Ubiquitin-like protein involved in ERα proteolysis</td>
<td>Fan et al., 2003</td>
</tr>
<tr>
<td>SRC1</td>
<td>AF-2</td>
<td>HATs</td>
<td>Onate et al., 1995</td>
</tr>
<tr>
<td>SRC2/TIF2</td>
<td>AF-2</td>
<td>HATs</td>
<td>Voegel et al., 1996</td>
</tr>
<tr>
<td>SRC3/AIB1</td>
<td>AF-2</td>
<td>HATs</td>
<td>Suen et al., 1998</td>
</tr>
<tr>
<td>p68</td>
<td>AF-1</td>
<td>RNA helicase</td>
<td>Endoh et al., 1999</td>
</tr>
<tr>
<td>CARM1</td>
<td>none</td>
<td>Indirect interaction with ERα through binding to p160, Methylation of histones</td>
<td>Stallcup et al., 2003</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>AF-2</td>
<td>Recruitment of SRC-family coactivators to ERα</td>
<td>Zwijsen et al., 1998</td>
</tr>
</tbody>
</table>

_CoRs_, corepressors; _CoAs_, coactivators.
1.3.5 ER signalling

In the absence of ligands, ERs exist in a monomeric form, predominantly found in the nucleus and sequestered within a large and stabilizing heterocomplex of chaperone proteins including the heat-shock proteins Hsp90, Hsp70, and the Hsp-interacting protein p23 (Pratt & Toft, 1997). ERs can regulate the expression of their target genes through three distinct but “cross-talking” mechanisms that can be referred as (i) the classical genomic, (ii) the non-classical genomic, and (iii) the non-genomic/cell membrane mechanisms (Ring & Dowsett, 2004).

1.3.5.1 Classical genomic action of ERs

The lipophilic nature of oestrogen allows the steroid hormone to diffuse freely through the cell plasma membrane and reach the nucleus where it binds to the ER. Oestrogen binding induces a conformational change of the receptor, which also become hyperphosphorylated at several serine and tyrosine residues (Nicholson et al., 1999), ultimately resulting in a rapid dissociation of the receptors from the Hsp90-based chaperone complex. This is rapidly followed by receptor homo- or hetero-dimerisation which, in the case of the classical genomic action of oestrogen, is followed by the binding of ERs to oestrogen response elements (EREs). The latter are present on the promoter region of oestrogen-responsive genes (Griekspoor et al., 2007; White & Parker 1998). ER binding to EREs is quickly followed by the recruitment of coactivators and components of the RNA polymerase II transcription initiation complex for gene activation (Figure 1.5A). A perfect ERE consensus consists of two inverted repeats of the sequence “5’-GGTCA-3’” separated by any 3 nucleotides, and onto which ERs bind with the highest affinity. However, perfect EREs are very rare (present in only three E2-regulated genes of the human genome) and it has been shown that many E2-responsive genes contain imperfect or half-EREs, which still bind ERs with high affinity (Sanchez et al., 2002). A genome-wide screen for high affinity EREs has suggested that the occurrence of EREs is high with an estimated number of ~70,000 motifs in the human genome (Bourdeau et al., 2004). In the same study, some functional EREs have been located as far as -10 kb upstream of transcriptional start sites although most ERE motifs were found to be
mostly enriched in the vicinity of transcriptional start sites (-1 to +2kb region). Additionally, large scale chromatin immunoprecipitation analysis (ChIP) on the E2-responsive gene pS2 in MCF-7 cells has shown that the recruitment of cofactors by ERs occurs as an ordered and cyclical event (Métivier et al., 2003). However, as suggested by the same authors this orderly and sequential recruitment of coactivators is likely to be different across E2-responsive genes.
Figure 1.5  **Classical (A) and non-classical (B) genomic action of ERs.** In the classical mode, E2-activated ERs bind to ERE sites and recruit a coactivator multiprotein complex that includes p160 and CBP/p300 and which is linked to the transcription apparatus for activation of gene transcription. In the non-classical mode, ER-mediated gene transcription occurs via binding of the receptors to transcription factors like fos/jun heterodimers, which are bound to AP-1 sites. GTFs, general transcription factors; TBP, TATA-binding protein.
1.3.5.2 Non-Classical genomic action of ERs

In addition to signal directly through EREs, ERs can also regulate transcription by binding to other DNA-bound transcription factors (tethering), which characterize the non-classical nuclear action of ERs (Figure 1.5B). For instance, E2-bound-ERα can bind to the API (activating protein 1) transcription factor consisting of the heterodimer c-Fos/c-Jun, and activate the AP-1 mediated transcription of genes such as cyclin D1, ovalbumin, collagenase, and the growth factor ligand IGF-1 (Cheung et al., 2005; Björnstorm & Sjöberg, 2005). Similarly, ERα can also interact with the transcription factor Spl (Specificity protein 1) to regulate transcription of genes controlled by GC-rich promoters such as c-fos (Kim et al., 2005). Overall, this “tethering” mechanism allows oestrogen to regulate the expression of a larger number of oestrogen-responsive genes including those that do not contain EREs. Interestingly, some Spl binding sites are located in proximity to ERE half-sites, both of which need to be occupied for maximal transcription of genes such as PR (progesterone receptor) and TGFα (tumour growth factor α) (Björnstorm & Sjöberg, 2005). A large scale microarray analysis in MCF-7 cells has revealed that oestrogen-mediated cell proliferation is associated with the down-regulation of ~70% of all E2-responsive genes. Those down-regulated genes were identified as transcriptional repressors, genes involved in cell cycle inhibition or of pro-apoptotic functions. In contrast, the up-regulated genes were involved in cell cycle progression, DNA synthesis and growth factor signalling (Frasor et al., 2003).

1.3.5.3 Non-genomic action of ERs

Non-genomic effects of oestrogen relate to cellular events that occur within seconds or minutes of oestrogen administration, which cannot be attributed to transcriptional activation of genes via nuclear ERs because the latter event usually occurs in a matter of hours or even days. Some of the well-known rapid physiological effects of E2 include pituitary prolactin secretion, vasodilatation of coronary arteries in association with nitric oxide release, and activation of the Ca\(^{2+}\) channels. E2 is also known to rapidly activate membrane growth factor receptors.
such as IGF-IR (insulin growth factor 1 receptor) and EGFR (epidermal growth factor receptor), which lead to the activation of their downstream MAPK (mitogen-activated protein kinase) and Akt signalling pathways (Levin, 2005; Nicholson et al., 2005).

In oestrogen-dependent breast cancer cells, it is thought that the non-nuclear actions of E2 are mediated by membrane and/or cytoplasmic ERs which, upon E2 binding, physically interact with a large signalling protein complex that rapidly initiate the growth factor-induced activation of MAPK and Akt signalling (Song et al. 2005; Song & Santen 2006). As proposed by Song & Santen (2006), E2-activated ERα at or near the membrane is thought to trans-activate EGFR and IGF-1R through interaction with the non-tyrosine kinase c-Src, which rapidly associates with many signalling molecules. The latter include G proteins, the scaffold protein MNAR (modulator of non-genomic activity of oestrogen), the p85α regulatory domain of PI3K (phosphoinositol-3 kinase) and the Shc protein. As discussed by Warner & Gustafsson (2006), there are still controversies as to whether those rapid E2 effects are mediated at the plasma membrane by the classical/nuclear ERs, primarily because the latter have no transmembrane domains. As mentioned by Björnstorm & Sjöberg (2005), the receptors could undergo palmitoylation for plasma membrane targeting. ERα has also been found to interact with the scaffold protein caveolin-1, which is found enriched in specialized membrane invaginations, called caveolae. In addition to membrane ERs, the issue that a pool of the classical receptors might be present in the cytoplasm has also been raised (Levin, 2005). Indeed, a small fraction of unliganded nuclear ERα has been identified in the cytosol and shown to constantly shuttle between the nucleus and the cytoplasm (Maruvada et al., 2003). Additionally, it has been suggested that oestrogen may act through a structurally distinct estrogen receptor, named GPR30 (Filardo & Thomas., 2005). Overall and as recommended by Warner & Gustafsson (2007), definitive identification of nuclear ERs in the membrane or/and the cytoplasm still remains to be fully established.
1.3.5.4 Convergence of genomic and non-genomic actions of ERs

It is now widely acknowledged that signalling initiated from membrane/cytoplasmic ERs (non-genomic) can cross-talk with nuclear ERs (genomic) through growth factor receptor tyrosine kinases such as EGFR and IGF-1R (Björnstorm & Sjöberg, 2005; Levin, 2005). As described previously, the E2-induced interaction between membrane/cytoplasmic ERs and components of the growth factor signalling pathways leads to downstream activation of MAPK and Akt. Importantly, both MAPK and Akt further up-regulate the transcriptional activity of E2-bound nuclear ERs by mediating, respectively, the phosphorylation of Ser118 and Ser167 residues present within the AF1 domain of the receptors. In the absence of oestrogen, these and other residues can still be phosphorylated in response to EGF or IGF-1 (Kato et al., 1995). Additionally, the membrane-induced activation of MAPK and Akt also leads to phosphorylation of their target transcription factors, such as AP-1 and Sp1, which are tethered to nuclear ERs at target gene promoters. Overall, signalling from the membrane/cytoplasmic ER through kinase cascades provide a rapid cellular response which is relayed to and maintained by nuclear ERs which provide a fine degree of control on gene transcription and breast epithelial cell proliferation and survival (Levin, 2005).
1.4 Regulation of ERα expression in breast cancer

As described previously, ERα levels are a crucial determinant of cellular growth potential for the majority of breast cancers. Therefore, regulation of ERα concentration is a key component in limiting oestrogen responsiveness in target cells (Pinzone et al., 2004). Studies attempting to shed light on the molecular mechanisms that modulate ERα levels have revealed that its regulation is complex and multidimensional. ERα expression is controlled at (i) the transcriptional level through interaction between cis-elements of the promoters and trans-acting factors; (ii) at the post-transcriptional level through modulation of mRNA stability and translation efficiency; and finally (iii) at the post-translational level via ERα protein degradation/turnover.

1.4.1 Transcriptional regulation

Historically, the proximal promoters A and B have been one of the first regulatory regions of the ERα gene to be identified and described as key players for ERα overexpression in breast cancer (Koš et al., 2001). Therefore, most of what is known about the molecular mechanisms regulating ERα gene transcription comes from studies identifying the cis-acting elements within these two promoters and their interaction with cell-specific transcription factors which are under the control of growth-factor signalling pathways. Castles et al. (1997) were amongst the first to suggest that ERα protein is able to modulate the activity of its own gene expression in a ligand-dependent manner, and therefore may contribute to its overexpression in some breast tumours. The same authors have shown that a ~200 bp fragment, located just upstream of the transcription start site (+1), contained important cis-acting elements whereby ERα is able to auto-regulate itself without directly binding to the DNA but through protein-protein interaction with other transcription factors.

It is now known that in breast cancer the majority of ERα promoter activity resides within the proximal promoters A and B, precisely between -245 bp and +212 bp relative to the originally described transcription start site (Green et al., 1986). This region, also known as the minimal promoter, contain several transcriptional motifs
including a TATA-box for the binding of the TFIID protein via its TATA-box binding protein subunit (TBP); and importantly a GC box to which binding of the transcription factors Sp1 and Sp3 has been shown to be crucial for the activation of ERα gene transcription in breast cancer (deGraffenried et al., 2002). A model for transcription from the ERα minimal promoter has been proposed for the first time (deGraffenried et al., 2004; Figure 1.6). In this model, ERα protein auto-regulates its expression through interaction, at the minimal promoter, with a multi-protein complex engaged by the binding of Sp1 and the USF-1 transcription factor (upstream stimulatory factor 1) to a GC box and an imperfect E box, respectively. This complex is thought to subsequently recruit the RNA polymerase II holoenzyme for initiation of ERα transcription.

A transcriptional enhancer element (named ER-EHO) containing binding sites for the AP-1 transcription factor has been located within the more distant promoter D, and has been described as a dominant cis-acting element for activation of ERα transcription in breast cancer cells (Tang et al., 1997). As suggested by deGraffenried & co-workers (2004), ER-EHO may enhance ERα transcription through binding of AP-1 and as yet unidentified other proteins to the ERα/Sp1/USF1-based multi-protein complex present in the minimal promoter. Adding to the complexity of the regulation of ERα transcription is the identification that p53 can also activate the ERα minimal promoter through a protein-protein interaction with factors that bind the -70 to -40 bp region of promoter A, such as the TATA binding protein (Angeloni et al., 2004). Another potentially important transcription factor that can selectively contribute to promoter-specific ERα expression in breast cancer cells is ERBF-1. Enhancer elements for this transcription factor were located within the distal promoter C, precisely at -1.9 kbp (Tanimoto et al., 1999). The authors showed that only breast cancer cells expressing ERBF-1, such as MCF-7, can generate high levels of the ERα mRNA variant C.
Figure 1.6 Model of regulation of ERα gene transcription at the minimal promoter. The minimal promoter (-245 to +212 bp) contains important cis-acting regulatory elements including a TATA box for the recruitment of the RNA polymerase II holoenzyme components, a GC box and a flanking imperfect E box for the binding of Sp1 and USF-1, respectively. A multi-protein complex containing at least USF-1, Sp1 and ERα interacts with the transcription machinery and is able to strongly activate transcription of the ERα gene. A 35 bp-long enhancer element located within the D promoter region may increase ERα transcription through binding of transcription factors (TFs) such as AP-1. GTFs: general transcription factors (adapted from deGraffenried et al., 2004).
Chapter 1. Introduction

The transcription factor Foxo3a has also been implicated in the regulation of ERα gene transcription. Foxo3a is a member of the mammalian Forkhead Box (Fox) family of transcription factors, which act as transcriptional regulators of the cell cycle progression (Costa, 2005). Using a panel of 5 breast cancer cell lines, Guo & Sonenshein (2004) have identified for the first time that the nuclear localization of Foxo3a, which is regulated by Akt, correlates with ERα expression. Using ChIP analysis and reporter gene assays, the same authors have demonstrated that Foxo3a can bind directly to two functional Forkhead Responsive Elements (FHREs) located within the promoter C, and induce ERE-mediated reporter activity as well as expression of ERα target genes in breast cancer cell lines.

Another potential transcription factor involved in the regulation of ERα gene transcription is AP2γ, formally known as ERF-1 (McPherson & Weigel, 1999; McPherson et al., 1997). The AP2 family of transcription factors consist of five members, AP2α-e, of which the highly homologous AP2α, AP2β and AP2γ are expressed in several solid tumours including breast cancer (Pellikainen & Kosma, 2007). AP2γ expression has been shown to trans-activate ERα gene transcription in human mammary epithelial cells and induce DNase I-hypersensitive sites localized to AP2 binding sites just upstream of the start codon within the ERα promoter A. (Schuur et al., 2001). In the same study, other binding sites for AP2γ have been found in the vicinity of the promoters C and E, and have also been shown to coincide with DNase I-hypersensitive sites occurring specifically in ERα-positive cells.

The molecular mechanisms, by which ERα transcription is regulated through chromatin remodelling in breast cancer, have been demonstrated for the first time in vivo with the tumour suppressor pRb2/p130 (Macaluso et al., 2003; Macaluso et al., 2005; Macaluso et al., 2007). The latter has been shown to bind directly to a region within the promoter A and engage a multi-protein complex with the transcription factor E2F4/5 and most importantly with specific chromatin modifying enzymes including the histone methyltransferase SUV39H1, DNMT1 (DNA methyltransferase 1), HDAC1 and the HAT p300 (Macaluso et al., 2003). Both DNMT1 and HDAC1 are important factors involved in the epigenetic silencing of many genes including ERs (Yan et al., 2001). Macaluso et al. (2003) have proposed that in MCF-7 cells, the presence and high activity of p300 within the pRb2/p130-
based macromolecular complex induces the activation of ERα transcription by maintaining the chromatin in an “open” state, and therefore allows access of the basal transcription factors to the ERα promoter. In contrast, the same authors have shown that in the ER-negative cells MDA-MB-231, the pRb2/p130-based multiprotein complex represses ERα gene transcription through epigenetic mechanisms that include the recruitment of DNMT1, and concomitant dislocation of p300, which results in chromatin condensation and therefore repress gene expression.

1.4.2 Post-transcriptional regulation

ERα expression is also regulated at the post-transcriptional levels through modulation of the receptor mRNA stability, which represents a major mechanism for regulation of gene expression. Rates of mRNA degradation in the cytoplasm determine protein levels. Like many other steroid hormones, oestradiol autoregulates its receptor gene expression by either stabilizing or destabilizing the mRNA in a tissue- and developmental-stage specific manner (Ing, 2005; Pinzone et al., 2004). In breast cancer, it has long been established that treatment of the MCF-7 cell line with oestradiol destabilizes the receptor mRNA and results in a decrease of ERα mRNA half-life from approximately 5h in control cells to less than an hour in the presence of the hormone (Berthois et al., 1990; Saceda et al., 1998).

The exact molecular mechanisms by which oestradiol regulates ERα mRNA stability are still unclear. However, it has been shown that the long 3'-untranslated region (3'UTR) of the ERα mRNA has a destabilizing function by mediating cleavage of the ERα mRNA by various endoribonucleases (RNases) and therefore reduce the ERα mRNA half-life (Kenealy et al., 2000). It is believed that in the context of hormonal-mediated regulation of mRNA stability, oestradiol may regulate the expression of RNA-binding proteins that can either protect or expose sites of the ERα mRNA against RNase activities (Ing, 2005). A study by Keen et al. (2005) has suggested that the protein phosphatase 2A (PP2A) may be important in the regulation of ERα mRNA stability by mediating the binding of a yet-to-be identified factor to the 3'UTR of the ERα transcript in MCF-7 cells. The same authors have proposed
that in the absence of PP2A activity, degradation of ERα mRNA occur as a result of the proteasomal degradation of this stabilizing factor. PP2A is a ubiquitously expressed holoenzyme and a member of a large protein phosphatase family, which is involved in the regulation of cell proliferation, differentiation and apoptosis (Schönthal, 2001).

In addition to modulation of mRNA stability, ERα expression is also determined by the translation efficiency of ERα transcripts (Ing, 2005). As mentioned earlier, the multiple promoters present within the ERα gene promoters generate mRNA variants that differ in their 5′-UTRs (see section 1.5.1). It is known that the structure of 5′-UTRs can strongly influence initiation of translation through the presence of important regulatory motifs such as upstream open reading frames (uORFs). The latter can significantly reduce translation efficiency of the downstream main ORF by restricting the access of ribosomes to the authentic start codon (Hughes, 2006; Pesole et al., 2001; Wang & Rothnagel, 2004). The 5′-UTRs of the various ERα mRNA variants contain between 1 and 6 uORFs and have been shown to differentially regulate ERα expression in breast cancer. Whereas UTRs from the ERα mRNA variants T strongly inhibit translation of the downstream main ORF, UTRs from the A and C transcripts have been shown to confer the highest translation efficiency, which is in agreement with their predominant expression in breast cancer (Koš et al., 2002).

1.4.3 Post-translational regulation

The rate at which ERα protein is synthesized and degraded (turnover) is also a major factor controlling ERα steady state levels and maintaining receptor homeostasis. In oestrogen-dependent cell lines, such as MCF-7, unliganded ERα is rapidly turned over with a half-life of approximately 5h. As well as activating ERα-mediated transcription of target genes, oestrogen binding to the receptor also results in a rapid ubiquitination and degradation of ERα via the ubiquitin-proteasome pathway, reducing ERα half-life to less than 3h (Laços et al., 2005). Different ligands can exert opposite effects on steady-state levels of ERα. While the pure anti-
oestrogen ICI 182,780 (Falsodex®, fulvestrant) causes rapid degradation of the receptor with a resulting half-life of less than 1h (Long & Nephew, 2006), tamoxifen stabilizes ERα causing receptor accumulation in the nucleus (Laños et al., 2003).

The proteasome-mediated ERα degradation is now known to be necessary for the transcriptional activity of ERα as shown by the loss of oestrogen-mediated activation of gene transcription concomitant with the use of the proteasome inhibitor MG132 (Lonard et al., 2000). Using FRAP analysis (fluorescence recovery after photobleaching) and ChIP, Reid et al. (2003) have shown that a proportion of newly synthesized and unliganded ERα have high nuclear mobility and continuously cycles on E2-responsive genes, with the nuclear matrix-associated proteasome playing a central role in clearing the receptor. This cycle is thought to allow continuous sensing of E2 levels. Following E2 binding and ERα-mediated transcription, ERα is no longer needed and is therefore rapidly degraded by the proteasome in order to limit oestrogen responsiveness (Reid et al., 2003). In this cycle, co-factors of ERα that are involved in the recruitment of the transcriptional machinery are also components of the proteasome degradation pathway and include the ubiquitin ligase and coactivators E6-AP and BRCA1 (Chu et al., 2007). Post-translational modifications of ERα, such as phosphorylation, also play an important part in regulating the cellular levels of the receptor. For example, it has been shown that phosphorylation of the serine residue Ser118 of ERα, which can serve as a phosphoacceptor site for MAPKs (Kato et al., 1995), is an essential determinant of E2-induced degradation of the receptor (Valley et al., 2005).
1.5 Anti-hormone therapy: response and resistance

1.5.1 Selective Oestrogen Receptor Modulators (SERMs)

Approximately 70% of all breast cancers are dependent for their growth and development on oestrogen and a functional ERα (ERα-positive tumours; Ali & Coombes, 2002). Therefore, most ERα-positive breast tumours can be effectively treated with agents that reduce the growth stimulatory effect of oestrogen. For more than 30 years, the anti-oestrogen tamoxifen has been used as the gold standard agent for the first line treatment of early and advanced ERα-positive breast cancers in both pre- and post-menopausal women (Jordan, 2007). However, a proportion of ERα-positive tumours (~30%) do not respond to tamoxifen (de novo resistance). The use of tamoxifen as a chemopreventive drug became a clinical reality after the NASBP P-1 (National Surgical Adjuvant Breast and Bowel Project) study showed that administration of tamoxifen at a daily dose of 20 mg for 5 years reduced the incidence of invasive and non-invasive breast cancer by approximately 50% in high risk pre- and post-menopausal women (Fisher et al., 1998). Thus in 1999, tamoxifen became the first drug to be approved by the US food and drug administration (FDA) for the prevention of breast cancer in women at increased risk for the disease (Lewis & Jordan, 2007).

Once in the body, tamoxifen is activated to its active metabolites (including 4-hydroxytamoxifen and endoxifene), which binds to ERs with much higher affinity and competes with oestrogen for binding to the LBD (Ring & Dowset, 2004). This results in the blocking of the AF2 region of the receptor and inhibition of ERα-mediated transcription through recruitment of co-repressors such as NCoR and SMRT (Webb et al., 2003). By failing to inhibit the AF1 region, tamoxifen exhibits beneficial oestrogen-like effects in tissues like bones, thus preventing osteoporosis in post-menopausal women (Jordan, 2007). However, the partial-agonist activity of tamoxifen in the uterus has been shown to increase the risk of endometrial cancer in women 50 years of age and older (Fisher et al., 1998). Other undesirable side effects of tamoxifen also include hot flushes and thromboembolic events. The observed oestrogenic and antioestrogenic actions of tamoxifen in different tissues have led to the reclassification of the drug from a non-steroidal anti-oestrogen to a selective
oestrogen receptor modulator (SERM, Figure 1.7). Another SERM that has also been shown to maintain bone density in postmenopausal women is raloxifene, which has been used for the treatment and prevention of osteoporosis for the last 10 years (Jordan, 2007). Interestingly, in the multiple outcomes of raloxifene evaluation (MORE) study for the prevention of osteoporosis in postmenopausal women, raloxifene therapy for 3 years has also been shown to reduce the risk of invasive breast cancer by 75% without an increased risk of endometrial cancer (Cumming et al., 1999). This study has paved the way for the NSABP-STAR trial (study of tamoxifen and raloxifene), which essentially aimed at testing raloxifene as a chemopreventive agent for breast cancer and compared its benefits and side effects with tamoxifen in high-risk postmenopausal women (Vogel et al., 2006). Results in this study were very promising showing that raloxifene is as effective as tamoxifen in reducing the risk of invasive breast cancer but with reduced risks of endometrial cancer and thromboembolic events.
Figure 1.7 Chemical structures of 17β-oestradiol, fulvestrant, tamoxifen and raloxifene.
1.5.2 Selective Oestrogen Receptor Downregulators (SERDs) and Aromatase inhibitors

As a result of the partial oestrogenic effects of SERMs, other anti-hormone agents have been developed. Using an alternative endocrine therapy when resistance develops can delay the need for cytotoxic chemotherapy, which leads to a significantly reduced quality of life and is generally considered to be a last option for patients with advanced breast cancer. Therefore, the sequential use of endocrine agents is a well acknowledged treatment strategy for advanced breast cancer (Gradishar, 2004). Many of the ER-positive breast tumours resistant to tamoxifen still respond to second line therapies that can include the pure anti-oestrogen fulvestrant or aromatase inhibitors (AIs), indicating that oestrogen still remain an important factor in the growth of tamoxifen resistant tumours (Riggins et al., 2007).

Fulvestrant (also known as Faslodex® or ICI 182,780) is the first of a new type of drugs known as selective oestrogen receptor downregulators (SERDs) or pure anti-oestrogen agents (Figure 1.7). They bind to ER with much higher affinity than tamoxifen, and downregulate the cellular levels of the receptor by increasing its degradation rate via the 26S proteasome complex. When complexed to ERs, fulvestrant prevents receptor dimerisation and disrupt nucleo-cytoplasmic shuttling, thereby blocking nuclear localization of the receptor. Most importantly, fulvestrant has no oestrogen agonist activity since it is able to block both the AF1 and AF2 sites of the receptor, therefore resulting in complete abrogation of ER-mediated transcription (Lynn, 2004; Osborne et al., 2004). Phase III clinical trials have shown that fulvestrant can be used effectively for the treatment of advanced breast cancers that have progressed on tamoxifen but still retain the ER (Howell et al., 2002; Osbourne et al., 2002). As a result, fulvestrant is now an FDA-granted alternative endocrine agent for the treatment of ER-positive metastatic breast cancer in postmenopausal women who have relapsed on prior anti-oestrogen therapy including tamoxifen (Howell, 2006).

