Mechanisms of Hormone-Independence in Human Breast Cancer Cells

by

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A thesis submitted to the Faculty of Graduate Studies in Partial Fulfillment
of the Requirement for the Degree of Doctorate of Philosophy

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Abstract

Breast cancer is a hormonally responsive cancer and hormones, including estrogen, are required for breast cancer growth. The evolution of breast cancer into an estrogen-independent growth phenotype is thought to be an important step in the progression of breast cancer to hormone-independence and endocrine therapy resistance. Understanding the factors that contribute to the development of an estrogen-nonresponsive growth phenotype is of major importance in terms of breast cancer therapeutics. Resistance to endocrine therapies may be due to a number of factors, including loss of estrogen receptor-alpha (ER-α) expression, but most tumours that have developed resistance to endocrine therapy remain ER-α positive. The mechanisms responsible for the development of estrogen-independence in the presence of continued expression of ER-α are poorly understood. In order to address this, a breast cancer cell model of apparent estrogen-independence was developed. An estrogen-nonresponsive cell line, T5-PRF, was developed from T5 (ER-α positive and estrogen-responsive) human breast cancer cells by chronically depleting the cells of estrogen in long-term culture. The T5-PRF cells are insensitive to the growth-stimulatory effects of estrogen while still retaining expression of the ER-α.

The tissue matrix consists of linkages and interactions of the nuclear matrix (NM), cytoskeleton and extracellular matrix. This system is a dynamic structural and functional component of the cell that maintains and coordinates cell function and gene expression. The ER-α is localized to the NM and studies suggest that alterations in NM proteins may influence gene expression. Three proteins (identified as cytokeratins 8, 18 and 19) present in the NM-intermediate filament (NM-IF) fraction were found to be regulated by estrogen in T5

human breast cancer cells. However, T5-PRF, estrogen-nonresponsive cells, overexpressed these three proteins compared to T5 cells, and these proteins were no longer regulated by estrogen in T5-PRF cells. Treating T5 cells with antiestrogens resulted in a significant reduction in these proteins, while no effect was seen in T5-PRF cells, supporting the conclusion that these three NM-IF proteins are regulated by estrogen in T5 human breast cancer cells and may play a role in estrogen action in human breast cancer cells.

T5-PRF cells were also found to have significantly increased ligand-independent ER-α transcriptional activity. In addition, an ER-α mRNA variant with an inframe deletion of exons 3 and 4 was detected in T5-PRF, but not T5, human breast cancer cells. Recombinant expression of this ER-α variant in T5 human breast cancer cells increased estrogen-dependent and -independent reporter gene expression, suggesting that the presence of this ER-α mRNA variant may contribute to an estrogen-independent growth phenotype. Furthermore, T5-PRF human breast cancer cells contained elevated mitogen-activated protein kinase (MAPK) activity. The MAPK signal transduction pathway can be activated by estrogen in human breast cancer cells and may be involved in the regulation of ER-α transcriptional activity through both ligand-dependent and -independent pathways, suggesting that increased activity of MAPK may contribute to the ligand-independent activity of the ER-α in T5-PRF cells.

In conclusion, T5-PRF human breast cancer cells contain several changes compared to parental T5 cells, which may all contribute to an estrogen-nonresponsive growth phenotype and affect the transcriptional activity/regulation of the ER-α.

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List of Abbreviations

Acronyms/Abbreviations:

16α-OH	16alpha-Hydroxyestrone
AF	Activation function
AIB1	Amplified in breast cancer 1
ANOVA	Analysis of variance
AP-1	Activator protein-1
AT, HAT	Acetyltransferase, histone AT
ATP	Adenosine triphosphate
bp	Base pair
BRCA1/2	Breast cancer 1/2
C	Carboxyl
cAMP	Cyclic adenosine monophosphate
CAT	Chloramphenicol acetyltransferase
CBP	CREB binding protein (CBP/p300)
CHO	Chinese hamster ovary
CK	Cytokeratin
CM	Dulbecco's modified essential medium with 5% FCS
CS	Phenol red free dulbecco's modified essential medium with 5% charcoal stripped fetal calf serum
CT	Cholera toxin
CTP	Cytosine triphosphate
DBD	DNA binding domain
DMEM	Dulbecco's minimal essential medium
DNA, cDNA	Deoxyribonucleic acid, complementary DNA
DT	Doubling time
E2, estradiol	17β-Estradiol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF, EGFR	Epidermal growth factor, EGF receptor
ER, ER-α, ER-β, vER	Estrogen receptor, ER-alpha, ER-beta, variant ER
ERE	Estrogen-responsive element
ERAP140	Estrogen receptor-associated protein 140
ERF-1	Estrogen receptor factor 1
ERK	Extracellular signal-related kinase
FBS	Fetal bovine serum
FCS, CS-FCS	Fetal calf serum, charcoal stripped-FCS
GF	Growth factor
GR, GRIP	Glucocorticoid receptor, GR-interacting protein
Grb2	Growth factor receptor-bound protein 2
GTP	Guanosine 5'-triphosphate

h	Human
HBD	Hormone binding domain
HDAC	Histone deacetylase
HMG1	High mobility group protein 1
hnRNP	Heterogenous nuclear ribonucleoprotein
hs	Horse serum
Hsp	Heat-shock protein
Hydroxytamoxifen, TOT	4-*trans*-monohydroxytamoxifen
ICI	Imperial Chemical Industries, ICI 164,384
IEF	Isoelectric focusing
IF	Intermediate filament
IGF, IGF-IR	Insulin-like growth factor, IGF-I receptor
LBD	Ligand binding domain
m	Mouse
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated ERK-activating kinase
M_r	Relative molecular mass
N	Amino
N-CoR	Nuclear receptor corepressor
NM	Nuclear matrix
NM-IF	Nuclear matrix-intermediate filament
NR	Nuclear receptor interaction domain (LXXLL, L= lysine, X= any amino acid)
P	Phosphate
PD	PD 98059
p/CIP	p300/CBP interacting protein
PCAF	p300/CBP associated factor
PCR	Polymerase chain reaction
PKA	Protein kinase A
PKC	Protein kinase C
PL	Pore-lamina
PPAR-γ	Peroxisome proliferator-activated receptor-gamma
PR	Progesterone receptor
PRF	Phenol red free
RAP46	Receptor associated protein 46
RAR-α	Retinoic-acid receptor-alpha
RFLP	Restriction fragment length polymorphism
RIP140	Receptor-interacting protein 140
RNA, mRNA, pre-mRNA	Ribonucleic acid, messenger RNA, precursor-mRNA
RT-PCR	Reverse transcription-polymerase chain reaction
RXR	Retinoid-X-receptor
sem	Standard error of the mean
SH2	Src-homology 2

SMRT	Silencing mediator of retinoid and thyroid receptors
SOS	Son of sevenless
SRC-1	Steroid receptor coactivator-1
snRNP	Small nuclear ribonucleoprotein particle
TBP, TAF	TATA-box binding protein, TBP-associated factor
TFIIB, TFIID, TFIIE, TFIIF	Transcription factor II B/D/E/F
TGF, TGFα, TGFβ	Transforming growth factor, TGF alpha, TGF beta
TIF	Transcription intermediary family
tk	Thymidine kinase
TLC	Thin-layer chromatography
TnT	*In vitro* transcription/translation
TR	Thyroid hormone receptor
WT	Wild-type

Amino acids:

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Glu	Glutamic acid
Gly	Glycine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Pro	Proline
Ser	Serine
Thr	Threonine
Tyr	Tyrosine
Val	Valine

Chemicals:

AEBSF	4-[2-Aminoethyl]benzenesulfonyl flouride hydrochloride
BES	N,N,bis (2-Hydroxyethyl)-2-aminoethanesulfonic acid
BSA	Bovine serum albumin
CAPS	3-[Cyclohexylamino]-1]propanesulfonic acid
CHAPS	3-[(3-Cholamindopropyl)dimethyl-ammonio]-1-propanesulfonate
DMBA	7,12-Dimethyl-benz[a]anthracene
DNAse I	Deoxyribonuclease I
EDTA	Disodium ethylenediamine tetraacetate disodium salt
EGTA	[Ethylenebis (oxyethylenenitrilo)]tetraacetic acid

HCl	Hydrochloric acid
Hepes	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)
IBMX	Isobutylmethylxanthine
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate monobasic
MBP	Myelin basic protein
$MgCl_2$	Magnesium chloride
MMLV-RT	Moloney murine leukemia virus-reverse transcriptase
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
Na_2HPO_4	Sodium phosphate dibasic
NaH_2PO_4	Sodium phosphate monobasic
NaOH	Sodium hydroxide
NMU	N-nitrosomethylurea
ONPG	o-Nitrophenyl β-D-galacto-pyranoside
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonyl fluoride
PNK	Polynucleotide kinase
PD 98059	2'-Amino-3'-methoxyflavone
R5020	Promegestone
RNAsin	Ribonuclease inhibitor
SDS, SDS-PAGE	Sodium dodecylsulfate, SDS-polyacrylamide gel electrophoresis
TPA	12-*O*-Tetradecanoylphorbol-13-acetate
Tris	Tris (hydroxymethyl) aminomethane
Triton X-100	Octyl phenoxy polyethoxythethanol
Tween 20	Polyoxyethylene-sorbitan monolaurate
$ZnCl_2$	Zinc chloride

Nucleotides:

G	Guanine
A	Adenine
T	Thymine
C	Cytosine

Units:

cm^2	Centimeters squared
fmol	Femtomoles
g, mg, μg, ng	Grams, milligrams, micrograms, nanogram
h	Hour
kb	Kilobases

kDa	Kilodaltons
L, mL, μL	Litre, millilitre, microlitre
°C	Degrees Celsius
mm	Millimeter
M, mM, μM, nM	Moles/litre (Molar), millimolar, micromolar, nanomolar
pmol	Picomole
rpm	Revolutions per minute
U	Units
V	Volts
vol	Volume
wt	Weight

Introduction

Breast Cancer

Significance

Breast cancer, the most common cancer among women, is the second leading cause of cancer death among all women and the leading cause of death among women aged 40-55 (1,2). A woman's overall lifetime chance of developing breast cancer is one in eight (1). Breast cancer incidence continues to rise and this year alone in Canada approximately 19,312 new cases of breast cancer will be diagnosed and there will be approximately 5,267 deaths due to breast cancer (3) (Figure 1). This is a worldwide health problem. For example, in 1996, 910,000 new breast cancer cases were diagnosed and 376,000 women died from breast cancer worldwide (4).

Risk Factors

While often controversial, there are factors associated with an increased risk of developing breast cancer and probably the most accepted risk factor is gender, with male breast cancers accounting for less than 1% of the overall breast cancer incidence (5). A woman's age is an important risk factor, with 78% of breast cancers occurring in women over 50 years of age (1). Hormone exposure, especially estrogen exposure, is an established risk factor and early menarche and late menopause are associated with an increased risk, while surgical menopause (bilateral oophorectomy) is associated with a decreased risk of breast cancer incidence (6,7). While still controversial, the use of oral contraceptives has been associated with an increased risk of breast cancer. A recent analysis of studies on hormonal contraceptive use and breast cancer incidence demonstrated that for current users there was

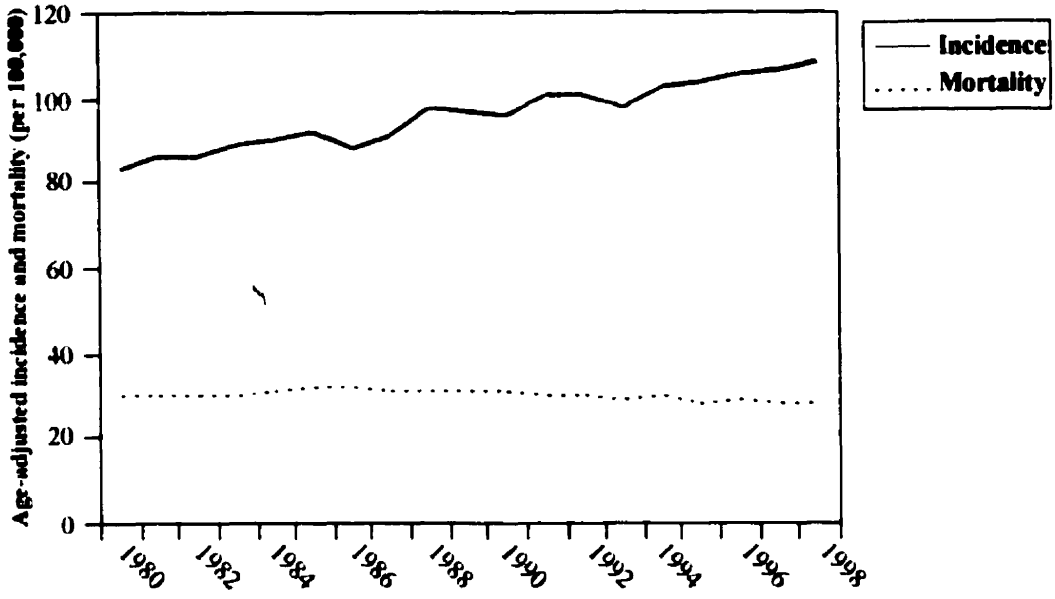


Figure 1. Breast cancer incidence and mortality rates. Rates are expressed as per 100,000 Canadian women and are adjusted for age. Data for 1994-1998 are estimated. Source: National Cancer Institute, 1998.

a small increase in risk (relative risk 1.24) but breast cancer risk decreases during the 10 years after discontinuing contraceptive use until the risk becomes the same as those women that have never used oral contraceptives (8). The use of hormone replacement therapy by postmenopausal women increases a woman's risk of breast cancer 2-3% per year of use (9). Adipose tissue is the major source of estrogen in the postmenopausal woman and increased weight gain during postmenopausal years has been linked to increasing risk of breast cancer incidence (10). Conversely, premenopausal women who are overweight have a slightly lower risk of breast cancer incidence, likely due to the fact that premenopausal obesity is associated with amenorrhea, which could lower total estrogen exposure (10,11). Nulliparity and late first pregnancy are associated with increased risk, while an early first pregnancy is associated with a reduced risk of breast cancer development, thought to be due to the degree of differentiation of the breast tissue (9,12). The level of exposure to steroid hormones during the prenatal period may also influence the risk of breast cancer (13).

Family history plays a significant role in breast cancer risk and a woman whose mother or sister developed breast cancer at an early age is most at risk. While 95% of human breast cancers are sporadic, 5% are hereditary and inherited forms of breast cancer tend to develop at an earlier age (30s and 40s) (14,15). Mutations in specific genes have been identified which are believed to be involved in genetic susceptibility to breast cancer. The first breast cancer susceptibility gene discovered was BRCA1 (breast cancer 1) located on chromosome 17q21 (16). Approximately one in 200 women will inherit a mutated BRCA1 gene and these women will have an 80-90% chance of developing breast cancer as well as an increased risk of developing ovarian cancer (17). Although the function of BRCA1 is not clear, there is

evidence to suggest that it may be involved in the negative regulation of mammary epithelial cell growth (18,19). No mutations in BRCA1 have been found in sporadic breast cancers, despite an intensive search, leading to questions regarding its role in the majority of breast cancers (20). It may be that mutations occur in non-coding regions that were not examined or that mutations in some other regulatory protein that affects the level and/or function of BRCA1 may be more important in these types of breast cancers. A second breast cancer susceptibility gene, BRCA2, has been identified and is localized to chromosome 13q12-13 (21). Again, the function of BRCA2 in breast cancer development is unclear, but studies suggest that it may be a protein involved in deoxyribonucleic acid (DNA) repair (22,23). While it is not yet known what the normal function of either of the proteins, corresponding to the BRCA1 or BRCA2 genes, is or how mutations in these genes lead to the development of breast cancer, researchers have estimated that together BRCA1 and BRCA2 may account for 40-50% of all hereditary breast cancers (24).

Women who have had a prior cancer in one breast have a 2-3 fold greater risk of developing a new breast cancer in the other breast (25,26). Exposure to ionizing radiation has also been associated with increased risk of breast cancer development, especially exposure in younger women (27).

Lifestyle factors such as diet, alcohol consumption and exercise, are thought to influence breast cancer risk. The incidence of breast cancer is highest among women in Canada, the United States and Northern Europe and much lower in Japan and Hong Kong. When women from low risk populations migrate to higher risk areas, within two or three generations the breast cancer incidence rates increase, suggesting the importance of lifestyle

and/or environmental factors in breast cancer risk (28). The relationship between dietary fat and breast cancer risk has been examined, with several studies suggesting no relationship (29,30), while others suggest the type of fat intake may influence breast cancer risk (31,32). Alcohol consumption may also be a risk factor, but again, the relationship is not clear and some studies assessing alcohol intake and breast cancer risk suggest no association (33) while others suggest there is an increased risk associated with alcohol consumption (34,35).

Although there have been many factors associated with an increased risk for breast cancer, and hormonal and hereditary factors play a clear role, approximately 75% of women with breast cancer have no measurable risk factors (36), demonstrating our lack of understanding regarding the factors involved in the etiology of breast cancer.

Treatment

Breast cancer is viewed as a systemic disease and the majority of treatments involve a whole body approach. Surgical procedures to attempt to remove the tumour mass are the first line in managing this disease. Adjuvant treatment is frequently given to patients after the initial surgery to remove the tumour mass. The most commonly used adjuvant therapies are chemotherapy, irradiation and hormone therapy. Steroid hormone receptor levels are determined to aid in treatment choice and the level of estrogen receptor (ER) and/or progesterone receptor (PR) is also correlated with prognosis, in that tumours with higher receptor levels generally have a more favourable prognosis (37). While endocrine therapies such as aromatase inhibitors (38,39), gonadotropin releasing hormone analogues (40), progestins (41) and antiprogestins (42,43) can be successfully used to treat breast cancer, for the purposes of this thesis I will discuss only the antiestrogens in terms of endocrine therapies

of breast cancer.

At the time of clinical detection approximately 70% of primary breast cancers contain ER levels equal to or above 10 femtomoles (fmol)/milligram (mg) protein and are thus considered ER positive (37). Antiestrogen therapy for human breast cancer targets the ER and as such generally requires that the breast cancer cells depend on estrogen for proliferation. Tamoxifen (Nolvadex), an antiestrogen, is presently the most popular choice of hormonal therapy for ER positive patients. Tamoxifen acts by binding to the ER and in certain tissues such as the breast, results in blocking the growth stimulatory effects of estrogen (44). Tamoxifen is a nonsteroidal partial antiestrogen in that in certain tissues, such as the uterus (45), bone (46) and the cardiovascular system (47), it can act as an estrogen mimic. The effects of tamoxifen are cytostatic and therefore use must be prolonged in order to effectively suppress tumour growth (48). Tamoxifen has been used extensively to treat both advanced breast cancer and as treatment in early-stage disease and is successful in treating both pre- and post-menopausal women (49-51). Tamoxifen is used as adjuvant treatment following surgery and also as a palliative treatment in women with advanced breast cancer (44). Tamoxifen is successful in increasing overall and disease-free survival especially in patients over 50 (50,51) and the use of tamoxifen also appears to prevent the development of second breast cancers (50,52). Tamoxifen is generally well tolerated with most side-effects limited to its antiestrogenic qualities (50,51).

More recent research has been done to study the potential benefit of using tamoxifen as a prophylactic measure in those women who do not presently have clinically detectable breast cancer, but are at high risk for its development. The fact that tamoxifen use for the

treatment of primary breast cancer resulted in a decreased incidence of contralateral breast cancer spawned the idea that tamoxifen may be a potential breast cancer preventative agent (52). The Breast Cancer Prevention Trial recently released the exciting data that tamoxifen treatment for 4 years caused a 45% reduction in tumour incidence in women who were at increased risk for breast cancer development, compared to women who were taking placebo (53). While tamoxifen offered a significant protective role in terms of breast cancer development, the fact that it is an estrogen agonist in the uterus, and is associated with an increased risk of endometrial cancer (53), is of concern. Raloxifene is another partial antiestrogen that maintains some of the advantageous estrogenic properties of tamoxifen on the bone and cardiovascular system, but has the added advantage that it has no known estrogenic effects in the uterus or breast. Currently a trial is underway to evaluate the use of raloxifene as a breast cancer chemopreventative agent (54).

At the time of clinical detection approximately 70% of human breast tumours are classified as ER positive and of these approximately 60% will respond to endocrine therapy (37,55). Therefore, approximately 40% of ER positive primary tumours are resistant to endocrine therapy and even of those tumours that initially respond, the majority will ultimately become resistant. Hormone-independence is a sign of a more aggressive phase of the disease and is obviously a problem in terms of efficacy of antiestrogen treatment (56,57). Patients who develop tumours resistant to tamoxifen therapy will often respond to a second line of endocrine treatment (58). Other "pure" antiestrogens have been developed that apparently do not exhibit ER agonist behaviour (59,60). These pure steroidal antiestrogens, such as Imperial Chemical Industries (ICI) 164,384 and ICI 182,780, can inhibit the growth of breast

cancer cells *in vitro* and *in vivo* (59,60). Clinical evidence supports the use of these steroidal antiestrogens and patients have shown a response to ICI 182,780 after becoming tamoxifen resistant (61,62).

Steroid hormone receptors

Steroid hormones modulate a multitude of diverse biological activities including, development and growth, cell differentiation and metabolism, as well as pathological conditions. The ability of cells and tissues to respond to steroid hormones is due to the presence of the corresponding hormone receptor. The nuclear receptor superfamily of ligand-dependent transcription factors includes not only the receptors for the steroid hormones, but receptors for a range of other hormones, vitamins, and orphan receptors whose ligands have not yet been identified (63). This superfamily is characterized by conservation in both structure and function among the various receptors (63). The biological effect of ligand binding to its cognate receptor is governed by multiple points along a pathway to ultimately culminate in changes in gene expression. A complex interplay amongst all the factors must occur, in a cell- and tissue-specific manner, in order to result in regulated expression of hormone-responsive genes and much research has been aimed at understanding the multiple events involved in the control of steroid receptor function.

Structure of the estrogen receptor

The ER is a member of the steroid/thyroid hormone receptor superfamily (63). The ER family is comprised of the classical ER, hereafter referred to as ER-α, and the newly discovered ER-β (64,65). For the purpose of this thesis I will discuss only those features that are pertinent in terms of ER-α structure and function.

The full length complementary (c) DNA for ER-α was first cloned and sequenced from MCF-7 human breast cancer cells (66-68). The gene was found to span over 140 kilobases (kb) of DNA and contain 8 exons (Figure 2) and 7 introns encoding a protein of approximately 66 kilodaltons (kDa) (67,69). The 7 introns of the human (h) ER-α gene are extremely large and range in size from approximately 3.5 to greater than 32 kb (69). The original cDNA clone (HEO) for hER-α contained a point mutation in the hormone binding domain (glycine at position 400 substituted with valine) (69) producing a protein that has a decreased affinity for estradiol at 25°C, but not at 4°C (70).

The hER-α messenger ribonucleic acid (mRNA) is approximately 6.2 kb with a 232 nucleotide long 5'-untranslated region and a 4,305 nucleotide long 3'-untranslated region (67,68). Three different promoters have been identified in the hER-α gene that are responsible for generating mRNA transcripts that differ in the length and sequence of the 5'-untranslated region. The promoter P1 is located upstream of the mRNA cap site (+1) and generates an mRNA with a 5'-untranslated region of 232 bases (67), P0 is located several kb further upstream of P1 (approximately -1900) and results in a 5'-untranslated region of 178 bases (71), while a third, restricted in use to the liver, is found approximately 12 kb upstream of the mRNA cap site (72). In human breast cancer cells, the P1 promoter is predominantly used with approximately 10-30% of transcripts originating from the P0 promoter (73,74). In normal human mammary epithelium the P1 promoter appeared to be exclusively used, leading to the suggestion that in human breast cancer cells, where apparent overexpression of ER-α occurs, both promoters, P0 and P1, are used, whereas in normal breast cells only P1 would

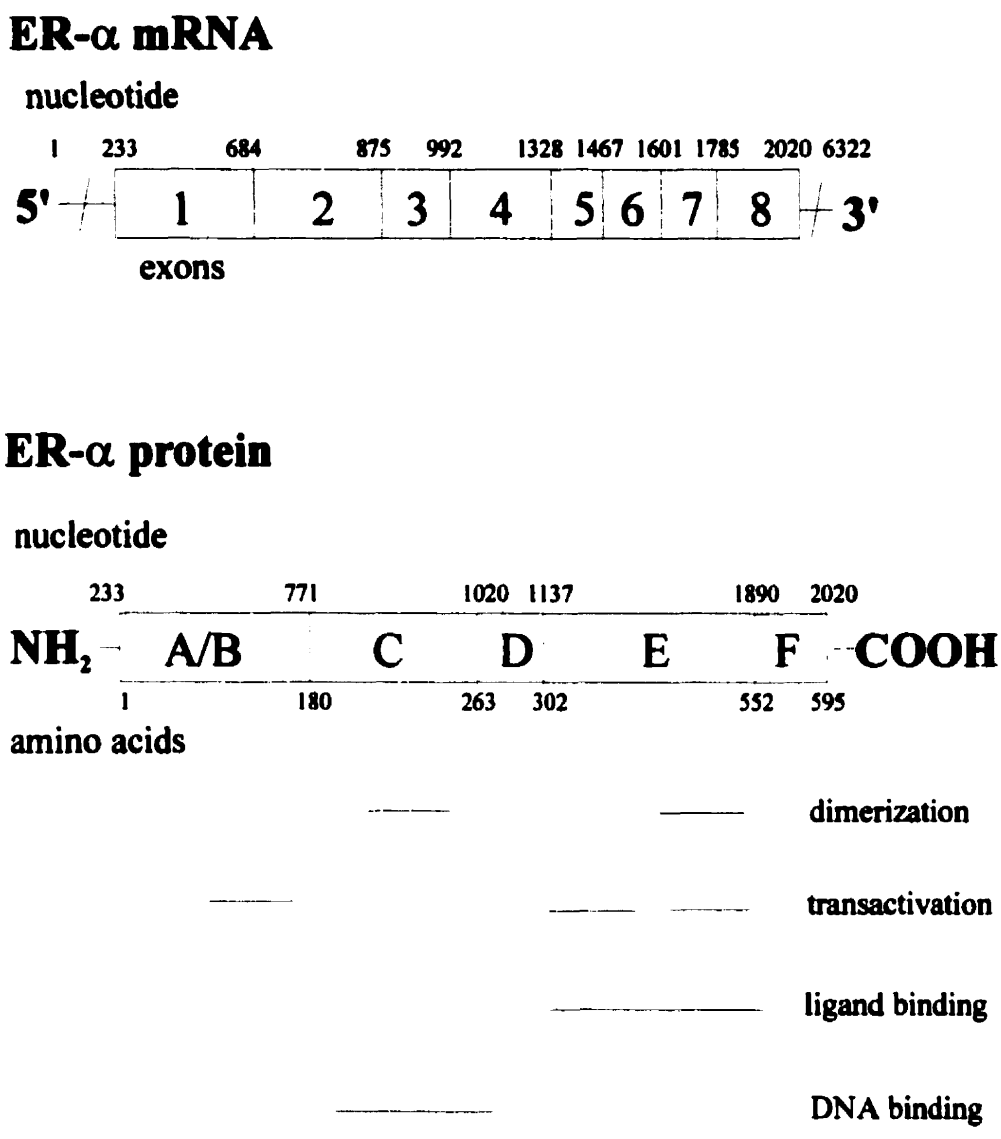


Figure 2. ER-α mRNA structure and protein functional domains. Functions corresponding to specific domains are listed below the protein structure. Nucleotide and amino acid numbering is according to (67).

be functional (73). However, other researchers found both promoters were used in human breast cancer cell lines as well as in normal breast tissue (74,75).

Many studies have revealed distinct functional units of the ER-α encoded by specific exons of the gene (Figure 2). The amino (N)-terminal domain is referred to as the A/B domain and associated with this region is a constitutive, hormone-independent transcriptional activation function (AF-1) (76). The next domain, C, contains the DNA binding domain (DBD) (77) which consists of two zinc-binding motifs that are responsible for binding to, and recognition of, hormone-responsive elements (HREs) in the promoter regions of responsive genes (78,79). Using two-dimensional nuclear magnetic resonance techniques, Schwabe and coworkers have described the solution structure of the hER-α DBD (78). The two zinc-binding motifs of the DBD fold to form a single structural unit consisting of two perpendicular helices. At the N-terminus of each helix is a loop with a zinc ion held at the base by four cysteine residues (78). This group has examined the crystal structure of the hER-α DBD bound to DNA to demonstrate that residues found in the first zinc finger interact with bases in the DNA, while residues in both zinc fingers make contacts to phosphates in the DNA backbone (79). Domain C also contains a dimerization domain and residues in the second zinc-binding motif are involved in dimerization (79,80). Domain D is the hinge region (77) which contains regions thought to be involved in nuclear localization (81). The hormone binding domain (HBD) is located in the E region (77), which also contains a strong region involved in receptor dimerization (80) and a second, ligand-dependent, transcriptional activation function (AF-2) (76). More recently a third putative transactivation domain (AF-2a) has been identified that encompasses amino acids 282-351 of the hER-α (82). A

hormone-inducible nuclear localization function has also been reported for the HBD (81) and it also contains regions involved in binding to heat shock protein (hsp)90 (83). The carboxyl (C)-terminal, domain F, has no clear function and while it is not absolutely required for transcriptional activity (77), deletion of this domain (amino acids 555-595) in hER-α affects the transcriptional response to both estrogen and the antiestrogens hydroxytamoxifen and ICI 164,384 in some cell types (84).

ER-α mRNA and protein regulation

The level of any particular protein is under complex control and is a result of transcription of the corresponding gene, level of cellular mRNA, translation of the mRNA into protein and stability of the translated protein. Levels of cellular mRNA are controlled by both its rates of synthesis and degradation and regulation of mRNA stability is an important aspect in the control of gene expression (85).

Regulation of ER-α levels has been shown to occur at both transcriptional and posttranscriptional levels. In MCF-7 cells estrogen treatment has been shown to result in a down-regulation of ER-α protein and mRNA expression (86-88). After estrogen treatment, transcription from the hER-α gene exhibits a marked transient decrease, to an almost undetectable level after 2 hours of treatment, followed by an increased level of transcription, despite the fact that mRNA levels remain low (86,88). This suggests that the mechanisms of ER-α downregulation may be due to both decreased transcription and posttranscriptional mechanisms.

Studies suggest that the ER-α may have a direct role in the regulation of its levels. Researchers have shown that hydroxytamoxifen has no effect on hER-α mRNA levels alone,

but is able to reverse estrogen-induced down-regulation (87,88). Studies have also demonstrated that the effect of estradiol on hER-α levels is likely not dependent on new protein synthesis, as the protein synthesis inhibitor cyclohexamide did not prevent estrogen-induced suppression of ER-α levels (88). Santagati *et al* demonstrated that oligonucleotides corresponding to the 5' end of the hER-α mRNA were able to interfere with estrogen-induced hER-α protein downregulation in MCF-7 cells and the hER-α was able to specifically bind to these oligonucleotides *in vitro* (89). These results suggest a direct role of the receptor in regulating its levels, perhaps via autologous downregulation of the ER-α gene by transcriptional repression (89). Conversely, in ER-α negative HeLa (human cervical carcinoma) cells, transiently expressed hER-α did not display any negative transcriptional regulation of a reporter gene containing an oligonucleotide effective in suppressing ER-α levels, suggesting other mechanisms of regulation may be involved (89). More recent work has demonstrated that in MCF-7 cells estrogen decreases the ER-α mRNA half-life (90). This effect is dependent on ongoing translation and may be mediated by an estrogen-regulated nuclease activity associated with ribosomes (90).

Researchers have also examined the role of other signal transduction pathways on ER-α levels and found that 12-*O*-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C (PKC), or the calcium ionophore A23187, resulted in a decrease in hER-α protein and mRNA levels in MCF-7 cells (91,92). TPA was found to decrease hER-α mRNA half-life, independent of new protein synthesis, but dependent on ongoing RNA synthesis, suggesting the requirement for a catalytic RNA species or an RNA-protein complex (92).

These data suggest that both TPA and estradiol may act through similar mechanisms to result in ER-α down-regulation.

Researchers have demonstrated that the steroidal antiestrogens ICI 164,384 and ICI 182,780 decrease ER-α levels, likely due to decreased stability of the ER-α protein (93,94).

More recent research has demonstrated that estrogen regulation of hER-α protein levels may occur through a proteasome-dependent degradation pathway (94a). Additionally, estrogen treatment does not always result in down-regulation of ER-α levels. For example, in T47D human breast cancer cells that express low levels of ER-α, estrogen treatment has been shown to result in an increase in ER-α mRNA levels (87).

RNA splicing

Splicing of precursor (pre)-mRNA is a nuclear process in which the non-coding introns are removed from the pre-mRNA and the remaining coding exonic sequences are joined (95). Splicing is catalyzed by the spliceosome, a large complex composed of small nuclear ribonucleoprotein particles (snRNPs) and non-snRNP proteins (including serine-arginine-rich (SR) proteins) which assemble onto the pre-mRNA template in an ordered fashion (95,96). What controls and organizes splicing in the nucleus is poorly understood. It has been shown that within the nucleus splicing factors localize to discrete-domains (speckles) with a nucleus typically containing 20-40 of these speckles (95). It appears that only a small amount of available splicing factors are found associated with active sites of transcription suggesting that splicing factors may be actively recruited to sites of transcription (97).

Alternative splicing, whereby one or more exons may be included and/or removed from the final mRNA transcript, occurs in numerous genes and can be regulated in a tissue-

specific manner, but the mechanisms that regulate it are unknown (98). The level of splicing factors can vary in a tissue-specific manner (99) and Caceres and coworkers have demonstrated that when the levels of the antagonistic splicing factors SF2/ASF (a member of the SR protein family) and heterogeneous nuclear ribonucleoprotein (hnRNP) A1 were varied, the alternative splicing pattern of several reporter genes was altered, demonstrating that the intracellular levels of splicing factors can influence splicing patterns (100).

SR proteins are believed to play a role in splice site selection and in spliceosome assembly. A region of basic amino acids within the C-terminal serine/arginine rich domain (RS-domain) of the SR proteins is necessary for targeting SR proteins to nuclear speckles (101). RS-domains are phosphorylated and phosphorylation of the SR proteins has been shown to alter their subnuclear-localization (95). Phosphorylation and dephosphorylation of SR proteins as well as snRNP proteins is important functionally and is required for formation of a functional spliceosome and completion of the splicing reaction (102). Using minigene constructs containing CD44 pre-mRNA, Konig *et al* demonstrated that exonic RNA elements can couple signal transduction to alternative splicing. These researchers demonstrated cell-specific activation of alternative splicing and that activation of Ras or PKC signalling pathways could also activate alternative splicing of the minigene constructs (103). These researchers hypothesize that this coupling of signalling pathways to alternative splicing may be how tissue-specific splice patterns occur and more importantly how splice patterns may be switched during physiological and pathophysiological conditions. The kinases and phosphatases that are important *in vivo* for splicing and factors involved in its regulation are unknown. Alternative splicing of the hER-α gene has been demonstrated in many laboratories,

including Dr. L.C. Murphy's, and will be discussed in detail in a later section.

Factors influencing ER-α activity

The biological response to receptor activation is ultimately a culmination of multiple effector systems. The basal state of ER-α activity is a state in which no defined activation systems, such as ligand, are initiated or present. Ligand-activated transcription will depend on multiple parameters, including; nature of ligand (agonist versus antagonist), cell-, promoter- and tissue-type, as well as the balance of proteins present that can act to influence receptor activity. In addition, ligand-independent activation of the receptor can occur through non-classical effector pathways. The following section will discuss some of the mechanisms involved in determining the activity of the ER-α. While an attempt has been made to classify these, it should be noted that each can influence overall activity and can overlap to influence each other.

Ligand

Estrogen

The natural ligand for the ER-α is estrogen and 17β-estradiol (estradiol) is considered the major estrogen in the female (104). Estradiol binds tightly to the ER-α with an equilibrium binding constant (Kd) in the range of 10^{-9}-10^{-10} M (moles/litre) (105). As described previously, studies have localized the ligand-binding portion of the ER-α to the C-terminus of the receptor. The classic model of steroid function was that ligand binding activated the receptor, resulting in translocation to the nucleus, DNA binding and gene activation (106). More current research has demonstrated that ER-α (including human) resides in the nucleus even in the absence of hormone (107,108). Experiments in yeast have suggested that

estradiol binding to the hER-α may also not be a prerequisite for *in vivo* DNA binding at higher receptor concentrations (109), but in mammalian cells, does result in tight association of the receptor complex with DNA and nuclear structures, including the nuclear matrix (110,111). Binding of hormone to the receptor has been shown to alter the conformation of the receptor and this conformational change is required for transcriptional activation of the ER-α (112,113). Researchers suggest that the role of ligand may be to overcome the effects of inhibitory factors complexed to the ER-α, such as heat shock proteins, and promote interactions of the receptor with coactivators and the general transcription machinery (113,114). These interactions and their potential role in ER-α activity will be discussed in greater detail in a later section.

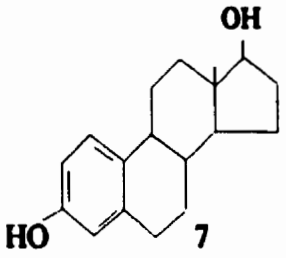
Recently, the 3-dimensional structure of the estrogen/hER-α/LBD (amino acids serine 301-threonine 553) complex has been solved (115). The LBD is folded into a three-layered structure, comprised mainly of antiparallel α-helices (helices H3-H12). Estrogen was shown to bind in a hydrophobic cavity formed by several secondary structures (including parts of H3 (methionine 342-leucine 354), H6 (tryptophan 383-arginine 394), H8 (valine 418-leucine 428), H11 (methionine 517-methionine 528) and H12 (leucine 539-histidine 547), and a small two-stranded antiparallel β-sheet (leucine 402-leucine 410)). The binding of hormone occurs through specific hydrogen bonds and complementarity of the binding cavity to the non-polar estrogen. Estrogen binds diagonally across the cavity between helices 11, 3 and 6. Hydrogen bonds formed between estrogen and hER-α/LBD occur at glutamine 353 (within H3), arginine 394 (within H6) and histidine 524 (within H11) and non-polar contacts occur with isoleucine 424 (within H8), glycine 521 (within H11) and leucine 525 (within H11). Helix 12

fits over this LBD cavity against H3, H5/6 and H11, forming a lid over the cavity, exposing the AF-2 function of the ER-α and residues important in its function. The structure of the antiestrogen, raloxifene, complexed with the hER-α/LBD was also solved. Raloxifene binds to the same sites as estrogen but the bulky-side chain of the antiestrogen (Figure 3) displaces H12 causing it to protrude out of the LBD pocket. Additionally, the bulky side chain common to other steroidal and non-steroidal antiestrogens (Figure 3) would be expected to result in a similar displacement of H12 (115).This is significant, since H12 is essential for AF-2 function and coactivator interactions as will be discussed in a proceeding section. These researchers also noted that the lysine at position 362 (the analogous residue of mouse (m)ER-α has been shown to be required for estrogen-dependent transactivation and coactivator recruitment (116)) is buried by the raloxifene induced re-orientation of H12 (115). This study provides a structural basis for the antagonistic actions of antiestrogens and confirms previous work demonstrating structural alterations of the ER-α complex with estrogen versus antiestrogen (112,113).

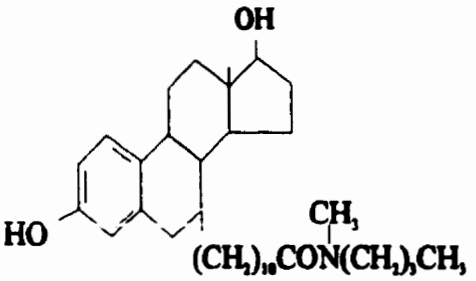
Antiestrogen

Antiestrogens are defined according to their abilities to act as either partial agonists (i.e., nonsteroidal antiestrogens such as tamoxifen) or complete antagonists (i.e., steroidal 'pure' antiestrogens such as ICI 164,384) (117). Both classes of antiestrogens have in common a bulky side-chain (Figure 3) important for their antiestrogenic activity (118,119) and compete with estradiol for high-affinity binding to the ER-α, leading to cell cycle blockade of breast cancer cells *in vitro* (59,120,121) and reduced breast tumour growth *in vivo* (49,62). Despite these commonalities, the mechanisms of action of the two classes of

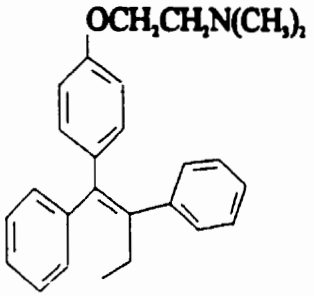
Estradiol



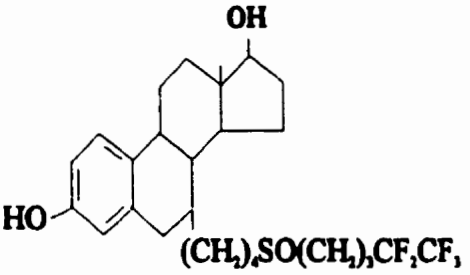
ICI 164,384



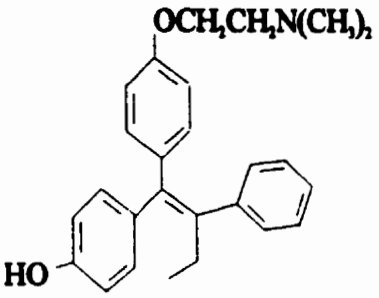
Tamoxifen



ICI 182,780



4-Hydroxytamoxifen



Raloxifene

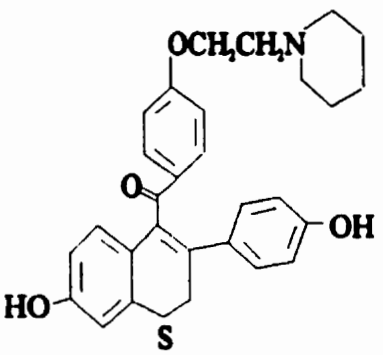


Figure 3. Schematic drawing of estrogen, nonsteroidal antiestrogens and steroidal antiestrogens. The position of the 7 α-substituted position of estradiol is shown. Adapted from (117,119).

antiestrogens appear to differ.

Nonsteroidal antiestrogens

Tamoxifen, as discussed earlier, is the most widely used clinical antiestrogen. 4-*trans*-Hydroxytamoxifen (hydroxytamoxifen), thought to be the clinically active metabolite of tamoxifen, competitively inhibits estradiol binding to the hER-α with an affinity 3-fold higher than estradiol (121,122). Hydroxytamoxifen inhibits the growth of ER-α positive human breast cancer cells *in vitro*, causing a reversible blockade of the cell cycle (120). Hydroxytamoxifen exhibits some agonist activities, which are dependent on the cell type and promoter context (123).

The complex of hydroxytamoxifen bound ER-α retains the ability to bind DNA but in many cases is unable to form a transcriptionally active ER-α (80,123). Studies have demonstrated that the conformation of the hER-α complexed with hydroxytamoxifen differs from that of estradiol complexed with hER-α (112). *In vitro* assays have demonstrated that hydroxytamoxifen/hER-α/ERE complexes migrate more slowly than the estrogen/hER-α/ERE complex (80,124). In addition, studies have demonstrated that in cell contexts where the AF-2 activity of the hER-α is not required hydroxytamoxifen can function as a partial agonist, whereas in AF-2 cell and promoter requiring contexts AF-2 activity is blocked and hydroxytamoxifen functions as an antiestrogen (112,123,125). It is thought that it is the inability of antiestrogens like tamoxifen to induce a transcriptionally active conformation that results in the inability to activate transcription (112,113). Indeed, as discussed previously, the recent elucidation of the structure of the raloxifene complexed ER-α LBD supports this hypothesis and provides a structural basis for the lack of transcription on antagonistic

promoters in that the AF-2 region of the hER-α LBD is significantly altered in structure (115). Further, more recent studies provide evidence to suggest that the antagonistic effects of tamoxifen reside in its ability to recruit corepressor proteins to the ER-α complex as will be discussed in more detail in a following section.

The expression of growth promoting factors such as, epidermal growth factor (EGF), transforming growth factor alpha (TGFα), and the insulin-like growth factors (IGF) -I and -II or growth inhibitory factors such as members of the TGFβ family, is thought to play a role in the growth of both normal and cancerous breast tissue (126). Tamoxifen-induced changes in the levels of these growth factors may also be involved in its antiproliferative effects. Tamoxifen can inhibit the estrogen-induced production of TGFα by human breast cancer cells (127) and tamoxifen treatment can lower TGFα production in some ER-α positive, but not ER-α negative, human breast tumours (128). IGF-1 is a mitogen for human breast cancer cells *in vitro* (126) and circulating levels of IGF-I are decreased in breast cancer patients during tamoxifen therapy (129). TGFβ is growth inhibitory to human breast cancer cells in culture regardless of ER-α status (130,131) and it has been reported that there are increases in serum levels of TGFβ2 and increases in TGFβ1 in the stroma of breast tumour biopsies from tamoxifen treated ER-α negative and positive breast cancer patients (131,132). Researchers have hypothesized that TGFβ may act in a paracrine fashion to alter the growth of ER-α positive or ER-α negative breast cancer cells and could explain why a small portion (~10%) of ER-α negative patients respond to tamoxifen therapy (44,132,133). Paradoxically, overexpression of TGFβ1 in tumour biopsies has been associated with disease progression

(134) and more recently, researchers have demonstrated that antibodies that blocked TGFβ signalling, did not block tamoxifen-mediated growth inhibition of MCF-7 and T47D human breast cancer cells (135), leading to questions regarding the role of TGFβ as a mediator of tamoxifen inhibition of breast cancer growth.

Steroidal antiestrogens

The use of tamoxifen in breast cancer therapy is limited by the eventual development of resistance to treatment (136). This led to the search for novel antiestrogens with enhanced antagonist, and less agonist, action and the development of a series of steroidal antiestrogens, such as ICI 164,384 and ICI 182,780 (59) (Figure 3). The steroidal antiestrogens have 7α-alkylamide side-chains that are important for their antiestrogenic activity (119). The steroidal antiestrogens compete with estrogen for binding to the ER-α and result in cell cycle blockade (59,120). ICI 164,384 and ICI 182,780 are apparently devoid of agonist activity *in vivo* (59,60,137-139) and *in vitro* (59,60,140).

