The Role of Androgen Receptor Signalling in Endocrine Resistant Breast Cancer

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Abstract

Breast cancer (BCa) is the most prevalent cancer among women in the UK. The majority of BCas are endocrine sensitive and develop through the action of oestrogens, facilitated through the transcription factor Oestrogen Receptor alpha (ERα). Treatment for these patients usually involves endocrine therapies (Aromatase Inhibitors and anti-oestrogens), which are successful in many patients, but therapy resistance represents a major clinical issue. The Androgen Receptor (AR) is a transcription factor that is more highly expressed than ERα in BCa, and mediates the functions of androgens. In early forms of ERα-positive disease, AR is a positive indicator of prognostic outcome and suppresses ERα signalling. However, in ERα-negative disease AR has been demonstrated to drive cancer progression and recent evidence has suggested that AR can drive endocrine resistance.

Reporter assays, gene expression analysis and Chromatin Immunoprecipitation assays demonstrated that AR and ERα inhibit each other's activity and that anti-oestrogens can reverse this inhibition, resulting in an active AR. Importantly, long term colony formation assays demonstrated that androgen could induce anti-oestrogen resistant growth, but anti-androgens prevented this from developing. Co-treatment of tumours with anti-oestrogens and anti-androgens could therefore be a viable option to block this mechanism of resistance. Cell line models of endocrine resistant disease were used to investigate AR signalling in therapy resistance. The results demonstrated that AR levels were enhanced in several lines and that all cell lines were sensitive to androgen for growth. Importantly, anti-androgens could inhibit androgen-induced growth in all models. Anti-androgens could therefore also be a viable option for the treatment of tumours that have become resistant to endocrine therapies. This study therefore furthers our understanding of the role of the AR in BCa progression and suggests that it is a valid therapeutic target to prevent and/or treat endocrine resistant disease.

Statement of Originality

Unless otherwise stated in the text, this thesis is the result of my own work.

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Abbreviations

1º: Primary

2º: Secondary

3β-diol: 5α-Androstane-3β, 17β-diol

17β-HSD: 17β-Hydroxysteroid Dehydrogenase

A: Androstenedione

AA: Abiraterone Acetate

Ab: Antibody

AF1: Activation Function 1

AF2: Activation Function 2

AGR-2: Anterior Gradient Protein 2 Homolog

AI: Aromatase Inhibitor

AIB1: Amplified in Breast Cancer 1

Akt: AKT Serine/Threonine Kinase (PKB)

ALGGEN: Algorithmics and Genetics Group

anti-PDL-1: Anti-Programmed Death-Ligand 1

AP-1: Activator Protein 1

APS: Ammonium persulphate

ARA70: Androgen Receptor Co-activator 70 kDa Protein

AR: Androgen Receptor

ARE: Androgen Response Element

AREG: Amphiregulin

ATM: Ataxia Telangiectasia Mutated

AURKA: Aurora Kinase A

BBS: (BES)-buffered saline

BCa: Breast Cancer

Bcl-2: B-cell lymphoma 2

BES: N,N-Bis(2-hydroxyethyl)-2-aminoethanesulphonic acid

BIC: Bicalutamide

BLC: Blomstrand Lethal Chondrodysplasia

BMI: Body Mass Index

BRCA1: Breast Cancer 1, Early Onset

BRCA2: Breast Cancer 2, Early Onset

BSA: Bovine serum albumin

$CaCl_2$: Calcium chloride

CARM1: Coactivator-Associated Arginine Methyltransferase 1

CDK3: Cyclin-Dependent Kinase 3

cDNA: Complementary DNA

ChIP: Chromatin Immunoprecipitation

ChIP-Seq: Chromatin Immunoprecipitation sequencing

CHK2: Checkpoint Kinase 2

CK14: Cytokeratin 14

CO_2: Carbon dioxide

Co-IP: Co-Immunoprecipitation

CPT: Cryptotanshinone

CLDN3: Claudin 3

CLDN4: Claudin 4

CRUK: Cancer Research UK

C-Terminal: Carboxyl-Terminal

CYP3A4: Cytochrome P450 3A4

DAX-1: *Dosage-sensitive sex reversal, Adrenal hypoplasia critical region, On chromosome X, Gene 1*

DBD: DNA Binding Domain

DC: Detergent Compatible

DCIS: Ductal Carcinomas *in situ*

DDC: Dopa Decarboxyla-se

ddH2O: Double distilled water

DHEA: Dehydroepiandrosterone

DHT: Dihydrotestosterone

DIO2: Deiodinase, Iodothyroni-ne Type II

DMSO: Dimethyl sulphoxide

DMEM: Dulbecco's Modified Eagle's Medium

DNA: Deoxyribonucleic acid

dNTPs: Deoxyribonucleotides

E1: Oestrone

E2: 17-β-Oestradiol

E3: Oestriol

E. coli: *Escherichia coli*

EDTA: Ethylenediaminetetraacetic acid

EGF: Epidermal Growth Factor

EGFR: Epidermal Growth Factor Receptor

EMT: Epithelial-to-Mesenchymal Transition

ERα: Oestrogen Receptor alpha

ERβ: Oestrogen Receptor beta

ERBB2: Erb-B2 Receptor Tyrosine Kinase 2 (HER-2)

ERE: Oestrogen Response Element

ESR1: *Oestrogen Receptor 1*

ESR2: *Oestrogen Receptor 2*

EtOH: Ethanol

EV: Empty Vector

FBS: Foetal bovine serum

FDA: Food and Drug Administration

FGF: Fibroblast Growth Factor

FGFR: Fibroblast Growth Factor Receptor

FOXA1: Forkhead Box A1

FSH: Follicle-Stimulating Hormone

FULV: Fulvestrant

FULVR: Fulvestrant Resistant

GCDFP15: Gross Cystic Disease Fluid Protein-15

GDI: - Guanosine Diphosphate Dissociation Inhibitor

GDP: Guanosine Diphosphate

GFP: Green Fluorescent Protein

GH: Growth Hormone

GnRH: Gonadotropin Releasing Hormone

GnRHa: Gonadotropin-Releasing Hormone agonist

GPER1: G-Protein-Coupled Oestrogen Receptor 1

GRB7: Growth Factor Receptor-Bound Protein 7

GREB1: Gene Regulated in Breast Cancer 1

GTA: General Transcription Apparatus

HCl: Hydrochloric acid

HDAC: Histone Deacetylase

HDAC1: Histone Deacetylase 1

HER-2: Human Epidermal Growth Factor Receptor 2

HOX: Homeobox

HOXB7: Homeobox B7

HOXC11: Homeobox C11

HRE: Hormone Response Element

HRP: Horseradish peroxidase

HRT: Hormone Therapy

HSD3B1: 3β-Hydroxysteroid Dehydrogenase Type 1

IGF1: Insulin-Like Growth Factor-1

IHC: Immunohistochemistry (IHC)

IL6: Interleukin 6

IPTG: Isopropyl β-D-1-thiogalactopyranoside

ISH: *in situ* Hybridisation

KCl: Potassium chloride

kDa: Kilodaltons

ki67: Marker Of Proliferation ki-67

KKLN: Killin

KRT5: Keratin 5

L19: RPL19 ribosomal protein

LB: Luria Broth

LBD: Ligand Binding Domain

L-Glutamine-PenStrep: L- Glutamine-Penicillin-Streptamycin

LH: Luteinizing Hormone

LHRH: Luteinizing Hormone Releasing Hormone

lncRNA: Long non-coding RNA

LTED: Long Term Oestrogen Deprived

MABC: Molecular Apocrine Breast Cancer

MAML2: Mastermind-Like Transcriptional Coactivator 2

MAPK: Mitogen-Activated Protein Kinase

MaSC: Mammary Stem Cell

MECT1: Mucoepidermoid Carcinoma Translocated 1

MeOH: Methanol

$MgCL_2$: Magnesium chloride

$MgSO_4$: Magnesium sulphate

MIB: Mibolerone

miRNA: MicroRNA

MKI67: Marker of Proliferation Ki-67

Moesin: Membrane-Organizing Extension Spike Protein

mRNA: Messenger RNA

mTOR: Mammalian Target Of Rapamycin

MYC: Myelocytomatosis Oncogene Cellular Homolog

MYST 3: MYST histone acetyltransferase (monocytic leukemia) 3

MRI: Magnetic Resonance Imaging

NaCl: Sodium chloride

NaOH: Sodium hydroxide

NCI: National Cancer Institute

NDRG1: N-myc Downstream-Regulated Gene 1

NGS: Next Generation Sequencing

NR: Nuclear Receptor

N-Terminal: Amino-Terminal

OHF: Hydroxyflutamide

OFS: Ovarian Function Suppression

O/N: Overnight

PALB2: Partner And Localiser of BRCA2

PAR-4: Prostate Apoptosis Response 4

PARP: Poly-ADP Ribose Polymerase

PBS: Phosphate-buffered saline

PCa: Prostate Cancer

PCR: Polymerase Chain Reaction

RFP: Red Fluorescent Protein

PFA: Paraformaldehyde

PI: Protease Inhibitor

PI3K: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase

pIGF-1R: Phosphorylated Insulin-like Growth Factor-1 Receptor

PIK3CA: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha

PKB: Protein Kinase B (Akt)

PMSF: Phenylmethylsulphonyl fluoride

RPMI: Roswell Park Memorial Institute

PPAR: Peroxisome Proliferator-Activated Receptor

PR: Progesterone Receptor

PSA: Prostate Specific Antigen

PSAP: Prosaposin

PTEN: Phosphatase and Tensin Homolog

PTH1R: Parathyroid Hormone 1 Receptor

PTHLH: Parathyroid Hormone-Related Protein

PV: Pathogenic Variant

PVDF: Polyvinylidene difluoride

qPCR: Real-Time quantitative PCR

RAR: Retinoic Acid Receptor

RIPA: Radioimmunoprecipitation assay buffer

RNA: Ribonucleic acid

RNA-Seq: RNA-Sequencing

Rpm: Revolutions per minute

RPMI: Roswell Park Memorial Institute

rRNA: ribosomal RNA

RT: Room Temperature

RXR: Retinoid X Receptor (RXR)

SARM: Selective Androgen Receptor Modulator

SDS: Sodium dodecyl sulphate

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEC14L2: SEC14 like Lipid binding 2

SERD: Selective Oestrogen Receptor alpha Downregulator

SERM: Selective Oestrogen Receptor alpha Modulator

SFCS: charcoal Stripped Foetal Calf Serum

SHBG: Sex Hormone-Binding Globulin

siRNA: small interfering RNA

SOB: Super Optimal broth with Catabolite repression

SPDEF: SAM Pointed Domain Containing ETS Transcription Factor

SRC1: p160 steroid receptor co-activator 1

SRC2: p160 steroid receptor co-activator 2

SRC3: p160 Steroid Receptor Co-Activator 3

T: Testosterone

TAE: Tris-acetate-EDTA

TAM: Tamoxifen

TAMR: Tamoxifen Resistant

TAE: Tris/Acetic acid/EDTA

TDM-1: Trastuzumab Emtansine

TE: Tris/EDTA

TEB: Terminal End Bud

TEMED: Tetramethylethylenediamine

TF: Transcription Factor

TFF1: Trefoil Factor 1

TKI: Tyrosine Kinase Inhibitor

Tm: Melting temperature

TNBC: Triple Negative Breast Cancer

TR: Thyroid hormone Receptor

Tris-HCl: Tris-Hydrochloric Acid

TSG: Tumour Suppressor Gene

UCSC: University of California, Santa Cruz

UV: Ultra Violet

VDR: Vitamin D Receptor

X-gal: 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside

ZBTB16: Zinc Finger And BTB Domain Containing 16

ZF: Zinc Finger

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1.1 Normal breast

1.1.1 Mammary gland function

The mammary gland is an organ that exclusively develops fully in female mammals, which functions in the production and secretion of milk to provide nutrition for their progeny (Inman *et al.*, 2015, Rezaei *et al.*, 2016). Mouse models have been instrumental in our understanding of the function, structure and development of the human mammary gland (Macias and Hinck, 2012, Inman *et al.*, 2015, Yeh *et al.*, 2003). The mammary gland is a compound, branched, tubulo-alveolar gland, which can be described as both apocrine and merocrine (Rezaei *et al.*, 2016). Its main structure consists of epithelial cells that stem from the nipple, branching out into ducts within a fat pad comprised of a large number of adipocytes packed tightly together (Macias and Hinck, 2012) (Figure 1.1.1). This fat pad also contains vascular endothelial cells, fibroblasts and immune cells (Macias and Hinck, 2012). Once the gland is fully developed at puberty, it undergoes cyclical patterns of growth and involution, due to hormone changes during the menstrual cycle triggering proliferation, differentiation or apoptosis of cells (Inman *et al.*, 2015). The ability of the mammary gland to repeatedly undergo proliferation, differentiation and apoptosis can be explained via the presence of Mammary Stem Cells (MaSCs), which can self-renew and differentiate into the variety of cells required (Macias and Hinck, 2012, Yang *et al.*, 2016). Oestrogen and progesterone and their relevant receptors, Oestrogen Receptor alpha (ERα) and Progesterone Receptor (PR), are highly important for normal gland function, however the receptors are only

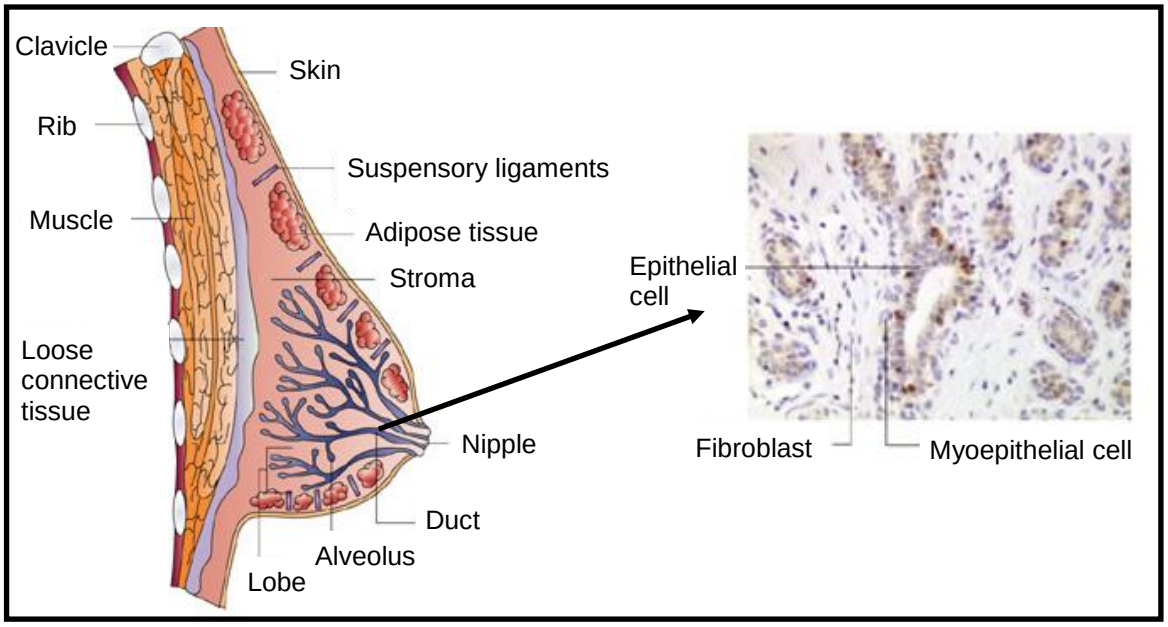


Figure 1.1.1 The general structure of the human mammary gland

The breast is a secretory gland, comprising of 15 – 20 lobes that stem from the nipple, branching out into ducts within a fat pad comprised of adipose tissue. A tissue section to display the ductal regions in more detail is included. A duct contains initially a layer of epithelial cells, followed by a further layer of myoepithelial cells, which function together to result in milk production and secretion.

(Figure is adapted from Ali and Coombes, 2002).

expressed by a small proportion of mammary epithelial cells (Margan *et al.*, 2016, Macias and Hinck, 2012).

During pregnancy, ductal branching vastly increases and epithelial end-bud structures are developed into alveoli, from which milk can be synthesised, mainly regulated by the action of progesterone and prolactin (Macias and Hinck, 2012). These luminal epithelial cells are the basis of the ductal structure bedded within fibroblast stroma, surrounded by an outer layer of contractible basal myoepithelial cells, which force milk out from the luminal cells (Macias and Hinck, 2012, Rezaei *et al.*, 2016). Additionally, during pregnancy adipose tissue within the fat pad decreases and vascularisation increases (Macias and Hinck, 2012, Rezaei *et al.*, 2016). Oxytocin release from the pituitary gland is triggered via stimulation of mechanoreceptors during nursing, initiating milk secretion via the nipple for lactation (Yang *et al.*, 2016, Rezaei *et al.*, 2016). Milk is comprised of water, proteins, lipids and carbohydrates as well as a variety of minerals and vitamins, and is required for the development and health of the new-born (Rezaei *et al.*, 2016). Prior to this ‘mature’ milk, in the initial days after birth the mammary gland produces colostrum, consisting of vast quantities of immunoglobulins, which is important in providing passive immunity for the baby (Rezaei *et al.*, 2016). Once milk production is no longer required, mass apoptosis occurs to allow the gland to regress back close to its original form (Macias and Hinck, 2012) .

1.1.2 Mammary gland development

Initial human mammary gland development occurs in both male and female embryogenesis (Inman *et al.*, 2015, Macias and Hinck, 2012). The embryo develops mammary lines within the first pregnancy trimester. This produces two placodes, which

develop through stages such as mammary bud and nipple sheath formation, to result in the rudimentary mammary gland structure that remains present at birth (Macias and Hinck, 2012). The Parathyroid Hormone-Related Protein (PTHLH) is highly important during this initial development to produce the rudimentary ductal system, and mutations to the gene encoding this protein (*Parathyroid Hormone 1 Receptor*, *PTH1R*) which impair its correct functioning lead to a condition termed Blomstrand Lethal Chondrodysplasia (BLC). BLC is a highly uncommon classification of dwarfism, where the embryo is unable to develop fully, including in the production of intact nipples, rudimentary mammary ducts or correct bone structure, and results in death prenatally or closely following birth (Macias and Hinck, 2012).

Breast development resumes in women during puberty, regulated through the action of hormones including Growth Hormone (GH), oestrogen, androgen, Insulin-Like Growth Factor-1 (IGF1) and progesterone (Ali and Coombes, 2002, Macias and Hinck, 2012). Hormone regulation, particularly oestrogen and androgen levels, is key for this developmental stage, demonstrated by the occurrence of gynecomastia in men when these levels are abnormal (Macias and Hinck, 2012). Normal pubertal mammary gland development involves the resumed growth of the rudimentary ductal epithelial cells into the surrounding fat pad to produce the fully developed organ (Figure 1.1.1), termed branching morphogenesis. This process is led by epithelial cells known as Terminal End Bud (TEB) structures that are strongly proliferative and resemble mesenchymal cells, which indicates that some Epithelial-to-Mesenchymal Transition (EMT) occurs (Inman *et al.*, 2015, Macias and Hinck, 2012, Yang *et al.*, 2016).

1.2 Breast Cancer

1.2.1 Epidemiology

Breast cancer (BCa) is currently recorded as the most highly prevalent cancer among women in the UK, constituting approximately 30 % of female cancer cases diagnosed (Cancer Research UK, 2014a, Siegel *et al*., 2018), with 1,735,350 new cases predicted to be diagnosed in 2018 in the USA alone (Siegel *et al.*, 2018). Although BCa survival rates have significantly increased in the last 10 years, Breast Cancer has been described as currently the second most common cause of cancer-related mortality in women in the UK (Cancer Research UK, 2014b) and USA (Siegel *et al*., 2018). In 2014 in the UK alone, there were 55,222 BCa diagnoses (Wu *et al.*, 2015), and this disease resulted in the deaths of 11,360 women and 73 men (Cancer Research UK, 2014b).

1.2.2 Molecular subtypes and grading

BCa is a highly heterogeneous disease, both within and between patients, therefore for the purposes of disease management, the identification of similarities between patients is highly important (Barnard *et al.*, 2015). Historically, BCas have been classified into four main molecular subtypes: Luminal A, Luminal B, HER-2 Enriched and Basal-Like. Tumours are classified according to the expression of Oestrogen Receptor alpha (ERα), Progesterone Receptor (PR) and Human Epidermal Growth Factor Receptor 2 (HER-2, or Erb-B2 Receptor Tyrosine Kinase 2, ERBB2) (Yersal and Barutca, 2014, Rakha and Green, 2016) (Table 1.2.1). These classifications can be used in combination with other factors to guide treatment options and to predict a patient's subsequent clinical response and prognosis (Prat *et al.*, 2015). However, at least three

Table 1.2.1 The four main classifications of Breast Cancer

Breast Cancer is classified according to the expression (+) or lack of expression (-) of Oestrogen Receptor Alpha (ERα), Progesterone Receptor (PR) and Human Epidermal Growth Factor Receptor 2 (HER-2). TNBC indicates Triple Negative Breast Cancer.

Subtype	ERα	PR	HER-2
Luminal A	+	+	-
Luminal B	+	+/-	+
HER-2-enriched	-	-	+
Basal-like/TNBC	-	-	-

additional subtypes have been recently identified: Claudin-Low, Molecular Apocrine and Normal Breast-Like (Prat *et al*., 2015, Badve *et al.*, 2011). In this section, the main features associated with each of these subtypes will be summarised, however these characteristics are not conclusive, and there is overlap between subtypes (Prat *et al.*, 2015).

1.2.2.1 Luminal A and B Breast Cancers

Luminal cancers are mainly identified by the expression of ERα encoded by the *Oestrogen Receptor 1* (*ESR1*) gene. Therefore these cancers are otherwise known as ERα-positive disease, and most BCas (approximately 70 %) are categorised into this subtype (Rakha and Green, 2016, Ali and Coombes, 2002). Cancers grouped into these classifications have an increased expression of genes associated with ERα and with the luminal epithelium (luminal-associated genes) hence the term ‘Luminal’ being used to describe these subtypes (Rakha and Green, 2016). Luminal A BCas are characterised by having a lower expression of several genes which promote cellular proliferation and are involved in cell cycle progression, such as *Marker of Proliferation Ki-67* (*MKI67*) and *Aurora Kinase A* (*AURKA*). Therefore, these cancers display reduced proliferation, as well as an elevated expression of some luminal-associated genes, notably including *Progesterone Receptor* (*PR*) and *Forkhead Box A1* (*FOXA1*), compared to luminal B BCas (Prat *et al.*, 2015). Luminal B cancers generally express HER-2 and express very little or no PR (Rakha and Green, 2016). Additionally, the Luminal A subtype contains fewer mutations within the genome than Luminal B (Prat *et al.*, 2015). These factors may explain the identification of Luminal A BCas across many studies to show, not only a better prognosis than Luminal B cancers, but the most favourable prognosis across all of the subtypes (Prat *et al.*, 2015, Bartmann *et al.*, 2017, Rakha and Green, 2016).

1.2.2.2 HER-2 Enriched Breast Cancers

HER-2 Enriched BCas are mainly characterised by an elevated expression of the *ERBB2* gene encoding HER-2 (due to amplification or over-expression) and its associated genes (Prat *et al.*, 2015, Rakha and Green, 2016). Additionally to this, HER-2-enriched cancers have an increased expression of some genes involved in cellular proliferation for instance *Growth Factor Receptor-Bound Protein 7* (*GRB7*), expression of luminal-associated genes including *ESR1* (encoding ERα) and a decreased expression of basal-associated genes for instance *Keratin 5* (*KRT5*) (Prat *et al.*, 2015). Additionally, this subtype contains the greatest number of mutations within the genome than the others (Prat *et al.*, 2015).

1.2.2.3 Basal-Like Breast Cancers

Basal-Like cancer is the most distinct BCa subtype (Prat *et al.*, 2015). It has greater levels of proliferation due to its increased expression of cellular proliferation genes, for instance *MKI67* (Rakha and Green, 2016, Prat *et al.*, 2015). Additionally, these tumours have an elevated expression of genes which are expressed by basal skin cells and the Basal-Like mammary cells, including keratins such as *KRT5* (Prat *et al.*, 2015, Rakha and Green, 2016). Furthermore, this subtype has an intermediate expression of HER-2-associated genes and a highly decreased expression of luminal-associated genes (Prat *et al.*, 2015). These cancers contain the next greatest number of mutations within the genome to HER-2 Enriched BCas (Prat *et al.*, 2015).

1.2.2.3.1 Triple Negative Breast Cancers (giving rise to Claudin-Low, Normal Breast-Like and Molecular Apocrine)

Triple Negative Breast Cancers (TNBCs) are cancers which do not express ERα, PR or HER-2, and are normally classified as Basal-Like tumours, however this is not always the case as these tumours are a very diverse group (Alluri and Newman, 2014, Badve *et al.*, 2011). TNBC has been found to be associated with *Breast Cancer 1, Early Onset* (*BRCA1*) mutations, patients with cancer onset at a younger age and cancers which are more aggressive, often with a poor prognosis (Prat *et al.*, 2015, Alluri and Newman, 2014). For instance, a recent study on 886 female patients over a time period of 53 months following Breast Cancer diagnosis, identified those with TNBC to have the lowest overall survival (Bartmann *et al.*, 2017).

Some TNBCs can be divided into the three additional molecular subtypes: Claudin-Low, Normal Breast-Like and Molecular Apocrine, however more alternative subtypes from this class have been identified (Yersal and Barutca, 2014, Badve *et al.*, 2011, Rakha and Green, 2016). Claudin-low BCas are attributed to a decrease in expression of genes responsible for cell-cell adhesive properties and the function of tight cell junctions such as *Claudin 3* (*CLDN3*) and *Claudin 4* (*CLDN4*), an increase in EMT genes and a higher proportion of cells displaying stem cell characteristics (Yersal and Barutca, 2014, Badve *et al.*, 2011). These cancers, like other TNBCs, tend to have a poor prognosis (Badve *et al.*, 2011).

Molecular Apocrine Breast Cancer (MABC) or Luminal Androgen Receptor is an additional sub-set of tumours within TNBC which express AR, and are driven by the AR pathway (Section 1.8.4) (Fioretti *et al*., 2014, Badve *et al.*, 2011, Rakha and Green, 2016).

However, some MABC tumours have been reported to be HER-2-Enriched. For instance, Lehmann-Che *et al.* demonstrated that patients with this form of the disease have aggressive tumours with an over-expression of HER-2 and/or Gross Cystic Disease Fluid Protein-15 (GCDFP15) (Badve *et al*., 2011, Lehmann-Che *et al*., 2013).

A proportion of TNBCs, which have been termed Normal-Breast Like, have been found to lack the Basal-Like gene expression patterns and instead contain cells that appear to behave as stromal and normal mammary epithelial cells (Badve *et al.*, 2011, Rakha and Green, 2016). Currently, it has not been possible to clearly characterise Normal-Like BCas, and therefore it is thought that diagnosis of this classification may be the result of tumour tissue samples containing a high level of contamination with non-cancerous mammary tissue (Hon *et al.*, 2016, Weigelt *et al.*, 2010, Yersal and Barutca, 2014).

1.2.3 Risk factors

There are a large number of established risk factors that are associated with BCa. The most significant is being female in gender, as demonstrated by the disease being diagnosed in approximately 140 times more women than men in 2014 (Cancer Research UK, 2014a). An increased risk of BCa in women has been associated with many factors, including: early age at menarche; nulliparity; late age of first pregnancy; shorter durations of breastfeeding in pregnancy; late onset of menopause; high Body Mass Index (BMI) in postmenopausal and low BMI in premenopausal women; familial risk; high levels of alcohol consumption; oral contraceptive use; geographical location; history of benign breast disease and use of menopausal Hormone Therapy (HRT) (Barnard *et al.*, 2015, McPherson *et al.*, 2000).

Familial risk has long been identified to be caused by Pathogenic Variants (PVs) in the *BRCA1* and *Breast Cancer 2, Early Onset* (*BRCA2*) gene loci (Miki *et al.*, 1994, Ford *et al.*, 1994, Ford *et al.*, 1998, Buys *et al.*, 2017), with *BRCA1* mutations associated with TNBC (Prat *et al.*, 2015). However, the development of Next Generation Sequencing (NGS) techniques has led to the identification of additional hereditary PVs in genes associated with an elevated BCa risk. A recent study tested 35,409 female BCa patients with a panel of 25 of cancer genes, including *BRCA1* and *BRCA2* (Buys *et al.*, 2017). They demonstrated that 9.3% of participants had the presence of at least one PV, and more than 50% of these PVs were in genes other than *BRCA1* and *BRCA2*, most notably in *Ataxia Telangiectasia Mutated* (*ATM*), *Checkpoint Kinase 2* (*CHK2*) and *Partner And Localiser of BRCA2* (*PALB2*). The authors suggest that using a panel of genes may help to stratify patients with a higher risk of developing BCa (Buys *et al.*, 2017).

Which risk factors are relevant and their degree of relevance can vary according to molecular subtype (Barnard *et al.*, 2015). Bernard *et al.* evaluated 38 studies investigating the association of established BCa risk factors with the Luminal A, Luminal B, HER-2 Enriched and Basal-Like subtypes. They demonstrated that the majority of these risk factors are most relevant to Luminal A BCa and that some factors vary in association patterns according to the subtype (Barnard *et al.*, 2015). A recent study evaluated the association of a variety of established BCa risk factors with ERα expression (Kerlikowske *et al.*, 2017). They statistically evaluated information from the mammography results of 1,279,433 women in the United States, obtained from the National Cancer Institute (NCI)-funded Breast Cancer Surveillance Consortium. The authors reported that the strength of association of several risk factors vary according to ERα status. For instance, an association between increasing BMI and BCa onset in pre-

and perimenopausal women was reported to be more significant in ERα-positive disease (Kerlikowske *et al.*, 2017).

1.2.4 Detection and monitoring

Different methods can be utilised for the detection and diagnosis of BCa including mammography, ultrasound, Magnetic Resonance Imaging (MRI) of the breast and biopsy of the tumour (PDQ® Adult Treatment Editorial Board, 2017). The introduction of regular mammography tests for women has resulted in early diagnosis to enable early intervention and prevent BCa-related deaths (Tilstra and McNeil, 2017), however there are some disadvantages associated with its use. For example, higher breast density can reduce its accuracy, and an increased frequency of mammography tests in women would be generally more useful in the identification of Ductal Carcinomas *in situ* (DCIS) (early stage BCa confined to the milk ducts) than invasive BCa (Tilstra and McNeil, 2017). Upon detection of BCa, ERα, PR and HER-2 expression in tumours is assessed using immunohistochemistry (IHC), with HER-2 testing being additionally confirmed with *in situ* Hybridisation (ISH) (Rakha and Green, 2016). This allows molecular classification of the tumour to determine the treatment options available. Additionally, microarray tools can also be used to diagnose several rare BCas, such as Mucoepidermoid Carcinoma, characterised by a translocation between chromosomes 11 and 19 resulting in the *Mucoepidermoid Carcinoma Translocated 1-Mastermind-Like Transcriptional Coactivator 2* (*MECT1-MAML2*) gene fusion (Rakha and Green, 2016).

The Marker Of Proliferation ki-67 (ki67) proliferation index is an additional tool to predict BCa prognosis, also evaluated via IHC (Rakha and Green, 2016, Penault-Llorca and Radosevic-Robin, 2017). The expression of Ki67, a nuclear protein that is altered

during the cell cycle and is most abundant during mitosis, can be used as a marker of proliferation (Penault-Llorca and Radosevic-Robin, 2017). The role of ki67 is unclear, but it has been suggested to have a direct role in cell division and ribosomal RNA (rRNA) synthesis. The ki67 proliferation index represents the percentage of cells tested which are positive for this marker, and can be used to determine whether a cancer is Luminal A or B (Penault-Llorca and Radosevic-Robin, 2017) (Section 1.2.2.1).

1.2.5 Treatment

Treatment for BCa is complex as it is such a heterogenous disease (treatment options are summarised in Figure 1.2.1). The most common treatment for localised DCIS BCa consists of surgical removal of the tumour combined with radiation and endocrine therapy (if suitable) (Section 1.5). This seemingly aggressive form of treatment for DCIS is used as a precaution to prevent it from developing into invasive cancer (Tilstra and McNeil, 2017). However, recently it has been suggested that DCIS is a risk factor instead of a precursor for invasive BCa, so a less aggressive treatment strategy could be more appropriate (Tilstra and McNeil, 2017). Additionally, there is often a need for adjuvant or neoadjuvant therapy to account for any micrometastases, and surgery is not an option for cases when the cancer has spread from its primary site (Ali and Coombes, 2002, Vorobiof, 2016). In these cases, endocrine therapies, which aim to block ERα activity, are often preferred for patients with metastatic ERα-positive Luminal disease. Endocrine therapies include anti-oestrogens, Aromatase Inhibitors (AIs) and Gonadotropin-Releasing Hormone agonists (GnRHas), which can be used either separately or together (Vorobiof, 2016, Ali and Coombes, 2002) (Section 1.5).

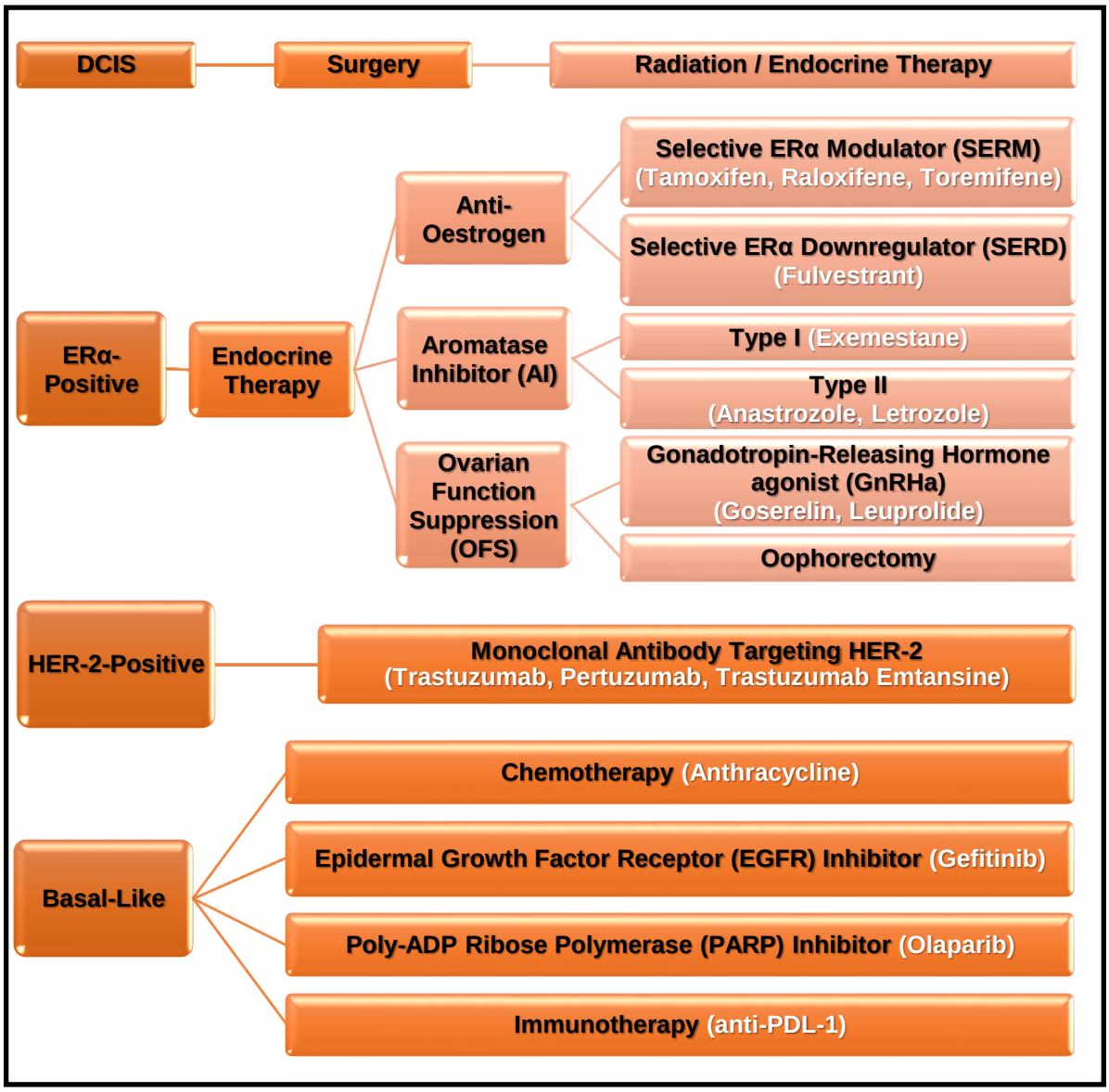


Figure 1.2.1 A summary of Breast Cancer treatment options

A schematic to represent the common treatment options selected for patients with different classifications of Breast Cancer: Ductal Carcinomas *in situ* (DCIS), Oestrogen Receptor alpha (ERα)-positive (Luminal cancers), Human Epidermal Growth Factor Receptor 2 (HER-2)-Positive (HER-2-Encriched) and Basal-Like.

The development of Trastuzumab (Herceptin), a monoclonal antibody that directly targets HER-2, has improved the prognosis for patients with HER-2-Enriched BCa (Vorobiof, 2016). This has resulted in the development of alternative monoclonal antibodies to target HER-2, including Pertuzumab (Perjeta) which is usually administered alongside chemotherapy and Trastuzumab, and antibody drug conjugates such as Trastuzumab Emtansine (TDM-1), which have been demonstrated to show benefits in many patients (Vorobiof, 2016). Currently, the irreversible HER-2/EGFR Tyrosine Kinase Inhibitor (TKI) drug Neratinib (HKI-272) is also being investigated and trialed as a potential treatment for HER-2-enriched BCa. Neratinib has shown promising results, but its use has been associated with negative side effects such as diarrhea and vomiting, so further research to reduce these complications is currently underway (Vorobiof, 2016, Ben-Baruch *et al.*, 2015).

Some Basal-Like TNBC patients respond well to chemotherapy such as Anthracycline, however a lack of response often indicates a very poor prognosis (Badve *et al.*, 2011). Basal-Like BCa patients may also benefit from drugs which target the Epidermal Growth Factor Receptor (EGFR) as this is normally over-expressed, and those with *BRCA1* mutations from treatment with Poly-ADP Ribose Polymerase (PARP) enzyme inhibitors (Yersal and Barutca, 2014). Additionally, recent advances in treatment for several cancers including bladder cancer has been through the use of immunotherapies, such as Anti-Programmed Death-Ligand 1 (anti-PDL-1) drugs. These are now being demonstrated through clinical trials to have a potential benefit for TNBC patients (Vorobiof, 2016, Pusztai *et al.*, 2016). Due to the heterogeneity observed in BCa disease, and the ongoing implementation of high-throughput genomic techniques which

could adjust the current disease molecular classifications, treatment is becoming increasingly individualised to patients (Rakha and Green, 2016).

1.3 Nuclear Receptors

1.3.1 The Nuclear Receptor family

Nuclear Receptors (NRs) are a family of proteins that function as ligand-dependent transcription factors (TFs) (Sever and Glass, 2013). The ligands involved in these pathways have the ability to pass through the cell membrane to interact intracellularly with their specific receptors (Sever and Glass, 2013). In humans, 48 NRs are currently recognised, including Oestrogen Receptors α and β (ERα and ERβ) (Section 1.4) and the Androgen Receptor (AR) (Section 1.7), and their roles as TFs regulate a large number of different functions, for instance cellular proliferation and metabolism (Sever and Glass, 2013, Maruthanila *et al.*, 2016, Pietri *et al.*, 2016). NRs have a common basic structure consisting of four-five main functional domains: an N-terminal whose sequence widely differs between NRs, often containing a domain termed Activation Function 1 (AF1) that mediates transcriptional activity; a second activation function site (Activation Function 2, AF2), a central DNA Binding Domain (DBD) partially comprising two Zinc Fingers (ZFs) and a Ligand Binding Domain (LBD), separated by a hinge region (Sever and Glass, 2013, Huang *et al*., 2010, Huss and Kelly, 2004, Maruthanila *et al*., 2016), which is displayed in Figure 1.3.1. NRs often have the ability to homodimerise with each other. For instance, the Retinoid X Receptor (RXR) has been demonstrated to interact with the Peroxisome Proliferator-Activated Receptor (PPAR), Retinoic Acid Receptor (RAR), Vitamin D Receptor (VDR), and Thyroid hormone Receptor (TR) (Dawson and Xia, 2012).

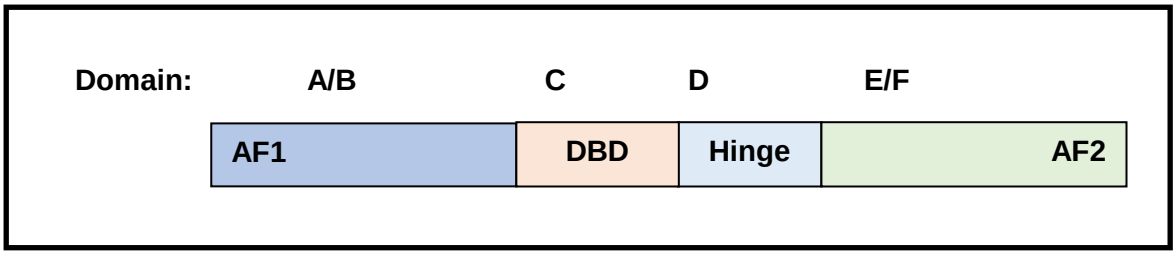


Figure 1.3.1: The general structure and domains in Nuclear Receptors

The general Nuclear Receptor (NR) structure is comprised of an A/B domain which includes an Activation Function 1 (AF1) that mediates transcriptional activity, a C domain which includes DNA Binding Domain (DBD) partially comprising two Zinc Fingers, and a E/F domain with a Ligand Binding Domain (LBD), separated by a D domain (Hinge).

(Figure is adapted from Huss and Kelly, 2004).

1.3.2 Type I Nuclear Receptor activation pathway

NRs can be organised into four classifications according to their activation pathways (Figure 1.3.2). The Oestrogen Receptors ERα and ERβ, PR and AR belong to the Type I NR category (Sever and Glass, 2013) (Figure 1.3.2a). In the absence of ligand, Type I NRs are held in the cytoplasm in an inactive state bound to chaperone proteins and co-repressors (co-factors that bind to TFs and repress activity). Once activated by ligand binding, the NR undergoes a conformational change, releasing it from this complex (Pietri *et al.*, 2016, Sever and Glass, 2013). This reveals the nuclear localisation domain and thereby allows the receptor to translocate to the nucleus and to homodimerise. Following this, the NR dimer binds to specific DNA sequences termed Hormone Response Elements (HREs) located in the promoter or enhancer regions of genes, resulting in their transcriptional activation (Figure 1.3.2a) (Sever and Glass, 2013, Brooke and Bevan, 2009, Dehm and Tindall, 2007, Maruthanila *et al*., 2016). It produces this effect by binding via two ZF motifs and via the recruitment of co-activators (proteins that enhance transcription factor activity), such as Amplified in Breast Cancer 1 (AIB1, otherwise known as p160 Steroid Receptor Co-Activator 3, SRC3) and the general transcription machinery (Lahusen *et al.*, 2009, Sever and Glass, 2013).

Type II receptors include the Thyroid Hormone Receptor. This class of NRs are located constantly within the nucleus of the cell bound to their HREs, however prior to ligand binding they are held in an inactive state bound within a complex including co-repressors (Figure 1.3.2b). Upon ligand binding, co-repressors release from this complex and co-activators bind in exchange, initiating transcriptional activation (Sever and Glass, 2013). Type III NRs have an activation pathway that strongly resembles that of the Type

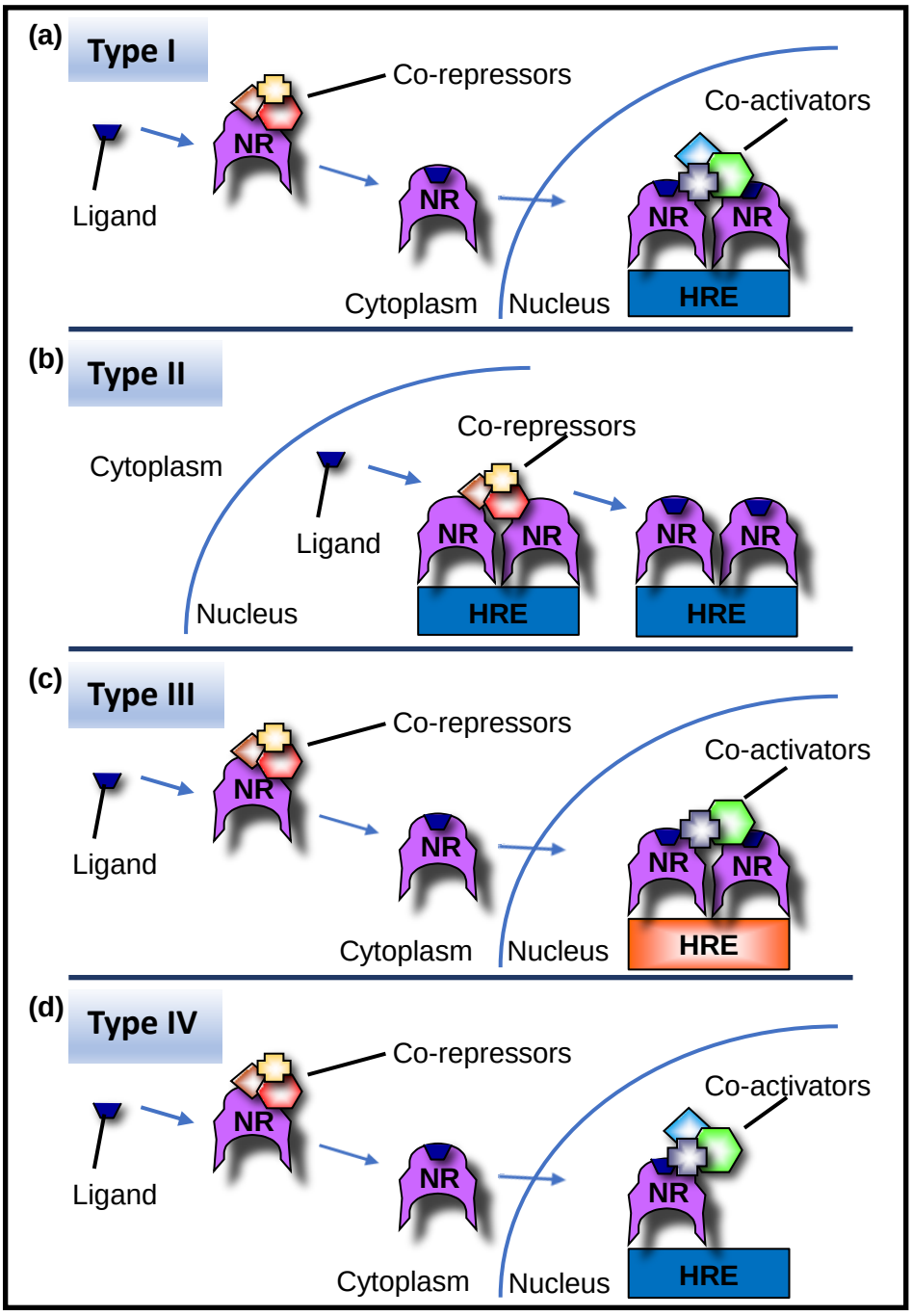


Figure 1.3.2 Alternative Nuclear Receptor pathways

Nuclear Receptors (NRs) have 4 different activation pathways. (a) Type I NRS are held in an inactive state in the cytoplasm bound to co-repressors. Following ligand binding, the NR undergoes a conformational change, releasing it from this complex and translocates to the nucleus. It interacts as a dimer to Hormone Response Elements (HREs) and recruits co-factors to activate gene transcription. (b) Type II NRs are held in an inactive state in the nucleus bound to their HREs and co-repressors. Upon ligand binding, co-repressors are released and co-activators bind, initiating transcriptional activation. (c) Type III NRs have an activation pathway that resembles Type I, but their HREs have an alternative structure. (d) Type IV NRs interact as single monomer units to half of a HRE site (Sever and Glass, 2013).

I class, however their HREs have an alternative structure (Figure 1.3.2c) and Type IV NRs interact as monomers to half of a HRE site (Figure 1.3.2d) (Sever and Glass, 2013).

1.4 Oestrogen Receptor alpha

1.4.1 The Oestrogens Receptors: structure and function

Oestrogens are sex hormones, which like other steroid sex hormones, normally exist in the bloodstream in a complex with Sex Hormone-Binding Globulin (SHBG) (Chuffa *et al.*, 2017, Hewitt *et al.*, 2016). Oestrogens are important in the correct functioning of the female reproductive system, such as the development of the mammary gland (Section 1.1.2), as well as for other non-related functions, including the regulation of bone density and the cardiovascular system (Ali and Coombes, 2002, Knowlton and Lee, 2012, Chuffa *et al.*, 2017). In premenopausal women, oestrogens are predominantly synthesised in the ovaries, as well as in the corpus luteum and placenta when relevant, and released into the bloodstream. Oestrogens are also produced to a lesser degree in other organs, for instance the heart and brain (Cui *et al.*, 2013). In postmenopausal women, however, the ovaries no longer produce oestrogens and these extragonadal oestrogen sources become more vital. In these women, oestrogens are produced to function locally, for instance by adipose tissue (Cui *et al.*, 2013). The main oestrogens produced in women are Oestrone (E1), 17-β-Oestradiol (E2), and Oestriol (E3). The most highly abundant circulating form of oestrogen as well as the most potent is E2 (Bean *et al.*, 2014, Speirs and Walker, 2007). E1 becomes more important postmenopause and E3 throughout pregnancy (Cui *et al.*, 2013).

E2 function is mainly applied through the action of two NRs: Oestrogen Receptors α and β (ERα and ERβ), encoded by *ESR1* and *Oestrogen Receptor 2* (*ESR2*) genes respectively (Bean *et al.*, 2014, Speirs and Walker, 2007). These genes are positioned in alternative chromosomes, *ESR1* at 6q25.1 and *ESR2* at 14q22-24 (Speirs and Walker, 2007). The structures of ERα and ERβ both follow the common basic NR structure (Figure 1.3.1). A comparison of the sequences of the two receptors displays a highly conserved DBD, but the other regions are less conserved (Figure 1.4.1). For ERα functioning, AF2 activation occurs following oestrogen binding to the receptor, whereas AF1 activation is regulated by ligand-independent phosphorylation of the receptor. These activation domains can function either independently or together (Ali and Coombes, 2002, Huang *et al.*, 2010, Huss and Kelly, 2004). AF2 is key for ERα action in breast epithelium, but in other tissue types, such as the uterus, AF1 activation is more important for ERα function. ERα is widely known as more important than ERβ in BCa and has been shown to facilitate BCa onset and progression (Ali and Coombes, 2002) (Section 1.4.3).

1.4.2 The ERα pathway and signalling

Most oestrogen signalling in mammary tissue occurs through ERα via the classical ERα signalling pathway, which can be categorised as a Type I NR activation pathway (Sever and Glass, 2013) (Figure 1.4.2). This enables it to interact via its DBD with Oestrogen Response Elements (EREs), which contain the specific consensus motif: GGTCAnnnTGACC. This motif consists of a palindromic series of bases, inversely replicated and connected via 3 variable bases (Hewitt *et al.*, 2016). It has been demonstrated that FOXA1 is vital as a pioneer factor in facilitating interactions between ERα and EREs (Hewitt *et al.*, 2016, Hurtado *et al.*, 2011).

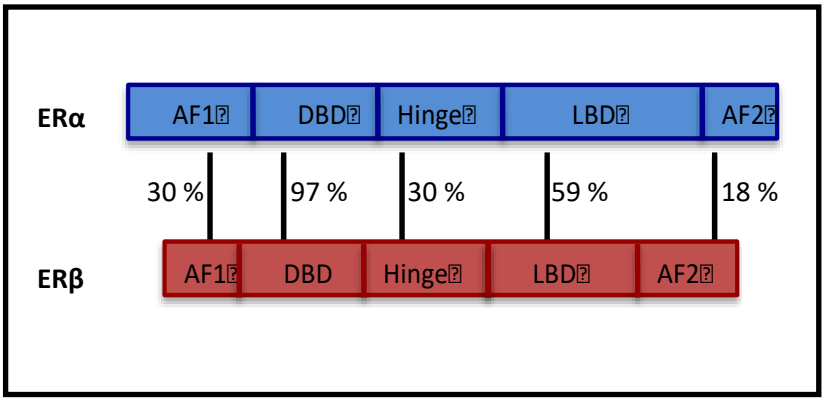


Figure 1.4.1: Homology comparison of the functional domains of Oestrogen Receptors alpha and beta

The functional regions of the proteins are displayed: Activation Function 1 (AF1); DNA Binding Domain (DBD); hinge; Ligand Binding Domain (LBD); and Activation Function 2 (AF2). The percentage of similarity between the amino acid sequences of these five functional regions of the two proteins is also indicated

(Figure is adapted from Speirs and Walker, 2007).

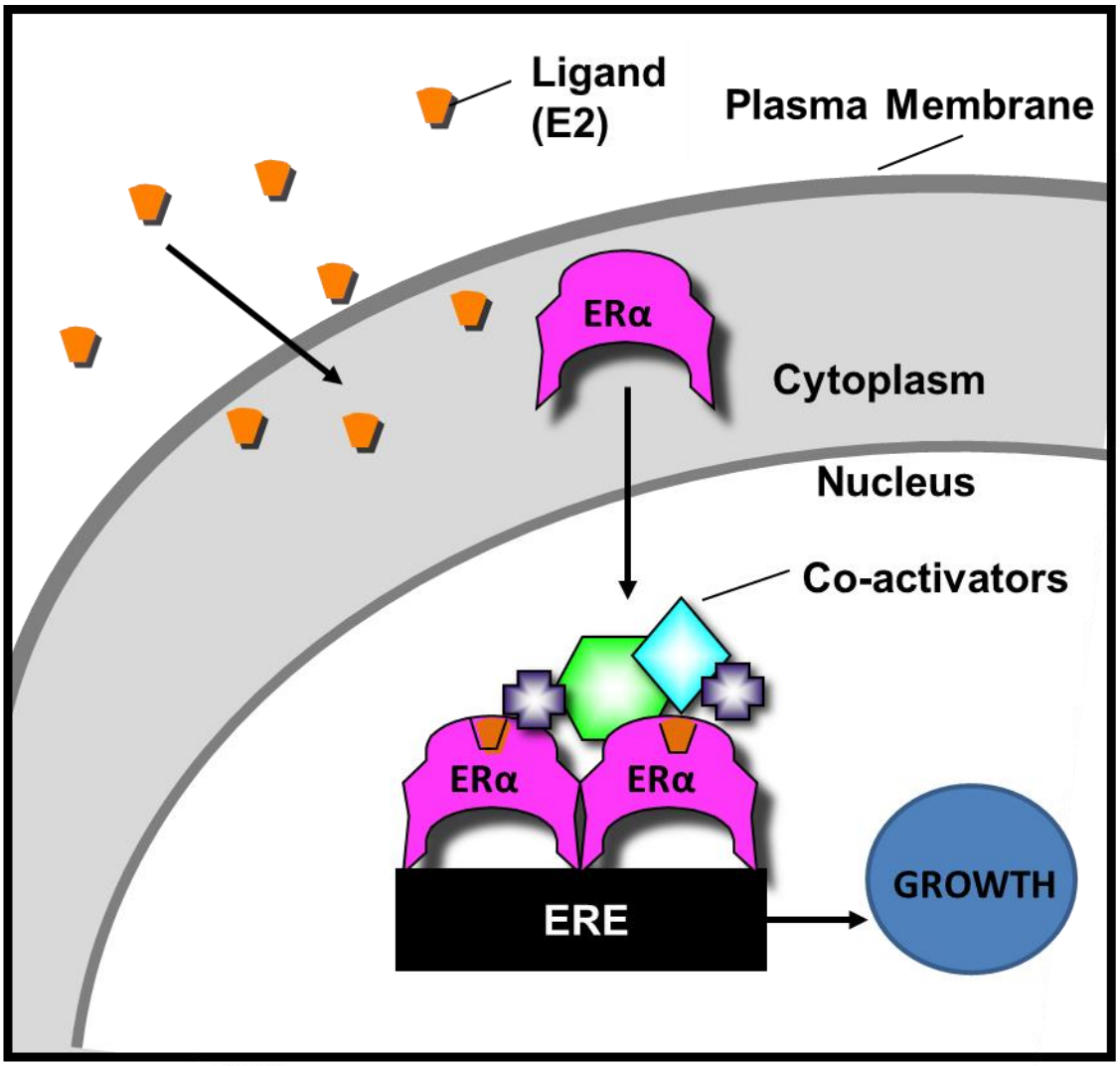


Figure 1.4.2 The Oestrogen Receptor alpha pathway of gene regulation

The classical oestrogen signalling pathway is ligand-dependent. Following ligand (most commonly 17β-Oestradiol, E2) binding, Oestrogen Receptor α (ERα) activates gene expression via a direct interaction of the ERα dimer to Oestrogen Response Elements (EREs) as part of a complex with co-activators (Musgrove and Sutherland, 2009, Hewitt *et al.*, 2016).

ERα target gene regulation can additionally occur via other mechanisms. For instance, it has been demonstrated to regulate gene expression through the receptor 'tethering' to other DNA-bound TFs (for example Activator Protein 1, AP-1), thereby bypassing the requirement for EREs (Hewitt *et al.*, 2016, Heldring *et al.*, 2011). A further example is through rapid non-transcriptional signalling, often described as 'non-genomic' (Lipovka and Konhilas, 2016, Hewitt *et al.*, 2016). This involves ERα interacting directly with specific proteins upon ligand activation, for instance its activation of the Mitogen-Activated Protein Kinase (MAPK) signalling pathway (Lipovka and Konhilas, 2016, Chuffa *et al.*, 2017). Some rapid E2 effects have been attributed to G-Protein-Coupled Oestrogen Receptor 1 (GPER1) (Liu *et al.*, 2015, Lipovka and Konhilas, 2016) and ERα36, a variant of ERα that was identified in 2005 and given its name due to its 36 KDa size (Wang *et al.*, 2005), which are both also associated with the cell membrane (Chuffa *et al.*, 2017). Additionally, ERα signalling can occur ligand-independently, and this is hypothesised to be via phosphorylation of ERα directly or of other proteins in its normal complex, such as co-activators (Hewitt *et al.*, 2016).

1.4.3 Endocrine sensitive Breast Cancer

It is widely accepted that hormones are important in the development and progression of the majority of cases of BCa. As stated previously (Section 1.2.2), approximately 70 % of BCas are ERα-positive (Luminal cancers), and are consequently dependent upon oestrogens for growth, providing insight into why this disease is much more common in women than men (Keen and Davidson, 2003, Rakha and Green, 2016, Ali and Coombes, 2002). This effect was first indicated in 1896, when George Beatson described his findings that the removal of the ovaries from three female BCa patients with inoperable tumours resulted in disease remission (Beatson, 1896). ERα is now widely

accepted to be a major factor in driving cancer progression in ERα-positive disease (Chuffa *et al.*, 2017). Therefore, these cancers are often termed endocrine sensitive, and therapies for these patients often aim to block ERα activity (Section 1.5).

ERα drives BCa progression via the regulation of various cellular processes. One example is through its regulation of kinase cascades. For instance, a recent study demonstrated that treatment with Cryptotanshinone (CPT) inhibits BCa cell proliferation and the Phosphatidylinositol-4,5-Bisphosphate 3-Kinase/AKT Serine/Threonine Kinase/Mammalian Target Of Rapamycin (PI3K/Akt/mTOR) signalling pathway, but that this effect is exclusive to endocrine sensitive cells with a functioning ERα (Pan *et al.*, 2017). Additionally, ERα promotes BCa progression through its regulation of proteins involved in apoptosis, such as its downregulation of the pro-apoptotic factor Prostate Apoptosis Response 4 (PAR-4) (Casolari *et al.*, 2011).

1.4.4 Oestrogen Receptor beta

ERβ has some differing functions compared to ERα, for instance it is not key for cellular proliferation in the mammary gland, but it is however involved in oestrogenic effects in the correct functioning of the immune, cardiovascular and nervous systems (Warner *et al.*, 2017). Also by contrast to ERα, ERβ has been suggested to act as a Tumour Suppressor Gene (TSG) in BCa (Warner *et al.*, 2017). It has been demonstrated to produce an anti-proliferative effect on BCa cell growth and to promote apoptosis, and ERβ levels have been reported to decrease in BCa, indicating it to have an opposing role to that of ERα (Huang *et al.*, 2014, Chuffa *et al.*, 2017, Hartman *et al.*, 2009, Williams *et al.*, 2008). However, some research has produced conflicting results. For example, it has been demonstrated that ERβ over-expression in endocrine sensitive MCF7 and T47D

BCa cells resulted in ERβ heterodimerising with ERα, and yet there was no significant negative effect on ERα signalling or cell proliferation (Jonsson *et al.*, 2014). Additionally, it has been reported that ERβ expression does not correlate with a favourable prognosis in endocrine sensitive patients (Tan *et al.*, 2016).

1.5 Targeting ERα in therapy

As discussed earlier (Section 1.2.5), endocrine therapies, which aim to block ERα activity and/or halt the synthesis of E2, are often preferred for patients with metastatic ERα-positive luminal disease. A schematic of these therapies is displayed in Figure 1.2.1. Endocrine therapies can be divided into three main categories: anti-oestrogens, Aromatase Inhibitors (AIs) and Gonadotropin-Releasing Hormone Agonists (GnRHas), which can be used either separately or together (Vorobiof, 2016, Ali and Coombes, 2002). Endocrine therapies have been shown to significantly increase disease-free and overall survival in BCa patients (Vorobiof, 2016).

1.5.1 Anti-oestrogens

The aim of anti-oestrogens is to target ERα to block its action. Anti-oestrogens can be grouped into two subclasses: Selective ERα Modulators (SERMs) such as Tamoxifen, Raloxifene and Toremifene and Selective ERα Downregulators (SERDs) for instance Fulvestrant (Kaklamani and Gradishar, 2017, Cauley *et al.*, 2001). SERMs can be described as nonsteroidal cytostatic agents, which inhibit ERα by competitively binding as ligands, and have tissue and target gene-specific effects (Kaklamani and Gradishar, 2017, Martinkovich *et al.*, 2014). SERM binding to ERα in tumour and mammary cells results in dimerisation of the receptor as in the normal signalling pathway, however the

SERM promotes the formation of an inhibitory nuclear complex that reduces the levels of DNA synthesis and oestrogen signalling (Kaklamani and Gradishar, 2017). The consequences of SERMs with binding ERα vary in a tissue specific manner, and this additionally varies between different SERMs (Kaklamani and Gradishar, 2017). SERMs have the ability to function as antagonists in BCa cells and as partial agonists in various other tissues, for instance in the bone or endometrium (Kaklamani and Gradishar, 2017). This combined ability to produce agonistic and antagonistic effects is produced by many factors which differ between SERMs, including: variations in the affinity of a SERM for each of the receptor's subtypes (ERα and ERβ); alterations in the expression of these subtypes and different co-factors between tissues; and the resulting conformational changes to ERα, following SERM binding, can affect co-factor interactions that have gene specific regulatory effects (Martinkovich *et al.*, 2014).

Currently, the most widely used anti-oestrogen in all stages of BCa treatment is the SERM Tamoxifen (TAM), which was the first extensively used anti-oestrogen to be introduced more than 30 years ago (Vorobiof, 2016, Lumachi *et al.*, 2015). SERMs have also been displayed to decrease the risk of BCa onset in women that are at a high risk of contracting the disease, and therefore have the potential to be useful as preventative therapies. This effect has been demonstrated using TAM, and it was approved for administration for this use in 1998. However this was not found to be beneficial for long term usage, as side effects associated with prolonged administration of this drug included endometrial stimulation and an increased risk of development of endometrial cancer (Fisher *et al.*, 1998). Raloxifene has also been found to reduce BCa risk and, unlike TAM, does not appear to promote endometrial cancers, being approved for this use in 2007 by

the U.S. Food and Drug Administration (FDA) (Cauley *et al.*, 2001, Martinkovich *et al.*, 2014, Waters *et al.*, 2012).

SERDs, in comparison, are described as pure ERα antagonists. They act solely as anti-oestrogens for ERα by blocking and reducing its activity, advancing the speed of its degradation, and inhibiting oestrogen-stimulated cellular proliferation (Kaklamani and Gradishar, 2017, Lumachi *et al.*, 2015). A SERD that is commonly prescribed to patients with endocrine sensitive disease is Fulvestrant (FULV). FULV competitively interacts with ERα in the same fashion as TAM but with a vastly increased affinity (100-fold), and as a pure ERα antagonist it does not stimulate cell growth in endometrial tissue (Kaklamani and Gradishar, 2017, Martinkovich *et al.*, 2014). Post ERα binding, FULV halts the transcription of genes regulated by the ERα pathway, by stopping it from dimerising and blocking its nuclear translocation, as well as by advancing the degradation of the receptor via proteasomes (Kaklamani and Gradishar, 2017, McDonnell *et al.*, 2015). FULV has proved valuable for use with advanced metastatic and TAM-resistant disease. However, its use has been limited in BCa as it is administrated through an intramuscular injection, resulting in patients experiencing a ‘lag’ in time for it to reach a consistent concentration. Therefore, work is being conducted to produce novel SERDS that are administered orally (Martinkovich *et al.*, 2014, McDonnell *et al.*, 2015, Xiong *et al.*, 2017).