In contrast to SERMs and fulvestrant whose mode of action relies on binding to ERs, aromatase inhibitors (AIs) act by preventing the formation of oestrogen from androgens in postmenopausal women through inhibition of the aromatase enzymes.
In postmenopausal women, breast tumours are able to locally produce high levels of oestrogen, which can be 10-20 times higher than the corresponding plasma oestrogen levels (Geilser, 2003). Anastrozole (arimidex®), letrozole (femara®) and exemestane (aromasin®) are the 3 FDA-approved AIs for the treatment of ER-positive breast cancer in postmenopausal women (Choueiri et al., 2004). AIs are now gradually replacing tamoxifen as a first-line therapy because of their superior efficacy for the treatment of metastatic breast cancer in postmenopausal women (Dixon & Bundred, 2006). The superior efficacy of AIs over tamoxifen has also been shown for the treatment of early breast cancer in postmenopausal women by several important phase III clinical trials. For example, the Arimidex and Tamoxifen Alone or in Combination (ATAC) trial has shown that upfront use of anastrozole for 5 years instead of tamoxifen is a better therapeutic option for postmenopausal women with early (localised) ER-positive breast cancer, improving disease-free survival by approximately 20% (Howell et al., 2005).

Although the newer more effective AIs are now gradually being introduced in the clinic, tamoxifen still remains the most commonly used anti-hormonal therapy for breast cancer for reasons that include its low cost and long-span clinical history (As discussed later in this thesis, Chapter 4).

1.5.3 Anti-hormone resistance

Although anti-hormone agents, as represented by SERMs, SERDs and AIs, have significantly improved patient care over almost four decades, the major consequence of prolonged treatment with endocrine therapy is the development of drug resistance. Because tamoxifen is the most commonly used anti-hormonal agent, understanding resistance to tamoxifen is of obvious clinical importance. Tamoxifen treatments result in tumour remission and improve the overall survival in approximately 70% of ER-positive breast cancers. However nearly all patients with metastatic disease and as many as 40% of patients receiving adjuvant tamoxifen therapy (early breast cancer) eventually relapse and acquire resistance to the drug despite continued expression of ERα (Ali & Coombes, 2002; Ring & Dowsett, 2004). Resistance to tamoxifen can also be de novo, which is mostly represented by
patients whose breast cancers lack expression of ERs at the time of diagnosis (approximately 30% of all breast cancer). However, de novo resistance also occurs in 30% of ERα-positive breast cancers (Riggins et al., 2007). The efficacy of tamoxifen is also greatly limited in ER-positive patients that do not express progesterone receptors (Clarke et al., 2003). As reviewed in several publications, different mechanisms that could contribute to tamoxifen resistance have been proposed including ERα mutation (Graham et al., 1990), increased prevalence of ERα protein variants (Poola & Speirs, 2001), alteration of the expression and activity of ERα co-factors (Smith et al., 1997) and of cell cycle regulators (Butt et al., 2005), as well as reduced intra-tumoral levels of metabolically activated tamoxifen (Osborne et al., 1991). Two other mechanisms that have been identified for tamoxifen resistance are utilisation of alternative growth regulatory pathways and loss of ERα expression, as described below.

1.5.3.1 Growth factor signalling

One of the most important and well documented mechanisms for tamoxifen resistance is aberrant activation of growth factor signalling pathways, which “cross-talk” with ERs in order to circumvent the anti-proliferative and pro-apoptotic effects normally induced by anti-hormonal drugs like tamoxifen (Knowlden et al., 2003; Kumar et al., 1996; Kurokawa et al., 2000, Benz et al., 1993). One important and well documented signalling pathway involved in mediating tamoxifen resistance is EGFR/HER-2/MAPK, whose expression levels and/or activity have been found elevated in several in vitro models of tamoxifen-resistant cell lines (Fan et al., 2007; Knowlden et al., 2003; Long et al., 1992). The contributing role of EGFR signalling in tamoxifen resistance has been demonstrated in vitro with the use of Gefitinib (Iressa®), which selectively inhibits EGFR activity resulting in the significant inhibition of cell proliferation in TamR cells (Nicholson et al., 2005). An increase in EGFR/HER-2 signalling is also evident in clinical materials (Gee et al., 2005).
Chapter 1. Introduction

EGFR is a member of the ErbB receptor tyrosine kinase family involved in regulation of cell proliferation, and which also comprises 3 other members, namely erbB2/HER-2, erbB3/HER3 and erbB4. ErbB2 has no known ligand while ErbB3 harbours a defective tyrosine kinase domain (Harari & Yarden, 2000). It has been shown that an EGFR/HER-2-driven autocrine regulatory loop occurs in TamR cells in which EGFR/MAPK-induced phosphorylation of tamoxifen-bound ERα at Ser118 results in the increased expression of EGFR ligands such as TGFα, and most predominantly amphiregulin (AR; Hutcheson et al., 2003; Britton et al., 2006). Precisely, Britton et al. have shown that the EGFR/Erk1/2-increased phosphorylation of ERα at Ser118 results in recruitments of the coactivator p68 RNA helicase and SRC1 at the AR gene, whose promoter region contain ERE sites (Britton et al., 2006). As a result, transcription of AR is increased and this provide a constant positive feedback loop by further enhancing the activity of EGFR and inducing preferential heterodimerization with HER-2. Interestingly, HER-2 is amplified and/or overexpressed in 20-30% of breast cancers and is thought to be associated with a more aggressive phenotype and a poor clinical outcome to tamoxifen therapy (Harari & Yarden, 2000). Encouragingly, recent in vivo studies using xenograft models of ERα-positive/HER-2–overexpressing MCF-7 cells, have shown that the combinatory inhibition of EGFR (with gefitinib/iressa®) and HER-2 (with trastuzumab/herceptin®) result in a significant delay of the development of tamoxifen resistance (Arpino et al., 2007).

The role of growth factor receptors and the autocrine actions of their ligands in mediating the growth of tamoxifen resistant cells has also been observed with IGF-IR and its activating ligand IGF-II. IGF-IR is known to be a key factor in promoting the growth of hormone-sensitive breast cancer cells by cross-talking with ERs (Nicholson et al., 2005, Cesarone et al., 2006). In vitro studies have demonstrated that a "cross-talk" mechanism exists between IGF-IR and EGFR in models of anti-hormone resistance including TamR cells (Knowlden et al., 2005; Santen et al., 2005). One primary consequence of this cross-talk is the further enhancement of EGFR signalling in TamR cells. In this cross-talk, the autocrine release of IGF-II ligands in TamR cells further activate EGFR signalling through the IGF-IR and the non tyrosine kinase c-Src. More precisely, IGF-II-induced activation of c-Src
through IGF-IR results in phosphorylation of EGFR at tyrosine 845 (Y845) and enhancement of EGFR signalling. Interestingly, clinical breast cancer samples originating from ER/EGFR-positive tamoxifen-resistant patients have shown expression of the activated forms of IGF-IR (Gee et al., 2005). Overall, aberrant activation of EGFR signalling (and to a lesser extent IGF-IR signalling) results in hyper-activation of downstream effectors such as the MAPKs Erk1/2 and Akt (Figure 1.8). The latter components increase the phosphorylation/activity of ERα at Ser118 and Ser167, respectively, thus reducing tamoxifen sensitivity. This so-called “cross-talk” mechanism between ERα and growth factor signal pathways is a well acknowledged contributory factor of tamoxifen resistance (Osborne et al., 2005; Massarweh & Schiff, 2006; Jordan et al., 2004). Furthermore, it was also shown that the specific conformation of tamoxifen-bound ERα can result in phosphorylation of the receptor at Ser305 by the protein kinase A (PKA), converting tamoxifen from an inhibitor to a stimulator of cell proliferation (Michalides et al., 2004).

As mentioned previously, coregulator proteins interacting with ERs at target genes can significantly influence ER-mediated transcription. It is now widely recognised that alteration of the corepressors to coactivator ratio, which determine the direction of transcription (activation or inhibition), can contribute to tamoxifen resistance (Kumar et al., 2005; Takimoto et al., 1999). A clinically relevant example is the coactivator SRC3/AIB1, which is overexpressed in more than 50% of breast cancers and can be activated by MAPKs (Anzick et al., 1997). In vitro studies have shown that overexpression of AIB1 in cultured cells enhances the partial-agonist activity of tamoxifen, more particularly in cells that also exhibit overexpressed levels of the HER-2 receptor (Osborne et al., 2003). Interestingly, Osborne et al. (2003) have also showed that patients receiving adjuvant tamoxifen therapy while having high levels of HER-2/AIB1 had a lower disease free survival than patients with low levels of HER-2 and/or AIB1. For this reason, the same authors have suggested that overexpression of HER-2 represent a poor prognostic factor for tamoxifen therapy only if high levels of AIB1 are also present in patients.
Figure 1.8  **Model depicting signaling molecules implicated in anti-hormone resistance.** Inappropriate activation of growth factor (GF) signaling pathways can result in anti-hormone resistance. CoAs, coactivators; GTFs, general transcription factors (adapted from Nicholson & Johnston, 2005).
1.5.3.2 Loss of ERα expression

Lack of ERα expression at the time of diagnosis represent the major cause of de novo resistance to tamoxifen, affecting approximately 30% of all breast cancers. Although most ERα-positive breast tumours retain ERα expression over the course of endocrine therapy, some can progress to ERα-negative tumours. In the metastatic setting, for example, approximately 30% of patients lose ERα expression on tamoxifen relapse (Holloway et al., 2004). In endocrine therapy, loss of ERα expression is a major clinical problem because ERα-negative breast tumours are associated with a more aggressive phenotype and increased disease spread, which ultimately means a worse clinical outcome for the patients (Gruvberger et al., 2001).

There is a continuing debate as to the origins of ERα-negative breast cancers. On one hand, evidence present in the literature supports the idea that ER-negative breast tumours may evolve either from ERα-positive cells that stop expressing the receptor as tumours “naturally” progress and enlarge or in response to prolonged anti-hormonal treatments like tamoxifen (adaptive mechanism). Kuukasjarvi & coworkers (1996) have reported the loss of ERα expression in metastatic lesions in the absence of intervening treatments. In contrast, other studies carried out from tumour biopsies (before and after intervening tamoxifen treatments) have demonstrated a significant decrease of ERα levels in a proportion of tumours that have relapsed on tamoxifen (Johnston et al., 1995). In contrast to the adaptive mechanism, it is also believed that a selective mechanism may also underlie both de novo and acquired ERα-negative breast cancers. Breast tumours are notoriously heterogeneous diseases and may therefore contain a small and stable population of ERα-negative progenitor cells, which can become independent of the oestrogen environment within the breast tumours and achieve a growth advantage over ERα-positive cells as tumours progress or, in the context of anti-hormone therapy, these ERα-negative progenitor cells may be naturally selected by tamoxifen, which primarily decrease proliferation of ERα-positive cells (Allred et al., 2004; Bachleitner-Hofmann, 2002).
The mechanisms underlying both de novo and acquired ERα-negative breast cancers remain poorly defined. However, epigenetic silencing of the ERα gene has been shown to be one contributing factor in approximately 25% of de novo ERα-negative breast cancers (Yan et al., 2001). Two extensively studied epigenetic mechanisms that might lead to loss of ERα expression is methylation of CpG islands by DNMTs and histone deacetylation by HDACs. CpG islands represent cytosine/guanine (GC)-rich areas, which are located within the promoter regions of about 40% of mammalian genes (Egger et al., 2004). As described in section 1.6.1, the formation of a transcriptional repression complex including DNMT1 and HDAC1 has been shown to be present in the proximal promoters of the ERα gene and cause ERα silencing in ERα-negative breast cancer cell lines (Macaluso et al., 2003 and 2007). Hypermethylation of CpG islands within the promoters A and C of the ERα gene is now known to be directly correlated with lack of ERα expression in some ERα-negative breast cancer cells including MDA-MB-231 as well as in human primary ERα-negative breast cancers. Interestingly, the demethylating agent 5-aza-2-deoxycytidine, which inhibits DNMT1, has been shown to re-induce ERα expression and function in ERα-negative cell lines (Yan et al., 2001; Yang et al., 2001). In addition, the loss of critical positive transcription factors involved in ERα transcription, such as ERBF-1, has also been suggested to contribute to ERα loss in vitro (Yoshida et al., 2000).

Because epigenetic silencing of the ERα gene is a mechanism that can only explain a fraction of ERα-negative breast cancers, other mechanisms are likely to be involved in loss of ERα expression. It is now well acknowledged that sustained and hyperactivated growth factor signalling may promote significant reduction or even loss of ERα expression (Gee et al., 2004; Massarweh & Schiff, 2006; Oh et al., 2001). In fact, it has been known for some time that an inverse relationship exist between EGFR/HER-2 expression and ER levels in clinical breast cancer, with overexpression of these growth factor receptors also being associated with decreased sensitivity to endocrine therapy and poor prognosis (Ciocca et al., 1992; Nicholson et al., 1994; Konecny et al., 2003). These clinical observations have been confirmed by a number of in vitro studies. Stoica & co-workers have shown that exogenous challenge of the highly ER-positive MCF-7 cells with the ligands EGF, IGF-1 and
TGFβ results in the significant downregulation of ERα mRNA and protein via mechanisms involving EGFR, IGF-IR, and PI3K/Akt (Stoica et al., 1997; Stoica et al., 2000a; Stoica et al., 2000b; Stoica et al., 2003a). TPA-induced activation of the protein kinase C (PKC) has also been shown to result in downregulation of ERα expression in MCF-7 cells (Martin et al., 1995). Also, overexpression of PKC isoforms α in ERα-positive cells has been associated with a more aggressive phenotype including enhanced cell proliferation and anchorage-independent growth (Ways et al., 1995, Tonetti et al., 2000). Overexpression of PKC isoforms α and δ have now been associated with the growth of tamoxifen-resistant cell lines (Frankel et al., 2007).

Additional evidence for a negative regulation of ERα expression by hyperactivated growth factor signalling pathways have been drawn by transfection studies in MCF-7 cells. Oh et al. (2001) have shown that transient transfection of constitutively active HER-2, MAP3K (Raf1) and MAPK kinase (MEK1) in MCF-7 cells results in hyperactivation of the downstream MAPKs Erk1/2 and concomitant downregulation of ERα (mRNA and protein), which is reversible by pharmacological inhibition of MEK or transfection with dominant negative Erk1/2. In this transfection study, loss of ERα expression has been shown to result in oestrogen-independence growth and significant reduction in anti-hormone sensitivity. In an attempt to define the molecular mechanism underlying loss of ERα, Holloway et al. have demonstrated that downregulation of ERα induced by hyperactivated Erk1/2 is mediated, at least in part, by the nuclear transcription factor NF-κB (Holloway et al., 2004). Interestingly, NF-κB activity is found elevated in ER-negative breast cancer cell lines such as MDA-MB-231 (Nakshatri et al., 1997).
1.6 Src family kinases

1.6.1 Structure and function

Src kinase, the first oncogene to be discovered, was initially identified by Peyton Rous in the early 20th century as the transforming agent in chicken sarcomas (Martin, 2004). It is now known to be a member of the largest family of non-receptor tyrosine kinases which, in humans, includes Fyn, Yes, Yrk, Lyn, Hck, Fgr, Blk and Lck. Src-family kinases (SFKs) all share a common structure of six distinct functional domains (Figure 1.9): an N-terminal Src Homology (SH) 4 domain containing a myristylation site that is involved in anchorage of SFKs to the cell membrane, a poorly-conserved unique region, the SH3 and SH2 domains which act as “docking” sites for the intracellular substrates of SFKs. The SH1 domain harbours the tyrosine kinase activity of SFKs at tyrosine residue 419 (Y419), the auto-phosphorylation of which is required for the full kinase activity of SFKs. The C-terminal negative-regulatory tail contains a highly conserved tyrosine at position 530 (in human) whose phosphorylation causes a conformational change and inactivation of SFKs (Yeatman, 2004).

1.6.2 Src in breast cancer

A considerable amount of data have been generated supporting the role of Src as a key signalling molecule in many important cellular pathways including cell proliferation, differentiation, survival, motility, and angiogenesis (Yeatman, 2004). Elevated Src kinase activity is also known to regulate the development and metastatic progression of many types of human cancer including breast cancer (Summy et al., 2003). Also, Src kinase activity is greatly increased in breast cancer tissue compared to normal breast tissue (Verbeek et al., 1996). Interestingly, however, c-Src by itself has not been shown to be a dominant transforming oncogene in human cancers (Shalloway et al., 1984). Most importantly, Src acts as key mediator of cross-talk between ERα and the EGFR family members, a cross-talk known to be important in the acquisition of endocrine resistance (see section 1.5.3.1). Indeed, Src can physically interact with both membrane-ERα and EGFR family receptors thus relaying the non-genomic action of oestrogen, as described in section 1.3.5.3 (Song & Santen 2006). As such, Src acts as an important mediator of many...
downstream effects of receptor tyrosine kinases (RTKs). Furthermore, previous studies have shown that c-Src transfection potentiates EGFR-induced oncogenesis (Maa et al., 1995). A Src-dependent phosphorylation of EGFR at tyrosine 845 is important for such neoplastic induction (Morgan et al., 2008). Indeed, activation of the tyrosine kinase domain leads to auto- and transphosphorylation of the intracellular domain of EGFR. Phosphorylated tyrosine residues then serve as docking sites for Src, which subsequently phosphorylate EGFR at tyrosine 845 and activate a complex network of downstream cell-signaling components. These include paxillin, phosphoinositol-3-kinase (PI3K), Crk-associated substrate (CAS), and STATs (Silva, 2004; Thomas & Brugge, 1997). Furthermore, Src can also be activated by various cytoplasmic proteins including focal adhesion kinases (FAKs), which play a prominent role in relaying integrin signalling (Brunton et al., 2005).
Figure 1.9   Structural organisation of human Src family kinases (adapted from Yeatman, 2004). UR, unique region.
1.6.3 Therapeutic targeting of Src in breast cancer

The role of Src in proliferation, invasion, angiogenesis and metastasis, has led to the development of Src inhibitors in breast cancer. Blocking Src activation may slow disease progression and potentially play an important role in the adjuvant setting to prevent disease recurrence and the development of metastases. In preclinical models of acquired tamoxifen resistance, elevated levels of EGFR activity have been shown to be accompanied by an increase in Src activity and sensitivity to the Src inhibitor AZD0530 (Hiscox et al., 2006).

AZD0530 (AstraZeneca, Wilmington, Delaware) is a highly selective, dual-specific, and orally available small-molecule currently in phase I studies (Hennequin et al., 2006). It inhibits Src and Abl kinase activity by competing for binding at the ATP binding pocket of Src/Abl proteins. AZD0530 was shown to inhibit both anchorage-dependent and -independent growth in several cell lines and to also increase cell sensitivity to growth inhibition by tamoxifen (Herynk et al., 2006). There are two other orally-active competitive ATP inhibitors currently in Phase I clinical trials: Dasatinib (BMS354825, Bristol Myers Squibb) and SKI-606 (Wyeth Research, Lombardo et al., 2004).

Dasatinib is currently approved for second-line treatment of chronic myelogenous leukaemia (CML) and Philadelphia chromosome-positive acute lymphoblastic leukaemia. Encouragingly, preclinical studies in breast cancer have shown that breast cancer cell lines representing the basal/triple-negative subtype are uniquely sensitive to growth inhibition by Dasatinib (Finn et al., 2007). Furthermore, treatment of the ER-negative MDA-MB-231 human breast cancer cells with SKI-606 also resulted in a dose-dependent inhibition of cellular proliferation and invasion (Jallal et al., 2007).

A number of other Src-inhibitory small molecules exist, which inhibit Src protein-protein interactions by blocking the SH2 and SH3 domains of Src (Oneyama et al., 2002), while some other Src-inhibitory agents, such as the benzoquinone antibiotics, interfere with the stability of Src protein by inhibiting the molecular chaperone Heat Shock Protein 90 (Hsp90, Beliakoff & Whitesell, 2004).
1.7 AIMS

ERα expression remains a strong predictor of good primary response to tamoxifen. However, one great challenge in breast cancer prevention and treatment is to prevent a drift towards more aggressive cancer cell behaviour as exemplified by both the de novo and acquired ERα-negative forms of the disease. Current hormonal therapies like tamoxifen are almost entirely ineffective in ERα-negative breast cancers, which are ultimately treated with cytotoxic chemotherapies. Although the development of acquired resistance to tamoxifen is mainly associated with maintained ERα expression, previous in vivo studies carried out from tumour biopsies (before and after intervening tamoxifen treatments) have demonstrated a significant decrease of ERα levels in tumours that have relapsed on tamoxifen. Although less commonly observed, the same studies have also shown that a proportion of breast tumours evolve from an ER-positive to an ER-negative status on relapse to tamoxifen in the adjuvant and metastatic settings (Johnston et al., 1995; Kuukasjarvi et al., 1996). The exact molecular mechanisms underlying ERα expression and loss over the course of anti-hormone treatments are still unclear. In light of the aforementioned clinical observations regarding ERα loss, this project aimed to develop an in vitro model of tamoxifen resistant cells in order to investigate whether long term tamoxifen treatment can induce loss of ERα expression in vitro thereby allowing one to study the underlying mechanisms. The present work was associated with two main study arms, which were:

- Analysis of ERα expression using in vitro models of MCF-7-derived tamoxifen resistant breast cancer cells (TamRs), which were cultured in the presence of 4-hydroxy-tamoxifen (4-OHT) up to a 30 month period. Using quantitative PCR, the expression of total ERα mRNA and its constituent mRNA variants were quantified in the endocrine-sensitive MCF7 cells, and TamR cells. Expression of ERα protein was also assessed by western blot and immunocytochemical analyses.

- Investigation of the molecular mechanisms involved in the regulation of ERα expression in TamR cells by means of pharmacological challenges.
Chapter 2

~ Material & Methods ~
Unless otherwise stated, all tissue culture medium and their chemical constituents were purchased from Gibco®-Invitrogen (Paisley, UK). All tissue culture plastic ware was purchased from Nunc (Roskilde, Denmark), supplied by Fisher Scientific (Loughborough, UK). All general molecular grade chemicals, organic solvents and molecular biology reagents were obtained from Sigma-Aldrich® (Poole, Dorset, UK) unless otherwise stated.

2.1 Routine cell culture maintenance

All cell culture procedures were carried out in MDH Class II laminar-flow safety cabinets (BIOQUELL UK Ltd, Andover, UK). Cells were routinely grown in 75 cm² flasks and incubated at 37°C in a humidified 5% CO₂ atmosphere using Sanyo MCO-17AIC incubators (Sanyo Gallenkamp, Loughborough, UK). Culture media were changed every 3 to 4 days and cell confluency was routinely assessed using the Nikon Eclispe TE200 phase-contrast microscope (Nikon Ltd, Kingstom-upon-Thames, UK). All cell lines were passaged when 80% confluency was reached. Throughout this study, the cell lines MCF-7, FasR and Tam/TKI-R were cultured for a maximum number of 20-25 passages (~ 12 weeks). TamR cells were passaged to a maximum of 10 times (~ 8 weeks) to avoid overlapping between the different TamR sub-variants (see section 2.1.1.2). When required, frozen cell stocks were thawed as described in section 2.1.2.2.

2.1.1 Cell lines

2.1.1.1 MCF-7 cells

The endocrine-sensitive MCF-7 wild-type cell line was kindly provided by AstraZeneca Pharmaceuticals (Cheshire, UK). MCF-7 cells were routinely maintained in phenol red-containing RPMI 1640 medium (rRPMI), supplemented with 5% (v/v) foetal calf serum (FCS), penicillin (100 u/ml), streptomycin (10 μg/ml), and fungizone (2.5 μg/ml). For all experimental procedures, MCF-7 cells were washed three times with pre-warmed Dulbecco’s PBS and transferred in phenol-red-free RPMI (wRPMI) supplemented with 5% charcoal-stripped FCS.
(SFCS), L-glutamine (200 mM), the aforementioned antibiotic and anti-fungal agents. The charcoal stripping procedure was carried out in order to deplete any steroidal compounds present in the serum (see section 2.1.2.3).

2.1.1.2 TamR cells

The tamoxifen-resistant cells (TamR) were originally derived from MCF-7 cells, which were continuously exposed to $10^{-7}$ M 4-hydroxytamoxifen (4-OHT, AstraZeneca Pharmaceuticals) over a period of 6 months in wRPMI medium containing 5% SFCS and the aforementioned L-glutamine, antibiotic and anti-fungal agents (referred to as wRPMI + 5% SFSC). After an initial period of growth inhibition (~ 2 months), outgrowth of resistant cells occurred (Knowlden et al., 2003). In the present study, TamR-3, TamR-11 and TamR-18 cells corresponded to cells that were exposed to 4-OHT for 3-, 11-, and 18 months, respectively. Long-term TamR-24 and TamR-30 cells were generated by continuous exposure of TamR-18 cells with 4-OHT for an extended period of 6 months and 12 months, respectively. All TamR cells were routinely maintained in wRPMI + 5% SFSC medium supplemented with 4-OHT ($10^{-7}$ M, in ethanol).

2.1.1.3 FasR cells

The Faslodex-resistant cell line (FasR) was originally derived from continuous exposure of MCF-7 cells to $10^{-7}$ M fulvestrant (ICI 182, 780/faslodex®, AstraZeneca Pharmaceuticals) in wRPMI + 5% SFCS over a period of approximately 6 months (McClelland et al., 2001). In the present study, long-term FasR cells, which have been cultured in the presence of ICI 182, 780 ($10^{-7}$ M in ethanol) for 24 months, were used.
2.1.4 Tam/TKI-R cells

Tam/TKI-R cells were originally established by continuous exposure of TamR-11 cells to the specific EGFR inhibitor gefitinib/Iressa® (1 μM in DMSO, AstraZeneca Pharmaceuticals) for 6 months in wRPMI + 5% SFCS (Jones et al., 2004). In the present study, Tam/TKI-R cells were maintained wRPMI + 5% SFCS supplemented with 4-OHT (10^{-7} M) and gefitinib (1 μM).

2.1.2 Routine technical procedures for cell culture

2.1.2.1 Cell passaging

At ~ 80% confluency, medium was removed from the flask and cells were detached by incubation with 10 ml of pre-warmed trypsin/EDTA (0.05%/0.02% v/v in PBS) for 3-5 minutes in the incubator, or until cells were in suspension. Trypsin/EDTA was neutralized by adding an equal volume of cell medium in the flask (rRPMI + 5% FCS, or wRPMI + 5% SFCS depending on cell lines). The cell suspension was transferred to a sterile universal container and cells were then pelleted by centrifugation at 1,000 rpm for 5 minutes (Mistral 3000i centrifuge, Sanyo Gallenkamp, Loughborough, UK). Using a pipette, the cell pellet was thoroughly but gently re-suspended in 10 ml of the appropriate medium. Cells were then seeded into fresh flasks at a split ratio of 1:10 (MCF-7, TamR-3, TamR-11, TamR-18), 1:20 (TamR-24), or 1:30 (TamR-30) in 15 ml medium (per 75 cm² flask).

