ANDROGEN SIGNALLING IN HUMAN BREAST CANCER CELLS

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by

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Summary

Several lines of evidence support an important role for male sex steroid hormones, or androgens, in breast cancer. The androgen receptor (AR), which mediates the action of androgens, is expressed in up to 90% of primary breast cancers. This frequency is higher than that for the estrogen and progesterone receptors (ERα, PR), which mediate the action of the female sex steroid hormones estrogen and progesterone - traditionally regarded as the key regulators of breast cancer cell growth. Epidemiological studies have shown an association between high levels of circulating androgens and a reduced risk of breast cancer, suggesting a protective effect of androgens. This is further supported by studies demonstrating that androgens can suppress the growth of breast cancer cells in culture and the clinical efficacy of androgenic drugs for the treatment of metastatic breast cancer. The masculinising side effects of androgens, however, have limited their clinical utility and hormonal therapies which exploit estrogen signalling pathways, such as anti-estrogens (eg tamoxifen), are currently prescribed as a first-line treatment for advanced breast cancers. Unfortunately, not all breast cancers, particularly those which do not express ERα, respond to anti-estrogens, creating a need for alternative treatment strategies. Although the anti-proliferative effects of androgens on breast cancer cells are well established, the precise mechanisms that mediate these effects are poorly understood. A better understanding of androgen signalling pathways in breast cancer cells would facilitate the development of novel androgenic or AR-based therapies that regulate breast cancer growth but circumvent the side effects of androgens.

Androgen signalling pathways have primarily been studied in prostate cells which have an essential requirement for androgens for normal growth and function. The AR is a nuclear transcription factor which, following ligand binding, dimerisation and nuclear translocation, binds to androgen responsive elements (AREs) located in the regulatory regions of target genes. The AR then interacts with other components of the transcriptional machinery to activate or repress gene expression, leading to cell proliferation, differentiation, morphogenesis or apoptosis. The precise role of androgen signalling, and the downstream pathways activated by the AR, in breast cancer cells are considerably less well defined than in prostate cells. The objectives of this thesis, therefore, were to further characterise androgen signalling pathways in breast cancer

cells by examination of the mechanisms regulating AR function, the expression of androgen responsive genes and cross-talk with estrogen signalling pathways.

The MDA-MB-453 breast cancer cell line expresses AR but not ERα or PR, making it a valuable model in which to study the effects of androgen action without interference from the activity of other steroid hormone receptors which are critical for breast cancer cell growth. Interestingly, *in vitro* studies have shown that the proliferation of MDA-MB-453 cells is stimulated by 5α-dihydrotestosterone (DHT) and inhibited by the synthetic progestin, medroxyprogesterone acetate (MPA), both of which bind with high affinity to the AR. These divergent proliferative responses may arise from functional defects of the endogenous AR in this cell line, which contains a point mutation in the ligand binding domain. Reporter gene assays, performed in AR negative cells transiently transfected with an androgen responsive luciferase reporter construct and either wild type or variant AR, demonstrated that although the variant AR retained transactivation potential it was less sensitive to DHT and MPA than the wild type AR. In contrast, analysis of endogenous variant AR activity with two independent androgen responsive reporters in the MDA-MB-453 cell line suggested that DHT and MPA may differ in their ability to induce gene expression depending on the promoter context. This was supported by cDNA array analysis, which indicated that gene expression profiles are considerably different between DHT and MPA treated MDA-MB-453 cells. These results suggest that the activation or repression of different sets of target genes by the AR may contribute to the divergent proliferative effects of DHT and MPA.

One of the genes identified in the cDNA array screen performed with MDA-MB-453 cells was the breast cancer susceptibility gene, BRCA1, which was markedly downregulated by MPA but its expression was not significantly altered by DHT. Further analysis of BRCA1 expression in MDA-MB-453 cells, using real time RT-PCR, demonstrated that BRCA1 was rapidly downregulated by MPA via a mechanism that involves the AR. Putative ARE sequences were identified in the BRCA1 gene although the precise role that these response elements play in the downregulation of BRCA1 by the AR is yet to be resolved. A better understanding of the mechanisms associated with the expression of androgen responsive genes in breast cancer cells has been achieved through the study of the prostate specific antigen (PSA) gene. The induction of PSA by androgens has been extensively characterised in prostate cells. Chromatin immunoprecipitation (ChIP) assays, which measure the binding of proteins to DNA in

its natural chromatin integrated context, demonstrated that AR occupancy and histone H3 acetylation (a marker of transcriptional activity at a given locus) at AREs situated in the PSA regulatory region increased with DHT treatment in breast cancer cells. Furthermore, time course experiments suggested that the increases in AR occupancy and histone H3 acetylation occurred prior to the accumulation of PSA mRNA. Further experiments using ChIP assays may be used to define the key transcription factors involved in regulating the expression of androgen responsive genes, such as PSA or BRCA1, in breast cancer cells.

In addition to directly regulating the expression of genes contributing to breast cancer cell proliferation, the AR may also indirectly regulate proliferation through interaction with other signalling molecules, such as ERα. The interactions between AR and ERα were examined using the T-47D breast cancer cell line, which expresses functional AR and ERα. Treatment with estrogen alone stimulated T-47D cell proliferation while androgens inhibited basal and estrogen-induced proliferation. The effect of androgens on estrogen-stimulated proliferation may be mediated by two possible mechanisms: (i) through inhibition of ERα activity, or (ii) through opposing downstream events in estrogen signalling pathways. Transient transfection assays using an estrogen responsive reporter gene demonstrated that the constitutively active AR amino terminal domain inhibits ERα activity in a dose dependent manner in T-47D cells. Furthermore, androgens inhibited estrogen-dependent induction of the endogenous PR gene via a mechanism involving AR and ERα. The balance, therefore, between androgen and estrogen signalling pathways may be critical in the regulation of breast cancer cell growth.

Collectively, these studies support a model in which androgens inhibit breast cancer cells directly, via regulating the expression of AR target genes, and indirectly, via an interaction between AR and ERα. The current studies suggest that BRCA1 may play a critical role in mediating the inhibitory effects of AR on breast cancer cells, and previous studies have shown that BRCA1 enhances AR activity but represses ERα. These observations highlight the need for further study specifically examining the regulation of BRCA1 expression by the AR, but also the expression of other as yet uncharacterised androgen responsive genes which mediate the proliferative effects of androgens. A greater understanding of the role of AR signalling in breast cancer cells may provide a rationale for the development of new therapeutic approaches.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give my consent to this copy of my thesis, when deposited in the University library, being made available for loan and photocopying.

Nicole L. Moore

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Publications arising from this Thesis

❖ ***Articles published in scientific journals:***

Mutations at the Boundary of the Hinge and Ligand Binding Domain of the Androgen Receptor Confer Increased Transactivation Function, Buchanan G, Yang M, Harris J, Nahm H, Han G, Moore N, Bentel J, Matusik R, Horsfall D, Marshall V, Greenberg N and Tilley WD, Molecular Endocrinology (2001), 15: 46-56

❖ ***Articles in preparation:***

AR gene mutation in the MDA-MB-453 breast cancer cell line exhibits ligand-specific activation characteristics, Moore NL, Buchanan G, Yang M, Davidson B, Lambert PF, Birrell SN, Marshall VR and Tilley WD

Inhibition of advanced breast cancer growth by medroxyprogesterone acetate is prevented by loss of function androgen receptor gene mutations: the regulation of estrogen signaling by an activated androgen receptor, Yang M, Buchanan G, Peters AA, Moore NL, Pickering MA, Harris JM, Butler LM, Bentel JM, Horsfall DJ, Birrell SN and Tilley WD

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❖ ***Abstracts published in the proceedings of scientific meetings:***

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Inhibition of estrogen signalling by the androgen receptor: a potential mechanism of inhibition of breast cancer cell growth, Moore NL, Buchanan G, Butler LM, Birrell SN and Tilley WD, American Association for Cancer Research, 94th Annual Meeting, Washington DC, USA, July 2003

Inhibition of estrogen signalling by the androgen receptor: a potential mechanism of inhibition of breast cancer cell growth, Moore NL, Buchanan G, Butler LM, Birrell SN and Tilley WD, Gordon Research Conference on Hormonal Carcinogenesis, Meridian, New Hampshire, USA, July 2003

Abbreviations used in this Thesis

Genes and proteins:

AcH3	acetylated histone H3
AIB1	amplified in breast cancer 1
AP	alkaline phosphatase
AR	androgen receptor
ARA	androgen receptor associated protein
BRCA1	breast cancer susceptibility gene 1
BRCA2	breast cancer susceptibility gene 2
CBP	CREB binding protein
CREB	cyclic AMP regulatory element binding protein
ER	estrogen receptor
FGF8	fibroblast growth factor 8
FGFR4	fibroblast growth factor receptor 4
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCDFP	gross cystic disease fluid protein
GR	glucocorticoid receptor
GRIP1	glucocorticoid receptor interacting protein 1
HAT	histone acetyltransferase
HDAC	histone deacetylase
HRP	horseradish peroxidase
HSD	hydroxysteroid dehydrogenase
HSP	heat shock protein
IGFBP5	insulin-like growth factor binding protein 5
JAK	janus kinase
KLK	kallikrein
MAPK	mitogen activated protein kinase
MR	mineralocorticoid receptor
NCoR	nuclear corepressor
PB	probasin
pCAF	p300/CBP associated factor
PKA	protein kinase A
PKC	protein kinase C
PI 3'-kinase	phosphatidylinositol 3'-kinase
PR	progesterone receptor

PSA	prostate specific antigen
RAR	retinoic acid receptor
RXR	retinoic X receptor
SHBG	sex hormone binding globulin
SMRT	silencing mediator of retinoic and thyroid hormone receptors
SRC1	steroid receptor coactivator 1
STAT3	signal transducer and activator of transcription 3
TR	thyroid hormone receptor

Steroid hormones and receptor antagonists:

Bic	bicalutamide
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone-sulphate
DHT	5α-dihydrotestosterone
E_1	estrone
E_2	17β-estradiol
FSH	follicle stimulating hormone
LH	leuteinising hormone
LHRH	leuteinising hormone release hormone
Mib	mibolerone (7α, 17α-dimethyl-19-nortestosterone)
MPA	medroxyprogesterone acetate
OHF	hydroxyflutamide
OHT	trans-4-hydroxytamoxifen

General abbreviations:

A	adenosine
AD	activation domain
AF	activation function
ANOVA	analysis of variance
ARE	androgen responsive element
BHQ	black hole quencher
BSA	bovine serum albumin
C	cytidine
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CSPD	disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro) tricyclo {3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate

CSS	charcoal stripped serum
C_T	cycle threshold
dATP	deoxyadenosine triphosphate
DBD	DNA binding domain
dCTP	deoxycytidine triphosphate
DEAE	diethylaminoethyl
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DIG	digoxygenin
DMBA	dimethylbenz(a)anthracene
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
E	reaction efficiency
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ERE	estrogen responsive element
FCS	foetal calf serum
G	guanosine
HRP	horse radish peroxidase
IL-6	interleukin-6
K_d	dissociation constant
LBD	ligand binding domain
luc	luciferase
MOPS	3-(N-morpholino) propane sulphonic acid
mRNA	messenger RNA
NTD	amino-terminal transactivation domain
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
P-SCAN	peak quantification with statistical comparative analysis
PVDF	polyvinylidine difluoride
RLU	relative light units
RNA	ribonucleic acid

RNase	ribonuclease
RNasin	ribonuclease inhibitor
RPA	ribonuclease protection assay
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal RNA
RT	reverse transcription/transcriptase
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
siRNA	short interfering RNA
T	thymidine
TAE	tris acetate EDTA
TBE	tris borate EDTA
TBS	tris buffered saline
TBST	tris buffered saline-tween 20
TE	tris EDTA
TEMED	tetramethylethylenediamine
tk	thymidine kinase
UV	ultraviolet
wt	wild type

Units:

bp	base pairs
Ci	curie(s)
°C	degrees Celsius
Da	Dalton(s)
g	gram(s)
hr	hour (s)
kb	kilobases
L	litre
min	minute(s)
M	molar (moles per litre)
sec	second(s)
U	units
V	volts

CHAPTER 1

HORMONAL CONTROL OF HUMAN BREAST CANCER

1.1 – Introduction

1.1.1 – Overview

The onset of puberty, the menstrual cycle, pregnancy, lactation and menopause results in breast tissue being exposed to continuous change in the hormonal milieu. The mature breast is comprised of a branching duct system embedded in adipose and stromal tissue. The growth of epithelial cells, which line the walls of the ducts, is primarily regulated by the female sex steroid hormones estrogen (Ali and Coombes, 2002) and progesterone (Clarke and Sutherland, 1990; Graham and Clarke, 1997), however other hormones such as prolactin (Ben Jonathan *et al*, 2002; Clevenger *et al*, 2003) and the male sex steroid hormones, or androgens, (Birrell *et al*, 1998; Liao and Dickson, 2002; Labrie *et al*, 2003) are also important. The cyclical nature of hormonal exposure makes the epithelial cells of breast tissue particularly susceptible to malignant transformation, and may, in part, explain the high incidence of breast cancer affecting women living in western countries such as Australia. In 1999, approximately one in 11 Australian women developed breast cancer by age 74, accounting for 28% of all new cancer cases and 17% of cancer deaths in Australian females (Australian Institute of Health and Welfare and Australasian Association of Cancer Registries, 2002).

The majority of breast cancers arise from malignant transformation of ductal epithelial cells (Anderson *et al*, 1998). Carcinoma *in situ* remains confined to the epithelium of the originating duct or lobule. Some cancers do not progress beyond this stage, however most patients have more invasive tumours that spread beyond the basement membrane into surrounding tissue. Malignant cells can then metastasise via the lymph channels or the bloodstream, eventually depositing in distant sites such as the axillary lymph nodes as well as lung, heart, bone, liver and brain tissue. Most metastatic

tumours inevitably progress and there are limited therapeutic options for these women. A better understanding of hormonal influences on carcinogenesis and tumour progression will facilitate the development of improved treatment and possible prevention strategies for breast cancer.

1.1.2 – Risk factors

1.1.2.1 – Genetic

Approximately 5-10% of all breast cancers are due to inherited mutations in susceptibility genes (Claus *et al*, 1991; Miki *et al*, 1994; Wooster *et al*, 1995). The best characterised of these genes are *BRCA1* and *BRCA2* (Miki *et al*, 1994). Inherited mutations in either of these genes are together associated with approximately 80% of familial breast cancers (Rosen *et al*, 2003), and women carrying a mutation in either *BRCA1* or *BRCA2* have an approximate 85-90% chance of developing breast cancer by age 70 (Ford *et al*, 1994; Wooster *et al*, 1995). Mutations in *BRCA1* and *BRCA2* are rare in sporadic breast cancers (Futreal *et al*, 1994; Lancaster *et al*, 1996; Papa *et al*, 1998; van der Looij *et al*, 2000) although a potential role for the loss of BRCA1 function in sporadic breast cancer has been suggested by studies showing reduced expression of *BRCA1* in sporadic breast cancer cells compared to non-malignant cells (Magdinier *et al*, 1998; Taylor *et al*, 1998; Ozcelik *et al*, 1998; Wilson *et al*, 1999; Yoshikawa *et al*, 1999; Lee *et al*, 1999b; Yang *et al*, 2001). The role of BRCA1 in breast cancer is discussed in further detail in Chapter 7. Mutations in other genes such as *p53*, the gene for ataxia-telangiectasia (*ATM*) and the gene for Cowden's disease (*PTEN*) are also associated with increased risk of breast cancer in certain families (Swift *et al*, 1987; Malkin *et al*, 1990; Ford and Easton, 1995; Savitsky *et al*, 1995; Li *et al*, 1997; Liaw *et al*, 1997; Rhei *et al*, 1997; Khanna, 2000; Chenevix-Trench *et al*, 2002).

1.1.2.2 – Hormonal

A large number of epidemiological studies have investigated the relationships between hormones which control breast development (ie estrogen, progesterone, prolactin and androgens) and the risk of breast cancer. In the case of androgens, which are the primary focus of this thesis, an increased risk for breast cancer appears to be associated with low endogenous androgen levels in premenopausal women, while an increased risk for breast cancer in postmenopausal women has been associated with high endogenous androgen levels (Adams, 1998; Lillie *et al*, 2003). A more detailed review of epidemiological data pertaining to androgens in breast cancer is presented in Section 1.2.2.

Estrogens have been positively associated with an increased risk for breast cancer. A recent meta-analysis of six prospective studies showed that endogenous serum estradiol (E_2) concentrations were 15% higher in postmenopausal women who subsequently developed breast cancer compared to unaffected women (Thomas *et al*, 1997c). Exogenous estrogens, administered as part of oral contraceptive or hormone replacement regimens, can also increase risk with long term use and in women with inherited predisposition to breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer, 1997; Ursin *et al*, 1997; Ursin *et al*, 1998; Burke, 2000; Henderson and Feigelson, 2000; Ross *et al*, 2000; Rossouw *et al*, 2002). Furthermore, epidemiological studies have established associations between a number of lifestyle-related and reproductive factors, which can modulate the cumulative lifetime exposure of the breast epithelium to estrogens, and breast cancer risk. Early age at menarche, late age at menopause, nulliparity and late age at first pregnancy, which can each increase lifetime exposure to estrogens, are associated with an increased risk for breast cancer (MacMahon *et al*, 1970; Trichopoulos *et al*, 1972; McPherson *et al*, 2000; Clemons and

Goss, 2001). Obesity and alcohol consumption, which can increase serum estrogen levels, are also established risk factors for breast cancer (Smith-Warner *et al*, 1998). In contrast, physical exercise, which can reduce circulating estrogen levels by slowing (or delaying the onset of) ovulatory cycles, has been established as a protective factor for breast cancer (Bernstein *et al*, 1994; Thune *et al*, 1997; Feigelson, 2003).

Lactation, which is in part controlled by progesterone and prolactin (Neville and Morton, 2001), has also been associated with a reduced risk of breast cancer (Yoo *et al*, 1992; Romieu *et al*, 1996; Freudenheim *et al*, 1997; Enger *et al*, 1998; Newcomb *et al*, 1999). However the precise role of progesterone and prolactin in modulating breast cancer risk remains unclear. Associations between endogenous progesterone and breast cancer risk have been difficult to establish as natural fluctuation of serum progesterone levels during the menstrual cycle has led to inconsistent results (Bernstein, 2002). Some clinical studies have reported a moderately increased risk of breast cancer in women taking combined estrogen/progestin hormone replacement therapy compared to those taking estrogen replacement alone, although a clear association between exogenous progesterone and breast cancer risk is yet to be established (reviewed in Eden, 2003). Many studies have reported no association between endogenous prolactin levels and the development of breast cancer (Bernstein and Ross, 1993) although a more recent study, using improved techniques for the measurement of serum prolactin, demonstrated an increased risk for breast cancer in women with high prolactin levels (Hankinson *et al*, 1999).

Together, these studies provide compelling evidence that hormones, particularly androgens and estrogens, play an important role in the promotion and progression of breast cancer.

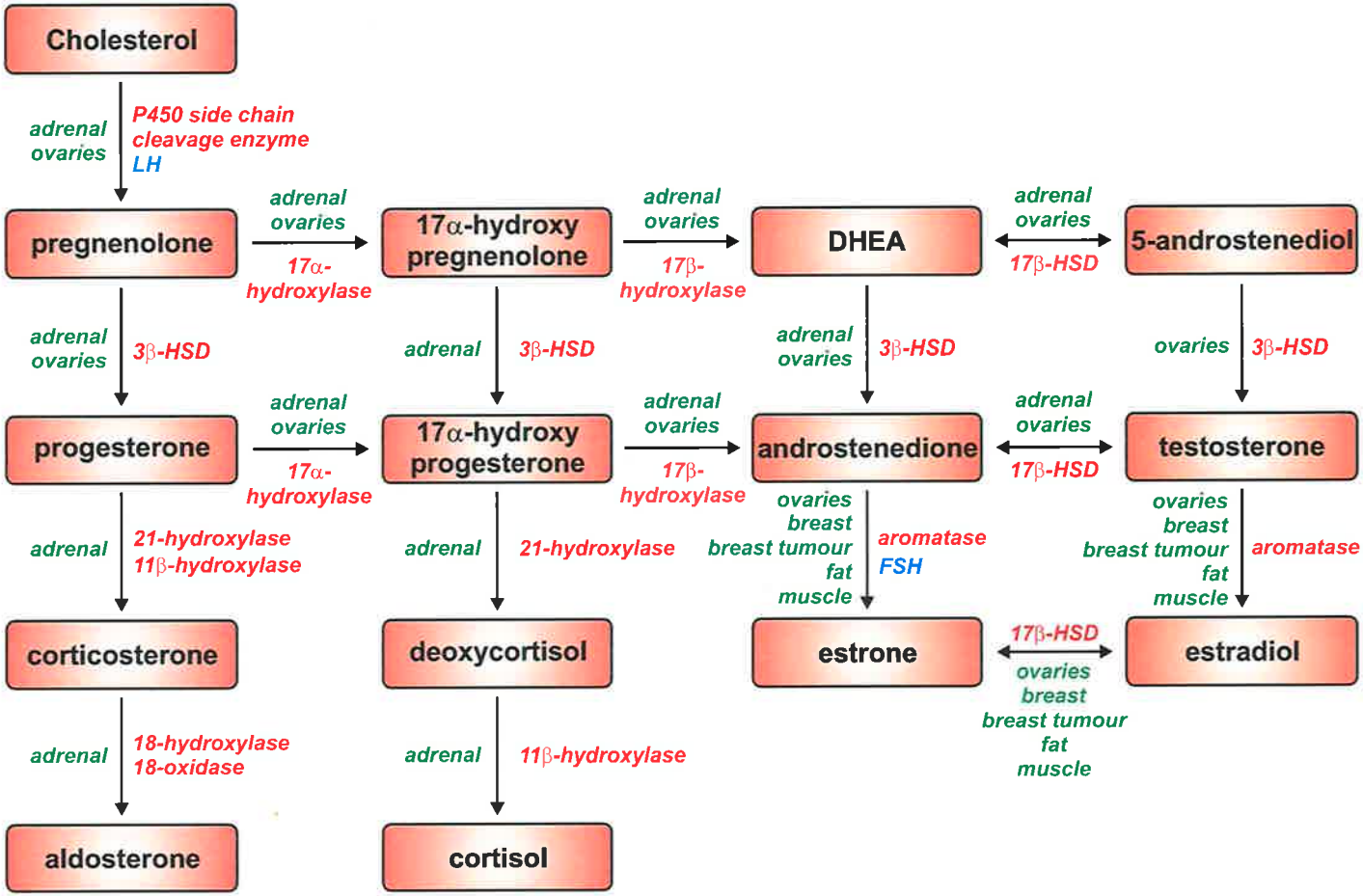
1.1.3 – Estrogens and breast cancer

Much attention has been focussed on the stimulatory effect of estrogens on breast cancer. More than 100 years ago George Beatson demonstrated that bilateral oophorectomy results in remission of advanced breast cancer in premenopausal women (Beatson, 1896), suggesting that hormones produced by the ovaries are critical regulators of breast cancer growth. More recent studies have shown that estrogens stimulate the proliferation of breast cancer cell lines *in vitro* and can induce tumour growth in animal models (Davidson and Lippman, 1989; Lupulescu, 1995; Couillard *et al*, 1998; Clemons and Goss, 2001). In addition to the stimulatory effect estrogens have on breast epithelial cell proliferation, metabolites of estrogen (catecholestrogens and quinones) also result in formation of DNA adducts, demonstrating a direct carcinogenic effect (Yager and Liehr, 1996; Cavalieri *et al*, 1997; Jefcoate *et al*, 2000; Clemons and Goss, 2001).

