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Differential Control of TGM2 Expression by Oestrogen Receptor/ SERMs.

Damian Lally
Technological University Dublin

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By

Damian Lally

School of Biological Sciences,

Dublin Institute of Technology,

Kevin Street, Dublin

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Abstract

Breast cancer is a malignancy of the epithelial cells comprising the mammary gland. As the ER is necessary for the growth of approximately 70% of breast cancers, pharmaceutical efforts have focused on uncovering modulators of the ERα such as anti-estrogens and Selective Oestrogen Receptor Modulator (SERMs). SERMs differ from pure ER antagonists in their capacity to display tissue-selective and C promoter dependent agonist-antagonist activities. Tamoxifen (TAM) is a SERM that is used in the treatment of hormonally responsive breast cancer. TAM is the most commonly used treatment for patients with ERα positive breast cancer.

In this study we examined the role of Oestrogen Receptor (ER) signaling in the regulation of tissue Transglutaminase 2 gene (*TGM2*). *TGM2* encodes tissue Transglutaminase (TG2), a multifunctional enzyme with many cellular functions, such as matrix remodelling, stabilization of apoptotic cells and cell adhesion and migration which are thought to be implicated in inhibition of tumour growth and prevention of metastasis. Ligand activated ER has been shown to induce the transcription of the *TGM2*.

Our results show mRNA expression of *TGM2* by E2/ER in breast cancer cells is maintained in the presence of TAM but not the SERM Raloxifene. As we would expect this gene to be inhibited by TAM, this reveals an added layer of complexity to the pharmacology of TAM. To investigate this finding further we studied the effects of compounds which are structurally related to TAM, and found that compounds such as Endoxifen and 4-Hydroxytamoxifen have similar effects to TAM. To analyse the sequence requirements for ER induced activation of *TGM2* transcription we cloned the 5' regulatory region into a luciferase vector. Further study is required in this area to better understand the significance of *TGM2* expression in breast cancer cells.

Declaration page

I certify that this thesis which I now submit for examination for the award of M.Phil is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for another award in any Institute. The work reported on in this thesis conforms to the principles and requirements of the Institute's guidelines for ethics in research.

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Candidate

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List of Abbreviations

AF	Activation Function
AhR	Aryl Hydrocarbon Receptor
AI	Aromatase Inhibitor
ANF	α-Napthoflavone
AP-1	Activator Protein 1
ATRA	All trans retinoic acid
BAZED	Bazedoxifene
Bgal	β-galactosidase
BNF	β-Napthoflavone
CBP	CREB-binding protein
CoA	Coactivator
COMT	Catchol *O*-Methyltransferase
CoR	Corepressor
CoRNR	Corepressor of Nuclear Receptor
CPRG	Chlorophenol Red β-D-Galactopyranoside
C_T	Threshold Cycle
CYP	Cytochrome P450
DBD	DNA Binding Domain
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl-sulphoxide
DNA	Deoxyribonucleic acid
DR	Direct Repeats
dsDNA	Double-Stranded DNA
DTT	Dithiothreitol
E1	Estrone
E2	Estradiol

E3	Estriol
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
ENDOX	Endoxifen
ER	Oestrogen Receptor
ERE	Oestrogen Response Element
ERK	Extracellular Signal-Regulated Kinase
ERK/MAPK	Extracellular signal-Regulated Kinase/Mitogen-Activated Protein Kinase
FBS	Fetal Bovine Serum
FSH	Follicle Stimulating Hormone
GTA	General Transcription Activator
GTP	Guanine Tri-phosphate
H12	Helix 12
HRE	Hormone Response Element
HSD	Hydroxysteroid Dehydrogenases
ICI	ICI 182,780
IL-6	Interleukin-6
IMM	Inner Mitochondrial Membrane
LASOFOX	Lasofoxifene
LBD	Ligand Binding Domain
Luc	Luciferase
MAPK	Mitogen-Activated Protein Kinase
MEM	Minimum Essential Media
ML	Molecular Ladder
NEAA	Non-essential amino acids
NR	Nuclear Receptor

NRRE	Nuclear Receptor Response Element
NRT	No-Reverse Transcriptase
OMM	Outer Mitochondrial Membrane
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI3K	Phosphotidylinositol-3-Kinase
PPAR	Peroxisome Proliferator-Activated Receptor
PRMT1	Protein Arginine Methyltransferase 1
qPCR	Qualitative PCR
RAL	Raloxifene
RAR	Retinoic Acid Receptor
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase PCR
SAP	Shrimp Alkaline Phosphatase
SEM	Standard Error of the Mean
SERD	Selective Oestrogen Receptor Down-regulator
SERM	Selective Oestrogen Receptor Modulator
SP1	Specificity Protein 1
SRC	Steroid Receptor Co-activator
STAR	Steroid Acute Regulatory Protein
TAM	Tamoxifen
TG2	Transglutaminase (protein)
TGM2	Tissue Transglutaminase II gene
TR	Thyroid Receptor
TRAP220	Thyroid Hormone Receptor Activating Protein of 220kDa
UGT	Uridine Diphosphate-Glucuronosyltransferases
V	Vehicle

VDR	Vitamin D3 Receptor

1 Introduction

1.1 General Principles of Cell Signalling

Multicellular organisms require elaborate mechanisms of communication whereby signals from one cell or group of cells influence the behaviour or function of another group of cells. This can involve direct cell-cell contact in which the signalling molecule present on the surface of a cell can directly contact a specific protein called a receptor on the surface on a target cell, initiating a cascade of events that alters the function of the target cell (Paracrine). The second mechanism of intercellular signalling involves secretion of a signalling molecule by a cell and the binding of the molecule to a receptor located either on the plasma membrane or intracellularly in the target cell (Endocrine) [1].

Signaling molecules may act locally on the same cell (autocrine signalling) or on neighbouring cells (paracrine signalling) or at a distance as with endocrine signalling. Because secreted signalling molecules must travel in a hydrophilic extracellular environment, many signalling molecules are hydrophilic and unable to cross the plasma membrane. These molecules therefore bind to cell surface receptors and transmit signals intracellularly. Hydrophilic signalling molecules include proteins, peptides, amino acids, nucleotides, and certain dissolved gases including nitric oxide and carbon monoxide. Alternatively, lipophilic signalling molecules, including fatty acids, steroid hormones, vitamins D3, and retinoids, can diffuse across the plasma membrane lipid bilayer and bind to intracellular receptors found within the cytosol or the nucleus. Because these signalling molecules are lipophilic, they travel through the extracellular milieu bound to carrier proteins. Within the target cell lipophilic molecules bind to intracellular receptors called nuclear receptors.

The nuclear receptor superfamily constitutes a family of intracellular receptors that effect transcriptional programs that play roles in a number of biological processes by responding to hormonal and metabolic signals.

1.2 Nuclear receptor signalling

1.2.1 The Nuclear Receptor Superfamily

In humans, the nuclear receptor superfamily consists of 49 different receptors (Figure 1.1). Many nuclear receptors are ligand-activated transcription factors that are expressed in a cell-restricted manner. Nuclear receptors (NR) can bind a host of endogenous ligands including steroid hormones, fatty acids, vitamin D3, xenobiotics, cholesterols, and bile acids, some NRs are orphan receptors whose endogenous ligand has not been identified.

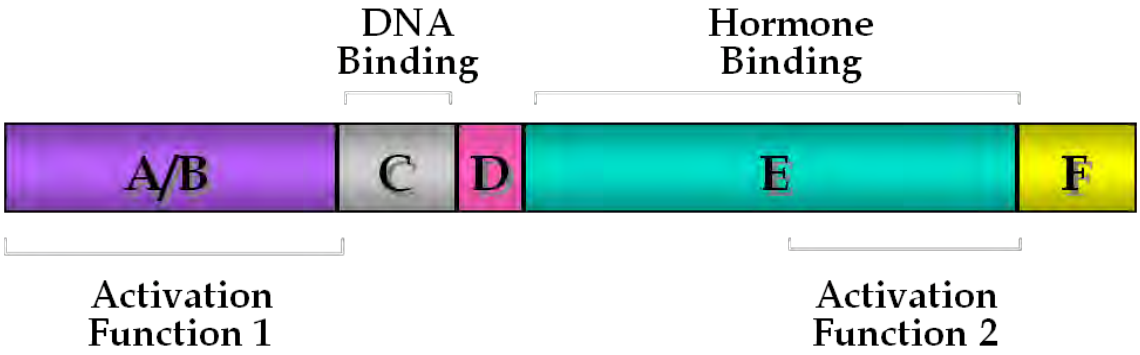
Figure 1.1 Nuclear Receptor gene family

AR	ERRα	LRH	PPARα	reverbα	SF1
CAR	ERRβ	LXRα	PPARγ	reverbβ	SHP
COUPα	ERRγ	LXRβ	PPARδ	RORα	TIX
COUPβ	FXR	MR	PR	RORβ	TRα
COUPγ	GCNF1	NGF1-Bα	PXR	RORγ	TRβ
DAX	GR	NGF1-Bβ	RARα	RXRα	TR2α
ERα	HNF4α	NGF1-Bγ	RARβ	RXRβ	TR2β
ERβ	HNF4γ	PNR	RARγ	RXRγ	VDR

Adapted with permission from Prof. D McDonnell, Department of Pharmacology and Cancer Biology, Duke University, NC.

Nuclear receptors share a common modular structure that includes 5-6 conserved domains, A through F (Figure 1.2) [2]. The A/B domain of the receptor is located at the N-terminus of the protein, and it contains a ligand-independent activation function 1 (AF-1) domain. This domain can activate transcription in an autonomous manner. The A/B domain is the most variable of the domains and its length varies widely between 23 amino acids for the vitamin D3 receptor (VDR) to over 550 amino acids for the androgen, mineralocorticoid, and glucocorticoid receptors [3]. The C domain of the receptor constitutes the DNA Binding Domain (DBD) of the receptor. This domain confers upon the receptor sequence-specific DNA recognition through two zinc finger motifs. The D domain of the receptor is a hinge region between the highly structured domains that precede and follow it. This domain contains a nuclear localization sequence, which may play a role in the dynamic nuclear-cytoplasmic shuttling of nuclear receptors that occurs in response to ligand binding [4]. The E domain of the receptor is the Ligand Binding Domain (LBD), which is involved in ligand-binding and dimerisation. The LBD also contains an activation domain called activation function 2 (AF-2), which is a region of the protein involved in recruiting coactivator molecules that are essential for ligand-dependent transcriptional activity [5]. Some receptors, including the peroxisome proliferator-activated receptor (PPAR) family possess an F domain at the very C-terminus [6]. This domain is evolutionarily not well-conserved, and the roles of the F domain are largely unknown.

Figure 1.2 Modular structure of nuclear receptors



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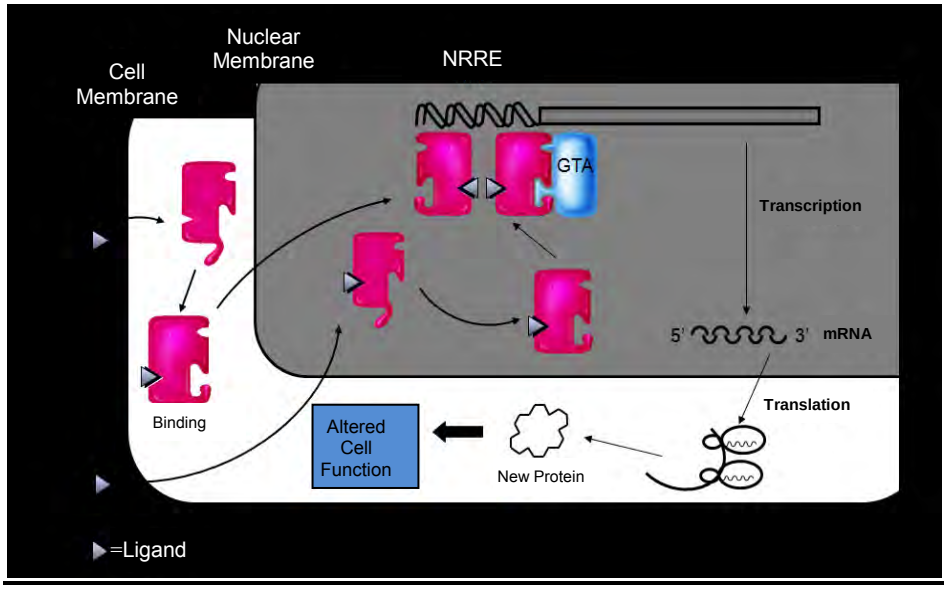
1.2.2 General Mechanisms of Nuclear Receptor Signalling

In an unliganded-state, nuclear receptors can be localised to either the cytoplasm or nucleus. Upon ligand binding, nuclear receptors dissociate from any associated heat shock chaperone proteins to which they are bound, translocate into the nucleus, and homo- or heterodimerise on specific DNA nuclear receptor response elements (NRREs) within the promoters of target genes (Figure 1.3). The DNA binding domain, located in the centre of the protein, contains nine cysteine residues, eight of which can chelate two zinc molecules, forming two zinc fingers that bind to two adjacent major grooves in the DNA[7]. Nuclear receptors recognize variations of the hexameric motif 5'-PuGGTCA (Pu = A or G) [3]. Some nuclear receptors, including the retinoid acid receptor (RAR), Vitamin D receptor (VDR), and thyroid receptor (TR) bind to direct repeats of the hexameric sequence separated by 1-5 nucleotides, termed DR1 through DR5 elements. The oestrogen receptor prefers a 5'-PuGGTCA half site sequence, whereas GR prefers a 5'-PuG(G/A)ACA sequence.

Nuclear receptors regulate gene transcription via several different mechanisms [8]. Through binding of various ligands, nuclear receptors can repress or activate transcription by recruiting corepressor and coactivator proteins, respectively [9].

Binding of agonist to the nuclear receptor alters the conformation of the AF-2 domain, facilitating the displacement of corepressors and the recruitment of coactivators. Most coactivators contain a canonical LXXLL amino acid motif that interacts with the AF-2 of the nuclear receptor. Recruitment of coactivators is essential for nuclear receptor-mediated gene transcription; their role is to facilitate transcription by remodelling the chromatin and recruiting RNA polymerase II and the general transcriptional machinery. Binding of antagonist to the nuclear receptor facilitates the recruitment of co-repressors; which leads to recruitment of histone deacetylases, condensation of chromatin and dampening of transcriptional activity.

Figure 1.3 The mechanism of action of intracellular receptors



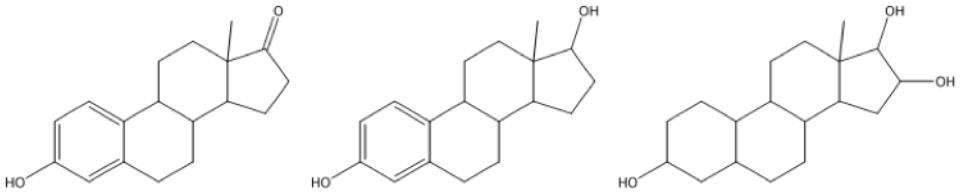
Ligand passes through the cell membrane and binds steroid receptors in the cytoplasm or nucleus. Binding of ligand induces a conformational change in the receptor structure permitting dimerisation and binding to specific nuclear receptor responsive elements (NRRE) in the DNA structure. The recruitment of general transcription activators (GTA) to the complex allows for the induction of gene transcription and production of a protein. Adapted with permission from [10].

1.2.3 Oestrogen Signalling

Oestrogen is probably the most widely studied of all hormones, the term „oestrogen' refers to a group of chemically similar hormones, estrone (E1), estradiol (E2), and estriol (E3) (Figure 1.4). These hormones are uniquely responsible for the growth and development of the female sexual characteristics. Estradiol and estrone are produced primarily in the ovaries in pre-menopausal women, while estriol is produced by the placenta during pregnancy. Oestrogen acts on cells in a wide variety of tissues including breast, uterus, brain, bone, liver, and heart to modulate cell activity. For example, oestrogen controls growth of the uterine lining during the first part of the menstrual cycle, and cause changes in breast during adolescence and pregnancy and regulates various other metabolic processes such as bone growth and cholesterol levels.

1.2.4 Oestrogens

Figure 1.4: The structures of the three oestrogens

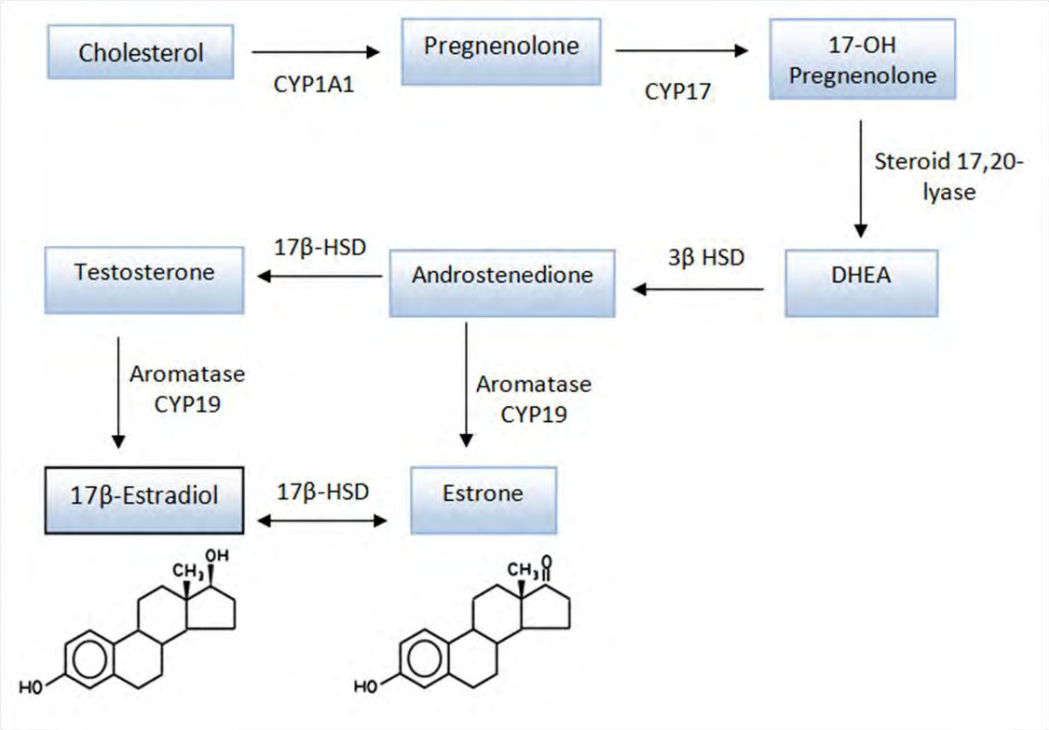


E1: estrone E2: 17β-estradiol E3: estriol

The three oestrogens, estrone (E1), estradiol (E2), and estriol (E3), are the most abundant and well-studied endogenous agonists for ER (Figure 1.4). The biosynthesis of oestrogen in the ovary is initiated in the early follicular stage of the reproductive cycle in response to follicle stimulating hormone (FSH), a pituitary hormone whose receptor is expressed in ovarian granulosa cells. Upon receipt of the signal, cholesterol is mobilized from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) by steroid acute regulatory protein (STAR). At the IMM, cholesterol is converted to pregnenolone by the action of the cholesterol side chain cleavage

enzyme cytochrome P450 (CYP) 11A1 (CYP11A1). From there, pregnenolone is metabolized by numerous CYPs, hydroxysteroid dehydrogenases (HSDs), and hydroxysteroid reductases to produce oestrogens, as well as other steroid hormones (Figure 1.5).

Figure 1.5: Steroidogenesis

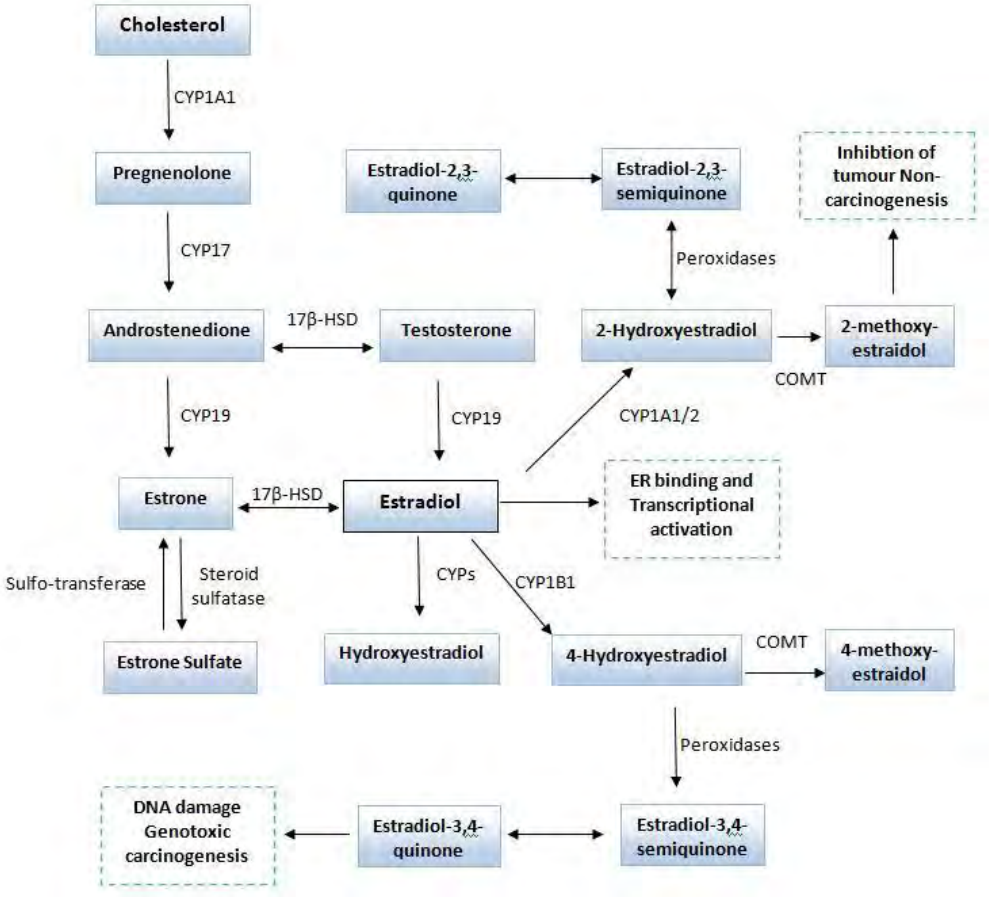


A diagram of the reactions in the steroidogenic pathway that contribute to the synthesis of endogenous oestrogens, highlighting the enzymes that catalyze these reactions.

Oestrogens can be metabolized through several mechanisms, including oxidation by CYPs, sulfation by sulfotransferases, glucuronidation by uridine diphosphate-glucuronosyltransferases (UGT), and *O*-methylation by catchol *O*-methyltransferases (COMT) (Figure 1.6) [11]. The primary site of E2 metabolism is the liver, where CYP1A2, CYP3A4, and CYP1B1 mediate 2 – and 4-hydroxylation of E2. Outside the liver (in the breast, uterus, placenta, brain, and pituitary) CYP1A1, and to some extent CYP3A4, converts E2 to the 2-hydroxylated form. COMT rapidly methylates 2-hydroxy-E2 to avoid free radical formation and is thus considered to be anti-

tumourigenic. In the breast and uterus, CYP1B1 actively produces high levels of 4-hydroxy-E2. Unfortunately, 4-hydroxy-E2 is rapidly converted between semiquinone and quinone forms, which generate free radicals that cause DNA damage, and thus CYP1B1 metabolism of E2 is considered genotoxic. There is evidence to suggest that E2 can control the expression of its metabolic enzymes, although the data is not clear [11]. Oestrogens function through ER to control particular biological processes, as discussed below.