2.1.2.2 Cryo-preservation of cell lines

Freezing procedures were carried out as follows: cells were trypsinized as previously described and re-suspended in 10 ml of the appropriate medium and counted as described in section 2.1.5. Cells were then pelleted by centrifugation (1,000 rpm, 5 minutes) and re-suspended at a density of 1x10^6 cells/ml in the appropriate medium supplemented with 10% FCS or SFCS and containing 7.5% DMSO (v/v). 1 ml cell aliquots were then prepared in cryo-vials and immediately put at -80°C for 24 hours. Cells were then transferred to liquid nitrogen for long-
term storage. For thawing procedures, cryo-vials were removed from liquid nitrogen and thawed as quickly as possible to limit cell exposure to DMSO. Vials were then sprayed with 70% ethanol in a sterile laminar-flow cabinet and allowed to evaporate completely prior to opening of the vials. Cells were transferred to a sterile universal container, washed with 9 ml of the appropriate medium and pelleted by centrifugation (1,000 rpm, 5 minutes). Cells were re-suspended in the appropriate medium containing 5% FCS or SFCS and transferred into 12.5 cm² flasks for overnight incubation at 37°C. Medium was changed and cells cultured as normal.

2.1.2.3 Charcoal stripping procedure for 100 ml FCS

A charcoal solution (2 g activated charcoal, 0.01 g dextran T70 in 18 ml dH₂O) was stirred vigorously for at least one hour. FCS was adjusted to pH = 4.2 using HCl (5 M) and allowed to equilibrate for 30 minutes at 4°C. 5 ml of charcoal solution was added to 100 ml FCS and the solution stirred gently for 16 hours at 4°C. Charcoal was then removed by centrifugation (12,000 rpm for 40 minutes) and the supernatant coarse-filtered through Whatman filter paper No. 4 to ensure total removal of charcoal. The solution was re-adjusted to pH = 7.2 using NaOH (5 M) and filter-sterilized using a 0.2 µM Supor Vacucap® membranes (Gellman Laboratory Pall, Ann Arbor, USA). Charcoal-stripped FCS was aliquotted in sterile universal container and stored at -20°C.

2.1.2.4 Cell counting

To seed cells for experimental analysis, cell monolayers were washed twice in pre-warmed PBS, trypsinized and re-suspended in the appropriate medium, as described in section 2.1.2.1. Cells were then passed through a sterile 25G syringe needle in order to obtain a single-cell suspension. 50 µl of this suspension was added to 10 ml of Isoton® II solution and cell number was determined using a Coulter™ Multisizer II (Beckam Coulter UK Ltd, High Wycombe, UK). Cells were then seeded into flasks or dishes in the appropriate medium at the required cell densities.
For basal growth curve analysis and growth responses to faslodex and gefitinib, cells were seeded into a 24-well plate at the appropriate densities and carried out as described in *Chapter 2-Results* (Figure 3.17 and Figure 3.18). Following growth of cells, the medium was removed from wells prior to the addition of 1 ml trypsin/EDTA per well. Plates were incubated at 37°C in the aforementioned Sanyo MCO-17AIC incubator for 5 minutes, or until the cells detached from wells. Cells were transferred into a 5 ml syringe by passing through a 25G needle three times in order to obtain single-cell suspensions. 3 x 1 ml of Isoton® solution was added in each well and also transferred to the same 5ml syringe to make up a 4 ml cell suspension. The latter was mixed with 6 ml of Isoton® solution in a counting cup to give a total 10 ml cell suspension, which was counted using the Coulter™ Multisizer II according to the manufacturer’s instructions. The counts obtained were multiplied by 20 to give the total number of cells per well.

2.1.3 Treatments

Throughout this study, cells were subjected to treatments with various compounds including hormones, anti-hormones, and signal transduction inhibitors (STIs). Details of these treatments (durations and compound concentrations) are indicated in figure legends throughout *Chapter 3-Results*.

2.2 Gene expression analysis

2.2.1 Total RNA extraction

All the centrifugation steps were performed at 13,000 rpm for 10 minutes at 4°C (Labofuge 400R centrifuge, Heraeus, Germany). Cells were grown in the appropriate routine medium in 100 mm dishes at a seeding density of 1 x 10^6 or 0.5 x 10^6 (TamR-30 cells) unless otherwise stated in *Chapter 2-Results*. Total RNA extractions were performed using the TRI REAGENT® according to the manufacturer’s instructions (Sigma-Aldrich®, Poole, UK). Briefly, cells were
washed twice with pre-warmed PBS and lysed directly on the dish by adding 1 ml of TRI REAGENT®. After 5 minutes, the viscous solution was transferred to sterile eppendorf tubes (Elkay, Galway, Ireland) to which 0.2 ml of chloroform was added (per ml of TRI REAGENT®). The solutions were vigorously but gently mixed for 15 seconds and allowed to stand at room temperature for 10 minutes prior to centrifugation. The upper aqueous phase (containing total RNA) was transferred into a fresh eppendorf tube to which 0.5 ml isopropanol (per ml of TRI REAGENT®) was added to precipitate RNA. Samples were vigorously mixed and incubated at room temperature for 10 minutes prior to centrifugation. RNA pellets were then washed with 1 ml 75% ethanol (per ml of TRI REAGENT®) followed by centrifugation. The supernatant was discarded and RNA pellets were air-dried for 10 minutes and dissolved in 20-50 μl of nuclease-free water (Sigma-Aldrich®). Samples were routinely stored at -70°C.

2.2.2 Nucleic acid quantification by spectrophotometry

Nucleic acids were diluted 1:200 in Tris/EDTA buffer (10 mM Tris-Cl, 1 mM EDTA; pH = 8) and transferred to quartz cuvettes. Nucleic acid concentrations were determined by measuring the optical density (OD) of the diluted nucleic acid solutions at 260 nm and 280 nm using the Cecil CE 2041 spectrophotometer (Cambridge, UK). RNA or DNA concentrations (µg/ml) were calculated according to the formulas below. Pure DNA and RNA samples are expected to have a $A_{260}/A_{280}$ ratio of approximately 1.8 and 2.0, respectively (Sambrook et al., 1989).

$$(A_{260}) \times (50 \mu g/ml) \times \text{dilution factor} = \mu g/ml \text{ DNA}$$

$$(A_{260}) \times (40 \mu g/ml) \times \text{dilution factor} = \mu g/ml \text{ RNA}$$

2.2.3 cDNA synthesis by reverse-transcription PCR (RT-PCR)

Complementary DNA strands (cDNA) were generated from total RNA using the Molony-murine leukaemia virus (MMLV)-reverse transcriptase enzyme (Invitrogen, Paisley, UK) and random hexamer oligonucleotides (Amersham
Bioscience, Buckinghamshire, UK). RT-PCR reactions were carried out on ice in a final volume of 20 μl as follows: 1 μg total RNA was adjusted to a volume of 7.5 μl with nuclease-free dH₂O and mixed with 11 μl of a master-mix containing:

- 2 μl random hexamers (100 μM stock, Pharmacia Biotechnologies, Hertz, UK)
- 5 μl dNTP mix (2.5 mM stock containing 0.625 mM of each dNTP, Invitrogen),
- 2 μl DTT (0.1 M stock),
- 2 μl 10X PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM MgCl₂, 0.01% v/v gelatine).

Reaction mixtures were incubated at 95°C for 5 minutes (denaturation step) using the PTC-100 thermocycler (MJ Research Ltd., Massachusetts, USA) and were then rapidly cooled on ice prior to brief centrifugation. 1 μl of MMLV enzyme (200 U/μl) and 0.5 μl of RNasin™ RNAse inhibitor (40 U/μl) were added to the reaction mixtures, bringing the final volume to 20 μl. RT-PCR reactions were run in the PTC-100 thermocycler using the following standard cycle conditions: step 1, 22°C/10 minutes; step 2, 42°C/40 minutes; step 3, 95°C/5 minutes.

2.2.4 Real time quantitative PCR (qPCR) analysis of total ERα and ERα mRNA variants

Using cDNA samples as templates, expression levels of total ERα mRNA and ERα mRNA variants were quantitatively assessed by qPCR analysis with the DyNAmo™ SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland). All real-time qPCR reactions were carried out in the DNA Engine® Opticon 2 system (MJ Research Ltd., Massachusetts, USA), which combined a continuous fluorescence detector and a traditional thermal cycler. All qPCR reactions were set up on ice in a Labconco Purifier PCR enclosure (GRI, Rayne, UK).
2.2.4.1 Primers

Primer pairs for β-actin and total ERα were as previously reported and were originally designed to span introns/exon borders for the detection of any potential genomic DNA contamination (Knowlden et al., 1997). Primer sequences are shown in Table 2.1. Primers for specific qPCR amplifications of each ERα mRNA variant were designed similarly to previous studies (Donaghue et al., 1999; Flouriot et al., 1998). Primer pairs consisted of a forward oligonucleotide designed to specifically bind a region within the 5'UTR (untranslated region) that is unique to each ERα mRNA species; and a common reverse oligonucleotide (used in all qPCR reactions) designed to anneal to a region within the 5' end of the protein coding region exon 1 (Figure 2.1). Primers were designed with the online Primer3-web 0.3.0 software package using the default settings (Rozen & Skaletsky, 2000). The input DNA sequences corresponding to the different ERα promoters (A to F) were retrieved from the Ensembl gene sequence ENSG00000091831 (ESR1, http://www.ensembl.org). Specificity of all primers was checked using the NCBI-BLASTN program (Altschul et al., 1990).
<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence</th>
<th>Ampli con size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>forward 5'-GGA GCA ATG ATC TTG ATC TT-3'</td>
<td>204</td>
</tr>
<tr>
<td></td>
<td>reverse 3'-CCT TCC TGG GCA TGG AGT CCT-5'</td>
<td></td>
</tr>
<tr>
<td>total ERα</td>
<td>forward 5'-GGAGACATGAGAGCTGCAAC-3'</td>
<td>432</td>
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<tr>
<td></td>
<td>reverse 3'-CCAGCAGCAGTGGACAGT-5'</td>
<td></td>
</tr>
<tr>
<td>variant A</td>
<td>forward 5'-ATGAGCTGGAGGAGGAGTT-3'</td>
<td>242</td>
</tr>
<tr>
<td></td>
<td>reverse* 3'-AGGGCAGAAGGCTCGAACC-5'</td>
<td></td>
</tr>
<tr>
<td>variant B</td>
<td>forward 5'-GGCACATAAGGCAGAGTT-3'</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td>reverse* 3'-AGGGCAGAAGGCTCGAACC-5'</td>
<td></td>
</tr>
<tr>
<td>variant C</td>
<td>forward 5'-TTCACTGAGGAGGAGTT-3'</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>reverse* 3'-AGGGCAGAAGGCTCGAACC-5'</td>
<td></td>
</tr>
<tr>
<td>variant D</td>
<td>forward 5'-AGCAGACCCCTGAGAAGTT-3'</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>reverse* 3'-AGGGCAGAAGGCTCGAACC-5'</td>
<td></td>
</tr>
<tr>
<td>variant E</td>
<td>forward 5'-ACCAGATATCAGCAGCAGTT-3'</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>reverse* 3'-AGGGCAGAAGGCTCGAACC-5'</td>
<td></td>
</tr>
<tr>
<td>variant F</td>
<td>forward 5'-GATCGATGATTCCTCTCTCT-3'</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>reverse* 3'-AGGGCAGAAGGCTCGAACC-5'</td>
<td></td>
</tr>
</tbody>
</table>

* common reverse primer
Figure 2.1 Location of primers specific for ERα mRNA variants. (A) Schematic location of primers on the ERα gene promoter and protein coding region. Short coloured arrows indicate forward primers specific for ERα mRNA variants and the short black arrow represents the common reverse primer used in all qPCR reactions for the amplification of ERα transcript variants. As indicated by the short dotted arrows, universal primers were used to amplify total levels of ERα transcript. (B) Genomic sequence of the 5'-end of the ERα gene (Ensembl gene ID: ENSG00000091831) with the exact location and sequence of ERα variant-specific primers. Each promoter region is highlighted in grey. The exact location and sequence of the transcript-specific primers is shown in bold red font. The common reverse primer is located at the 5’-end of exon 1 as indicated by underlined black bold font. The translation start site is indicated by an asterisk.
2.2.4.2 Preparation of cDNA standards

For quantitative assessment of total and variant-ERα mRNA expression in the various cell lines, standard curves were generated during each qPCR run and corresponded to a range of known concentrations of total ERα cDNA amplicons. The latter were produced by semi-quantitative PCR (section 2.2.5) using total ERα primers and analyzed by agarose gel electrophoresis (section 2.2.6) prior to their purification by gel extraction according to the manufacturer’s instructions (QIAquick Gel Extraction Kit, Quiagen Ltd., Crawley, UK). Total ERα cDNA amplicons were quantified by spectrophotometry using the GeneQuant RNA/DNA Calculator (Biochrom Ltd., Cambridge, UK) and were then serially diluted in nuclease-free dH2O to generate a known concentration range of total ERα cDNA standards, which were included in each qPCR run. The same protocol was also used to generate cDNA standards for quantitative measurements of β-actin signals, against which all qPCR data were normalized. Using a serial dilution factor of 10, the optimized concentration range was 10 pg/μl-0.0001 pg/μl and 100 pg/0.001 pg/μl for total ERα cDNA and β-actin standards, respectively.

2.2.4.3 Real-time qPCR reaction set ups

Real-time quantification of total and variant-ERα mRNA expression in unknown samples was carried out using the total ERα cDNA standards. Real-time qPCR reactions for the assessment of β-actin signals (using the β-actin cDNA standards) in the same unknown samples were performed alongside but in different wells since SYBR Green is unable to discriminate between different double-stranded DNA fragments. All qPCR reactions were set up in 96-well multiplate low-profile PCR plates (Bio-Rad Laboratories Ltd., Hertz, UK), in a total volume of 25μl containing the following reagents: 12.5 μl of 1X DyNAmo PCR solution (containing dNTPs, PCR buffer, hot-start Taq polymerase and SYBR Green dye), 0.375 μl forward primer (20 μM stock), 0.375 μl reverse primer (20 μM stock), 11.3 μl nuclease-free dH2O, and 0.5 μl cDNA template (standard or unknown sample). To reduce experimental errors, a master mix (containing all the reagents except cDNA) was routinely prepared and distributed evenly (24.5 μl) across the wells prior to the
addition of 0.5 μl of the appropriate cDNA template. Negative controls (24.5 μl + 0.5 μl nuclease-free dH2O) and blanks (12.5 μl 1X DyNAmo PCR solution + 12.5 μl nuclease-free dH2O) were systematically included in all qPCR runs. Wells were sealed tightly using flat cap strips (Bio-Rad Laboratories Ltd., Hertz, UK) and mixed prior to brief centrifugation for 30 seconds at 2,000 rpm (Labofuge 400R centrifuge, Heraeus, Germany). qPCR Cycling conditions were as follows:

Step 1: initial denaturation (hot-start) 95°C/15 minutes
Step 2: denaturation 94°C/1 minute
Step 3: annealing 58°C/30 seconds x 40
Step 4: extension 72°C/1 minute
Step 6: melting curve analysis from 50°C to 95°C

2.2.4.4 Data analysis

At the end of each qPCR run, a “Data” graph of Fluorescence (or log fluorescence) versus Cycle number, and a “Standards” graph of Log Quantity versus c(t) Cycle were automatically generated and plotted by the DNA Engine® Opticon 2 software (Figure 2.2). The c(t) or cycle threshold refers to the cycle at which a sample’s fluorescent signal surpass background fluorescence “noise”. As described by the manufacturer’s instructions, the c(t) line was manually positioned on the “Data” graph of baseline and blank subtracted data prior to any quantitation of cDNA amplicons in standards and samples. The c(t) line was positioned just above background noise (whereby fluorescent signals start to increase) and adjusted so that the R² value associated with the “Standards” graph be at least 0.996 in order to ensure the accuracy of quantity data. The “Standards” graph of known initial amount of cDNA templates was then automatically used by the DNA Engine® Opticon 2 software in order to calculate the quantity (pre-set in pg/μl) of initial cDNA template in unknown samples. PCR product homogeneity in standards and unknown samples was routinely checked by “Melting curve” analysis, which was performed at the end of the cycling protocol (step 6). The “Melting curve” analysis was set up so that continuous fluorescent reading in samples was performed every 1°C (for 10 seconds)
from 50°C to 95°C. The "Melting curve" was automatically plotted at the end of each qPCR run by DNA Engine® Opticon 2 software.

Figure 2.2 Example of a (A) Standard and (B) Data graph for total ERα cDNA standards, as generated by the Opticon 2 software. The concentration range of total ERα cDNA amplicons (run in duplicate) are indicated (10-0.0001 pg/μl). C(t), cycle treshold line.
2.2.5 Semi-quantitative PCR

All semi-qPCR reactions were carried out in the aforementioned PCR enclosure. PCR reactions were performed in a total volume of 25 µl by adding 0.5 µl of cDNA templates to the following master-mix in a sterile 0.5 ml eppendorf tube:

- 18.6 µl nuclease-free dH2O (adjusted to 18 µl for β-actin amplification)
- 2.5 µl 10X PCR buffer
- 2 µl dNTPs (2.5 mM stock)
- 0.6 µl forward-primer (20 µM stock) or 0.3 µl (for β-actin)
- 0.6 µl reverse-primer (20 µM stock) or 0.3 µl (for β-actin)
- 0.2 µl BIOTAQ™ DNA polymerase (5 U/µl; Bioline Ltd., London, UK)

Reaction mixtures were thoroughly but gently mixed and briefly centrifuged (IEC Micromax RF, Thermo Electron, Hampshire, UK). Semi-qPCR reactions were performed in the aforementioned PTC-100 thermocycler pre-set with a heated lid to prevent any volume loss through evaporation. PCR running conditions were as follows:

step 1 (initial denaturation): 94°C/5 minutes
step 2 (denaturation): 94°C/1 minutes x 25 (β-actin)
step 3 (annealing): 58°C/1 minutes x 28 (total ERα)
step 4 (Extension): 72°C/1 minutes x 35 (ERα variants)
step 5 (final extension): 72°C/5 minutes

61
2.2.6 Agarose gel electrophoresis

Electrophoretic separations of nucleic acids were run in agarose gels cast in 1X Tris-Acetate EDTA/TAE buffer (40 mM Tris-acetate, 1 mM EDTA) in the presence of 0.2 µg/µl ethidium bromide. For a 3% agarose gel, 3 g of agarose (Bioline Ltd., London, UK) was dissolved in 100 ml 1X TAE buffer and the solution heated in a microwave for ~2 minutes. The solution was allowed to cool prior to the addition of 2 µl ethidium bromide (10 mg/ml), gently mixed and poured into a tray containing the appropriate comb. The polymerized gel was placed into the Mini-Sub® Cell GT electrophoretic tank (Bio-Rad Laboratories Ltd., Hertz, UK) filled with 1X TAE buffer. Appropriate volumes of PCR products were mixed with 1X DNA loading buffer (60% w/v sucrose, 0.05% w/v bromophenol blue) and dispensed into the wells. 5 µl of a molecular-weight DNA marker was also included in the gel for assessment of DNA fragment sizes (HyperLadder IV, Bioline Ltd., Hertz, UK). Electrophoreses were carried out at 70 V constant voltage for approximately 40-60 minutes (Bio-Rad Power-PAC 1000). Gels were then visualized on a standard UV transilluminator and pictures taken using the AlphaDigiDoc™ System 1000 (Genetic Technologies Inc., Miami, USA).

2.3 Protein expression analysis

2.3.1 Protein cell lysis

Cells were grown in the appropriate routine medium in 60 mm dishes at a seeding density of 5 x 10^5 cells/dish (MCF-7, TamR-3, TamR-11, TamR-18, TamR-24 cells) or 0.25 x10^5 cells/dish (TamR-30 cells), unless otherwise stated in Chapter 3. Culture medium was then removed and cells were washed twice with pre-warmed PBS prior to cell lysis with ice-cold Triton-X100 lysis buffer pH 7.5 (50 mM Tris base, 150 mM NaCl, 5 mM EGTA, 1% v/v triton-X100) containing a freshly-added cocktail of protease and phosphatase inhibitors at the following final concentrations: sodium orthovanadate (Na_3VO_4, 2mM), sodium fluoride (NaF, 200 mM), sodium molybdate (10 mM), phenylmethylsulfonyl fluoride (PMSF, 1 mM), phenylarsine oxide (20 µM), aprotinin (10 µg/ml) and leupeptin (10 µg/ml). A total volume of
250 μl of lysis buffer was added per 60 mm dish, which were left on ice for approximately 5 minutes. Cells were scrapped off the dish with a sterile cell scraper (Fisher Scientific, Loughborough, UK) and transferred to fresh eppendorf tubes, which were left on ice for approximately 10 minutes. Tubes were then centrifuged at 13, 000 rpm for 15 minutes at 4°C (IEC Micromax RF, Thermo Electron, Hampshire, UK), and supernatants (crude cell extracts) were aliquoted for storage at -20°C.

### 2.3.2 Determination of protein concentration

The concentration of solubilised proteins in crude cell extracts was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories Ltd., Hertz, UK). As described by the manufacturer's instructions, serial dilutions of the protein standard BSA (1 mg/ml stock solution) were prepared in duplicates as shown in Table 2.2. Crude cell extracts were diluted 1:200 in dH₂O, also in duplicates. 800 μl of standard or sample dilution was thoroughly mixed with 200 μl of Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories Ltd., Hertz, UK) in a semi-micro cuvette (Bio-Rad), which was incubated for 5-15 minutes at room temperature. Absorbance of standards and samples was then measured at a wavelength of 595 nm using the Cecil CE 2041 spectrophotometer, which automatically generated a standard curve with a relative quantification of protein concentration (in μg/μl) corrected with the dilution factor.

<table>
<thead>
<tr>
<th>BSA final concentration (μg/ml)</th>
<th>Volume BSA, 1 mg/ml (μl)</th>
<th>Volume dH₂O (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1000</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>995</td>
</tr>
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<td>980</td>
</tr>
<tr>
<td>25</td>
<td>25</td>
<td>975</td>
</tr>
</tbody>
</table>
2.3.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

All SDS-PAGE experiments were carried out using the Bio-Rad Mini-Protean® 3 Cell system. Protein samples (50 µg) were denatured by boiling for 5 minutes in appropriate volumes of 2X Laemmli loading buffer (8% w/v SDS, 40% glycerol, 0.25 M Tris base, 0.02% w/v bromophenol blue, and 0.3 M DTT added freshly). Samples were collected by pulse-centrifugation at 13,000 rpm. Proteins were loaded into a 7.5% resolving gel overlaid with a 4% stacking gel, as described by the manufacturer’s instructions (see Table 2.3 for polyacrylamide gel preparations). The electrophoresis tank was half-filled with 1X Tris/Glycine running buffer, which was routinely prepared from a 10X stock solution (Tris base, 250 mM; glycine, 192 mM; SDS, 1% w/v, solution adjusted to pH 8.3). Gel electrophoresis was run under a constant voltage of 170 V for approximately 90 minutes (Bio-Rad Power-PAC 1000), or until the bromophenol blue dye front reached the bottom of the gel. The Full Range Rainbow™ molecular weight marker was routinely included in the gel alongside protein samples (Amersham Bioscience, Buckinghamshire, UK).

Table 2.3 Composition of resolving and stacking SDS-polyacrylamide gels

<table>
<thead>
<tr>
<th>Reagents</th>
<th>7.5% resolving gel (10 ml)</th>
<th>4% stacking gel (10 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH2O</td>
<td>4.8 ml</td>
<td>6.1 ml</td>
</tr>
<tr>
<td>1.5 M Tris-Hcl, pH 8.8 (Bio-Rad)</td>
<td>2.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>0.5 M Tris-Hcl, pH 6.8 (Bio-Rad)</td>
<td>-</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>30% acrylamide/bis-acrylamide solution (Sigma-Aldrich®)</td>
<td>2.5 ml</td>
<td>1.3 ml</td>
</tr>
<tr>
<td>10% w/v SDS</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>10% w/v APS</td>
<td>100 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>TEMED (added last)</td>
<td>6 µl</td>
<td>10 µl</td>
</tr>
</tbody>
</table>
2.3.4 Western blotting

Proteins were transferred to the PROTAN® nitrocellulose membranes (0.2 μM pores, Scheilcher & Schuell, Dassel, Germany) using the Mini Trans-Blot® transfer system powered by the Bio-Rad Power-PAC 1000. Membranes were washed under shaking using the platform rocker STR6 (Stuart Scientific Bibby Sterilin Ltd., Stone, UK). Both gels and membranes were equilibrated in 1X transfer buffer (25 mM Tris base, 192 mM glycine, 20% v/v methanol) for 10 minutes under gentle shaking. Two Whatman 3MM filters and two fibre pads were also pre-soaked in 1X transfer buffer and assembled in cassettes together with the equilibrated gel and membrane according to the manufacturer’s instructions. Any air bubbles that formed between the different layers were removed by gentle pressure with a serological pipette as they can disrupt protein transfer from gel to membrane. The cassette was then placed into the transfer apparatus according to the manufacturer’s instructions. A frozen cooling unit and stir bar were place inside the buffer tank, which was filled with 1X transfer buffer. Protein transfer was carried out at constant voltage (100 V) for 1 hour under constant stirring. After transfer, membranes were briefly washed in 1X TBS-Tween buffer, which was routinely prepared from a 10X stock solution (Tris, 100 mM; NaCl, 1 M; Tween 20, 0.5% v/v; solution adjusted to pH 7.6). Membranes were then reversibly stained with Ponceau-S solution (Ponceau S, 0.1% w/v; Acetic Acid, 5% v/v) to verify that proteins were successfully transferred and also to check for equal protein loading across samples. Ponceau S staining was removed by washing membranes with 1X TBS-Tween buffer for 5 mins with shaking. Membranes were blocked in 5% w/v non-fat dried milk (prepared in 1X TBS-Tween buffer) for at least 2 hours with gentle shaking, followed by a brief wash in 1X TBS-Tween buffer. Membranes were subsequently subjected to immunodetection or were alternatively stored at 4°C in 1X TBS-Tween buffer.
2.3.5 Immunoblotting

Blocked membranes were washed in 1X TBS-Tween for 5 mins and were then incubated with the appropriate dilution of primary polyclonal antibody overnight at 4°C. All primary antibodies were prepared in 1X TBS-Tween containing 5% v/v of Western Blocking Reagent (Roche Diagnostics, Mannheim, Germany) and 0.05% (w/v) sodium azide, except for phospho-specific antibodies against EGFR (Tyr1068), which were diluted in 1X TBS-Tween containing 1% w/v non-fat dried milk and 0.05% (w/v) sodium azide. Dilutions of all primary antibodies used in this study are shown in Table 2.4.