1.5.2 Gonadotropin-Releasing Hormone agonists and Oophorectomy

Another method to inhibit endocrine sensitive breast cancer is to block the synthesis of E2, and the method by which this is done is dependent upon whether the patient is pre- or post- menopause. In premenopausal women, E2 (and progesterone) synthesis predominantly occurs in the ovaries during follicular development, regulated by

the action of the pituitary gonadotrophins Follicle-Stimulating Hormone (FSH) and Luteinizing Hormone (LH) whose production is regulated by Gonadotropin Releasing Hormone (GnRH) (Ali and Coombes, 2002, Lumachi *et al.*, 2015, Nourmoussavi *et al.*, 2017, Kaklamani and Gradishar, 2017). Therefore, Ovarian Function Suppression (OFS) can be utilised for BCa treatment in these patients to vastly decrease E2 levels (Lumachi *et al.*, 2015, Nourmoussavi *et al.*, 2017). This can be achieved through surgical removal of the patient's ovaries (oophorectomy), but is more commonly conducted now using GnRH agonists (GnRHas) such as Goserelin and Leuprolide (Keen and Davidson, 2003, Lumachi *et al.*, 2015, Vorobiof, 2016).

GnRHas work by reducing LH production and thereby halting follicular activity and the ovarian synthesis of oestrogens (Ali and Coombes, 2002). A recent review evaluated many studies of the use of OFS in premenopausal BCa patients in combination with other endocrine therapies (Nourmoussavi *et al.*, 2017). They found that in low-risk ERα-positive patients, OFS did not tend to improve patient outcomes over the use of TAM alone (Nourmoussavi *et al.*, 2017). However in higher-risk patients (i.e. those who have already undergone chemotherapy or are less than 35 years old) the use of OFS with TAM or AIs (Section 1.5.3) in patients resulted in 4.5-7.7 % less disease relapse than using solely TAM (Nourmoussavi *et al.*, 2017). However, the disadvantages associated with the administration of GnRHas in this way include the increase in cost and that early menopause has been linked to a risk of premature mortality in the long run, for instance through a higher risk of ischaemic heart disease (Nourmoussavi *et al.*, 2017, Lokkegaard *et al.*, 2006, Svejme *et al.*, 2012).

1.5.3 Aromatase Inhibitors

In postmenopausal women, E2 production no longer occurs in the ovaries and synthesis of E2 in peripheral, extra-gonadal tissues becomes the main source of E2 (Nourmoussavi *et al.*, 2017, Patani and Martin, 2014). This E2 is synthesised from androgens mediated via the action of aromatase, a steroid hydrolase cytochrome P450 enzyme, mainly through the synthesis of E1 from androstenedione and E2 from testosterone (Section 1.7.3) (Kaklamani and Gradishar, 2017, Patani and Martin, 2014). This method of biosynthesis has long been suggested to be exploited in BCa by the production of high quantities of aromatase in tumour cells (Harada, 1997). Therefore, in postmenopausal BCa patients, Aromatase Inhibitors (AIs) are the most commonly administered treatment, which block aromatase activity and therefore reduce E2 synthesis, thus decreasing circulating oestrogen levels and E2 synthesised within the tumour (Kaklamani and Gradishar, 2017). This from of treatment is commonly described as oestrogen deprivation (Patani and Martin, 2014). This endocrine therapy is not appropriate for premenopausal women, as in these patients AIs result in an elevated production of gonadotrophins, and so raise circulating oestrogen levels (Lumachi *et al.*, 2015).

AIs can be classified into two differing subtypes, commonly referred to as Types I and II (Kaklamani and Gradishar, 2017). Type I AIs are steroidal and include Exemestane. These directly compete with endogenous substrates to irreversibly bind aromatase and therefore result in its irreversible inactivation, even after administration of this treatment has finished (Patani and Martin, 2014, Kaklamani and Gradishar, 2017). Type II AIs, for instance Anastrozole and Letrozole, however, are non-steroidal and reversibly bind aromatase to halt E2 production (Patani and Martin, 2014).

1st and 2nd generation AIs had problems with their specificity and resulted in some interference with the activity of additional steroid hydroxylases (Dowsett *et al.*, 1990, Patani and Martin, 2014). 3rd generation AIs however, the most commonly used of which are Anastrozole, Exemestane and Letrozole, have a much higher specificity for aromatase and an improved efficacy (Vorobiof, 2016, Patani and Martin, 2014). However, disadvantages can still be associated with the administration of AIs, such as possible elevated bone resorption during treatment, meaning an elevated chance of bone fractures in patients (Kaklamani and Gradishar, 2017).

1.6 Endocrine resistance

Although endocrine therapies have proved highly successful in BCa treatment, many patients experience resistance to treatment termed endocrine resistance, either initially (*de novo*, or primary) or developed during the course of therapy (acquired or secondary) and relapse, often resulting in advanced metastatic disease (Kaklamani and Gradishar, 2017, Vorobiof, 2016, Reinert *et al.*, 2017). *De novo* resistance is normally described as when a patient relapses within 2 years when endocrine therapy is used as an adjuvant treatment or 6 months when the therapy is used as first-line (Reinert *et al.*, 2017). Resistance is therefore normally described as acquired when a patient experiences relapse post these time periods (Reinert *et al.*, 2017). It is currently estimated that 20-30 % of BCa patients will experience endocrine resistance, and many mechanisms by which this occurs have been described (Vorobiof, 2016), several of which are outlined in this section.

In the majority of TAM resistance cases ERα expression is maintained, and some patients have responded to alternative endocrine therapies, such as FULV (Osborne,

1998, Howell *et al.*, 1995, Howell *et al.*, 1996, Schiff *et al.*, 2003). This indicates that ERα is still driving cancer growth, but its regulation has been modulated. In these endocrine resistant cases, many studies have suggested that TAM has begun to stimulate rather than inhibit cancer progression, so a rapid detection of resistance development is much needed (Schiff *et al.*, 2003). Additionally, analysis of 6 AI-resistance cell line models developed using the same method resulted in multiple resistance mechanisms, sometimes within the same model, indicating that endocrine resistance in BCa is complex and can alter in patients, and thereby could be difficult to target (Hayashi and Kimura, 2015). Therefore, the endocrine resistance mechanisms outlined in this section often overlap, as demonstrated in Figure 1.6.1.

1.6.1 ERα mutations

Mutations to the *ESR1* gene encoding ERα are highly uncommon in early stages of BCa (Alluri *et al.*, 2014, Reinert *et al.*, 2017), with a recent study conducting whole exome sequencing on *ESR1* in different BCa tumours identifying no mutations in the primary ERα-positive tumour samples (Yanagawa *et al.*, 2017). However, *ESR1* mutations were identified in more advanced forms of the disease and are believed to promote endocrine resistance (Kaklamani and Gradishar, 2017, Ma *et al.*, 2015, Angus *et al.*, 2017). For example, Yanagawa *et al.* detected 6 different *ESR1* mutations (3 being novel) in 5 out of 47 recurrent tumours from patients with ERα-positive metastatic disease undergoing endocrine therapy (Yanagawa *et al.*, 2017). A schematic to represent the commonly occurring mutations in advanced BCa is displayed in Figure 1.6.2.

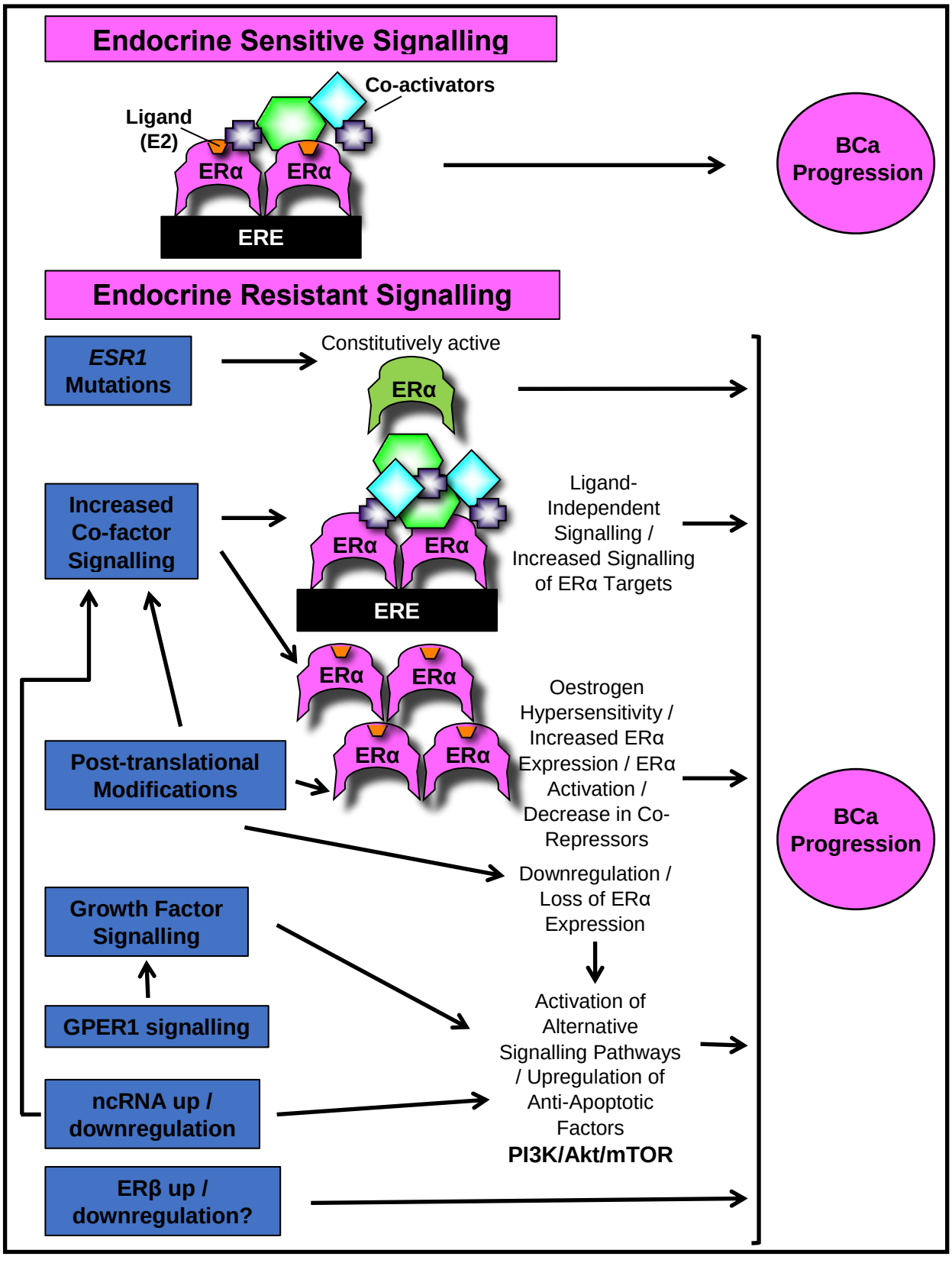


Figure 1.6.1 The overlap of mechanisms for endocrine resistance in Breast Cancer (BCa)

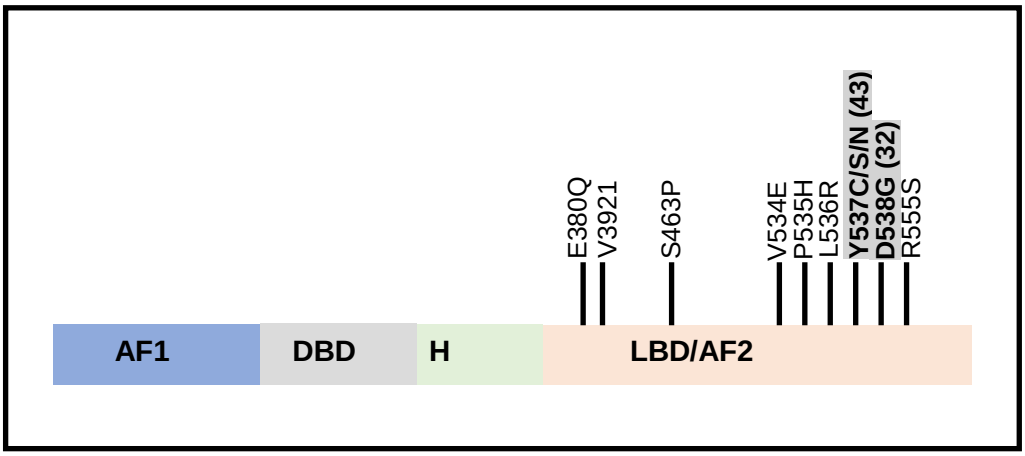


Figure 1.6.2 The most common mutations to the *ESR1* gene found in advanced Breast Cancer following endocrine therapy

A schematic representation of the *Oestrogen Receptor 1* (*ESR1*) gene encoding the Oestrogen Receptor alpha (ERα) with the locations of mutations that have been most commonly identified in advanced disease indicated. The two mutations that occur in the highest frequencies are highlighted (grey). The main structural domains are indicated: Activation Functions 1 and 2 (AF1/2), the DNA Binding Domain (DBD), the Hinge (H) domain containing sequences important for the receptor dimerization and nuclear localization and the Ligand Binding Domain (LBD).

(Figure is adapted from Angus *et al*., 2017 and Ma *et al*., 2015)

Often these *ESR1* mutations alter the structure/function of the LBD to constitutively activate ERα. For instance, Robinson *et al.* displayed that 6 out of 11 patients with ERα-positive metastatic BCa had *ESR1* mutations that altered the structure of the ERα LBD, resulting in the receptor becoming constitutively active and therefore able to function ligand-independently (Robinson *et al.*, 2013). Additionally, in a separate study, ERα LBD mutations were identified in 17.5 % of samples obtained from 80 patients with ERα-positive metastatic disease, mainly resulting in a production of the ERα agonist conformation to promote its ligand-independent activity and decrease anti-oestrogen efficacy (Toy *et al.*, 2013). Y537S and D538G mutations, which have also been demonstrated to produce a constitutive activation of the ERα, appear to be the most commonly occurring mutations (Figure 1.6.2) (Fanning *et al.*, 2016, Reinert *et al.*, 2017). Investigations into treatments that can target mutant forms of ERα are underway, including the novel SERD AZD9496 (Weir *et al.*, 2016), for which a clinical trial for its first use in patients is currently being conducted to assess the correct dosage, safety, tolerability, pharmacokinetics and biological activity, as compared for patients with and without *ESR1* mutations (ClinicalTrials.gov Identifier: NCT02248090).

1.6.2 Oestrogen hypersensitivity and over-expression of co-factors

Altered expression or activity of ERα co-regulators can promote endocrine resistance (Kaklamani and Gradishar, 2017). Co-factors are highly involved in regulating the transcriptional activity of ERα and its signalling pathway. These co-factors include a group of highly researched transcriptional co-activators, the p160 Steroid Receptor Co-activator (SRC) family consisting of SRC1, SRC2 and SRC3. The over-expression of these factors has been displayed in several human cancers, including BCa (Xu *et al.*, 2009). Increased levels of co-activators have been linked to endocrine resistant disease,

and appears to hypersensitise the pathway. For instance, it has been demonstrated that in patients treated with TAM post-surgery, increased levels of SRC3 (otherwise known as Amplified in Breast Cancer-1, AIB1) were significantly associated with a decreased chance of disease free survival and increased chance of endocrine resistance (Osborne *et al.*, 2003). The role of SRC3 in endocrine resistance has been demonstrated in other studies, including by Alkner *et al.* who found its elevated expression in Metachronous Contralateral Breast Cancer (a subsequent tumour which has developed over 6 months post original BCa diagnosis in the other breast) following past endocrine therapy with TAM (Alkner *et al.*, 2016). The involvement of various other co-activators in endocrine resistant BCa have been indicated, such as Homeobox B7 (HOXB7) (Jin *et al.*, 2015) and Coactivator-Associated Arginine Methyltransferase 1 (CARM1) (Hiken *et al.*, 2016). The method via which oestrogen hypersensitivity and co-factor over-expression occurs appears to be via post-translational modifications (Section 1.6.3).

1.6.3 Post-translational modifications

Post-translational modifications (e.g. phosphorylation, methylation and ubiquitination) of ERα and/or its co-regulator proteins and their encoding genes are important in BCa development and progression. They have been demonstrated to alter ERα signalling and thereby can affect the response to treatment with endocrine therapies and potentially promote the onset of resistance (Kaklamani and Gradishar, 2017, Abdel-Hafiz, 2017, Hayashi and Kimura, 2015).

1.6.3.1 Post-translational modifications of ERα and/or its co-regulators

Hayashi and Kimura (2015) described the phosphorylation of ERα resulting in ligand-independent ERα activity as a potential endocrine resistance mechanism, which has been supported by several studies. For instance, one group produced a set of oestrogen-deprived cell line derivatives of MCF7 endocrine sensitive cells (to mimic AI-resistance) that were stably transfected with an ERE-Green Fluorescent Protein (GFP) reporter (Fujiki *et al.*, 2014). Despite oestrogen deprivation, half of these resistance models still demonstrated strong ERα activity, as well as displaying an elevated expression of ERα and several of its associated target genes. They discovered an increase in phosphorylation of ERα on the amino acid residue Ser167 and Protein Kinase B (PKB, more commonly known as Akt) on Thr308, suggesting that the Akt signalling pathway was key in this resistance mechanism (Fujiki *et al.*, 2014). Work conducted by other groups has supported this mechanism (Campbell *et al.*, 2001, Yamashita *et al.*, 2005).

1.6.3.2 Post-translational modifications that directly affect gene expression

Work conducted by Yu *et al*. has suggested the involvement of MYST Histone Acetyltransferase (Monocytic Leukemia) 3 (MYST3), a protein which functions in histone acetylation, in epigenetic activation of the *ESR1* gene. They observed that *MYST3* gene over-expression was associated with a poorer prognosis in endocrine sensitive BCa, and demonstrated the ability of MYST3 to interact with the proximal promoter of *ESR1* and enhance expression of the receptor (Yu *et al.*, 2017). Therefore, this process could play a role in endocrine resistance.

Alterations in gene methylation have also been linked to therapy resistance, whereby genes that promote cancer progression are hypomethylated and those that would suppress it are hypermethylated (Lubecka *et al.*, 2016). Recently, one group performed a genome-wide analysis of DNA methylation and expression using cell line models to mimic AI endocrine resistance (Hiken *et al.*, 2017). The authors demonstrated that *Prostaglandin E2 Receptor 4* (*PTGER4*) expression was upregulated following gene demethylation in these cell lines, and subsequent knockdown analyses indicated the importance of its expression for ligand-independent cellular proliferation in these models. They demonstrated that this mechanism was at least in part due to PTGER4 enhancing the activity of the ERα co-factor CARM1, which interacts with ERα and promotes the expression of ERα target genes in the absence of oestrogen (Hiken *et al.*, 2017). It appears that different post-translational modifications can occur in endocrine resistance and can produce alternative effects. For instance, Tsuboi *et al*. produced two cell line models of FULV resistant BCa and observed differing methylation patterns between them, in particular in the promoter regions upstream of *ESR1*, and consequently there were differing characteristics of FULV resistance in the two models (Tsuboi *et al.*, 2017).

1.6.3.3 Post-translational modifications of ERα co-regulators and *ESR1* can be linked

An alternative mechanism of endocrine resistance that has been described is through the downregulation or loss of ERα expression, which has been estimated to arise in approximately 15–20 % of endocrine resistant tumours (Citro *et al.*, 2015). Work conducted by Citro *et al*. suggested one mechanism via which this could occur is through mitogenic (such as Epidermal Growth Factor, EGF) activation of the PI3K/AkT/mTOR signalling pathway results in phosphorylation of the Histone Deacetylase 1 (HDAC1)

protein, which facilitates deacetylation of *ESR1* to downregulate its expression (Citro *et al.*, 2015). HDAC1 has been previously demonstrated to control transcriptional activation of *ESR1* via interacting with its promoter and altering the chromatin structure (Macaluso *et al.*, 2003). The role of Histone Deacetylases (HDACs) in endocrine resistance has also been attributed to their part in the suppression of ERα co-repressor activity (Legare and Basik, 2016), and the use of Histone Deacetylase Inhibitors in BCa therapy is currently being explored (Damaskos *et al.*, 2017, Hegedus *et al.*, 2017, Trapani *et al.*, 2017).

1.6.4 Enhanced growth factor signalling

Another mechanism thought to drive endocrine resistance is through ligand-independent stimulation of ERα via activation of growth factor signalling pathways. Commonly this involves either the Epidermal Growth Factor (EGFR)/HER-2 or Insulin-Like Growth Factor (IGFR) Receptor pathways (Abdel-Hafiz, 2017). EGFR is classified as a member of the ErbB family of Receptor Tyrosine Kinases, alongside HER-2, and EGFR and HER-2, which are known to commonly heterodimerise (Foley *et al.*, 2010). It has been suggested that the downstream effects of EGFR and other ErbB signalling induces phosphorylation of the ERα co-activator SRC3 and therefore enhances oestrogen signalling (Foley *et al.*, 2010).

GPER1 is a variant of ERα, which has been linked rapid E2 signalling (Liu *et al.*, 2015, Lipovka and Konhilas, 2016) and much research has suggested the role of GPER1 in driving endocrine resistance, including through the activation of the EGFR signalling pathway (Ignatov *et al.*, 2010, Ignatov *et al.*, 2011). The expression of GPER1 and EGFR was found to be directly correlated in metastatic BCa samples. Further, GPER1 translocation to the cell membrane and subsequent regulation of EGFR signalling has

been demonstrated to promote TAM resistance in cell line models (Mo *et al.*, 2013). Research has been conducted to investigate the efficacy of the EGFR inhibitor Gefitinib in conjunction with endocrine therapy. However the results showed the that combination of these therapeutics provided little benefit compared to endocrine therapy alone (Smith *et al.*, 2007, Abdel-Hafiz, 2017, Lluch *et al.*, 2014). This is at least in part believed to be due to the lack of clear predictor biomarkers as indicators of response (Abdel-Hafiz, 2017).

A recent study demonstrated that the alternative growth factors Fibroblast Growth Factors (FGFs), in particular FGF7 (mediated by the Fibroblast Growth Factor Receptor FGFR2), can induce proliferation of BCa cells during TAM treatment (Turczyk *et al.*, 2017). The authors demonstrated that this pathway resulted in alternative post-translational modifications (via phosphorylation and ubiquitination) to the ERα, resulting in its transcriptional activation and receptor degradation, which is not normally induced via TAM treatment. Additionally, this FGF7/FGFR2 signalling increased the expression of the anti-apoptotic factor B-cell lymphoma 2 (Bcl-2) (Turczyk *et al.*, 2017). FGF7/FGFR2 signalling also led to PI3K/Akt/mTOR activation (Turczyk *et al.*, 2017). The PI3K/Akt/mTOR signalling pathway is involved in cell survival, and is frequently reported to be activated in BCa (Kaklamani and Gradishar, 2017). Therefore, as other mechanisms have additionally involved this pathway (Citro *et al.*, 2015) targeting it in conjunction with endocrine therapies could be investigated further.

Alternatively, it has been suggested that growth factors and their receptors could act independently of the ERα to drive endocrine resistance, via activating the receptor's target genes and thereby bypassing the ERα signalling pathway (Kaklamani and Gradishar, 2017, Osborne and Schiff, 2011, Gluck, 2014). For instance, a study by

Moerkens *et al*. demonstrated that TAM-induced suppression of E2-stimulated BCa cell growth could be abrogated via EGFR signalling (Moerkens *et al.*, 2014).

1.6.5 Non-coding RNA

Non-coding RNA (ncRNA) are non-protein coding transcripts that can be grouped into several subgroups, two of which that have been described as the most important in the regulation of gene expression are microRNA and long non-coding RNA (Hayes and Lewis-Wambi, 2015).

MicroRNA (miRNA) is a class of ncRNA that are relatively small (18 - 22 bp in size), which post-transcriptionally regulate a gene's expression via either the inhibition of its translation or through the degradation of its transcripts, and their aberrant regulation has been associated with endocrine resistance (Muluhngwi and Klinge, 2015, Hayes and Lewis-Wambi, 2015). Research has implicated the role of miRNA in promoting endocrine resistance through many mechanisms, including: promoting the decreased expression of ERα protein (miR-221/222) (Zhao *et al.*, 2008) and adjusting the expression of proteins important in the cell cycle, including p27 suppression (miR-221/222 and miR-519a) (Miller *et al.*, 2008, Wei *et al.*, 2014). Interestingly, recent research is more focused not on how miRNAs drive endocrine resistance, but that the suppression of some miRNAs could promote endocrine resistance. Muluhngwi *et al*. indicated miR-29b-1 to have a tumour suppressive role in a cell line model of TAM resistance, whereby its over-expression reduced cell growth through the suppression of genes involved in mitochondrial bioenergetics (Muluhngwi *et al.*, 2017). Additionally, miR-873 was demonstrated to reduce expression of the ERα target gene *Cyclin-Dependent Kinase 3* (*CDK3*). miR-873 levels were shown to be decreased in a model of TAM resistance, and induced

expression of this miRNA enhanced TAM sensitivity (Cui *et al.*, 2015). An further example is that the loss of miRNA-200, through p53 mutation in endocrine sensitive cells, can promote endocrine resistance through regulation of expression of the oncogene *Membrane-Organizing Extension Spike Protein* (*Moesin*) (Alam *et al.*, 2017).

Long non-coding RNA (lncRNA) is an alternative classification of ncRNA that are much larger than miRNAs (over 200 bp in size) (Hayes and Lewis-Wambi, 2015). They are known to be transcribed from a wide variety of positions on the genome, including promoter or enhancer regions of genes or their own unique genomic locations. In contrast to miRNAs, lncRNAs have a vast variety of methods for gene regulation, including functioning as protein–DNA scaffolds and protein decoys, on top of their role in regulating the translation of genes (Hayes and Lewis-Wambi, 2015). Little research has been conducted on the role of lncRNAs in endocrine resistance, however, a recent study demonstrated that targeting and inhibiting the action of the lncRNA ROR can abrogate resistance to TAM treatment (Li *et al.*, 2017). Additionally, lncRNAs (as well as miRNAs) have been implicated in progressing endocrine resistance through their regulation of Homeobox (HOX) genes, a family of transcription factors with multiple functions, including in the regulation of the cell cycle and cellular differentiation (Jin and Sukumar, 2016). Therefore, this mechanism may become of more interest.

1.6.6 Oestrogen Receptor beta

As described previously (Section 1.4.4), ERβ has been suggested have a tumour suppressive role in endocrine sensitive BCa, producing an anti-proliferative effect on BCa cells, and a reduction in its expression has been associated with BCa progression and a decrease in tumour suppressor proteins such as p53 (Huang *et al.*, 2014, Chuffa *et al.*,

2017, Bado *et al.*, 2017, Hartman *et al.*, 2009, Williams *et al.*, 2008, Lu and Katzenellenbogen, 2017). However, conflicting results have also been published that suggest that ERβ expression in BCa cell lines does not abrogate ERα signalling (Jonsson *et al.*, 2014), nor is ERβ expression in tumours correlated with a favourable prognosis in endocrine sensitive patients (Tan *et al.*, 2016). Additionally, increasing evidence has now associated increased ERβ expression with endocrine resistance and a poorer prognosis in ERα-positive patients treated with endocrine therapies (Gao *et al.*, 2005, Speirs *et al.*, 1999, Guo *et al.*, 2016). However, conflicting studies have indicated ERβ as an indicator of positive response to endocrine therapy (Madeira *et al.*, 2013) and low ERβ expression has been associated with TAM resistance (Esslimani-Sahla *et al.*, 2004), therefore the role of ERβ in endocrine resistance is disputed.

1.7 Androgen Receptor

1.7.1 Structure and function

The Androgen Receptor (AR) is a ligand-dependent NR that applies the functions of the male steroid hormones androgens (Rahim and O'Regan, 2017). Androgens are involved in the regulation of many functions, including the onset of erythropoiesis, metabolism and male sexual differentiation (Murashima *et al.*, 2015, Schweizer and Yu, 2017). In women, androgens are synthesised in the ovaries, adrenal glands and adipose tissues (Rahim and O'Regan, 2017, Schweizer and Yu, 2017). The androgens most abundant in circulation in women are Androstenedione (A), Testosterone (T) and Dihydrotestosterone (DHT), however of these three, only T and DHT are able to fully activate AR (Burger, 2002, Rahim and O'Regan, 2017). The AR protein is 919 amino acids in size and is encoded by the *AR* gene located on chromosome Xq11 (Gao *et al.*,

2005, Narayanan and Dalton, 2016). Its structure follows that of most NRs (Figure 1.3.1), consisting of an N-terminus, a DBD, and a small hinge region followed by a LBD. Like ERα, it contains two activation sites: AF1, which is located in the N-terminus and AF2 that is found in the LBD, with the latter being ligand-dependent (Gao et al., 2005, Sever and Glass, 2013) (Figure 1.7.1).

1.7.2 The AR Pathway

Like ERα, ligand binding to the AR promotes a conformational change promoting receptor dimerisation, translocation to the nucleus, binding to Androgen Response Elements (AREs) present in the regulatory regions of target genes, and promotes gene expression (Figure 1.7.2). AR activity is modulated by interactions with co-repressor and co-activator molecules, which are recruited to AREs along with the general transcription machinery (Sever and Glass, 2013, Brooke and Bevan, 2009, Dehm and Tindall, 2007).

1.7.3 Steroidogenesis and the production of androgens and their relation to oestrogens

Steroidogenesis is highly important in women for the regulation of female sexual differentiation and development, and in the consequential functioning of their mammary glands and other female organs. The most widely researched hormones in women are oestrogens. However, androgens are secreted in significantly higher quantities than oestrogens (Burger, 2002). Additionally, it has been indicated that in pre-menopausal women AR is actually more highly expressed than ERα and helps to regulate the development of the normal functioning mammary gland (Yeh *et al*., 2003, Fioretti *et al*.,

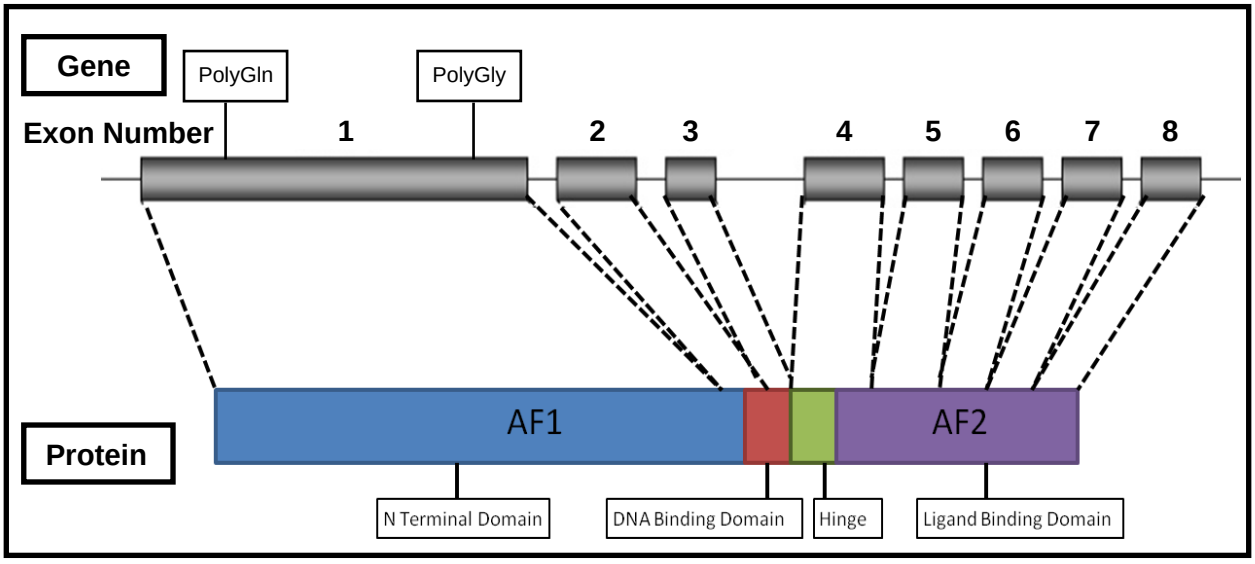


Figure 1.7.1 Structural representation of the Androgen Receptor protein and gene

The *Androgen Receptor* (*AR*) gene comprises 8 exons. AR is a modular protein consisting of two Activation Function sites (AF1 and AF2), an N terminal domain, a DNA Binding Domain (DBD), a hinge region and a Ligand Binding Domain (LBD). Polyglutamine (PolyGln) and polyglycine (PolyGly) tracts are located in exon 1.

(Figure is adapted from Gao *et al.*, 2005)

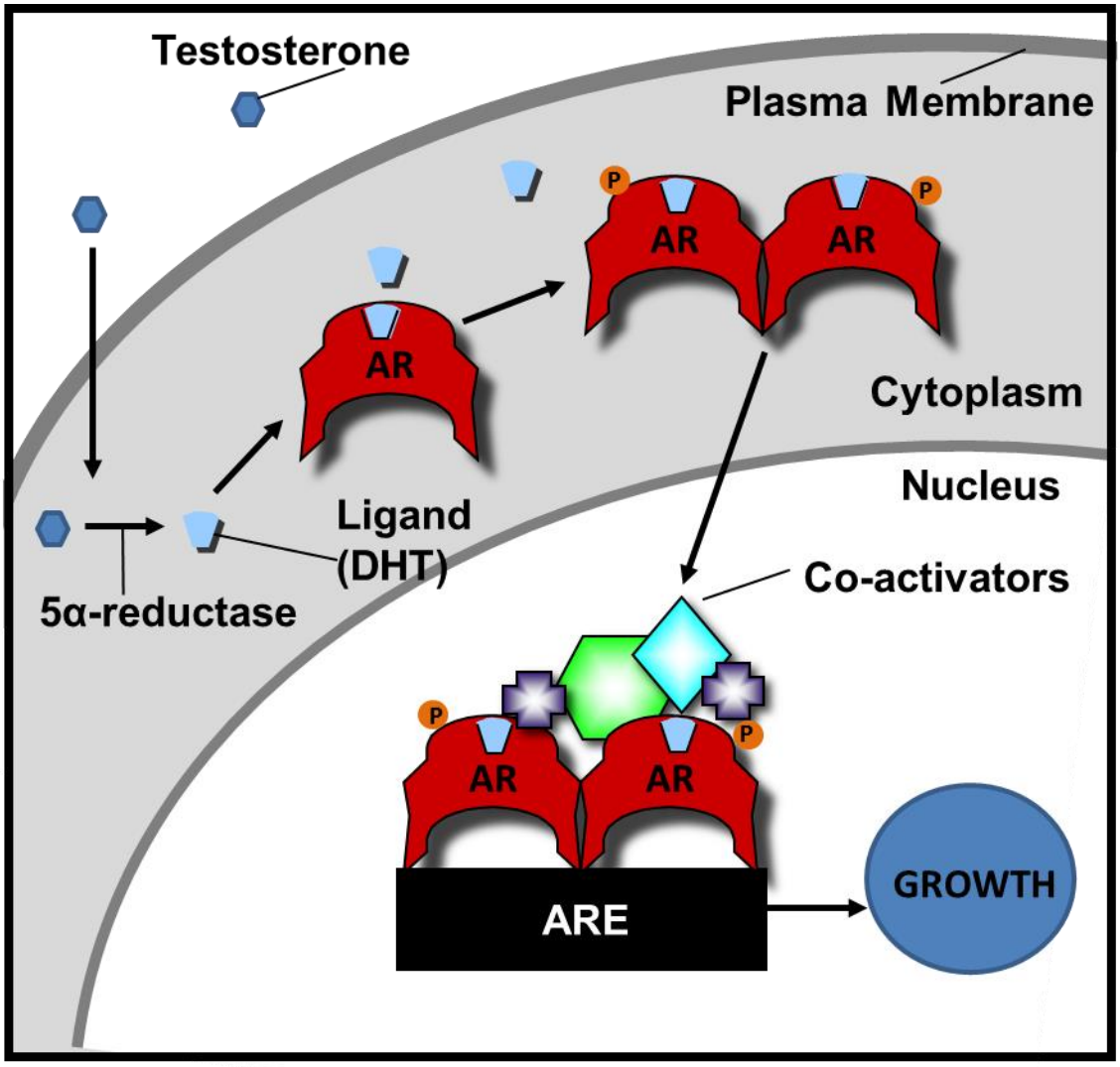


Figure 1.7.2 Schematic representation of the AR pathway

Testosterone enters an androgen-responsive cell and is converted into Dihydrotestosterone (DHT) via the action of 5α-reductase. DHT binds to the Androgen Receptor (AR). This promotes Phosphorylation (P) and dimerisation of AR, which then and binds to specific sequences of DNA within the promoter regions of target genes, known as Androgen Response Elements (AREs). Co-activators bind to AR to promote the recruitment of the General Transcription Apparatus (GTA) (Feldman and Feldman, 2001).

2014). Androgen production directly affects the ERα pathway, as two of the main androgens that are produced in women are precursors for E2: T, which is converted to E2 directly by aromatase; and A, which is converted by aromatase to E1, after which it can then be used to synthesise E2 via the action of 17β-Hydroxysteroid Dehydrogenase (17β-HSD) (Figure 1.7.3) (Kaklamani and Gradishar, 2017, Patani and Martin, 2014, Hong and Chen, 2011, Rahim and O'Regan, 2017).

1.8 AR and Breast Cancer

1.8.1 AR expression in Breast Cancer

The AR is more highly expressed than ERα in BCa, being present in 60-90 % of cases (Rahim and O'Regan, 2017, Vera-Badillo *et al.*, 2014). For example, IHC conducted on BCa tumour samples from 189 patients demonstrated that 80 % of the tumours expressed AR, across all of the different disease classifications (Niemeier *et al.*, 2010). However, one study conducted a large systematic review of BCa research between 1946 and 2012, and found that AR expression was much more common in ERα-positive BCas. They reported that AR expression occurred in 74.8 % of ERα-positive as compared to 31.8 % of ERα-negative tumours (Vera-Badillo *et al.*, 2014). A study that combined and re-analysed data from nine separate studies, in a total of 663 women who proceeded to develop BCa and 1,765 women who did not, displayed that increased androgen levels positively correlated with risk of onset of BCa (Key *et al.*, 2002). Therefore, research has been conducted into the role of androgens and the AR in women with differing BCa subtypes.

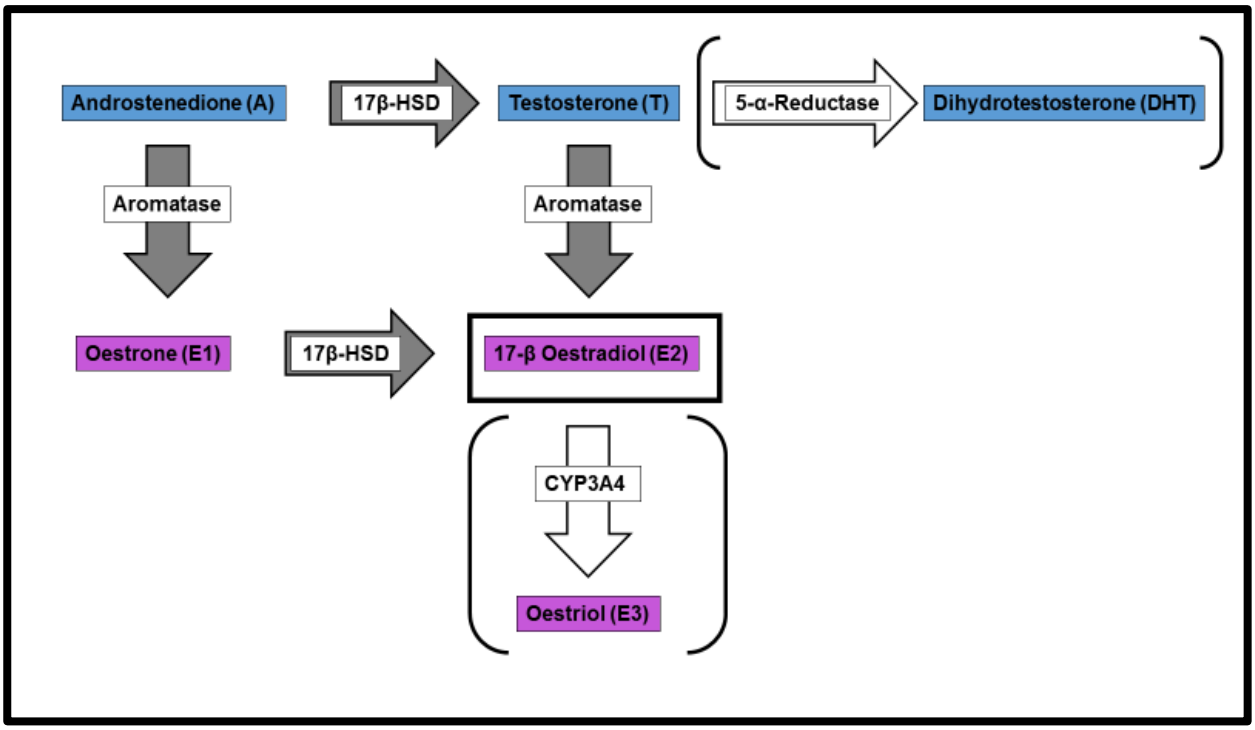


Figure 1.7.3: Synthesis of oestrogens from androgens

Schematic representation of the steroidogenic pathways leading to oestrogen synthesis (the most highly abundant circulating form being 17-β Oestradiol, E2) from androgenic precursors. 17β-HSD indicates 17β-Hydroxysteroid Dehydrogenase and CYP3A4 indicates Cytochrome P450 3A4.

1.8.2 AR in ERα-positive Breast Cancer

In ERα-positive disease, AR appears to be an indicator of positive prognostic outcome, which has been hypothesised to be due to the AR having an inhibitory effect on ERα-driven cancer progression (Rahim and O'Regan, 2017, Fioretti *et al.*, 2014, Tarulli *et al.*, 2014). Studies have demonstrated a significant association between AR expression in ERα-positive BCa with indicators of a positive clinical outcome, for instance decreased tumour size, lower graded tumours and PR expression (Tsang *et al.*, 2014, Niemeier *et al.*, 2010). One study found that, in cases of low AR expression (in which less than 75 % of cells expressed AR) the risk of patient mortality increased by 4.6-fold (Peters *et al.*, 2009). Therefore, the ratio of ERα and AR expression and the abundance of their relevant ligands has been deemed as potentially important for BCas that are both AR- and ERα-positive, however this connection is complicated as androgens can be used as precursors for oestrogens (Figure 1.7.3) (Rahim and O'Regan, 2017).

This protective effect of AR in ERα-positive BCa is plausible as androgens are known to inhibit breast growth during and following puberty (Tarulli *et al*., 2014), and 20 years ago it was demonstrated that over-expression of AR in the MCF7 ERα-positive BCa cell line had an inhibitory effect on cell proliferation (Szelei *et al.*, 1997). This was also demonstrated in a more recently study, where treatment with DHT and Mibolerone (MIB, a synthetic analogue of DHT that cannot be metabolised to oestrogen) inhibited E2-stimulated cellular proliferation in ERα-positive cell lines MCF7 and T47D, with the effect being more pronounced in the T47D cell line which is additionally positive for PR (Cops *et al.*, 2008). This tumour suppressive role was confirmed in T47D cells to be directly through the action of AR, as additional treatment with Bicalutamide (BIC), an anti-androgen to inhibit AR action, could reverse this effect (Cops *et al.*, 2008). Additionally, it

has been demonstrated that treatment with androgens can induce apoptosis in T47D cells (Kampa *et al.*, 2005).

Several mechanisms through which the AR inhibits oestrogen-induced BCa growth have been proposed. Firstly, this has been demonstrated to be brought about by AR upregulation of tumour suppressor proteins such as Killin (KKLN), Phosphatase and Tensin Homolog (PTEN) and ERβ (Wang *et al.*, 2013, Rizza *et al.*, 2014). One study demonstrated that following ligand binding in MCF7 cells, AR induces *Dosage-sensitive sex reversal, Adrenal hypoplasia critical region, On chromosome X, Gene 1* (*DAX-1*) expression via interacting with an ARE, which resulted in a reduction of aromatase expression and activity, supressing cancer progression (Lanzino *et al.*, 2013). Furthermore, AR signalling has been shown to enhance the effects of AIs in ERα-positive disease. For example, one study in MCF7 cells stably transfected with aromatase demonstrated that targeting AR using siRNA or BIC inhibited the anti-proliferative effects induced via treatment with the AI Letrozole (Macedo *et al.*, 2006).

1.8.3 AR and ERα cross-talk

It has been established that AR and ERα suppress each other's activity, and this cross-talk has been suggested to occur by a variety of alternative mechanisms (Figure 1.8.1) (Fioretti *et al*., 2014). Interestingly, it has been demonstrated that AR can supress ERα signalling via interaction with EREs (Peters *et al.*, 2009). Using reporter assays, the authors demonstrated that AR can potently inhibit ERα activity and only the presence of the AR DBD was sufficient for this inhibitory effect (Peters *et al.*, 2009).

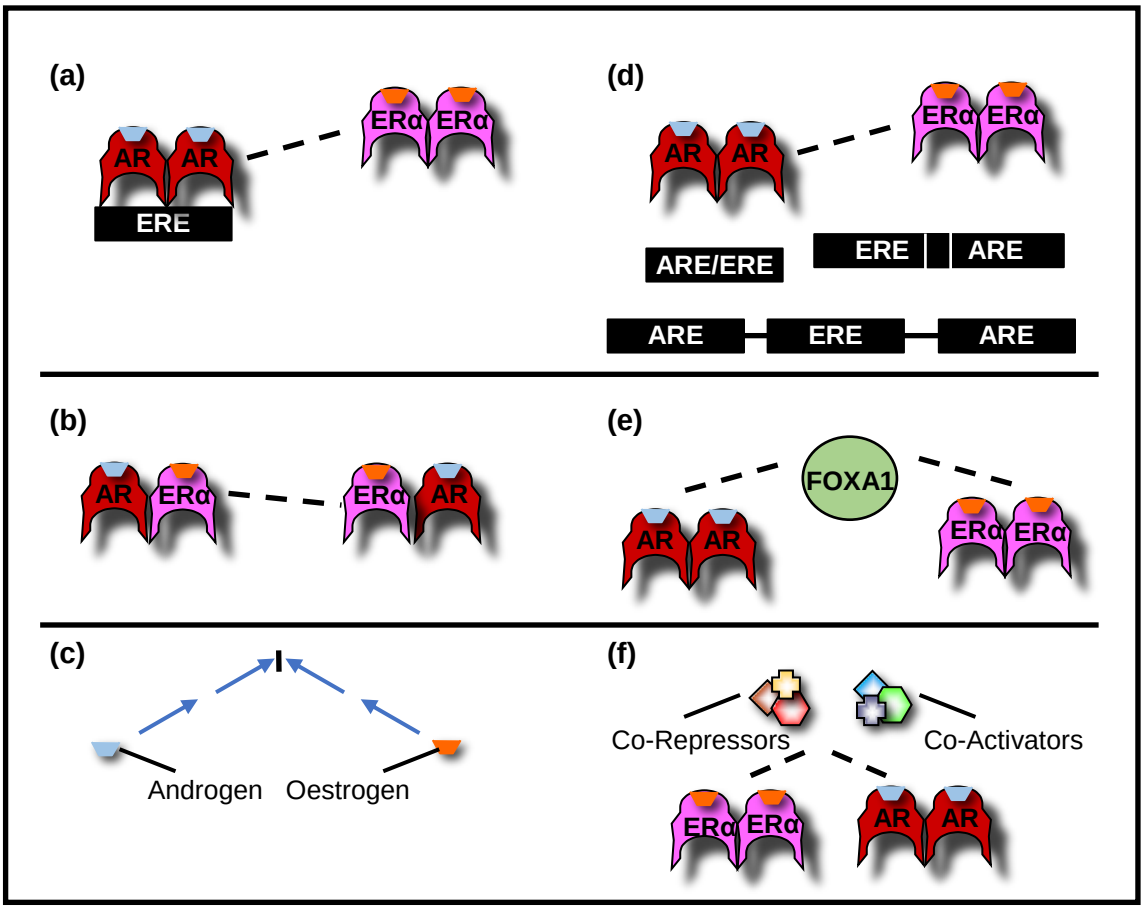


Figure 1.8.1: Proposed mechanisms of AR and ERα cross-talk from current literature

Studies have demonstrated that Androgen Receptor (AR) and Oestrogen Receptor alpha (ERα) suppress each other's activity by a variety of mechanisms: (a) AR can supress ERα signalling via interaction with Oestrogen Response Elements (EREs). (b) ERα and AR can heterodimerise, resulting in suppression of the transcriptional activity of both receptors. (c) AR and ERα pathways overlap, and so oestrogen and androgen antagonise each other's target genes. (d) AR and ERα binding sites are closely located, overlap in location or are common for both receptors, so that the binding of one receptor could block the other from interacting with a response element, or antagonise its desired regulatory effect. (e) The receptors compete for common pioneer factors, such as Forkhead Box A1 (FOXA1). (f) AR and ERα compete for common co-factors. ARE represents Androgen Response Elements.

Yeast and mammalian two-hybrid systems have been used to demonstrate that ERα and AR can heterodimerise, resulting in suppression of the transcriptional activity of both receptors (Panet-Raymond *et al.*, 2000). ChIP and microarray analyses of the ZR-75-1 ERα-positive cell line have demonstrated that the regulation of several E2 and DHT target genes could be antagonised via their co-treatment, suggesting an overlap of the ERα and AR signalling pathways. Need *et al*. further demonstrated that AR and ERα binding sites were often closely located, overlapped or shared for a common gene, which indicates that the binding of one receptor could potentially block the other from interacting with a response element, or antagonise its desired regulatory effect (Need *et al.*, 2012). Additionally, AR and ERα activity is known to be influenced by shared co-factors such as Androgen Receptor Co-activator 70 kDa Protein (ARA70) (Fioretti *et al*., 2014, Lanzino *et al*., 2005). Competition for these limiting factors could also result in inhibitory cross-talk between the receptors. Finally, the action of both receptors is highly influenced via the pioneer factor FOXA1 (Hurtado *et al.*, 2011, Robinson *et al.*, 2011), which has binding sites that overlap substantially with both receptors (Need *et al.*, 2012). These interactions between AR and ERα can explain the inhibitory effect displayed by AR on ERα signalling in ERα-positive BCa.

1.8.4 Molecular Apocrine Breast Cancer

As described previously (Section 1.2.2.3.1), MABC is a sub-classification of ERα-negative BCas (TNBC or HER-2-Enriched) which express AR and are driven by the AR pathway (Fioretti *et al*., 2014, Badve *et al*., 2011, Rakha and Green, 2017, Vranic *et al*., 2017). MABC was initially defined in 2005 by Farmer *et al.*, who demonstrated these ERα-negative AR-positive tumours have increased androgen-signalling with a distinct gene expression profile (Farmer *et al.*, 2005). Following this, a study demonstrated that MABC

tumours express ERα target genes that are associated with endocrine sensitive disease (Doane *et al.*, 2006). They additionally described that the MDA-MB-453 ERα-negative and AR-positive BCa cell line displayed androgen-stimulated growth, and that treatment of these cells with androgens resulted in the activation of genes that significantly overlapped with the oestrogen-associated genes displayed in MABC tumours (Doane *et al.*, 2006). The inference of these results is that AR can mimic ERα signalling to promote BCa progression in the absence of functioning ERα (Fioretti *et al*., 2014).

MABCs are characterised as aggressive tumours and are associated with a poor patient prognosis (Lehmann-Che *et al.*, 2013, Liu *et al.*, 2016), as well apocrine phenotypical features, a lack of necrosis and a negative correlation with Basal-Like markers, for instance Cytokeratin 14 (CK14) (Tsang *et al.*, 2014). Growing evidence has emerged to implicate the role of AR in mediating disease progression in MABC patients. High AR expression and increased levels of AR signalling have been reported in some ERα-negative and AR-positive BCa cell line models, including MDA-MB-453 and SUM185PE, and the growth of these cells was decreased following treatment with the anti-androgen BIC (Lehmann *et al.*, 2011). Known AR target genes for instance *Anterior Gradient Protein 2 Homolog* (*AGR-2*) and *SAM Pointed Domain Containing ETS Transcription Factor* (*SPDEF*) have been found to be significantly upregulated in MABC tumours (Lehmann-Che *et al.*, 2013), and AR nuclear localisation has been reported in all MABC primary tumours tested (Barton *et al.*, 2015), both suggesting AR transcriptional activity. Barton *et al*. additionally demonstrated that AR upregulates the EGFR ligand Amphiregulin (AREG) in AR-positive TNBC cells, which suggests that AR is promoting cancer progression at least in part through the EGFR pathway (Barton *et al.*, 2015). The authors also demonstrated that AR inhibition via treatment with the anti-androgen

Enzalutamide could abrogate proliferation and migration of AR-positive TNBC cell lines (Barton *et al.*, 2015).

It has been demonstrated that the pioneer factor FOXA1 is key for AR function in MABC, as silencing this gene inhibits AR binding DNA, cellular proliferation and the expression of genes associated with the unique MABC gene signature (Robinson *et al.*, 2011). Over-expression of FOXA1 was subsequently found to be correlated with MABC tumours, however 31 % of Basal-Like tumours also expressed this factor (Lehmann-Che *et al.*, 2013). Interestingly, the authors additionally reported that HER2 and/or GCDFP15 over-expression were also frequent in MABCs, suggesting a potential role of these proteins in facilitating the progression of this BCa subtype (Lehmann-Che *et al.*, 2013). In addition to this, it has been suggested that AR could promote MABC progression via exploiting the PI3K signalling pathway, which has a vital role in cellular proliferation and survival. *Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha* (*PIK3CA*) gene mutations were reported in 19 % of MABC tumours, but absent from Basal-Like tumours (Lehmann-Che *et al.*, 2013). Subsequently, Lehmann *et al*. (2014) reported *PIK3CA* mutations were significantly enriched in AR-positive TNBCs, and the authors demonstrated that PI3K inhibitors could have value if administered in combination with an AR antagonist in MABC (Lehmann *et al.*, 2014). This suggests that AR and its cross-talk with PI3K signalling may be important for promoting cancer progression in this subtype of the disease.

It has been suggested that MABCs should be identified through AR messenger RNA (mRNA) and not through IHC, as research from two groups reported that only half of tumours which fall into this category by mRNA analysis were identified via IHC (Doane *et al.*, 2006, Lehmann-Che *et al.*, 2013). Lehmann-Che *et al*. therefore proposed

combining Real-Time quantitative Polymerase Chain Reaction (qPCR) with IHC of GCDFP and HER-2 in order to identify MABC tumours, and demonstrated in their study that this correctly identified 94 % of MABC tumours, rather than 58 % through AR IHC (Lehmann-Che *et al.*, 2013). AR and HER-2 signalling pathways appear to be closely linked (Rahim and O'Regan, 2017) and GCDFP15 is an AR target whose expression has been found to be strongly associated with AR expression (Darb-Esfahani *et al.*, 2014). An additional study used this proposed IHC staining of HER-2 and GCDFP15 to identify MABC tumours, and found that 52 % of the ERα-negative tumours investigated tested positive for HER-2/GCDFP/both and were distinct from the other ERα-negative tumours, yet only 38 % were AR positive through IHC (Tsang *et al.*, 2014).

1.8.5 Androgen levels in endocrine resistance

Research has been suggested that high levels of circulating testosterone are associated with BCa that is endocrine sensitive (Secreto *et al.*, 2009). In addition to this, several studies have reported that an increase in androgen levels occurs during treatment with endocrine therapy. For instance, it has been demonstrated that treatment with the AI Exemestane results in an increase in levels of DHT and testosterone in ERα-positive BCa tumours (Takagi *et al.*, 2010) and that testosterone levels increased within the serum of patients with ERα-positive disease following treatment with the AI Letrozole (Rossi *et al.*, 2009). In addition to this, increased serum levels of testosterone were reported to be significantly associated with patients who developed TAM resistance (Berrino *et al.*, 2005) and one group identified the development of growth dependency on 5α-Androstane-3β, 17β-diol (3β-diol) a metabolite of DHT (converted by 3β-Hydroxysteroid Dehydrogenase Type 1, HSD3B1) in endocrine sensitive cells as a potential mechanism of AI resistance (Hanamura *et al.*, 2013). However, contradictory reports have indicated that endocrine

therapies do not produce this effect, for instance the study conducted by Rossi *et al*. (2009) that indicated increased testosterone levels within patient serum following Letrozole treatment, also reported that serum testosterone levels were unaffected following TAM treatment (Rossi *et al.*, 2009); but an alternative study reported increased androgen levels in plasma following TAM treatment but not AI treatment (Baumgart *et al.*, 2014). Therefore, role of androgen levels during endocrine treatment and in endocrine resistance development are currently unclear.

1.8.6 AR in endocrine resistance

It has now been indicated that AR drives some cases of endocrine resistance. Several studies conducted by the Fuqua group and others have provided some insights into this resistance mechanism (De Amicis *et al.*, 2010, Rechoum *et al.*, 2014, Ciupek *et al.*, 2015). Firstly, the Fuqua group conducted a microarray analysis of five tumour samples from patients with TAM-resistant disease and four control samples of TAM-sensitive disease, and identified elevated AR expression in the TAM-resistant samples (De Amicis *et al*., 2010). To recreate this effect *in vitro*, the group exogenously over-expressed AR in the MCF7 BCa cell line. In agreement with the data from the clinical samples, elevated AR expression promoted TAM resistance, and this effect could be abrogated via treatment with the anti-androgen BIC (De Amicis *et al*., 2010). The study also demonstrated that AR over-expression resulted in TAM treatment inducing the transcriptional activation of ERα, which was abrogated via BIC, suggesting that AR facilitates TAM becoming an agonist in TAM resistance (De Amicis *et al*., 2010). To explore this further, the group demonstrated that AR can facilitate TAM resistance via activation of ERα through the EGFR signalling pathway (Ciupek *et al.*, 2015). Ciupek *et al.* demonstrated that TAM acted as an ERα agonist in cells with an AR over-expression,

resulting in an increase in its transcriptional activity and cellular proliferation, and this effect could be stopped via treatment with the anti-androgen Enzalutamide or the EGFR-inhibitor Gefitinib (Ciupek *et al.*, 2015). This group previously linked a reduction in Rho Guanosine Diphosphate(GDP)-Dissociation Inhibitor (GDI) expression with TAM resistance, where they also observed TAM agonist activity (Ciupek *et al.*, 2015, Barone *et al.*, 2011). Rho GDI is a negative regulator of the Rho GTPase pathway, which is involved in the regulation of many cellular functions including the rearrangement of the actin-cytoskeleton and cellular motility, and alterations to this pathway have been associated with BCa tumours with features indicating a poor prognosis, for example local recurrence (Ciupek *et al*., 2015, Barone *et al*., 2011). How this TAM agonist activity occurs in these models is currently undetermined, however they proposed that AR signalling and Rho GDI resistance mechanisms may be linked to produce this effect (Ciupek *et al.*, 2015).

The balance between AR and ERα expression is thought to be important in the development of endocrine resistance through AR (Rondon-Lagos *et al.*, 2016). For example, it was reported that BCa tumours that have a ratio of AR:ERα of more than 2:1 are four times more likely to become resistant to TAM than tumours that have a less than 2:1 ratio (Cochrane *et al.*, 2014, Carreno *et al.*, 2007).

AR signalling has also been linked to AI resistance. One group stably exogenously over-expressed aromatase alone and in combination with AR in MCF7 cells (MCF7 Arom and MCF7 AR Arom, consecutively) (Rechoum *et al.*, 2014). Cells were treated with androstenedione, an androgen which can be converted into oestrogen via the action of aromatase (Figure 1.7.3). Treatment with the AI Anastrazole resulted in the inhibition of androstenedione-stimulated proliferation as well as ERα transcriptional activity in the cells

which solely over-expressed aromatase, yet had no significant effect in those also over-expressing AR (Rechoum *et al.*, 2014). They demonstrated that treatment of MCF7 AR Arom cells with anti-androgens could increase their sensitivity to Anastrazole, as well as treatment with drugs targeting ERα (FULV), Phosphorylated Insulin-like Growth Factor-1 Receptor (pIGF-1R) or Akt, suggesting the importance of these proteins also in this mechanism (Rechoum *et al.*, 2014). AR and ERα appeared to cooperate in this mechanism of AI resistance, and MCF7 Arom and MCF7 AR Arom had differentially expressed ERα and AR target genes compared to MCF7 cells, which implies that treating patients that are both ERα- and AR-positive with anti-androgens as well as anti-oestrogens could be beneficial (Rechoum *et al.*, 2014). In accordance with these results, a different research group performed IHC on tissue samples from BCa patients who had recurrence and developed AI resistance following surgery (Fujii *et al.*, 2014). Tissue sections were obtained during the initial surgery and following recurrence. The authors reported that the recurrent tissues had a significant decrease in ERα and PR expression, and an increase in Prostate Specific Antigen (PSA) expression. Additionally, elevated AR expression was demonstrated in the recurrent tissues from 62 % of patients, although this was not significant (P=0.22) (Fujii *et al.*, 2014). They created a model of AI resistance by growing T47D cells in hormone-depleted media. These cells were subsequently found to have a loss of ERα expression and oestrogen responsiveness, and increased AR expression and AR signalling (Fujii *et al.*, 2014). Interestingly, however, the same research group linked AI resistance to androgens activating ERα and decreased AR expression (Hanamura *et al.*, 2013).

An alternative study has indicated the importance of Prosaposin (PSAP) in AR driving the growth of cell line models of AI and TAM resistance (Ali *et al.*, 2015). This

study demonstrated that PSAP stimulates cell migration and can promote AR recruitment to HREs in AI resistant cells, and that treatment with the anti-androgen Enzalutamide could abrogate AR activation of PSAP (Ali *et al.*, 2015). The authors identified a significant increase in PSAP levels in the serum of BCa patients prior to surgery who experienced subsequent recurrence, and suggested the use of PSAP as a biomarker in endocrine therapy treated patients to potentially identify those likely to develop resistance as a result of AR signalling (Ali *et al.*, 2015).

In light of this, work carried out by the Brooke group has displayed that AR levels are increased in some models of endocrine resistance and that AR promotes the proliferation of endocrine resistant cell lines (Fioretti and Brooke, unpublished). Therefore, the AR could become a potential new drug treatment in BCa patients who have TNBC or endocrine resistant ERα-positive BCa.

1.9 Targeting AR in cancer

1.9.1 Prostate Cancer

Much knowledge of the AR and how to therapeutically target it comes from studies in Prostate Cancer (PCa). Cancer of the prostate, an important constituent of the male reproductive system, is currently the most prevalent cancer in men in the UK, contributing to approximately 26 % of all male cancer diagnoses (Cancer research UK, 2014c). It is well established that AR drives PCa growth and progression through activation of the AR pathway (Brooke and Bevan, 2009, Crumbaker *et al.*, 2017).

1.9.2 Anti-androgens and their use in Prostate Cancer

Approximately 25 % of PCa cases are confined to the prostate gland (Brooke and Bevan, 2009). In these cases active surveillance, followed by radical prostatectomy, external-beam radiotherapy or brachytherapy is the preferred treatment option (Attard *et al.*, 2016, Brooke and Bevan, 2009). However, for the remaining 75 % of cases the disease will have advanced locally further than the prostate capsule, or metastasised, and hence surgery is no longer possible (Brooke and Bevan, 2009). In these cases, endocrine therapy is often used to block the AR pathway. This consists of chemical castration, where analogues of Luteinizing Hormone-Releasing Hormone (LHRH, e.g. Leuprolide and Goserelin) block the androgen synthesis through the pituitary-hypothalamus signalling axis (Brooke and Bevan, 2009, Pelekanou and Castanas, 2016). This form of chemical castration effectively decreases circulatory testosterone levels, however it is much less effective at decreasing the concentration of adrenally produced androgen precursors, for instance Dehydroepiandrosterone (DHEA), which can be converted to DHT. Therefore this therapy is commonly combined with anti-androgens, ligands that interact with the AR and keep it in an inactive form (Brooke and Bevan, 2009, Gillatt, 2006). Originally, the steroidal anti-androgen Cyproterone acetate was administered to patients (Culig, 2014). Since, non-steroidal anti-androgens have been developed, including first generation anti-androgens such as BIC and Flutamide, as well as the second generation anti-androgen Enzalutamide (Pelekanou and Castanas, 2016). Enzalutamide has a higher relative binding affinity for AR than BIC (Culig, 2014). It differs from the first generation anti-androgens as it halts the AR pathway via not only competing with ligands to block AR action, but it additionally stops AR nuclear translocation and AR interaction with DNA and co-factors (Nadal and Bellmunt, 2016, Rahim and O'Regan,

2017). Interestingly, it has also been demonstrated that the anti-oestrogen FULV can reduce AR signalling and androgen-induced proliferation in the LNCaP PCa cell line (Bhattacharyya *et al.*, 2006). Therefore, treatment options are readily available which target the AR pathway, which could be repurposed for the treatment of endocrine resistant BCa.

1.9.3 Administration of androgens in Breast Cancer therapy

It is well established that steroidal androgens can result in breast tumour regression, and their administration was utilised in BCa patients in 1940s prior to the development of anti-oestrogens and AIs (Tarulli *et al.*, 2014, Narayanan and Dalton, 2016). Steroidal androgens were reviewed to result in tumour regression in patients by 30–50 %, with the effects being more significant in AR-positive BCas (Tarulli *et al.*, 2014, Narayanan and Dalton, 2016). The movement to oestrogen-targeting therapies came as a result of the masculine side-effects associated with using androgens for treatment, and the realisation that through steroidogenesis androgens could be precursors to oestrogens (Narayanan and Dalton, 2016, Garay *et al.*, 2012).