Figure 1.6 Oestradiol Metabolism

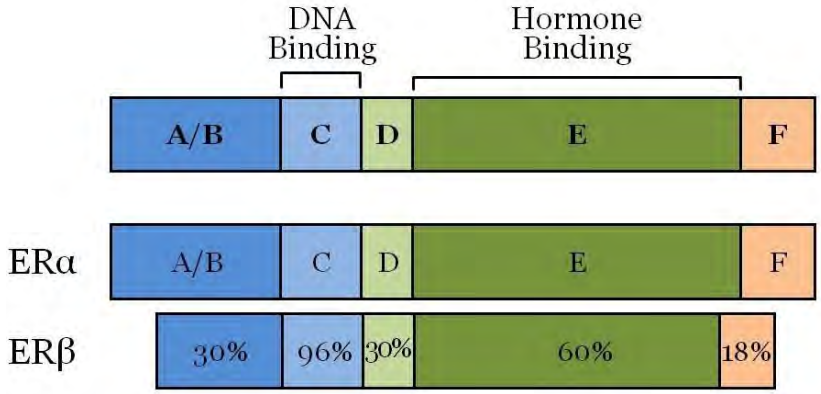


The creation and metabolism of E2 is catalyzed by many members of the CYP family. E2 metabolites fall into two main categories, those that are genotoxic and those that may inhibit tumourigenesis [11].

1.2.5 The Oestrogen Receptor

ER is a member of the nuclear receptor (NR) super-family of ligand-activated transcription factors. There are two genetic subtypes of ER, ERα and ERβ, which exhibit overlapping but distinct activities and expression patterns [12-15]. They share the same modular structure: an N-terminal A/B domain, the C domain containing the zinc-finger DNA binding domain (DBD), a hinge region (D domain), the E domain which houses the ligand binding domain (LBD), and the C-terminal F domain (Figure 1.7). Given their high degree of homology in the LBD and DBD, it is not surprising that ERα and ERβ regulate mostly identical DNA response elements and bind many of the same endogenous and exogenous ligands [14, 16]. Tissue distribution patterns and knockout mouse models of ER indicate that ERα and ERβ are not redundant, and instead suggest unique tissue-specific functions for each receptor subtype [12, 17]. ERα is more ubiquitously expressed throughout the body, whereas the pattern of ERβ is restricted to the testis, ovary and thymus [12]. Together, ERα and ERβ control many important biological processes, including ovulation, mammary gland development, proper bone mineral density, cardiovascular function, aspects of mating behaviour, and the immune system [17].

Figure 1.7 Schematic of ER structure



Schematic illustration of ER modular structure, highlighting the amino acid similarity between ERα and ERβ. Adapted with permission from [18].

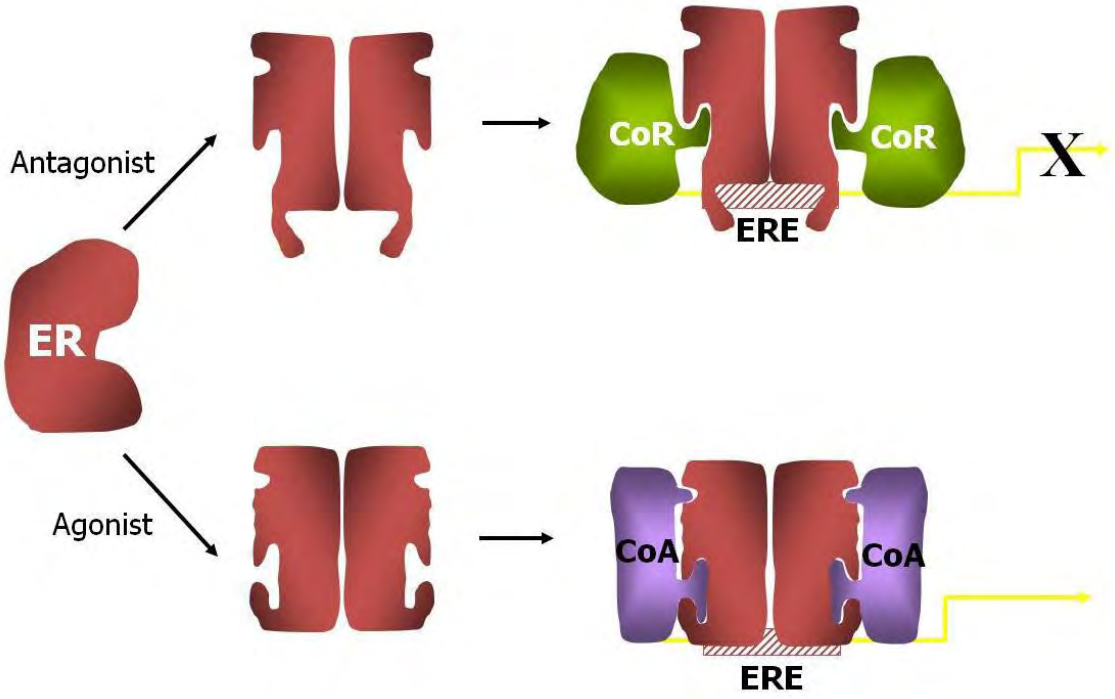
Residing in either the cytoplasm or nucleus, signalling through ER is initiated by either ligand binding or by ligand-independent processes such as phosphorylation. The transcriptional activity of ER is mediated by the activation functions (AFs), of which there are two in ERα (AF-1 and AF-2) but only one in ERβ (AF-2). AF-2 is located within the LBD and is primarily responsible for ligand-dependent activation of transcription, whereas AF-1 is thought to transduce ligand-independent transcriptional activation. When both are present, as in ERα, AF-1 and AF-2 can function synergistically or independently, depending on cell and promoter context, thus adding a layer of complexity [19]. The distinct biologies of ERα versus ERβ may stem from the divergent A/B and F domains, particularly since no obvious AF -1 domain is found in ERβ.

1.2.5.1 Activation of transcription

To activate transcription, the general transcriptional machinery must be recruited to DNA. Although it was once considered that ER directly interacted with this machinery, it is now apparent that the protein-protein interaction surfaces on ER recruit coregulatory proteins, such as coactivators and corepressors, which bridge this

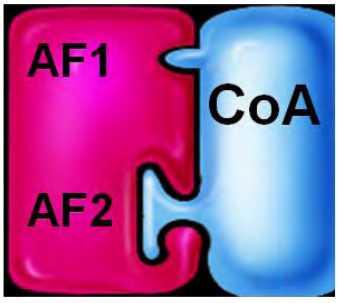
interaction (Figure 1.8). Coregulatory proteins interact primarily with ER at the AF-2 domain, where a hydrophobic pocket is formed predominantly by helix 12 (H12) (Figure 1.9)

Figure 1.8 Diagram of ER agonist and antagonist activity



The binding of agonist to the ER induces a conformational change in the receptor structure which facilitates dimerisation and recruitment of coactivators to the receptor complex. This allows for the activation of transcription of E2 regulated genes. The binding of agonist to the ER facilitates dimerisation and recruitment of corepressors which does not lead to gene transcription. Corepressor (CoR), coactivator (CoA). Adapted with permission from [20].

Figure 1.9 Structure of the nuclear receptor/coactivator complex



The interaction of ER AF-1 and AF-2 with coactivator (CoA). Adapted with permission from [20].

This pocket binds the hydrophobic sequence LxxLL found in coactivators. [21] Coactivators can promote transcription through several mechanisms: the acetylation of histones by steroid receptor coactivators (SRC) -1, -2, and -3, thyroid hormone receptor activating protein of 220kDa (TRAP220), and CREB-binding protein (CBP/p300); the methylation of histones by protein arginine methyltransferase 1 (PRMT1); RNA processing by RNA helicases such as p68; and the coupling of ERα to degradation machinery through the ubiquitin ligases E6-associated protein and ribosome production factor 1. [22-23] The other class of coregulatory proteins, the corepressors, contain a similar domain, the CoRNR box motif, that binds to the hydrophobic pocket in ER [24]. In general, corepressor proteins either recruit or exhibit intrinsic histone deacetylase activity, thereby actively repressing transcription (Figure 1.10).

Figure 1.10 ER-associated cofactors

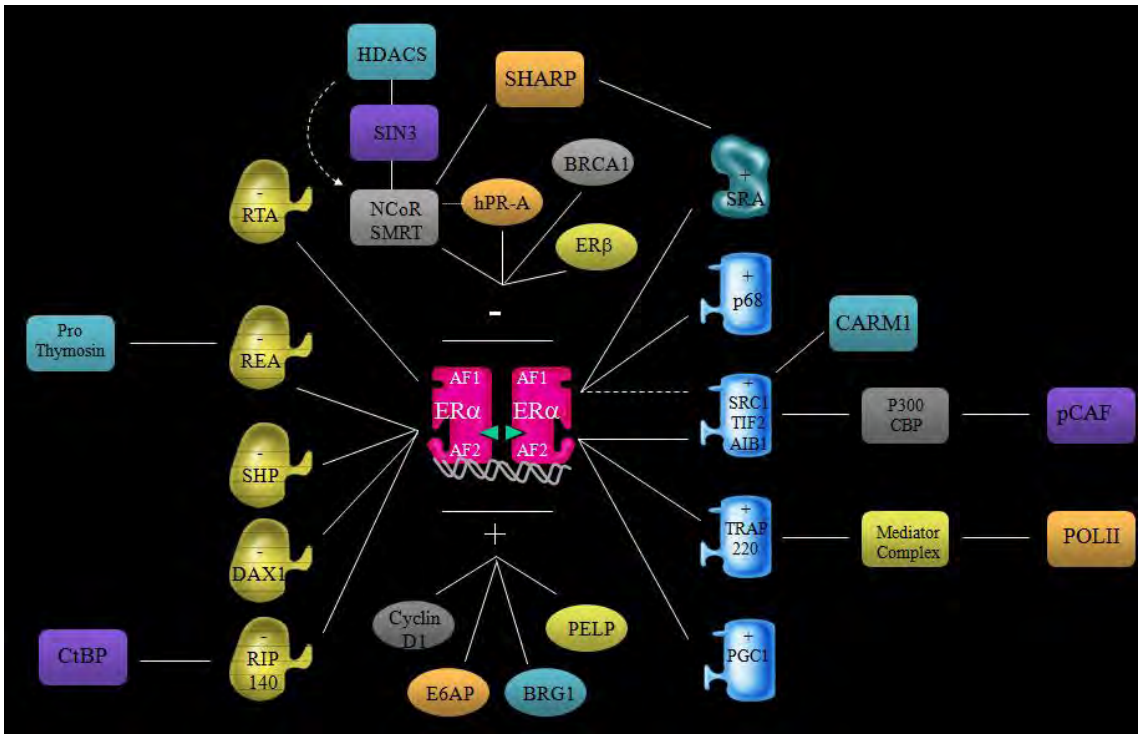


Diagram showing coactivators (+), corepressors (-) and other associated proteins. Oestrogen receptor shape influences cofactor binding preferences [10]. Adapted with permission from [25].

After binding to ligand, ER undergoes a conformational change that is critical in dictating the downstream biological response. Previously, it was thought that there were only two conformations that ER could adopt; an active and an inactive one. However, the discovery that different ligands confer unique responses made this model unlikely. Instead, it appears that each ligand induces a unique conformational change that allows for differential presentation of protein-protein interaction surfaces that are used to couple ER to other signalling pathways, therefore determining the cellular response to a particular ligand [26].

After binding to oestrogen and undergoing a conformational change, ER dimerises and binds to DNA within the regulatory regions of target genes [27]. Direct ER binding to DNA occurs at oestrogen response elements (EREs), whereas ER can also interact indirectly with DNA through binding to Fos and Jun at AP-1 (activator protein 1) elements [28] or to specificity protein 1 (Sp1) family members at GC-rich DNA regions [29]. The specific ERE sequences influence the affinity of ER binding by inducing a unique conformation in the ER structure which may contribute to the promoter specific activities of the ER on different ERE-containing genes [30]. The canonical ERE was determined to have the consensus sequence 5'-GGTCAnnnTGACC-3' [31-32]. The symmetry of the sequence facilitates the binding of ER as a homodimer [33]. However, only a handful of the most highly oestrogen-responsive genes actually contain perfect consensus EREs. Many genes have been found to contain sequences that appear to be EREs, but most of these vary from the consensus by one or more nucleotides. Studies of ER binding showed that one or more changes from the consensus sequence resulted in lower ER-ERE affinity and that sequences immediately flanking the ERE impact ER-ERE binding [34]. One study has performed a genome-wide analysis of oestrogen receptor binding sites, identifying a set

of 3665 unique ER binding sites. This study has shown that the percentage of upregulated genes in breast tumours with an ER binding site within 100kb is 56 - 59%. Also almost one-third of early-oestrogen upregulated genes have ER binding sites within 50kb of the transcription start site [35], while there at least 236 genes with a consensus ERE within -10 to +5 kb of the 5' end [36].

Besides binding ERE sequences on the gene promoter, ERα or ERβ can activate transcription through different types of DNA enhancer elements such as AP-1 sites. This requires ligand-bound ER and the AP-1 transcription factors Fos and Jun. ERα or ERβ both have different transactivation behaviour at Ap-1 sites.

After initiating transcription, ERα undergoes ligand-mediated degradation through the 26S proteosome. The activity of particular coactivators, such as AIB1 (amplified in breast cancer 1), has been shown to be necessary for ERα degradation under certain contexts [37], therefore bringing forth the hypothesis that DNA-bound ERα recruits the proteins that target it for destruction. The complete identity of these proteins, and whether they interact with ERα in the same way as coactivators, has yet to be fully elucidated. Further, ERβ does not undergo ligand-dependent degradation, despite efficient transcription, suggesting perhaps that another surface outside the coactivator binding groove may be responsible for recruitment of the degradation machinery.

Besides its well-studied role in modulating transcription of target genes, ER may also regulate other biological processes in both the nuclear and cytoplasmic compartments through both ligand-dependent and –independent mechanisms. These include association with the phosphotidylinositol-3-kinase (PI3K), insulin-like growth factor 1 (IGF-1), and extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathways. IGF-1, like E2 is a potent mitogen which is involved in

a large array of processes that control proliferation and differentiation in mammalian cells. One study has shown that E2 activates IGF-1 receptor and ERK1/2 via ERα leading to activation of the IGF-1 signalling cascade [38-40]. Activation of IGF-1 receptor leads to the selective recruitment of downstream signaling molecules and results in activation of the Ras/Raf/MAPK signaling cascade. Phosphorylation of ERα at serine 118 is required for full action of AF-1, as ER activation by growth factors involves the MAPK pathway; this is another potential interaction site of the two pathways [41]. This cross-talk between IGF-1 receptor and ER signalling pathways results in synergistic growth stimulation.

It has also been established that oestrogens induce rapid increases in cAMP as well as activation of phospholipase C [42]. The time course of these acute events supports the hypothesis that they do not involve the „classical' gene activation action of oestrogens. Through interaction with these pathways, ER activity can be regulated by direct phosphorylation or by phosphorylation of its coactivator and corepressor proteins.

The exact role of these phosphorylation events, and other potential post-translational modifications, on the signalling competency of ERα and its coactivators remains to be fully elucidated. These pathways may play a pivotal role in oestrogen-independent breast cancer cell growth and tamoxifen-resistance.

1.3 Breast Cancer

1.3.1 General information

In 2007 there were 2463 new cases of breast cancer in Ireland, the number of new cases has been rising steadily each year (+2.4%). However mortality rates have dropped by about 2% per annum (1994-2004), likely due to increased early detection, better therapeutics, and successful prevention strategies. Breast cancer is the most common cause of cancer death in women, at over 660 deaths per year (National Cancer Registry).

Breast cancer is a malignancy of the epithelial cells comprising the mammary gland. Many risk factors have been identified for breast cancer, including lifetime exposure to endogenous hormones, obesity, increased age, family history, and alcohol consumption. The primary treatments for breast cancer are surgery, radiation therapy, and systemic chemical therapy, which include chemotherapeutics and endocrine/hormone therapy.

1.3.2 The role of oestrogen and ER in breast cancer

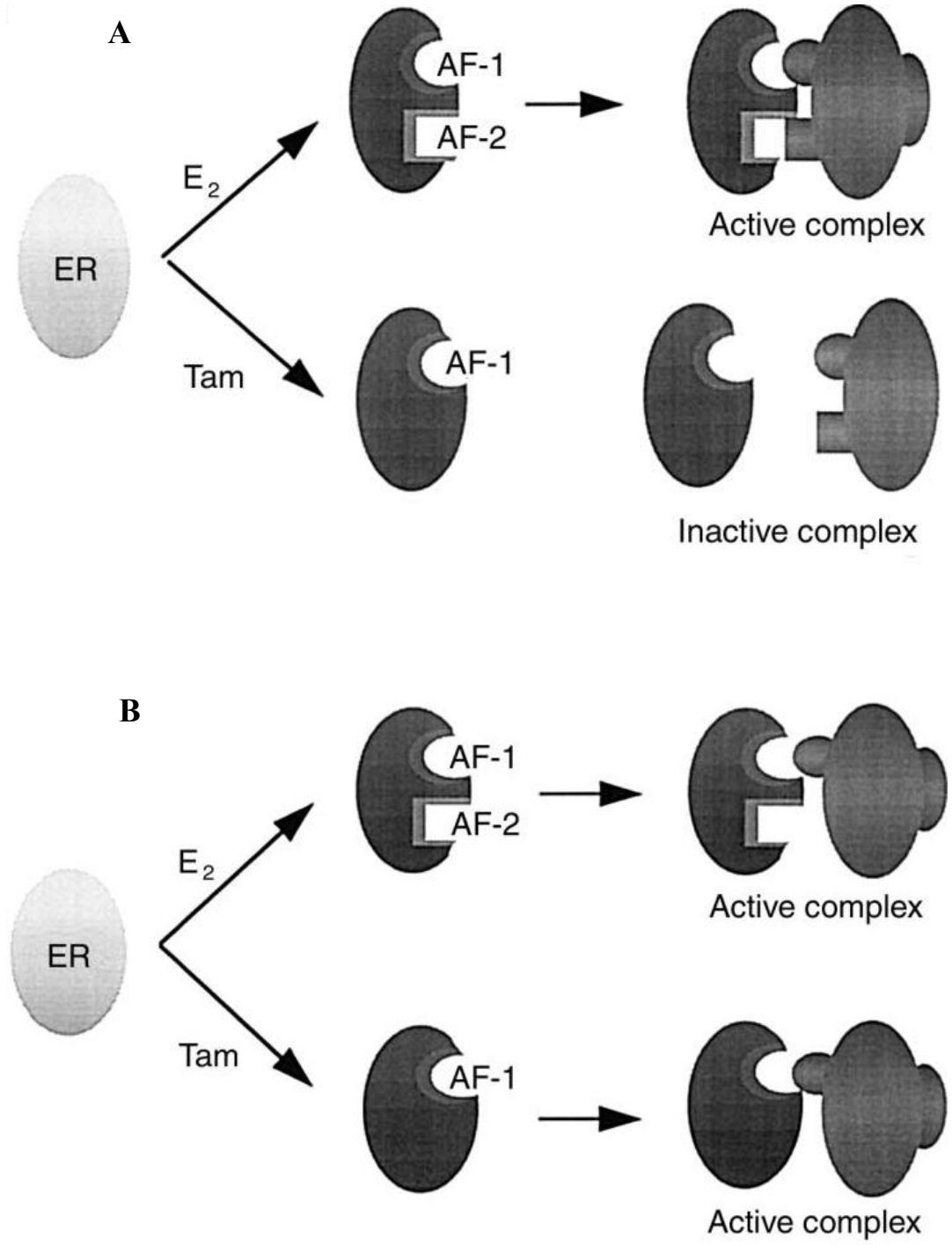
Oestrogens and ER have been implicated in many cancers, but none so clearly as breast cancer. In 1896 it was first suggested that ovariectomy induced regression of metastatic breast cancer [43], and by 1900 it became clear that this surgery had a one-third response rate in metastatic breast cancer [44]. The discovery of oestrogen in ovarian follicular fluid set the stage for the development of compounds that either mimic or inhibit classic oestrogenic responses [45]. Breast cancer cells may be either positive or negative for the ER; most ER positive breast cancer cells require the ER for growth and survival. ER positive breast cancers exhibit stronger clinical responses to hormonal treatment and have a more differentiated morphologic appearance [46-47].

In mammary epithelial cells, E2 signalling through ERα promotes cellular proliferation, which when unchecked can lead to pathologic disease. Even after menopause, when systemic E2 production is largely decreased, adipose tissues continue to make E2 and many breast tumours acquire the ability to locally produce E2 through expression of the aromatase enzyme (CYP19) [48-49]. In addition to promoting cell proliferation, E2/ERα engages the ERK/MAPK and PI3K signalling pathways to increase cell proliferation and survival. As discussed earlier, some metabolites of E2 are DNA damaging agents that may increase DNA mutagenesis, leading to increased probability of cell transformation [49-50].

1.3.3 Breast Cancer pharmacology

Given the implication of E2/ERα signalling in the aetiology of breast cancer, pharmaceutical efforts have uncovered many natural and synthetic modulators of ERα activity and E2 synthesis with a wide range of activities. The Selective ER Modulators (SERMs) are characterized by three important qualities: they bind competitively to ER, they induce a unique conformational change in ER that facilitates differential cofactor interactions, and they exhibit promoter- and tissue-specific activity (Figure 1.10). SERMs allow the ER to adopt a structure which is intermediate between that observed following the binding of agonist or antagonist, which may or may not lead to gene transcription.

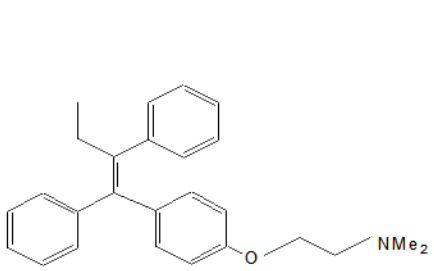
Figure 1.11 ER activation complex showing the selective nature of Tamoxifen in different cells.



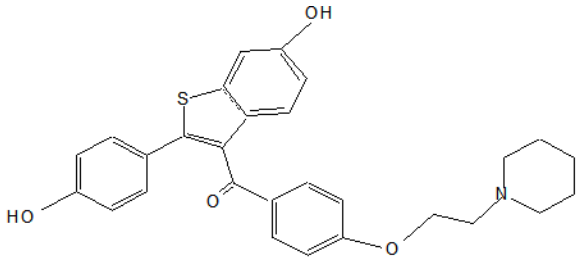
The activities of AF-1 and AF-2 are manifested in a cell-specific manner. (**A**) In some cells the interaction of both ER-AF domains with a cellular cofactor is required for transcriptional activity. In these cell contexts, estradiol (E2), but not tamoxifen, functions as an agonist. (**B**) In other cell contexts, ER-AF-1 alone is required for ER transcriptional activity. Therefore Tam is an AF-1 agonist. Reprinted with permission from [51].

Tamoxifen (TAM) and raloxifene (RAL) are SERMs that have clinical utility in breast cancer (Figure 1.12), and the tissue-specific activity profiles of these compounds as compared to E2 are shown in Table 1.1. In the context of the breast, both TAM and RAL are considered ER antagonists based on their ability to block coactivator recruitment to ER and thus transcriptional activation, yet they have different biological consequences as assessed by their individual abilities to decrease the risk for either invasive or non-invasive breast cancer. Whereas TAM decreases the risk for both invasive and non-invasive breast cancer, RAL only decreases the risk for invasive breast cancer, suggesting perhaps that differences exist in their mechanism of action in the breast [52]. Raloxifene has been shown to decrease the risk of invasive breast cancer by 76% during 3 years of treatment. This was attributable to a 90% reduction in the risk of ER-positive breast cancer, while there was no apparent decrease in the risk of ER-negative breast cancer [53]. A distinction between TAM and RAL is highlighted in the uterus, wherein TAM is an ER agonist and thereby increases the risk for endometrial/uterine cancer and endometriosis, but RAL is an ER antagonist [54]. Therefore SERMs display tissue-selective pharmacology, acting as an agonist in some tissues and antagonists in other tissues. Also E2, TAM, or ICI (ICI 182,780) bound ERα will all activate transcription through AP-1 whereas only TAM or ICI bound ERβ activates transcription through Ap-1 [55]. This reveals a potential control mechanism for transcriptional regulation of E2-responsive genes and also adds a layer of complexity to the differential pharmacology of TAM

Figure 1.12 Structures of Tamoxifen and Raloxifene



Tamoxifen



Raloxifene

Reprinted with permission from [51].