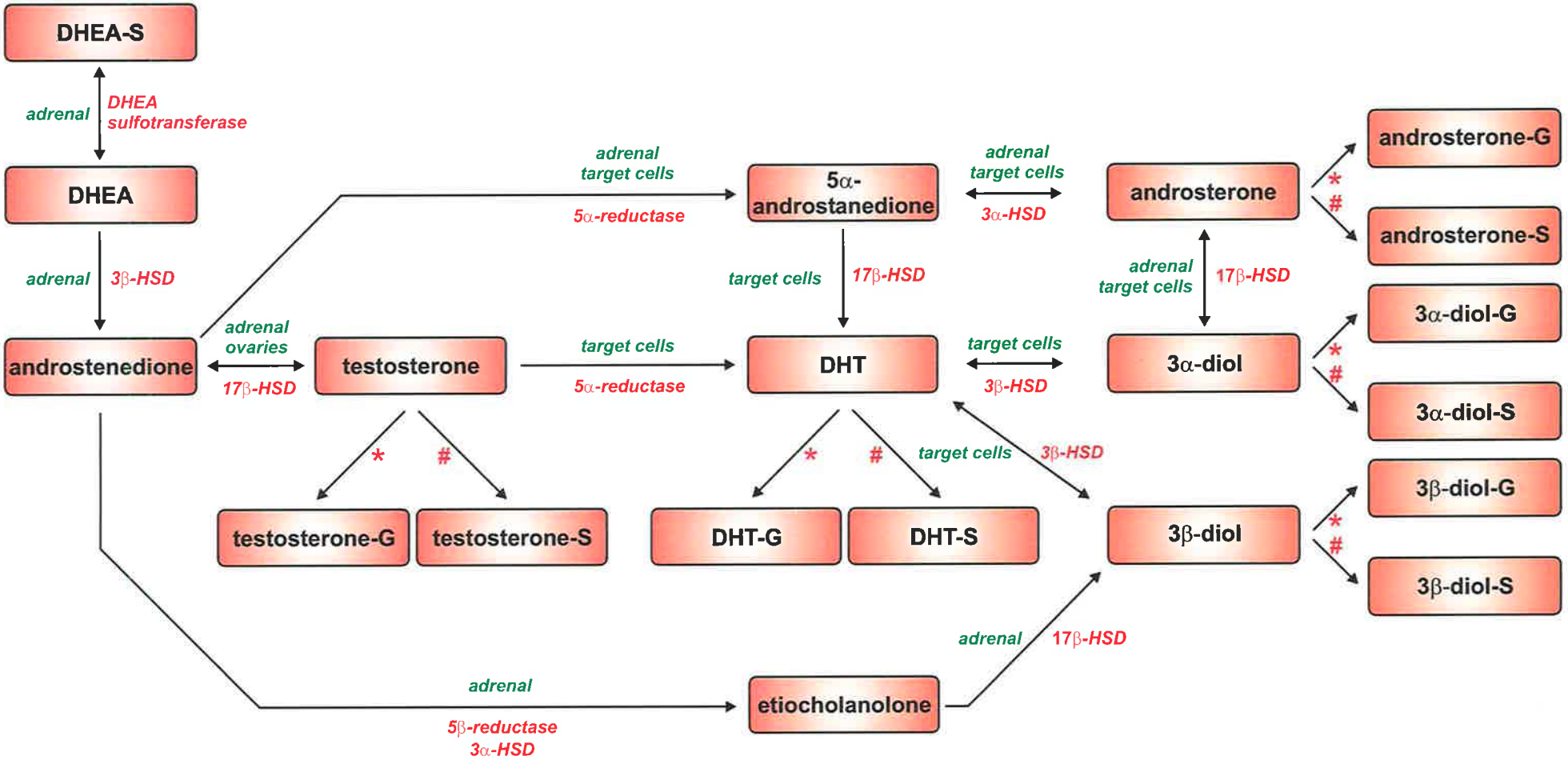
In premenopausal women, the pituitary gonadotrophins luteinising hormone (LH) and follicle stimulating hormone (FSH) stimulate the ovaries to produce estradiol (E_2) and estrone (E_1) from the androgens testosterone and androstenedione respectively in reactions catalysed by the aromatase complex (Wilson *et al*, 1998; Stanczyk and Bretsky, 2003) (Figure 1.1 (a)). Estrogens are also produced at peripheral sites, such as the breast, adipose tissue, brain, bone and muscle in premenopausal women (Stanczyk and Bretsky, 2003), where they act in a paracrine or intracrine fashion. Following menopause, ovarian estrogen levels fall but its synthesis from aromatisation of androgenic precursors continues at peripheral sites (Castagnetta *et al*, 1996; Simpson and Davis, 2001; Stanczyk and Bretsky, 2003) – local estrogen levels in the normal and malignant breasts of postmenopausal women are actually higher than serum levels in premenopausal women (Lu *et al*, 1996; Castagnetta *et al*, 1996; Pasqualini *et al*, 1996;

Figure 1.1: Steroid biosynthesis and metabolism pathways in the female body. Conversion sites are shown in green, catalysing enzymes are indicated in red and influences from pituitary hormones are shown in blue. Double headed arrows indicate reversible reactions, which are balanced by oxidative and reductive pathways. *(a)* All steroid hormones are derived from precursor cholesterol molecules in reactions involving cytochrome P450 enzymes. Mineralocorticoids, such as aldosterone, are produced from progesterone while glucocorticoids, such as cortisol, are produced from 17α-hydroxyprogesterone. In pre-menopausal women the corpus luteum, granulosa and theca cells of the ovaries produce the majority of circulating estrogens, the levels of which are enhanced by contributions from breast, fat and muscle tissue. Androgens are produced by the adrenal and ovaries. Following menopause, the ovarian contribution to hormone production is significantly decreased, however estrogens and androgens continue to be produced at peripheral sites. *(b)* Intracellular metabolism of androgens by reduction, hydroxylation or conjugation. Testosterone and dihydrotestosterone metabolism occurs in target tissues while DHEA and androstenedione metabolism occurs in the adrenal gland. Abbreviations: DHEA = dehydroepiandrosterone, DHT = 5α-dihydrotestosterone, HSD = hydroxysteroid dehydrogenase, FSH = follicle stimulating hormone, LH = luteinising hormone, G = glucuronide, S = sulphate. Figure adapted from Stanczyk and Bretsky, 2003 and Wilson *et al*, 1998.

(a) Steroid hormone biosynthesis in females



(b) Metabolism of androgens in the adrenal gland and target cells in females



* - glucuronidated conjugates formed by glucuronyl transferase in target cells
\# - sulphated conjugates formed by sulphotransferase in target cells

Yue *et al*, 1998). This provides a mechanism for continued estrogenic stimulation of breast cancer cell growth following the cessation of ovarian function.

Estrogens act via high affinity binding to estrogen receptors (ERα or ERβ), members of the nuclear receptor superfamily of transcription factors which also includes receptors for other steroid hormones (the androgen receptor (AR), progesterone receptor (PR), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (Figure 1.2)), thyroid hormones, retinoids and vitamin D as well as various 'orphan' receptors for which no ligand has been identified (reviewed in Evans, 1988; Laudet, 1997; Whitfield *et al*, 1999)). ERα and ERβ consist of three major functional domains: the amino terminal transactivation domain (NTD), a centrally located DNA binding domain (DBD), a short hinge region and a carboxy terminal ligand binding domain (LBD) (Figure 1.2). Binding of estrogen to the ER-LBD initiates a signalling cascade which regulates the expression of estrogen responsive genes and the control of breast epithelial cell growth, differentiation and survival (Ali and Coombes, 2002).

1.1.4 – Hormonal therapies for breast cancer

Current hormonal therapies for advanced breast cancer exploit the dependence of breast epithelial cells on estrogen signalling pathways. The anti-estrogen, tamoxifen, which blocks ER activity, is effective for the systemic management of advanced breast cancers and the adjuvant treatment of early stage cancers (Osborne, 1998; Johnston, 2001). Clinical trials have also demonstrated potential benefits of tamoxifen for breast cancer prevention (Fisher *et al*, 1998; Veronesi *et al*, 1998; Powles *et al*, 1998; Cuzick *et al*, 2002). Other hormonal therapies act by reducing estrogen biosynthesis. LH releasing hormone (LHRH) agonists, such as goserelin, cause a decrease in LH secretion by the pituitary through a feedback loop and subsequently block follicular activity and

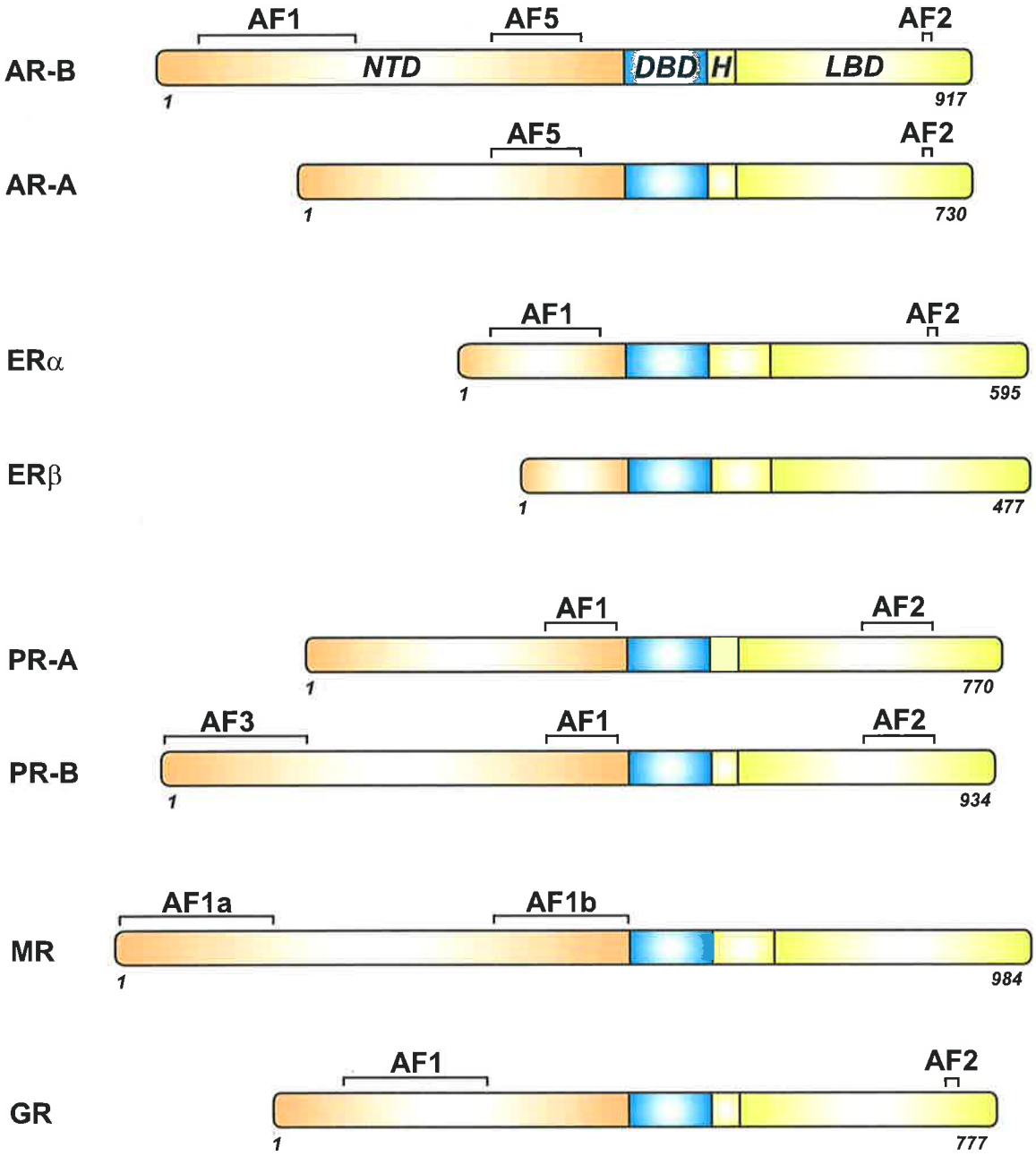


Figure 1.2: Schematic representation of functional domains of human steroid hormone receptors. The amino terminal transactivation domain (NTD) is shaded in orange, the DNA binding domain (DBD) in blue, the hinge (H) in yellow and the ligand binding domain (LBD) in green. Numbers represent the size of each receptor in amino acids. Activation function (AF) domains are indicated where defined. AR = androgen receptor, ER = estrogen receptor, PR = progesterone receptor, MR = mineralocorticoid receptor, GR = glucocorticoid receptor.

estrogen production by the ovaries (Klijn *et al*, 2001). Synthetic progestins, such as medroxyprogesterone acetate (MPA) and megestrol acetate, also effectively diminish ovarian estrogen biosynthesis via a reduction in FSH and LH secretion (Clarke and Sutherland, 1990). Aromatase inhibitors, such as anastrozole, target the enzyme mediating the synthesis of estrogens from androgens (Figure 1.1 (a)). Despite the initial effectiveness of these drugs, many patients unfortunately progress with hormone resistant tumours. This emphasises the need for development of alternative treatment strategies that target other critical regulators of breast cancer cell growth.

1.2 – Androgens and Breast Cancer

1.2.1 – Androgen biosynthesis

An additional important, and often overlooked, hormonal regulator of breast cancer cell growth is the androgen signalling pathway. Androgens, such as testosterone, dehydroepiandrosterone (DHEA) and androstenedione, are C_{19} steroids produced in females by the ovaries, adrenal glands and at peripheral sites (Wilson *et al*, 1998) (Figure 1.1 (a)). In premenopausal women, the ovaries secrete 25-30% of total circulating testosterone, the major physiologically active androgen in the circulation (Labrie *et al*, 2003; Lillie *et al*, 2003). A further 25% is secreted by the adrenal gland and the remainder is produced from androstenedione and DHEA at peripheral sites such as the breast. The serum testosterone concentration in women peaks at a level of approximately 1.3nM at 20-30 years of age, however a decline of approximately 50% occurs by age 40 (Zumoff *et al*, 1995; Davison and Davis, 2003b). In studies investigating the change in serum testosterone levels across the menopausal transition, either no significant change (Longcope *et al*, 1986; Burger *et al*, 2000) or a 15% decrease (Rannevik *et al*, 1995) was observed after menopause. These results support

previous observations that the ovaries continue to secrete testosterone following menopause (Judd *et al*, 1974; Longcope *et al*, 1980).

Testosterone produced by the ovaries and adrenals is transported in the plasma bound to sex hormone binding globulin (SHBG) or albumin. Approximately 66% of serum testosterone is bound to SHBG, 30% is bound to albumin and 1-3% is free (Dunn *et al*, 1981). Testosterone enters cells by diffusion across the cell membrane into the cytoplasm, where it is modified by 5α-reductase, reducing it to the biologically active form 5α-dihydrotestosterone (DHT) (Figure 1.1 (b)). DHT may also be produced in the breast from 5α-androstanedione by 17β-hydroxysteroid dehydrogenase (17β-HSD). As high levels of testosterone and DHT are produced locally in target tissues, measurement of serum testosterone levels may not accurately reflect total androgenic activity. Instead, serum or urinary levels of DHT metabolites such as androsterone-glucuronide, 3α-diol-glucuronide and 3β-diol-glucuronide (Figure 1.1 (b)) may provide a more accurate measure of total body androgen levels. By measuring these metabolites, total androgen levels in women are estimated to be approximately 66% of those observed in men of the same age (Labrie *et al*, 1997a; Labrie *et al*, 1997b; Labrie *et al*, 2003).

The relatively high levels of endogenous androgens estimated by measurement of androgen metabolites suggest an important biological role for androgens in females. Indeed, androgens are required for maintenance of bone tissue and enhance the stimulatory effects of estrogens on bone development (Steinberg *et al*, 1989; Kasperk *et al*, 1989; Davis *et al*, 1995; Raisz *et al*, 1996; Slemenda *et al*, 1996; Davis, 1999). Furthermore, the use of androgens in hormone replacement therapy has been associated with increased well-being, quality of life and libido as well as having beneficial cardiovascular and cognitive effects (Sherwin and Gelfand, 1985; Sherwin, 1988; Davis

et al, 1995; Davis, 1999; Shifren *et al*, 2000; Worboys *et al*, 2001; Davis and Tran, 2001; Davison and Davis, 2003b).

Androgens are also important for normal mammary development and homeostasis. Treatment of rats with androgens during pregnancy leads to suppression of nipple formation in female offspring (Elger and Neumann, 1966; Goldman *et al*, 1976; Forsberg *et al*, 1977; Liao and Dickson, 2002). Conversely, culture of a male rudimentary mammary gland in the absence of androgens results in the development of a female gland (Kratochwil, 1971). These results suggest an inhibitory effect of androgens on the developing breast, which is consistent with epidemiological studies showing a protective effect of androgens against breast cancer development (Section 1.2.2). In the mature breast, however, various studies with animal models have shown both inhibitory and stimulatory effects of androgens on lobuloalveolar and ductal development and epithelial proliferation (Turkington and Topper, 1967; Pashko *et al*, 1981; Luo *et al*, 1997b; Sourla *et al*, 1998; Zhou *et al*, 2000; Jayo *et al*, 2000; Liao and Dickson, 2002). Despite extensive studies of the regulation of breast cancer cell growth by androgens, both clinically (Section 1.2.3) and experimentally (Section 1.2.4), the precise role of androgens in the development and maintenance of normal breast tissue is poorly understood.

1.2.2 – Androgens and breast cancer risk

A large number of epidemiological studies have investigated the association between endogenous androgen levels and breast cancer risk. In retrospective studies, decreased levels of androgens and their metabolites (Figure 1.1 (b)) have been detected in serum or urine from premenopausal women with breast cancer compared to controls (Zumoff *et al*, 1981; Helzlsouer *et al*, 1992; Lee *et al*, 1999a), results which suggest a protective

effect of androgens in premenopausal women. This is further supported by studies of premenopausal women with polycystic ovary syndrome, which is associated with abnormally high androgen levels (Fox *et al*, 1991), who show an approximate 50% decrease in breast cancer incidence compared to the normal population (Coulam *et al*, 1983; Gammon and Thompson, 1991; Pugeat *et al*, 1993; Secreto and Zumoff, 1994; Anderson *et al*, 1997). Epidemiological studies of male breast cancer provide further insight into the association between androgens and breast cancer risk. The incidence of male breast cancer is rare compared to female breast cancer, accounting for approximately 1% of all cases (Rose, 1988; Hecht and Winchester, 1994). Clinical studies have shown that male breast cancer is associated with androgen deficiency (Thomas *et al*, 1992). Additionally, low endogenous androgen levels in men, or androgen blockade, are associated with breast growth (Staiman and Lowe, 1997). The apparent protective effect of androgens against breast cancer development that is suggested by these studies is in line with *in vitro* studies showing a suppressive effect of androgens on breast cancer cell proliferation (Section 1.2.4). However some studies have reported an association between high levels of androgens and increased risk for breast cancer (Secreto *et al*, 1984; Secreto *et al*, 1989; Yu *et al*, 2003), or no significant association at all (Wysowski *et al*, 1987; Thomas *et al*, 1997b), in premenopausal women. Differences in the experimental approach are likely to account for the sometimes contradictory results as these studies vary considerably in their design (prospective *versus* retrospective), the androgens measured (serum free or total testosterone, serum adrenal androgens, urinary testosterone, urinary adrenal androgens or urinary androgen metabolites) and adjustments made (eg for parity or endogenous estrogen levels). In premenopausal women who do develop breast cancer, low levels of serum androgens are associated with rapid growth, increased rate of recurrence and a poor response to endocrine therapy (Bulbrook and Thomas, 1989).

In prospective studies, Bulbrook *et al* (1971) found an association between low levels of urinary androgen metabolites and increased breast cancer risk in premenopausal women. Extended follow-up of the same cohort, with increased numbers of breast cancer cases accumulated over a 37 year period, found that women under age 50, who were mostly premenopausal, with urinary androsterone in the highest tertile had a relative risk for breast cancer of 0.42 (95% confidence interval 0.43-2.45) compared to women with levels in the lowest tertile (Wang *et al*, 2000). This also supports a protective role for androgens against breast cancer in premenopausal women. However in women 50 years and above in this cohort, who were mostly postmenopausal, levels of urinary androsterone in the highest tertile was associated with a relative risk of 1.7 (95% confidence interval 2.08-3.94) compared to women with levels in the lowest tertile (Wang *et al*, 2000). Collectively, results from this long term study of premenopausal and postmenopausal women imply that low androgen levels may be related to an earlier age at diagnosis. Other prospective (Zumoff *et al*, 1981; Dorgan *et al*, 1996; Berrino *et al*, 1996; Dorgan *et al*, 1997; Zeleniuch-Jacquotte *et al*, 1997; Thomas *et al*, 1997a; Cauley *et al*, 1999) and retrospective (Gordon *et al*, 1990; Secreto *et al*, 1991) studies have also observed a significant association between elevated levels of androgens and increased breast cancer risk in postmenopausal women. There are some studies which have reported either no significant association between androgens and breast cancer risk (Wysowski *et al*, 1987; Garland *et al*, 1992; Lipworth *et al*, 1996; Hankinson *et al*, 1998) or an association between low androgen levels and increased breast cancer risk (Thijssen *et al*, 1975) in postmenopausal women. These discrepancies in the reported results are again likely to be attributed to differences in experimental design as outlined above. However the emerging trend from the studies presented above is that an increased risk for breast cancer is associated with low androgen levels in premenopausal

women and high androgen levels in postmenopausal women (Adams, 1998; Lillie *et al*, 2003).

1.2.3 – Targeting androgen action in the clinical management of breast cancer

The clinical efficacy of androgenic drugs for the treatment of breast cancer is further evidence that androgens are critical regulators of breast cancer cell growth. Androgenic agents such as testosterone propionate and fluoxymesterone acetate were used as treatments for breast cancer until the 1960s. While these drugs induced tumour regression with an efficacy comparable to that of current hormonal therapies (Fels, 1944; The Cooperative Breast Cancer Group, 1961), severe masculinising side effects such as hirsutism and acne have limited their clinical utility. In addition, androgenic agents have been shown to improve response to anti-estrogen therapies (Tormey *et al*, 1983; Ingle *et al*, 1988; Ingle *et al*, 1991) and induce remission in patients whose tumours progress following first round anti-estrogen therapy (Manni *et al*, 1981). Androgens were initially believed to suppress tumour growth through antagonism of estrogen action and also via suppression of pituitary gonadotrophin release and subsequent inhibition of ovarian estrogen secretion (Manni *et al*, 1981). However androgens have also been demonstrated to directly inhibit breast cancer cell proliferation (Section 1.2.4) by acting through their cellular target, the AR (Section 1.3).

Studies of the mechanism of action of the synthetic progestin MPA, when used in a clinical setting, provide further evidence of the significance of androgen signalling pathways in breast cancer cells. Although initially thought to act exclusively through the PR, masculinising side effects observed in women treated with MPA suggested that it has some androgenic properties (Bullock and Bardin, 1977). The androgenic activity of MPA has been further demonstrated by clinical (Birrell *et al*, 1995b) (Section 1.3.1)

and *in vitro* studies (Poulin *et al*, 1989a; Poulin *et al*, 1991; Hackenberg *et al*, 1993a; Bentel *et al*, 1999) (Section 1.2.4.1) which show that the AR can mediate MPA action in breast cancer cells.

1.2.4 – Proliferative effects of androgens

1.2.4.1 – Cell lines

In addition to their growth inhibitory effects *in vivo*, androgens also regulate the proliferation of breast cancer cell lines *in vitro*. As described below, a proliferative response to androgens is only observed in breast cancer cell lines expressing the AR, however the effect of androgens may be either stimulatory or inhibitory.

The effects of androgens on basal proliferation of commonly used breast cancer cell lines are summarised in Table 1.1. T-47D, ZR-75-1 and MCF-7 are the most widely used AR positive cell lines of those listed. Most authors agree that the natural androgens DHT and testosterone and the synthetic androgen mibolerone inhibit proliferation of T-47D and ZR-75-1 cell lines (Poulin *et al*, 1988; Simard *et al*, 1990; Labrie *et al*, 1990a; de Launoit *et al*, 1991; Birrell *et al*, 1995a; Ortmann *et al*, 2002). The degree of inhibition of basal proliferation by androgens ranges from 30-65% relative to controls after 10-20 days exposure in these studies. Variation in the potency of androgens is likely due to the dose and type of androgen used, initial cell seeding density, length of the assay and clonal variation of cell lines. In the MCF-7 cell line both stimulatory (Zava and McGuire, 1978; Hackenberg *et al*, 1988; Hackenberg *et al*, 1993b; Birrell *et al*, 1995a; Maggiolini *et al*, 1999) and inhibitory (Simard *et al*, 1990; Szelei *et al*, 1997; Ortmann *et al*, 2002; Ando *et al*, 2002) responses to androgens have been reported, with variation possibly due to differences in assay conditions described above. In ZR-75-1 and MCF-7 cells, the inhibitory effects of androgens have been

Table 1.1: Steroid hormone receptor profiles* of common breast cancer cell lines and their proliferative responses** to androgenic ligands (DHT, testosterone, mibolerone) and the synthetic progestin MPA *in vitro*.