Clinically, the administration of androgens in BCa therapy appears to have begun with the use of the synthetic steroidal androgen Fluoxymesterone and the progestin Medroxyprogesterone acetate which has reported androgenic properties (Africander *et al.*, 2014, Ghatge *et al.*, 2005). Both of these compounds have demonstrated inhibition on BCa proliferation, and were subsequently found to enhance the effects of TAM treatment (Reviewed: Narayanan and Dalton, 2016). The aim behind this treatment is to activate the AR to promote inhibitor cross-talk with ERα.

1.9.4 Anti-androgens in Molecular Apocrine disease and endocrine resistance

As discussed previously (Section 1.8.4), in contrast to ERα-positive disease, AR expression in MABC can drive tumour growth (Fioretti *et al*., 2014, Badve *et al*., 2011, Rakha and Green, 2017, Vranic *et al*., 2017), and evidence now supports the hypothesis that AR can drive endocrine resistance (Section 1.8.6). The anti-androgen Flutamide was previously tested on advanced and metastatic BCa patients in 1988 and showed no clinical benefit, however molecular subtype was not into consideration (Rahim and O'Regan, 2017, Zhao and He, 1988, Perrault *et al.*, 1988). Therefore, clinical trials have now been conducted which have been selective in identifying MABC patients, and have shown promising results. For instance, one study identified AR expression in 51 out of 424 patients (12 %) with TNBC. These AR-positive patients were consequently enlisted in a Phase II clinical trial for daily treatment with the anti-androgen BIC. A complete/partial response or stable disease was observed in 19 % of these patients after 6 months of treatment, and across all patients who received BIC treatment few adverse side-effects were seen and none serious (mainly grade I toxicities, including fatigue and hot flushes) (Gucalp *et al.*, 2013). However, as this therapy was not beneficial in all the AR-positive patients, other pathways are presumably driving tumour growth and therefore further research is needed to understand this more fully. Additionally, a case study was recently published describing an AR-positive patient with metastatic TNBC, who had been undergoing palliative chemotherapy, who subsequently displayed a complete response to BIC following 4 months of treatment and remained in remission for at least 12 months (Arce-Salinas *et al.*, 2016).

Current on-going clinical trials with anti-androgens can be observed in Table 1.9.1. In particular Enzalutamide, alone and in combinations with other drugs, is being trialled

Table 1.9.1 A summary of current clinical trials that are recruiting/ongoing/awaiting published results targeting Androgen Receptor (AR) in different molecular subtypes of Breast Cancer

Results obtained from ClinicalTrials.gov. Trials are Phase II unless otherwise stated.

Drug	Subtype	Reference
Bicalutamide	ER-, PR-, AR+, metastatic	NCT00468715
Enobosarm	ER+ and AR+	NCT02463032, NCT02746328
Enobosarm	ER+ metastatic	NCT01616758
Enobosarm	TNBC, AR+, advanced	NCT02368691
Enobosarm (and pembrolizumab)	TNBC, AR+, metastatic	NCT02971761
Enzalutamide (alone and with Exemestane)	1:Primary, ER+ 2: Primary, TNBC, AR+	NCT02676986
Enzalutamide (and with Paclitaxel)	TNBC, AR+	NCT02689427
Enzalutamide (and with Fulvestrant)	ER+, HER-2-, Advanced	NCT02953860
Enzalutamide (alone and with Exemestane/Fulvestrant/Anastrazole: Phase I)	AR+, incurable	NCT01597193
Enzalutamide	TNBC, AR+	NCT01889238
Enzalutamide (with Exemestane)	ER+ or PR+ or both	NCT02007512
Enzalutamide	Early stage, AR+, TNBC	NCT02750358
Enzalutamide (alone and with Paclitaxel)	TNBC	NCT02929576
Enzalutamide (and with Fulvestrant) Preoperative	ER+, HER-2-	NCT02955394
Enzalutamide (and with Trastuzumab)	AR+, HER-2+, advanced/metastatic	NCT02091960
Enzalutamide (and with Taselisib)	TNBC, AR+	NCT02457910

in BCa patients, including in patients with incurable and advanced disease, some of whom have presumably failed to respond or developed resistance to endocrine therapies. A clinical trial has been recently published, which investigated the pharmacokinetic interactions, safety, and tolerability of the use of Enzalutamide and established the correct dosage for its use in combination with endocrine therapy (for patients with less advanced cancer) and as a singular treatment (for patients with more advanced cancer) for ERα- and PR- positive BCa patients (Schwartzberg *et al.*, 2017). The authors suggested that as well as targeting AR, Enzalutamide could aid endocrine therapy via inducing Cytochrome P450 3A4 (CYP3A4), a protein which often metabolises endocrine therapies. The results demonstrated that Enzalutamide was safe and generally well-tolerated in BCa patients, and that the pharmacokinetic interactions of Enzalutamide treatment alone was comparable to male patients with PCa. They additionally reported that Enzalutamide treatment in combination with the AI Exemestane would require doubling the concentration of Exemestane administered (Schwartzberg *et al.*, 2017). However, the authors did not explore clinical benefit. Additionally, Enzalutamide is showing much promise in various other tumour types, for example Ovarian, Primary Peritoneal and Fallopian Tube Cancer (ClinicalTrials.gov Identifier: NCT01974765), and therefore the results of these further trials outlined in Table 1.9.1 will provide further insight into whether targeting AR therapeutically with Enzalutamide is of benefit to ERα- positive and negative BCa patients expressing AR.

In addition, Selective Androgen Receptor Modulators (SARMs) have been developed, which can act as agonists or antagonists specifically in for example breast, muscle and bone (Narayanan and Dalton, 2016). The novel SARM Enobosarm has received a lot of interest (Dobs *et al.*, 2013). For instance, one study indicated that

Enobosarm treatment of an MABC xenograft model (MDA-MB-453-AR cells) reduced tumour proliferation and size, and decreased metastasis-promoting paracrine factors, such as Interleukin 6 (IL6) (Narayanan *et al.*, 2014). A case study on a patient with ERα-, PR- and AR- positive disease who was enrolled in a recent clinical trial for treatment with Enobosarm, demonstrated the first incidence of a positive clinical response to a SARM following failure and developed resistance to a vast number of ERα-targeted endocrine therapies (Vontela *et al.*, 2017). As Enobosarm can interact with aromatase and 5α-reductase but cannot be metabolised by them, Vontela *et al*. suggested therefore that as well as acting as an anti-androgen in postmenopausal patients with ERα-positive BCa, it could potentially decrease E2 synthesis via directly competing with oestrogen-precursors (Vontela *et al.*, 2017). Currently, many clinical trials are investigating the efficacy of Enobosarm, for instance to explore its benefit in metastatic and locally advanced cases of ERα-positive and AR-positive BCa, as well as MABC (Table 1.9.1). The SARM is additionally being trialled for ERα-positive disease, as it may have benefit to patients who experience alternative endocrine resistance pathways to AR signalling.

Additionally, targeting AR through treatments other than anti-androgens, could prove useful in some AR-positive BCa patients. Abiraterone Acetate (AA), a 17α-hydroxylase and CYP17 inhibitor (and thereby an androgen synthesis inhibitor, often used for treatment in castrate-resistant PCa patients), was used in a Phase II clinical trial in AR-positive TNBC patients, in combination with Prednisone (Bonnefoi *et al.*, 2016). Prednisone was combined with AA in order to try and prevent secondary mineralocorticoid excess, and to try and enhance the effect of AA treatment due to the well-established link of Prednisone to decreasing androgen production in PCa patients (Tannock *et al.*, 1989, Auchus *et al.*, 2014, de Bono *et al.*, 2010, Bonnefoi *et al.*, 2016).

Bonnefoi *et al*. demonstrated that 20 % of patients enrolled in this study experienced a clinical benefit within 6 months of the start of the trial, including one patient with a complete response (Bonnefoi *et al.*, 2016). Currently, more clinical trials are underway in BCa patients with AA, including one with ERα- or AR-positive metastatic BCa patients (ClinicalTrials.gov Identifier: NCT00755885) and one specifically for ERα-positive patients who have relapsed following AI treatment (ClinicalTrials.gov Identifier: NCT01381874). Furthermore, Seviteronel, a CYP17A1 inhibitor that is also being trialled in PCa patients, is being explored for ERα-positive as well as AR-positive TNBC patients (ClinicalTrials.gov Identifiers: NCT02130700, NCT02580448).

1.10 Project objective

Clinical trials have demonstrated that targeting androgen signalling can have a good response in some BCa patients, but not others. Therefore, there is a need to better understand why some patients respond better to this treatment method than others. Further, it is important to identity methods to stratify patients into those that are more likely to benefit from such therapies. In order to do this, we need to better understand the role of the AR in BCa and its role in endocrine resistance, so that we can identify how to better use current therapeutics. Therefore, the aim of this project is to explore the hypothesis that there is a clinical benefit in therapies that target the AR to prevent the development of endocrine resistance and for a subset of patients who have developed endocrine resistance.

Chapter 2: Materials and Methods

The research in this thesis was conducted ethically.

2.1 Reagents, general media, buffers and solutions

Table 2.1.1: Preparations of reagents, general media, buffers and solutions

Name	Recipe	Sterilisation	Storage
General stock solutions			
1 % Bovine serum albumin (BSA) in PBS-T (1 % BSA-PBS-T)	0.1 g BSA lyophilised powder (Sigma-Aldrich) in a total volume of 10 mL PBS-T.	0.22 µm filter sterilise	4 °C, used within 24 hrs of making
0.08 % Crystal violet	32 mg crystal violet (Sigma-Aldrich) in a final volume of 40 mL double distilled water (ddH_2O).	0.22 µm filter sterilise	RT
0.5 M Ethylenediaminetet-raacetic acid (EDTA)	186.12 g EDTA disodium salt (Fisher Scientific) to a final volume of 1 L using ddH_2O, adjusted to pH 8.0 using 5 M NaOH stock solution.	N/A	RT
4 M Hydrochloric acid (HCl)	19.6 mL of 32 % HCl (Fisher Sceintific) with 30.4 mL of ddH_2O.	N/A	RT
4 % Paraformaldehyde (PFA)	4 g of PFA (Sigma-Aldrich) dissolved in PBS to a final volume of 100 mL, whilst heated on a stirring plate within a fume cupboard.	N/A	-20 °C, in 5-10 mL aliquots
1 x Phosphate buffered saline (PBS)	10 (Dulbecco A) tablets (Oxoid Limited) dissolved in ddH_2O to a final volume of 1 L.	Autoclave	RT
PBS-0.1 %-Tween (PBS-T)	0.5 mL of Tween®-20 (Sigma-Aldrich) in a total of 500 mL PBS.	N/A	4 °C
0.2 M Phenylmethanesul-phonylfluoride (PMSF)	0.35 g of PMSF (Sigma-Aldrich) to a total of 10 mL using ddH_2O.	N/A	-20 °C
5 M Sodium hydroxide (NaOH)	20 g of NaOH pellets (Fisher Scientific) to a final volume of 100 mL with ddH_2O.	N/A	RT
Agarose gel electrophoresis			
1 % and 1.4 % Agarose gels	1 g (1 %) or 1.4 g (1.4 %) of agarose (Fisher Scientific) dissolved in 100 mL of 1 X TAE via boiling. This is briefly allowed to cool prior to the addition of 5 µL of ethidium bromide (10 mg/mL, Sigma-Aldrich) and casting. For analysis of ChIP sonication, a more	N/A	RT, or gels can be wrapped and stored at 4 °C

	fine-tooth comb was utilised than for general applications.		O/N if necessary

Name	Recipe	Sterilisation	Storage
1 X Tris-acetate-EDTA (TAE)	40 mM Tris base (4.846 g, Fisher Scientific), 1.114 mL glacial acetic acid (Fisher Scientific) and 1 mM EDTA (2 mL of 0.5 M stock), in a total of 1 L ddH_2O.	N/A	RT

Bacterial culture

100 mg / mL Ampicillin stock	1 g ampicillin sodium salt (Sigma-Aldrich) to a final volume of 10 mL ddH_2O. Added to LB broth/agar to a final concentration of 100 µg/mL.	0.22 µm filter sterilise	-20 °C in 1 mL aliquots
1 M Glucose stock	90.08 g of Glucose (Fisher Scientific) in a final volume of 500 mL ddH_2O.	0.22 µm filter sterilise	RT
20 mg / mL Isopropyl β-D-1-thiogalactopyranoside (IPTG) stock	200 mg of IPTG powder (Sigma-Aldrich) dissolved to a final volume of 1 mL in ddH_2O.	N/A	-20 °C in 50 µL aliquots
50 mg / mL Kanamycin stock	0.5 g kanamycin (Sigma-Aldrich) added to LB broth/agar to a final concentration of 50 µg/mL.	0.22 µm filter sterilise	-20 °C in 1 mL aliquots
Luria Broth (LB)	20 g LB (Lennox, larger granules, Fisher Scientific) dissolved in a total of 1 L of ddH_2O, with the pH adjusted to 7.2 where necessary. Supplemented if required using antibiotics.	Autoclave	4 °C
LB Agar plates	8.75 g of LB Agar (Sigma-Aldrich) to a final volume of 250 mL ddH_2O, supplemented if required with antibiotics. Melted prior to use and poured to make agar plates whilst still molten.	Autoclave	4 °C
LB/ampicillin/IPTG/X-gal plates	Mix 40 µL Xgal stock solution with 4 µL of IPTG stock solution per plate. To a prepared LB Agar plate supplemented with ampicillin (equilibrated to RT after 4 °C storage), spread 44 µL of Xgal-IPTG solution over the plate surface, and leave to dry agar side up at 37 °C for approximately 2 hrs prior to use.	N/A	Prepared immediately before use
1 M Magnesium chloride ($MgCl_2$) stock	101.655 g of $MgCl_2$ (Fisher Scientific) in a final volume of 500 mL ddH_2O.	Autoclave	RT
1 M Magnesium sulphate ($MgSO_4$) stock	120.366 g of $MgSO_4$ (Fisher Scientific) in a final volume of 500 mL ddH_2O.	Autoclave	RT

Name	Recipe	Sterilisation	Storage
Super Optimal Broth (SOB) media	20 g of Tryptone (Oxoid), 5 g of Yeast Extract (Oxoid), 0.58 g of Sodium chloride (10 mM, NaCl, Sigma-Aldrich), 0.18 g Potassium chloride (2.5 mM, KCl, Sigma-Aldrich), 10 mL of 1 M $MgCl_2$ stock (10 mM) and 10 mL of 1M $MgSO_4$ stock (10 mM), dissolved in ddH_2O up to 1 L.	Autoclave	4 °C
Super Optimal broth with Catabolite repression (SOC) media	20 g of Tryptone (Oxoid), 5 g of yeast extract (Oxoid), 0.58 g of NaCl (10 mM, Sigma-Aldrich), 0.18 g KCl (2.5 mM, Sigma-Aldrich), 10 mL of 1 M $MgCl_2$ stock (10 mM), 10 mL of 1M $MgSO_4$ stock (10 mM), and 20 mL of 1 M Glucose stock, dissolved in ddH_2O up to 1 L.	Autoclave (prior to adding glucose)	4 °C
20 mg/mL 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) stocks	100 mg of X-gal powder (Sigma-Aldrich) dissolved to a final volume of 5 mL in DMSO (Sigma-Aldrich).	N/A	-20 °C in 120 µL aliquots, kept in the dark

Mammalian cell culture

Cell line/s	Media	Supplements	Storage
Normal culture media			
MCF7, COS-1	Dulbecco's Modified Eagle's Medium (DMEM) (Gibco®, Life Technologies™)	Per 500 mL of media: 50 mL Foetal bovine serum (10% FBS, Hyclone®, Thermo Scientific); 5 mL of L-Glutamine-Penicillin-Streptomycin solution (Sigma-Aldrich) resulting in a final concentration of 2 mM L-glutamine, 100 U penicillin and 0.1 mg/mL streptomycin, henceforth described as L-Glutamine-PenStrep.	4 ºC, once made used within 1 month.
ZR-75-1, T47D	Roswell Park Memorial Institute medium (RPMI) (Lonza)	Per 500 mL of media: 50 mL (10 %) FBS (Hyclone®, Thermo Scientific) and L-Glutamine-PenStrep.	4 ºC, once made used within 1 month.
MCF7-TAMR, MCF7-LTED, MCF7-FULVR	Phenol-red free DMEM (Gibco®, Life Technologies™)	Per 500 mL of media: L-Glutamine-PenStrep and 50 mL (10 %) Double charcoal Stripped Foetal calf serum (FCS, First Link (UK) Ltd.) that was treated via overnight gentle mixing at 4 ºC with 5 g of dextran coated charcoal (Sigma-Aldrich) per 500 mL of FCS which is then removed via filtration. This form of FBS is henceforth described as stripped FCS (SFCS).	4 ºC, once made used within 1 month.

Cell line/s	Media	Supplements	Storage
T47D-TAMR, T47D-LTED	Phenol-red free RPMI (Gibco®, Life Technologies™)	Per 500 mL of media: L-Glutamine-PenStrep and 50 mL (10 %) SFCS.	4 °C, once made used within 1 month.

Hormone-depleted culture media

MCF7, MCF7-TAMR, MCF7-LTED, MCF7-FULVR	Phenol-red free DMEM (Gibco®, Life Technologies™)	Per 500 mL of media: L-Glutamine-PenStrep and 25 mL (5 %) SFCS.	4 °C, once made used within 1 month.
ZR-75-1, T47D, T47D-TAMR, T47D-LTED	Phenol-red free RPMI (Gibco®, Life Technologies™)	Per 500 mL of media: L-Glutamine-PenStrep and 25 mL (5 %) SFCS.	4 °C, once made used within 1 month.
COS-1	Phenol-red free DMEM (Lonza)	Per 500 mL of media: L-Glutamine-PenStrep and 10 mL (2 %) SFCS	4 °C, once made used within 1 month.

Freezing media

MCF7, COS-1, ZR-75-1, T47D	90 % FBS, 10 % Dimethyl sulphoxide (DMSO, Sigma-Aldrich).	N/A	-20 °C, in 10 mL aliquots.
MCF7-TAMR, MCF7-LTED, MCF7-FULVR, T47D-TAMR, T47D-LTED	90 % SFCS, 10 % DMSO (Sigma-Aldrich).	N/A	-20 °C, in 10 mL aliquots.

Name	Recipe	Sterilisation	Storage
Transfections			
N,N-Bis(2-hydroxyethyl)-2-aminoethanesulphonic acid (BES)-buffered saline (BBS) 2 x solution	50 mM BES (10.66 g, Sigma-Aldrich), 280 mM NaCl (16.36 g, Sigma-Aldrich), 1.5 mM Sodium phosphate dibasic (Na_2HPO_4, 0.21 g, Sigma-Aldrich), to a final volume of 1 L using ddH_2O, adjusted to pH 6.95 using 5 M NaOH stock solution.	0.22 µm filter sterilise	-20 °C, in 50 mL aliquots
2.5 M Calcium Chloride ($CaCl_2$)	138.73 g of anhydrous granular $CaCl_2$ (Sigma-Aldrich) to a total of 500 mL in ddH_2O.	0.22 µm filter sterilise	-20 °C, in 50 mL aliquots

Western blotting

Name	Recipe	Sterilisation	Storage
10 % Ammonium persulphate (APS)	1 g of APS (Sigma-Aldrich) dissolved in a total volume of 10 mL ddH_2O.	N/A	-20 °C, in 160 µL aliquots
Blocking solution	2.5 g (5 %) dried skimmed milk powder (Marvel) to a total of 50 mL in PBS-T.	N/A	4 °C, used within 24 hrs.
10 % Polyacrylamide Gel	Per gel, a 10 % resolving gel was made, consisting of 1.65 mL Acrylamide/Bis-acrylamide 30 % solution (Sigma-Aldrich), 1.9 mL of 1 M Tris/HCl at pH 8.9, 1.4 mL of ddH_2O and 50 µL of 10 % SDS. Immediately prior to pouring, 10 µL of 10 % APS stock and 2.5 µL N,N,N',N'-Tetramethylethylenediamine (TEMED, Sigma-Aldrich) were added. Additionally a stacking gel was made, consisting of 425 µL of Acrylamide/Bis-acrylamide 30 % solution (Sigma-Aldrich), 937.5 µL of 1 M Tris/HCl at pH 6.8, 1.0875 mL of ddH_2O and 25 µL of 10 % SDS. Immediately prior to pouring, 10 µL of 10 % APS and 2.5 µL TEMED were added.	N/A	4 °C, kept moist and used within a week.
Radioimmunoprecipitation assay (RIPA) buffer	0.5 mL of 1 M Tris-Cl (pH 8.0) stock (10 mM), 20 mg of EDTA (1 mM, Fisher Scientific), 0.5 mL of Triton X-100 (1 %, Sigma-Aldrich), 50 mg of Sodium deoxycholate (0.1 %, Sigma-Aldrich), 0.5 mL of 10 % SDS stock solution (0.1 %) and 0.41 g of NaCl (Sigma-Aldrich). Supplemented with 5 µL of 0.2 M PMSF stock and 10 µL of Halt Protease Inhibitor (PI) Cocktail (ThermoScientific) per 1 mL of RIPA just prior to use.	0.22 µm filter sterilise	4 °C
1 x Running Buffer	3 g of Tris base (25 mM, Fisher Scientific), 14.45 g of Glycine (0.2 M, Fisher Scientific) and 0.5 g of SDS (0.05 %, Fisher Scientific) were dissolved in a total volume of 1 L ddH_2O.	N/A	RT
10 % Sodium dodecyl sulphate (SDS)	50 g of SDS (Fisher Scientific) dissolved in a total volume of 500 mL ddH_2O.	N/A	RT

Name	Recipe	Sterilisation	Storage
Semi-Dry Transfer Buffer	5.63 g Glycine (150 mM, Fisher Scientific), 1.22 g Tris base (20 mM) and 100 ml of Methanol (20 %, MeOH, Fisher Scientific), dissolved in a total volume of 500 mL ddH_2O.	N/A	4 °C, used within 1 month
1 M Tris	12.114 g of Tris base (Fisher Scientific) dissolved to a final volume of 100 mL, adjusted to pH 6.8/8.0/8.9 using 4 M HCl stock solution.	Autoclave	RT

2.2 Bacterial cultures, transformation and DNA preparation

2.2.1 Bacterial strains and cultures

For transformation procedures using ligated vectors, the max efficiency DH5α (Invitrogen) strain of competent *Escherichia coli* (*E. coli*) was utilised. JM109 High Efficiency competent cells were selected for cloning with the pGEM®-T Easy Vector system (Promega) (Section 2.3). All bacterial work was conducted under sterile conditions, and where bacterial suspensions were incubated with shaking, this was conducted at 225 rpm.

2.2.2 Transformation

DH5α cells were transformed using the standard protocol outlined by Invitrogen with a few minor adjustments. In brief, 50 µL of DH5α cells were thawed on ice and gently mixed with 50 ng of plasmid DNA. The mixture was incubated on ice for 30 mins before heat shocking for 45 secs using a 42 ºC water bath. The cells were immediately incubated on ice for a further 2 mins and then incubated in 950 µL of pre-warmed SOC media for 1 hr at 37 ºC with shaking as a recovery period. The required volume of cell suspension was spread onto LB Agar plates with the appropriate antibiotic selection (ampicillin/kanamycin) (Table 2.1) and incubated overnight (O/N) at 37 ºC.

2.2.3 DNA preparation

A single transformed bacterial colony was selected from each bacterial plate using a pipette tip sterilised by autoclaving. This was used to inoculate 5 mL of LB

supplemented with the appropriate antibiotic for 12 hrs (37 ^{o}C, with shaking). Initially, small scale isolation of plasmid DNA was conducted using the Plamid Miniprep Kit (Qiagen), following the standard protocol. DNA harvested in this way was used for plasmid verification via sequencing (Source Bioscience) if cloning was conducted, or through a diagnostic digestion using fast digestion restriction enzymes (Thermo Scientific), according to the manufacturer's guidelines, assessed via 1 % agarose gel electrophoresis (Table 2.1). Glycerol stocks of plasmids were created by combining 200 µL of glycerol (Fisher Scientific) with 800 µL of cell suspension from a bacterial culture and stored at -80 ^{o}C.

Isolation of plasmid DNA was subsequently conducted on a larger scale. Initially, glycerol stocks were streaked onto LB Agar plates (with the correct antibiotic selection) and grown O/N. Again, a single bacterial colony was used to inoculate 5 mL of LB broth supplemented with the appropriate antibiotic, this time for 16 hrs (37 ^{o}C, with shaking). This suspension was transferred to 200 mL of LB and incubated O/N (37 ^{o}C, with shaking). Plasmids were harvested using the Plasmid Midiprep Kit (Qiagen), according to the standard protocol. Following mini and midi DNA preparation, the NanoDrop® ND-1000 UV/VIS Spectrophotometer (Nanodrop, LabTech) was used to quantify the plasmid concentration and assess purity, according to the manufacturer's guidelines.

2.3 Plasmids

Table 2.3.1 Plasmids used throughout this study

The source or reference of these primers is indicated.

Plasmid	Source/Reference
pSV-AR	(Brinkmann *et al.*, 1989)
Bos-β-galactosidase	C. Bevan
pGL3-TAT-GRE-LUC (ARE-luciferase)	(Jenster et al., 1997)
3 x ERE TATA LUC (ERE-luciferase)	Addgene
pSG5-ERα	M. Parker
pSG5-Empty	Stratagene
pGL4.18	Promega
pGL4.18-ERE$_{-19,247-18,610}$	R.A. Bryan/ G. Brooke
pGL4.18-ERE$_{-18,889-18,783}$	R.A. Bryan/ G. Brooke
pGL4.18-ERE$_{+150,441-758}$	R.A. Bryan/ G. Brooke
pGL4.18-ERE$_{+151,438-726}$	R.A. Bryan/ G. Brooke
pEGFP-NI-AR	G. Brooke
pcDNA3.1-RFP-ERα	R.A. Bryan/ G. Brooke

2.3.1 Cloning

Identified EREs in the *AR* promoter were cloned for intended use with luciferase reporter assays and ERα was cloned into the pcDNA3.1-Red Fluorescent Protein (RFP) plasmid for use with fluorescence microscopy. Initially, the ERE sites and ERα sequence were amplified by PCR using REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich), with primers designed to add either *Kpn*I and *Xho*I ($ERE_{+150,441-758}$ and $ERE_{+151,438-726}$), *Kpn*I and *Bgl*II ($ERE_{-19,247-18,610}$ and $ERE_{-18,889-18,783}$) or *Bam*HI and *Eco*RI (ERα-RFP) restriction sites (Table 2.3.2). Amplified regions for the ERE suites were sub-cloned into the pGEM®-T Easy Vector (Promega) and transformed into JM109 High Efficiency Competent cells, according to the manufacturer's guidelines. Transformation cultures were spread onto prepared LB/ampicillin/IPTG/X-Gal plates (Table 2.1), and incubated O/N. A single white bacterial colony was selected per plate and used to inoculate 5 mL of LB supplemented with ampicillin for 12 hrs (37 ^{o}C, with shaking). Subsequently, isolation of plasmid DNA was conducted using the Plasmid Miniprep Kit (Qiagen), following the standard protocol (As in Section 2.2.3). This step was not conducted for ERα-RFP. Isolated plasmids were digested using *Kpn*I and *Xho*I ($ERE_{+150,441-758}$ and $ERE_{+151,438-726}$), *Kpn*I and *Bgl*II ($ERE_{-19,247-18,610}$ and $ERE_{-18,889-18,783}$) or *Bam*HI and *Eco*RI (ERα-RFP) fast digestion restriction enzymes (ThermoFisher Scientific), according to the manufacturer's guidelines. The pGL4.18 (Promega) or pcDNA3.1-RFP plasmids were also digested using the relevant enzymes, and then subsequently treated using FastAP Thermosensitive Alkaline Phosphatase (ThermoFisher Scientific), according to the standard protocol. The resulting products of these reactions were separated using 1 % agarose gel electrophoresis, from which they were extracted and purified using the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's guidelines.

Table 2.3.2 Sequences of primers used to amplify cloning sites and to add on restriction sites

Sequences are displayed in 5' to 3' direction. The recommended oligo sequence for the restriction enzyme site by New England BioLabs is indicated (Capital letters), with the exact restriction enzyme sites also indicated (white).

Primer Name	Sequence
ERE$_{+150,441-758}$ FP (*KpnI* restriction site)	GGGGTACCCCttgactcaggcggatgca
ERE$_{+150,441-758}$ RP (*XhoI* restriction site)	CCGCTCGAGCGGctggcttcttctcctggag
pGL4.18-ERE$_{+151,438-726}$ FP(*KpnI* restriction site)	GGGGTACCCCtaagccattaatacaccaatcgtatt
pGL4.18-ERE$_{+151,438-726}$ RP (*XhoI* restriction site)	CCGCTCGAGCGGcctgctcatatgaaattgcagag
ERE-$_{19,247-18,610}$ FP (*KpnI* restriction site)	GGGGTACCCCTttgatttctaaggccagataactg
ERE-$_{19,247-18,610}$ RP (*BglII* restriction site)	GAAGATCTTCactctaatgcctcagagccaag
ERE$_{-18,889-18,783}$ FP (*KpnI* restriction site)	GGGGTACCCCTctcaccttcctgatcagcc
ERE$_{-18,889-18,783}$ RP (*BglII* restriction site)	GAAGATCTTCgaagaaacaccttctctcctcca
pcDNA3.1-RFP-ERα FP (*BamHI* restriction site)	CGGGATCCCGaatgaccatgaccctccaca
pcDNA3.1-RFP-ERα RP (*EcoRI* restriction site)	GGAATTCCtcagaccgtggcagggaa

Plasmid and inserts were ligated using a Rapid DNA Ligation kit (ThermoFisher Scientific), following the standard protocol. The resulting ligated plasmids were transformed into DH5α (Section 2.2.2) and the plasmid DNA isolated (Section 2.2.3). The cloning procedure was confirmed initially through a diagnostic digestion using the same fast digestion restriction enzymes as for the cloning procedure, and 1 % agarose gel electrophoresis was conducted to confirm the correct size plasmid and insert. If this was correct, the plasmid was additionally verified via sequencing (Source Bioscience).

2.4 Mammalian cell culture

Two established adherent human BCa cell lines were utilised: MCF7 and T47D. These cell lines are models of endocrine sensitive BCa and express ERα and AR. Additionally the established cell line COS-1 was utilised, as these cells are negative for both ERα and AR (Brooke, unpublished). These three cell lines were obtained from the American Type Culture Collection and were cultured in their relevant media, described in Table 2.1. The endocrine resistant cell line derivatives were produced via the method outlined in Figure 2.1 by Dr Greg Brooke. These were cultured using the hormone-depleted media described in Table 2.1 and in the presence of 100 nM Fulvestrant/Tamoxifen where necessary (Figure 2.4.1).

All cells were cultured under incubation conditions of 37 ^{o}C and 5 % Carbon dioxide (CO_2), with regular microscopy observations to monitor cell confluence and health. Once the cell confluence reached approximately 70–80 %, cell passaging was completed (twice weekly).

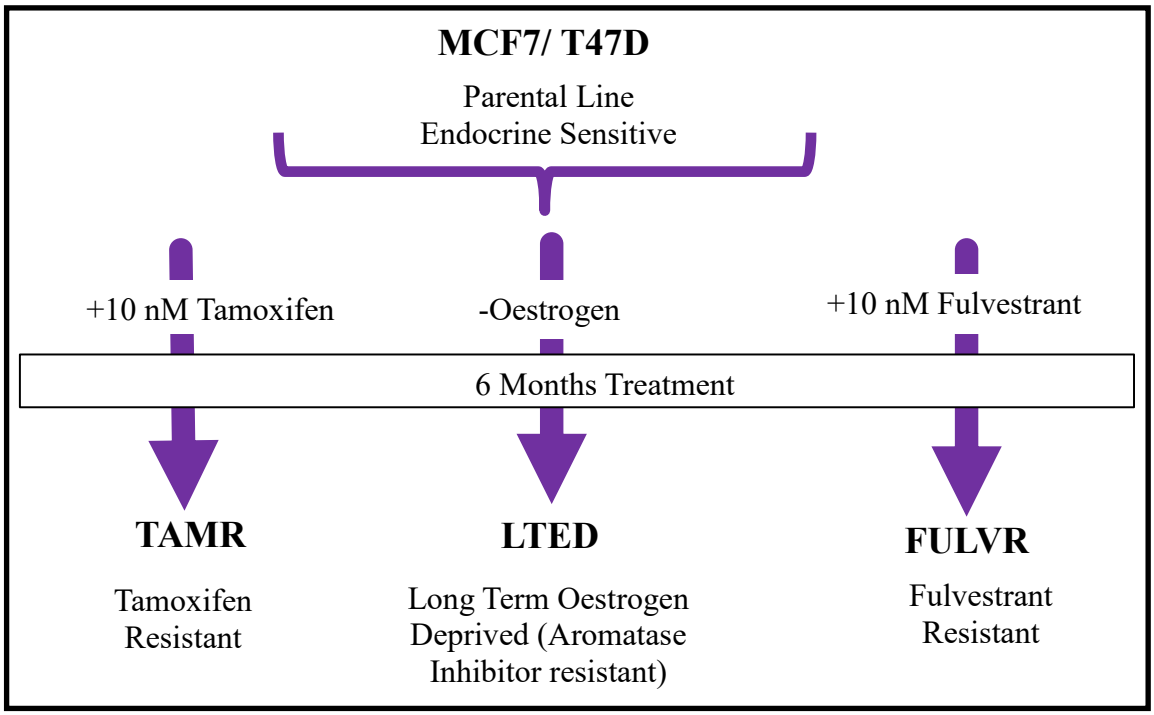


Figure 2.4.1 Outline of the production of various endocrine resistant cell lines using MCF7 and T47D endocrine sensitive Breast Cancer parental lines (conducted by Dr Greg Brooke)

Cells were grown for 6 months using hormone-depleted phenol red free Dulbecco's Modified Eagle Medium (DMEM) (MCF7) or Roswell Park Memorial Institute (RPMI) (T47D) supplemented with charcoal stripped foetal calf serum (FCS), penicillin streptomycin and glutamine (hormone-depleted). Additionally 100 nM Tamoxifen was added in the Tamoxifen Resistant (TAMR) derivative and 100 nM Fulvestrant for the Fulvestrant Resistant (FULVR) derivative. Following this, a cell colony was picked and continued to be grown under the specified conditions.

2.4.1 Freezing and defrosting cells

To make frozen cell stocks, cells were passaged, pelleted (1,500 rpm for 3 mins) and re-suspended using pre-warmed freezing mixture (Table 2.1). The mixture was transferred into cryotubes (1 mL per tube), wrapped in insulating material and maintained at -80 ^{o}C for short term storage, but transferred to liquid nitrogen for longer term storage. To defrost frozen stocks, 1 mL of frozen cells were defrosted at 37 ^{o}C, then transferred immediately into 10 mL of pre-warmed medium. The resulting cell suspension was centrifuged (1,500 rpm for 3 mins), the supernatant removed and the cells re-suspended in the required volume of relevant media (Table 2.1) and returned to culture conditions (Section 2.4.1).

2.5 Transient transfection of mammalian cells

2.5.1 Calcium phosphate

The Calcium phosphate method was conducted as outlined previously (Chen and Okayama, 1987). Per well of a 24 well plate, the required DNA was mixed and diluted to 45 µL using ddH_2O (Refer: Section 2.6). Subsequently, 5 µL of 2.5 M $CaCl_2$ and 50 µL of 2 x BBS were added, and mixed gently via bubbling with a Gilson pipette. The resulting transfection mix was incubated at RT for 15 mins, before being added to the well in a drop-wise manner.

2.5.2 jetPRIME

Alternatively, transections were conducted for use with confocal microscopy. Cells were seeded at a low confluency (approximately 20 %) on cover-slips in the relevant hormone-depleted media, and incubated for 24 hrs. Following this, cells were transfected using jetPRIME transfection reagent (Polypus Transfection) according to the standard protocol. After 48 hrs, cells were treated with the required hormones for 2 hrs and washed three times using PBS, prior to fixing. Fixing was accomplished using 200 µL of 4 % Paraformaldehyde (PFA) for 15 mins, whilst rocking gently at RT and coverslips were fixed onto microscope slides and visualisation accomplished as outlined in Section 2.9.

2.5.3 siRNA knockdown

Cells were plated in 96 well or 6 well plates in the relevant hormone-depleted media for 24 hrs prior to transfection. On-target small interfering RNA (siRNA) pool targeting AR/ERα or control non-target (NT) siRNA (Dharmacon, catalogue numbers L-003400-00-0005, L-003401-00-0005 and D-001206-13-05 respectively) to a final concentration of 20 nM (96 well) or 50 nM (6 well) was transfected using Lipofectamine RNAiMAX Reagent, according to the manufacturer's guidelines (Invitrogen). The knockdown was confirmed using immunoblotting and qPCR.

2.6 Reporter assays

COS-1 cells were plated in the relevant hormone-depleted media (Table 2.1) at approximately 60 % confluency in 24 well plates and incubated for 24 hrs prior to transfection. Cells were transfected using 50 ng pSV-AR, pSG5-ERα or Empty Vector

(EV); 10 ng β-galactosidase; and 1 µg ARE-/ERE-luciferase reporter (Table 2.3.1) using the Calcium phosphate Method (Section 2.5.1). 24 hrs post transfection, cells were washed twice using pre-warmed hormone-depleted media, before being replaced with fresh hormone-depleted media containing the desired concentration of hormone/drug/vehicle. After a 24 hr incubation period, cells were washed twice using pre-chilled PBS and lysed by adding 60 µL of 1 x Reporter Lysis Buffer (Promega) and being transferred to -80 ^{o}C until frozen. Luciferase assays (Promega) were conducted on 20 µL of defrosted lysate alongside the β-galactosidase assay Galacto-Light (Life Technologies) on 5 µL for normalisation, according to each manufacturer's guidelines. Luminescence was quantified using the FLUOstar Omega plate reader (BMG Labtech).

2.7 Gene expression analysis

To prepare samples, the relevant cells were seeded at approximately 70 % confluency in either 6 or 12 well plates and cultured in hormone-depleted media (Refer: Table 2.1) for 120 hrs. Following this, cells were treated with the required ligand or drug concentration for either an 8 or 24 hr time period.

2.7.1 RNA extraction

Following treatment, cells were washed twice using ice cold PBS and lysed in TRIsure reagent (Bioline). RNA extraction was conducted according to the standard protocol. Visualisation of the RNA pellet was aided using Glycoblue (Ambion). Once pelleted, an additional 75 % Ethanol (EtOH) wash step for the RNA was added, and pellets were re-suspended post air-drying in 30 µL (6 well) or 20 µL (12 well) RNase free, sterile ddH_2O. RNA was quantified and its quality assessed using the NanoDrop® ND-

1000 UV/VIS Spectrophotometer (Nanodrop, LabTech), according to the manufacturer's guidelines.

2.7.2 cDNA synthesis

Reverse transcription was conducted to synthesise complementary DNA (cDNA) using either the Transcriptor First Strand cDNA Synthesis Kit (Roche) or High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific), according to each manufacturer's guidelines. 500 ng of RNA was utilised per cDNA synthesis reaction and the resulting cDNA was diluted 1:4.

2.7.3 Primer design

To design primers for gene expression analysis, mRNA and genomic sequences of the gene were obtained using the National Centre for Biotechnology Information (NCBI) and converted from a FASTA to a tabulated format using the FaBox (1.4.1) Fasta2excel converter. To design primers for use with ChIP analysis, the sequences to input were obtained from the relevant DNA site under investigation using the University of California, Santa Cruz (UCSC) Genome Browser, following assessment of TF binding using the PROMO tool for the identification of putative transcription factor binding sites (Algorithmics and Genetics Group, ALGGEN, Universitat Politècnica de Catalunya). These sequences were input into PerlPrimer Software and designed for use with qPCR. The parameters were set to create primers 20-25 bp in length with an amplicon size near to 100 bp, ideally spanning the intron/exon boundary, with the other parameters kept as recommended.

2.7.4 Real-Time quantitative PCR (qPCR)

Real-Time quantitative PCR (qPCR) was performed on 2 µL of cDNA with the LightCycler® 480 SYBR Green I Master (Roche) or Fast SYBR™ Green Master Mix (Applied Biosystems) where specified, in the reaction conditions according to each manufacturer's specifications. qPCR was conducted using the LightCycler® 480 (Roche) and a melt curve was observed for each reaction. Gene expression was normalised using the *RPL19 ribosomal protein* (*L19*) reference gene and analysed using the delta-delta Ct ($\Delta\Delta$Ct) method. The primers used are displayed in Table 2.7.1.

2.8 Protein analysis

2.8.1 Cell collection

Cells were washed twice using ice cold PBS and detached via scraping in fresh PBS. Cells were subsequently centrifuged (13,000 rpm for 1 min at 4 ^{o}C), the supernatant discarded and the remaining cell pellet snap frozen and stored at -80 ^{o}C. When required, cell pellets were re-suspended in Radioimmunoprecipitation buffer (RIPA) supplemented with Halt Protease Inhibitor (PI) Cocktail (ThermoScientific) and Phenylmethanesulphonylfluoride (PMSF) to a final concentration of 1 µM (Table 2.1) (100 µL of RIPA was utilised per well of a 6 well plate). Lysates were sonicated for 3 cycles on 'high' of 30 secs on and 30 secs off using Biorupter® Plus (Diagenode). Following this, samples were centrifuged (13,000 rpm for 10 mins at 4 ^{o}C) and the supernatant was transferred to a fresh pre-chilled 1.5 mL tube.

Table 2.7.1 Sequences of gene expression primers for use with qPCR

Sequences are displayed in 5' to 3' direction, and the source of the primers is included.

Gene	Forward Primer Sequence	Reverse Primer sequence	Source
Myelocytomatosis Oncogene Cellular Homolog (MYC)	GGCTCCTGGCAAAAGGTCA	CTGCGTAGTTGTGCTGATGT	(Qinyu *et al.*, 2013)
Deiodinase, Iodothyronine Type II (DIO2)	ACTCGGTCATTCTGCTCAAG	TCACCCAATTTCACCTGTTTGT	(Fujii *et al*., 2014)
Dopa Decarboxylase (DDC)	GACTGGACCCTTGTCGAAACT	TCTTCACCAACTTTCACTGTTCC	(Fujii *et al*., 2014)
Gene Regulated in Breast Cancer 1 (GREB1)	ATGGGAAATTCTTACGCTGGAC	CACTCGGCTACCACCTTCT	(von der Heyde *et al.*, 2015)
N-myc Downstream-Regulated Gene 1 (NDRG1)	CTCCTGCAAGAGTTTGATGTCC	TCATGCCGATGTCATGGTAGG	(Wu *et al.*, 2015)
RPL19 ribosomal protein (L19)	GCGGAAGGGTACAGCCAAT	GCAGCCGGCGCAAA	(Millour *et al.*, 2010)
SEC14 like Lipid binding 2 (SEC14L2)	CCGAAACACTGAAGCGTCTTT	CTCCTTCCAATTTGCTCCCAG	R.A. Bryan
Trefoil Factor 1 (TFF1)	CATGGAGAACAAGGTGATCTG	CACTGTACACGTCTCTGTCTG	R.A. Bryan
Zinc Finger And BTB Domain Containing 16 (ZBTB16)	CTGGATAGTTTGCGGCTGAG	ATGTCAGTGCCAGTATGGGT	R.A. Bryan

2.8.2 DC protein assay

The protein concentration was quantified using the Detergent Compatible (DC) Protein assay (Bio-Rad), using 5 µL of sample against standard concentrations of BSA as a guideline, according to the manufacturer's protocol. Protein concentrations were measured at absorbance λ = 650 nm using the FLUOstar Omega plate reader (BMG Labtech). Samples were diluted to an equal volume of protein per set (10–20 µg/10 µL of sample) with 4 x Laemmli protein sample buffer (Bio-rad) (2.5 µL/10 µL of sample).

2.8.3 SDS-PAGE

Prior to running, lysates were vortexed and incubated at 95 ^{o}C for 5-10 mins. Immediately following this, samples were transferred to ice and vortexed once cool. 15-20 µL of lysate was loaded per well of a 10 % polyacrylamide gel, against 5 µL of the Page Ruler Prestained Protein Ladder (Thermo Scientific) unless otherwise stated in results. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted at 120 V with pre-made running buffer.

2.8.4 Immunoblotting

Proteins were transferred onto a Polyvinylidene difluoride (PVDF) membrane (Immobilion P, Millipore Inc., hydrated in preparation using 100 % Methanol, MeOH) via semi-dry transfer. This was conducted at 15 V and 100 mA for 2 hrs, using semi-dry electro blotting apparatus (Bio-Rad) and pre-made transfer buffer. Following this, membranes were incubated with blocking solution for 15 mins, followed by probing with the required primary (1^{o}) antibody (Ab) (Table 2.8.1a) for 1 hr at RT or O/N at 4 ^{o}C, with

gentle rocking. Three washes were conducted using PBS-T for 5 mins, prior to the relevant secondary (2º) Ab incubation (Table 2.8.1a) for 1 hr at RT, both with rocking. Three washes were conducted again using PBS-T for 5 mins, and an additional wash with PBS was conducted for 5 mins, each with rocking. Proteins were subsequently visualised via chemiluminescence using Luminata™ Forte (Millipore) with the Fusion FX imager (Vilber Lourmat).

2.9 Cell staining and confocal imaging

Cells were seeded at a low confluency (approximately 20 %) on cover-slips in the relevant hormone-depleted media, and incubated for 24 hrs. Following this, cells were either transfected (Section 2.5.2) or treated with the required hormones for 2 hrs and washed three times using PBS, prior to fixing. Fixing was accomplished using 200 µL of 4 % PFA for 15 mins, whilst rocking gently at RT. Subsequently, cells were washed three more times with PBS for 5 mins (shaking), and fixed using 200 µL of ice cold 100 % MeOH for 10 mins at -20 ºC.

For staining, cells were initially incubated with 250 µL of 1 % BSA in PBS-T for 30 mins at RT, with gentle shaking. 1º Ab incubation was conducted for AR or ERα (Table 2.8.1b) in 100 µL 1 % BSA PBS-T, for 1 hr at RT (or O/N at 4 ºC), shaking gently. Cells were washed three times for 5 mins again, and 2º Ab incubation was conducted as per Table 2.8.1b, in 100 µL of 1 % BSA PBS-T for 1 hr at RT in the dark, shaking gently. Cells were washed an additional three times for 5 mins, and coverslips were fixed onto microscope slides with Fluoroshield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI, Abcam) and sealed using Fixogum (Marubu). Cells could then be visualised using confocal microscopy.

Table 2.8.1 Antibodies

A description of the antibodies used for **(a)** immunoblotting **(b)** Confocal Microscopy and **(c)** Chromatin Immunoprecipitation (ChIP) experiments. ChIP concentrations were used as recommended by the Zymo-Spin™ ChIP Kit.

(a) Immunoblotting

Protein	Primary Antibody	Secondary Antibody
Androgen Receptor (AR)	AR (N-20) sc-816 (Santa Cruz, diluted 1:1,00)	Anti-Rabbit IgG (whole molecule)-Peroxidase (Sigma-Aldrich, diluted 1:2,000)
Beta-Actin (β-Actin)	Beta Actin Ab8226 (Abcam diluted 1:3,000)	Anti-Rabbit IgG (whole molecule)-Peroxidase (Sigma-Aldrich, diluted 1:2,000)
Beta-Tubulin (β-Tubulin)	Beta Tubulin T5168 (Sigma-Aldrich diluted 1:3,00)	Anti-Mouse IgG (whole molecule)-Peroxidase (Sigma-Aldrich, diluted 1:2,000)
Oestrogen Receptor alpha (ERα)	ERα HC-20 sc-543 (Santa Cruz diluted 1:2,00)	Anti-Rabbit IgG (whole molecule)-Peroxidase (Sigma-Aldrich, diluted 1:2,000)

(b) ChIP

Protein	Primary Antibody	Secondary Antibody
Androgen Receptor (AR)	Ms mAb to AR 441 (Abcam, diluted 1:200 for MCF7 derivatives and 1:150 for T47D derivatives)	Goat pAb to Ms IgG (Alexa 488, Abcam, diluted 1:4,000)
Oestrogen Receptor alpha (ERα)	ERα HC-20 sc-543 (Santa Cruz, diluted 1:400 for MCF7 derivatives and 1:300 for T47D derivatives)	Goat pAb to Rb IgG (Alexa 488, Abcam, diluted 1:4,000)

(c) Confocal Microscopy

Protein	Primary Antibody	Secondary Antibody
Androgen Receptor (AR)	AR (N-20) sc-816 (Santa Cruz)	Dynabeads Protein A (Life Technologies)
Oestrogen Receptor alpha (ERα)	ERα (HC-20) sc-543 Santa Cruz)	Dynabeads Protein A (Life Technologies)

2.10 Proliferation assays

Cells were grown in a 96-well plate with the relevant hormone-depleted culture media (Table 2.1), and treated using the desired concentration of hormone/drug treatment/vehicle for 72–120 hrs (both specified per experiment). Cell proliferation was then assessed using WST-1 Cell Proliferation Reagent (Abcam), according to the manufacturer's protocol. Growth was quantified by measuring the resulting absorbance λ = 440 nm on the FLUOstar Omega plate reader (BMG Labtech).

2.11 Colony formation assays

Cells were seeded at a low confluency in 6 well plates (approximately 10 %) in the required hormone-depleted media (Table 2.1). Cells were cultured in the presence of the relevant ligand/drug concentration or vehicle, with twice weekly media changes with fresh treatments, for 4 weeks. The wells were washed three times using PBS, followed by fixing at RT with 500 µL of 4 % PFA for 1 hr. Subsequently, cells were washed a further three times with PBS and left O/N to air dry fully. Fixed cells could then be stained using 500 µL of 0.08 % crystal violet at RT for 1 hr, with gentle rocking. Wells were washed a further three times using ddH_2O and following air drying O/N were visualised using a the Epson Perfection 1250 scanner.

2.12 ChIP

Chromatin Immunoprecipitation (ChIP) was conducted to assess AR and ERα DNA binding in MCF7 or MCF7-TAMR cells. To prepare samples, cells were cultured in hormone-depleted media (Table 2.1) for 120 hrs and treated with EtOH (vehicle)/1 nM

MIB/1 nM E2/E2 + MIB for 4 hrs. ChIP assays were then conducted via the Zymo-Spin™ ChIP Kit (Zymo Research), according to the manufacturer's guidelines. Briefly, cells were treated with formaldehyde to crosslink DNA-protein complexes and this reaction was quenched using Glycine to a final concentration of 250 µM. Cells were then lysed and sonicated to fragments approximately 200 bp in size (analysed using 1% agarose gel electrophoresis). Following this, 4 µg per assay of AR and ERα antibodies (Table 2.8.1c) were used to pull down DNA target sites. The following adjustments to the protocol were used: samples were sonicated for 25 cycles on 'high' of 30 secs on and 30 secs off using Biorupter® Plus (Diagenode) and 3 x 10^6 rather than 1 x 10^6 sonicated cells were utilised per assay. AR and ERα binding sites were assed via qPCR (Refer: Section 2.7.3) with primers for validated and suspected binding sites against control regions (Table 2.12.1).

Table 2.12.1 Sequences of primers to identify Androgen and Oestrogen Response Elements

Sequences are displayed in 5' to 3' direction. Positive (+ve) and negative (-ve) regions are indicated for each Androgen Response Element (ARE) or Oestrogen Response Element (ERE) on the *Androgen Receptor* (*AR*), *Trefoil Factor 1* (*TFF1*) or *Zinc Finger And BTB Domain Containing 16* (*ZBTB16*) genes. The source of the primers is included.

Description	Forward Primer Sequence	Reverse Primer sequence	Source
***TFF1* ERE +ve**	TATGAATCACTTCT GCAGTGAG	GAGCGTTAGATA ACATTTGCC	(Periyasamy *et al.*, 2015)
***TFF1* ERE –ve**	GTGATTCTCCTGA CTTAACC	TGGCGCAGTGGC TCACGCTG	(Periyasamy *et al.*, 2015)
***ZBTB16* ARE +ve**	ATGCCCTGCGTCT GTACTCATT	TGTTCTGATGAG ATCTGCACGCCT	(Robinson *et al.*, 2011)
***ZBTB16* ARE -ve**	GTCCTGTCTCCCA TTCCAGA	GAGAAGCCCAAT CGCAATAA	R.A. Bryan
***AR* ERE$_{-19,247-18,610}$**	TGGCTTGGGACTT TAGCCTC	TGAGGTGACCTG GTTTAGCC	R.A. Bryan
***AR* ERE -ve**	CAGCATTGCATAG CCAGAAA	AAAGCCTTCCAC AGCTTTCA	R.A. Bryan

Chapter 3. Results: Androgen Receptor and Oestrogen Receptor alpha cross-talk in endocrine sensitive Breast Cancer

3.1 Introduction

Breast cancer (BCa) is currently the most highly prevalent cancer among women in the UK (Cancer Research UK, 2014a). In 2014 in the UK alone, there were 55,222 BCa diagnoses (Wu et al., 2015). The majority of BCas are endocrine sensitive: Oestrogen Receptor α (ERα)-positive (Luminal) cancers that are dependent upon oestrogens for progression (Chuffa *et al.*, 2017, Rakha and Green, 2016, Ali and Coombes, 2002). ERα is a Type I Nuclear Receptor (NR), a family of ligand-dependent transcription factors that translocate to the nucleus to exert their effects (Sever and Glass, 2013). The most abundant circulating form of oestrogen as well as the most potent is 17-β-Oestradiol (E2) (Bean *et al*., 2014, Speirs and Walker, 2007).

For endocrine sensitive patients where the disease has spread from its primary site or metastasised, treatment often involves endocrine therapies (Vorobiof, 2016). These include anti-oestrogens, which bind to ERα and block its action. The anti-oestrogens used in this study are the Selective ERα Modulator (SERM) Tamoxifen (TAM) and the Selective ERα Downregulator (SERD) Fulvestrant (FULV), with the latter additionally enhancing ERα degradation (Kaklamani and Gradishar, 2017, Cauley *et al.*, 2001, Lumachi *et al.*, 2015). Additionally, Aromatase Inhibitors (AIs) are commonly administered, which block aromatase activity to reduce E2 synthesis, and therefore decrease circulating oestrogen levels (Kaklamani and Gradishar, 2017). However, resistance to anti-oestrogens and AIs commonly occurs, termed endocrine resistance,

and often results in advanced metastatic disease for which few treatment options are available (Kaklamani and Gradishar, 2017, Vorobiof, 2016, Reinert *et al.*, 2017).

The Androgen Receptor (AR) is also a Type I NR, and is activated in response to androgens (Rahim and O'Regan, 2017). Androgens are secreted in significantly higher quantities than oestrogens in women (Burger, 2002) and directly affect the ERα pathway, as two of the main androgens that are produced in women (androstenedione and testosterone) are precursors for E2 (Kaklamani and Gradishar, 2017, Patani and Martin, 2014). It has additionally been reported that AR is more highly expressed than ERα in BCa tissues (Rahim and O'Regan, 2017, Vera-Badillo *et al.*, 2014). In ERα-positive disease, AR appears to be an indicator of positive prognostic outcome, which has been hypothesised to occur by AR having an inhibitory effect on ERα-driven cancer progression (Rahim and O'Regan, 2017, Fioretti *et al*., 2014, Tarulli *et al*., 2014). It has been established that AR and ERα suppress each other's activity, and this cross-talk (Fioretti *et al*., 2014) could explain the inhibitory effect displayed by androgen signalling in ERα-positive BCa. However, in ERα-negative disease it has been suggested that AR can mimic ERα signalling to promote BCa progression in the absence of a functioning ERα (Fioretti *et al*., 2014). Further, recently AR signalling has been indicated as a driver in some cases of endocrine resistance (De Amicis *et al.*, 2010, Rechoum *et al.*, 2014, Ciupek *et al.*, 2015, Ali *et al.*, 2015).

The aim of this chapter was to explore, using the endocrine sensitive cell line MCF7, how therapeutic interventions may alter this cross-talk and promote the onset of endocrine resistance.

3.2 ERα regulates AR in endocrine sensitive BCa

3.2.1 ERα and AR expression is correlated in BCa, and siRNA knockdown of ERα decreases AR expression in endocrine sensitive cells

Data obtained via interrogation of the METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) dataset (Pereira *et al.*, 2016) using the cBioPortal for cancer genomics (Gao *et al.*, 2013, Cerami *et al.*, 2012), indicated that several Type I NRs are co-expressed in BCa, and the strongest association was observed between ERα and AR (Table 3.2.1 and Figure 3.2.1). From this it can be inferred that one of these receptors could regulate the other or that both receptors are regulated by a common factor in endocrine sensitive (ERα-positive) disease. To expand upon this, immunoblotting analysis was conducted on lysates from a cell line model of endocrine sensitive disease (MCF7) following siRNA knockdown of AR or ERα (Figure 3.2.2). This revealed that ERα knockdown in MCF7 cells also resulted in a 51.5 % reduction in AR expression, however ERα expression remained constant following AR knockdown. Therefore, it was concluded that there is a possibility that ERα regulates AR expression.

3.2.2 ERα interacts with an ERE upstream of *AR* and can transcriptionally activate an ERE within the *AR* gene

To investigate this further, analysis was conducted on ERα Chromatin Immunoprecipitation-Sequencing (ChIP-Seq) data from MCF7 cells provided by Carroll *et al.* (2006) using the UCSC Genome Browser to identify the presence of an Oestrogen Response Element (ERE) within a 637 bp region 19,247-18,610 bp upstream of the *AR* gene. Subsequently, the PROMO tool for the identification of putative transcription factor

Table 3.2.1 Comparison of Steroid Receptor expression in 2,433 Breast Cancers obtained from the METABRIC dataset

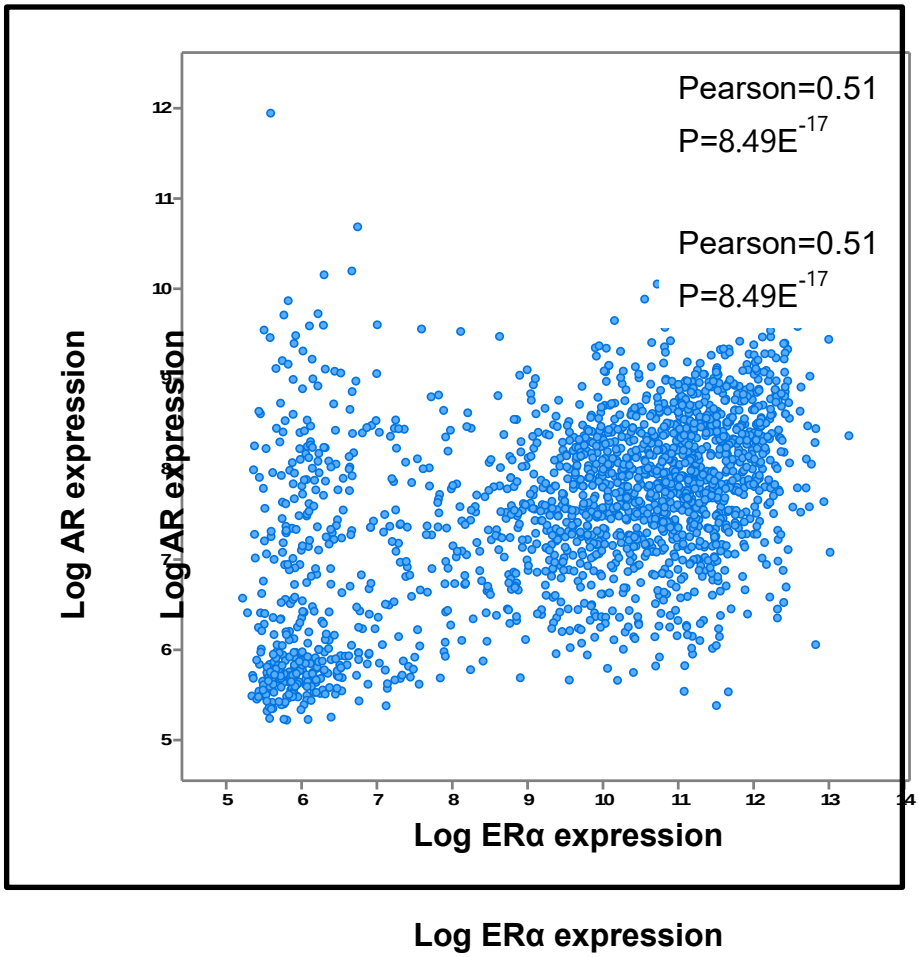
The co-occurrence or mutual exclusivity of expression of Type I Nuclear Receptors (Steroid Receptors) was obtained via interrogation of the METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) dataset (Pereira *et al.*, 2016) using the cBioPortal for cancer genomics (Gao *et al.*, 2013, Cerami *et al.*, 2012). The receptors investigated were: Progesterone Receptor (PR), Oestrogen Receptor alpha (ERα), Oestrogen Receptor beta (ERβ), Glucocorticoid Receptor (GR), Androgen Receptor (AR) and Mineralocorticoid Receptor (MR). Rows have been colour-coded to indicate a tendency towards co-occurrence or mutual exclusivity and significant differences have been indicated in bold. P-values were obtained through the software via the Fisher Exact Test and the Log Odds Ratio is a quantification of co-occurrence or mutual exclusivity in the Breast Cancers analysed.

Gene A	Gene B	P-Value	Log Odds Ratio
AR	**ERα**	**<0.001**	**2.702**
ERα	**ERβ**	**0.001**	**1.472**
GR	**MR**	**0.003**	**1.219**
GR	**ERβ**	**0.012**	**1.049**
ERα	**MR**	**0.018**	**1.149**
PR	MR	0.073	1.006
AR	PR	0.096	<-3
AR	ERβ	0.123	0.572
MR	ERβ	0.170	0.579
PR	ERβ	0.187	<-3
AR	MR	0.224	0.422
AR	GR	0.273	0.346
ERα	PR	0.299	<-3
PR	GR	0.514	0.170
ERα	GR	0.598	-0.141

Key

☐ tendency towards co-occurrence

☐ tendency towards co-mutual exclusivity



Log ERα expression

Figure 3.2.1 AR and ERα are significantly co-expressed in 2,433 Breast Cancers obtained from the METABRIC dataset

The pattern of co-expression of Androgen Receptor (AR) and Oestrogen Receptor alpha (ERα) mRNA in Breast Cancers obtained via interrogation of the METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) (Pereira *et al.*, 2016) using the cBioPortal for cancer genomics (Gao *et al*., 2013, Cerami *et al*., 2012). The Pearson correlation coefficient test was calculated to test for co-occurrence.

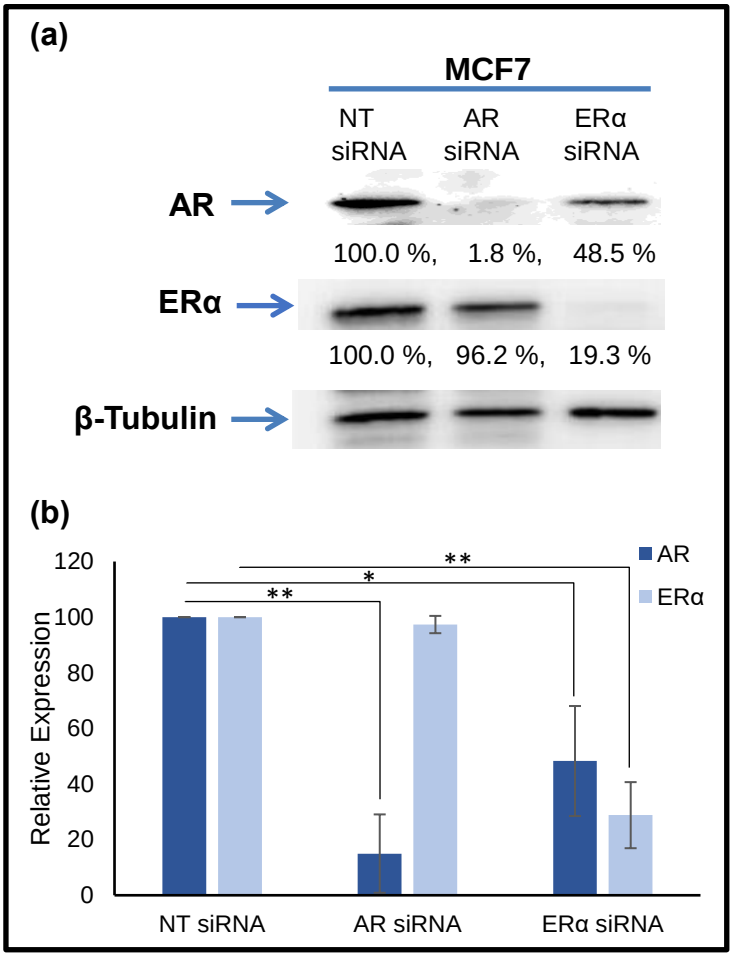


Figure 3.2.2 siRNA knockdown of ERα decreases AR expression in endocrine sensitive cells

(a) Androgen Receptor (AR) or Oestrogen Receptor alpha (ERα) levels were depleted using siRNA in MCF7 cells alongside a Non-Targeting (NT) siRNA control, then collected after 72 hours for Western blotting analysis of AR expression. The percentage expression of ERα or AR following knockdown as compared to the NT siRNA control samples were analysed using Fusion X Software with β-Tubulin as a loading control. **(b)** The average expression of AR and ERα from one replicate in MCF7 cells and two replicates in MCF7-TAMR cells is displayed following AR or ERα depletion using siRNA ±SE. T-Test *P<0.05, **P<0.005.

binding sites (Algorithmics and Genetics Group, ALGGEN, Universitat Politècnica de Catalunya) was used to identify a consensus ERE within this region (AGGTCA), the sequence of which corresponds with a described consensus binding motif for ERα (Carroll *et al*., 2006). To verify this as a functional ERE, a 637 bp region containing the ERE (ERE-$_{19,247\text{-}18,610}$) and a shorter 106 bp region 18,889-18,783 bp upstream of *AR* (ERE-$_{18,889\text{-}18,783}$) (Figure 3.2.3a), were cloned into the pGL4.18 Luciferase Reporter plasmid (Promega) and successful cloning confirmed via sequencing.