Table 1.1: The relative agonist activities of SERMs

	Breast	Uterus	Bone	Cardiovascular System	Brain / Vasomotor
Estradiol	++	++	++	++	++
Tamoxifen	-	+	+	+	-
Raloxifene	-	-	+	+	-

The relative activities of E2, Tamoxifen and Raloxifene in various cell types. [52-53]

The active form of Tamoxifen, 4-Hydroxytamoxifen (4OHT), has been shown to be exhibit 30 to 100-fold higher potent anti-oestrogenic activity than that of Tamoxifen [56]. For this reason Tamoxifen is often referred to as a pro-drug that requires conversion to its hydroxylated metabolite to exert its activity. The secondary metabolite of 4OHT, Endoxifen (4-hydroxy-N-desmethyl-tamoxifen), is as potent as 4OHT with respect to ER binding and inhibition of E2 induced cell proliferation. Endoxifen is formed by the cytochrome P450 2D6 (CYP2D6) and has been shown to be present in a 6 fold higher concentration than 4OHT in patients receiving Tamoxifen. Therefore this may suggest that Endoxifen could be a more important contributor to Tamoxifen activity than 4OHT [57].

Bazedoxifene is a SERM which is currently under development by Wyeth Pharmaceuticals for the prevention and treatment of osteoporosis. Bazedoxifene is shown to transactivate the ER, and positively affect the skeletal and lipid profile without stimulating the uterine endometrium, causing breast cancer proliferation, or negatively impacting the central nervous system [58]. Lasofoxifene is a nonsteroidal SERM which is currently under development by Pfizer for the prevention and treatment of osteoporosis and vaginal atrophy. Lasofoxifene binds ERα and ERβ selectively (100 fold selectivity against all other NRs) and with high affinity [59].

Another class of ER ligands that has been developed clinically is the selective ER down-regulators (SERDs), which include ICI and GW5638. SERDs, similar to SERMs, bind competitively to ER, but have the additional activity of inducing rapid re-compartmentalisation and degradation of ERα protein. This activity contributes to the efficacy of SERDs such as ICI as a second-line therapy for patients who have failed at least one endocrine therapy, such as in Tamoxifen-resistant breast cancer. GW5638 has been shown to function as oestrogen in skeletal and cardiovascular systems while opposing the actions of oestrogen in the breast and uterus. Importantly, it also inhibits the growth of tumours that are resistant to (or stimulated by) Tamoxifen.

Besides inhibiting ER signalling at the level of ligand binding, another way to impact ER signalling is to reduce the concentration of its agonist, E2. As mentioned above, E2 synthesis from testosterone requires the aromatase enzyme, which also catalyzes the conversion of androstenedione to E1. Thus, aromatase inhibitors (AIs), such as anastrozole, exemestane, and letrozole, have been developed [60]. However, only 40 – 50 % of patients respond to AIs, suggesting *de novo* or acquired resistance mechanisms of circumventing this particular block in E2 synthesis. One hypothesis is that breast tissue expresses high levels of steroid-sulfatase expression, which converts

E1-3-sulfate into the un-sulfaconjugated form, whereby subsequent action of 17β-HSD creates E2 [61]. Other rationales maintain that perhaps not all ER-positive breast cancers rely on E2 and may instead rely on other endogenous oestrogenic compounds [62], or that distinct molecules and signalling pathways, such as the epidermal growth factor receptor (EGFR)/MAPK pathway [63], compensate in the face of an AI to continue to induce cell proliferation and survival.

The addition of targeted therapies to traditional chemotherapeutic regimens has provided significant benefit with relatively mild side effects. For example, treatment of early-stage ERα positive breast cancer with Tamoxifen, results in a 30% reduction in annual mortality. Use of an aromatase inhibitor, such as Letrazole, shows comparable benefit with an improved side effect profile; the most serious common side effect, cardiovascular events, occurs in less than 1% of patients. The success of anti-oestrogen therapies in the treatment of breast cancer highlights the importance of E2 and ERα in the development and progression of this disease, but both *de novo* and acquired resistance suggest that there is still much to learn. A more thorough understanding of the signalling pathways and how they interact and intersect will allow for the development of superior targeted therapies as well as better biomarkers that predict risks and likelihood of response to a given therapeutic.

1.3.4 Breast cancer cell lines as in vitro models

The study of breast cancer cell lines powerfully informs both our molecular understanding of this disease and the development of novel therapeutic agents. Current research aims to closely analyse tumour heterogeneity and tumour-stromal interactions in *in vitro* models of cancer. The majority of the breast cancer cell lines were derived from metastatic tumours as the highly malignant nature of these cells facilitates their long-term proliferation. Three cell lines that were developed in the 1970's (MCF-7, T-

47D and MDA-MB-231) were used in the vast majority of published breast cancer studies. However there are some disadvantages in using cell lines as these are prone to genotypic and phenotypic drift during their continual culture. One study highlighted many discrepancies in MCF-7 cells obtained from different laboratories, demonstrating variations in cell growth rate, hormone receptor content, karyotype and clonogenicity [64]. Another pitfall of using these breast cancer cell lines is that most of these cells are derived from tumour metastasis rather than the primary legion. Thus research based on such cell lines will be biased towards more rapidly progressive types of breast cancer and to late stage disease rather than lower grade and earlier stage breast cancer. An alternative to using cell lines is to prepare primary cultures derived directly from a breast tumour which has the advantage of being able to compare the characteristics of the culture with those of the original tumour. However primary cultures have slow population doubling times and a finite lifespan also these cells may behave differently in culture compared to their response when they are part of a tissue/organ. Because of the ease of use of cell lines they remain the model of choice for breast cancer research, however it is important to understand their limitations and take these into consideration when designing experiments and interpreting results.

The general attributes of breast cancer cell lines (such as ER positive or negative) necessarily informs the choice of a particular cell line in the experiment as well as the interpretation of experimental results [65]. MCF-7 cells are used in the majority of the experiments in this study as these cells express ERα but do not express ERβ.

1.4 Tissue Transglutaminase

1.4.1 Physiological Functions

Tissue or type 2 transglutaminase (TG2,1 EC 2.3.2.13) is a ubiquitously expressed multifunctional enzyme belonging to the transglutaminase family which is coded by the *TGM2* gene [66]. Tissue Transglutaminase protein (referred to as TG2) is a 687 amino acid protein and 77329 Da in size. Its primary function is catalyzing the Ca^{2+} -dependent acyl transfer reactions between carboxamide groups of glutamine residues and amino groups in lysine residues in peptides, forming cross-links in proteins. Transglutaminases are expressed in a variety of tissues and differ in their pattern of expression, substrate specificity and their physiological regulation. Some members of the transglutaminases such as Factor XIIIa, which is involved in blood clotting, are secreted from the cell and are involved in cross-linking of plasma proteins. Other transglutaminases, such as keratinocyte transglutaminases, are intracellular enzymes which play a major role in cross-linking proteins in the differentiation and cornification of skin cells [67]. Tissue transglutaminase (TG2) is an intracellular transglutaminase which may be secreted from the cell where it is involved in processing the matrix that occurs during osteogenesis, wound healing and other remodelling processes [68]. However the enzyme's method of secretion is not fully understood. Like Factor XIII and several other extracellular proteins, tissue transglutaminase does not contain a leader peptide or obvious secretory signal and it remains to be determined how the enzyme becomes deposited in the extracellular compartment. One study has shown that increased expression and activation of TG2 leads to increased externalisation of the protein, whereas inactive TG2 was not externalised. This study also showed that exposure of the active site is key in determining enzyme secretion [69].

Evidence also exists to show that TG2 has a role in apoptosis, where both high levels of the protein synthesis and enzymatic activity are observed. Activation of TG2 crosslinking activity during both physiological and pathological apoptosis leads to assembly of an intracellular protein scaffold contributing to the stabilization of dying cells, before their clearance by phagocytosis. TG2 therefore stabilizes the integrity of the apoptotic cells, preventing the release of harmful intracellular components into the extracellular space. In non-apoptotic cells the binding of GTP prevents the activation of TG2, allowing the cell to survive in the presence of high TG2 protein levels [70]. During apoptosis the drop in intracellular GTP and an increase in Ca^{2+} lead to the activation of TG2. Cell surface-bound Tissue Transglutaminase is also thought to be involved in cell adhesion and cell migration processes by a mechanism independent of its cross linking activity [71].

1.4.2 Oestrogen Receptor control of *TGM2* expression

It has been previously shown that E2 activates the *TGM2* gene in cells which contain either ERα or ERβ. This ER-induced activation is inhibited by ICI 182780 [72]. It is thought that the ER activates *TGM2* through an ERE located in its promoter. The proximal *TGM2* promoter contains an 8/10 match with the canonical ERE (GCGGTCAAGG CTACCTG) (Figure 1.13).

As discussed earlier the ER may also mediate gene transcription from an AP1 enhancer element that requires ligand and the AP1 transcription factors Fos and Jun for transcriptional activation. The *TGM2* promoter contains a 6 out of 7 match to the canonical AP-1 site (TGAGCA) (Figure 1.13).

Figure 1.13 *TGM2* 5' flanking region with putative ERE and AP-1 sites

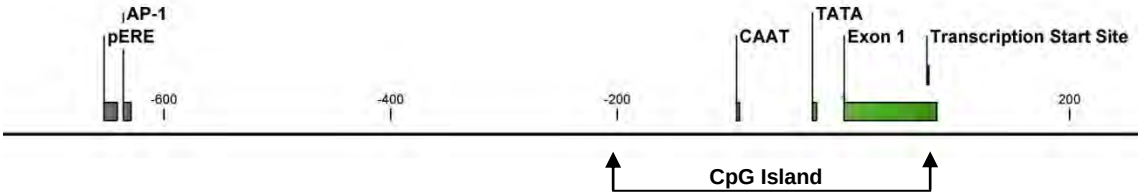


Diagram of TGM2 5' flanking region showing putative ER binding sites, CpG methylation site, translation start site and transcription start site.

The proximal promoter of this gene includes two CpG-rich regions, well recognised sites of DNA methylation. These sites are concentrated in two clusters of CpG dinucleotides; one is located in the core promoter (nucleotides -205 to +75) (Figure 1.13) and the other is located approximately 1.3kb upstream. One study has shown that methylation of the proximal CpG region results in a marked decrease in transcriptional activity while demethylation increases transcription [73]. This study describes a mechanism for the negative regulatory control of *TGM2* by DNA methylation. Release from the inhibition of this regulatory mechanism may play an important role in controlling the level of transglutaminase expression in a tissue-specific manner.

1.4.3 Role of TG2 as a tumour suppressor

The growth and development of new tissue in physiological and pathological conditions relies on the generation of new blood supply. The control of inappropriate angiogenesis in diseases such as cancer has been a keen area of research, leading to the development of novel anti-angiogenic agents. While some of the functions of TG2 protein are similar to those required for angiogenesis, the role of TG2 in this process is poorly understood. It has been shown that TG2 is down-regulated in epithelial cells undergoing capillary angiogenesis; also *TGM2* gene knock-out mice show no vascular abnormalities [74]. These findings fit in with the proposed role of TG2 in extracellular

matrix stabilisation, since angiogenesis requires local destabilisation of the matrix. One study has shown that application of exogenous TG2 blocks angiogenesis in a dose-dependent manner without causing cell death. The same study also shows that intratumour injections of TG2 inhibited tumour growth and increased survival in mice bearing carcinoma tumours. Also tumours grafted into *TGM2* knockout mice ($TGM2^{-/-}$) showed a significant increase in tumour growth compared to $TGM2^{+/+}$ mice [75].

1.4.4 TG2 in chemotherapy resistant breast cancer

Chemotherapeutic drugs such as Doxorubicin are used in the treatment of breast cancer. This drug is known to intercalate DNA thus preventing transcription; it also inhibits the topoisomerase type II enzyme stopping the process of replication. Resistance to chemotherapy is a major obstacle to successful treatment of breast cancer. Although chemotherapy drugs are outside the focus of this study, the implications of TG2 protein in drug resistance is relevant. A direct link has been established between the development of drug resistance and metastatic phenotypes in breast cancer and increased expression of TG2. One study demonstrated that TG2 protein expression is up-regulated in drug-resistant and metastatic breast cancer cells, and it could serve as a prognostic marker for the development of these phenotypes [76].

As discussed earlier, many reports have supported the involvement of TG2 in apoptosis; its overexpression primed cells for apoptosis whereas its inhibition by antisense RNA rendered the cells resistant to induction of apoptosis [77]. Contrary to this, recent evidence indicates that increased expression of TG2 may prolong cell survival by preventing apoptosis [78]. It has been proposed that pro-apoptotic and anti-apoptotic effects of TG2 strongly depend on its location within the cell. One study has shown that cytosolic TG2 is pro-apoptotic while nuclear TG2 diminishes apoptosis [79].

It has been shown that TG2 protein levels in drug-resistant tumour cells is about 10 times higher than that in drug-sensitive tumour cells [80]. However it is thought that these cells are to be able to survive in the presence of high TG2 due to deficient intracellular calcium levels [81].

1.4.5 Structure of *TGM2* gene

The *TGM2* gene is composed of 13 exons covering a size of 35kb on the long arm of chromosome 20 (20q12) which is expressed as a 3.6kb mRNA in human endothelial cells (Figure 1.14) [82].

Figure 1.14 Layout of exons in *TGM2*

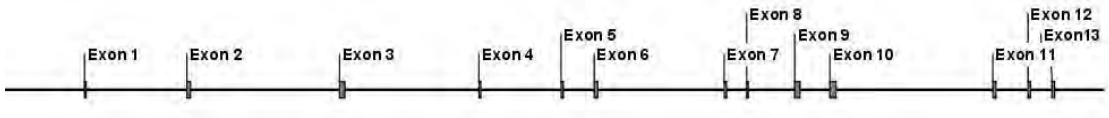


Diagram of *TGM2* exons drawn to scale.

2 Aims

The aim of this study was to investigate the differential promoter-specific pharmacology of Tamoxifen (TAM) in the context of breast cancer cells using *TGM2* as a model. We analysed the effects of SERMs which are structurally and functionally related to TAM. We also aimed to compare the inhibitory activities of TAM on a group of ER regulated genes in various breast cancer cell types. To this end we cloned the sequence upstream of the *TGM2* gene promoter containing the putative nuclear receptor response elements and insert it into a luciferase plasmid for use in transient transfection assays.

Specifically we aimed to:

- Study effects of Tamoxifen and Endoxifen on *TGM2* mRNA expression
- Analyse the requirements of ER or other cellular factors in *TGM2* activation
- Search the *TGM2* 5' flanking region for possible ER binding sites
- Clone a sequence from this 5' region into a luciferase vector
- Determine the degree of transcriptional regulation of the isolated *TGM2* promoter region by ER and E2/TAM

3 Materials and Methods

3.1 Materials

3.1.1 Materials used for cell culture

- Cell culture dishes (100 x 20mm): BD Falcon (Franklin Lakes, NJ) (cat. 353003)
- Cell culture plates (12 well): Corning Incorporated (Corning, NY) (cat. T-2989-6)
- Dulbecco's Modified Eagle Medium (DMEM): Invitrogen (Carlsbad, CA), Nutrient Mixture F-12 Ham 1X (DMEM/F12 1:1) (cat. 11330) Lot number: 488586, supplemented with 8% charcoal stripped fetal calf serum, 0.1 mmol/L nonessential amino acids and 1 mmol/L NaPyr.
- DMEM (Phenol red free): Invitrogen Gibco, Nutrient Mixture F-12 Ham 1X (DMEM/F12 1:1) (cat. 21041) Lot number: 495171, supplemented with 8% charcoal stripped fetal calf serum, 0.1 mmol/L nonessential amino acids and 1 mmol/L NaPyr.
- RPMI 1640 (Invitrogen Gibco): supplemented with 8% fetal bovine serum (FBS) (Hyclone Laboratories), 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids (Invitrogen) (cat.10-040-CV) Lot number: 10040424
- RPMI 1640, Phenol red free (Invitrogen Gibco): supplemented with 8% fetal bovine serum (FBS) (Hyclone Laboratories), 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids (Invitrogen) (cat. 11835) Lot number: 492502
- Minimum Essential Media (MEM): Invitrogen Gibco supplemented with 8% fetal bovine serum (FBS) (Hyclone Laboratories), 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids (Invitrogen) (cat.11095) Lot number: 539196
- MEM (phenol red free): Invitrogen Gibco supplemented with 8% fetal bovine serum (FBS) (Hyclone Laboratories), 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids (Invitrogen)

- Oestradiol (E2): obtained from Sigma Aldrich (St. Louis, MO)
- Trypsin: Sigma– (0.25% Trypsin – EDTA solution) Lot number: 8J0422
- 100% Ethanol: Pharmaco-AAPER (Brookfield, CT)
- Dimethyl-sulphoxide (DMSO): Sigma Lot number: 25K2409
- Tamoxifen (TAM), 4-hydroxytamoxifen (4OHT) obtained from Sigma Aldrich
- ICI 182,780 (ICI) obtained from Tocris Bioscience (Ellisville, MI)
- Endoxifen obtained from Ross Weatherman, Purdue University
- Bazedoxifene, Lasofoxifene and Raloxifene obtained from Wyeth (Madison, NJ) as a donation to research.
- α-Napthoflavine, β-Napthoflavine obtained from Sigma Aldrich
- All Trans Retinoic Acid (ATRA) obtained from Sigma Aldrich (cat. R2625)
- Interleukin – 6 (Il-6) obtained from Sigma Aldrich (cat. I3268)
- 50ml tubes: Greiner Bio-One (Monroe, NC) (cat. 227261) Lot number: 08420195
- TE buffer: (10mM Tris & 1mM EDTA)
- PBS: Cellgro (Manassas, VA) Dulbeccos Phosphate Buffered Saline (cat. 21-031-CV) Lot number: 21031303
- Cells: MCF-7, MDA 231, BT483, HeLa and Skbr-3 obtained from American Type Culture Collection (ATCC, Manassas, VA)

- Cell lines used in this study, all of which are epithelial tumour cells, are shown below:

Table 3.1 Human cell line used in current study

Cell line	ATCC No	Attributes
MCF-7	HTB-22	ER positive, breast
MDA-MB-231	HTB-26	ER negative, breast
BT483	HTB-121	ER positive, breast
Skbr-3	HTB-30	ER negative, breast
HeLa	CCL-2	ER negative, cervical

- pRST7-ERα expression plasmid (Accession no. X03635)

3.1.2 Materials used for RNA isolation

- 2-Mercaptoethanol: Sigma Lot number: M3148
- DEPC water: Omnipur Lot number: 1108B425
- RNA isolation kit: Bio-Rad (Hercules, CA) Aurum Total RNA Minikit (cat. 732-6820) Lot number: 7326820

3.1.3 Materials used for Reverse Transcriptase (RT) PCR

- Isolated RNA
- Spectrophotometer: Beckman (Fullerton, CA) DU640
- PCR Tubes and Strip Caps (8 Strip): Phenix (Candler, NC) (cat. MPX-445) Lot number: 080930-800
- cDNA Synthesis Kit: Bio-Rad iScript (cat.170-8890) Lot number: 92079967
- Bio-Rad iCycler

3.1.4 Materials used for Quantitative PCR

- cDNA samples
- qPCR plates: (96 well) Bio-Rad
- Bio-Rad Microseal „B' Film (cat. MSB1001)
- Sybr Green supermix: Qiagen (Valencia, CA)
- Bio-Rad iCycler optical system with associated software

Table 3.2 qPCR primers used for qualitative analysis of mRNA

	Forward (5'-3')	Reverse (5'-3')
36B4	GGACATGTTGCTGGCCAATAA	GGGCCCGAGACCACTGTT
TGM2	TCACCTTCAGTGTCGTGACC	TCCTCTTCCGAGTCCAGGTA
RET	GGATTAAAGCTGGCTATGGCA	TGGCTTGTGGGCAAACTTGTG
pS2	TCCCCTGGTGCTCTATCCTAATAC	GCAGTCAATCTGTGTTGTGAGCC
WISP2	CCTACACACACAGCCTATATC	CCTTCTCTTCATCCTACCC
CYP1A1	TGCAGAAGATGGTCAAGGAG	AGCTCCAAAGAGGTCCAAGA

36B4 qPCR primers span exons 4 & 5, *TGM2* primers span exons 2, 3 & 4, *RET* primers span exons 9 & 10, *pS2* primers span exons 2 and 3, *CYP1A1* primers span exons 2 & 3.