Cell line	**Receptor Expression**			**Proliferative Response to Ligands**		**References**
	AR	ER	PR	androgens	MPA	
T-47D	+	+	+	↓	↓	Reddel *et al*, 1985; Reece *et al*, 1988; Sutherland *et al*, 1988; Haagensen *et al*, 1992; Hall *et al*, 1992; Classen *et al*, 1993; Birrell *et al*, 1995a; Bentel *et al*, 1999; Ortmann *et al*, 2002
ZR-75-1	+	+	+	↓	↓	Reddel *et al*, 1985; Poulin *et al*, 1988; Sutherland *et al*, 1988; Dumont *et al*, 1989; Labrie *et al*, 1990a; de Launoit *et al*, 1991; Birrell *et al*, 1995a; Bentel *et al*, 1999; Magklara *et al*, 2002; Ortmann *et al*, 2002
MCF-7	+	+	+	↑ or ↓	↓	Lippman *et al*, 1976; Hackenberg *et al*, 1988; Sutherland *et al*, 1988; Birrell *et al*, 1995a; Ortmann *et al*, 2002; Andò *et al*, 2002
BT-474	+	+	+	?	↓	Reddel *et al*, 1985; Sutherland *et al*, 1988; Magklara *et al*, 2002
MDA-MB-453	+	–	–	↑	↓	Hall *et al*, 1992; Hall *et al*, 1994; Birrell *et al*, 1995a; Bentel *et al*, 1999
MFM-223	+	–	–	↓	↓	Hackenberg *et al*, 1991; Hackenberg *et al*, 1992; Hackenberg *et al*, 1993a; Magklara *et al*, 2002;
MDA-MB-231	–	–	–	–	–	Reddel *et al*, 1985; Sutherland *et al*, 1988; Classen *et al*, 1993; Birrell *et al*, 1995a
BT-20	–	–	–	–	–	Reddel *et al*, 1985; Sutherland *et al*, 1988; Birrell *et al*, 1995a; Bentel *et al*, 1999; Magklara *et al*, 2002

* +, receptor positive; –, receptor negative

** - no proliferative response; ↑, basal proliferation stimulated; ↓, basal proliferation inhibited; ?, not reported

associated with inhibition of cell cycle progression (de Launoit *et al*, 1991; Szelei *et al*, 1997; Ando *et al*, 2002). Androgens can also stimulate proliferation of MDA-MB-453, EFM-19 and EVSA-T cells (Hackenberg *et al*, 1988; Marugo *et al*, 1992; Hall *et al*, 1994; Birrell *et al*, 1995a) but inhibit MFM-223 cells (Hackenberg *et al*, 1991; Hackenberg *et al*, 1993c). AR negative breast cancer cell lines, such as MDA-MB-231 and BT-20, show no proliferative response to androgens (Birrell *et al*, 1995a). These studies suggest that AR expression is required for a proliferative response to androgens, however the level of AR expression or the expression of other steroid hormone receptors (Table 1.1) does not appear to be associated with the magnitude or direction of the response. The apparent cell type specific responses to treatment with androgens may be explained by the expression profile of steroid metabolising enzymes or AR cofactors (Section 1.3.7) within cell lines and sub-lines.

Numerous studies have investigated the effects of androgens on the proliferation of breast cancer cells cultured in the presence of estrogens. These experimental systems may be a closer reflection of the situation *in vivo*, where circulating androgens and estrogens can both regulate breast cancer cells. DHT and testosterone significantly inhibit E_2-stimulated proliferation of T-47D, ZR-75-1 and MCF-7 cells (Poulin *et al*, 1988; Reese *et al*, 1988; Labrie *et al*, 1990a; Labrie *et al*, 1990b; de Launoit *et al*, 1991; Boccuzzi *et al*, 1993; Ando *et al*, 2002). The magnitude of the inhibitory effect in these studies ranged from 50-100% relative to cells treated with E_2 only. The adrenal androgens androstenedione, DHEA and DHEA-sulphate (DHEA-S) have also been shown to inhibit E_2-stimulated proliferation of these cell lines, although with a lower potency than DHT and testosterone (Boccuzzi *et al*, 1993; Ando *et al*, 2002). The antagonism of E_2 stimulated cell proliferation by androgens is discussed further in Chapter 3.

Although not a classical androgen, the synthetic progestin MPA has also been shown to exclusively inhibit basal proliferation of T-47D, ZR-75-1, MCF-7, EFM-19, SK-BR-3, MFM-223 and MDA-MB-453 breast cancer cell lines *in vitro* via a mechanism involving the AR (Sutherland *et al*, 1988; Poulin *et al*, 1989a; Hackenberg *et al*, 1990; Poulin *et al*, 1991; Classen *et al*, 1993; Hackenberg *et al*, 1993a; Bentel *et al*, 1999) (Table 1.1). Inhibition ranged from 30-100% relative to controls in these studies. Treatment with MPA is also associated with arrest at the G1 phase of the cell cycle (Sutherland *et al*, 1988). In contrast, there is no proliferative effect of MPA on the steroid receptor negative cell lines MDA-MB-231 and BT-20 (Classen *et al*, 1993; Bentel *et al*, 1999). These studies have shown that higher concentrations of MPA (typically 10-100nM) are required to maximally inhibit cell proliferation compared to DHT and testosterone (typically 0.1-10nM). These concentrations are comparable to the high doses of MPA required for clinical regression of breast cancers. Furthermore, MPA has been reported to bind to the wild type AR with a lower affinity (K_d = 1.7-3.6nM) than DHT (K_d = 0.2-0.5nM) (Hackenberg *et al*, 1993a; Kemppainen *et al*, 1999), suggesting that higher concentrations of MPA are required for maximal proliferative response. MPA has also been shown to completely inhibit proliferation of ZR-75-1, MCF-7, EFM-19 and T-47D cells cultured in the presence of E_2 (Sutherland *et al*, 1988; Poulin *et al*, 1989a; Hackenberg *et al*, 1990; Poulin *et al*, 1991).

Early studies proposed that the effects of androgens on breast cancer cell proliferation were mediated by a mechanism involving the ER. This hypothesis was supported by demonstration that androgens, at high concentrations, could bind to the ER (K_d > 100nM), preventing activation by estrogens (Rochefort and Garcia, 1976). Androgens have also been shown to downregulate expression of ER (Zava and McGuire, 1978; Poulin *et al*, 1989b), which suggests another possible mechanism for

reducing the mitogenic effects of estrogens. More recent studies have suggested that the proliferative effects of androgens are mediated by their cognate receptor, the AR. This is supported by the response of breast cancer cells to AR antagonists, such as hydroxyflutamide (OHF), bicalutamide and nilutamide, as well as antisense oligonucleotides directed against the AR, which can each block the proliferative effects of androgens (Poulin *et al*, 1988; Hall *et al*, 1994; Birrell *et al*, 1995a; Ando *et al*, 2002). Furthermore, most studies observed significant proliferative responses with androgen concentrations of 0.1-1nM, which is consistent with the binding affinity of DHT, testosterone and mibolerone for the AR (Kemppainen *et al*, 1999). These concentrations are lower than that required for binding to the ER, suggesting that the effects of physiological concentrations of androgens are primarily mediated by the AR and not by the ER.

AR antagonists have also been used to demonstrate that the proliferative effects of MPA are mediated, at least in part, by the AR (Poulin *et al*, 1989a; Hackenberg *et al*, 1993a; Bentel *et al*, 1999). This is further validated by the observed inhibitory effect of MPA on MDA-MB-453 and MFM-223 breast cancer cells, which express high levels of AR but no detectable PR or ER (Hackenberg *et al*, 1993a; Bentel *et al*, 1999). Of particular interest are the divergent proliferative effects of DHT and MPA on MDA-MB-453 cells (Birrell *et al*, 1995a; Bentel *et al*, 1999). As these cells express the AR, but no other sex steroid hormone receptors, the AR mediates the action of both ligands in this cell line. However the molecular mechanisms by which the AR can mediate these divergent proliferative responses have not yet been defined. Further characterisation of the molecular responses to DHT and MPA in MDA-MB-453 cells is presented in Chapters 5 and 6.

1.2.4.2 – Animal models

As observed in breast cancer cell lines, antagonism between estrogens and androgens has also been investigated in animal models of breast cancer. This was first described by Lacassagne (1936), who demonstrated that the occurrence of mouse mammary tumours stimulated by E_1 was considerably delayed by testosterone propionate. More recent studies have shown that ZR-75-1 xenografts stimulated by exogenous estrogen (E_2 or E_1) in ovariectomised athymic nude mice regress upon administration of DHT, MPA or DHEA (Dauvois *et al*, 1991; Couillard *et al*, 1998). Likewise, E_2-stimulated growth of dimethylbenz(a)anthracene (DMBA)-induced mammary tumours in ovariectomised rats is inhibited by DHT (Dauvois *et al*, 1989). DHT also increases the number of complete tumour regressions, decreases the progression of existing tumours and the decreases the incidence of new tumours in this model (Dauvois *et al*, 1989). In ovariectomised rhesus monkeys, E_2-induced mammary epithelial cell proliferation, examined by immunohistochemical detection of the Ki-67 antigen, is inhibited by treatment with testosterone (Zhou *et al*, 2000). These results suggest that antagonism of estrogen action by androgens may also occur in non-malignant cells. In general, the effects of androgens on normal mammary epithelial cell proliferation have been poorly described. This is discussed in further detail in Section 9.2.2.2.

Few studies using *in vivo* animal models of breast cancer have investigated the effects of androgens in the absence of estrogens. Early studies showed that testosterone propionate inhibits the growth of DMBA-induced mammary tumours in rats (Zava and McGuire, 1977). More recently, a decrease in the incidence and size of DMBA-induced mammary tumours was observed in response to the adrenal androgen DHEA (Li *et al*, 1994; Luo *et al*, 1997a; Luo *et al*, 1997b). MPA has also been shown to inhibit mammary tumour growth in this model (Li *et al*, 1993; Labrie *et al*, 1993).

1.3 – Androgen Receptor (AR) Action

1.3.1 – AR expression in the breast

The action of androgens is mediated by high affinity binding to the AR in target cells. AR immunoreactivity is observed in the nuclei of breast epithelial cells, with weak to no immunoreactivity detected in surrounding stromal cells (Kuenen-Boumeester *et al*, 1992; Isola, 1993; Hall *et al*, 1996; Hall *et al*, 1998; Zhuang *et al*, 2003; Moinfar *et al*, 2003). The presence of AR within breast tumour cells provides evidence that they can potentially respond to manipulation of androgen signalling pathways. The AR, found in 70-90% of primary breast tumours, is expressed at a higher frequency than either ER (55-80%) or PR (40-70%) (Lea *et al*, 1989; Kuenen-Boumeester *et al*, 1992; Isola, 1993; Moinfar *et al*, 2003). Although the levels of each of these receptors is lower in metastatic tumours, AR is still expressed at the highest frequency (75%) and approximately 25% of metastatic tumours contain AR as their sole sex steroid receptor (Lea *et al*, 1989; Moinfar *et al*, 2003). The loss of receptor expression upon tumour progression can be explained by de-differentiation of tumour cells (Helin *et al*, 1989) and it has been postulated that the AR may be the last receptor to be lost during progression to a receptor negative phenotype.

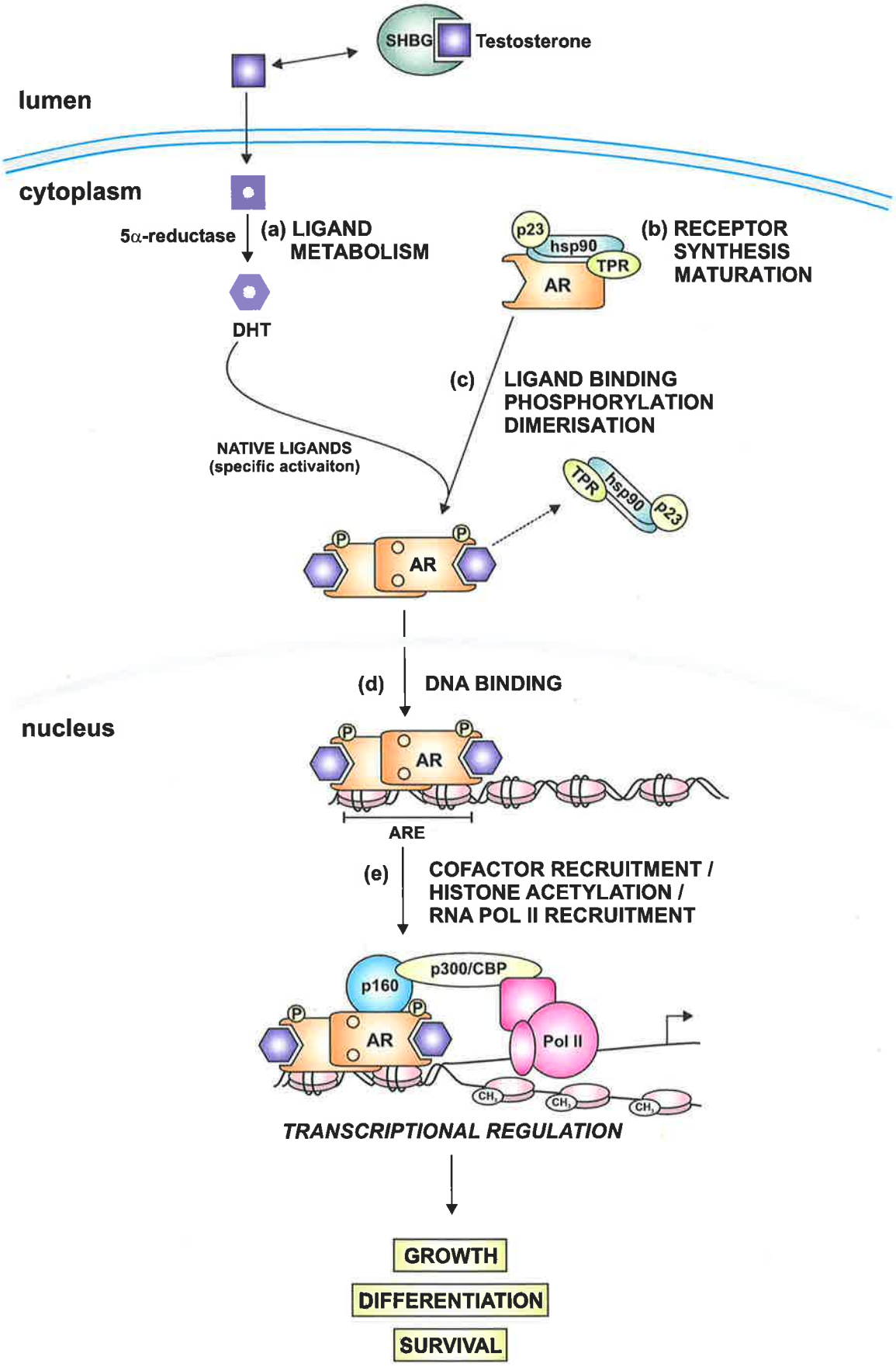
AR levels in tumour cells are highly predictive of the response to various endocrine therapies. Presence of AR in a metastatic tumour greatly improves the response rate to tamoxifen, provided that the tumours also contain ER, while postmenopausal women with AR negative primary tumours have a shorter survival than those with AR positive primary tumours (Nomura *et al*, 1980; Bryan *et al*, 1984; Langer *et al*, 1990; Kuenen-Boumeester *et al*, 1996). Response to therapy with synthetic progestins is also associated with AR levels (Teulings *et al*, 1980). In a cohort of women who had

relapsed on treatment with tamoxifen and responded to second round therapy with MPA, AR levels in the primary tumour were significantly higher than in tumours from patients who did not respond to MPA (Birrell *et al*, 1995b). Other tumour characteristics, such as ER and PR levels, size and nodal involvement were not correlated with the response to MPA. Furthermore, the likelihood and duration of response to second round treatment with MPA was predicted by the level of AR expression in the primary tumour. These observations support *in vitro* data demonstrating that the inhibitory effect of MPA on breast cancer cell proliferation is mediated by the AR (Section 1.2.4.1).

1.3.2 – The androgen signalling axis

The androgen signalling axis collectively encompasses cellular mechanisms leading to the regulation of target gene expression and subsequent control of cellular behaviour. Testosterone, transported in the circulation bound to SHBG or albumin, enters cells by diffusion across the cell membrane into the cytoplasm where it is converted by 5α-reductase to the more potent metabolite, DHT (Figure 1.3 (a)). Following translation of the AR, association with a multi-protein maturation complex containing heat shock proteins (such as Hsp40, Hsp70 and Hsp90) and chaperones (such as p23 and Hop) increases its affinity for androgens (Pratt and Toft, 1997) (Figure 1.3 (b)). In the absence of ligand the AR is localised in the cytoplasm (Tyagi *et al*, 2000). DHT binding induces a conformational change in the AR ligand binding domain (LBD) leading to release of the maturation complex, receptor dimerisation and translocation to the nucleus (Tyagi *et al*, 2000) (Figure 1.3 (c)), which may be facilitated by ligand induced conformational changes exposing the nuclear localisation signal. Binding of the ligand-AR complex to androgen response elements (AREs) in the promoter regions of target genes (Figure 1.3 (d)) stimulates recruitment of coregulatory proteins and

Figure 1.3: Mechanisms of ligand dependent induction of androgen responsive gene expression. Testosterone, transported in the circulation bound to SHBG, diffuses across the membrane into the cytoplasm of target cells. *(a)* Here, it is converted by 5α-reductase to is more potent derivative 5α-dihydrotestosterone (DHT). *(b)* AR proteins undergo synthesis and maturation in the cytoplasm; association with a multiprotein chaperone complex is required for high affinity ligand binding. *(c)* DHT binding induces conformational changes in the receptor, dissociation of the maturation complex, receptor phosphorylation, dimerisation and nuclear translocation. *(d)* The AR dimer binds to AREs situated in the regulatory regions of androgen responsive genes. *(e)* This is followed by recruitment of cofactors, relaxation of DNA structure by histone acetylation and recruitment of other components of the general transcription machinery, including RNA polymerase II (Pol II). Phosphorylation of Pol II enables RNA synthesis from genes regulating cell growth differentiation and survival. Following ligand dissociation, the AR is exported back to the nucleus, where it reassociates with ligand and undergoes further cycles of nuclear import and export. Figure adapted from Buchanan, 2002.


SHBG
Testosterone
lumen
cytoplasm
5α-reductase
(a) LIGAND METABOLISM
DHT
p23
hsp90
TPR
AR
(b) RECEPTOR SYNTHESIS MATURATION
(c) LIGAND BINDING PHOSPHORYLATION DIMERISATION
NATIVE LIGANDS (specific activaiton)
P
AR
(d) DNA BINDING
nucleus
ARE
(e) COFACTOR RECRUITMENT / HISTONE ACETYLATION / RNA POL II RECRUITMENT
p300/CBP
p160
Pol II
CH3
TRANSCRIPTIONAL REGULATION
GROWTH
DIFFERENTIATION
SURVIVAL

components of the general transcriptional machinery, including transcription factor IIB (TFIIB), TFIID and RNA polymerase II, resulting in the stimulation of gene transcription (Figure 1.3 (e)). Following ligand dissociation, the AR is exported back to the cytoplasm where it can undergo further rounds of ligand binding and nuclear import and export (Tyagi *et al*, 2000). This model characterises the mechanisms associated with induction of gene transcription by the AR. However, studies performed in this thesis (Chapter 6) suggest that the androgen signalling axis may also repress gene expression. The mechanisms by which this occurrs are currently poorly understood.

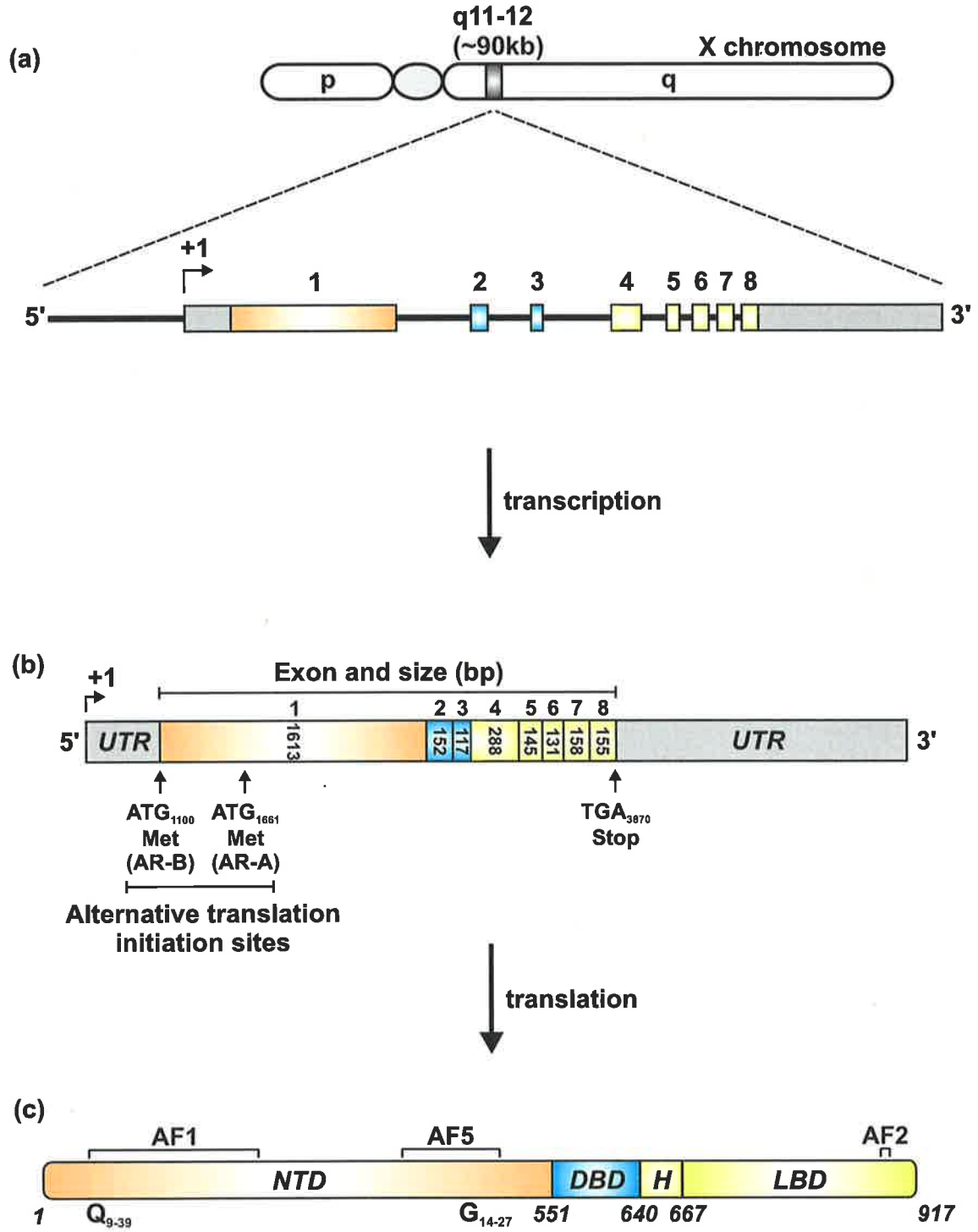
Characterisation of the genes regulated by the AR in breast cancer cells will enable a better understanding of the mechanisms by which androgens modulate cellular behaviour. There are currently very few genes that have been identified as direct targets of the AR in breast cancer cells. The best characterised of these genes are prostate specific antigen (PSA) and the gross cystic disease fluid proteins (GCDFP-15, GCDFP-24/Apolipoprotein D and GCDFP-44), which are induced by androgens in breast cancer cell lines (Murphy *et al*, 1987; Chalbos *et al*, 1987; Dumont *et al*, 1989; Simard *et al*, 1990; Simard *et al*, 1992; Hall *et al*, 1994; Yu *et al*, 1994b; Zarghami *et al*, 1997; Hsieh *et al*, 1997; Lapointe *et al*, 1999; Magklara *et al*, 2000). However the roles of PSA and the GCDFPs in breast cancer cell growth, differentiation and survival are unclear. The oncoprotein bcl-2, which regulates cell death by inhibiting apoptosis and binding other proteins involved in apoptotic mechanisms (reviewed in Reed, 1997), is significantly downregulated by DHT in ZR-75-1 cells (Lapointe *et al*, 1999; Kandouz *et al*, 1999). This is correlated with a proapoptotic effect of DHT on ZR-75-1 cells (Kandouz *et al*, 1999). Although the absence of AREs in the bcl-2 gene promoter suggests that the AR may indirectly regulate bcl-2 expression, these observations nevertheless provide evidence for a mechanism by which androgens may inhibit breast cancer cell

proliferation. The identification of other AR target genes potentially involved in modulating breast cancer cell proliferation is addressed in Chapter 6, while the mechanisms associated with induction of androgen responsive genes in breast cancer cells have been further investigated, using PSA as a model gene, in Chapter 8.

1.3.3 – AR gene and protein structure

The human AR is encoded by a single copy gene more than 90kb in length located on the X chromosome at Xq11-12 (Brown *et al*, 1989; Brinkmann *et al*, 1992) (Figure 1.4 (a)). The gene is divided into eight exons, with exon 1 encoding the amino terminal transactivation domain (NTD), exons 2 and 3 encoding the DNA binding domain (DBD) and exons 4-8 encoding the hinge region and the ligand binding domain (LBD) (Lubahn *et al*, 1988; Chang *et al*, 1988; Tilley *et al*, 1989). The AR mRNA is 10.5kb in length (Figure 1.4 (b)), with 3.0kb coding for a 917 amino acid protein with a molecular weight of 98.9kDa (Tilley *et al*, 1989) (Figure 1.4 (c)). A second AR species of 730 amino acids and 80kDa (AR-A) is an amino terminal truncated form of the full length receptor (AR-B) resulting from translation initiation at an internal ATG codon positioned 187 codons downstream of the standard translation initiation site (Wilson and McPhaul, 1994) (Figure 1.2). Post translational modifications, such as phosphorylation, increase the apparent molecular weight of the AR-B (~110kDa) and AR-A (~87kDa) when analysed by SDS polyacrylamide gel electrophoresis. Both isoforms of the AR co-exist in genital skin fibroblasts, with the B isoform approximately ten times more abundant than the A isoform. The A isoform has been detected in various adult tissues (Wilson and McPhaul, 1996) but not in the breast. Throughout this thesis, discussion will focus on the function and expression of the predominant B isoform.