COS-1 cells were co-transfected with pSG5-ERα or Empty Vector (EV) and pGL4.18-ERE-$_{19,247\text{-}18,610}$ or pGL4.18-ERE-$_{18,889\text{-}18,783}$, an Empty pGL4.18 (negative control) or a known oestrogen responsive luciferase reporter (ERE-LUC) as a positive control. Luciferase assays were conducted to assess activity in the presence/absence of ERα ligand (E2) (Figure 3.2.3b). The results produced demonstrated that ERα activity was highly responsive to E2 in the positive control (ERE-LUC), whereas no activity was evident for the negative control. Neither of the *AR*-promoter regions cloned showed transcriptional activity (Figure 3.2.3b) and hence it appears that this ERE is not functional. To investigate this result further, ChIP assays were conducted in MCF7 cells treated +/- E2. Primers were designed to test whether ERα interacts within this region of DNA or a negative control region 2,066 bp upstream of this site (confirmed via the PROMO tool to not contain any ERα binding sites) (Figure 3.2.4a). These were tested alongside a known ERE in the promoter region of the *Trefoil Factor 1* (*TFF1*) gene 340-354 bp upstream and a negative control region approximately 685-520 bp upstream (Figure 3.2.4b). *TFF1* otherwise known as *Presenilin 2* (*PS2*), is a strongly established positively regulated ERα target gene in BCa (Lin *et al*., 2004, Brown *et al*., 1984, Westley *et al*., 1984, Welboren *et al*., 2007). In contrast to the reporter assays, this region was demonstrated to be significantly enriched over 3-fold following E2 stimulation (Figure 3.2.4c).

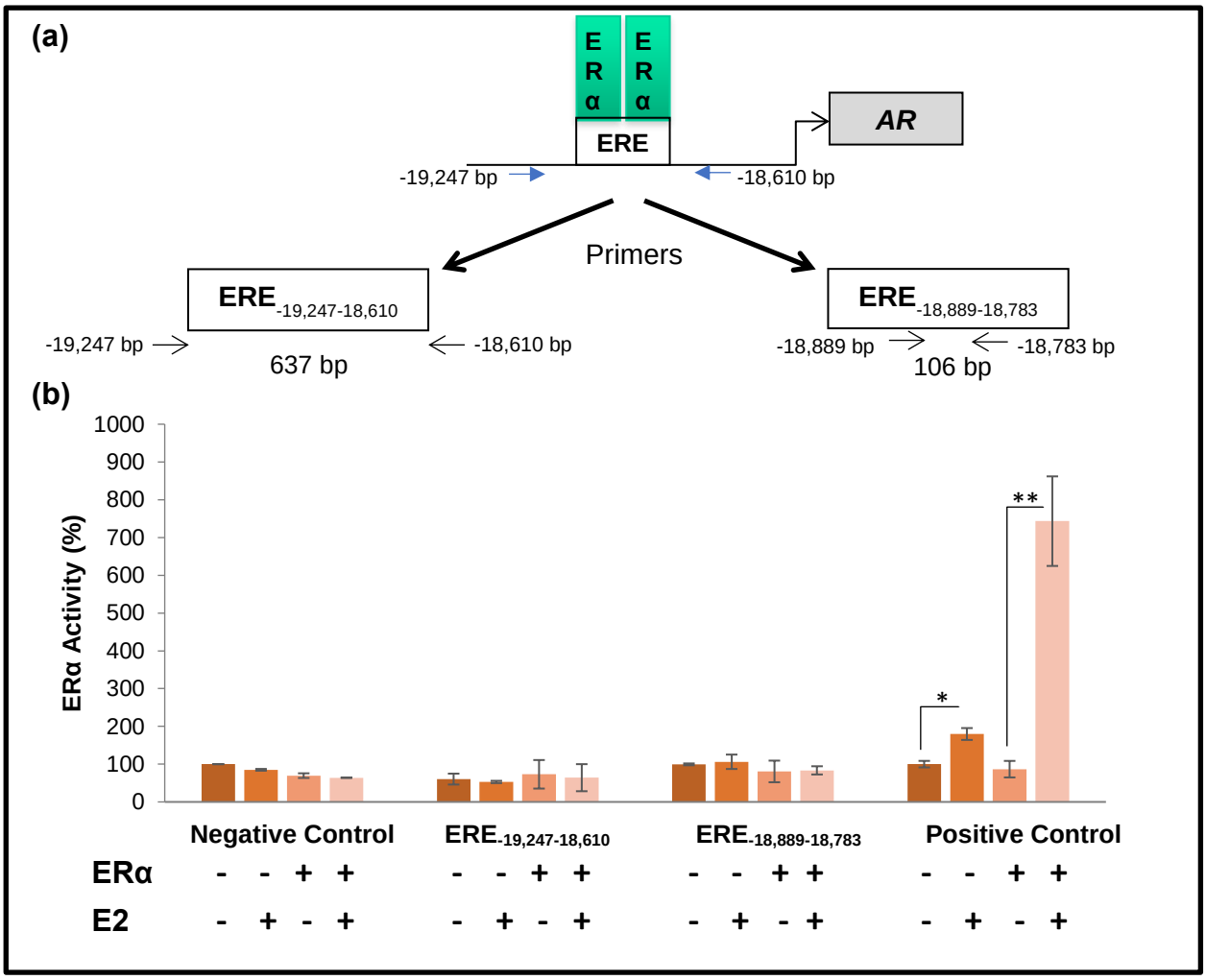


Figure 3.2.3 ERα does not activate a predicted ERE upstream of the *AR* gene

(a) Schematic of an Oestrogen Response Element (ERE) identified upstream of *Androgen Receptor* (*AR*) from ChIP-Sequencing data on MCF7 cells provided by Carroll *et al.* (2006) using the University of California, Santa Cruz (UCSC) Genome Browser. The primers used to clone this ERE into the pGL4.18 Luciferase Reporter plasmid are indicated for the full length region ($ERE_{-19,247-18,610}$) as well as a shorter region within this containing the AGGTCA sequence ($ERE_{-18,889-18,783}$) which corresponds with a described consensus binding motif for ERα. **(b) COS-1 cells** were co-transfected with plasmids encoding Oestrogen Receptor α (ERα) or empty vector (EV) and either $ERE_{-19,247-18,610}$, $ERE_{-18,889-18,783}$, an Empty Vector pGL4.18 plasmid (negative control) or a known ERE luciferase reporter (positive control). Cells were treated for 24 hours with 1 nM 17-β-Oestradiol (E2) ligand or vehicle (Ethanol, EtOH). ERα activity was measured using luciferase and normalised to β-galactosidase activity. Mean of 3 independent duplicates ±SE. T-Test *P<0.05, **P<0.005.

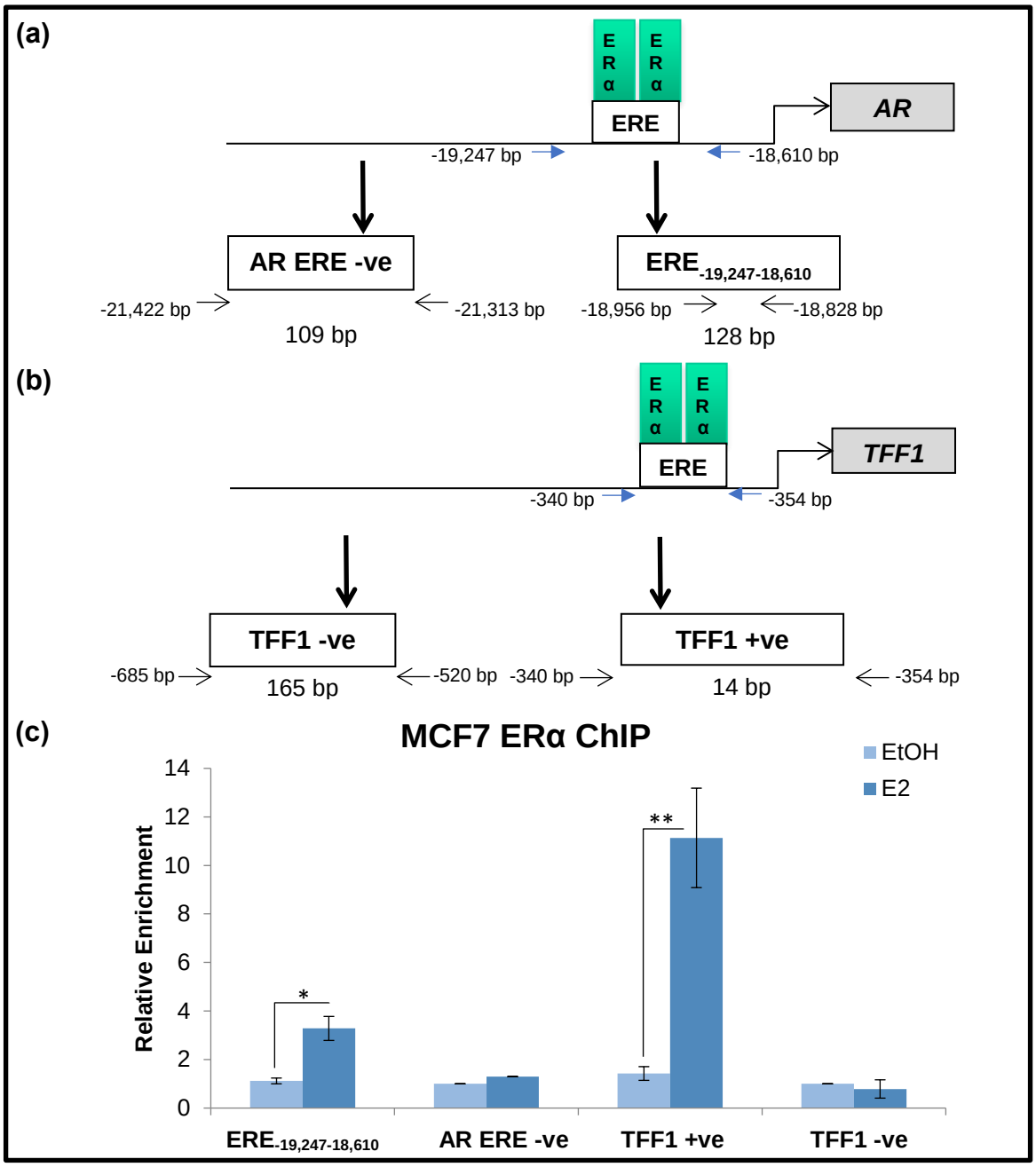


Figure 3.2.4 ERα is recruited ligand-dependently to a predicted ERE upstream of the *AR* gene

(a) Schematic of an Oestrogen Response Element (ERE) identified upstream of *Androgen Receptor* (*AR*) from ChIP-sequencing data on MCF7 cells provided by Carroll *et al.* (2006) using the University of California, Santa Cruz (UCSC) Genome Browser. Primers are indicated to detect this ERE ($ERE_{-19,247-18,610}$) or a negative control region upstream (AR ERE -ve). **(b)** Schematic of a known ERE in the promoter region of *Trefoil Factor 1* (*TFF1*) gene (TFF1 +ve) and its relative control region approximately 2 kb upstream (TFF1 -ve). **(c)** MCF7 cells were treated with Ethanol (EtOH), or 1 nM 17-β-Oestradiol (E2) and crosslinked with formaldehyde prior to DNA shearing using sonication. Chromatin Immunopreceipitation (ChIP) was performed with an ERα-specific antibody. Subsequent qPCR analysis was conducted to amplify $ERE_{-19,247-18,610}$, AR ERE -ve, TFF1 +ve and TFF1 -ve. Results are displayed relative to the relevent negative regions. Mean of 3 independent duplicates ±SE. T-Test *P<0.05, **P<0.005.

To follow on from these results, two alternative EREs located between exons 3 and 4 on the *AR* gene were identified in ChIP-Seq studies by Prof S. Ali (Imperial College London, *Pers. Comm.*) in both MCF7 and ZR-75-1 endocrine sensitive cell lines. These regions were additionally cloned into the pGL4.18 Luciferase reporter plasmid (Promega). The first region was 273 bp in size, located 150,441-150,758 bp from the start site ($ERE_{+150,441-758}$) and contained two ERα binding sequences (gtGTCAcctTGgaCa and AGGTtcctgTGgCCa); and the second was 287 bp, located 151,438-151,726 bp from the start site ($ERE_{+151,438-726}$) and included a perfect palindromic ERE sequence (AGGTCAtgcTGACCT) (Figure 3.2.5a). Luciferase reporter assays were conducted in the same manner (Figure 3.2.5b). The results demonstrated that, again, ERα in the presence of E2 activated the positive control (ERE-LUC), whereas no activity was evident for the negative control (pGL4-Empty). Interestingly, ERα activity was significantly responsive to E2 1.9-fold in the $ERE_{+151,438-726}$ which contains the perfect palindromic ERE sequence, confirming this as a functional site (Figure 3.2.5b). This suggests that ERα can regulate *AR* through inducing transcription at this region. However, ERα activity was not responsive to E2 for $ERE_{+150,441-758}$, but appeared to be constitutively active (Figure 3.2.5b), suggesting that an alternative transcription factor could be regulating *AR* expression through this region.

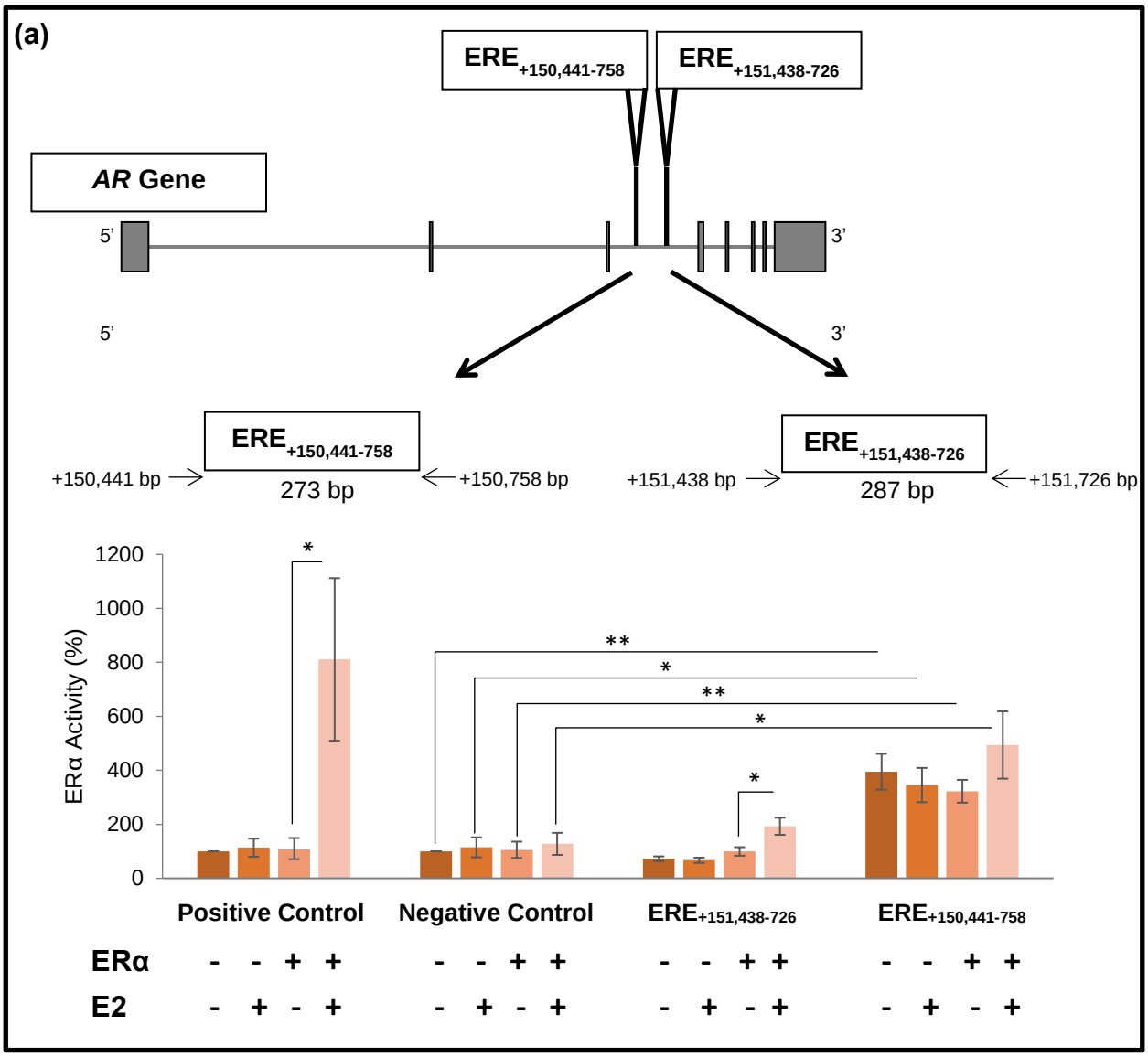


Figure 3.2.5 ERα activates a predicted ERE on the *AR* gene and another is constitutively active

(a) Schematic of two Oestrogen Response Elements (EREs) identified within *Androgen Receptor* (*AR*): ERE+150,441-758 and ERE+151,438-726. Primers are indicated to clone both EREs. **(b) COS-1 cells** were co-transfected with plasmids encoding Oestrogen Receptor α (ERα) or Empty Vector (EV) and either ERE+150,441-758, ERE+151,438-726, an Empty Vector pGL4.18 plasmid (negative control) or a known ERE luciferase reporter (positive control). Cells then treated for 24 hours with 1 nM 17-β-Oestradiol (E2) ligand or vehicle (Ethanol, EtOH). ERα activity was measured using luciferase and normalised to β-galactosidase activity. Mean of 4 independent duplicates ±SE. T-Test *P<0.05, ** P<0.005.

3.2.3 Identification of transcription factors that could be regulating *AR* through a proposed ERE on the *AR* gene

To investigate whether an alternative transcription factor to ERα is regulating AR through $ERE_{+150,441-758}$, the cloned region was analysed for alternative transcription factor binding utilising the PROMO tool (Algorithmics and Genetics Group, ALGGEN, Universitat Politècnica de Catalunya). The results produced indicated binding sites for 44 alternative transcription factors to ERα, which were subsequently investigated via the METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) dataset (Pereira *et al.*, 2016) using the cBioPortal for cancer genomics (Gao *et al*., 2013, Cerami *et al*., 2012) for their association with AR in BCa. The results produced demonstrated a significant association in expression between 20 of these transcription factors and AR (Table 3.2.2), the two most significant being X-box binding protein 1 (XBP1) and MYB Proto-Oncogene, transcription factor (c-Myb) (Figure 3.2.6). Interestingly, Forkhead box protein A1 (FOXA1), a known pioneer factor that has been described as key for AR functioning (Robinson *et al.*, 2011), was also found to be significantly associated with AR expression (Figure 3.2.6). Therefore, ERα may regulate AR, but other transcription factors such as XBP1, c-Myb and FOXA1 are also likely to be involved and these warrant further investigation.

Table 3.2.2 Correlation analysis of the AR with transcription factors that might regulate its expression

Several transcription factors were found to interact with a region within the *Androgen Receptor* gene ($ERE_{+150,441-758}$) using the PROMO tool (Algorithmics and Genetics Group, ALGGEN, Universitat Politècnica de Catalunya). The co-occurrence or mutual exclusivity of these receptors in Breast Cancers was obtained via interrogation of the METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) dataset (Pereira *et al.*, 2016) using the cBioPortal for cancer genomics (Gao *et al.*, 2013, Cerami *et al.*, 2012). Rows have been colour-coded to indicate a tendency towards co-occurrence or mutual exclusivity and only significant differences have been included. P-values adjusted for multiple comparisons were obtained through the software via the Fisher Extact Test and the Log Odds Ratio is a quantification of co-occurrence or mutual exclusivity in the Breast Cancers analysed.

Gene A	Gene B	P-Value	Log Odds Ratio
XBP-1	AR	<0.001	2.802
c-Myb	AR	<0.001	2.004
NFATC1	AR	<0.001	1.88
P53	AR	<0.001	1.084
NFIA	AR	<0.001	2.49
IRF1	AR	<0.001	1.222
FOXA1	AR	<0.001	>3
C/EBPα	AR	<0.001	1.605
MEF-2A	AR	<0.001	1.653
AR	NFIC	<0.001	1.564
AR	Elk-1	0.001	1.046
VDR	AR	0.003	1.099
STAT1	AR	0.006	1.197
c-Ets-1	AR	0.009	1.039
Gtf2i	AR	0.01	0.955
YY1	AR	0.013	0.964
STAT4	AR	0.014	0.95
PROKR1	AR	0.017	0.921
AR	E2F-1	0.022	0.867
TFAP2A	AR	0.027	0.828

Key

- ☐ tendency towards co-occurrence
- ☐ tendency towards co-mutual exclusivity

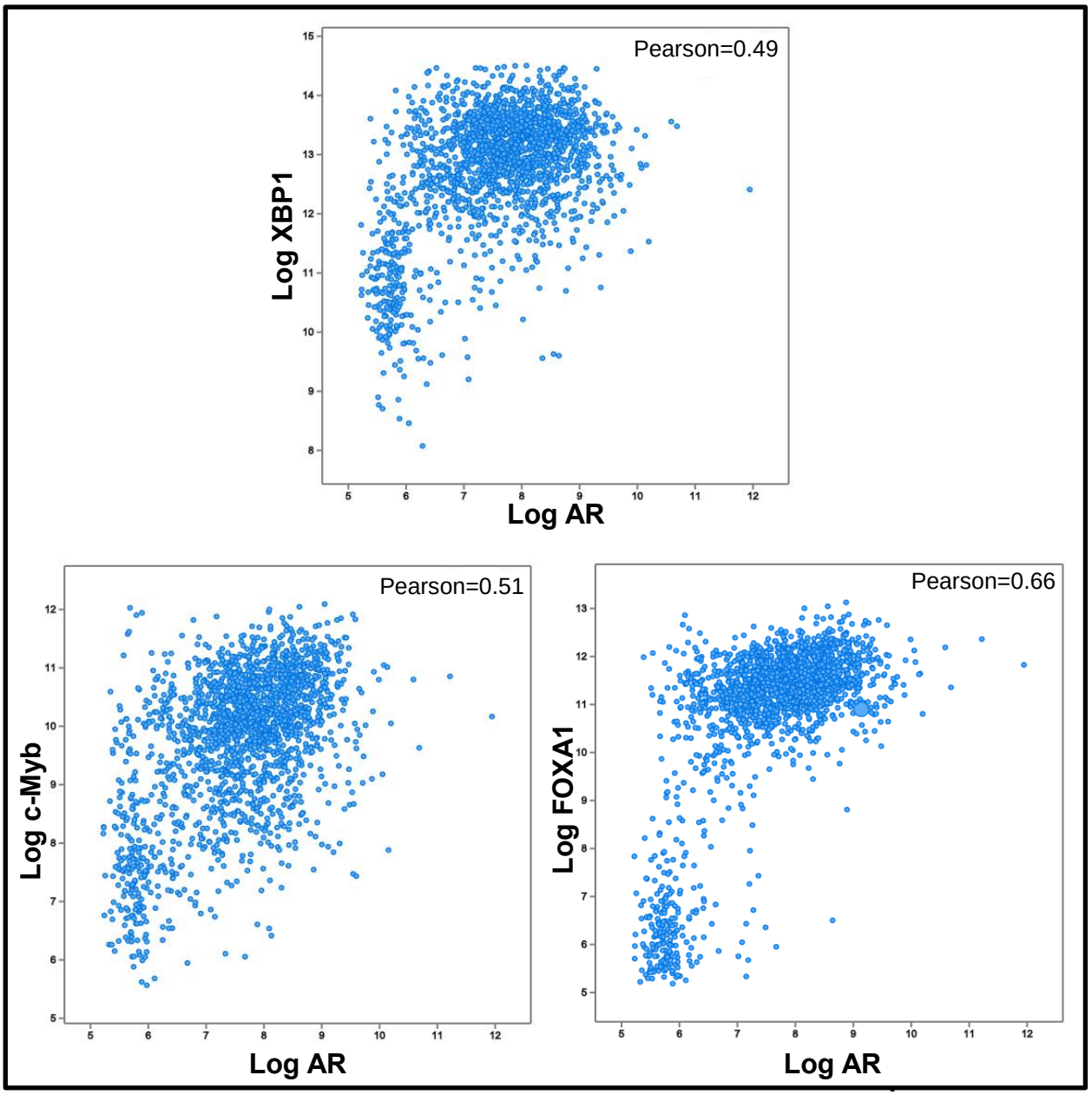


Figure 3.2.6 AR is significantly co-expressed with other transcription factors in 2,433 Breast Cancers obtained from the METABRIC dataset

The pattern of co-expression of Androgen Receptor (AR) with X-box binding protein 1 (XBP1), MYB Proto-Oncogene, transcription factor (c-Myb) and Forkhead box protein A1 (FOXA1) mRNA in Breast Cancers obtained via interrogation of the METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) dataset (Pereira *et al.*, 2016) using the cBioPortal for cancer genomics (Gao *et al*., 2013, Cerami *et al*., 2012). The Pearson correlation coefficient test was calculated to test for co-occurrence.

3.3 The effect of anti-oestrogen treatment on ERα and AR activity

It appears that ERα may regulate AR activity. It is also known that AR and ERα cross-talk at the protein level (Panet-Raymond *et al.*, 2000). To investigate this cross-talk and the effect of anti-oestrogens upon this, reporter assays were performed. Initially, the effect of the anti-oestrogens upon each receptor was analysed. It has been suggested that the anti-oestrogen Fulvestrant (FULV) may also have anti-androgen activity, having been demonstrated to suppress AR-induced growth in the LNCaP Prostate Cancer (PCa) cell line (Bhattacharyya *et al.*, 2006), therefore, it was of interest to see if these inhibitors are also able to block AR activity. The anti-oestrogens Tamoxifen (TAM, a SERM) and FULV (a SERD) were chosen for study. COS-1 cells (negative for both of these receptors) were transfected with ERα or AR and an oestrogen responsive luciferase reporter (ERE-LUC) or a luciferase reporter under the control of an Androgen Response Element (TAT-GRE-EIB-LUC-1), respectively. Cells were also transfected with a β-galactosidase control expression vector (PDM-LAC-Z-β-GAL). Receptor activity was measured using luciferase assays following treatment with a dose range of TAM (Figure 3.3.1) or FULV (Figure 3.3.2) in the presence of the relevant ligand or EtOH (vehicle). The AR ligand used was Mibolerone (MIB), a synthetic analogue of DHT that cannot be metabolised to oestrogen, and the ERα ligand was E2.

The results demonstrated that ERα activity increased with E2 treatment and that this effect was potently inhibited by the addition of TAM or FULV, even at the lowest concentration tested of 0.001 μM (Figures 3.3.1a and 3.3.2a). AR activity was increased with MIB treatment and this was unaffected by TAM or FULV except at the highest concentration tested of 10 μM (Figures 3.3.1b and 3.3.2b). To support these results, immunoblotting was performed on the lysates from these assays (Figures 3.3.1-2). The

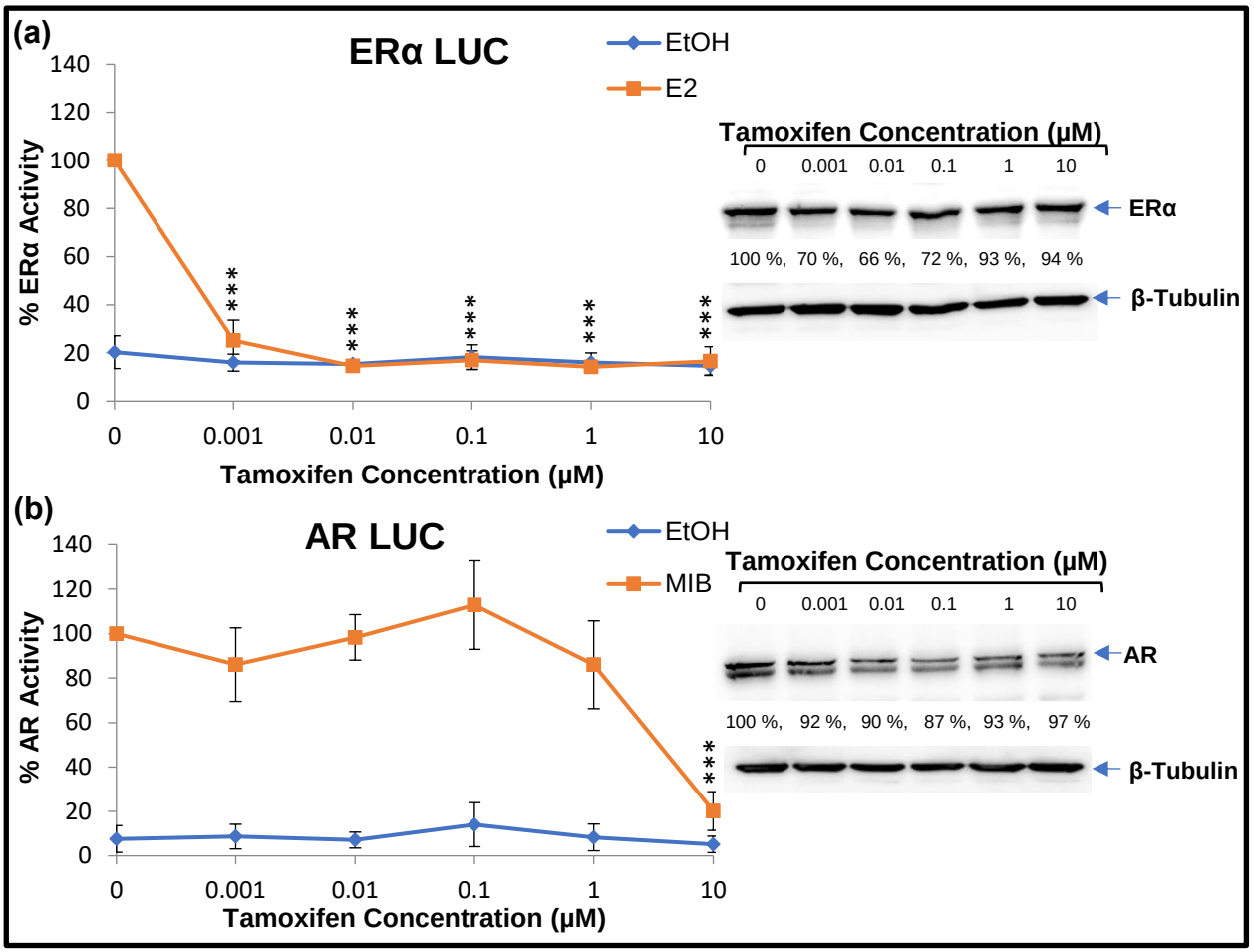


Figure 3.3.1 ERα and AR activity and expression in response to Tamoxifen

COS-1 cells were transfected with plasmids encoding **(a)** Oestrogen Receptor α (ERα) and an oestrogen responsive luciferase reporter (ERE-LUC) or **(b)** Androgen Receptor (AR) and a luciferase reporter under the control of an Androgen Response Element (TAT-GRE-EIB-LUC-1). Cells were also transfected with a β-galactosidase control vector (PDM-LAC-Z-β-GAL). Cells were treated for 24 hours with 1 nM AR ligand (Mibolerone, MIB) or ERα ligand (17-β-Oestradiol, E2), along with varying concentrations of the anti-oestrogen Tamoxifen. ERα or AR activity was measured using luciferase and normalised to β-galactosidase activity. Mean of at least 3 independent duplicates ±SE. T-Test comparing receptor activity in the presence of ligand +/- Tamoxifen. ***P<0.0005. Western Blotting was conducted on the ligand treated samples to assess ERα and AR expression, with β-Tubulin included as a loading control. The percentage expression of ERα or as compared to the untreated samples was analysed using Fusion X Software.

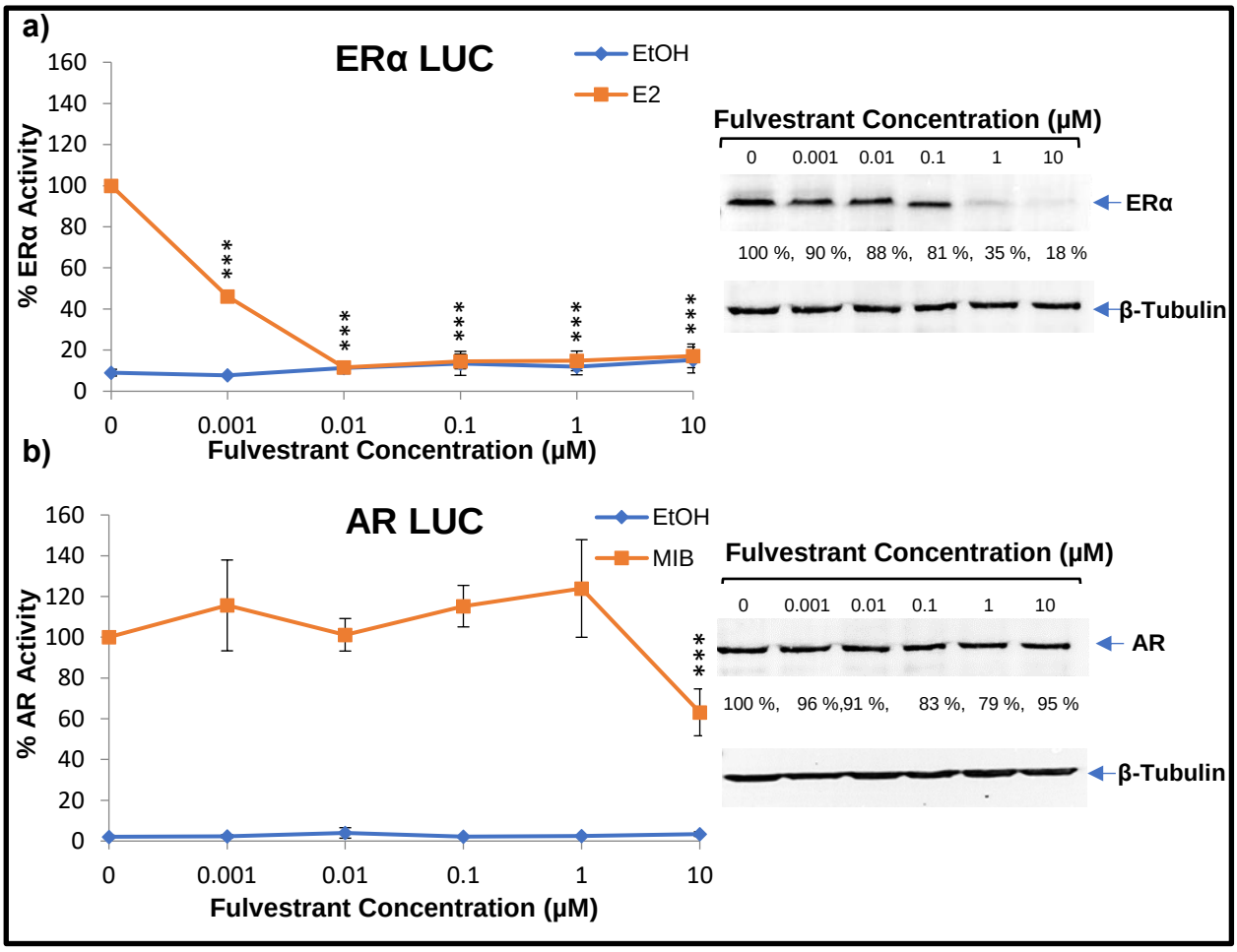


Figure 3.3.2 ERα and AR activity and expression in response to Fulvestrant

COS-1 cells were transfected with plasmids encoding **(a)** Oestrogen Receptor α (ERα) and an oestrogen responsive luciferase reporter (ERE-LUC) or **(b)** Androgen Receptor (AR) and a luciferase reporter under the control of an androgen response element (TAT-GRE-EIB-LUC-1). Cells were also transfected with a β-galactosidase control vector (PDM-LAC-Z-β-GAL). Cells were treated for 24 hours with 1 nM AR ligand (Mibolerone, MIB) or ERα ligand (17-β-Oestradiol, E2), along with varying concentrations of the anti-oestrogen Fulvestrant (FULV). ERα or AR activity was measured using luciferase and normalised to β-galactosidase activity. Mean of 3 independent duplicates ±SE. T-Test comparing receptor activity in the presence of ligand +/- FULV. ***P< 0.0005. Western Blotting was conducted on the ligand treated samples to assess ERα and AR expression, with β-Tubulin included as a loading control. Densitometry analysis was completed using the Fusion FX software to identify the percentage of AR and ERα in FULV treated samples as compared to untreated. The percentage expression of ERα or AR as compared to the untreated samples was analysed using Fusion X Software.

results produced demonstrated that upon TAM treatment both ERα and AR levels remained relatively constant (Figures 3.3.1a-b). In contrast, increasing FULV treatment resulted in a decrease in ERα levels (Figure 3.3.2a) but AR expression remained relatively constant (Figure 3.3.2b). As a control, the activity of AR in the presence of the anti-androgen Bicalutamide (BIC) was investigated (Figure 3.3.3). As expected, AR activity was increased with MIB treatment and this was abrogated by BIC from the 1 μM concentration. This is in accordance with other work, as for PCa research a 10 μM BIC concentration or higher is often used (Barboro *et al.*, 2013, Nunes *et al.*, 2017).

3.4 Anti-oestrogen treatment blocks AR/ERα cross-talk, resulting in enhanced AR activity

It has previously been demonstrated that AR and ERα cross-talk supresses the activity of both receptors (Panet-Raymond *et al*., 2000, Fioretti *et al*., 2014, Lanzino *et al*., 2005). Several mechanisms have been proposed to explain this inhibitory activity. For example, a direct interaction between the receptors and competition for common co-factors have been demonstrated to repress receptor activity (Panet-Raymond *et al*., 2000, Fioretti *et al*., 2014, Lanzino *et al*., 2005). Additionally, work conducted by Peters *et al.,* 2009 has demonstrated that AR can supress ERα signalling via interaction with EREs, and reporter assays demonstrated that AR can inhibit ERα activity. However, there has been no investigation focussing on the effect of endocrine therapies upon this cross-talk.

Initially it was confirmed that AR and ERα can inhibit each other's activity. COS-1 cells were transfected with AR and/or ERα and an oestrogen responsive luciferase

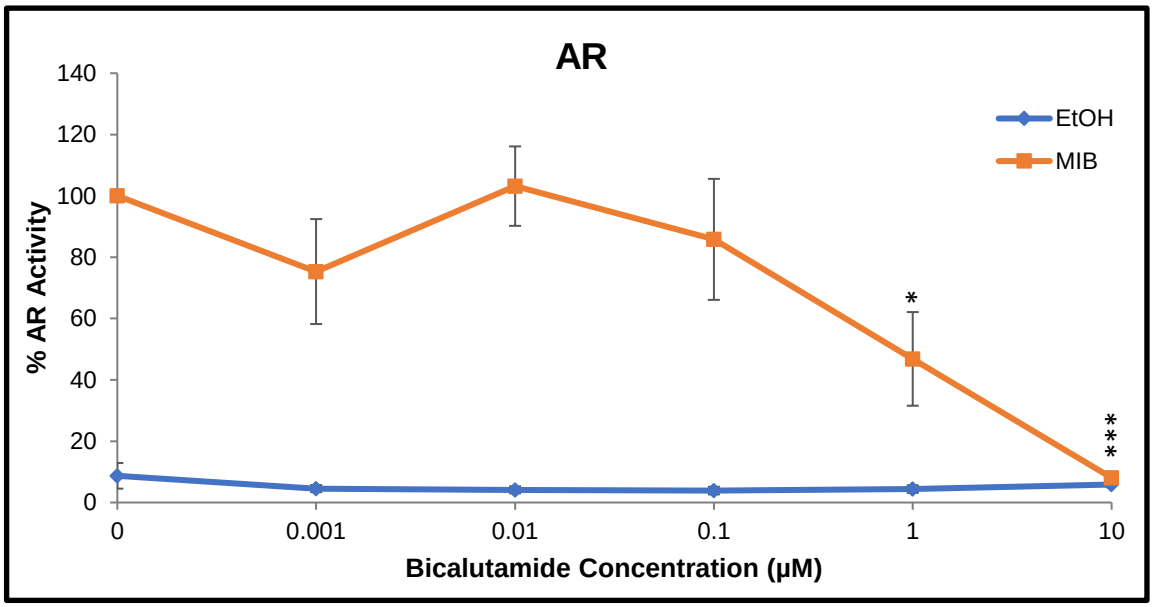


Figure 3.3.3 Bicalutamide inhibits ligand-dependent activation of AR

COS-1 cells were transfected with plasmids encoding Androgen Receptor (AR) and a luciferase reporter under the control of an androgen response element (TAT-GRE-EIB-LUC-1). Cells were also transfected with a β-galactosidase control vector (PDM-LAC-Z-β-GAL). Cells were treated for 24 hours with 1 nM AR ligand (Mibolerone, MIB), along with varying concentrations of the anti-androgen Bicalutamide (BIC). AR activity was measured using luciferase and normalised to β-galactosidase activity. Mean of 3 independent duplicates ±SE. T-Test comparing receptor activity in the presence of ligand +/- BIC. *P<0.05, ***P<0.0005.

reporter (ERE-LUC) or a luciferase reporter under the control of an ARE (TAT-GRE-EIB-LUC-1). Cells were additionally either co-transfected with the other receptor or an Empty Vector (EV control) and a β-galactosidase control expression vector (PDM-LAC-Z-β-GAL). Receptor activity was measured using luciferase assays in the presence of the relevant ligands (MIB/E2 respectively) (Figure 3.4.1). The results demonstrated that co-expression of AR and ERα significantly supressed ERα by 2.8-fold (Figure 3.4.1a) and AR activity by 4.4-fold (Figure 3.4.1b).

To investigate the effect of anti-oestrogen therapies on this AR/ERα cross-talk, luciferase assays were conducted using COS-1 cells as above. Cells were treated with the relevant ligand/both ligands and with different concentrations of either TAM (Figure 3.4.2) or FULV (Figure 3.4.4) prior to luciferase assays being conducted. As an inhibitory effect on ERα activity was previously observed from the lowest concentrations of both anti-oestrogens (Figures 3.3.1a and 3.3.2a), 1 nM and 10 nM FULV/TAM concentrations were selected for this experiment. To support these results, immunoblotting of the lysates from these assays were also conducted (Figures 3.4.3 and 3.4.5).

As expected, ERα activity was inhibited by both TAM (Figure 3.4.2a) and FULV (Figure 3.4.4a). The presence of AR reduced ERα activity, but both TAM and FULV - induced ERα inhibition was still observed (Figures 3.4.2b and 3.4.4b). A small increase in AR activity following anti-oestrogen treatment was observed in the absence of ERα, but this was not always significant (Figure 3.4.2c and 3.4.4c). The presence of ERα reduced AR activity, and this effect was reversed with the addition of TAM (Figure 3.4.2d) or FULV (Figure 3.4.4d). Interestingly, AR activity increased approximately 250 % in response to 10 nM FULV treatment. Therefore, ERα inhibits AR activity and this inhibition

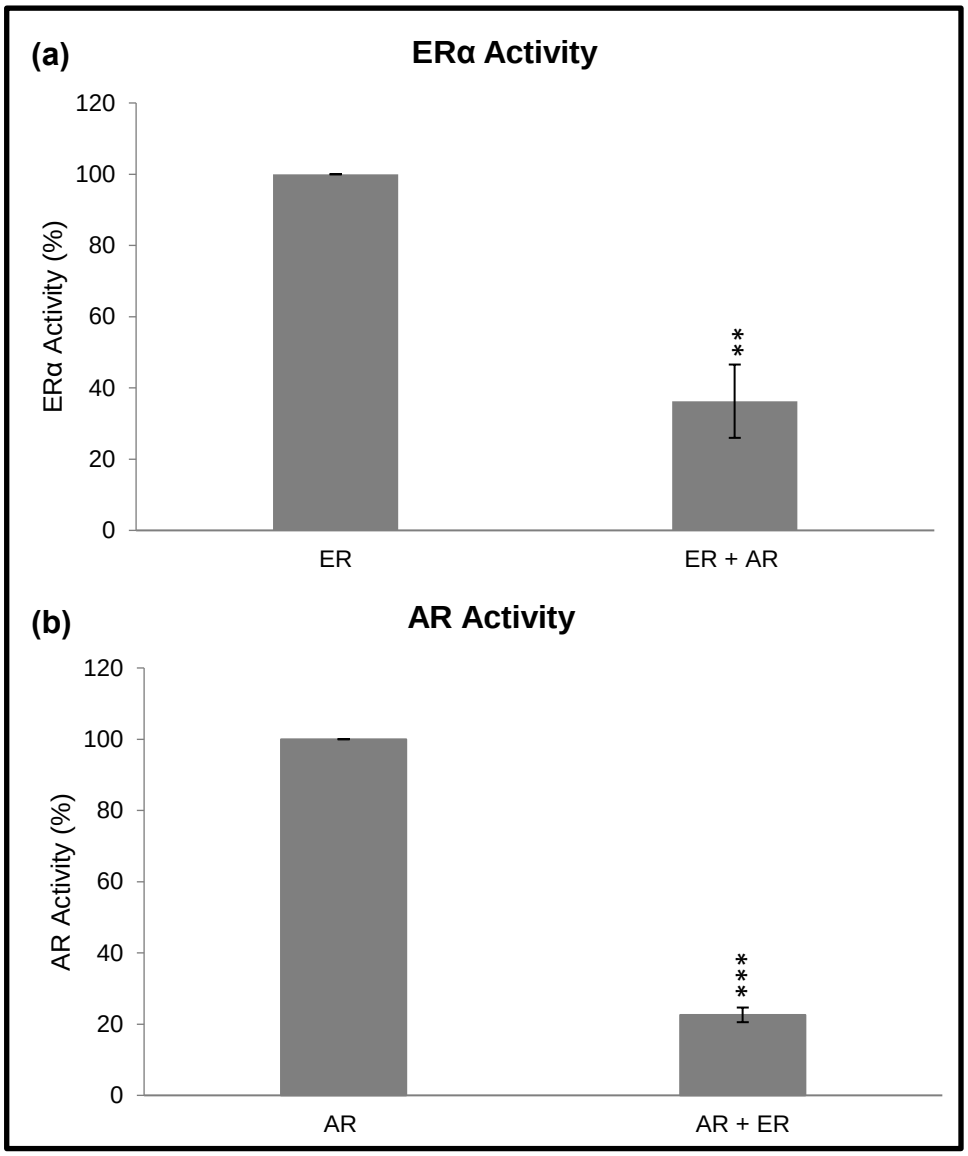


Figure 3.4.1 ERα and AR inhibit each other's activity

COS-1 cells were seeded in hormone-depleted media and transfected with plasmids encoding **(a)** Oestrogen Receptor α (ERα) and an oestrogen responsive luciferase reporter (ERE-LUC) or **(b)** Androgen Receptor (AR) and a luciferase reporter under the control of an Androgen Response Element (TAT-GRE-EIB-LUC-1). Cells were also co-transfected with either the alternative receptor or an Empty Vector (EV) and a β-galactosidase control vector (PDM-LAC-Z-β-GAL). Following transfection, cells were incubated for 24 hrs prior to treatment with the relevant ligands 1 nM Mibolerone (MIB) for AR/1 nM 17-β-Oestradiol (E2) for ERα/both. **(a)** ERα and **(b)** AR activity was measured using luciferase assays, normalised to β-galactosidase activity and expressed as a percentage of AR/ERα activity in the absence of the alternative receptor. Mean of 3 independent duplicates ±SE. T-Test **P<0.005, ***P<0.0005.

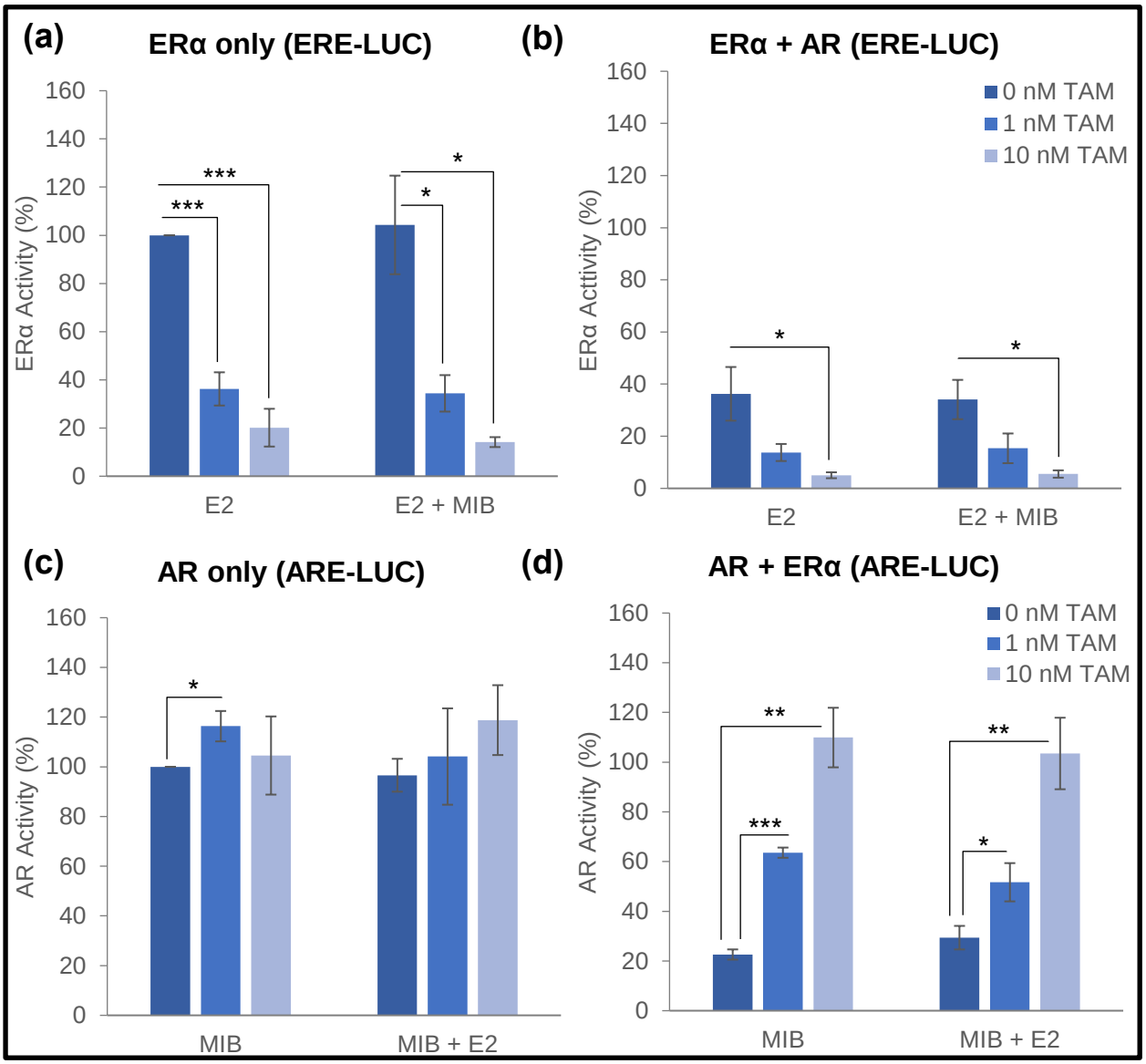


Figure 3.4.2 Tamoxifen reverses the inhibitory effect of ERα upon AR signalling

COS-1 cells were seeded in hormone-depleted media and transfected with plasmids encoding **(a,b)** Oestrogen Receptor α (ERα) and an oestrogen responsive luciferase reporter (ERE-LUC) or **(c,d)** Androgen Receptor (AR) and a luciferase reporter under the control of an Androgen Response Element (TAT-GRE-EIB-LUC-1). Cells were also co-transfected with either the alternative receptor **(b,c)** or an Empty Vector (EV) **(a,c)** and a β-galactosidase control vector (PDM-LAC-Z-β-GAL). Following transfection, cells were incubated for 24 hrs prior to treatment with 1 nM Mibolerone (MIB)/1 nM 17-β-Oestradiol (E2)/both, along with varying concentrations of Tamoxifen (TAM). **(a and b)** ERα activity in response to ± AR and **(c and d)** AR activity in response to ± ERα activity were measured using luciferase assays. Luciferase activity was normalised to β-galactosidase expression and expressed as a percentage of AR/ERα activity in the absence of the alternative receptor and in the presence of MIB/E2. Mean of at least 3 independent duplicates ± SE. T-Test *P<0.05, **P<0.005, ***P<0.0005.

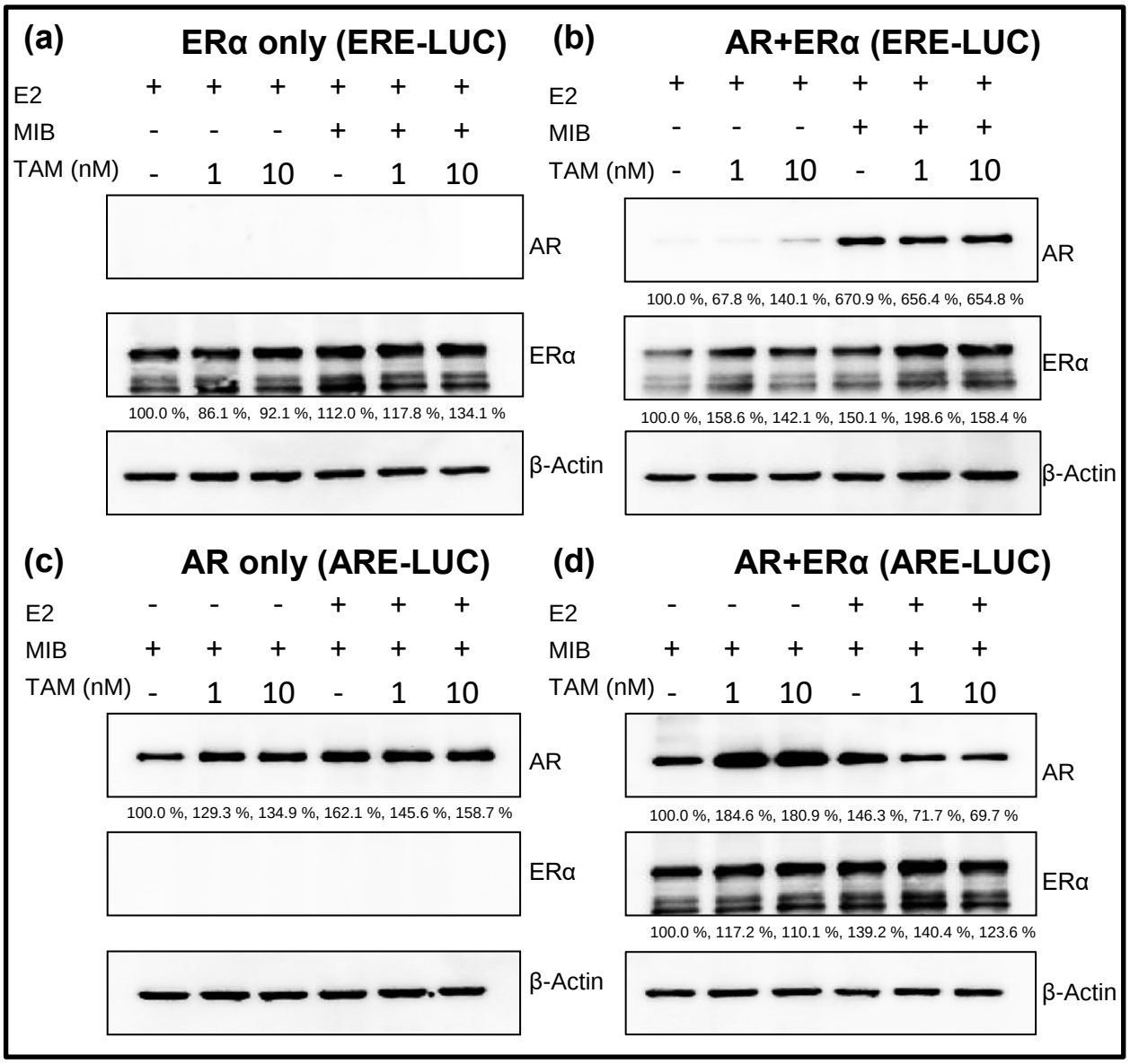


Figure 3.4.3 ERα and AR expression in response to Tamoxifen

COS-1 cells were seeded in hormone-depleted media and double transfections were conducted with plasmids encoding **(a,b)** Oestrogen Receptor α (ERα) and an oestrogen responsive luciferase reporter (ERE-LUC) or **(c,d)** Androgen Receptor (AR) and a luciferase reporter under the control of an androgen response element (TAT-GRE-EIB-LUC-1). Cells were also transfected with either the other receptor **(b,c)** or an Empty Vector (EV) **(a,c)** and a β-galactosidase control vector (PDM-LAC-Z-β-GAL). Following transfection, cells were incubated for 24 hrs prior to treatment with 1 nM Mibolerone (MIB)/1 nM 17-β-Oestradiol (E2)/both, along with varying concentrations of Tamoxifen (TAM). Western blotting was conducted to assess ERα and AR expression, with β-Actin included as a loading control. Densitometry analysis was completed using the Fusion FX software to quantify AR and ERα levels in TAM treated samples as compared to untreated.

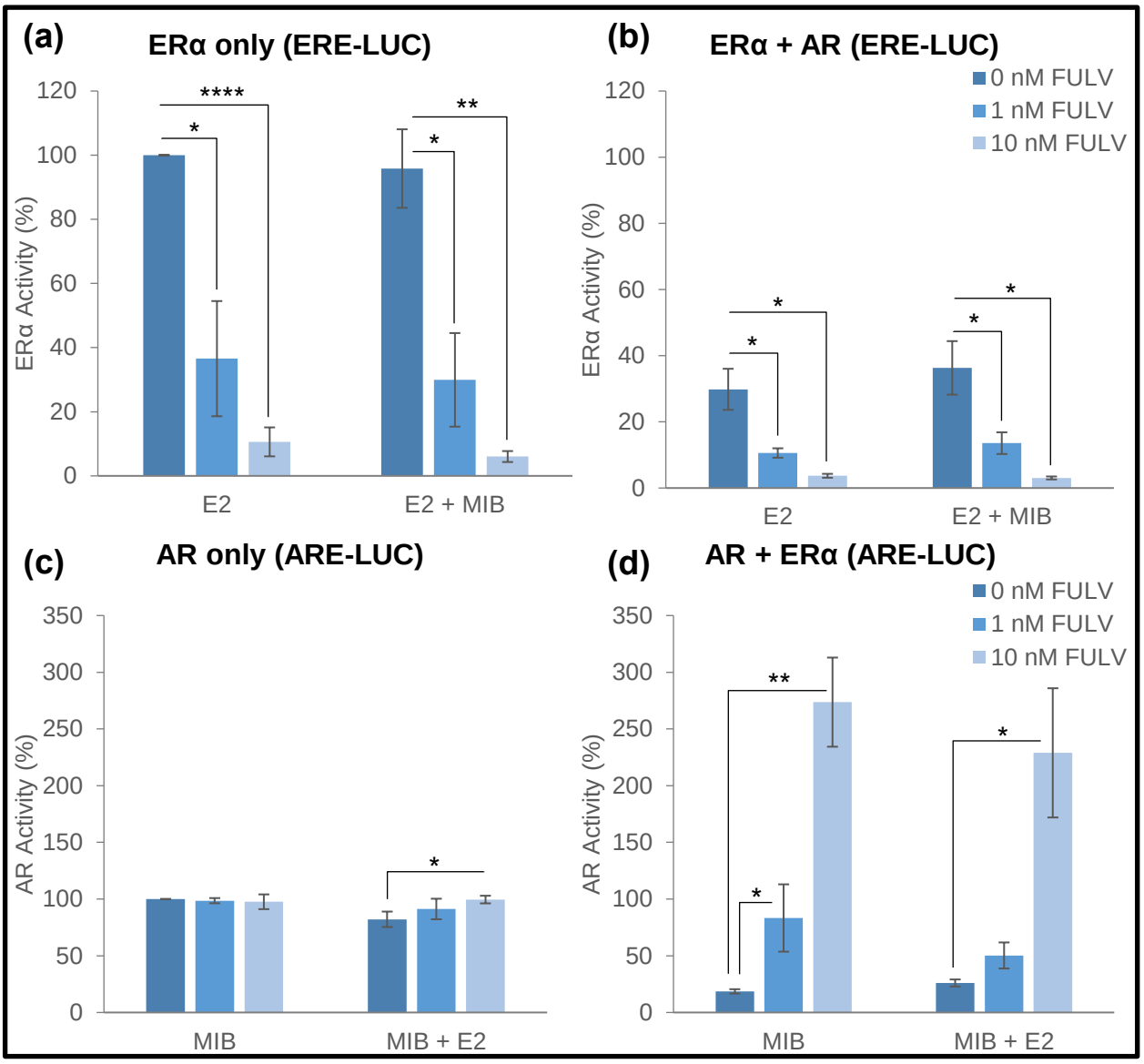


Figure 3.4.4 Fulvestrant reverses the inhibitory effect of ERα upon AR signalling

COS-1 cells were seeded in hormone-depleted media and transfected with plasmids encoding **(a,b)** Oestrogen Receptor α (ERα) and an oestrogen responsive luciferase reporter (ERE-LUC) or **(c,d)** Androgen Receptor (AR) and a luciferase reporter under the control of an Androgen Response Element (TAT-GRE-EIB-LUC-1). Cells were also co-transfected with either the alternative receptor **(b,c)** or an Empty Vector (EV) **(a,c)** and a β-galactosidase control vector (PDM-LAC-Z-β-GAL). Following transfection, cells were incubated for 24 hrs prior to treatment with 1 nM Mibolerone (MIB)/1 nM 17-β-Oestradiol (E2)/both, along with varying concentrations of Fulvestrant (FULV) and **(a and b)** ERα activity in response to ± AR. **(c and d)** AR activity in response to ± ERα activity were measured using luciferase assays. Luciferase activity was normalised to β-galactosidase expression and expressed as a percentage of AR/ERα activity in the absence of the alternative receptor and in the presence of MIB/E2. Mean of at least 3 independent duplicates ± SE. T-Test, *P<0.05, **P<0.005, ***P<0.0005.

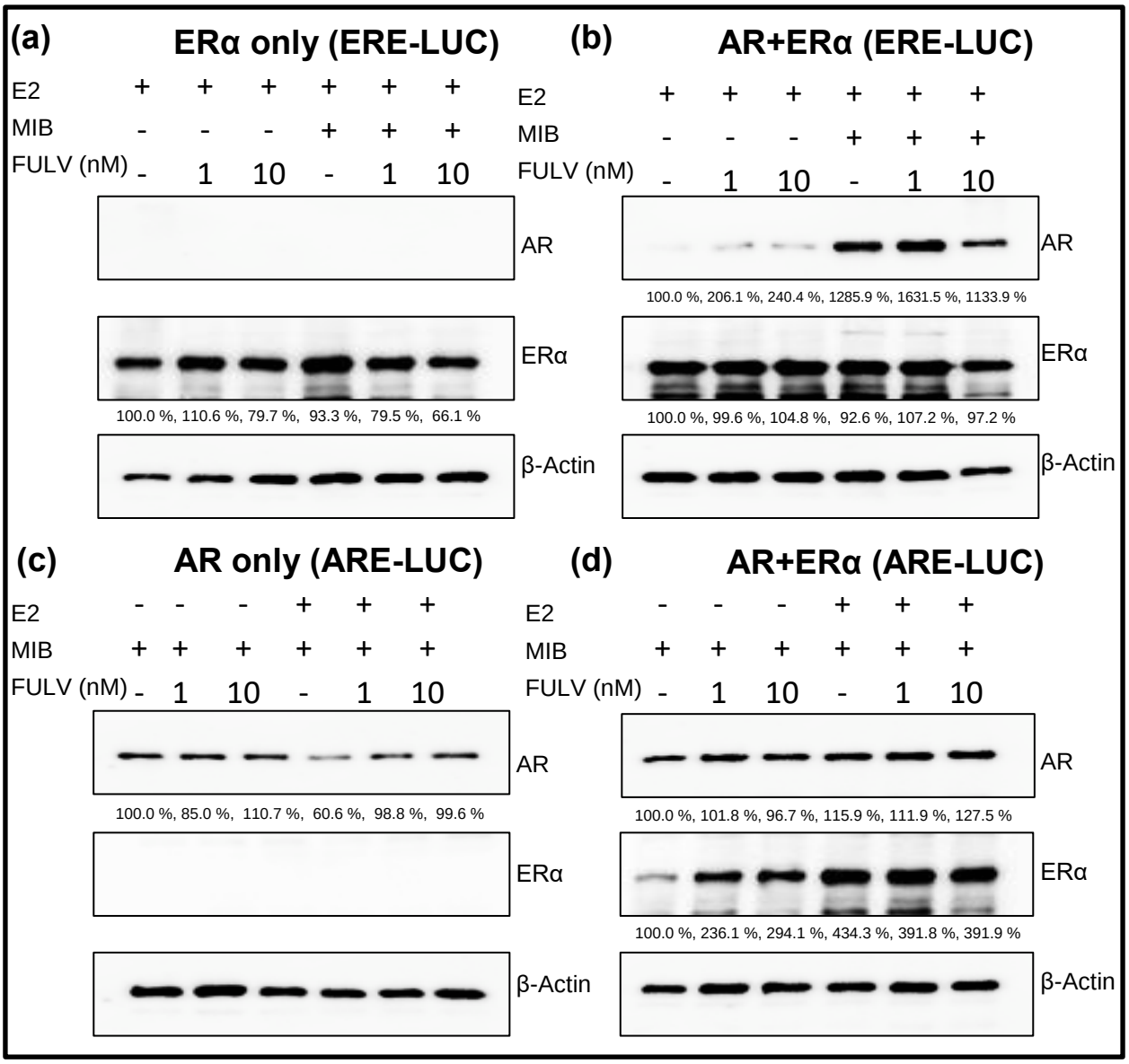


Figure 3.4.5 ERα and AR expression in response to Fulvestrant

COS-1 cells were seeded in hormone depleted media and double transfections were condcuted with plasmids encoding **(a,b)** Oestrogen Receptor α (ERα) and an oestrogen responsive luciferase reporter (ERE-LUC) or **(c,d)** Androgen Receptor (AR) and a luciferase reporter under the control of an androgen response element (TAT-GRE-EIB-LUC-1). Cells were also transfected with either the other receptor **(b,c)** or an Empty Vector (EV) **(a,c)** and a β-galactosidase control vector (PDM-LAC-Z-β-GAL). Following transfection, cells were incubated for 24hrs prior to treatment for 24 hours with 1 nM Mibolerone (MIB)/1 nM 17-β-Oestradiol (E2)/both, along with varying concentrations of Fulvestrant (FULV). Western Blotting was conducted to assess ERα and AR expression, with β-Actin included as a loading control. Densitometry analysis was completed using the Fusion FX software to identify the percentage of AR and ERα in TAM treated samples as compared to untreated.

can be abrogated through TAM treatment, and FULV appears to lead to hyperactivation of the AR (Figure 3.4.4d).