After incubation with the primary antibody, membranes were washed three times in 1X TBS-Tween for 10 minutes and incubated at room temperature for 1 hour with HRP-linked anti-rabbit IgG antibody (Cell signalling Technology™, New England BioLabs®, UK). The latter was diluted 1:10,000 in 1X TBS-Tween containing 5% v/v of Western Blocking Reagent. For β-actin signals, an anti-mouse IgG HRP-linked antibody was used and diluted 1:20,000 (Cell signalling Technology™). Membranes were then washed three times in 1X TBS-Tween for 10 minutes and processed for luminol-based chemiluminescence detection of the protein of interest using Supersignal™ West Pico, Supersignal™ West Dura or Supersignal™ West femto (Pierce & Warriner Ltd., Cheshire, UK) according to the abundance of the target proteins in cells. Each Supersignal™ chemiluminescent reagent was prepared according to the manufacturer’s instructions and ~250-500 μl was applied to each membrane and allowed to incubate at room temperature for 5 minutes. X-Ray films were then exposed to membranes placed in autoradiography cassettes for times ranging from 15 seconds to 3 hours depending on signal strength. X-Rays were developed using an X-O-graph Compact X2 developer (X-O-graph Imaging system, Tetbury, UK) and scanned using the AlphaDigiDoc™ System 1000 (Genetic Technologies Inc., Miami, USA).
Table 2.4 Primary antibodies for western blot analysis.

All antibodies were polyclonal anti-rabbit IgGs except for β-actin (anti-mouse monoclonal IgG) and total PKCα (anti-rabbit monoclonal IgG).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Company</th>
<th>Catalogue No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα (H-184)</td>
<td>1:10,000</td>
<td>Santa Cruz Biotechnology, CA, USA</td>
<td>sc-7207</td>
</tr>
<tr>
<td>EGFR-Tyr1068</td>
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<td>#2234</td>
</tr>
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<td>sc-03</td>
</tr>
<tr>
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<td>1:2,000</td>
<td>BioSource International, Inc., CA, USA</td>
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</tr>
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<td>Cell signalling Technologies™</td>
<td>#2242</td>
</tr>
<tr>
<td>IGF-IR-Tyr1316</td>
<td>1:1,000</td>
<td>AstraZenece, Macclesfield, UK</td>
<td>gift</td>
</tr>
<tr>
<td>Total IGF-IR</td>
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<td>Santa Cruz Biotechnology, CA, USA</td>
<td>sc-712</td>
</tr>
<tr>
<td>PKCα-Ser657</td>
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<td>Upstate Biotechnologie Inc. (NY, USA)</td>
<td>#06-822</td>
</tr>
<tr>
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<td>Transduction Laboratories (KY, USA)</td>
<td>P-16520</td>
</tr>
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</tr>
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</tr>
<tr>
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<tr>
<td>Total Foxo3A</td>
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<td>Cell signalling Technologies™</td>
<td>#9467</td>
</tr>
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</table>
2.3.6 Immunocytochemical analysis

Cells were routinely grown on sterile 3-aminopropyltriethoxysilane (TESPA)-coated coverslips, which were put in 35 mm dishes. Cells were grown to 60-70% confluency in the appropriate routine medium as described in section 2.1.1 or allowed to grow for 24 hours prior to treatments with the Src inhibitor AZD0530, as described in Chapter 2-Results (Figure 3.32). Unless otherwise stated in Chapter 2-Results, cells were seeded at 1 x 10^5 cells/dish (MCF-7, TamR-3, TamR-11, TamR-18 cells) or 0.5 x 10^5 cells/dish (TamR-24 and TamR-30 cells). The PBS solution used for antibody dilutions and washing steps was prepared in house (5L solution: 42.5 g NaCl, 7.15 g K_2HPO_4, and 1.25 g KH_2PO_4 in dH_2O). Prior to immunodetection, cells were fixed by the ER-ICA or formal saline method depending on the protein target. Visualisation of immunostainings and subsequent HScore analysis were carried out using an Olympus BH-2 light microscope connected to an Olympus DP12 digital camera.

2.3.6.1 ER-ICA fixation

Coverslips were removed from culture medium and immersed in a freshly prepared 3.7% v/v formaldehyde solution (30 ml of 37% formaldehyde in 270 ml PBS) and allowed to incubate for 15 minutes at room temperature. Coverslips were then washed in PBS for 5 minutes at room temperature prior to incubation in methanol for 5 minutes (between -10°C and -30°C), followed by acetone for 3 minutes (between -10°C and -30°C). They were then washed in PBS for 5 minutes at room temperature, transferred to 35 mm dishes filled with a sucrose storage medium (42.8 g sucrose, 0.33 g MgCl_2, 250 ml PBS, 250 ml glycerol) and stored at -20°C.

2.3.6.2 Phenol formal saline fixation

Coverslips were removed from culture medium and fixed for 5 minutes at room temperature with a 2.5% phenol formal solution (2 ml per coverslip), which was prepared by dissolving the appropriate amount of phenol in a 3.7% formal saline solution (NaCl, 4.5 g; 37% formaldehyde, 50 ml; dH_2O, 450 ml). Cells were then
washed with 100% ethanol (1 x 5 minutes followed by a brief rinse in 100% ethanol) and PBS (1 x 5 minutes followed by a brief rinse in PBS). Coverslips were stored in the sucrose storage medium at -20°C for at least 24 hours before use for immunodetection.

### 2.3.6.3 ERα

Cells were ER-ICA fixed, as described previously. Detection of nuclear ERα was carried out using the Vector-6F11 mouse monoclonal antibodies (Vector Laboratories, Burlingame, CA, USA), which was diluted 1:200 in PBS. First, the sucrose storage medium was discarded and coverslips were briefly washed in dH₂O followed by a PBS wash (3 x 3 minutes). A PBS-Tween solution (0.02% v/v Tween-20) was applied to the coverslips for 3 minutes in order to facilitate even spreading of the diluted antibody solution. Excess PBS-Tween solution was then removed and 50 μl of the diluted Vector-6F11 antibody was applied to each coverslip, which were then incubated at 23°C in a humidified atmosphere for 90 minutes. Following a wash in PBS (1 x 1 minute) and PBS-Tween (2 x 5 minutes), coverslips were incubated with 1 drop/coverlip of EnVision HRP-linked anti-mouse antibodies (Dako Ltd., Ely, UK) for 75 minutes at room temperature. Coverslips were washed in PBS (1 x 1 minute) and PBS-Tween (2 x 5 minutes), and incubated with 70μl/coverlip of EnVision D!B chromagen solution (Dako ltd.) for 10 minutes, at room temperature. Coverslips were washed in dH₂O (2 x 5 minutes) prior to counter-staining in methylgreen (0.02% v/v) for 5-10 minutes. Coverslips were rinsed in dH₂O and air-dried prior to mounting onto slides using DPX mountant.

### 2.3.6.4 AP2γ

Nuclear AP2γ was detected using the specific 6E4 anti-mouse monoclonal antibodies (Gee et al., 2008). Cells were ER-ICA fixed and the sucrose storage medium was removed from coverslips by a brief wash with dH₂O, and PBS (3 x 3 minutes). As described in section 2.3.6.3, a PBS-Tween solution (0.02% v/v Tween-20) was applied to the coverslips for 3 minutes and discarded. 60 μl (per coverslip) of 6E4 antibodies diluted 1:50 (in PBS) was applied to the coverslips and incubated
overnight at 23°C in a humidified atmosphere. Coverslips were washed in PBS (3 x 4 minutes) and PBS-Tween (1 x 4 minute) and were then incubated at room temperature for 30 minutes with EnVision HRP-linked anti-mouse antibodies (1 drop/coverslip, DAKO). 70μl (per coverslip) of EnVision DAB chromagen solution was applied and allowed to incubate for 10 minutes at room temperature. Coverslips were washed in dH₂O (2 x 5 minutes) prior to counter-staining in methyl-green (0.02% v/v) for 5-10 minutes. Coverslips were rinsed in dH₂O and air-dried prior to mounting onto slides using DPX mountant.

2.3.6.5 Total Foxo3A, phospho-Foxo3A (Ser 253)

The same protocol described for ERα (section 2.3.6.3) was followed. Total levels of Foxo3A were detected using anti-rabbit Foxo3a antibodies (Cell signalling Technologies™, #9467) diluted 1:250 in PBS. Phosphorylated/inactive Foxo3A was detected with anti-rabbit phospho-Foxo3a (Ser253) antibodies (Cell signalling Technologies™, #9466) diluted 1:20 in PBS. EnVision HRP-linked anti-rabbit secondary antibodies were used (Dako Ltd., Ely, UK).

2.3.6.6 Total EGFR, phospho-EGFR (Tyr1068)

The sucrose storage medium was removed from coverslips, which were washed in dH₂O and PBS, as described in section 2.3.6.3. A PBS-Tween solution was applied for a few seconds, discarded and coverslips were incubated overnight with Neomarkers EGFR anti-mouse primary antibodies diluted 1:140 (in PBS) for the detection of total EGFR (Lab Vision, Fisher Scientific, Loughborough, UK), or with EGFR (Tyr1068) phospho-specific anti-rabbit antibodies diluted 1:40 (in PBS containing 1% v/v BSA; BioSource International, Inc., CA, USA). Coverslips were washed in PBS (1 x 1 minute) and PBS-Tween (2 x 5 minutes) prior to incubation with 1 drop/coverslip of EnVision HRP-linked anti-mouse antibodies (Dako Ltd., Ely, UK) for 60 minutes at room temperature. Coverslips were washed in PBS (1 x 1 minute) and PBS-Tween (2 x 5 minutes), and incubated with 70μl/coverslip of EnVision DAB chromagen solution (Dako ltd.) for 10 minutes, at room temperature. Coverslips were washed in dH₂O (2 x 5 minutes) prior to counter-staining in methyl-
green (0.02% v/v) for up to 15 minutes. Coverslips were rinsed in dH₂O and air-dried prior to mounting onto slides using DPX mountant.

2.4 Haematoxylin & Eosin staining

The sucrose storage medium was discarded and coverslips were briefly washed in dH₂O followed by a PBS wash (3 x 3 minutes). A 10% Haematoxylin solution (Raymond A Lamb, Eastbourne UK) was applied to the coverslips for 5 minutes followed by a brief rinse in dH₂O. A 1% Eosin solution (Raymond A Lamb) was applied for 10 minutes; coverslips were briefly rinsed with dH₂O and allowed to dry at room temperature prior to mounting onto slides using DPX mountant.

2.5 Cell migration assay

Cell migration assays were performed using 6.5 mm Transwell® inserts (Corning Life Sciences) with an 8 μm membrane pore size. Under sterile conditions, membranes were coated with fibronectin by placing Transwell® inserts in wells of a 24-well plate (supplied by the manufacturer) containing 200μl of a fibronectin solution (10 μg/ml in wRPMI containing no supplements). The plate was incubated at 37°C for 2 hours; inserts were washed in sterile PBS and air-dried. Cells were trypsinized as described previously and seeded (in duplicate) into the inner compartment of the Transwell® inserts at a density of 5 x 10⁴ cells/insert in 200 μl of the appropriate routine medium. 650 μl of the same routine medium was added to the outer compartments (wells) of the Transwell® inserts and the 24-well plate was incubated at 37°C for 48 hours in a Sanyo MCO-17AIC incubator. The medium containing non-migratory cells was removed from the inner compartment, which was swabbed with a cotton bud. Migratory cells were fixed by incubating the inserts in a 3.7% (v/v) formaldehyde solution (prepared in PBS) for 10 minutes. Fixed migratory cells were washed in PBS and stained with crystal violet (0.5% w/v in dH₂O) for 15-30 minutes. Excess crystal violet stain was removed; inserts were washed with PBS and air-dried at room temperature. Migratory stained cells were counted at x20 magnification using an Olympus BH-2 light microscope.
2.6 Cell invasion assay

Invasion assays were carried out using the aforementioned 6.5 mm/8 μm membrane pore size Transwell® inserts (Corning Life Sciences), which were coated with 50 μl of Matrigel™ basement membrane matrix solution diluted 1:3 in ice-cold wRPMI containing no supplements (Matrigel™ sock solution: 12 mg/ml, BD Biosciences). The 24-well plate, containing the Matrigel™-coated inserts, was incubated at 37°C for 2 hours to allow the Matrigel™ to set. Cells were trypsinized as described previously and seeded (in duplicate) into the inner compartment of the Transwell® inserts at a density of 5 x 10⁴ cells/insert in 200 μl of the appropriate routine medium. 650 μl of the same routine medium was added to the outer compartments (wells) of the Transwell® inserts and the 24-well plate was incubated at 37°C for 72 hours in a Sanyo MCO-17AIC incubator. The medium was removed from the inner compartment (non-invasive cells) and the polymerised Matrigel™ removed with a cotton bud. Invasive cells on the membrane of the inserts were fixed in a 3.7% (v/v) formaldehyde solution (prepared in PBS) for 10 minutes, followed by a wash with PBS and air-dried at room temperature. The membranes were excised from the inserts using a scalpel blade and mounted, cell-side up, onto glass sides using 1 drop of VectaShield® Hard-set™ mounting medium with DAPI nuclear stain (Vector Laboratories, Burlingame, CA, USA). Slides were wrapped in foil and stored at 4°C. The number of DAPI-stained invasive cells on the membranes was assessed using the DMIRE2 Leica fluorescent microscope (Leica Microsystem, Arizona, USA), fitted with a digital camera (Orca-285, Hamamatsu, Japan).
2.7 **Statistical analysis**

For direct comparisons between two cell lines or between controls and treatments, the statistical significance was calculated using independent, two-tailed Student's *t*-test. Overall differences between control and treatment groups (or between more than two cell lines) were examined by one-way ANOVA with post-hoc tests. For immunocytochemical analysis, the Mann–Whitney U test was employed to compare median HScore values between cell lines. Differences were considered significant at the *P* < 0.05 level for all tests.

2.8 **AliBaba2.1**

Approximately 1-kb of upstream DNA sequences of the different ERα promoters was screened for transcription factor binding sites using the web-based AliBaba2.1 software ([http://www.gene-regulation.com](http://www.gene-regulation.com)). DNA sequences were pasted in the text box and the default parameters were used for analysis of transcription factor binding sites.
Chapter 3

~ Results ~
3.1 Validation of primers used for real-time qPCR analysis of variant-ERα mRNA expression

3.1.1 Verification of primer-specific PCR products by semi-qPCR and melting curve analysis

Throughout this study, the expression of total ERα mRNA and its constituent variants were quantified in various anti-hormone responsive and resistant cell lines using real-time quantitative PCR (qPCR) based on SYBR® Green fluorescence. As depicted in Figure 2.1 of Material and Methods, amplifications of the different ERα isoforms were carried out using sets of primers with forward oligonucleotides specifically annealing to the 5'UTRs, which are unique to each ERα mRNA variant. A common reverse primer was also used and was designed to anneal to the 5' end of ERα exon 1. Also, expression levels of total ERα mRNA were assessed using “universal” primers specific to a region within exon 5 of the ERα gene (Knowlden et al., 1997). These “universal” primers were not flanking intron-exon boundaries and therefore potential contamination from amplified genomic DNA could occur during PCRs. However, routine use of these primers in this study and others (Knowlden et al., 1997; McClelland et al., 2001) has shown that genomic contamination was not apparent in PCR products. In order to experimentally evaluate the effectiveness and specificities of all primers, conventional semi-quantitative PCR reactions were carried out using cDNA prepared by RT-PCR from MCF-7 cells and run on a 3% agarose gel. As shown in Figure 3.1A, each PCR reaction produced a single fragment of the expected molecular weight for total ERα mRNA and the mRNA isoforms A-F. The specificity of all primers and the homogeneity of the PCR products were further verified by melting curve analysis, which was automatically carried out by the analysis software of the DNA Engine® Opticon 2 system used in this study, as described in Material and Methods (section 2.2.5). All ERα isoform were distinguished by the melting point of their corresponding PCR products. The melting temperature of nucleic acids is affected, amongst other factors, by length and GC contents. As exemplified in Figure 3.1B, real-time PCR amplicons corresponding to total ERα mRNA and ERα mRNA variants were consistently generated with a distinguishable melting curve signature containing a single peak of
fluorescence. This strongly suggests PCR product homogeneity and the absence of primer-dimers or other non-specific products that could affect the efficiency of the PCR reactions.
Figure 3.1  Validation of primer sets for total ERα mRNA and ERα mRNA variants by (A) semi-quantitative PCR and (B) melting curve analysis in MCF-7 cells. (A) cDNA was prepared by RT-PCR from MCF-7 cells grown to 70% confluency, as described in Material and Methods. Semi-qPCR reactions were carried out using universal primer sets for total ERα, and primers specific for ERα mRNA variants A-F. PCR products were then separated on a 3% agarose gel containing ethidium bromide. The expected size of each PCR product is (in bp): total ERα, 432; A, 242; B, 190; C, 150; D, 116; E, 102; F, 100. M, Molecular weight marker. (B) cDNA from MCF-7 cells were also subjected to real-time qPCR during which a melting curve was generated. The representative graph shows the negative first derivative of the fluorescence intensity (-dl/dT) versus temperature. As indicated, each ERα transcript is associated with a specific melting curve containing a single fluorescent peak.
3.1.2 E2-induced downregulation of ERα mRNA expression in MCF-7 cells

Transcriptional regulation of the ERα gene by oestrogen (E2) has long been established in MCF-7 cells, in which treatment with the steroid hormone results in a time dependent decrease of ERα mRNA steady-state levels (Saceda et al., 1988). This reduction has later been shown to affect all ERα mRNA isoforms in a similar manner in MCF-7 cells (Flouriot et al., 1998). In light of these observations and in order to further validate the identity of all amplified ERα transcripts, the effect of E2 on expression levels of all ERα mRNA isoforms was measured in MCF-7 cells. Real-time qPCR was performed with cDNA templates prepared from MCF-7, which were cultured in the presence or absence of 17β-oestradiol for 24 h (10^-9 M). Previous studies have shown that this time point can result in a significant decrease in steady-state levels of ERα mRNA isoforms (Flouriot et al., 1998; Berthois et al., 1990). As expected, oestrogen treatment resulted in approximately 40% reduction in expression levels of total ERα mRNA, and 40-60% decrease of the different ERα mRNA variants (Figure 3.2). Transcripts generated from the proximal (A/B) and distal (C) promoters have been shown to be the most predominantly expressed in breast cancer cells overexpressing ERα protein. Accordingly in this study, the ERα mRNA isoforms A, B and C were detected as the most prevalent variants in MCF-7 cells. The liver- and bone-specific variants E and F, respectively, were expressed at lower levels whereas transcripts generated from the promoter D were almost non-detectable.
Figure 3.2  Effects of oestrogen on steady state expression levels of (A) total ERα mRNA and (B) ERα mRNA variants in MCF-7 cells. MCF-7 cells were grown to 70% confluency in wRPMI + 5% SFCS prior to treatment with 10⁻⁹ M 17β-oestradiol (MCF-7 +E2) for 24 hours. Control treatments (MCF-7 -E2) were carried out in EtOH (vehicle for 17β-oestradiol). Error bars represent the S.D. of at least three independent experiments, each carried out in duplicate. *, $P < 0.01$, **, $P < 0.001$, versus MCF-7 (-E2).
3.2 Loss of ERα expression in response to anti-hormone treatments: Proof of principle with fulvestrant in vitro

As described in Chapter 1 (section 1.5.3.2), a proportion of initially ERα-positive breast tumours become ERα-negative on tamoxifen relapse, and for those that remain ERα-positive, a significant reduction in ERα expression has been revealed (Johnston et al., 1995; Kuukasjarvi et al., 1996; Holloway et al., 2004). In light of these clinical observations, this study addressed the questions of whether, and through which molecular mechanisms, could long-term tamoxifen treatment induce loss of ERα expression (adaptive mechanism) using in vitro models of tamoxifen-resistant cells.

Previous in vitro studies have already provided proof of principle that loss of ERα expression can occur following long-term anti-hormone treatment with the pure anti-oestrogen fulvestrant (Faslodex®, ICI 182, 780; Liu et al., 2006). The same authors have generated a faslodex-resistant cell line with an irreversible ERα-negative phenotype, by culturing the ERα-positive MCF-7 cells with faslodex-containing medium for approximately 18 months. In order to validate the observations made by Liu et al., ERα mRNA and protein expression levels were also assessed using in house faslodex-resistant cells (FasR). The latter were generated by continuous exposure of the MCF-7 breast cancer cells with fulvestrant (10⁻⁷ M) over a period of 24 months (Julia Gee, personal communication). As shown in Figure 3.3, ERα expression could not be detected in FasR cells both at the mRNA and protein levels, as determined by real-time qPCR and immunocytochemistry (ICC), respectively. The ERα-negative phenotype exhibited by FasR cells was irreversible as withdrawal of faslodex from culture medium over a 6 months period did not result in re-expression of ERα (Dr. Martin Giles, personal communication). These results are in agreement with studies by Liu et al. and provide in vitro evidence that ERα expression can be lost in response to long term anti-hormone treatments of initially ERα-positive cells.
Figure 3.3 Analysis of total ERα expression levels in MCF-7 and FasR cells. (A) Real-time PCR analysis of total ERα mRNA expression levels. Total RNA was extracted from cells grown to 70% confluency in their routine medium, as described in Material and Methods. Expression levels of total ERα were normalized against β-actin expression, and results of the experiments are reported as relative mRNA expression levels. Error bars represent S.D. of at least three independent experiments, each carried out in duplicate. (B) Immunocytochemical analysis of total ERα nuclear protein. MCF-7 and FasR cells were grown to 70% confluency on glass coverslips in their routine medium followed by ER-ICA fixation, as described in Material and Methods. Total expression levels of nuclear ERα protein were detected with polyclonal mouse antibodies. The picture is representative of two independent experiments (Original magnification 20x).
3.3 Analysis of ERα expression in TamR cells

3.3.1 TamR-18 cells

A starting point for investigating whether prolonged exposure to tamoxifen can induce loss of ERα in vitro was the assessment of the receptor expression using the in house cell model of acquired-tamoxifen resistance TamR-18. As described in Material and methods, TamR-18 cells were generated by continuous exposure of MCF-7 cells with 4-OHT over a period of 18 months. Total ERα mRNA and protein expression levels were measured in TamR-18 cells and compared to those of the parental cells. As shown in Figure 3.4A, TamR-18 cells showed a significant decrease in total ERα mRNA expression levels compared to MCF-7 cells. In this study, it was initially hypothesised that progression from an endocrine-responsive to a resistant phenotype may be accompanied with a change in the prevalence of ERα mRNA isoforms. This hypothesis was based on previous studies, which have established that there was a tissue and cell specificity in the level of expression of the different isoforms (Gannon et al., 2001; Flouriot et al., 1998). Therefore, steady-state levels of all the ERα transcript variants were also analysed in TamR-18 cells by real-time qPCR.

Results showed that downregulation of ERα expression affected all the variants in TamR-18 cells in which, like in MCF-7 cells, the isoforms A/B and C were identified as the most predominant variants (Figure 3.4B). Most notably, however, expression levels of transcript variants C were significantly reduced by more than 70% in TamR-18 compared to MCF-7 cells ($P < 0.001$). It should also be noted that the overall decrease in expression of all the ERα mRNA variants in TamR-18 is reminiscent of that observed in MCF-7 cells treated with $17\beta$-oestradiol, suggesting that both cells may use similar molecular mechanisms to regulate ERα gene expression. Also in correlation with the mRNA data was the reduction in expression levels of total ERα protein in TamR-18 cells, as measured by Western blot analysis (Figure 3.5A). This was further confirmed by immunocytochemical analysis, in which the intensity of nuclear ERα staining was noticeably reduced in TamR-18 compared to MCF-7 cells (Figure 3.5B). Expression levels of β-actin protein remained constant for all Western blot samples, which verified equivalent sample
loading. Taken together, these results show that emergence of tamoxifen resistance \textit{in vitro} is accompanied with a small attenuation of ER\(\alpha\) expression over an 18 months period. Furthermore, immunocytochemical analysis of nuclear ER\(\alpha\) indicates that this attenuation occurred across the entire cell population rather than being the result of an out-growth of an ER\(\alpha\)-negative cohort. It is important to note that despite this downregulation, ER\(\alpha\) protein levels still remain high in TamR-18 cells with the receptor retaining an important functional role in regulating cell growth (Hutcheson \textit{et al.}, 2003).
Figure 3.4 Real-time qPCR analysis of steady state mRNA levels of (A) total ERα and (B) ERα transcript variants in MCF-7 and TamR cells. Total RNA was extracted from 70% confluent cells grown in the appropriate routine medium as described in Material and Methods. Error bars represent the S.D. of at least four independent experiments, each carried out in duplicate. *, $P < 0.05$, **, $P < 0.001$, versus TamR-18.
Figure 3.5  (A) Western blotting and (B) immunocytochemical analysis of total ERα protein expression in MCF-7 and TamR-18 cells. Western blotting analysis was carried out from whole cell extracts prepared from 70% confluent cells grown in their routine medium. Total ERα expression was detected using rabbit polyclonal antibodies specific to the full length form of ERα (66 kDa). β-actin signals were used as internal controls to monitor equal protein loading. Immunocytochemical analysis was performed on ER-ICA fixed cells grown to 70% confluency. Original magnification, x20. Data are representative of at least three independent experiments for each methodology.