3.1.5 Materials used for PCR of genomic DNA

- DNA isolation: Qiagen DNeasy kit (cat. 69564) Lot number: 42151724
- MCF-7 cells
- Isolated DNA
- Cloned Pfu Turbo: Stratagene (La Jolla, CA) (cat. 600153) Lot number: 0006037068
- Pfu buffer (10X): Stratagene (cat. 600153.82) Lot number: 0650189
- PCR grade water

- GC-rich PCR: Clontech (Mountain View, CA) Advantage GC kit (cat. 639114) Lot number: 8051025
- dNTPs (50X)
- Agarose: Omnipur lot. 0265B068
- TAE buffer
- Electrophoresis chamber
- Ethidium Bromide solution: Bio-Rad– (10mg/ml) (cat. 161-0433)
- DNA ladder (1kb): Bio-Rad (cat. 170-8204)) Lot number: L1708204
- DNA ladder (100bp) GIBCO BRL (cat. 15628-019) Lot number: 1082294
- Primers obtained from Sigma Aldrich (designed with a 5' XhoI binding site) as shown in Table 3.3

Table 3.3 Sequences of primers targeting regions of *TGM2* gene

Forward-1645	5'-ATCGCTCGAG GAGCAGTTTCTGCAACAATC-3'
Forward -900	5'-ATCGCTCGAG GTCTGTTTTTGCAGGTGTGT-3'
Forward -561	5'-ATCGCTCGAG GCCAGCCGTGTTTGGTG-3'
Reverse +50	5'-ATCGCTCGAG CCACTGGCGGCGAGAC-3'
Reverse -305	5'-ATCGCTCGAG CCCTCATAGAAACACACAACG-3'
Reverse -540	5'-ATCGCTCGAG CCACAGTTACACCAAACACG-3'

F-690	5'-ATCGCTCGAG TCTGGACACCTGCTCATCTG-3'
R-480	5'-ATCGCTCGAG GCAGACACACCAAGACAGGG-3'
F-1200	5'-ATCGCTCGAG GGCTGCTATGGTCATTTCC-3'
R-exon9	5'-ATCGCTCGAG GTCCCTGGCAGAGGTAGAAA-3'
F+exon9	5'-ATCGCTCGAG GGGTATGTGCCCTCAGAGTCTT-3'
R+700	5'-ATCGCTCGAG GGGCTAAGGCTGTGTTTTACT-3'
F-UBS	5'-ATCGCTCGAG GGCTTTGAGATTGGACTGC-3'
R-UBS	5'-ATCGCTCGAG GGCCTGGACTTAGTTCATTACA-3'

F_UBS-560	5'-AACCCTCGAG GTGGTAGGAACAAAGCCATTG-3'
R_UBS+550	5'-AACCCTCGAG CACCAGCTCCATTCTGAACA-3'
F_ERE-1120	5'-GGAACTCGAG CTGTGAGGGAGGGATTCTTT-3'
R_ERE+440	5'-GGAACTCGAG CGGACAGGGACACACAACTA-3'

All primers have an XhoI binding site motif (CTCGAG) (in red) with a 4 bp spacer (in blue) tagged to the 5' end. Primers are named by the distance in bp to their relevant putative ER binding region; either within the 5'binding region, 3kb upstream or within exon 9. (F, Forward; R, Reverse; UBS, upstream binding sequence)

3.1.6 Materials used for cloning of DNA fragments

- PCR purification kit: GeneScript (Piscataway, NJ) (cat. L00198) Lot number: PP00107
- DNA extraction kit: GeneScript gel (cat. L00199) Lot number: GE00107
- XhoI restriction enzyme: New England Biolabs (Ipswich, MA) (cat. R0146S) Lot number: 54
- HindIII restriction enzyme: New England Biolabs (cat. R014S) Lot number: 66
- BglII restriction enzyme: New England Biolabs (cat. R0144S) Lot number: 40
- KpnI restriction enzyme: New England Biolabs (cat. R0142S) Lot number: 51
- SacI restriction enzyme: New England Biolabs (cat. R0156S) Lot number: 49
- RE buffer #1: New England Biolabs (cat. B7001S) Lot number: 1007
- RE buffer #2: New England Biolabs (cat. B7002S) Lot number: 03064
- RE buffer #3: New England Biolabs (cat. B7003S) Lot number: 1107A
- RE buffer #4: New England Biolabs (cat. B7004S) Lot number: 0030805
- pGL4.26 vector (luc2/minP/Hygro): Promega (Madison, WI) (cat. E8441)
- Shrimp Alkaline Phosphatase (SAP)
- SAP buffer
- T4 DNA ligase: New England Biolabs (cat. M0202L) Lot number: 1910801
- T4 DNA ligase buffer: New England Biolabs (cat. B0203S) Lot number: 0303
- SOC media: Invitrogen (cat. 460821) Lot number: 470041
- LB broth
- DH5α cells
- XL2-Blue Ultracompetent cells: Stratagene (cat.200150) Lot number: 0006039741
- Miniprep kit: GeneScript (cat. L00193) lot. MM00107

- Plasmid Maxiprep kit: Qiagen Qiafilter (10) (cat. 12262) Lot number: ANL1012/BRL023/L02/133

3.1.7 Materials used for transfections

- Opti-MEM Reduced Serum Media (cat.11058) Lot number: 544027
- Lipofectin Transfection Reagent: Invitrogen (cat. 18324-020)
- Luciferase substrate: Lucferin
- β-gal substrate: chlorophenol red β-D-galactopyranoside (CPRG) Oz Biosciences (cat: GC10002)
- Dithiothreitol (DTT) obtained from Sigma Aldrich (D9779)
- Microplate Analyser: Fusion™ Universal (PerkinElmer)
- 96 well plates: Corning (cat.3596)
- 96 well luminometer plates: Optiplate
- Expression vectors:
    - pCMV-βGal
    - pRST7ERα
    - pRST7ERβ
- Reporter constructs:
    - 3xERE-TATA-luc
    - TGM2-luc
- Software programs
    - Microsoft Excel
    - CLC Main Workbench 5
    - Geospiza Finch TV 1.4

3.2 Methods

3.2.1 Mammalian Cell Culture

All cell lines were obtained from American Type Culture Collection (Manassa, VA) and cultured in media from Invitrogen (Carlsbad, CA). HeLa cells were maintained in MEM, MCF7 cells in DMEM/F12 and SKBR3 and BT483 cells in RPMI 1640, each supplemented with 8% charcoal-stripped foetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), 1mM sodium pyruvate, and 0.1mM non-essential amino acids (Invitrogen). All cell lines were propagated in a 37˚C incubator with 5% CO_2.

3.2.2 Hormone/inhibitor treatment assays

One ml of $2x10^5$ cells/ml in phenol red free media was plated in each well in 12 well plates. All ligand stocks were dissolved in ethanol or DMSO before use in cell culture. After 2-day incubation cells were treated with a 10x concentration of hormone or inhibitor diluted in spent media. 100µl of the 10x concentration of the appropriate hormone/inhibitor treatment were added to each well and incubated for 8 hours (unless otherwise stated). E2 was used in 10ng/ml concentration while all pharmacological agents were used in concentrations ranging from 10nM to 1µM. After incubation cells were washed in PBS and lysed with RNA lysis solution (Bio-Rad).

3.2.3 RNA isolation, cDNA preparation and quantitative PCR

Total RNA was isolated from cells using Bio-Rad Aurum Total RNA Mini Kit according to the manufacturer's instructions, which included a DNase step. One µg of RNA was reverse transcribed into cDNA using the Bio-Rad iScript cDNA synthesis kit according to the manufacturer's instructions. In all cases, a cDNA reaction lacking reverse transcriptase was performed to monitor possible genomic DNA contamination. The Bio-Rad iCycler Real-time PCR System was used to amplify and quantitate the levels of target gene cDNA. Quantitative PCR (qPCR) reactions were performed using

8µl of diluted cDNA (1:15), 10uM specific primers and Qiagen SYBR green supermix in a total volume of 13ul. The following cycling protocol was used for all reactions: 1 cycle at 95°C for 5 min, followed by 40 cycles consisting of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. A final cycle at 72°C for 7 min followed. A melt curve analysis from 70-90°C (0.5°C/10 sec increments) was performed for all reactions to detect primer dimers or other transcripts. A single PCR product was detected in all cases. Each cDNA reflecting each biological sample were run in triplicate and expressed as an average +/- SEM.

3.2.4 Plasmids

3.2.4.1 Expression plasmids

The pRST7-ERα plasmid is a CMV - driven expression plasmid containing amino acids 1-595 of the full length human ERα [19]. The pRST7-ERβ plasmid is a CMV driven expression plasmid containing amino acids 1-477 of the full length human ERβ [83]. pCMV-β-Gal (obtained from Clontech, Palo Alto, CA) is a mammalian vector expressing a β-galactosidase gene driven by the human cytomegalovirus immediate early gene promoter.

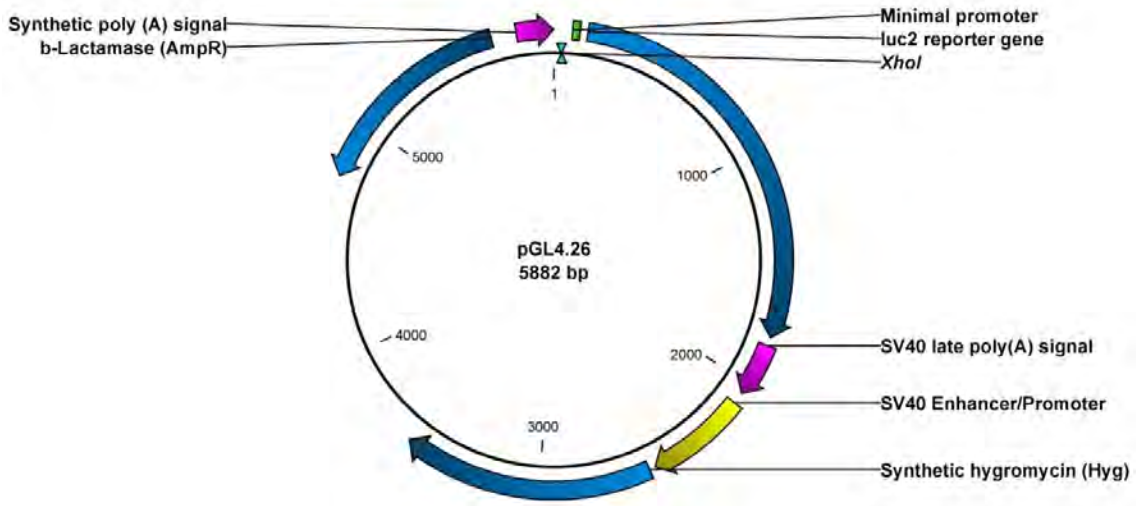
3.2.4.2 Reporter constructs

The pTGM2-luc plasmid (Figure 3.2) was constructed as follows: an empty pGL4.26 plasmid (Figure 3.1) (obtained from Promega) was digested with XhoI and ligated to a 1.7kb fragment (previously digested with XhoI) spanning the *TGM2* regulatory element isolated from gDNA. The sequences of the oligonucleotides were: forward 5'-GGAACTCGAGCTGTGAGGGAGGGATTCTTT-3' and reverse 5-GGAACTCGAGCGGACAGGGACACACAACTA-3'. The following PCR protocol was used: 1 cycle at 95°C for 2 min, followed by 32 cycles consisting of 95°C for 30 sec, 62°C for 40 sec and 72°C for 2 min. A final cycle at 72°C for 5 min followed. The

ligated plasmid was transformed into XL2-Blue ultracompetent cells and grown at 37°C overnight on Ampicillin plates. Colonies were re-cultured in 10mls of LB broth + 200µl Ampicillin (1mg/ml) at 37°C overnight. Plasmid isolation was performed on 1ml of transformed XL2 cells according to the manufacturer's instructions. Isolated plasmids were XhoI digested to identify clones with the insert. The correct orientation was confirmed by analysis of the restriction fragment length using HindIII and BglII. A large plasmid isolation was used to isolate 1ml of purified pTGM2-pGL4.26 plasmid.

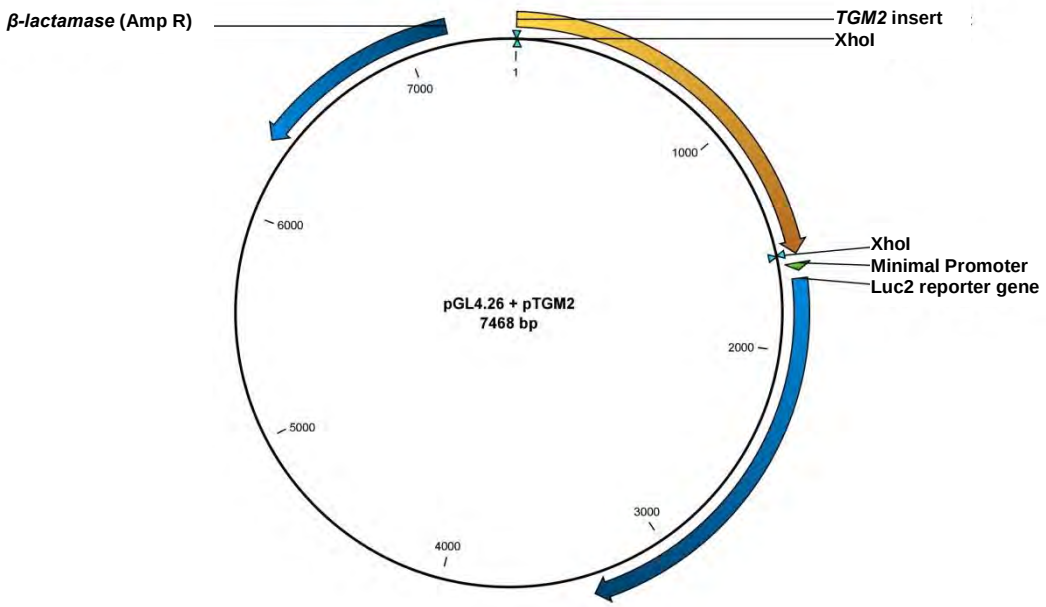
3xERE-TATA-luc is a luciferase reporter vector containing three tandem repeats of the consensus ERE upstream of the human TATA promoter sequence inserted into the multiple cloning site of pGL3-basic vector.

Figure 3.1 pGL4.26 vector, obtained from Promega



Map of pGL4.26 plasmid obtained from Promega, showing β-lactamase gene (Ampicillin resistance), luciferase reporter gene (luc2), multiple cloning region containing XhoI restriction site, minimal promoter (TATA box) and mammalian selectable marker (hygromycin).

Figure 3.2 TGM2-luc plasmid



Map of cloned TGM2-luc plasmid with Ampicillin resistance gene (β-lactamase), luciferase reporter gene (luc2) and the TGM2 insert (5' flanking region)

3.2.5 Mammalian Cell Culture and Transfections

All cells were plated in phenol red free media in 24-well plates 24h prior to transfection. Cells were transfected in OptiMEM I Reduced Serum Medium (Invitrogen) with 3μg of total plasmid per triplicate sample in 24-well, using Lipofectin® according to manufacturer's protocol (Invitrogen). 100ng of the pCMV-ßGal normalization vector was used with 0, 10 or 20ng of ERα expression vector and luciferase reporter constructs making up the remaining. DNA-Lipofectin mix was added and incubated for 4 h. All ligand stocks were dissolved in ethanol or DMSO before use in cell culture, and cells were treated with ligands added to phenol-red-free media. A saturating concentration of ligand was added to the cells 20-24 hr prior to assays: 10nM E2, 1μM 4OHT, 1μM ICI, 100ng/μl IL-6 or 1μM ATRA (All Trans Retinoic Acid). Cells were lysed 24-28hrs after transfection and assayed for luciferase and β-galactosidase activities using a Fusion™ Universal Microplate Analyzer (PerkinElmer). Results are expressed as normalized luciferase activity (NLA, normalized with βgal for transfection efficiency) ± SD per triplicate sample of cells. Statistic analysis was performed on these results using the t-test to compare the difference between two groups of data.

4 Results

4.1 Background

4.1.1 Oestrogen Receptor Signaling in the Breast

Oestrogens function as mitogens in most ER-positive breast cancers. Upon ligand binding, ER undergoes a conformational change that results in dimerisation, DNA binding, recruitment of transcriptional coregulators, and modulation of target gene expression. The implication of oestrogens and ER in breast cancer has led to the pharmaceutical development of SERMs and anti-oestrogens. SERMs exhibit tissue- and promoter-specific agonist and antagonist behaviour. The unique and compound-specific conformational change in ER induced by SERM binding allows different protein-protein interaction surfaces to be exposed, leading to differential recruitment of co-regulatory proteins and thus diverse biological outcomes. The ER regulates a large set of genes in the breast cancer cell. Genes activated by the ER are generally involved in proliferation and cell survival while genes inhibited by ER are generally involved in halting the cell cycle. Therefore SERMs prevent the growth of breast cancer cells by inhibiting ER-regulated genes.

It is not fully known however how a patient on TAM develops a *de novo* or an acquired resistance to the drug. Therefore understanding the biology of ER/TAM control of gene expression is critical to understanding how resistance to TAM may develop. As described in the introduction, TG2 expression has been shown to be up-regulated in drug-resistant and metastatic breast cancer cells and is thought to contribute to TAM resistance [84].

4.2 ER control of TGM2 expression

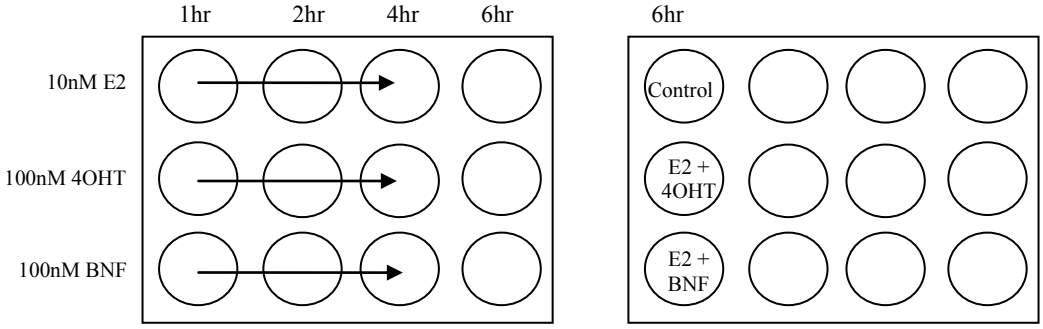
It has been previously shown that E2 activates the *TGM2* gene. As discussed earlier *TGM2* is involved in many processes within the cell. As *TGM2* is an ER-regulated gene, we would expect this gene to be inhibited by TAM.

In this study, we focused on defining the molecular mechanisms underlying TAM inhibition of *TGM2* in the context of breast cancer cells.

4.2.1 Time-course of ligand treatments on *TGM2* expression

Our first objective was to evaluate the activation of *TGM2* by E2 in MCF-7 cells over a 6 hr time-course. The concentrations of each ligand required were examined in the next experiment; however for this experiment we used physiologically relevant ligand concentrations. [85] Also cells were treated with 4OHT and β-Napthoflavone (BNF) over a six hour time-course as these were used in later experiments. BNF is an Aryl Hydrocarbon receptor agonist and is used is this study to investigate the effects of TAM on AhR-controlled genes. This experiment aims to identify the length of incubation required for optimum gene activation and mRNA production. *pS2* (trefoil factor 1), an ER-regulated gene highly expressed in breast cancer cells, was used here as a positive control. Figure 4.1 shows the plate setup for this experiment.

Figure 4.1 Plate setup for MCF-7 cell culture



1ml of 2x10^5 MCF-7 cells/ml were plated in 15 wells of two 12 well plates. Cells were diluted in phenol red free DMEM//F12 media (+8% CFS with NEAA and Na Pyruvate) Cells were incubated for 48hrs to achieve confluency. Dilutions of ligands were made up in spent media and added to the appropriate wells to make to make a final volume of 1ml/well.

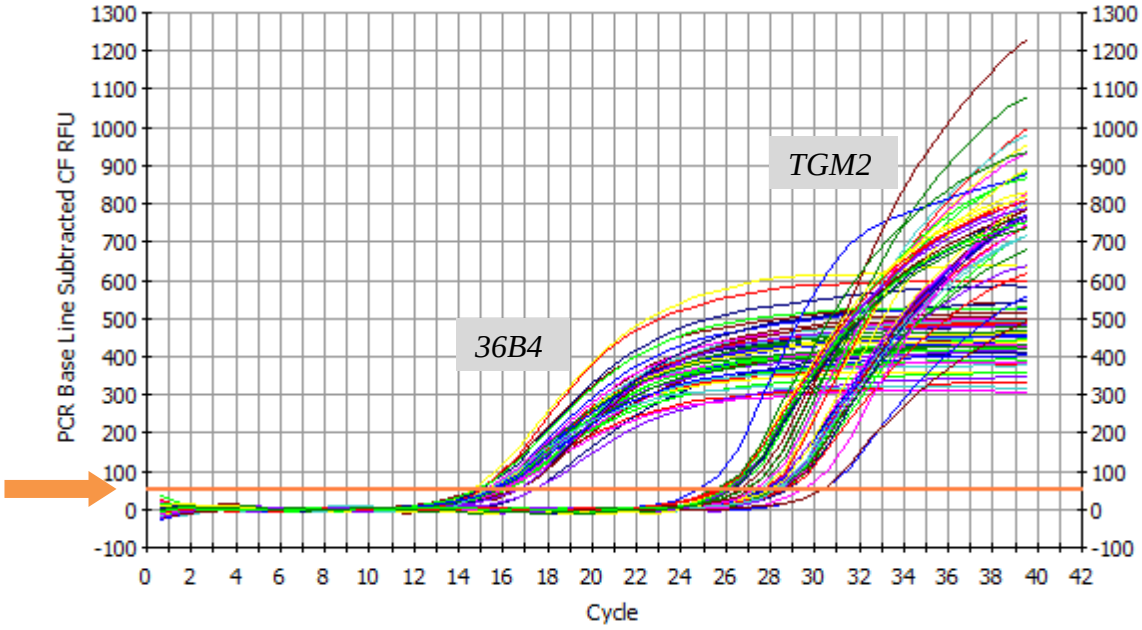
After the incubation period, RNA was isolated from each cell culture well and quantified. Table 7.1 UV quantification of isolated RNA (Raw data) shows the UV quantification values for each of the RNA samples. The volume of isolated RNA

required to give 1µg/well in the iScript Reverse Transcriptase reaction was calculated by dividing 1µg by the concentration of RNA (µg/ml).

Each well in the Reverse Transcriptase (RT) reaction contained 1µl Reverse Transcriptase enzyme (iScript), 1µg of RNA and made up to a final volume of 16µl with RNase-free water (Table 7.2). A no-RT (NRT) control also was set up as a negative control which contained RNA but no RT enzyme. The RT samples were run on a single PCR cycle according to manufacturer's instructions.

The cDNA produced in the RT reaction was subjected to real-time PCR (qPCR) for quantification. In addition to the 15 samples and NRT, a set of standards were set up. This was done by pooling 1µl from each cDNA sample (15µl) and diluting the cDNA in a set of serial dilutions to give 1x, 0.2x, 0.1x and 0.02x concentrations. Each sample was run in triplicate for each of the primers analysed. An example of the qPCR results are presented in Figure 4.2, which shows the data for *36B4* and *TGM2* analysis. *36B4* is a house-keeping gene which is transcribed at a constant rate, regardless of cell treatments. It is analysed as an internal standard for the purpose of normalising qPCR data.

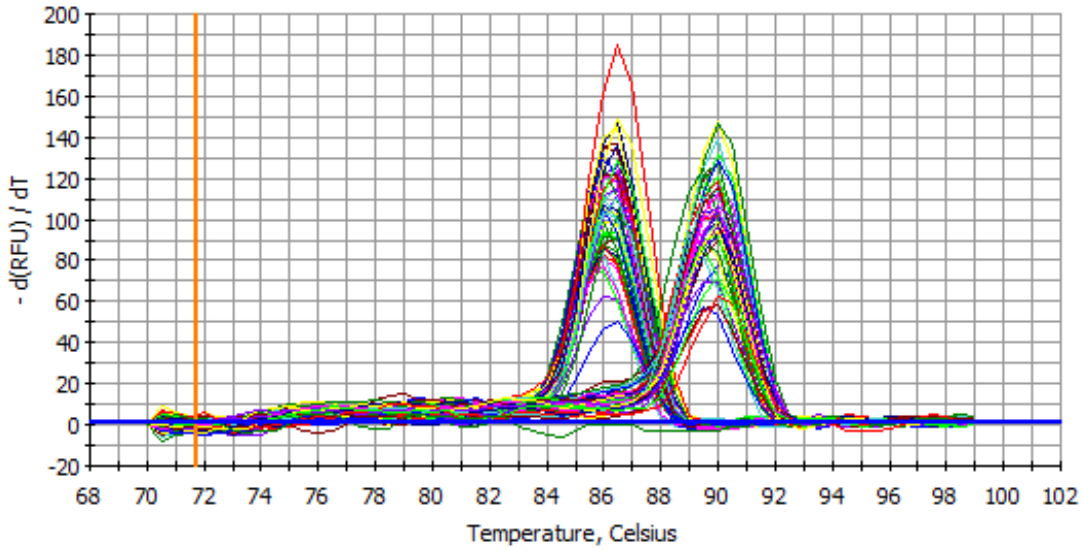
Figure 4.2 PCR quantification curve for *36B4* and *TGM2* primers



PCR cycle number (X-axis) and fluorescence (Y-axis). The graph shows the increasing uptake of Sybr green (fluorescent dye) as it binds amplified dsDNA in each well. Sybr green fluoresces intensely while intercalated in the minor groove of double stranded DNA. The cycle at which the amount of fluorescence exceeds a threshold value (orange line) is known as the C_T (Threshold cycle). The C_T relates to the concentration of cDNA present at the start of the run. The first set of curves to cross the threshold line represents analysis of *36B4*, followed by curves for *TGM2* analysis. The earlier the curve crosses the threshold, the higher the amount of cDNA present at the start of the run.

The first set of data on the graph to cross the threshold value is 36B4, followed by TGM2. This means that 36B4 is in higher concentration than TGM2. The CT values for each sample are exported to a spreadsheet for analysis. For each primer used in the qPCR experiments a melt curve (Figure 4.3) and a standard curve (Figure 4.4) is performed. Upon completion of the PCR reaction a melt curve was performed to test for the presence of primer dimers or contaminating primers. The presence of a single peak on the melt curve for each primer confirms the presence of a single PCR product. The results of this analysis are presented in Figure 4.3.