As demonstrated previously in Figures 3.3.1a and 3.3.2a, in the absence of AR, ERα expression remained relatively constant with TAM treatment (Figure 3.4.3a) but was degraded by FULV (Figure 3.4.5a). However, in the presence of AR, FULV was unable to decrease ERα expression (Figures 3.4.5b, d) and TAM treatment appeared to stabilise ERα expression (Figures 3.4.3b, d). As expected from the reporter assays (Figure 3.4.2a), E2-induced ERα expression was not affected by MIB treatment when ERα only was transfected (Figure 3.4.3a and 3.4.5.a). When the receptors were co-expressed in the presence of ARE-LUC, co-treatment with E2 stabilises ERα expression, but this effect was much greater in Figure 3.4.5 than Figure 3.4.3.

It was demonstrated previously (Figures 3.3.1b and 3.3.2b) that AR expression remained relatively constant upon both TAM and FULV treatment at 1 nM and 10 nM anti-oestrogen concentrations, and this was confirmed in Figure 3.4.3c and 3.4.5c. Additionally, AR expression remained relatively constant with FULV treatment, regardless of ERα co-expression (Figures 3.4.5b, d). When ERα and AR were co-expressed, AR expression remained relatively constant with TAM treatment when an ERE-LUC was present (Figure 3.4.3b), but when an ARE-LUC was present TAM treatment increased AR expression in the presence of MIB and co-treatment with E2 abrogated this effect (Figure 3.4.3d).

To ensure that the activity seen in Figures 3.4.1, 2 and 4 was not due to AR or ERα binding to and activating the other receptor's luciferase reporter, control experiments were performed where COS-1 cells were transfected with the ARE/ERE luciferase

reporters and Empty Vector (EV), AR and ERα separately and treated with the relevant ligand or ethanol (EtOH) as a control (Figure 3.4.6). The results demonstrated that the ARE/ERE luciferase reporters can only be significantly activated by the relevant receptor upon ligand binding.

In conclusion, AR and ERα inhibit each other's activity. Treatment with anti-oestrogens (FULV/TAM) inhibit ERα activity, regardless of the presence of AR. FULV/TAM do not inhibit AR activity until higher concentrations (10 μM), however ERα inhibition of AR activity can be reversed through TAM/FULV treatment.

3.5 The role of Androgen Receptor in the development of endocrine resistance

3.5.1 Androgen promotes endocrine resistance in long term but not short term growth assays

Growth assays were conducted to investigate if E2- or MIB-induced cellular proliferation of endocrine sensitive MCF7 cells is affected by anti-oestrogen (TAM or FULV) or anti-androgen (BIC) treatment (Figure 3.5.1). The results demonstrated that, as expected, MCF7 cells were responsive to E2 for growth, and slightly less responsive when co-treated with MIB, but this reduction was not significant. MIB treatment alone did not stimulate MCF7 cell growth. Following TAM treatment, E2-stimulated proliferation was not significantly reduced, but was significantly decreased with FULV treatment, and the presence of MIB could not rescue this effect, as was expected from the earlier reporter assays (Section 3.4). The addition of the anti-androgen BIC did not significantly enhance or abrogate the inhibitory action of the anti-oestrogen treated samples (Figure 3.5.1).

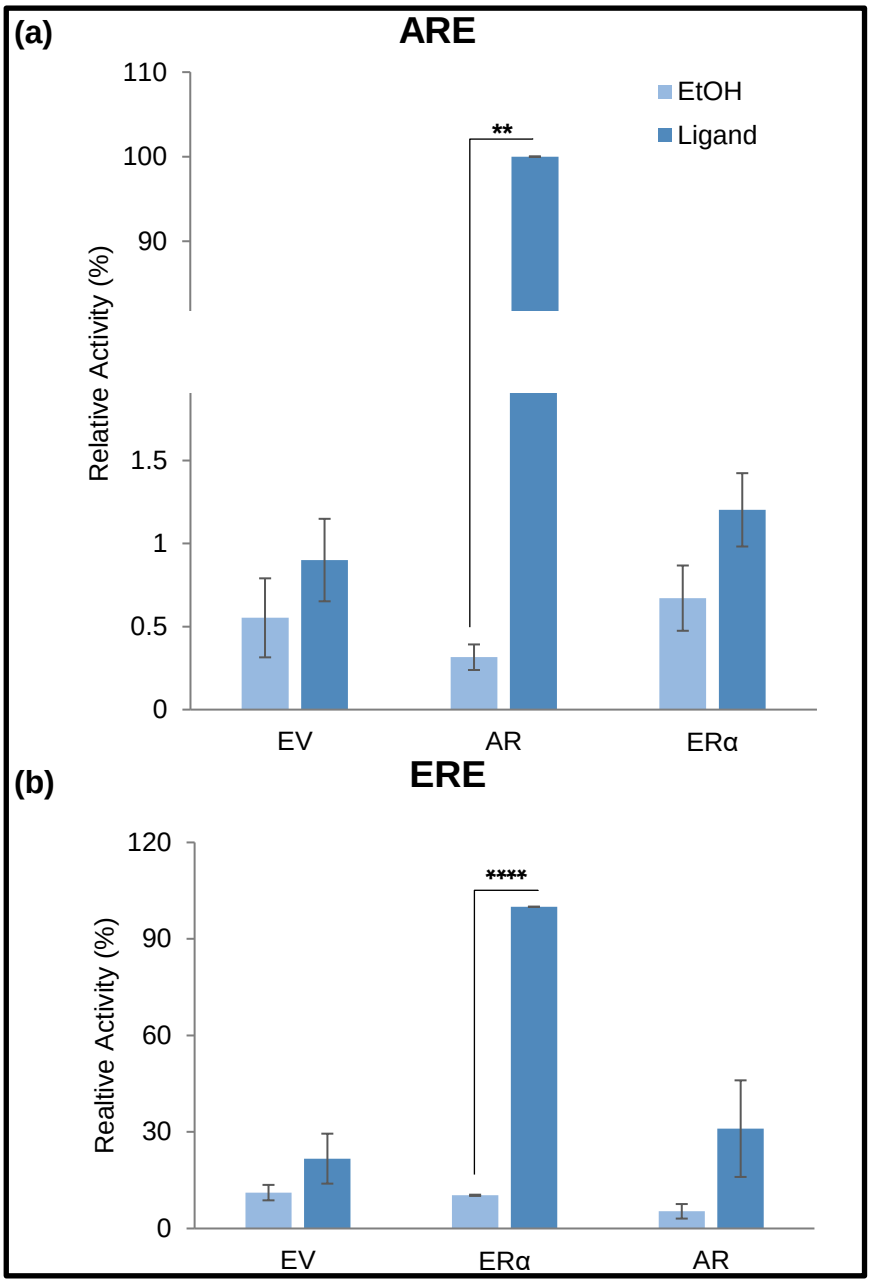


Figure 3.4.6 Investigation of the specificity of an Oestrogen Response Element (ERE) and an Androgen Response Element (ARE) luciferase reporter

COS-1 cells were seeded in hormone depleted media and transfected with plasmids encoding Androgen Receptor (AR) and Oestrogen Receptor α (ERα) or Empty Vector (EV) and **(a)** a luciferase reporter under the control of an ARE (TAT-GRE-EIB-LUC-1) or **(b)** an oestrogen responsive luciferase reporter (ERE-LUC). Cells were also transfected with a β-galactosidase control vector (PDM-LAC-Z-β-GAL). Following transfection, cells were incubated for 24hrs prior to treatment for 24 hours with the relevant ligand,1 nM Mibolerone (MIB for AR/1 nM 17-β-Oestradiol (E2) for ERα. Activity was measured using luciferase and normalised to β-galactosidase expression and expressed as a percentage of AR/ERα activity with the correct response element in the presence of the relevant ligand. Mean of 3 independent duplicates ± SE. T-Test, **P<0.005, ****P<0.00005.

To expand upon this work, colony formation assays were conducted in MCF7 cells over a period of 4 weeks using the same concentrations and combinations of hormones +/- anti-oestrogen treatments and stained using crystal violet (Figure 3.5.2). Additional treatments of MIB +/- TAM/FULV were included. These results demonstrated that MCF7 cells were responsive to E2 for growth, and this effect was not abrogated by co-treatment with MIB. Interestingly, over this longer term assay, MCF7 cells were responsive to MIB for growth, although E2-induced growth was more pronounced. Treatment with TAM/FULV inhibited E2- or MIB-induced growth, but co-treatment with both MIB and E2 was able to partially reverse this effect. Importantly, in contrast to a previous study that suggested BIC could antagonise the anti-proliferative effect of an AI (Macedo *et al.*, 2006), the anti-androgen BIC was able to block this androgen-induced growth and aid anti-oestrogen treatment.

3.5.2 Anti-oestrogen treatment effects on ERα and AR target gene expression in endocrine sensitive cells

To investigate target gene expression in endocrine sensitive cells and how endocrine therapies affects this, MCF7 cells were incubated in hormone-depleted media for 120 hours prior to treatment with 1 nM E2 ± 1 nM MIB ± the anti-oestrogens TAM or FULV for 8 hours, and qPCR performed. The regulation of two ERα target genes *Trefoil Factor 1*, (*TFF1*) and *Myelocytomatosis Oncogene Cellular Homolog* (*MYC*) (Figure 3.5.3); two AR target genes *N-myc Downstream-Regulated Gene 1* (*NDRG1*) and *Zinc Finger And BTB Domain Containing 16* (*ZBTB16*) (Figure 3.5.4); and a gene regulated by both *Gene Regulated in Breast Cancer 1* (*GREB1*) (Figure 3.5.5) were investigated. The references describing these target genes to explain why they were selected are

Table 3.5.1 The regulation of AR and ERα target genes in Breast and Prostate Cancers

References linking the Androgen Receptor (AR) and Oestrogen Receptor alpha (ERα) target genes to Prostate Cancer (PCa) and Breast Cancer (BCa) are provided.

Gene	AR / ERα Regulated	
	Breast Cancer	Prostate Cancer
***Gene Regulated in Breast Cancer 1* (*GREB1*)**	**Positively regulated ERα target gene, important for oestrogen-induced BCa cell proliferation that has also been displayed as a ERα co-factor** (Chand *et al.*, 2012, Mohammed *et al.*, 2013, Rae *et al.*, 2005, Deschenes *et al.*, 2007, Hodgkinson and Vanderhyden, 2014)	**Androgen-regulated gene important for PCa growth, but has been associated with organ-confined PCa and good prognosis** (Rae *et al.*, 2006, Antunes *et al.*, 2012, Ngan *et al.*, 2009, Hodgkinson and Vanderhyden, 2014)
***Myelocytomatosis Oncogene Cellular Homolog* (*MYC*)**	**Positively regulated, oestrogen-induced ERα target gene, which promotes BCa proliferation and whose deregulation is associated with BCa progression** (Wang *et al.*, 2011, Musgrove *et al.*, 2008, Zhu *et al.*, 2014, Dubik *et al.*, 1987, Xu *et al.*, 2010)	***MYC* overexpression deregulates AR in PCa and is linked to androgen-independent PCa cell growth** (Barfeld *et al.*, 2017, Bernard *et al.*, 2003)
***N-Myc Downstream-Regulated Gene 1* (*NDRG1*)**	**Negatively associated with BCa progression** (Wang *et al.*, 2006)	**AR target gene, positively regulated by androgen in PCa cells, with a TSG function in PCa progression** (Nelson *et al.*, 2002, Ngan *et al.*, 2009, Masuda *et al.*, 2005, Li *et al.*, 2015b, Bandyopadhyay *et al.*, 2003)
***Trefoil Factor 1* (*TFF1*)**	**Positively regulated ERα target gene in BCa, involved in BCa cell migration and anti-apoptotic effects** (Lin *et al.*, 2004, Brown *et al.*, 1984, Westley *et al.*, 1984, Welboren *et al.*, 2007, Prest *et al.*, 2002, Pelden *et al.*, 2013)	**Appears to promote PCa progression, but not so far linked to ERα or AR and its role is unclear** (Abdou *et al.*, 2008, Ather *et al.*, 2004, Vestergaard *et al.*, 2010)
***Zinc Finger and BTB Domain Containing 16* (*ZBTB16*)**	**Androgen-regulated AR target gene in MABC** (Robinson *et al.*, 2011)	**Androgen Receptor target gene in PCa cells** (van de Wijngaart *et al.*, 2009)

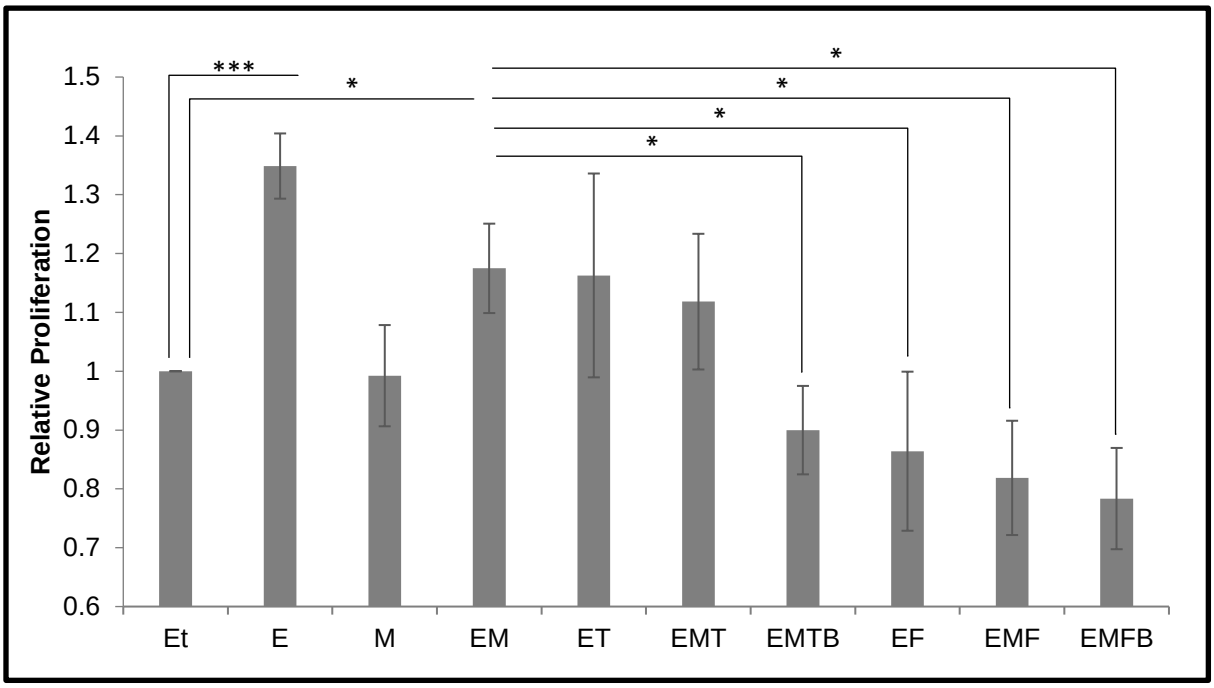


Figure 3.5.1: The effect of anti-oestrogen treatment on oestrogen and androgen stimulated growth in MCF7 cells

Cells were treated for 120 hours in hormone-depleted media with 1 nM of the synthetic androgen Mibolerone (M) or 17-β-Oestradiol (E), separately and in combination (EM) +/- 100 nM Tamoxifen (T), Fulvestrant (F) or Bicalutamide (B). Ethanol (EtOH) treatment was used as a vehicle control. Proliferation was assessed using WST-1 assays. Mean of at least 3 independent experiments, ±SE. T-test *P<0.05, ***P<0.005.

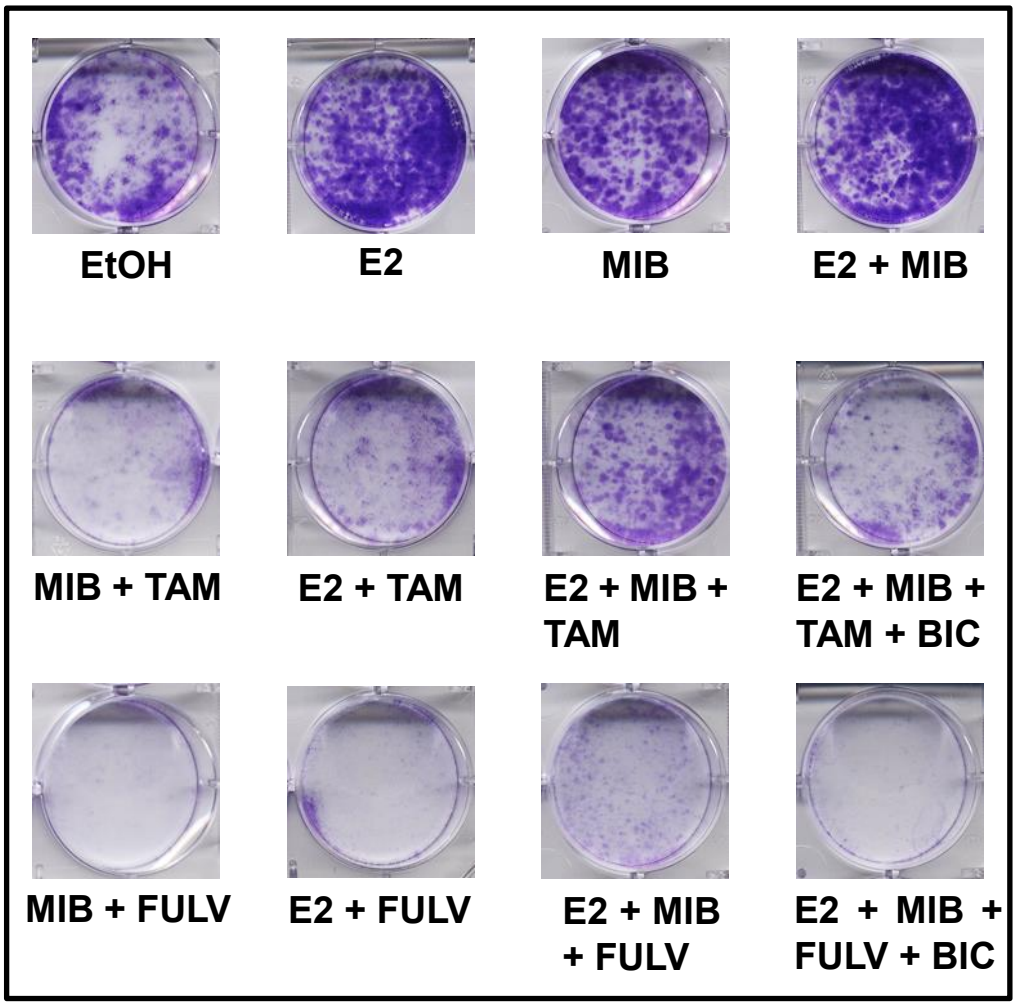


Figure 3.5.2: The effect of anti-oestrogen and anti-androgen treatment on long term oestrogen and androgen stimulated growth in MCF7 cells

Cells were treated for 4 weeks in hormone-depleted media with 1 nM of the synthetic androgen Mibolerone (MIB) or 17-β-Oestradiol (E2), separately and in combination +/- 100 nM Tamoxifen (TAM), Fulvestrant (FULV) or Bicalutamide (BIC). Ethanol (EtOH) treatment was used as a vehicle control. A colony formation assay was conducted and the cells fixed with 4 % Paraformaldehyde and stained using 0.08 % crystal violet.

summarised in Table 3.5.1. The concentration of the anti-oestrogens utilised in this study was 100 nM, consistent with recent studies (e.g. Periyasamy *et al*., 2015) and a pre-treatment with them 24 hours prior to hormone treatment was also explored.

E2 treatment significantly increased the expression of both the ERα targets, *TFF1* 3.4-fold and *MYC* 2.1-fold (Figures 3.5.3a-b). Co-treatment of MIB with E2 was unable to compete with the E2-induced gene expression for either *TFF1* or *MYC* (Figures 3.5.3a-b), as also demonstrated in earlier reporter assays (Figures 3.4.2 and 4). As expected, E2-induced expression of these ERα targets was inhibited via both anti-oestrogens (Figures 3.5.3a-b). MIB was not able to enhance *TFF1* (Figure 3.5.3a) or *MYC* expression (Figure 3.5.3b).

The expression of the AR target gene *ZBTB16* was not induced via E2 treatment (Figure 3.5.4a). MIB strongly induced *ZBTB16* expression by approximately 1,000 % and co-treatment with E2 inhibited this increase by 54.9 %. Importantly, the anti-oestrogen treatments reversed this inhibitive action of oestrogen, enhancing AR activity, but pre-treatment with anti-oestrogens appears to reduce this effect (Figure 3.5.4a). Additionally, the expression of the alternative AR target *NDRG1* was not induced following MIB treatment, however co-treatment with E2 still inhibited its expression by 55.1 % and pre-treatment with anti-oestrogens were able to restore its expression (Figure 3.5.4b).

Finally, the expression of a target gene for both AR and ERα *GREB1* was explored (Figure 3.5.5). Both E2 and MIB significantly enhanced *GREB1* expression by 12.5-fold and 3.8-fold respectively. This gene is therefore more responsive to oestrogen than androgen. Co-treatment with MIB did not reduce the E2-induction of this gene. All anti-oestrogen treatments were able to significantly reduce E2-induced *GREB1* expression, regardless of MIB co-treatment (Figure 3.5.5).

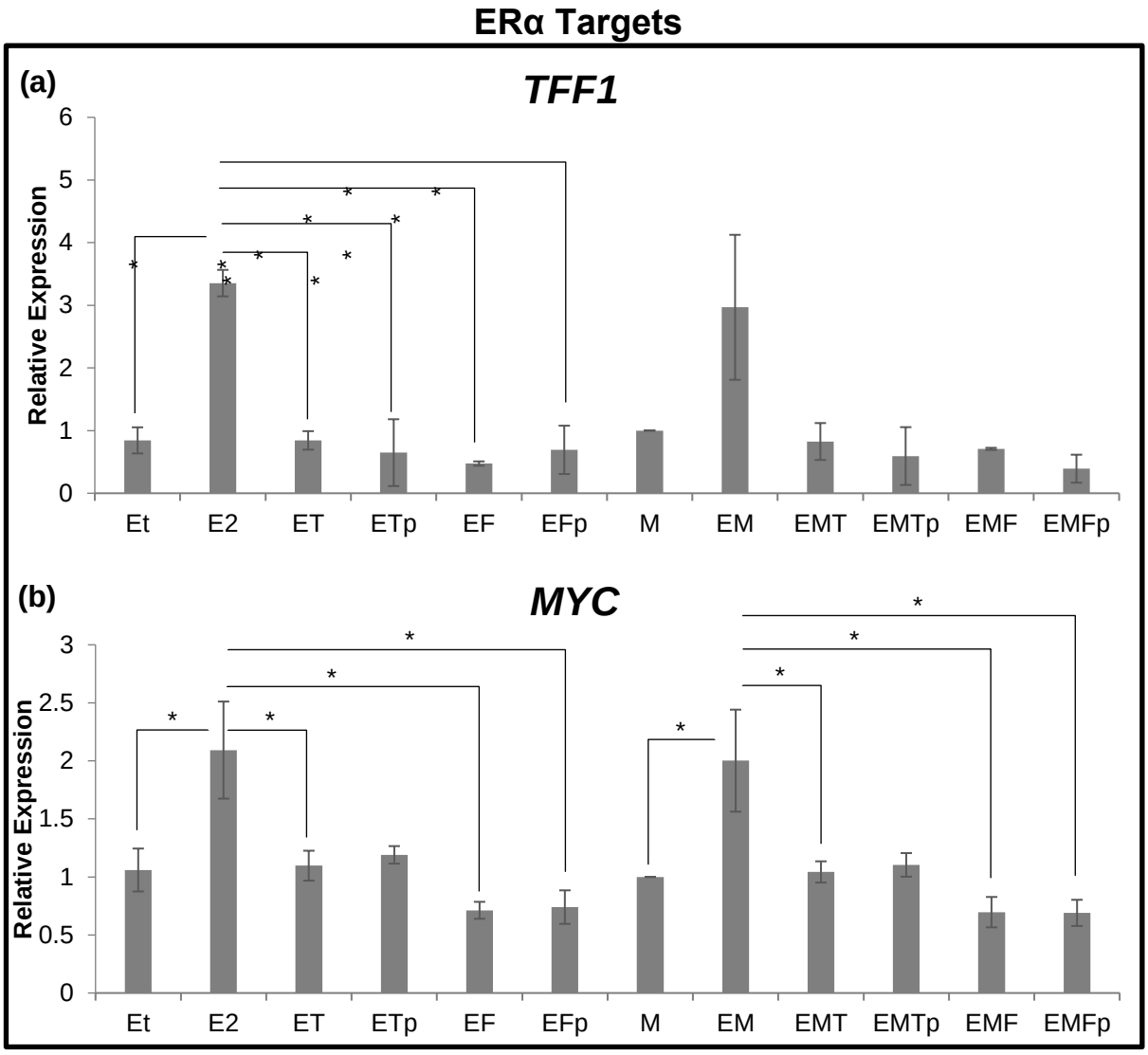


Figure 3.5.3 Expression of ERα target genes in MCF7 cells

MCF7 cells were incubated in hormone depleted media for 120 hours, treated with 1 nM of the relevant hormones 17-β-Oestradiol (E2) ± Mibolerone (M) or Ethanol (Et) as a control ± 100 nM Fulvestrant (F) or Tamoxifen (T) for 8 hours. Some samples were pre-treated with Fulvestrant/Tamoxifen 24 hours prior to hormone treatments (Fp/Tp respectively). RNA was extracted, reverse transcribed into cDNA and qPCR was conducted using SYBR green to measure the expression level of Oestrogen Receptor alpha (ERα) targets **(a)** *Trefoil Factor 1* (*TFF1)* and **(b)** *Myelocytomatosis Oncogene Cellular Homolog* (*MYC)*. Expression was normalised to *RPL19 ribosomal protein*, *L19,* and expression is relative to the MIB-treated MCF7 sample. Mean of 3 independent experiments are displayed ±SE. T-Test *P<0.05.

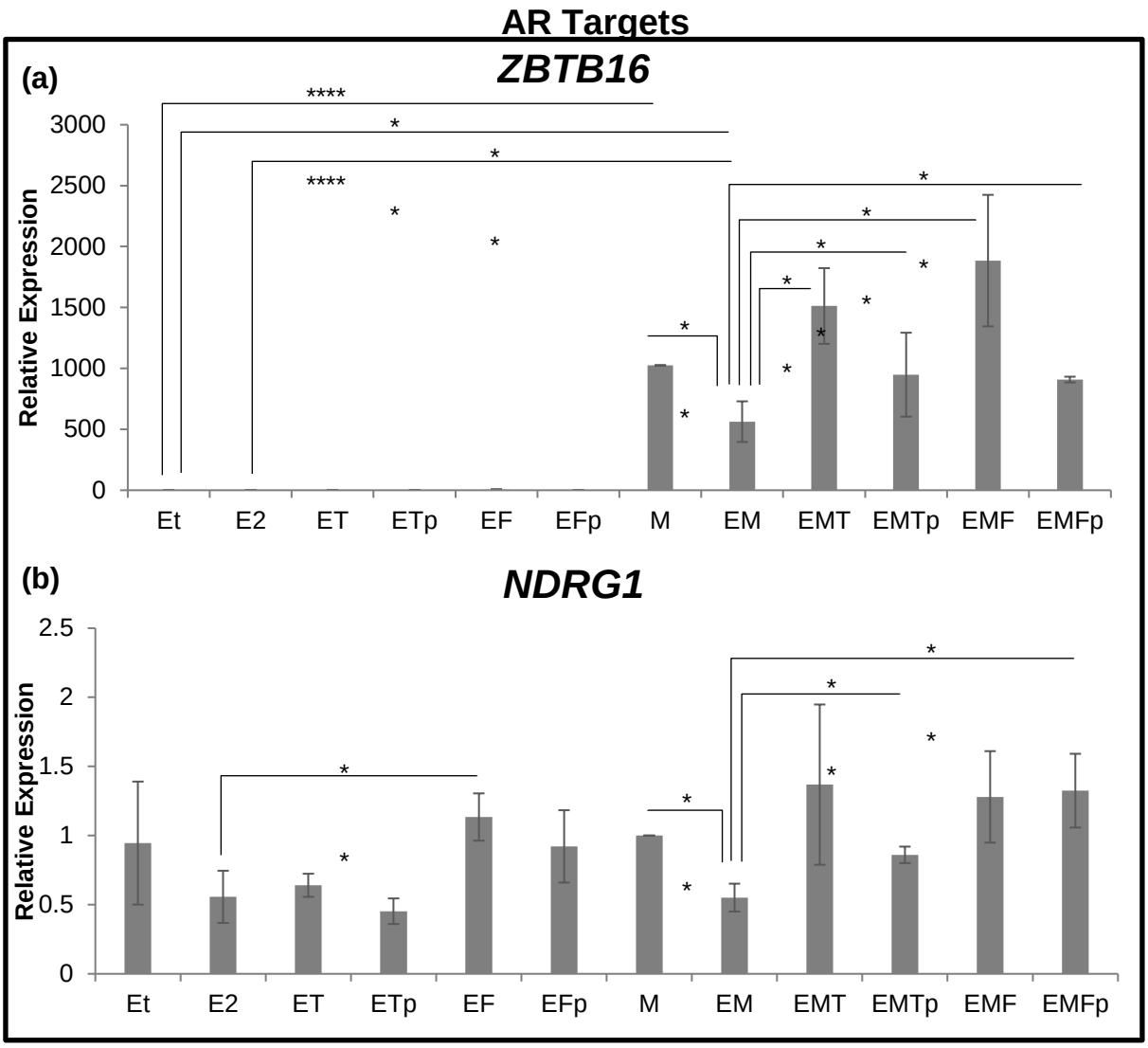


Figure 3.5.4 Expression of AR target genes in MCF7 cells

MCF7 cells were incubated in hormone depleted media for 120 hours, treated with 1 nM of the relevant hormones 17-β-Oestradiol (E2) ± Mibolerone (M) or Ethanol (Et) as a control ± 100 nM Fulvestrant (F) or Tamoxifen (T) for 8 hours. Some samples were pre-treated with Fulvestrant/Tamoxifen 24 hours prior to hormone treatments (Fp/Tp respectively). RNA was extracted, reverse transcribed into cDNA and qPCR was conducted using SYBR green to measure the expression level of Androgen Receptor (AR) target genes **(a)** *Zinc Finger And BTB Domain Containing 16* (*ZBTB16*) and **(b)** *N-myc Downstream-Regulated Gene 1* (*NDRG1*). Expression was normalised to *RPL19 ribosomal protein*, *L19,* and expression is relative to the **(a)** EtOH or **(b)** MIB-treated MCF7 sample. Mean of 3 independent experiments are displayed, ±SE. T-Test *P<0.05, ***P<0.0005.

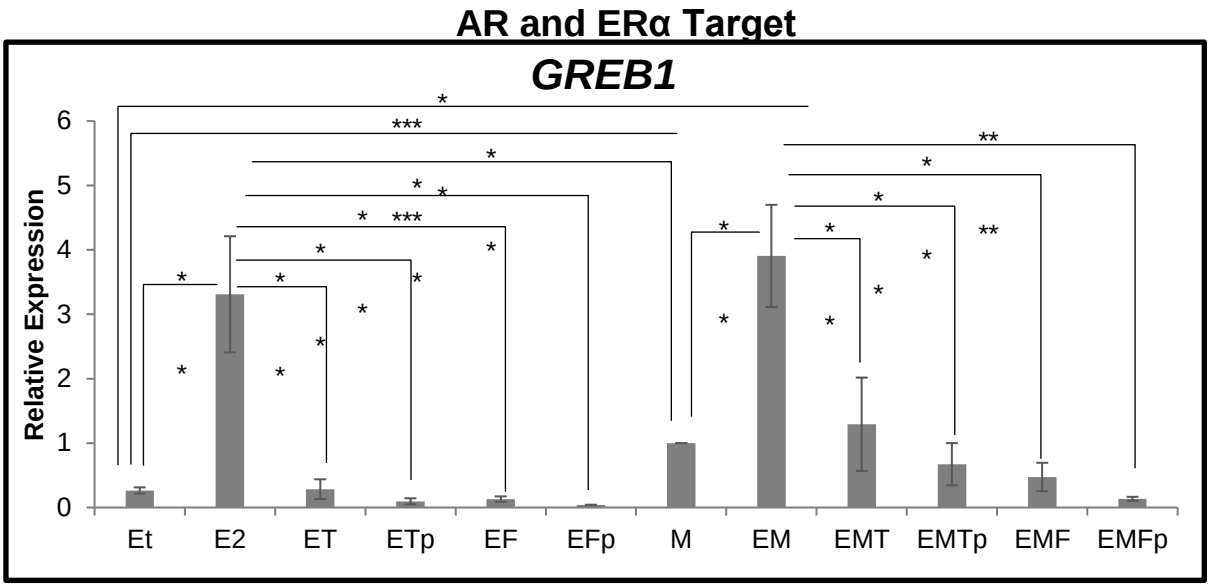


Figure 3.5.5 Expression of an AR and ERα target gene in MCF7 cells

MCF7 cells were incubated in hormone depleted media for 120 hours, treated with 1 nM of the relevant hormones 17-β-Oestradiol (E2) ± Mibolerone (M) or Ethanol (Et) as a control ± 100 nM Fulvestrant (F) or Tamoxifen (T) for 8 hours. Some samples were pre-treated with Fulvestrant/Tamoxifen 24 hours prior to hormone treatments (Fp/Tp respectively). RNA was extracted, reverse transcribed into cDNA and qPCR was conducted using SYBR green to measure the expression level of Androgen Receptor (AR) and Oestrogen Receptor alpha (ERα) target *Gene Regulated in Breast Cancer 1* (*GREB1*). Expression was normalised to *RPL19 ribosomal protein*, *L19,* and expression is relative to the MIB-treated MCF7 sample. Mean of 3 independent experiments are displayed, ±SE. T-Test *P<0.05, ***P<0.0005.

In conclusion, the results demonstrated that the addition of anti-oestrogens decreases the hormone-induced expression of ERα regulatory target genes and genes regulated by AR and ERα, yet reverses the inhibitory action of E2 upon AR target genes. This demonstrates that treating endocrine sensitive disease with anti-oestrogens inhibits ERα, which could reduce this cross-talk and therefore increase AR activity.

3.5.3 E2 abrogates MIB-induced AR enrichment at an ARE, and anti-oestrogens can partially rescue this effect

It has been previously shown that AR/ERα cross-talk inhibits signalling and that anti-oestrogens can reverse this. To investigate the possible mechanism of this cross-talk, Chromatin Immunoprecipitation (ChIP) assays were used to assess ligand-dependent AR recruitment to DNA in MCF7 cells, and how this is altered by anti-oestrogen treatment. MCF7 cells were treated with ± 1 nM MIB ± 1 nM E2 for 4 hours ± pre-treatment with 100 nM TAM/FULV 24 hours prior to hormone treatments. Cells were crosslinked using formaldehyde and sonicated to approximately 200 bp (Figure 3.6a), and ChIP performed using an antibody specific for the AR. qPCR was conducted to quantify DNA enrichment at a known ARE located between exons 3 and 4 of the *ZBTB16* gene (ZBT ARE) and a relative negative control region (ZBT ARE -ve) 2,443 bp upstream (Figures 3.5.6c-d). A schematic to demonstrate the location of these sites is included (Figure 3.5.6b). As expected, AR bound to the ARE region in response to MIB and this binding was reduced by E2 treatment (Figures 3.5.6c-d). This effect could be partially rescued via pre-treatment with TAM (Figures 3.5.6c) or FULV (Figure 3.5.6d).

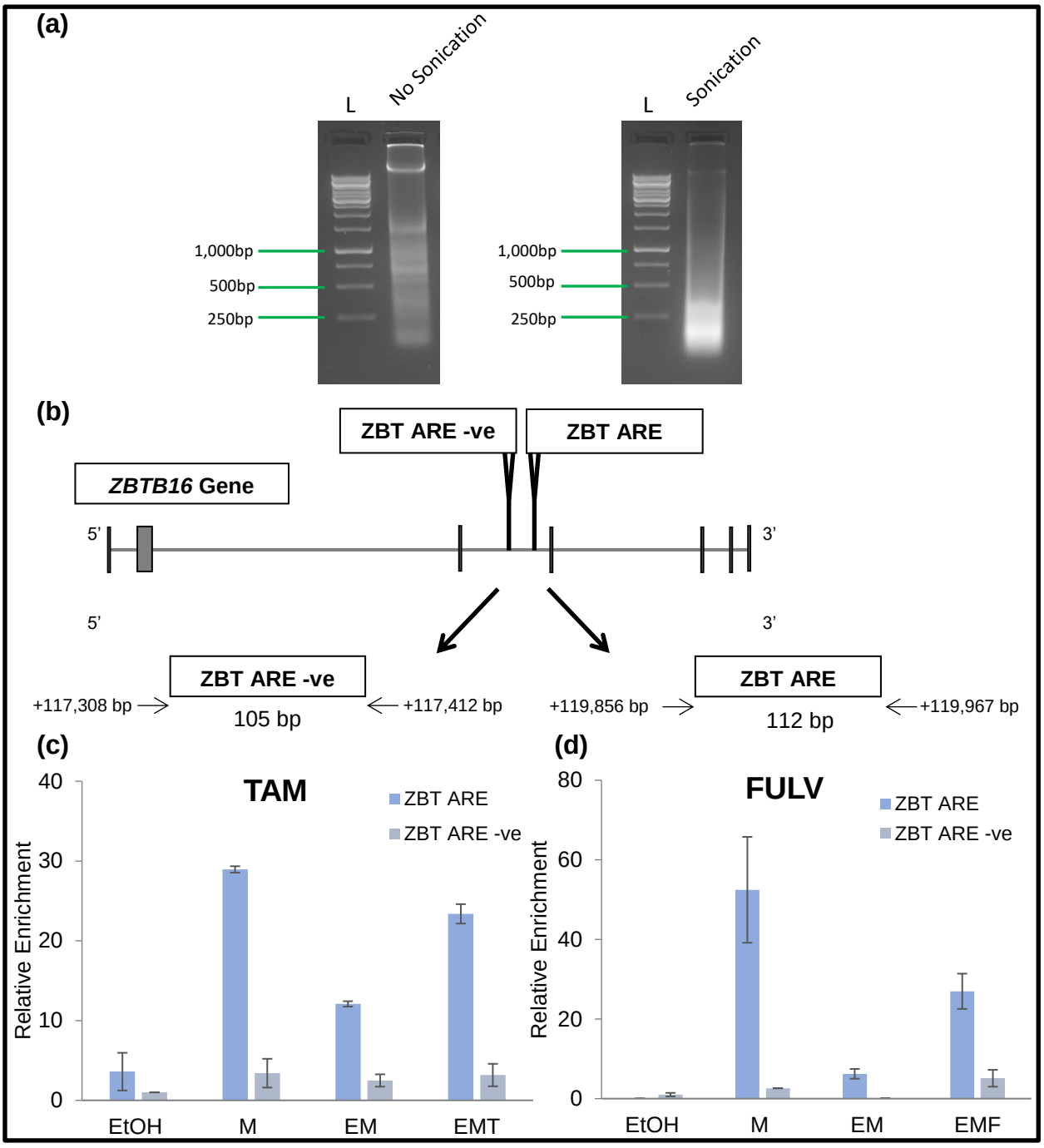


Figure 3.5.6: E2 reduces MIB-induced AR enrichment at an ARE, and this effect can be partially rescued via Tamoxifen or Fulvestrant

MCF7 cells were treated with ± 1 nM MIB ± 1 nM E2 for 4 hours ± pre-treatment with 100 nM TAM **(c)** / FULV **(d)** 24 hours prior to hormone treatments. Ethanol (EtOH) was used as vehicle control. Cells were crosslinked using formaldehyde and sonicated to approximately 200 bp **(a)**. ChIP assays were performed using an antibody specific for the AR. Subsequent qPCR was conducted to identify DNA enrichment at a known Androgen Response Element (ARE) upstream of the *Zinc Finger And BTB Domain Containing 16* (*ZBTB16*) gene (ZBT ARE) and its relative negative control region (ZBT ARE -ve) **(c, d)**. Enrichment values are relative to the ZBT ARE -ve EtOH sample. Mean of 2 independent experiments are displayed for **(c)** and a representative replicate for **(d)** ±SE. A schematic of ZBT ARE and its negative region is included **(b)**.

3.6 Discussion

3.6.1 ERα regulates AR in endocrine sensitive disease

The majority of BCas are endocrine sensitive, for which cancer progression is driven by ERα (Chuffa et al., 2017, Rakha and Green, 2016, Ali and Coombes, 2002). ERα is a ligand-dependent transcription factor, and upon ligand (commonly E2) binding, it translocates to the nucleus and interacts as a dimer with specific DNA sequences termed Oestrogen Response Elements (EREs) to regulate transcription (Sever and Glass, 2013, Brooke and Bevan, 2009, Dehm and Tindall, 2007, Maruthanila *et al*., 2016).

It has been demonstrated that ERα and AR expression is often correlated in BCa (Table 3.2.1 and Figure 3.2.1) as has been previously reported by others (Vera-Badillo *et al.*, 2014, Collins *et al.*, 2011, Lin *et al.*, 2015). This co-expression suggests that the receptors could both be regulated by a common factor, or that one receptor regulates the expression of the other. Knockdown of each receptor separately and subsequent immunoblotting analysis revealed that ERα knockdown also resulted in a reduction in AR expression, indicating that ERα might regulate AR expression (Figure 3.2.2). Consequently, three potential ERE sites were identified: one upstream of *AR* ($ERE_{-19,247-18,610}$), and two were located between exons 3 and 4 of the *AR* gene ($ERE_{+150,441-758}$ and $ERE_{+151,438-726}$). Luciferase reporter assays were conducted to investigate whether these were functional sites (Figures 3.2.3 and 3.2.5). The results demonstrated that, interestingly, ERα activity was significantly responsive to E2 in $ERE_{+151,438-726}$ which contains a perfect palindromic ERE sequence (Figure 3.2.5b) suggesting that ERα might regulate AR expression via this site.

On the other hand, for the two additional sites investigated, one upstream of *AR* ($ERE_{-19,247-18,610}$) and one located between exons 3 and 4 of the *AR* gene ($ERE_{+150,441-758}$), ERα activity was not responsive to E2 for either site under the conditions employed (Figures 3.2.3b and 3.2.5b). However, $ERE_{+150,441-758}$ appeared to be constitutively active, regardless of ERα expression or E2 treatment (Figure 3.2.5b), suggesting that an alternative transcription factor could be inducing expression through this region. Bioinformatics analysis of this region revealed transcription factor response elements for 20 alternative transcription factors, whose expression were significantly associated with AR in BCa (Table 3.2.2). The two most significant proteins were XBP1, involved in immune and cellular stress responses (He *et al.*, 2010) and previously linked to BCa including the promotion of endocrine resistance (Gomez *et al.*, 2007, Gupta *et al.*, 2016); and c-Myb, involved in hematopoiesis (Lorenzo *et al.*, 2011, Bengtsen *et al.*, 2015) and has been previously implicated in promoting BCa progression and metastasis through enhancing the Wnt/β-catenin/Axin2 signalling pathway (Li *et al.*, 2016) (Figure 3.2.6). Additionally, it was discovered that another transcription factor with binding sites in this region and significantly associated with AR in BCa was FOXA1, a known pioneer factor that has been demonstrated as key for AR functioning in Molecular Apocrine (ERα-negative, AR-positive) BCa progression (Robinson *et al.*, 2011). These results could be explored further in the future to identify which factors might be regulating AR expression, which might subsequently lead to an increase in AR levels during therapy resistance. For example, by conducting siRNA knockdown of the factors or ChIP assays to confirm which factors have a role in this regulation could be beneficial.

Although $ERE_{-19,247-18,610}$ appeared to be non-functional from the luciferase reporter assays, ChIP analysis revealed that ERα was indeed recruited to this site. A

potential explanation for this apparent lack of consistency between the two results could be explained by a study by Pan *et al.* (2008). The authors demonstrated that a distal ERE for the *TFF1* gene acts as a transcriptional enhancer to enable maximum gene expression, and this is achieved via long-range chromosomal interactions with a proximal *TFF1* ERE (Pan *et al.*, 2008). The inference from this is that either this site could require the presence of another ERE for other transcription factors or pioneer factors for its transcriptional activation, that are unable to bind to the short region of DNA cloned for these studies.

3.6.2 Anti-oestrogen effects on ERα or AR activity

The effects of endocrine therapies on ERα and AR activity was investigated using two commonly prescribed anti-oestrogens: TAM and FULV. As expected, both anti-oestrogens successfully inhibited E2-induced ERα activity, from the lowest concentrations of the drugs tested (Figures 3.3.1a and 3.3.2a). Immunoblotting analysis of these lysates demonstrated that ERα expression remained relatively constant with TAM treatment, but FULV decreased its expression (Figures 3.3.1a and 3.3.2a). These differences can be explained by the differences in the mechanism of these anti-oestrogens. TAM (a SERM) has tissue-specific activity, and in tumour and mammary cells competitively binds to ERα. TAM promotes receptor dimerisation, similar to oestrogen, however instead of promoting the formation a transcriptional complex, it promotes the formation of an inhibitory nuclear complex that reduces the levels of DNA synthesis and oestrogen signalling (Kaklamani and Gradishar, 2017). On the other hand, FULV (a SERD) acts as a pure ERα antagonist, competitively interacting with ERα, which additionally to blocking and reducing its activity, advances the speed of its degradation

(Kaklamani and Gradishar, 2017, Lumachi *et al.*, 2015, Lanvin *et al.*, 2007, Osborne *et al.*, 2004), as demonstrated by immunoblotting in Figure 3.3.2a.

MIB-induced AR activity was unaffected by anti-oestrogen treatment except at the highest concentration tested of 10 µM (Figures 3.3.1b and 3.3.2b). Previously it has been suggested that FULV may also have anti-androgen activity, having been demonstrated to downregulate AR expression and suppress AR-induced growth in the LNCaP PCa cell line (Bhattacharyya *et al.*, 2006). However, the AR in this cell line is known to have a mutation causing an alteration in its LBD (Veldscholte *et al.*, 1992), therefore it was unknown whether FULV would have an effect on wildtype AR activity. Interestingly, MIB-induced wildtype AR activity in our study was significantly decreased by FULV treatment only at the highest concentration tested of 10 µM (Figure 3.3.2b), which corresponded with the concentration used by Bhattacharyya *et al.* (2006), and therefore their study agrees with these findings. However, our findings demonstrated that AR expression was not decreased by 24-hour FULV treatment at this concentration, therefore the 48-hour treatment period used by Bhattacharyya *et al.* (2006) could be required to demonstrate this effect. Furthermore, Bhattacharyya *et al.* (2006) described the mechanism by which FULV leads to reduced AR levels in LNCaP cells is not via a direct interaction with AR or an induction of AR proteasomal degradation as may be expected. Instead, the authors demonstrated that FULV reduces AR expression at the transcriptional level (Bhattacharyya *et al.*, 2006). Our results, however, have indicated that ERα regulates AR (Sections 3.2 and 3.6.1), and therefore it could be inferred that in fact FULV is targeting and degrading ERα, and thereby down-regulating AR expression.

3.6.3 Anti-oestrogen treatment abrogates AR/ERα cross-talk in endocrine sensitive disease

In endocrine-sensitive disease, AR has been widely demonstrated to have an inhibitory effect on BCa progression and its expression is associated with indicators of a positive clinical outcome (Tsang *et al.*, 2014, Niemeier *et al.*, 2010, Fioretti *et al.*, 2014). It has previously been demonstrated that AR and ERα cross-talk supresses the activity of both receptors (Panet-Raymond *et al*., 2000, Fioretti *et al*., 2014, Lanzino *et al*., 2005). Mechanisms of AR and ERα cross-talk include a direct interaction between the receptors (Panet-Raymond *et al.*, 2000) and competition for binding to the regulatory regions of target genes (Peters *et al.*, 2009). In support of this, Figure 3.4.1 indicates that when both the AR and ERα are co-expressed they compete, and inhibit each other's activity. This competitive role of the receptors could explain why co-expression is associated with a good prognosis.

To explore the effect of endocrine therapies upon AR/ERα cross-talk, the effect of TAM and FULV on AR and ERα activity when both receptors were co-transfected was investigated. AR expression had no impact on the inhibitory effects of the anti-oestrogens on ERα activity. However, TAM reversed ERα suppression of AR activity, and FULV treatment actually increased AR activity to levels 2.7-fold higher than those induced by androgen alone (Figures 3.4.2 and 3.4.4).

This more potent activity induced by FULV does not appear to be as a result of ERα degradation, because at the concentrations tested, ERα levels (although showing a slight decrease) still remained fairly constant. Additionally, it does not appear that this effect is due to the higher affinity of FULV to ERα than TAM as described by others

(Kaklamani and Gradishar, 2017, Martinkovich *et al.*, 2014, Osborne *et al.*, 2004), as a comparison of Figures 3.3.1a and 3.3.2a demonstrate that at the 1 nM or 10 nM concentrations FULV does not have a more potent inhibition of ERα than TAM. Therefore, this mechanism is presumably due to the difference in the mechanism via which these two anti-oestrogens work, as described previously in Section 3.6.2.

In Molecular Apocrine (ERα-negative, AR-positive) BCa, AR has a putative oncogenic effect and its expression is associated with aggressive tumours (Doane *et al.*, 2006; Robinson *et al.*, 2011; Lehmann-Che *et al.*, 2013). This suggests that in the absence of a functioning ERα, AR signalling can be oncogenic in certain circumstances. In accordance with this, our results demonstrate that in the absence of functional ERα, as a result of endocrine therapy, AR activity is increased.

To extend these results into a more physiologically relevant system, the effects of anti-oestrogens upon the expression of ERα target genes *TFF1* and *MYC* (Figure 3.5.3); AR target genes *NDRG1* and *ZBTB16* (Figure 3.5.4); and a gene regulated by both *GREB1* (Figure 3.5.5) was investigated in MCF7 cells.

TFF1 is a strongly established positively regulated ERα target gene in BCa (Lin *et al.*, 2004, Brown *et al.*, 1984, Westley *et al.*, 1984, Welboren *et al.*, 2007). Target gene analysis in MCF7 cells revealed that *TFF1* expression was induced following E2 treatment, and this increase was inhibited by the anti-oestrogens FULV and TAM (Figure 3.5.3a). This demonstrates that, as expected, ERα target gene expression is responsive to E2 treatment and anti-oestrogens in endocrine sensitive disease. This was additionally confirmed in an alternative ERα target gene *MYC* (Wang *et al.*, 2011) (Figure 3.5.3b). Interestingly, co-treatment of MIB with E2 was unable to compete with the E2-induced

gene expression for either *TFF1* or *MYC* (Figures 3.5.3a-b), as also demonstrated in earlier reporter assays (Figures 3.4.2 and 4), suggesting that AR/ERα cross-talk is ligand independent.

The *ZBTB16* gene has been shown by Robinson *et al*. (2011) to be an androgen-regulated AR target in Molecular Apocrine BCa. Therefore, its androgen-regulation in endocrine sensitive disease (MCF7 cells) was explored. *ZBTB16* expression was strongly induced via MIB treatment (by 1,024.8 %), and co-treatment with E2 inhibited this increase by 54.9 %. Importantly, all of the anti-oestrogen treatments reversed this inhibitive action of oestrogen, enhancing AR activity (Figure 3.5.4a). This suggests that ERα inhibits AR signalling, and that anti-oestrogen inhibition of ERα results in an increase in AR signalling, in accordance with other results (Sections 3.6.3 and 3.6.7). To explore the mechanism via which this occurs, ChIP assays were conducted to investigate the effect of E2 and TAM/FULV treatment on ligand-dependent AR recruitment to a known ARE upstream of the *ZBTB16* gene. The results produced demonstrated that AR bound to the ARE region in response to MIB, binding was inhibited by E2 treatment (Figures 3.5.6c-d), and that this effect could be partially reversed via treatment with TAM (Figure 3.5.6c) or FULV (Figure 3.5.6d). It has been demonstrated previously that AR and ERα can compete for common sites (Peters *et al.*, 2009), which could explain the mechanism via which this ERα antagonism is occurring.

NDRG1 expression was not significantly altered following androgen treatment, however co-treatment with E2 still inhibited its expression by 55.1 %, and pre-treatment with anti-oestrogens were able to significantly induce its expression (Figure 3.5.4b). *NDRG1* is gene that is positively regulated by androgen in the LNCaP PCa cell line (Nelson *et al.*, 2002, Ngan *et al.*, 2009) that has been identified as an AR target gene

(Masuda *et al.*, 2005). However, contrastingly to these findings, many studies have also indicated that *NDRG1* acts as a Tumour Suppressor Gene in PCa, associating it's downregulation with enhanced cellular proliferation and invasion (Li *et al.*, 2015b) and metastasis (Bandyopadhyay *et al.*, 2003). Genes regulated in PCa by AR are therefore not necessarily regulated by AR in BCa, which could be due to differences in co-factors/pioneer factors and/or genomic modifications.

GREB1 is a known target gene for both AR (Rae *et al.*, 2006) and ERα (Chand *et al.*, 2012). *GREB1* expression was induced via both E2 or MIB, 12.5-fold and 3.8-fold respectively, so this gene was much more responsive to oestrogen than androgen (Figure 3.5.5). All anti-oestrogen treatments were able to significantly reduce E2-induced *GREB1* expression, regardless of MIB co-treatment (Figure 3.5.5). This suggests that ERα activity is key for this gene's expression, and during inhibition of ERα, AR regulation of this gene does not increase. Therefore, it could be inferred that for the expression of *GREB1* AR and ERα are not competing and therefore its expression is inhibited by anti-oestrogen treatment.

In conclusion, anti-oestrogen treatment inhibited the oestrogen-induced expression of ERα regulatory target genes, but androgen can abrogate this effect for certain targets. On the other hand, anti-oestrogen treatment enhanced the AR target genes, demonstrating that treating endocrine sensitive disease with anti-oestrogens inhibits ERα, which subsequently results in an increase in AR activity on AR target genes. However, anti-oestrogen treatment inhibited the gene regulated by both AR and ERα, which suggests that genes where it appears that AR and ERα are not competing are inhibited by anti-oestrogen treatment.

3.6.4 Anti-oestrogen treatment enhances androgen-induced growth in endocrine sensitive BCa

The effects of androgen (MIB) and oestrogen (E2) on MCF7 proliferation were explored. It is well established that endocrine sensitive cell lines are highly responsive to E2 for growth (Keen and Davidson, 2003, Rakha and Green, 2016, Ali and Coombes, 2002, Pan *et al.*, 2017). Growth assays demonstrated that the proliferation of MCF7s, as expected, was stimulated by E2, but co-treatment with MIB reduced this E2-induced growth (Figure 3.5.1). This is in support of previous work conducted where treatment with the androgens DHT and MIB inhibited E2-stimulated cellular proliferation in MCF7 cells and in the additional ERα- and AR- positive cell line T47D (Cops *et al.*, 2008). This is likely to be a result of the AR/ERα cross-talk discussed in Section 3.6.3, where AR appears to have an opposing role to ERα and supresses BCa progression (Rahim and O'Regan, 2017, Fioretti *et al*., 2014, Tarulli *et al*., 2014, Lanzino *et al*., 2013). This also supports past studies that have demonstrated the potential therapeutic benefit of the administration of androgens in ERα-positive BCa (Poulin *et al.*, 1988, Cops *et al.*, 2008, Kampa *et al.*, 2005, Dauvois *et al.*, 1991). However, in contrast to these results, long term colony formation assays conducted on MCF7 cells demonstrated that following 4 weeks of treatment, MIB could stimulate MCF7 cell growth (Figure 3.5.2). This colony formation assay could be seen as a model of the development of AI resistance as the cells were grown in E2-depleted media, suggesting that MIB could begin to stimulate growth as a resistance mechanism. An inference that could be concluded from this is that the antagonistic effect of AR on ERα-induced growth can be overcome over time with exposure to MIB, and AR can in some cases begin to drive BCa cell growth.

The effects of anti-oestrogen (TAM/FULV) treatment on E2/MIB-stimulated growth in MCF7 cells was investigated in both short term proliferation assays (Figure 3.5.1) and long term colony formation assays (Figure 3.5.2). FULV effectively inhibited E2-stimulated growth in both assays, but TAM only over the longer time period (Figures 3.5.1-2). Treatment with androgen was not able to rescue anti-oestrogen induced inhibition of oestrogen-stimulated growth in the short term growth assays (Figure 3.5.1). However, when cells were grown for 4 weeks, androgen was able to rescue both FULV- and TAM-induced inhibition of oestrogen-stimulated growth (Figure 3.5.2). Therefore, it appears that AR is able to drive resistance to these therapies in an endocrine sensitive cell line. From this it can be inferred that when ERα function is inhibited through endocrine therapy, over time androgen signalling can begin to stimulate growth. This is in accordance with the results produced from reporter assays and gene expression analysis suggesting that TAM and FULV treatment remove the repressive cross-talk of ERα with AR and therefore AR activity is enabled (Figures 3.4.2d and 3.4.4d). However, both TAM and FULV inhibited MIB-stimulated growth in the colony formation assays (Figure 3.5.2), suggesting that the presence of an inhibited ERα is key for enhanced AR signalling to occur. However, it is unclear why in the absence of E2 that this effect is not seen as it would be expected that ERα would sequester shared co-repressors such as the Silencing Mediator of Retinoic Acid and Thyroid Hormone Receptor (SMRT) (Blackmore *et al.*, 2014, Liao *et al.*, 2003). The data instead suggests that the presence of E2 and an anti-oestrogen is essential for this effect.

Importantly, treatment with an anti-androgen (BIC) could prevent androgen-induced FULV/TAM resistance in the colony formation assays (Figure 3.5.2). Additionally, even in the shorter term growth assays (Figure 3.5.1), BIC enhanced the abrogation of

growth in both TAM and FULV treated samples. This confirmed that the MIB-induced effect was produced via the AR, and indicates that combining anti-androgens with anti-oestrogens in endocrine sensitive BCa treatment could help to prevent activation of AR signalling and potentially halt this resistance mechanism from occurring.

Chapter 4. Results: Androgen Receptor regulation of endocrine resistant Breast Cancer

4.1 Introduction

As discussed in Chapter 3, the majority of BCas are ERα-positive, endocrine sensitive cancers, dependent on oestrogens such as E2 for progression (Chuffa *et al*., 2017, Rakha and Green, 2016, Ali and Coombes, 2002). Endocrine therapies are often administered to these patients, including anti-oestrogens to block ERα action (for instance TAM and FULV), and AIs (for example Anastrozole) to reduce oestrogen levels (Kaklamani and Gradishar, 2017, Cauley *et al*., 2001, Lumachi *et al*., 2015). However endocrine resistance is a major issue, and often results in advanced metastatic disease for which few treatment options are available (Kaklamani and Gradishar, 2017, Vorobiof, 2016, Reinert *et al*., 2017).

Chapter 3 explored how the AR (an alternative ligand-dependent transcription factor, activated by androgens) can become activated in response to endocrine therapies, which aids in the development of endocrine resistance. The aim of this Chapter was to explore this mechanism of resistance further, by investigating the role of AR and ERα signalling in endocrine resistant cell lines. Two endocrine sensitive cell lines were utilised (MCF7 and T47D) and their TAM Resistant (TAMR), FULV Resistant (FULVR) and Long Term Oestrogen Deprived (LTED, a model of AI resistance) derivatives (Figure 2.4.1).

4.2 AR and ERα expression alters in endocrine resistance

To investigate AR and ERα expression in MCF7 and T47D cells, and how this is altered in their endocrine resistant derivatives, immunoblotting was performed (Figure 4.2.1a). The results demonstrated that ERα expression was decreased in the endocrine resistant derivatives as compared to their endocrine sensitive parental lines, apart from the T47D-TAMR line where it increased by 1.8-fold. AR expression was found to be 2.6-fold higher in MCF7-LTED cells and by 3.9-fold in T47D-TAMR cells as compared to the relevant parental lines, whereas AR and ERα expression remained relatively constant in MCF7-TAMR cells (Figure 4.2.1a). However, both AR and ERα expression were strongly decreased in T74D-LTED and MCF7-FULVR derivatives, indicating that these cells may not be dependent upon AR or ERα signalling (Figure 4.2.1a). In light of these results, and as TAM is the most widely used anti-oestrogen in treatment (Vorobiof, 2016, Lumachi *et al.*, 2015), MCF7-TAMR and MCF7-LTED derivatives were selected for in depth study of AR signalling.

To explore the correlation between ERα and AR expression across these cell lines, these results were plotted onto a graph (Figure 4.2.1b). Although AR and ERα expression differs in the endocrine resistant cell lines, the general pattern observed was that AR and ERα expression was correlated across all the cell lines. These results are in accordance with data presented previously from the METABRIC dataset, which indicated an association between ERα and AR in BCa (Table 3.2.1 and Figure 3.2.1).

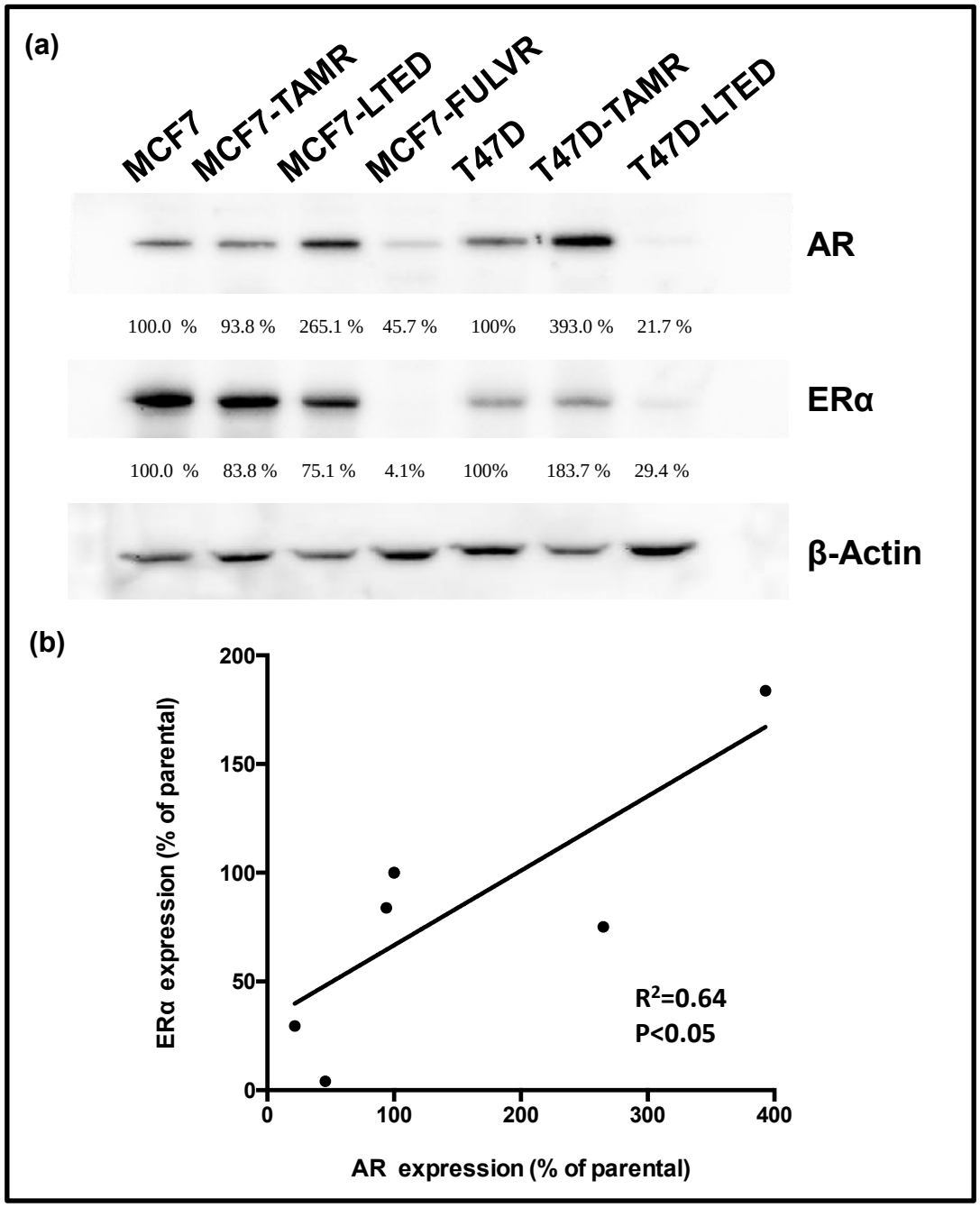


Figure 4.2.1 AR and ERα expression is altered in endocrine resistant Breast Cancer cell line models

Parental endocrine sensitive MCF7 and T47D cells and their endocrine resistant derivatives: Tamoxifen Resistant (TAMR), Long Term Oestrogen Deprived (LTED) and Fulvestrant Resistant (FULVR) were grown in DMEM (MCF7) or RPMI (T47D) supplemented with 10 % foetal calf serum, penicillin streptomycin and glutamine for 72 hours. Cells were lysed and immunoblotting conducted to examine Androgen Receptor (AR) and Oestrogen Receptor α (ERα) expression, with β-Actin included as a loading control. Densitometry analysis was conducted using the Fusion FX software to identify the percentage of AR and ERα in the resistant cell lines as compared to their relevant parental cell lines.