The mechanism by which these compounds interfere with ER-α activity is unclear. The steroidal antiestrogens rapidly reduce ER-α protein levels in cultured cells, including MCF-7 cells, uterine tissue, and human breast tumours (61,93,94,141). Experiments have demonstrated that the steroidal antiestrogen-induced decrease in mER-α protein is not due to alterations in mRNA levels, but is a result of increased turnover of the mER-α protein, with the half-life reduced from approximately 5 hours in the presence of estrogen to less than 1 hour in the presence of ICI 164,384 (93). *In vitro* experiments have demonstrated that these compounds can inhibit the ability of ER-α (human and mouse) to bind to DNA (142,143) but other studies have failed to demonstrate an inhibition of DNA binding of ER-α

from various sources, including human breast cancer cells, in the presence of steroidal antiestrogens (112,114,124,143,144). Arbuckle and coworkers have demonstrated using *in vitro* translated mER-α, that the presence of steroidal antiestrogen during translation inhibited receptor DNA binding activity (143). Researchers suggest that the steroidal antiestrogens act to prevent the formation of ER-α receptor dimers but cannot interfere with preformed dimers, which may explain the conflicting data on ER-α obtained from different sources, including the use of *in vitro* translated hER-α incubated with ICI 164,384 after, versus during, translation (142-144). Additional support for this hypothesis comes from the fact that in the presence of steroidal antiestrogens, DNA binding of mER-α was restored using an ER-α antibody that restored DNA binding to dimerization-deficient receptor mutants (142,143). It is thought that the reduction in the cellular ER-α levels in the presence of steroidal antiestrogens is likely a result of impaired receptor dimerization which leads to a less stable protein (93). Additional evidence suggests that the steroidal antiestrogens may interfere with receptor function by preventing nuclear uptake which was also suggested to lead to an increase in ER-α degradation (145).

Hormone-responsive elements

The ER-α binds to DNA as a dimer (80) and in most cases gene activation requires interaction of the hormone receptor complex to regulatory DNA sequences, generally located in the 5'-promoter region of target genes. A comparison of hormone-responsive elements (HREs) from several estrogen-responsive genes has revealed what is termed a 'consensus' nucleotide sequence for the estrogen-responsive element (ERE) (Table 1). This 13 base pair (bp) sequence is organized as a palindrome with two unequally conserved half-sites separated

Table 1. Estrogen-responsive genes and EREs*

Gene	Species	Estrogen-responsive element
Consensus		5'-GGTCAnnnTGACC-3'
Vitellogenin A1	Xenopus	GGTCAnnnTGACC
Vitellogenin A2	Xenopus	GGTCAnnnTGACC
Vitellogenin B1a	Xenopus	AGTTAnnnTGACC
Vitellogenin B1b	Xenopus	AGTCAnnnTGACC
pS2	Human	GGTCAnnnTGGCC
PR E1(-2480/-2459)	Rabbit	GTACAnnnTGACC
E2(-433/-419)	Rabbit	GGTCAnnnCGATT
E3(+698/+723)	Rabbit	GGTCGnnnTGACT
Uteroglobin	Rabbit	GGTCAnnnTGCCC
Ovalbumin	Chicken	TGACC

* this table represents examples of estrogen-responsive genes and their EREs and is not an exhaustive list. References (146,148,150-152,154).

by three non-conserved nucleotides (5'-GGTCAnnnTGACC-3') (146) and functions as an enhancer in a position- and orientation-independent manner (147). Most estrogen-regulated genes do not contain a perfect consensus ERE, but instead contain one or more EREs that are imperfect palindromes in that they diverge from the consensus sequence by one or more nucleotides, while others may contain multiple half-site EREs. For example, in the estrogen-responsive *Xenopus* vitellogenin B1 gene, three EREs are located in the promoter region (-596/-42) (148). Deletion of the ERE (-555/-543) is dispensable for hormone-responsive reporter gene activity in MCF-7 cells, while the other two (-334/-322 and -314/-302) are required for estrogen-inducibility (148). The two imperfect EREs located at position -334 and -302 have been shown to synergize with each other and with upstream activator elements, or a consensus ERE, in synthetic promoters (147,149). The chicken ovalbumin gene contains within its promoter a series of four half-palindromic motifs that act synergistically (150) and the gene for human pS2 contains a single copy of an imperfect palindromic ERE (151). The PR is induced by estrogen and three ERE elements have been identified within the rabbit PR gene. Interestingly, of the three, only one is able to activate a heterologous gene in transfection experiments alone, and this ERE is located at a position (+698/+723) that overlaps with the initiation of translation (152). The other two EREs located further upstream, were inactive alone, but when placed in tandem were able to activate transcription (152). It has also been demonstrated *in vitro* that EREs can act synergistically with additional copies of EREs (149,153) and can act synergistically with HREs of other steroid receptors (154) and with DNA-binding sites for other transcription factors (149). As well, nonclassical EREs consisting of Sp1-DNA binding elements may function as estrogen-responsive elements

(154a). While in most cases ER-α is thought to bind DNA as a homodimer (80), it can bind as a monomer to a half-palindromic ERE *in vitro* (155) and can form heterodimers with the recently identified ER-β (156).

Generally, it is believed that in order for ER-α to exert its effects on a target gene, direct binding of the receptor to regulatory elements in the promoter region are required, but studies have demonstrated that the ability of steroid hormone receptors to modulate transcription does not necessarily require the receptors bind DNA. PRc, an N-terminally truncated hPR isoform lacking the first zinc-finger of the DBD, has no transcriptional activity of its own but has been shown to enhance progestin-induced transcriptional activity (157). ER-α can activate transcription from AP-1 (activator protein 1) dependent promoters through a DNA binding-independent pathway (158,159). At these sites the hER-α DBD is not required for estrogen-induced transcriptional activation, but direct interaction of ER-α with c-jun and c-fos (AP-1) proteins is the likely mechanism (158,159). More recently, researchers have demonstrated the existence of a DNA response element for the antiestrogen raloxifene, an estrogen agonist in bone, in the TGFβ3 gene (160). These researchers found that removal of the DBD of the hER-α did not prevent activation of the TGFβ3 gene by raloxifene and further demonstrated that an adapter protein is likely required (160).

ER-α associated proteins

The hER-α, as previously described, contains three domains thought to be involved in transcriptional activation, two of which have been studied in detail. These, AF-1 and AF-2, function in a cell-, promoter-, and tissue-specific fashion and are regions involved in interactions with ER-α accessory proteins (76,77,125). Studies have demonstrated that AF-1

is a ligand-independent, constitutive activation function, while the activity of AF-2 is ligand-dependent (123,161). In some situations the activity of AF-1 or AF-2 predominate, and in others, the activities of both AF-1 and AF-2 are required (76,77,125). In addition, the AF-2 domain contains a highly conserved α-helix (Helix 12) that is required for ligand-dependent transcriptional activation (162,163). As discussed previously, the recent elucidation of the structure of the estrogen versus antiestrogen bound ER-α LBD revealed that the region essential for AF-2 function is significantly displaced in the presence of antiestrogen (115).

'Squelching' experiments first suggested that AF-containing regions of hormone receptors, including the ER-α, required additional cellular factors for efficient transcriptional activation. Studies demonstrated that estrogen-dependent hER-α activation could compete transcriptional activation by PR and glucocorticoid receptors (GR) (164). In addition, using hER-α mutants, researchers demonstrated that inhibition of progestin-induced transcription could be achieved using the AF function containing N-terminal A/B or LBD of hER-α and did not require the DBD (164). Indeed, studies have demonstrated that AF regions of steroid hormone receptors, including the ER-α, interact with a number of proteins that are thought to regulate transcriptional activation.

There have been numerous proteins identified that interact with the ER-α and are believed to be involved in regulating some aspect of receptor function. It is likely that many more interacting proteins will be discovered before we can fully understand the role they play in regulating transcription by steroid hormone receptors such as ER-α. For the purpose of clarity this section is broken down into heat-shock proteins, basal transcription factors/coactivators, corepressors and other associated proteins.

Heat-shock proteins

The ER-α can be recovered from estrogen-free cell cytosols in an inactive multimeric complex (165,166). This complex contains ER-α associated with a group of proteins, that may include, heat-shock proteins (hsp 90, hsp70, hsp27) and p59 (166-168). Hsp90, a molecular chaperone involved in the correct folding of newly translated proteins (169), is ubiquitously expressed and found mainly in the cytoplasm with low levels in the nucleus (166,170). Chemical cross-linking of steroid-free hER-α from intact MCF-7 cells has demonstrated an association of the receptor with hsp90 and p59 (168) and hsp90 is thought to interact with several regions throughout the ligand binding and DNA-binding domains of hER-α (83). *In vivo* studies have shown that the interaction between hsp90 and hER-α resulted in nuclear localization of hsp90 in the absence of ligand and a decrease in the level of nuclear hsp90 occurred in the presence of estrogen or tamoxifen (170). While the precise role of hsp90 is unclear, it is believed to maintain steroid hormone receptors inactive in the absence of hormone and may enable efficient response to hormone (166), although it is not absolutely necessary for hER-α hormone-dependent transcriptional activation (171).

Hsp70 has been detected in hER-α complexes isolated using various biochemical techniques (167) but other researchers have not detected an association of hsp70 with hER-α (168) and it is thought that while hsp70 is likely a component of the nonactivated receptor complex, it may not make direct contacts with the receptor itself (166). The association of hsp70 with hER-α containing complexes was found to be significantly reduced following treatment with hydroxytamoxifen or estrogen but not with ICI 182,780 (167). *In vitro* electrophoretic mobility shift assays using recombinant hER-α demonstrated that hsp70 may

play a role in hER-α DNA interactions (167), while other *in vitro* studies using calf uterine ER-α suggest it does not (172). These differences may be due to species- and source-specific differences of the ER-α used in these studies.

A 29 kDa protein has also been found in association with the ER-α, which is likely hsp27 (173,174). The function of hsp27 in ER-α action is unknown, but hsp27 levels are associated with ER-α expression and are low in normal breast tissue and high in estrogen-responsive breast tumours (175), suggesting it may play an important role in ER-α function.

While the function of these proteins is unclear, it is thought that in the absence of hormone they may maintain the receptor in a transcriptionally inert state and additional proteins may also exist that are involved in ligand-free repression of hER-α (166,171).

Basal transcription factors/coactivators

Receptor binding to its HRE is, in most cases, a prerequisite for gene regulation, but alone it is not sufficient. Evidence suggests that steroid hormone receptors interact with both general and sequence-specific transcription factors, directly or indirectly, to influence transcription at the target promoter. In cases where the direct interaction of the ER-α with the target DNA sequence is not required (i.e., AP-1 and raloxifene response elements) it is clear that the requirement for another protein(s) likely exists. It has also been demonstrated *in vitro* that the mER-α can directly contact the basal transcription machinery and enhance the formation of stable pre-initiation complexes at target promoters to increase transcription in a cell-free system (176). The LBD of the hER-α has been shown to interact *in vitro* with

TFIIB, a component of the basal transcription apparatus (177). TBP (TATA-box binding protein) has been demonstrated to interact with both the AF-1 and AF-2 domains of hER-α *in vitro* (178). A specific subpopulation of TFIID containing TAF (TBP-associated factor) $_{II}$30 is required by hER-α for *in vitro* transcriptional activation and it was demonstrated that $TAF_{II}30$ could interact *in vitro* with AF-2, but not AF-1, of hER-α (179). The *in vitro* interaction of hER-α with TFIIB, $TAF_{II}30$ or TBP has been shown to occur in the presence or absence of estrogen (179,180).

While the ability of the receptor to regulate transcription may require direct contacts with components of the transcription initiation complex, indirect or direct contacts with coactivators or transcription intermediary factors are also important. That cofactors are required for steroid hormone receptor transactivation was first suggested by 'squelching' experiments as discussed (164). Subsequently, a steroid receptor coactivator protein, SRC-1, has been identified that enhances hER-α transcription in the presence of estrogen (181). SRC-1 was able to reverse the 'squelching' between hER-α and hPR in transient transfection experiments, confirming it to be a limiting cofactor required for efficient transactivation (181). SRC-1 binds to the ligand binding/AF-2 domain of hER-α *in vitro* in the presence of estrogen but not in the absence of estrogen or in the presence of tamoxifen, ICI 164,384 or ICI 182,780 (182). A truncated hER-α lacking amino acids 534-595, that is not transcriptionally active, does not interact with SRC-1 *in vitro* (182), but the transcriptional activity of a transiently expressed hER-α containing point mutations that disrupt AF-2, but not ligand-binding, activity was still enhanced by SRC-1 in HepG2 (human hepatocellular carcinoma) cells from a reporter gene containing three imperfect EREs, demonstrating that AF-2 activity

is not required for SRC-1 coactivation *in vivo* in this cell and promoter context (183). Transiently expressed SRC-1 has also been shown to enhance the agonist activity of hydroxytamoxifen on hER-α expressed in HepG2 cells (183). An *in vitro* interaction of the N-terminal of hER-α and SRC-1 has also been demonstrated and in Rat-1 cells the constitutive activity of the AF-1 region of the hER-α (amino acids 1-182) could be blocked by microinjection of SRC-1 antibodies (184). Additionally, the AF-1 activity of hER-α transiently expressed in HeLa cells can be stimulated with SRC-1 (185). Together these data suggests that SRC-1 can functionally interact with both the N- and C-terminal portions of the hER-α.

SRC-1 binds not only steroid hormone receptors, but also TBP and TFIIB, suggesting it may tether nuclear hormone receptors to the basal transcription machinery (186). SRC-1 has been shown to contain histone acetyltransferase (HAT) activity (187), linking the recruitment of coactivators to the chromatin and alterations in chromatin structure as a mechanism for transcriptional activity. Acetylation of specific lysine residues within the N-terminal tail of histones is thought to mediate alterations in chromatin structure and the acetylation of the histone tails has been demonstrated to correlate with transcriptional activation (188-190).

Subsequent studies have demonstrated that SRC-1 belongs to a family of related proteins (SRC-1/p160) (191). Transcription intermediary family 2 (TIF2) is a member of the SRC-1 family, and in transient transfection analyses increases estrogen-activated hER-α transcriptional activity and interacts in an estrogen-dependent manner with the LBD of hER-α *in vitro* and the full length hER-α *in vivo* (192). The mouse homologue of TIF2 (GR-

interacting protein 1, GRIP1) has been demonstrated to function as a coactivator for the AF-1 region of the hER-α transiently expressed in HeLa cells (185). AIB1 (amplified in breast cancer 1), another member of the SRC-1 family, was cloned from chromosomal regions amplified in some breast and ovarian cancers and has been shown to be overexpressed in some breast tumour biopsy samples relative to normal breast tissue and in some ER-α positive, not negative, human breast cancer cell lines (193). AIB1 enhanced estrogen-dependent hER-α transcriptional activation and was shown to interact *in vitro* with hER-α in a ligand-dependent fashion (193). Interestingly, AIB1 is identical in sequence to a protein, ACTR, which contains acetyltransferase (AT) activity, suggesting that AIB1 may be able to acetylate histones when recruited to chromatin (193,194).

Other ER-α interacting proteins have been identified that can likely function as more general coactivator proteins for a variety of transcription factors. For example, Verrier *et al* have demonstrated that high mobility group protein 1 (HMG1) binds to hER-α *in vitro* (195) and that baculovirus expressed hER-α does not bind to an ERE *in vitro* even in the presence of estradiol, but does bind in the presence of HMG1 (195). HMG1 was not able to stimulate hER-α transcription *in vitro* in the presence of estrogen, but the addition of $TAF_{II}30$ in the presence of HMG1 stimulated transcription 20-fold in the absence of estrogen and a further 5-fold in the presence of estrogen (195). HMG1 is a ubiquitously expressed protein believed to play a role in chromatin decondensation and transcriptional activation and is a substrate for AT activity (196-198).

CREB binding protein (CBP) and p300 (CBP/p300) are related nuclear phosphoproteins that can act as a coactivator for the ER-α. CBP/p300 has been shown to

interact with the hER-α in an estrogen-dependent manner *in vitro* (199) and can enhance both estrogen-stimulated and basal hER-α-dependent transcriptional activity in transient transfection analyses (200). CBP/p300 has AT activity and can acetylate nuclear histones as well as the basal transcription factors TFIIF and TFIIEβ *in vitro* (201,202). In a recent study, Martinez-Balbas *et al* were able to demonstrate that the AT domain of CBP can stimulate transcription *in vivo* and there was a direct correlation with the *in vitro* ability of CBP to acetylate histones and *in vivo* transcriptional activation (189). CBP/p300 can interact *in vitro* with the SRC-1 related protein p/CIP and immunodepletion studies have demonstrated that p/CIP is required for hER-α action *in vivo* (203). Additionally, p/CIP can interact with hER-α in a ligand-dependent manner *in vitro* (203). CBP/p300 can also bind to SRC-1 (199) and both CBP/p300 and SRC-1 can interact with the histone acetylase PCAF (p300/CBP-associated factor) and in this way multiple proteins with AT activity are likely recruited to the chromatin template (187,204).

Additionally, other putative ER-α coactivator proteins have been identified whose role in ER-α function has not been clearly established. RIP140 (receptor-interacting protein 140), interacts with the mER-α LBD *in vitro* in the presence of estrogen, but not the antiestrogens tamoxifen, ICI 164,384 and ICI 182,780 (205,206). Mutations in the AF-2 domain that abolish transcriptional activity also prevented or decreased the interaction of RIP140 with mER-α in the presence of estrogen *in vivo* (206,207). These data suggest a role for RIP140 in ER-α ligand-dependent transcriptional activity, but a function for RIP140 as a coactivator has not been clearly established. In transient transfection experiments in mammalian cells RIP140 has some coactivator activity and can increase mouse and human ER-α transcription

in the presence of estrogen approximately 2-4 fold, but increasing levels of RIP140 result in repression of receptor activity (116,206,208). Similarly, an ER-α interacting protein, TIF1 has been identified and shown to require the AF-2 domain for *in vitro* estradiol-dependent interaction with the hER-α (209) and in transient expression systems can interact *in vivo* with hER-α in the presence of estrogen (210) but a role as a coactivator protein has not been established. Recently, Brx, a novel 170 kDa protein related to the Dbl oncogene, has been shown to interact with the hER-α *in vitro* in the presence and absence of estradiol and required the LBD for *in vitro* interaction (211). Overexpression of Brx in Ishikawa endometrial cells resulted in a ligand-dependent increase in transcriptional activity of transiently expressed hER-α, suggesting this protein may also function as an ER-α coactivator (211).

The precise interaction sites on the ER-α with coactivators is unknown. As discussed, mutations in the AF-2 region of the receptor LBD impairs the ability of several coactivators to interact with ER-α. Replacement of a lysine at position 366 with alanine in mER-α (corresponds to lysine 362 in hER-α) reduces AF-2 function and the ability of SRC-1, TIF1 and TIF2 to interact with mER-α, but had no effect on RIP140 binding *in vitro* (116). Mutation of residues within the AF-2 (methionine 543, leucine 544, leucine 539) of the hER-α prevented *in vitro* interaction of TIF2 (192). Mutation of any one of three residues (lysine 362, valine 376 and glutamic acid 542) within the AF-2 of the hER-α have been shown to abolish transcriptional activity and diminish *in vitro* binding of GRIP1 (212). It has been postulated that coactivators bind to a small hydrophobic cleft formed by the AF-2 region of

hormone-activated receptor via positioning of helix 12 against the scaffold of helices 3, 5 and 6 (212). Together with the recently elucidated structure of estrogen or raloxifene bound hER-α/LBD, it would appear that specific residues either exposed or buried depending on the ligand-induced conformation will be important in determining coactivator binding as well as which coactivators (if multiple are present within the cell) can interact with the receptor. Interestingly, more recent research has demonstrated that *in vitro* individual coactivator proteins interact differently with the LBD of the hER-α and can compete with each other for *in vitro* interaction (208). This would suggest that *in vivo* competition between different ER-α interacting proteins could occur and furthermore suggests that different intracellular levels of these proteins could determine ER-α activity.

The AF-2 domain of ER-α recognizes a specific motif, termed the NR box (203,213) that is found within the SRC-1/p160 family of coactivators (TIF2/GRIP1; AIB1/p/CIP/ ACTR; SRC-1) (193,203,213) and also within proteins such as TIF1 and RIP140 and other coactivator proteins such as CBP and p300 (203). Recent studies indicate that two NR box motifs of a single SRC-1 are required for interaction with heterodimers of retinoid-X receptors (RXRs) and retinoic-acid receptor-α (RAR-α) (214). Additionally, the crystal structure of a homodimer of ligand-bound peroxisome proliferator-activated receptor-γ (PPAR-γ) LBD complexed with SRC-1 (amino acids 623-710 containing NR box motifs 1 and 2) revealed that each NR box motif of SRC-1 bound to one PPAR-γ molecule of the homodimer (215). This may be a general feature of steroid hormone receptors and suggests that nuclear receptor homo or heterodimers have the ability to contact multiple NR box containing proteins. The findings that both the N- and C-terminal (AF-1 and AF-2) regions

of the hER-α can interact at least *in vitro* with coactivator proteins supports this and suggests that multiple proteins could interact simultaneously with different regions of the ER-α. Additionally, research suggests that the AF-1, as well as the AF-2, function through coactivator proteins. Interestingly, the AF-1 activity of hER-α has been shown to be capable of functioning through p160 proteins including, SRC-1, TIF1 and GRIP1 (185,216), and it was found that AF-1 of hER-α interacts with a region of GRIP1 distinct from that of AF-2 (185). This would suggest that the ER-α is capable of multiple contacts with an individual coactivator protein as well as the potential for simultaneous interactions with more than one coactivator. It is likely through these multiple interactions that a variety of functionally different receptor complexes can be recruited in a ligand- and cell-specific manner.

Corepressors

The corepressor proteins, N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid receptors), were first identified as interacting with the thyroid hormone receptor (TR) and RAR to repress the activity of unliganded receptors (217,218). In addition, homodimers of TR or TR-RXR heterodimers have been shown to be required for corepressor binding and two receptor C-termini are required for corepressor binding on DNA, demonstrating a requirement for receptor dimers for transcriptional repression (219). As in the case of coactivators, a strong link between chromatin structure and the function of corepressor proteins has been made. Corepressors have been shown to exist in a complex containing the corepressor protein Sin3 and the histone deacetylases (HDAC) RPD3 or HDAC1 (220-223). Heinzel *et al* demonstrated that all three components of the complex of N-CoR, Sin3 and RPD3 are essential for transcriptional repression *in vivo* (220). The

deacetylation of the histone tails is thought to compact the chromatin to repress gene expression and recruitment of deacetylases to the chromatin by repressor proteins is thought to mediate their effects on gene expression (220,221).

In vitro hER-α has been shown to interact with N-CoR and SMRT in a ligand-independent manner (183,224), and in immunoprecipitates from MCF-7 human breast cancer cells, N-CoR was shown to interact with the hER-α strongly in the presence of tamoxifen and weakly in ligand-free conditions (184). Microinjecting antibodies against N-CoR and SMRT in MCF-7 cells converted tamoxifen to an agonist but this had no effect on the response to estrogen as measured by transfection of an estrogen-responsive reporter gene (184). Conversely, the partial agonist activity of tamoxifen on hER-α has been shown to be suppressed by either N-CoR or SMRT overexpression (183,225). More recently, an N-CoR binding protein, SAP30, has been identified which is required for N-CoR mediated repression of tamoxifen bound hER-α (226). Together these data suggest that the antagonistic activity of tamoxifen may be due to its ability to recruit corepressor containing complexes to the transcription complex and the partial agonism seen in some cases may be due to decreased levels or interaction of antagonist-bound receptor with corepressors.

In HepG2 cells transiently expressed SMRT was also able to decrease basal hER-α activity by 60% and *in vitro* ligand-independent interactions between hER-α and SMRT have been demonstrated (183). Interestingly, agents that can activate the ER-α in the absence of ligand (i.e., EGF and PKC activation) were shown to decrease the interaction of N-CoR with hER-α in MCF-7 cells in the presence of tamoxifen and tamoxifen was unable to inhibit this ligand-independent activity (184).

The data support a model in which the ER-α when bound by antagonist (or perhaps in the absence of ligand) interacts with the corepressor complex to effectively mask the transcriptional activation functions. Once the receptor interacts with agonist-ligand (or is activated by ligand-independent pathways) the corepressor interactions are relieved, allowing interactions with coactivator complexes. More recently, some interesting data have been published to suggest that the nature of the ERE itself can alter the conformation of hER-α *in vitro* (227). This adds another layer of complexity to ER-α regulation and further suggests that the promoter itself may be able to 'guide' corepressor/coactivator interactions by altering the structure of the ER-α.

Other associated proteins

Other researchers have identified proteins capable of interacting with the ER-α and in some cases effects on ER-α activity have been demonstrated. Recent studies have demonstrated that cyclin D1, an important component of the cell cycle machinery, is capable of activating endogenous ER-α transcriptional activity in T47D human breast cancer cells in the absence and presence of estrogen, as well as transiently expressed hER-α in HeLa cells (228). Additionally, *in vitro* binding of cyclin D1 to the E/F region of the hER-α was demonstrated, supporting the hypothesis that cyclin D1 acts through direct physical contacts with the ER-α (228).

The transcription factor Sp1 and hER-α have been shown to interact *in vitro* in a ligand-independent fashion and enhance Sp1-DNA binding. Transcriptional enhancement by hER-α of an Sp1 binding site-containing Hsp27 promoter construct was demonstrated to be

ligand-dependent, but interestingly, did not require the ER-α-DNA binding domain (229).

Estrogen receptor-associated protein 140 (ERAP140) was isolated from MCF-7 cells as a protein that bound to the LBD of hER-α (182) and RAP46 (receptor associated protein 46) is a protein that interacts with hER-α in a ligand-independent manner *in vitro* (230). No clear function for either of these ER-α interacting proteins has been identified.

A 27 kDa protein, L7/SPA, has been identified using a yeast two-hybrid screening strategy, that interacts with hER-α hinge/LBD and was found to enhance the transcription of tamoxifen, but not estrogen or ICI 164,384, occupied hER-α (225).

A 55 kDa protein, that binds to the ER-α, is thought to aid the nuclear transport of the ER-α in the goat uterus (231). This protein can also interact with the cytoskeletal elements actin and tubulin, suggesting a role of the cytoskeleton in nuclear transport of ER-α (231).

Landel and coworkers isolated proteins that interacted with hER-α from MCF-7 and chinese hamster ovary (CHO) cell extracts (167). In addition to the ER-α associated protein hsp70, a protein of 55 kDa was identified as a protein disulfide isomerase and two proteins, p48 and p45, were also described, whose identities are unknown. While the function of these proteins is not known, the researchers did demonstrate that maximum interaction of hER-α with an ERE *in vitro* occurred in the presence of all four proteins (167).

It is clear that numerous proteins that may be able to interact *in vivo* with the ER-α have been identified and it is likely that more ER-α interacting proteins will be identified before we can fully understand the role these proteins play in ER-α function. Cell- and tissue-

specific expression of these proteins is likely going to be of significance in understanding their role as well as the effects of ligand on *in vivo* interaction and function of the respective proteins.

Phosphorylation

The ER-α, like other members of the steroid-thyroid hormone receptor superfamily, is a phosphoprotein and several protein kinases, including mitogen-activated protein kinase (MAPK), have been implicated in ER-α phosphorylation (232-239). In MCF-7 human breast cancer cells, the hER-α is phosphorylated at serine 118 and serine 167 in response to estradiol binding (238,240). In COS-1 monkey kidney cells, hormone-induced phosphorylation at serine 104, serine 106 and serine 118, on recombinantly expressed hER-α have been identified, with little or no phosphorylation at serine 167 (239). These differences in hormone-inducible phosphorylation sites detected by different research groups are unclear but may be due to differences in the cell types and source of hER-α used in these studies.

Phosphorylation is thought to play a role in regulating many aspects of steroid hormone receptor function including DNA binding and transcriptional activation. In a yeast system, hER-α mutated to an alanine at serine 167 was 75% less transcriptionally active than wild-type hER-α and casein kinase II phosphorylated hER-α *in vitro* at serine 167 to enhance DNA binding (234,236). Mutation of serine 118 to alanine in hER-α had no effect on *in vitro* DNA binding but did reduce the transcriptional activity, compared to wild-type hER-α, of transiently expressed receptors in COS-1 and HeLa cells but not in chicken embryo fibroblasts (241). Studies using mER-α demonstrated that while transcriptionally defective receptors were phosphorylated, the level of phosphorylation was 80% that of wild-type receptor (242).

In addition, DNA-binding defective mER-α mutants were markedly reduced in the level of phosphorylation (15% that of wild-type), suggesting that a majority of mER-α phosphorylation depends on an intact DNA-binding domain (242). Denton and coworkers demonstrated that dephosphorylation of hER-α eliminated its affinity for an ERE *in vitro* (243). Therefore the data suggest that a majority of ER-α phosphorylation may be due to transcriptionally active/DNA bound receptor.

It has also been demonstrated that serine 118 and tyrosine 537 on hER-α are phosphorylated independently of estradiol binding in MCF-7 cells (236,237). Agents that can modulate the phosphorylation of the ER-α, including growth factors, can activate the ER-α in the absence of ligand (244,245). MAPK has been demonstrated to phosphorylate hER-α on serine 118 *in vitro* and activation of MAPK *in vivo* via estrogen-independent mechanisms (i.e., EGF) results in phosphorylation of hER-α on serine 118 (232,236,246) and can result in transcriptional activation of hER-α, requiring phosphorylation of serine 118 (240,246). *In vivo*, EGF has also been shown to result in phosphorylation of hER-α expressed in COS-1 cells at serine 167 and evidence suggests that pp90rsk1, a downstream component of the MAPK cascade, phosphorylates hER-α at serine 167 *in vitro* and *in vivo* (233). Additionally, Src family tyrosine kinases can phosphorylate recombinant hER-α *in vitro* at tyrosine 537 independent of estrogen (237). Together these data suggest that modulation of ER-α phosphorylation may be involved in ligand-independent activation of the ER-α. In transient transfection experiments, mutation of tyrosine 537 to serine or asparagine in hER-α resulted in constitutive transactivation and ligand-independent interaction of the mutant ER-α with

the coactivator SRC-1, suggesting phosphorylation of this site may be involved in regulating response to ligand (247,248). Data also suggest that estradiol-induced phosphorylation of hER-α on serine only occurs on tyrosine 537-phosphorylated ER-α species and tyrosine-dephosphorylation of hER-α eliminated hER-α-ERE interactions *in vitro* (249). Studies also suggest that phosphorylation of hER-α on tyrosine 537 may be important for dimerization of the receptor since addition of hER-α antibodies to tyrosine-dephosphorylated hER-α restored ER-α-ERE interactions *in vitro* (249). Additionally, Src-phosphorylation of the hER-α restored ERE interactions *in vitro* (249). These data suggest that phosphorylation of tyrosine 537 on the ER-α may be required for binding to an ERE and stabilization of receptor dimers, after which phosphorylation on serine residues can occur.

It is presently unclear if any differences in phosphorylation of the ER-α exist that could account for differences in response to estrogen or antiestrogens. It has been shown that the antiestrogens ICI 182,780, ICI 164,384 and hydroxytamoxifen are able to induce the phosphorylation of the hER-α (239,240). Phosphopeptide patterns of wild-type hER-α transfected in COS-1 cells were similar in the presence of estrogen, hydroxytamoxifen or ICI 164,384 treatment (239), but overall phosphorylation of hER-α transfected in COS-1 cells was lower in the presence of these antiestrogens compared to estrogen (241). Joel and coworkers demonstrated that ICI 182,780 induces an upshift of hER-α on Western blots associated with serine 118 phosphorylation similar to that seen with estradiol (240). These observations suggest that an increase in overall ER-α phosphorylation does not necessarily correlate with transcriptional activation and more subtle site-specific differences may be

important. Interestingly, estradiol treatment of MCF-7 cells resulted in dephosphorylation of a single ^{32}-P-labelled ER-α phosphopeptide, suggesting that dephosphorylation of specific sites within the ER-α may also be important in regulating response to ligands (250).

While together these data suggest that phosphorylation may modulate transcriptional activity and DNA binding of ER-α, a clear understanding of the functional role for phosphorylation has not been established. Estrogen-stimulated, as well as ligand-independent reporter gene activity, can be reduced by inhibiting protein kinase activity in rat uterine cells, demonstrating the likely importance of phosphorylation in both ligand-dependent and -independent ER-α activation (244).

Role of hormones in breast cancer

Approximately 100 years ago Beatson demonstrated one of the first links between hormones and breast cancer when he reported that excision of the ovaries induced regression of breast cancer in two women (251). Almost 70 years later Jensen correlated the presence of a specific ER with the response to endocrine therapy (252). While in the past 100 years, much knowledge has been gained towards understanding steroid hormones, their receptors and their action in normal tissue as well as their role in breast cancer, we still do not fully understand the role hormones play in the initiation or progression of human breast cancer. The fact that male breast cancer accounts for less than 1% of the total annual incidence of breast cancer (5) underlies the importance of the hormonal milieu as a significant factor in breast cancer susceptibility.

As previously discussed, steroid hormones are implicated in the etiology of breast cancer. Risk factors, that are related to an increased time of hormone exposure such as a

younger age at first menarche and older age of menopause (6,253) and use of hormonal replacement therapy in postmenopausal women (9) are associated with an increased risk. Growth factors have also been implicated in tumour growth and development, and estrogen is thought to act in part via the production of locally acting growth promoting substances (126).

Estrogen and breast cancer

Estrogens are important mitogens in terms of breast cancer cell growth both *in vivo* and *in vitro* (254,255). Further, reducing breast exposure to ovarian steroids significantly reduces breast cancer risk (6,7). Estrogen appears to play an important role in terms of breast cancer development and promotion. Rodent mammary tumour models are often used to study the effects of estrogens and antiestrogens on mammary cancers. Hormone-dependent tumours can be produced in rodents through the transplantation of breast cancer cells or treatment with chemical carcinogens such as N-nitrosomethylurea (NMU) or 7,12-dimethyl-benz[a]anthracene (DMBA) (255). In rodent models of chemically induced mammary tumours estrogen is required for tumour formation and tamoxifen has been shown to inhibit the growth of tumours in these types of systems (256,257). When human breast cancer cells are implanted into athymic mice, estrogen alone stimulates tumour formation and antiestrogens can inhibit the growth of estrogen-induced tumours (258,259). These studies demonstrate the importance of estrogen in the growth of mammary cancer.

In the clinical setting, the response of breast cancer to endocrine therapy is highly correlated with the presence of the ER-α, which is overexpressed in approximately 70% of human breast tumours (37). Some of the most compelling evidence for the role of estrogen

in the development of breast cancer comes from a recent study on the prophylactic effects of the antiestrogen tamoxifen (The Breast Cancer Chemoprevention Trial) (53). In this study, women who did not already have breast cancer, but where at a significant risk for developing breast cancer, were administered tamoxifen for four years. The incidence of breast cancer in the women receiving tamoxifen treatment was reduced by approximately 45% compared to women receiving placebo. Since tamoxifen is thought to act mainly via preventing the activity of the ER-α, this is very strong evidence that estrogen, acting through its receptor, is likely responsible for many human breast cancers.

The availability of breast cancer cell lines as *in vitro* models has greatly facilitated the study of breast cancer and an understanding of the hormonal control. There are a few estrogen-responsive breast cancer cell lines with the MCF-7 cell line being the best characterized (260). These cells express the ER-α and demonstrate an absolute requirement for estrogen for tumour formation in athymic mice (259). Estrogen is a potent mitogen for human breast cancer cells in culture and antiestrogen (including hydroxytamoxifen and ICI 164,384) inhibits estrogen-induced cell proliferation and expression of estrogen-regulated genes (61,254). The discovery that estrogenic contaminants of phenol red, a component of cell culture medium, could stimulate the growth of human breast cancer cells in culture was further proof that estrogen alone could stimulate breast cancer cell growth *in vitro* (261).

Metabolites of estrogen have also been demonstrated to have biological function both dependent on and independent of the ER-α. The metabolic oxidation of estradiol can occur at several carbon atoms of the cholesterol backbone to produce either estrone, which can be converted to 16α-hydroxyestrone (16α-OH), or the catechol estrogens (2-hydroxyestradiol

and 4-hydroxyestradiol) (262). 16α-OH can bind to the hER-α and stimulate MCF-7 cell growth in culture (263). Levels of 16α-hydroxylase, the enzyme involved in forming 16α-OH, are increased in breast cancer patients (264). Catechol estrogens have also been shown to bind to the ER-α and increase proliferation and PR levels in MCF-7 cells in culture (265).

Despite the fact that much of the endocrine treatment of breast cancer is directed at blocking or interfering with the growth promoting effects of estrogen, it is still not fully understood how estrogen is involved in breast cancer development. As discussed, *in vivo* and *in vitro* studies have demonstrated that estrogen is a potent mitogen for both breast tumours and breast cancer cells in culture. It is the mitogenic effect of estrogens (acting through the ER-α) that is believed to account for the carcinogenicity of estrogen. Although the mechanisms are not clear, it has been proposed that increased proliferation may result in an accumulation of genetic damage or that estrogen stimulation may prevent the inhibition of cell proliferation by overriding growth inhibitory factors through increased synthesis of growth promoting substances (255). Additionally, it has been hypothesized that metabolites of estrogen may bind to and damage DNA directly, thereby promoting or initiating tumourigenesis (262).

Protein kinases in breast cancer

Growth factors interact with their transmembrane receptor tyrosine kinases resulting in autophosphorylation of the cytoplasmic domains leading to the activation of a multicomponent signaling cascade that ultimately leads to the nucleus and affects gene transcription (266) (Figure 4). As discussed, the ER-α is thought to be functionally regulated via phosphorylation and estrogen is thought to act in part by the production of paracrine

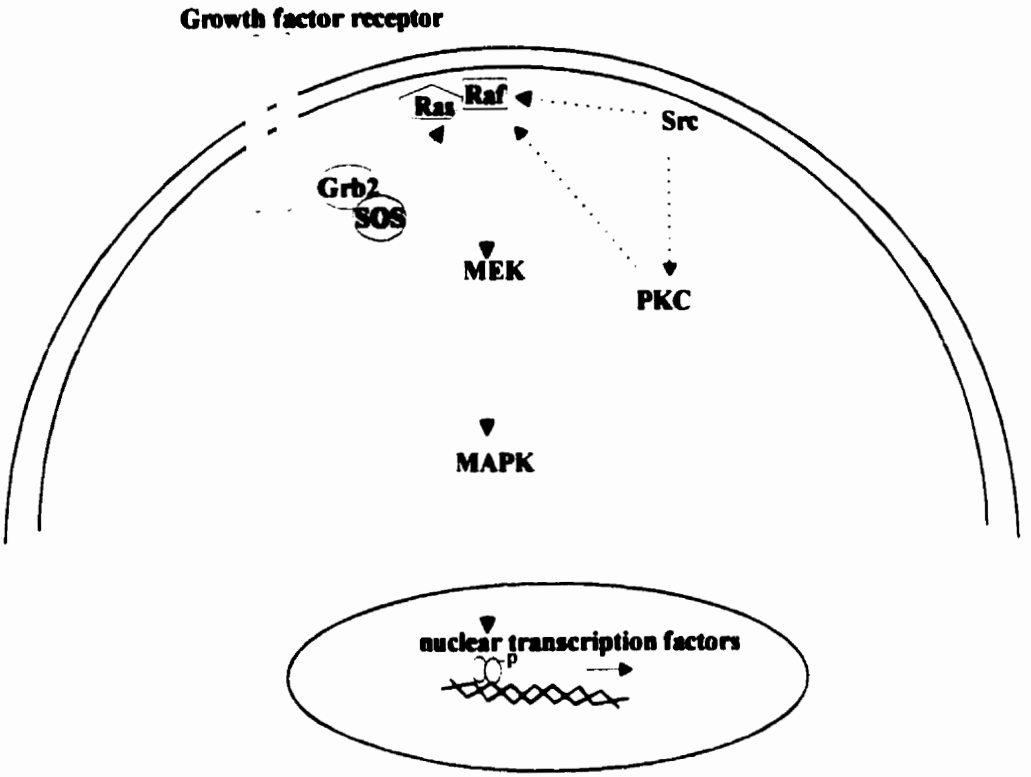


Figure 4. The MAPK signalling cascade. Growth factors (i.e. EGF) activate receptor tyrosine kinases, leading to the activation of the MAPK ERK1 and ERK2 and phosphorylation of substrates, including nuclear transcription factors such as ER-α. Additionally, kinases such as PKC and Src can activate MAPK. Details of the pathway are outlined in the text. A dashed arrow indicates that the activation may be indirect. This figure is not intended to imply that growth factor receptors cannot activate other signalling cascades directly or indirectly.

and/or autocrine growth regulatory substances many of which can interact with cell-surface tyrosine kinase receptors to activate protein kinase cascades.

Breast tumours have been shown to contain elevated tyrosine kinase activities compared to benign breast tumours and normal breast tissues (267,268). Human breast tumours have been shown to contain elevated levels of Src tyrosine kinase activity compared to normal tissue (268) and one study has estimated that 70% of the elevated tyrosine kinase activity found in breast cancer may be due to increased Src-like activity (269). Elevated levels of MAPK activity and expression have been associated with the malignant phenotype and have been demonstrated in breast tumours compared to normal tissue and benign breast conditions (270). MAPKs are serine/threonine kinases that are intermediates in a signal transduction cascade that can be activated by tyrosine kinases such as Src and the EGF receptor (EGFR). These data suggest that an increase or de-regulation of growth controlling signals, such as those contributed by MAPK, may be involved in the etiology and pathogenesis of breast cancer, as well as affecting ER-α activity.

Mitogen-activated protein kinases

Mitogen-activated protein kinases (MAPK or extracellular signal-related kinase (ERK)) are a family of protein kinases involved in transmitting signals from a variety of stimuli from the cell membrane to the nucleus. ERK1 and ERK2, the first mammalian MAPK isoforms identified, are highly homologous proteins of 44 kDa and 42 kDa, respectively. Once activated ERK1 and ERK2 phosphorylate a variety of proteins, including transcription factors, to effect changes in gene expression, resulting in cell growth, differentiation or apoptosis (266). A cascade of protein kinases regulate and activate MAPK via

phosphorylation on both threonine and tyrosine residues (266,271). It is known that multiple MAPK cascades exist, but the most widely studied pathway involves activation of MAPKs, ERK1 and ERK2, via growth factor receptor tyrosine kinases such as EGFR (266).

Tyrosine phosphorylated residues of growth factor receptors interact with SH2 (Src-homology 2) domains of adapter proteins such as Grb2 (growth factor receptor-bound protein 2) (271). Guanine nucleotide exchange factors (i.e., son of sevenless (SOS)) are next recruited to the membrane where they activate Ras by promoting the association of Ras with guanosine 5'-triphosphate (GTP) (272). GTP-bound Ras interacts with the serine/threonine kinase, Raf, which is activated via recruitment to the plasma membrane by Ras and upon activation is hyperphosphorylated (266).

The next downstream signal in the MAPK cascade is MEK (mitogen-activated, ERK-activating kinase), a target of activated Raf (271). The MEK isoforms 1 and 2 are believed to be highly specific for ERK1 and ERK2 and phosphorylate the MAPKs on threonine and tyrosine residues (at the sequence threonine-glutamic acid-tyrosine), both of which are required for full activation (273). ERK1 and ERK2 recognize and phosphorylate a wide range of substrates, including transcription factors and cytoskeletal components and are located in both the cytoplasm and the nucleus (266).

As previously described, the ER-α, is believed to be functionally regulated via phosphorylation by several protein kinases including MAPK. Serine 118 of the hER-α has been shown to be phosphorylated by MAPK in response to receptor activation by estradiol and growth factors, including EGF, and mutation of this site to an alanine residue severely diminished ER-α transcriptional ability (232,246).

In several cell types, including MCF-7 human breast cancer cells, estradiol has been shown to rapidly increase MAPK activity (274-277). That this activation requires the ER-α was demonstrated in cells via transient transfection experiments showing an absolute requirement for ER-α for activation and in MCF-7 cells the addition of the antiestrogen ICI 182,780 blocked estrogen-induced MAPK activation (276). A recent report was able to show growth factor, but not estrogen, induced activation of MAPK in MCF-7 cells (240). The discrepancy between this report and a previous report demonstrating estrogen activation of MAPK in MCF-7 cells (276) is unclear, but may be due to differences in the experimental conditions under which activation was assayed between the two papers. Growth arrested MCF-7 cells can be stimulated mitogenically by estrogen, also without activation of ERK1 and 2 (278), again suggesting that estradiol treatment does not necessarily result in the activation the MAPKs, ERK1 and 2.

In T47D human breast cancer cells progestins can activate the MAPK pathway. Transient transfection studies demonstrated that progestin-induced MAPK activation required the ER-α and the antiestrogens ICI 182,780 and hydroxytamoxifen were capable of blocking progestin induction of MAPK (279). Immunoprecipitation experiments demonstrated an association of hER-α with PR and also c-Src (279). Both estradiol and progestin have been shown to activate c-Src (276,279) and in immunoprecipitates from T47D cells c-Src interacted with hER-α in the presence, but not in the absence, of either estradiol or progestin (279). Together these data suggest that c-Src may be an initial target of estradiol or ER-α mediated activation of the MAPK pathway. Additionally, the tumour promoter thapsigargin has been shown to activate MAPK through Src stimulation of Raf-1 kinase (280), providing

a further link between elevated Src-like activity in human breast cancer cells and the activation of MAPK which can lead to activation of the ER-α.