Figure 4.3 Melt curve for *36B4* and *TGM2* primers



The melt curve shows the temperature dependent dissociation between two DNA strands, in this case the amplified product. The dissociation of the DNA during heating is measureable by the large reduction in fluorescence that results. The graph shows the change in fluorescence (y-axis) vs. temperature (x-axis) for *36B4* and *TGM2* amplified products. The melt curve is useful for determining the specificity of the primers used as primers may induce primer-dimer formation or amplify other non-specific products. The presence of a single peak for each primer indicates that no primer dimer or other non-specific products have been produced.

The results of the melt curve indicate that the PCR reaction has no contamination and that single product was formed. A standard curve is also performed for each qPCR reaction in this study which is used to examine PCR efficiency. The standard curve is presented in Figure 4.4. The graph shows there is a direct correlation ($r = 0.998$) between the relative log concentrations of the samples/standards and the threshold cycle. The unknown sample (in red) is the NRT which has practically no detectable cDNA.

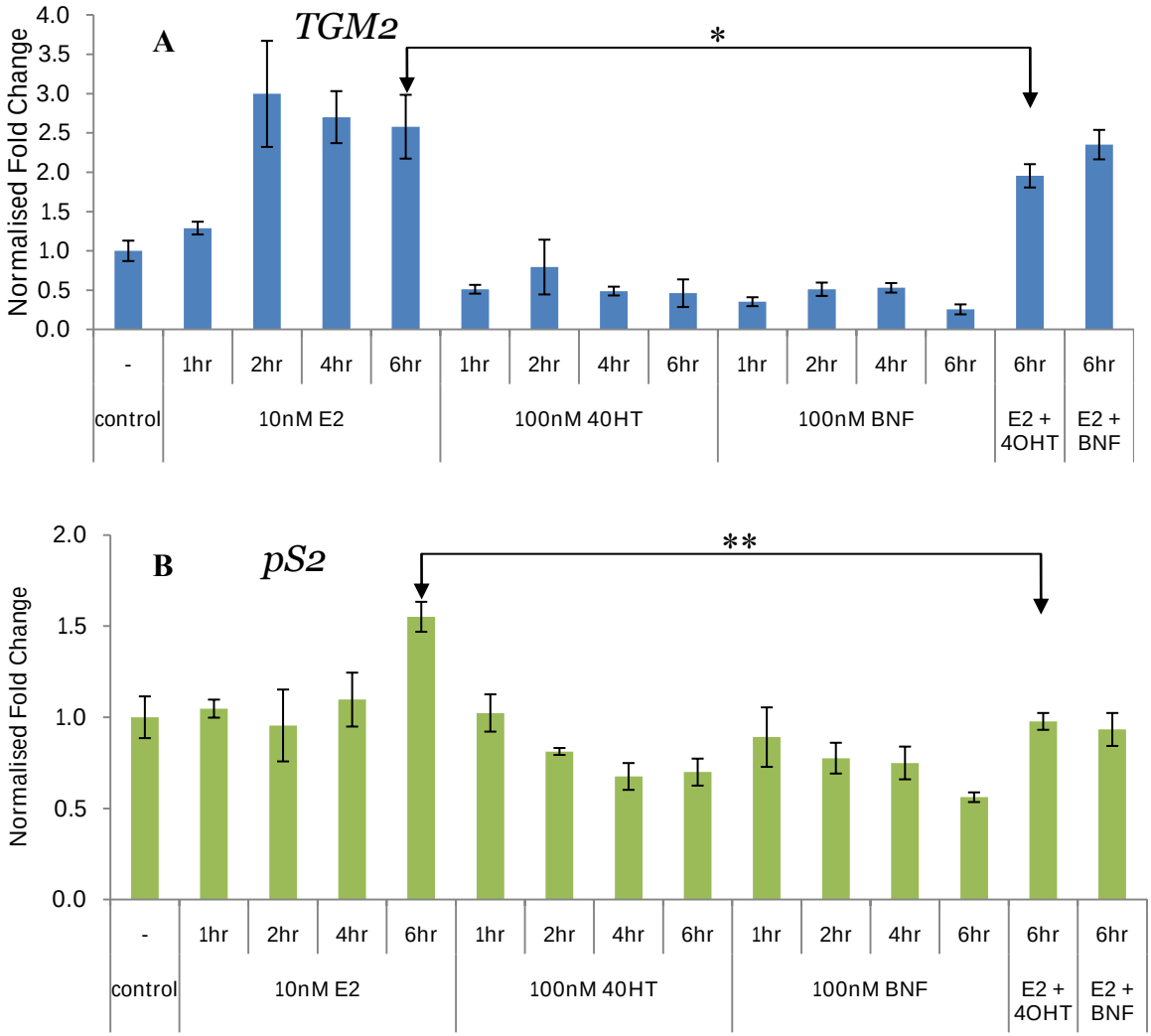
Figure 4.4 qPCR Standard curve for 36B4

The relative starting concentrations of cDNA (x-axis) are graphed against the threshold cycle (y-axis) for each of the standards. The graph shows the standards (in blue) analysed in this experiment. The NRT samples were run as a negative control (in red). The slope of the curve is directly related to the efficiency of the PCR reaction. At a PCR efficiency of 100% the template DNA doubles after each cycle during exponential amplification.

The relative gene expression for each sample was calculated according to the $2^{-\Delta\Delta C_T}$ method using Microsoft Excel [86]. This method normalises the C_T from each sample to its untreated control, which is then normalised again to its respective *36B4* C_T value to give a $\Delta\Delta C_T$ value. *36B4* is used in this study to normalise all other genes as its expression remains unchanged regardless of cell treatments. The fold change in gene induction is given by the formula $2^{-\Delta\Delta C_T}$.

The results for the assay described in Figure 4.1 are presented in Figure 4.5. In this experiment the cells were treated with no ligand (control), E2, 4OHT, BNF, or combinations of the ligands prior to RNA isolation, RT-PCR and qPCR for *36B4*, *TGM2* and *pS2*.

Figure 4.5 Time-course of gene expression in MCF-7 cells



MCF-7 cells (2x10^5 /well) were treated with vehicle (control), 10nM E2, 100nM 4OHT, 100nM BNF for 1, 2, 4 or 6hr intervals. Cells were harvested and cDNA prepared from isolated RNA was subjected to qPCR analysis. qPCR was performed to analyze *TGM2*, *pS2* and *36B4* gene expression. Data are normalized to the *36B4* gene and expressed as mean fold change over vehicle ± SEM for triplicate amplification reactions. All qPCR data in this study was calculated using the $2\text{-}\Delta\Delta C_T$ method to produce a normalised fold change [86]. Asterisks: unpaired two-tailed T-test, * *p value* > 0.05 (no significant difference), ** *p value* < 0.01.

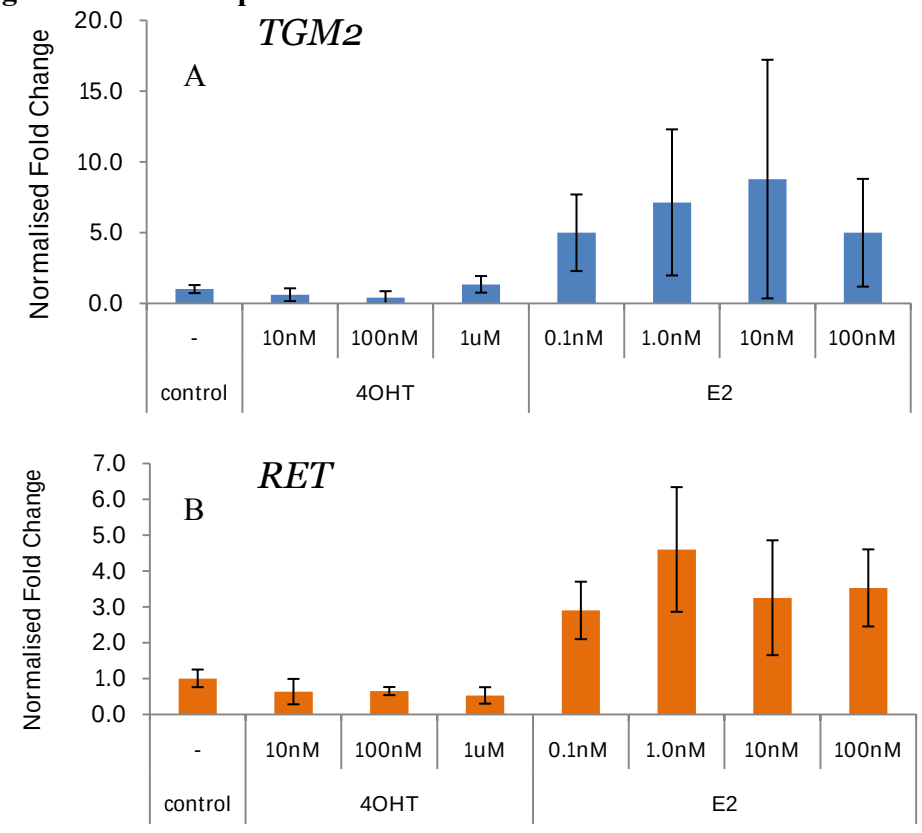
The qPCR results show that incubating the cells with ligand for 6 hrs after ligand treatment is sufficient for *TGM2* (**A**) and *pS2* (**B**) mRNA up-regulation. BNF or 4OHT do not activate transcription of these genes. Treatment of E2 in combination with 4OHT inhibits E2 induced *pS2* mRNA expression but not *TGM2*. Statistical analysis using the Students t-test shows no significant difference ($p<0.01$) between *TGM2*

mRNA expression in either E2-treated or E2/4OHT co-treated cells. This finding suggests that 4OHT is unable to inhibit E2-induced *TGM2* mRNA expression. The next objective was to identify the concentration of ligands that are required for optimum gene transactivation.

4.2.2 To determine optimum ligand concentrations for *TGM2* mRNA expression

Determining the required concentration of ligands for activation of gene transcription is also required for future pharmacological experiments. RET (RET proto-oncogene) is used here as a positive control (ER-regulated gene). All qPCR experiments were performed as outlined in experiment 4.2.1.

Figure 4.6 Dose-response curve of *TGM2* induction.



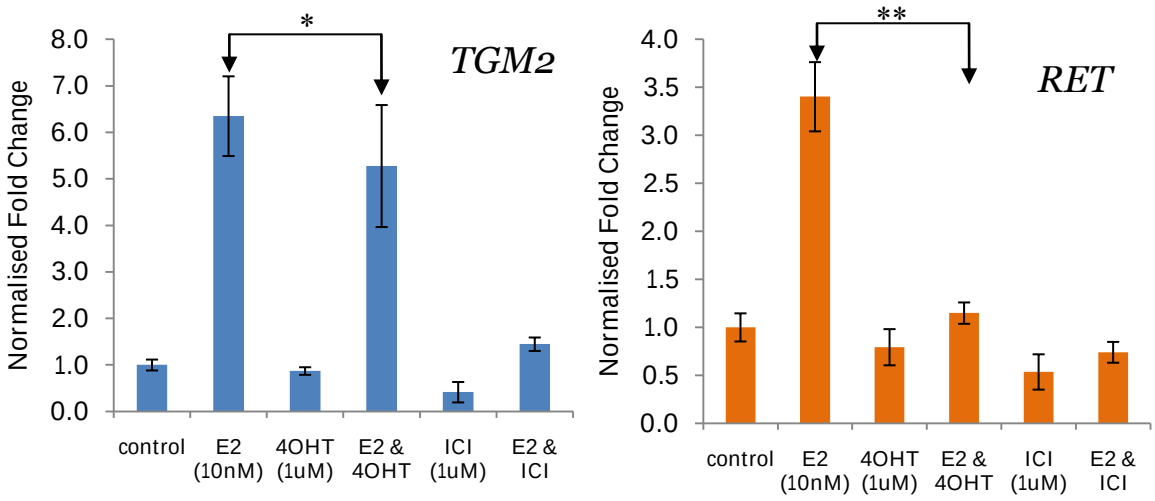
MCF-7 cells were treated with increasing concentrations of E2(0.1 - 100nM), 4OHT (10 - 1000nM). Cells were harvested and cDNA prepared from isolated RNA was subjected to qPCR analysis. Data are normalized to *36B4* and expressed as mean fold change over vehicle ± SEM for triplicate amplification reactions.

The qPCR results for *TGM2* show a high standard error which may suggest a problem with the PCR protocol or polymerase enzyme stability. The DNA polymerase enzyme may have been allowed to reach room temperature prior to the start of the qPCR run, resulting in imprecise data. The results do show however that between 1-100nM E2 is sufficient for activation of *RET* and *TGM2*. A concentration of 10nM E2 and 1µM 4OHT and an incubation time of 6 hours was chosen for future experiments.

4.2.3 TAM fails to inhibit ER-activation of *TGM2*

Results presented in Figure 4.5 indicate that 4OHT fails to inhibit E2-induced up-regulation of *TGM2*; this experiment was repeated using 4OHT and a SERD, ICI as a pharmacological control. RET is used as a control E2-regulated gene. ICI works through a different mechanism than TAM, by inducing rapid degradation of ER.

Figure 4.7 E2-induced activation of *TGM2* is not inhibited by TAM



MCF-7 cells were treated with vehicle (control), 10nM E2 with either 1µM 4OHT or 1µM ICI for 6hr. Cells were harvested and cDNA prepared from isolated RNA was subjected to qPCR analysis. Data are normalized to *36B4* and expressed as mean fold change over vehicle ± SEM for triplicate amplification reactions from one representative experiment. Asterisks: unpaired two-tailed Student's T-test, * *p value* > 0.05 (no significant difference), ** *p value* < 0.01.

The results show that both *RET* and *TGM2* are both induced by E2. However 4OHT treatment in combination with E2 resulted in a significant decrease in RET expression, while no significant decrease in *TGM2* expression was observed. Statistical analysis using the Student's t-test shows that there is a significant difference ($p < 0.01$) in *RET* gene expression between E2 and E2/4OHT treated cells whereas there is no difference ($p > 0.05$) in *TGM2* expression between E2 and E2/4OHT treated cells. This suggests that TAM is unable to inhibit E2-mediated activation of *TGM2*. ICI however is able to fully inhibit E2-induced activation of both *TGM2* and RET.

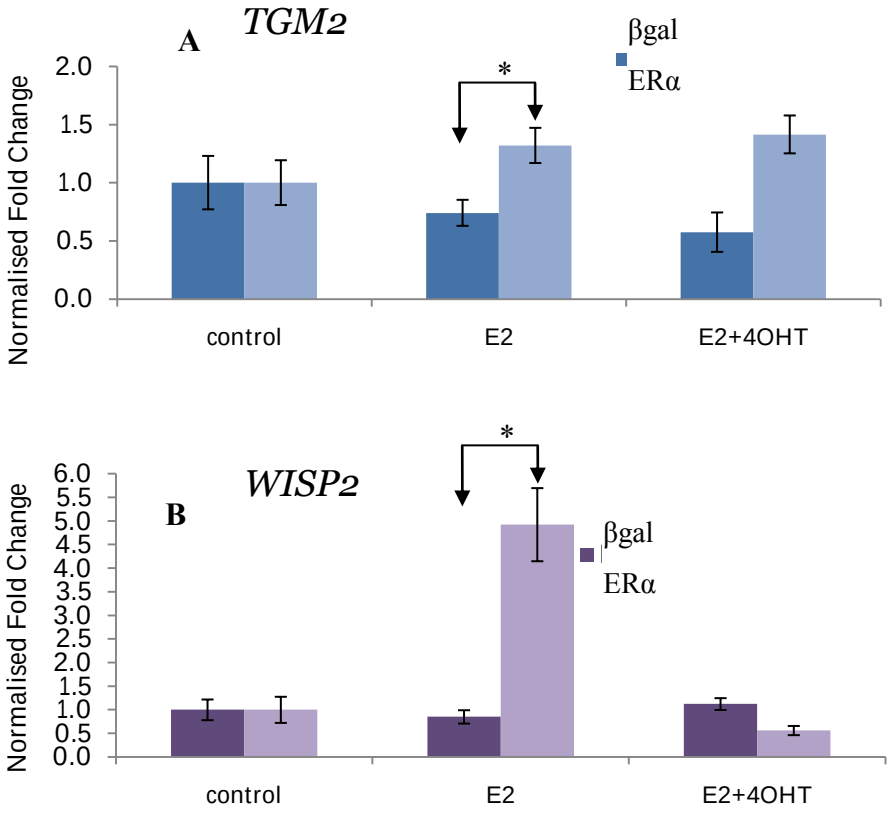
SERDs work by inducing rapid re-compartmentalisation and degradation of ERα protein while SERMs block ligand induced activation of the ER. This indicates that TAM is binding the ER and preventing transcription of RET but not *TGM2*. ICI is able to inhibit *TGM2* transcription since it leads to degradation of the ER. This left us with the task of investigating why *TGM2* is not inhibited by TAM when other ER-regulated genes are, and what is the possible biological significance of this.

4.2.4 Is ER required for *TGM2* up-regulation?

In the following set of experiments we examined the role of some of the cellular factors required for the up-regulation of *TGM2*. We have already shown that E2 is required for activation of this gene (Figure 4.5). This experiment investigates the role of ERα in *TGM2* activation.

Transfection of an ERα plasmid into MDA-231 cells is used to identify the role of ERα in *TGM2* induction. MDA-231 (ER negative) breast cancer cells were transfected with the ERα expression vector. Cells are treated with E2 and with 4OHT in combination with E2. qPCR was performed using primers for *WISP2* (WNT1 inducible signaling pathway protein 2), an ER responsive gene, *36B4*, and *TGM2*. βgal is used as a negative control as it does not have a canonical ER binding site and is not activated by oestrogen signalling.

Figure 4.8 ERα transfection of MDA-231 cells



MDA-231 cells treated with either ERα expression vector or βGal (negative control) and treated with 10nM E2 in combination with either control (V) or 1μM 4OHT. Cells were harvested and cDNA prepared from isolated RNA was subjected to qPCR analysis. Data are normalized to the *36B4* gene and expressed as mean fold change over vehicle ± SEM for triplicate amplification reactions. Asterisks: unpaired two-tailed T-test, * *p value* < 0.01.

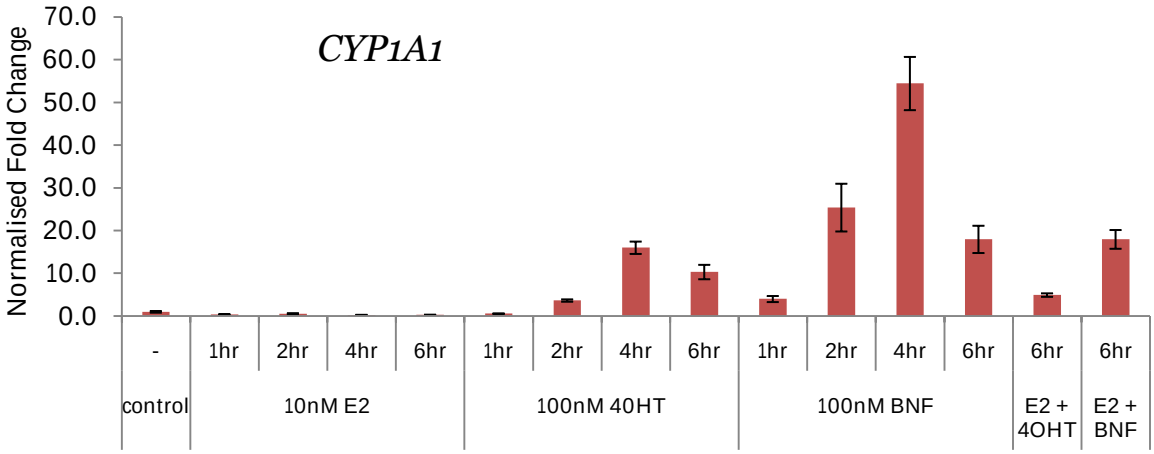
WISP2 qPCR data show good E2-induced activation of transfected ERα confirming that ER activates *WISP2* and ER transfection was successful. Statistical analysis using the Student's t-test shows there is a significant difference ($p < 0.01$) between *TGM2* induction in cells treated with E2 which have the ERα over cells treated with βgal. The results however do show poor activation of *TGM2* which may be due to inadequate RT/qPCR efficiency or insufficient ERα transfection. These results further confirm that *TGM2* is an E2-regulated gene. The addition of 4OHT to cells treated with E2 and transfected with the ERα plasmid induces an inhibition of *WISP2* while having no effect on *TGM2* mRNA expression. These results also confirm data from previous experiments where TAM is unable to inhibit E2-induced up-regulation of TGM2.

To understand why this gene is not inhibited by TAM we must examine the involvement of other cofactors which may be preventing this inhibition. The following experiment examines the possible role of the Aryl Hydrocarbon Receptor (AhR) in preventing TAM inhibition of *TGM2*. We hypothesized that activated AhR prevents ER inhibition of the *TGM2* gene by a process which is distinct from other ER regulated genes. The following experiment investigates the role of TAM on the AhR and the effects of activated AhR on transcription of the *TGM2* gene. The aim of this is to investigate if TAM activation of AhR is responsible for the continued up-regulation of *TGM2* in the presence of E2 and TAM through the process of AhR-ER cross-talk.

4.2.5 Tamoxifen activates the Aryl Hydrocarbon receptor (AhR)

It has been previously shown that TAM can activate *CYP1A1,* an AhR (Aryl-Hydrocarbon Receptor) regulated gene independently of the ER [87]. MCF-7 cells are treated with E2, 4OHT and BNF over a 6 hour time-course. BNF (β-Napthoflavone), an AhR agonist, is used here as a positive control.

Figure 4.9 Dose-response curve of *TGM2* induction.



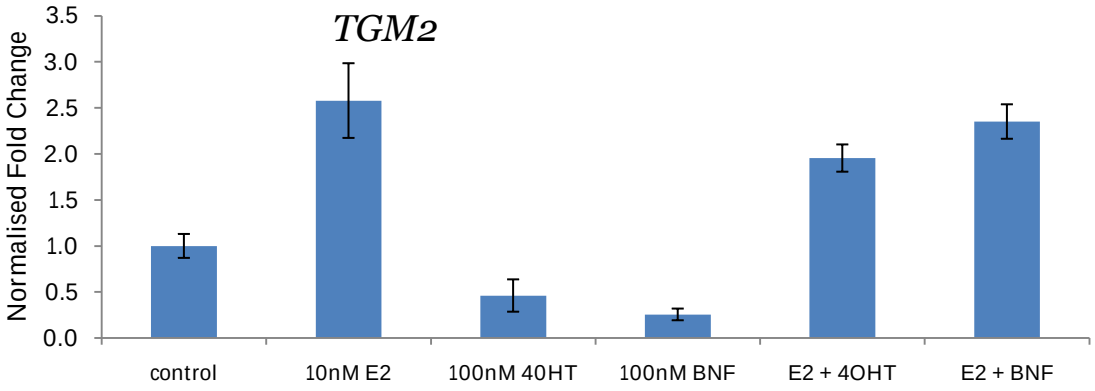
MCF-7 cells (2x10^5 /well) were treated with vehicle (control), 10 nM E2, 100nM 4OHT, 100nM βNF for 1,2,4 or 6hr intervals. Cells were harvested and cDNA prepared from isolated RNA was subjected to qPCR analysis. Data are normalized to the *36B4* gene and expressed as mean fold change over vehicle ± SEM for triplicate amplification reactions.

Results presented in Figure 4.9 show that 4OHT and βNF (positive control) will activate the *CYP1A1* gene (at an optimum incubation of 4 hr) confirming that 4OHT does activate AhR as an off-target effect. This is where a drug binds to and activates a receptor which is not its intended target.

In previous experiments we have shown that ER is required for *TGM2* gene expression. However because TAM activates AhR, we must also investigate if AhR is capable of maintaining *TGM2* gene expression in the presence of TAM. To do this we treated MCF-7 cells with ligands which activate the AhR and measured subsequent *TGM2* mRNA expression.