4.3 An alteration in the regulation of AR and ERα target genes occurs in response to hormones and anti-oestrogens in endocrine resistant BCa cells

To investigate how anti-oestrogen treatment affects gene regulation in endocrine resistance, analysis of the regulation of various AR and ERα target genes previously conducted in MCF7 cells (Figures 3.4.2.1-3) were expanded to the MCF7-TAMR and MCF7-LTED resistant cell lines (Figures 4.3.1-5). The expression of target genes were measured following 8 hour treatment with E2, MIB or both (EtOH treatments were included as a control). Two ERα target genes were explored (*TFF1* and *MYC*), a target gene for both AR and ERα (*GREB1*) as well as two AR target genes (*NDRG1* and *ZBTB16*) (Figures 4.3.1-5). The references describing these target genes to explain why they were selected are summarised in Table 3.5.1. The MCF7 endocrine sensitive results from Section 3.4 have been included in these figures to allow for direct comparison and the main results have been summarised in Table 4.3.1.

E2 treatment significantly increased the expression of the ERα target gene *TFF1* in both MCF7 and MCF7-TAMR cells, 3.4-fold and 2.9-fold respectively, but had no significant impact in MCF7-LTED cells (Figures 4.3.1a-c). Co-treatment of MIB with E2 was unable to compete with the E2-induced *TFF1* expression for any of the cell lines (Figures 4.3.1a-c), in agreement with earlier reporter assays (Figures 3.4.2 and 4). As expected, E2-induced expression of *TFF1* was inhibited via both anti-oestrogens in MCF7 cells (Figure 4.3.1a), however this was unexpectedly also the case for MCF7-TAMR cells (Figure 4.3.1b). However, although these patterns also appear to occur in MCF7-LTED cells, anti-oestrogen treatments displayed no significant effect on *TFF1* expression in this cell line (Figure 3.5.2.1c). These results suggest that in TAM-resistant cells (MCF7-TAMR), some ERα target genes are still regulated by oestrogen, and anti-oestrogens

Table 4.3.1: Expression changes in AR and ERα target genes in different Breast Cancer cell lines in response to hormone and drug treatments
↑ and ↓ indicates an increase or decrease in gene expression respectively, in response to 17-β -Oestradiol (E2), Mibolerone (MIB), Tamoxifen (TAM) or Fulvestrant (FULV) treatments. Two Oestrogen Receptor alpha (ERα) target genes (*TFF1*, and *MYC*), two Androgen Receptor (AR) target genes (*ZBTB16* and *NDRG1*) and a target gene for both (*GREB1*) were investigated in MCF7 cells and its TAM Resistant (TAMR) and Long Term Oestrogen Deprived (LTED) derivatives.

(a) *Trefoil Factor 1* (*TFF1*)

	Effect of Treatment			
Cell Line	**E2**	**MIB**	**TAM**	**FULV**
MCF7	↑	-	↓ from E2 induction	↓ from E2 induction
MCF7-TAMR	↑	-	↓ from E2 induction	↓ from E2 induction
MCF7-LTED	-	-	-	-

(b) *Myelocytomatosis Oncogene Cellular Homolog* (*MYC*)

	Effect of Treatment			
Cell Line	**E2**	**MIB**	**TAM**	**FULV**
MCF7	↑ and ↑ from MIB induction	-	↓ from E2 induction, regardless of MIB treatment	↓ from E2 induction, regardless of MIB treatment
MCF7-TAMR	-	-	-	↓ from E2 induction, regardless of MIB treatment
MCF7-LTED	-	↓	-	↓ from E2 induction, regardless of MIB treatment

(c) *Zinc Finger And BTB Domain Containing 16* (*ZBTB16*)

	Effect of Treatment			
Cell Line	**E2**	**MIB**	**TAM**	**FULV**
MCF7	↓ from MIB induction	↑ and ↑ from E2 induction	↑ from EM induction	↑ from EM induction
MCF7-TAMR	↓ from MIB induction	↑ and ↑ from E2 induction	-	↑ from EM induction
MCF7-LTED	-	↑	-	-

(d) *N-Myc Downstream-Regulated Gene 1* (*NDRG1*)

	Effect of Treatment			
Cell Line	**E2**	**MIB**	**TAM**	**FULV**
MCF7	↓ from MIB induction	-	-	↑ from E2 induction
MCF7-TAMR	-	-	-	-
MCF7-LTED	↓ from MIB induction	↑	-	-

(e) *Gene Regulated in Breast Cancer 1* (*GREB1*)

	Effect of Treatment			
Cell Line	**E2**	**MIB**	**TAM**	**FULV**
MCF7	↑ and ↑ from MIB induction	↑	↓ from E2 induction, regardless of MIB treatment	↓ from E2 induction, regardless of MIB treatment
MCF7-TAMR	↑	-	↓ from E2 induction	↓ from E2 induction
MCF7-LTED	↑ and ↑ from MIB induction	↑	↓ from E2 induction	↓ from E2 induction, regardless of MIB treatment

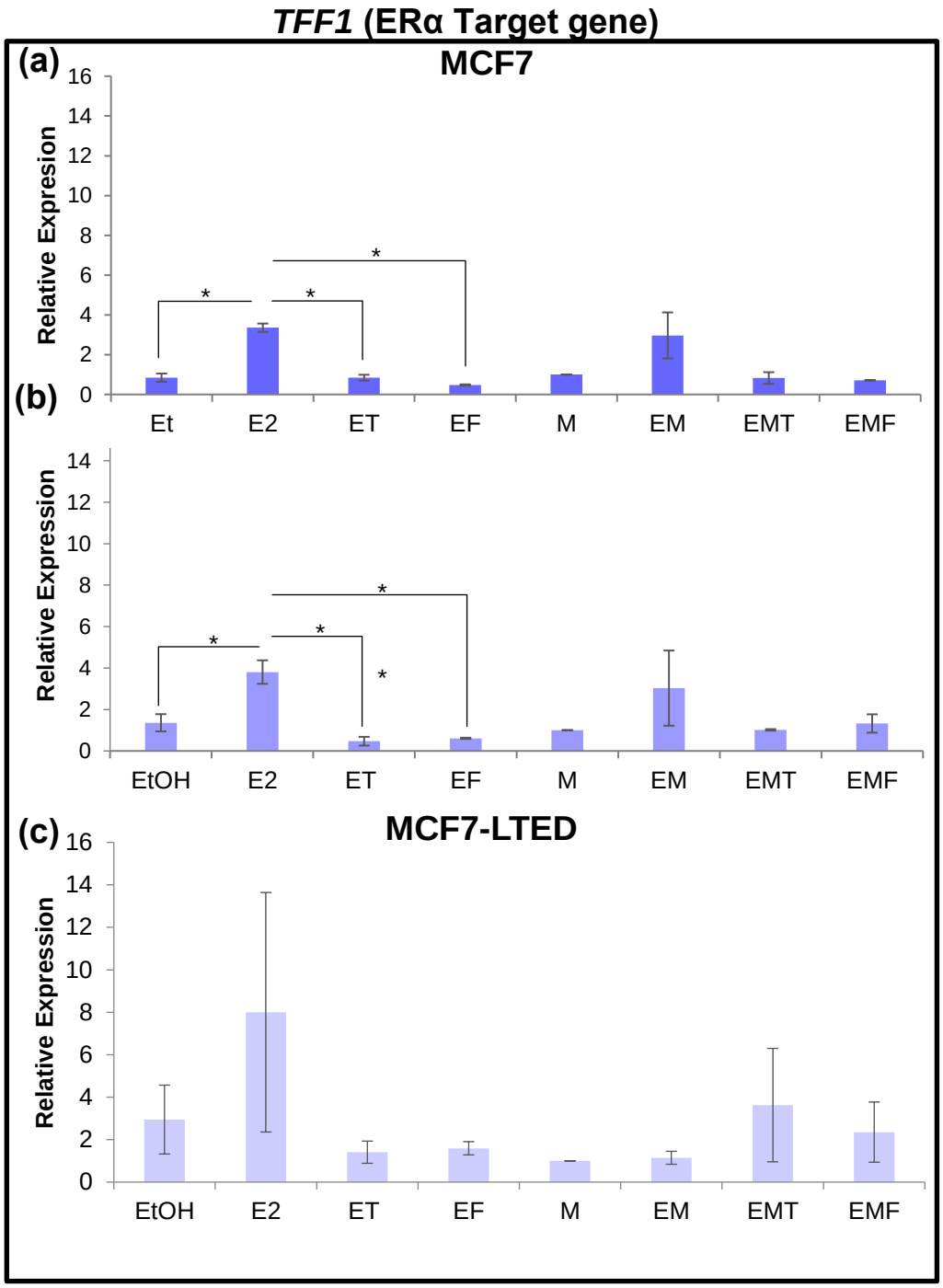


Figure 4.3.1 Effect of oestrogen, androgen and anti-oestrogen treatment on the expression of the ERα target gene *TFF1* in MCF7 cells and its endocrine resistant derivatives

MCF7 cells **(a)** and their Tamoxifen Resistant (TAMR) **(b)** and Long Term Oestrogen Deprived (LTED) **(c)** derivatives were incubated in hormone depleted media for 120 hours, treated with 1 nM of the relevant hormones 17-β-Oestradiol (E2)/Mibolerone (M)/both/ ethanol (EtOH) as a control for 8 hours, with or without the presence of 100 nM Fulvestrant (F)/Tamoxifen (T). RNA was extracted, reverse transcribed into cDNA and qPCR was conducted using SYBR green to measure the expression level of the Oestrogen Receptor alpha (ERα) target gene *Trefoil Factor 1* (*TFF1*). Mean of 3 independent experiments are displayed, ±SE. T-Test P<0.05*, P<0.005**.

could still have some benefit, but androgen inhibits this effect. However, this may not be the case for MCF7-LTED cells, so endocrine resistance could differ between the two cell line models.

For the alternative ERα target gene *MYC*, E2 treatment significantly increased its expression in MCF7 cells (2.1-fold) but not MCF7-TAMR or MCF7-LTED cells (Figures 4.3.2a-c). Again, co-treatment of MIB with E2 was unable to compete with the E2-induced *MYC* expression in MCF7 cells, and did not decrease or induce its expression in the resistant lines (Figures 4.3.2a-c). However, MIB treatment in MCF7-LTED negatively regulated *MYC* expression, but this effect was lost via co-treatment with E2. As expected, E2-induced expression of *MYC* was inhibited via both anti-oestrogens in MCF7 cells (Figure 4.3.2a), however *MYC* expression was significantly reduced via FULV but not TAM treatment in MCF7-TAMR and MCF7-LTED cells (Figures 4.3.2b-c). The addition of MIB treatment had no effect upon the anti-oestrogen inhibition to E2-induced expression of *MYC* in any of the cell lines (Figures 4.3.2a-c). These results suggest that some ERα target genes lose oestrogen responsiveness in endocrine resistance, however FULV could still inhibit ERα action and downregulate their expression.

The expression of an AR target gene, *ZBTB16*, was induced via MIB treatment 1,025-fold in MCF7 cells (Figure 4.3.3a). This effect also occurred in the endocrine resistant cell lines, however it was more strongly induced in MCF7-TAMR cells (24,442-fold), and induction was weaker in MCF7-LTED cells (330.6-fold) (Figures 4.3.3b-c). The expression of *ZBTB16* was not induced via E2 treatment in any of the cell lines, even in the presence of anti-oestrogens (Figures 4.3.3a-c), and co-treatment with E2 significantly reduced MIB-induced *ZBTB16* expression in MCF7 and MCF7-TAMR cells, by 54.9 % and 43.2 %, respectively, and abrogated the significant increase produced by MIB

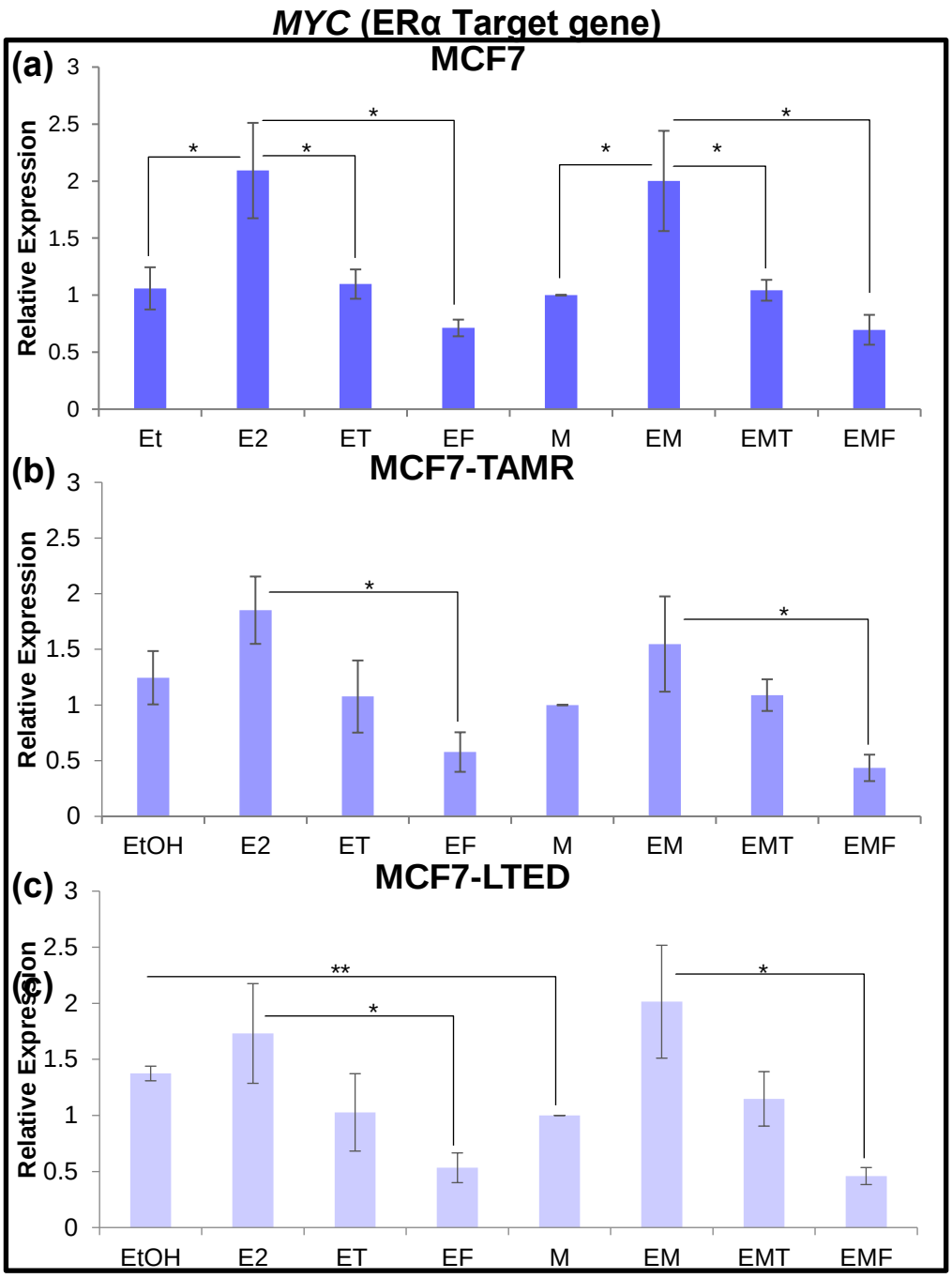


Figure 4.3.2 Effect of oestrogen, androgen and anti-oestrogen treatment on the expression of the ERα target gene *MYC* in MCF7 cells and its endocrine resistant derivatives

MCF7 cells **(a)** and their Tamoxifen Resistant (TAMR) **(b)** and Long Term Oestrogen Deprived (LTED) **(c)** derivatives were incubated in hormone depleted media for 120 hours, treated with 1 nM of the relevant hormones 17-β-Oestradiol (E2)/ Mibolerone (M)/both/ ethanol (EtOH) as a control for 8 hours, with or without the presence of 100 nM Fulvestrant (F)/Tamoxifen (T). RNA was extracted, reverse transcribed into cDNA and qPCR was conducted using SYBR green to measure the expression level of the Oestrogen Receptor alpha (ERα) target gene *Myelocytomatosis Oncogene Cellular Homolog* (*MYC*). Mean of 3 independent experiments are displayed, ±SE. T-Test P<0.05*, P<0.005**.

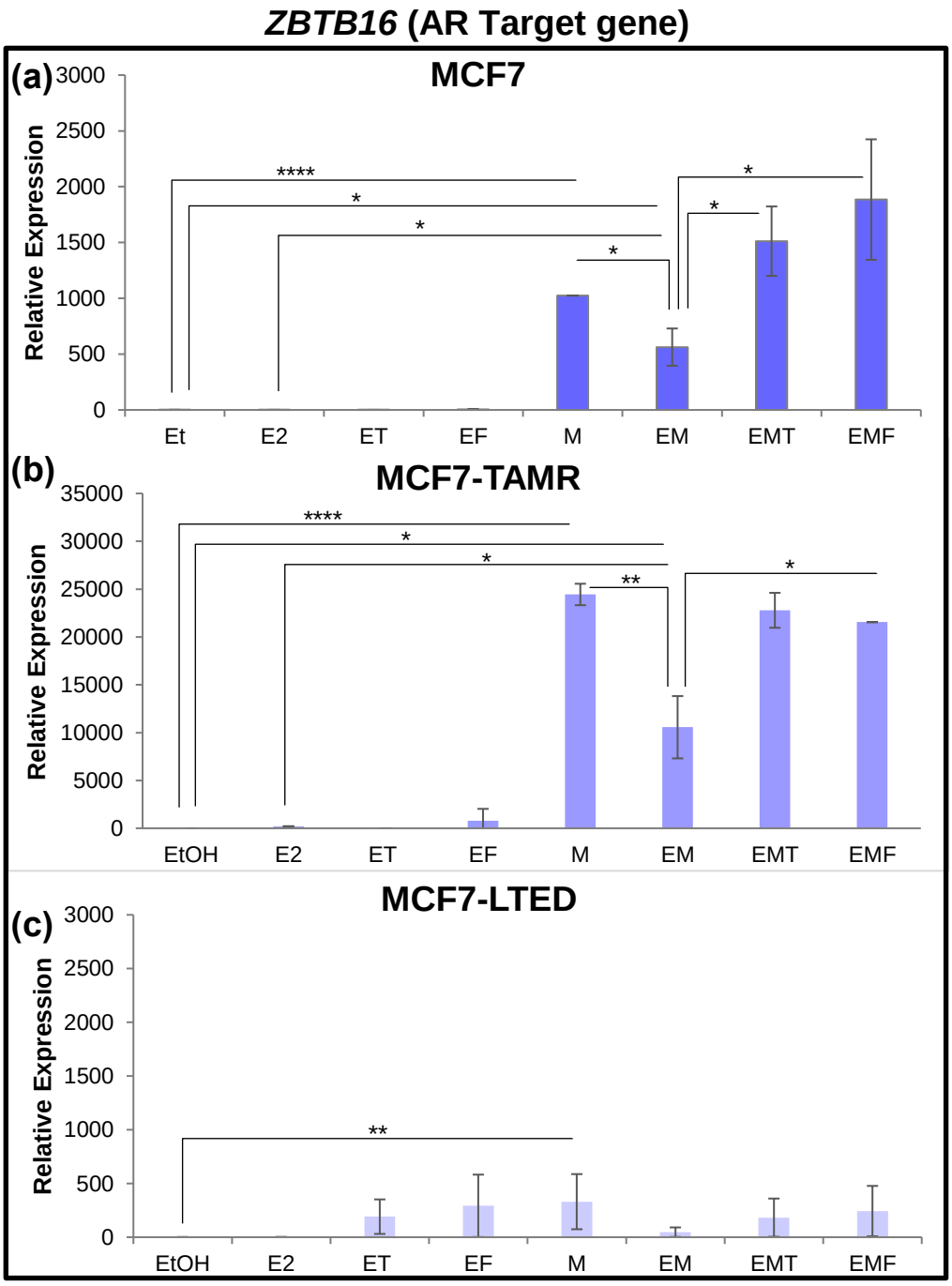


Figure 4.3.3 Effect of oestrogen, androgen and anti-oestrogen treatment on the expression of the AR target gene *ZBTB16* in MCF7 cells and its endocrine resistant derivatives

MCF7 cells **(a)** and their Tamoxifen Resistant (TAMR) **(b)** and Long Term Oestrogen Deprived (LTED) **(c)** derivatives were incubated in hormone depleted media for 120 hours, treated with 1 nM of the relevant hormones 17-β-Oestradiol (E2)/ Mibolerone (M)/both/ ethanol (EtOH) as a control for 8 hours, with or without the presence of 100 nM Fulvestrant (F)/Tamoxifen (T). RNA was extracted, reverse transcribed into cDNA and qPCR was conducted using SYBR green to measure the expression level of the Androgen Receptor (AR) target gene *Zinc Finger And BTB Domain Containing 16* (*ZBTB16*). Mean of 3 independent experiments are displayed, ±SE. T-Test P<0.05*, P<0.005**.

treatment in MCF7-LTED cells (Figures 4.3.3a-c). Importantly, this inhibitive action of E2 was significantly reversed via treatment with the anti-oestrogens in MCF7 cells (Figure 4.3.3a) and FULV treatment in MCF7-TAMR cells (Figure 4.3.3b). This effect was also observed in MCF7-LTED cells, however it was not significant (Figure 4.3.3c). Therefore, MIB induces expression of this gene in all models. However, in the endocrine resistant and sensitive models, oestrogen treatment inhibits androgen induction of this gene, and anti-oestrogen treatments could reverse this effect. This indicates that in endocrine resistance ERα still demonstrates inhibitory action on AR activity, and that anti-oestrogens abrogate this effect and make the AR more active.

The expression of the alternative AR target *NDRG1* was not induced following MIB treatment in MCF7 or MCF7-TAMR cells, however it could be significantly induced 3.1-fold by MIB treatment in MCF7-LTED cells (Figures 4.3.4a, c). Co-treatment of E2 with MIB inhibited *NDRG1* expression in both MCF7 and MCF7-LTED cells by 55.1 % and 49.0 %, respectively (Figures 4.3.4a, c). Co-treatment with FULV significantly increased E2-induced *NDRG1* expression in MCF7 cells, and this was lost in MCF7-LTED cells (Figures 4.3.4a, c). *NDRG1* expression was unaffected by the hormone and drug treatments in MCF7-TAMR cells, suggesting that regulation of this gene has been lost in this endocrine resistant model (Figure 4.3.4b). These results suggest that the regulation of the AR target gene *NDRG1* is induced by FULV treatment in endocrine sensitive cells, and that androgen regulation of *NDRG1* is “switched on” in a model of AI resistance (MCF7-LTED), but oestrogen abrogates this regulation. However, the regulation of this gene is weaker than that of *ZBTB16*, and it is not regulated in a model of TAM resistance (MCF7-TAMR).

The expression of *GREB1*, a target gene for both ERα and AR, was significantly induced via E2 treatment in MCF7 (12.5-fold), MCF7-TAMR (10.3-fold) and MCF7-LTED

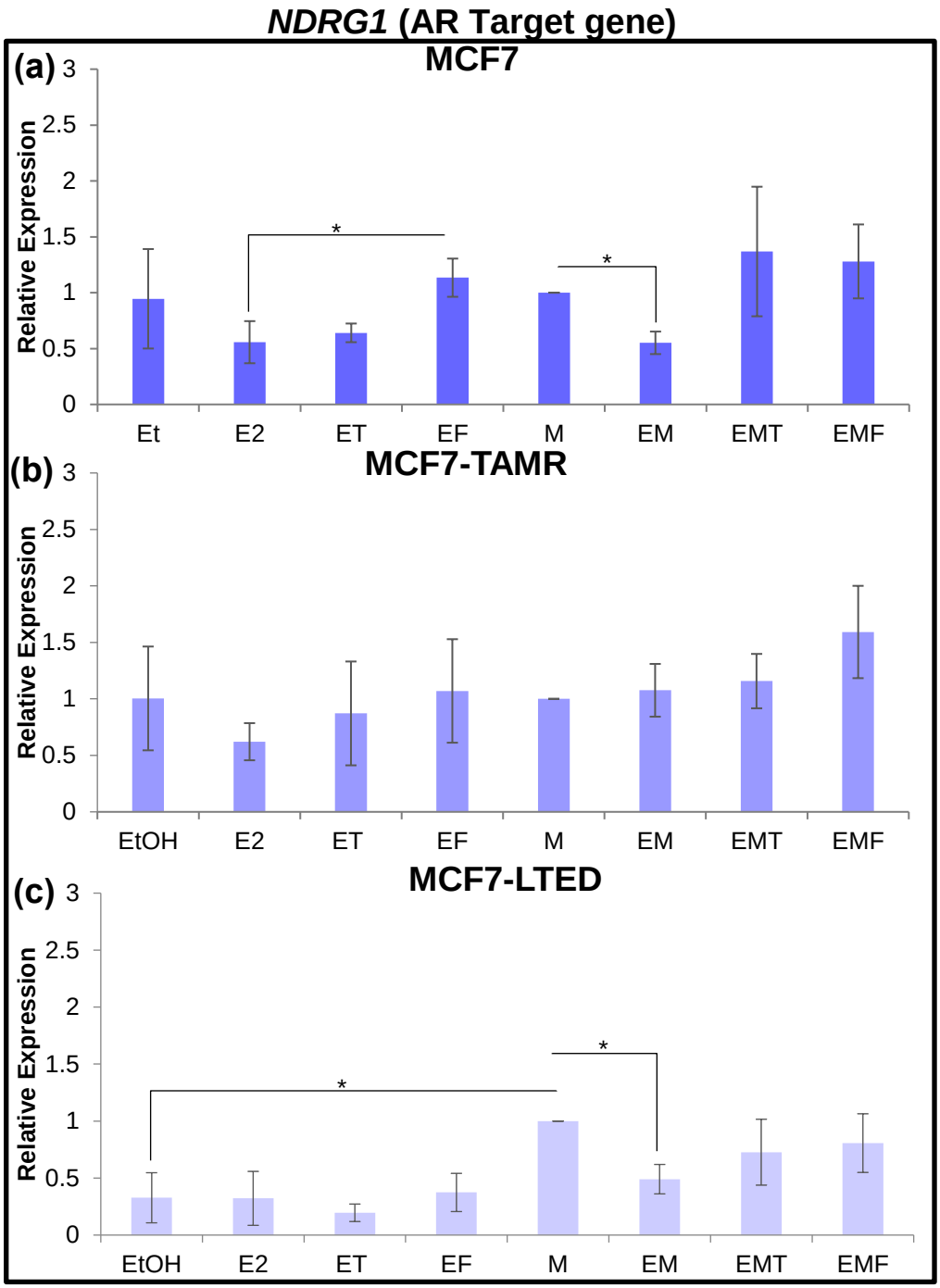


Figure 4.3.4 Effect of oestrogen, androgen and anti-oestrogen treatment on the expression of the AR target gene *NDRG1* in MCF7 cells and its endocrine resistant derivatives

MCF7 cells **(a)** and their Tamoxifen Resistant (TAMR) **(b)** and Long Term Oestrogen Deprived (LTED) **(c)** derivatives were incubated in hormone depleted media for 120 hours, treated with 1 nM of the relevant hormones 17-β-Oestradiol (E2)/ Mibolerone (M)/both/ ethanol (EtOH) as a control for 8 hours, with or without the presence of 100 nM Fulvestrant (F)/Tamoxifen (T). RNA was extracted, reverse transcribed into cDNA and qPCR was conducted using SYBR green to measure the expression level of the Androgen Receptor (AR) target gene *N-Myc Downstream-Regulated Gene 1* (*NDRG1*). Mean of 3 independent experiments are displayed, ±SE. T-Test P<0.05*.

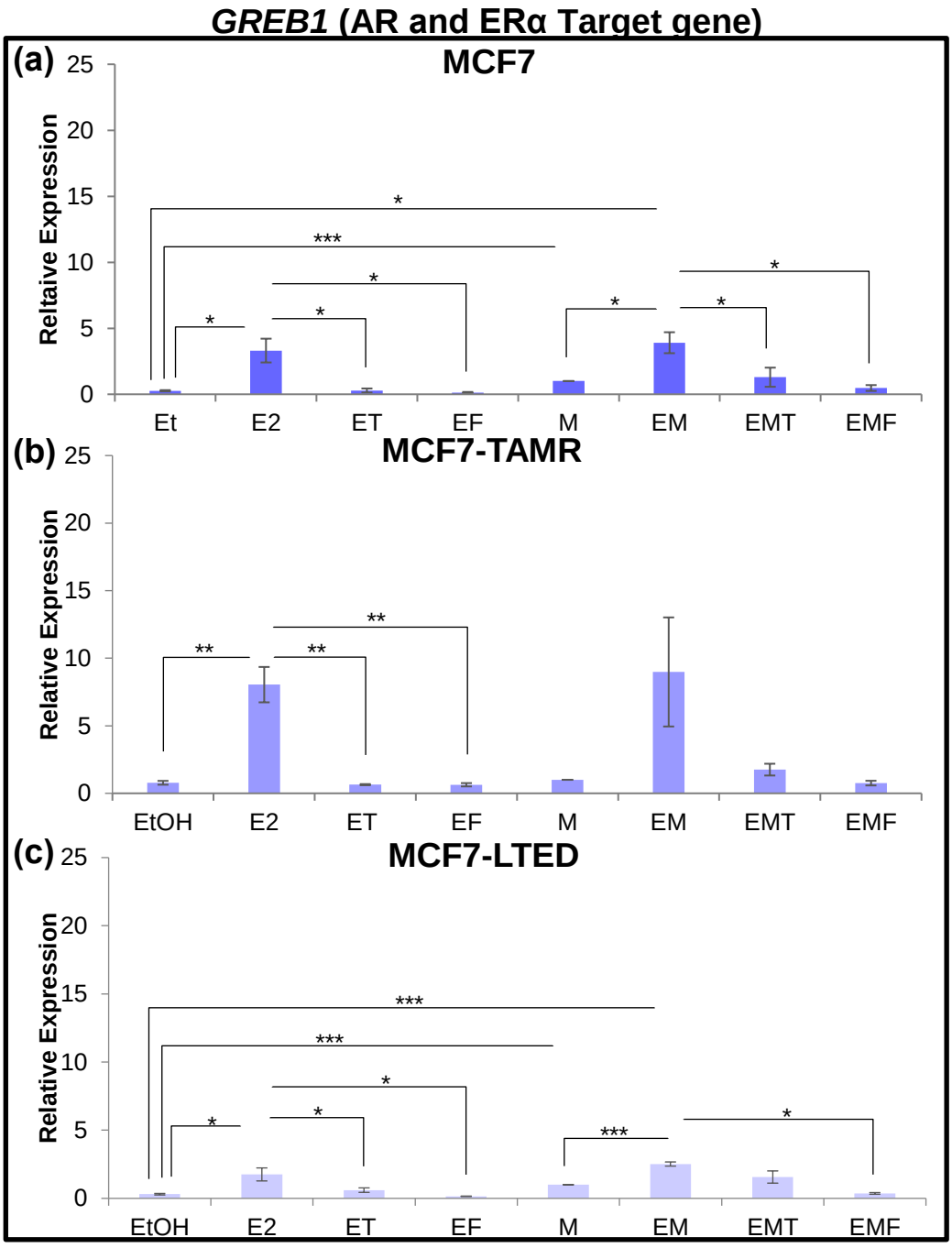


Figure 4.3.5 Effect of oestrogen, androgen and anti-oestrogen treatment on the expression of an ERα and AR target gene *GREB1* in MCF7 cells and its endocrine resistant derivatives

(a) MCF7 cells and their **(b)** Tamoxifen Resistant (TAMR) and **(c)** Long Term Oestrogen Deprived (LTED) derivatives were incubated in hormone depleted media for 120 hours, treated with 1 nM of the relevant hormones 17-β-Oestradiol (E2)/ Mibolerone (M)/both/ ethanol (EtOH) as a control for 8 hours, with or without the presence of 100 nM Fulvestrant (F)/Tamoxifen (T). RNA was extracted, reverse transcribed into cDNA and qPCR was conducted using SYBR green to measure the expression level of the Oestrogen Receptor alpha (ERα) and Androgen Receptor (AR) target gene *Gene Regulated in Breast Cancer 1* (*GREB1*). Mean of 3 independent experiments are displayed, ±SE. T-Test P<0.05*, P<0.005**, P<0.0005***.

cells (5.6-fold), and to a lesser degree by MIB in MCF7 (3.8-fold) and MCF7-LTED (3.2-fold) cells, but not in MCF7-TAMR cells (Figures 4.3.5a-c). In all 3 cell lines, treatment with the anti-oestrogens was able to significantly reduce E2-induced *GREB1* expression (Figures 4.3.5a-c). However, co-treatment of MIB resulted in a partial abrogation of anti-oestrogen inhibition to E2-induced expression of *GREB1* in MCF7-TAMR cells, as these differences were no longer significant (Figure 4.3.5b), however E2-induced *GREB1* expression was still significantly reduced via both anti-oestrogens in MCF7 cells and via FULV in MCF7-LTED cells in the presence of MIB (Figures 4.3.5a, c). Therefore, *GREB1* appears to be more strongly regulated by ERα than AR in all of our cell line models, and anti-oestrogen treatment only slightly increases the androgen-regulation of this gene, including in the resistant models.

In conclusion, the main results from this section have demonstrated that the endocrine resistant cell line models have altered hormone-regulated expression of AR and ERα regulatory target genes in comparison to their endocrine sensitive parental line. Some ERα target genes lose oestrogen responsiveness in endocrine resistance, however anti-oestrogen treatment (particularly FULV) can still inhibit ERα action and downregulate their expression, suggesting that this receptor is still, in part, promoting BCa growth. Androgen treatment can abrogate the effect of this anti-oestrogen inhibition in the endocrine resistant and sensitive cells, suggesting that androgen can drive growth and endocrine resistance when ERα is inhibited. In conjunction with this, in some cases oestrogen treatment was found to downregulate AR target gene expression, and anti-oestrogen treatment could reverse this effect. The mechanism of resistance alters between these TAM-resistance and AI-resistance models as they have differing

responses to hormone and anti-oestrogen treatments for the genes, including that not all androgen-regulated genes are upregulated.

4.4 AR and ERα localisation alters in endocrine sensitive and resistant cells

It is known that AR and ERα cross-talk supresses each other's transcriptional activity (Fioretti *et al.*, 2014) and it has previously been demonstrated that this cross-talk is at least in part as a result of a direct interaction of the receptors (Panet-Raymond *et al*., 2000). Additionally, AR and ERα are both ligand-dependant transcription factors that exert their effects in the nucleus (Sever and Glass, 2013). Confocal microscopy was therefore conducted to investigate whether AR and ERα colocalise.

Initially, ERα-Red Fluorescent Protein (ERα-RFP) and AR-Green Fluorescent Protein (AR-GFP) plasmids were transfected into COS-1 cells, which are negative for both ERα and AR, and the localisation of the receptors were investigated following treatment with Ethanol (EtOH) as a control, ERα ligand (E2), AR ligand (MIB) or both (Figure 4.4.1). 4', 6-diamidino-2-phenylindole (DAPI) was used to stain cell nuclei. In the absence of hormone treatment, AR was predominantly cytoplasmic and ERα nuclear. However, upon E2 treatment AR nuclear localisation and ERα cytoplasmic localisation became more pronounced, although they remained predominantly cytoplasmic and nuclear, respectively. Following MIB treatment (+/- E2), ERα localisation remained pronounced in the nucleus. AR nuclear co-localisation with ERα was much more pronounced with MIB treatment, and following co-treatment with E2 and MIB. Nuclear translocation of AR, in response to MIB, was partially blocked by E2, with some cytoplasmic localisation evident (Figure 4.4.1).

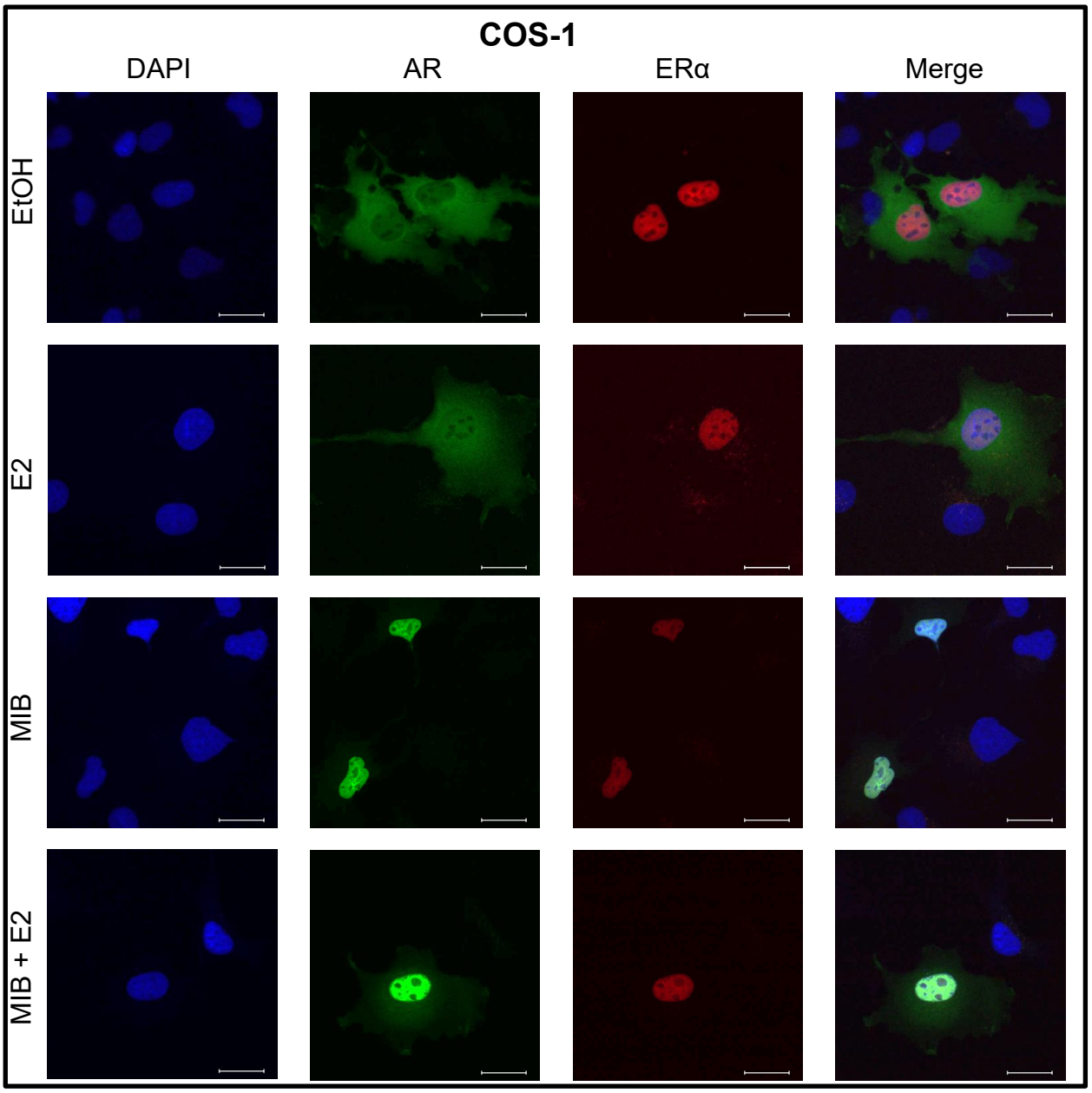


Figure 4.4.1 Exogenous AR and ERα localisation

COS-1 cells were transiently transfected with plasmids encoding Androgen Receptor fused to Green Fluorescent Protein (AR-GFP) and Oestrogen Receptor alpha fused to Red Fluorescent Protein (ERα-RFP), cultured in hormone depleted media for 24 hours and treated with EtOH (vehicle) or 1 nM 17-β-Oestradiol (E2) and/or Mibolerone (MIB) for 2 hours. Cells were fixed using 4 % paraformaldehyde. 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus (blue). Cells were visualised using confocal microscopy. Scale bar = 10 μm.

To identify whether AR and ERα colocalise in endocrine sensitive disease and how this changes in endocrine resistance, confocal microscopy was used to identify the localisation of these receptors in MCF7 and T47D cells (Figures 4.4.2-3) and how this changes in their TAMR and LTED endocrine resistant derivatives (Figures 4.4.4-7). Cells were treated with the same hormone treatments as in Figure 4.4.1. Antibodies specific for AR and ERα were used followed by secondary antibodies labelled with Alexa Fluor 488 (green, AR) or 568 (red, ERα) to visualise receptor localisation. DAPI was used to stain cell nuclei. The localisation of AR and ERα was noted under all conditions and is summarised in Table 4.4.1.

In the absence of hormone, AR was predominantly cytoplasmic and ERα was predominantly nuclear for both MCF7 and T47D cells (Figures 4.4.2-3). Upon E2 treatment however, AR nuclear localisation and ERα cytoplasmic localisation additionally became more pronounced, displaying co-localisation (Figures 4.4.2-3). In T47D cells, AR and ERα remained both nuclear and cytoplasmic, following all hormone treatments (Figure 4.4.3). In MCF7 cells however, AR nuclear localisation was more pronounced with MIB treatment, but mainly cytoplasmic upon co-treatment with E2 (Figure 4.4.2).

This was then expanded to the endocrine resistant derivatives of MCF7 cells: MCF7-TAMR and MCF7-LTED (Figures 4.4.4-5). In both resistant lines, in the absence of hormone AR was predominantly cytoplasmic and ERα was predominantly nuclear, as previously demonstrated in the parental line. As for MCF7 cells, upon E2 treatment, MCF7-LTED AR nuclear localisation and ERα cytoplasmic localisation additionally became more pronounced, displaying co-localisation (Figure 4.4.5). By contrast, in MCF7-TAMR cells, AR remained predominantly cytoplasmic (Figure 4.4.4). Unlike the parental cell line, AR and ERα were both nuclear and cytoplasmic with MIB treatment for

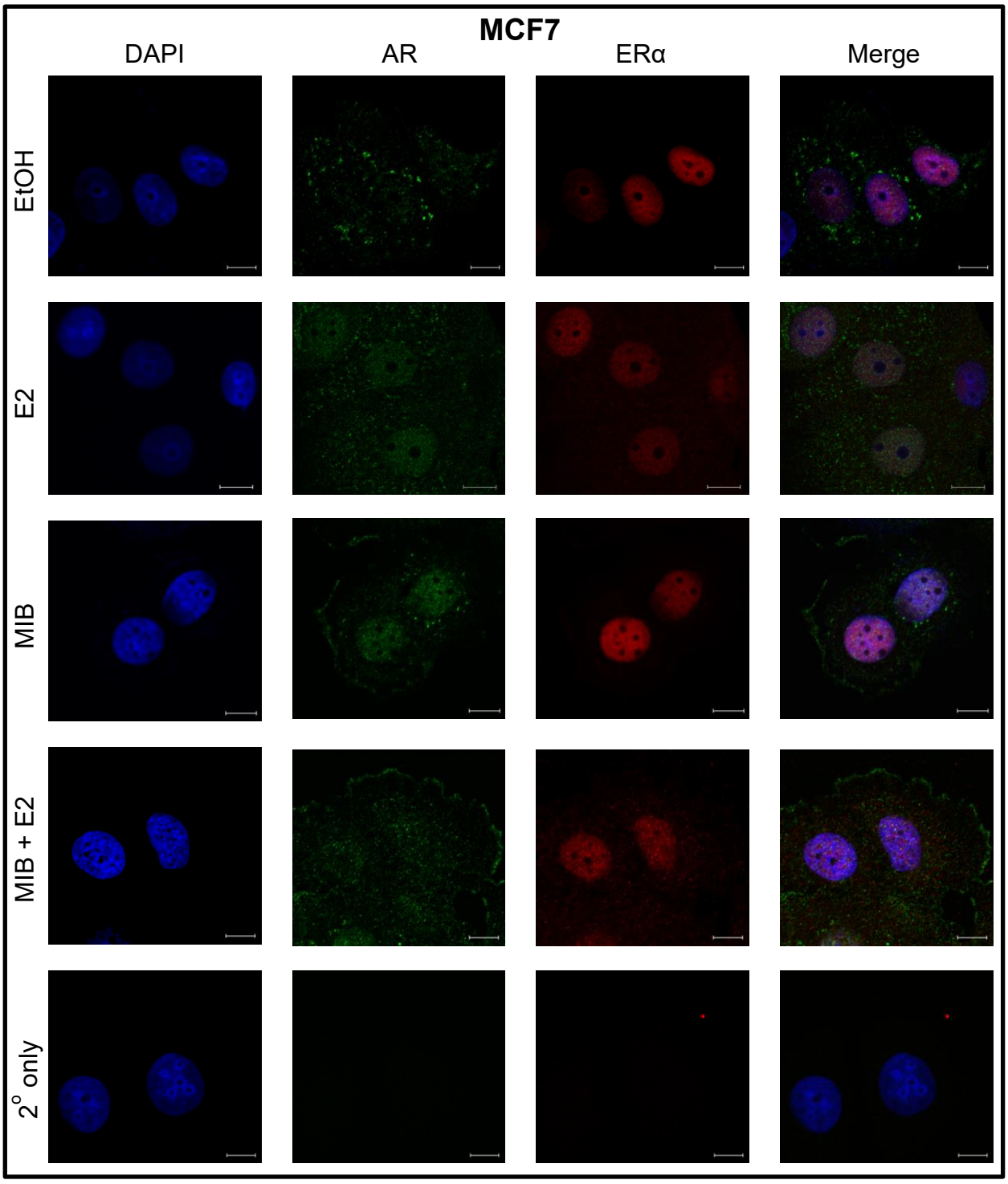


Figure 4.4.2 AR and ERα localisation in MCF7 cells

Cells were cultured in hormone depleted media for 24 hours and treated with EtOH (vehicle) or 1 nM 17-β-Oestradiol (E2) and/or Mibolerone (MIB) for 2 hours. Cells were fixed using 4 % paraformaldehyde and methanol and probed with anti-Androgen Receptor (AR) and/or anti-Oestrogen Receptor alpha (ERα) following with secondary antibodies labelled with Alexa Fluor 488 (green, AR) or 568 (red, ERα) to visualise receptor localisation. 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus (blue). A secondary antibody only stain (2^{o}) is displayed as a control. Cells were visualised using confocal microscopy. Scale bar = 10 μm.

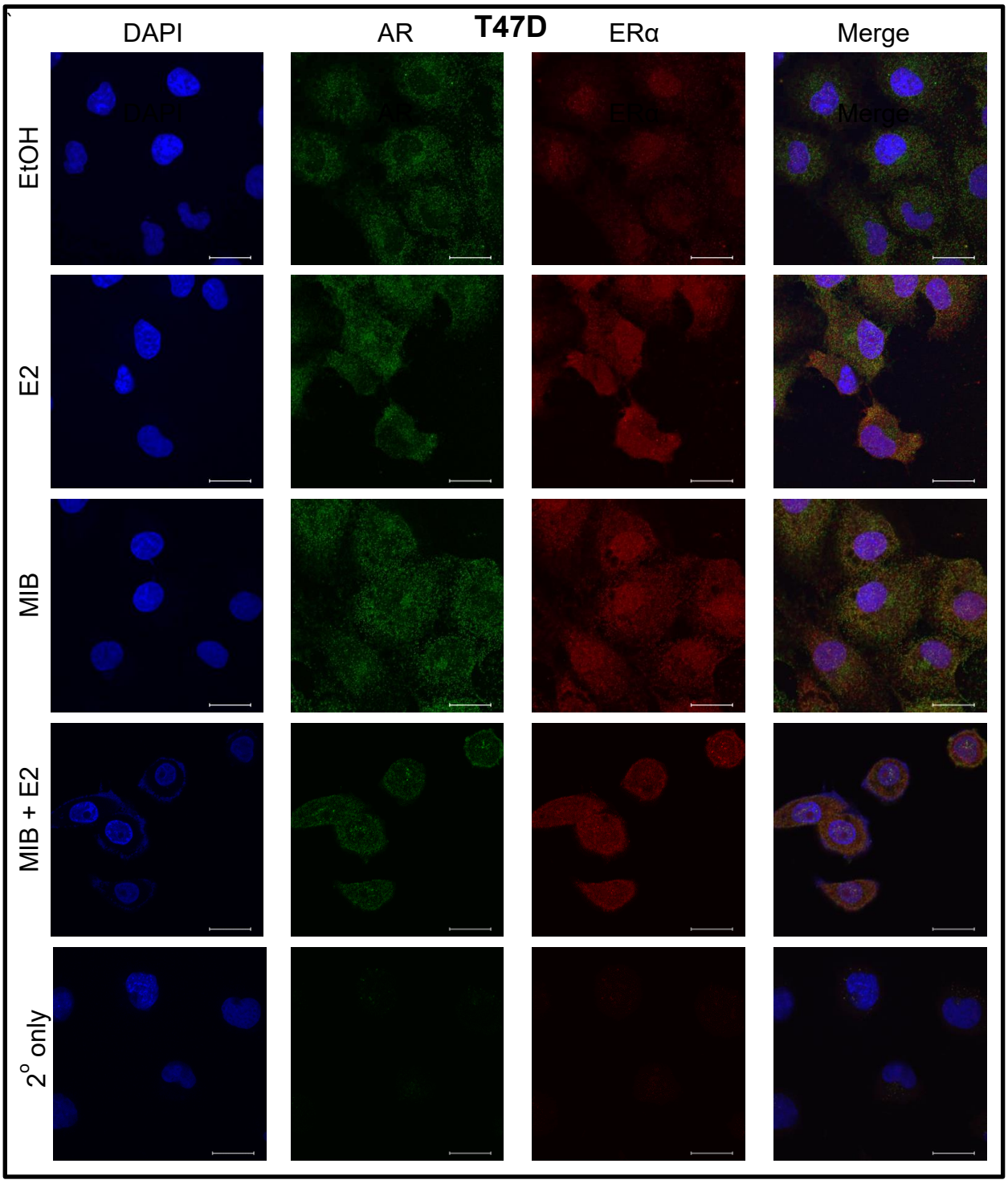


Figure 4.4.3 AR and ERα localisation in T47D cells

Cells were cultured in hormone depleted media for 24 hours and treated with EtOH (vehicle) or 1 nM 17-β-Oestradiol (E2) and/or Mibolerone (MIB) for 2 hours. Cells were fixed using 4 % paraformaldehyde and methanol and probed with anti-Androgen Receptor (AR) and/or anti-Oestrogen Receptor alpha (ERα) following with secondary antibodies labelled with Alexa Fluor 488 (green, AR) or 568 (red, ERα) to visualise receptor localisation. 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus (blue). A secondary antibody only stain (2°) is displayed as a control. Cells were visualised using confocal microscopy. Scale bar = 10 μm.

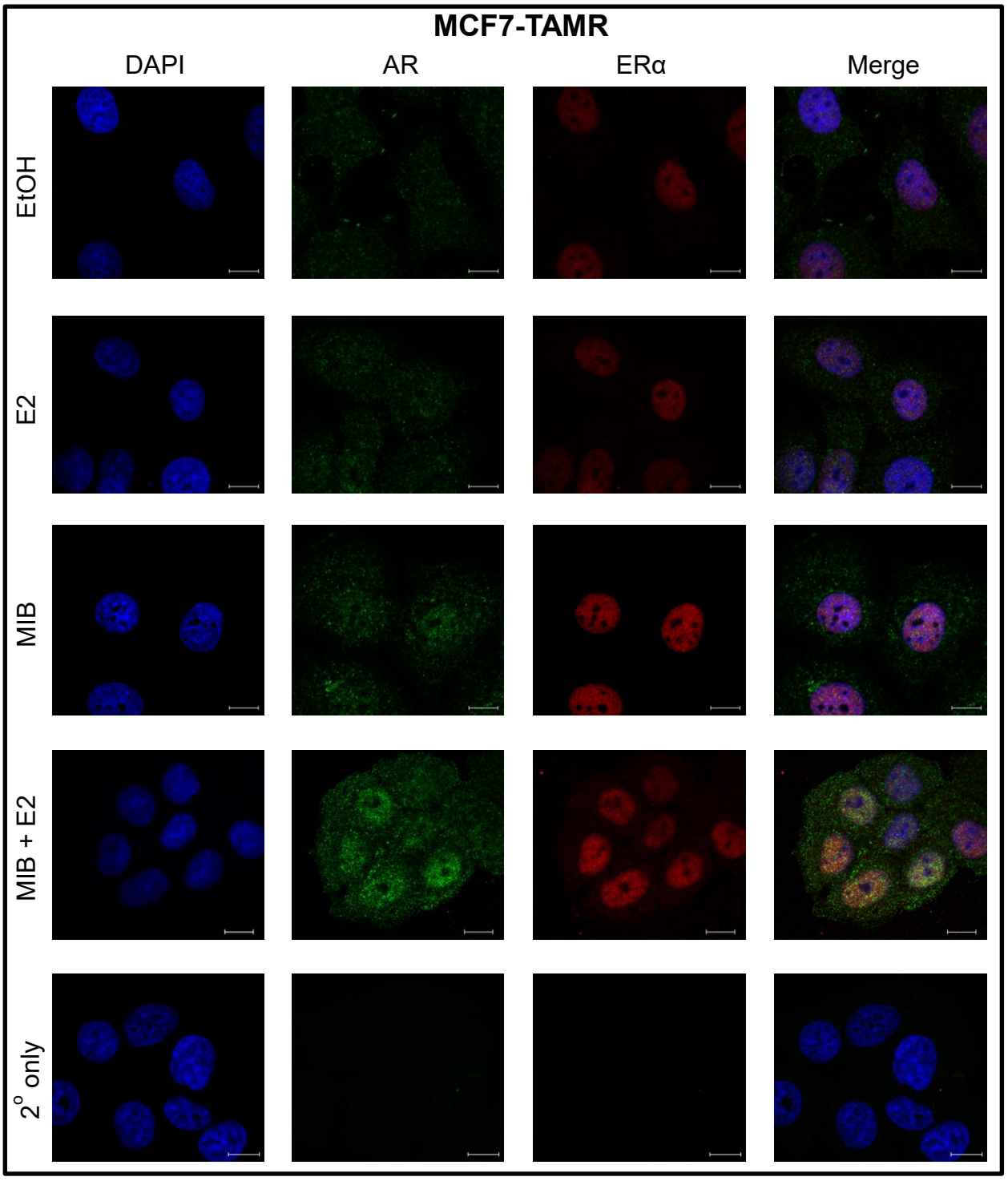


Figure 4.4.4 AR and ERα localisation in MCF7-Tamoxifen Resistant (TAMR) cells Cells were cultured in hormone depleted media for 24 hours and treated with EtOH (vehicle) or 1 nM 17-β-Oestradiol (E2) and/or Mibolerone (MIB) for 2 hours. Cells were fixed using 4 % paraformaldehyde and methanol and probed with anti-Androgen Receptor (AR) and/or anti-Oestrogen Receptor alpha (ERα) following with secondary antibodies labelled with Alexa Fluor 488 (green, AR) or 568 (red, ERα) to visualise receptor localisation. 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus (blue). A secondary antibody only stain (2°) is displayed as a control. Cells were visualised using confocal microscopy. Scale bar = 10 μm.

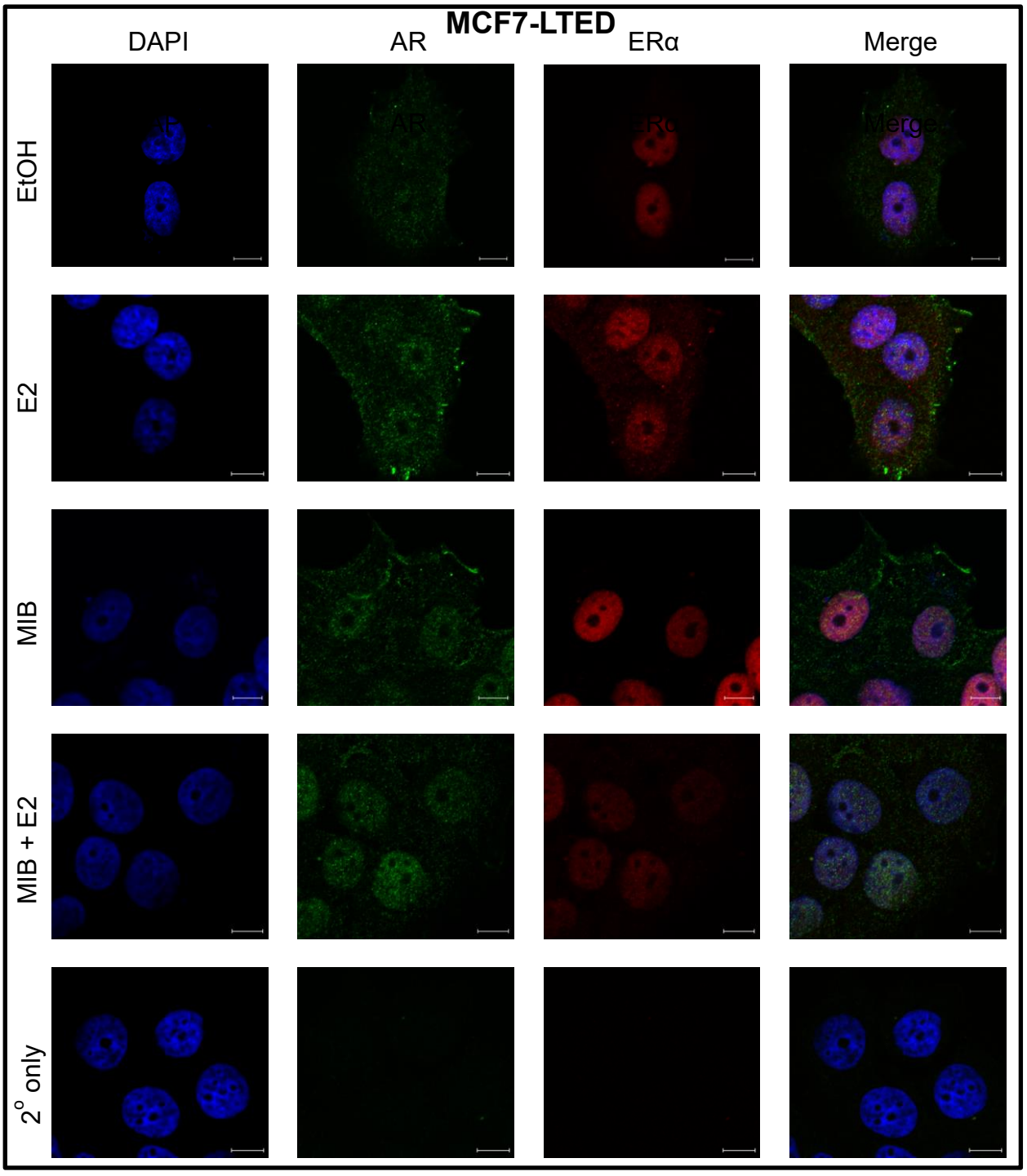


Figure 4.4.5 AR and ERα localisation in MCF7-Long Term Oestrogen Deprived (LTED) cells

Cells were cultured in hormone depleted media for 24 hours and treated with EtOH (vehicle) or 1 nM 17-β-Oestradiol (E2) and/or Mibolerone (MIB) for 2 hours. Cells were fixed using 4 % paraformaldehyde and methanol and probed with anti-Androgen Receptor (AR) and/or anti-Oestrogen Receptor alpha (ERα) following with secondary antibodies labelled with Alexa Fluor 488 (green, AR) or 568 (red, ERα) to visualise receptor localisation. 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus (blue). A secondary antibody only stain (2°) is displayed as a control. Cells were visualised using confocal microscopy. Scale bar = 10 μm.

MCF7-TAMR and MCF7-LTED (Figures 4.4.4-5). Co-treatment with E2 resulted in AR shifting to predominantly nuclear localisation in MCF7-LTED cells, but AR remained both nuclear and cytoplasmic in MCF7-TAMR cells (Figures 4.4.4-5).

Finally, this experiment was conducted in the endocrine resistant derivatives of T47D cells: T47D-TAMR and T47D-LTED (Figures 4.4.6-7). In contrast to their parental line, AR was predominantly nuclear prior to hormone treatment and colocalised with ERα in T47D-TAMR and T47D-LTED cells. In accordance with T47D cells, E2 treatment resulted in both nuclear and cytoplasmic localisation of AR and ERα in T47D-TAMR cells (Figure 4.4.6). By contrast, in T47D-LTED cells E2 treatment resulted in more pronounced cytoplasmic localisation of both AR and ERα (Figure 4.4.7). Interestingly, in T47D-TAMR cells, both AR and ERα were predominantly cytoplasmic upon treatment with MIB, in the presence or absence of E2 (Figure 4.4.6), in contrast to T47D and T47D-LTED cells where the receptors were both cytoplasmic and nuclear (Figures 4.4.2,7).

To conclude, AR and ERα localisation varies between two endocrine sensitive models, and differs from these parental lines in their endocrine resistant derivatives, however the receptors are commonly found to colocalise upon hormone treatments. The results from this section are summarised in Table 4.4.1.

4.5 The responsiveness of AR and ERα regulatory target genes to hormones differs in endocrine resistant cells

In Section 4.3, the regulation of AR and ERα target genes in response to treatment with hormones and anti-oestrogens was analysed to investigate how it changes in endocrine resistance. This was conducted using MCF7, MCF7-TAMR and MCF7-LTED

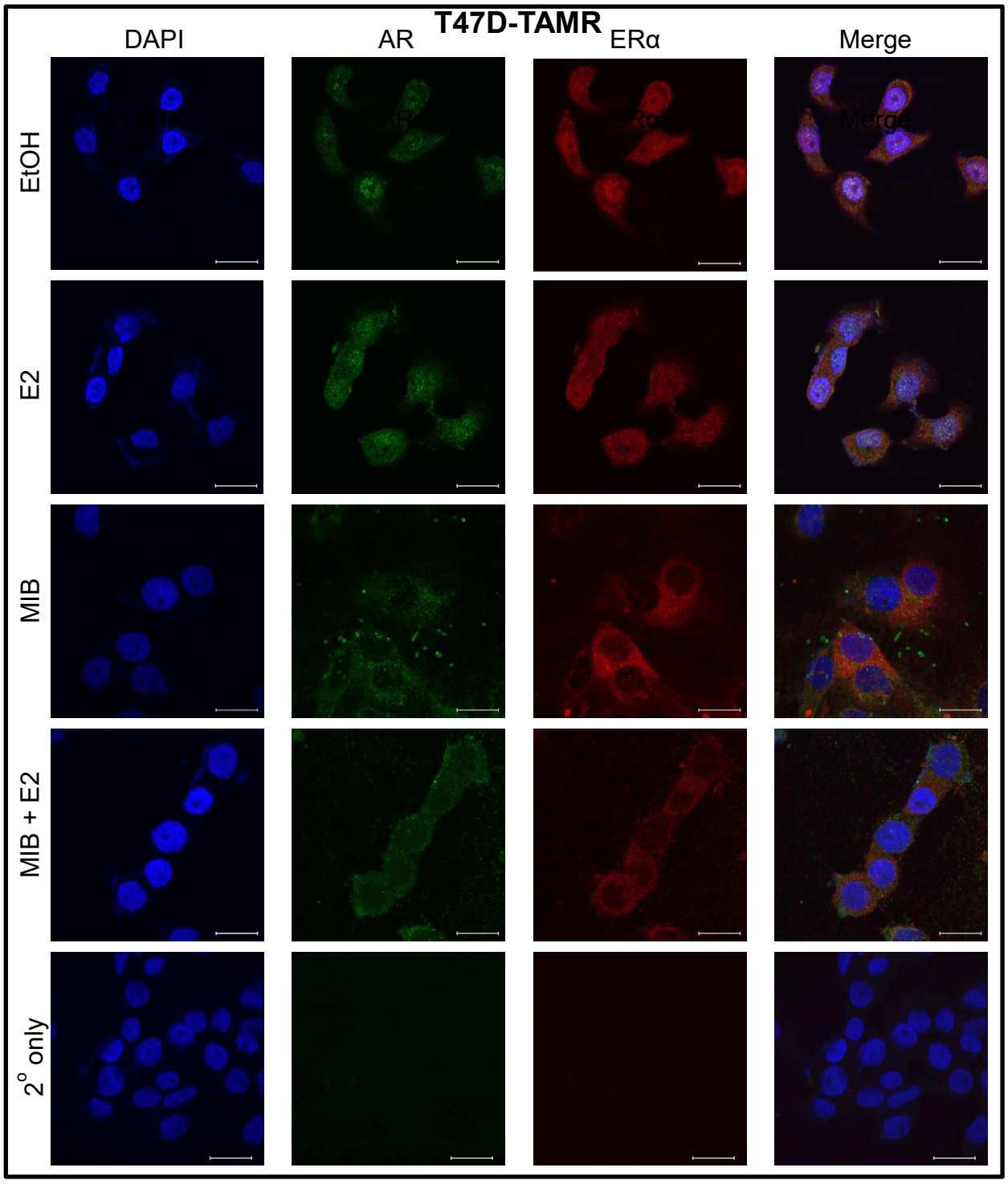


Figure 4.4.6 AR and ERα localisation in T47D-Tamoxifen Resistant (TAMR) cells

Cells were cultured in hormone depleted media for 24 hours and treated with EtOH (vehicle) or 1 nM 17-β-Oestradiol (E2) and/or Mibolerone (MIB) for 2 hours. Cells were fixed using 4 % paraformaldehyde and methanol and probed with anti-Androgen Receptor (AR) and/or anti-Oestrogen Receptor alpha (ERα) following with secondary antibodies labelled with Alexa Fluor 488 (green, AR) or 568 (red, ERα) to visualise receptor localisation. 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus (blue). A secondary antibody only stain (2°) is displayed as a control. Cells were visualised using confocal microscopy. Scale bar = 10 μm.