Growth factors and their receptors

The observations that growth factors such as EGF could mimic the effects of estrogen led to the idea that such signalling systems may play a role in ER-α action (126,245). Polypeptide growth factors, such as EGF and IGF-I and -II, are potent mitogens of human breast cancer. The fact that estrogens can also regulate the expression of the ligands and receptors for growth factors suggests that they can also act in an autocrine or paracrine fashion to mediate estrogen-induced proliferation. As discussed in previous sections, growth factors (such as EGF) can activate the MAPK pathway resulting in the phosphorylation and activation of ER-α. The receptor for EGF belongs to a family of receptor tyrosine kinases, consisting of the epidermal growth factor receptor (EGFR/erbB-1), erbB-2 (HER-2/neu), erbB-3, and erbB-4 receptors (126,281). A role in breast cancer development has been suggested for some members of this family. For example, the gene for erbB-2 has been shown to be amplified and/or overexpressed in 30% of human breast tumours (282-284) and in transgenic mice, expression of c-erbB-2 in the mammary glands led to the development of mammary tumours (285). In human breast cancer cell lines, overexpression of erbB-2 has been associated with elevated levels of MAPK activity (286) and in growth arrested human breast cancer cell lines, restimulation of cell growth via the addition of heregulin-β2 (a ligand for erbB-3 and erbB-4) required the activation of MAPK (287). Studies have suggested that erbB-2 amplification or overexpression may be of prognostic value in human breast cancer. Amplification of c-erbB-2 and erbB-2 protein levels have been correlated with lymph node

involvement, ER-α status, nuclear grade and tumour size, in human breast tumour biopsy samples (284,288). However, a recent report suggests that erbB-2 expression is not associated with response to tamoxifen treatment or survival in ER-α positive metastatic breast tumours (289).

The EGFR is a transmembrane receptor with tyrosine kinase activity (290). The EGFR has been found to be overexpressed in some breast carcinomas and the presence of EGFR is inversely correlated with expression of ER-α (291). High levels of EGFR in human breast tumours have also been correlated with failure to respond to antiestrogen treatment (292,293). The inverse correlation between ER-α and EGFR expression can also be seen in breast cancer cell lines (294). A ligand for the EGFR, EGF, is expressed in human breast tumour biopsy samples and some breast cancer cell lines in culture, including MCF-7 (295,296) and addition of EGF to breast cancer cells in culture can decrease growth inhibition in response to hydroxytamoxifen treatment (127). EGF has also been shown to be able to stimulate MCF-7 tumour growth in nude mice (297). Interestingly, EGF is able to activate the ER-α in the absence of estrogen and upregulation of EGFR expression may be a mechanism that can lead to the loss of estrogen-dependent growth as will be discussed in more detail in following sections. TGFα also binds to and activates the EGFR (298) and is expressed by a variety of ER-α positive and negative human breast cancer cell lines and primary human breast tumours (299). Estrogen can induce TGFα expression in MCF-7 cells in culture and in MCF-7 tumours in nude mice (299) and exogenous TGFα can increase the proliferation of MCF-7 cells in culture in the absence of estrogen (140). TGFα levels have been shown to be reduced in tumours from tamoxifen treated ER-α positive breast cancer

patients (128) and tamoxifen and hydroxytamoxifen treatment inhibits the expression of TGFα in breast cancer cells in culture (127). These data suggest that TGFα expression may be involved in estrogen-regulation of breast cancer cell growth. Overexpression of TGFα in the mammary glands of transgenic mice can lead to accelerated progression of carcinogen-induced tumours and hyperproliferation that can result in mammary cancers after pregnancy, suggesting TGFα may be involved in the early stages of mammary cancer (300-302). Transfection of TGFα into an immortalized normal human breast epithelial cell line (MCF10A) resulted in transformation *in vitro* (303) while transfection in MCF-7 cells had little effect on cell growth (304), supporting a role of TGFα in breast cancer initiation.

The insulin-like growth factors, IGF-I and IGF-II, can stimulate the proliferation of human breast cancer cells in culture (305,306) and exogenous IGF-I has been shown to stimulate MCF-7 tumour growth in nude mice (297). IGF-I and IGF-II mRNAs have been detected in breast tumour biopsy samples and *in situ* hybridization has demonstrated that it is mainly the stromal compartment of breast tissue specimens that expresses IGF-I and IGF-II, suggesting a paracrine role for these growth factors (307,308). Breast cancer cell lines do not apparently express IGF-I (307) and most, including MCF-7, do not express IGF-II (308). Circulating levels of IGF-I have been found to be decreased in breast cancer patients during tamoxifen therapy (129), suggesting that tamoxifen may exert some growth inhibitory effects by decreasing production of endogenous IGF-1 levels.

The mitogenic effects of IGF-I and -II are mediated by the type I IGF receptor (IGF-IR) which possesses tyrosine kinase activity and can activate the Ras-MAPK pathway (309). IGF-IR is expressed in almost all breast tumour biopsy samples and breast cancer cell lines

(309) and several studies have reported overexpression of IGF-IR in breast tumours compared to normal breast tissue (310,311). Resnik *et al* demonstrated that IGF-IR expression was 14-fold higher in malignant breast tissue than in normal breast tissue and IGF-IR autophosphorylation and kinase activity were also elevated (310). In human breast tumours the expression of IGF-IR is positively correlated with ER-α expression (311) and estrogen increases IGF-IR levels in MCF-7 cells (312).

IGF-II can bind not only to the IGF-IR, but also the insulin receptor and IGF-II receptor (IGF-IIR) (281). The insulin receptor, which can activate the MAPK pathway, has also been shown to be elevated in human breast tumours compared to normal tissue (313) and in some human breast cancer cell lines compared to a nonmalignant human breast epithelial cell line (314). The role of signalling through the IGF-IIR in human breast cancer is unclear but there is evidence to suggest it may play a role in breast tumour invasion (315).

Together the data suggest that these growth factors and their signalling pathways may be implicated in breast cancer growth and development but many questions regarding their role in tumour progression and receptor function remain unanswered. Peptide growth factor signalling pathways can crosstalk with the ER-α and it has been demonstrated that growth factors can result in ligand-independent activation of the ER-α (316) and these growth factor signalling pathways have been implicated in estrogen-independent growth as will be discussed in a later section.

Tissue matrix system

How a cell behaves or responds to any particular stimulus is controlled in an overall sense by a network of signals derived from both soluble and insoluble cellular components

such as growth factors, the extracellular matrix, the cytoskeleton and the nuclear matrix, to effect changes in gene expression. This system of dynamic linkages and interactions is referred to as the tissue matrix system and consists of links between the nuclear matrix (NM), the cytoskeleton and the extracellular matrix (ECM), and forms a structural and functional connection from the cell periphery to the DNA (317). The cytoskeleton is composed, in part, of intermediate filaments (IFs) and cytokeratins are members of the intermediate filament family of proteins (318). Direct connections (via intermediate filaments) between the cell periphery and the NM have been demonstrated and this nuclear matrix-intermediate filament (NM-IF) system is altered by tumour promoters and in oncogene-transformed cells (319-321). Direct evidence of a continuous network connecting the plasma membrane structure and cytoskeleton with the nucleoskeleton of eukaryotic cells is provided by data demonstrating that vimentin is anchored directly to the nuclear lamina via lamin B (322,323). Evidence also suggests that the intermediate filaments (including the lamins and cytokeratins) not only exist at the nuclear periphery but are also found as part of the internal NM (324-326).

Architectural alterations (defined by NM proteins, and/or interactions with the cytoskeleton or ECM) within, or associated with, the nucleus may influence or control what genes or subsets of genes are actively transcribed. IFs can interact with RNA and DNA (327) and cytokeratins can interact with nuclear DNA (326). In cultured murine mammary epithelial cells, the ECM has been shown to regulate tissue-specific gene expression (328) and modulating the cytoskeleton in murine mammary epithelial cells in culture with agents that disrupt actin and cytokeratin filament networks can alter milk protein synthesis (329,330). Reversion of the malignant phenotype using antibodies directed at ECM components of

human breast cancer cells in culture has also been demonstrated (331). Together these data demonstrate that not only is the cytoskeleton physically connected to, and may even be considered, an intimate part of the nucleus/NM structure, but that the nucleus can respond to signals from the structural organization of the cytoskeleton and ECM in order to modulate gene expression.

Nuclear matrix

The NM is a protein and RNA containing network within the nucleus that comprises the nuclear pore lamina, an internal fibrogranular RNA and protein containing network, and residual nucleolus (332). The NM exists not only as a structural entity but is thought to play an inherent role in many important nuclear processes including; DNA organization and replication, gene transcription and processing, and steroid hormone action (333-337). The expression of several NM proteins has been shown to be cell, tissue, differentiation, and hormonal state specific (338-340). Differences in NM protein expression exist between normal tissues and their cancerous counterparts and more specifically, differences have been shown between normal breast tissue and breast tumour tissue (341,342). Additionally, specific NM proteins have been shown to be associated with ER-α status in human breast cancer cell lines and human breast tumours (343). Several steroid hormone receptors, including the ER-α, have been shown to localize to the NM in steroid target tissue (111,337). Cell-free binding assays have confirmed that this localization of the mER-α is due to the presence of specific acceptor sites in the NM to which steroid-receptor complexes bind with high affinity and tissue specificity (344). Patterns of NM protein expression are hypothesized to be involved in changes in gene expression and it is believed that specific proteins of the NM

can influence gene expression (345). How protein changes in the NM could influence gene expression is unclear, but transcriptionally active genes have been shown to be associated with the NM, whereas inactive genes are not (334).

Cytoskeleton

The cytoskeleton is composed of an interacting network of actin microtubules, microfilaments and IFs (317). The IFs are a heterogeneous group of proteins that can interact directly with the nucleus and plasma membrane, providing a link between the ECM and the nucleus (317). IFs are expressed in a tissue- and differentiation-specific manner and the cytokeratin IFs are characteristic of epithelial cells (318). ER-α positive human breast cancer cells that are epithelial in origin generally express the cytokeratins 8, 18 and 19 (318).

The cytokeratins (like other IF proteins) form a cage-like structure around the nucleus and a dense network throughout the cytoplasm (346). Studies have shown that IF proteins, including cytokeratins, can associate directly with membrane phospholipids, notably phosphatidyl-inositol-4,5-bisphosphate, and may be involved in second messenger signalling pathways (317,347). Research has also suggested a possible role for IFs in steroid biosynthesis. It has been shown in a human adrenal tumour cell line that vimentin filaments are required for the intracellular movement and esterification of low density lipoprotein-derived cholesterol (348). Cytokeratin expression has also been correlated with tumour progression and grade, suggesting that cytokeratin expression may be involved in the tumour phenotype (349,350).

Hormone-independence

Breast cancer is a hormonally-responsive cancer and hormones including estrogen, are

required for breast cancer growth. As discussed previously, estrogens promote the growth of human breast cancer, and as such, most therapies are aimed at blocking the growth promoting effects of estrogen. Breast cancers are classified according to their requirement for proliferation as being either hormone-dependent or hormone-independent, based ultimately on the response to endocrine therapy of metastatic disease (351). At the time of clinical detection approximately 70% of human breast cancers contain ER-α, but only 60% of these will respond to endocrine therapy and are thus classified as hormone-dependent. Therefore, approximately 40% of ER-α positive tumours are resistant to endocrine therapy. Even of those tumours that originally respond to endocrine therapy, the majority will eventually develop resistance to this type of therapy.

The evolution of breast cancer into an estrogen-independent growth phenotype marks the beginning of a more aggressive phase of the disease and is a major problem in the efficacy of endocrine therapies such as antiestrogens (56,57). Understanding the factors that contribute to the development of a hormone-independent phenotype is of major importance in terms of breast cancer therapeutics.

Several breast cancer cell lines in culture also require estrogen for growth. Long-term culture in estrogen-deplete conditions can result in these cells becoming independent of the requirement for estrogen for growth. Indeed, the development of estrogen-nonresponsive growth in human breast cancer is thought to be one of the initial steps in the progression to hormone-independence (352). Although many possibilities exist for the development of endocrine resistance, and multiple factors are likely involved, the mechanisms are still unknown. In some cases hormone-independence and resistance can occur due to loss of ER-α

expression, but most tumours which have developed resistance to endocrine therapy remain receptor positive (353). Since many endocrine resistant tumours continue to express ER-α, defects in the ER-α structure and function or activation of the ER-α via ligand-independent mechanisms may result in resistance of tumours to endocrine therapy. It is evident from the preceding discussions that many factors can influence the activity of the ER-α and therefore there are many areas that could be perturbed leading to hormone-independence. Some of these have previously been discussed and several of the more pertinent mechanisms will be discussed in more detail in the proceeding sections.

Hormone-nonresponsive growth

In vitro studies to examine the effects of long-term growth in estrogen-free conditions became possible following the discovery that contaminants of phenol red, a pH indicator used in cell culture medium, were in fact estrogenic (261). This allowed researchers to study the effects of estrogen-deprivation via the use of cells cultured in the absence of phenol red and serum-steroids (354). Long-term growth of ER-α positive breast cancer cells (MCF-7, T47D and ZR-75-1) in estrogen-free conditions has been used to study the development of hormone-independence (355-363). Long-term growth in estrogen-free conditions can result in the cells becoming apparently unresponsive to estrogen in terms of growth and can in some instances result in the cells no longer responding to growth inhibition by antiestrogen. Jiang and coworkers obtained a clone of MCF-7 cells (5C), after long-term culture in estrogen-deplete conditions, that was unresponsive to both estrogen and the antiestrogens hydroxytamoxifen and ICI 164,384 in terms of growth (355). 5C cells contained wild-type ER-α at levels similar to parental cells but synthesis of PR in response to estrogen was

reduced as was estrogen-responsive reporter-gene expression (355).

Other researchers have shown that although long-term growth in estrogen-free conditions can result in apparently estrogen-independent growth, PR synthesis and steroidal and nonsteroidal antiestrogen inhibition of growth and reporter-gene expression is often retained (354,356-358,361). Estrogen-independent, ER-α positive sublines of MCF-7 cells have been isolated both *in vitro* (BSK cell lines) and *in vivo* in the nude mouse (MIII cell line) through prolonged growth in estrogen-free conditions (356,357). Katzenellenbogen *et al* have shown that while long-term estrogen-deprived MCF-7 cells (BSK-3) no longer responded to estrogen with increased proliferation, hydroxytamoxifen was still able to inhibit the proliferation of these cells (356). PR expression was still responsive to estrogen in BSK-3 cells, demonstrating a dissociation of estrogen-nonresponsive growth and expression of an endogenous estrogen-regulated gene (356). Clarke and coworkers demonstrated that both the BSK and MIII sublines were able to form tumours in ovariectomized nude mice in the absence of estrogen (357). Brunner *et al* continued study of the MIII subline and isolated a second line, LCC1, from a rapidly proliferating MIII tumour in ovariectomized nude mice (364). While the LCC1 cells are apparently estrogen-independent in terms of *in vitro* growth and display increased metastatic potential compared to the MIII cells, hydroxytamoxifen is still growth inhibitory to these cells (364). LCC1 cells contain ER-α levels equivalent to parental cells and a high constitutive level of PR that is still estrogen inducible (364). LCC1 cells were further selected *in vitro* for resistance to hydroxytamoxifen to produce the cell line, LCC2 (365). LCC2 cells retain ER-α and PR levels comparable to the parental cell line and PR remains estrogen-inducible (365). Hydroxytamoxifen is not growth inhibitory to the LCC2

cells *in vitro* or *in vivo* in nude mice, but these cells retain sensitivity to the steroidal antiestrogen ICI 182,780 *in vitro* (365). The LCC1 cells were also selected for *in vitro* resistance to the steroidal antiestrogen ICI 182,780, to generate the LCC9 cell line (366). The LCC9 cells are also resistant to *in vivo* growth inhibition by ICI 182,780 and additionally have developed cross-resistance to hydroxytamoxifen *in vitro* and *in vivo*. The level of ER-α in LCC9 cells is comparable to the parental cell line, but the basal PR levels is elevated and no longer responsive to estrogen treatment (366). While this study suggests that resistance to steroidal antiestrogen can result in a general loss of estrogen-responsiveness and cross-resistance to the nonsteroidal antiestrogen hydroxytamoxifen, other studies demonstrate this is not always the case. Several reports have demonstrated that the *in vitro* development of resistance to ICI 182,780 or tamoxifen via long-term growth of MCF-7 cells in the presence of either antiestrogen did not result in lack of general estrogen-responsiveness and the resistant lines required estrogen for *in vivo* tumour formation in nude mice (367,368). In contrast to the LCC2 and LCC9 antiestrogen-resistant lines, both the tamoxifen-resistant (MCF-7/TAM^R) and the ICI 182,780-resistant (MCF-7/182^R) lines maintained expression of the ER-α but demonstrated a complete lack of PR expression (367,368). In addition, the MCF-7/TAM^R line was not cross-resistant to steroidal antiestrogens, nor was the MCF-7/182^R line cross-resistant to tamoxifen-mediated growth-inhibition (367,368). Therefore, like loss of estrogen-responsive growth, antiestrogen-resistant growth does not necessarily result in a general lack of estrogen-responsiveness and again demonstrates a dissociation between growth responses and regulation of gene expression. This is likely a result of the multiple factors involved in the development of estrogen- and/or antiestrogen-nonresponsive growth

and the complexity of the regulation of the function of the ER-α.

The effects of long-term estrogen-deprived growth of T47D and ZR-75-1 human breast cancer cells has also been examined (358,361-363). Murphy and coworkers examined the effects of long-term estrogen-free culture conditions on T47D (T47D:C) human breast cancer cells (362,363). T47D:C cells no longer express detectable ER-α protein or mRNA, but still retain a low level of PR protein expression that was not responsive to estrogen (363). A clone of the T47D:C cells (4) was also found to be both ER-α and PR negative (363). Estradiol, hydroxytamoxifen, and ICI 164,384 had no effect on the growth of these cells (T47D:C and T47D:C4) likely as a result of lack of ER-α expression (362,363). Another group of researchers examined the effects of long-term culture in estrogen-deplete conditions on T47D cells (358). Again, this resulted in estrogen-nonresponsive growth *in vitro* with the cells remaining responsive to growth inhibition to hydroxytamoxifen treatment (358). In contrast to the previous study, these cells had 3-fold higher ER-α levels and high basal PR levels which, similar to parental cells, could only be marginally upregulated with estrogen treatment (358). This group also examined the effects of long-term estrogen-deprivation on ZR-75-1 cells and again demonstrated that this resulted in the apparent loss of estrogen-responsive growth *in vitro* but a retention of hydroxytamoxifen growth inhibition. This was accompanied by a 2-fold increase in ER-α levels and in one clone (4) PR levels were similar to parental cells and remained fully estrogen-inducible, while in another clone (11A) PR levels were elevated and less estrogen-inducible (358). van den Berg and Lynch have also examined the effects of long-term estrogen-deplete culture conditions on ZR-75-1 cells and again have demonstrated that this results in apparently estrogen-nonresponsive *in vitro* growth (361).

While ER-α protein expression was no longer detectable in these cells using ligand-binding techniques, tamoxifen is still somewhat growth inhibitory to these cells. Basal PR levels were elevated above parental estrogen-stimulated values in the estrogen-nonresponsive cells and were no longer estrogen-inducible (361).

These data demonstrate the complexity of the acquisition of a hormone-independent phenotype and underscore the fact that multiple mechanisms likely exist. In most cases there is a dissociation between estrogen-nonresponsive growth, antiestrogen-resistance and regulation of estrogen-responsive genes. While in some cases loss of hormone-responsive growth can be attributed to loss of ER-α expression, in most cases the cells retained expression of ER-α. This would suggest that alterations in the function of the ER-α are responsible for the estrogen-nonresponsive growth phenotype seen in these studies.

Variant ER-α mRNAs

Naturally occurring mutant or variant forms of the ER-α have been identified, as have polymorphic forms of the ER-α gene. ER-α gene polymorphisms have been detected in primary and metastatic breast tumours (248,369-373) but in most cases correlations with clinical parameters have not been found (372). A PvuII restriction fragment length polymorphism (RFLP) was found to correlate with patient age but not ER-α expression in one study (369), while another found no correlation with age or ER-α expression (374) and a third correlated this RFLP with ER-α expression (375). Garcia and coworkers have identified a genetic polymorphism in the B-coding region of hER-α mRNA, that correlates with lower levels of hormone-binding activity in some ER-α positive breast tumours

(373,376) and a high incidence of spontaneous abortion in women with ER-α positive breast cancer (377).

In primary breast cancers naturally occurring mutations of the ER-α gene appear to be quite rare (105,378) and point mutations in the ER-α gene occur in only approximately 1% of primary breast tumours (370,372). Mutations that alter the amino acid sequence of ER-α have been detected in both primary and metastatic breast cancer (248,370-373,379,380) (Table 2).

There has only been one reported case in humans of a germline mutation in the wild-type ER-α. This was a 28 year old male who is very tall, with incomplete epiphyseal closure and osteoporosis (381). The ER-α in this man was found to contain a point mutation in exon 2 resulting in a stop codon that would result in a severely truncated ER-α protein without any HBD or DBD, consistent with the fact this individual had no detectable response to estrogen administration (381).

Many laboratories, including Dr.L.C. Murphy's, have detected variant ER-α mRNA transcripts in breast cancer biopsy samples, breast cancer cell lines, and normal breast tissue (382-386). Currently, three main types of altered or variant ER-α mRNAs have been identified in human breast cancers: exon deleted, truncated, and exon duplicated or inserted ER-α-like transcripts. Altered ER-α mRNA is found, most often, along with the wild-type ER-α mRNA transcript and in some cases, variant transcripts occur in high abundance relative to normal ER-α mRNA in some human breast cancer biopsy samples (385,387). A brief description of some of the different types of variant ER-α mRNA transcripts follows and

Table 2. ER-α mutations and polymorphisms in human breast tumours

Nucleotide change	Codon/protein change	Exon
T1119----C	Leu296----Pro	4
A1290----T	Glu352----Val	4
A1418----G	Met396----Val	5
C439-----G	Asn69----Lys	1
T1609----A	Tyr537----Asn	8
G140----C	Ser47----Thr	1
T1591----G	Lys531----Glu	8
C701----T	truncated at 156	2
Gdel at 1463	Asp411--Thr, truncated at 417	5
T del at 1526	Ser432 truncated at 436	5
TT insert at 982	Met250--Ile, truncated at 251	3
1271-1318 exon 6 replaced with 1148-1190 of exon 5	truncated at 454	6
G1646----A	Lys472 none	7
C1059----T	Gly276 none	4
C1747---G	Ala505 none	7
T1963---C	His577 none	8
A2014----G	Thr594 none	8
T262----C	Ser10 none	1
G493----C	Ala87 none	1
C961----T	Arg243 none	3
C1207---G	Pro325 none	4

Adapted from (379,380)

figure 5 demonstrates the structure of these transcripts compared to the wild-type hER-α mRNA.

Single exon deleted

An ER-α mRNA variant lacking exon 2 (ERd2) has been detected in breast tumours, normal breast tissue and breast cancer cell lines (382,388-391). ERd2 would encode a truncated 16 kDa protein comprising only the A/B domain of wild-type ER-α. Variant ER-α mRNA with an inframe deletion of exon 3 (ERd3) has been detected in human breast cancer cell lines, breast tumours and normal human breast cells (382,387,391-393). The putative protein of 61 kDa would be missing the second zinc finger of the DBD and *in vitro* recombinantly expressed ERd3 cannot bind DNA (391). The ERd4 mRNA contains an inframe deletion of exon 4, which could encode a 54kDa protein lacking the hinge region (D) and a portion of the HBD of wild-type ER-α. The ERd4 protein is unable to bind to DNA or hormone *in vitro* (394). ERd4 mRNA has been detected in normal breast tissue, breast tumours and breast cancer cell lines (382,384,395-398). The exon 5 deleted ER-α mRNA (ERd5) could encode a 40 kDa truncated protein, lacking a majority of the HBD (399). Initially ERd5 was detected in ER-α negative/PR positive tumours (399) but has subsequently been detected in normal breast tissue as well as ER-α positive breast tumours and ER-α positive and negative breast cancer cell lines (382,387,400,401). The exon 7-deleted ER-α mRNA (ERd7) is the most abundant ER-α deleted variant in breast tumours (384,387) and has also been detected in normal breast tissue and breast cancer cell lines (382,384,395). ERd7 would encode a truncated protein of approximately 51 kDa, missing the C-terminal portion of the HBD, including the AF-2 region, and the F domain. This putative protein

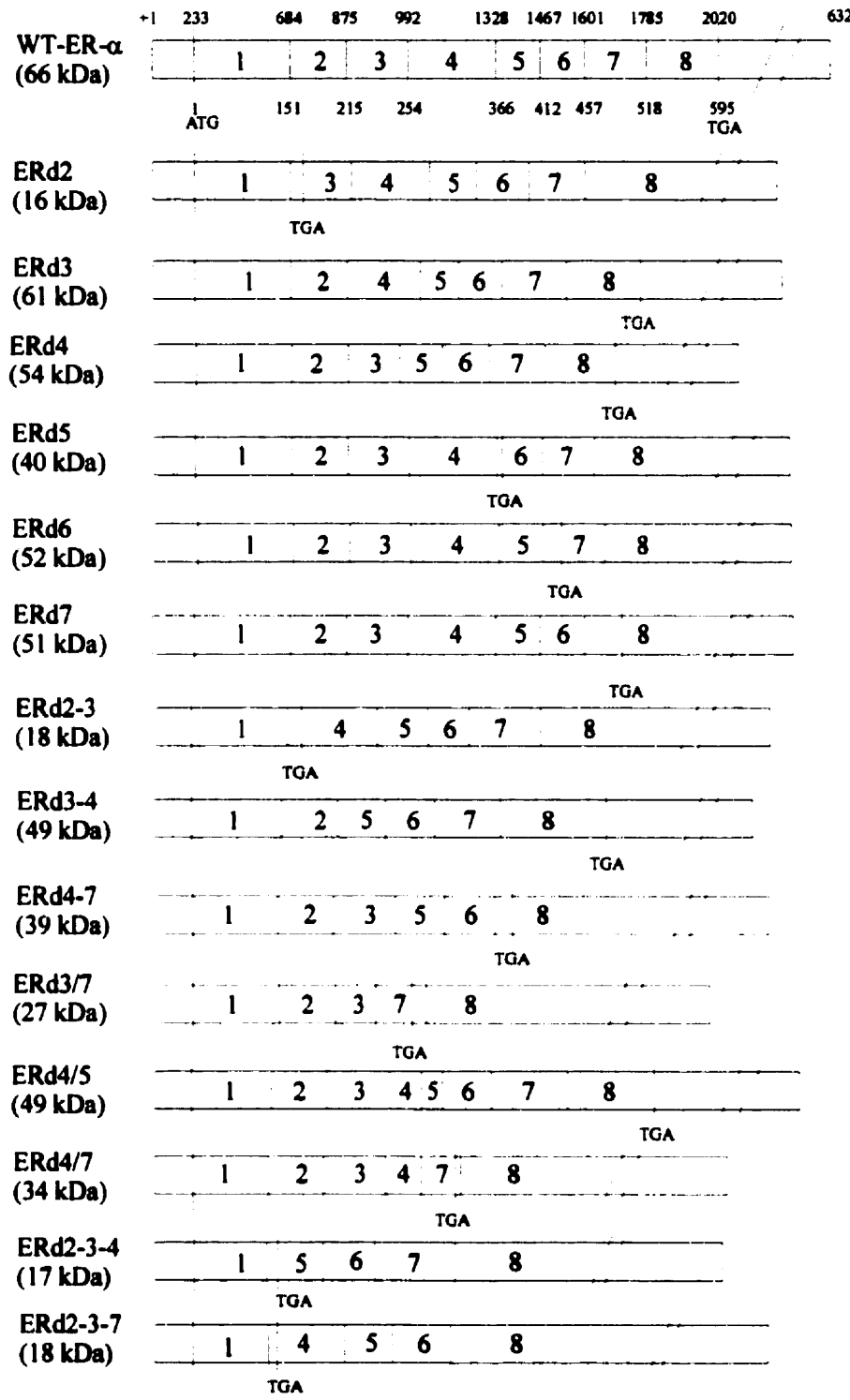


Figure 5. Schematic diagram of variant ER-α cDNA compared to wild-type ER-α (WT-ER-α) cDNA. The numbering on top of WT-ER-α cDNA refers to nucleotide position (67). Numbering below refers to amino acid position (67). Adapted from (380).

would be identical to wild-type ER-α up to amino acid 456 after which it would contain 10 novel amino acids.

Multiple exon deleted and other deleted

ER-α mRNA splice variants lacking two or more exons have also been detected in breast tumours, normal breast tissue and breast cancer cell lines (382,384,387-389,395,398). ER-α mRNA with an inframe deletion of both exons 3 and 4 has been detected in both normal breast tissue and breast tumours (384,387,388). An ER-α splice variant lacking both exons 4 and 7 was initially detected in tamoxifen-resistant MCF-7 human breast cancer cells (389) and has been detected in human breast tumours (384). This variant mRNA could result in the generation of a 39 kDa protein with an inframe exon 4 deletion and an out of frame exon 7 deletion, resulting in 10 novel amino acid residues prior to a premature termination of translation (389).

ER-α variant transcripts containing deletions that are not entire exons, but vary in size from a single nucleotide to several hundred nucleotides, have also been identified (370,382,384,402,403) (Table 2 and figure 5). In a tamoxifen-resistant metastatic human breast tumour an ER-α-like mRNA whose corresponding cDNA contained a deletion of a thymidine residue in exon 6 has been detected. This single nucleotide deletion would result in a frameshift in the HBD giving rise to a premature translation termination at amino acid 437, generating a protein that contains wild-type ER-α sequence up to amino acid 432 followed by 5 unique amino acids (370). In breast tumours an ER-α splice variant that consists of a deletion within exon 4 to sequences within exon 7 has been identified (ERd4/7). This could potentially encode a 34 kDa protein, lacking the majority of the HBD and

containing 32 unique amino acids after wild-type ER-α sequence ending at amino acid 277 (403). An ER-α variant mRNA transcript deleted from within exon 3 to within exon 7 (ERd3/7) has been detected in human breast tumours. The putative 27 kDa protein would lack the hinge region and most of the HBD (384). The expression of ERd3/7 mRNA was found to be associated with tumours containing high levels of wild-type ER-α (384). In T47D human breast cancer cells, an ER-α variant mRNA containing an inframe deletion of 460 bases from with exon 4 to within exon 5 (ERd4/5) has been identified. This could encode a putative 49 kDa protein with a 153 amino acid deletion from the DBD to the mid-HBD (402). These same researchers also identified a variant ER-α mRNA transcript in T47D cells that contained a frame-shift mutation in the HBD that resulted from a deletion of a guanine residue at nucleotide 1463. This could lead to the expression of a variant ER-α protein truncated at amino acid 417 in the HBD with 7 unique amino acids at the C-terminus (402).

Insertions/duplications

Altered ER-α transcripts containing duplications of one or more exons have also been identified in breast tumours. An altered ER-α mRNA with a duplication of exon 6 could encode a 51 kDa protein truncated after the exon 6 repeat lacking most of the HBD including AF-2 (404). An exon 3 and 4 duplicated ER-α mRNA has also identified in breast tumours (404) and an ER-α-like mRNA containing an inframe duplication of exons 6 and 7 has been detected in an estrogen-independent subline of MCF-7 cells (405).

An altered ER-α mRNA transcript with an inframe 69 base pair insertion between exon 5 and 6 has been identified in human breast tumours (404). Subsequent studies suggest

the insertion of this sequence, normally present in intron 5 of the ER-α gene, resulted from a point mutation in the ER-α gene of the breast tumour, generating a splice site donor sequence 3' to this 69 bp sequence (406).

Truncations

The detection of ER-α mRNA variants is performed primarily using polymerase chain reaction (PCR) techniques, but initially Murphy and Dotzlaw identified, by Northern blotting, several truncated ER-α mRNA in human breast cancer biopsy samples expressed at high abundance relative to normal ER-α mRNA in some samples (385). Cloning and sequencing of one mRNA transcript (clone 4) revealed it to contain exons 1 and 2 of wild-type ER-α mRNA, after which it contained LINE-1 related sequences (407). The cDNA corresponding to this transcript would encode a 24 kDa protein containing the A/B region and the first zinc binding motif of the DBD of the wild-type ER-α followed by 6 unique amino acids (407). Clone 4 mRNA has been detected in breast tumours, normal breast tissue and breast cancer cell lines (382,384,385).

Expression of variant ER-α proteins

Presently, it is unknown if altered/variant ER-α mRNAs are stably translated *in vivo* but, while still controversial, evidence is emerging to support the existence of ER-α variant proteins which could correspond to some ER-α variant mRNAs in some cell lines and tissues *in vivo* (386,408-413). In BT-20, ER-α negative, human breast cancer cells a protein consistent in size with that predicted to be encoded by the ERd5 mRNA found in these cells, has been detected by Western blotting (409). An 80 kDa ER-α-like protein has been detected

in an estrogen-independent MCF-7 subline that likely corresponds to an exon 6 and 7 duplicated ER-α mRNA detected in the same cells (405).

Immunohistochemical data also suggest the existence of ER-α variant proteins. Huang and coworkers examined primary breast tumours for ER-α protein expression using two ER-α antibodies recognizing individual epitopes in the N- and C-terminal ends of the wild-type ER-α protein (398). The data demonstrated a discordance between the ER-α levels measured by the two antibodies, suggesting the existence of truncated ER-α-like proteins in the discordant tumours. ER-α mRNA expression was also evaluated in these tumours, and in cases that had inconsistent antibody staining, variant ER-α mRNAs, ERd2-3-7, ERd2-3-4, ERd3/7 and clone 4, were elevated (398). Immunohistochemical studies of human breast tumours have identified ER-α that are unable to locate to the nucleus in the presence of hormone (413) and in some ER-α positive tumours, ER-α unable to bind to an ERE has been detected (408,411). Desai and coworkers, using an antibody specific to ERd5 protein, obtained immunohistochemical evidence to suggest that a protein corresponding to this variant ER-α protein is expressed in some breast tumours (414).

In some human breast cancer biopsy samples truncated DNA-binding forms of ER-α-like proteins have been detected (411) and truncated ER-α-like protein has also been detected in ER-α positive/PR negative breast tumours that bound to an ERE in gel mobility shift assays (386). Using site-directed monoclonal ER-α antibodies targeted to specific functional domains of the ER-α, Traish and coworkers have provided evidence to suggest that breast tumours may contain ER-α with defects in specific functional domains (415).

Unfortunately the lack of specific antibodies to the majority of these putative proteins and the fact that many of them are similar in size to heavy chain immunoglobulins makes definitive identification and absolute proof of the existence of such proteins a difficult task. Nevertheless, while still controversial, the data do provide evidence that variant/altered forms of the ER-α protein could exist *in vivo* and in some cases correlate with the presence of specific variant ER-α mRNA transcripts.

Functional significance of variant ER-α mRNA

While the pathophysiological significance of ER-α variant expression is unclear, altered expression of some ER-α variant mRNAs has been shown to be associated with both breast tumourigenesis and breast cancer progression (382,386,389,416,417). The expression of clone 4 relative to wild-type ER-α mRNA was found to be significantly elevated in breast tumour tissue compared to normal breast tissue (382,416). Increased expression of clone 4 variant ER-α mRNA has been associated with breast tumours with characteristics of poor prognosis and markers of reduced endocrine sensitivity (417), suggesting that increased expression of some variant ER-α mRNAs relative to wild-type ER-α could be involved in breast cancer development and/or progression. ERd5 mRNA levels have been found to be increased in breast tumour compared to normal breast tissue (414) and the expression of ERd5 mRNA relative to wild-type ER-α mRNA has been found to be significantly higher in some breast tumours compared to normal tissue (382,400). ERd5 mRNA has also been detected in ER-α negative/PR positive human breast tumours (400) and a study suggests that ERd5 mRNA may be elevated in ER-α negative tumours which express PR or the estrogen-responsive protein pS2 (418). This type of data led to suggestions that ERd5 may be involved

in breast tumour progression, but low to undetectable levels of this variant have been demonstrated when compared to multiple variant ER-α transcripts in a range of human breast tumours (384), suggesting that increased expression of ERd5 may not be a general phenomenon. Furthermore, levels of ERd5 mRNA have been associated in tumours from patients with increased disease-free survival, suggesting that it may be an indicator of good prognosis (414). Leygue and coworkers examined ER-α mRNA splice variants in 100 breast tumours and increased expression of ER-α mRNA splice variants, ERd2-3-4 and ERd3/7, was associated with higher grade and increased wild-type ER-α, respectively (384). Increased levels of ERd7 mRNA are often found in ER-α positive/PR negative breast tumours (386), suggesting it may interfere with normal ER-α function. ERd4 mRNA expression was shown to be more common in PR positive tumours and was associated with markers of good prognosis (384) and the level of ERd3 mRNA compared to wild-type ER-α mRNA has been shown to be reduced in breast tumour tissue compared with normal tissue (393). It is presently still unclear what role altered ER-α variant mRNA expression may play in breast tumour development or progression, but the findings that increased or decreased expression of ER-α variant mRNAs has been found in different breast tumour phenotypes suggests the potential for alterations in the balance of variant mRNA expression to affect wild-type ER-α function.

Several studies, using transient transfection analyses, have shown that individual ER-α variant proteins, if expressed *in vivo*, may have the ability to interfere with wild-type ER-α activity (386,391,399,405,409,414,419). For some ER-α variants, conflicting results have

been obtained (391,420) which may be due to cell- and promoter-specific events previously described for various structural/functional domains of the wild-type ER-α. Similarly, overexpression of a single ER-α variant using stable transfection technology has resulted in different results in different laboratories (419,421). For example, in a yeast transient expression system, ERd3 variant had no intrinsic transcriptional activity nor did it affect the activity of co-expressed wild-type ER-α (420). This is in contrast to data obtained in mammalian cells. In HeLa cells, transiently expressed ERd3 displayed no intrinsic transcriptional activity but was able to inhibit the transcriptional activity of co-expressed wild-type ER-α (391). In addition, under conditions in which ERd3 protein was unable to bind an ERE in gel mobility shift assays, it was able to inhibit the ability of wild-type ER-α to bind an ERE (391). These studies suggest that ERd3 is able to function in a dominant-negative fashion to interfere with wild-type ER-α activity. Stable transfection of ERd3 in MCF-7 cells resulted in reduced *in vivo* invasiveness and anchorage-independent growth, suggesting that it is able to suppress the transformed phenotype (393). In yeast cells and chicken embryo fibroblasts, transiently expressed ERd5 displayed ligand-independent transcriptional activity (399,419). Stable transfection of an ERd5 expression vector into MCF-7 cells demonstrated little effect in one instance (419), while in another, it resulted in estrogen-independence and tamoxifen-resistance (421). While the reasons for the discrepancies between these reports are unclear, a study has demonstrated a correlation between reduced estrogen-responsiveness and increased ERd5 mRNA expression in stocks of MCF-7 cells throughout North America, suggesting that levels of endogenous ERd5 may influence hormone-responsiveness and could influence the results obtained by different laboratories using different stocks of MCF-7 cells

(422). Conflicting data have also been obtained for ERd7, in which it had no activity when transiently expressed with wild-type ER-α in HeLa cells, (391) but in a yeast transient expression system was able to act in a dominant-negative fashion and inhibit wild-type ER-α activity (386). Cell-specific differences are likely to account for the differences obtained in these two studies.

Important in interpreting the data on the individual ER-α variants is the fact that not only are these mRNAs often expressed along with the wild-type ER-α, but multiple ER-α variant mRNA species are often expressed together. Therefore, it is likely that the combined expression of several ER-α mRNA species may play a role, as yet poorly defined, in the overall activity of the ER-α in any particular cell.

It has been speculated that some variant ER-α mRNAs could encode proteins involved in the development of hormone-independence. Jordan and coworkers isolated a subclone of MCF-7 human breast cancer cells (MCF-7:2A) through long-term growth in estrogen-free conditions that exhibits apparently estrogen-nonresponsive growth (410). These cells express an 80 kDa ER-α-like protein detected using Western blotting techniques (410). Analysis of ER-α mRNA transcripts in the MCF-7:2A cells revealed the presence of an ER-α variant mRNA with an inframe duplication of exons 6 and 7 (405). This variant mRNA could encode a protein of 80 kDa, likely corresponding to the 80 kDa protein detected by Western blotting, which may be involved in the evolution of the estrogen-independent growth phenotype of these cells (405). Subsequent studies on the exon 6 and 7 duplicated ER-α variant demonstrated it was incapable of ligand binding, but could bind to an ERE *in vitro* and overexpression of this variant in T47D cells resulted in inhibition of wild-type ER-α

transcriptional activity in transient transfection analyses (423). Sublines of T47D cells have also been found to contain abnormal ER-α mRNA and it has been proposed that heterodimers of wild-type and mutant ER-α proteins could override the estrogen requirement of the wild-type receptors resulting in an ER-α positive, but estrogen-resistant phenotype (353). In a mouse model of hormone-independence, loss of expression of the 65 kDa wild-type ER-α, and increased expression of 50 and 35 kDa ER-α-like proteins was seen as the tumours progressed from hormone-dependent to hormone-independent (412).

Again, there are conflicting results obtained when researchers have attempted to examine a role for variant ER-α mRNA in tamoxifen-resistance. As described, different groups have obtained differing results after stably expressing an ERd5 transgene in MCF-7 cells. One group demonstrated little effect of ERd5 over-expression (419) while, another (421) found ERd5 overexpression resulted in estrogen-independence and tamoxifen-resistance. Similarly, a study found that the levels of ERd5 mRNA transcripts were lower in tamoxifen-resistant MCF-7 cell lines than in the parent cell line (389), while other studies found no difference in ERd5 mRNA levels in tamoxifen-resistant MCF-7 sublines compared to wild-type (392) or in tamoxifen-resistant tumours compared to primary breast cancers (418). One study found no difference in levels of ERd2 in tamoxifen-resistant MCF-7 sublines compared to wild-type cells (392) while in another, researchers found that ERd2 was overexpressed in tamoxifen-resistant MCF-7 cells compared to parental cells (389). Once again, the reasons for the different results are unclear, but as multiple parameters affect wild-type ER-α activity, it is likely that multiple parameters can also affect the activity of variant ER-α proteins, and again the expression of multiple variant ER-α mRNA species along with

wild-type ER-α is likely to influence the overall ER-α activity.

Ligand-independent activation of the ER-α

Research has demonstrated that the ER-α can be activated in a ligand-independent fashion (244,245,316). The ability to activate the ER-α in the absence of ligand could confer a growth advantage to ER-α positive breast cancer cells and aid in the development of a hormone-independent phenotype. Studies have shown that several growth factors such as EGF, TGFα and IGF-1 are able to activate the ER-α in the absence of estrogen (244-246,316,424). For example, EGF was able to activate hER-α transiently expressed in HeLa cells in the absence of estrogen (246,424) and in rat uterine cells, IGF-1 was able to activate the endogenous ER-α in the absence of estrogen (244). Importantly, ICI 164,384 was able to block this ligand-independent activation of the ER-α, confirming that these signals do indeed cross-talk with the ER-α (244-246,316). Researchers have also demonstrated that ligand-independent activation of the rat uterine ER-α by growth factor signalling pathways can result in increases in intracellular PR levels, an endogenous estrogen-responsive gene (425).

Agents that can modulate phosphorylation of the ER-α (239,426) have also been shown to activate the ER-α in the absence of ligand. Activation of protein kinase A (PKA), by agents that raise intracellular cyclic adenosine monophosphate (cAMP) (cholera toxin (CT) and isobutylmethylxanthine (IBMX) or 8-bromo-cAMP), or activation of PKC, via TPA, can activate endogenous rat uterine ER-α, endogenous hER-α in BG-1 (human endometrial carcinoma) cells, and hER-α transiently transfected into HeLa cells, in the absence of estrogen

and the antiestrogen ICI 164,384 can inhibit this response (244,246,316,424). Inhibition of cAMP-dependent PKA blocked ligand-independent activation of rat uterine ER-α and hER-α transfected into HeLa cells by IGF-1 and cAMP (IBMX+CT) (244,424) but not EGF (424), suggesting that IGF-1 is acting through a PKA-dependent pathway in this system. Inhibition of PKC inhibited TPA-induced hER-α transactivation in BG-1 cells but did not inhibit the effects of TGFα, EGF or IGF-1 (316) but inhibition of PKC did reduce EGF activation of hER-α transiently expressed HeLa cells by 40-50% (246). This suggests that in some cell types EGF may be acting through a PKC-dependent pathway that may account for some, but not all, of its effects. Additionally, research has demonstrated that treatment of HeLa cells with okadaic acid (an inhibitor of protein phosphatases 1 and 2A) could also activate transiently expressed hER-α in the absence of ligand and again ICI 164,384 could abolish this response (246), supporting a role for phosphorylation in ligand-independent ER-α activity. Activation of PKA (IBMX+CT) or PKC (TPA) resulted in only very weak ligand-independent activation of endogenous hER-α in MCF-7 human breast cancer cells or hER-α transfected into CHO cells, but these agents could synergize with estrogen to increase ER-α mediated transcriptional activation in a cell- and promoter-specific fashion (427). Additionally, EGF did not result in ligand-independent activation of hER-α transfected into COS-1 cells (424). Taken together, the data suggest that the growth factor response (i.e., EGF versus IGF-1) may be mediated by different signalling pathways and is likely to influence ER-α activity in a cell- and promoter-specific manner.

Studies using mouse and human ER-α mutants have demonstrated that the ability of

the ER-α to respond to EGF, required the N-terminal A/B domain of the receptor, but not the LBD (245,316,424) and PKC activation of mER-α required the N-terminal AF-1 domain (428). In contrast, cAMP activation of the hER-α in HeLa cells required the LBD, but not the N-terminal A/B domain (424) and when hER-α is expressed in COS-1 cells phosphorylation of ER-α by IBMX+CT with an N-terminal deleted receptor was maintained (239). As discussed, the hER-α contains several functionally significant phosphorylation sites. Several of these sites (serine 104, 106, 118, and 167) are found in the N-terminal region of the hER-α, while a tyrosine 537 is phosphorylated in the C-terminal region of the molecule. Together this suggests that not only do different growth factor signalling pathways activate ER-α, but the signal transduction pathways and their targeted phosphorylation sites on the ER-α are likely to be different.