Figure 4.10 shows the effects of the AhR-activating ligands, TAM and BNF on *TGM2* mRNA expression. These results show that treatment of MCF-7 cells with AhR ligands alone is not sufficient maintain to mRNA expression of *TGM2*. The data from this experiment show that activated AhR alone is unable to activate this gene.

Figure 4.10 Effects of AhR ligands on TGM2 activation.

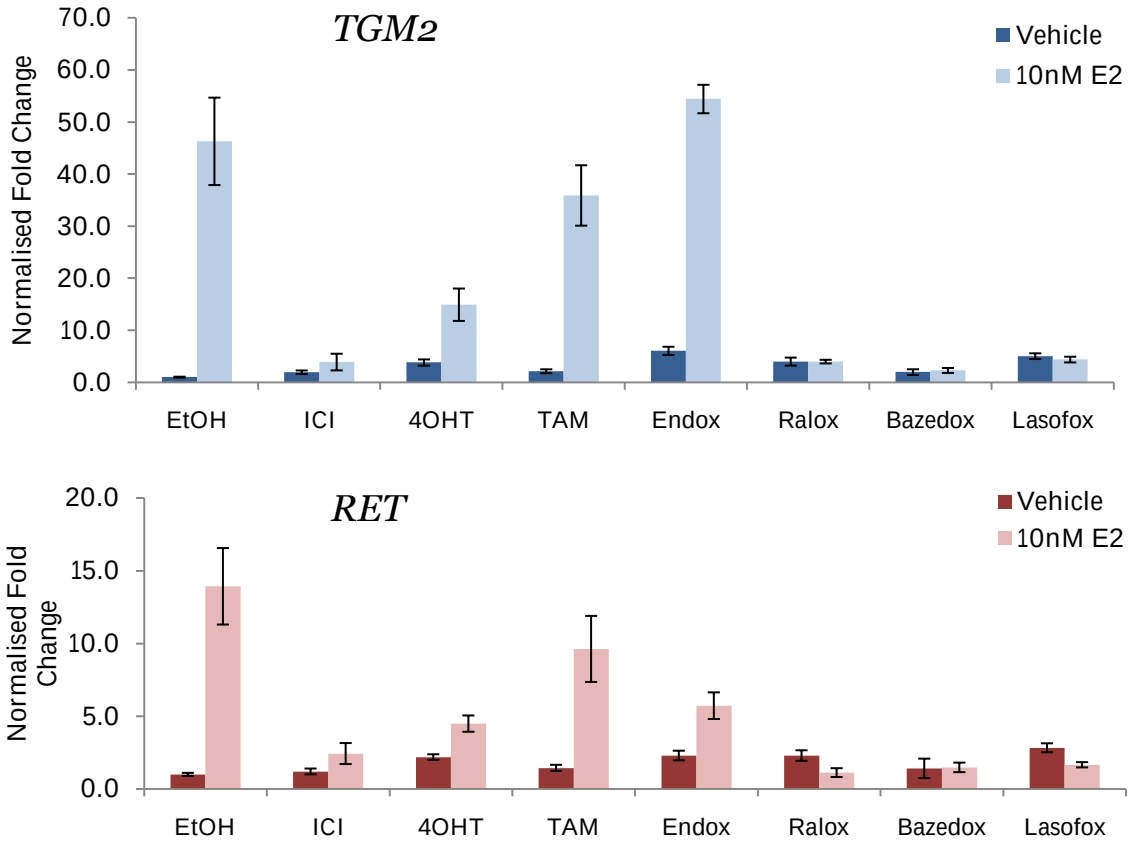


MCF-7 cells (2x10^5 /well) were treated with vehicle (control), 10 nM E2, 100nM 4OHT, 100nM βNF for a 6hr incubation. Cells were harvested and cDNA prepared from isolated RNA was subjected to qPCR analysis. Data are normalized to the *36B4* gene and expressed as mean fold change over vehicle ± SEM for triplicate amplification reactions.

4.2.6 Array of SERMs on *TGM2* activation

Although we have shown that TAM cannot inhibit E2 induced *TGM2* mRNA expression, we sought to determine whether structurally related breast cancer drugs show similar results. In this experiment we analysed the effects of an array of SERMs and SERDs on E2-mediated activation of *TGM2*.

Figure 4.11 Effect of SERMs on *TGM2* activation



MCF-7 cells were treated with vehicle (control) or 10 nM E2 and either 1µM ICI, 4OHT, Tamoxifen, Endoxifen, Raloxifene, Lasofoxifene or Bazedoxifene and incubated for 8hr. Cells were harvested and cDNA prepared from isolated RNA was subjected to qPCR analysis. Data are normalized to 36B4 and expressed as mean fold change over vehicle ± SEM for triplicate amplification reactions.

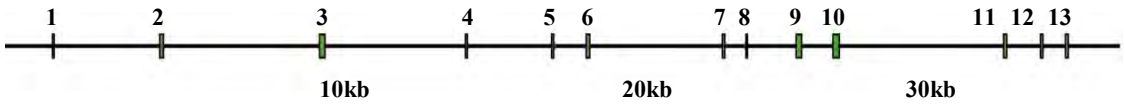
These data are the first indication that Endoxifen (Endox) is also unable to inhibit *TGM2*. As stated earlier Endoxifen, a secondary metabolite of 4OHT is as potent as 4OHT with respect to ER binding and inhibition of E2 induced cell proliferation. However in this experiment we did not achieve full inhibition of the control gene RET; this may be due the poor solubility of some of these drugs in media. Also Tamoxifen is known to undergo spontaneous rearrangement from its *trans* to *cis* forms resulting in a weak anti-oestrogen. A dramatic solvent effect has been observed on the rate of the process of molecular rearrangement [88]. This may explain the lowered efficacy of Tamoxifen in these set of results. To help overcome these solubility issues we used serial dilutions when diluting ligands, while all ligands were kept on ice.

4.3 Cloning TGM2 response elements

At this point we have displayed the differential pharmacology of TAM on ER activation of *TGM2* in the context of breast cancer cells. The cell culture assays have shown us that TAM is unable to inhibit E2/ER-induced activation of *TGM2* while other agents such as ICI fully inhibit this activation. The next step in this study is to investigate the genetic requirements for E2 activation of *TGM2*. This involves cloning a short segment of the promoter region into a Luciferase plasmid for use in cell transfection assays. By designing primers to different response elements on the *TGM2* promoter we can clone these regions and investigate the precise sequence requirements of the ER/E2/TAM activation complex.

The first step is to perform a bioinformatics study on the *TGM2* DNA sequence. The entire genomic sequence for chromosome 20 was downloaded (www.bioinfo.ut.ee/HMgenome/CEU) and the exons/introns located along with the putative ERE.

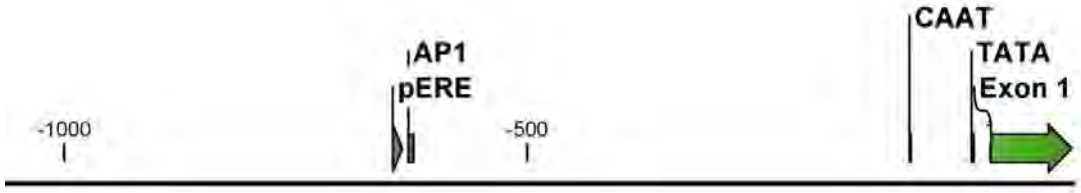
Figure 4.12 Layout of exons on *TGM2*



Exons within the *TGM2* gene are labelled 1 to 13, drawn to scale.

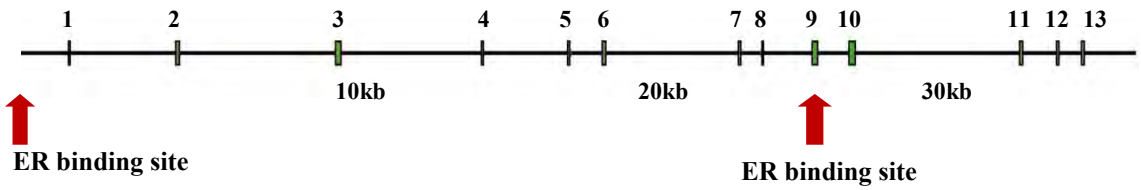
The putative ERE and AP-1 sites were found by performing a nucleotide sequence search using Bio-informatics software (CLC Main Workbench 5.0)

Figure 4.13 Putative ER binding regions in the *TGM2* promoter



A further literature search highlighted two other possible ER binding regions on the *TGM2* gene based on a genome-wide study of ER binding sites (Figure 4.14). [35] These regions which were identified by chip-on-chip analysis located the binding sites to within 600bps. One is located 3kb upstream of the transcription start site (Figure 4.16), while the other is located within exon 9 (Figure 4.17). There are also three SP-1 sites located just upstream of the gene promoter which may also indirectly drive ER-mediated transcription.

Figure 4.14 Location of ER binding sites

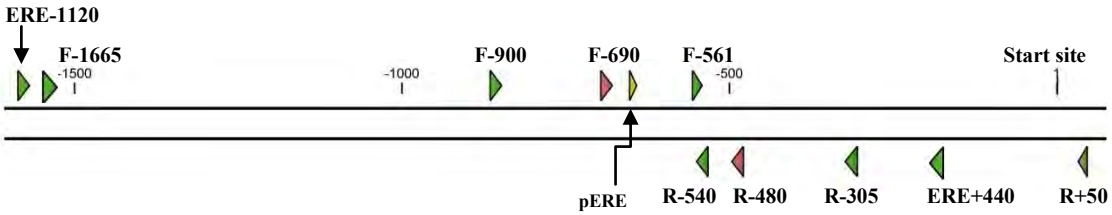


Data concerning the locations of these ER binding sites was downloaded from the supplemental data supplied with the published data on the Harvard University website (http://research.dfci.harvard.edu/brownlab/datasets/index.php?) [35]

The next step was to design a series of primers to cover the *TGM2* regulatory region and the two putative ER binding sites shown above. All primers were produced with an Xho1 cutting site on the 5' end for ligating the PCR products into the Luciferase vector. The list of primers is outlined in the Materials and Methods section (Table 3.3). These were named by their relative position to the transcription start site (Figure 4.15).

Different combinations of these forward and reverse primers can be used to amplify the required sequences.

Figure 4.15 Location of primers on *TGM2* 5' flanking region



Primers were designed to a length of 20bps with a GC content of 45-55% and an annealing temperature of 55-60°C. All primers have an Xho1 binding site attached.

Figure 4.16 Location of primers on upstream putative ER binding site

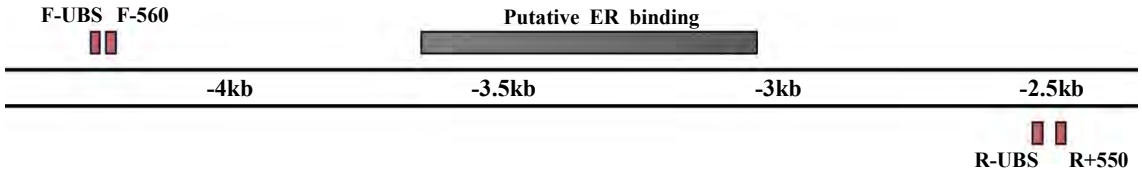
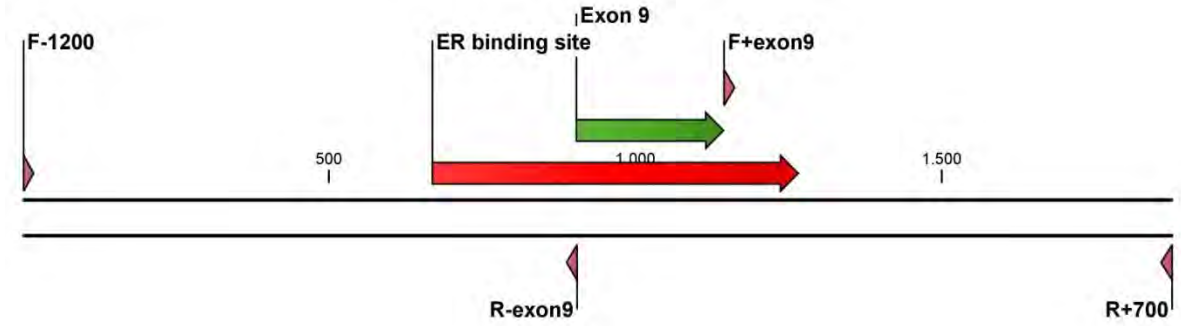


Figure 4.17 Location of primers on Exon 9/ER binding site

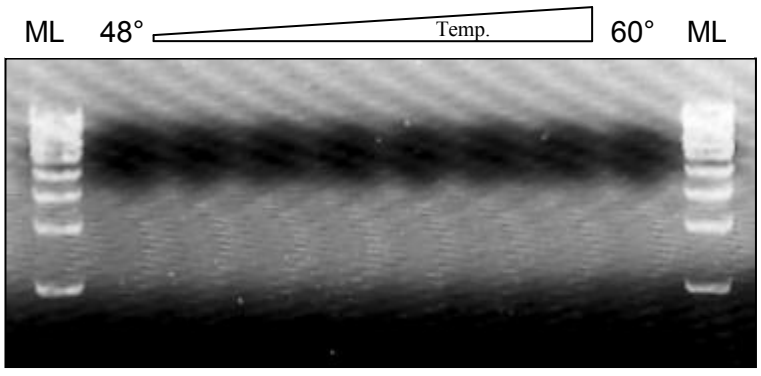


Primers were designed spanning the putative ER binding region and exon 9. An extra set of primers located on either side of the exon gave the option of isolating the region upstream or downstream of the exon. Primers are named relative to their position to the putative ER binding site.

4.4 PCR of genomic DNA

Having designed the primers, PCR reactions were set up in order to amplify the particular DNA elements. Genomic DNA was isolated from MCF-7 cells. PCR was first performed with the primers targeting the full length (1.7kb) *TGM2* regulatory region as in Figure 4.15. The PCR protocols used are described in the Materials and Methods. PCR amplification of the full length *TGM2* regulatory region was first performed using the primers "Forward-1645" and "Reverse +50" in a temperate gradient PCR cycle (Figure 4.15). Repeating this protocol with the addition of varying amounts of DMSO (Dimethyl Sulfoxide) (1-7%) and the use of either Pfu Turbo enzyme or GC Advantage 2 polymerase did not result in any visible bands (Figure 4.18). In the process of optimising PCR conditions, PCR was repeated for all combinations of forward and reverse primers surrounding the putative ERE (Figure 4.15) and run on agarose gel, however no visible specific bands were produced.

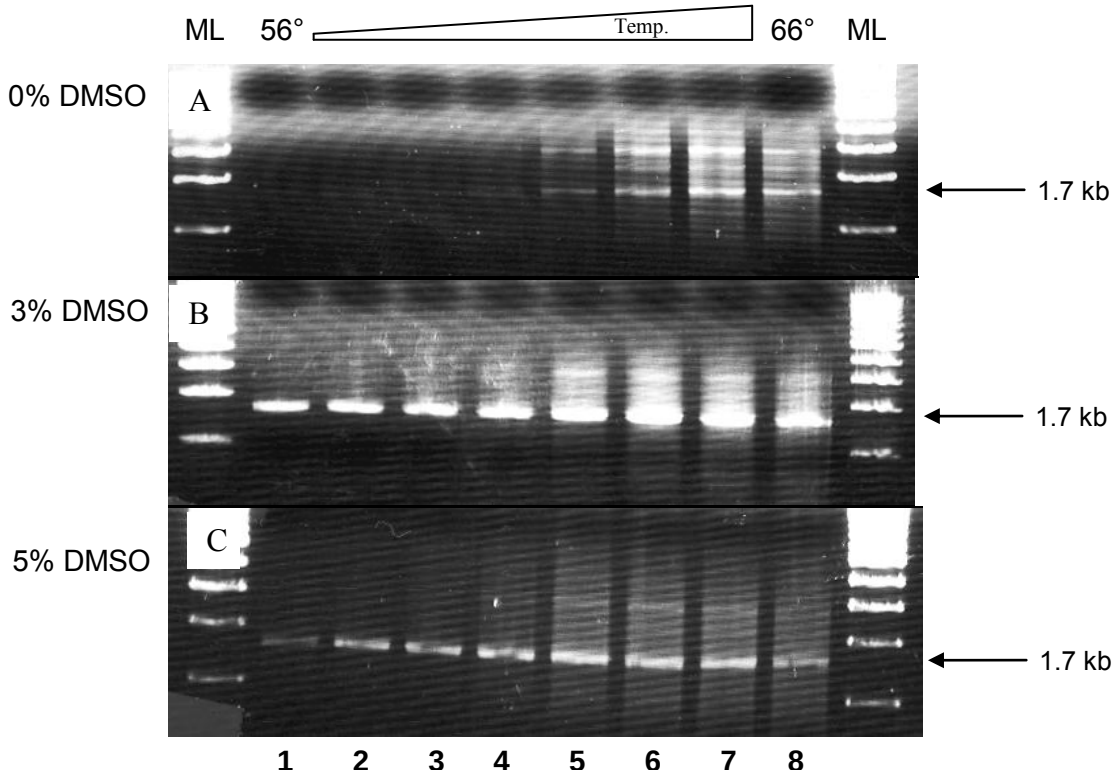
Figure 4.18 PCR amplification of full length TGM2 regulatory region



Agarose gel of PCR amplified gDNA using the temperature gradient 48-60°C. ML, 1kb DNA molecular weight ladder.

After redesigning primers for this region ("ERE-1120" and "ERE+440") (Figure 4.15), this protocol was repeated with the new set of primers with increasing concentrations of DMSO (0, 3 and 5%) and at temperature gradients of 56-66°C.

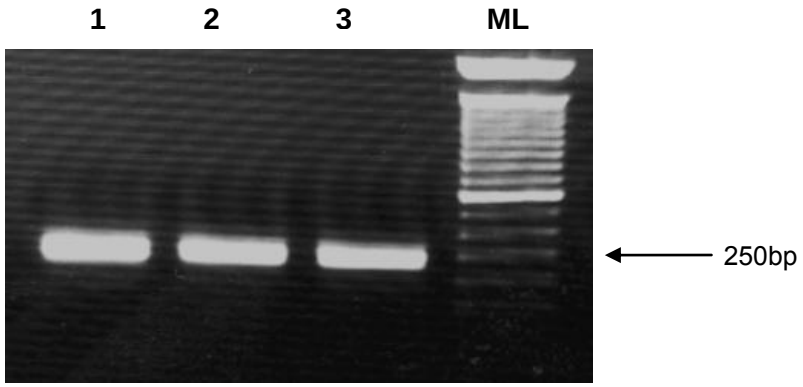
Figure 4.19 PCR amplification of full length TGM2 regulatory region



0% DMSO (A) shows unspecific bands, PCR preformed at gradient temperature (56-66°C). Addition of 3% or 5% DMSO (B) and (C) gives a specific band at 1.7Kbps. This sequence is be referred to as pTGM2 (*TGM2* promoter) in the following experiments.

The specific bands were cut from the gel and the DNA isolated. This sequence contains the 5' *TGM2* regulatory region containing the putative ERE and AP-1 sites as shown in Figure 4.13. With the isolated DNA as a template (pTGM2), we used a separate set of primers ("Forward 561" to "Reverse 305") which target a short sequence within the template sequence in a PCR reaction (Figure 4.15). This was done to confirm that the correct sequence was amplified from genomic DNA.

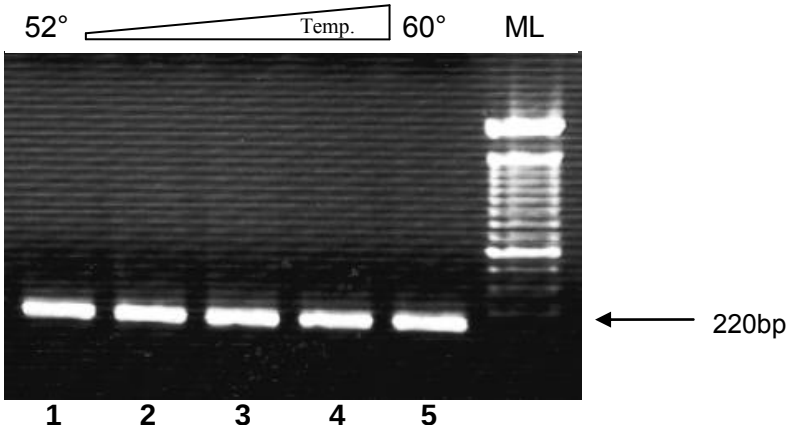
Figure 4.20 PCR amplification of a 250bp sequence using pTGM2 as a template



The amplified sequence from primers "ERE-1120" and "ERE+440" (pTGM2) was used as a DNA template in this reaction. Primers used were "Forward 561" to "Reverse 305". The presence of PCR products (shown in triplicate) proves that the correct 1.7kb sequence was amplified (Figure 4.19)

PCR was performed using the primers "Forward-690 and Reverse-480" and the pTGM2 sequence as a template (Figure 4.15). The target sequence contains the putative ERE, located about 650bps upstream of the transcription start site (Figure 4.13). PCR was performed at a temperature gradient of 52-60°C.

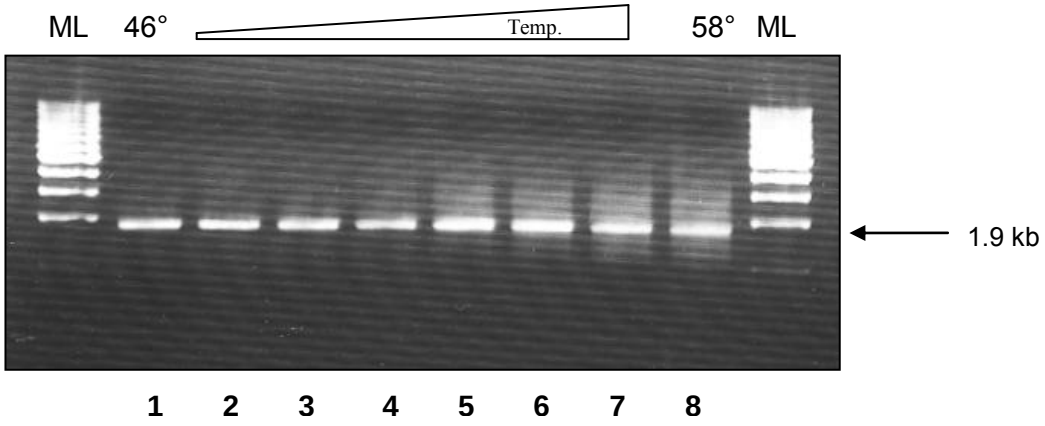
Figure 4.21 PCR amplification of putative ERE at position -650bp



PCR products were run on 1% Agarose gel with a 100bp molecular ladder. Product size is 220bps including the XhoI binding sites attached to either end. This sequence contains the putative ERE as described earlier.

In order to amplify the downstream putative ER binding site (Figure 4.17), PCR was performed on gDNA using primers targeting a 1.9kb sequence on exon 9 using the primers "Forward-1200" and "Reverse+700". A temperature gradient from 46-58°C was set up on the PCR thermocycler in order to find the optimum primer annealing temperature (Figure 4.22).

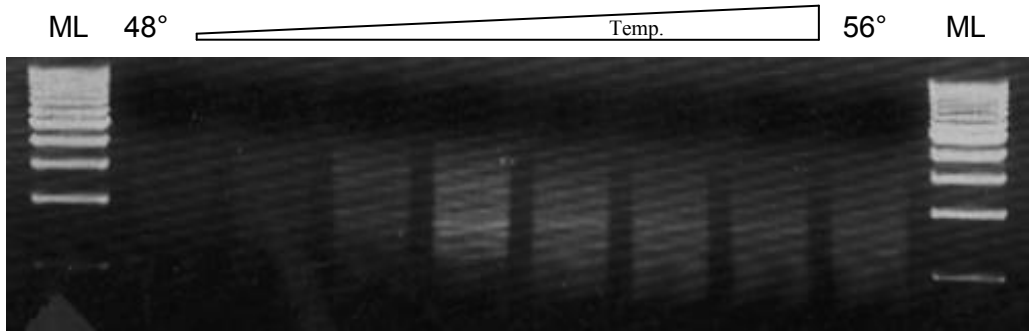
Figure 4.22 PCR amplification of 1.9kb of *TGM2* exon 9



PCR reaction was performed at a temperature gradient of 46-58°C. Products were run on 0.75% Agarose gel (+Ethidium Bromide) with 1kb molecular ladder.