Figure 1.4: AR gene and protein structure. *(a)* Schematic representation (not to scale) of the AR gene on chromosome Xq11-12 showing the eight exons (numbered), which are separated by up to 16kb of intronic sequences. *(b)* AR mRNA transcript showing coding exons and 5' and 3' untranslated regions (UTR). Translation is primarily directed from the first of two initiating ATG codons. *(c)* Structure of the predominant 917 amino acid (AR-B) form of the AR. Indicated are the amino-terminal transactivation domain (NTD, orange), the DNA binding domain (DBD, blue), the hinge region (H, yellow), and ligand binding domain (LBD, green), spanning amino acids as indicated. The positions of two polymeric amino acid stretches in the NTD, Q_n and G_n, and the activation functions, AF1 and AF5 in the NTD and AF2 in the LBD are shown. Figure adapted from Buchanan, 2002.


(a)
q11-12
(~90kb)
X chromosome
p
q
+1
1
2
3
4
5 6 7 8
5'
3'
transcription
(b)
+1
Exon and size (bp)
1
2 3 4 5 6 7 8
5'
UTR
1613
152
117
288
145
131
158
155
UTR
3'
ATG1100
Met
(AR-B)
ATG1661
Met
(AR-A)
TGA3870
Stop
Alternative translation
initiation sites
translation
(c)
AF1
AF5
AF2
NTD
DBD
H
LBD
1
Q9-39
G14-27
551
640
667
917

Conservation of critical functional domains and sequence motifs between members of the nuclear receptor superfamily suggests that their genes evolved from a common ancestor. Phylogenetic analysis has grouped family members into six distinct classes (Laudet, 1997). Ancestral members of each of these classes evolved by gene duplication before the arthropod/vertebrate split. The ER was the first member of class III which comprises steroid hormone receptors (Figure 1.2). Further rounds of gene duplication after the evolution of vertebrates gave rise to other members of the steroid receptor family (Thornton, 2001). Phylogenetically, the AR is most closely related to the PR – both are thought to have evolved from an ancestral receptor which could bind both androgens and progestins (Thornton, 2001).

1.3.4 – Amino terminal transactivation domain (NTD)

1.3.4.1 – Activation domains in the AR-NTD

The AR-NTD shows poor homology with the corresponding region of other members of the steroid receptor family and is unique amongst steroid hormone receptors in that it is the predominant region directing transactivation activity. The AR-NTD spans amino acids 1-551 and contains two activation function domains, AF1 and AF5 (Figure 1.4 (c)). Two LxxLL-like (where L = leucine and x = any amino acid) pentapeptide motifs, one within AF1 (^{23}FQNLF^{27}) and one within AF5 (^{432}WHTLF^{436}), mediate a ligand-induced interaction with the AF2 domain in the LBD which is essential for AR activity (Ikonen *et al*, 1997; Berrevoets *et al*, 1998; He *et al*, 1999; He *et al*, 2000). This so-called N/C interaction may occur in an inter-or intra-molecular fashion and confers increased stability and DNA binding affinity to the ligand-receptor complex. The AF1 may also mediate interactions with LxxLL motifs found in p160 coactivator molecules (Section 1.3.7), which may further facilitate N/C interactions by acting as bridging molecules between the NTD and the LBD (Voegel *et al*, 1998; Onate *et al*, 1998; Feng

et al, 1998; Darimont *et al*, 1998; Ma *et al*, 1999; Irvine *et al*, 2000). The N/C interaction is discussed in further detail in Section 5.1.

1.3.4.2 – Polymorphic regions in the AR-NTD

Exon 1 of the AR gene also contains two polymorphic trinucleotide satellite repeats, CAG and GGC, which can modulate AR activity. The lengths of the polyglutamine (polyQ, encoded by CAG) and polyglycine (polyG, encoded by GGC) tracts vary from 9-39 and 14-27 residues respectively in the normal population (Edwards *et al*, 1992; Giovannucci *et al*, 1997) (Figure 1.4 (c)). Variation in the length of these regions has led to discrepancies in the reported size and numbering of the AR[1]. *In vitro* transactivation assays have shown that polyQ length is inversely related to the transactivation potential of the AR (Mhatre *et al*, 1993; Chamberlain *et al*, 1994; Kazemi-Esfarjani *et al*, 1995; Tut *et al*, 1997; Beilin *et al*, 2000). The modulation of AR transactivation potential with varying polyQ lengths may be due to altered interaction with cofactor molecules (Hsiao *et al*, 1999; Irvine *et al*, 2000; Buchanan, 2002).

1.3.4.3 – AR polyQ length and breast cancer

Variation in the length of the AR polyQ tract is associated with development of certain diseases. Long polyQ tracts (>28 repeats) are associated with an increased risk of impaired spermatogenesis (Tut *et al*, 1997) and polyQ tracts greater than 40 repeats are commonly found in patients with Kennedy's disease (La Spada *et al*, 1991). Short polyQ tracts (<20 repeats) are associated with a higher relative risk of prostate cancer development (Irvine *et al*, 1995; Stanford *et al*, 1997; Giovannucci *et al*, 1997; Ingles *et al*, 1997; Hakimi *et al*, 1997). There is conflicting data on the association between AR

[1] The numbering system used in this thesis is according to that of Tilley *et al*, 1989.

polyQ length and the risk of breast cancer development. In one study breast cancer risk doubled in women with a sum of polyQ length (on each allele) greater than 40 repeats (Giguere *et al*, 2001). An additional study has demonstrated that women carrying *BRCA1* mutations are at increased risk of developing breast cancer, and at an earlier age, if they have at least one AR allele with a polyQ length greater than 28 repeats (Rebbeck *et al*, 1999). Furthermore, longer polyQ repeat lengths have been associated with an increased risk of breast cancer in women who have previously used oral contraceptives (Suter *et al*, 2003). Increased polyQ length has also been observed in a study of male breast cancers (Wu *et al*, 1999). These results suggest that decreased AR activity resulting from increased polyQ length may diminish the protective effects of androgen signalling on breast cancer development. In contrast, other studies have failed to establish a significant association between polyQ length and breast cancer risk (Spurdle *et al*, 1999; Dunning *et al*, 1999; Young *et al*, 2000; Kadouri *et al*, 2001) or age of diagnosis (Given *et al*, 2000). Another study has shown that short polyQ repeat lengths were found to occur more frequently in more aggressive breast cancers (Yu *et al*, 2000). The discrepancies between these studies may arise from differences in the sample populations, in particular the age at which cancer was diagnosed and whether there was a family history of breast cancer. Additionally, as the AR is on the X chromosome, one allele is suppressed by X inactivation in female breast cancer cells. The studies described above have determined AR polyQ length using DNA extracted from tumour samples and this method does not distinguish which allele is expressed in the tumour. Therefore, the conflicting reports on the association between polyQ length and breast cancer may also be explained by the fact that polyQ length measured in these studies may not accurately reflect AR activity in tumour cells. These observations, in particular the potential for AR polyQ length to modify the penetrance of *BRCA1*

mutations, provide compelling evidence that the AR, in addition to androgens themselves, plays a critical role in breast tumourigenesis.

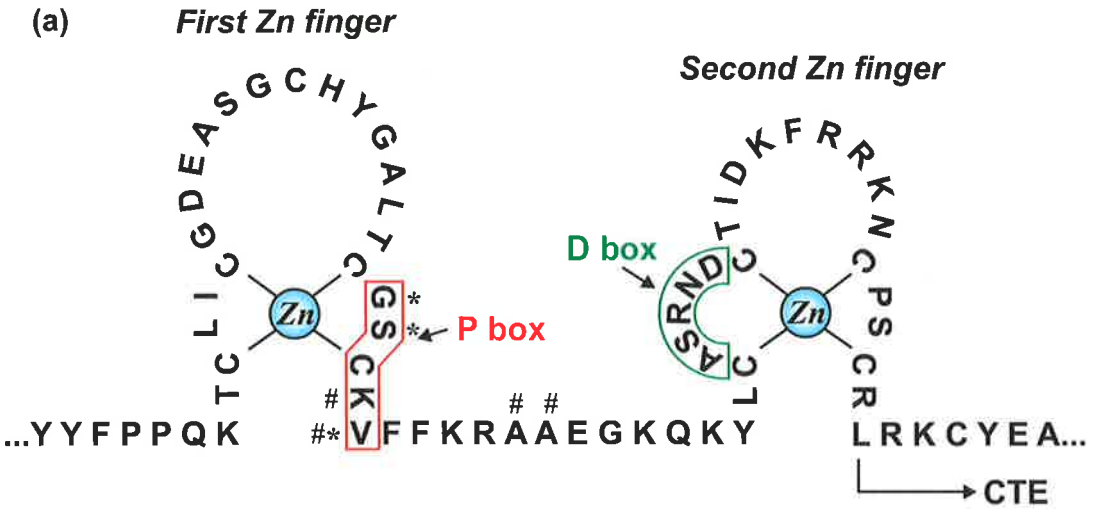
1.3.5 – DNA binding domain (DBD)

The DBD, spanning amino acids 552-640 (Figure 1.4 (c)), is cysteine rich and is highly conserved across steroid hormone receptors (Lubahn *et al*, 1988; Chang *et al*, 1988; Tan *et al*, 1988; Tilley *et al*, 1989). It contains two zinc finger motifs formed by interactions between cysteine residues and zinc molecules (Figure 1.5 (a)). Studies of the GR suggest that the amino terminal zinc finger, containing the P box, is involved in recognition of specific responsive elements in the regulatory regions of target genes and interacts with the major groove of the DNA (Umesono and Evans, 1989; Perlmann *et al*, 1993). The carboxy terminal zinc finger, containing the D box, is involved in recognition of spacer sequences (see below) and in receptor dimerisation (Luisi *et al*, 1991; Dahlman-Wright *et al*, 1991; Dahlman-Wright *et al*, 1993).

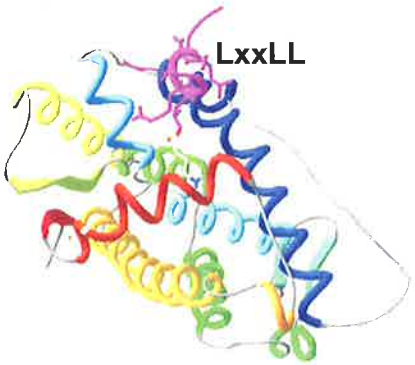
Androgen response elements (AREs), consisting of hexameric half-sites arranged as inverted repeats separated by a 3bp spacer, show high homology with response elements for the PR and GR. Multiple AREs are often present in gene promoters, either in close proximity or at distant sites, to achieve maximal androgen inducibility (Adler *et al*, 1992; Rennie *et al*, 1993; Grad *et al*, 1999). Two classes of AREs have been identified: ARE class I (R<u>G</u>AA<u>C</u>AngnT<u>G</u>**T**N<u>C</u>**T**, where R = A or G and N = any nucleotide) and ARE class II (R<u>G</u>**GA**<u>C</u>AnnaA<u>G</u>**CCAA**) (Reid *et al*, 2000). Zinc fingers of the DBD form hydrogen bonds with guanine bases at the underlined positions. Additional consensus features in these AREs are indicated in bold type. There is, however, considerable variation in the natural ARE sequences that exist in the regulatory regions of androgen responsive genes.

Figure 1.5: *(a)* Schematic representation of the AR-DBD. The AR-DBD contains two zinc finger motifs, formed by interactions between cysteine residues and zinc molecules, and a short C-terminal extension (CTE) that forms part of the hinge region. The first zinc finger, which contains the P box, recognises specific androgen response elements (AREs) and binds to the major groove of DNA. The second zinc finger contains the 'D box' which mediates dimerisation between steroid receptor monomers. Sequences in the CTE bind to the minor groove of DNA on the opposite side of the helix to the first zinc finger and mediate sequence specificity of steroid receptors. * denotes amino acids that determine the specificity of DNA binding, # denotes amino acids likely to make base-pair contacts in the ARE half-site. *(b)* Ribbon diagram of the AR-LBD based on the published crystal structure showing α-helix and β-sheet structures, and the LxxLL peptide (purple) of the p160 coactivator, GRIP1, modelled into the hydrophobic cleft formed by AF2. *(c)* Topology and electrostatic surface potential diagram of the AR-LBD showing position of the bound LxxLL peptide of GRIP1. Homology modelling of the AR *(b, c)* was performed by Dr Jonathan Harris (Queensland University of Technology). *(d)* Schematic representation of the crystal structure of the LBD of nuclear receptors, exemplified by the retinoic acid receptor. Cylinders represent α-helices, numbered 1-12, and arrows represent β-sheets. *(Left)* Helix 12 is positioned away from the ligand binding pocket in the unliganded or antagonist-bound receptor. *(Right)* Agonist binding induces conformational change in receptor structure and helix 12 folds across the entrance to the ligand binding pocket. Figure adapted from Buchanan, 2002 and Wurtz *et al*, 1996.

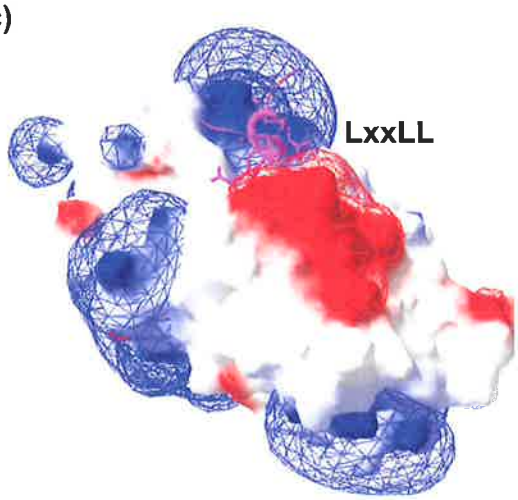
(a)


First Zn finger
Second Zn finger
D box
P box
Zn
Zn
...YYFPPQK
#*VFFKRAAEGKQKY
LRKCYEA...
CTE

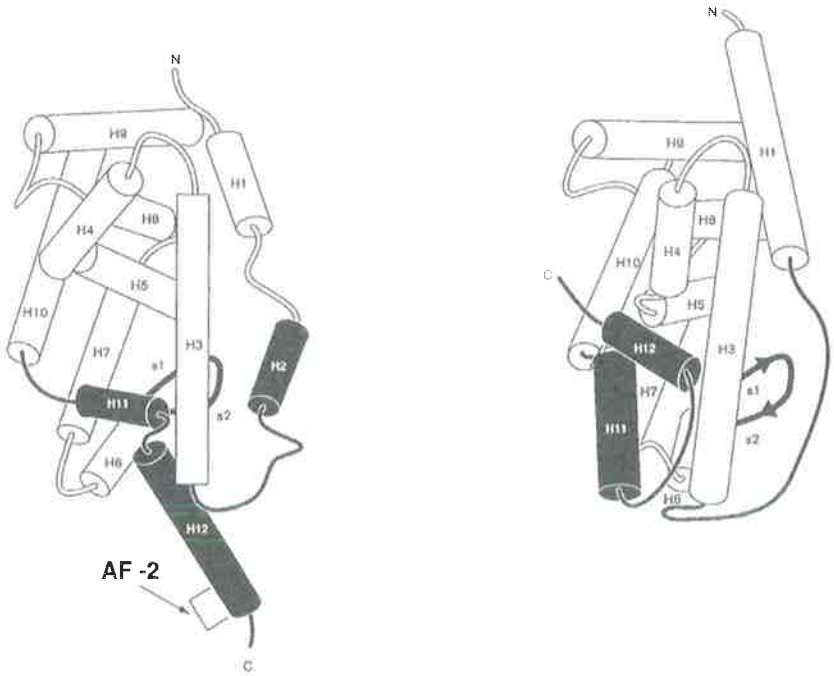
(b)


LxxLL

(c)


LxxLL

(d)


N
H9
H1
H4
H8
H5
H10
H7
H3
H2
H11
s1
s2
H6
H12
AF -2
C
N
H9
H1
H8
H10
H4
H5
H12
H3
H7
s1
H11
s2
H6

The hinge (amino acids 641-667) is a short, flexible region found between the DBD and LBD (Figure 1.4 (c)) and contains a nuclear localisation signal sequence (Jenster *et al*, 1991). It may have a repressive effect on AR activity as receptors containing mutations or deletions in the hinge region exhibit increased transactivation function (Wang *et al*, 2001a; Buchanan *et al*, 2001b).

1.3.6 – Ligand binding domain (LBD)

The sequence of the AR-LBD (amino acids 668-917, Figure 1.4 (c)) determines its ability to distinguish androgenic ligands from other structurally similar steroid hormone molecules. Testosterone and its more potent metabolite, DHT, have the highest affinity for the AR (K_d = 0.2-0.5nM), although testosterone dissociates much more rapidly (Grino *et al*, 1990; Kemppainen *et al*, 1999). Synthetic androgens such as R1881 (K_d = 0.6nM) and mibolerone (K_d = 0.53nM) also bind with high affinity to the AR (Kemppainen *et al*, 1999). The recently resolved crystal structure of the holo-AR-LBD determined that it is comprised of 11 alpha helices and four beta sheets arranged to form a three-dimensional ligand binding pocket (Matias *et al*, 2000; Sack *et al*, 2001) (Figure 1.5 (b), (c)). This structure is similar to the LBDs of other steroid hormone receptors (ERα, ERβ, PR, vitamin D receptor, retinoic acid receptor (RAR), thyroid hormone receptor (TR) and peroxisome proliferator activated receptors) (Renaud *et al*, 1995; Wagner *et al*, 1995; Bourguet *et al*, 1995; Brzozowski *et al*, 1997; Shiau *et al*, 1998; Moras and Gronemeyer, 1998; Ribeiro *et al*, 1998; Tanenbaum *et al*, 1998; Williams and Sigler, 1998; Nolte *et al*, 1998; Uppenberg *et al*, 1998). Steroid binding is mediated by direct interaction between the ligand backbone and 18 amino acids in the AR-LBD (Matias *et al*, 2000). The crystal structure of the apo-AR has not yet been determined however its structure can be predicted from that of other steroid hormone receptors, such as RAR, TR and ER. In the unliganded or antagonist-bound receptor, helix 12 is

positioned away from the ligand binding pocket (Figure 1.5 (d, left)). Agonist binding induces conformational changes that result in the repositioning of helix 12 across the entrance to the ligand binding pocket (Renaud *et al*, 1995; Wagner *et al*, 1995; Brzozowski *et al*, 1997; Shiau *et al*, 1998; Ribeiro *et al*, 1998; Tanenbaum *et al*, 1998) (Figure 1.5 (d, right)). Ligand induced repositioning of helix 12 results in the formation of a hydrophobic cleft, termed AF2, that can interact with LxxLL motifs in the AR-NTD (the N/C interaction) or in cofactor molecules (Heery *et al*, 1997; Ding *et al*, 1998; He *et al*, 1999; He *et al*, 2000) (Figure 1.5 (b) and (c)).

In addition to binding ligands the AR-LBD contains leucine zipper like structures which may be important in receptor dimerisation, a region that interacts with heat shock protein 90 and the AF2 region (Figure 1.4 (c)). AF2 is highly conserved amongst nuclear receptors, however unlike other members of the receptor family it has only weak intrinsic activity in the AR (Moilanen *et al*, 1997). The AF2 is required to interact with AF1, via cofactor molecules (Section 1.3.7), to confer high level activation (He *et al*, 1999).

1.3.7 – Cofactor interactions

Cofactor molecules mediate the interactions between nuclear receptors and the general transcriptional machinery – coactivators stimulate activation of target gene expression by nuclear receptors while corepressors inhibit this process. The most well characterised cofactors belong to the p160 family, including steroid receptor coactivator 1 (SRC1), glucocorticoid receptor interacting protein 1 (GRIP1[2]) and amplified in breast

[2] GRIP1 also known as transcriptional intermediary factor 2 (TIF2)

cancer 1 (AIB1[3]), which are recruited by agonist-bound AR. The AR can interact with conserved LxxLL motifs within p160 coactivators via the AF2 region (Figure 1.5 (b), (c)), although this interaction is relatively weak compared to other steroid hormone receptors (He *et al*, 1999). Instead, interactions with p160 molecules are primarily mediated by AF1 and AF5 in the AR (Heery *et al*, 1997; Ding *et al*, 1998; Voegel *et al*, 1998; Onate *et al*, 1998; Feng *et al*, 1998; Darimont *et al*, 1998; He *et al*, 1999; Ma *et al*, 1999; Glass and Rosenfeld, 2000). The p160 molecules actively recruit secondary coactivators, such as cyclic AMP response element binding protein (CBP), p300 and p300/CBP associated factor (pCAF), to the AR transcription complex (Kamei *et al*, 1996; Chakravarti *et al*, 1996; Anzick *et al*, 1997; Hong *et al*, 1997; Torchia *et al*, 1997; Chen *et al*, 1997; Korzus *et al*, 1998; Voegel *et al*, 1998; Blanco *et al*, 1998) (Figure 1.3 (e)). Many coactivators possess histone acetyltransferase (HAT) activity and their interaction with the AR results in acetylation of lysine residues on histones in the immediate vicinity of the DNA-bound receptor (Ding *et al*, 1998; Struhl, 1998). Acetylation results in altered affinity of histones for DNA, leading to reorganisation of chromatin structure and assembly of essential components of the basal transcriptional machinery, including RNA polymerase II (Mizzen *et al*, 1996; Ogryzko *et al*, 1996; Bannister and Kouzarides, 1996; Torchia *et al*, 1997; Pazin and Kadonaga, 1997; Kurokawa *et al*, 1998; Ogryzko *et al*, 1998; Xu *et al*, 1999).

Another family of coactivators appears to enhance AR activity. The first of these coactivators described was androgen receptor associated protein 70 (ARA70), a 70kDa protein which enhances transcriptional activity of AR while only slightly increasing transactivation of other steroid receptors (Yeh and Chang, 1996). Other AR specific

[3] AIB1 also known as activator of thyroid and retinoic acid receptors (ACTR), thyroid receptor activator molecule 1 (TRAM1), receptor associated coactivator 3 (RAC3) or p300/CBP interacting protein (pCIP)

cofactors, such as ARA54 (Kang *et al*, 1999), ARA55 (Fujimoto *et al*, 1999), ARA160 (Hsiao and Chang, 1999) and ARA24 (Hsiao *et al*, 1999), have been identified in prostate cells. BRCA1 and BRCA2 have also been shown to enhance DHT-induced AR transcriptional activity in the presence and absence of p160 coactivators, ARA70, ARA55 and CBP (Yeh *et al*, 2000; Park *et al*, 2000b; Shin and Verma, 2003). Part of the tumour suppressive effects of BRCA1 and BRCA2 may therefore be to promote anti-proliferative AR signalling pathways in breast cancer cells.

Alternatively, repression of gene transcription can occur by recruitment of corepressors, such as nuclear corepressor (NCoR) and silencing mediator of retinoic and thyroid hormone receptors (SMRT) (Horlein *et al*, 1995; Chen and Evans, 1995). Corepressors are associated with histone deacetylases (HDACs), enzymes which reduce histone acetylation and therefore inhibit gene transcription (Rosenfeld and Glass, 2001). NCoR and SMRT interact with nuclear receptors via two domains at the carboxy terminus, each containing LxxI/HIxxxI/L motifs which are related to the LxxLL motifs of coactivators (Aranda and Pascual, 2001). While RAR and TR can actively recruit corepressors and inhibit basal gene transcription in the absence of ligand, recruitment of corepressors to the ER, PR and AR is thought to only occur in the presence of receptor antagonists (Shang *et al*, 2000; Hu and Lazar, 2000; Shang *et al*, 2002). The current model for receptor activation proposes that ligand-induced exchange of corepressors for coactivators prompts the switch from gene repression to gene activation (Rosenfeld and Glass, 2001). However recent data from our laboratory (Buchanan, 2002), and others (Dotzlaw *et al*, 2002), challenges this model of AR corepression as SMRT has been demonstrated to inhibit AR activity in the absence of ligand, suggesting that SMRT can potentially associate with the unliganded AR. Likewise, SMRT has been shown to inhibit DHT-induced AR activity via disruption of the N/C interaction and competition

with p160 coactivators, which appears to be mediated by a direct interaction between the DHT-AR complex and SMRT (Agoulnik *et al*, 2003; Liao *et al*, 2003). Cheng *et al* (2002) have also observed that NCoR can interact with the AR and inhibit its activity in the presence of DHT.

p160 coactivators, the ARA coactivators, CBP, p300, pCAF, NCoR and SMRT are expressed at varying levels in breast cancer cell lines and tumours (Yeh and Chang, 1996; Hanstein *et al*, 1996; Anzick *et al*, 1997; Xu *et al*, 2000; Kurebayashi *et al*, 2000; Magklara *et al*, 2002; Vienonen *et al*, 2003) suggesting that the specific pool of cofactors available can modulate AR activity in a cell-specific manner. However a precise association between expression of particular cofactors and expression levels of AR target genes or proliferative response is yet to be established in breast cancer cells.