EGF results in phosphorylation of serine 118 on the hER-α (232,246), which is required for ligand-independent activation of the receptor (246). EGF activation of hER-α transfected into HeLa or SKBr3 (human breast cancer) cells was found to be via a MAPK pathway and MAPK phosphorylates serine 118 of the ER-α *in vitro* (232,246). These data support the hypothesis that EGF-induced ER-α activation may be via a MAPK pathway that results in phosphorylation of serine 118 in the N-terminal A/B domain of the ER-α. Interestingly, it has previously been shown that transfection of oncogenic v-ras (which activates MAPK) DNA into MCF-7 cells can result in these cells becoming estrogen-independent both *in vitro* and *in vivo* (429), suggesting that activation of the Ras-MAPK pathway could result in both ligand-independent activation of the ER-α and estrogen-

independent growth.

Mutations in the ER-α have also been identified which can result in ligand-independent transcriptional activity. For example, as discussed previously, an ER-α with a tyrosine 537 to asparagine mutation has been detected in one metastatic human breast cancer (248). A hER-α containing this mutation displayed transcriptional activity in the absence of estrogen when transiently expressed in HeLa or MDA-MB-231 (ER-α negative human breast cancer) cells (248). Tamoxifen and ICI 164,384 were both able to partially suppress the constitutive activity of this receptor in a promoter-dependent context (248). Researchers have also demonstrated that mutation of the tyrosine 537 in the hER-α to an alanine or serine resulted in a receptor with constitutive transcriptional activity when transiently expressed in MDA-MB-231 or CHO cells and hydroxytamoxifen and ICI 164,384 were able to block this activity (180,247). Interestingly, these researchers demonstrated an interaction of the tyrosine 537 mutant receptors *in vitro* with the coactivators SRC-1, TIF1 and RIP140, that, in contrast to wild-type hER-α, occurred in the absence, as well as presence of estrogen (180,247). Additionally, tamoxifen was able to prevent this *in vitro* interaction (180,247). As described, this residue is a site of phosphorylation in the hER-α, further implicating phosphorylation as a mechanism of control of ER-α activity. Estrogen-independent interactions with coactivators, such as SRC-1, may explain the constitutive transcriptional activity of the receptor. It would thus appear that residues, such as tyrosine 537, may be required to maintain the receptor inactive in the absence of estrogen and that an alteration affecting this region (perhaps by mimicking the effects of phosphorylation) can result in recruitment of coactivator proteins in the absence of estrogen. In the absence of estrogen the

tyrosine 537 to serine mutant hER-α displayed a conformation (as assessed by proteolytic digestion) that was similar to that seen with wild-type hER-α treated with estrogen (180), confirming that receptor conformation is an important determinant of its ability to interact with coactivators and exhibit ligand-independent transcriptional activity. A hER-α mutated at glutamate 380 to glutamine, has also been shown to display ligand-independent transcriptional activity when transiently expressed in MDA-MB-231 and CHO cells and the ability to interact *in vitro* with SRC-1 in the absence of ligand (180), again supporting an important role for coactivator interactions in receptor function. Transient transfection studies have also demonstrated that SRC-1 can increase the ligand-independent activity of hER-α in HeLa cells induced by increases in cAMP (183), suggesting that an alteration in the interaction of the ER-α with a coactivator protein could also result in ligand-independent as well as estrogen-independent activation of the ER-α. These data suggest that alterations in the ability of the ER-α to interact with coactivator proteins could be involved in altered ER-α activity in human breast tumours. Additionally, increased or altered expression of ER-α coactivator proteins may be involved in breast cancer development and progression. The coactivator AIB1 (a member of the SRC-1 coactivator family) is amplified in breast cancer (193). While decreases in the levels of SRC-1 protein have been found in breast tumours and breast cancer cell lines compared to normal breast tissue, lower levels of SRC-1 protein were found in patients that did not respond to tamoxifen treatment compared to those that did (430).

Loss of ER-α expression

At the time of clinical detection one third of tumours are classified as ER-α negative

in terms of ligand binding and immunoreactivity and rarely respond to antiestrogen treatment. As discussed previously, this could be accounted for by the presence of altered ER-α proteins that no longer bind ligand, or contain the epitopes necessary to be detected by antibody, but there are ER-α negative human breast tumours that do not express ER-α mRNA transcripts (431,432) and do not apparently contain mutations within, or loss of, the ER-α gene (105,372). This would suggest that lack of ER-α expression in human breast tumours is due, in many cases, to lack of ER-α gene expression. One mechanism which could block or prevent the transcription of the ER-α gene is methylation of the cytosine-guanine-rich CpG island which can result in transcriptional silencing of genes. Studies examining the 5' region of the hER-α gene have shown a correlation with methylation of the CpG island and lack of ER-α expression in both primary breast cancers and human breast cancer cell lines (431,433). Furthermore, Ferguson *et al* treated two ER-α negative human breast cancer cell lines, MDA-MB-231 and Hs578t, with demethylating agents and demonstrated the re-expression of both ER-α mRNA and functional ER-α protein (434).

A loss of transcription of the ER-α gene could also be due to a loss or decrease of ER-α gene specific transcription factors. A protein, ERF-1 (estrogen receptor factor 1), has been cloned from MCF-7 cells that binds to the promoter of the hER-α gene and is absent in some ER-α negative breast cancer cell lines (435,436) but it is unclear if this or other ER-α gene specific transcriptional regulatory proteins are specifically involved in the lack of ER-α expression in human breast tumours.

Apparent loss of ER-α expression could also occur via the outgrowth of an ER-α

negative population of cells. Studies have demonstrated that ER-α negative outgrowth can occur and long-term growth in estrogen-free conditions can also result in ER-α negative populations of cells (362,363). For example, as described, a clone of the T47D human breast cancer cell line, C4, derived by long term culture in estrogen-free conditions no longer expresses ER-α mRNA or protein and is insensitive to growth stimulation by estrogen and inhibition by hydroxytamoxifen (362). Additionally, studies using T47D cells have demonstrated that single cell clones are heterogeneous and are composed of populations of cells that are ER-α positive and estrogen-responsive, ER-α negative, and ER-α positive and estrogen-resistant (402). Human breast tumours are heterogeneous and contain populations of ER-α positive and negative cells (437), but it is not know if populations of ER-α negative cell outgrowth occurs in human tumours *in vivo* to result in an ER-α negative tumour, or if cells previously ER-α positive lose ER-α expression. Johnston and coworkers in studying tamoxifen-resistant human breast tumours concluded that while the development of tamoxifen-resistance was associated with a lowering in the overall level of immuno-histochemically detected ER-α (89% of responders to primary tamoxifen treatment were ER-α positive versus 61% of tamoxifen-relapsed tumours in the same patients) it was not associated with loss of ER-α expression (438) and as previously mentioned a significant proportion of hormone-independent human tumours retain ER-α expression, suggesting that in many cases it is the ability of the cells (or tumours) to overcome the requirement of estrogen for ER-α function that may be involved in hormone-independence and estrogen-nonresponsive growth.

Tamoxifen stimulated growth

Tamoxifen inhibits the estrogen-stimulated growth of MCF-7 tumours in nude mice but long-term tamoxifen treatment eventually results in resumed tumour growth (439,440). Serial transplantation of these tamoxifen-resistant tumours into athymic mice results in tumours that can be stimulated with tamoxifen as well as estrogen (439,440). Similarly, in MCF-7 cells in culture, long-term growth in the presence of hydroxytamoxifen can result in growth stimulation, rather than inhibition, in response to hydroxytamoxifen treatment (441). Tamoxifen-stimulated growth is not associated with a loss of ER-α expression (439-441) and the steroidal antiestrogen ICI 164,384 is able to inhibit the growth of tamoxifen-stimulated MCF-7 cells *in vivo* (138) and *in vitro* (441). In tamoxifen-stimulated MCF-7 cells in culture PR levels were no longer estrogen-responsive but estrogen-responses to ER-α, pS2 and reporter-gene expression were maintained, demonstrating that loss of estrogen-responsive gene expression is not a general phenomenon (441). There is also evidence to suggest that tamoxifen-stimulated tumours may develop in humans (442). In breast cancer patients whose tumour has become refractory to tamoxifen treatment, cessation of tamoxifen therapy can, in some cases, result in tumour regression (443).

Researchers have suggested that the stimulation of tumour growth by tamoxifen might be due to metabolism of tamoxifen to estrogenic metabolites (440). As mentioned previously hydroxytamoxifen, is the main tamoxifen antiestrogenic metabolite (122), but tamoxifen can be metabolized to produce several compounds that have estrogenic properties (136,440). The E isomer of metabolite E has been shown to be a potent estrogen (444) and while it has been detected in some tamoxifen-resistant tumours (445), serum from tamoxifen treated patients

did not demonstrate changes in metabolites or their profiles even after long-term treatment (446). Tamoxifen-stimulated MCF-7 tumour growth and development has also been shown to occur in nude mice in the presence of tamoxifen analogues that are not capable of conversion to estrogenic compounds (447,448) suggesting that other possibilities for tamoxifen-resistance also exist.

It has been reported that some tamoxifen-stimulated tumours contain 90% less intratumoural tamoxifen levels compared to tamoxifen-inhibited tumours (440), suggesting that alterations in drug uptake or efflux may be involved in some cases of antiestrogen resistance. In contrast, Wolf and coworkers compared the levels of tamoxifen in MCF-7 tumours in athymic mice that were stimulated or inhibited by tamoxifen and found similar concentrations in the two tumour types (447).

Antiestrogen binding sites distinct from the ER-α have been identified and these binding sites appear to be specific for the triphenylethylene antiestrogens such as tamoxifen (449). A clone of MCF-7 cells has been described that is resistant to tamoxifen and appears to have no detectable antiestrogen-binding sites (450), while another group have reported an antiestrogen-resistant MCF-7 subline expresses high levels of antiestrogen binding sites (451). This group has suggested that excess antiestrogen-binding sites, distinct from the ER-α, may prevent antiestrogen accessing ER-α, thereby resulting in resistance (451). Currently, it is unclear what role these antiestrogen binding sites play in tamoxifen-resistant growth.

Alterations in the agonist/antagonist activity of the ER-α in response to antiestrogens may also be involved in the development of tamoxifen resistance. Discussed in an earlier section, the ER-α has two well-studied AF domains involved in the response to agonist and

antagonist ligands. Tamoxifen, while most often an antagonist to human breast tumours and breast cancer cells in culture, is an agonist in other tissues and cell types and on different promoters within the same cell. An interesting study demonstrated that stimulating the PKA pathway in MCF-7 cells by treatment with agents that raised cAMP levels (8-bromo-cAMP or IBMX+CT), or by transient expression of the catalytic subunits of PKA, markedly stimulated the agonistic activity of tamoxifen (452). This effect was found to be promoter specific and interestingly, the activity of the pure antiestrogen ICI 164,384 was not affected by these types of treatments (452). These data suggest that cross-talk between signal transduction pathways may be important in both ligand-independent ER-α activation (as described) and stimulation of tamoxifen's agonistic activities. More importantly, in HeLa cells, ligand-independent activation of hER-α by elevating cAMP levels could not be inhibited by tamoxifen (183), suggesting that if such signalling pathways are elevated, both ligand-independent and tamoxifen-stimulation of the ER-α may occur and tamoxifen treatment may be unable to inhibit this type of activity. The ability of hydroxytamoxifen to activate or inhibit ER-α mediated gene expression could also be modulated by the relative expression of coactivators or corepressors as described (183). hER-α corepressor proteins have been identified that bind *in vitro* to the receptor only in the presence of tamoxifen, suggesting that the antagonistic properties of tamoxifen may be dependent upon recruitment of such proteins. Interestingly, a protein L7/SPA has been shown to bind to the hER-α only in the presence of tamoxifen and in contrast to corepressor proteins, this protein is able to enhance the agonist activity of tamoxifen when transiently expressed in HeLa cells (225). As previously described, altering the balance of corepressor and coactivator proteins can alter the agonist and

antagonist properties of hydroxytamoxifen on hER-α, dependent on cell context (183) and alterations in the levels of some coactivator proteins has been described in human breast tumours as discussed. This suggests that alterations in cellular levels of ER-α interacting proteins in human breast tumours, including corepressors, could influence the biocharacter of tamoxifen. To date extensive studies have not been performed to examine the relative levels of the different hER-α coactivator and corepressor proteins in hormone-dependent versus -responsive human breast tumours and breast cancer cell lines but this type of data will no doubt be forthcoming.

Mutations within the hER-α could also result in tamoxifen-resistance and/or tamoxifen-stimulated tumour growth. As described variant forms of hER-α mRNA have been identified that have the potential for biological activity of their own or interfere with the activity of wild-type hER-α. While it is still unclear if these could account for estrogen-insensitivity and tamoxifen-resistance *in vivo*, studies have been performed to suggest the possibility exists. Montano and coworkers constructed an hER-α containing a point mutation (leucine 540 to glutamine) within the hormone binding domain and demonstrated that it is transcriptionally activated by the antiestrogens, hydroxytamoxifen and ICI 164,384, but not estrogen, when expressed in ER-α negative MDA-MB-231 breast cancer cells (453). While this particular mutation has not been detected in human breast tumours to date, mutations in similar regions have been detected (see Table 2). Interestingly, Wolf and Jordan isolated a hER-α with a point mutation in the LBD (aspartate 351 to tyrosine) in a tamoxifen-stimulated MCF-7 tumour from mice (454) and hER-α mRNAs with point mutations have been identified from metastatic human breast tumours, as described (248,370,371). Together the

data suggests the potential for a single point mutation to result in tamoxifen-resistance or tamoxifen-stimulated tumour growth.

Alterations in autocrine/paracrine interactions

As discussed earlier, breast cancer cells express and secrete a variety of growth factors whose expression can be altered in response to estrogen and antiestrogen treatment. As described, long-term growth in the absence of estrogen results in estrogen-nonresponsive growth of T47D and ZR-75-1 ER-α positive human breast cancers cells and studies have shown that this is accompanied by changes in mRNA expression for several growth factors (358,455). In T47D, ER-α positive, estrogen-nonresponsive sublines, increases in TGFα, TGFβ1 and TGFβ2 mRNA were found compared to parental cell lines (358,455) and in contrast to the parental cells whose growth is unaffected by exogenous TGFβ, estrogen-nonresponsive T47D cells were shown to be growth stimulated *in vitro* in response to exogenous TGFβ (455). In contrast, estrogen-nonresponsive, ER-α positive, ZR-75-1 cells contained decreased levels of TGFβ1 and TGFα mRNA and TGFβ1 was shown to inhibit the growth of both ZR-75-1 and the estrogen-independent sublines (358). These studies again demonstrate the multifactorial nature of the development of estrogen-independent growth and underline the fact that several mechanisms may be involved.

In MCF-7 cells TGFβ1 treatment inhibits DNA synthesis as measured by decreases in [^{3}H]thymidine incorporation (441). In contrast, in hydroxytamoxifen resistant MCF-7 cells (MCF/TOT), [^{3}H]thymidine incorporation was unaffected by TGFβ1 treatment (441). Additionally, in MCF/TOT cells elevated mRNA levels for the TGFβ isoforms, β1, β2 and β3, were detected with corresponding increases in secreted TGFβ protein compared to the

parent MCF-7 cells (441). As discussed, induction of growth-inhibitory TGFβ has been proposed as a mechanism involved in tamoxifen's effects on human tumour growth and overcoming this potential inhibitory effect may be an additional mechanism by which cells can become resistant to tamoxifen in terms of growth inhibition. While estrogen can increase the expression of TGFα in human breast cancer cells and it may be involved in mediating the mitogenic effects of estrogen (299), stable transfection of TGFα, resulting in overexpression in MCF-7 cells, did not result in estrogen-independent growth *in vitro* or *in vivo* (304). This would suggest that at least alone, increases in TGFα expression may not be responsible for estrogen-nonresponsive growth of MCF-7 cells.

Despite the demonstration by several research groups that changes in growth factor expression can accompany both estrogen-independent and tamoxifen-resistant growth the exact role that growth factor expression plays in hormonal-independence is still unclear (127).

The progression to hormone-independence in a mouse model of mammary cancer has been demonstrated to be associated with an increase in EGFR levels. This progression was also associated with a decrease in ER-α levels and EGFR ligands (456). In human breast tumours an inverse relationship between ER-α and EGFR levels exists and an increase in EGFR may be associated with resistance to endocrine treatment (292,293). Stable overexpression of EGFR in estrogen-dependent, ZR-75-1, human breast cancer cells (ZR/HERc) has been shown to result in estrogen-independent growth and hydroxytamoxifen resistance in the presence of exogenous EGF in one instance (457) but not in another (458). Another group of researchers have stably transfected MCF-7 cells with the cDNA for EGFR and found that the cells stably express high levels of EGFR only in the absence of estrogen

and cells expressing a high level of EGFR displayed an increased ability to grow *in vitro* in the absence of estrogen (459). These results suggest that the differences in response to EGFR overexpression may depend on the cellular background, growth conditions under which the cells are assayed, or endogenous production of ligands (i.e., EGF and TGFα) for the EGFR in the different cell types (which were not examined in these studies).

While the above studies have demonstrated that overexpression of a growth factor, or its receptor, which can activate the MAPK cascade can result in estrogen-independent growth *in vitro* other studies have demonstrated that expression of a component of the MAPK cascade (i.e., Raf or Ras) can also result in estrogen-independent growth *in vitro* and in some cases tumourigenesis *in vivo* in the absence of estrogen. Transfection and overexpression of oncogenic v-ras cDNA into MCF-7 cells can result in the cells becoming estrogen-independent in terms of growth both *in vitro* and *in vivo* (429) and the cells were found to secrete elevated levels of growth factors including TGFα, TGFβ and an IGF-I-like protein (460). These researchers also demonstrated that the ras-transformed breast cancer cells, or the conditioned media from these cells, were able to partially support *in vivo* tumour formation by the nontumourigenic parental cells in the absence of estrogen, suggesting the production of active, diffusible substances by the ras-transformed cells (461). Interestingly, overexpression of a constitutively active Raf-1 kinase in MCF-7 cells allows for growth of the cells in the absence of estrogen and is incompatible with growth in the presence of estrogen (462). Additionally estrogen was found to be directly responsible for down-regulation of Raf-1 expression (462). These effects of growth in estrogen-containing conditions are similar to those seen when researchers transfected EGFR into MCF-7 cells

(i.e., down-regulation of EGFR expression) and suggests that perhaps the continued growth of human breast cancer cells in estrogen-deplete conditions can in some cases allow for activation/expression of previously estrogen-downregulated signalling pathways. These data demonstrate the potential importance of the MAPK pathway in cell growth regulation and possibly tumourigenesis and as mentioned previously, MAPK has been shown to be overexpressed in breast tumours compared to normal breast tissue.

Supersensitivity to estrogen

As mentioned, the observation that contaminants of phenol red were estrogenic to human breast cancer cells in culture led to the use of estrogen-free culture conditions to study the effects of long-term estrogen deprivation. The caveat to these types of studies is the removal of endogenous steroid in fetal calf serum, a component of the cell culture medium. Charcoal stripping of fetal calf serum is used, and presumed, to remove all of the steroid present although residual estrogens may remain (254) or could be contaminants from plastics used in cell culture flasks (359). Masumara and colleagues have demonstrated that MCF-7 cells grown in the absence of estrogen developed supersensitivity to residual estrogens in the charcoal stripped serum (359). These cells appeared estrogen insensitive in that they were maximally stimulated by the low levels of estrogen present in the culture medium. The supersensitivity was demonstrated by the fact that doses of ICI 164,384 6-fold lower than that required for the parent cells efficiently inhibited the growth of these cells by 50% (359). Additionally, these 'supersensitive' cells contained 5-fold higher levels of ER-α than the parental MCF-7 cells (359) which could also be involved in the increased sensitivity these cells display to estrogen.

It is likely that multiple mechanisms are involved in the development of a hormone-independent growth phenotype both in cell culture models and *in vivo*. The development of estrogen-nonresponsive growth is thought to be one of the initial stages in the development of a hormone-independent breast tumour phenotype and signifies a more aggressive stage of the disease. Unfortunately the mechanisms involved in the progression to estrogen-independent growth are not clear and understanding the factors that contribute to this phenotype are important in terms of breast cancer therapeutics and in an understanding in the mechanisms of ER-α function.

Research Aim

As discussed, there are multiple factors involved in ER-α activity and likely in the generation of hormone-resistance and estrogen-nonresponsiveness. The goal of this project was to examine the mechanisms involved in the generation of hormone-independence in a human breast cancer cell culture model with the hypothesis that apparent estrogen-independence in ER-α positive breast cancer cells is associated with altered activity of the ER-α, likely via multiple mechanisms which may interfere with normal ligand-dependent ER-α activity. T47D5 (T5) human breast cancer cells are ER-α positive and an MCF-7 derived cell line that require estrogen for maximum proliferation in culture. An estrogen-nonresponsive subline, T5-PRF, was developed through long-term culture of the parental T5 cells in phenol-red free growth medium containing charcoal-stripped fetal calf serum. These two cell lines (T5 and T5-PRF) were used to identify potential mechanisms involved in the generation of estrogen-nonresponsive growth in the continued presence of ER-α expression, with an emphasis placed on ER-α and factors influencing its activity.

Materials and Methods

Reagents

Dulbecco's minimal essential medium (DMEM) and phenol red free DMEM (PRF-DMEM) powder were purchased from GIBCO/BRL (Burlington, Ontario). Trypsin/EDTA, penicillin-streptomycin (10,000 units/mL) and L-glutamine (200 mM) were purchased from GIBCO/BRL (Burlington, Ontario). Fetal bovine serum (FBS) was purchased from Upstate Biotechnology Incorporated (UBI) (Lake Placid, New York) and all other cell culture ingredients were purchased from Flow Laboratories (Mississauga, Ontario). Tissue culture flasks were from Corning (New York, New York) and cell culture dishes were from Nunclon (Canadian Life Technologies, Burlington, Ontario). Cholera toxin and hydrocortisone were obtained from Sigma Chemical Co. (St. Louis, Missouri). Horse serum and epidermal growth factor were purchased from UBI (Lake Placid, New York). Activated charcoal was purchased from Sigma Chemical Co. (St. Louis, Missouri) and dextran T70 from Pharmacia (Uppsala, Sweden).

ICI 164,384 was a gift from Imperial Chemical Industries (ICI), now called Zeneca (Macclesfield, Cheshire). 4-Hydroxytamoxifen, 17β-estradiol and dexamethasone were obtained from Sigma Chemical Co. (St. Louis, Missouri). [^{3}H]-Estradiol, [^{14}C]-chloramphenicol, [^{35}S]-methionine, [α-^{35}S]dATP, R5020 and [^{3}H]-R5020 (Promegestone, [17α-methyl-^{3}H]-(R 5020)) were purchased from NEN (Lachire, Quebec). [^{32}P]dCTP and [γ-^{32}P]ATP and were purchased from ICN (St. Laurent, Quebec).

Monoclonal rat anti-ER-α antibody, H226, was a generous gift from Dr. G. Greene (University of Chicago, Chicago, Illinois). Anti-rat peroxidase conjugated antibody (A-5795)

was from Sigma Chemical Co. (St. Louis, Missouri). Monoclonal mouse anti-phospho-MAPK antibody (9105S) was from New England Biolabs (Beverly, Massachusetts). Polyclonal rabbit anti-ERK1 (C-16) antibody (sc-93) was from Santa Cruz Biotechnologies, Inc. (Santa Cruz, California). Monoclonal mouse anti-ER-α antibodies (Clone AER314 and Clone AER308) were from Neomarkers, Inc. (Freemont, California). Anti-mouse peroxidase conjugated antibody (115-035-071) was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, Pennsylvania). Anti-rabbit peroxidase conjugated antibody (172-1019) was from BioRad (Hercules, California). Monoclonal mouse anti-lamin antibodies (119D5-F1 and 131C3) and lamin standards were generous gifts from Dr. Y. Raymond (Montreal, Quebec). Enhanced chemiluminescence (ECL) detection kit was purchased from Amersham International (Buckinghamshire, England). Molecular weight prestained protein markers were purchased from BioRad (Hercules, California).

Myelin basic protein was from Life Technologies, Inc (Burlington, Ontario). Cyclic AMP-dependent protein kinase inhibitor peptide (H_2N-Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-NH_2) was purchased from Bachem (Torrance, California). PD 98059 (2'-Amino-3'-methoxyflavone) and BES (N,N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid) were purchased from Calbiochem (La Jolla, California). CAPS (3-[cyclohexylamino]-1-propanesulfonic acid), ONPG (o-Nitrophenyl β-D-galacto-pyranoside), Tween 20 (polyoxyethylene-sorbitan monolaurate), Triton X-100 (octyl phenoxy polyethoxyethanol) and Nonidet P-40 (NP-40) were purchased from Sigma Chemical Co. (St. Louis, Missouri). EDTA (disodium ethylenediamine tetraacetate disodium salt) and sodium thiosulfate were purchased from Fisher Scientific Company (Nepean, Ontario).

Kodak XAR film was purchased from Eastman Kodak Company (Rochester, New York). Hyperfilm was purchased from Amersham International (Buckinghamshire, England). Nitrocellulose (NitroPlus, 0.45 micron) paper was purchased from Micron Separations Incorporated (Westborough, Massachusetts). Rabbit reticulocyte lysate *in vitro* transcription/translation kit (TnT) was purchased from Promega (Madison, Wisconsin). TRIzol reagent was purchased from Life Technologies, Inc (Burlington, Ontario). Silicagel 1B thin layer chromatography sheets were purchased from Mallinckrodt Baker Incorporated (Phillipsburg, New Jersey). Whatman P81 discs and #1 filter paper were purchase from Whatman Incorporated (Clifton, New Jersey). Ecolite liquid scintillation cocktail was purchased from ICN (Costa Messa, California). Ampholines were purchased from Pharmacia (Uppsala, Sweden).

Deoxyribonuclease I (DNAse I) from bovine pancreas (2,000 Kunitz units/vial) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, Missouri). Aprotinin, leupeptin, and 4-[2-aminoethyl]benzenesulfonyl fluoride hydrochloride (AEBSF) were purchased from Boehringer Mannheim (Indianapolis, Indiana). Ribonuclease inhibitor (RNAsin) was purchased from Promega Corp. (Madison, Wisconsin). Random hexamers and moloney murine leukemia virus-reverse transcriptase (MMLV-RT) were purchased from GIBCO/BRL (Burlington, Ontario).

The original TA cloning kit was purchased from Invitrogen (San Diego, California). pOR8 (ER-α cDNA), pHEGO (ER-α expression vector) and pSG5 (eukaryotic expression vector) were gifts from Dr. P. Chambon (Strasbourg, France). pT7βhER (ER-α expression vector) was a gift from Dr. S. Tsai (Baylor College of Medicine, Houston, Texas). pCH110

(β-galactosidase expression vector) was purchased from Pharmacia (Uppsala, Sweden). ERE-tk-CAT (pBL-CAT8+), *Xenopus laevis* vitellogenin B1 estrogen response element reporter gene was obtained from Dr. Wahli (Lausanne, Switzerland).

Cell Culture

All cells were routinely kept in 150 cm^2 flasks and grown in a 37°C incubator, containing a humidified atmosphere of 5% CO_2. T5 cells, previously called T47D5, were originally thought to be a T47D subline, however, DNA fingerprinting analysis showed that they were an MCF-7 subline (105). T5, T47D, HBL 100, and MDA-MB-231 human breast cancer cells were routinely cultured in DMEM containing 5% vol/vol fetal calf serum (FCS), 0.3% wt/vol glucose, 2 mM L-glutamine and 100 units/mL penicillin-streptomycin (5% CM). Cells were passaged at 70-80% confluency using Earle's EDTA solution (5.3 mM KCl, 117 mM NaCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 5.6 mM D-glucose, 1 mM EDTA). A chronically-estrogen-depleted T5 subline (T5-PRF) was developed by long-term growth of these cells in PRF (phenol red free)-DMEM supplemented with 5% vol/vol twice charcoal dextran stripped FCS and 0.3% wt/vol glucose, 2 mM L-glutamine, and 100 units/mL penicillin-streptomycin (5% CS). T5-PRF cells were routinely cultured in 5% CS. MCF10A1 human breast epithelial cells (463) were routinely grown in DMEM containing 5% vol/vol horse serum (hs), 0.3% wt/vol glucose, 2 mM L-glutamine and 100 units/ mL penicillin-streptomycin, 0.1 μg/mL cholera toxin, 20 ng/mL hEGF, 10.4 μg/mL bovine insulin and 1 μM hydrocortisone (DMEM-special). Cells were passaged at 70-80% confluency using trypsin/EDTA. Transient transfections using T5, T5-PRF and MDA-MB-231 cells were performed in 5% CS. Transient transfections using MCF10A1 cells were performed in PRF-

DMEM containing 5% vol/vol twice charcoal dextran stripped hs, 0.3% wt/vol glucose, 2 mM L-glutamine and 100 units/mL penicillin-streptomycin (PRF-DMEM-hs) and cells were passaged once prior to transfection in PRF-DMEM containing 5% vol/vol twice charcoal dextran stripped hs, 0.3% wt/vol glucose, 2 mM L-glutamine, 100 units/mL penicillin-streptomycin, 0.1 μg cholera toxin, 20 ng/mL hEGF, 10.4 μg/mL bovine insulin and 1 μM hydrocortisone (PRF-DMEM-special).

Growth curves

Growth experiments were performed by setting up cells at 10^4 cells/35-mm dish. Estrogen growth experiments were performed in 5% CS, while antiestrogen growth experiments were performed in 5% CM. Two days later, fresh medium was added, which contained the appropriate concentration of drug to be tested from 1000 X stock solutions in ethanol. After 5 days the cells were harvested in triplicate using trypsin/EDTA and counted using an electronic cell counter (Coulter Electronics, Burlington, Ontario). Results were expressed as proliferation rate (percentage control) using the equation; Doubling time (DT) = $2 \log_n/\log (Tn/Ti)$, where Ti is initial cell number, Tn is final cell number and n is the time (days) between Ti and Tn. Proliferation rate as a percentage of control was then calculated from the equation: Proliferation rate = doubling time (control) X 100/doubling time.

Receptor assays

PR assays were performed using whole cell ligand binding assays and T5 cells were passaged twice in 5% CS before being set up at $1x10^6$ cells/35 mm well one day prior to receptor assays. [^{3}H]-R5020 and [^{3}H]-R5020 plus 100 fold molar excess unlabelled R5020 were used to determine PR total and nonspecific binding, respectively. The working stock

of [^{3}H]-R5020 was made by drying down the appropriate volume of [^{3}H]-R5020 in ethanol under air nitrogen, and then adding the appropriate volume of binding buffer (PRF-DMEM plus 0.3% wt/vol glucose, 2 mM L-glutamine and 100 units/ mL penicillin-streptomycin, 0.1% wt/vol bovine serum albumin and 100 nM dexamethasone) to give a final concentration of 20 nM [^{3}H]-R5020. All assays were performed in the presence of 100 nM dexamethasone to prevent binding of R5020 to the glucocorticoid receptor. This solution was stored in the dark and left overnight at 4°C. The following morning an aliquot was counted using liquid scintillation to determine the concentration. The excess R5020 solution was made by drying down under nitrogen enough unlabelled R5020 to give a final concentration of 1 μM to be taken up in the appropriate volume of the [^{3}H]-R5020 total solution. This solution (excess) was left at room temperature (in the dark) for several hours. For each assay, total and excess measurements were performed on triplicate wells and cell counts were performed on triplicate wells. To perform the assay, the medium was aspirated from the wells and 500 μL of total or 500 μL excess solution was added to each of three wells, while another three were treated with binding buffer only (these were for counting cell numbers). The plates were then placed in a 37°C incubator for 1 hour, after which the plates were placed on ice, the binding solutions aspirated and 1 mL of cold wash solution (5% wt/vol bovine serum albumin in Isoton) was added to each well. This was aspirated from the wells and the wash was repeated. One mL of trypsin/EDTA solution was added to each well to be used for counting and after several minutes the solution containing the detached cells was removed and added to 7 mL of isoton in a counting vial. The wells were rinsed twice with 1 mL DMEM and this was also added to the counting vial. The solution was syringed twice using a 21 gauge needle and the cells

counted using an electronic cell counter (Coulter Electronics). To the cells treated with total or excess R5020 solution was added 500 μL of solubilization solution (0.5% vol/vol Triton-X 100 in 1 M NaOH) and left overnight at room temperature. The next day a 250 μL aliquot of solubilized cells was added to a counting vial, neutralized with 62.5 μL of 4 N HCl and 5 mL of liquid scintillant (EcoLight) was added and the vials counted using a liquid scintillation counter. ER-α levels were determined in a similar fashion with [^{3}H]17β-estradiol and 17β-estradiol used in place of [^{3}H]R5020 and R5020 and dexamethasone omitted from the binding buffer solution.

Isolation of nuclear matrix-intermediate filament fraction

Cell pellets (either fresh or stored at -70°C) were resuspended in TNM buffer (100 mM NaCl, 300 mM sucrose, 10 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$, 1% (vol/vol) thiodiglycol) at 10°C. PMSF was added from a 100 mM stock to a final concentration of 1 mM. This cell solution was transferred to a glass homogenizer on ice using a pasteur pipette and the cells were homogenized 5 strokes at room temperature. The cells were transferred using a pasteur pipette to a large Sorval centrifuge tube and incubated on ice for 5 minutes. Triton X-100 was added from a 25% vol/vol stock solution to a final concentration of 0.5%. The cells were sheared, 3 passes, through a 22 gauge needle on ice. Samples were centrifuged at 1,000 rpm for 6 minutes at 4°C. The supernatant (cytosol fraction) was saved to a fresh tube and the pellet was resuspended in TNM buffer containing 1 mM PMSF, homogenized 7-10 strokes and centrifuged at 1,000 rpm for 6 minutes at 4°C. This was repeated and before the final centrifugation a sample of the nuclei was removed and placed in 2 M NaCl/5 M urea and the optical density at A_{260} (absorbance, 260 nanometres) measured. After the final

centrifugation, the nuclei pellets were resuspended in digestion buffer (50 mM NaCl, 300 mM sucrose, 10 mM Tris-HCl pH 7.4, 3 mM $MgCl_2$, 1% (vol/vol) thiodiglycol, 0.5% (vol/vol) Triton X-100) at a concentration of 20 A_{260}/mL and digested with DNAse I (168 U/mL) for 20 minutes at room temperature. Ammonium sulfate was added dropwise from a 4 M stock, to a final concentration of 0.25 M, and the nuclear matrix was pelleted by centrifugation at 5,000 rpm for 10 minutes at 4°C. The supernatant (chromatin fraction = S1) was saved to a fresh tube and the pellet was resuspended in digestion buffer and re-extracted by slowly adding NaCl with mixing to a final concentration of 2.0 M from a 4.0 M stock solution. This was left on ice for 30 minutes and pelleted by centrifugation at 5,000 rpm for 10 minutes at 4°C. The supernatant (S2) was saved to a fresh tube and the pellet was again re-extracted with 2 M NaCl and 1% (vol/vol) 2-mercaptoethanol for 30 minutes on ice. The resulting insoluble NM-IF was isolated by centrifugation at 5,000 rpm for 10 minutes at 4°C. The supernatant (S3) was saved to a fresh tube and the pellet (NM-IF) containing nuclear matrix proteins and associated intermediate filaments (320) was resuspended in 8 M urea. All samples were stored at -20°C. Protein levels were assayed using BioRad (Bradford) protein assay kit (Mississauga, Ontario).

Preparation of nuclear matrix-intermediate filament samples for electron microscopy

Immediately after isolation of NM-IF the samples were resuspended in 100 mM Hepes solution pH 7.4 and glutaraldehyde was added (prediluted with 100 mM Hepes pH 7.4) to a final concentration of 2.5% (vol/vol). The samples were left overnight at 4°C and the following day were pelleted by centrifugation at 5,000 rpm for 10 minutes at 4°C and the NM-IF pellet was washed twice with 100 mM Hepes buffer (pH 7.4) to inactivate the

glutaraldehyde. The samples were resuspended in increasing concentrations of ethanol (30, 50, 75, 95 and 100 %, made by diluting absolute ethanol in distilled autoclaved water) by incubating the samples on a rotating bed for approximately 1 hour at each ethanol concentration at room temperature. The samples were pelleted in between successive dehydrations at 5,000 rpm at room temperature. The sample was resuspended in 100 μL of 100% ethanol for shipping. Electron microscopy was performed by Dr. M. Henzel (Calgary, Alberta).

SDS-PAGE, Coomassie staining and quantitation

Subcellular fractions were analyzed under reducing conditions by electrophoresis on sodium dodecyl sulfate-12 % polyacrylamide gels (SDS-PAGE) with 4 % stacking gel at 200 V for 45 minutes at room temperature according to the Laemmli method (464). Gels were stained in 0.05% Coomassie Blue R-250 (BioRad). Quantification of cytokeratin levels was performed on Coomassie Blue stained gels using scanning densitometry and the lamin bands were used as loading controls. Scans were performed using a ColorOne Scanner (MacIntosh) and images and data analysed using Image (National Institutes of Health) and Ofoto 2.0 (LightSource Computer Images Inc.) software packages.

Protein purification

NM-IF samples were prepared and samples were resuspended in 7 M urea, 20 mM Tris-HCl, pH 8. Samples were chromatographed on a 1 mL Porus PI (anion-exchange) column at a protein concentration of 8 mg/column. Proteins were eluted with a linear gradient of 0-0.5 M NaCl in 15 mL and 0.6 mL collected/fraction. Fractions were assayed by SDS-PAGE and Coomassie Blue staining and samples containing protein bands of interest were

pooled.

Two-dimensional electrophoresis

First-dimension isoelectric focusing was performed according to the method of O'Farrell (465) and the second-dimension (2-D) SDS-PAGE according to the method of Doucet (466). Gels were stained using the silver staining technique (467). For the first dimension isoelectric focusing (IEF) the IEF gel monomer solution contained per 20 mL; 11g urea, 0.3g CHAPS, 100 µL NP-40, 0.25 mL pH 5-7 ampholines, 0.25 mL pH 6-8 ampholines, 0.5 mL pH 3.5-9.5 ampholines and 3 mL of 30 % acrylamide/0.8% bis-acrylamide. The solution was filtered through a 0.45 micron SFCA filter (Nalgene) before storing in 1 mL aliquots at -70°C. The IEF sample solutions contained per 10 mL; 1 g SDS and 0.232 g dithiothreitol and was stored in 1 mL aliquots at -70°C. Samples for IEF were in 7 M urea and to 40 µL sample was added 10 µL IEF sample solution per gel. First dimension tube gels were run for 2 hours at 200 V, 2 hours at 500 V and 16 hours at 800 V in a buffer system composed of 100 mM NaOH catholyte solution (upper chamber) and 10 mM phosphoric acid anolyte solution (lower chamber).

Tube gels were extruded onto parafilm boats and overlayed with 1 mL 1 X sample reducing buffer (5 X contains per 10 mL; 1.5 g SDS, 0.77 g dithiothreitol, 5 mL 0.05% bromophenol blue, and 3.5 mL 1 M Tris-HCl pH 6.7) and incubated at room temperature for 30 minutes. The second-dimension (size separation) resolving gel contained per 80 mL; 29.3 mL distilled water, 27 mL 30 % acrylamide/0.3 % bis-acrylamide, 4 mL glycerol, and 16 mL 5 X resolving gel buffer (500 mL = 61 g Tris and 19 g glycine) which was degassed for 15 minutes before adding 3.2 mL 10 % SDS and then filtered through a 1 micron glass fibre filter

(Gelman). The stacking gel contained per 10 mL; 5.7 mL distilled water, 1.3 mL 30 % acrylamide/0.3 % bis-acrylamide, 0.5 mL glycerol and 2 mL 5 X stacking gel buffer (100 mL = 4.2 g Tris and 0.74 g EDTA , pH 6.7) which was degased for 15 minutes before adding 0.4 mL 10 % SDS and then filtered through a 1 micron glass fibre filter (Gelman). Tube gels were loaded onto the second dimension slab gel and run at 300 V for 2.5 hours with an upper buffer composed of 60 mL concentrated running buffer (1 L = 61 g Tris, 56 g glycine and 5 g SDS) in 300 mL distilled water and a lower buffer composed of 400 mL concentrated running buffer in 4 L distilled water.

For silver staining, gels were fixed for 30 minutes in 50 % methanol/10 % acetic acid with gentle shaking at room temperature. Gels were then incubated in sensitizer (500 mL = 34 g sodium acetate, 1 g sodium thiosulfate, 150 mL ethanol and 2.5 mL 25 % (vol/vol) glutaraldehyde) for 30 minutes at room temperature with gentle shaking. Gels were washed 3 times with water for 10 minutes each with gentle shaking before incubating the gels with silver solution (500 mL = 50 mL 2.5 % silver nitrate and 200 µL formaldehyde) for 30 minutes at room temperature with gentle shaking. The gels were then washed twice with water for approximately one minute each before incubating with developer (500 mL = 12.5 g sodium carbonate and 100 µL formaldehyde) for 10-15 minutes at room temperature with gentle shaking. The developing reaction was terminated by incubating the gels in stop solution (500 mL = 7.4 g EDTA) for approximately 10 minutes at room temperature with gentle shaking.

Western blotting and immune detection

NM-IF samples

Lamin and ER-α detection

NM-IF samples were run on SDS-12 %PAGE with 4 % stacking gels at 200 V for 45 minutes at room temperature under reducing conditions according to the Laemmli method (464). Gels were equilibrated for 30 minutes at room temperature in modified buffer O (468) (5 % (vol/vol) 2-mercaptoethanol, 2.3 % (wt/vol) SDS and 62.5 mM Tris-HCl pH 6.8). Gels were transferred to nitrocellulose using CAPS transfer buffer (25 mM CAPS pH 10 and 20 % (vol/vol) methanol) for 1 hour at 120 V at 4°C. Nitrocellulose blots were air-dried and baked at 65°C for 30 minutes. Blots were blocked overnight at 4°C in 5 % (wt/vol) skimmed milk in Tris-buffered saline (TBS, 1 L 5 X stock = 12.1 g Tris, 146.3 g NaCl pH 7.5) containing 0.2 % Tween-20 (TBS-T). Blots were incubated with primary antibody (for ER-α detection: anti-rat H226 ER-α antibody; for lamin detection: anti-mouse 119D5-F1 (lamin B1) and 131C3 (lamin A/C) antibodies) at a dilution of 1 μg/mL in 1 % TBS-T for 1 hour at room temperature. Blots were washed with TBS-T and then incubated with the appropriate peroxidase-conjugated secondary antibody at a dilution of 1 in 1000 in 1 % TBS-T for 1 hour at room temperature. Blots were washed 4 times for 15 minutes each in TBS-T. Detection was carried out using the ECL detection system (Amersham, Buckinghamshire, England) according to the manufacturer's instructions.

Cell extracts

ER-α detection

Whole cell extracts (dissolved in 8 M urea) were analysed using SDS-10 % PAGE with a 4 % stacking gel at 200 V for 45 minutes at room temperature under reducing conditions according to the Laemmli method (464). Gels were equilibrated for 30 minutes at

room temperature in ice cold CAPS transfer buffer (10 mM CAPS pH 11, 20% (vol/vol) methanol) and transferred to nitrocellulose using CAPS transfer buffer for 1 hour at 120 V at 4°C. Blots were blocked for 1 hour at room temperature in 5 % (wt/vol) skimmed milk/TBS-T. Blots were incubated with either: ER-α specific primary antibody, H226, (which recognizes an epitope encoded in exon 1/2 of the wild-type ER-α), the ER-α specific antibody, AER308 (which recognizes an epitope encoded in exon 4 of the wild-type ER-α), or the ER-α specific antibody AER314 (which recognizes an epitope encoded in exon 2 of the wild-type ER-α) overnight in 1 % (wt/vol) skimmed milk/TBS-T at 4°C at a concentration of 1:1000. Blots were then incubated with the appropriate peroxidase-conjugated secondary antibody (see Materials) for 1 hour at room temperature in 1 % (wt/vol) skimmed milk/TBS-T at a concentration of 1:1000. Blots were washed 4 times for 15 minutes each in TBS-T and detection was carried out using the ECL detection system (Amersham, Buckinghamshire, England) according to the manufacturer's instructions.

MAPK detection

Cell extracts were obtained by resuspending cell pellets in MAPK extraction buffer (100 mM β-glycerophosphate, 1 mM sodium orthovanadate pH 10 (made to pH 10, then boiled until solution turns clear and pH again adjusted to 10), 2 mM EGTA, 20 mM Tris-HCl pH 7.4, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 0.1 mM AEBSF, 1 mM PMSF, 1 mM dithiothreitol, 0.2 mM benzamidine) and subjecting cells to 3 cycles of freeze-thawing followed by a 10 minute room temperature centrifugation at 5,000 rpm. Equivalent amounts of protein were run on SDS-7.5 % PAGE with a 4 % stacking gel at 200 V for 45 minutes at room temperature under reducing conditions according to the Laemmli method (464). Gels

were equilibrated for 30 minutes at room temperature in ice cold CAPS transfer buffer. Gels were transferred to nitrocellulose using CAPS transfer buffer at 120 V for 1 hour at 4°C. Blots were air-dried and baked for 30 minutes at 65°C. Blots were blocked overnight in 5 % (wt/vol) skimmed milk/TBS-T. Blots were incubated with mouse anti-phospho-MAPK antibody for detection of dually phosphorylated MAPK (NEB, Beverly, Massachusetts; 1:1000 in 1 % (wt/vol) skimmed milk/TBS-T) or rabbit anti-ERK1 (C-16) for detection of total MAPK protein (Santa Cruz Biotechnology; 1:1000 in 1% skimmed milk/TBS-T) for 4 hours at room temperature. Blots were incubated with the appropriate secondary antibody (see Materials) for 1 hour at room temperature, 1:1000 in 1% skimmed milk/TBS-T. Detection was carried out using the ECL detection system (Amersham, Buckinghamshire, England) according to the manufacturer's instructions.

PD 98059 treated ER-α detection

The detection of ER-α after treating T5 and T5-PRF cells with the MEK inhibitor PD 98059 was carried out as described (240). This method was used initially in attempts to detect serine 118 phosphorylation of the wild-type ER-α and subsequently it was realized that PD 98059 decreased ER-α levels. This method is based on the Laemmli method using 7 % 16 cm X 16 cm acrylamide gels run under reducing conditions. T5 and T5-PRF cells were grown in 5% CS and cell extracts were obtained by addition of 0.25 mL of 2 X SDS-PAGE sample buffer (0.12 M Tris-HCl, pH 6.8, 4 % SDS, 20 % glycerol, 0.2 M dithiothreitol, 0.008 % bromophenol blue) warmed to near 100°C directly to plates and scraping cells into microfuge tubes kept on a 95°C heating block. Cells were then incubated at 95°C for 10 minutes before assay of protein levels (BioRad reagent) and equal amounts of protein loaded onto gels. Gels

were run for 1.5 hours at 200 V.