The PCR products from the most specific bands (at 46-50°C) were isolated and Xho1 digested for ligating into the Luciferase vector in later experiments. To isolate the upstream putative ER binding site (Figure 4.16), PCR was performed using the primers "Forward UBS" and "Reverse UBS" at a gradient of 48-56°C.

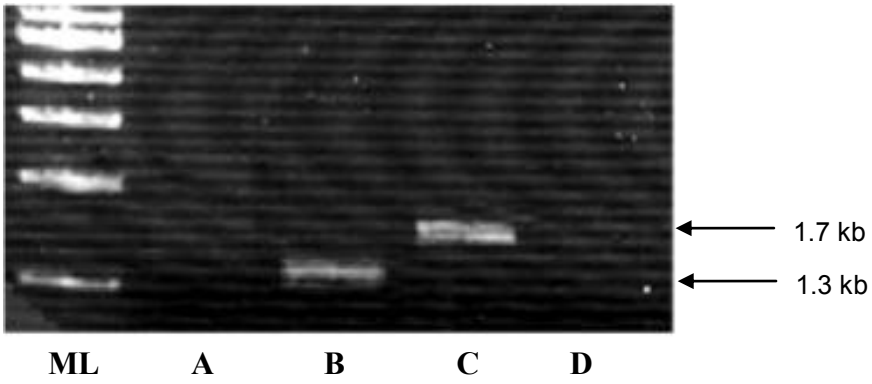
Figure 4.23 PCR amplification of upstream putative ER binding site



PCR of the upstream putative ER binding region using the primers "Forward UBS" and "Reverse UBS" did not result in any specific DNA product.

Primers to this region (Figure 4.16) were redesigned (F-560 and R+550) and the PCR was repeated using different combinations of forward and reverse primers (Figure 4.24). PCR reactions B and C produced a visible DNA product. Since the expected size of the product is 1.7kb, the DNA sequence in reaction B is more than likely not the correct target sequence.

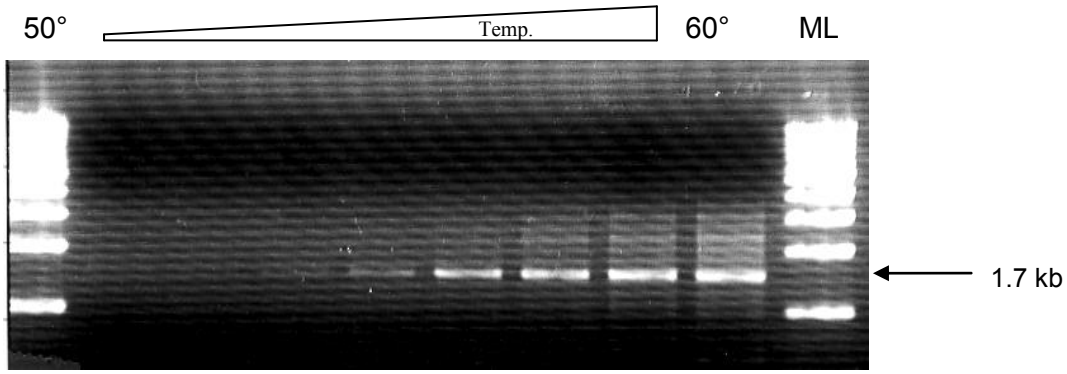
Figure 4.24 PCR amplification of upstream putative ER binding region



Four sets of PCR reactions (A-D) were set up using the primers: (A) Forward UBS-560 and Reverse UBS+550 (B) Forward-UBS and Reverse UBS+550 (C) Forward UBS-560 and Reverse-UBS (D) Forward-UBS and Reverse-UBS. The PCR reaction was run at a constant temperature of 52°C and PCR products were run on 1% agarose gel. The same reaction was set up with 1% DMSO, this achieved the same results as shown above.

As the primers in reaction (C) gave a specific DNA product of the expected size (1.7kb), these were used in subsequent experiments. A PCR reaction was set up using the primers “Forward UBS-560” and “Reverse-UBS” and run at a gradient of 50-60°C using gDNA as a template.

Figure 4.25 UV visualization of agarose gel

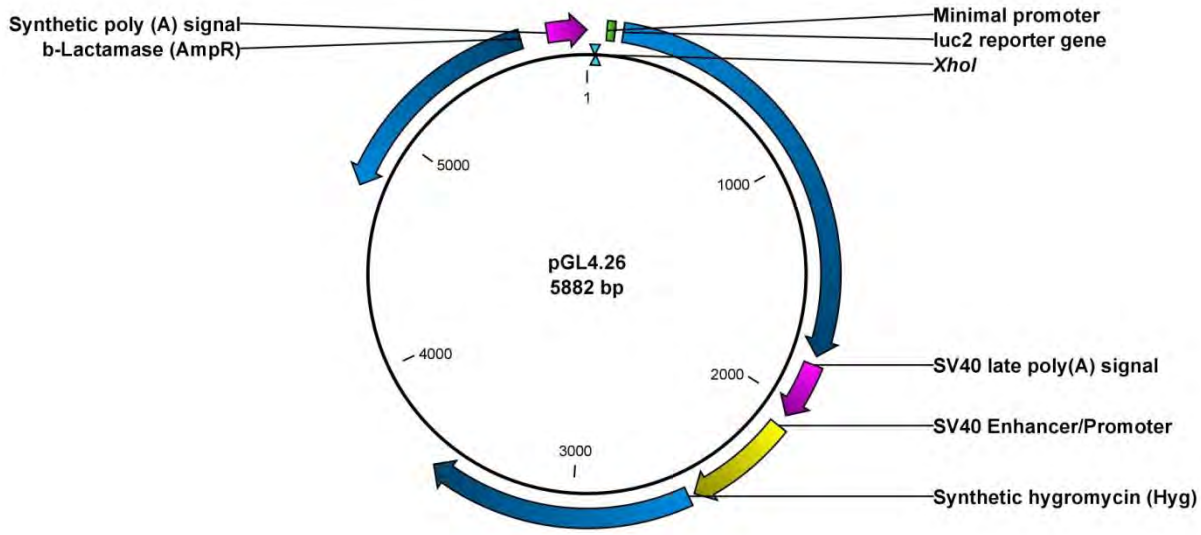


The PCR products were run on a 1% agarose gel with a 1kb molecular ladder. The DNA from the specific bands was cut from the gel and isolated using a DNA gel isolation kit. The isolated DNA which is referred to as UBS (upstream binding sequence) was Xho1 digested for use in future ligation experiments.

4.5 Ligations

The pGL4-26 vector (Promega) was used in all ligation experiments, this contains a minimal promoter, an Ampicillin resistance gene (β-lactamase) and a luc2 (Luciferase) reporter gene. (Figure 4.26)

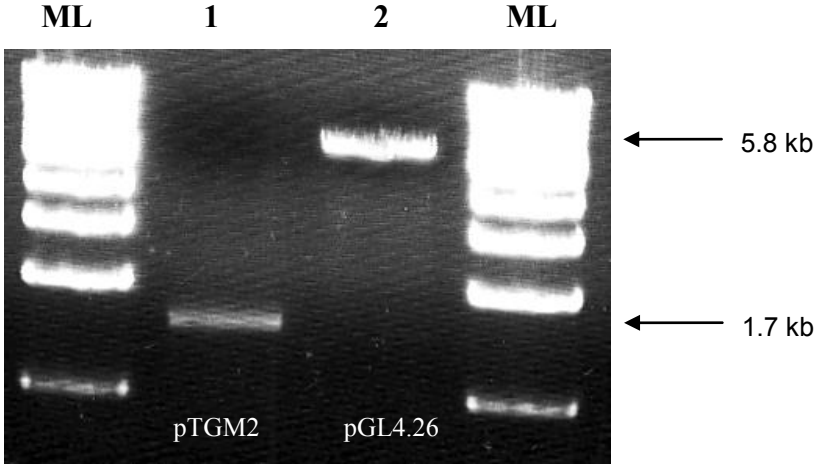
Figure 4.26 Promega pGL4.26 plasmid with minimal promoter



Sizes and locations of the specific plasmid components were obtained from the pGL4 luciferase manual. The map was constructed using Bio-informatics software.

The pGL4.26 plasmid and the full length (1.7kb) TGM2 regulatory sequence insert (Figure 4.19) were digested overnight with Xho1 restriction enzyme. pGL4.26 was treated with Shrimp Alkaline Phosphatase (SAP) to remove 5' phosphates, preventing re-ligation of the plasmid. Both DNA strands were run on a 1% agarose gel to determine volumes required for ligation reaction. (Figure 4.27).

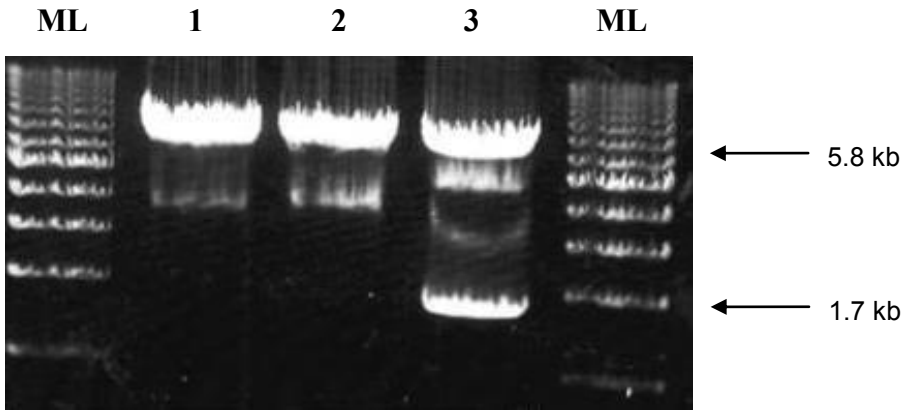
Figure 4.27 Xho1 digested pTGM2 and pGL4.26



Digested pTGM2 and pGL4.26 were run on a 1% agarose gel with a 1kb molecular ladder. The intensity of the bands indicate their relative concentrations, which is used to determine the volumes required for the ligation reaction

Multiple ligation reactions were set up with T4 DNA ligase (1.5µl), 10x ligase buffer (1.5µl), the insert (6µl) and varying volumes of plasmid (0.5µl to 2.0µl) to a final volume of 15µl. Also a vector-only ligation was set up as a negative control. The ligation mixtures were incubated at room temp for 1-2hrs prior to transforming in XL2 ultra-competent cells (Stratagene) according to manufacturer's instructions. Transformed cells were plated on Ampicillin plates and incubated at 37°C overnight. All colonies (3) were re-cultured in 10mls of LB broth with added Ampicillin, while the vector-only control did not grow any colonies. Plasmid isolation was performed on the 4 cultured broths to isolate the ligated plasmid. Each plasmid isolate was quantified, Xho1 digested, and run on 0.75% agarose gel.

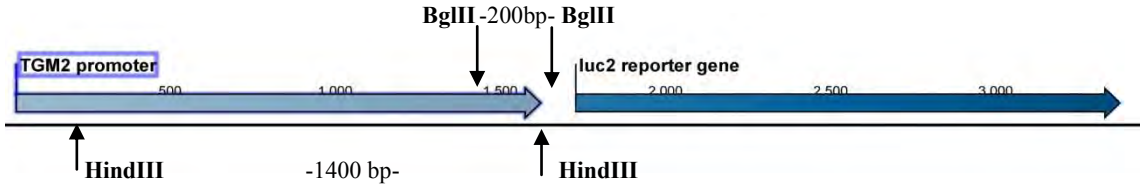
Figure 4.28 UV visualisation of three isolated plasmids



1µg of each isolated plasmid was run on 0.75% agarose gel. Sample number 3 has both the vector (5.8kb) and the insert (1.7kb) while both 1 and 2 have the plasmid only.

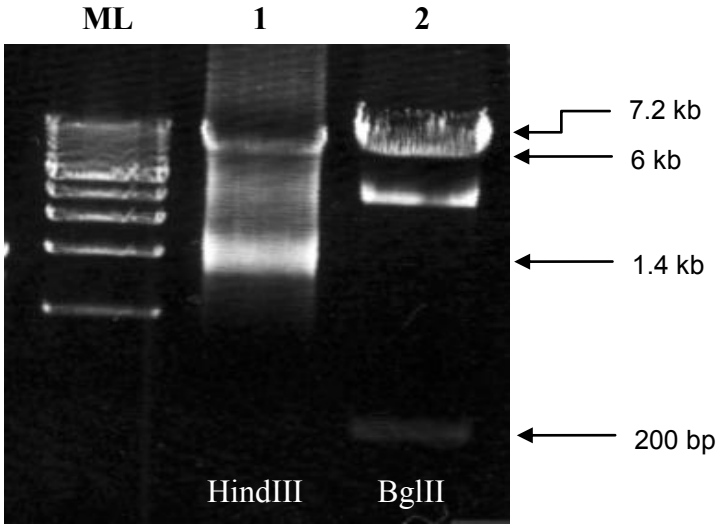
The plasmid from sample number 3 was shown to contain the insert (referred to as pTGM2-luc in future experiments). The next step is to determine whether or not the insert is in the correct orientation. Two restriction enzyme digests were set up using HindIII and BglII as each of these enzymes cut both the insert and the vector in one position only. By analysing the size of the fragments, the orientation of the insert can be determined. Plasmid isolation using a Maxiprep kit was performed to isolate a large volume of the plasmid (pTGM2-luc). The isolated plasmid was quantified for use in transfection studies.

Figure 4.29 Locations of HindIII and BglII cutting sites on TGM2-luc



The diagram shows the pTGM2 insert, the multiple cloning site and the luciferase gene. As the distance between the two HindIII sites is 1.4kb and the total plasmid size is 7.4kb, a HindIII digest will produce fragment sizes of 1.4 kb and 6 kb. Also a BglII digest will produce fragment sizes of 7.2 kb and 200 bp. These exact fragment sizes are only formed when the pTGM2 sequence is inserted in the correct orientation.

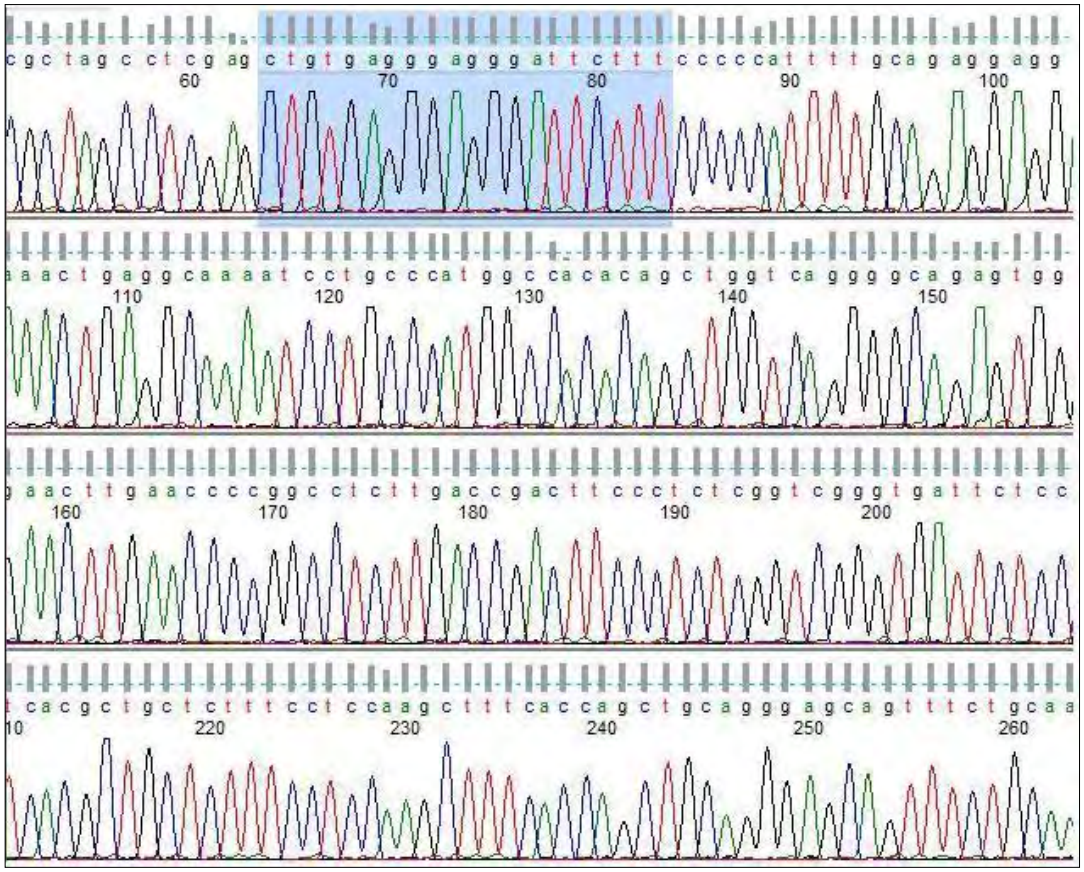
Figure 4.30 HindIII and BglII restriction digest of isolated pTGM2-luc



The isolated plasmid (pTGM2-luc) was digested with HindIII (Lane 1) and BglII (Lane 2) in a single digest reaction. The fragments sizes from the HindIII digest were 6kb and 1.4kb while the fragment sizes from the BglII digest were 7.2kb and 200bp. Therefore we can conclude the insert is in the correct orientation.

The pTGM2-luc plasmid was then sequenced to confirm that the correct sequence from the *TGM2* promoter region is incorporated into the plasmid (Figure 4.31).

Figure 4.31 Chromatogram of sequenced pTGM2-luc plasmid



This figure shows part of the chromatogram of sequenced pTGM2-luc viewed using Geospiza Finch TV 1.4. Highlighted in blue is the primer sequence used to amplify the *TGM2* insert, indicating the start of the inserted sequence. Not shown is the putative ERE which is downstream of the sequenced region.

The sequence data was aligned with the *TGM2* sequence using the Bio-informatics software CLC Main Workbench 5.0. Figure 4.32 shows a perfect alignment between the sequenced insert and the actual *TGM2* target sequence.

Figure 4.32 Alignment between sequenced insert and expected sequence

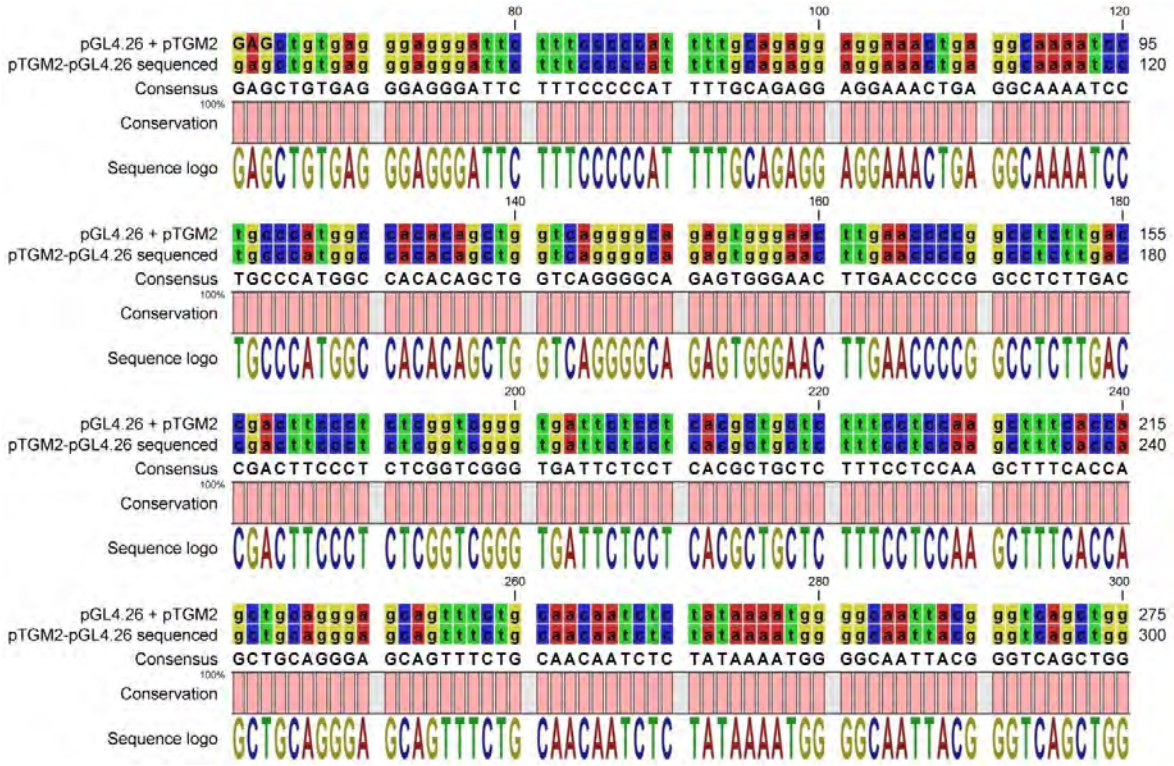


Figure shows comparison between sequenced insert (pTGM2-luc) and genomic sequence. Alignments were analysed using Bio-informatics software.

We decided to focus on the cloned TGM2-luc plasmid and proceed with transfection studies using this plasmid to ascertain the transcriptional regulation of *TGM2* by ER in presence and absence of E2/TAM.

4.6 Transfections

At this point 1.7kb of the *TGM2* promoter region has been cloned into a Luciferase plasmid. In order to study the regulation of *TGM2* by ER with E2 or TAM, the pTGM2-luc plasmid (Figure 4.33) was transfected into HeLa cells.

Figure 4.33 pGL4.26 luciferase vector with 1.7kb *TGM2* insert

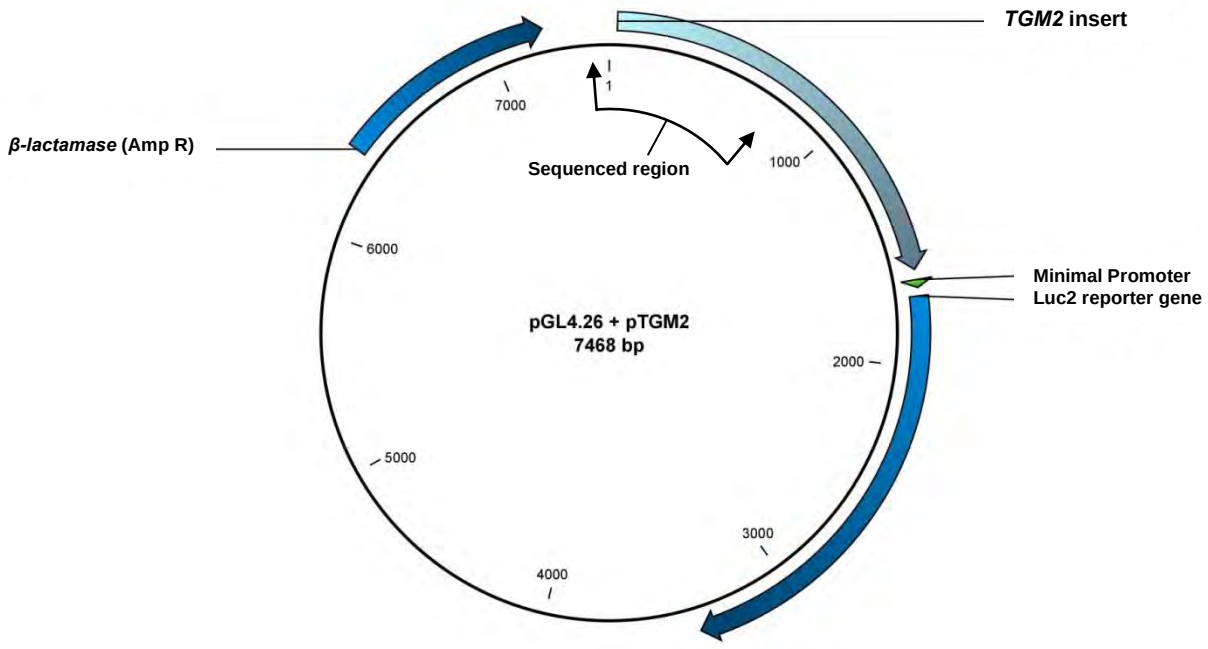


Diagram of the constructed luciferase plasmid containing the luciferase gene, minimal promoter and *TGM2* promoter insert, indicating sequenced region.