1.3.8 – Ligand independent activation

Recent evidence suggests that, in addition to activation by androgenic ligands, the AR can also be activated in the absence of androgens by a range of non-steroidal agents such as growth factors (insulin-like growth factor 1, keratinocyte growth factor and epidermal growth factor) (Culig *et al*, 1994), the cytokine interleukin-6 (IL-6) (Ueda *et al*, 2001), the protein kinase A (PKA) activator forskolin (Nazareth and Weigel, 1996; Ueda *et al*, 2001), the differentiation agent butyrate (Nazareth and Weigel, 1996) (Gleave *et al*, 1998; Sadar and Gleave, 2000) or the tyrosine kinase receptor HER-2/neu (Yeh *et al*, 1999; Craft *et al*, 1999). These agents activate intracellular protein kinase signalling pathways involving MAPK, JAK and STAT3 (Table 1.2), leading to phosphorylation of the AR at serine residues (Gioeli *et al*, 2002). It is currently unknown whether AR phosphorylation through ligand independent mechanisms is sufficient for AR activation or if other factors/alterations are necessary. In the case of

Table 1.2: Protein kinase signalling pathways mediating ligand independent AR activation by non-steroidal agents in prostate cells.

Non-steroidal AR activator	**Kinase signalling pathways modulating AR activity**	**References**
IL-6	JAK, STAT3, MAPK, PKA, PKC, PI 3'-kinase, Akt,	Hobisch *et al*, 1998; Chen *et al*, 2000; Ueda *et al*, 2001; Culig *et al*, 2002; Ueda *et al*, 2002; Lee *et al*, 2003; Yang *et al*, 2003
Forskolin	PKA	Nazareth and Weigel,1996; Ueda *et al*, 2001
Butyrate	PKA	Sadar and Gleave, 2000
HER-2/neu	PI 3'-kinase, Akt, MAPK	Craft *et al*, 1999; Yeh *et al*, 1999; Wen *et al*, 2000
Growth factors (Insulin-like growth factor 1, keratinocyte growth factor, epidermal growth factor)	unknown	Culig *et al*, 1994

JAK = janus kinase, STAT3 = signal transducer and activator of transcription, MAPK = mitogen activated protein kinase, PKA = protein kinase A, PKC = protein kinase C, PI 3'-kinase = phosphatidylinositol 3'-kinase.

IL-6, the STAT3 and MAPK pathways mediate ligand independent stimulation of AR activity while the PI 3'-kinase and Akt signalling pathways have been shown to mediate suppression of AR activity (Yang *et al*, 2003). These studies suggest that multiple pathways may interact to determine the overall level of AR activity induced by IL-6. Ligand independent activation of the AR can induce transcription of androgen responsive genes (Yeh *et al*, 1999; Sadar, 1999) and cell proliferation in prostate cancer cells *in vitro* and *in vivo* (Yeh *et al*, 1999; Sadar, 1999; Ueda *et al*, 2001; Lee *et al*, 2003). Ligand independent AR activity can also modulate the effects induced by androgenic ligands. Despite these observations it is still unclear whether ligand independent and ligand dependent activity induce the same cellular responses with respect to the recruitment of cofactors and the specificity of target genes.

Ligand independent activation of the AR has only previously been described in prostate cells and there are currently no reports which specifically investigate ligand independent activation of the AR in breast cancer cells. Some of the agents described above have been shown to modulate proliferation of breast cancer cells, indicating that they may play a role in breast tumourigenesis. Activation of JAK/STAT and MAPK pathways by IL-6 in breast cancer cells inhibits proliferation and increases migration (Chen *et al*, 1991; Speirs *et al*, 1993a; Blais *et al*, 1995; Badache and Hynes, 2001). Furthermore, IL-6 expression in breast cancers is inversely associated with histological grade (Fontanini *et al*, 1999). A role for IL-6 in modulating steroid receptor activity in breast cancer has been demonstrated by studies showing that IL-6 directly activates ER in cells cultured from primary breast tumours (Speirs *et al*, 2000). IL-6 has also been shown to modulate the expression of GCDFP-15 and GCDFP-24 in breast cancer cells (Blais *et al*, 1995), although these studies did not demonstrate specific involvement of the AR in mediating the effects of IL-6. Overexpression of HER-2/neu, a result of gene

amplification, is detected in up to 30% of breast cancers (Slamon *et al*, 1987). HER-2/neu is a transmembrane protein with tyrosine kinase activity. In breast cancer cell lines, overexpression of HER-2/neu results in ER phosphorylation, induction of estrogen responsive genes and stimulation of estrogen independent proliferation (Pietras *et al*, 1995). These observations may explain, at least in part, the poor prognosis for breast cancers overexpressing HER-2/neu. The therapeutic agent Herceptin, an antibody which blocks HER-2/neu activity, may inhibit breast cancer cell growth *in vitro* and *in vivo* through the suppression of ligand independent ER activity (Wang *et al*, 2001b). It has been recently demonstrated that breast tumours overexpressing HER-2/neu have reduced expression of AR or ARA70 (Kollara *et al*, 2001). This provides evidence for cross-talk between HER-2/neu and AR signalling pathways in breast cancer cells and suggests that loss of AR signalling may confer a growth advantage to HER-2/neu overexpressing cells.

1.4 – Objectives of this Thesis

There is considerable evidence supporting a critical role for androgen signalling pathways in the regulation of breast cancer cells. However the precise mechanisms that mediate androgen and AR action in breast cancer cells are poorly characterised. In particular, the mechanisms by which androgens may inhibit proliferation of some breast cancer cell lines but stimulate others, the AR mediated mechanisms of action of the synthetic progestin MPA, and the mechanisms by which androgens can inhibit E_2-stimulated breast cancer cell proliferation remain unclear. The androgen signalling pathway may potentially regulate breast cancer cells via different mechanisms, such as by modulating the expression of androgen responsive genes, or by modulating the action of other critical factors that control breast cancer cell growth, such as the ER.

A greater understanding of androgen signalling may facilitate the development of novel therapies for breast cancer that target androgen action. Therefore the overall objective of this study was to further define the mechanisms associated with the androgen signalling axis in breast cancer cells, particularly the effects of androgens on gene expression and interactions between the AR and other signalling pathways. This was achieved by addressing the following specific aims:

1. To investigate the molecular mechanisms by which androgens can inhibit the mitogenic effects of estrogens on breast cancer cells,
2. To characterise AR activity in breast cancer cell lines in response to the native ligand, DHT, and the synthetic progestin, MPA,
3. To identify AR target genes that potentially mediate the proliferative effects of androgens, and
4. To examine the mechanisms associated with the regulation of androgen responsive gene expression in breast cancer cells.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 – Materials

The suppliers of all materials used throughout this thesis are indicated below.

Chemicals, solutions and general reagents were purchased from the following companies:

AGFA (Mortsel, Belgium): developer (parts A and B), rapid fixer

Amersham Biosciences (Buckinghamshire, England): Hybond™-N nitrocellulose transfer membrane, Hybond™-P PVDF transfer membrane, Hyperfilm™ ECL™, protein G sepharose slurry,

Asia Pacific Specialty Chemicals (Seven Hills, NSW, Australia): charcoal, isopropanol, sodium chloride

Baxter Healthcare Corporation (Deerfield, IL, USA): saline, sterile H_2O

BDH (Kilsyth, Victoria, Australia): sucrose, Nonidet P40

Beckman Coulter Inc (Fullerton, CA, USA): ReadySafe™ liquid scintillation fluid

Bio-Rad Laboratories (Hercules, CA, USA): 40% acrylamide/bis solution 37.5:1, Bio-Spin 6 chromatography columns, Chelex 100 molecular biology grade resin, coomassie blue, extra thick blot paper

Diploma (Melbourne, Victoria, Australia): skim milk powder

Geneworks (Thebarton, SA, Australia): 100bp DNA ladder, 1kb DNA ladder

Invitrogen (San Diego, CA, USA): DMEM/F-12 powdered medium, human Cot-1 DNA, Lipofectamine™ 2000, OptiMem reduced serum liquid medium, MicroHyb hybridisation solution, poly dA, SeeBlue Plus2 pre-stained protein standard, sheared salmon sperm DNA

JRH Biosciences (Lenexa, KS, USA): phenol red free RPMI liquid medium 1640 (1x solution containing 2.05mM L-glutamine), RPMI liquid medium 1640 (1x solution containing 2.05mM L-glutamine), trypsin-EDTA (1x solution containing 0.05% trypsin, 0.02% EDTA in Hank's balanced salt solution)

Merck (Kilsyth, Victoria, Australia): chloroform, ethanol, glacial acetic acid, methanol, sodium molybdate

Nalge Nunc International (Rochester, NY, USA): Cell scrapers (32cm)

Packard (Mount Waverley, ACT, Australia): Optical plates

Progen Industries (Brisbane, Qld, Australia): 6% acrylamide/bis solution (19:1)

Promega Corporation (Madison, WI, USA): agarose, dATP, dCTP, dGTP, dTTP, passive lysis 5x buffer, RNase free H_2O, λ-HindIII DNA ladder

Roche (Mannheim, Germany): CSPD, glycogen, proteinase K

SIGMA (St Louis, MO, USA): ammonium persulphate, ampicillin, aprotinin, bromophenol blue, deoxycholate, dextran, DTT, EDTA, ethidium bromide, ficoll 400, formaldehyde, formamide, glycerol, glycine, Igepal CA-630, leupeptin, lithium chloride, luria agar base, luria broth base, magnesium sulphate, β-mercaptoethanol, mineral oil, MOPS, PBS, phenol:chloroform:isoamyl alcohol (25:24:1), phenylmethylsulfonylfluoride, protease inhibitor cocktail, SDS, sodium acetate, sodium bicarbonate, sodium fluoride, sodium orthovanadate, TEMED, tetrasodium pyrophosphate, Tris base, triton X-100, tween 20, xylene cyanol FF

Stratagene (La Jolla, CA, USA): StrataClean™ resin

TRACE Scientific Ltd (Melbourne, Victoria, Australia): foetal calf serum

Cell lines were purchased from the *American Type Culture Collection (Manassas, VI, USA).*

Cos-1 cells are derived from African green monkey (*Cercopithecus aethiops*) kidney fibroblasts transformed with SV40.

LNCaP is a prostate epithelial cell line isolated from a lymph node metastasis from a 50 year old male with prostate carcinoma.

MDA-MB-453 is a breast epithelial cell line derived from a pleural effusion from a 48 year old female with metastatic carcinoma of the breast.

PC-3 is a prostate epithelial cell line derived from a bone metastasis of an advanced (grade IV) prostatic adenocarcinoma from a 62 year old male.

T-47D is a breast epithelial cell line derived from a pleural effusion from a 54 year old female with infiltrating ductal carcinoma of the breast.

Hormones and receptor antagonists were purchased from the following companies:

Astra Zeneca (Cheshire, UK): bicalutamide

Imperial Chemical Industries (Cheshire, UK): trans-4-hydroxytamoxifen

Schering Plough (Baulkham Hills, NSW, Australia): hydroxyflutamide

SIGMA (St Louis, MO, USA): 5α-dihydrotestosterone, 17β-estradiol, medroxyprogesterone acetate

Upjohn Pharmaceuticals (Kalamazoo, MI, USA): mibolerone

Radiochemicals were purchased from the following companies:

Amersham Biosciences (Buckinghamshire, England): α-[^{33}P]dCTP (Redivue), 5α-dihydro[1,2,4,5,6,7-^{3}H]testosterone (^{3}H-DHT)

New England Nuclear (Boston, MA, USA): methyl-17α-hydroxyprogesterone acetate, 6α[1,2-^{3}H(N)] (^{3}H-MPA)

Plasmid constructs were either provided or derived from parental plasmids from the following sources. Detailed descriptions and vector maps are located in Appendix 1.

Expression constructs:

pCMV3.1-AR:wt was obtained from Prof Wayne Tilley, and is described in Tilley *et al* (1989).

pCMV3.1-AR(ms-):wt was constructed by Dr Grant Buchanan from the pCMV3.1-AR:wt plasmid, as described in Appendix 1.

pCMV3.1-AR(ms-):Q865H was constructed from the pCMV3.1-AR(ms-):wt plasmid as described in Section 5.2.2.

pcDNA3.1(+) was purchased from *Invitrogen (San Diego, CA, USA)*

pcDNA-AR(NTD/DBD) was obtained from Drs Gerhard Coetzee and Ryan Irvine (Norris Comprehensive Cancer Center, University of Southern California, CA, USA), and is described in Park *et al* (2000b).

HEGO/pSG5 (ER-α) (Tora *et al*, 1989) was obtained from Prof Pierre Chambon (College de France, Illkirch, France)

Mammalian two-hybrid constructs:

pM, pVP16, pM-53 and pVP16-T vectors and were purchased from *BD Biosciences Clontech (Palo Alto, CA, USA)*

pVP16AD-AR:NTD (Ma *et al*, 1999) was obtained from Dr Michael Stallcup (University of Southern California, Los Angeles, CA, USA).

pM-AR:LBD (wt) was obtained from Dr Michael Stallcup (Ma *et al*, 1999).

pM-AR:LBD (Q865H) was constructed by Ms Kathleen Saint from pCMV3.1-AR(ms-):Q865H and pM-AR:LBD (wt) plasmids as described in Appendix 1.

Luciferase Reporters:

pGL3-Basic was purchased from *Promega Corporation (Madison, WI, USA).*

tk81-PB_3-luc was obtained from Dr Robert Matusik (The Vanderbilt Prostate Cancer Center, Nashville, TN, USA) and is described in Kasper *et al* (1999).

PSA_{630} (promoter)-luc was constructed by Dr Miao Yang from the PSA630-CATSAT plasmid (Brookes *et al*, 1998), which was obtained from Dr Peter Molloy (CSIRO Division of Molecular Science, Sydney Laboratory, North Ryde, NSW, Australia). Construction of this plasmid is described in Appendix 1.

PSA_{630} (promoter + enhancer)-luc was constructed by Dr Miao Yang from the PSE/PSA-CATSAT plasmid (Brookes *et al*, 1998), which was obtained from Dr Peter Molloy. Construction of this plasmid is described in Appendix 1.

ERE-tk-luc (Lascombe *et al*, 1998) was obtained from Prof Alessandro Weisz (Seconda Universita di Napoli, Italy).

pGK1 (Webb *et al*, 1998) was provided by Dr Michael Stallcup.

pRL-tk was purchased from *Promega Corporation (Madison, WI, USA).*

Other plasmids:

pBluescript was purchased from *Stratagene (La Jolla, CA, USA)*

Competent **bacterial cells** (One Shot® INVαF') were purchased from *Invitrogen (San Diego, CA, USA)*

Human GeneFilters™ ("Named Genes") **cDNA microarrays** were purchased from *Research Genetics (Huntsville, AL, USA)*

Kits were purchased from the following companies:

Ambion Inc (Austin, TX, USA): RPA II kit

Amersham Biosciences (Buckinghamshire, England): ECL™ Western Blotting detection reagents

Applied Biosystems (Foster City, CA, USA): TaqMan® Reverse Transcription Reagents

Bio-Rad Laboratories (Hercules, CA, USA): Protein Assay kit

Promega Corporation (Madison, WI, USA): Dual-Luciferase Reporter Assay System, Luciferase Assay System, SV Total RNA Isolation System

QIAGEN (Hilden, Germany): RNeasy mini kit, QIAquick PCR purification kit, QIAquick gel extraction kit, QIAprep Spin miniprep kit, Plasmid maxi kit

Roche (Mannheim, Germany): DIG RNA labelling kit, DIG Wash and Block Buffer set

Antibodies were purchased from the following companies:

Chiron (Clayton, Victoria, Australia): AR rabbit polyclonal (U407)

Chemicon International (Temecula, CA, USA): HRP-conjugated sheep polyclonal anti-rabbit IgG, HRP-conjugated sheep anti-mouse IgG, HRP conjugated donkey anti-sheep/goat IgG

Roche (Mannheim, Germany): Anti-Digoxygenin-AP Fab fragments (sheep polyclonal)

Santa Cruz (Santa Cruz, CA, USA): actin (I-19) goat polyclonal, AR (N-20) rabbit polyclonal

Upstate (Lake Placid, NY, USA): Anti-acetyl-Histone H3 (AcH3) rabbit polyclonal

hPRa6 and hPRa7 antibodies were obtained from Dr Christine Clarke (Department of Medical Oncology, University of Sydney Westmead Hospital, NSW, Australia).

Enzymes were purchased from the following companies:

Applied Biosystems (Foster City, CA, USA): SYBR green 2x PCR reaction mix, TaqMan PCR mastermix

Biotech (West Perth, WA, Australia): Taq DNA polymerase (supplied with 10x PCR buffer)

Finnzymes (Espoo, Finland): DyNAzyme EXT™ DNA polymerase (supplied with 10x PCR buffer)

Invitrogen (San Diego, CA, USA): Superscript II RNase H- reverse transcriptase (supplied with 5x RT buffer and 0.1M DTT), T4 DNA ligase (supplied with 10x ligase buffer)

New England Biolabs (Beverly, MA, USA): Bst BI, Nar I, Xba I (supplied with appropriate reaction buffers)

Promega Corporation (Madison, WI, USA): RNasin ribonuclease inhibitor

Roche (Mannheim, Germany): DNase I

Oligonucleotide primers (sequences indicated in Appendix 2) were purchased from the following companies:

Invitrogen (San Diego, CA, USA): $Oligo(dT)_{12-18}$

Geneworks (Thebarton, SA, Australia): Random hexamers, Mycoplasma sense, Mycoplasma antisense, β-actin sense, β-actin antisense, 18S rRNA sense, 18S rRNA antisense + SP6, BRCA1 sense, BRCA1 antisense + SP6, BRCA1 5349 sense, BRCA1 5523 antisense, N111, N222, N122, N2330, 144, 244, N155, Hind III AS, N166, x4AS, ARCS1, ARCAS3, S, Q865H, Xba I antisense, K718E, ARCS3, AR917

Norris Cancer Center Microchemical Core Facility (Los Angeles, CA, USA): GAPDH sense, GAPDH antisense, PSA sense, PSA antisense, PSA ARE I sense, PSA ARE I antisense, PSA ARE III sense, PSA ARE III antisense, PSA irrelevant region sense, PSA irrelevant region antisense

Dual labelled real time PCR probes were purchased from *Biosearch Technologies (Novato, CA, USA):* PSA ARE I, PSA ARE III, PSA irrelevant region, GAPDH mRNA, PSA mRNA (sequences indicated in Appendix 2)

Equipment was purchased from the following companies:

Alpha Innotech Corporation (San Leandro, CA, USA): AlphaImager 2200 gel documentation system

Amersham Biosciences (Buckinghamshire, England): PhosphorImager SI and cassettes

Beckman (Palo Alto, CA, USA): scintillation counter, spectrophotometers, ultra centrifuges

Bio-Rad Laboratories (Hercules, CA, USA): Imaging Densitometer, iCycler iQ real time PCR detection system, Mini-PROTEAN II electrophoresis system, Mini Trans-Blot modules, Power Pac power supply units, Sub-cell electrophoresis systems, Trans-Blot SD semi-dry transfer cell

BMG Labtechnologies (Offenburg, Germany): LUMIstar Galaxy plate reading luminometer

Corbett Research (Mortlake, NSW, Australia): Rotor-Gene 3000 real time PCR thermal cycler

Dynex (Chantilly, VA, USA): MLX plate reading luminometer

Eppendorf (Hamburg, Germany): microcentrifuges

Fisher Scientific (Tustin, CA, USA): Sonic Dismembrator Model 60

MJ Research, Inc (Waltham, MA, USA): thermal cycler

Packard (Mount Waverley, ACT, Australia): Top Count™ plate reading luminometer

Stratagene (La Jolla, CA, USA): UV cross linker

Thermo Hybaid (Middlesex, UK): hybridisation oven, hybridisation roller bottles, PCR thermal cycler

Software was obtained from the following companies:

Alpha Innotech Corporation (San Leandro, CA, USA): AlphaEaseFC™ version 3.1.2

Amersham Biosciences (Buckinghamshire, England): ImageQuant™ version 3.3

Bio-Rad Laboratories (Hercules, CA, USA): Quantity One, QuickTime 4.0

BMG Labtechnologies (Offenburg, Germany): LUMIstar Galaxy version 4.30-0

Corbett Research (Mortlake, NSW, Australia): Rotor-Gene version 4.6 build 84

Dynex (Chantilly, VA, USA): Revelation MLX 4.06

Microsoft Corporation (Seattle, WA, USA): Excel 2002

National Institute of Health (Bethesda, MD, USA): P-SCAN version 1.2.010626 (2002)

Packard (Mount Waverley, ACT, Australia): Top Count™ NXT version 2.12

SPSS Inc (Chicago, IL, USA): SPSS version 11.0

The MathWorks, Inc (Natick, MA, USA): MATLAB version 5.3

2.2 – Buffers and Solutions

The composition of all buffers and solutions used throughout this thesis is described below. All solutions were made using dH_2O and stored at room temperature unless otherwise indicated.

CSS

500mL dextran coated charcoal was centrifuged at 5000rpm for 30mins. The pellet was resuspended in 500mL FCS and incubated at room temperature for 2hrs with occasional mixing. Tubes were centrifuged at 5000rpm for 30mins at 4°C and the FCS supernatant was added to charcoal pellets from an additional 500mL dextran coated charcoal. Following a second incubation at room temperature for 2hrs with occasional mixing,

tubes were re-centrifuged as above and the FCS supernatant was filtered through a 0.2µm filter. Aliquots were stored at -20°C.

Coomassie Blue Stain

0.02% Coomassie blue, 45% methanol, 7% acetic acid

Coomassie Blue Destain

45% methanol, 7% acetic acid

Cytosol buffer

10mM Tris, 1.5mM EDTA, 10mM sodium molybdate, 10% glycerol, pH 7.4

Stored at 4°C

DTT was added to 1mM immediately prior to use for cytosol extraction.

Denaturing PAGE gel solution

6% acrylamide/bis solution (19:1), 1x TBE buffer, 7M urea

ammonium persulfate (0.025%) and TEMED (0.1%) were added immediately prior to pouring.

DEPC-H_2O

0.1% DEPC in sterile dH_2O

One mL DEPC was added to 1L sterile dH_2O, the solution was mixed thoroughly, incubated at room temperature overnight and autoclaved.

Dextran coated charcoal

0.5% charcoal, 55mM dextran, 20% glycerol

This solution was made using cytosol buffer (without DTT).

Dilution buffer

0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA pH 8.1, 16.7mM TrisHCl pH 8.1, 167mM sodium chloride, 1x protease inhibitor

DMEM

One vial of DMEM/F-12 powdered medium and 37g sodium bicarbonate were dissolved in 9L dH2O, the pH was adjusted to 7.2 and the volume was made up to 10L. The solution was filtered through a 0.2μM filter and stored at 4°C.

10x DNase I buffer

1M sodium acetate, 0.5M magnesium sulphate, pH 5.2

This solution was made with DEPC-H_2O, sterilised by autoclaving and stored at -20°C.

Elution buffer

1% SDS, 0.1M sodium bicarbonate

High salt immune complex wash buffer

0.1% SDS, 1% Triton X-100, 2mM EDTA pH 8.1, 20mM TrisHCl pH 8.1, 500mM sodium chloride

Lithium chloride immune complex wash buffer

0.25M lithium chloride, 1% Igepal CA-630, 1% deoxycholate, 1mM EDTA pH 8.1, 10mM Tris pH 8.1

5x loading buffer for agarose gels

15% ficoll 400, 0.25% xylene cyanol, 0.25% bromophenol blue

Low salt immune complex wash buffer

0.1% SDS, 1% Triton X-100, 2mM EDTA pH 8.1, 20mM TrisHCl pH 8.1, 150mM sodium chloride

Lysis buffer (for mycoplasma detection)

1x TE, 0.5% Tween 20, 0.5% Nonidet P40, 0.25% Chelex 100

0.4mg/mL proteinase K (added immediately prior to use)

10x MOPS

0.4M MOPS, 0.1M sodium acetate, 0.01M EDTA, pH 7.0

Made with DEPC-H_2O, sterilised by autoclaving and stored shielded from light.

10x MOPS loading buffer

0.6x MOPS, 7.4% formaldehyde, 57% formamide, 0.1mg/mL ethidium bromide

Made with DEPC-H_2O and freshly prepared for each use.

5x PCR reaction buffer

500µM dATP, 500µM dCTP, 500µM dGTP, 500µM dTTP, 6.25mM Tris, 5x PCR buffer (supplied with enzyme)

Made with sterile dH_2O and stored at -20°C.

6x protein loading dye

0.35M Tris-Cl, 10.4% SDS, 30% glycerol, 0.6M DTT, 0.012% bromophenol blue

A 4x solution of Tris-Cl/SDS was prepared by dissolving 3.025g Tris-Cl in 20mL sterile H_2O, adjusting pH to 6.8, and adding 0.2g SDS and sterile H_2O to 50mL. Seven mL of Tris-Cl/SDS was then combined with 3mL glycerol, 1g SDS, 0.93g DTT and 1.2mg bromophenol blue.

RIPA buffer

10mM Tris (pH 7.4), 150mM sodium chloride, 1mM EDTA, 1% triton X-100.

Protease inhibitors were added to the following final concentrations immediately prior to use: 2mM phenylmethylsulfonylfluoride, 10μg/ml leupeptin, 10μg/ml aprotinin

Phosphatase inhibitors were added to the following final concentrations immediately prior to use: 100mM sodium fluoride, 10mM tetrasodium pyrophosphate, 2mM sodium orthovanadate

RNA dilution buffer

6x SSC, 7.6% formaldehyde

Made with DEPC-H_2O and freshly prepared for each use.