Transient transfections and CAT assays

T5, T5-PRF and MDA-MB-231 cells were passaged once in 5 % CS and set up in 100 mm dishes at 0.5 x 10^6 cells per dish in 5 % CS the day before transfection. MCF10A1 cells were passaged once in PRF-DMEM-special and set up in 100 mm diameter dishes at 2 x 10^6 cells per dish in PRF-DMEM-special two days before transfection. The following day the medium was changed to PRF-DMEM-hs and cells were transfected the following day, using the calcium phosphate/glycerol shock method (469) overnight using an equal volume of 2 X BBS buffer (50 mM BES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 6.95) combined with an equal volume of DNA solution (containing 3.2 µL of 2M $CaCl_2$ for every µg of plasmid DNA brought to the desired volume with sterile double distilled water), followed by a 2 minute glycerol shock (20 % vol/vol in PRF-DMEM). Cells were washed twice with 1 X PBS (1L 10 X stock = 2 g KCl, 2 g KH_2PO_4, 80 g NaCl, 11 g Na_2HPO_4, pH 7.1) and given fresh medium plus or minus drug of the appropriate concentration or vehicle alone. After 24 hours of treatment the cells were harvested. The dishes were placed on ice and the medium aspirated. The dishes were then washed twice with 1 X PBS and 1 mL of TEN (40 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl) was added to dishes. The cells were scraped from the dishes into a microfuge tube and the dishes rinsed with 0.5 mL of TEN. The cells were centrifuged for approximately 3 minutes at 4°C and 100 µL of 0.25M Tris-HCl pH 8 was added to the cell pellet. The cell lysate was obtained by 3 cycles of freezing and thawing followed by a 5 minute centrifugation at 5,000 rpm at 4°C. The supernatant was saved to a fresh microfuge tube and samples stored at -20°C for chloramphenicol acetyltransferase

(CAT) assay (470). Transfection efficiency was determined by cotransfection of pCH110 (β-galactosidase expression vector, Pharmacia) and assay of β-galactosidase activity (471). T5 and T5-PRF cells were transfected with 5 μg ERE-tk-CAT (148) to determine ER-α transcriptional activity, along with 5 μg pCH110. For experiments in which the activity of ERd3-4 was examined in T5 cells, transfections were performed using 5 μg ERE-tk-CAT, 5 μg pCH110, plus or minus ERd3-4 expression vector (0.1-1 pmol) or vector DNA alone (pSG5, 1 pmol) in addition, vector DNA was added to all transfection mixes to ensure equal amounts of DNA were added to each dish. MDA-MB-231 and MCF10A1 cells were transfected with 5 μg ERE-tk-CAT, 5 μg pCH110, plus or minus 0.5 pmol HEGO (wild-type ER-α expression vector, kindly provided by Dr. P. Chambon) with increasing amounts of ERd3-4 expression vector (0.5-2 pmol) or vector DNA alone (pSG5, 2 pmol) in addition, vector DNA was added to all transfection mixes to ensure equal amounts of DNA were added to each dish. Plasmids were isolated using either the standard cesium chloride gradient technique (472) or Qiagen plasmid isolation kit (Qiagen, Toronto, Canada) .

Long-Range ER-α RT-PCR

Total RNA was extracted using the TRIzol reagent (GIBCO/BRL, Grand Island, New York) and reverse transcribed (RT) to make cDNA for polymerase chain reaction (PCR) amplification. Reverse transcription was carried out using 1 μg of denatured RNA in a final volume of 15 μl (382). RNA was reverse transcribed in the presence of 1 mM deoxyadenosine triphosphate (dATP), 1 mM deoxythymidine triphosphate (dTTP), 1 mM deoxyguanosine triphosphate (dGTP), 1 mM deoxycytidine triphosphate (dCTP), 5 mM

dithiothreitol, 1 unit/μL ribonuclease inhibitor (RNAsin), 20 μM random hexamers, 50 mM Tris-HCl pH 7.5, 3 mM $MgCl_2$, 75 mM KCl and 5 units/μL MMLV-RT. Reverse transcription was carried out for 10 minutes at room temperature followed by a further incubation of 1 hour at 37°C and was stored at -20°C. Long-range PCR amplification was performed on RT products using a primer pair that consisted of 1/8U ER-α primer (5'-TGCCCTACTACCTGGAGAACG-3', sense; located in wild-type ER-α exon 1; nucleotides 615-637) and 1/8L ER-α primer (5'-GCCTCCCCCGTGATGTAA-3'; antisense; located in wild-type ER-α exon 8; nucleotides 1995-1978). The nucleotide positions given correspond to the published sequences of the human ER-α cDNA (67). PCR amplifications were performed using 1 μL of reverse transcription product (cDNA) in a final volume of 10 μL in the presence of 20 mM Tris-HCl pH 8.4, 2 mM $MgCl_2$, 50 mM KCl, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dCTP, 0.2 mM dGTP, 4 ng/μL of each primer, 1 unit of Taq DNA polymerase (Promega, Madison, Wisconsin) and 10 nM [α-^{32}P]dCTP (384). PCR was performed using 40 cycles (5 minutes at 94°C, 1 minute at 60°C, 2 minutes at 72°C, 1 minute at 94°C) on a Thermocycler (The Perkin-Elmer Corp., Foster City, California) (382).

PCR products were separated on 3.5% polyacrylamide gels containing 7 M urea which were run for 3.5 hours at approximately 1600 V. 5 μL of formamide buffer (80 % (vol/vol) deionized formamide, 1 mM EDTA pH 7.5, 0.1 % (wt/vol) xylene cyanol, 0.1 % (wt/vol) bromophenol blue) was added to each PCR tube, the samples were boiled and 5 μL of the sample was loaded onto the gel for separation. After separation, the gel was place on Whatman #1 filter paper, dried and exposed to film (Kodak XAR) overnight to visualize PCR

products.

Identification of PCR products

Bands of interest were cut from the dried gel after exposure to film, extracted, subcloned and sequenced (382). The gel fragment was placed in a 1.5 mL microcentrifuge tube to which 100 μL of distilled water was added and this was overlayed with 2 drops of mineral oil. The tube was heated at 100°C for 15 minutes and the eluate was transferred to a clean microfuge tube (473). 5 μL of this eluate in a total of 50 μL was used for further reamplification under the same conditions previously described for long-range RT-PCR with the exception that [α-^{32}P]dCTP was omitted from the final reaction mixture. The amplified product was purified using the Wizard PCR preps kit (Promega Corp., Madison, Wisconsin) and subcloned using the TA cloning vector kit (Invitrogen Corp., San Diego, California) according to the manufacturer's instructions. In brief this procedure involved ligating a portion of the amplified and purified PCR product into the TA cloning kit vector (pCR2.1) overnight at 14°C (ligation reaction contained: 5 μL sterile water, 1 μL 10 X ligation buffer, 2 μL pCR2.1 vector, 1 μL PCR product and 1 μL T4 DNA ligase). Following ligation, 2 μL of the reaction was incubated with competent bacterial cells for the purpose of transformation (30 minutes on ice followed by a 30 second heat shock at 42°C, 2 minutes on ice and 1 hour at 37°C). The bacteria were plated onto LB agar containing 25 mg/mL ampicillin and incubated overnight at 37°C. Transformant colonies were selected and miniprep plasmid DNA isolations were performed using standard laboratory techniques (472) for the purpose of sequencing. This procedure was used to subclone 3 separate RT-PCR bands from 3 separate RNA isolations and 3 colonies from each of the TA cloning vector kit isolations were

sequenced to confirm the identity of the RT-PCR band. Sequencing was performed using the T7 sequencing kit (Pharmacia, Uppsala, Sweden) based on the dideoxy (Sanger) chain termination method according to manufacturer's instructions with 5 μg of alkali-denatured plasmid DNA in the presence of [α-^{35}S]dATP and the appropriate primer. Sequencing reaction products (3 μL) were run on 6% acrylamide gels containing 7 M urea for approximately 1.5 hours at 2000 V using a BioRad sequencing gel apparatus. Gels were transferred to Whatman #1 filter paper, dried for approximately 2 hours at 80°C using a BioRad gel dryer and exposed to film (Kodak XAR) in order to visualize sequencing reaction products. In order to sequence the full length of the cDNA clones the following primers were used in separate sequencing reactions for each plasmid DNA obtained; M13 forward (5'-CTGGCCGTCGTTTTAC-3', supplied with the TA cloning kit), M13 reverse (5'-CAGGAAACAGCTATGAC-3', supplied with the TA cloning kit), 1/8L and 1/8U, P1L (5'-CTGGCTACATCATCTCGGTTCCGCAT-3'; antisense; located in wild-type ER-α exon 6) and P2U (5'-TCCTGATGATTGGTCTCGTCTGGCGC-3'; sense; located in wild-type ER-α exon 5).

Construction of variant ER-α expression vector

The RT-PCR product corresponding to the exon 3/4 deleted ER-α cDNA was cloned into the TA cloning vector (described above) (Invitrogen Corp., San Diego, California). Stu I digestion of this plasmid released an exon 3/4 deleted fragment which was used to replace the corresponding region of the wild-type ER-α from pOR8 (70), which contains a glycine to valine point mutation at amino acid 400. Stu I sites are in exon 2 and exon 7 of wild-type ER-α and the subcloned d3/4 PCR fragment resulted in a correction of the wild-type

sequence of glycine at amino acid 400 (in exon 5 in pOR8). The full length EcoR I ER-α fragment from HEGO (an expression plasmid containing wild-type ER-α coding region cloned into the eukaryotic expression vector pSG5 (70)) was then excised and replaced with the corresponding fragment from pOR8 containing the exon 3-4 deleted ER-α cDNA. The identity of the expression plasmid containing the exon 3-4 deleted ER-α (ERd3-4) was confirmed by restriction enzyme digestion and sequence analysis using the dideoxy method and T7 sequencing kit as described above.

***In vitro* transcription and translation**

In vitro transcription/translation (TnT) reactions were performed using a coupled transcription/translation system (TnT coupled Reticulocyte Lysate System, Promega Corp., Madison, Wisconsin). Reactions were performed according to the manufacturer's instructions with the addition of 1 μM $ZnCl_2$ to the reaction mixture to stabilize the DNA binding domain of the wild-type ER-α.

Ligand binding assay using TnT reaction products

The principle of the assay used to determine ligand binding to *in vitro* translated proteins is similar to that described for the whole cell receptor binding assays. Total and excess solutions where made by drying down the appropriate concentration of labelled and unlabelled [^{3}H]17β-estradiol or [^{3}H]-tamoxifen. For estrogen binding assays, [^{3}H]17β-estradiol was dried under air nitrogen to give a final stock of 40 nM in TE/BSA buffer (10 mM Tris-HCl pH 7.4, 1.5 mM EDTA, 0.2 % (wt/vol) BSA). To "total" tubes was added 10 μL of TnT lysate, 90 μL TE buffer (10 mM Tris-HCl pH 7.4, 1.5 mM EDTA), 50 μL [^{3}H]17β-estradiol solution and 50 μL TE/BSA. To 'excess' tubes was added 10 μL TnT

lysate, 90 μL TE buffer, 50 μL [^{3}H]-estradiol and 50 μL excess unlabelled estradiol (from a stock of 4 μM in TE/BSA). Samples were set up in microfuge tubes and left overnight in ice-water at 4°C. The following day 500 μL of charcoal/dextran (0.25 grams washed activated charcoal and 0.025 grams dextran T70 in TE buffer) was added to each tube and left for 30 minutes at 4°C. Samples were centrifuged for 10 minutes at 4°C and 250 μL of supernatant is added to 5 mL liquid scintillant for counting using a liquid scintillation counter.

Gel shift assay

Gel shift assays were performed using 4% acrylamide, 0.5 X TBE gels, run at 200 V for approximately 40 minutes at 4°C. A double-stranded ERE from the vitellogenin B1 gene (5'-GTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTT-3', synthesized at the DNA synthesis Lab., University of Calgary) was used as a probe for the gel mobility shift assay. 2-4 μl of TnT lysate (~ 1-2 ng protein) was used for each gel shift reaction as well as 0.5 ng labelled probe. The ERE was end-labelled by incubating 25 ng ERE, 4 μL 10 X polynucleotide kinase (PNK) heating buffer (0.2 M Tris-HCl, pH 9.5, 10 mM spermidine and 1 mM EDTA) and 35 μL distilled water at 70°C for 10 minutes. The sample was then cooled for 5 minutes on ice before adding 5 μL 10 X blunt end kinase buffer (0.5 M Tris-HCl, pH 9.5, 0.1 M $MgCl_2$, 50 mM dithiothreitol and 50 % glycerol), 5 μL [γ-^{32}P]-ATP and 2 μL T4 PNK (10 units/μL) and incubating at 37°C for 30 minutes. The labelled oligonucleotide was isolated using a G-25 Sephadex Quick Spin Column (Boehringer Mannheim, Indianapolis, Indiana). The appropriate amount of TnT reaction (marked in figure legends) was used in the gel shift reactions and T7 RNA polymerase heat-killed reaction refers to the use of a TnT reaction sample in which all the appropriate components are present but the T7

RNA polymerase enzyme was boiled for 5 minutes prior to use. Gel shift reactions were composed of 1 μL labelled ERE (0.5 ng), TnT reaction, and 8 μL 1 X stock buffer (200 μL contains: 50 μL 5 X stock (50 mM Hepes pH 7.9, 500 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 10 mM dithiothreitol and 50 % glycerol), 5 μg bovine serum albumin, 2.5 % vol/vol FICOL, and 25 μg poly dI-dC) and placed on ice for 15 minutes with or without drug treatment. Gel shift protocol was kindly provided by Dr. T. Miller (NorthWestern University, Chicago, Illinois).

MAPK assay

In vitro MAPK activity was measured using myelin basic protein (MBP) as a substrate (474). For experiments performed in serum-free conditions, cells were plated in 5% CS, and the following day the medium was changed to 5% CS minus serum and changed every day for 7 days. To measure basal MAPK activity, T5 cells were passaged twice in 5% CS and both T5 and T5-PRF cells were set up in 5% CS in 100 mm dishes at approximately 0.2 x 10^6 cells and harvested in MAPK buffer 3 days later. Cell extracts were obtained by the previously described method of three cycles of freezing and thawing and the resulting supernatant was used for *in vitro* MAPK assays. Reaction mixtures contained, 6 μL Buffer A (0.5 M Tris-HCl pH 7.5, 100 μg bovine serum albumin, 0.1 M $MgCl_2$), 8 μL Buffer B (37.5 μL Buffer A in a 100 μL solution containing 20 μM PKI peptide and 125 μM Calmidazolium), 6 μL of a 2 mg/mL stock solution of MBP (or water for blank control tubes), 100 μM ATP and 15 μCi [γ-^{32}P]ATP. The reaction was initiated by adding 10 μL of cell lysate in MAPK buffer containing 1 μg of protein to each tube and reactions were allowed to proceed for 10 minutes at 30°C. Reactions were terminated by spotting 20 μL of the sample onto Whatman P81

phosphocellulose paper and washing for 2 hours in phosphoric acid buffer (200 mM), changing wash buffer every 30 minutes. P81 paper was placed into a liquid scintillant vial, 5 mL of liquid scintillant was added and samples were counted using a liquid scintillation counter. Reactions that had no added MBP served as controls for background activity.

Results

I. Identification of estrogen regulated NM-IF proteins

Rationale

As discussed, the nuclear matrix (NM) is thought to play an important role in many nuclear processes and the ER-α has been localized to the NM. Initially, it was of interest to use a cell culture model to examine NM protein composition in an estrogen-nonresponsive versus estrogen-responsive breast cancer cell line. Norm Huzel, a technician in Dr.L.C.Murphy's laboratory, initially developed an estrogen-nonresponsive human breast cancer cell line, T5-PRF, through long-term growth of T5 estrogen-responsive human breast cancer cells in the absence of estrogen. This was accomplished by culturing cells in phenol-red free DMEM and charcoal-stripped fetal calf serum. This subline, T5-PRF, was further characterized and compared to the parental T5 cells.

Results

T5 human breast cancer cells are ER-α positive and estrogen treatment in culture results in increased proliferation of these cells. The T5-PRF subline was derived from the T5 cell line through growth in the absence of estrogen for at least 60 passages before examining the effects of estrogen treatment on the growth of these cells in culture. In the absence of estrogen, T5-PRF and T5 cells have comparable doubling times (in days) of 1.37 $\pm$ 0.17 (mean $\pm$ sem, n=4) and 1.26 $\pm$ 0.05 (mean $\pm$ sem, n=4) respectively. The addition of 10 nM estradiol decreased the doubling time of the T5 parental cells to 1.05 $\pm$ 0.07 (mean $\pm$ sem, n=4) days. This is in contrast to the T5-PRF cells in which the addition of 10 nM estradiol resulted in a doubling time of 1.43 $\pm$ 0.14 (mean $\pm$ sem, n=4) days. This is reflected in figure

6A in which estradiol treatment, even at higher doses, had little or no effect on the growth of T5-PRF cells compared to parental T5 cells in which estradiol treatment at all doses tested resulted in an increase in proliferation ($p < 0.0001$, ANOVA, comparing T5 to T5-PRF estrogen treatments).

The antiestrogens ICI 164,384 and 4-monohydroxytamoxifen were able to inhibit the growth of both T5 and T5-PRF cells, although the T5-PRF cells appeared less sensitive to growth inhibition by ICI 164,384 this was not of statistical significance (Figure 6B and C).

As mentioned, T5 cells are ER-α positive and to examine if the lack of growth response to estrogen in the T5-PRF cells was due to loss of ER-α expression, ligand-binding techniques were used to quantitate the level of ER-α expression. These results are shown in Table 3 and demonstrate that T5-PRF cells still retain expression of the ER-α, although at a level approximately 50% of that seen in the parental T5 cells. Therefore, the T5-PRF cell line, while still retaining ER-α expression, is estrogen-nonresponsive in terms of growth in culture, providing a model in which to study mechanisms involved in the acquisition of an estrogen-nonresponsive growth phenotype, thought to be one of the initial steps in the progression to hormone-independence (352).

It has been previously demonstrated that the ER-α is associated with the NM (344) and patterns of NM protein expression have been hypothesized to be involved in changes in gene expression (345). Therefore it was of interest to examine the pattern of NM proteins expressed in both the T5 and T5-PRF cell lines to determine if differences did exist. As described in the methods section, using an established protocol for NM isolation generated

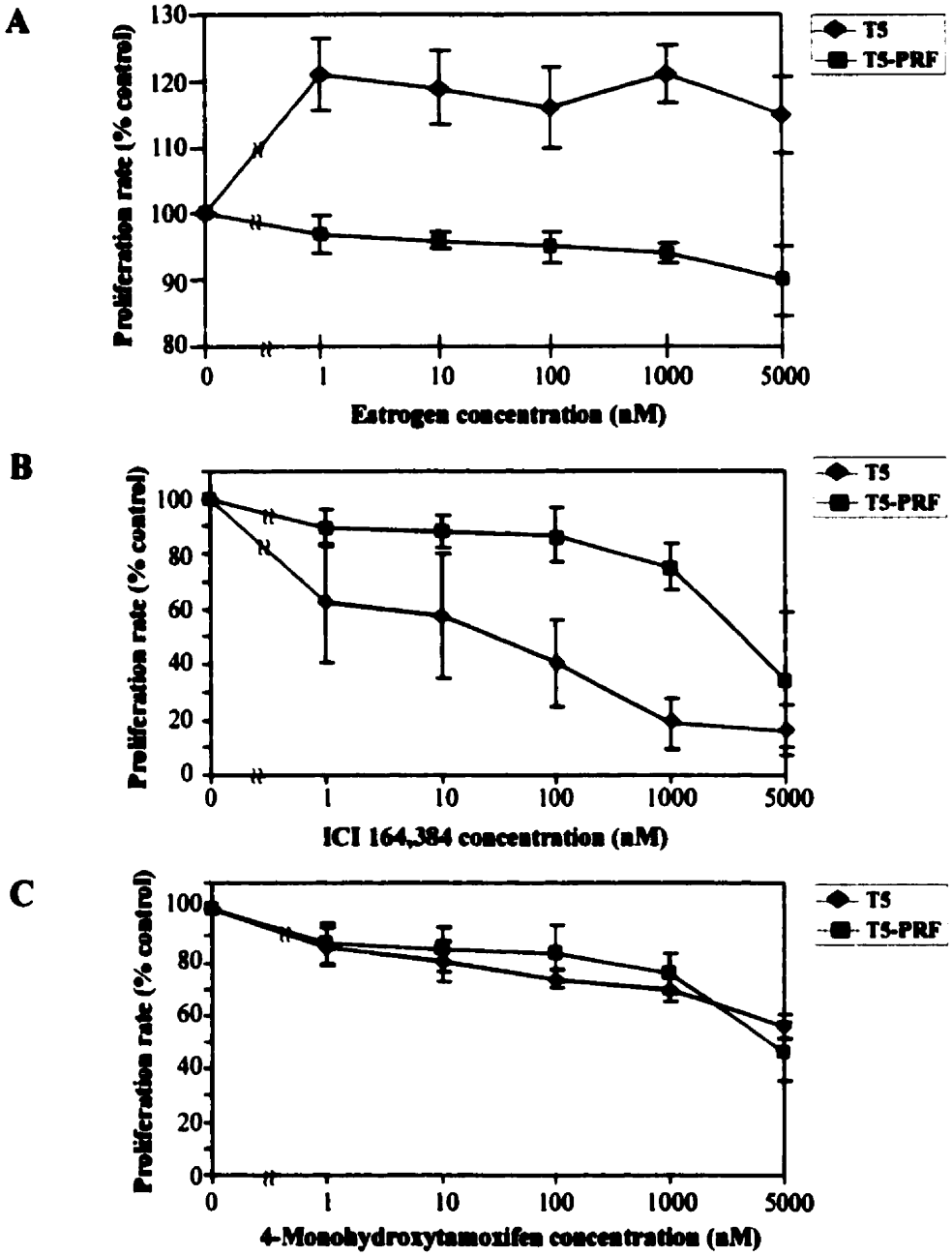


Figure 6. Effect of estrogen and antiestrogen on proliferation of T5 and T5-PRF human breast cancer cells. The effects of increasing concentrations of 17β-estradiol on cells grown in estrogen-deplete conditions (A) and antiestrogen (ICI 164,384 (B) and 4-monohydroxytamoxifen on cells grown in estrogen-replete conditions (C)) on proliferation rate (percentage control). Approximately 10^4 cells were plated in duplicate on day -2. On day 0, three dishes were counted and cells were treated with medium containing estradiol or the appropriate antiestrogen to a final concentration of 0, 1, 10, 100, 1000, and 5000 nM. Five days later cells were harvested and results expressed as proliferation rate as a percentage of control (see Methods for formulae). Results represent the mean ± sem, n = 4 independent experiments.

Table 3. ER-α levels in T5 and T5-PRF human breast cancer cells#.

Cell line	ER-α fmol/10^6 cells (± sem)
T5	235.0 ± 51.3
T5-PRF	119.4 ± 24.1*

#Results are expressed as ER-α fmol/10^6 cells ± sem, n= 5 independent experiments.

* $p<0.05$, Student's t-test

a protein profile referred to as the NM with attached IF (NM-IF) (320). The IF, as described in the introduction, forms a cage-like structure around the nucleus, but more recent evidence suggests that IF proteins, including cytokeratins, also exist within the nucleus, associated with NM structures and DNA (326). The T5 cell line, grown in the presence of estrogen, was initially used to work up the protocol for NM-IF isolation. The isolation of a NM structure is achieved after digestion of the nuclei with DNAse I followed by salt extraction to remove chromatin components. Initially, the method used for NM-IF preparation stopped at 0.25 M ammonium sulphate. However, the T5 NM-IF fraction still contained significant amounts of histone contamination (Figure 7, lane 4). To further improve the purity of the NM-IF fraction, sequential salt extractions of the NM-IF preparation were performed and resulted in a significant reduction in histone contamination (Figure 7, lane 7). Significantly, it was noted that three proteins of approximately 41, 45, and 54 kDa appeared to be enriched in the final NM-IF fraction of estrogen-treated T5 human breast cancer cells (Figure 7, lane 7). To ensure that the NM-IF procedure maintained the integrity of the NM-IF structure, samples were sent to Dr. M. Henzel at the University of Calgary for electron microscopy. Figure 8 is an electron micrograph of the NM-IF structure that demonstrates an intact structure. It can be seen that a filamentous network is present throughout the NM, the surrounding pore-lamina complex and the residual nucleoli can also be seen, consistent with results previously reported using other cell types (332).

Studies have demonstrated that the ER-α binds to the NM in the presence of hormone and have suggested that the association of the receptor with the NM is linked to hormone function (337,475). Western blotting was performed using NM-IF samples to confirm that

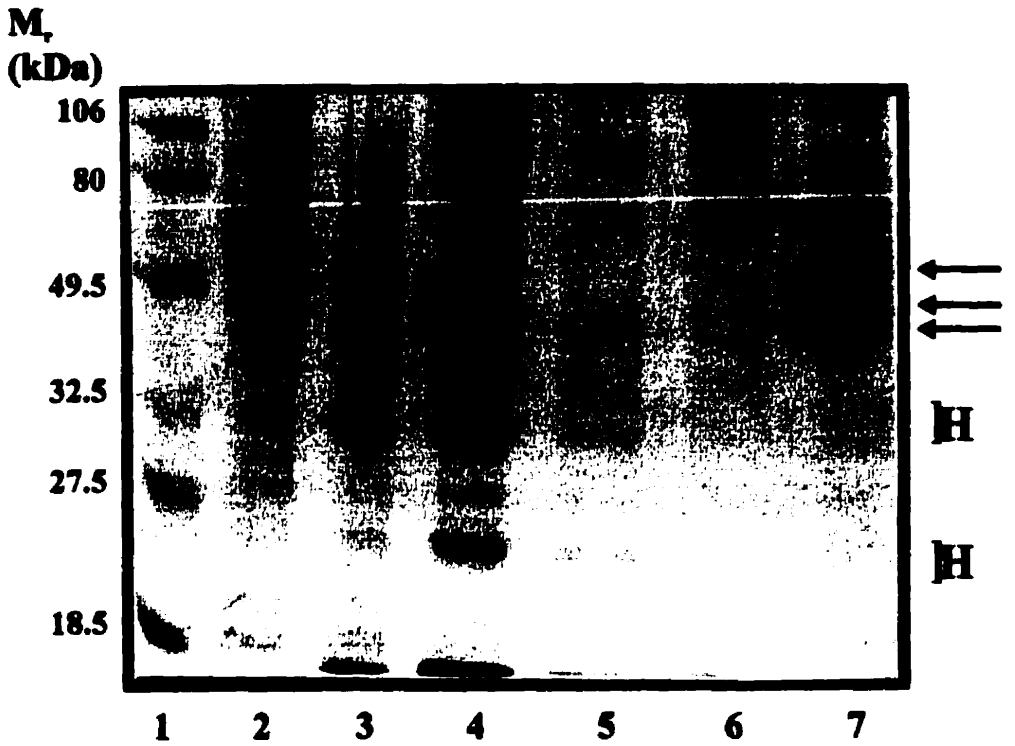


Figure 7. NM-IF protein profile in T5 human breast cancer cells. T5 cells were grown in the presence of 10 nM 17β-estradiol for 72 hours. NM-IF was isolated as described under Methods. Lane 1. Molecular mass (M_r) standards (BioRad); Lane 2. cytosol; Lane 3. whole nuclei; Lane 4. S1= 0.25 M ammonium sulphate NM-IF pellet; Lane 5. S2= 2 M NaCl solublized fraction; Lane 6. S3= 2 M NaCl/1% 2-mercaptoethanol solubilized fraction; Lane 7. NM-IF pellet. Subcellular fractions were run on SDS-12% acrylamide gels and were stained with Coomassie Blue to visualize protein bands. Arrows denote 54, 45, and 41 kDa protein bands. H= histones.

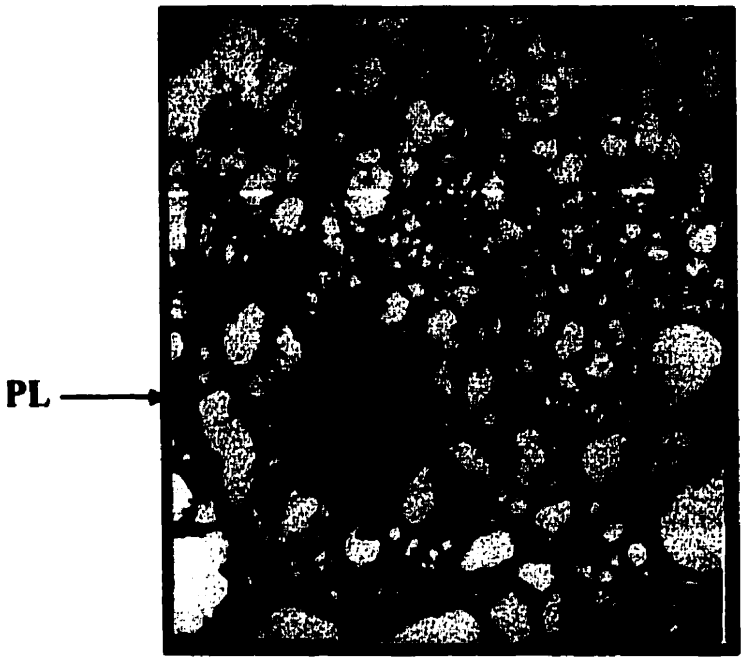


Figure 8. Electron microscopy of NM-IF preparation. The figure represents an electron micrograph of a resinless section of an NM-IF preparation from T5 human breast cancer cells. The components of the NM-IF are the residual nucleolus, pore-lamina complex (arrow PL) and the internal fibrogranular network. This photograph was generously taken by Dr. M. Henzel, University of Calgary. PL = pore-lamina. Magnification = 12000X.

the ER-α of T5 cells was indeed associated with the NM-IF fraction. Figure 9A demonstrates that the majority of the ER-α in T5 cells, after estrogen treatment, can be found in the NM-IF fraction and is resistant to salt extraction (compare lane 7 with lanes 4, 5, and 6). As a negative control, MDA-MB-231 (ER-α negative) human breast cancer cells were also examined in a similar fashion and figure 9B demonstrates no detectable ER-α in these cells. The association of ER-α with the NM-IF fraction was examined in T5-PRF estrogen-nonresponsive cells (grown routinely in estrogen-deplete conditions) and it was demonstrated that the ER-α was associated with the NM-IF fraction in these cells (Figure 9C). This study also confirmed the fact that the level of ER-α was lower in these cells compared to the parental T5 cells grown in estrogen-deplete conditions (compare the intensity of the band in lane 9 to lane 5, figure 9C). It also appears that the ER-α from both T5 and T5-PRF is less resistant to salt-extraction in this experiment (Figure 9C), since there is apparently more ER-α detectable in the chromatin fractions (Figure 9C, lanes 4 and 8). This is likely due to the lack of estrogen-treatment before isolation of the NM-IF (compare figure 9A, lanes 4-6, estrogen-treated T5 cells to figure 9C, lanes 4 and 8, non-estrogen-treated T5 and T5-PRF, respectively). As well, the ability to detect ER-α in the cytosol fraction (Figure 9A, lane 2 and figure 9C, lanes 2) may be due to leakage of nuclei during the isolation procedure.

The total pattern of NM-IF protein composition, as determined using 1 dimensional SDS-acrylamide gel electrophoresis and Coomassie blue staining, was compared between the parent T5 cells and T5-PRF human breast cancer cells. The NM-IF was isolated from cells cultured in the absence or presence of estrogen. As described, T5 cells were grown routinely in estrogen-replete conditions, containing 5% fetal calf serum and phenol-red containing

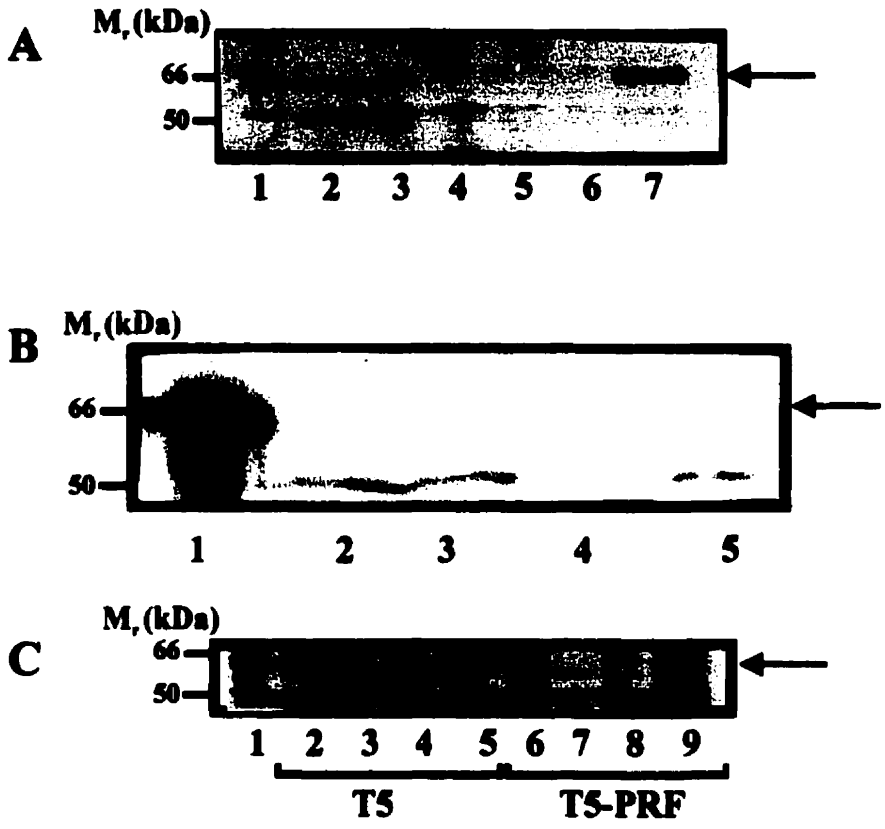


Figure 9. Association of ER-α with the NM-IF. NM-IF and subcellular fractions were isolated as described in Methods. Samples were run on SDS-12% acrylamide gels under reducing conditions. Western blotting and immune detection was performed as described in Methods using the ER-α antibody H226. **A.** T5 cells treated with 10 nM 17β-estradiol for 72h prior to harvesting and NM-IF isolation. Lane 1. *In vitro* transcribed/translated ER-α positive control; Lane 2. Cytosol fraction; Lane 3. Nuclei; Lane 4. S1 = 0.25 M ammonium sulphate solubilized fraction; Lane 5. S2 = 2 M NaCl solublized fraction; Lane 6. S3 = 2 M NaCl and 1% 2-mercaptoethanol solubilized fraction; Lane 7. NM-IF pellet. **B.** MDA-MB-231 ER-α negative human breast cancer cells. Lane 1. *In vitro* transcribed/translated ER-α positive control. Lane 2. Cytosol; Lane 3. Nuclei; Lane 4. Chromatin fractions (S1, S2, and S3 pooled); Lane 5. NM-IF fraction; **C.** T5 and T5-PRF human breast cancer cells grown in the absence of estrogen. Lane 1. *In vitro* transcribed/translated ER-α positive control; Lane 2. T5 cytosol; Lane 3. T5 nuclei; Lane 4. T5 chromatin fractions (S1, S2 and S3 pooled); Lane 5. T5 NM-IF fraction; Lane 6. T5-PRF cytosol; Lane 7. T5-PRF nuclei; Lane 8. T5-PRF chromatin fractions (S1, S2, and S3 pooled); Lane 9. T5-PRF NM-IF. Arrow indicates position of wild-type ER-α . Gels were loaded based on initial cell number in pellet before extraction such that each lane represents 0.5 x 10^6 cell equivalents. Molecular mass marker positions are denoted on the left (BioRad).

medium (5% CM). The NM-IF composition of T5 cells grown in this fashion is shown in figure 10, lane 1. When T5 cells grown in 5% CM were treated with 10 nM 17β-estradiol for 72 hours (these conditions were used to demonstrate ER-α association with the NM-IF) an increase in intensity of proteins of 54, 45 and 41 kDa was observed (Figure 10, lane 2 versus lane 1). While increases in the levels of these proteins were observed with 24 and 48 hour treatments, a 72 hour treatment resulted in consistently higher levels and was therefore used throughout. T5-PRF cells were grown routinely in estrogen-deplete conditions (medium without phenol-red and containing 5% charcoal-stripped fetal calf serum, 5% CS) and have been grown long-term in these conditions and are thus chronically-estrogen-depleted. T5-PRF cells grown under these conditions contain an elevated level of the three aforementioned proteins in the absence of estrogen (Figure 10, lane 3 versus lane 1) and estrogen treatment of T5-PRF cells did little to alter the levels of these proteins (Figure 10, lane 4 versus lane 3). To determine if growth in estrogen-deplete (5% CS) or -replete (5% CM) conditions was responsible for the changes in levels of these NM-IF proteins, T5 cells were cultured in acute (1 passage) estrogen-deplete conditions. Acute estrogen withdrawal of T5 parental cells resulted in a significant reduction in the level of these protein bands which could be increased after 10 nM 17β-estradiol treatment for 72 hours (Figure 10, lane 5 and lane 6). T5 cells that had been cultured in estrogen-deplete conditions for 10 passages, referred to as short-term chronic estrogen-depleted cells, were also examined. These cells contained elevated levels of the NM-IF proteins compared to acute-estrogen-deplete T5 cells (Figure 10, lane 7 versus lane 5) but the level was still reduced compared to long-term chronic estrogen-depleted T5-PRF cells (Figure 10, lane 7 versus lane 3). Additionally, in short-term chronically estrogen-

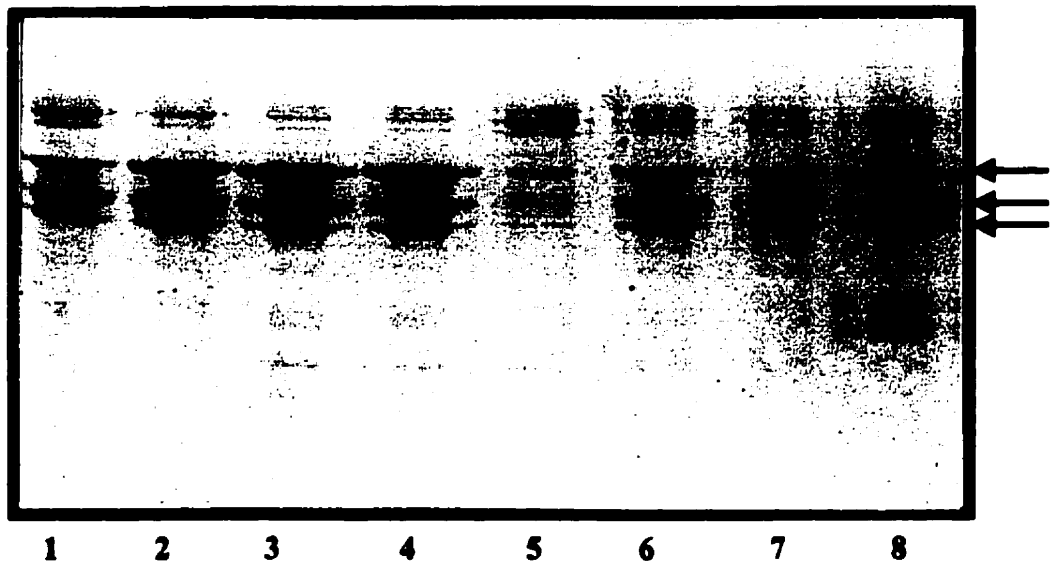


Figure 10. NM-IF in estrogen-replete and estrogen-deplete conditions. NM-IF was obtained as described in Methods. Lane 1. T5 NM-IF (ethanol vehicle); Lane 2. T5 NM-IF (10 nM estrogen 72 h); Lane 3. T5-PRF NM-IF (ethanol vehicle); Lane 4. T5-PRF NM-IF (10 nM estrogen 72 h); Lane 5. acute estrogen-deplete T5 NM-IF (ethanol vehicle); Lane 6. acute estrogen-deplete T5 NM-IF (10 nM estrogen 72 h); Lane 7. short-term estrogen-deplete T5 NM-IF (ethanol vehicle); Lane 8. short-term estrogen-deplete T5 NM-IF (10 nM estrogen 72 h). Subcellular fractions were run on SDS-12% acrylamide gels and stained using Coomassie blue to visualize protein bands. 5 μg of protein was loaded per lane. Arrows denote 54, 45 and 41 kDa bands. h=hours. Representative of 3 independent experiments.

depleted T5 cells, estrogen treatment still resulted in increased levels of the 54, 45 and 41 kDa protein bands (Figure 10, lane 8 versus lane 7). This suggests that the progression of T5 cells towards an estrogen-nonresponsive growth phenotype may be associated with increased expression and loss of estrogen-regulation of NM-IF proteins, specifically 54, 45 and 41 kDa proteins. The level of these three proteins in the NM-IF appears to be regulated by estrogen in the estrogen-responsive parental T5 cells, but not the estrogen-nonresponsive T5-PRF cells. The NM-IF proteins in the short-term estrogen-depleted T5 cells still respond to estrogen in terms of increased NM-IF abundance, but in the T5-PRF cells, high levels occur in the absence of estrogen and estrogen treatment no longer increases the level of these proteins.

The effect of estrogen on these NM-IF proteins was examined in more detail using T5 cells grown in the absence of estrogen for one passage (acute) to reduce the level of these proteins associated with the NM-IF fraction. The results of a dose-response experiment, 72 hours after estrogen treatment, are shown in Figure 11. This demonstrates that as little as 0.1 nM estrogen resulted in maximally increased levels of these proteins in the NM-IF of acute estrogen-depleted T5 cells. Comparing the abundance of these proteins to the lamins (Figure 11), it can be seen that the lamins are the most abundant NM-IF proteins in vehicle treated acute estrogen-depleted conditions, but following estrogen treatment, the 54, 45 and 41 kDa bands are much more abundant. Figure 11 also demonstrates the approximate percent changes in expression of the three NM-IF proteins as a percentage of control. To perform this semi-quantitation, the Coomassie Blue stained gels were scanned and the intensity of the combined lamin bands was used as a loading control to correct the intensity of the combined scanned

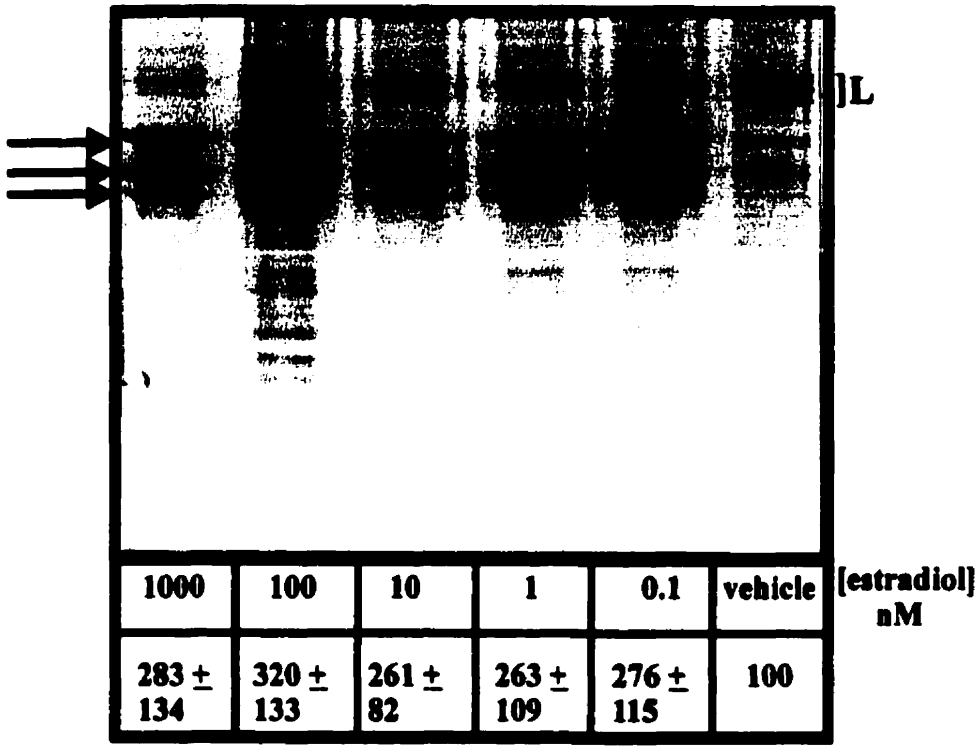


Figure 11. Estrogen dose-response effects on NM-IF protein levels in acute estrogen-depleted T5 cells. Cells were treated with 17β-estradiol at the appropriate concentration or ethanol vehicle for 72 hours. NM-IF was isolated as described in Methods. Samples were run on SDS-12% acrylamide gels and stained with Coomassie Blue to visualize protein bands. 5 μg of protein was loaded per lane. Arrows show 54, 45 and 41 kDa bands. L= lamins. Numbers below 17β- estradiol concentrations represent 54, 45 and 41 kDa protein levels as a percentage of control, mean ± sem, n=4. $p<0.05$, ANOVA. Lamin bands were used as a loading control.

data for the 54, 45 and 41 kDa bands (data were obtained using NIH Image and Ofoto 2.0 software packages). Once the data were normalized for loading using the lamins, the results were expressed as percentage of vehicle treated control cells. Data represent the mean $\pm$ sem of four independent experiments and demonstrate that estrogen treatment results in an approximately 2-3 fold increase in expression of the 54, 45 and 41 kDa bands (Figure 11 bottom panels, $p<0.05$, ANOVA). The identification of the lamins was performed using Western blotting and immune detection of the NM-IF fraction in parallel with purified lamins as standards (Figure 12).