Both ER positive and ER negative cancer cells were used in the transfection studies. In order to investigate the requirements of ER in the activation of *TGM2*, a transfection assay was set up using ER negative HeLa cells. In this assay a CMV-driven ERα plasmid was co-transfected with either pTGM2-luc, ERE-luciferase (3xERE-TATA-luc) reporter (positive control) or the empty vector (pGL4.26) as a negative control. All cells in the transfection experiments are co-transfected with 100ng CMV-β-gal (Figure 4.34). The β-galactosidase substrate Chlorophenol red-β-D-galactopyranoside (CPRG) was added to the cell lysates and incubated at 37°C. The absorbance of each well was measured at 570nm, which was used to normalise the

luciferase activity for transfection efficiency. The results of this transfection are presented in Figure 4.35.

Figure 4.34 pCMV-β-gal plasmid used to normalise transfections

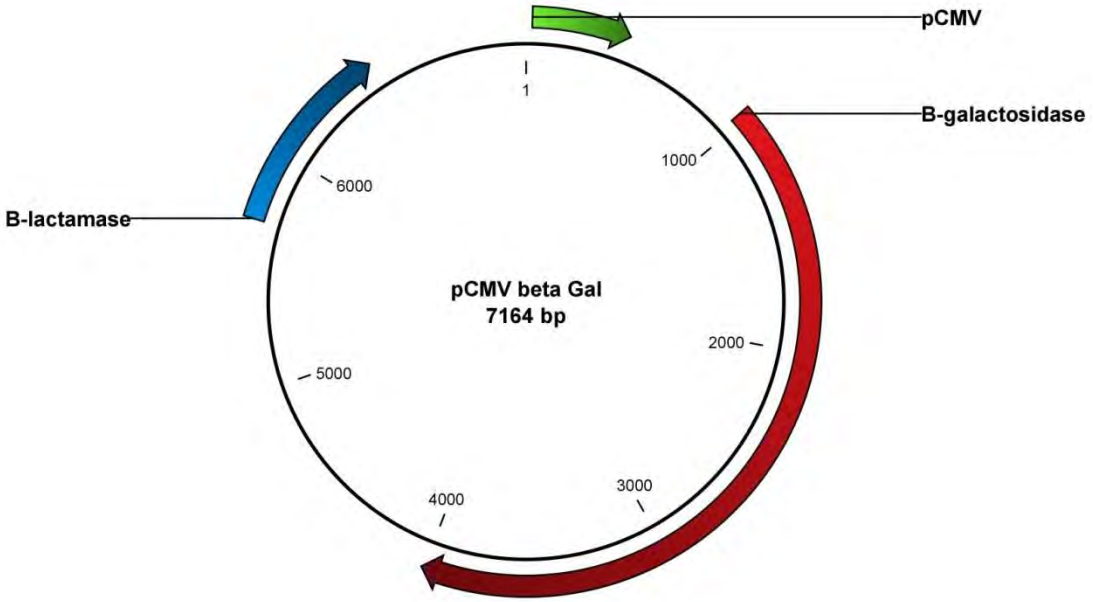
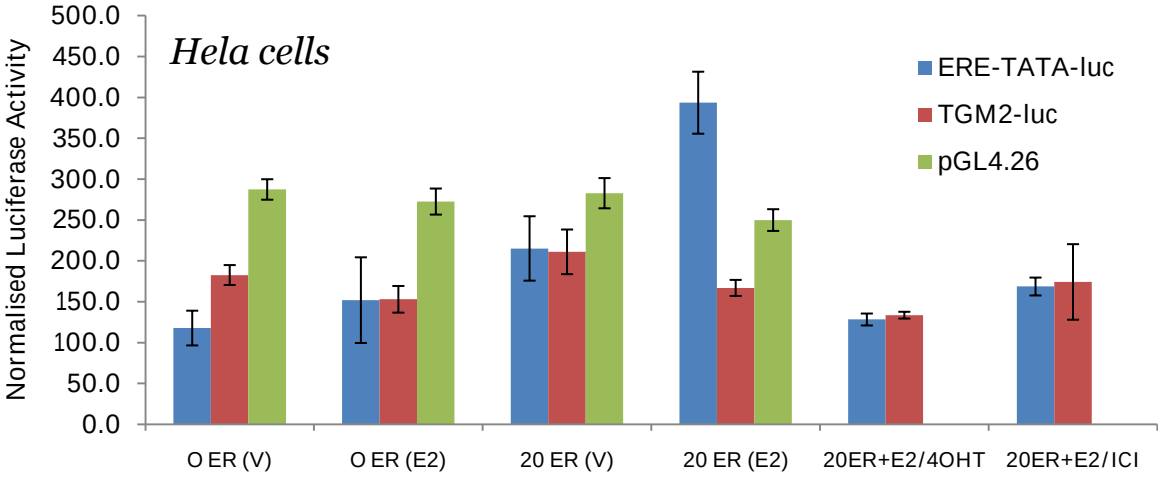


Diagram of the β-gal plasmid with a β-lactamase gene and a β-galactosidase gene driven by a CMV promoter.

Figure 4.35 Effects of E2 and ER on *TGM2* activation

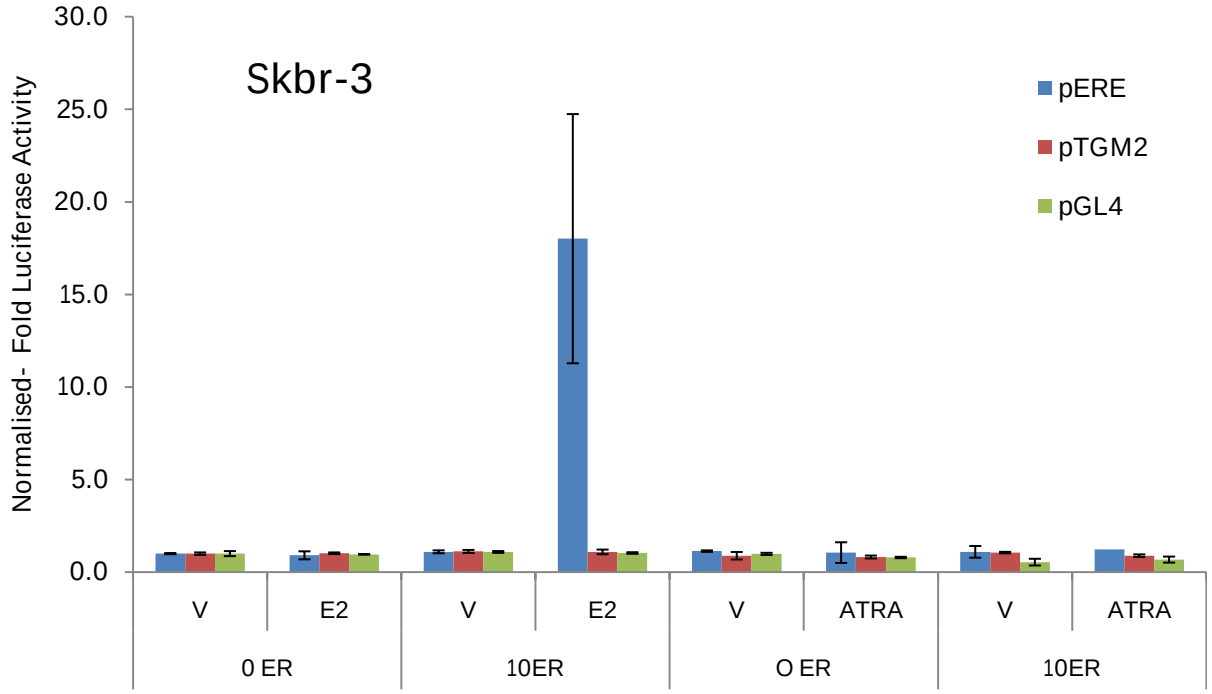


HeLa cells were transfected with 2880ng plasmid (ERE-TATA, pGL4.26 or TGM2-luc), 0 or 20ng ERα, along with 100ng CMV-βgal. Total DNA was 3µg/triplicate. After 24hrs cells were treated with vehicle (V) or 10nM Estradiol (E2) with 1µM 4OHT or 1µM ICI and then harvested and assayed for luciferase and β-gal activity. The effect of ERα on the activation of *TGM2* was evaluated. Results are expressed as normalized luciferase activity (normalized with β-galactosidase for transfection efficiency) +/- standard deviation per triplicate sample of cells. Note: pGL4.26 transfection was not carried out for the E2/4OHT or E2/ICI treated cells.

From the graph we can see there is no significant activation of the *TGM2* plasmid ($p=0.32$). However there is significant activation of the positive control ERE-TATA-luc by E2 ($p<0.01$) and repression of ERE-TATA-luc by 4OHT ($p<0.01$). This experiment was repeated a further 3 times in HeLa cells without any significant activation of pTGM2-luc.

This experiment was repeated in both HeLa cells and Skbr-3 cells using the ligand ATRA (All-Trans Retinoic Acid), a known activator of *TGM2* [89]. The results of this experiment are presented in Figure 4.36, which shows significant activation of the control plasmid ERE-TATA-luc by E2. However ATRA did not produce any significant activation of the *TGM2* plasmid. One possible explanation for this is that as the location of the retinoid response element on *TGM2* has not been fully established it may be further upstream than the sequence included in this plasmid.

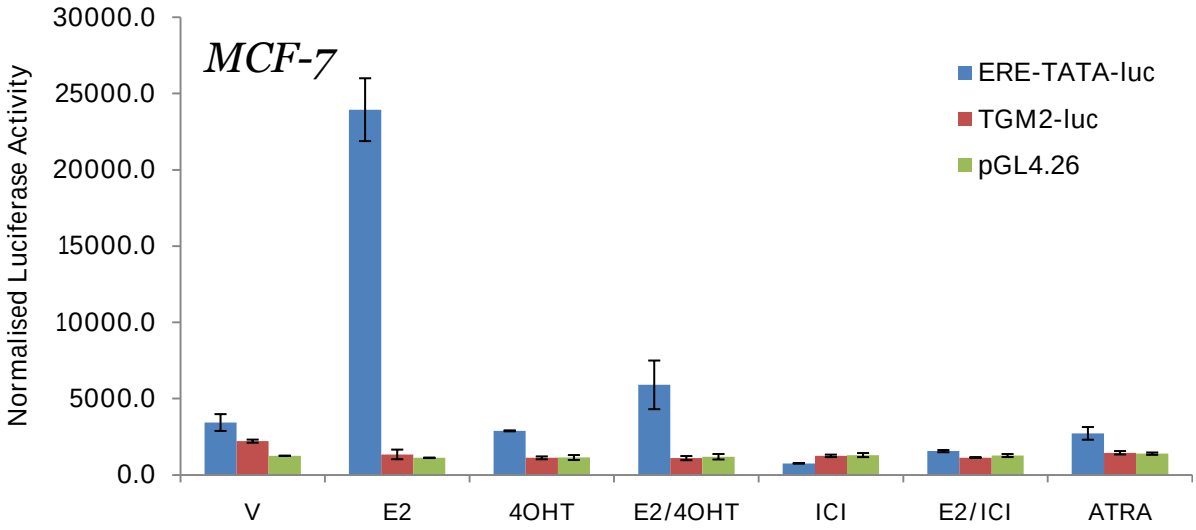
Figure 4.36 Treatment of transfected cells with ATRA



Skbr-3 cells were transfected with 2880ng plasmid (ERE-TATA, pGL4.26 or TGM2-luc), 0 or 10ng ERα, along with 100ng CMV-βgal. Total DNA was 3µg/triplicate. After 24hrs cells were treated with vehicle (V) or Oestradiol (E2) or ATRA and then harvested and assayed for luciferase and β-gal activity. The effect of ERα on the activation of *TGM2* was evaluated. Results are expressed as normalized luciferase activity (normalized with β-galactosidase for transfection efficiency) +/- standard deviation per triplicate sample of cells and expressed as mean fold change over vehicle-treated cells.

Oestrogen Receptor positive cells (MCF-7) were used in the following transfection experiment. ER positive cells were used here as previous experiments failed to show TGM2-luc activity. To out rule poor ER transfection efficiency as a cause of this, cells containing the ER are used. MCF-7 cells were transfected with the TGM2-luc, ERE-TATA-luc and pGL4.26 plasmids. Following transfection cells were treated with the ER ligands, E2, TAM, and ICI (Figure 4.37). The aim of this experiment is to analyse the effects of ER ligands and the oestrogen receptor on the transcriptional activity of cloned TGM2-luc plasmid.

Figure 4.37 Transfection of TGM2-luc in MCF-7 cells



MCF-7 cells were transfected with 2900ng plasmid (ERE-TATA, pGL4.26 or TGM2-luc) and 100ng CMV-βgal. Total DNA was 3µg/triplicate. After 24hrs cells were treated with vehicle (V), 10nM Oestradiol (E2), or 1uM All-Trans Retinoic Acid (ATRA) with 1uM 4OHT or 1uM ICI, and then harvested and assayed for luciferase and β-gal activity. The effect of E2 or ATRA on the activation of *TGM2* was evaluated. Results are expressed as normalized luciferase activity (normalized with β-galactosidase for transfection efficiency) +/- standard deviation per triplicate sample of cells.

Although there is good activation of the ERE-TATA-luc with E2, there is no significant activation of the TGM2-luc plasmid ($p>0.05$). Also ERE-TATA-luc induction is inhibited by 4OHT, as shown here by a decrease in luciferase activity ($p<0.01$). This experiment was repeated giving the same results as shown here. The

putative ERE within the pTGM2-luc plasmid being tested may not be functional. This may explain the lack of luciferase activity in pTGM2-luc transfected cells.

5 Discussion

Tamoxifen (TAM) has been used clinically for more than 30 years to treat breast cancer, and for more than 10 years to reduce the risk of breast cancer in women at high risk of developing this disease. The activities of TAM and its metabolites 4-Hydroxytamoxifen (4OHT) and Endoxifen, in breast cancer have primarily been attributed to their ability to inhibit ER signaling. Although clearly an ER antagonist in the breast, TAM has agonist activity in other ER target tissues and as such preserves bone mineral density but increases the risk for endometriosis and endometrial cancer, blood clots, and stroke. However, it is not entirely clear how TAM, RAL, and other SERMs exhibit tissue-specific or promoter-specific activities through a single signaling pathway. This study aims to investigate the differential promoter-specific pharmacology of TAM in the context of breast cancer cells using the *TGM2* gene as a model.

The results of this study reveal the differential control of the *TGM2* gene by the oestrogen receptor and TAM in the context of breast cancer cells. Figure 4.7 shows that ER/E2 induces expression of *RET* and *TGM2* but co-treatment with TAM will inhibit *RET* but not *TGM2*. These results are interesting as we would expect all ER-regulated genes to be inhibited by TAM in breast cancer cells. Therefore *TGM2* may be the first in a class of ER-regulated gene differentially regulated by TAM.

A further investigation into these findings leads us to examine the requirement of ER in the activation and inhibition of *TGM2*. An ER plasmid was transfected into ER negative cells breast cancer cells (MDA-231), using a β-gal plasmid as a control. ER-transfected cells showed a significantly higher induction of *TGM2* when treated with E2 ($p<0.01$) over cells transfected with the β-gal plasmid (Figure 4.8). However although there was good activation of the *WISP2* ER-regulated gene (control), there was only a slight but significant increase in *TGM2* induction. One possible cause of this may be poor transfection efficiency, where an insufficient amount of ER required for *TGM2* activation was present in the cell.

To explain the failure of TAM to inhibit ER-induced activation of *TGM2* we investigated the hypothesis that activated AhR is preventing inhibition of this gene by a process of ER-AhR cross-talk. Figure 4.9 confirms that TAM is a potent activator of AhR, as shown by the large fold increase in *CYP1A1* (AhR-responsive gene) induction. Figure 4.10 shows the effects of the AhR agonists, TAM and BNF on *TGM2* mRNA expression. These results clearly show that activated AhR alone is not sufficient to maintain expression of *TGM2*. Although AhR is activated by TAM, it does not lead to transcription of *TGM2* therefore we can out rule the involvement of AhR in *TGM2* gene induction.

Analysis of other related SERMs revealed that Endoxifen and 4OHT also show similar patterns to Tamoxifen in inhibiting ER mRNA induction of *TGM2* while the SERM Raloxifene was very effective in inhibiting this induction. The fact that Endox and 4OHT both have similar patterns to TAM is not surprising given that they are structurally related to TAM.

Although RAL functions in the same way as TAM, they have different patterns of activation and inhibition in different cells. RAL acts as an agonist in bone and an

antagonist in the uterus while TAM acts as an agonist in the bone and uterus (Table 1.1). Our findings show that RAL and TAM have different inhibition patterns of the *TGM2* gene in breast cancer cells. The prevailing question in this study is why two similar but distinct SERMs cause different activation patterns with the same gene in the same cells. While we know that upon E2 treatment, ER and other cofactors are recruited to gene regulatory regions to modulate gene expression, but depending on the promoter context and cellular background, TAM and E2 can regulate gene expression in a manner similar to or different from RAL/E2. It has been shown using phage-display/ELISA technology that the structures of the TAM-ER and RAL-ER complexes are not identical and show important differences that may contribute to differences in their patterns of gene regulation [26]. This would explain why RAL but not TAM can act as an antagonist in the uterus. It does not fully explain however why TAM has different patterns on the *TGM2* gene in breast cancer cells while inhibiting all other ER-regulated genes. SERMs act in a promoter and cell-context specific manner. We hypothesise that *TGM2* must have an oestrogen receptor responsive element in its regulatory region which is slightly different to that in other ER-regulated genes. Therefore binding of RAL/ER to *TGM2* promoter would allow for the binding of co-repressors which inhibits gene transcription whereas binding of TAM-ER to the gene does not recruit co-repressors, instead allowing for the recruitment of a co-activator complex leading to transcription.

We set out to investigate the effects of TAM and its related compounds on the *TGM2* gene expression in breast cancer cells. Our data highlights the differential pharmacology of TAM in the regulation of *TGM2*. However now the prevailing question is what makes *TGM2* different from other ER-regulated genes. In order to accomplish a better understanding of TAM/ER action in breast cancer, *TGM2* may be

used as a model gene which does not conform to the expected characteristics of an ER-regulated gene. To investigate *TGM2* further, we analysed the genomic sequence of its regulatory region.

To understand the basis for the differential regulation of this gene we analysed 2kbs of the sequence upstream of the *TGM2* promoter. Two possible ER regulatory regions were located, one putative ERE (8 out of 10 match) and one AP-1 site (6 out of 7 match) (Figure 1.13). These binding sites are a slight deviation from the canonical binding site. This slight difference in sequence may be a contributing factor to the differential pharmacology seen in these results. If the ER is indeed binding to these sequences we would not expect a typical binding site. ERα is known to activate AP-1 controlled genes when bound by either E2 or TAM in the cytoplasm [90]. However our results show E2 is required for *TGM2* up-regulation. This finding may out rule ER - AP-1 as a mode of *TGM2* activation.

Transfections were performed in MCF-7, HeLa and Skbr-3 cell types using the cloned TGM2-luc plasmid. Each experiment was designed to investigate specific requirements of TGM2 activation and inhibition. Our constructed plasmid did not show any significant luciferase activity in any of the experiments. As we have confirmed that the plasmid has the correct insert and orientation (Figure 4.31), these results may indicate that the ER is not actually binding to the sequence within the cloned 1.7kb *TGM2* 5' flanking region. However it is possible that the required coactivators or transcription factors are not present in sufficient quantity to induce transcription or perhaps other NRREs are required. As discussed earlier, two other proposed ER binding sites are present in the vicinity to the *TGM2* gene, which was previously identified using chip-on-chip data shown in Figure 4.14 [35]. It is entirely possible that the ER is driving transcription of this gene through one of these putative binding sites as the ER can act at

a large distance from the promoter [91]. Further study is required to investigate the role of these putative ER binding sites in *TGM2* regulation.

Although other studies have examined *TGM2* involvement in apoptosis, cell development and tumour migration and development, this is the first study on the hormonal regulation of *TGM2* in the context of SERMs in breast cancer. Our findings indicate important differences in the regulation of *TGM2* by E2 and SERMs that might impact on the activity of *TGM2* in breast cancer cell migration and metastasis.

In conclusion our data show the differential pharmacology of *TGM2* in relation to TAM and Endoxifen. Cell culture experiments have demonstrated the role of ERα in *TGM2* activation. We have identified two putative ER binding sites with the 5' flanking region, an ERE and an AP-1 site and cloned this region into a luciferase vector. Finally our results suggest that the ER may not be binding to this region and instead my activate transcription from a more distal binding site.

The continued mRNA expression of *TGM2* in the presence of ER/TAM may have clinical significance due to its role in tumour suppression and prevention of metastasis, as discussed earlier. Further research is required to determine the role of *TGM2* in tumour cells.

6 References

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7 Appendix

Table 7.1 UV quantification of isolated RNA (Raw data)

Sample ID	Net Abs 260.0nm	Net Abs 280.0nm	260.0/280.0	Dil Factor	Conc. µg/ml
1	0.1668	0.0744	2.2419	25.000	166.8
2	0.1396	0.0613	2.2773	25.000	139.6
3	0.1905	0.0847	2.2491	25.000	190.5
4	0.1285	0.0557	2.3070	25.000	128.5
5	0.1220	0.0553	2.2061	25.000	122.0
6	0.1248	0.0548	2.2774	25.000	124.8
7	0.1359	0.0594	2.2879	25.000	135.9
8	0.1434	0.0625	2.2944	25.000	143.4
9	0.1316	0.0579	2.2729	25.000	131.6
10	0.1547	0.0672	2.3021	25.000	154.7
11	0.1425	0.0627	2.2727	25.000	142.5
12	0.1328	0.0601	2.2097	25.000	132.8
13	0.1718	0.0753	2.2815	25.000	171.8
14	0.1620	0.0732	2.2131	25.000	162.0
15	0.1639	0.0728	2.2514	25.000	163.9

RNA samples were diluted 1/25 in TE buffer prior to quantification. All RNA samples in these studies have a 260:280nm ratio of between 2.0 – 2.5

Table 7.2 Volumes required for iScript RT-PCR reaction

sample	treatment	Time	µg/ml RNA	µl/1µg RNA	µl H_20
1	control	-	166	6	9
2	10nM E2	1hr	139	7.2	7.8
3		2hr	190	5.3	9.7
4		4hr	128	7.8	7.2
5		6hr	112	8.9	6.1
6	100nM 40HT	1hr	124	8.1	6.9
7		2hr	135	7.4	7.6
8		4hr	143	7	8
9		6hr	131	7.6	7.4
10	100nM BNF	1hr	154	6.5	8.5
11		2hr	142	7	8
12		4hr	132	7.6	7.4
13		6hr	171	5.8	9.2
14	E2 + 4OHT	6hr	162	6.2	8.8
15	E2 + BNF	6hr	163	6.1	8.9