RNA loading dye

50% glycerol, 1mM EDTA, pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol

Made with DEPC-H_2O

5x running buffer for immunoblot

0.12M Tris, 1M Glycine, 0.5% SDS

SDS lysis buffer

1% SDS, 10mM EDTA pH 8.1, 50mM TrisHCl pH 8.1, 2x protease inhibitor

SDS PAGE separating gel

6-7.5% acrylamide/bis solution (37.5:1), 375mM Tris, pH 8.8, 0.001% SDS, 0.004% ammonium persulfate, 0.1% TEMED

SDS PAGE stacking gel

4% acrylamide/bis solution (37.5:1), 125mM Tris, pH 6.8, 0.005% SDS, 0.004% ammonium persulphate, 0.1% TEMED

20x SSC

3M sodium chloride, 0.3M sodium citrate dihydrate, pH 7.0

50x TAE

2M Tris, 17.5M acetic acid, 50mM EDTA

10x TBE

890mM Tris, 890mM boric acid, 32mM EDTA

10x TBS

0.5M Tris, 1.5M sodium chloride, pH 7.4

TBST

1x TBS, 0.2% Tween 20

TE buffer

10mM Tris, pH 7.5, 1mM EDTA

Transfer buffer

25mM Tris, 192mM Glycine, 20% methanol (added fresh for each use)

Trypan blue

0.01% trypan blue dissolved in sterile saline.

2.3 – Methods

Core experimental procedures used throughout this thesis are described in a general context in this section. A more detailed explanation of specific methods used is described in the methods section of each individual chapter.

2.3.1 – Cell culture

All cell lines were maintained in RPMI medium 1640 containing 5% FCS at 37°C in an atmosphere containing 5% carbon dioxide. Manipulation of cells was performed under aseptic conditions in a laminar flow cabinet. When cells were approaching confluence, medium was removed from the flasks and cells were washed gently with 1mL/cm^2 sterile saline. Cells were incubated at 37°C with 1mL/cm^2 trypsin-EDTA until detached. The cell suspension was then diluted in an equal volume of medium and an aliquot was passaged into fresh medium. Cells were routinely passaged every seven days with replacement of medium every 2-3 days. When seeding for an experiment, cells were harvested with trypsin-EDTA as described above and the concentration of cells was determined using a hæmocytometer. The suspension was diluted in medium and seeded into appropriate culture flasks or plates as indicated.

2.3.2 – Mycoplasma detection

Cells were routinely tested for mycoplasma contamination using a PCR based approach. Two mL of culture medium was collected from confluent cells and centrifuged at 2000rpm for 5mins at 4°C. The resulting pellet was resuspended in 100µL lysis buffer (for mycoplasma detection) and incubated at 56°C overnight. Lysates were boiled at 95°C for 15mins before adding 5µL of StrataClean™ resin, mixing gently and centrifuging at 13000rpm for 2mins. PCR reactions were performed using 2µL of each

supernatant, 100ng each of mycoplasma sense and mycoplasma antisense primers (primer sequences are described in Appendix 2) and 1.5mM magnesium chloride as described in Section 2.3.11. Template DNA was denatured at 94°C for 5mins, followed by 35 cycles of 94°C for 1min, 67°C for 30secs, 72°C for 1min and a final elongation step at 72°C for 10mins. PCR products were electrophoresed on a 2% agarose gel as described in Section 2.3.12 and mycoplasma contamination was indicated by amplification of a 715bp band. Tests were performed by Dr Ben Davidson, Mr Duncan Holds and Ms Ali Ochnik. No evidence of mycoplasma contamination was detected with the cells used throughout these studies.

2.3.3 – Preparation of steroid stocks

Steroid hormones and receptor antagonists were dissolved in 100% ethanol to a concentration of 10^{-2}M (DHT, mibolerone, E_2, OHF, OHT) or 10^{-3}M (MPA, bicalutamide) and stored in glass vials at -20°C. Concentrated stocks were further diluted in ethanol as required. When treating cells with steroids, existing medium was replaced with fresh medium containing 1/1000 dilutions of steroid stocks or 1/1000 dilution of ethanol vehicle (0.1%). Receptor antagonists were typically used at a 1000 fold molar excess over steroid up to a concentration of 5μM, and were diluted 1/1000 (or 1/100 if required at high concentration) in medium.

2.3.4 – Preparation of plasmid DNA

Plasmid minicultures were prepared by inoculation of a single bacterial colony containing the plasmid of interest into 5mL luria broth containing 100μg/mL ampicillin and overnight incubation at 37°C with shaking at 180rpm. Small scale preparations of plasmid DNA were then performed using the QIAquick spin miniprep kit, according to instructions. Plasmid maxicultures were prepared by 1/1000 dilution of a starter culture,

prepared as described above but with incubation for 6-8hrs, in luria broth containing 100μg/mL ampicillin and overnight incubation at 37°C with shaking at 180rpm. Large scale preparations of plasmid DNA were then performed using the Plasmid maxi kit, according to instructions. The concentration and purity of plasmid DNA was measured as described in Section 2.3.5, and DNA was stored at -20°C.

2.3.5 – Estimation of nucleic acid concentration

The concentration of nucleic acid samples was determined spectrophotometrically. Preparations were diluted in H_2O and the optical density at 260nm and 280nm was measured. Concentrations were calculated based on one OD unit representing a concentration of 40ng/μL for RNA and 50ng/μL for double stranded DNA (Sambrook *et al*, 1989). OD_{260}/OD_{280} ratios were also calculated to estimate the purity of each sample, with ratios between 1.8 to 2.0 expected for pure RNA or plasmid DNA (Sambrook *et al*, 1989).

2.3.6 – *In vitro* reporter gene assay

Reporter gene assays were performed as described in Buchanan *et al* (2001b). Cells were seeded at a density of 1-2 x 10^4 cells/well (in a volume of 200μL/well) in 96 well tissue culture plates and allowed to attach overnight. Reporter (100ng/well), expression (2.5-10ng/well if required) and transfection control[4] (pRL-tk, 1ng/well) plasmids were diluted in OptiMem to a total volume of 25μL/well. Lipofectamine™ 2000 (0.2-0.4μL/well) was also diluted in OptiMem to a volume of 25μL/well. The plasmid and Lipofectamine™ mixes were then combined in equal volumes and incubated at room

[4] As discussed in section 5.3.3.3, no significant difference in renilla reporter gene activity was detected across 96 well plates transfected with the pRL-tk transfection control plasmid. These results imply that transfection efficiency is equal between wells, and pRL-tk was therefore not used for all transient transfection assays.

temperature for 30mins. Following washing of cells with 200µL/well OptiMem, 100µL fresh OptiMem and 50µL of plasmid/Lipofectamine™ 2000 mix were added to each well. Following transfection for 3-5 hours at 37°C, cells were treated with 200µL/well steroid (or ethanol vehicle) diluted 0.1% in medium containing 5% CSS (and 1mg/mL BSA where indicated). After incubation at 37°C for 24-48hrs, cells were lysed by addition of 50µL/well passive lysis 1x buffer and incubation at room temperature for 30-60mins. Cell lysates were then assayed for luciferase activity using the Luciferase Assay System by combination of 25µL cell lysate and 50µL luciferase assay reagent in a 96 well optical plate. Luminescence was measured immediately using a plate reading luminometer.

2.3.7 – RNA extraction

Total RNA was extracted from cell line pellets using the RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. RNA was eluted from the column with 30µL RNase free H_2O. The concentration of RNA was measured as described in Section 2.3.5 and RNA samples were stored at -70°C.

2.3.8 – Electrophoresis of RNA

RNA was electrophoresed on a 1% agarose-MOPS gel containing 6.7% formaldehyde. Two to five µg of each RNA sample was combined with 8µL 10x MOPS loading buffer, the RNA was heated at 65°C for 5 mins, chilled on ice and then combined with 2µL of RNA loading dye. RNA samples were electrophoresed in 1x MOPS at 80V and visualised under 302nm UV light using an AlphaImager gel documentation system. To minimise degradation of RNA, all gels and solutions were prepared in autoclaved glassware, and gel apparatus exclusive for RNA applications was used.

2.3.9 – DNase I treatment

Total RNA (1-15μg) was combined with 5μL 10x DNase I buffer, 2.5μL 0.1M DTT and 10U DNase I in a total volume of 50μL. Following incubation at 37°C for 2 hours, RNA was subsequently purified by extraction with phenol:chloroform:isoamyl alcohol (pH 6.7) and precipitated with 2.5 volumes of 100% ethanol, 0.3M sodium acetate pH 5.2 and 20μg glycogen overnight at -70°C. RNA was centrifuged at 13000rpm for 15mins at 4°C, washed with 70% ethanol, air dried at room temperature for 15mins and resuspended in 5-15μL RNase free H_2O.

2.3.10 – Reverse transcription

DNase treated RNA samples were reverse transcribed in 20μL reactions with Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Reactions were preformed using 1μL of RNA, 100ng random hexamers and 200U reverse transcriptase. A control reaction lacking reverse transcriptase was also performed for each RNA sample to verify the absence of genomic DNA contamination. An additional control reaction without RNA template was included to confirm the purity of reagents used. cDNA samples were stored at -20°C.

2.3.11 – PCR

cDNA, genomic DNA or plasmid DNA fragments were generally amplified in 25μL reactions containing 1x PCR reaction buffer, 2mM magnesium chloride, 5pmol each of sense and antisense primers and 0.55U of *Taq* DNA polymerase. A negative control reaction containing sterile H_2O instead of template was also included for each primer set in each PCR run. PCR reactions were overlaid with mineral oil to prevent evaporation during thermal cycling and incubated in a Hybaid thermal cycler pre-heated to 95°C, in

order to minimise amplification of non-specific products. Template DNA was denatured at 95°C for 5mins, followed by 30-40 cycles of denaturation at 95°C for 1min, annealing at 55-67°C (optimised for each primer set) for 1min and extension at 72°C for 1-2mins. A final incubation at 72°C for 20mins was included to allow complete extension of unfinished PCR products. If required for automated DNA sequencing or for downstream manipulations such as mutagenesis, restriction enzyme digestion or riboprobe synthesis, PCR products were purified using the QIAquick PCR purification kit according to instructions.

2.3.12 – Electrophoresis of DNA

DNA samples were electrophoresed on 1-2% agarose-TAE gels containing 1μg/mL ethidium bromide and run in 1x TAE buffer. The percentage of agarose in the gel varied depending on the size of the DNA fragments being analysed, with 1% agarose used for high molecular weight DNA (eg plasmids) and 1-2% agarose for low molecular weight DNA (eg PCR products). DNA was visualised under 302nm UV light using an AlphaImager gel documentation system.

2.3.13 – DNA sequencing

Prior to sequencing of PCR products, amplification of a single band from each primer set was confirmed by agarose gel electrophoresis. PCR products were then purified using the QIAquick PCR purification kit. For plasmids to be sequenced, DNA was prepared as described in Section 2.3.4. Automated DNA sequencing was carried out by the Flinders Medical Centre sequencing facility. Sequencing reactions were primed with the original primers (for PCR products) or with primers flanking the region of interest (for plasmid DNA).

2.3.14 – Immunoblotting

Gels were prepared by pouring a SDS PAGE separating gel and overlaying with a SDS PAGE stacking gel. Equal amounts of protein samples were combined with protein loading dye and denatured at 95°C for 5mins. Proteins were electrophoresed, alongside molecular weight markers, in 1x running buffer at 50V through the stacking gel and 150V through the separating gel for 90mins.

Proteins were transferred to nitrocellulose or PVDF membranes using either wet or semi-dry conditions. The gel and membrane were soaked in ice cold transfer buffer for 10mins. Wet transfers were performed with a Mini Trans-Blot module (Bio-Rad), used according to the manufacturer's instructions, at 25-35V overnight. Semi-dry transfers were performed with a Trans-Blot SD semi-dry transfer cell (Bio-Rad) at 15-20V for 30-60mins.

Membranes were blocked in TBST containing 3% skim milk powder for 1hr to overnight. This was followed by sequential incubation in primary antibody and HRP-conjugated secondary antibody (each diluted in TBST containing 1% milk powder) for 1-2hrs each. Membranes were rinsed three times for 5mins with TBST in between each blocking and antibody incubation step. All incubations and washes were performed at room temperature with agitation. Bound antibody was detected using ECL™ western blotting detection reagents, according to instructions, and then exposed to Hyperfilm™ ECL™. Films were developed in a dark room by soaking in developing solution until the desired signal intensity was reached, and removal of emulsion with fixing solution. If required, total protein was detected by staining membranes with Coomassie Blue Stain for 5-15mins with gentle agitation followed by rinsing in Coomassie Blue Destain until bands became clear.

2.3.15 – Statistical tests

Recommendations for statistical analysis of data were kindly provided by Ms Kristyn Willson (Department of Public Health, The University of Adelaide, SA), and were analysed using the following statistical tests, unless otherwise stated. Pairwise comparisons were analysed using an independent samples t-test. Comparisons between multiple groups were analysed using a one-way analysis of variance (ANOVA), with Tukey's post-hoc test. Tests were performed using SPSS statistical analysis software and statistical significance was accepted at $p < 0.05$.

CHAPTER 3

PROLIFERATIVE EFFECTS OF ANDROGENS

3.1 – Introduction

The importance of estrogen signalling for the maintenance of cellular integrity and stimulation of epithelial cell proliferation in the breast is well established (Davidson and Lippman, 1989; Lupulescu, 1995; Bocchinfuso and Korach, 1997; Clemons and Goss, 2001). Past and present hormonal therapies for breast cancer have exploited this dependence on estrogen signalling to circumvent the mitogenic effect of estrogens (Beatson, 1896; Brodie and Njar, 2000; Klijn *et al*, 2001; Johnston, 2001). Clinical approaches for reducing the effects of estrogens include ovariectomy or the use of LHRH agonists, anti-estrogens and aromatase inhibitors (Section 1.1.4). Androgens are also effective inhibitors of breast cancer cell proliferation in both clinical and experimental contexts (Fels, 1944; Tormey *et al*, 1983; Ingle *et al*, 1988; Ingle *et al*, 1991; Birrell *et al*, 1998; Labrie *et al*, 2003). In addition to inhibiting basal breast cancer cell proliferation *in vitro*, androgens can also inhibit the mitogenic effects of estrogens (Poulin *et al*, 1988; Reese *et al*, 1988; Dauvois *et al*, 1989; Labrie *et al*, 1990a; Labrie *et al*, 1990b; Dauvois *et al*, 1991; de Launoit *et al*, 1991; Boccuzzi *et al*, 1993; Couillard *et al*, 1998; Ando *et al*, 2002). However the mechanisms associated with inhibition of estrogen-induced breast cancer cell growth by androgens, and the specific role of the AR in mediating the effects of androgens, are poorly understood.

In order to further investigate androgen action in estrogen responsive cells, a suitable *in vitro* model was required. The inhibitory effect of the native androgen, DHT, on estrogen-stimulated proliferation has previously been studied in a variety of breast cancer cell lines. In T-47D cells stimulated with 0.1nM E_2, a maximal 75% inhibition of proliferation was observed with 0.1nM DHT (Reese *et al*, 1988). In MCF-7 cells, proliferation induced by 1nM E_2 is also suppressed by DHT, with a maximal 68%

inhibition attained with 100nM DHT (Ando *et al*, 2002). Similarly, proliferation of ZR-75-1 cells induced by E_2 (1nM) is inhibited by DHT in a dose dependent manner, with complete inhibition observed in the presence of 10nM DHT (Poulin *et al*, 1988; Labrie *et al*, 1990b; Labrie *et al*, 1992). In each of those studies, concentrations of DHT greater than 100nM did not induce further inhibition of E_2-induced proliferation and, in some cases, the inhibitory effect was diminished at higher DHT concentrations. Although DHT consistently inhibits E_2-induced cell proliferation, there is considerable variation in the magnitude of inhibition. Reese *et al* (1988) and Andò *et al* (2002) examined the effect of DHT at one time point only (eight days and six days respectively) whereas Poulin *et al* (1988) and Labrie *et al* (1990b) used time course assays conducted over a period of up to 22 days. Their experiments suggest that the inhibitory effect of DHT is greater, and closer to 100%, when cells are almost confluent. Additional experimental factors, such as cell seeding density and medium conditions, may also contribute to variations in the reported effects of DHT on breast cancer cell proliferation.

Another possible explanation for the variation in potency of DHT is that the inhibitory effect on breast cancer cell proliferation may be a function of its stability in cultured cells. In MCF-7 cells, DHT levels rapidly decline in the first few hours of incubation, resulting in accumulation of metabolites such as 3α-diol, 3β-diol and their glucuronidated derivatives (see Figure 1.1 (b)), which have less potent androgenic activity than DHT (Rochefort and Garcia, 1983; Roy *et al*, 1992). In LNCaP cells, the half-life of DHT has been estimated at two hours, whereas synthetic androgens such as R1881 and mibolerone are comparatively resistant to metabolism (Murthy *et al*, 1986; Berns *et al*, 1986).

The T-47D breast cancer cell line was chosen as a model to investigate androgen and estrogen action in this study. T-47D cells express ER, AR and PR (Horwitz *et al*, 1978; Birrell *et al*, 1995a; Dotzlaw *et al*, 1997; Ortmann *et al*, 2002), reflecting the receptor profile of the majority of clinical breast cancers. T-47D cells are known to have functional estrogen and androgen signalling pathways, as demonstrated by the induction of estrogen and androgen responsive genes (Westley and May, 1987; Liberato *et al*, 1993; Yu *et al*, 1994b). Basal proliferation of T-47D cells is stimulated by estrogens but inhibited by androgens (Sutherland *et al*, 1988; Reese *et al*, 1988; Sutherland *et al*, 1992; Liberato *et al*, 1993; Birrell *et al*, 1995a) and physiological concentrations of DHT have been shown to inhibit E_2-stimulated T-47D cell proliferation by up to 75% (Reese *et al*, 1988). These observations suggest that T-47D cells are a suitable experimental model in which to study the molecular mechanisms of AR signalling that lead to antagonism of estrogen-induced proliferation by androgens. However as there is considerable variation in the reported effects of androgens and estrogens on breast cancer cells, it is therefore necessary to carefully define the experimental conditions under which sensitivity to androgens and estrogens is conferred. T-47D cells also show an increased sensitivity to transient transfection of reporter and expression plasmids in comparison to some other receptor positive breast cancer cell lines (unpublished observations). This indicates the potential to manipulate gene expression in these cells, which may facilitate definition of molecular mechanisms by which androgens inhibit the effects of estrogens.

The specific aims of this chapter were to further investigate the effects of androgens on proliferation of the T-47D breast cancer cell line, cultured in the presence and absence of 17β-estradiol (E_2). Two non-aromatisable androgenic ligands were used in these studies: the natural ligand, DHT and the synthetic non-metabolisable androgen,

mibolerone. The use of mibolerone, which has previously been used in this laboratory to inhibit basal T-47D cell proliferation (Birrell *et al*, 1995a), eliminates potential problems associated with ligand metabolism. Additionally, as it is not fully understood whether androgens inhibit estrogen-stimulated cell proliferation through decreasing cell proliferation rate or by decreasing cell survival (ie increasing apoptosis), the viability of T-47D cells treated with androgens in the presence and absence of estrogens was also assessed. The role of the AR in mediating the anti-estrogenic effects of these androgens was investigated using the AR antagonist, bicalutamide.

3.2 – Methods

3.2.1 – Cell proliferation assay

T-47D breast cancer cells were seeded in 24 well plates at varying cell densities as indicated and allowed to attach for 48-72hrs. Fresh medium (phenol red free RPMI containing either 5% foetal calf serum (FCS) or 5% charcoal stripped serum (CSS)) supplemented with steroids or ethanol vehicle (diluted 0.1%) was added every three days. Cells from individual wells were harvested every three days and counted using a hæmocytometer. Viable cells, assessed by trypan blue exclusion, were plotted as the mean cell number/well ± SEM of triplicate wells. The mean number of dead cells in each well, assessed by trypan blue uptake, was plotted as a percentage of the total number of cells/well.

3.2.2 – Reporter gene assay

Transactivation activity of ER and AR in response to E_2 or DHT was measured using luciferase reporter gene assays. Plasmid DNA was prepared as described in Section 2.3.4. T-47D cells were seeded in 96 well plates at a density of

2.0 x 10^4 cells/well in phenol red free RPMI medium containing 5% CSS. The following day, cells were transfected for five hours with either the ERE-tk-luc or the PSA_{630} (promoter + enhancer)-luc reporter construct (100ng/well) using Lipofectamine™ 2000 as described in Section 2.3.6. Cells were then treated with steroids and receptor antagonists as indicated. After 48hrs, cells were lysed and luciferase activity (in relative light units, RLU) was measured using the Luciferase Assay System and a Top Count plate reading luminometer. Four replicate wells of each treatment were measured and results are expressed as the mean RLU ± SEM.

3.3 – Results

3.3.1 – Determination of optimal seeding density for T-47D cells

Initial proliferation assays were performed in order to determine the seeding density required for optimal proliferation of T-47D cells. Assays for the studies in this chapter were performed under different serum conditions: cells were either cultured in medium containing 5% FCS or 5% CSS. The use of FCS in the culture medium enabled the response to androgens and estrogens to be investigated while cells were undergoing maximal proliferation. CSS, which is stripped of endogenous steroid hormones and, to some extent, growth factors, was used to more precisely control exposure to steroid hormones and to minimise the effects of steroids found in FCS. As the basal cell proliferation rate was expected to differ between these conditions, the seeding density for optimal basal cell proliferation was determined in each case.

The optimal seeding density for T-47D cells cultured in phenol red free RPMI containing 5% FCS without exogenous steroid was 2.5 x 10^4 cells/well. At this seeding density, cells proliferated throughout the assay period, resulting in a 24-fold increase in

the number of viable cells/well by day nine. Cells were sub-confluent at day nine and dead cells in each well were scored at less than 10% of the total cell number. At a lower seeding density (1.25 x 10^4 cells/well), cells reached an exponential growth phase between days six and nine and a higher percentage of dead cells was observed in the early stages of the assay (25% at day 0 and 20% at day 3). At a seeding density of 5.0 x 10^4 cells/well, cells reached confluence by day six.

The optimal seeding density was determined to be 5.0 x 10^4 cells/well for T-47D cells cultured in phenol red free RPMI containing 5% CSS without exogenous steroid. At this density, cells proliferated throughout the assay period, resulting in an 18-fold increase in the number of viable cells/well by day nine. At a density of 2.5 x 10^4 cells/well the cell proliferation rate decreased considerably, with a 6-fold increase in the number of viable cells/well observed at day nine. Cells seeded at 7.5 x 10^4 cells/well were approaching confluence at day nine. The percentage of dead cells remained below 10% throughout the assay period for cells at all seeding densities.

3.3.2 – Proliferative response of T-47D cells to E_2

The proliferative response of T-47D cells to E_2 was dependent on the serum conditions used. Cells were cultured in phenol red free RPMI containing 5% FCS or 5% CSS, with or without the addition of E_2.