As stated, T5-PRF chronically estrogen-depleted cells were grown routinely in the absence of estrogen. This is accomplished by growing the cells in cell culture medium that lacks the pH indicator phenol red (which contains estrogenic contaminants) and also by growing the cells in 5% charcoal-stripped fetal calf serum to remove endogenous steroids. T5 parental cells are grown routinely in medium that contains phenol red and 5% fetal calf serum. The increased levels of the 54, 45 and 41 kDa proteins that is seen in the chronically estrogen-depleted T5-PRF cells may be a result of the absence of estrogen, or may be due to some other component of fetal calf serum that is removed during the charcoal-stripping process. To examine the effects of fetal calf serum on the level of the three proteins associated with the NM-IF, acute estrogen-depleted T5 cells and T5-PRF cells were grown in the presence of estrogen or 5% fetal calf serum for 72 hours. The results shown in Figure 13 demonstrate that estrogen appears to be responsible for the increased level of these proteins in the NM-IF fraction.

The effect of antiestrogens on the expression of the 54, 45 and 41 kDa proteins was

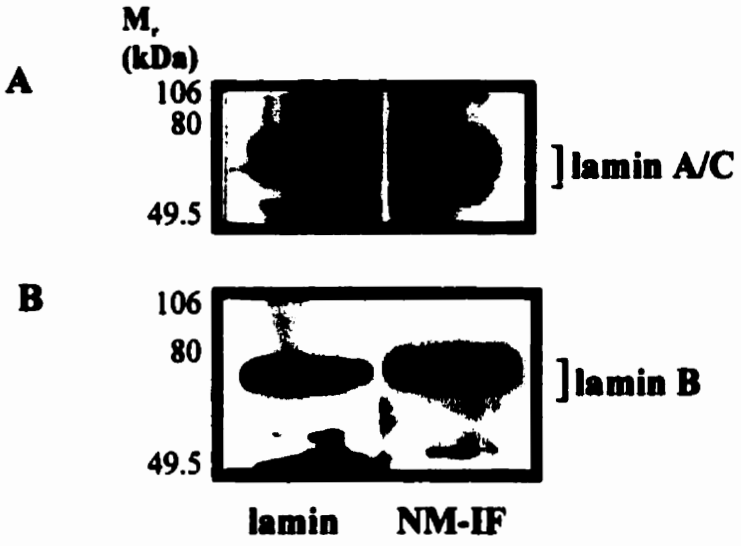


Figure 12. Immune detection of lamin proteins. NM-IF and purified lamins were run on SDS-12% acrylamide gels under reducing conditions.Western blotting and immune detection were performed as described in Methods. Purified lamins were run as positive controls (a generous gift from Dr. Y. Raymond (Montreal, PQ). Antibodies to lamins A/C (119D5-F1) and lamin B (131C3), panel A and B respectively, were also generously provided by Dr. Y. Raymond. Molecular mass marker positions are denoted on the left (BioRad).

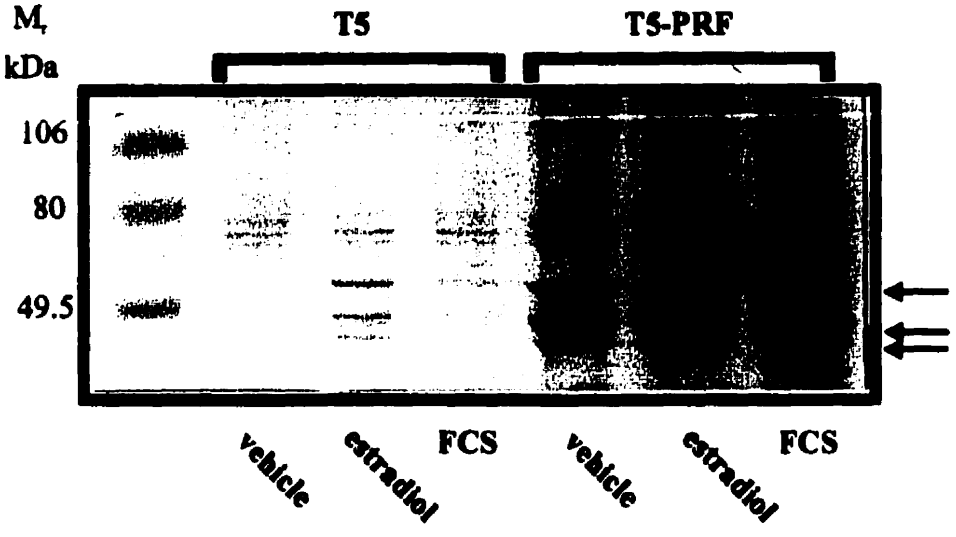


Figure 13. Effects of fetal calf serum on NM-IF proteins. T5 cells were grown for one passage in the absence of estrogen (acute estrogen-depletion) and T5-PRF cells were grown routinely in the absence of estrogen. Cells were treated with 10 nM 17β-estradiol or 5% fetal calf serum (FCS) for 72 hours before harvesting. NM-IF preparation was as described under Methods. Samples were run on SDS-12% acrylamide gels and stained using Coomassie Blue to detect protein bands. 10 μg protein was loaded per lane. Molecular mass standards are shown (BioRad). The 54, 45 and 41 kDa bands are denoted with arrows. n=2 independent experiments.

next examined. Treating T5 cells, grown in the presence of estrogen (5% CM), for 72 hours with 100 nM 4-monohydroxytamoxifen (TOT) resulted in a marked reduction in the NM-IF levels of these proteins (Figure 14A). The amounts of these three proteins associated with the NM-IF fraction of long-term chronically estrogen-depleted T5-PRF cells did not appear to be affected by antiestrogen treatment (Figure 14B). Thus, although antiestrogens inhibit the proliferation of these cells, the inhibitory effect on the abundance of the three NM-IF proteins seen in the parent T5 cell line is no longer observed. The dose-dependence of antiestrogen treatment was examined in T5 cells grown in estrogen-replete conditions (5% CM). Similar to that observed with estrogen dose effects, the abundance of these proteins in the NM-IF fraction was sensitive to alteration by antiestrogen. With as little as 10 nM ICI or 0.1 nM TOT, significant reductions in the levels of these proteins in the NM-IF were seen (Figure 15A, B, $p<0.05$, ANOVA, for both ICI and TOT). Interestingly, in T5 parental cells, there is also a dissociation in the ability of the antiestrogens to inhibit cell growth and their ability to decrease the level of the NM-IF proteins. The antiestrogen ICI is a more potent antiestrogen than TOT in terms of growth inhibition (Figure 6B, C) but is less potent than TOT in terms of effects on NM-IF protein levels (Figure 15A, B).

The effects of estrogen treatment on the level of these three proteins in terms of whole cell extracts were also examined. The data presented in Figure 16 demonstrate that even at the whole cell level, estrogen treatment increased the abundance of these proteins in T5 but not T5-PRF cells, suggesting that estrogen has an effect on increasing the total expression of these proteins, and the results do not merely reflect an increased association with the NM-IF fraction.

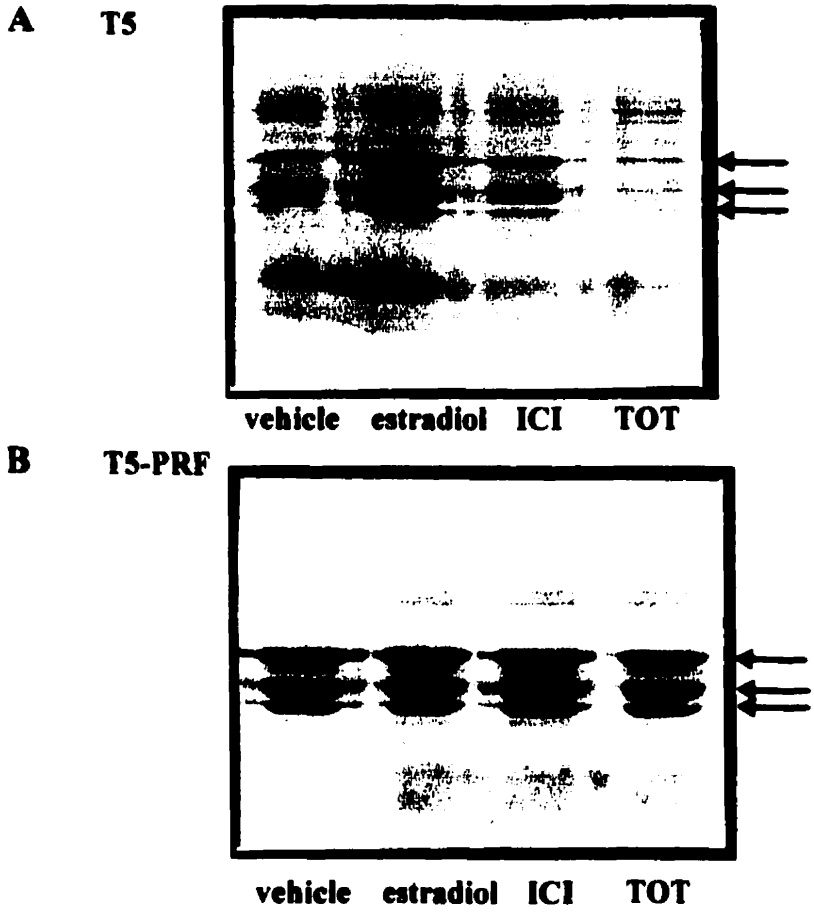
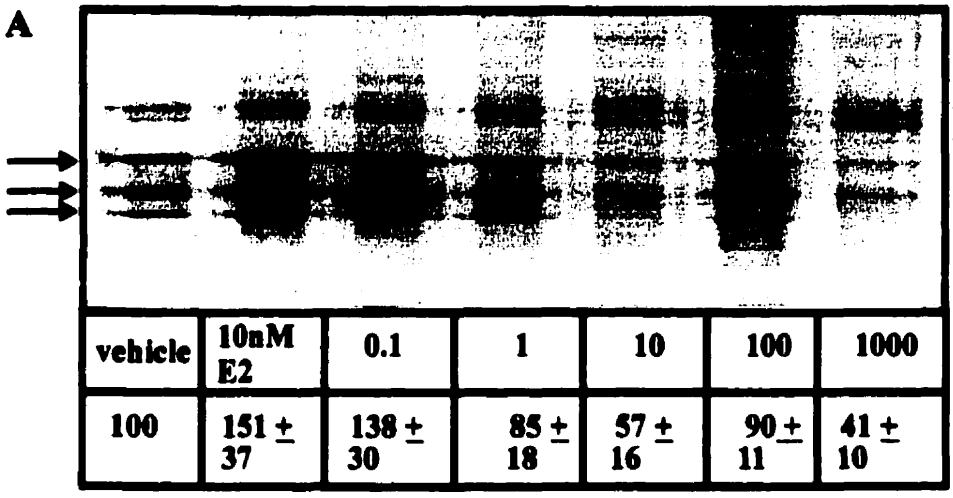
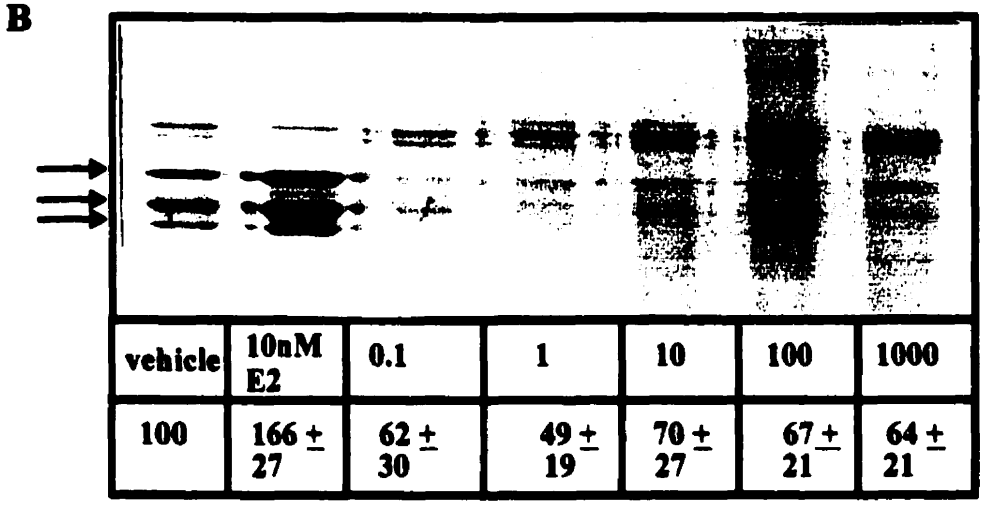


Figure 14. Effects of antiestrogens on NM-IF proteins in T5 and T5-PRF human breast cancer cells. T5 cells were grown in estrogen-replete conditions (5% CM) and treated with ethanol vehicle, 10 nM 17β-estradiol or 100 nM of the antiestrogens ICI 164,384 (ICI) or 4-monohydroxytamoxifen (TOT) for 72 hours prior to harvesting and isolation of NM-IF fractions. T5-PRF cells were routinely grown in the absence of estrogen and treated in a similar fashion to T5 parental cells. NM-IF fractions were run on SDS-12% acrylamide gels and stained with Coomassie Blue to detect protein bands. Arrows denote the position of the 54, 45 and 41 kDa NM-IF protein bands. 5 μg protein was loaded per lane. n = 3, independent experiments.



vehicle	10nM E2	0.1	1	10	100	1000	[ICI] nM
100	151 ± 37	138 ± 30	85 ± 18	57 ± 16	90 ± 11	41 ± 10	



vehicle	10nM E2	0.1	1	10	100	1000	[TOT] nM
100	166 ± 27	62 ± 30	49 ± 19	70 ± 27	67 ± 21	64 ± 21	

Figure 15. Dose-dependent effects of antiestrogen on NM-IF proteins in T5 human breast cancer cells. T5 cells were plated in estrogen-replete conditions (5% CM) and following antiestrogen treatments cells were harvested and NM-IF prepared as described in Methods. NM-IF samples were run on SDS-12% acrylamide gels and stained with Coomassie Blue to visualize protein bands. Cells were treated with (A) ICI 164,384 (ICI) or (B) 4-monohydroxytamoxifen (TOT) at the appropriate concentration. Control cells were treated with vehicle and for comparison cells were also treated with 10 nM 17β-estradiol. All treatments were for 72 hours. Arrows denote 54, 45 and 41 kDa protein bands. 5 μg protein was loaded per lane.L= lamins. Numbers below the treatment concentration represent 54, 45 and 41 kDa protein bands as a percentage of control, mean ± sem, n=3 independent experiments. $p<0.05$, ANOVA, for both ICI and TOT dose-responses. Lamin bands were used as loading controls.

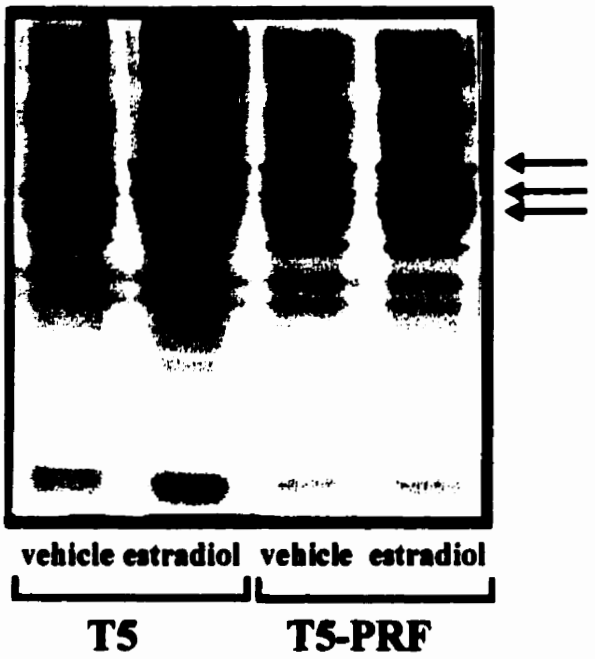


Figure 16. Whole cell extracts of T5 and T5-PRF human breast cancer cells. Cells were treated with 17β-estradiol or vehicle control for 72 hours. Cells were dissolved in 8 M urea and samples were run on SDS-12% acrylamide gels and stained with Coomassie Blue to detect protein bands. Positions of 54, 45 and 41 kDa bands are marked with arrows. 10 μg protein was loaded per lane. n=2 independent experiments.

Concomitant with these studies, Helmut Dotzlaw, a technician in Dr. L.C.Murphy's laboratory, had noted that the level of these proteins was altered by progestin treatment in the estrogen-responsive and ER-α positive human breast cancer cell line T47D. H. Dotzlaw further chromatographically enriched for these proteins and isolated spots from 2-dimensional (2-D) gels which were then sent out for microsequencing (WM Keck Foundation, New Haven, Connecticut). This resulted in the identification of these proteins as the cytokeratins 8 (54 kDa), 18 (45 kDa) and 19 (41 kDa). These protein bands were confirmed to be the same proteins identified in the NM-IF of T5 human breast cancer cells after column purification of these proteins from T5 cells followed by 2-D gel analysis of fractions (apparent molecular size and isoelectric point comparisons) from T47D, T5 and mixing experiments of the two sets of column fractions from each cell line (Figure 17). Additionally, whole cell extracts obtained from T5 cells before and after estrogen treatment were also subjected to 2-D gel analysis and stained with Coomassie Blue to demonstrate these spots increased after estrogen treatment (Figure 18).

To determine if a correlation existed between ER-α expression and expression of cytokeratin (CK) 8, 18 and 19, total NM-IF composition was compared between ER-α positive and ER-α negative human breast cell lines. Figure 19 clearly shows a marked difference in total NM-IF composition between ER-α positive and negative cell lines. In particular, a notable decrease or absence of the CK8, 18 and 19 bands was observed in the two ER-α negative breast cell lines examined.

In conclusion, these data demonstrate an altered expression and regulation by estrogen of NM-IF associated cytokeratins 8, 18 and 19 in estrogen-nonresponsive T5-PRF human

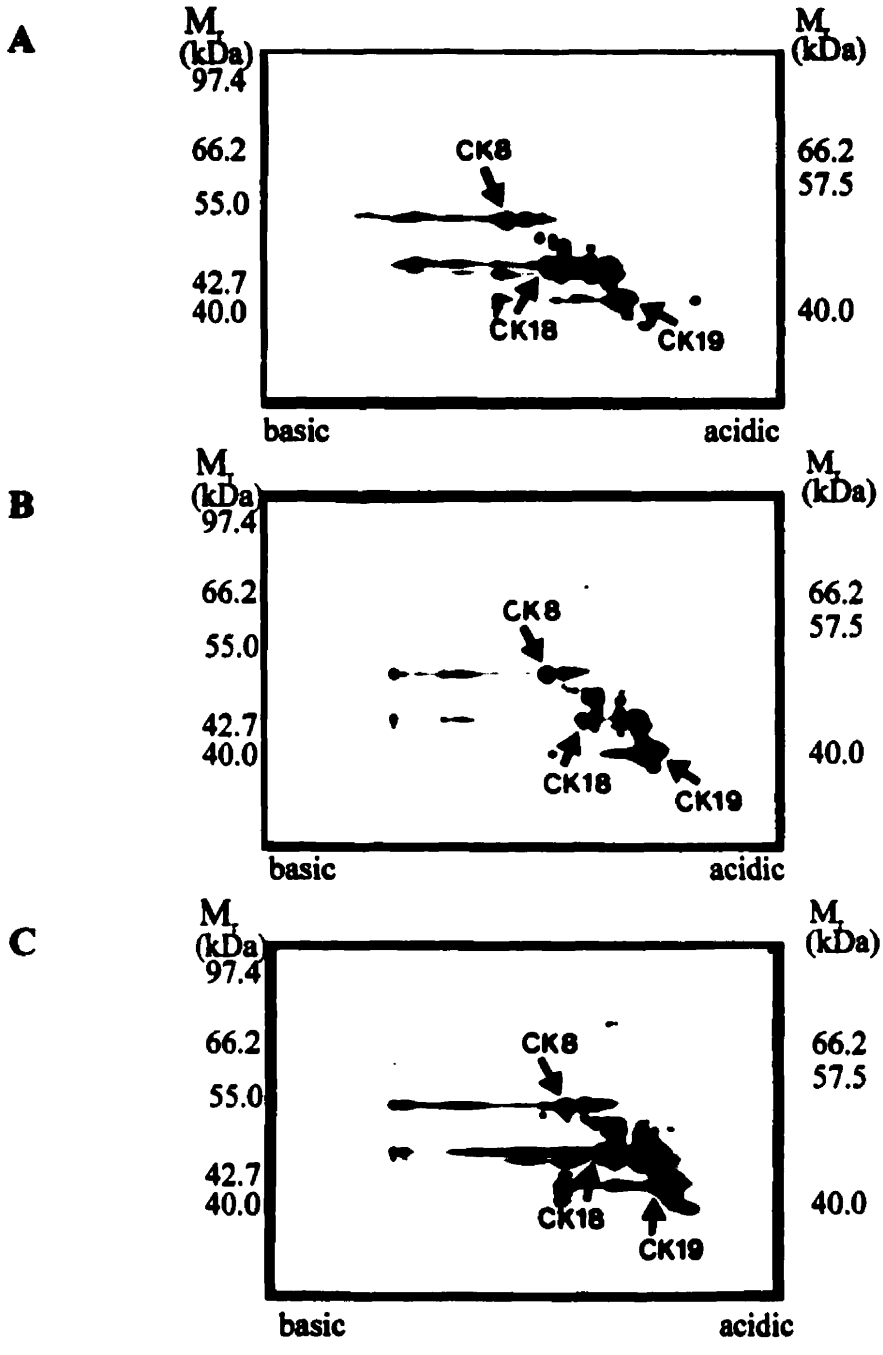


Figure 17. Two-dimensional electrophoresis of T5 and T47D column fractions. **A.** T5 column fraction. **B.** T47D column fraction. **C.** T5 and T47D mix. 15 μg protein was loaded per gel. Gels were silver stained to visualize protein bands. Column purification, 2-D electrophoresis and silver staining were performed as described under Methods. Position of molecular mass markers (BioRad) is shown. Positions of the cytokeratin bands are marked.

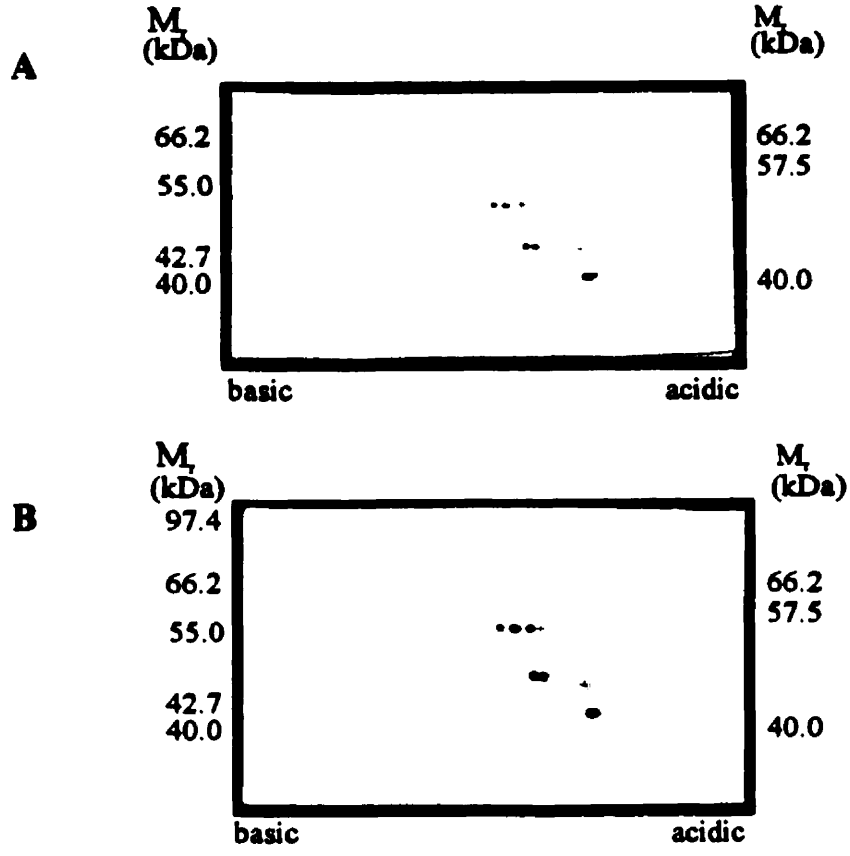


Figure 18. Two-dimensional gel electrophoresis of T5 whole cell extracts. 2-D gel analysis was performed as described in Methods. **A.** T5 cells were grown in the absence of estrogen for 72 hours and cells were harvested and lysed in 8 M urea. **B.** T5 cells were treated with 10 nM 17β-estradiol for 72 hours before harvesting cells and lysing in 8 M urea. 30 μg protein loaded per gel. Gels were stained with Coomassie Blue to visualize protein bands. n = 3 independent experiments.

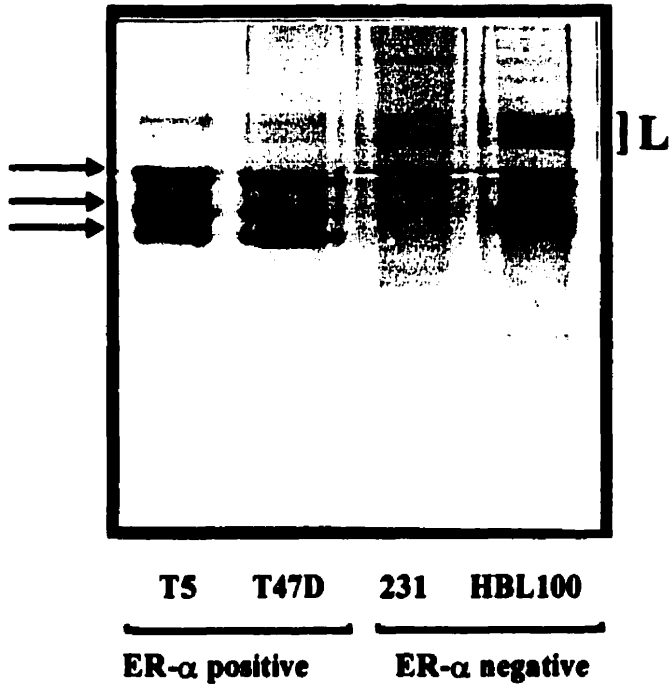


Figure 19. ER-α positive versus ER-α negative NM-IF protein composition. NM-IF composition was compared between ER-α negative and ER-α positive human breast cell lines. Cells were grown in estrogen-replete conditions (5% CM) and NM-IF isolated as described in Methods. Samples were run on SDS-12% acrylamide gels and were stained with Coomassie Blue to visualize protein bands. 231= MDA-MB-231; ER-α negative human breast cancer cell line. HBL100; ER-α negative breast epithelial cell line. Arrows denote the 54 (CK8), 45 (CK18) and 41 (CK19) kDa bands. CK, cytokeratin. L= lamins. 5 μg protein loaded per lane. n = 2, independent

breast cancer cells. However, although antiestrogens inhibit the proliferation of T5-PRF cells, they do not decrease the levels of the cytokeratins 8, 18 and 19 in the NM-IF as they do in the parental T5 cell line, suggesting that both estrogen and antiestrogen sensitivity in terms of regulating NM-IF levels of these proteins, have been lost in T5-PRF cells.

II. Variant ER-α expression in estrogen-nonresponsive T5-PRF human breast cancer cells.

Rationale

The previous studies had demonstrated that T5-PRF cells were no longer responsive to estrogen in terms of growth in culture and appear to have lost responsiveness to a set of proteins that are regulated by estrogen and antiestrogen in the parental T5 human breast cancer cells. It was reasoned that the activity of the ER-α in T5-PRF cells may be altered and if so a possible mechanism may be alterations of ER-α structure and/or function. Studies were ongoing in Dr. L.C.Murphy's laboratory to identify variant ER-α mRNA transcripts in breast tumours and therefore a technique established by Dr.E.Leygue in our laboratory was used to compare expression of variant ER-α mRNA transcripts between T5 and T5-PRF breast cancer cells.

Results

To investigate further the mechanism(s) responsible for the estrogen-nonresponsiveness of T5-PRF cells, ER-α transcriptional activity was examined by transient transfection assays using an estrogen-responsive reporter gene. Figure 20 demonstrates a typical chloramphenicol acetyltransferase (CAT) assay. Cells were transfected with the

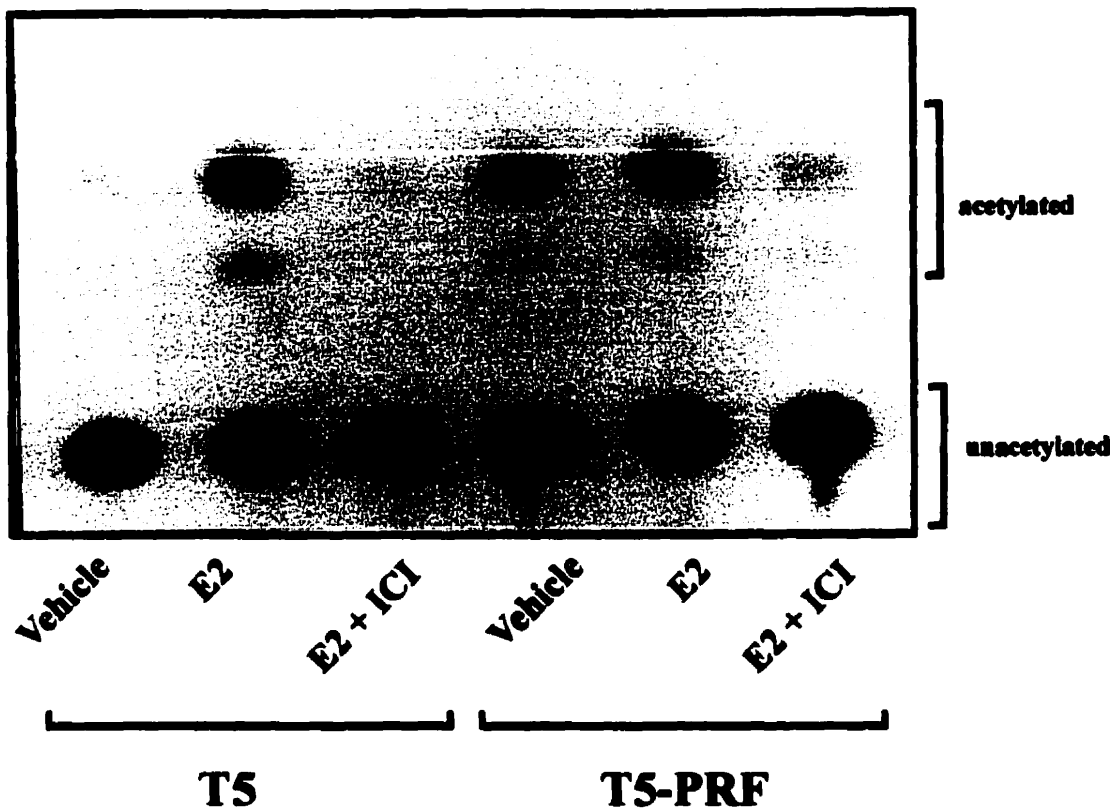


Figure 20. Chloramphenicol acetyltransferase (CAT) assay. A typical chloramphenical acetyltransferase assay (CAT) assay is shown. E2 = 10 nM 17β-estradiol; ICI = 1 μM ICI 164,384. Transfections and CAT assays were performed as described in Methods. Acetylated and unacetylated spots are labelled. Spots are cut out by overlaying a transparency sheet over the autoradiogram and marking where the spots are in relation to the origin. The transparency is then placed over the thin layer chromatography sheet (TLC) and spots cut out and counted using liquid scintillation counting techniques. Results are calculated by expressing acetylated counts as a percentage of total (acetylated and nonacetylated) counts and experiments are combined to generate histographical representations of the data.

estrogen-responsive reporter gene, ERE-tk-CAT (148), and the β-galactosidase expression vector pCH110 (Pharmacia) to control for transfection efficiency. After transfection, cell extracts were prepared and the β-galactosidase activity in the samples was measured. The β-galactosidase activity was used to equalize the volume of extract used for the CAT assay. If the reporter promoter (an ERE in this case) is active it will result in the production of the chloramphenicol acetyltransferase enzyme (not normally produced in mammalian cells) and the enzyme activity can be measured by the acetylation of [^{14}C]chloramphenicol, resulting in a change in mobility on thin-layer chromatography sheets (TLC). These sheets are exposed to film and the resulting spots can be visualized (Figure 20). To quantitate an assay, a transparency film is overlayed on the autoradiogram and marked, this is then placed over the TLC sheet and the spots can be cut out and the radioactivity counted using liquid scintillation techniques. The results are calculated as a percentage of acetylated versus total (acetylated and nonacetylated) spots (marked on Figure 20) and can then be expressed as a fold increase or decrease over control (basal, arbitrarily set at 1.0). The histogram in figure 21A shows the fold difference in CAT activity between the T5 and T5-PRF human breast cancer cell lines. As expected, estrogen treatment increases CAT activity in T5 and to a lesser extent in T5-PRF cells, while the antiestrogen ICI 164,384 inhibits the estrogen-induced transcriptional activity of the ER-α in both cell lines. In the absence of added estrogen there is a low basal ER-α activity in the parental T5 cells, however in the estrogen-nonresponsive T5-PRF cells, the basal ER-α activity was 3.6 ± 0.5 (mean $\pm$ sem, n=7) fold higher than that seen in T5 cells ($p<0.05$). Consistent with the increased basal CAT activity in T5-PRF cells being mediated by ER-α, treating cells under basal conditions with ICI 164,384 alone almost completely

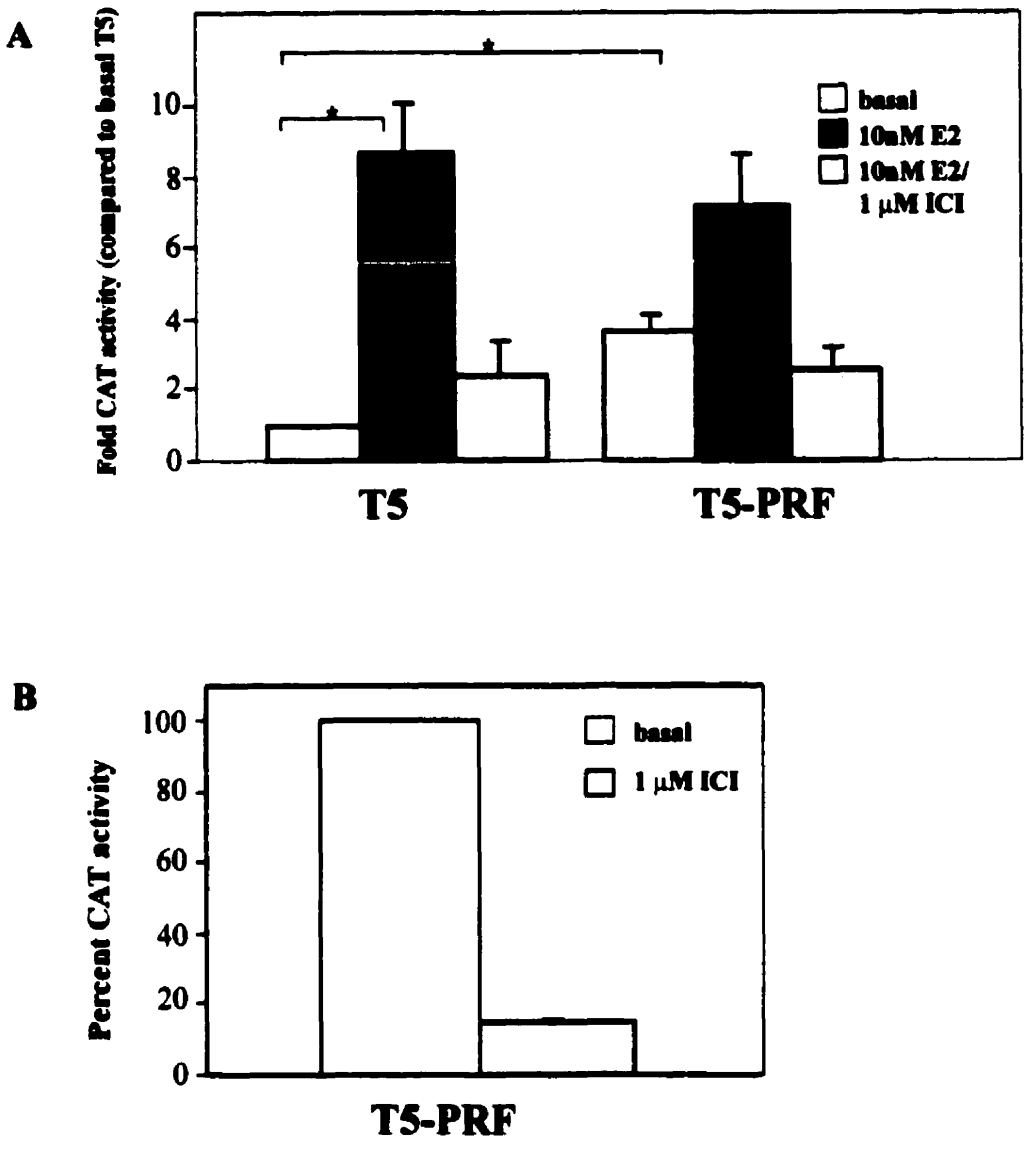


Figure 21. ER-α transcriptional activity. A. T5 and T5-PRF cells were transfected and CAT assays performed as described in Methods. Results are expressed as fold CAT activity compared to T5 basal (arbitrarily set at 1.0). Cells were grown in estrogen-deplete conditions (5% CS) and treated with vehicle alone (basal), 10 nM 17β-estradiol (E2) or 10 nM E2 plus 1 μM ICI 164,384 (ICI) for 24 hours. *$p<0.05$, Student's t-test (compared to T5 basal). Results represent mean ± sem, n=7 independent experiments. **B.** T5-PRF cells were transfected and CAT assays performed as described in Methods. Cells were treated with ICI 164,384 alone under basal (estrogen-deplete) conditions. Results represent percentage CAT activity with T5-PRF basal set as 100%, n=2.

abolished the increased basal transcriptional activity (Figure 21B).

As discussed in the Introduction, PR expression is a marker of ER-α activity, therefore PR levels were examined to determine if the increase in basal ER-α activity in T5-PRF cells was reflected in an endogenous estrogen-responsive gene. Under basal (i.e. no added estrogen) conditions the T5-PRF cells have significantly higher PR levels (~3 fold) than the parent T5 cells passaged twice in 5% CS (as defined in Methods) before receptor assays (464 ± 12 fmol/10^6 cells, n=4 versus 148 ± 28 fmol/10^6 cells, n=3, mean $\pm$ sem, see figure 22).

Since there was an observed increased basal ER-α activity from both an estrogen-responsive reporter gene and an endogenous estrogen-responsive gene (PR) in T5-PRF cells despite a decreased level of endogenous ER-α as determined by ligand-binding analyses and Western blotting, it was reasoned that the intrinsic activity of wild-type ER-α in these cells was increased or some ER-like activity existed that was not detectable by ligand binding experiments. Alterations in the structure or presence of variant forms of the ER-α with ligand-independent activity could be one mechanism for the observed results. Long-range ER-α reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed on total RNA isolated from T5 and T5-PRF cells to examine the pattern of deleted variant ER-α mRNA expression. RT-PCR analysis was performed using a primer pair specific for exons 1 and 8 of the wild-type human ER-α sequence, allowing detection of any variant ER-α mRNA species containing both exons 1 and 8 of wild-type ER-α sequence. Figure 23A shows the PCR products obtained and the presence of a 928 base pair (bp) band whose relative expression is markedly increased in T5-PRF estrogen-independent cells. To confirm the

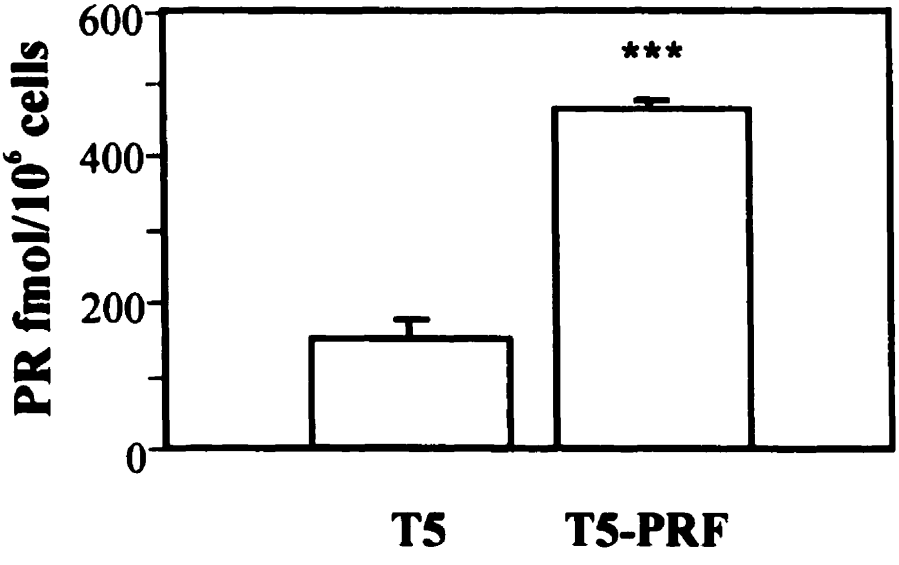


Figure 22. Progesterone receptor levels. PR levels were determined by whole cell binding as described in Methods. T5 and T5-PRF cells were grown in estrogen-deplete conditions (basal, 5% CS). PR levels are expressed as fmol PR/10^6 cells and results represent mean ± sem, n=3 independent experiments, *** = $p<0.0001$ basal T5-PRF PR levels versus basal T5 PR levels, Student's t-test.

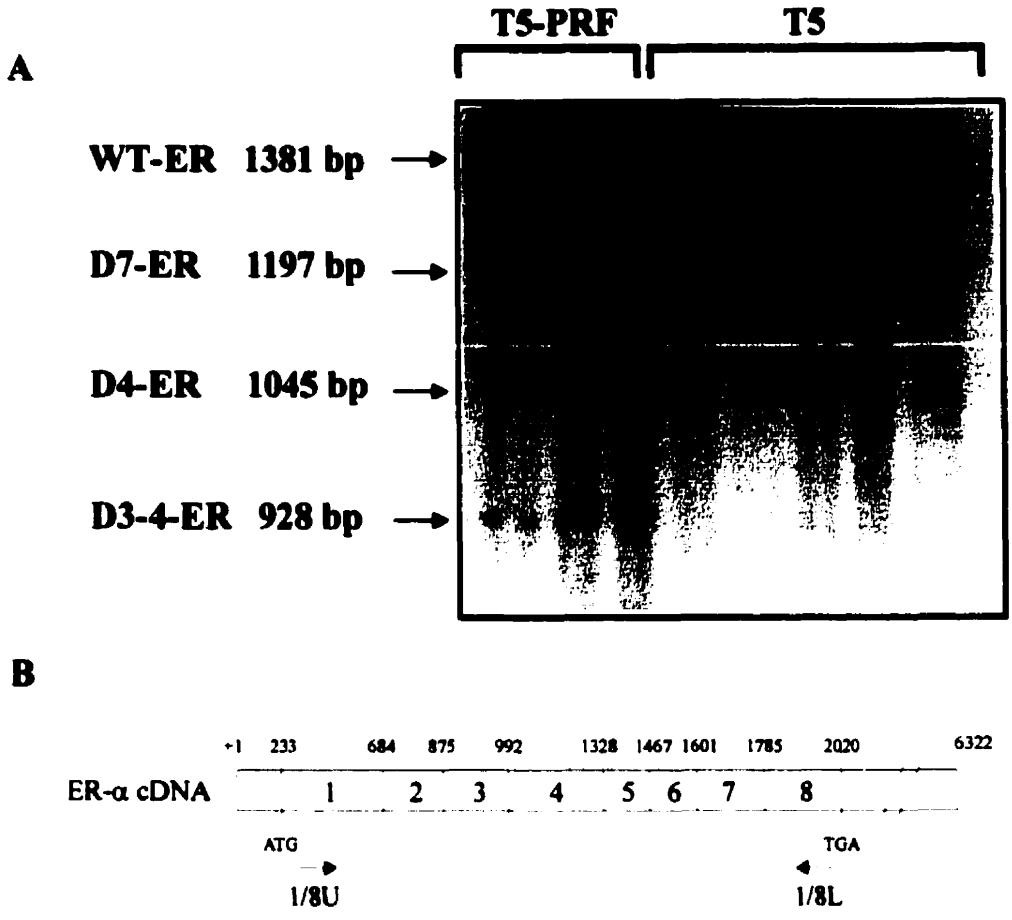


Figure 23. Long-range ER-α RT-PCR. A. Total RNA was extracted from T5 and T5-PRF human breast cancer cells, reverse transcribed and PCR amplified using 1/8U and 1/8L ER-α primers as described in Methods. Labelled PCR products were separated on 3.5% acrylamide-urea gels and visualized using autoradiography. WT-ER = wild-type ER-α, D7-ER = exon 7 deleted ER-α, D4-ER = exon 4 deleted ER-α and D3-4-ER = exon 3 and 4 deleted ER-α, based on size compared to labelled markers. The 928bp product was excised from a gel and subcloned (in triplicate) and three colonies from each independent subcloning were sequenced to confirm the identity of d3-4 cDNA. **B.** Structure of WT-ER-α cDNA and primer set used to amplify and detect PCR products.

identity of this variant, the cDNA corresponding to the 928 bp band was subcloned and sequenced, as described under Methods. The nucleotide sequence of the cDNA was found to represent a variant ER-α mRNA containing a deletion of both exons 3 and 4 (Figure 24A). The exon 3 and 4 deleted ER-α (ERd3-4) is in frame and is predicted to encode a protein of 443 amino acid residues with a predicted molecular mass of 49 kDa (analyzed using MacVector software program). This putative ER-α-like protein would be missing the second zinc finger of the ER-α DNA binding domain, the hinge region and part of the ligand binding domain (Figure 24B).