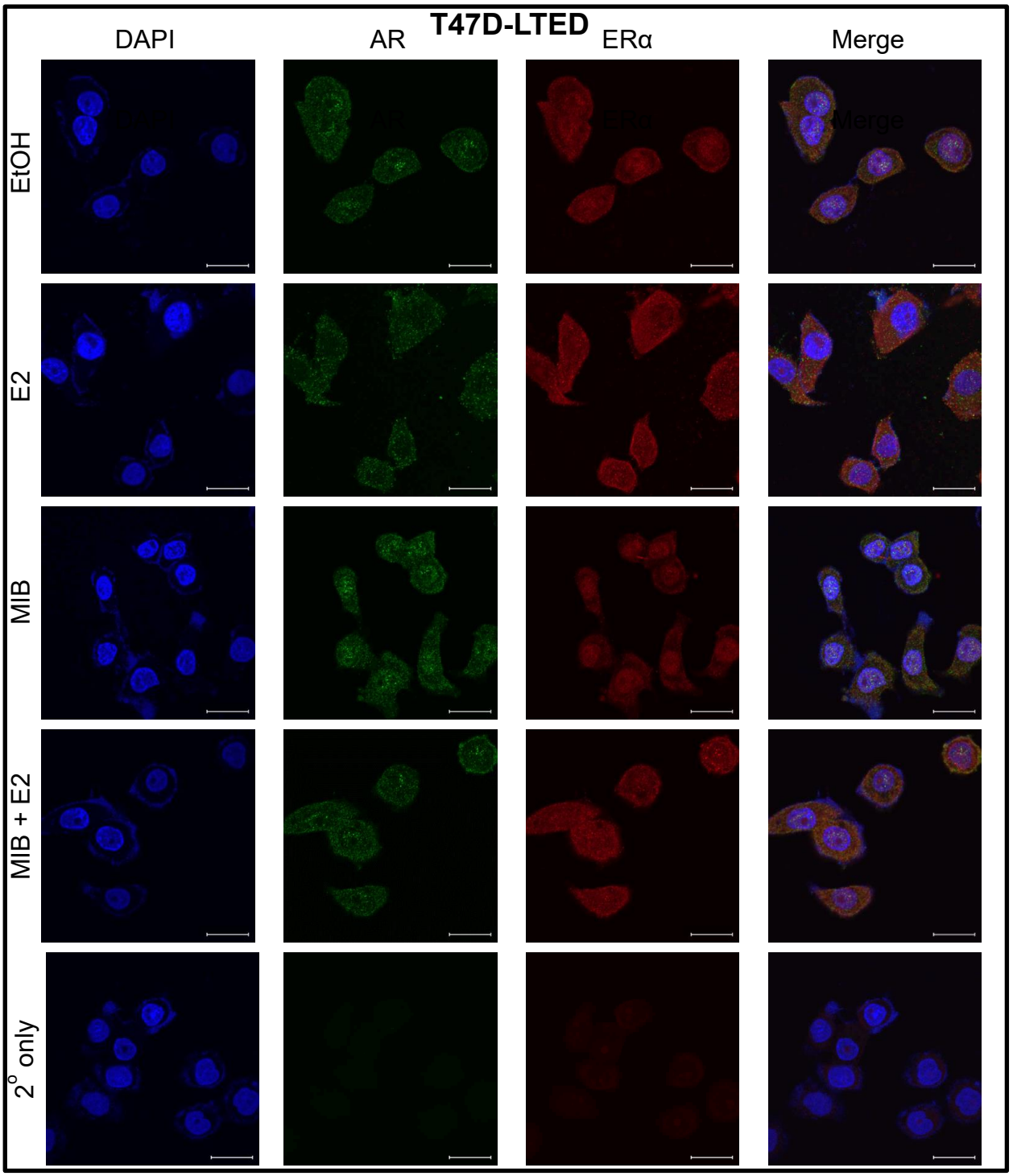


Figure 4.4.7 AR and ERα localisation in T47D-Long Term Oestrogen Deprived (LTED) cells

Cells were cultured in hormone depleted media for 24 hours and treated with EtOH (vehicle) or 1 nM 17-β-Oestradiol (E2) and/or Mibolerone (MIB) for 2 hours. Cells were fixed using 4 % paraformaldehyde and methanol and probed with anti-Androgen Receptor (AR) and/or anti-Oestrogen Receptor alpha (ERα) following with secondary antibodies labelled with Alexa Fluor 488 (green, AR) or 568 (red, ERα) to visualise receptor localisation. 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus (blue). A secondary antibody only stain (2°) is displayed as a control. Cells were visualised using confocal microscopy. Scale bar = 10 μm.

Table 4.4.1 Endogenous AR and ERα cellular localisation following androgen or oestrogen treatment

Table indicates whether **(a)** AR or **(b)** ERα were predominantly Nuclear (N), Cytoplasmic (C) or both (N + C) following 2 hour treatment with androgen (Mibolerone, MIB) or oestrogen (17-β-Oestradiol, E2) in MCF7 and T47D endocrine sensitive cell lines and their Tamoxifen Resistant (TAMR) and Long Term Oestrogen Deprived (LTED) derivatives.

(a) AR Localisation				
	EtOH	**E2**	**MIB**	**E2 + MIB**
COS-1 (Exogenous)	C	C	N	N + C
MCF7	C	N + C	N	C
MCF7-TAMR	C	C	N + C	N + C
MCF7-LTED	C	N + C	N + C	N
T47D	C	N + C	N + C	N + C
T47D-TAMR	N	N + C	C	C
T47D-LTED	N	C	N + C	N + C

(b) ERα Localisation				
	EtOH	**E2**	**MIB**	**E2 + MIB**
COS-1 (Exogenous)	N	N	N	N
MCF7	N	N + C	N	N + C
MCF7-TAMR	N	N	N	N + C
MCF7-LTED	N	N + C	N	N
T47D	N	N + C	N + C	N + C
T47D-TAMR	N	N + C	C	C
T47D-LTED	N	C	N + C	N + C

cells. The aim of this section was to investigate if the sensitivity to E2 or MIB is altered in the endocrine resistant lines as compared to their endocrine sensitive parental line. The same target genes were selected as investigated previously (Table 3.5.1), as well as three additional AR target genes: *Dopa Decarboxylase* (*DDC*), *SEC14 Like Lipid Binding 2* (*SEC14L2)* and *Deiodinase*, *Iodothyronine Type II* (*DIO2*) (Figures 4.5.1-6). *DDC* is an androgen regulated gene that has been identified as an important AR co-activator in PCa (Margiotti *et al.*, 2007, Wafa *et al.*, 2003). *SEC14L2* has been identified as an androgen-regulated gene associated with PCa risk and involved in PCa cellular proliferation (Bolton *et al.*, 2007, Chan *et al.*, 2016, Ni *et al.*, 2005). Interestingly, *SEC14L2* has been reported to have low expression in MCF7 (Wang *et al.*, 2009), but can be upregulated via androgen treatment in T47D (Takagi *et al.*, 2010). By contrast, although *DIO2* has been reported to be rapidly upregulated via DHT treatment in PCa, its function is involved in the regulation of Thyroid hormone Receptor (TR) activity through the production of the more potent thyroid hormone triiodothyronine from thyroxine, the process of which is upregulated via E2 (Detti *et al.*, 2013, Xu *et al.*, 2006).

As demonstrated in Figure 4.3.1, the ERα target *TFF1* was upregulated by E2 treatment in all 3 cell lines, however this was only significant for MCF7 (Figure 4.5.1a). Interestingly, in all 4 treatments, MCF7 cells had a significantly higher *TFF1* expression than MCF7-TAMR cells, suggesting that this gene is silenced in this model of endocrine resistance. Additionally, the expression of the alternative ERα target gene *MYC* was upregulated in MCF7 cells via E2 treatment, and co-treatment of MIB with E2 was unable to compete with the E2-induced *MYC* expression (Figure 4.5.1b). Although significant differences between treatments were lost in the endocrine resistant lines, a similar *MYC* expression and no significant differences between the 3 cell lines for each hormone

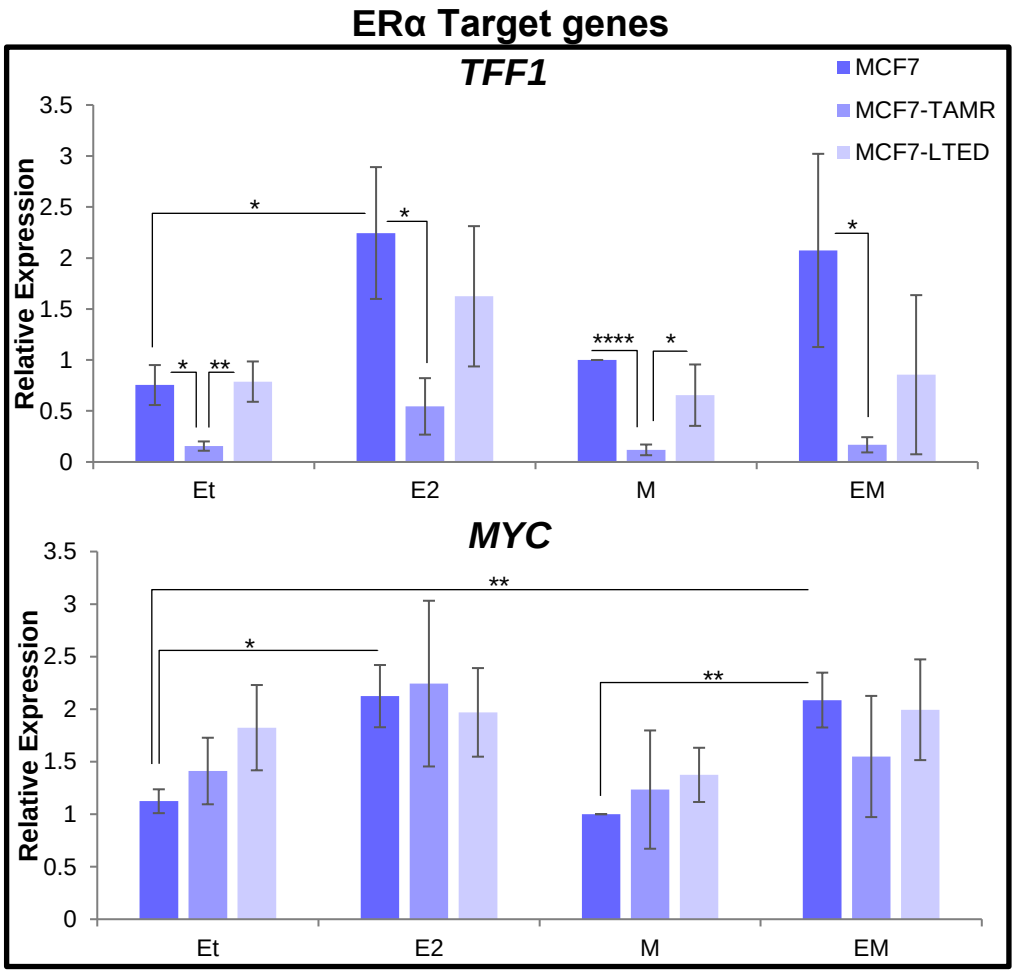


Figure 4.5.1 Direct comparison of expression of ERα target genes *TFF1* and *MYC* in MCF7, MCF7-TAMR and MCF7-LTED cells

MCF7 cells, their Tamoxifen Resistant (TAMR) and Long Term Oestrogen Deprived (LTED) derivatives were incubated in hormone depleted media for 120 hours, and treated with 1 nM of the relevant hormones 17-β-Oestradiol (E2)/ Mibolerone (M)/both/ ethanol (EtOH) as a control for 8 hours. RNA was extracted, reverse transcribed into cDNA and qPCR was conducted using SYBR green to measure the expression level of the Oestrogen Receptor alpha (ERα) target genes **(a)** *Trefoil Factor 1* (*TFF1)* and **(b)** *Myelocytomatosis Oncogene Cellular Homolog* (*MYC)*. Expression was normalised to *RPL19 ribosomal protein*, *L19,* and expression is relative to the MIB-treated MCF7 sample for each gene. Mean of 5 independent experiments, ±SE. T-Test *P<0.05, **P<0.005, ***P<0.0005, ****P<0.00005.

treatment were observed. This suggests that an alteration to the regulation of *MYC* is not important for these endocrine resistant lines.

The expression of 5 AR target genes were subsequently evaluated (Figures 4.5.2-3). *ZBTB16* expression was induced by androgen in all cell lines (although this was not significant for MCF7-LTED) (Figure 4.5.2a). Androgen sensitivity (i.e. fold change in *ZBTB16* expression in response to MIB) was higher in MCF7-TAMR cells (2,621.1-fold) than MCF7 cells (1,981.7-fold), but total expression levels were higher in MCF7 (Figure 4.5.2a). *ZBTB16* expression was barely detectable in MCF7-LTED cells compared to the other cell lines (Figure 4.5.2a). Co-treatment with E2 reduced MIB-induced *ZBTB16* expression in all cell lines, however this effect was most noticeable in MCF7 cells (48.1 %) and only significant in this cell line. These results indicate that although *ZBTB16* expression is reduced in our endocrine resistant models, its androgen regulation has become stronger in the MCF7-TAMR line, indicating that AR activity has increased in this cell line. Additionally, oestrogen significantly reduced the androgen-induced *ZBTB16* expression in MCF7 cells, however this effect was not significant in the resistant lines, indicating that ERα inhibition of this expression is decreased in endocrine resistance. These results also indicate that the regulation of this gene is more important in MCF7-TAMR cells than in MCF7-LTED cells.

On the other hand, *NDRG1* expression was weakly induced via MIB treatment (1.8 - 2.6-fold) and co-treatment with E2 reduced this effect in all cell lines, however this was only significant in MCF7 cells (Figure 4.5.2b). Under all treatment conditions, *NDRG1* expression was highest in MCF7 cells, followed by MCF7-LTED then MCF7-TAMR cells (Figure 4.5.3b). These results indicate that *NDRG1* expression is lost in endocrine resistance.

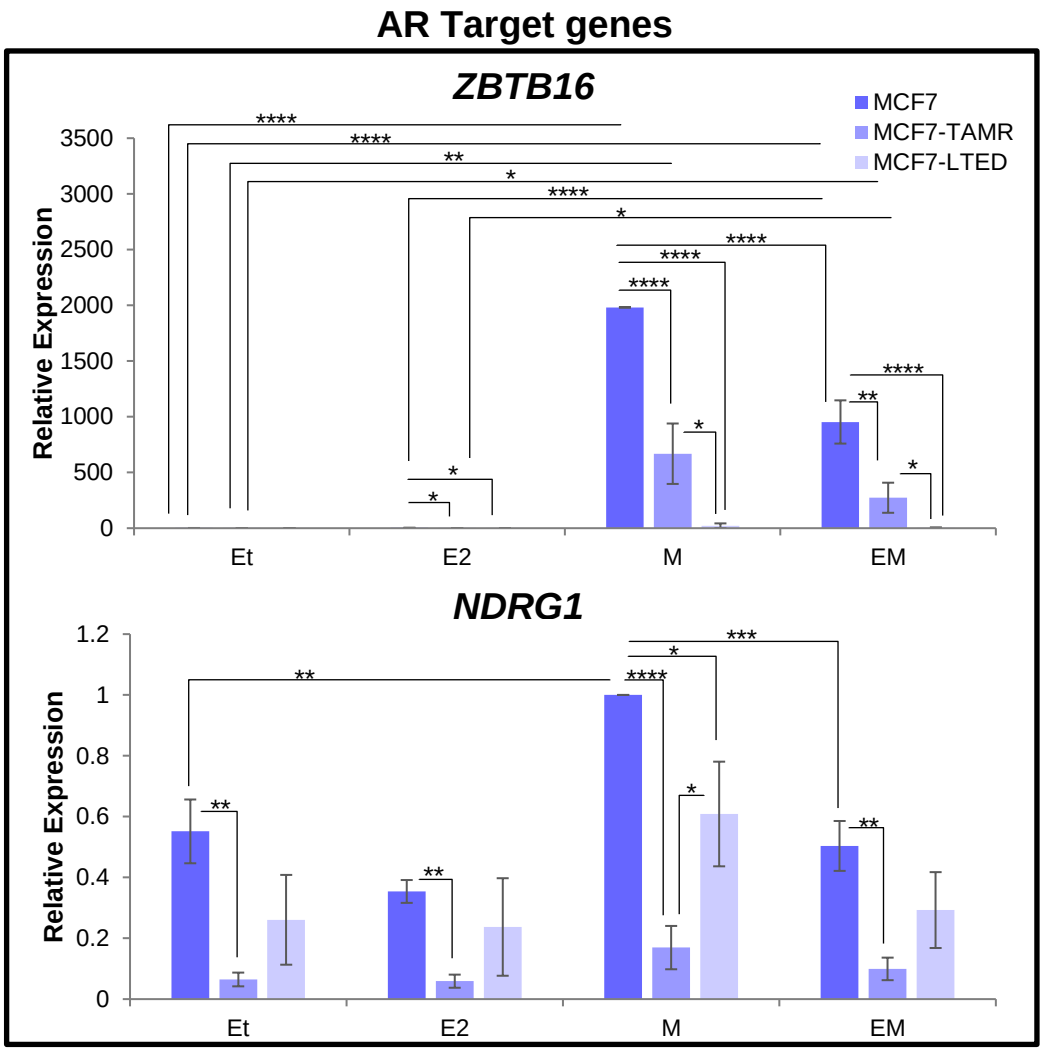


Figure 4.5.2 Direct comparison of expression of AR target genes *ZBTB16* and *NDRG1* in MCF7, MCF7-TAMR and MCF7-LTED cells

MCF7 cells, their Tamoxifen Resistant (TAMR) and Long Term Oestrogen Deprived (LTED) derivatives were incubated in hormone depleted media for 120 hours, and treated with 1 nM of the relevant hormones 17-β-Oestradiol (E2)/ Mibolerone (M)/both/ ethanol (EtOH) as a control for 8 hours. RNA was extracted, reverse transcribed into cDNA and qPCR was conducted using SYBR green to measure the expression level of the Androgen Receptor (AR) target genes **(a)** *Zinc Finger And BTB Domain Containing 16* (*ZBTB16*) and **(b)** *N-Myc Downstream-Regulated Gene 1* (*NDRG1*). Expression was normalised to *RPL19 ribosomal protein*, *L19,* and expression is relative to the MIB-treated MCF7 sample for each gene. Mean of at least 5 independent experiments, ±SE. T-Test *P<0.05, **P<0.005, ***P<0.0005, ****P<0.00005.

By contrast, *DDC* expression was significantly induced by MIB treatment in all cell lines, however this effect was much stronger in MCF7 cells (41.5-fold) than in MCF7-TAMR (3.4-fold) and MCF7-LTED cells (10.7-fold), and co-treatment with E2 reduced this effect, however for MCF7 and MCF7-LTED cells this was not deemed significant (Figure 4.5.3a). However, *DDC* expression was significantly higher with MIB treatment in the resistant cell lines than MCF7 cells, and MCF7-LTED cells had the highest *DDC* expression of the 3 cell lines, upon treatment with EtOH, MIB or E2 + MIB. The elevated expression of this gene in the absence of androgen in the resistant lines indicate that an alternative factor or mechanism could be upregulating *DDC* expression in these cells, and therefore this may be an important gene in these resistant cell lines, in particular in MCF7-LTED cells. Interestingly, in the absence of MIB treatment, *DDC* expression was also significantly upregulated 5.8-fold via E2 treatment in MCF7 cells, but this effect was lost in its resistant derivatives (Figure 4.5.3a). Therefore, this could be additionally an ERα target gene in endocrine sensitive disease, the regulation of which is lost in endocrine resistance, and warrants further investigation.

DIO2 expression was only significantly induced via MIB treatment in MCF7 cells (Figure 4.5.3b). Additionally, MIB-induced *DIO2* expression was significantly higher in MCF7 cells than the other two cell lines (Figure 4.5.3b). This suggests that *DIO2* is an AR target gene upregulated by androgen in endocrine sensitive cells, the regulation of which is lost in endocrine resistance.

Lastly, in MCF7 and MCF7-LTED cells, *SEC14L2* expression was significantly induced via MIB treatment (16.8-fold and 26.8-fold, respectively), and this induction was unaffected by co-treatment with E2, with similar expression values observed for both cell lines under each treatment (Figure 4.5.3c). However, no significant differences were

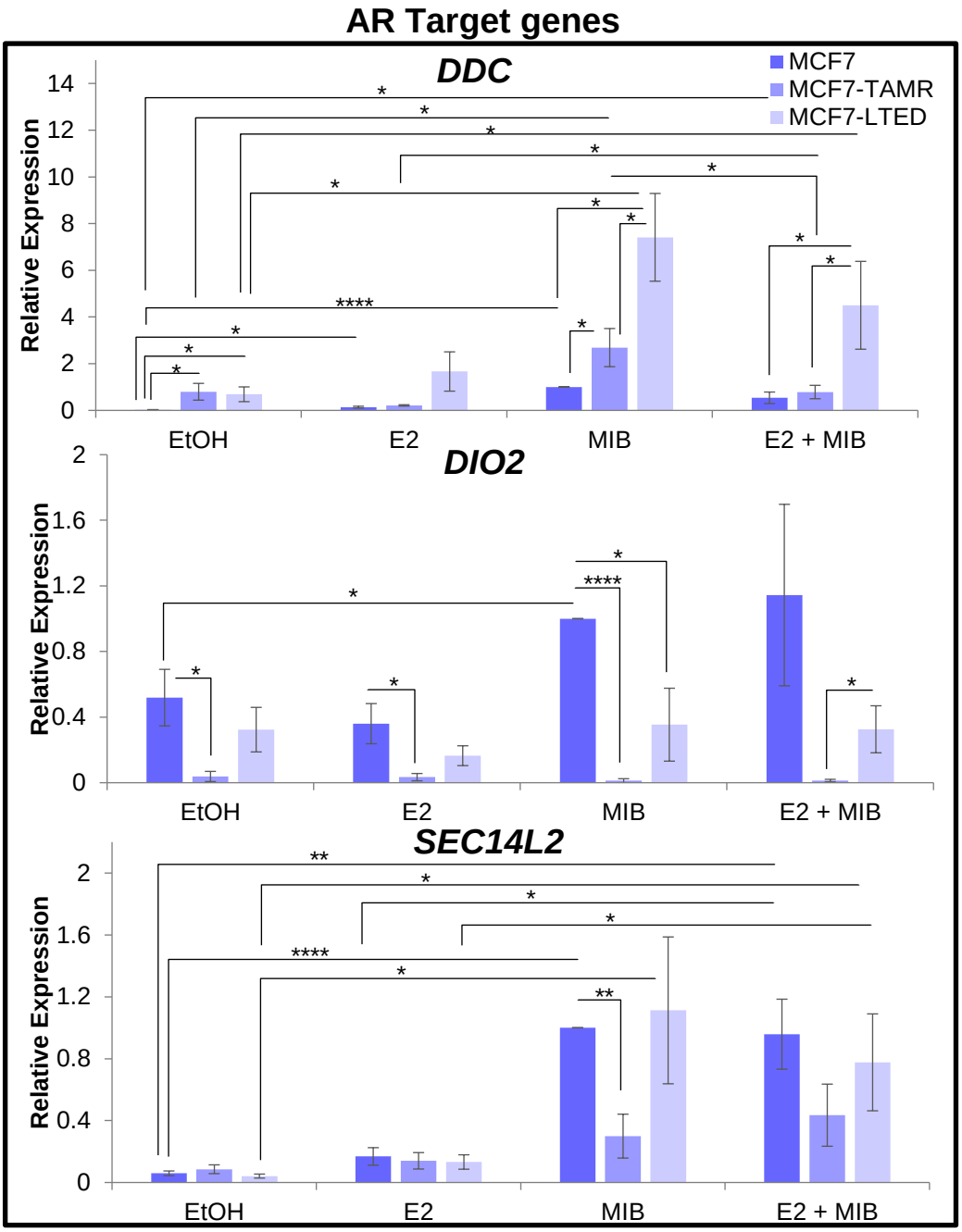


Figure 4.5.3 Direct comparison of expression of AR target genes *DDC*, *DIO2* and *SEC14L2* in in MCF7, MCF7-TAMR and MCF7-LTED cells

MCF7 cells, their Tamoxifen Resistant (TAMR) and Long Term Oestrogen Deprived (LTED) derivatives were incubated in hormone depleted media for 120 hours, and treated with 1 nM of the relevant hormones 17-β-Oestradiol (E2)/ Mibolerone (M)/both/ ethanol (EtOH) as a control for 8 hours. RNA was extracted, reverse transcribed into cDNA and qPCR was conducted using SYBR green to measure the expression level of the Androgen Receptor (AR) target genes **(a)** *Dopa Decarboxylase* (*DDC*) **(b)** *SEC14 Like Lipid Binding 2*, (*SEC14L2*) and **(c)** *Deiodinase*, *Iodothyronine Type II*, (*DIO2*). Expression was normalised to *RPL19 ribosomal protein*, *L19,* and expression is relative to the MIB-treated MCF7 sample for each gene. Mean of 4 independent experiments, ±SE. T-Test *P<0.05, **P<0.005, ***P<0.0005, ****P<0.00005.

observed for *SEC14L2* expression in MCF7-TAMR cells between hormone treatments (Figure 4.5.3c). This indicates that *SEC14L2* is an AR target gene upregulated in MCF7 cells, and that this regulation is slightly upregulated through endocrine resistance in MCF7-LTED cells, however it is lost in the other resistance model MCF7-TAMR.

As observed previously (Figure 4.2.5), in all cells lines the expression of the AR and ERα target *GREB1* was induced via E2 treatment and to a lesser degree in response to MIB, however these differences were not always significant in this experiment (Figure 4.5.2). Interestingly, *GREB1* expression was stronger in MCF7 and MCF7-LTED cells than for MCF7-TAMR cells with all treatments, although these differences were not always significant. This suggests that *GREB1* regulation is lost in the MCF7-TAMR cell line, but its regulation remains in MCF7-LTED cells.

To conclude, the regulation of ERα target genes or a target gene for both ERα and AR is either unaltered or deceased in the endocrine resistant cell lines, suggesting that ERα regulation has become less important for cancer progression in these models of resistance. However, the expression of some of the AR targets, including *ZBTB16* and *DDC*, have become more sensitive to androgen treatment in at least one of the endocrine resistance lines compared to the parental line, demonstrating that AR activity/sensitivity has increased in relation to the regulation of these genes in endocrine resistance. Additionally, ERα inhibition of AR target gene expression can be lost in endocrine resistance. These effects are cell line specific, indicating that the two models of endocrine resistance vary. Additionally, the regulation of some AR target genes does not appear to be important in either model of endocrine resistance, such as *NDRG1* and *DIO2*, indicating that although AR activity increases in these models of resistance, not all AR targets are upregulated.

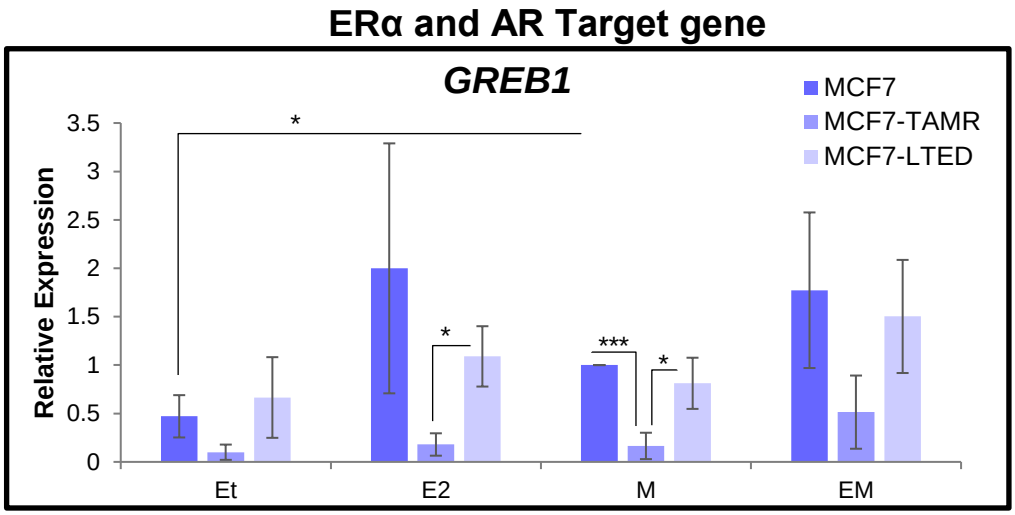


Figure 4.5.4 Direct comparison of expression of an ERα and AR target gene *GREB1* in MCF7, MCF7-TAMR and MCF7-LTED cells

MCF7 cells, their Tamoxifen Resistant (TAMR) and Long Term Oestrogen Deprived (LTED) derivatives were incubated in hormone depleted media for 120 hours, and treated with 1 nM of the relevant hormones 17-β-Oestradiol (E2)/ Mibolerone (M)/both/ ethanol (EtOH) as a control for 8 hours. RNA was extracted, reverse transcribed into cDNA and qPCR was conducted using SYBR green to measure the expression level of the Oestrogen Receptor alpha (ERα) and Androgen Receptor (AR) target gene *Gene Regulated in Breast Cancer 1*, (*GREB1*). Expression was normalised to *RPL19 ribosomal protein*, *L19,* and expression is relative to the MIB-treated MCF7 sample for each gene. Mean of 6 independent experiments, ±SE. T-Test *P<0.05, **P<0.005, ***P<0.0005, ****P<0.00005.

4.6 AR drives endocrine resistant cell growth

Previously conducted colony formation assays (Figure 3.5.2) suggested that androgens may be able to promote endocrine resistance. Therefore, it was of interest as to whether the cell line models of endocrine resistance have lost their oestrogen responsiveness and become more sensitive to androgens for growth. Growth assays were initially conducted to confirm that in the parental MCF7 endocrine sensitive cell line, cellular proliferation was driven through oestrogen and not androgen (Figure 4.6.1a). The results demonstrated that MCF7 cell growth was significantly enhanced by E2 treatment from the lowest concentration tested (0.1 nM), but only the highest concentration of MIB (100 nM) was found to significantly increase its growth (Figure 4.6.1a). However, in the endocrine resistant cell line derivatives of MCF7, a reduced (MCF7-TAMR and MCF7-FULVR) or no (MCF7-LTED) response to E2 treatment was observed, as compared to the parental cells (Figures 4.6.1b-d). Additionally, it was demonstrated that these resistant cells were more sensitive to MIB for growth compared to E2 (Figures 4.6.1b-d). For instance, the proliferation of MCF7-TAMR cells was significantly enhanced from 0.1 nM MIB (Figure 4.6.1b).

To expand upon these results, growth assays were additionally conducted in the T47D endocrine sensitive cell line and its endocrine resistant cell line derivatives T47D-TAMR and T47D-LTED (Figures 4.6.2a-c). T47D cells were more sensitive to MIB for growth than MCF7 cells, however the line was still more sensitive to E2 than MIB (Figure 4.6.2a). Both T47D-TAMR and T47D-LTED cell lines were responsive to E2 for growth, but to a lesser extent compared to T47D cells (Figure 4.6.2b-c). Similar to the MCF7 resistant models, T47D-TAMR were more sensitive to MIB than the parental T47D cells at the 100 nM concentration ($P=0.04$) however its oestrogen and androgen sensitivity

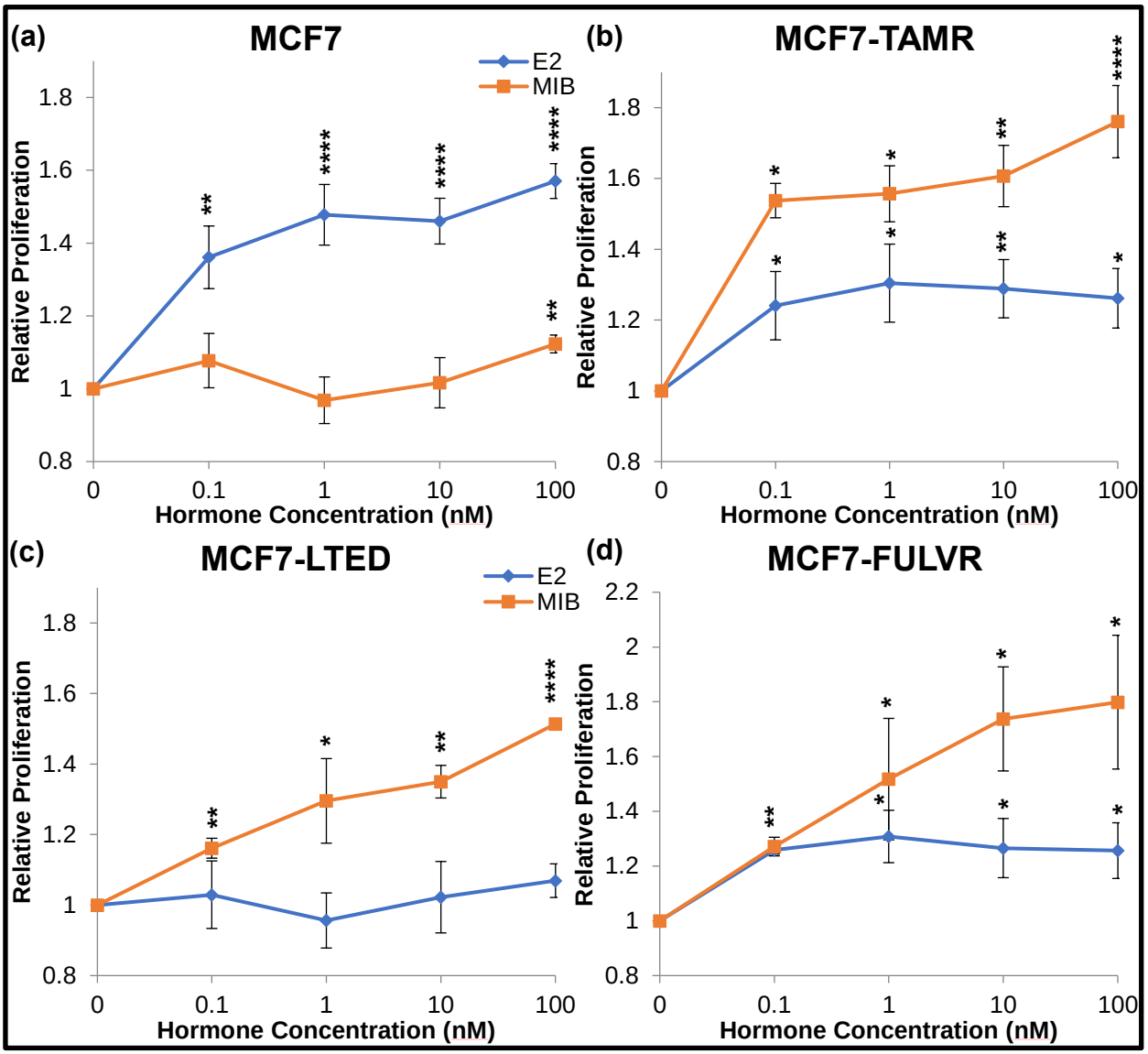


Figure 4.6.1: Endocrine Resistant derivatives of MCF7 cells are more sensitive to androgen than oestrogen for growth

(a) MCF7 cells and their endocrine resistant derivatives **(b)** Tamoxifen Resistant (MCF7-TAMR), **(c)** Long Term Oestrogen Deprived (MCF7-LTED) and **(d)** Fulvestrant Resistant (MCF7-FULVR) cells were treated for 72 hours with different concentrations of the synthetic androgen Mibolerone (MIB) and 17-β-Oestradiol (E2). Proliferation was assessed using WST-1 assays. Mean of at least 3 replicate experiments are displayed, ±SE. T-Test was conducted between 0 nM and the following concentrations, *P<0.05, **P<0.005, ***P<0.0005, ****P<0.00005.

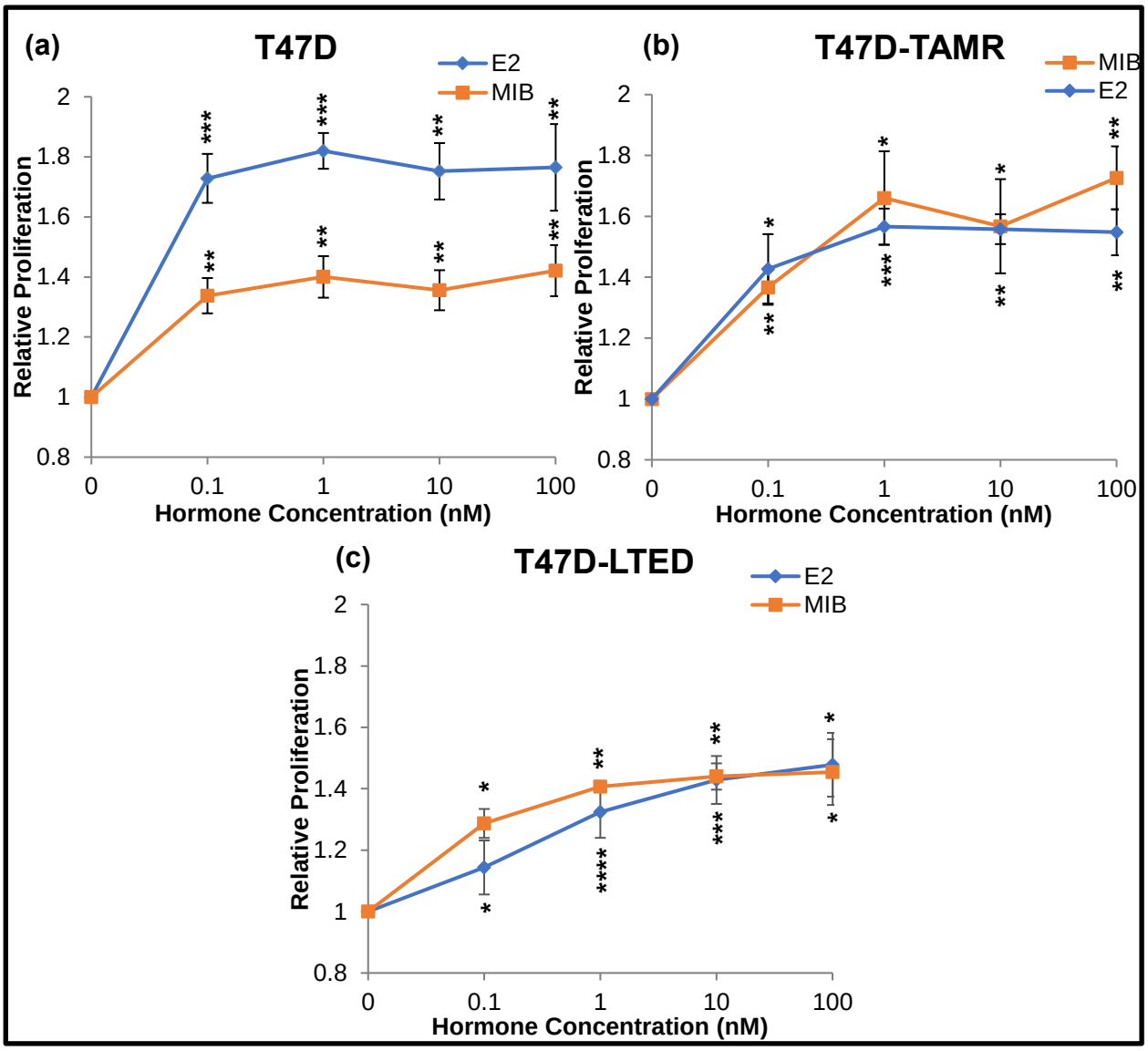


Figure 4.6.2: Endocrine Resistant derivatives of T47D cells have altered sensitivity to androgen and oestrogen for growth

(a) T47D cells and their endocrine resistant derivatives **(b)** Tamoxifen Resistant (T47D-TAMR) and **(c)** Long Term Oestrogen Deprived (T47D-LTED) cells were treated for 72 hours with different concentrations of the synthetic androgen Mibolerone (MIB) and 17-β-Oestradiol (E2). Proliferation was assessed using WST-1 assays. Mean of at least 3 replicate experiments are displayed, ±SE. T-Test was conducted between 0 nM and the following concentrations, *P<0.05, **P<0.005, ***P< 0.0005, ****P<0.00005.

were similar (Figure 4.6.2b). T47D-LTED cells, by contrast, had a similar sensitivity to MIB to the parental line (Figure 4.6.2c). The majority of the endocrine resistant cell lines therefore have partially lost their oestrogen responsiveness, and are instead more sensitive to androgens for growth.

To confirm the role of AR in this androgen-induced growth, *AR* and *ESR1* (ERα) levels were reduced using siRNA by 98.2 % and 80.7 % respectively in MCF7 cells and 100 % and 85.3 % respectively in MCF7-TAMR cells (Figure 4.6.3a). Interestingly, *AR* levels decreased by 51.5 % (MCF7) and 86.1 % (MCF7-TAMR) with ERα knockdown (Figure 4.6.3a). Proliferation assays were subsequently conducted following MIB and E2 treatment (Figures 4.6.3b-d). In MCF7 cells, growth was weakly increased by E2 and MIB treatment (not significant, Figure 4.6.3b, c). ERα knockdown resulted in a decrease in MCF7 cell growth regardless of hormone treatment (Figure 4.6.3b), although this effect was not significant. These results are in accordance with proliferation assays conducted previously, where it was demonstrated that inhibiting ERα activity with anti-oestrogens did not cause a significant induction of androgen-stimulated growth (Figure 3.5.1). In MCF7-TAMR cells, growth was significantly stimulated by MIB and this effect was abrogated following *AR* knockdown (Figure 4.6.3d), indicating that androgen-induced growth is dependent on the AR in this cell line. These results suggest that androgens are driving growth in many cases of endocrine resistance through the action of AR, and thereby anti-androgen treatment in these cases could be of benefit. It should be noted that the ligand-responsiveness of the cells in this experiment was weaker than for other experiments, suggesting that the siRNA transfection protocol was partially inhibitory to growth.

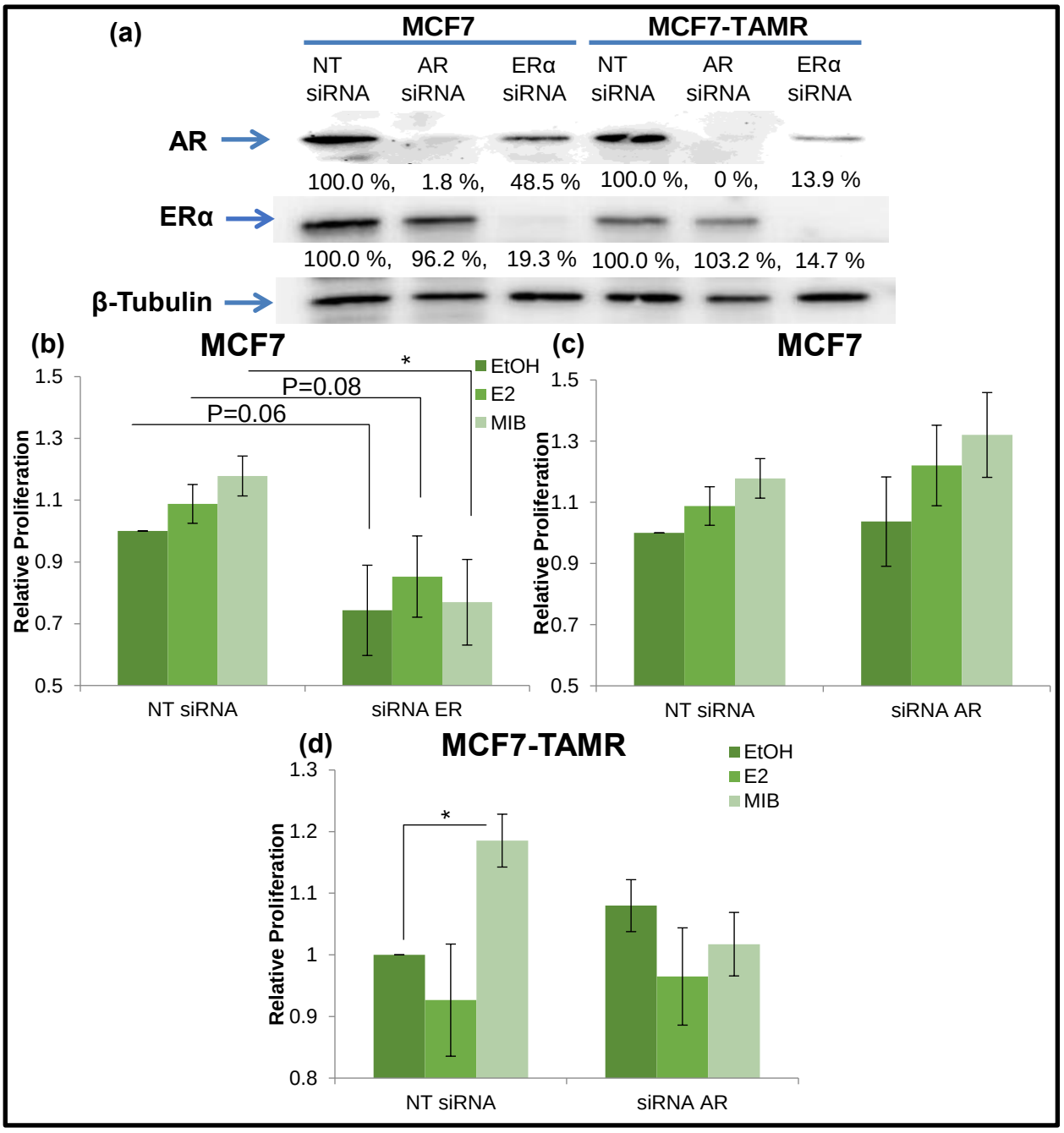


Figure 4.6.3 ERα knockdown abrogates MCF7 cell growth, and AR knockdown abrogates androgen-stimulated growth in MCF7-TAMR cells

Androgen Receptor (AR) or Oestrogen Receptor alpha (ERα) levels were depleted using siRNA in MCF7 or MCF7-Tamoxifen Resistant (TAMR) cells alongside a Non-Targeting (NT) siRNA control for 72 hours. **(a)** Cells were collected for Western blotting analysis. The percentage expression of ERα or AR following knockdown as compared to the NT siRNA control samples were analysed using Fusion X Software with β-Tubulin as a loading control. MCF7 results were additionally shown in Figure 3.2.2. **(b-d)** Cells were treated for a further 72 hours following transfection, with ethanol (EtOH) vehicle, 1 nM Mibolerone (MIB) or 1 nM 17-β-Oestradiol (E2). Proliferation was assessed using WST-1 assays for **(b)** MCF7 cells following ERα knockdown and **(c)** AR knockdown, and **(d)** MCF7-TAMR cells following AR knockdown. Mean of at least 3 experiments are displayed, ±SE. T-Test *P<0.05.

4.6.1 Anti-androgen treatment can successfully inhibit the proliferation of models of endocrine resistance

Following the knockdown growth assays, it was of interest to investigate whether targeting AR through therapeutics could be used to inhibit androgen-induced growth in endocrine resistant cells. Therefore, further proliferation assays were conducted on MCF7 and T47D cells, and their TAMR and LTED derivatives following treatment with 1 nM MIB +/- the anti-androgens BIC and Hydroxyflutamide (OHF) (Figures 4.6.4-5).

In MCF7 cells, as also demonstrated previously (Figure 4.6.1a), this concentration of MIB (1nM) did not stimulate cellular proliferation, however BIC also significantly reduced cell growth in this cell line as compared to the ethanol (EtOH) treated samples (Figure 4.6.4a). In MCF7-TAMR and MCF7-LTED cells, as also demonstrated previously, MIB significantly induced cellular proliferation, and this effect was reduced via anti-androgen treatment, although OHF in MCF7-TAMR cells did not reach significance (P=0.054) (Figures 4.6.4b-c). BIC treatment additionally resulted in a significant reduction in cell growth in the absence of MIB in both MCF7-TAMR and MCF7-LTED cell lines, however OHF simulated MCF7-TAMR proliferation in the absence of MIB (Figures 4.6.4b-c).

MIB stimulated growth in T47D cells and its T47D-TAMR and T47D-LTED derivatives (Figures 4.6.5a-c), as seen previously (Figures 4.6.2a-c). Treatment with both anti-androgens reduced this androgen-stimulated growth, including in the endocrine sensitive cells, however this effect did not reach significance for OHF treatment in T47D-TAMR cells (P=0.063) (Figures 4.6.5a-c). Interestingly, OHF simulated T47D-LTED proliferation in the absence of MIB (Figure 4.6.5c) as seen in the MCF7-TAMR cell line

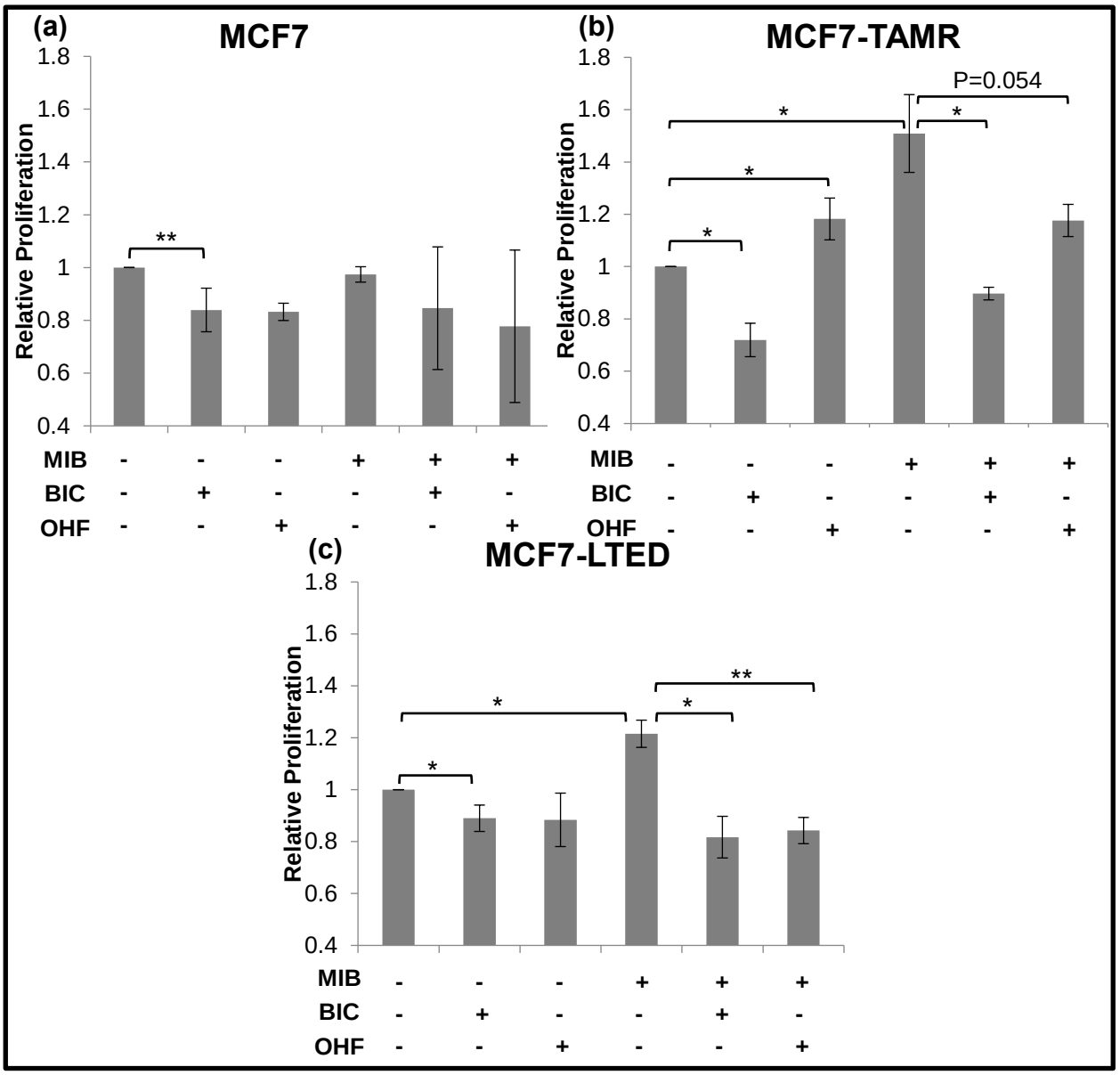


Figure 4.6.4 Androgen-stimulated growth in endocrine resistant derivatives of MCF7 cells is inhibited by treatment with anti-androgens

(a) MCF7, **(b)** MCF7-Tamoxifen Resistant (TAMR) and **(c)** MCF7-Long Term Oestrogen Deprived (LTED) cells were treated for 72 hours with 1 nM Mibolerone (MIB) or Ethanol (EtOH) as a control, 10 μM anti-androgen Bicalutamide (BIC) / Hydroxyflutamide (OHF) in hormone-depleted media. Proliferation was assessed using WST-1 assays. Mean of three replicate experiments are displayed, ±SE. T-Test *P<0.05, **P<0.005.

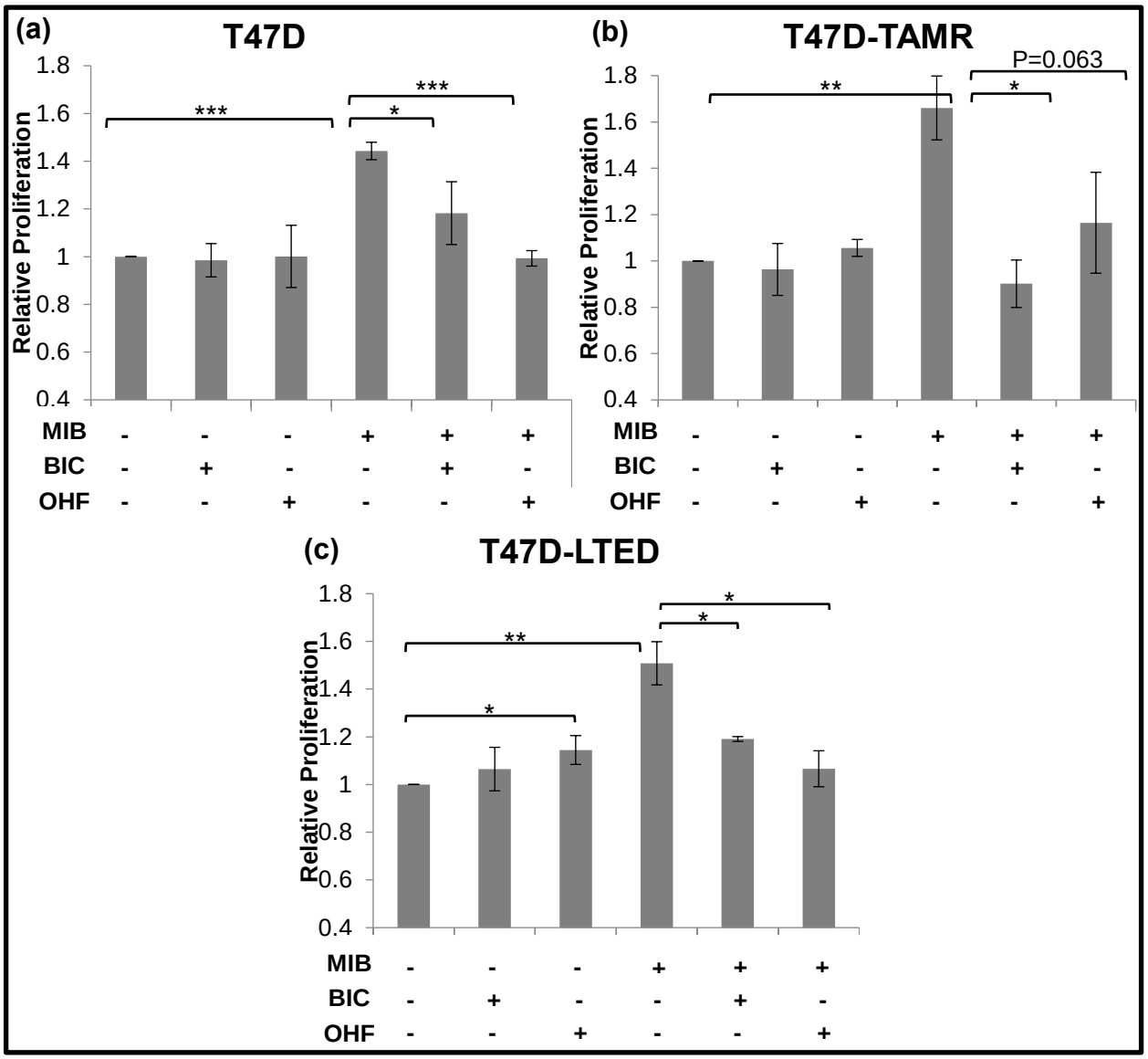


Figure 4.6.5 Androgen-stimulated growth in T47D cells and its endocrine resistant derivatives is inhibited by treatment with anti-androgens

(a) T47D, **(b)** T47D-Tamoxifen Resistant (TAMR) and **(c)** T47D-Long Term Oestrogen Deprived (LTED) cells were treated for 72 hours with 1 nM Mibolerone (MIB) or Ethanol (EtOH) as a control, 10 µM anti-androgen Bicalutamide (BIC) / Hydroxyflutamide (OHF) in hormone-depleted media. Proliferation was assessed using WST-1 assays. Mean of three replicate experiments are displayed, ±SE. T-Test *P<0.05, **P<0.005.

(Figure 4.6.1.1b). These results suggest that anti-androgen treatment could be beneficial in tumours for both endocrine sensitive and endocrine resistant disease that are thought to be showing androgen-induced growth.

4.7 The effect of anti-oestrogen and anti-androgen treatments upon AR and ERα expression is altered in endocrine resistance

It has been demonstrated that the response of AR and ERα target gene expression to hormone and anti-oestrogen treatments is different in endocrine resistant cells as compared to endocrine sensitive cells (Section 4.2), and that anti-androgen treatment can inhibit endocrine resistant cell growth (Section 4.6.1). Therefore, it was investigated how anti-oestrogen and anti-androgen treatments affect ligand-induced changes to AR and ERα expression in MCF7 cells and its MCF7-TAMR and MCF7-LTED derivatives (Figures 4.7.1a-c).

In MCF7, AR levels stabilised in response to MIB and treatment with anti-androgens (OHF and BIC) abrogated this effect. Interestingly, in the absence of MIB treatment, the anti-oestrogen TAM decreased AR levels, yet the alternative anti-oestrogen FULV produced a slight increase in AR levels (Figure 4.7.1a). Interestingly, co-treatment with FULV increased MIB-induced AR levels 3.2-fold, however co-treatment of MIB with TAM resulted in little variation in AR levels (Figure 4.7.1a). ERα levels, on the other hand, decreased following both MIB or anti-androgen treatment, yet the combination of MIB with anti-androgens slightly abrogated (BIC) or cancelled out this effect (OHF) (Figure 4.7.1a). TAM treatment increased ERα expression 2.4-fold, whereas FULV decreased its expression, and the addition of MIB reduced both of these effects (Figure 4.7.1a).

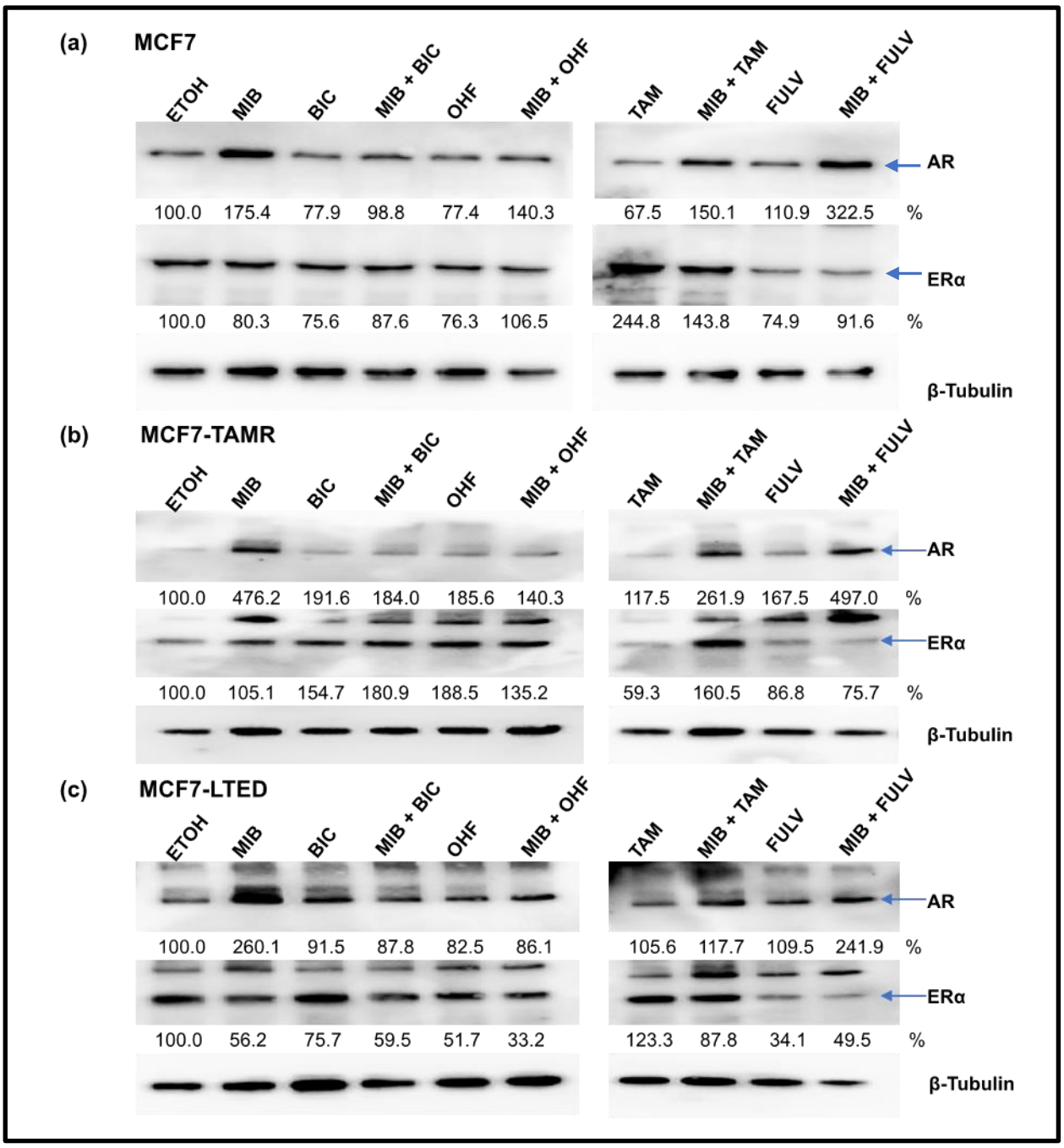


Figure 4.7.1 AR and ERα expression in MCF7 cells and its endocrine resistant derivatives, following different hormone and drug treatments

(a) MCF7 cells and their **(b)** Tamoxifen Resistant (TAMR) and **(c)** Long Term Oestrogen Deprived (LTED) derivatives were grown in hormone depleted media for 72 hours, then treated for 24 hours with different combinations of 1 nM Mibolerone (MIB) or vehicle (Ethanol, EtOH) and 1 μM anti-androgens Hydroxyflutmatide (OHF) or Bicalutamide (BIC), or 100 nM anti-oestrogens Tamoxifen (TAM) and Fulvestrant (FULV). Cells were lysed and immunoblotting conducted to examine androgen receptor (AR) and oestrogen receptor α (ERα) expression, with β-Actin included as a loading control. Densitometry analysis was conducted using the Fusion FX software to identify the percentage of AR and ERα expression as compared to vehicle.