3.3.2.1 – E_2 does not stimulate T-47D cell proliferation in medium containing 5% FCS

No difference in the growth rate of T-47D cells was observed between vehicle treated controls and cells treated with E_2 (0.01-100nM) over the nine day assay period (Figure 3.1 (a)). The number of dead cells counted per well was less than 10% of the total cell number throughout the assay (Figure 3.1 (b)). The duration of this assay was extended

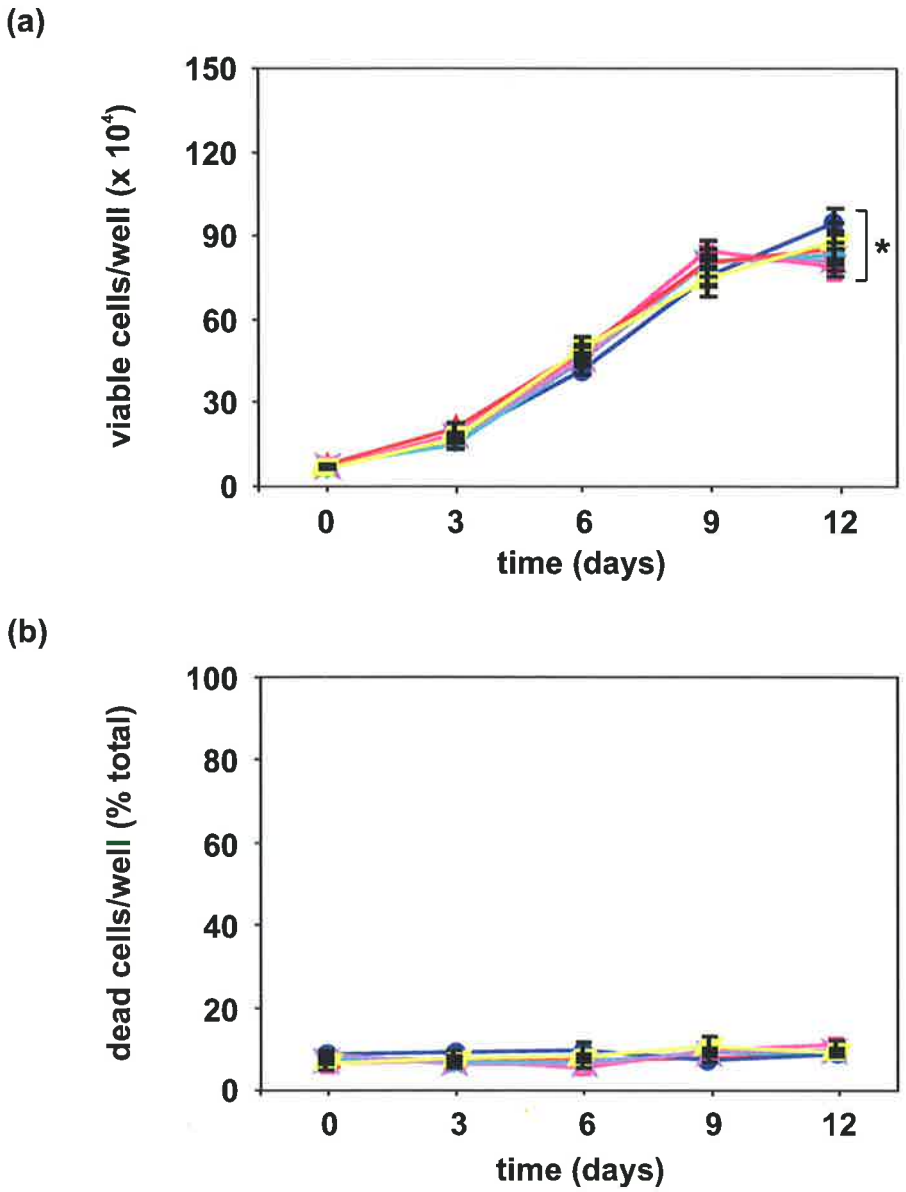


Figure 3.1: Effect of E_2, in medium containing FCS, on proliferation and viability of T-47D breast cancer cells. Cells were seeded at a density of 2.5 x 10^4 cells/well in 24 well plates and grown for 12 days in phenol red free RPMI + 5% FCS containing increasing concentrations of E_2 as indicated: (●) control (0.1% ethanol), (▲) 0.01nM E_2, (▰) 0.1nM E_2, (✖) 1nM E_2, (◆) 10nM E_2, (■) 100nM E_2. Cells were counted every 3 days. *(a)* Number of viable cells/well at each time point, assessed by trypan blue exclusion. *(b)* Dead cells, expressed as a percentage of the total number of cells/well at each time point. Results are plotted as the mean +/- SEM of triplicate wells. *ANOVA; $p = 0.75$ between all treatment groups.

to 12 days in order to ascertain whether proliferation continued after the standard day nine cut-off point. No further increase in cell number was observed after day nine, suggesting that results from these proliferation assays would be most informative prior to cells becoming confluent after approximately nine days in culture.

3.3.2.2 – E_2 stimulates T-47D cell proliferation in medium containing 5% CSS

Cells were seeded at a density of 2.5 x 10^4 cells/well for this experiment. As indicated in Figure 3.2 (a), cell number was maintained at this density, in the absence of E_2, without significant proliferation or cell death throughout the assay. These conditions therefore provided potential to observe maximal growth stimulatory effects of E_2. Cell proliferation was enhanced at all concentrations of E_2 compared to vehicle treated controls (Figure 3.2 (a)), with a maximal 8.7-fold increase in the number of viable cells observed at day nine. No significant differences in the number of viable cells at day nine were observed with the different concentrations of E_2. The number of dead cells counted per well remained less than 10% of the total cell number over the assay period (Figure 3.2 (b)).

3.3.3 – Functional activity of estrogens present in FCS

In the experiments described above, E_2 did not stimulate T-47D cells cultured in medium containing 5% FCS. Furthermore, maximal stimulation of T-47D cells cultured in medium containing 5% CSS was observed with the lowest concentration of E_2 used (0.01nM). To determine whether endogenous E_2 present in FCS was conferring maximal stimulation of proliferation, the transactivation activity of the endogenous ER in T-47D cells in response to E_2 was tested under different serum and hormone conditions. In T-47D cells transiently transfected with the ERE-tk-luc estrogen responsive luciferase reporter, treatment with E_2 (0.01nM and 10nM) in medium

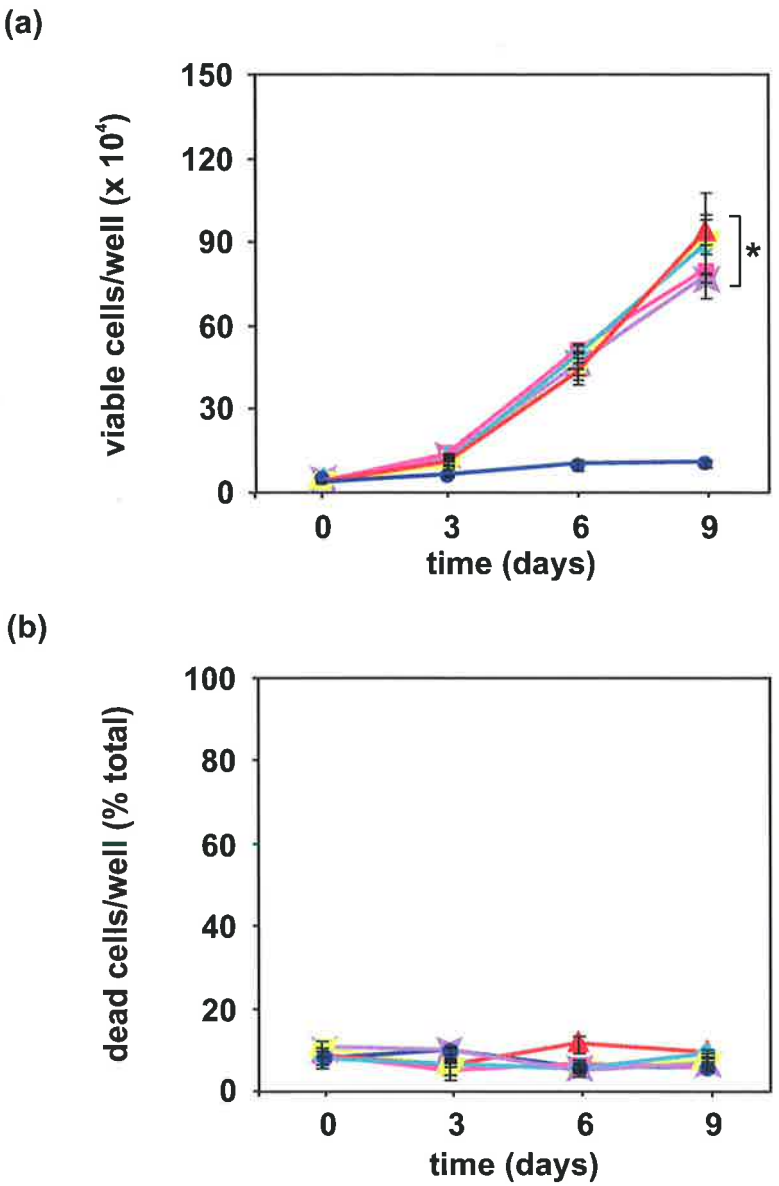


Figure 3.2: Effect of E_2, in medium containing charcoal stripped serum, on proliferation and viability of T-47D breast cancer cells. Cells were seeded at a density of 2.5 x 10^4 cells/well in 24 well plates and grown for 9 days in phenol red free RPMI + 5% CSS containing increasing concentrations of E_2 as indicated: (●) control (0.1% ethanol), (▲) 0.01nM E_2, (▰) 0.1nM E_2, (✖) 1nM E_2, (◆) 10nM E_2, (■) 100nM E_2. Cells were counted every 3 days. *(a)* Number of viable cells/well at each time point, assessed by trypan blue exclusion. *(b)* Dead cells, expressed as a percentage of the total number of cells/well at each time point. Results are plotted as the mean +/- SEM of triplicate wells. * ANOVA; $p < 0.002$: E_2 (0.01-100nM) *versus* control, $p > 0.813$ between E_2 treatment groups.

containing 5% CSS induced comparable activation of the endogenous ER, with a maximal 15-fold induction of activity compared to vehicle treated controls (Figure 3.3). This induction of ER activity was blocked by a 500-fold molar excess of the anti-estrogen OHT. In medium containing 5% FCS and no additional exogenous E_2, a 6-fold increase in ER activity was induced compared to that observed in medium containing 5% CSS and no exogenous E_2. ER activity in response to estrogens contained in FCS was blocked by 1μM OHT. ER activity in the presence of FCS was approximately 40% of maximal ER activity, suggesting that there are sufficient, albeit low, levels of E_2 present in FCS to activate the ER.

3.3.4 – Proliferative response of T-47D cells to DHT

The proliferative response of T-47D cells to DHT was also dependent on serum conditions. Cells were cultured in phenol red free RPMI containing 5% FCS or 5% CSS, with or without the addition of DHT (0.01-100nM).

3.3.4.1 – DHT suppresses T-47D cell proliferation in medium containing 5% FCS

DHT treatment inhibited proliferation of T-47D cells in a dose dependent manner (Figure 3.4 (a)). A maximal 41% reduction in the number of viable cells was observed with 100nM DHT compared to vehicle treated controls at day nine. The number of dead cells counted per well remained less than 10% of the total cell number over the assay period, suggesting that the inhibition of T-47D cell proliferation by DHT was not due to induction of cell death (Figure 3.4 (b)).

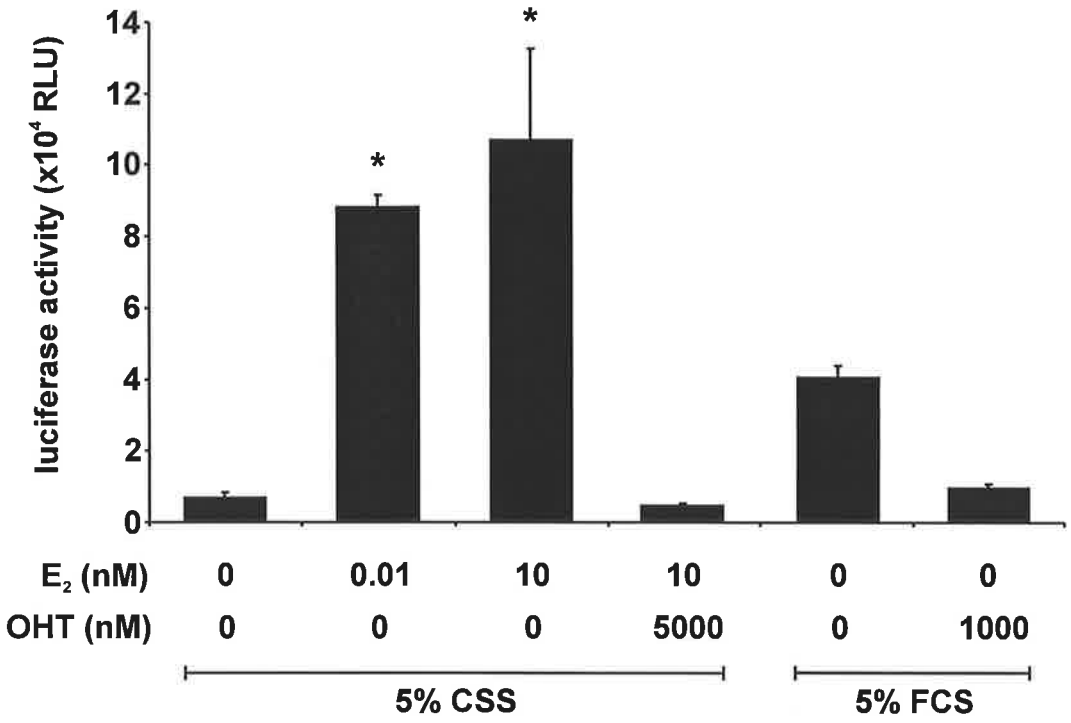


Figure 3.3: Transactivation activity of the endogenous ER in T-47D breast cancer cells. Cells were seeded in 96 well plates (2.0×10^4 cells/well) and transfected with 100ng/well of the ERE-tk-luc reporter plasmid using Lipofectamine 2000. Five hours after transfection, cells were treated with phenol red free RPMI medium containing 5% CSS and E_2 (or vehicle, 0.1% ethanol) or 5% FCS as indicated, in the presence or absence of the anti-estrogen trans-4-hydroxytamoxifen (OHT). After 48hrs, the medium was removed and cell lysates were assayed for luciferase reporter gene activity using the Luciferase Assay System. Each bar represents the mean +/- SEM of 4 replicate wells. * ANOVA; $p < 0.001$: E_2 *versus* control.

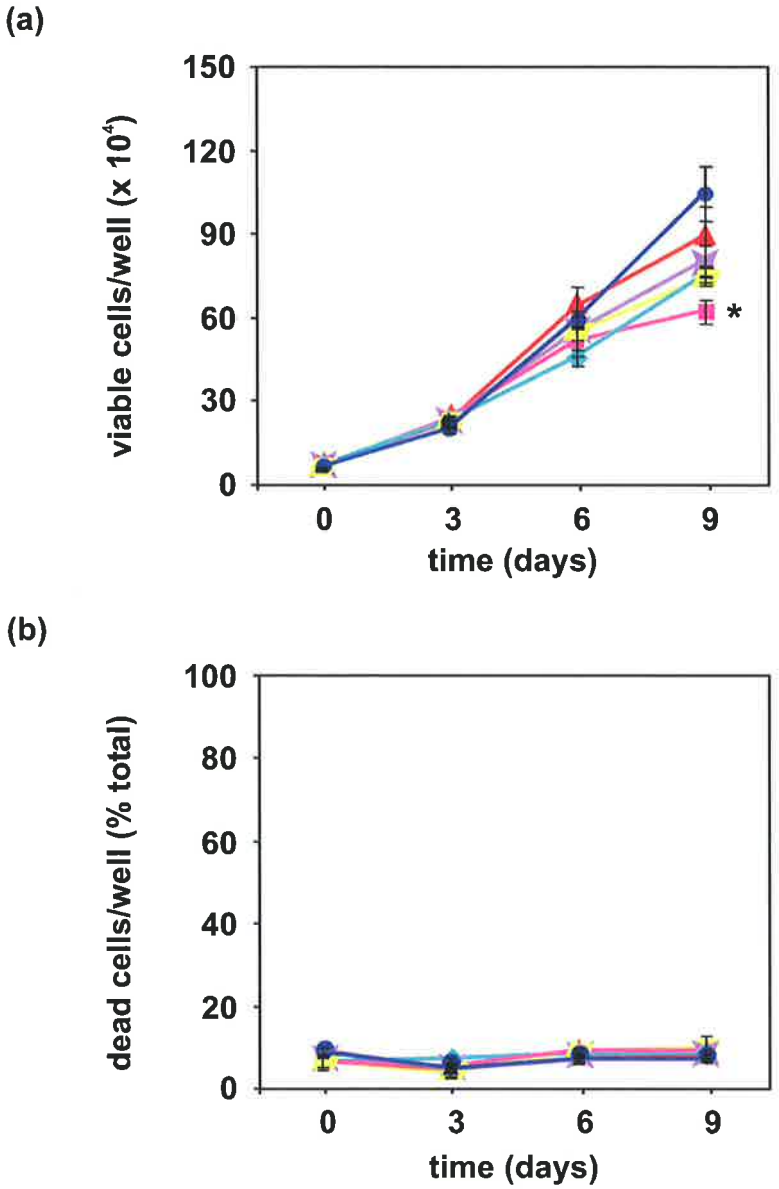


Figure 3.4: Effect of DHT, in medium containing FCS, on proliferation and viability of T-47D breast cancer cells. Cells were seeded at a density of 2.5 x 10^4 cells/well in 24 well plates and grown for 9 days in phenol red free RPMI + 5% FCS containing increasing concentrations of DHT as indicated: (●) control (0.1% ethanol), (▲) 0.01nM DHT, (▰) 0.1nM DHT, (✖) 1nM DHT, (◆) 10nM DHT, (■) 100nM DHT. Cells were counted every 3 days. *(a)* Number of viable cells/well at each time point, assessed by trypan blue exclusion. *(b)* Dead cells, expressed as a percentage of the total number of cells/well at each time point. Results are plotted as the mean +/- SEM of triplicate wells. * ANOVA; p =0.009: DHT (100nM) *versus* control.

3.3.4.2 – DHT does not suppress T-47D cell proliferation in medium containing 5% CSS

Little change in the number of viable T-47D cells/well was observed throughout the assay for all concentrations of DHT tested (Figure 3.5 (a)). There was no significant difference in the number of viable cells at day nine between vehicle treated and DHT (0.01-10nM) treated cells. Interestingly, 100nM DHT increased the number of viable cells 2.4-fold compared to controls at day nine. The number of dead cells counted per well remained less than 10% of the total cell number over the assay period (Figure 3.5 (b)).

3.3.5 – ER transactivation by DHT

Results presented in Figure 3.5 suggest that DHT, at high concentrations (100nM) and under the appropriate culture conditions, can stimulate T-47D cell proliferation. Previous studies have also demonstrated that, despite its lower affinity for the ER, high concentrations of DHT (100-1000nM) can stimulate breast cancer cell proliferation (Lippman *et al*, 1976; Noguchi *et al*, 1987; Simard *et al*, 1997; Maggiolini *et al*, 1999) and activate the ER (Ruh *et al*, 1975; Rochefort and Garcia, 1976; Zava and McGuire, 1978; Poulin and Labrie, 1986; Markiewicz and Gurpide, 1997). Reporter gene assays were therefore performed on T-47D cells, transfected with the ERE-tk-luc reporter, to determine whether 100nM DHT can activate the endogenous ER in these cells. E_2 (1nM) in medium containing 5% CSS caused a significant 34-fold increase in ER activity compared to control however no significant induction of ER activity was observed with 100nM DHT (Figure 3.6 (a)). In T-47D cells transfected with the androgen responsive PSA_{630} (promoter + enhancer)-luc reporter, 100nM DHT induced a significant 200-fold increase in endogenous AR activity compared to vehicle treated

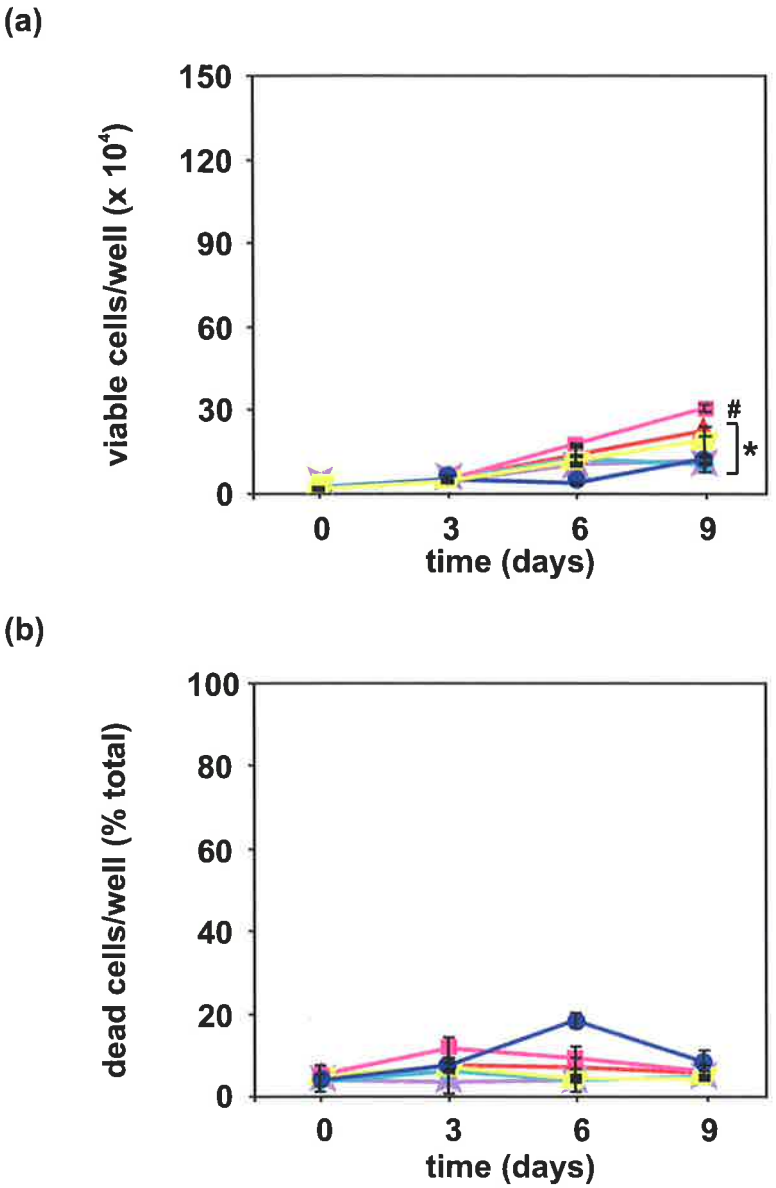


Figure 3.5: Effect of DHT, in medium containing charcoal stripped serum, on proliferation and viability of T-47D breast cancer cells. Cells were seeded at a density of 2.5 x 10^4 cells/well in 24 well plates and grown for 9 days in phenol red free RPMI + 5% CSS containing increasing concentrations of DHT as indicated: (●) control (0.1% ethanol), (▲) 0.01nM DHT, ( ) 0.1nM DHT, ( ) 1nM DHT, (◆) 10nM DHT, (■) 100nM DHT. Cells were counted every 3 days. *(a)* Number of viable cells/well at each time point, assessed by trypan blue exclusion. *(b)* Dead cells, expressed as a percentage of the total number of cells/well at each time point. Results are plotted as the mean +/- SEM of triplicate wells. * ANOVA; $p > 0.144$: DHT (0.01-10nM) *versus* control, # ANOVA; $p = 0.003$: DHT (100nM) *versus* control.

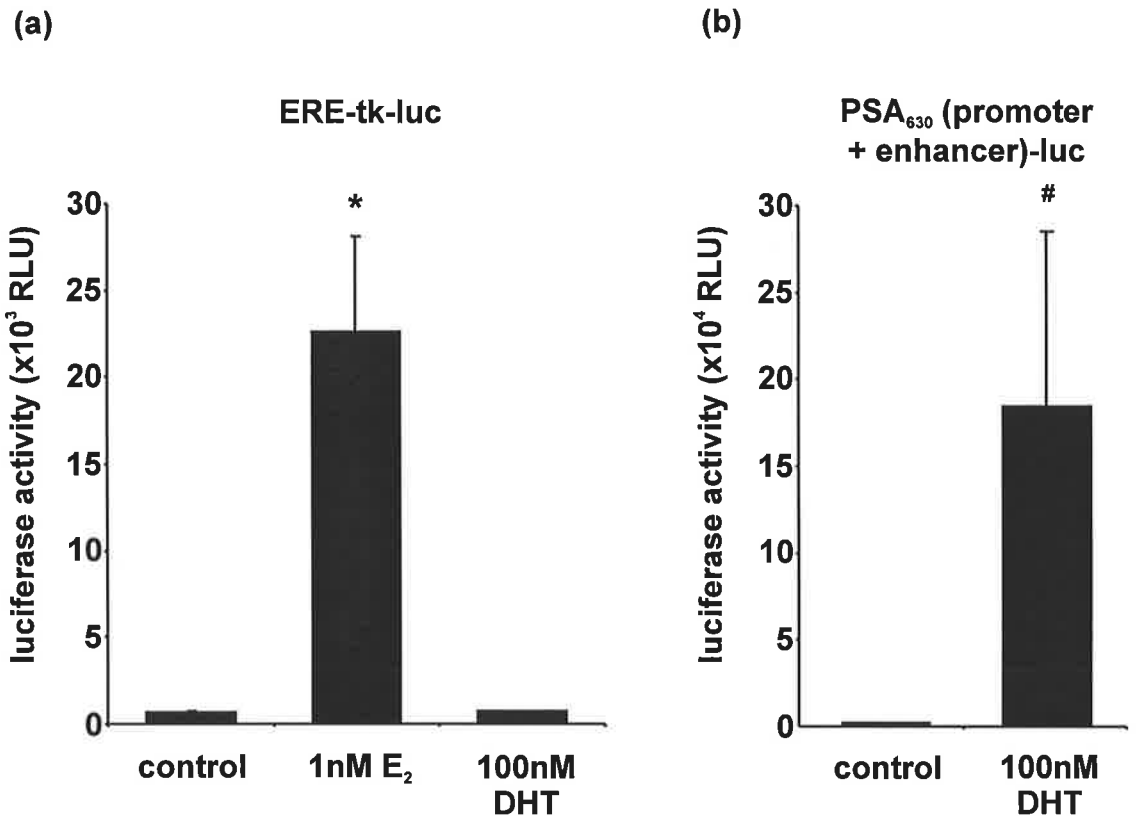


Figure 3.6: Transactivation activity of endogenous estrogen and androgen receptors in T-47D cells. Cells were seeded in 96 well plates (2.0×10^4 cells/well) and transfected with 100ng/well of the *(a)* ERE-tk-luc reporter plasmid or *(b)* PSA_{630} (promoter + enhancer)-luc reporter plasmid using Lipofectamine 2000. Five hours after transfection, cells were treated with phenol red free RPMI medium containing 5% CSS and E_2 (1nM), DHT (100nM) or vehicle (0.1% ethanol). After 48hrs, the medium was removed and cell lysates were assayed for luciferase reporter gene activity using the Luciferase Assay System. Each bar represents the mean +/- SEM of a minimum of 3 replicate wells. * ANOVA; $p < 0.001$: E_2 (1nM) *versus* control, # ANOVA; $p = 0.041$: DHT (100nM) *versus* control.

controls (Figure 3.6 (b)), indicating that the activity of DHT is specific for the AR in these cells.