To address the potential function of this variant ER-α, eukaryotic expression vectors containing the ERd3-4 cDNA were constructed (as described under Methods) and shown to express a protein of the expected size (Figure 25A) using *in vitro* transcription/translation techniques. Western blotting and immune detection were performed on the *in vitro* transcription/translation products generated in the absence of radiolabel. The ERd3-4 protein was recognized by the ER-α antibody H226 that recognizes an epitope encoded in exon 1/2 (A/B region) of wild-type ER-α (Figure 25B, lanes 1 and 2). Using an antibody (AER308) that recognizes an epitope located in exon 4 of the wild-type ER-α, the band corresponding to the ERd3-4 is not seen, while wild-type ER-α is still detected (Figure 25B, lanes 3 and 4).

Ligand-binding analysis was also performed on *in vitro* translated ERd3-4 and wild-type ER-α (HEGO) using [^{3}H]17β-estradiol or [^{3}H]tamoxifen as described under Methods. Figure 26 demonstrates that under conditions in which wild-type ER-α can bind to both estrogen and antiestrogen ERd3-4 displays little or no ability to specifically bind either ligand.

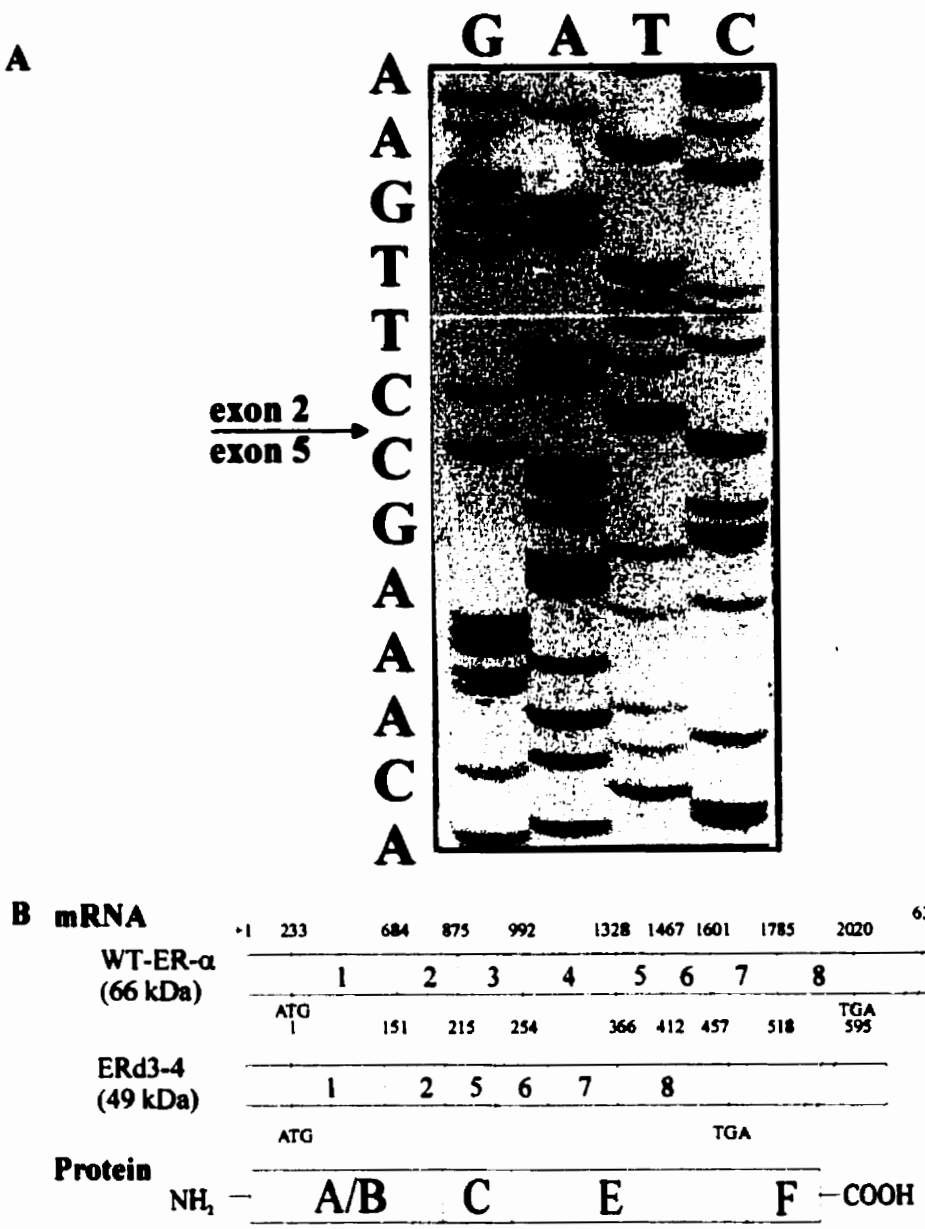


Figure 24. Sequence and structure of exon 3 and 4 deleted ER-α. A. Sequence of the exon 2/5 junction. The 928 bp RT-PCR product was subcloned and sequenced as described in Methods. Sequencing reactions were run on 6% acrylamide gels and exposed to film to visualize reaction products. **B.** Structure of the ERd3-4 compared to wild-type ER-α. The ERd3-4 mRNA would encode a protein of 49 kDa that would be missing the second zinc finger of the ER-α DNA binding domain, the hinge region and a portion of the ligand binding domain.

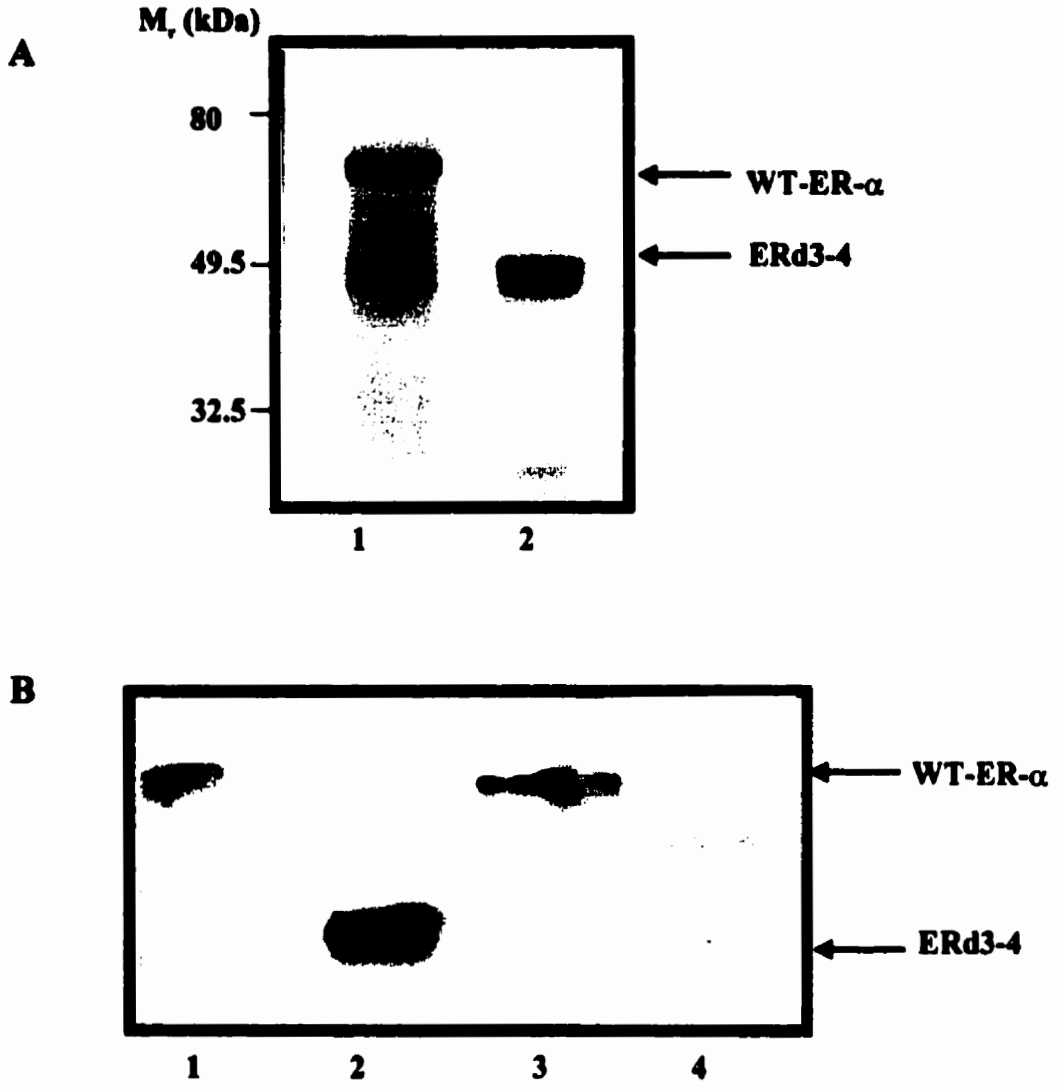


Figure 25. ***In vitro*** **transcription/translation and Western blotting of ERd3-4 protein. A.** Expression vectors containing wild-type ER-α (lane 1) and ERd3-4 cloned into pSG5 (lane 2) were subjected to *in vitro* transcription/translation reactions in the presence of [^{35}S]-methionine. Reaction products were run on an SDS-10% acrylamide gel, dried and exposed to film overnight. **B.** *In vitro* transcription/translation products (1 μL wild-type (WT)-ER-α and 5 μL ERd3-4) were run on an SDS-10% acrylamide gel and Western blotting performed as described in Methods. Lanes 1 and 2 were visualized using the ER-α antibody H226 (epitope located in wild-type ER-α exon 1/2) and lanes 3 and 4 using ER-α antibody 308 (epitope located in wild-type ER-α exon 4).

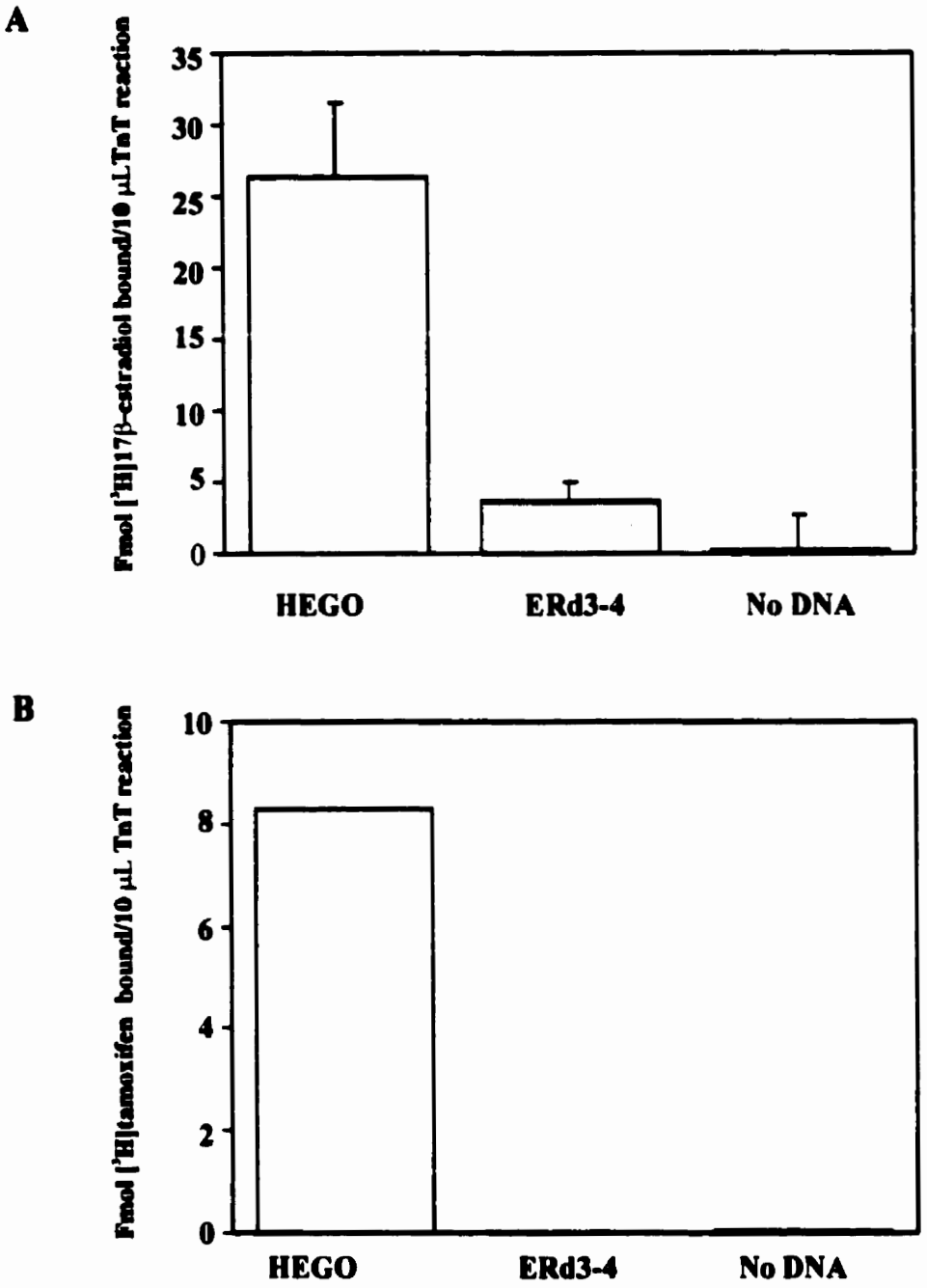
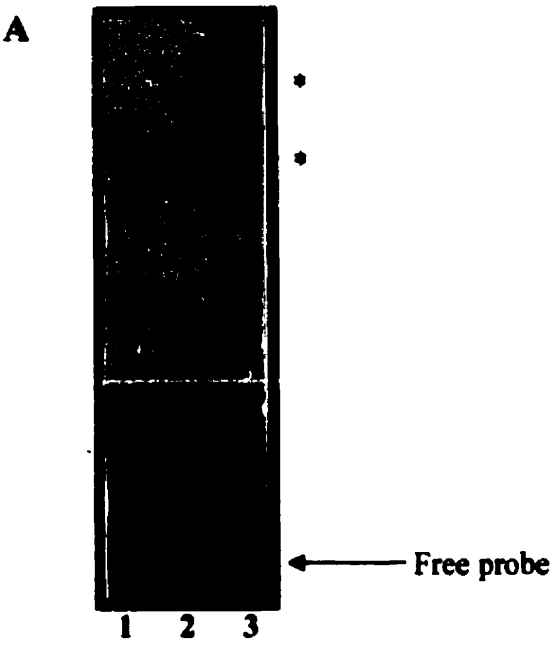


Figure 26. Ligand binding of *in vitro* transcribed/translated wild-type ER-α and ERd3-4 protein. *In vitro* transcription/translation reactions and ligand binding analysis were performed as described in Methods. **A.** Binding of [^{3}H]17β-estradiol. Histogram represents mean ± sem, n=3 independent experiments. Results are expressed as fmol [^{3}H]17β-estradiol specifically bound per 10 μL of *in vitro* transcription/translation reaction (TnT). **B.** Binding of [^{3}H]tamoxifen. Results are expressed as fmol [^{3}H]tamoxifen specifically bound per 10 μL of *in vitro* TnT reaction, n=1.

The ERd3-4 protein would also be missing the second zinc finger of the DNA binding domain and as such would not be expected to bind to DNA. Under conditions in which *in vitro* transcribed/translated wild-type ER-α bound to an oligonucleotide containing the vitellogenin B1 ERE as determined by gel mobility shift assays, ERd3-4 did not demonstrate any specific DNA binding in the absence of ligand (Figure 27A). To examine if ligand could affect the DNA binding ability of ERd3-4 or if heterodimers between wild-type ER-α and ERd3-4 could be detected further experiments were performed. Again, ERd3-4 alone did not demonstrate an ability to bind to the vitellogenin B1 ERE in the absence of ligand (Figure 27B, lane 3 versus lane 2). Added to this reaction and that of the wild-type ER-α, was *in vitro* transcription/translation mix obtained in the presence of heat killed T7 RNA polymerase to prevent transcription. This was an additional control to ensure that components of the rabbit reticulocyte lysate reaction mix or plasmid DNA did not affect DNA binding. When equal amounts of wild-type ER-α and ERd3-4 were used in the absence of estradiol no differences in shifted bands could be observed (Figure 27B, lane 4 compared to lane 2). Additionally, equal amounts of wild-type ER-α and ERd3-4 were incubated in the presence of estrogen or the antiestrogens ICI 164,384 or 4-monohydroxytamoxifen (Figure 27B, lanes 5, 6 and 7). Again, no additional bands could be detected to suggest the presence of wild-type ER-α and ERd3-4 heterodimers under these experimental conditions. The reduction in the amount of ERE bound to wild-type ER-α in figure 27B, lanes 5, 6 and 7 compared to lanes 2 and 4 is likely due to the fact that ethanol vehicle was not included in the reaction mixtures used in lanes 2 and 4.

To examine if the ERd3-4 protein could be expressed *ex vivo*, transfections into ER-α



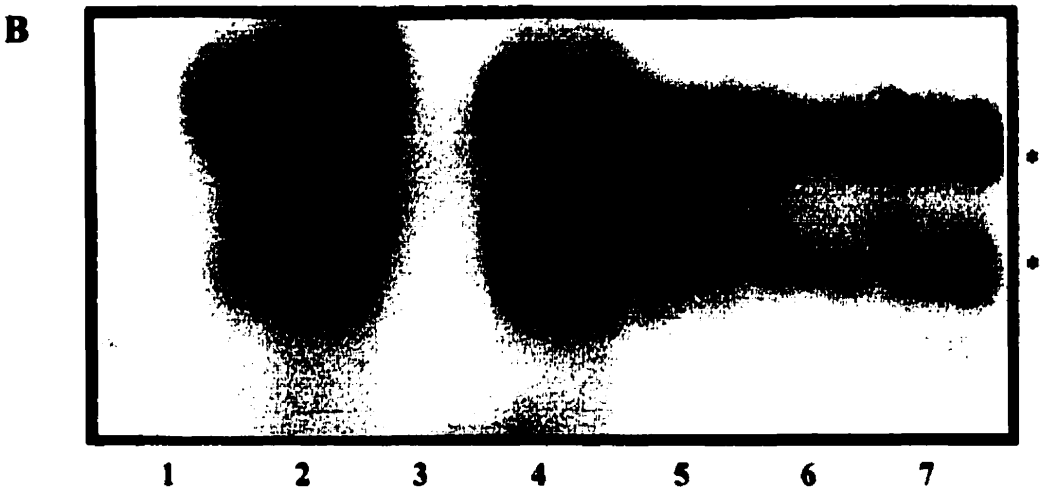


Figure 27. Gel mobility shift assay. *In vitro* transcription/translation reactions were performed as described in Methods and incubated with a [^{32}P] labelled vitellogenin B1 ERE 35bp oligonucleotide. **A.** Gel shift assay performed using *in vitro* transcribed/translated proteins (2μL) in the absence of estrogen. Lane 1. Free probe; Lane 2. ERd3-4; Lane 3. wild-type (WT) ER-α. **B.** Gel shift assay performed using *in vitro* transcribed/translated proteins. Lane 1. Free probe; Lane 2. WT-ER-α protein plus ERd3-4 reaction in presence of heat killed T7; Lane 3. ERd3-4 protein plus WT-ER reaction in presence of heat killed T7; Lane 4. WT-ER-α and ERd3-4; Lane 5. WT-ER-α and ERd3-4 in the presence of 10 nM 17β-estradiol; Lane 6. WT-ER-α and ERd3-4 in the presence of 1μM ICI 164,384; Lane 7. WT-ER-α and ERd3-4 plus 1μM 4-monohydroxytamoxifen. 2 μL of *in vitro* transcription/translation reaction were used in each instance. • = ER/ERE complex.

negative MCF10A1 human breast epithelial cells were performed. MCF10A1 cells were transfected with expression vectors for wild-type ER-α, ERd3-4 or vector (pSG5) DNA alone. Cells were harvested and extracts used for Western blotting and immune detection demonstrated that ERd3-4 could be expressed *ex vivo* as detected by the ER-α antibody H226 (Figure 28).

Despite the observations that this variant protein appeared incapable of binding ligand or DNA it was still of interest to examine potential effects on wild-type ER-α transcriptional activity since the transient transfection experiment had demonstrated it could be expressed *ex vivo*. To this end, transient transfections using the ER-α negative breast cell lines, MDA-MD-231 and MCF10A1 were carried out. Under conditions in which transiently transfected wild-type ER-α was transcriptionally active and able to induce CAT activity in a ligand-dependent fashion, the ERd3-4 did not demonstrate any transcriptional activity on its own (Figure 29A and B). This is unlikely to be due to low levels of expression of this transgene, since after transfection of 5 μg of ERd3-4 expression vector (approximately 1.5 pmol) into MCF10A1 cells a protein corresponding in size to the expected ERd3-4 protein was detectable (Figure 28, lane 3). To determine if ERd3-4 and wild-type ER-α could interact to influence transcription of an estrogen-responsive reporter gene, cotransfections of wild-type ER-α and ERd3-4 into MDA-MB-231 and MCF10A1 ER-α negative breast cell lines were carried out (Figure 29A and B). HEGO transfected alone showed the expected estrogen-dependent activity (MDA-MD-231, 7.7 $\pm$ 3.8 and MCF10A1 7.3 $\pm$ 4.2, mean $\pm$ sem, fold increase over basal), while ERd3-4, as mentioned, had no transcriptional activity alone in the presence of estrogen. However, when increasing amounts of ERd3-4 were transfected with

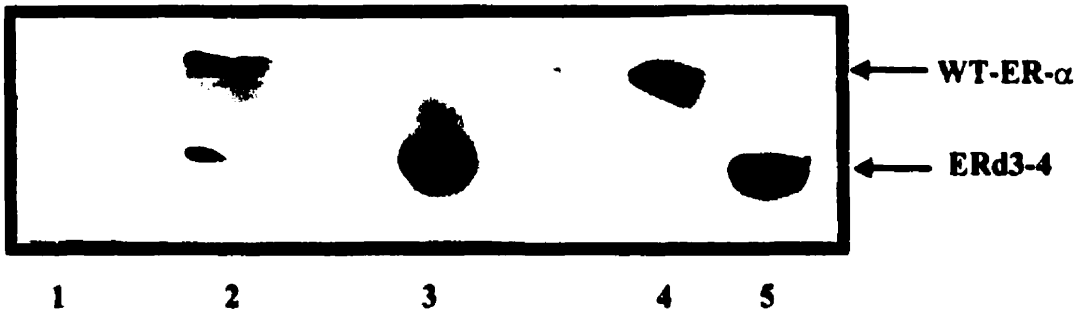


Figure 28. *Ex vivo* expression of ERd3-4 protein. MCF10A1 ER-α negative human breast epithelial cells were transfected with the appropriate expression vector, cells were lysed in 8M urea, and 10 μg protein run on an SDS-10% acrylamide gel. Cells were transferred to nitrocellulose and Western blotting performed using ER-α antibody H226. Lane 1. Control cells tranfected with 5 μg of vector (pSG5) DNA alone; Lane 2. Cells transfected with 5 μg HEGO (WT-ER-α) expression vector; Lane 3. Cells transfected with 5 μg ERd3-4 expression vector; Lane 4. *In vitro* transcribed/translated WT-ER-α (1 μL); Lane 5. *In vitro* transcribed/translated ERd3-4 (2 μL). n=2, independent experiments.

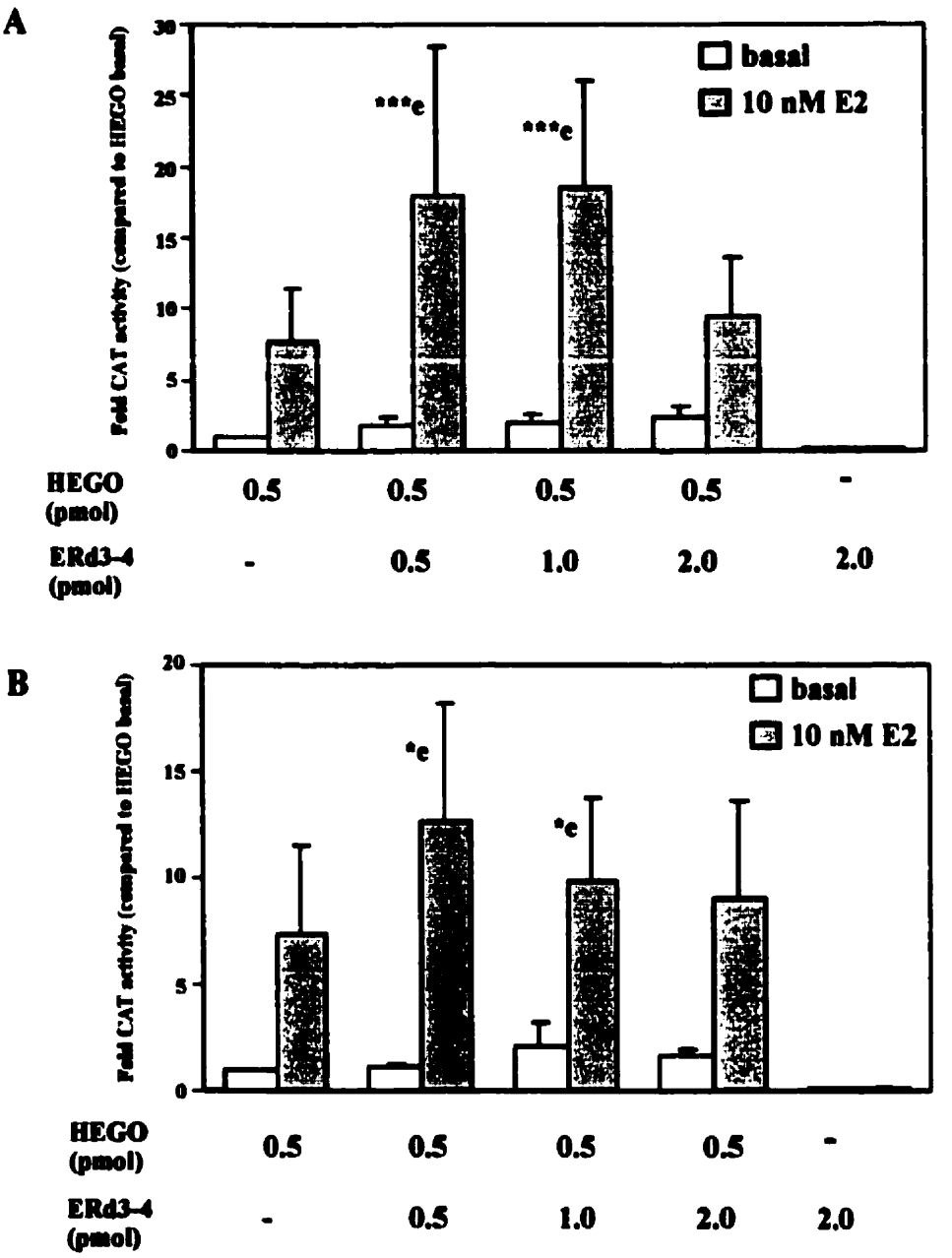


Figure 29. Activity of ERd3-4 in ER-α negative human breast cells. A. MDA-MB-231 human breast cancer cells were transfected with 5 μg ERE-tk-CAT, 1μg pCH110, 0.5pmol HEGO ± 0.5-2pmol ERd3-4 ± vector DNA to give a total of 17 μg DNA/dish. Cells were treated with 10 nM 17β-estradiol (E2) for 24 h or vehicle alone as control (basal). Results are expressed as fold CAT activity compared to basal HEGO activity arbitrarily set as 1. Histograms represent mean ± sem, n=6-7. ***e=$p<0.0001$, ANOVA, results compared to estradiol treated HEGO alone. B. MCF10A1 human breast epithelial cells were similarily transfected. Histograms represent mean ± sem, n=4. *e=$p<0.05$, ANOVA, result compared to estradiol treated HEGO alone.

a constant amount of HEGO, ERd3-4 significantly increased estrogen-dependent activity of wild-type ER-α . When equal amounts of ERd3-4 and wild-type HEGO were transfected into MDA-MB-231 and MCF10A1 cells a significant increase in the estrogen-dependent activity was seen compared to HEGO estrogen-dependent activity (17.9 ± 10.5 and 12.7 ± 5.5 respectively, mean fold increase over HEGO basal ± sem, $p<0.0001$ and $p<0.05$). The lowered estrogen-dependent activity of HEGO at a higher dose of ERd3-4 (2 pmol) may be due to 'squelching' effects, perhaps due to the ability of ERd3-4 to sequester proteins required for transcriptional activity of wild-type ER-α. There is a significant interexperimental variation between transfections which results in a large standard error of the mean, but within each experiment it can be seen in Table 4 that there is a consistent increase in transcriptional activity with the addition of ERd3-4. Increasing amounts of cotransfected ERd3-4 was associated with increases in basal transcription in both MDA-MB-231 and MCF10A1 cells (Figure 29A and B) but this did not reach a level of statistical significance.

Since the ERd3-4 was originally detected in the estrogen-nonresponsive T5 subline, T5-PRF, it was of interest to examine the effects of introducing this variant ERd3-4 into the parental estrogen-responsive T5 cells. Transient transfections of ERd3-4 into T5 cells were performed as described under Methods. When ER-α transcriptional activity in T5 cells was measured in the presence of ERd3-4, statistically significant increases in both basal and estrogen-dependent CAT activity were seen (Figure 30). Transfection of 1 pmol of ERd3-4 into T5 cells caused a significant increase in CAT activity both in the presence of estrogen (39.3 ± 11.1 fold increase over basal in the presence of ERd3-4 versus 23.8 ± 3.2 fold increase over basal in the absence of ERd3-4, mean ± sem, $p<0.05$) and in the absence of

Table 4. Fold CAT activity in breast cells

I. MDA-MB-231 breast cancer cells

Individual experiments

HEGO 0.5pmol		ERd3-4 0.5		1.0		2.0		pmol
-	E2	-	E2	-	E2	-	E2	
1	3.8	1.5	6	2	5.8	4.8		
1	4.2	4.2	70.2	1.3	54.6	1	4	
1	1.6	0.3	6	0.5	6	0.3	3.2	
1	5.4	1	6.9	1.4	7.4	1	4.6	
1	3.1	1	6.1	4.5	8.8	5.2	3.9	
1	6	1	12	1.4	7.1	1.5	10.6	
1	30	3.3		2.9	39.9	3.3	30.3	

Fold cumulative data (mean ± sem)

-	E2	-	E2	-	E2	-	E2
1.0	7.7 ±3.8	1.7 ± 0.6	17.9 ±10.5	2.0 ±0.5	18.5 ±7.6	2.4 ±0.8	9.4 ±4.3

II. MCF10A1 breast epithelial cells

Individual experiments

HEGO 0.5pmol		ERd3-4 0.5		1.0		2.0		pmol
-	E2	-	E2	-	E2	-	E2	
1	1.7	1.0	3.0	1.0	2.7	1.1	3.3	
1	3.4	1.0	5.9	1.0	8.1	1.5	5.2	
1	4.3	1.0	14.1	5.2	8.0	2.0	4.7	
1	19.9	1.4	27.6	1.3	20.8	1	22.6	
1						2.3		

Fold cumulative data (mean ± sem)

-	E2	-	E2	-	E2	-	E2
1.0	7.3 ±4.2	1.1 ± 0.1	12.7 ±5.5	2.1 ±1.1	9.9 ±3.8	1.6 ±0.3	9.0 ±4.6

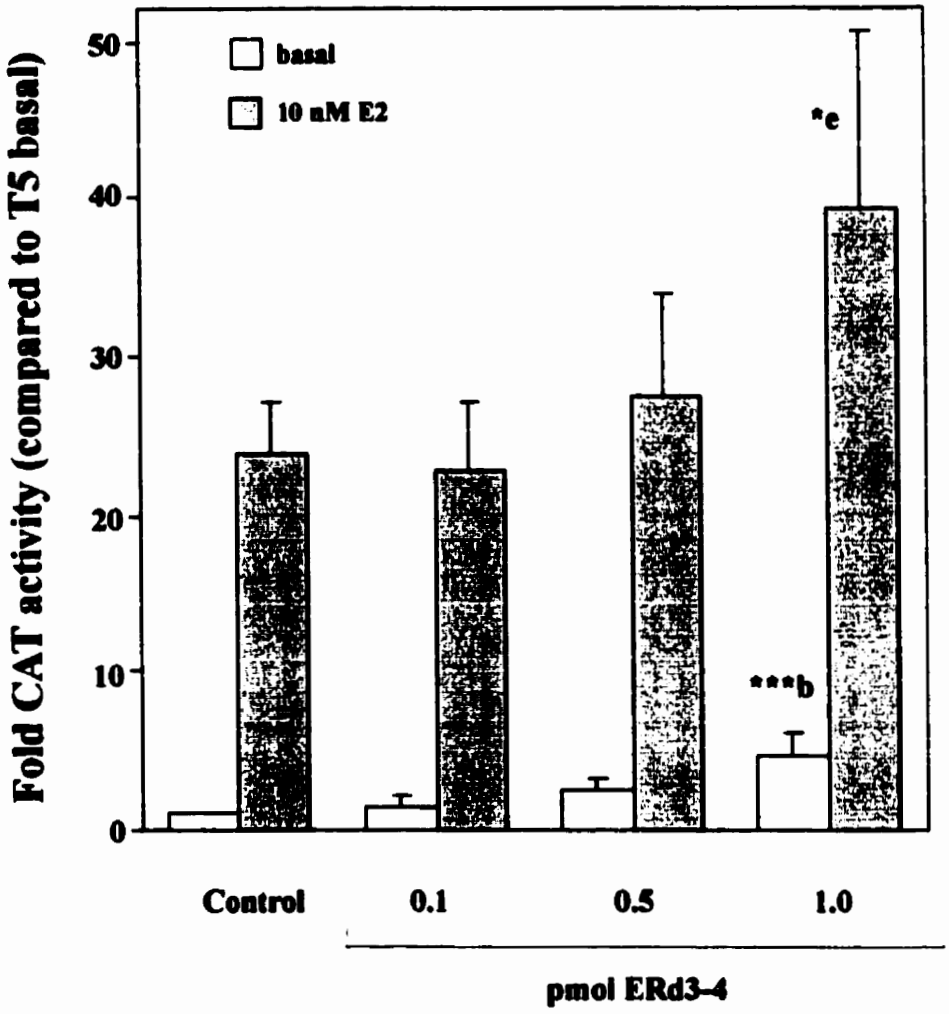


Figure 30. Activity of ERd3-4 in T5 human breast cancer cells. Cells were grown in PRF-DMEM (estrogen-deplete conditions) as described in Methods and transfected with 5 μg ERE-tk-CAT expression vector, 5 μg pCH110 along with the appropriate amount of ERd3-4 expression vector or vector DNA alone. Cells were treated with vehicle (basal) or 10 nM 17β-estradiol (E2) for 24h, harvested and CAT assays performed as described in Methods. Results represent mean ± sem, n=3-5, ***b=p<0.0001, ANOVA, result compared to basal ERE-tk-CAT activity, *e=p<0.05 ANOVA, result compared to estradiol treated ERE-tk-CAT activity.

added estrogen (4.7 ± 1.6 fold increase over basal (no ERd3-4), mean ± sem, $p<0.0001$), despite the fact this protein apparently does not bind DNA or ligand. Table 5 lists the fold change cumulative data for the transient transfection analyses in T5 human breast cancer cells.

The statistical analyses for the transfection data were performed by M. Cheang, University of Manitoba, Biostatistical Consulting Unit using ANOVA.

The question as to what role any particular variant ER-α mRNA may play *in vivo* is most likely dependent on the expression of the corresponding protein. While it is still unclear if ER-α variant naturally occurring mRNAs are stably translated *in vivo*, there has been evidence (discussed in the Introduction) to suggest that variant ER-α proteins could exist *in vivo*. It was therefore an obvious step to attempt to demonstrate the existence of a protein that could correspond to the ERd3-4 mRNA in T5-PRF cells. Unfortunately detection of ER-α variant proteins is difficult due to a lack of the appropriate tools (i.e., specific antibodies). Furthermore, ER-α variant proteins such as ERd3-4 are of a similar size to immunoglobulin heavy chain proteins which can hamper detection of these proteins using immunoprecipitation and Western blotting immune detection techniques (380). Additionally, it can be seen by examining figure 9, that there is a strong 50 kDa immunoreactive band detected in both T5 and T5-PRF cell lines, thought to consist of a proteolytic product of the ER-α as well as non-specific interactions, that has hampered the ability to definitively identify a band that could correspond to ERd3-4 protein. To attempt to circumvent this problem, 2-D gels were used with the hope that separation by both size and isoelectric point would aid in the identification. It was also reasoned that using an antibody that would recognize both wild-type ER-α and ERd3-4 (mouse AER314, Neomarkers, Santa Cruz, California) and one that recognized wild-

Table 5. T5 transfected with ERd3-4 cumulative data

Fold cumulative data (mean ± sem)

vector alone		ERd3-4 (pmol)					
		0.1		0.5		1.0	
-	E2	-	E2	-	E2	-	E2
1.0	23.8 ±3.2	1.6 ± 0.4	22.6 ±4.6	2.5 ±0.8	27.4 ±6.4	4.7 ±1.6	39.3 ±11.1

type ER-α and not ERd3-4, (mouse AER308, Neomarkers, Santa Cruz, California) distinctions could be made. Unfortunatgly, despite exhaustive attempts, no clear cut data were obtained that could support the existence of a protein specifically expressed in T5-PRF human breast cancer cells that corresponded to that predicted for the ERd3-4 mRNA.

In conclusion, T5-PRF human breast cancer cells have increased basal ER-α activity and contain a variant ER-α mRNA, ERd3-4, that when expressed in both ER-α negative human breast cell lines and parental ER-α positive T5 human breast cancer cells can increase both basal and estrogen-dependent ER-α transcriptional activity. While it is unclear if this variant mRNA is stably translated in T5-PRF cells, the data support the hypothesis that expression of this protein could be involved in the altered ER-α activity seen in the T5-PRF cells.

III. MAPK activity in T5 and T5-PRF human breast cancer cells.

Rationale

The mitogen-activated protein kinase (MAPK) signal transduction pathway, as discussed in the Introduction, plays an essential role in cell cycle progression and can be activated by many growth factor/mitogen pathways including estrogen. MAPK activity has been shown to be elevated in primary breast cancer compared to benign breast tissue and has also been shown to be overexpressed in metastatic cells within lymph nodes of breast cancer patients (270). MAPK has also been implicated in ligand-independent activation of the ER-α. Serine 118 of the ER-α has been shown to be phosphorylated by MAPK in response to receptor activation by growth factors and mutation of this site severely diminished ER-α

transcriptional ability (232,246). Since T5-PRF cells contain an elevated basal ER-α transcriptional activity despite the fact they contain 50% less ER-α than parental T5 cells, it was of interest to examine the possibility that an alteration in a pathway that may be involved in ligand-independent activation of the ER-α (i.e., MAPK) could be altered in T5-PRF compared to T5 human breast cancer cells.

Results

The initial studies were performed using an *in vitro* MAPK assay that was routinely used in Dr. G. Arthur's laboratory (University of Manitoba). C. Richard, a student in Dr. G. Arthur's laboratory, was kind enough to demonstrate and provide the protocol which was used in their laboratory and is described under Methods. This *in vitro* assay measures MAPK activity in cell extracts, obtained in the presence of inhibitors of other protein kinases, using myelin basic protein as a substrate to measure its phosphorylation in the presence of [γ-^{32}P]ATP. The initial experiments demonstrating an elevated ER-α transcriptional activity in T5-PRF cells were performed under basal (i.e., estrogen-deplete) culture conditions with cells grown in 5% CS, therefore, *in vitro* MAPK activity was initially examined under these conditions. *In vitro* MAPK activity was significantly higher in T5-PRF cells compared to parental T5 cells (Figure 31). MAPK activity was 2.6 ± 0.3 (mean ± sem, n=3) fold higher ($p<0.05$) than the activity assayed in parental T5 cells. MAPK (ERK1 and ERK2 isoforms) are phosphorylated on both tyrosine and threonine residues and the phosphorylation at these two sites results in the activation of MAPK (266,476). To determine if the increased MAPK activity in T5-PRF cells was reflected in an increase in the total amount of MAPK protein, and/or an increase in the active MAPK pool, Western blotting on cell extracts was performed.

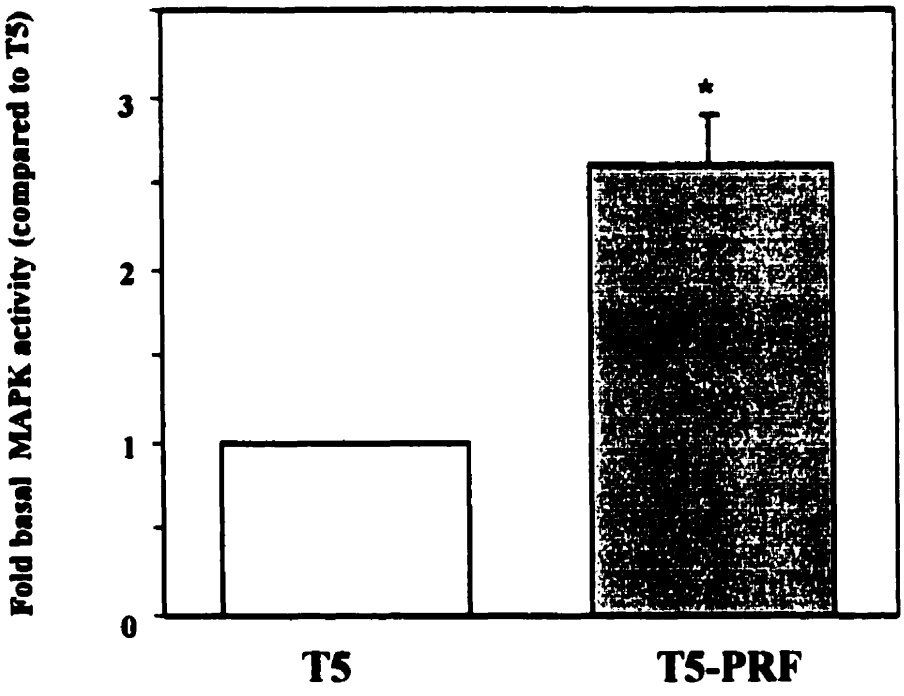


Figure 31. ***In vitro*** **basal MAPK activity.** T5 and T5-PRF cells were grown in estrogen-deplete conditions and extracts for measurement of *in vitro* MAPK activity were obtained as described in Methods. Myelin basic protein was used as a substrate to measure kinase activity and the assay was allowed to proceed for 10 minutes at 30°C. Histograms represent fold difference in MAPK activity after arbitrarily expressing the activity of T5 parent cells as 1.0. T5-PRF cells have a 2.6 ± 0.3 fold increase in kinase activity, n=3. *$p<0.05$, Student's t-test.

Using an antibody that recognizes total MAPK protein it was seen that both T5 and T5-PRF cells express large amounts of both the ERK1 and ERK2 isoforms of MAPK (Figure 32B, lanes 1 and 3). Using an antibody that specifically recognizes only the dually-phosphorylated active forms of ERK1 and ERK2 it was demonstrated that T5-PRF cells contained elevated levels of active MAPK protein compared to T5 cells in estrogen-deplete conditions (Figure 32A, lanes 1 and 3). PD 98059 is a specific inhibitor of MAPK activity due to its specific inhibition of MAPK kinase (also referred to as MEK) (477). Treating both cell lines with 50 μM PD 98059 for 1 hour resulted in a marked inhibition of the dually-phosphorylated MAPK (Figure 32A, lanes 2 and 4) with little or no effect on total MAPK protein levels (Figure 32B, lanes 2 and 4), supporting the conclusion that T5-PRF cells have elevated levels of activated MAPK.

Serum-starvation of cells in culture is often used as a means to reduce active MAPK. Under conditions in which T5 and T5-PRF cells were serum starved for 7 days, T5-PRF cells still maintained elevated levels of *in vitro* MAPK activity (Figure 33). The level of *in vitro* MAPK activity in T5-PRF cells was 1.5 $\pm$ 0.2 fold (mean $\pm$ sem, n=3) fold higher than T5 parental cells ($p<0.001$). Total MAPK protein levels were similar between the two cell lines (Figure 34B, lanes 1 and 3) but activated MAPK protein levels were much higher in T5-PRF cells (Figure 34A, lanes 1 and 3). As expected, treating T5-PRF cells with 50 μM PD 98059 for 1 hour under serum-free conditions decreased the level of active MAPK detected by Western blotting (Figure 34A, lane 4) with little effect on total MAPK protein levels (Figure 34B, lane 4). These data suggest that T5-PRF cells contain elevated and perhaps constitutively active MAPK activity compared to T5 cells.

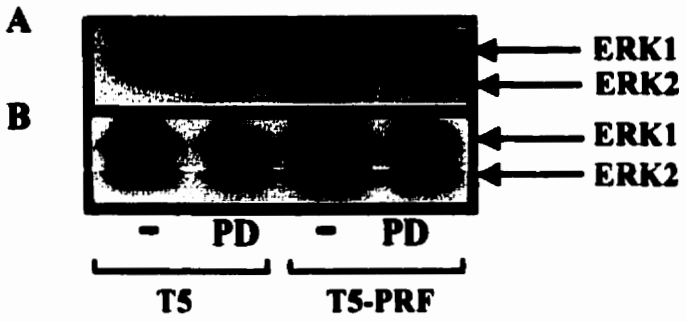


Figure 32. Western blot of MAPK protein levels under basal conditions. A. 30 μg of protein from whole cell extracts were run on SDS-7.5% acrylamide gels. Western blotting and immune detection was performed as described in Methods. Immune detection was using an anti-phospho-MAPK antibody (NEB). **B.** 5 μg of protein from whole cell extracts were run on SDS-7.5% acrylamide gels. Western blotting and immune detection were performed as described in Methods. Immune detection was using an anti-ERK1 antibody (Santa Cruz). PD = MEK inhibitor PD 98059, 50 μM for 1 hour. ERK1 and ERK2 bands are denoted by arrows.