HScore analysis (median values): HScore (MCF-7) = 195; HScore (TamR-18) = 169. $P < 0.05$ for ERα levels in MCF-7 versus TamR-18.
3.3.2 Effects of short-term tamoxifen treatment on ERα expression levels

To establish whether loss of ERα was an early response to tamoxifen therapy, expression of ERα was examined in tamoxifen-resistant cell lines cultured in the continuous presence of 4-OHT for 3 (TamR-3) and 11 months (TamR-11). Real-time qPCR analysis revealed that total ERα mRNA expression was significantly downregulated in TamR-3 and TamR-11 cells compared to MCF-7 cells, and to levels similar to those observed in TamR-18 cells (Figure 3.6A). All the ERα mRNA variants were also significantly downregulated in all of the tamoxifen-resistant variants compared to MCF-7 cells (Figure 3.6B). In agreement with the mRNA data, Western blot analysis showed a noticeable decrease in expression levels of total ERα protein after 3- and 11 months exposure with tamoxifen. Similar to ERα mRNA, ERα protein levels in TamR-3 and TamR-11 cells were equivalent to those measured in TamR-18 cells (Figure 3.7A). As expected, parallel immunocytochemical analysis and subsequent HScore analysis showed a significant reduction in the intensity of nuclear-localized ERα staining in all the TamR cells compared to the parental MCF-7 cells (Figure 3.7B).
Figure 3.6 Effects of short-term tamoxifen treatments on steady state levels of (A) total ERα mRNA and (B) ERα mRNA variants. Parental MCF-7 cells and the sequentially acquired TamR cell lines were grown to 70% confluency in their routine medium and harvested for total RNA extraction for subsequent RT-PCR and real-time qPCR analysis of ERα mRNA levels (n = 3 ± S.D.). *, P < 0.001 versus MCF-7.
Figure 3.7  (A) Western blotting and (B) immunocytochemical analysis of total ERα protein levels in TamR cells. Data are representative of at least three independent experiments for each methodology. Original magnification, x 20.

HScore analysis (median values): HScore (MCF-7) = 195; HScore (TamR-3) = 159; HScore (TamR-11) = 166; HScore (TamR-18) = 169; HScore (TamR-24) = 145. $P < 0.05$ for ERα levels in MCF-7 versus each TamR cell line.
3.3.3 Effects of long-term tamoxifen treatment on ERα expression levels

The effects of long-term tamoxifen therapy on ERα levels were also examined by using TamR-24 and TamR-30 cell models. As described in Material and Methods, TamR-24 and TamR-30 cells were generated by continuous culture of TamR-18 cells in the presence of 4-OHT for 6- and 12 months, respectively. As shown in Figure 3.8A, real-time qPCR analysis revealed that total ERα mRNA levels were significantly reduced in TamR-24 cells compared to TamR-18 cells ($P < 0.01$, TamR-24 versus TamR18). This downregulation was further exacerbated in TamR-30 cells, in which total ERα mRNA levels were significantly reduced compared to TamR-24 cells ($P < 0.05$ TamR-30 versus TamR-24). Expression levels of all ERα mRNA variants were also significantly downregulated in the long-term TamR cells (Figure 3.8B). Interestingly, however, mRNA isoforms C was the most severely affected and expressed to almost non-detectable levels in TamR-24 and TamR-30 cells, thus suggesting that transcription from the promoter C may be particularly important in maintaining adequate levels of ERα gene expression in these cells. In corroboration with the mRNA data, total levels of ERα protein were drastically reduced in TamR-24 and TamR-30 cells, as demonstrated by Western blot analysis (Figure 3.9A). A significant decrease in the intensity of nuclear ERα staining was also observed in the long-term TamR cells, particularly so in TamR-30 cells, as demonstrated by immunocytochemical analysis (Figure 3.9B). As mentioned previously, this decline in nuclear ERα staining was seen across the vast majority of the ERα-positive cells, further indicating that loss of ERα during tamoxifen therapy is unlikely to be due to an out-growth of ERα-negative progenitor cells.
Figure 3.8  Real time qPCR analysis of (A) total ERα and (B) ERα mRNA variant levels in the late TamR cells. Error bars represent the S.D. of three independent experiments, each carried out in triplicates. *, $P < 0.01$ versus TamR-18; **, $P < 0.001$ versus TamR-18; †, $P < 0.05$ versus TamR-24.
Figure 3.9 Effects of long-term tamoxifen treatments on expression levels of total ERα protein, as demonstrated by (A) western blotting and (B) immunocytochemical analysis (original magnification, x20). Data are representative of at least four (western blotting) or three (ICC) independent experiments.

HScore analysis (median values): HScore (MCF-7) = 191; HScore (TamR-18) = 148; HScore (TamR-24) = 128; HScore (TamR-30) = 79. *P* < 0.05 for ERα levels in MCF-7 versus each TamR cell line; *P* < 0.05 for ERα levels in TamR-30 versus TamR-18, and TamR-30 versus TamR-24.
3.3.4 Sensitivity to fulvestrant and basal growth rate of TamR-30 cells

Based on the observations that both TamR-24 and TamR-30 cells contain very low levels of ERα, it was hypothesised that these cells might be drifting towards an ER-negative phenotype. In order to investigate this further, the influence of the ER down-regulator fulvestrant on growth of TamR-24 and TamR-30 cells was examined and compared to that of TamR-18 cells. The latter have previously been shown to respond sensitively to the growth inhibitory properties of fulvestrant at the optimal concentration of $10^{-7}$ M (Hutcheson et al., 2003). In this study, the growth of TamR cells in response to increasing concentrations of fulvestrant ($10^{-8}$ M and $10^{-7}$ M) was assessed by means of cell counts on day 7 from initial treatments with the pure anti-oestrogen. Although the growth of TamR-24 and TamR-30 cells was significantly reduced compared to their respective controls at the optimal concentration ($P < 0.05$), results clearly demonstrated that these cells have become less sensitive to the growth inhibitory action of fulvestrant compared to TamR-18 cells, whose growth was reduced by nearly 60% compared to ~20% for TamR-24 and TamR-30 cells at the $10^{-7}$ M dose (Figure 3.10). In view of this data, it would be of interest to further analyze ERα levels following a 7-day treatment regime of TamR cells with fulvestrant.

ERα-negative cells often exhibit increased rates of cell proliferation, which is driven by increased growth factor signalling particularly Erk1/2 (Oh et al., 2001). In view of this and to provide further evidence for a drift of TamR-24 and TamR-30 cells towards an ER-negative phenotype, the basal growth rate of these cells was assessed and compared to that of TamR-18 cells. Proliferation of TamR cells in the presence of 4-OHT was compared by means of growth curves over a period of 11 days. Results showed that the growth rate of TamR-30 cells was considerably and significantly higher than that observed for TamR-18 and TamR-24 cells, by over 200% on day 11 of cell proliferation (Figure 3.11). Surprisingly, TamR-24 cells exhibited an almost similar growth pattern to that of TamR-18 cells.
Figure 3.10 Comparative assessment of TamR-18, TamR-24, and TamR-30 cell growth in response to ICI 182,780. Cells were preliminarily grown to 70% confluency in their routine medium and re-seeded in 24-well plates at a density of 30,000 cells/well. Cells were allowed to grow for 24 hours in their routine medium (supplemented with 4-OHT) before treatment with increasing concentrations of ICI 182,780 or the vehicle EtOH (control) for 7 days (medium was changed at day 3). Results are expressed as % of control values on day 7 and are the means ± S.D. of three separate experiments carried out in triplicate wells for each dose. *, P < 0.05 versus control; †, P < 0.05 versus TamR-18 at the $10^{-8}$ M and $10^{-7}$ M doses.
Figure 3.11 Basal growth rates of TamR-18, TamR-24 and TamR-30 cells. Cells were grown to 70% confluency in their routine medium, trypsinized and re-seeded in 24-well plates at a density of 40,000 cells/well (TamR-18) or 20,000 cells/well (TamR-24, TamR-30). The experiment was carried out in routine medium, which was changed every 4 days. Cell number was assessed 1-, 4-, 6-, 8- and 11 days after seeding, as described in Material and Methods. Results are expressed as % of control values on day 1, which were derived from the mean cell number ± S.D. of three separate experiments carried out in triplicate wells for each time point. *, P <0.001 TamR-30 versus TamR-18, and TamR-30 versus TamR-24.
3.4 Molecular mechanisms for ERα loss in TamR cells

3.4.1 Role of EGFR signalling

As described in Chapter 1 (section 1.5.3.2), there is compelling evidence in the literature to suggest that hyperactivated growth factor signalling, can result in downregulation of ERα expression. Most notably, preclinical and clinical data have shown that overexpression of EGFR and hyper-activation of its downstream signalling elements is associated with ERα negativity. In view of this, the potential involvement of EGFR signalling in attenuating ERα levels was investigated in TamR cells. Basal expression and activity of EGFR signalling was first assessed in the early TamR cell lines (TamR-3, TamR-11, and TamR-18) which, as described previously, exhibited reduced levels of ERα mRNA and protein. Whole cell lysates from MCF-7 and TamR cells were prepared and subjected to Western blot analysis for the detection of total and activated EGFR, HER2, Erk1/2 and Akt. HER2 has previously been shown to be the preferred dimerization partner of EGFR (Knowlden et al., 2003).

As shown in Figure 3.12, a progressive increase in phosphorylated EGFR was observed as the exposure with 4-OHT was extended over the 18 month period. Although specific antibodies were used against activated EGFR, the latter were experimentally difficult to detect under basal growth conditions. Therefore, in house positive control samples were routinely used and prepared from TamR-18 cells treated with the EGFR-activating ligand EGF for 5 min (10 ng/ml). Total levels of EGFR protein appeared relatively similar across the different TamR cells but, as expected, were increased in comparison with MCF-7 cells. Levels of both total and activated HER2 were also similar across the different TamR cells but all were similarly increased compared to MCF-7 cells, which is in agreement with previous studies (Knowlden et al., 2003). Consistent with the enhanced activity of EGFR was a clear and progressive increase in levels of phosphorylated Akt and Erk1/2. In agreement with the Western blot data, immunocytochemical analysis of total EGFR protein also revealed a noticeable increase in membrane and cytoplasmic staining for this protein in TamR-18 cells compared to MCF-7 cells (Figure 3.13). Immunostaining of activated EGFR (Tyr1068) revealed that staining was
predominantly cytoplasmic. The cytoplasmic staining of EGFR (Tyr1068) was likely due to the fact that upon activation, the growth factor receptor is rapidly internalized into the endocytic compartment of the cells for subsequent recycling or degradation in lysosomes (Harari & Yarden, 2000).
Figure 3.12 Basal expression levels and activity of EGFR, HER2, Erk1/2 and Akt in MCF-7 cells and the sequentially acquired TamR cells. Results are representative of at least three independent experiments except for p-EGFR (Tyr1068), n=1.
Figure 3.13 Immunocytochemical analysis of basal EGFR activity and expression in MCF-7 and TamR-18 cells. Cells were fixed using the phenol formal saline method and stained with phospho-specific antibodies against EGFR (Tyr1068), as described in Material and Methods. Original magnification, x40; n = 1.
In view of the increased activity of EGFR in TamR-18 cells, the effects of the specific EGFR tyrosine kinase inhibitor (TKI) gefitinib (Iressa®/ZD1839) on ERα mRNA and protein expression was investigated. Using an optimal concentration of 1µM, this compound has successfully been used by other studies to modulate EGFR activity in TamR-18 cells (Britton et al., 2002; Knowlden et al., 2003; Hutcheson et al., 2007). As shown in Figure 3.14A, treatments of TamR-18 cells with TKI for 24 h resulted had no significant effect on levels of total and variant-ERα mRNA. Of note, only the breast cancer-specific ERα mRNA variants (A/B/C) were measured from this point onward since they were, as stated previously, the predominant isoforms in TamR-18 cells. In contrast to the mRNA data, a noticeable reduction in ERα protein expression was observed by Western blot analysis (Figure 3.14B).

In order to validate the efficacy of gefitinib in inhibiting EGFR signalling, the activity of the tyrosine kinase receptor and its downstream signalling components were also assessed by Western blotting. As expected, levels of phosphorylated EGFR, Erk1/2 and Akt were completely abrogated upon TKI treatments in TamR-18 cells (Figure 3.14B), and this was not due to a reduction in total levels of these proteins. Extending the gefitinib treatment regime over a 48 h period resulted in a significant reduction in both total and variant-ERα mRNA expression levels in TamR-18 (Figure 3.15A). Expression levels of total ERα protein clearly corroborate the mRNA data and were considerably reduced upon treatment with TKI after 48 h. Assessment of phospho-EGFR, Akt and Erk1/2 again clearly showed the strong inhibitory effects of gefitinib on activity of the EGFR signalling pathway (Figure 3.15B).
Figure 3.14 Effects of 24-hour gefitinib treatments on ERα expression and EGFR signalling in TamR-18 cells. (A) Real-time qPCR analysis of total ERα mRNA and ERα mRNA variants A-C. Cells were grown to 70% confluency in routine medium before treatment with gefitinib (Gef, 1 μM) or with the vehicle DMSO (control) for 24 hours. Error bars represent the S.D. of three independent experiments, each performed in duplicate. (B) Western blot analysis of total ERα protein expression and basal activity of EGFR cell signalling components. Phospho-specific antibodies against EGFR (Tyr1068), Erk1/2 (Thr202/Tyr204), and Akt (Ser473) were used as described in Material and Methods. Blots were also probed for total EGFR, Erk1/2, Akt, ERα and β-actin signals. Results are representative of three separate experiments.
Figure 3.15 Effects of 48-hour gefitinib treatments on ERα expression and EGFR signalling in TamR-18 cells. (A) Real-time qPCR analysis of total ERα mRNA and ERα mRNA variants A-C. Cells were grown to 70% confluency in routine medium before treatment with gefitinib (Gef, 1 μM) or with the vehicle DMSO (control) for 48 hours. Error bars represent the S.D. of three independent experiments, each performed in duplicate. (B) Western blot analysis of total ERα protein expression and basal activity of EGFR cell signalling components. Results are representative of three separate experiments. *, P < 0.05 versus control.
The effect of long-term EGFR inhibition on ERα expression was also investigated with TamR-18 cells being treated with gefitinib over a period of 10 days. As shown in Figure 3.16, steady-state levels of total and variant-ERα mRNAs remained significantly reduced after 10 days of gefitinib treatment although much less markedly when compared to the 48 h period. These results indicate that ERα mRNA levels may start to recover upon sustained EGFR inhibition. This was further suggested by Western blot analysis of ERα protein levels, where expression appeared to be re-instated in the gefitinib-treated samples compared to the control samples (Figure 3.17). Although there was abrogation of basal phosphorylated EGFR, there appears to be a partial recovery of Akt and Erk1/2 signalling activity after a 10 day-gefitinib treatment. Furthermore, total levels of the same proteins were also up-regulated, thus reflecting a compensatory mechanism in response to the sustained inhibitory action of gefitinib on EGFR signalling.
Gefitinib (10 days)

Figure 3.16 Effects of 10-day gefitinib treatments on ERα mRNA expression levels in TamR-18 cells. Cells were grown to 70% confluency in their routine medium and re-seeded into 60mm dishes (0.25 million cells/dish). Cells were allowed to grow for 24 hours before treatment with gefitinib (1μM) or with the vehicle DMSO (control) for 10 days. Real-time qPCR analysis of total ERα mRNA and ERα mRNA variants A-C is shown. Error bars represent the S.D. of three separate experiments, each performed in duplicate. *, $P < 0.05$ versus control.
Figure 3.17 Effects of 10-day gefitinib treatments on ERα protein levels and basal activity and expression of EGFR cell signalling components in TamR-18 cells. Cell culture conditions and treatments with gefitinib were as described in Figure 3.16. Results are representative of three separate experiments.
In view of the results observed with long-term gefitinib treatments, it was interesting to investigate whether ERα expression was maintained in TamR-18 cells that have been continuously exposed to Gefitinib and have subsequently gained resistance to the growth inhibitory effects of this agent. Therefore, ERα mRNA and protein levels were measured in the double tamoxifen/gefitinib-acquired resistant cell lines Tam/TKI-R. These cells were previously generated by continuous exposure of TamR-18 cells with Gefitinib (1µM) over a period of 6 months (Jones et al., 2004). Real-time qPCR analysis revealed that expression levels of total ERα mRNA is significantly higher in Tam/TKI-R cells compared to TamR-18 cells, and to almost equivalent levels to those observed in MCF-7 cells (Figure 3.18A). Surprisingly, the increase in total ERα mRNA levels was only reflected by an increase of the ERα mRNA variant A in Tam/TKI-R cells, suggesting for the first time in this study that a potential promoter-specific regulation of the ERα gene expression may occur in these cells. In agreement with the mRNA data, immunocytochemical analysis showed that there was also a considerable increase in ERα nuclear staining of wild-type (MCF-7) equivalence in Tam/TKI-R cells compared to TamR-18 cells (Figure 3.18B).
Figure 3.18 Analysis of total ERα expression levels in MCF-7, TamR-18 and the double resistant cells Tam/TKI-R. (A) Real-time PCR analysis of total and variant ERα mRNA levels. Error bars represent the S.D. of four separate experiments, each performed in duplicate. (B) Immunocytochemical analysis of total ERα protein expression (n=1, original magnification, x20). *, P < 0.05 versus TamR-18.
Overall, the gefitinib experiments would strongly suggest that EGFR signalling, although being considerably increased in TamR-18 cells, is not involved in the downregulation of ERα expression. In order to investigate this further, the activity of EGFR and its modulation by gefitinib was examined in the long-term TamR cells. Western blot analysis revealed that basal phosphorylated EGFR was increased in TamR-30 compared to TamR-24 and TamR-18 cells which only exhibited a faint signal of the activated growth factor receptor, while MCF-7 cells exhibited no apparent signals (Figure 3.19A). Because EGFR expression levels are very often inversely related to ERα levels in clinical breast cancers, an increase in total EGFR signals was initially expected in TamR-30 cells compared to TamR-18 cells. However, Western blot analysis showed that total levels of EGFR remained similar between TamR-18, -24, and -30 cells, but were increased compared with MCF-7 cells. Furthermore, no robust changes in levels of total and activated HER2 was observed between TamR-18, TamR-24 and TamR-30 cells, as demonstrated by Western blot analysis (Figure 3.19A). As expected and in agreement with previous studies (Knowlden et al., 2003), all three TamR cells (-18, -24 and -30) contained elevated levels of phosphorylated HER2 compared to the parental MCF-7 cells, and this appeared to be due to an increase in total expression of the protein. As depicted in Figure 3.19B, immunocytochemical analysis of total EGFR further confirmed the Western blot data with both TamR-18 and TamR-30 cells exhibiting no obvious changes in cytoplasmic and membrane staining of the tyrosine kinase receptor.

Given that phosphorylated EGFR (Tyr1068) was most noticeably increased in TamR-30 cells, the effect of gefitinib treatments on ERα mRNA and protein levels was assessed. As shown in Figure 3.20, real-time qPCR and Western blot analysis showed, respectively, that ERα mRNA and protein expression was not affected by the gefitinib treatment regime in TamR-30 cells, thus further demonstrating that EGFR signalling does not contribute to ERα downregulation in TamR cells.
Figure 3.19  (A) Western blotting analysis of basal expression and activity of EGFR and HER2 in MCF-7 and the late TamR cells. (B) Immunocytochemical analysis of total EGFR levels in TamR-18 and TamR-30 cells. (A) Cells were grown to 70% in their routine medium and harvested for total protein extraction as described in Material and Methods. Phospho-specific antibodies against EGFR (Tyr1068) and HER2 (Tyr1248) were used to detect the activated form of the proteins. Blots were also probed for total EGFR and HER2. A positive control sample for the detection of p-EGFR(Tyr1068) was used (+). Results are representative of two separate experiments. (B) Immunocytochemical detection of total EGFR. The image is representative of two separate experiments (original magnification, x40).
Figure 3.20 Effects of gefitinib on expression levels of total ERα (A) mRNA and (B) protein in TamR-30 cells. Cells were seeded into 75cm2 flasks (750,000 cells/flask) and allowed to grow for 24 hours in their routine medium before treatment with gefitinib (1 µM) or the vehicle DMSO (control) for 3 days. Error bars represent the S.D. of three independent experiments, each performed in duplicate.
Despite EGFR signalling not being involved in the progressive loss of ERα expression, it was interesting to investigate the role of EGFR in the growth of TamR cells. Previous studies have demonstrated that the increased activation of EGFR correlates with the enhanced proliferative activity of TamR-18 cells compared to MCF-7 cells (Knowlden et al., 2003). Given that TamR-30 cells exhibited higher levels of activated EGFR (Tyr1068) in comparison with TamR-18 cells, it was therefore feasible to suggest that TamR-30 cells may also exhibit an increased reliance on EGFR signalling to mediate cell growth compared to TamR-18 cells. To investigate this further, the effect of increasing concentrations of gefitinib on growth of TamR-18 and TamR-30 cells was examined. As shown in Figure 3.21, no difference in sensitivity to gefitinib was observed between TamR-18 and TamR-30 after 5 days. However, the growth of both cell lines was significantly reduced by more than 50% compared to their respective control at the optimal gefitinib concentration (1 μM). These results suggest that although EGFR signalling remains important for the growth of both TamR-18 and TamR-30 cells, the enhanced EGFR activity exhibited by TamR-30 cells is not associated with an increased reliance of these cells on the growth factor receptor to mediate cell growth. Therefore other yet-to-be identified signalling pathways are likely be involved.
Figure 3.21 Basal growth response of TamR-18 and TamR-30 cells to increasing concentrations of gefitinib. Cells were preliminarily grown to 70% confluency in their routine medium and re-seeded in 24-well plates at a density of 30,000 cells/well. Cells were allowed to grow for 24 h before treatment with increasing concentrations of gefitinib (0.1-1 μM) or DMSO (control) for 5 days; medium was changed at day 3. Results are expressed as % of control values on day 5 and are the means ± S.D. of three separate experiments carried out in triplicate wells for each dose. *, P < 0.05 versus control.
3.4.2 Role of epigenetic mechanisms

Early experiments were performed in TamR-18 cells (prior to the development of TamR-24 and TamR-30 cells) to investigate whether methylation of the ERα gene promoter could be a potential contributory factor to the downregulation of ERα expression in these cells. Therefore, the effects of the DNA-demethylating agent 5-azadeoxycytidine (5-AZA) on levels of total and variant-ERα mRNAs were examined in TamR-18 cells. As shown in Figure 3.22, Real-time qPCR analysis demonstrated no effects of 5-AZA on expression levels of total ERα mRNA and ERα mRNA variants A-C, therefore suggesting that methylation of the ERα gene is an unlikely mechanism responsible for the significant reduction of ERα expression in TamR-18 cells.

The effects of tamoxifen withdrawal on the receptor levels were also examined in TamR-24 and TamR-30 cells. 4-OHT was omitted from routine medium and cells were maintained in this condition for 4 weeks in order to ensure total clearance of 4-OHT from the residual levels of ERα present in the cells. As depicted in Figure 3.23, tamoxifen withdrawal from culture medium resulted in a significant increase of total ERα mRNA levels in both TamR-24 and TamR-30 cells. In TamR-24 cells, this increase was accompanied by a significant increase in expression of all three ERα mRNA variants. In TamR-30 cells, steady-state levels of the ERα mRNA variants A and C were also significantly recovered following 4-OHT withdrawal. Western blot analysis of ERα expression also revealed a noticeable recovery of ERα protein levels in TamR-24 and TamR-30 cells, to approximately TamR-18 equivalents (Figure 3.24A). A strong increase in nuclear staining of ERα was also observed by immunocytochemical analysis in TamR-24 and TamR-30 cells, to levels comparable to that of TamR-18 cells but not MCF-7 cells, in which nuclear ERα staining remained considerably stronger (Figure 3.24B). These results clearly indicate that the reduction of ERα expression in TamR-24 and TamR-30 cells was not permanent, therefore further excluding the possibility of epigenetic modifications (particularly DNA methylation) as a mechanism for ERα loss in these cells.
Figure 3.22 Effects of 5-AZA treatments (5 days) on expression levels of (A) total ERα mRNA and (B) ERα mRNA variants in TamR-18 cells. Cells were seeded on 60 mm dishes (0.5 million cells/dish) and allowed to grow in their routine medium for 24 h. Cells were then treated with an initial 5 μM-dose of 5-AZA (+ 5-AZA) or the vehicle DMSO for controls without 5-AZA (-5-AZA). After 48 h, media was changed and cells were then maintained in culture for a further 3 days in the presence of 1 μM 5-AZA. Cells were harvested for total RNA extraction, and subsequent real-time qPCR analysis of ERα mRNA expression was performed (n = 1).
Figure 3.23 Effects of 4 weeks-tamoxifen withdrawal on ERα mRNA expression levels in (A) TamR-24 and (B) TamR-30 cells. Cells were preliminarily grown to 70% confluency and passaged by trypsinization in their routine medium (containing 4-OHT) as described in Material and Methods. Cells were allowed to grow for 24 h before 4-OHT was omitted from the medium (-Tam). Cells were then maintained in culture without tamoxifen for 4 weeks prior to cell harvest for total RNA extraction. Expression levels of both total ERα mRNA and ERα transcript variants were then assessed by real-time qPCR. Error bars represent the S.D. of three independent experiments, each carried out in triplicate. *, P < 0.05 versus TamR-24 (-Tam); †, P < 0.05 versus TamR-30 (-Tam).
Figure 3.24  Effects of 4 weeks-tamoxifen withdrawal on total levels of ERα protein in TamR-24 and TamR-30 cells. Cell culture conditions were as described in Figure 3.23. (A) Western blot analysis of total ERα protein expression. Data are representative of two separate experiments. (B) Immunocytochemical analysis of total ERα protein levels (n=1); Original magnification, x20.
3.4.3 Role of alternative growth factor signalling pathways

In an attempt to identify the mechanisms underlying loss of ER\( \alpha \) expression in TamR cells, the basal expression and activity of other cell signalling components were analysed. A cross-talk mechanism between IGF-IR and EGFR, whereby the IGF-IR regulates basal-activated EGFR signalling and cell proliferation, has been shown in TamR cells (Knowlden et al., 2005). Given that levels of EGFR (Tyr1068) were increased in TamR-30 cells, it was interesting to assess basal levels of activated IGF-IR in these cells. In line with the increased levels of EGFR (Tyr1068) in TamR-30 cells as described earlier, basal levels of phosphorylated IGF-IR (Tyr1316) were also markedly enhanced in these cells (Figure 3.25). In contrast, levels of total IGF-IR were progressively and markedly reduced as cells progressed through to TamR-30 cells, further reflecting the loss of ER\( \alpha \) expression in these cells since IGF-IR is a well known ER-dependent gene product. The enhanced basal activity of the receptor tyrosine kinases EGFR and IGF-IR in TamR-30 cells was further validated by the marked increase in basal activity of their two common downstream signalling elements Akt and Erk1/2 which, as described in Chapter 1 (section 1.5.3.2), have been linked with the downregulation of ER\( \alpha \) expression in breast cancer. Interestingly, levels of total Akt were visibly reduced in TamR-24 and TamR-30 cells.