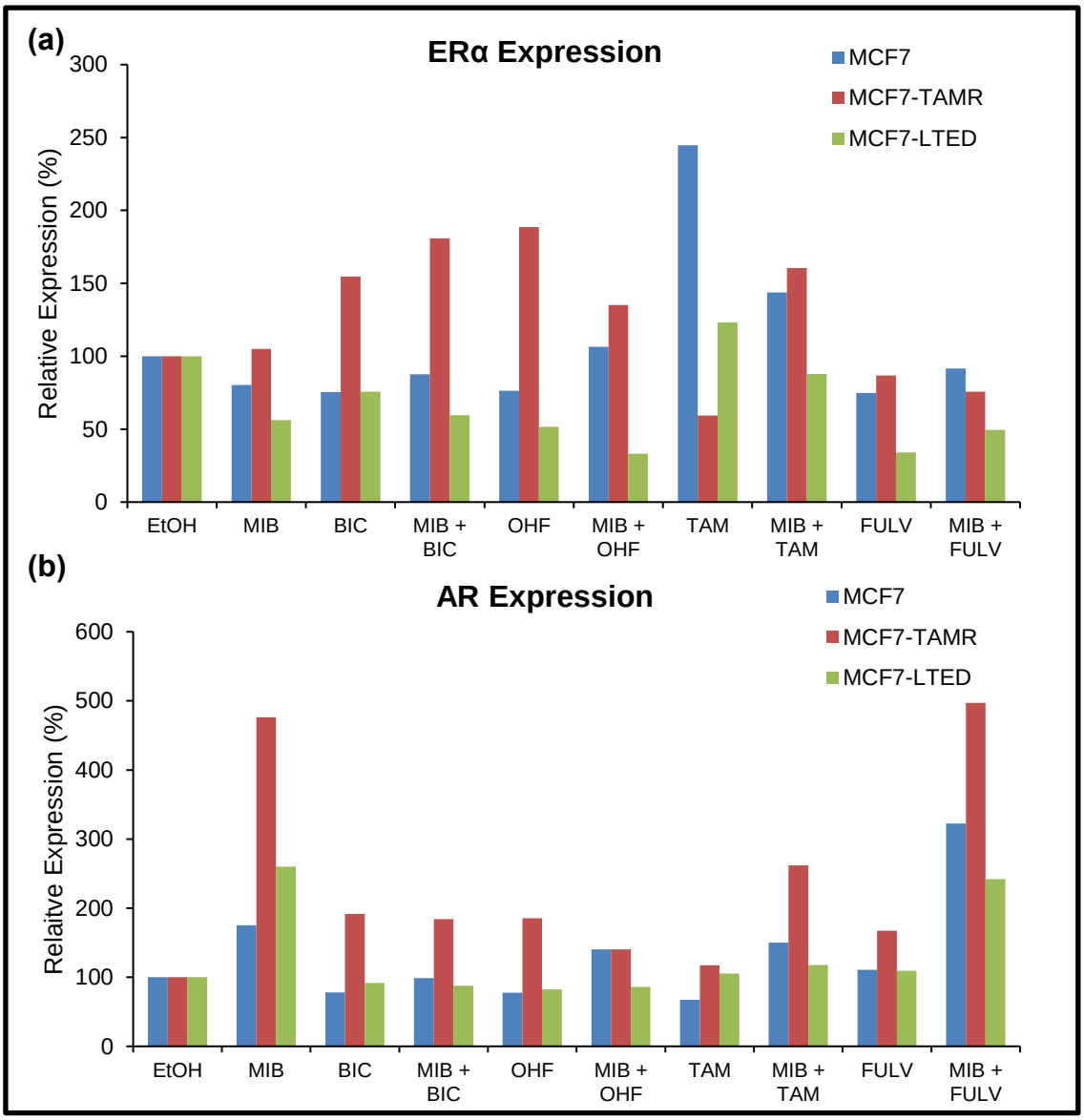


Figure 4.7.2 AR and ERα expression in MCF7 cells and its endocrine resistant derivatives, following different hormone and drug treatments

MCF7 cells and their Tamoxifen Resistant (TAMR) and Long Term Oestrogen Deprived (LTED) derivatives were grown in hormone depleted media for 72 hours, then treated for 24 hours with different combinations of 1 nM Mibolerone (MIB) or vehicle (Ethanol, EtOH) and 1 μM anti-androgens Hydroxyflutmatide (OHF) or Bicalutamide (BIC), or 100 nM anti-oestrogens Tamoxifen (TAM) and Fulvestrant (FULV). Cells were lysed and immunoblotting conducted to examine Androgen receptor (AR) and Oestrogen receptor α (ERα) expression, with β-Actin included as a loading control. Densitometry analysis was conducted using the Fusion FX software to identify the percentage of **(a)** ERα or **(b)** AR expression as compared to vehicle for each cell line.

In accordance with the endocrine sensitive cells, AR expression stabilised following MIB treatment in the endocrine resistant lines, however to a larger extent of 4.8-fold in MCF7-TAMR and 2.6-fold in MCF7-LTED as compared to 1.7-fold in the parental MCF7 line (Figures 4.7.1a-c). As seen in the endocrine sensitive cells, this effect was abrogated via anti-androgen treatment in MCF7-LTED cells, and in the absence of MIB both OHF and BIC reduced ligand-independent AR expression (Figure 4.7.1c). In MCF7-TAMR cells, treatment with anti-androgens also reduced MIB-induced AR levels, however in the absence of ligand anti-androgen treatment resulted in a 1.9-fold increase in AR expression for both OHF and BIC (Figure 4.7.1b). As demonstrated in MCF7 cells, TAM reduced the MIB increase in AR levels for MCF7-TAMR or MCF7-LTED (Figures 4.7.1a-c). In contrast, FULV had little effect upon MIB-induced AR levels in MCF7-TAMR and MCF7-LTED (Figures 4.7.1b-c).

ERα expression, consistent with MCF7 cells, decreased following both MIB or anti-androgen treatment in MCF7-LTED cells, however unlike in MCF7 cells where the combination of MIB with anti-androgen treatment appeared to abrogate this effect, it enhanced this effect in MCF7-LTED cells (Figure 4.7.1c). Alternatively, in the MCF7-TAMR line, ERα expression remained fairly constant following MIB treatment (105.1 %) and interestingly increased with anti-androgen treatment regardless of the presence of MIB (Figure 4.7.1b). As demonstrated in MCF7 cells, FULV treatment in both MCF7-TAMR and MCF7-LTED cells reduced ERα expression, however this was not abrogated via MIB treatment (Figure 4.2.2b-c). Similar to MCF7 cells, in MCF7-LTED cells TAM treatment resulted in a slight increase in ERα expression and this was not affected by MIB treatment (Figure 4.2.2c). Alternatively, in MCF7-TAMR cells TAM treatment reduced

ERα expression, but upon treatment with both MIB and TAM ERα expression increased 1.6-fold (Figure 4.2.2b).

In conclusion, AR expression was more responsive to MIB in the endocrine resistant cells than the endocrine sensitive cells, suggesting that the AR has an increased sensitivity to androgen in therapy resistance. Treatment with either anti-androgen or the anti-oestrogen TAM could at least partially abrogate this ligand-induced effect in all 3 cell lines. This indicates that anti-androgen treatment could be beneficial in both endocrine sensitive and endocrine resistant BCa. However, when ERα expression was decreased through FULV treatment in MCF7 cells, AR levels were enhanced, which could contribute to cancer progression. In MCF7 and MCF7-LTED cells, androgen or anti-androgen treatment decreased ERα expression, which suggests that AR signalling can also affect ERα expression. However, in MCF7-TAMR cells, ERα levels remained fairly constant following MIB treatment but increased with anti-androgen treatment. Previous data obtained from siRNA knockdown analysis demonstrated that a decrease in ERα levels also results in a decrease in AR levels (Figure 4.6.3a), however in this experiment a reduction in ERα levels through treatment does not appear to have a significant impact on AR expression in any of the cell lines.

4.8 AR and ERα display altered DNA binding in endocrine resistance

It has been previously demonstrated that AR binding to DNA is disrupted by E2 signalling in MCF7 cells (Figures 3.5.6c-d). To see if this was the case in endocrine resistance, ChIP was used to investigate ERα and AR binding in MCF7-TAMR cells. Cells were treated with EtOH as a control, 1 nM E2, 1 nM MIB or both (E2 + MIB) and crosslinked with formaldehyde prior to DNA shearing via sonication to approximately 200 bp. ChIP assays were subsequently conducted to pull down AR or ERα. qPCR analysis was conducted to amplify a known ERE in the promoter region of the *TFF1* gene 340-354 bp upstream (TFF1 +ve) and a negative control region 685-520 bp upstream (TFF1 –ve) for ERα pull down (Figure 4.8.1a) or a known ARE located between exons 3 and 4 of the *ZBTB16* gene (ZBT +ve) and its relative negative control region 2,443 bp upstream (ZBT -ve) for AR pull down (Figure 4.8.1b).

The results demonstrated that AR was recruited in a ligand-dependent manner to the ARE (Figure 4.8.1a) (as previously seen in MCF7 cells, Figures 3.5.6c-d). Co-treatment with E2 decreased AR recruitment to this site (Figure 4.8.1a). It was also demonstrated that ERα appeared to be constitutively bound to the ERE (Figure 4.8.1b), which ERα was recruited to in a ligand-dependent manner in MCF7 cells (Figure 3.2.4b). Therefore, TAM resistance has impacted the recruitment of ERα and AR to this gene. ChIP-Seq will be conducted on these samples in future work to provide more insight into where in the genome ERα and AR bind in these TAM resistant cells.

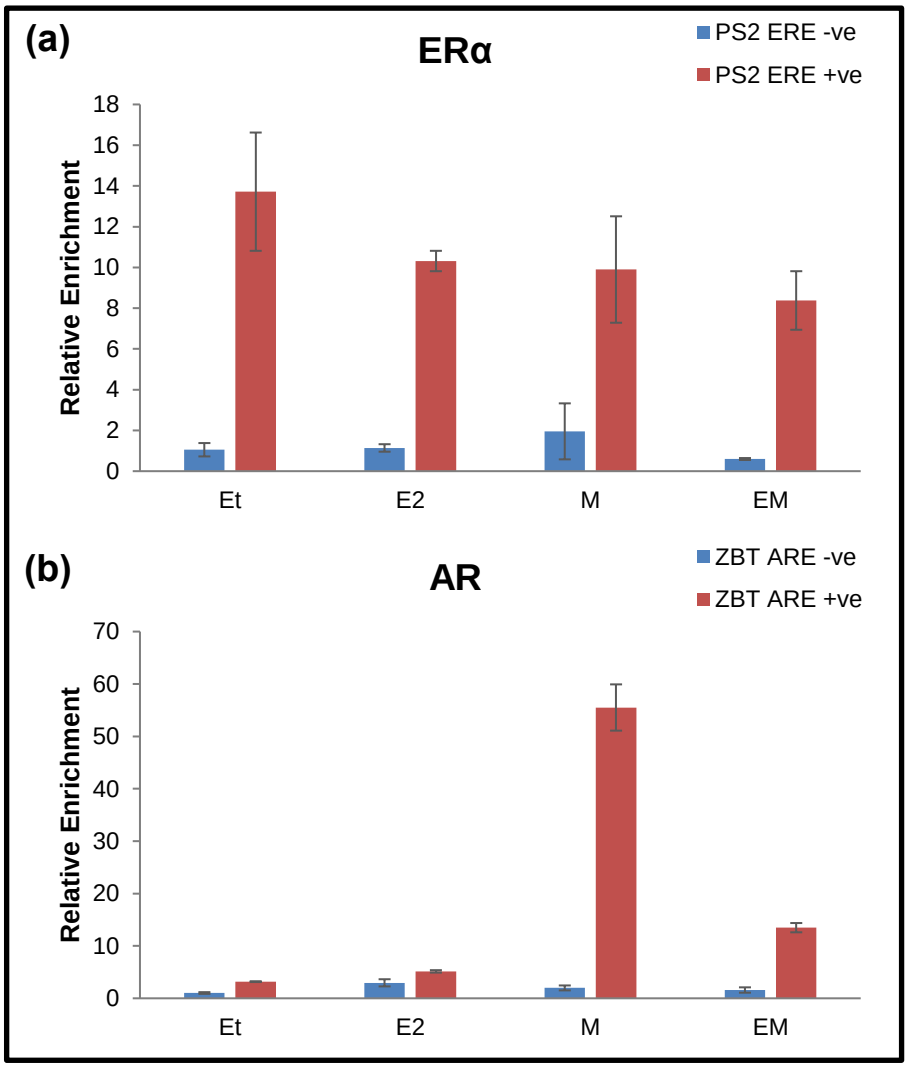


Figure 4.8.1 Investigation of ERα and AR recruitment in MCF7-TAMR cells

Tamoxifen Resistant (TAMR) derivative of MCF7 cells were treated with ethanol (EtOH), 1 nM 17-β-Oestradiol (E2), 1 nM Mibolerone (MIB) or both (E2 + MIB) and crosslinked with formaldehyde prior to DNA shearing via sonication to approximately 200 bp. Chromatin Immunopreceipitation (ChIP) assays were then conducted, to pull down **(a)** Androgen Receptor (AR) or **(b)** Oestrogen Recptor alpha (ERα). Subsequent qPCR analysis was conducted to amplify a known ERE in the promoter region approximately 400 bp upstream of *Trefoil Factor 1*, *TFF1*, (TFF1 +ve) and a negative control region approximately 2 kb upstream (TFF1 –ve) or a known androgen response element (ARE) upstream of the *Zinc Finger And BTB Domain Containing 16* (*ZBTB16*) gene (ZBT +ve) and its relative negative control region (ZBT -ve). A representative example from two replicate experiments are displayed, ±SE.

4.9 Discussion

4.9.1 AR and ERα expression are generally correlated in endocrine resistance

It has been previously demonstrated that ERα and AR expression is often correlated in BCa (Table 3.2.1 and Figure 3.2.1) as also reported by others (Vera-Badillo *et al.*, 2014, Collins *et al.*, 2011, Lin *et al.*, 2015). Therefore, immunoblotting was conducted to investigate AR and ERα expression in our cell line models of endocrine sensitive and resistant disease (Figure 4.2.1). The results demonstrated here that although there are differences in expression between the cell lines, AR and ERα expression was observed to be generally correlated across all the cell lines (Figure 4.2.1b). This suggests that in endocrine resistance the receptors could both be regulated by a common factor, or that one receptor regulates the expression of the other. However, in contrast to this it was later demonstrated that a reduction in ERα levels through treatment does not appear to have a significant impact on AR expression in MCF7, MCF7-TAMR or MCF7-LTED cells (Figure 4.7.1).

Previously, it was indicated that ERα regulates AR in endocrine sensitive disease (Section 3.6.1). It was demonstrated that ERα knockdown resulted in a reduction in AR expression in endocrine sensitive cells (Figure 3.2.2) and experiments suggested that ERα could regulate AR expression through an ERE on the *AR* gene ($ERE_{+151,438-726}$, Figure 3.2.5), and that ERα was recruited ligand-dependently to an alternative ERE upstream of *AR* ($ERE-_{19,247-18,610}$, Figure 3.2.4). Interestingly, when ERα levels were depleted in MCF7-TAMR cells, AR expression decreased by 86.1 %, an even greater reduction than the 51.5 % observed in MCF7 cells (Figure 4.6.3a). This demonstrated that in this mechanism of TAM resistance, ERα is still regulating AR expression.

Therefore, if AR is driving this form of endocrine resistance, the use of an alternative endocrine therapy to target the ERα and degrade its expression, such as FULV, could be beneficial. This is in line with work published by others, as it has been described that in the majority of TAM resistance cases ERα expression is maintained, and some patients have responded to alternative endocrine therapies, such as FULV (Osborne, 1998, Howell *et al.*, 1995, Howell *et al.*, 1996, Schiff *et al.*, 2003).

4.9.1.1 ERα and AR colocalise in models of endocrine resistance

The subcellular localisation of AR and ERα was initially investigated via transfecting COS-1 cells with ERα-RFP and AR-GFP (Figure 4.4.1). In the absence of hormone, AR was predominantly cytoplasmic and ERα nuclear. These results are in line with IHC conducted previously by another group that demonstrated that ERα is more commonly localised in the nucleus than the cytoplasm in BCa tumours (Li *et al.*, 2015a) and that prior to ligand binding AR is often cytoplasmic in PCa cells (Leung and Sadar, 2017, Brooke, 2005). However, upon oestrogen or androgen treatment the receptors became more colocalised in the nucleus (Figure 4.4.1), as these receptors are both ligand-dependant transcription factors that exert their effects in the nucleus (Sever and Glass, 2013). Subsequent investigation of endogenous AR and ERα in the endocrine sensitive cell lines (MCF7 and T47D) and their TAMR and LTED derivatives, demonstrated that AR and ERα localisation varies between the endocrine resistant models and the parental lines, however the receptors are commonly found to colocalise with hormone treatments (Figures 4.4.2-7). For instance, unlike in the other cell lines, in T47D-TAMR cells both AR and ERα were predominantly cytoplasmic upon treatment with MIB, in the presence or absence of E2 (Figure 4.4.6). This result could indicate an usual mechanism of regulation in this cell line, such as non-genomic AR signalling, which has

been demonstrated by others to occur in advanced, therapy-resistant PCa cell line models (Liao *et al.*, 2013, Leung and Sadar, 2017). ChIP assays in future work could therefore indicate whether AR is binding to DNA in this cell line.

4.9.2 Endocrine resistant cells have enhanced sensitivity to androgen and increased AR activity

Our resulted indicate that the resistant cell lines have an enhanced sensitivity to androgen. AR expression was stabilised via MIB treatment more strongly in MCF7-TAMR (4.8-fold) and MCF7-LTED cells (2.6-fold) than their parental line (1.7-fold) (Figures 4.7.1a-c), and the endocrine resistant lines have an increased sensitivity to MIB for growth (with the exception of T47D-LTED) (Figures 4.6.1-2). The role of AR in facilitating endocrine resistance has been investigated by others (De Amicis *et al*., 2010, Rechoum et al., 2014, Ciupek *et al*., 2015). For instance, an elevated AR expression has been associated with TAM resistant tumours and the exogenous overexpression of AR has been demonstrated to promote TAM resistance *in vitro* (De Amicis *et al*., 2010). Additionally, an increase in the AR activator PSAP has been demonstrated to promote AI resistance through promoting AR recruitment to HREs (Ali *et al.*, 2015). Therefore, there could be an increased importance of androgens and the AR in endocrine resistance.

4.9.2.1 AR and ERα signalling is altered in endocrine resistance

The regulation of ERα target genes *TFF1* and *MYC* (Table 3.5.1) was investigated in MCF7 cells and their endocrine resistant derivatives MCF7-LTED and MCF7-TAMR. *TFF1* was significantly induced via E2 treatment in MCF7 cells, however this induction was decreased in both models of endocrine resistance and hence not always significant

(Figures 4.3.1 and 4.5.1a). Additionally, it was demonstrated that total *TFF1* levels were higher in MCF7 cells than the resistant lines, in particular MCF7-TAMR cells (Figure 4.5.1a). ChIP assays additionally demonstrated that ERα recruitment to a known ERE in the promoter region of the *TFF1* gene was significantly induced via E2 treatment in MCF7 cells (Figure 3.2.4b), however in MCF7-TAMR cells ERα appeared to be constitutively bound to this ERE and was unaffected by E2 treatment (Figure 4.8.1b). So, TAM resistance has impacted ERα recruitment to this gene. In accordance with these results, E2 treatment significantly increased *MYC* expression in MCF7 cells, but not in the resistant lines (Figures 4.3.2a-c and 4.5.1b). Taken together, these results suggest that some ERα target genes lose oestrogen responsiveness in endocrine resistance.

As expected, E2-induced expression of *MYC* and *TFF1* was inhibited via both anti-oestrogens in MCF7 cells (Figures 4.3.1a and 4.3.2a). It was further demonstrated that FULV treatment could also significantly reduce *MYC* expression in both MCF7-TAMR and MCF7-LTED cells (Figures 4.3.2b-c) and *TFF1* expression in MCF7-TAMR cells (Figure 4.3.1b). These results demonstrate that ERα has remained at least partially active in these cells, and therefore treatment with an alternative anti-oestrogen such as FULV, which inhibits ERα activity via an alternative mechanism of action to TAM, could still be of benefit in these resistance models. This is in accordance with work conducted by others, where it has been demonstrated that ERα expression is commonly maintained in TAM resistance and that some patients have responded to alternative endocrine therapies, including FULV (Osborne, 1998, Howell *et al.*, 1995, Howell *et al.*, 1996, Schiff *et al.*, 2003). However, it is surprising that TAM was still able to significantly inhibit *TFF1* expression in MCF7-TAMR cells (Figure 4.3.1b). In order to see if this result is true for

more E2-regulated genes, global gene analysis for instance through RNA-Sequencing (RNA-Seq), is required.

GREB1 is both an AR and ERα target gene (Table 3.5.1), however in all cells lines the expression *GREB1* was induced via E2 treatment and to a lesser degree by MIB (Figures 4.3.5 and 4.5.2). In all 3 cell lines, treatment with anti-oestrogens was able to significantly reduce E2-induced *GREB1* expression, and MIB was only able to partially abrogate this effect in MCF7-TAMR cells (Figures 4.3.5a-c). This suggests that genes where it appears that AR and ERα are not competing are inhibited by anti-oestrogen treatment.

Interestingly, the patterns of expression observed for 5 AR target genes varied greatly between the genes and the cell lines. Two AR target genes become more sensitive to androgen treatment in one model of endocrine resistance but not the other (*ZBTB16* in MCF7-TAMR and *SEC14L2* in MCF7-LTED); one gene demonstrated a smaller induction from androgen treatment in the endocrine resistant cells than the endocrine sensitive cells, but increased total levels of expression in endocrine resistance (*DDC*); one gene demonstrated a decreased expression and responsiveness to androgen in endocrine resistance (*DIO2*)*;* and the expression of another gene appeared to be unaffected in endocrine resistance (*NDRG1*) (Figures 4.3.3-4 and 4.5.3-4).

It appears that the endocrine resistant cells have a unique androgen signalling pathway to endocrine sensitive cells, differing to that observed in PCa (Farmer *et al.*, 2005, Need *et al.*, 2012). *DDC* is an androgen regulated gene that has been identified as an important AR co-activator in PCa (Margiotti et al., 2007, Wafa et al., 2003); *SEC14L2* is as an androgen-regulated gene associated involved in PCa cellular proliferation (Bolton

et al., 2007, Chan et al., 2016, Ni et al., 2005); *DIO2* has been reported to be rapidly upregulated via DHT treatment in PCa (Xu et al., 2006); *ZBTB16* has been demonstrated as an AR target gene in PCa cells (van de Wijngaart et al., 2009); and *NDRG1* is an AR target gene identified to be positively regulated by androgen treatment in PCa cells (Masuda *et al.*, 2005, Nelson *et al.*, 2002, Ngan *et al.*, 2009). However, it was not observed in all endocrine resistant lines that the expression of these genes became more sensitive in response to androgen, and the total expression levels of the genes did not increase in all endocrine resistant models. MABC (ERα-negative, AR positive) tumours have been demonstrated to have increased androgen-signalling with a distinct gene expression profile (Farmer *et al.*, 2005). Therefore it was expected that the regulation of AR targets would not follow patterns demonstrated in PCa, and it was hypothesised that androgen signalling in endocrine resistance could appear more like that observed in MABC.

ERα activity, however, could influence the results for *DIO2* and *DDC* target gene analysis. *DIO2* has been demonstrated to function in the regulation of TR activity through the production of the more potent thyroid hormone triiodothyronine from thyroxine, the process of which is upregulated via E2 (Detti *et al.*, 2013, Xu *et al.*, 2006). Therefore there is a possibility that this gene is also regulated by ERα. Additionally, in the absence of MIB treatment, *DDC* expression was also significantly increased via E2 treatment in MCF7 cells, but this effect was lost in its resistant derivatives (Figure 4.5.4a). Therefore, this gene could be additionally an ERα target gene in endocrine sensitive disease, or part of the mechanism via which ERα is regulating AR could be through the regulation of *DDC*, the effect of which is lost in endocrine resistance. These results would therefore be worth exploring further.

Alternatively, lack of an increase in androgen induction of *DDC* in the resistant lines as compared to MCF7 cells was because the total *DDC* levels were significantly higher in the absence of hormone treatments in the resistant cell lines than MCF7 cells (Figure 4.5.4a). These results indicate that *DDC* expression could be being additionally regulated by an alternative factor or mechanism in these cells. DDC has been identified by others as an important AR co-activator in PCa (Margiotti *et al.*, 2007, Wafa *et al.*, 2003), therefore an alternative factor could be increasing its expression in order to promote AR activity in endocrine resistance, which warrants further investigation.

Interestingly, it also appears that AR signalling in endocrine resistance may differ to that in MABC. *ZBTB16* has been identified as an androgen-regulated AR target gene in MABC (Robinson *et al.*, 2011), and therefore this was hypothesised to be the case in endocrine resistant BCa. However, androgen induced expression of this target gene was increased in MCF7-TAMR cells but not MCF7-LTED cells. Additionally, the differences in regulation observed between the different resistance models could be from variations in the mechanism via which AR is driving endocrine resistance in these different models. For instance, alternative co-factors or pioneer factors could be influencing AR activity in these lines. Additionally, differing DNA modifications to AR could be present in these lines. Therefore, further analysis using ChIP-Seq and RNA-Seq experiments would give further insight into the differences in AR signalling between these resistant lines, and how androgen signalling differs in endocrine resistance to endocrine sensitive disease and PCa.

4.9.2.1.1 AR and ERα cross-talk in gene expression

Co-treatment of MIB was unable to compete with the E2-induced *TFF1* or *MYC* expression for any of the cell lines (Figures 4.3.1a-c and 4.3.2a-c), as also demonstrated in earlier reporter assays (Figures 3.4.2 and 4). However, co-treatment with E2 significantly reduced MIB-induced expression of *ZBTB16*, *NDRG1* and *DIO2* in MCF7 cells, and for *ZBTB16* and *NDRG1* this effect additionally occurred in at least one of the resistant lines (Figures 4.3.3, 4.3.4, and 4.5.4b). This was also observed for *DDC* in all cell lines, however it was not always significant (Figure 4.5.4a). Importantly, anti-oestrogen treatment could significantly reverse this inhibitive action of E2 on *ZBTB16* expression in some cases: via TAM or FULV treatment in MCF7 cells (Figure 4.3.3a) and by FULV treatment in MCF7-TAMR cells (Figure 4.3.3b). This effect was also observed in MCF7-LTED cells, however it was not significant (Figure 4.3.3c). This indicates that ERα cross-talk is having an inhibitory effect on AR activity, in endocrine resistant as well as endocrine sensitive cells, and that treatment with anti-oestrogens could abrogate these effects. This crosstalk was supported by confocal microscopy where it was demonstrated that when AR and ERα were exogenously transfected into COS-1 cells, nuclear translocation of AR in response to MIB was partially blocked by E2, with some cytoplasmic localisation evident (Figure 4.4.1). However, in the case of *NDRG1*, a gene much more weakly induced by androgen than *ZBTB16* in MCF7 cells, co-treatment with FULV significantly its increased E2-induced expression in MCF7 cells, and this was lost in MCF7-LTED cells (Figures 4.3.4a, c). This indicates that genes which are not strong AR targets in endocrine sensitive cells can be induced via endocrine therapy.

4.9.3 Changes to AR and ERα expression in endocrine resistance

4.9.3.1 An upregulation of AR expression is not essential for endocrine resistance

An elevated expression of AR has been reported in some TAM-resistant tumours, and it has been demonstrated that a ratio of AR:ERα higher than 2.0 increases the possibility of development of TAM resistance more than 4-fold (Cochrane *et al.*, 2014, Carreno *et al.*, 2007, De Amicis *et al.*, 2010). Additionally, exogenous overexpression of AR in MCF7 cells has been demonstrated to promote TAM resistance *in vitro* (De Amicis *et al.*, 2010, Ciupek *et al.*, 2015). Therefore, it was investigated as to whether AR expression was altered in our cell line models of endocrine resistance (Figure 4.2.1a). The results demonstrated that AR expression was 2.6-fold higher in MCF7-LTED cells and 3.9-fold in T47D-TAMR cells as compared to the relevant parental lines, which indicates that its elevated expression could help facilitate resistance in these lines (Figure 4.2.1a).

However, AR expression remained relatively constant in MCF7-TAMR cells as compared its parental line (Figure 4.2.1a), but these cells displayed an enhanced sensitivity to androgen for growth (Figure 4.6.1b) and siRNA knockdown growth experiments confirmed that this effect was produced through AR (Figure 4.6.3d). Additionally, it was demonstrated that AR expression is more responsive to MIB treatment in MCF7-TAMR cells and the expression of the AR target gene *ZBTB16* was more responsive to androgen treatment in this resistant line than demonstrated in the parental line (Figures 4.3.3 and 4.7.1). Therefore, an alternative mechanism to AR overexpression must be important in the development of this endocrine resistant line. Interestingly, both AR and ERα expression were strongly decreased in T74D-LTED cells (Figure 4.2.1a),

which additionally were later demonstrated to have a similar sensitivity to MIB to the parental line (Figure 4.6.2c). This suggestis that these cells retained androgen sensitivity despite these low AR levels, indicating that AR could drive growth in tumours which have developed this resistance mechanism. However, MCF7-FULVR was also displayed to have strongly decreased AR and ERα expression (Figure 4.2.1a), but was sensitive to androgen for growth (Figure 4.6.1d), which suggests that low levels of AR are sufficient to drive proliferation in these resistant cells.

4.9.3.2 ERα expression varies in endocrine resistant models

It has been demonstrated that the endocrine resistant cells have developed a reduced sensitivity or lost their response to oestrogen for growth, as compared to their parental lines (Figures 4.6.1-2). Therefore, it could be expected that ERα expression is downregulated or lost in these lines. Although ERα expression was decreased in all but one resistant cell line (T47D-TAMR, where it increased by 1.8-fold), this reduction was only slight in MCF7-TAMR and MCF7-LTED cells (16.2 and 24.9 %, respectively) (Figure 4.2.1a). This suggests that a loss of ERα expression is not required for the alternative signalling driving these resistance mechanisms to occur. It has been reported that ERα expression is often maintained in TAM resistance, and therefore these results are in line with work previously conducted (Osborne, 1998, Howell *et al.*, 1995, Howell *et al.*, 1996, Schiff *et al.*, 2003).

However, a more noticeable decrease in ERα expression was observed in two of the resistant lines: MCF7-FULVR (4.1 %) and T47D-LTED (29.4 %). A downregulation or loss of ERα expression has been estimated from published work to arise in approximately 15-20 % of endocrine resistant tumours, and this resistance mechanism has been linked

to promoting the activity of HDAC1 and the PI3K/AkT/mTOR signalling pathway (Citro *et al.*, 2015). Therefore, this could be an important factor in these endocrine resistant lines, and warrants further investigation. On the other hand, FULV treatment advances the degradation of ERα expression (Kaklamani and Gradishar, 2017, Lumachi *et al.*, 2015, Lanvin *et al.*, 2007, Osborne *et al.*, 2004), so this effect in MCF7-FULVR could be due to the constant exposure to FULV treatment in the maintenance of this resistant line.

4.9.4 AR drives endocrine resistant cell growth

It is well established that BCa proliferation is driven through oestrogens via the action of ERα in endocrine sensitive disease, for example through its regulation of the PI3K/AKT/mTOR signalling pathway (Beatson, 1896, Pan *et al*., 2017, Chuffa *et al*., 2017, Ali and Coombes, 2002). Studies have suggested that the AR, by contrast, has an inhibitory effect on ERα-driven cancer progression (Rahim and O'Regan, 2017, Fioretti *et al*., 2014, Tarulli *et al*., 2014) and it has been demonstrated that AR overexpression or androgen treatment abrogates E2-stimulated cellular proliferation in endocrine sensitive cells (Cops *et al.*, 2008, Szelei *et al.*, 1997). In support of this, our proliferation assays conducted on MCF7 and T47D cells demonstrated that endocrine sensitive cells respond with an elevated sensitivity to oestrogens over androgens for growth (Figure 4.6.1a and 4.6.2a), demonstrating the importance of the ERα pathway in driving cancer growth. Unlike MCF7 cells, however, whose growth was only significantly increased by the highest concentration of MIB (100 nM), T47D cells were sensitive to MIB for growth. As MIB is a synthetic analogue of DHT that cannot be metabolised to E2, this was not produced by oestrogenic effects. Additionally, AR was demonstrated to be more highly expressed in T47D cells than MCF7 cells (Figure 4.2.1a). This suggests that for endocrine sensitive tumours that have a high AR expression, AR signalling could produce a

sensitivity to androgen for growth, and therefore targeting this axis in treatment could be beneficial.

It has been reported that androgens are secreted in significantly higher quantities than oestrogens in women (Burger, 2002) and two of the main androgens that are produced in women are precursors for E2: androstenedione and testosterone (Figure 1.7.3.1). Several studies have indicated that treatment with different endocrine therapies can cause an increase in androgen levels (Takagi *et al*., 2010, Rossi *et al*., 2009), and that increased androgen levels are associated with endocrine resistance (Berrino *et al*., 2005, Hanamura *et al*., 2013, Baumgart *et al*., 2014). In addition to this, in MABC (ERα-negative, AR-positive BCa) it has been demonstrated that AR has an oncogenic effect and its expression is associated with aggressive tumours (Doane *et al.*, 2006; Robinson *et al.*, 2011; Lehmann-Che *et al.*, 2013). Therefore, it can be assumed that as androgens are readily available, in the absence of an active ERα the AR would be able to function more highly and drive endocrine resistant cell growth. In support of this, previously conducted colony formation assays (Figure 3.5.2) suggested that androgens may be able to promote the onset of endocrine resistance. Figures 4.6.1b-d demonstrated that the MCF7 endocrine resistant derivatives have a reduced (MCF7-TAMR and MCF7-FULVR) or no (MCF7-LTED) response to E2 and have become more sensitive to MIB for growth (Figures 4.6.1b-d). Therefore, AR signalling could be a common mechanism in resistance to different endocrine therapies.

Additionally, the T47D endocrine resistant derivatives have a reduced response to E2 for growth (Figures 4.6.2b-c). Although T47D-TAMR cells were more sensitive to MIB than the parental T47D cells, its oestrogen and androgen sensitivity were similar (Figure 4.6.2.b), and T47D-LTED cells displayed a similar sensitivity to MIB to the parental line

(Figure 4.6.2c). siRNA knockdown of AR abrogated MIB-stimulated growth in MCF7-TAMR cells, which demonstrated that androgen-induced growth is dependent on the AR in this cell line (Figure 4.6.3). It was expected that androgen-stimulated growth would be induced by ERα knockdown, as in the absence of functioning ERα we have demonstrated previously that the AR can become activated (Chapter 3). However, ERα knockdown resulted in a decrease in MCF7 cell growth regardless of hormone treatment (Figure 4.6.3a). This lack of AR-induced growth could be the result of the reduction in AR levels as a side effect of siRNA knockdown.

These results demonstrate that all of the endocrine resistant cell lines have a reduced sensitivity to oestrogen, and the majority an enhanced sensitivity to androgen for proliferation. There were variations between how the endocrine resistant models behaved in response to androgen or oestrogen treatment, however this was expected as work previously conducted has indicated that multiple alternative resistance mechanisms can develop using a resistance model developed in the same way (Hayashi and Kimura, 2015). Additionally, qPCR analysis of MCF7-TAMR and MCF7-LTED cells have demonstrated different androgen and oestrogen induced regulation of AR and ERα target genes within these endocrine resistance models (Section 4.9.2). However, these results indicate that androgens are a potential common driver of endocrine resistant growth through the action of AR in resistance pathways.

4.9.4.1 Anti-androgen treatment can successfully inhibit the proliferation of models of endocrine resistance

Growth assays have indicated that AR drives endocrine resistant cell growth. Therefore, it was hypothesised that anti-androgens which are currently used in the

treatment of PCa could be repurposed for use in endocrine resistant BCa. To explore this, proliferation assays were conducted to investigate the effects of the anti-androgens BIC and OHF on MIB-stimulated growth in MCF7 and T47D cells, and their TAMR and LTED endocrine resistant derivatives (Figures 4.6.1.1-2). The results demonstrated that BIC and OHF inhibited MIB-stimulated growth in all the resistant cell lines tested (Figures 4.6.1.1b-c and 4.6.1.2b-c). Interestingly, this included the T47D-LTED line (Figure 4.6.1.2c) which was demonstrated to have a much lower AR expression than the other lines (Figure 4.2.1a) as well as no enhanced sensitivity to MIB than its parental line (Figure 4.6.2c), suggesting that AR is still important for cancer progression in this line. BIC treatment additionally resulted in a significant reduction in cell growth in the absence of MIB in both MCF7-TAMR and MCF7-LTED (Figures 4.6.1.1b-c). Interestingly, both BIC and OHF treatment significantly reduced MIB-stimulated growth in T47D cells (Figure 4.6.1.2a), and BIC treatment significantly decreased MCF7 proliferation in the absence of MIB treatment (Figure 4.6.1.1a). As these results suggest that AR is a common factor in driving endocrine resistance in our cell line models (Figures 4.6.1, 4.6.2, 4.6.1.1 and 4.6.1.2), it could be hypothesised that anti-androgens could be used as a therapeutic to treat endocrine resistance.

Chapter 5: Conclusions

5.1 Targeting AR to prevent the development of endocrine resistance

The majority of BCa patients are post-menopausal women with ERα-positive, luminal tumours (Rakha and Green, 2016, Ali and Coombes, 2002). Treatment for these patients usually involves the administration of endocrine therapy which aims to block ERα activity. This includes anti-oestrogens (SERMs such as TAM and SERDs such as FULV), which aim to target ERα directly to block its action. Additionally, AIs such as Anastrozole are commonly administered which block aromatase activity to reduce E2 synthesis, thus decreasing circulating oestrogen levels and E2 synthesised within the tumour (Patani and Martin, 2014, Kaklamani and Gradishar, 2017). Endocrine therapy has been largely successful, however the development of endocrine resistance in patients with ERα-positive disease is a major issue in BCa treatment. It is currently estimated that 20-30 % of BCa patients will experience endocrine resistance, and many mechanisms by which this may occur have been described (Vorobiof, 2016).

Endocrine resistance development has been reported to occur by multiple mechanisms, including mutations to the gene encoding ERα, *ESR1*, resulting in constitutive activation of ERα (Fanning *et al.*, 2016, Reinert *et al.*, 2017), increased co-factor expression hyperactivating the ERα pathway (Osborne *et al.*, 2003, Jin *et al.*, 2015, Hiken *et al.*, 2016), phosphorylation of ERα resulting in its ligand-independent activity (Hayashi and Kimura, 2015), the stimulation of ERα via activation of growth factor signalling pathways (Abdel-Hafiz, 2017) and miRNA-mediated alterations to ERα expression or to the levels of proteins functioning in cell cycle regulation (Miller *et al.*, 2008, Wei *et al.*, 2014, Zhao *et al.*, 2008). Many of these mechanisms have been found

to overlap, with one group describing how their development of AI-resistance cell line models using the same method resulted in multiple resistance mechanisms, sometimes within the same model (Hayashi and Kimura, 2015).

The AR has been demonstrated to promote cancer progression in a subclass of ERα-negative AR-positive tumours (MABC disease) (Fioretti *et al*., 2014, Badve *et al*., 2011, Rakha and Green, 2017, Vranic *et al*., 2017, Farmer *et al*., 2005, Doane *et al*., 2006), and the AR has fairly recently been implicated in promoting the onset of endocrine resistance (Ali *et al.*, 2015, Fujii *et al.*, 2014, Rechoum *et al.*, 2014, Ciupek *et al.*, 2015, De Amicis *et al.*, 2010). To develop further understanding of this resistance mechanism, the initial aim of this thesis was to explore how AR signalling is altered in endocrine sensitive disease following treatment with endocrine therapies, from its previously demonstrated tumour suppressive action (Cops *et al.*, 2008, Kampa *et al.*, 2005, Wang *et al.*, 2013, Lanzino *et al.*, 2013), to drive the development of endocrine resistance.

The key results presented here demonstrated that ERα inhibits AR activity and through anti-oestrogen treatment this inhibition could be abrogated (TAM) and even promote a hyperactivated AR (FULV) (Figures 3.4.2d and 3.4.4d); colony formation assays indicated that long term androgen treatment could abrogate the growth inhibitory effects of anti-oestrogens on oestrogen-treated endocrine sensitive cells (Figure 3.5.2); anti-oestrogen treatment decreases the hormone-induced expression of ERα regulatory target genes, yet increases that of AR targets in endocrine sensitive cells (Figure 3.5.3-4); and that in endocrine sensitive cells, oestrogen treatment abrogates androgen-induced AR enrichment at an ARE, but anti-oestrogens can partially rescue this effect (Figure 3.5.6). Therefore, anti-oestrogen treatment could, in addition to inhibiting ERα activity, lead to a more active AR and so select for this mechanism of resistance. In

addition to these results, in Chapter 4 it was demonstrated that anti-androgens could significantly reduce androgen-stimulated growth in some endocrine sensitive cells (Figure 4.6.5a). Taken together, these results provide strong evidence to suggest that resistance to endocrine therapy can be promoted via enhanced AR activity. It is likely that this effect, which is produced through the inhibition of ERα signalling, is due to the removal of ERα/AR crosstalk that has been demonstrated here (Figure 3.4.1) and by others (Peters *et al.*, 2009, Need *et al.*, 2012, Fioretti *et al.*, 2014, Lanzino *et al.*, 2005).

Importantly, the development of this resistance mechanism could be prevented via co-treatment with anti-androgens, since treatment with the anti-androgen BIC enhanced the abrogation of growth in both TAM and FULV treated samples (Figure 3.5.1) and prevented androgen-induced FULV/TAM resistance in the colony formation assays (Figure 3.5.2). This work therefore indicates that the administration of anti-androgens as a combination therapy with endocrine therapies could help to prevent activation of AR signalling and potentially halt this resistance mechanism from occurring in endocrine sensitive patients. This model is summarised in Figure 5.1.1.

Clinical trials investigating the use of the anti-androgen Enzalutamide in combination with various endocrine therapies (including the AI Exemestane and the anti-oestrogen FULV) in ERα-positive patients expressing AR are currently underway (Table 1.9.4.1). Enzalutamide is a second generation anti-androgen that, in addition to competing with ligands to block AR action, stops AR nuclear translocation and AR interaction with DNA and co-factors (Nadal and Bellmunt, 2016, Rahim and O'Regan, 2017, Pelekanou and Castanas, 2016). These clinical trials are following recently published work whereby the authors established the pharmacokinetic interactions, safety, tolerability and correct dosage for the use of Enzalutamide combination with endocrine

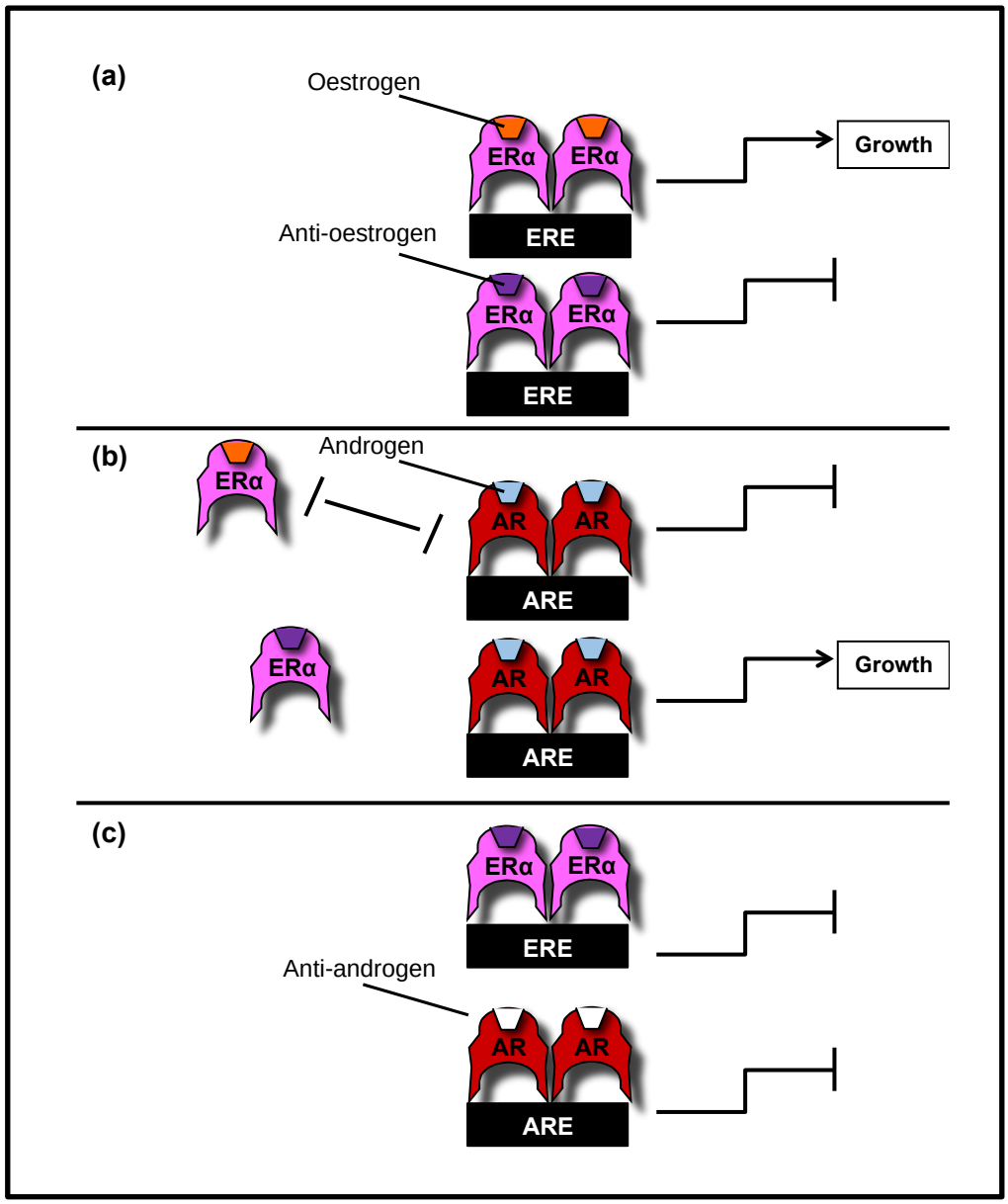


Figure 5.1.1 Targeting the Androgen Receptor therapeutically could abrogate the development of therapy resistance in endocrine sensitive patients

(a) In patients that are positive for Oestrogen Receptor alpha (ERα), ERα drives cancer growth via interactions with Oestrogen Response Elements (EREs), and anti-oestrogen treatment can inhibit this growth. **(b)** ERα inhibits the Androgen Receptor (AR), and anti-oestrogen treatment reverses this repression, so AR can promote endocrine resistance via interactions with Androgen Response Elements (AREs) to promote cancer progression. **(c)** Therefore, the combination of endocrine therapy with anti-androgens to inhibit AR action could prevent the onset of endocrine resistance.

therapy in ERα-positive BCa patients (Schwartzberg *et al.*, 2017). An additional benefit to using Enzalutamide in this co-therapy is that in addition to targeting AR to prevent resistance onset, Enzalutamide could enhance endocrine therapy action via the induction of Cytochrome P450 3A4 (CYP3A4), as Enzalutamide has been previously demonstrated to be a strong CYP3A4 inducer (Gibbons *et al.*, 2015). CYP3A4 is a protein which often metabolises endocrine therapies, for instance it produces the primary active metabolite for TAM, N- dimethyltamoxifen (Yiannakopoulou, 2012). The results of this clinical trial, comparing antiandrogens and antioestrogens as monotherapies or combination therapies, will therefore confirm whether my hypothesis for the use of anti-androgens in these patients is correct.

The use of the novel Selective Androgen Receptor Modulator (SARM) Enobosarm is currently being investigated in many BCa clinical trials, including as the sole treatment for patients with ERα-positive AR-positive tumours (Table 1.9.1). It has been demonstrated that Enobosarm cannot be metabolised by aromatase or 5α-reductase, and therefore as well as acting as an anti-androgen, Vontela *et al*. suggested in postmenopausal patients with ERα-positive BCa, it could potentially decrease E2 synthesis via directly competing with oestrogen-precursors (Vontela *et al.*, 2017). Therefore, colony formation assays to assess the effect of Enobosarm on endocrine sensitive cells could provide interesting results, and the combination of Enobosarm with endocrine therapy in patients with ERα- and AR-positive disease could be beneficial.

We are yet to see how effective anti-androgens will be in endocrine sensitive disease, but data from MABC suggests that these therapies will not be effective in all patients. Clinical trials conducted so far have indicated that targeting androgen signalling can have a good response in some patients. For instance, a recently published case study

described how one patient with metastatic MABC who had been undergoing palliative chemotherapy had a complete clinical response to BIC following 4 months of treatment, and remained in remission for at least 12 months (Arce-Salinas *et al.*, 2016). However, a Phase II clinical trial investigating the use of BIC in MABC patients, observed a complete/partial response or stable disease in just 19% of the patients enlisted after 6 months of treatment (Gucalp *et al.*, 2013). Furthermore, a Phase II clinical trial in MABC of Abiraterone Acetate (AA), a 17α-hydroxylase and CYP17 inhibitor (and thereby an androgen synthesis inhibitor, often used for treatment in castrate-resistant PCa patients) administered in combination with Prednisone (known to decrease androgen production in PCa patients) demonstrated a clinical benefit within 6 months in 20 % of patients (Bonnefoi *et al.*, 2016). Therefore, there is a need to better understand why some patients respond better to treatments that target the AR than others, to identify which patients are likely to benefit from this therapeutic strategy.

Due to the complexity of endocrine resistance and the various mechanisms of resistance described, as well as the mixed response observed to anti-androgen therapy in MABC patients, it would be beneficial to identify endocrine sensitive patients likely to have a positive clinical response to anti-androgen and anti-oestrogen combination therapy. One group demonstrated a significant increase in levels of Prosaposin (PSAP), a proposed AR activator, in the serum of BCa patients prior to surgery who experienced subsequent recurrence (Ali *et al.*, 2015). The authors suggested its use as a biomarker to potentially identify patients likely to develop endocrine resistance as a result of AR signalling. Therefore, this could also be a potential biomarker to indicate patients who would benefit from this combination therapy and this requires further investigation. Future research into identifying additional potential biomarkers would also be of interest, for

instance via obtaining tissue samples from ERα-positive patients during biopsy prior to treatment initiation. Comparison of samples from patients that are responsive and non-responsive to this combination of therapeutics could be used to identify such prognostic biomarkers.

To conclude, rather than the use of anti-oestrogens or AIs as monotherapies in endocrine sensitive BCa patients, I propose that to prevent the onset of endocrine resistance it may be of clinical benefit to combine these therapies with anti-androgens. Ongoing clinical trials to investigate the use of Enzalutamide in combination with endocrine therapies such as AI and FULV will provide further insight into this hypothesis. However, due to the variability of endocrine resistant mechanisms in BCa, I propose that the development of a biomarker(/s) that can stratify patient according to their predicted response would be essential in order to identify patients likely to benefit from this combination therapy.

5.2 Mechanisms via which AR could drive endocrine resistance

The initial aim of this thesis was to explore how AR can promote the onset of endocrine resistance, and our results proposed a potential clinical benefit to combine endocrine therapy with anti-androgen treatment (Chapter 3). However, in some cases patients experience *de novo* rather than acquired resistance, whereby patients have an innate resistance or are prone to develop resistance to endocrine therapy quickly (Reinert *et al.*, 2017). Therefore, the second aim of this thesis was to investigate the mechanism by which AR drives endocrine resistant cell growth in multiple resistance models, and to evaluate whether anti-androgens can block this form of cancer progression (Chapter 4).

Accumulating research has implicated the role of AR in endocrine resistance (Ali *et al.*, 2015, Fujii *et al.*, 2014, Rechoum *et al.*, 2014, Ciupek *et al.*, 2015, De Amicis *et al.*, 2010). However, how AR promotes endocrine resistance remains unclear, with research describing alternative mechanisms, for example: elevated AR expression has been shown to drive TAM resistance through activation of EGFR signalling, which results in TAM becoming agonistic and therefore stimulates ERα activity (De Amicis *et al.*, 2010, Ciupek *et al.*, 2015); overexpression of AR and aromatase causes an activation in ERα activity and differentially expressed ERα target genes as well as AR target genes, which promotes resistance to the AI Anastrazole (Rechoum *et al.*, 2014); increased AR and PSA expression, alongside a loss of ERα expression, promotes AI resistance through an enhanced sensitivity to androgen for growth and a differential expression of androgen-induced genes (Fujii *et al.*, 2014); and PSAP activates AR and can promote AR recruitment to HREs to drive AI resistance (Ali *et al.*, 2015). These results suggest that, as described previously, endocrine resistance mechanisms are complex and can vary greatly, therefore the role of AR in endocrine resistance is likely to vary between patients.

Therefore, a better understanding of the role of the AR in BCa and its role in endocrine resistance is important in order to identify how to better use therapeutics. To do this, we used cell line models of resistance to TAM, FULV and AI endocrine treatments, derived from two alternative endocrine sensitive cell lines. Our results demonstrated that AR signalling promoted proliferation in all of the models of endocrine resistance tested. All but one (T47D-LTED) of the endocrine resistant cell lines displayed an enhanced sensitivity to androgen for growth (Figures 4.6.1-2) and siRNA knockdown in MCF7-TAMR cells confirmed the role of the AR in this enhanced proliferation (4.6.3d). Additionally, AR levels were more responsive to androgen treatment in the resistant lines

tested (MCF7-TAMR and MCF7-LTED) than their parental line (Figures 4.7.1a-c). The alternative proposed mechanisms via which AR could potentially drive endocrine resistance across these cell line models are summarised in Figure 5.2.1.

ChIP and microarray analyses of the ZR-75-1 ERα-positive cell line have demonstrated that the regulation of several E2 and DHT target genes could be antagonised via their co-treatment, suggesting an overlap of the ERα and AR signalling pathways (Need *et al.*, 2012). Need *et al*. further demonstrated that AR and ERα binding sites were often closely located, overlapped or shared for a common gene, which indicates that the binding of one receptor could potentially block the other from interacting with a HRE, or antagonise its desired regulatory effect (Need *et al.*, 2012) (Figure 5.2.1a). Our results have demonstrated that E2 reduces MIB-induced AR enrichment at an ARE, and this effect can be partially rescued via TAM or FULV (Figure 3.5.6). Additionally, we have demonstrated that E2 can significantly reduce MIB-induced expression of AR target genes such as *ZBTB16* in MCF7 and MCF7-TAMR cells (Figures 4.3.3a-c) and that nuclear translocation of AR in response to MIB could be partially blocked by E2 (Figure 4.4.1). Therefore, AR could be competing with ERα for common binding sites in endocrine sensitive disease, so inhibition of ERα increases AR binding, promoting endocrine resistance (Figure 5.2.1a).

It has been previously demonstrated that AR can supress ERα signalling via interacting with EREs (Peters *et al.*, 2009). Therefore, in the absence of functioning ERα, it could be hypothesised that AR could interact with EREs and take over ERα signalling to drive endocrine resistance (Figure 5.2.1d). However, gene expression analysis indicated that AR is not regulating ERα target genes in the endocrine resistant cells (Figures 4.3.1-2), as in MABC cells it has been demonstrated that androgen treatment

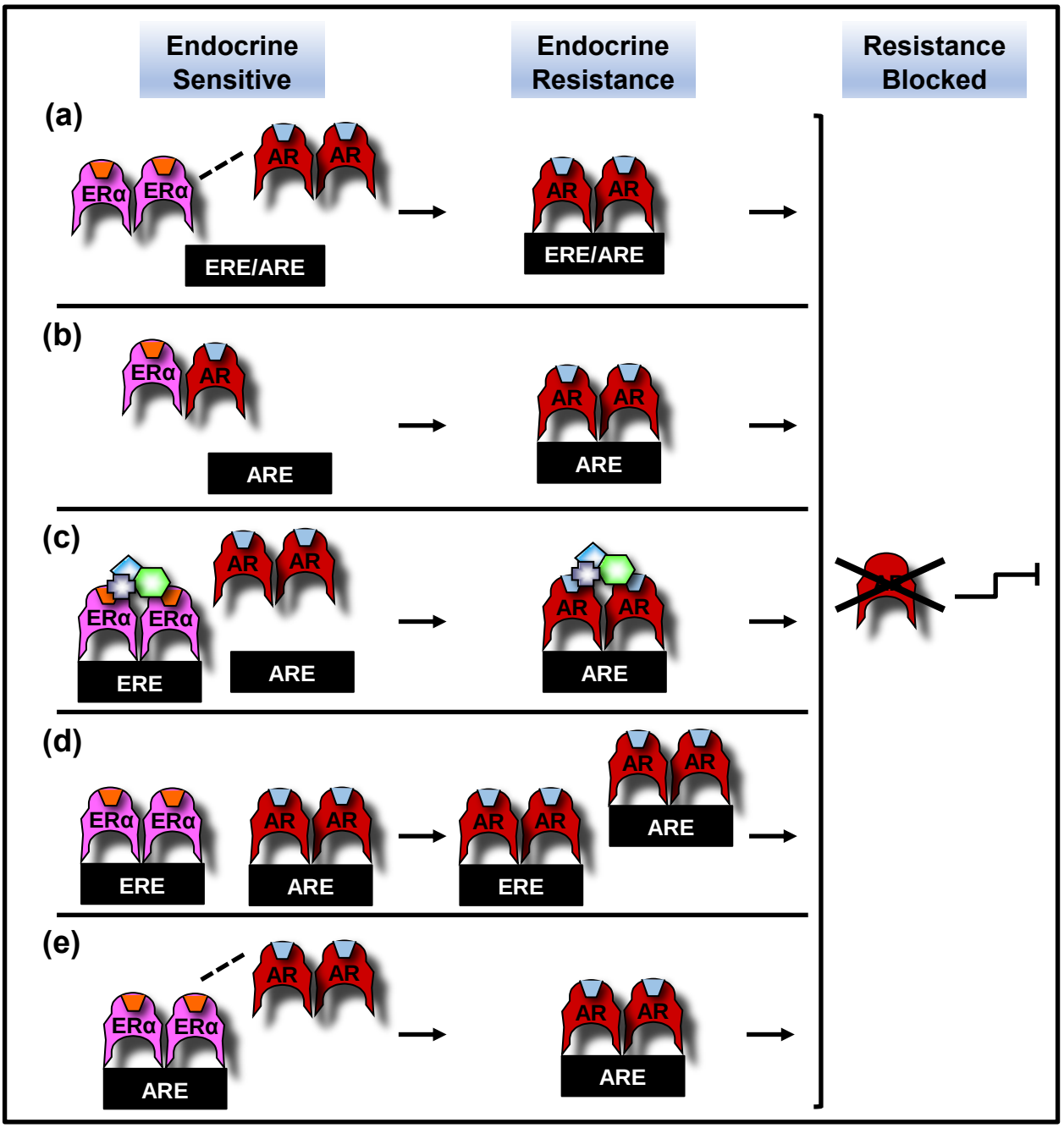


Figure 5.2.1 Proposed mechanisms via which the Androgen Receptor could drive endocrine resistant growth, which could be blocked via targeting it

(a) Androgen Receptor (AR) is competing with Oestrogen Receptor alpha (ERα) for common binding sites in endocrine sensitive disease, so inhibition of ERα increases AR binding, promoting endocrine resistance. **(b)** ERα is inhibiting AR action via a protein-protein interaction in endocrine sensitive disease, so inhibition of ERα increases AR signalling, promoting endocrine resistance. **(c)** ERα and AR are competing for shared co-activators in endocrine sensitive disease, and so inhibition of ERα increases AR activity. **(d)** In the absence of functioning ERα, AR interacts with Oestrogen Response Elements (EREs) and takes over ERα signalling to drive endocrine resistance. **(e)** In endocrine-sensitive disease, ERα is inhibiting AR from interacting with Androgen Response Elements (AREs) to act as a physical barrier, so when ERα is inhibited AR signalling increases to drive endocrine resistance.

results in the activation of oestrogen-associated genes (Doane *et al.*, 2006). It could be that alternative ERα target genes to those explored here are being regulated by AR in these resistant lines, and future ChIP-Seq and RNA-Seq analysis on AR binding in MCF7-TAMR cells would provide insight on this. Alternatively, it could be that in endocrine-sensitive disease, ERα could be inhibiting AR from interacting with AREs to act as a physical barrier, so that when ERα is inhibited AR signalling increases to drive endocrine resistance (Figure 5.2.1e). AR and ERα target gene expression demonstrated that the transcriptome is altered in endocrine resistance compared to the parental lines, and there were differences between the models of therapy resistance (Figures 4.3.1-5 and 4.5.1-4). Therefore, as seen in MABC (Farmer *et al.*, 2005, Need *et al.*, 2012), endocrine resistant cells appear to have a unique androgen signalling pathway, differing to that observed in PCa (for which AR signalling is also important), but additionally unique between the different types of endocrine resistance. Again, future analyses (through NGS) on these resistant lines could provide insight into this mechanism.

Yeast and mammalian two-hybrid systems have been used previously to demonstrate that ERα and AR can heterodimerise, resulting in suppression of the transcriptional activity of both receptors (Panet-Raymond *et al.*, 2000). Confocal imaging demonstrated that, in conjunction with this, AR and ERα localisation were commonly found to colocalise with hormone treatments (Figures 4.4.2-7). Therefore, there is a possibility that ERα is inhibiting AR action via a protein-protein interaction in endocrine sensitive disease, so inhibition of ERα increases AR signalling, promoting endocrine resistance (Figure 5.2.1b). In order to investigate this further, these imaging and 2-hybird assays would need to be performed in the presence of an anti-oestrogen to see if this blocks colocalisation or interaction between the receptors. The loss of this interaction

would be predicted to increase receptor activity. Co-Immunoprecipitation (co-IP) could additionally be used in future work in order to demonstrate whether AR and ERα interact in our endocrine sensitive models, and whether this is altered in the resistance models, in order to support this theory.

Previous studies have demonstrated that a ratio of AR:ERα higher than 2.0 increases the development of TAM resistance more than 4-fold (Cochrane *et al.*, 2014, Carreno *et al.*, 2007, De Amicis *et al.*, 2010). By contrast to this, immunoblotting demonstrated that the highest AR:ERα ratio observed in these endocrine resistance models was for MCF7-LTED (3.5), however MCF7-TAMR cells (which had an AR:ERα ratio of 1.1) were subsequently found to be more responsive to androgen for growth than this cell line derivative (Figures 4.2.1b and 4.6.1).

AR and ERα cross-talk is also influenced by shared co-factors and pioneer factors such as ARA70 (Fioretti *et al*., 2014, Lanzino *et al*., 2005). Competition for these limiting factors could also result in inhibitory cross-talk between the receptors, and therefore inhibition of ERα through endocrine therapies could produce an increase in AR activity (Figure 5.2.1c). This could explain the lack of AR overexpression as a mechanism for the increase in AR sensitivity for all resistance models with the exception of MCF7-LTED and T47D-TAMR (Figure 4.2.1a). Alternatively, inhibiting ERα with treatment could result in an increase in the availability of a common pioneer factor such as FOXA1 (Hurtado *et al*., 2011, Robinson *et al*., 2011) to the AR.

5.3 Targeting AR as a treatment option for endocrine resistance

Our results demonstrated that androgen-stimulated growth of both our models of TAM and AI resistance derived from two alternative endocrine sensitive cell lines could be successfully inhibited via anti-androgen (OHF/BIC) treatment (Figures 4.6.4b-c and 4.6.5b-c). These results are supported by work by others, who have demonstrated that Enzalutamide and BIC treatment are effective in inhibiting AR-driven endocrine resistance (De Amicis *et al*., 2010, Ali *et al*., 2015). Importantly, AR appears to be able to promote therapy resistance to different anti-oestrogens and AIs, and although our cell line models behave differently, and the mechanisms via which AR drives endocrine resistance in them appears to differ, they all responded to anti-androgen treatment. Therefore, targeting AR could be a viable option to treat resistance to various agents targeting ERα signalling.

Published research on endocrine resistance has demonstrated that the mechanisms by which endocrine resistance can occur can vary greatly. Therefore, as growth might not always be driven via AR in endocrine resistance, the response to anti-androgen treatment for endocrine resistant AR-positive disease could vary between patients (as found in MABC). Therefore, the identification of tumours that are dependent on AR for growth could aid in patient stratification. In order to do this, I propose that the development of a panel of biomarkers in order to indicate a positive response to this therapy would be highly beneficial, such as PSAP (as discussed in Section 5.1). Additionally, the proposed future ChIP-Seq and RNA-Seq analyses in MCF7-TAMR cells, the preparation of which is presented in Figure 4.8.1, will indicate genes regulated in response to androgen signalling in this cell line, and these studies can be expanded to the other resistant models to identify similarities.

5.4 Final Conclusion

In conclusion, I propose that there could be a clinical benefit from the administration of anti-androgen therapies for a subset of patients with tumours that express the AR in order to prevent the development of endocrine resistance or for the treatment of endocrine resistant disease. This is supported by a recent case study whereby the first incidence of a positive clinical response from a patient with ERα, PR and AR-positive BCa to a SARM following failure and developed resistance to a vast number of ERα-targeted endocrine therapies was recorded (Vontela *et al.*, 2017). However, clinical trials have indicated that therapeutics targeting AR may not always be beneficial to patients with AR-positive endocrine sensitive or resistant BCa. Ongoing clinical trials to investigate the use of Enzalutamide and Enobosarm in advanced patients with ERα-positive and AR-positive disease who have not responded or developed resistance to endocrine therapies will provide further insight into this hypothesis (Table 1.9.1). Due to the variability of endocrine resistance mechanisms in BCa, I propose that the development of a panel of prognostic biomarkers perhaps including PSAP to predict clinical response to these therapies would be highly beneficial.

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