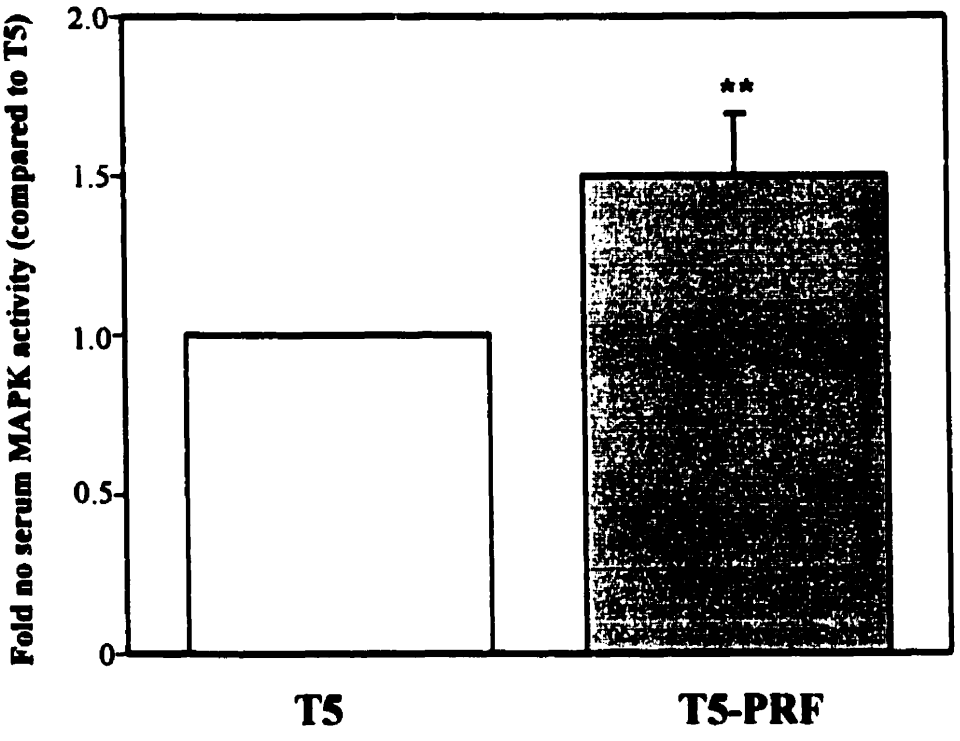


Figure 33. *In vitro* no serum MAPK activity. T5 and T5-PRF cells were set up in estrogen-deplete conditions in the presence of 5% charcoal-stripped fetal calf serum. The following day the medium was changed to no serum medium and changed each day for 7 days. Cells were harvested and cell extracts for *in vitro* MAPK assay obtained as described in Methods. Myelin basic protein was used as a substrate and the assay was allowed to proceed for 10 minutes at 30°C. Histograms represent fold difference in MAPK activity after setting the activity of T5 parent cells to 1.0. T5-PRF cells have a 1.5 $\pm$ 0.2 fold increase in kinase activity, n=3, **$p<0.001$, Student's t-test.

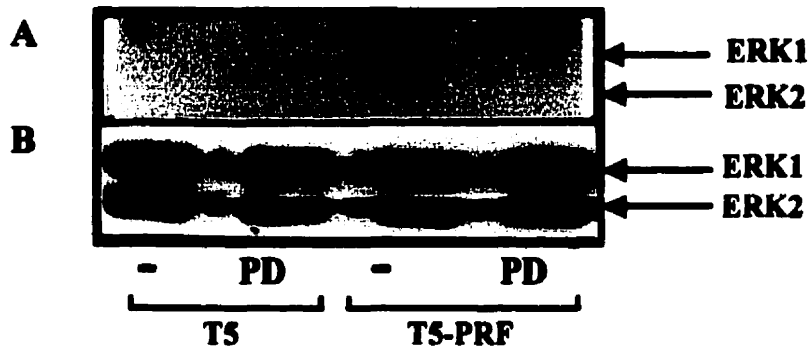


Figure 34. Western blot of MAPK protein levels under no serum conditions. A. 30 μg of protein from whole cell extracts were run on an SDS-7.5% acrylamide gel. Western blotting and immune detection were performed as described in Methods. Immune detection was using an anti-phospho-MAPK antibody (NEB). **B.** 5 μg of protein from whole cell extracts were run on an SDS-7.5% acrylamide gel. Western blotting and immune detection were performed as described in Methods. Immune detection was using an anti-ERK1 antibody (Santa Cruz). PD = MEK inhibitor PD 98059, 50 μM for 1 hour. ERK1 and ERK2 bands are denoted by arrows.

To further examine the role elevated MAPK activity might play in terms of the elevated basal (i.e., estrogen-independent) ER-α transcriptional activity in T5-PRF cells, transient transfections were carried out. It was previously shown that 50 μM PD 98059 for 1 hour was sufficient to reduce the levels of dually-phosphorylated MAPK under basal conditions (Figure 34A), but since the transient transfection experiments required approximately 24 hour treatments, conditions were determined under which PD 98059 was able to maintain low levels of dually-phosphorylated MAPK over this time period. Figure 35 demonstrates that adequate inhibition could be achieved by treating cells initially with 50 μM PD 98059 followed 2 hours later by an additional 50 μM treatment with the cells being harvested 20 hours later, resulting in an 86% inhibition of dually-phosphorylated MAPK. When T5-PRF cells were transfected with an estrogen-responsive CAT reporter gene and treated with PD 98059 there was a significant reduction of 44.2% ± 8.1 (mean ± sem, $p<0.05$, $n=3$) in the basal ER-α transcriptional activity seen (Figure 36). Interestingly there was also a significant and equivalent reduction in the estrogen-induced transcriptional activity of 46.3% ± 12.4, (mean ± sem, $p<0.05$, $n=3$) by PD 98059, suggesting a role for MAPK activation in both estrogen-independent and -dependent transcriptional activation. The effect of PD 98059 on the transcriptional activity of ER-α in T5 cells was also examined (Figure 37). A significant reduction in the estrogen-induced ER-α transcriptional activity of 69.1% ± 2.0, (mean ± sem, $p<0.05$, $n=3$) by PD 98059 was seen, again suggesting a role for MAPK in estrogen-induced ER-α activity. Additionally, evidence was obtained to suggest that the inhibition of active MAPK protein via PD 98059 results in a decrease in the level of ER-α protein (Figure 38). When T5 and T5-PRF cells were grown in estrogen-deplete conditions

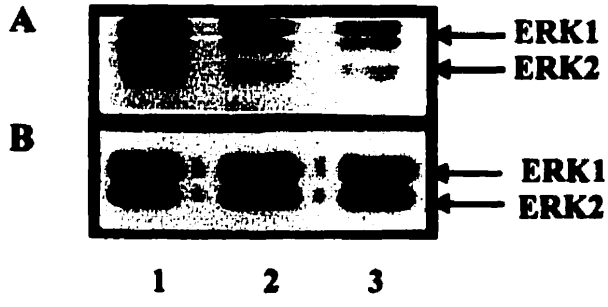


Figure 35. Western blot analysis of MAPK protein levels after PD 98059 treatment. T5-PRF cells were grown in estrogen-deplete conditions and extracts prepared and Western blotting performed as described in Methods. **Lane 1.** Basal MAPK protein levels. **Lane 2.** 50 μM PD 98059 treatment 24 hours. **Lane 3.** 50 μM PD 98059 treatment for 2 hours, followed by a second 50 μM 'hit'. Cells were harvested after an additional 22 hours. **A.** 30 μg of whole cell extract was run on an SDS-7.5% PAGE. Immune detection was performed using an anti-phospho-MAPK antibody (NEB). **B.** 5 μg of whole cell extract was run on an SDS-7.5% PAGE. Immune detection was performed using an anti-ERK1 antibody (Santa Cruz). Positions of ERK1 and ERK2 are denoted by arrows.

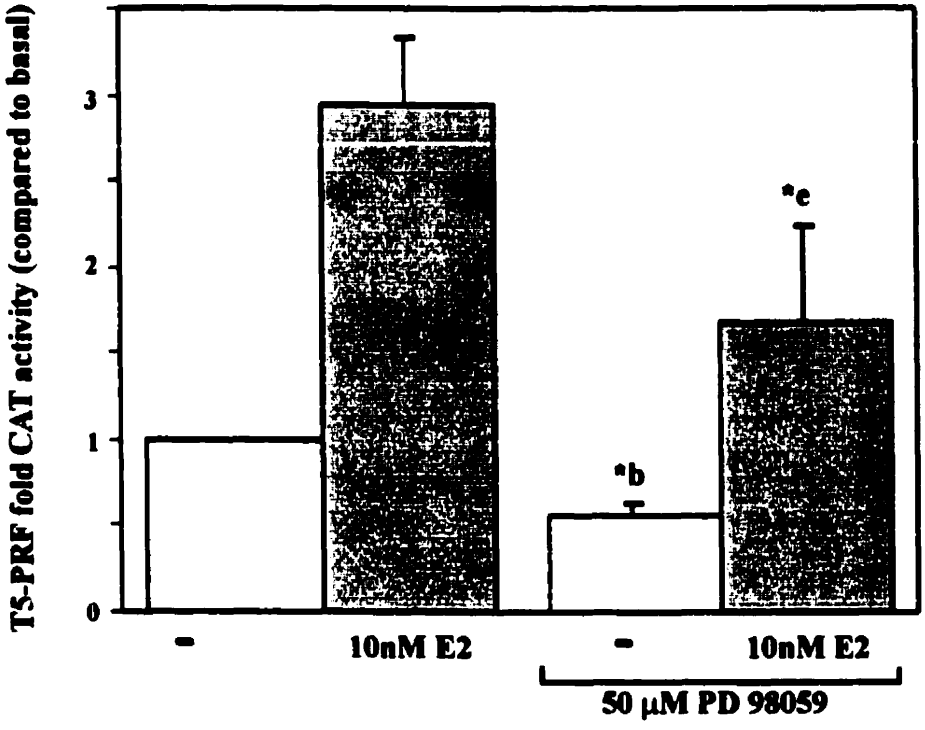


Figure 36. T5-PRF ER-α transcriptional activity in the presence of PD 98059. Transient transfections were performed as described in Methods. T5-PRF cells were grown in estrogen-deplete conditions with vehicle alone (basal) or 10 nM 17β-estradiol for 22 hours in the absence or presence of 50 μM PD 98059 (PD). PD treatment was given at the time of estrogen treatment and two hours later a second 50 μM PD treatment was given. Cells were harvested after 22 hours and CAT assays performed as described in Methods. Results are expressed as fold CAT activity compared to basal T5-PRF cells. Histograms represent mean ± sem, n=3. *b, compared to basal without PD, *e, compared to estrogen treatment without PD, $p<0.05$, Student's t-test.

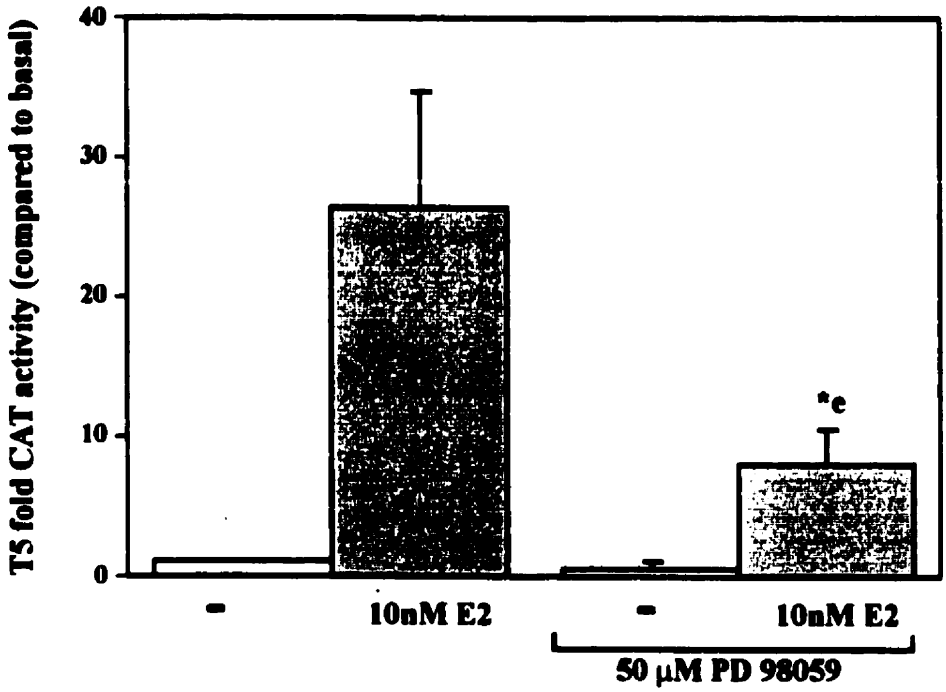


Figure 37. T5 ER-α transcriptional activity in the presence of PD 98059. Transient transfections were performed as described in Methods. T5 cells were grown in estrogen-deplete conditions with vehicle alone (basal) or 10 nM 17β-estradiol for 22 hours in the absence or presence of 50 μM PD 98059 (PD). PD treatment was given at the time of estrogen treatment and two hours later a second 50 μM PD treatment was given. Cells were harvested after 22 hours and CAT assays performed as described in Methods. Results are expressed as fold CAT activity compared to basal T5 cells. Histograms represent mean ± sem, n=3. *e, compared to estrogen treatment without PD, $p<0.05$, Student's t-test.

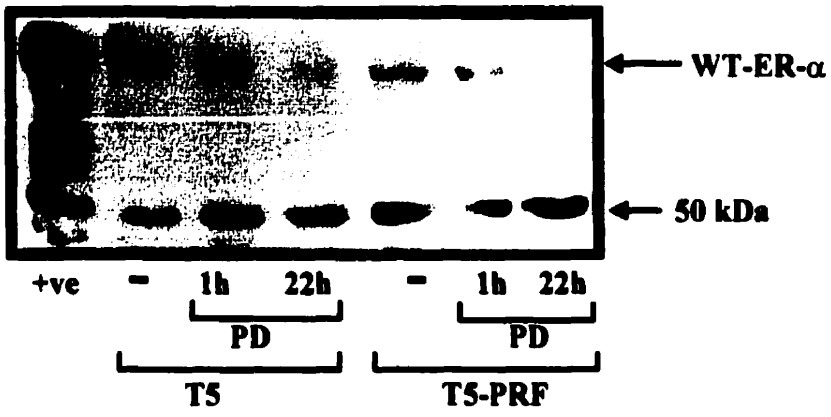


Figure 38. Effect of MEK inhibitor PD 98059 on ER-α levels in T5 and T5-PRF cells. Cell extracts were obtained and Western blotting performed as described under Methods. Immune detection was performed using the ER-α antibody H226. +ve= *In vitro* transcribed/translated wild-type ER-α protein. Cells were treated with 50 μM PD 98059 for 1 hour or 22 h before harvesting. 22 h treatment = 2 hour 50 μM treatment and after 2 h cells are given a second 50 μM dose and then left for an additional 20 h before harvesting. h= hour; - = control cells harvest at the time both the 1 h and 22 h PD treated plates were harvested. n=2, independent experiments.

and treated with 50 μM PD 98059 for 1 hour or 22 hours (2h plus second treatment for 20h as described) there is a marked reduction in the level of immunoreactive ER-α (Figure 38, compare intensity of band at - (22h control) to 22 PD band) in both T5 and T5-PRF cells after 22 hours of treatment.

In conclusion, T5-PRF human breast cancer cells contain elevated MAPK activity. Inhibition of MAPK activity results in a significant decrease in both basal and estrogen-dependent ER-α transcriptional activity in these cells and a significant decrease in estrogen-dependent transcriptional activity in T5 parental cells. In addition, inhibition of MAPK activity is associated with a reduction in immunoreactive ER-α protein levels in both T5 and T5-PRF cells. The increased activity and expression of MAPK may contribute to the estrogen nonresponsive growth phenotype and ligand-independent activity of ER-α in T5-PRF cells.

Discussion

I. Estrogen regulated NM-IF proteins

These studies resulted in the identification of three NM-IF proteins, cytokeratin (CK) 8, 18 and 19 in T5 human breast cancer cells that are regulated by estrogen. The abundance of these proteins in the NM-IF fraction is dramatically reduced upon acute withdrawal of estrogen from the cell culture medium, and re-addition of estrogen results in increased levels of these proteins in the NM-IF. The reduced levels of these three NM-IF proteins in the absence of hormone reflect the requirement of estrogen for upregulated expression of these NM-IF proteins (i.e., they are estrogen-responsive proteins, or that the association of these proteins with the NM increases upon estrogen treatment). The fact that the antiestrogens 4-monohydroxytamoxifen and ICI 164,384 down-regulate the levels of these three NM-IF proteins in T5 cells is consistent with the effect being mediated via the ER-α.

Chronic estrogen-depletion of T5 cells resulted in the development of a cell line, T5-PRF, that has overcome the requirement of estrogen for growth in culture, while remaining ER-α positive. Although loss of the ER-α can accompany and/or explain hormone-independence, loss of ER-α does not always occur in the hormone-independent phenotype. Katzenellenbogen and colleagues have demonstrated levels of ER-α comparable to parent MCF-7 cell lines, or greater ER-α expression, in hormone-independent MCF-7 cells obtained through prolonged growth in estrogen-deplete conditions (87,356). Clarke and coworkers isolated a series of hormone-independent MCF-7 sublines that still maintained ER-α expression at levels comparable to, or greater than, the parent cell line (357). In the T5 and T5-PRF model, continued expression of ER-α allowed the study of expression of estrogen-

responsive genes, under conditions comparable in both the parent and hormone-nonresponsive cell lines. Despite the depletion of estrogen in the cell culture medium, the NM-IF fraction from T5-PRF cells has elevated levels of all three proteins, CK8, 18 and 19. While in the parental T5 cell line addition of estrogen to the cell culture medium results in increased levels of the three NM-IF proteins, in the T5-PRF cells, the addition of estrogen at an equivalent dose does not increase the levels of these NM-IF proteins. How these cells could express elevated levels of these estrogen regulated proteins in the absence of estrogen remains to be determined, but perhaps the cells are able to activate the ER-α through pathways other than classical estrogen/ER-α interactions as the later studies suggest. Recent research has demonstrated that the ER-α can be activated in a ligand-independent fashion (244,245) and perhaps the ability to activate the ER-α in the absence of estrogen would confer a growth advantage to the cells and aid in the development of a hormone-independent phenotype. Furthermore, the data suggest that as the length of time in estrogen-deplete conditions is increased, there is a concomitant increase in the levels of CK8, 18 and 19 associated with the NM-IF.

The development of hormone-independence and endocrine resistance in human breast cancer is a multifactorial process and indeed there are many examples where the development of estrogen-independent growth and antiestrogen resistance are dissociable events in breast cancer cell line models (354,356,361,364). Similarly, this study found that the development of estrogen-independent growth in a breast cancer cell line model, through long term growth in estrogen-depleted medium, was not associated with antiestrogen resistance. Although antiestrogens inhibit the growth of T5-PRF cells, they do not decrease

the levels of CK8, 18 and 19 in the NM-IF as they do in the parental T5 cells, suggesting that both estrogen/antiestrogen sensitivity, in terms of regulating the NM-IF levels of these proteins, has been lost in this cell line.

That cytokeratins are estrogen regulated in T5 cells is consistent with previous studies. In MCF-7 cells in culture, estrogen and tamoxifen treatment both resulted in an apparent increase in cytokeratins detected by immunofluorescence (478). Studies in rat vaginal epithelium have also demonstrated estrogen-induced increases in cytokeratin expression (479). More recently, preliminary data suggest that estrogen regulates CK19 expression at the mRNA level (480). Consistent with these studies, in T5 cells, estrogen-induced increases in cytokeratin expression were observed at the whole cell level. This suggests that the estrogen-induced increase at the NM-IF level is due to increased expression, as well as perhaps an increased association of these proteins with the NM-IF fraction.

The ER-α negative cell lines, MDA-MB-231 and HBL100 are estrogen-nonresponsive cell lines and have very low levels of CK8, 18 and 19 in their NM-IF, suggesting that significant expression of these cytokeratins may be associated with an ER-α positive phenotype. Indeed, more recent studies have identified CK 8, 18 and 19 as differentially expressed genes in ER-α positive MCF-7 and T47D human breast cancer cell lines versus the ER-α negative MDA-MB-231 and HBL100 human breast cell lines (481,482).

The relationship between tumour growth and cytokeratin expression has been examined, with some studies suggesting a correlation between specific cytokeratin expression and tumour progression and development (349,483). Changes in the levels and subset of cytokeratin expression have been found with increasing tumour grade (350,484). Studies

have demonstrated increased expression of CK18 and 19 in a set of malignant, compared to benign, human breast tumours (350) and significant correlations with CK19 with increased breast tumour size and increased CK8 expression in node positive versus node negative human breast cancers (485). Evidence also suggests that estrogen-independence in breast cancer cells is associated with changes in expression of a set of estrogen-regulated genes (364). van Agthoven and coworkers have shown that in an estrogen-independent ZR-75-1 human breast cancer cell line changes in cell morphology occur along with increases in total cellular levels of CK 8, 18 and 19 (457,486). These studies suggest that cytokeratins may be a biomarker for tumour stage and perhaps changes in cytokeratin expression are associated with an altered tumour phenotype in breast cancer.

Our knowledge of the function of the NM-IF proteins is still very limited. The findings in this study that cytokeratins associated with the nuclear matrix are regulated by estrogen in human breast cancer cells suggests that these structural proteins may be important to estrogen action. Subsequent studies performed in Dr. J. Davie's laboratory have further demonstrated that CK 8, 18 and 19 are associated with nuclear DNA and that estrogen and ICI 164,384 regulate the level of association in T5 but not T5-PRF cells (326). Together these studies suggest that an alteration in the level and changes in regulation of NM-associated proteins may result in nuclear architectural changes that could reflect alterations in gene expression associated with a hormone-independent phenotype.

II. Variant ER-α expression in estrogen-nonresponsive T5-PRF human breast cancer cells.

Numerous studies, as discussed in the Introduction, have identified variant ER-α mRNAs in both normal and neoplastic breast tissue and cell lines. While still a controversial topic, evidence is emerging to support the existence of ER-α variant proteins, which could correspond to some ER-α variant mRNAs, in some cell lines and tissues *in vivo*. However, the pathophysiological significance of ER-α variant expression is unclear. Altered expression of some ER-α variant mRNAs was found associated with both breast tumourigenesis and breast cancer progression (382,386,416-418). Several studies, using transient transfection analyses, have shown that individual ER-α variant proteins can have both positive and negative effects on wild-type ER-α activity (386,391,399,405,409,410,414,419). Conflicting results for some ER-α variants have been obtained (391,420) which may be due to cell and promoter specific events previously identified for various structural/functional domains of the wild-type ER-α (76,125). Similarly, overexpression of a single ER-α variant using stable transfection technology has given different results in different laboratories (419,421). Moreover, direct correlation of any single ER-α variant with clinical tamoxifen resistance or tamoxifen resistance of breast cancer cells in culture has not been forthcoming. Since most of these comparisons have been performed using individual ER-α variants and do not take into account the entire spectrum of ER-α variants relative to each other, the conclusions remain controversial. However, when the relative pattern of expression of ER-α deleted variant mRNA was investigated in T5-PRF compared to parental T5 cells, there was a significant difference in the relative expression of a previously described exon 3 and 4 deleted ER-α variant mRNA (384,387,388). Although the question of whether this ER-α variant is

a cause of estrogen-independence or merely an effect of the selection process for estrogen-independence is not examined, the data, using transient transfection analyses tend to support a possible functional role of the putative exon 3 and 4 deleted ER-α protein encoded by the variant mRNA in the phenotype observed in T5-PRF breast cancer cells. It was shown that T5-PRF cells have significantly increased ligand-independent (basal) ER-α activity (reflected in ERE-tk-CAT activity and endogenous PR and CK levels). The ERd3-4 was able to confer increased ligand-independent (basal) and estrogen-responsive transcriptional activity when expressed in parental T5 cells and increased estrogen-responsive transcriptional activity when coexpressed with wild-type ER-α in ER-α negative human breast cell lines. The demonstrated effect of ERd3-4 to increase HEGO (wild-type ER-α) transcriptional activity in the ER-α negative cell lines suggests a putative functional role for this variant ER-α. Although such data suggest that the ERd3-4 variant can modulate the transcriptional activity of wild-type ER-α, the relevance of the expression levels of each protein achieved in the reconstituted transient expression system to the endogenous levels of ER-α and ERd3-4 variant expression in T5-PRF is unclear. Furthermore, differences in background of transcriptional coactivators and corepressors between naturally ER-α positive and negative cell lines (125), as well as the presence of other naturally occurring ER-α variants in naturally ER-α positive cell lines are all likely to impact on the final outcome of ER-α mediated transcriptional activity and underlie the differences seen between the transiently manipulated cells and the naturally occurring T5-PRF phenotype. Moreover, expression of ER-β and/or its variants may influence estrogen action. Both T5 and T5-PRF cells express low levels of

ER-β mRNA determined by reverse transcription polymerase chain reaction analysis ((345) H. Dotzlaw, unpublished), however, the functional significance of the levels remains unknown. Nonetheless, there was a significant effect on ER-α ligand-independent transcription in T5 cells at levels of co-transfected ERd3-4 that likely would not be higher than the endogenous ER-α in these cells, but the extrapolation of these data to the relative expression of wild-type ER-α and ERd3-4 variant in T5-PRF cells is presently unknown as I was unable to provide proof of the existence of a protein corresponding to the ERd3-4 mRNA *ex vivo* in these cells. It is of significance that an effect of ERd3-4 variant could be reproduced in the parental T5 cells, which would contain a more representative background of ER-α accessory proteins (i.e., coactivators and/or corepressors) as well as other variant forms of ER-α which would all contribute to the final ER mediated biological response. As well, the data do not exclude the possibility that other alterations have occurred in T5-PRF cells which in combination with an altered ER-α variant, may contribute to the estrogen-independent phenotype of T5-PRF cells.

It has previously been shown that breast cancer cells can adapt to low levels of estrogen by enhancing their sensitivity to estrogen (359). Estrogen-deprivation of MCF-7 human breast cancer cells resulted in estrogen hypersensitivity and maximal growth was achieved with an estrogen concentration 4-5 orders of magnitude lower than wild-type cells. These researchers also found that the concentration of ICI needed to inhibit the growth of these cells was approximately 6 orders of magnitude lower than wild-type cells, supporting the hypothesis in this model, that increased sensitivity to ER ligands had occurred. While supersensitivity to estrogen in T5-PRF cells cannot be entirely ruled out, the data on growth

response demonstrate that while T5-PRF cells are sensitive to growth inhibition by ICI 164,384, in contrast to the data of Masamura and coworkers, T5-PRF cells are less sensitive than the parental T5 cells (i.e., ID_{50} 100 nM and 5 μM for T5 and T5-PRF, respectively), suggesting that in this model other mechanisms are likely involved.

These data do not address the mechanism by which ERd3-4 enhances ER-α transcriptional activity, but several possibilities exist. The ER-α contains at least two separate regions that are required for optimal transcriptional activation (76,125). The amino-terminal region contains promoter and cell-type specific ligand-independent transcriptional activity (AF-1), while a second, AF-2, is located in the ligand-binding carboxyl-terminus of the receptor. ERd3-4 containing an intact AF-2 or AF-1 domain could interfere with, or sequester, an ER-α repressor protein resulting in increased ER-α transcriptional activity in the absence of ligand (171). This variant may also retain the ability to interact with other ER-α regulatory proteins such as coactivators or components of the basal transcription machinery. While ERd3-4 would be missing lysine 362 which has been demonstrated to be important for SRC-1 recruitment (116), an *in vitro* interaction of the N-terminal of hER-α and SRC-1 has been demonstrated (184) and the AF-1 activity of hER-α transiently expressed in HeLa cells can be stimulated with SRC-1 (185). This suggests that SRC-1 can functionally interact with both the N- and C-terminal portions of the hER-α and given the fact that ERd3-4 contains an intact AF-1 domain, may still retain the ability to interact with coactivator proteins. Additionally, research also has demonstrated that both the AF-1 and AF-2 domains of the hER-α can interact with TBP *in vitro* (178).

The crystal structure of the ER-α hormone binding domain has recently been

elucidated (115). Based on this structure, the ERd3-4 protein would contain many of the regions essential for transactivation, including the predominant helix 12 (encompassing amino acids 539-547). However, since ERd3-4 alone has no transcriptional activity (at least on a classical ERE regulated promoter) the structure must be sufficiently altered to prevent activity, or AF-2 can only be activated in a ligand-dependent manner, but ERd3-4 cannot bind ligand. Helix 12 in AF-2 is believed to be the main region involved in coactivator recruitment and it may be possible that ERd3-4 could enhance recruitment of coactivators to the basal transcription complex and this enhances ER-α activity.

ER-α also contains two domains involved in dimerization (79,80). A weak dimerization interface is present in the DNA-binding domain and a strong interface is located in the C-terminal ligand-binding domain (487). ERd3-4 containing an intact C-terminal dimerization domain, may form heterodimers with wild-type ER-α that have altered transcriptional regulatory properties through differing protein-protein interactions. Although the results of the gel mobility shift assays did not detect any such interactions, the use of rabbit reticulocyte lysates, may have precluded an additional protein(s) that if present *in vivo*, may facilitate an interaction between ER-α and ERd3-4.

Using a transient expression system, it was found that ERd3-4 caused increased ligand-independent wild-type ER-α activity and also enhanced the ligand-induced ER-α transcriptional activity, despite the fact that on its own this variant is not transcriptionally active on a classical ERE promoter, nor does it bind ligand *in vitro* to any significant degree. Studies have demonstrated that the ability of steroid hormone receptors to modulate transcription does not necessarily require that the receptors bind DNA. PRc, an N-terminally

truncated PR isoform, lacking the first zinc-finger of the DNA-binding domain, has no transcriptional activity of its own but has been shown to enhance progestin-induced transcriptional activity (157). An ER-α variant lacking exon 5 that has no transcriptional activity on its own has been shown to enhance both basal and estrogen-dependent transcriptional activity when coexpressed with wild-type ER-α (488). The DNA-binding domain of the ER-α does not appear to be necessary for raloxifene activation of the TGFβ3 gene (160) and ER-α can activate transcription from AP-1 dependent promoters through a DNA-binding-independent pathway (159). Sp1 and ER-α directly interact to enhance estrogen-induced transactivation of the Sp1-dependent Hsp27 gene promoter and the DNA-binding domain of the ER-α is not required (229). ER-α stimulation of the human RAR-α1 promoter has been demonstrated to occur independently of the ability of the ER-α to bind to DNA (489) and estrogen regulation of the brain creatine kinase promoter by the ER-α is thought to occur via a mechanism that likely does not require direct DNA binding (490).

The presence of alternative forms of ER-α capable of interacting with wild-type ER-α to increase ligand-independent activity could also confer a potential growth advantage to breast cancer cells. A recent study has shown that constitutively active, ligand-independent ER-α mutants undergo conformational changes and interactions with coactivators that mimic changes in ER-α that are usually regulated by ligand (180). Recently, researchers have shown that thyroid hormone receptor-β2 (TR-β2) is a ligand-independent activator of the gene encoding thyrotropin-releasing hormone and have mapped a region in the N-terminus of the receptor responsible for this activity (491). These researchers suggest that the mechanism of

ligand-independent activation involves direct interaction of the TR-β2 amino terminus with either transcriptional cofactors or the basal transcription machinery itself.

An increased relative expression of variant ER-α proteins containing intact AF domains, could result in increased interactions with the ER-α and/or other proteins involved in ER-α transcriptional activity. This could be a potential mechanism for estrogen-independent growth associated with the presence of one or more variant ER-α species and could explain the increased ER-α activity seen with the ERd3-4.

III. MAPK activity in T5 and T5-PRF human breast cancer cells.

Elevated levels of active MAPK were found in the T5-PRF estrogen non-responsive breast cancer cell line compared to the parental estrogen responsive T5 breast cancer cell line. The T5-PRF cell line, as described, also contains elevated levels of estrogen-independent ER-α transcriptional activity. The ER-α, like other members of the steroid hormone receptor superfamily, is a phosphoprotein. The function of phosphorylation is not clear, but it has been suggested to play a role in many aspects of receptor activity, including DNA binding and transcriptional activation. In MCF-7 human breast cancer cells, the ER-α is phosphorylated on serine-118, serine-154 and serine-167 in response to estradiol binding (238,240,250). It has also been demonstrated that serine-118 and tyrosine-537 on ER-α are phosphorylated independently of estradiol binding in MCF-7 cells (237,250). The activation of the MAPK pathway through an estrogen-independent mechanism (i.e., EGF stimulation) can result in transcriptional activation of the ER-α, and phosphorylation of the ER-α on serine-118 is required for this activity (232,240,246).

Estrogens are known mitogens for breast cancer cells, but how estrogen promotes cell proliferation is unknown. The MAPK signal transduction pathway plays an essential role in cell cycle progression and can be activated by many growth factor/mitogenic pathways including estrogen. In several cell types, including MCF-7 human breast cancer cells, estradiol has been shown to rapidly increase MAPK activity (276). That this activation requires ER-α was demonstrated in cells via transient transfection experiments showing an absolute requirement of ER-α for activation and the addition of the antiestrogen ICI 182,780 blocked estrogen-induction of MAPK activation in MCF-7 cells (276). A recent report was able to show growth factor, but not estrogen-induced, activation of MAPK in MCF-7 cells (240). The discrepancy between this report and a previous report demonstrating estrogen activation of MAPK in MCF-7 cells (276) is unclear, but may be due to differences in the experimental conditions under which activation was assayed between the two papers, as Migliaccio and coworkers (276) used cells grown in estrogen-deplete conditions for one week, versus the use of serum-starved cells by Joel and coworkers (240).

Peptide growth factor signalling pathways can cross-talk with the ER-α. Indeed, it has been demonstrated that growth factors such as EGF can result in ligand-independent activation of ER-α (246). Several studies have demonstrated that overexpression of a growth factor, or its receptor, which can activate the MAPK cascade, or a component of the MAPK pathway (e.g., Raf or Ras) can result in estrogen-independent growth in cells in culture and in some cases tumourigenesis *in vivo* in the absence of estrogen. For example, overexpression of a constitutively active Raf kinase in MCF-7 cells allows for growth in the absence of estrogen (462) and MCF-7 cells stably transfected with the ras oncogene were able to form

tumours *in vivo* in the absence of estrogen (429). These data demonstrate the importance of the MAPK pathway in cell growth regulation and likely tumourigenesis.

What role elevated MAPK activity might play in the estrogen-independent growth phenotype of T5-PRF cells is not clear. It is likely that elevated MAPK activity resulting in ligand-independent activation of the ER-α may allow maximal proliferation in the absence of estrogen. The transfection experiments using the MEK inhibitor PD 98059 suggest that the MAPK activity may play a role in the elevated basal ER-α activity seen in these cells, but may not account for all the increased basal activity. At a concentration of 50 μM, PD 98059 can almost completely abolish the level of active dually phosphorylated MAPK protein (an approximately 85% decrease), whereas a similar concentration resulted in slightly less than 50% reduction in basal ER-α transcriptional activity. This suggests that other mechanisms, along with elevated MAPK, may contribute to the ligand-independent ER-α transcriptional activity. Indeed, the T5-PRF cells contain the ER-α variant ERd3-4 which may also contribute to the increased basal ER-α activity. It is also likely that the residual dually phosphorylated MAPK protein remaining, even after treating cells with PD 98059, is sufficient to contribute to the basal ER-α activity.

These studies also demonstrate an inhibition of estrogen-dependent ER-α transcriptional activity after treating cells with PD 98059. This was of a similar magnitude as the effect on basal transcription (approximately 50%), supporting the hypothesis that MAPK plays an important role in both ligand-dependent and -independent ER-α transcriptional activity.

This research also suggests that the inhibition of MAPK through PD 98059 treatment

is associated with a reduction in immunoreactive ER-α protein levels. The mechanism involved in this is not known. It is likely that a reduction, or prevention of phosphorylation of ER-α on serine-118 via MAPK may cause increased turnover of the protein. Links between protein phosphorylation and protein turnover have been made. For example, researchers have demonstrated that phosphorylation of the transcription factor c-Jun by the MAPK, c-jun N-terminal kinase (JNK), increased the half-life of c-Jun (492). The decreased ER-α transcriptional activity after PD 98059 treatment may not be related to the decreased ER-α protein level as estrogen, as discussed in the Introduction, often can downregulate its own receptor levels, despite the fact that estrogen treatment results in increased ER-α transcriptional activity.

Elevated levels of MAPK activity and expression have been associated with the malignant phenotype and have been shown in breast tumours compared with normal tissue and benign breast conditions (270). Breast tumours also have been shown to contain elevated tyrosine kinase activities compared with benign breast tumours and normal breast tissues (267). These data suggest that an increase or deregulation of growth controlling signals, such as those contributed by MAPK, may be involved in the etiology and pathogenesis of breast cancer. During the course of breast cancer progression, tumours become hormone-independent and refractory to endocrine therapies directed at blocking the activity of ER-α. The development of estrogen-independent growth is believed to be an initial step in the progression to a hormone-independent phenotype, and estrogen-independent growth is a characteristic of a more aggressive breast cancer cell phenotype. The data shown support the hypothesis that elevated levels of MAPK activity are associated with increased ligand-

independent activity of the ER-α. Elevated MAPK activity may be one mechanism of estrogen-independent growth.

Conclusions

The research that contributes to this thesis support the hypothesis that the development of an estrogen-independent growth phenotype in human breast cancer cells is likely a multifactorial process. It is clear that in T5-PRF estrogen-nonresponsive human breast cancer cells, there are several changes that have been detected that can all likely contribute to an estrogen-nonresponsive growth phenotype (see Table 6). Studies have demonstrated the importance of cellular architecture contributed by the tissue matrix system to gene expression and that perturbations in this system can alter gene expression (328-330,493). This cellular framework serves not only as a structural entity but is likely intimately connected with all cellular processes including gene expression and signal transduction.

While these studies suggest an important link between NM-IF proteins, gene expression, signal transduction and the response of a cell to hormone treatment, it remains unclear what the initiating factor is. As a cell progresses from a hormone-responsive to a non-responsive growth phenotype it is unclear if a single initiating event common to all cells, or if a multitude of events in a single cell, or multiple but single events occur over a whole cell population, results in hormone-independent growth. In cases where the cells no longer express ER-α it is easier to visualize how this single event can result in hormone-independent growth. In the case where the cell continues to express the ER-α and many ER-α associated changes are present it becomes less clear as to what the initiating factor may be.

Table 6. Comparison of T5 and T5-PRF human breast cancer cell lines.

	Cell line	
	T5	T5-PRF
Growth response		
Estrogen	++	-
4-monohydroxytamoxifen*	++	++
ICI 164,384*	++	+
Basal receptor level		
ER-α	++	+
PR	+	++
Cytokeratin level		
Basal	+	+++
Estrogen	+++	+++
ICI 164,384	+	+++
4-monohydroxytamoxifen	+	+++
Basal ER-α transcriptional activity	-	++
ERd3-4 mRNA expression	-	++
MAPK activity	+	++

* refers to antagonist activity on cells grown in estrogen-replete conditions.

It is conceivable that deregulated splicing could occur during tumour progression. Recent research has demonstrated that splicing factors are phosphorylated and phosphorylation plays an important role in splice factor localization and activity (95,102). As well, Konig *et al* have demonstrated activation of signal transduction pathways can activate/influence alternative splicing (103). Using minigene constructs containing CD44 variant exon 5 (v5), these researchers found that the inclusion of v5 occurred with the activation of PKC (via TPA) or cotransfection of active ras in a T-lymphoma cell line that was shown to normally exclude v5. It may be that an increase in a signal transduction pathway such as Ras or PKC that can activate MAPK could initiate a series of changes involving ER-α variant expression through affecting splicing patterns. While it is not known if T5-PRF cells contain elevated Ras or PKC activity, it is of note that studies have demonstrated that approximately 70% of human breast tumours overexpress Ras protein (494) and, as discussed in the Introduction, numerous studies have implicated protein kinase signalling pathways in the development and progression of breast cancer.

An increase in MAPK activity could also affect the tissue matrix system by increasing cytokeratin expression. Data have demonstrated that activation of Ras and or Raf increases CK 18 expression (495). As well, cytokeratins are phosphoproteins and protein kinases including MAPK have been implicated in cytokeratin phosphorylation (496,497). While the function of phosphorylation of the cytokeratins is unclear, experimental data support a role in organization and solubility of the cytokeratins, as well as regulating their interactions with other proteins (497-499). Of interest is the fact that phosphorylation of CK 18 is essential for its interaction with 14-3-3 proteins (498), which have also been shown to directly interact

with Raf, and are thought to be required for its activity (500,501). Together these data further support a link between deregulated MAPK activity and changes to the tissue matrix system. T5-PRF cells overexpress cytokeratins compared to parental T5 cells and appear to have lost both estrogen and antiestrogen regulation of expression. This is in contrast to the reduction of the ligand-independent (basal) activity of the ER-α measured in the transient transfection analyses by ICI 164,384 treatment in T5-PRF cells. It may be that increased activity of a pathway such as MAPK can lead to increases in cytokeratin expression and 'short circuit' the need for ER-α regulation, accounting for the lack of effect of ICI 164,384 on the cytokeratin levels in T5-PRF cells.

Figure 39 describes a model which serves to demonstrate how the changes identified in T5-PRF cells may be involved in the development of an estrogen-nonresponsive growth phenotype. It can be envisioned that a change that results in the cell no longer requiring estrogen for growth (i.e., expression of a dominant-positive variant ER-α protein) can result in an increase in estrogen-regulated processes (i.e., MAPK activation, CK and PR expression). Alternatively, a deregulated and increased MAPK activity may result in not only increased ER-α activity and increased cytokeratin expression, but may also lead to altered splicing patterns of the ER-α. It is possible that any one of these changes would confer additional growth advantages to breast cancer cells and lead to further changes in gene expression that could ultimately result in estrogen-independent growth.

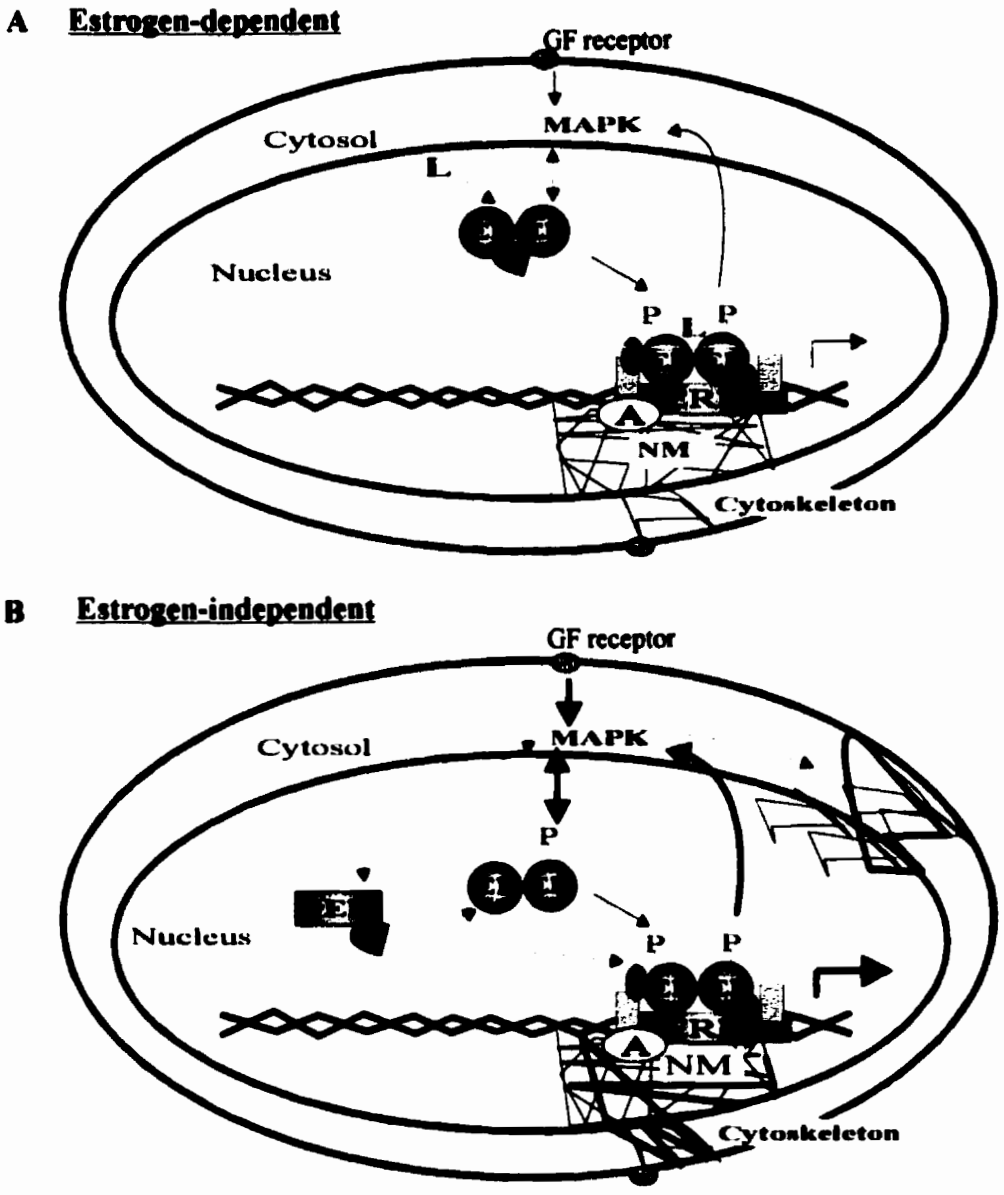


Figure 39. A. Estrogen-dependent human breast cancer cells. Ligand binding results in release of repressor proteins from the ER-α, phosphorylation of the receptor and interaction with NM, chromatin/ERE and coactivator/accessory proteins. **B. Estrogen-independent human breast cancer cells.** Elevated MAPK activity could result in altered variant ER-α expression and/or increased phosphorylation of the ER-α in the absence of ligand. Variant ER-α containing intact AF functions may sequester an ER-α repressor protein(s) and/or interact with the wild-type ER-α. Increased expression of NM-IF proteins may increase acceptor sites or promote increased interaction of ER-α with NM. Ligand-independent activation of the ER-α could further increase MAPK activity and result in an increased/deregulated expression of normally estrogen-regulated genes. A= accessory/acceptor protein, v = variant, NM = nuclear matrix, ER = estrogen receptor, GF = growth factor.

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