As described in Chapter 1 (section 1.5.3.2), an inverse relationship between PKC\( \alpha \) activity and ER\( \alpha \) expression is well known in breast cancer. Overexpression of PKC\( \alpha \) has also been involved in the development of tamoxifen resistance, ER\( \alpha \)-independent tumour growth as well as tumour aggressiveness and metastasis \textit{in vivo} (Ways et al., 1995; Tonetti et al., 2000; Frankel et al., 2006). Furthermore, elevated activity of the non-receptor tyrosine kinase Src has recently been associated with a significant proportion of ER\( \alpha \)-negative primary breast cancers, and the reduced stability of ER\( \alpha \) protein in ER\( \alpha \)-positive breast cancer cell lines (Chu et al., 2007). In light of these observations, the basal expression and activity of both PKC\( \alpha \) and Src was analyzed in TamR cells. As depicted in Figure 3.26, Western blot analysis revealed a slight but noticeable increase in levels of phosphorylated PKC\( \alpha \) in TamR-24 compared to TamR-18 cells. MCF-7 cells exhibited no apparent signals of the activated protein kinase. In contrast, a considerable increase of phosphorylated
PKCα was observed in TamR-30 cells, which also displayed the lowest levels of total expression of the protein, thus reflecting a hyper-phosphorylated state for PKCα in these cells. Src kinase activity was not apparent in MCF-7 cells but was progressively and noticeably increased as tamoxifen-resistance developed, and reached maximum levels in TamR-30 cells. The increase in phosphorylated Src was not due to an increase in expression levels of the protein.
Figure 3.25 Basal expression and activity of IGF-IR, Erk1/2 and Akt in MCF-7 and the late TamR cells. Cells were grown to 70% in their routine medium and harvested for total protein extraction as described in Material and Methods. Phospho-specific antibodies against IGF-IR (Tyr1316), Erk1/2 (Thr202/Tyr204) and Akt (Ser473) were used to detect the activated form of the proteins. Blots were also probed for total levels of the proteins. Results are representative of three separate experiments.
Figure 3.26  Basal expression and activity of PKCα and Src in MCF-7 and the late TamR cells. Phospho-specific antibodies against PKCα (Ser657) and Src (Tyr418) were used to detect the activated form of the proteins. Total expression levels of the same proteins were also assessed. Results are representative of three independent experiments.
In light of the increased activity of PKCα, Src, Akt and Erk1/2 in TamR-30 cells, and their reported link with downregulation of ERα expression in breast cancer, a panel of signal transduction inhibitors targeting specifically each of these proteins individually were used in TamR-30 cells in order to examine the effects on ERα mRNA and protein expression. ERα expression levels were first examined following a short-term inhibition (24 h) with the inhibitors. As shown in Figure 3.27A, inhibition of Src kinase activity by AZD0530 resulted in a significant increase of total ERα mRNA levels (p<0.001 control vs AZD0530). This increase could also be clearly seen at the protein levels, as demonstrated by Western blot analysis (Figure 3.27B). In contrast, no significant changes in total ERα mRNA levels were observed following treatment of TamR-30 cells with the PKC inhibitor bisindolylmaleimide IX (Bis), and the PI3K inhibitor wortmannin (Wort). Although not significant, a slight but visible reduction in ERα mRNA steady-state levels could be seen upon inhibition of Erk1/2 signalling by the MEK1/2 inhibitor U0126. In agreement with the mRNA data, ERα protein levels remained unchanged in TamR-30 cells treated with Bis, suggesting that PKCα may not be involved in downregulation of ERα levels in TamR-30 cells. Although no change in total ERα mRNA levels was observed with wortmannin, the latter induced a visible recovery of ERα protein but not to the same extent as with AZD0530 treatments. Interestingly, a total loss of ERα protein was observed following treatment with the inhibitor U0126, and this was confirmed by overexposure of the blots.
Figure 3.27 Short term effects of various signal transduction inhibitors on (A) ERα mRNA and (B) protein expression in TamR-30 cells. Cells were seeded into 60mm dishes (TamR-30 cells, 0.25x10⁵ cells/dish; TamR-18 cells, 0.5x10⁵ cells/dish) and grown to 70% in their routine medium before treatment with the various inhibitors for 24 hours. (A) real-time qPCR analysis of total ERα mRNA expression. Error bars represent the S.D. of three independent experiments, each carried out in duplicate. (B) Whole cell extracts were also prepared for western blot analysis of total ERα protein expression. C, control; AZD, AZD0530 (1µM); Bis, bisindolylmaleimide (0.5 µM); UO126 (10 µM); Wort, wortmannin (100nM). Western blot data are representative of two separate experiments. *, $P < 0.01$ versus control, C.
In an attempt to confirm more robustly the results presented in Figure 3.27, the pathway inhibition study was extended to a period of 3 days. As depicted in Figure 3.28A, a significant increase in total ERα mRNA levels was observed following treatments of TamR-30 cells with AZD0530 (p<0.001 control vs AZD0530, n=3). This increase was noticeably stronger (~6.5 fold vs. control) than that observed at the 24 h period with the same inhibitor (~2-fold vs. control). Again, no apparent changes in total ERα mRNA levels were observed upon inhibition with Bis, or wortmannin. There was a slight but non-significant reduction of ERα mRNA steady-state levels following treatment of TamR-30 cells with U0126. However, at the protein level, treatment with the inhibitor U0126 resulted in a total loss of ERα protein signals. In contrast to the observations made at the 24 h-period, treatment of TamR-30 cells with wortmannin for 3 days resulted in only a very slight increase of ERα protein levels. Most importantly, Western blot analysis confirmed the effects of AZD0530 on ERα mRNA expression, with total ERα protein levels being considerably increased in TamR-30 cells treated with the inhibitor, to levels similar to that of TamR-18 cells (Figure 3.28B). In line with the Western blot data, immunocytochemical analysis revealed that AZD0530-treated TamR-30 cells exhibited intense nuclear staining for ERα to levels surprisingly comparable to wild-type MCF-7 cells (Figure 3.29). In light of the results obtained with AZD0530, steady-state levels of ERα mRNA variants (A-C) were also assessed in TamR-30 cells treated with the Src inhibitor for 3 days. As shown in Figure 3.30, a significant increase in expression levels of all three transcripts was observed following Src inhibition in TamR-30 cells.

These results strongly implicate Src kinase activity as one potential mechanism underlying the considerable loss of ERα expression levels in TamR-30 cells. Akt activity may also be involved in the progressive reduction of ERα, at least at the post-translational level, since treatment with wortmannin did result in a slight but visible increase of ERα protein (particularly at 24 h) but not ERα mRNA expression. Although in disagreement with the literature, the U0126-induced downregulation of ERα protein expression would suggest that hyperactivation of Erk1/2 may have a positive regulatory function on ERα expression at least at the post-translational levels in TamR-30 cells.
Figure 3.28 Long term effects of various signal transduction inhibitors on (A) ERα mRNA and (B) protein expression in TamR-30 cells. Cells were seeded into 75cm² flasks (750,000 cells/flask) and allowed to grow for 24 hours in their routine medium before treatment with the various inhibitors for 3 days. Error bars represent the S.D. of three independent experiments, each performed in duplicate. Western blot data are representative of two separate experiments. *, P < 0.001 versus control, C.
Figure 3.29  Immunocytochemical analysis of total ERα protein expression in MCF-7 cells and TamR-30 cells treated or not with AZD0530 for 3 days. Cells were seeded onto coverslips (75,000 cells/cover slips) and allowed to grow for 24 h in their routine medium before treatment with 1 μM AZD0530 or with the vehicle DMSO (control: TamR-30 (-AZD)) for 3 days (except MCF-7 cells). Cells were then ER-ICA fixed for immunocytochemical detection of nuclear ERα (n=1).
Figure 3.30  Effects of 3 day-AZD0530 treatments on steady-state levels of ERα mRNA variants in TamR-30 cells. cDNA samples from TamR-30 cells treated with AZD for 3 days were also used to assess expression levels of ERα mRNA variants. Error bars represent the S.D. of at least 3 separate experiments carried out in duplicate. *, P < 0.05 versus TamR-30 (-AZD).
Because ERα expression was partially restored in TamR-30 cells when tamoxifen was omitted from culture medium (section 3.4.2), the effect of tamoxifen withdrawal on the kinase activity of Src, Akt and Erk1/2 was also examined. To this end, the same whole cell lysates used to detect total ERα protein after 4-weeks of tamoxifen withdrawal from culture medium (see Figure 3.27) were used. Western blot analysis revealed that levels of phosphorylated Src and phosphorylated Akt were reduced upon tamoxifen withdrawal in TamR-30 cells. In contrast, the levels of activated and total Erk1/2 proteins remained unchanged in the tamoxifen-withdrawn samples (Figure 3.31).

In order to confirm the inhibitory action of AZD0530 on Src kinase activity, the latter was analyzed before and after treatments of TamR-30 cells with the inhibitor. As expected, levels of phosphorylated Src were reduced to almost non-detectable levels following a 3 day treatment with the inhibitor (Figure 3.32A). Furthermore, TamR-cells with elevated Src activity have previously been shown to display poor cell-cell contacts when grown in culture in vitro (Hiscox et al., 2006). Therefore, the morphology of live TamR-30 cells was also examined following treatments with the Src inhibitor using an inverted microscope fitted with a Hoffman condenser. As illustrated in Figure 3.32B, inhibition of Src activity in TamR-30 cells restored cell-cell contacts and resulted in reorganization of the cells into a tightly packed epithelial cell colony similar to that of the parental MCF-7 cells. The increase in cell-cell contacts as a result of Src inhibition was further confirmed by Haematoxylin/Eosin staining (Figure 3.32C).
Figure 3.31 Effects of 4 weeks-tamoxifen withdrawal on activity and expression of Src, Akt and Erk1/2 in TamR-30 cells. Data is representative of two separate experiments.
Figure 3.32 Effects of AZD0530 treatments on Src activity and cell morphology in TamR-30 cells. (A) The inhibitory effects of AZD0530 (1 μM, 3 days) on Src activity were assessed by western blotting. Control samples were treated with the vehicle for AZD0530, DMSO. Data are representative of two separate experiments. (B) Representative HMC® images of TamR-30 cells with or without (C, control) AZD0530 (1 μM) for 3 days. (Original magnification, x20). (C) Haematoxylin & Eosin staining of TamR-30 cells grown in the presence or absence of AZD (1 μM, 3 days). Cell seeding densities were as described in Figure 3.29.
3.5 Analysis of cell motility and invasiveness in TamR-30 cells

Using the in house TamR-18 cells, previous studies have shown that the acquisition of endocrine resistance is associated with the development of a more aggressive cell phenotype essentially due to elevated Src activity. This was demonstrated by TamR-18 cells exhibiting increased cell motility and invasion capabilities in vitro (Hiscox et al., 2004; 2006). In view of this and the findings herein that TamR-24 and TamR-30 cells contain a noticeable increase of activated Src, it was expected that the invasion and motility would be enhanced in these cells compared to TamR-18.

Using fibronectin-coated porous membranes, the in vitro migratory capacity of TamR-24 and TamR-30 cells was first assessed and compared to that of TamR-18 cells over a period of 48 h, and under basal conditions. As shown in Figure 3.33, the migratory capacity of TamR-24 and TamR-30 cells was significantly increased compared to TamR-18 cells (TamR-18, 36 ± 8; TamR-24, 102 ± 20; TamR-30, 151 ± 16 mean cell number/field of view). TamR-30 cells were also significantly more migratory when compared to TamR-24 cells. A representative picture of crystal-violet stained cells that have migrated through the membrane is shown (Figure 3.33A).
**Figure 3.33** *In vitro* migration assay in TamR-18, TamR-24, and TamR-30 cells. Cells were seeded onto fibronectin-coated porous membranes (Transwell™), as described in Material and Methods. After 48 h, migratory cells were fixed, stained and counted. (A) Representative images of crystal-violet-stained migratory cells. Original magnification, x40. (B) Quantitative assessment of the number of migratory cells. The latter were counted in 10 random fields of view per membrane using an Olympus BH-2 light microscope, as described in Material and Methods (Original magnification, x20). Results are expressed as mean cell number/field of view ± S.D. of three separate experiments carried out in triplicates. *, *P* < 0.001 versus TamR-18; †, *P* < 0.001 versus TamR-24.
As depicted in Figure 3.34, basal cell invasion was also significantly increased in TamR-24 and TamR-30 cells compared to TamR-18 over a 3 day period (TamR-18, 25 ± 6; TamR-24, 64 ± 12; TamR-30, 103 ± 28 mean cell number/field of view). These results are in agreement with the causative role of Src activity in the development of a more aggressive and invasive cell phenotype, since the increase of cell motility and invasion in the late TamR cells correlate robustly with the increase of Src activity in those cells compared to TamR-18. The aggressive properties of TamR-30 cells were also visibly apparent when routinely grown in their culture medium, as shown by their rapid adhesion to uncoated Petri-dishes. Although it was only assessed qualitatively, the cell adhesion rate on plastic surfaces was visibly higher in TamR-30 cells compared to TamR-18 cells, as shown by the Hoffman Modulation Contrast (HMC®) images of the live cells taken at different time shots after seeding (Figure 3.35).
Figure 3.34  *In vitro* cell invasion assay in TamR-18, TamR-24, and TamR-30 cells. An artificial basement membrane (Matrigel) was used to assess the invasive capacity of the cells, as described in *Material and Methods*. After 72 h, invasive cells were fixed, stained and counted.  

(A) Representative images of DAPI-stained invasive cells. Original magnification, ×40 (inverted microscope DMIRE2 Leica, Arizona, USA).  

(B) Cell invasion was quantified by viewing DAPI-stained-cells on five separate fields of view per membrane and counting the number of cells in each field using magnification ×20. Data were then plotted as mean cell number/field of view ± S.D. of at least three separate experiments, each performed in duplicate. *, P < 0.001 versus TamR-18; †, P < 0.05 versus TamR-24.
Figure 3.35 Comparative assessment of cell spreading on uncoated plastic surfaces between TamR-18 and TamR-30 cells. Cells were grown to 70% confluency in their routine medium, trypsinized and re-seeded onto uncoated 60mm Petri-dishes (1x10^6 cells/dish) for incubation at 37°C for up to 24 h. Hoffman Modulation Contrast (HMC®) images of live cells were taken 1-, 3-, 6-, and 24 h after seeding using an inverted microscope (DMIRE2 Leica, Arizona, USA) and a digital camera (Orca-285, Hamamatsu, Japan). Original magnification, x20.
3.6 Potential Transcription factors regulating ERα expression in TamR cells: preliminary studies

3.6.1 AliBaba2.1 and Affymetrix database

The loss of critical transcription factors involved in the positive regulation of ERα transcription has been suggested as one potential mechanism that could contribute to the loss of ERα expression in vitro (Yoshida et al., 2000). Similarly, loss of ERα expression can also be due to the predominant expression of transcriptional repressors (Yoshida et al., 2000; Macaluso et al., 2007). In the present study, TamR-18 cells have been shown to exhibit a significant reduction of ERα mRNA steady-state levels compared to MCF-7 cells. In a preliminary attempt to identify transcription factors involved in the downregulation of ERα gene expression in TamR cells, potential transcription factor binding sites were mapped to both the proximal (A/B) and distal promoters (C) of the ERα gene using AliBaba 2.1 software (Grabe 2002; http://www.gene-regulation.com).

As described in Material and Methods, the nucleotide sequence of each promoter and approximately 1-kb of upstream DNA sequences (relative to the originally described transcription start site, +1) were subjected to the AliBaba2 algorithm. The list of potential transcription factors as predicted by AliBaba2 are represented in Table 3.1. The prediction of transcription factor binding sites performed by AliBaba2.1 software is based on the TRANSFAC® database, which contains amongst other data those of experimentally-proven binding sites for eukaryotic transcription factors (Matys et al., 2006). However, at the time of the analysis, the AliBaba2 program was based on an outdated version of TRANSFAC® (version 4.0). Therefore, the candidate list of transcription factors generated by AliBaba 2.1 was further extended with additional transcriptional regulators (outlined below), which were shown in the literature to regulate ERα transcription either through protein/DNA interactions or through protein-protein interactions at the ERα promoter.
Table 3.1 List of potential transcription factors (TFs) for ERα proximal (-1000 to +245)* and/or distal (-3000 to -1977)* promoters as predicted by AliBaba 2.1 software.

<table>
<thead>
<tr>
<th>TFs</th>
<th>GeneCard ID†</th>
<th>ERα promoter</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNF-1/TCF1</td>
<td>GC12P119900</td>
<td></td>
</tr>
<tr>
<td>MYC</td>
<td>GC08P128817</td>
<td></td>
</tr>
<tr>
<td>EGR2/Krox20</td>
<td>GC10M064241</td>
<td>Proximal</td>
</tr>
<tr>
<td>MYOD1</td>
<td>GC11P017697</td>
<td></td>
</tr>
<tr>
<td>C/EBPβ</td>
<td>GC20P048240</td>
<td></td>
</tr>
<tr>
<td>CREB2</td>
<td>GC02M175645</td>
<td></td>
</tr>
<tr>
<td>HSF1</td>
<td>GC08P145486</td>
<td>Distal</td>
</tr>
<tr>
<td>RARα</td>
<td>GC17P035718</td>
<td></td>
</tr>
<tr>
<td>SOX2</td>
<td>GC03P182912</td>
<td></td>
</tr>
<tr>
<td>HSF1</td>
<td>GC08P145486</td>
<td></td>
</tr>
<tr>
<td>C/EBPα</td>
<td>GC19M038482</td>
<td></td>
</tr>
<tr>
<td>HNF4α</td>
<td>GC20P042463</td>
<td></td>
</tr>
<tr>
<td>POU2F1</td>
<td>GC01P165456</td>
<td></td>
</tr>
<tr>
<td>POU2F3</td>
<td>GC11P119616</td>
<td></td>
</tr>
<tr>
<td>Pu.1</td>
<td>GC11M047332</td>
<td></td>
</tr>
<tr>
<td>NFκB1</td>
<td>GC04P103641</td>
<td>Proximal/Distal</td>
</tr>
<tr>
<td>AP2α</td>
<td>GC06M010503</td>
<td></td>
</tr>
<tr>
<td>FOS</td>
<td>GC14P074815</td>
<td></td>
</tr>
<tr>
<td>JUN</td>
<td>GC01M058958</td>
<td></td>
</tr>
<tr>
<td>IRF-1</td>
<td>GC05M131846</td>
<td></td>
</tr>
<tr>
<td>ZEB1</td>
<td>GC10P031648</td>
<td></td>
</tr>
<tr>
<td>SP1</td>
<td>GC12P052060</td>
<td></td>
</tr>
</tbody>
</table>

*, relative to the originally described transcription start site (Green et al., 1986); †, www.genecards.org
As described in Chapter 1 (section 1.4.1), these transcriptional regulators include the pRb2/SUV39H1/HDAC1/DNMT1/E2F4/E2F5 and p300/CBP complex (Macaluso et al., 2003; 2007), p53 (Angeloni et al., 2004) and AP2γ (Schuur et al., 2001); all of which have been shown to regulate ERα transcription as part of a multiprotein complex that bind to the proximal promoter. In addition, FoxO3a were also added to the list since it was shown to positively regulate ERα gene transcription through direct binding to the C promoter (Guo & Sonenshein, 2004). Next, the gene expression profiles of all the candidate transcription factors were examined using a gene array database in TamR-18 cells (vs. MCF-7 cells), which has previously been generated from the Affymetrix U133A Genechip (see Appendix A for protocol overview). A heat map representing the gene expression level of each candidate transcription factor is shown in Figure 3.36. As first cut-off criteria, only the expression profiles associated with a p-value of less than 0.1 were considered in order to select relevant candidate transcription factors, whose expression were either up- or downregulated in TamR-18 cells compared to MCF-7 cells.

As a result of the cut-off criteria, 7 transcriptional regulators were short-listed and these are shown in Table 3.2 (see Appendix B for Log-intensity plot corresponding to the gene expression of these transcription factors). Statistical significance (P < 0.05, MCF-7 versus TamR-18) was only found for Affymetrix probes corresponding to the transcription factors c-JUN (heat map number 47 and 54), C/EBPβ (heat map number 1) and the transcriptional co-activator CBP/p300 (heat map number 46). Using the Pubmed-Medline resource (http://www.ncbi.nlm.nih.gov/sites/entrez), a literature search was carried out in order to identify whether each of these transcription factors was a positive or negative regulator of ERα transcription. As outlined in Chapter 1, the transcription factors p53, FoxO3a, AP2γ and c-Jun (part of AP1 complex) have been reported to positively regulate ERα transcription. Also, the transcriptional coactivator CBP/p300 has been identified as part of a multi-protein complex found on the promoter A to activate transcription of the ERα gene (See Table 3.2 for references). Binding sites for both C/EBPα and C/EBPβ transcription factors on ERα proximal and distal promoters were suggested by AliBaba2 in the present study. However, no literature has been reported that implicate regulation of
ERα gene transcription with these transcription factors, whose gene expression were found elevated in TamR-18 compared to MCF-7 cells.

In light of the substantial amount of evidence that exist in the literature implicating AP2γ, p53 and FoxO3a in the positive regulation of ERα gene transcription, loss of one or several of these transcription factors could potentially be involved in the downregulation of ERα in TamR cells. Indeed, the Affymetrix database showed reduced gene expression levels of these transcription factors in TamR-18 compared to MCF-7 cells (Figure 3.36). However, due to time constraints, the gene expression profiles of these transcription factors could not be verified by semi-quantitative PCR. However, an optimized in house immunocytochemical assay for the detection of nuclear AP2γ was available and thus, expression levels of AP2γ was examined in the long-term TamR cells. FoxO3a were also assessed by both Western blot and immunocytochemical analysis in TamR cells.
Figure 3.36 Gene array expression profiles of potential transcriptional regulators of the ERα gene. The heat map represents comparative gene expression analysis between MCF-7 and TamR-18 cells, which was performed using median-normalised, log-transformed data using the online software package GeneSifter® (www.genesifter.net). Colour key is shown at the bottom of the map and represents the Log expression intensity with red and green colours indicating higher and lower relative expression, respectively. Underlined gene names represent potential regulators of ERα gene transcription as suggested by the literature (see Table 3.2 for references). *, P < 0.1; **, P < 0.05.
Table 3.2  Short-list of potential transcriptional regulators of ERα gene expression.
Red font indicate gene array expression profiles associated with $P < 0.05$ (MCF-7 versus TamR-18).

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene ID</th>
<th>Probe No.</th>
<th>Heat map No.</th>
<th>Direct regulator of ERα transcription in BrCa?</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53/TP53</td>
<td>NM_000546</td>
<td>211300_s_at 201746_at</td>
<td>38 43</td>
<td>Y, positive regulator of ERα transcription through protein-protein interaction on A/B promoter. Mutated form present in high proportion of ERα-negative advanced BrCa.</td>
</tr>
<tr>
<td>Foxo3A/ FKHR1</td>
<td>NM_001455</td>
<td>204132_s_at 210655_s_at</td>
<td>32 50</td>
<td>Y, positive regulator of ERα transcription (C promoter).</td>
</tr>
<tr>
<td>AP2γ/ERF1</td>
<td>NM_003222</td>
<td>205286_at 205287_s_at</td>
<td>27 42</td>
<td>Y, positive regulator of ERα transcription in vitro (A/B/C promoters) but lack of association between AP2γ and ERα expression in clinical BrCa.</td>
</tr>
<tr>
<td>c-Jun</td>
<td>NM_002228</td>
<td>213281_at 201464_x_at 201466_s_at 201465_s_at</td>
<td>39 47 52 54</td>
<td>Y, through binding of c-Jun to the ER-EH0 element (promoter D)</td>
</tr>
</tbody>
</table>

References

- Berns et al., 1996;
- Berns et al., 2000;
- Angeloni et al., 2004

- Guo & Sonenshein, 2004

- Schuur et al., 2001

- Tang et al., 1997;
Table 3.2  ...continued

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene ID</th>
<th>Probe No.</th>
<th>Heat map No.</th>
<th>Regulator of ERα gene transcription?</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/EBPβ</td>
<td>AL564683</td>
<td>212501_at</td>
<td>1</td>
<td>Not reported, but C/EBPβ shown to regulate transcription of other steroid receptors.</td>
<td>Dong et al., 2006; Langosch et al., 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Correlation between C/EBPβ-3 (LIP) expression and ERα-negative status.</td>
<td></td>
</tr>
<tr>
<td>C/EBPa</td>
<td>NM_004364</td>
<td>204039_at</td>
<td>3</td>
<td>Not reported. No correlation found between C/EBPa expression and ER status in primary BrCa.</td>
<td>Gery et al., 2005; Langosch et al., 2003</td>
</tr>
<tr>
<td>CBP/p300</td>
<td>NM_004380</td>
<td>211808_s_at</td>
<td>23 46</td>
<td>Y, through interaction with large multi-protein complex involved in epigenetic regulation of ERα transcription (promoter A)</td>
<td>Macaluso et al., 2005; 2007</td>
</tr>
</tbody>
</table>
3.6.2 AP2γ

Although no clear correlations has yet been found between AP2γ and ERα expression in clinical breast cancers, *in vitro* studies using human mammary epithelial cells have shown that AP2γ can activate ERα transcription through binding to the proximal promoter A (Schuur *et al*., 2001). Furthermore, a number of highly ERα-positive breast cancer cell lines are known to overexpress AP2γ (Schuur *et al*., 2001; Pellikainen & Kosma, 2007). In light of these observations and the fact that gene array analysis revealed that AP2γ transcript levels were downregulated in TamR-18 cells compared to MCF-7 cells, it was hypothesised that downregulation of nuclear AP2γ could, at least in part, underlie the significant reduction of ERα expression levels in the late TamR cells. As a preliminary attempt to investigate this hypothesis, total levels of nuclear AP2γ protein were examined by immunocytochemical analysis.

As shown in Figure 3.37 and in agreement with the gene array data, immunocytochemical assessment of AP2γ expression and subsequent HScore analysis showed a reduction, although not statistically significant, of nuclear AP2γ staining in TamR-18 and TamR-24 cells compared with MCF-7 cells. No significant difference in AP2γ nuclear staining was observed between TamR-18 and TamR-24 cells. In contrast, AP2γ expression was found to be significantly reduced in TamR-30 cells (median HScore, 132) compared with MCF-7 (median HScore, 170), TamR-18 (median HScore, 165) and TamR-24 cells (median HScore, 155).
Figure 3.37 Immunocytochemical analysis of AP2γ expression in MCF-7 cells and the late TamR cells. Cells were grown to 70% confluency and ER-ICA fixed for the detection of total nuclear AP2γ using the specific 6E4 antibodies, as described in Material and Methods. Data are representative of at least three independent experiments (Original magnification, x20).

HScore analysis (median values): HScore (MCF-7) = 170; HScore (TamR-18) = 165; HScore (TamR-24) = 155; HScore (TamR-30) = 132. P < 0.01 for TamR-30 versus MCF-7, TamR-30 versus TamR-18, TamR-30 versus TamR-24.
3.6.3 FoxO3a

As previously stated, FoxO3a has been shown to positively regulate ERα gene transcription through binding to a region within the promoter C (Guo & Sonenshein, 2004). Interestingly, gene array analysis in the present study showed that FoxO3a transcript levels were downregulated in TamR-18 cells compared to MCF-7 cells. It was initially hypothesised that reduced expression levels of FoxO3a proteins could, at least in part, be correlated with the progressive loss of ERα expression levels (and more particularly steady state levels of ERα mRNA variants C) in the different TamR cells. To examine this further, total levels of FoxO3a protein were analysed by Western blotting and immunocytochemical analysis in MCF-7, TamR-18 and the late TamR cells (TamR-24 and TamR-30). As shown in Figure 3.38A, Western blotting showed no robust changes in levels of total FoxO3a protein between the TamR cells, which exhibited similar levels of the protein compared to MCF-7 cells.

FoxO3a activity is negatively regulated by the PI3K signalling pathway, whereby Akt-mediated phosphorylation of FoxO3a on specific threonine and serine residues result in nuclear export and concomitant inhibition of the transcription factor since it is no longer able to interact with DNA (Brunet et al., 1999). Interestingly, levels of phosphorylated/active Akt were shown in the present study to be dramatically increased in TamR-30 cells (see Figure 3.25). In view of this, it was therefore feasible to anticipate that the very low ERα content exhibited by TamR-30 cells could correlate with low levels of nuclear FoxO3a, as a result of the Akt-mediated phosphorylation/nuclear exclusion of FoxO3a. In order to test this hypothesis further, immunocytochemical analysis of total FoxO3a was carried out using total Foxo3a antibodies that detect both nuclear/active- and cytoplasmic/inactive-Foxo3a. Results showed a very slight but visible decrease in FoxO3a nuclear staining in TamR-30 cells compared to MCF-7 and TamR-18 cells (Figure 3.38B). The cytoplasmic staining of Foxo3a was very weak in TamR cells and this could be due to the high dilution used with total Foxo3a antibodies (1:250).

It was also anticipated that cytoplasmic levels of phosphorylated/inactive FoxO3a (Ser 253) be increased in TamR-30 cells, since Akt activity was considerably elevated in these cells. Immunocytochemical analysis of phospho-FoxO3a (Ser 253)
confirmed the cytoplasmic localization of the inactive protein. However, the cytoplasmic staining of phospho-FoxO3a appeared similar across the different cell lines (Figure 3.39).
Figure 3.38 (A) Western blot and (B) immunocytochemical analysis of total Foxo3A expression in MCF-7 and the late TamR cells. Cells were grown to 70% confluency in their routine medium. Immunocytochemical detection of total Foxo3A was carried out using specific polyclonal antibodies on ER-ICA fixed cells, as described in Material and Methods. Data are representative of one (ICC) or two (WB) independent experiments.
Figure 3.39 (A) Western blot and (B) immunocytochemical analysis of phosphorylated/inactive Foxo3A expression in MCF-7 and the late TamR cells. Phospho-specific antibodies against Phosphorylated-Foxo3A (Ser253) were used, as described in Material and Methods (n=1).
Chapter 4

~ Discussion ~
4.1 Rationale and aims of this study

ERα is a major contributor to the biology of breast carcinogenesis and is still currently considered as one of the most powerful prognosis factors in breast cancer. Therefore, understanding the mechanisms controlling its expression during breast cancer development and following treatment with anti-hormones is of obvious clinical interest. Endocrine therapy is primarily aimed at blocking oestrogen-induced proliferation of ERα-positive breast cancer cells and is currently represented by three broad classes of agents: the SERMs (e.g.: tamoxifen), the SERDs (e.g.: fulvestrant) and the third-generation aromatase inhibitors (AIs; e.g.: anastrozole; Gradishar, 2004). A new therapeutic trend has been emerging in which AIs are progressively taking over from tamoxifen as the primary anti-hormonal agents in post-menopausal women with early or advanced ERα-positive breast cancer (Dixon & Bundred, 2006). This new trend has in part been initiated by data obtained from several phase III clinical trials, which have showed that AIs have a superior efficacy over tamoxifen when used as first-line endocrine agents for the treatment of early breast cancer in postmenopausal women. Most notably, the ATAC trial revealed that 5 years of anastrozole over tamoxifen improves disease-free survival by 20% in the adjuvant setting (Howell et al., 2005; Younus & Vandenberg, 2005). Nonetheless, AIs have yet to be fully established into the routine clinical practise and thus, tamoxifen still remains widely used for the management of ERα-positive breast cancers. Unlike AIs, tamoxifen has proved itself as a flexible agent, being administered to breast cancer patients irrespective of their age, nodal or menopausal status. Moreover, while little is known about the long-term benefits and drawbacks of AIs, there is a substantial body of data available for tamoxifen whose clinical history spans over three decades, thus making it a relatively safe endocrine agent in systemic therapy for breast cancer. It is also worth to mention that the low cost of tamoxifen is of great relevance for its implemented use in developing countries.

Despite all these advantages, the efficacy of tamoxifen, like many other endocrine agents, is too often limited by the onset of resistance (intrinsic and acquired). Several underlying mechanisms for tamoxifen resistance have been proposed in the literature including mutations of ER, increased expression of ER splice variants, altered metabolism of tamoxifen, altered expression and activity of ER cofactors and increased activity of non-genomic/membrane ER (Riggins et al., 2007; Ring & Dowsett, 2004). Two other
mechanisms that have also been identified, and which have been the subject of investigation in this study, are (i) the aberrant activation of alternative growth regulatory pathways, such as EGFR and HER-2, and (ii) loss of ERα expression.

In clinical disease, an inverse association between EGFR and/or HER-2 with ERα expression has been shown by many groups and is now a well acknowledged observation (Ciocca et al., 1992; Nicholson et al., 1994; Konecny et al., 2003; Gee et al., 2005). As previously stated in Chapter 1, loss of ERα expression is a major issue since it is associated with a very poor prognosis, a more aggressive and metastatic breast cancer phenotype. Although the majority of breast cancer patients retain ERα expression and function throughout endocrine therapy, concordant data from several clinical studies have shown that 15% to 30% of initially ERα-positive breast tumours can convert to an ERα-negative phenotype on tamoxifen relapse in the adjuvant or metastatic setting (Gutierrez et al., 2005; Johnston et al., 1995; Kuukasjarvi et al., 1996; Lower et al., 2005). A loss of ERα in as much as 50% of tumours at the time of progression on hormonal therapy has recently been quoted by the Johnston group (Johnston et al., 2008). In the adjuvant setting, Johnston et al. (1995) have also shown that there was a significant reduction in the quantitative expression of ERα in the ERα-positive recurrent tumour compared to the primary. In support of this, a recent clinical study has reported a proportion (~ 15%) of tamoxifen-relapsed breast tumours with “low-positive” ERα levels (Kennecke et al., 2008). Cumulatively, these clinical data would suggest that loss of ERα expression may occur as a direct consequence of tamoxifen therapy. The present study explored this possibility further by developing in vitro cell models of acquired resistance to tamoxifen in order to investigate whether ERα loss can indeed occur in response to prolonged tamoxifen treatments, and most importantly, elucidate the underlying molecular mechanisms together with their therapeutic implications.
4.2 *In vitro* models of anti-hormone-resistance with depleted ERα levels

In the present study, MCF-7-derived fulvestrant-resistant (FasR) cells provided the first proof-of-principle that sustained exposure to anti-hormones can induce loss of ERα expression. Indeed, continuous exposure of MCF-7 cells with the pure anti-oestrogen fulvestrant resulted in irreversible loss of ERα expression over a 24-month period. In agreement with the FasR cells in the present study, other studies have also reported a fully and irreversible ERα-negative phenotype following extended culture (~18 months) of MCF-7 cells in fulvestrant-containing medium (Liu *et al*., 2006). In contrast to the fulvestrant-resistant subline, long-term treatment of MCF-7 cells with 4-OHT did not result in total loss of ERα, even after a 30-month treatment regime, which generated TamR-30 cells. The FasR cell models seem therefore more susceptible to the generation of an ERα-negative phenotype than the acquired tamoxifen resistant variant. This observation in itself is not surprising considering that fulvestrant mode of action is based on its ability to rapidly downregulate ERα levels (Osborne *et al*., 2004), potentially via a nuclear matrix-associated proteasome mechanism (Long & Nephew, 2006). However, it has previously been shown that ERα expression (mRNA and protein) and function can be maintained in several MCF-7-derived models of fulvestrant resistance; yet ERα protein levels were still reduced by one-third compared to the parental cells (Larsen *et al*., 1997).

Although total loss of ERα was not achieved after 30 months exposure with 4-OHT (TamR-30), the data presented herein clearly indicate that acquisition of tamoxifen resistance in MCF-7 cells is associated with a significant reduction of both ERα mRNA and protein levels. Precisely, 18 months exposure with tamoxifen (TamR-18) was shown to reduce ERα expression by approximately 40%. Importantly, this fall was further exacerbated with a 24- (TamR-24) and 30 month (TamR-30) treatment regime, which led to a ~90% reduction in ERα mRNA and protein steady state levels. Using *in vitro* models of early resistance to tamoxifen (TamR-3, TamR-11), this study also revealed that the decline in ERα expression occurs relatively quickly during *in vitro* acquisition of the tamoxifen-resistant phenotype. This is in agreement with previous *in vitro* studies by Badia *et al*. (2000) who have reported that ERα expression was reduced by ~50% in all of their eight variants of MCF-7-derived tamoxifen-resistant cells, which were exposed with 4-OHT for 6 months. Preliminary *in house* experiments have shown that short-term
treatments of MCF-7 cells with tamoxifen for 48 hours resulted in an increase of both ERα mRNA and protein levels (Dr Martin Giles, personal communication). This is in agreement with previous studies, which have shown a tamoxifen-induced accumulation of ERα protein in the nucleus of MCF-7 cells treated with 4-OHT for up to 72 hours (Laios et al., 2003). In the same study, tamoxifen was shown to stabilize the receptor while oestrogen and faslodex caused rapid ERα degradation.

A number of tamoxifen resistant cell models that have been reported in the literature retain ERα expression at wild-type levels (Mullick & Chambon, 1990; Brunner et al., 1993; Badia et al., 2000). However, almost two decades ago, van den Berg & colleagues were the first to report the development of a tamoxifen-resistant phenotype associated with total loss of ERα protein (van den Berg et al., 1989). The same authors generated a ZR-75-1-derived cell line of tamoxifen resistance (ZR-75-9a1), in which a total but reversible loss of detectable ERα protein was observed in a tamoxifen concentration-dependent manner over a 12 month period. The high concentration of tamoxifen used to maintain ZR-75-9a1 cells (i.e.: 8 μM) is in sharp contrast with that used to maintain TamR-30 in the present study (i.e.: 1 μM), in which the more potent metabolite 4-OHT was used. Furthermore, the more rapid decline of ERα expression occurring in ZR-75-9a1 cells (i.e.: 12 months) compared to TamR-30 cells (i.e.: 30 months) can be attributed to the fact that the initial cellular levels of ERα in the parental ZR-75-1 cells are considerably lower than in MCF-7 cells (Dr. Julia Gee, personal communication). Interestingly, previous clinical studies have shown that the duration of response to endocrine therapy tended to be longer in “ER-rich” primary breast tumours than those with lower levels of the receptor (Campbell et al., 1981). It is thus feasible to suggest that ERα-positive breast tumours with lower initial levels of ERα may be more susceptible to become ERα-negative lesions culminating in an earlier anti-hormone relapse.

In contrast to this project’s findings, one study did claim that ERα protein was expressed at levels similar to those of parental MCF-7 cells in three tamoxifen-resistant variants (MTR1-3 cells), which were grown in the presence of 4-OHT (1μM) for up to 2 years (Kilker et al., 2004). Using western blot analysis, the authors demonstrated that ERα was readily detectable in all three MTR cells. However, a more critical evaluation of the authors’ immunofluorescence data reveals that MTR cells exhibited a noticeable
reduction in nuclear staining for ERα compared to the parental MCF-7 cells. Also in the same study, western blot analysis showed that cyclin D1, which is a classic ERα-regulated gene, was markedly reduced at the protein level in all three MTR cells compared to the parental MCF-7 cell line. Similarly in the current study, total levels of IGF-IR (another classic ERα-target gene) were shown to be downregulated as ERα levels were progressively lost during the development of the tamoxifen resistant cell models. Cumulatively, the immunofluorescence and cyclin D1 data would therefore suggest that the development of MTR cells was in fact associated with a downregulation of ERα expression, thus in line with the results presented in this thesis.

Conclusively, TamR-30 cells and the other reported cell models of tamoxifen resistance like ZR-75-9a1 provide in vitro evidence that loss of ERα can occur as a direct consequence of tamoxifen therapy. These in vitro models are therefore in line with the aforementioned clinical data which showed that a proportion of breast tumours become ERα-negative on tamoxifen relapse (Johnston et al., 1995; Kuukasjarvi et al., 1996; Gutierrez et al., 2005). However, it is important to mention that other factors causing ERα downregulation may also exist independently to tamoxifen therapy. For example, previous clinical data have shown that ERα was lost in approximately one third of metastatic tumours, which originated from ERα-positive primary lesions treated with adjuvant chemotherapy without tamoxifen (Lower et al., 2005). Sustained oestrogen deprivation in vitro (mimicking the biological effect of AIs) has also been associated with the emergence of an ERα-negative phenotype from the ERα-positive T47D cells (Murphy et al., 1990; Pink et al., 1996), and hypoxia has also been demonstrated to result in downregulation of ERα expression in several ERα-positive breast cancer cell lines and breast tumours (Cooper et al., 2004; Stoner et al., 2002). It is noteworthy to mention that loss of ERα expression could also occur as part of the natural biological evolution of breast carcinoma. Whilst this hypothesis remains difficult to extrapolate from clinical data for obvious ethical reasons, a few studies have reported loss of ERα in metastatic lesions that were associated with ERα-positive primary tumours in the absence of intervening treatments (Kuukasjarvi et al., 1996; Nedergaard et al., 1995; Zheng et al., 2001).
4.3 ERα promoter usage in TamR cells

In addition to assessing the overall transcriptional activity of the ERα gene (through measurements of total ERα mRNA levels), this study also examined ERα gene promoter usage through expression analysis of the different ERα mRNA variants in MCF-7 cells and in the tamoxifen-resistant cell models (TamR cells). The primary aim was to assess whether loss of ERα in TamR cells was a result of reduced usage of particular promoters. Based on the reports available in the literature to date, it appears that alternative promoter usage of the ERα gene has only been investigated in the endocrine-sensitive phase in vitro but not during acquisition of the tamoxifen resistant phenotype. Herein, assessment of ERα mRNA variant levels was first validated in oestradiol-treated MCF-7 cells which, in agreement with previous studies, exhibited reduced levels of all ERα mRNA variants but more significantly so for the breast-cancer specific variants A, B and C (Donaghue et al., 1999; Flouriot et al., 1998). Levels of the ERα mRNA variant C were as predominant as the variant A in control MCF-7 cells. This is in contrast to data from Flouriot et al. (1998) who have shown that ERα mRNA variants A were the most prevalent in MCF-7 cells. This discrepancy could be due to a natural variation in levels of ERα mRNA isoforms across cell lines from different laboratories. Furthermore, expression levels of ERα mRNA variant C were associated with a large error bar in the present study, thus reflecting variation within the cells tested. Worthy of mention is the expression profiles of ERα mRNA variants in TamR-18 cells, which closely resembled that of MCF-7 cells treated with oestrogen. Interestingly, a previous study showed that tamoxifen treatment of MCF-7 cells for 6 days downregulated total ERα mRNA expression to levels similar to those observed in oestradiol-treated MCF-7 cells (Larsen et al., 1997). Because the effect of oestradiol on ERα expression has long been attributed to a decreased transcription rate of the ERα gene in MCF-7 cells (Saceda et al., 1988; Pink & Jordan, 1996), it is feasible to suggest that oestrogen and tamoxifen may act through similar mechanisms to regulate ERα gene transcription; possibly involving a similar repertoire of transcription factors in both MCF-7 and TamR cells.

Data presented in this study showed that the time-dependent reduction of total ERα mRNA levels in response to prolonged tamoxifen treatments was attributable to a decreased expression of the entire population of ERα mRNA variants. However, this
reduction was most dramatic for ERα mRNA variants C whose steady-states were reduced to almost non-detectable levels following a 30 month treatment regime with tamoxifen. This would therefore suggest that transcription of the ERα gene from the C promoter may play an essential role in maintaining adequate levels of total ERα mRNA in TamR cells. This is in agreement with previous studies, which showed a good correlation between overall ERα protein expression and the increased mRNA expression from the distal promoter C in MCF-7 cells (Tanimoto et al., 1999; Donaghue et al., 1999). Interestingly, also, transcription from the promoter C was found to be dramatically increased in long-term oestrogen deprived MCF-7 cell models, which exhibited much higher ERα levels than their parental cells (Sogon et al., 2007). A more thorough examination of the ERα gene promoter activity (using for example a luciferase-based reporter gene assay) would be needed to conclusively confirm the predominant function of promoter C on ERα gene expression in TamR cells. Overall, the real-time PCR data obtained in this study would suggest that expression of the ERα gene does not occur through the selective use of a specific promoter in the tamoxifen-resistant cells. Indeed, downregulation of total ERα mRNA levels in TamR cells was associated with a declined expression of all the ERα mRNA variants. This is in line with previous studies carried out across different types of ERα-positive breast cancer cells, in which ERα promoters were either all upregulated (e.g.: ZR-75-1) or downregulated (e.g.: MCF-7) by oestrogen treatments (Donaghue et al., 1999).
4.4 Mechanisms of ERα loss in TamR cells

4.4.1 A reversible and “adaptive” mechanism

The significant reduction in ERα mRNA and protein levels observed in TamR-24 and TamR-30 cells was shown to be reversible upon tamoxifen withdrawal over a period of 4 weeks. This reversibility, thus, lends further support to this project’s hypothesis that tamoxifen therapy could be an active contributor of ERα depletion. Reversibility of ERα expression upon tamoxifen withdrawal was also previously been demonstrated in the aforementioned ZR-75-9a1 cells, which were generated as ERα-negative tamoxifen-resistant breast cancer cells (Long et al., 1992; van den Berg et al., 1989). Significantly, however, it is important to note that the recovery of ERα levels in TamR cells was only partial since expression levels of both ERα mRNA and protein were not restored to wild-type levels (i.e.: MCF-7) under tamoxifen-withdrawn conditions. This would therefore imply that additional factors other than tamoxifen exist that would contribute additively to ERα loss during tamoxifen therapy. Furthermore, data from the tamoxifen-withdrawn experiments clearly indicate that downregulation of ERα levels during prolonged tamoxifen treatments was not permanent, thus excluding epigenetic modifications as a mechanism responsible for the reduced ERα levels in TamR cells. This is further supported by the findings, herein, that ERα mRNA levels were not recovered with the DNA-demethylating agent 5-azadeoxycytidine (5-AZA) in TamR-18 cells. Interestingly, it was also shown that the irreversible loss of ERα in FasR cells was not linked to epigenetic silencing of the ERα gene, as suggested by DNA methylation studies on the proximal promoter of the ERα gene (Dr. Martin Giles, personal communication).

It is noteworthy to mention that the tamoxifen-resistant phenotype exhibited by TamR-24 and TamR-30 cells was not sustainable in tamoxifen-withdrawn conditions. Indeed, cell growth was noticeably reduced when TamR cells were routinely maintained in 4-OH-depleted medium for greater than 4 weeks. This observation is indicative of the acquired agonistic properties of tamoxifen and its role as a growth promoting agent in TamR cells (Michalides et al., 2004; Riggins et al., 2007). Interestingly, in vitro and in vivo data from a recent report have suggested that unliganded ERα may have inhibitory effects on cell growth through its interaction with the cyclin-dependent kinase inhibitor p21/WAF1 (Maynadier et al., 2008). Assuming that the cellular pools of ERα have become fully
unliganded in TamR cells under the tamoxifen-withdrawn culture conditions, it is feasible to speculate that a mechanism, similar to that described by Maynadier et al., may underlie the reduced growth of TamR-24 and TamR-30 cells when deprived of 4-OHT.

Immunocytochemical analyses have shown that the progressive downregulation of ERα levels (during the development of the tamoxifen resistant phenotype) affected the entire cell population and was unlikely to be due to a clonal outgrowth from an initial ERα-negative cohort within the parental MCF-7 cell line. This observation together with the fact that downregulation of ERα was reversible in TamR cells provide in vitro evidence in support of an “adaptive” mechanism for the loss of ERα expression in response to prolonged tamoxifen therapy. In support of this adaptive mechanism, Beeram & co-workers (2007) have recently reported that ERα was lost in 9 out of 16 (56%) tamoxifen resistant cells, which have been clonally derived from ERα-positive MCF-7 breast cancer cell colonies that were cultured in the presence of 4-OHT (1μM) over a 4 month-period. Furthermore, ERα mRNA levels were shown to be decreased by ~50% in tamoxifen-resistant cells that were also clonally derived from ERα-positive MCF-7 cells (Fan et al., 2006). It should be noted, however, that a “selective” mechanism in which anti-hormones would favour the growth of ERα-negative cells originally present in the primary tumour lesions, can not be entirely ruled out and has been quoted by others (Allred et al., 2004; Zheng et al., 2001).

4.4.2 An EGFR-independent mechanism

In the present work, it was clearly shown that a 24 hour- and 48 hour treatment regime with gefitinib induced downregulation of ERα mRNA and protein levels in TamR-18 cells. This would therefore imply that activation of EGFR signalling, with resultant stimulation of downstream components like Erk1/2 MAPKs, may have a positive regulatory effect on ERα expression in TamR cells. Also in this study, sustain inhibition of EGFR signalling over a 10-day period in TamR-18 cells resulted in what appeared to be a recovery of ERα expression (most notably at the protein level), indicative of a compensatory mechanism. Interestingly, the recovery of ERα expression also correlated with that of Erk1/2 activity (although not fully), thus lending further
support to the possibility that Erk1/2 may maintain ERα steady-states levels in TamR cells. This possibility was further confirmed by the findings that inhibition of Erk1/2 activity by U0126 resulted in total loss of ERα protein in TamR-30 cells. Taken together, these results are very surprising since the vast majority of reports present in the literature strongly favours the view that exaggerated EGFR/MAPK signalling promotes ERα downregulation. Most significantly, compelling data from the El-Ashry group have shown that hyperactivation of MAPK in EGFR or HER-2-overexpressing breast cancer cell lines can directly induce loss of ERα expression in vitro (Holloway et al., 2004; Oh et al., 2001). A hyperactivated-MAPK signature distinguishing ERα-negative from ERα-positive phenotypes has also been reported by the same group (Creighton et al., 2006). Also in contrast to the findings presented in this thesis, another study by the El-Ashry group has shown that ERα expression can be restored in ERα-negative breast cancer cell lines and ERα-negative tumours upon direct inhibition of hyperactive MAPK with the inhibitor U0126, but also via inhibition of the upstream growth factor receptor EGFR (Bayliss et al., 2007).

Despite the overwhelming data in the literature demonstrating an MAPK-induced downregulation of ERα expression, a few studies are encouragingly in agreement with observations made in the current project. For example, it has recently been demonstrated that a 48 hour-treatment of MCF-7 cells with U0126 also resulted in reduced levels of ERα protein (Chu et al., 2007). Furthermore, Marsaud et al. (2003) have shown that blockade of the Erk1/2 pathway by U0126 resulted in a proteasome-mediated degradation of 4-OHT-bound ERα protein in MCF-7 cells. Perhaps most significantly for this study, the same authors further suggested that ERα levels may be maintained by the low basal activity of MAPKs normally exhibited by MCF-7 cells.

As outlined in Chapter 1, it is known that post-translational modifications of the receptor, most notably phosphorylation on different serine residues of ERα, have a regulatory role on both ligand-dependent- and ligand-independent-degradation of the receptor (Alarid et al., 1999; Laios et al., 2005; Valley et al., 2005). For example, phosphorylations of Ser118 sites have been shown to contribute to ERα degradation by facilitating targeting of the receptor to the ubiquitin-proteasome pathway (Valley et al., 2005). Furthermore, MAPKs are known to be involved in the phosphorylation of such serine sites on ERα (Kato et al., 1995). In view of this and bearing in mind both Marsaud et al.'s findings
(2003) and those of the El-Ashry group, it is feasible to suggest that low levels of activated MAPKs may not efficiently initiate proteasome-mediated degradation of ERα, thus levels of the receptor appear to be maintained. However, hyperactivated MAPKs induce rapid receptor degradation, and thus reduce ERα steady-state levels. One would therefore expect Erk1/2 to induce degradation and thus downregulation of ERα levels in TamR cells since the latter have been shown in this study to contain considerably higher basal activity of Erk1/2 compared to MCF-7 cells. However, the U0126 experiment in TamR-30 cells suggested otherwise. Therefore, it is interesting to speculate that in TamR cells, levels of phosphorylated/activated Erk1/2 proteins (although relatively high compared to MCF-7 cells) may not have yet reached a "threshold point" above which they would contribute to rapid degradation of ERα. Indeed, the MAPK-induced loss of ERα suggested by the El-Ashry group is mainly based on experiments with constitutively active EGFR- and MEK-transfected MCF-7 cells in which the EGFR-Erk1/2 signalling reaches extreme levels of activity; far higher than that found in TamR cells, wherein the basal activity of EGFR signalling mainly depends on the autocrine production of EGFR ligands like amphiregulin (Britton et al., 2005; Hutcheson et al., 2003). In view of this and in order to further evaluate the potential role of the EGFR-Erk1/2 signalling in maintaining ERα levels in TamR cells; it would be interesting to assess the effect of exogenous EGFR-ligands on ERα expression.

4.4.3 A Src-dependent mechanism

Perhaps the most significant finding of this work is that the elevated Src kinase activity exhibited by TamR-30 cells appears to be an important regulator of ERα loss in these cells. Indeed, modulation of intracellular signalling pathways with various pharmacological inhibitors revealed that only the Src inhibitor AZD0530 rapidly and significantly restored both ERα mRNA and protein steady-state levels in TamR-30 cells. The recovery of ERα expression induced by AZD0530 was considerably higher and occurred much more rapidly (3 days) than that observed with withdrawal of tamoxifen from culture medium (4 weeks). ERα levels in TamR-30 cells were recovered to at least TamR-18-equivalent following Src inhibition for 3 days, as demonstrated by western blot analysis. However, the AZD0530-induced re-expression of ERα was shown to reach
wild-type (MCF-7) levels by immunocytochemical analysis. The latter method is certainly more sensitive than western blotting in detecting ERα expression: there was a considerable decrease in the number of ERα-negative cells in the AZD0530-treated TamR-30 cells compared to the non-treated cells. Also, a considerable increase in intensity of nuclear ERα staining was observed within the ERα-positive cells cohort. As described in Chapter 1 (section 1.6.2), Src is a key mediator of cross-talk between EGFR and ERα signalling. In view of this and the AZD0530-induced re-expression of ERα, it would be of interest to investigate the effects of Src inhibition on EGFR and HER-2 activities in TamR-30 cells. Previous studies have indeed shown that although AZD0530 does not affect phosphorylation of EGFR at Y1068, it partially reduces phosphorylation of the growth receptor at Y845 (Hiscox et al., 2006). It would be equally interesting to investigate Src activity in response to the inhibitory effects of gefitinib on EGFR activity which, as described in Chapter 3 (Figure 3.20), had no effects on ERα levels in TamR-30 cells. The studies by Hiscox et al. (2006) have previously demonstrated only a partial inhibitory effects of gefitinib on Src kinase activity and suggested that mechanisms other than elevated EGFR expression and activity may contribute to increased levels of activated Src in TamR cells.

The restoration of ERα with AZD0530 is in strong agreement with the study by Chu et al. (2007) who provided for the first time compelling in vitro and in vivo evidence for a Src-mediated regulation of ERα expression in ERα-positive and ERα-negative breast cancer cell. These authors have established that Src-mediated phosphorylation of ERα targets the receptor for its ubiquitin-proteasome degradation in a ligand-dependent manner. Using a cohort of 18 ERα-negative primary breast tumours, the authors have also demonstrated an inverse clinical correlation between Src kinase activity and ERα-negativity. Furthermore, the same authors demonstrated that ERα protein stability was reduced in cells exhibiting high Src kinase activity with lower ERα levels compared to MCF-7 cells being observed. Similarly, preliminary in house studies with the protein synthesis inhibitor cycloheximide have revealed that ERα was less stable and thus more rapidly degraded in TamR-18 cells compared to wild-type MCF-7 cells. In view of this and the fact that TamR-18 cells have higher basal Src activity than MCF-7 cells, it would be anticipated that treatment of TamR-18 cells with AZD0530 would result in an increase of ERα stability and expression in these cells.
Data from Chu et al. (2007) mainly advocate a role for Src in regulating ERα expression at the post-translational level (i.e.: protein degradation). Surprisingly, herein, the AZD0530-induced upregulation of ERα mRNA steady states levels in TamR-30 cells also points to a transcriptional control of ERα gene levels by Src. Expression levels of the ERα mRNA variants A-C were significantly increased upon AZD0530 treatments in TamR-30 cells. These results would therefore suggest that Src kinase activity may indirectly inhibit ERα gene transcription from the proximal (A/B) and distal (C) promoters. In order to more convincingly ascertain this possibility, it would be interesting to assess the activity of ERα promoter-based reporter genes (containing the firefly luciferase gene for example) that have been transfected into TamR-30 cells treated with the Src inhibitor. In support of a Src-dependent modulation of ERα gene expression, Longo et al. have provided convincing in vitro evidence for a Src-mediated regulation of the F promoter in the osteoblast-like Saos-2 cells (Longo et al., 2006). As outlined in Chapter 1, the F promoter is specifically activated in bones cells (Flouriot et al., 1998; Lambertini et al., 2003). The study by Longo et al. has also shown that PKCa was involved in mediating downregulation of ERα gene expression by acting upstream of Src in Saos-2 cells. Interestingly, an inverse relationship between PKCa overexpression/hyperactivation and ERα expression has been established in clinical breast cancer, breast cancer cell lines (Martin et al., 1995; Tonetti et al., 2000; Ways et al., 1995), and notably in models of tamoxifen-resistant cells (Frankel et al., 2006). In the present study, basal levels of activated PKCa were considerably increased in TamR-30 cells, as demonstrated by western blot analysis. However, treatments of TamR-30 cells with the widely used PKC inhibitor bisindolylmaleimide (Bis) did not restore ERα expression, thus suggesting that the PKC pathway may not be involved in the downregulation of ERα levels in TamR cells. Significantly, however, the inhibitory effect of Bis (0.5μM) on the basal activity of PKCa in TamR-30 cells could not be checked due to time constraint; thus a regulatory role for PKCa on ERα expression in TamR cells can not be definitively ruled out herein. Similarly, the inhibitory effects of U0126 and wortmanin on MAPK and Akt kinase activity, respectively, were not checked due to time constraints, thus other signalling pathways involved in downregulation of ERα levels cannot be ruled out.

The prominent role of Src in tamoxifen resistance has also recently been highlighted by the Santen group. The latter has shown that prolonged tamoxifen treatments in vitro (~12
months) caused a fraction of nuclear ERα to translocate to the cytoplasm and interact with 
EGFR in a Src-dependent manner (Fan et al., 2007; Yue et al., 2007). The extra-nuclear 
redistribution of ERα is thought to enhance the non-genomic action of ERα with 
EGFR/MAPK signalling to drive the growth of tamoxifen-resistance cells. The same 
authors have showed that inhibition of Src activity prevented cytoplasmic translocation of 
ERα and, importantly, restored the growth sensitivity of TamR cells to tamoxifen. Most 
significantly, however, total levels of ERα did not change during acquisition of the 
tamoxifen resistance in the studies by the Santen group. In contrast, the present work 
provides the first in vitro evidence, in a model of acquired tamoxifen-resistance, for a link 
between Src kinase activity and ERα loss. It should be noted that the Src inhibitor 
AZD0530 used in the current study not only affect Src but also other members of the non-
receptor tyrosine kinase family including Fyn and Yes which, because of their high 
structural and functional redundancy with Src, could also play a role in the depletion of 
ERα levels in TamR cells (Hennequin et al., 2006; Parsons & Parsons, 2004). siRNA 
experiments would certainly provide more clues regarding the extent to which each Src 
family kinase member may contribute to ERα downregulation in TamR cells. It is also 
noteworthy to mention that AZD0530 also inhibits the other non-receptor tyrosine kinase 
Abl (Hennequin et al., 2006). Therefore, a potential involvement of the Abl signalling 
pathway in the downregulation of ERα expression in TamR cells can not be entirely ruled 
out. While the role of Abl remains unclear in endocrine resistance, aberrant Abl kinase 
activity has been shown to be highly implicated in the invasion capacity of breast cancer 
cells; with Abl acting as downstream effectors of the ErbB receptors, IGF-IR and the Src 
family (Srinivasan et al., 2008; Srinivasan & Plattner, 2006).

Overexpression of the non-receptor tyrosine kinase Src has now been established as a 
crucial component of growth factor signalling pathways that regulate the development 
and metastatic progression of many types of human cancer including breast cancer 
(Yeatman, 2004; Song & Santen, 2006). Importantly, recent emerging in vitro data have 
now also attributed a prominent role for Src in mediating the growth and invasion 
capacities of ERα-positive tamoxifen-resistant cells. Previous in house publications have 
shown that acquisition of the tamoxifen resistant phenotype (up to the 18 month-period) 
is associated with an elevation in Src kinase activity concomitant with an aggressive and 
invasive phenotype in vitro (Hiscox et al., 2004; Hiscox et al., 2006). Overexpression of
activated c-Src has also been reported in other models of tamoxifen-resistant variants (Kilker et al., 2004; Planas-Silva et al., 2006). In the present study, the basal activity of Src, as determined by phosphorylation of the tyrosine residue Y418, was shown to be further enhanced when tamoxifen treatments were prolonged to a 30-month period, and as expected, this was associated with a further increase in cell motility and invasion. This would indicate that as cells progress towards ERα-negativity, this is associated with increase in aggressive behaviour mimicking what is seen with patients with ERα-negative breast cancers (Gruvberger et al., 2001; Gee et al., 2004).
4.5 Therapeutic implications of the AZD0530-induced restoration of ERα expression

Regardless of the molecular mechanisms governing ERα downregulation during tamoxifen therapy, re-expression of ERα must be that of a functional receptor that is still able to regulate growth in order to be clinically relevant. Therefore, it will be essential to conduct further studies investigating whether the AZD0530-induced upregulation of ERα expression in TamR-30 cells is accompanied with a restoration of the growth-inhibitory effect of tamoxifen or other anti-hormonal therapies. A growth response of TamR-30 cells in response to increasing concentrations of 4-OHT whilst in the presence of AZD0530 would be an appropriate initial experiment. Very encouragingly, in house preliminary studies have shown that sensitivity to tamoxifen could be restored in TamR-18 cells treated with AZD0530 (Hiscox et al., 2007). Importantly, the data presented in this thesis would indicate that inhibiting Src activity alongside anti-hormonal therapy may prove to be a valuable therapeutic strategy to retain ERα expression and function, thus preventing ERα-positive breast tumours to drift towards more aggressive cancer cell behaviour in clinical breast cancer. Furthermore, because epigenetic silencing of ERα can only be explained in ~25% of the de novo ERα-negative breast cancers, it is plausible to suggest that inhibition of Src kinase activity in a proportion of the remaining de novo ERα-negative tumours (for which cytotoxic chemotherapy is eventually used) might also be therapeutically beneficial in triggering anti-hormone response. Encouragingly, the aforementioned study by Chu et al. (2007) has shown that Src kinase activity was found elevated in as much as 78% of ERα-negative breast cancers.

In support of the results presented herein, pre-clinical data is now emerging which suggest that up-front combination therapies targeting Src activity and ERα have maximal inhibitory effects on growth of ERα-positive breast cancer cell lines, thus delaying or preventing emergence of a resistant phenotype (Herynk et al., 2006; Planas-Silva & Hamilton, 2007; Hiscox et al., 2008). Significantly, the study by Hiscox et al. (2008) has shown that combining AZD0530 and tamoxifen treatments in the ERα-positive MCF-7 and T47D cell lines prevented not only cell proliferation but also resulted in complete abrogation of their in vitro migratory and invasive behaviour. In light of this, it is anticipated that AZD0530 treatments would also reduce the highly motile and invasive
characteristics of TamR-30 cells. In an attempt to identify the mechanisms underlying cell growth suppression following dual targeting of Src and ERα, pre-clinical studies have demonstrated that Src inhibition resulted in reduced expression of the key positive cell cycle regulators cyclin D1 and c-myc (Planas-Silva & Hamilton, 2007; Hiscox et al., 2008). One potential explanation for the reduced expression of these cell cycle proteins could be the complete abrogation of EGFR signalling, which was shown to be essentially Src-dependent in a significant study by Knowlden et al. (2005). Importantly, in light of the novel data presented in this thesis, it is very plausible that the anti-proliferative effects of the AZD0530/tamoxifen combination treatments, as observed by the aforementioned studies, could now also be a consequence of an (AZD0530-induced) increase in ERα expression levels in TamR cells, thus restoring better response to tamoxifen.
4.6 Potential upstream signalling elements for the Src-mediated loss of ERα expression

While data presented in this thesis are strongly suggestive of a Src-dependent mechanism causing ERα downregulation during prolonged tamoxifen treatments, it remains to be determined which growth factor receptors or other factors, acting upstream of Src, are involved. As previously stated in this chapter, EGFR signalling appears not to be involved in the downregulation of ERα expression in TamR cells. However, it remains possible that HER-2, although shown to be the preferred ally of EGFR in TamR cells (Knowlden et al., 2003), may contribute to ERα loss independently of EGFR. HER-2-inhibition by trastuzumab/Herceptin has been shown to restore ERα levels in ERα-negative breast cancer cell lines and ERα-negative tumours (Bayliss et al., 2007). Furthermore, a preliminary clinical study has shown for the first time that ERα expression could be reinstated with trastuzumab in a small cohort of patients with ERα-negative breast tumours overexpressing HER-2 (Munzone et al., 2006). In light of these clinical data, it would be equally interesting to investigate the effect of HER-2 blockade by Herceptin on ERα expression in TamR-30 cells as it can be speculated that HER-2 together with another member of the ErbB family other than EGFR may be involved in ERα depletion. In support of this, Stoica et al. (2003b) have shown that activation of HER-2/HER-3 heterodimerization with the exogenous ligands heregulin-β1 (HRG-β1) results in downregulation of ERα mRNA and protein in MCF-7 cells, via the PI3K/Akt pathway. Such heterodimerization between HER-2 and HER-3 has been demonstrated in TamR-18 cells under HRG-β1 challenge (Hutcheson et al., 2007). In the present study, the basal activity of Akt was dramatically increased in TamR-30 cells and, interestingly, its inhibition by the PI3K inhibitor wortmanin did result in a slight but visible increase of ERα protein. It is therefore possible that Akt kinase activity may also have a contributory role in the downregulation of ERα levels in TamR cells. In support of this, Guo & Sonenshein (2004) have observed an increase of ERα protein levels when the low-ERα expressing NF639 cells were treated with wortmanin. Overall, it is very plausible that the Src-induced downregulation of ERα levels in TamR-30 cells could be mediated by the upstream signalling pathway elements HER2 and PI3K/Akt.
One upstream growth factor receptor that could potentially be involved in the Src-mediated downregulation of ERα is IGF-IR. Indeed, Knowlden et al. (2005) have provided, for the first time, experimental evidence for a unidirectional cross-talk from IGF-IR to EGFR in a Src-dependent manner in TamR-18 cells. In the same study, Src-dependent phosphorylation of EGFR at tyrosine 845 was shown to be central for basal and ligand-mediated activation of the EGFR signalling and the resultant cell growth of TamR cells. Other upstream signalling elements that could also be involved in the Src-mediated loss of ERα, but which have not been investigated herein are the integrins. These so called cell adhesion molecules, consisting of α and β subunits, are know to cooperate with growth factor receptors in the control of cell proliferation, cell survival and cell adhesion and migration (Hehlgans et al., 2007). β1 and β4 integrins have been shown to be essential in breast tumour progression and invasion by amplifying EGFR and HER-2 signalling (Guo et al., 2006; Park et al., 2006). Most notably, in an attempt to elucidate the incompletely understood mechanisms of Src activation in human cancers, Huveneers et al. (2007) have identified α,β3 integrins as a pivotal activator of Src kinase activity. Interestingly, in house preliminary studies have demonstrated a significant increase in α,β3 expression levels in TamR cells (Dr. Steve Hiscox, personal communication). In view of these preliminary data, it would be interesting to analyse the protein expression levels of these integrins in TamR-30 cells.
4.7 Potential transcription factors regulating ERα expression in TamR cells

It is likely that the Src-mediated downregulation of ERα mRNA expression described in this study is exerted indirectly by the Src kinase activity through yet-to-be unveiled transcription factors that are regulated by upstream Src-dependent signalling pathways. In an attempt to identify such transcription factors and elucidate more thoroughly the Src-mediated regulation of ERα expression in TamR cells, Affymetrix studies are currently undergoing using RNA samples prepared from TamR-30 cells treated with the Src inhibitor AZD0530 for 3 days. Meanwhile, in a preliminary attempt to identify the mechanisms involved in the downregulation of ERα expression in TamR cells, the present study examined the protein expression and localization of the AP2γ and FoxO3a transcription factors which, as described in section 1.4.1, have been shown to exert a positive influence on ERα expression in vitro through their direct binding to the ERα promoter.

A correlation between nuclear FoxO3a localization and ERα status has been described in several breast cancer cell lines (Guo & Sonenshein, 2004). However, immunocytochemical analysis in this study failed to convincingly demonstrate that decreased levels of nuclear/active FoxO3a could be linked to the downregulation of ERα expression in TamR cells. Therefore, a relationship between FoxO3a and ERα expression may not exist in these cells. In support of this, Madureira et al. (2006) demonstrated a poor correlation between FoxO3a and ERα expression using a panel of breast cancer cell lines that was considerably larger (n = 16) than the one used in the study by Guo & Sonenshein (n = 6). However, Madureira et al. did observe a much better correlation between FoxM1 and ERα expression (13/16) compared with FoxO3a (6/16). FoxM1 is another member of the mammalian Forkhead Box family of transcription factor and is established as a key regulator of the G1/S phase transition and progression into mitosis (Costa, 2005). Using siRNA and ChIP analyses, Madureira & colleagues (2006) have also shown that FoxM1, and not FoxO3a, is the primary positive regulator of ERα expression through direct binding to the proximal promoter of the ERα gene. Interestingly, the same authors also demonstrated that U0126-inhibition of the Erk1/2-dependent activation of FoxM1 resulted in a significant downregulation of ERα expression in MCF-7 cells. This is in agreement with the U0126-induced loss of ERα
protein levels described earlier in TamR-30 cells. In light of Madureira et al.'s findings, it would be very interesting to investigate whether a relationship between FoxM1 and ERα expression exist in TamR cells.

Although a convincing correlation between AP2γ and ERα expression has yet to be reported in clinical breast cancer, several in vitro studies have identified AP2γ as a positive regulator of ERα gene expression by directly interacting with the ERα promoter in human mammary epithelial cells and breast cancer cell lines (McPherson et al., 1997; McPherson et al., 1999; Schuur et al., 2001). In the present study, statistical analysis of immunocytochemically detected nuclear AP2γ failed to demonstrate that a reduction in nuclear/active AP2γ expression could be one contributing cause for the downregulation of ERα expression in TamR-18 and TamR-24 cells. However, this possibility can not be entirely ruled out in the case of TamR-30 cells, which exhibited a significant reduction in AP2γ nuclear staining compared to MCF-7 and the other TamR cells. Furthermore, it is feasible to suggest that that reduced expression of the AP2α isoform could also be contributing to ERα downregulation in TamR-30 cells. The AP2α isoform has also been associated with the positive control of ERα promoter activity in vitro (McPherson et al., 1999; Schuur et al., 2001). Moreover, in contrast to AP2γ, previous studies have found a significant correlation between nuclear AP2α (and also the AP2β isoform) and ERα expression in clinical breast cancer materials (Gee et al., 1999; Friedrichs et al., 2005; Turner et al., 1998). However, recent siRNA studies in several models of ERα-positive breast cancer cell lines revealed that endogenous expression of ERα was specifically regulated by AP2γ but not AP2α (Woodfield et al., 2007). In the same study, AP2γ was also found to be the key regulator of oestrogen-responsiveness while AP2α was involved in the regulation of genes involved in cell cycle inhibition and apoptosis. This is in agreement with previous studies in which AP2γ mRNA expression was upregulated in response to oestrogen treatment of ERα-positive breast cancer cell lines, whilst AP2α mRNA levels were downregulated (Orso et al., 2004). Clearly, the regulatory loop existing between ERα and AP2 transcription factors is complex. Therefore, a more thorough analysis of the protein expression and localization of the three well-known AP2 isoforms (AP2α, AP2β and AP2γ) would be needed in order to better assess AP-2’s contribution to ERα expression in TamR cells.
Deregulation of the expression and/or activity of the AP-1 transcription factor could be involved in ERα loss in TamR cells. AP-1 consists of homodimers of the JUN family of proteins (JunB and JunD) or the more stable heterodimers between JUN and members of the FOS family (c-Fos, Fra-1, Fra-2, FosB), which can not homodimerize. The AP-1 members can have opposite functions with c-Fos and c-Jun promoting growth, whereas JunB is a negative regulator of cell proliferation (Shaulian & Karin, 2001). As described in Chapter 1 (section 1.4.1), Tang et al. (1997) demonstrated the existence of a dominant transcriptional enhancer containing a functional AP-1 site mapped within the distal promoter D of the ERα gene. The same authors have shown that the DNA-protein complexes specifically formed on this enhancer element could be supershifted by polyclonal antibodies against c-Fos and c-Jun, thus suggesting that these proteins may be involved in activation of ERα gene transcription. However, Smith et al. (1999) have demonstrated that overexpression of c-Jun in MCF-7 cells resulted in the downregulation of ERα gene expression. Furthermore, recent in vivo experiments using transgenic mice with overexpressing HER-2 breast tumours have shown that inhibition of AP-1 activity could prevent the development of ERα-negative breast cancers (Shen et al., 2008). In light of these data and given that the AP-1 members can have antagonizing functions on cell proliferation, qualitative or quantitative changes in the protein composition of the AP-1 complex may change the overall transcriptional activity of the transcription factor, and determine whether AP-1 act as an inhibitor or activator of gene transcription. It would therefore be necessary to assess the expression and activity of all the AP-1 members if a role of this transcription factor in the regulation of ERα expression was to be investigated in TamR cells.

As described in Chapter 1 (section 1.4.1), p53 has been shown to positively regulate ERα gene transcription in MCF-7 cells (Angeloni et al., 2004). Deregulation of p53 expression could therefore also be involved in ERα loss in TamR cells. Interestingly, previous clinical studies have demonstrated a significant correlation between mutation of p53 and ERα-negativity (Bems et al., 1996). This correlation has recently been further highlighted by compelling in vivo studies by D’Assoro et al. (2008) who demonstrated that mice, carrying xenograft with MCF-7 cells overexpressing a dominant negative p53 mutant, developed poorly differentiated tumours lacking not only ERα expression, but also PR and HER2, characteristic of the triple negative breast cancer subgroup. Very
interestingly, the same authors proposed that ERα downregulation could be mediated by an Akt-induced activation of Mdm2 (a key negative regulator of p53 function found to be overexpressed in the xenograft model), whose ubiquitin-ligase activity has been shown to promote ERα protein degradation by another study (Duong et al., 2007). Given that Akt activity was considerably increased in TamR-30 cells, the mechanism of ERα downregulation proposed by D'Assoro et al. could be very relevant for the present study.
4.8 Summary and conclusion

Data presented in this thesis indicate that considerable ERα loss can occur in response to prolonged anti-hormonal treatment of breast cancer cells as part of an adaptive mechanism, and that this can lead to a more aggressive phenotype. Encouragingly, however, even after 30 months exposure to tamoxifen, the process is reversible by inhibition of Src kinase activity. Should future evidence reveal that this re-expressed ERα remains functional and interacts with growth factor signalling pathways in TamR-30 cells, the data from this study provides more evidence in support of combining a Src inhibitor with anti-hormonal therapy in order to circumvent the emergence of aggressive cancer cell behaviour. In view of the ubiquitous expression of Src and its regulatory role in many critical cellular processes in normal tissues, one would expect that Src inhibition may cause high toxicity in many patients. Although it is too early to determine the long-term effects of Src inhibitors in breast cancer patients, previous phase I clinical trials revealed that Src inhibitors are well tolerated and with low side effect (Summy & Gallick, 2006). Furthermore, data herein may not only be clinically relevant for ERα-positive patients loosing the receptor during endocrine therapy but also for patients with intrinsic ERα-negative breast tumours for whom alternative treatments are still very much needed. However, as rightfully quoted by Johnston et al. (2008), translation of these pre-clinical studies (advocating STIs/endocrine combinations therapies) into successful clinical trials will depend on the systematic identification of the drug target, through the use of relevant biomarkers within an appropriately selected cohort of patients.
Chapter 5

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5. References


García Pedrero JM, Zuazua P, Martínez-Campa C, Lazo PS, Ramos S; 2003. The naturally occurring variant of estrogen receptor (ER) ERdeltaE7 suppresses estrogen-dependent transcriptional activation by both wild-type ERalpha and ERbeta. *Endocrinology*; 144(7):2967-76.


178


Graham, ML 2nd; Krett, NL; Miller, LA; Leslie, KK; Gordon, DF; Wood, WM, et al.; 1990. T47Dco cells, genetically unstable and containing oestrogen receptor mutations, are a model for the progression of breast cancers to hormone resistance. *Cancer Res.*; 50(19): 6208—6217.


Holloway JN, Murthy S, El-Ashry D; 2004. A cytoplasmic substrate of mitogen-activated protein kinase is responsible for estrogen receptor-alpha down-regulation in


Kurokawa, H; Lenferink, AE; Simpson, JF; Pisacane, PI; Sliwkowski, MX; Forbes, JT, et al.; 2000. Inhibition of HER2/neu (ErbB-2) and mitogen-activated protein kinases enhances tamoxifen action against HER2-overexpressing, tamoxifen-resistant breast cancer cells. *Cancer Res;* 60(20):5887–5894.


Pink, JJ; Wu, SQ; Wolf, DM; Bilimoria, MM; Jordan, VC.; 1996. A novel 80 kDa human estrogen receptor containing a duplication of exons 6 and 7. *Nucleic Acids Res*; 83:1477-1482


Chapter 5. References


[Accessed July 2008]


APPENDIX A.

Protocol Overview for Affymetrix U133A Genechip

Microarray analysis was performed on triplicate RNA samples, which were extracted from MCF7 and TamR cell lines by the TRI REAGENT® procedure, as described in Chapter 2 (section 2.2.1). Total RNA was DNAse-digested and RNA integrity and quality was assessed by agarose gel electrophoresis and spectrophotometry. RNA samples were sent to a specialist microarray facility in Cardiff University (Central Biotechnology Services) for further RNA quality control and subsequent Affymetrix GeneChip hybridization using the Affymetrix Human Genome U133A GeneChips™, which has approximately 23,000-gene coverage, including in-built quality control genes on RNA samples. Specific hybridization data were generated using Affymetrix MAS-5 software. Data were then normalized, log-transformed, and the quality of replicate data confirmed statistically (including MVA plots). To enable full analysis, the hybridization data for each sample was then uploaded into the commercial analysis software GeneSifter®.
APPENDIX B.

Log-intensity plots and heat map representing gene expression levels of transcription factors short-listed in Table 3.2. Gene expression analysis was carried out using GeneSifter® software (http://www.genesifter.net/web/). The heat map and probe number for each transcription factor is shown with $P$ values (student $t$-test) and Affymetrix calls (P, present; A, absent, M, marginal).

38. P53: -211300_s_at

43. P53: -210746_at

32. FOXO3A: -204132_s_at

$T_{\text{test}}$: $p < 0.1$

Affy Call: P P
39. C-jun: 213281_at

T-test: p<0.1
Affy Call: P P

47. C-jun: 201464_x_at

T-test: *p<0.05
Affy Call: P M

52. C-jun: 201466_s_at

T-test: p<0.1
Affy Call: M A

54. C-jun: 201465_s_at

T-test: *p<0.05
Affy Call: A A
1. C/EPBβ:- 212501_at

T-test: *p<0.05

3. C/EBPα:- 204039_at

T-test: p<0.1
Affy Call := P

23. P300/CBP:- 211808_s_at

T-test: p<0.1
Affy Call := A A

46. P300/CBP:- 202160_at

T-test: *p<0.05
Affy Call := P P