3.3.6 – Mibolerone suppresses T-47D cell proliferation in medium containing 5% FCS

As inhibition of T-47D cell proliferation by DHT was only observed when cells were cultured in medium containing 5% FCS, the proliferative response to the non-metabolisable synthetic androgen mibolerone was determined using the same culture conditions. Culture with mibolerone inhibited T-47D cell proliferation in a dose dependent manner compared to controls, with a significant 56% reduction in viable cell number observed using 0.1nM mibolerone (Figure 3.7 (a)). No further inhibitory effect was achieved with concentrations of up to 100nM mibolerone. The number of dead cells counted per well again remained less than 10% of the total cell number over the assay period (Figure 3.7 (b)).

3.3.7 – DHT suppresses E_2- stimulated T-47D cell proliferation

Culture of T-47D cells with varying concentrations of DHT (0.01-100nM) inhibited E_2-stimulated proliferation when cells were cultured in medium containing 5% CSS (Figure 3.8 (a)). E_2 (1nM) alone stimulated proliferation of T-47D cells, with a 3.1-fold increase in the number of viable cells compared to control observed at day nine. In the presence of E_2 (1nM), DHT treatment inhibited cell proliferation in a dose dependent manner, resulting in a 36% reduction in viable cell number with 1nM DHT. A 1000-fold molar excess of the AR antagonist bicalutamide significantly abrogated the inhibitory effect of 1nM DHT on E_2-stimulated proliferation (Figure 3.9 (a)). DHT was a less effective inhibitor of E_2-stimulated cell proliferation than the anti-estrogen OHT, which caused a 93% reduction in the number of viable cells compared with E_2 alone

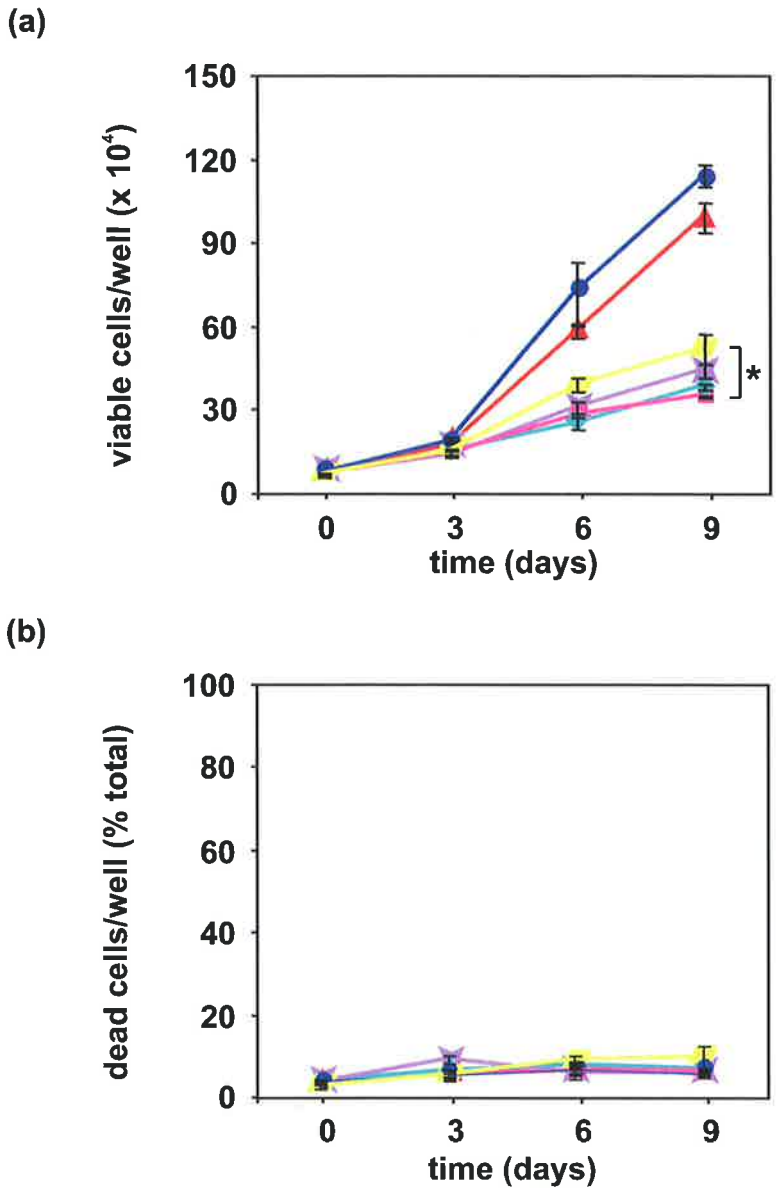


Figure 3.7: Effect of mibolerone, in medium containing FCS, on proliferation and viability of T-47D breast cancer cells. Cells were seeded at a density of 2.5 x 10^4 cells/well in 24 well plates and grown for 9 days in phenol red free RPMI + 5% FCS containing increasing concentrations of mibolerone as indicated: (●) control (0.1% ethanol), (▲) 0.01nM mibolerone, (●) 0.1nM mibolerone, (✖) 1nM mibolerone, (◆) 10nM mibolerone, (■) 100nM mibolerone. Cells were counted every 3 days. *(a)* Number of viable cells/well at each time point, assessed by trypan blue exclusion. *(b)* Dead cells, expressed as a percentage of the total number of cells/well at each time point. Results are plotted as the mean +/- SEM of triplicate wells. *ANOVA; $p < 0.001$: mibolerone (0.1-100nM) *versus* control.

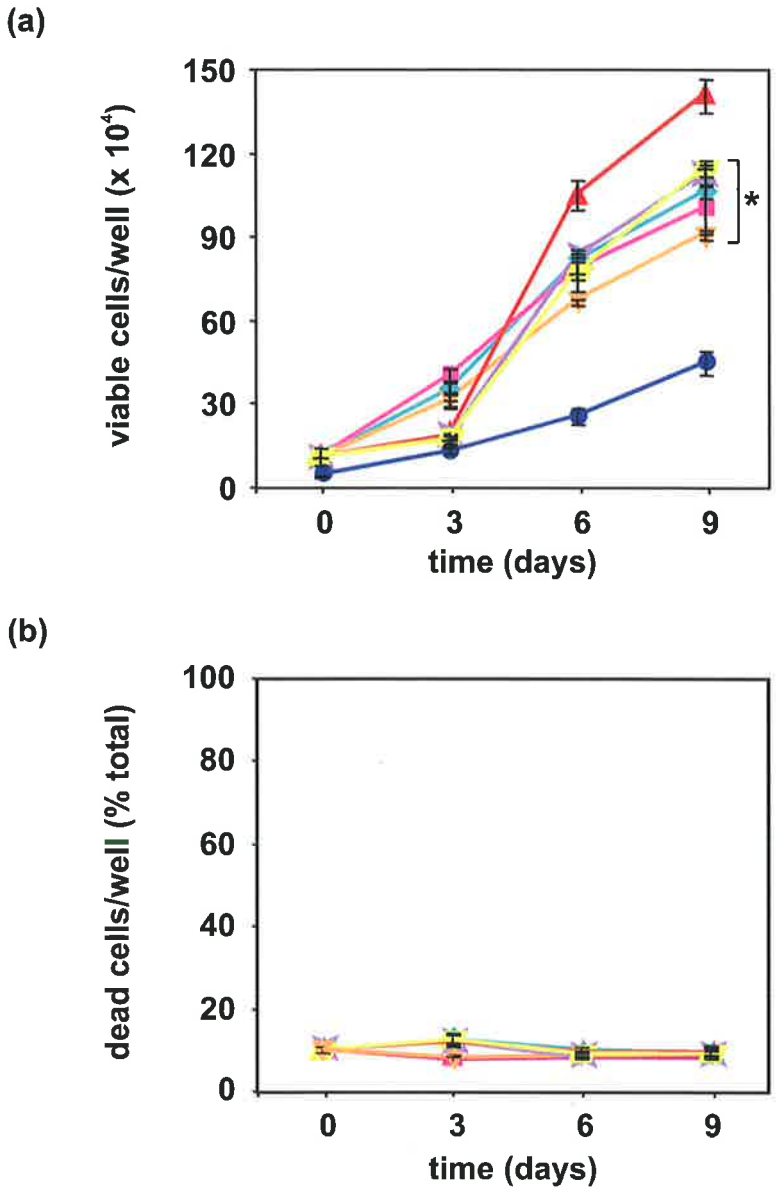


Figure 3.8: Effect of E_2 alone or in combination with DHT on proliferation and viability of T-47D breast cancer cells. Cells were seeded at a density of 5.0 x 10^4 cells/well in 24 well plates and grown for 9 days in phenol red free RPMI + 5% CSS containing E_2 and increasing concentrations of DHT as indicated: (●) control (0.1% ethanol), (▲) 1nM E_2, (▰) 1nM E_2 + 0.01nM DHT, (✖) 1nM E_2 + 0.1nM DHT, (◆) 1nM E_2 + 1nM DHT, (■)1nM E_2 + 10nM DHT, (▼) 1nM E_2 + 100nM DHT. Cells were counted every 3 days. *(a)* Number of viable cells/well at each time point, assessed by trypan blue exclusion. *(b)* Dead cells, expressed as a percentage of the total number of cells/well at each time point. Results are plotted as the mean +/- SEM of triplicate wells. * ANOVA; $p < 0.015$: E_2 (1nM) + DHT (0.01-100nM) *versus* E_2 (1nM).

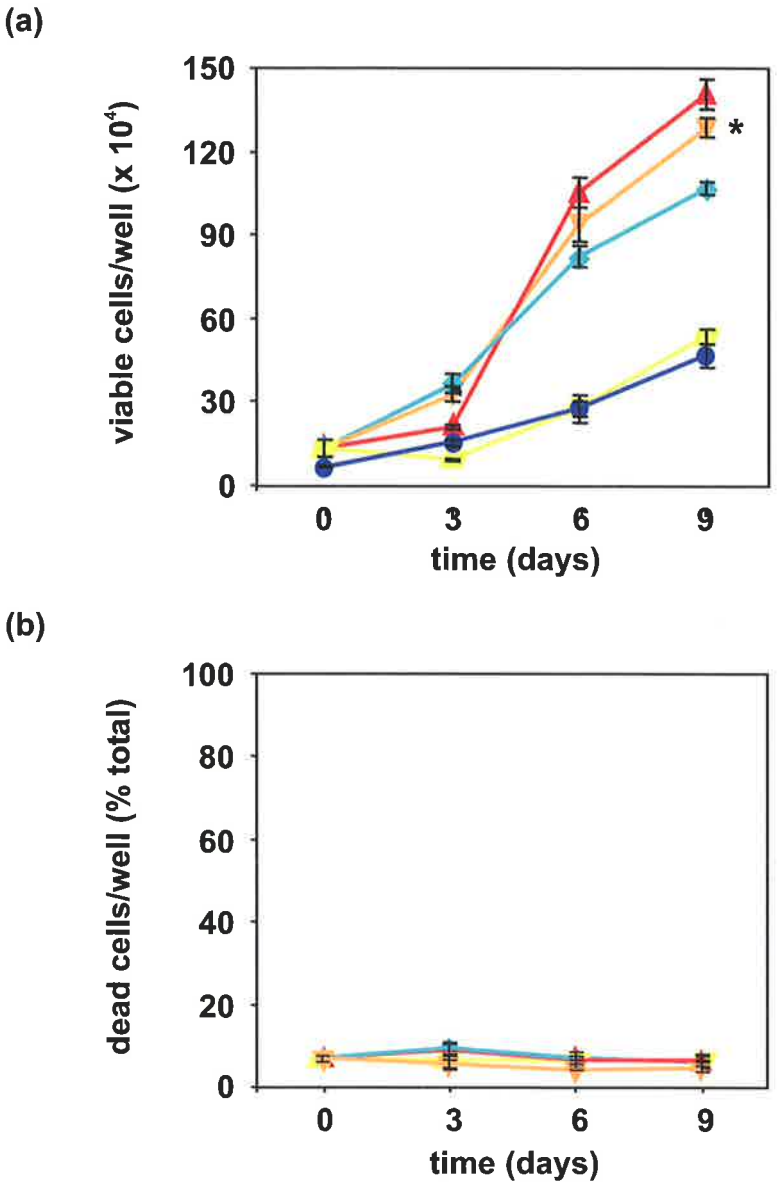


Figure 3.9: Effect of E_2 alone or in combination with OHT or DHT +/- bicalutamide on T-47D breast cancer cell proliferation and viability. Cells were seeded at a density of 5.0 x 10^4 cells/well in 24 well plates and grown for 9 days in phenol red free RPMI + 5% CSS containing steroids as indicated: (●) control (0.1% ethanol), (▲) 1nM E_2, (▰) 1nM E_2 + 1μM OHT, (◆) 1nM E_2 + 1nM DHT, (▼) 1nM E_2 + 1nM DHT + 1μM bicalutamide. Cells were counted every 3 days. *(a)* Number of viable cells/well at each time point, assessed by trypan blue exclusion. *(b)* Dead cells, expressed as a percentage of the total number of cells/well at each time point. Results are plotted as the mean +/- SEM of triplicate wells. * ANOVA; $p = 0.048$: E_2 (1nM) + DHT (1nM) + bicalutamide (1μM) *versus* E_2 (1nM) + DHT (1nM).

(Figure 3.9 (a)). The number of dead cells counted per well remained less than 10% of the total cell number over the assay period for all treatments (Figure 3.8 (b) and 3.9 (b)).

3.3.8 – Mibolerone suppresses E_2- stimulated T-47D cell proliferation

Mibolerone, under the same assay conditions, was a more potent inhibitor of E_2-stimulated T-47D cell proliferation than DHT (Figure 3.10 (a)). A maximal 80% reduction in the number of viable cells induced by E_2 (1nM) was observed with 0.1nM mibolerone after nine days. Mibolerone had comparable inhibitory effects to the anti-estrogen OHT as inhibition of E_2-induced cell proliferation by mibolerone was not significantly different from the inhibition caused by OHT (Figure 3.11 (a)). Surprisingly, however, the inhibitory effects of 1nM mibolerone were not significantly blocked by a 1000-fold molar excess of bicalutamide. The number of dead cells counted per well remained less than 10% of the total cell number over the assay period (Figure 3.10 (b) and 3.11 (b)).

3.4 – Discussion

The results presented in this chapter indicate that the potent androgens DHT and mibolerone inhibit both basal and E_2-stimulated proliferation of T-47D breast cancer cells. The inhibitory effect of the synthetic androgen mibolerone on basal and E_2-stimulated proliferation was more potent than that of the natural androgen DHT with respect to both the magnitude of inhibition and the concentration required for maximum response. Moreover, the inhibitory effect of mibolerone was comparable to that of the anti-estrogen OHT in the presence of E_2 (1nM). Assessment of cell survival in each assay indicated that inhibition by both DHT and mibolerone was not a result of increased induction of cell death. Thus it appears that androgens inhibit proliferation by

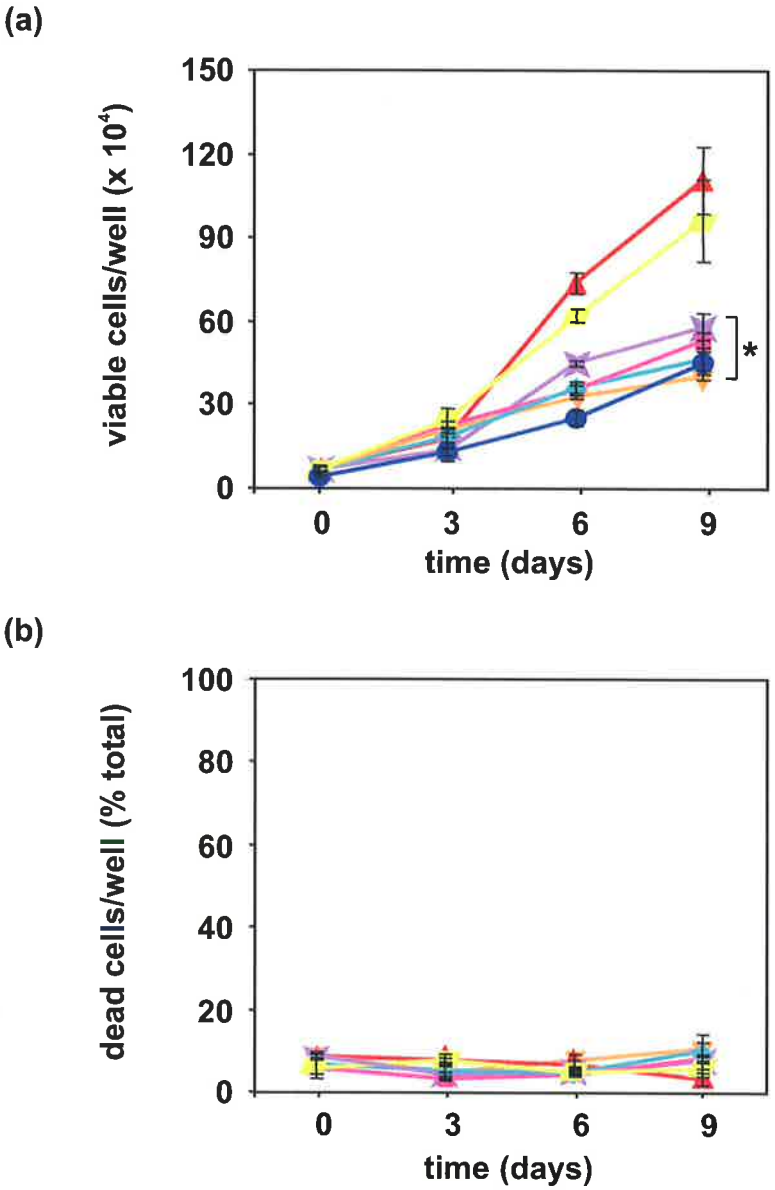


Figure 3.10: Effect of E_2 alone or in combination with mibolerone on proliferation and viability of T-47D breast cancer cells. Cells were seeded at a density of 5.0 x 10^4 cells/well in 24 well plates and grown for 9 days in phenol red free RPMI + 5% CSS containing E_2 and increasing concentrations of mibolerone as indicated: (●) control (0.1% ethanol), (▲) 1nM E_2, (▰) 1nM E_2 + 0.01nM mibolerone, (✖) 1nM E_2 + 0.1nM mibolerone, (◆) 1nM E_2 + 1nM mibolerone, (■) 1nM E_2 + 10nM mibolerone, (▼) 1nM E_2 + 100nM mibolerone. Cells were counted every 3 days. *(a)* Number of viable cells/well at each time point, assessed by trypan blue exclusion. *(b)* Dead cells, expressed as a percentage of the total number of cells/well at each time point. Results are plotted as the mean +/- SEM of triplicate wells. *ANOVA; $p < 0.003$: E_2 (1nM) + mibolerone (0.1-100nM) *versus* E_2 (1nM).

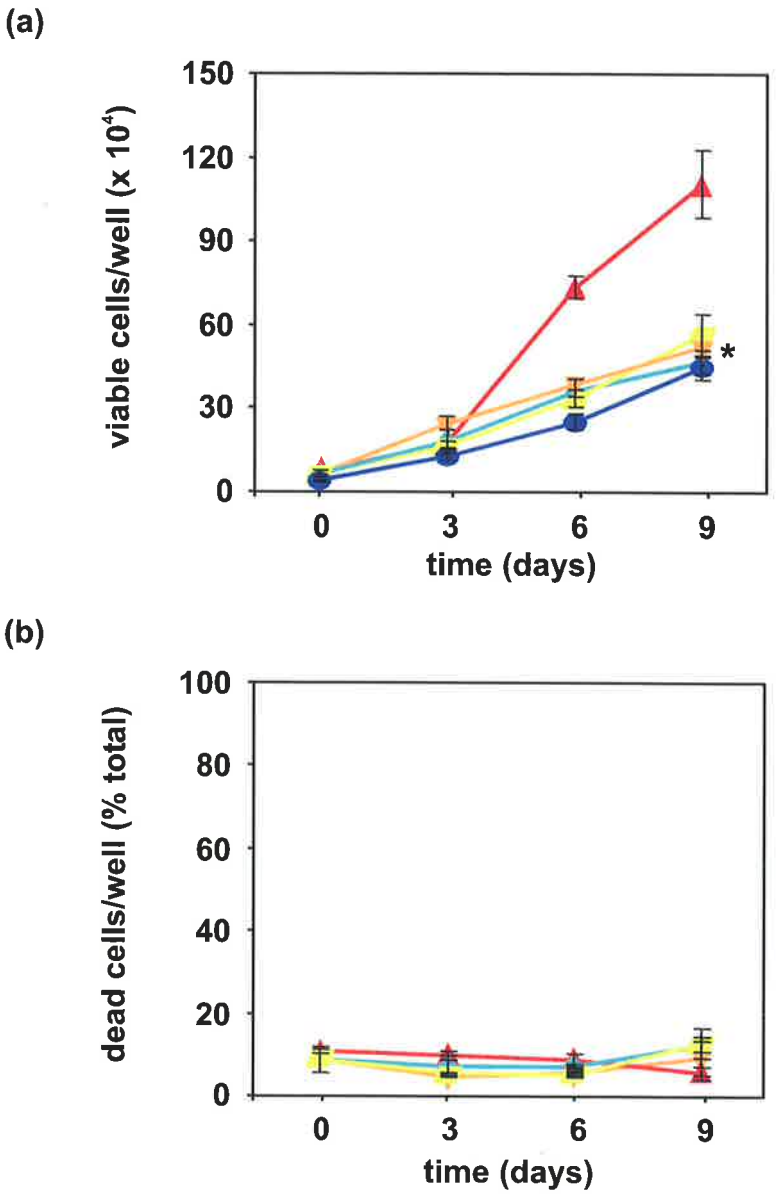


Figure 3.11: Effect of E_2 alone or in combination with OHT or mibolerone +/- bicalutamide on proliferation and viability of T-47D breast cancer cells. Cells were seeded at a density of 5.0 x 10^4 cells/well in 24 well plates and grown for 9 days in phenol red free RPMI + 5% CSS containing steroids as indicated: (●) control (0.1% ethanol), (▲) 1nM E_2, (▰) 1nM E_2 + 1μM OHT, (◆) 1nM E_2 + 1nM mibolerone, (▽) 1nM E_2 + 1nM mibolerone + 1μM bicalutamide. Cells were counted every 3 days. *(a)* Number of viable cells/well at each time point, assessed by trypan blue exclusion. *(b)* Dead cells, expressed as a percentage of the total number of cells/well at each time point. Results are plotted as the mean +/- SEM of triplicate wells. * ANOVA; p = 1.0: E_2 (1nM) + mibolerone (1nM) + bicalutamide (1μM) *versus* E_2 (1nM) + mibolerone (1nM).

inhibiting cell cycle progression or increasing cell cycle transit time. This hypothesis is supported by previous studies which have shown that MCF-7 and ZR-75-1 cells either accumulate in the G1 phase of the cell cycle or progress at a slower rate through the cell cycle in response to treatment with DHT or the synthetic androgen R1881 (de Launoit *et al*, 1991; Szelei *et al*, 1997; Ando *et al*, 2002).

Investigation of the effect of E_2 on proliferation of T-47D cells under different culture conditions suggested that proliferation of T-47D breast cancer cells is maximally stimulated by concentrations of E_2 equal to or less than 0.01nM. These observations are in agreement with previous studies which have shown maximal stimulation of T-47D cell proliferation with 0.001nM E_2, although cells were cultured in medium containing 2% FCS in that study which may have increased the level of estrogen exposure (Reese *et al*, 1988). In addition, maximal stimulation of ZR-75-1 cells cultured in phenol red free medium containing 5% CSS has been observed with 0.01nM E_2 (Poulin *et al*, 1988). In the current studies, maximal stimulation of T-47D breast cancer cell proliferation was also observed when cultured in phenol red free medium containing 5% FCS and no exogenous E_2, indicating that FCS contains sufficient levels of E_2 to induce maximal T-47D cell growth. The concentration of estrogen in medium containing 5% FCS has been previously estimated at 0.03-0.06nM (Esber *et al*, 1973). The precise level of estrogens in culture medium, however, may vary with manufacturing conditions and the use of phenol red indicators, which have estrogenic properties (Berthois *et al*, 1986; Rajendran *et al*, 1987). Although only an indirect estimate of the estrogenic activity of FCS, results from reporter gene assays in the current study imply that the concentration of estrogens in FCS is less than 0.01nM. However, it appears that these low levels of estrogen, in combination with other growth factors present in FCS, are sufficient to induce maximal proliferation of T-47D cells.

Discrepancies in the magnitude of inhibition of basal and E_2-induced proliferation by DHT and mibolerone in the current experiments may be due to differences in the stability of these ligands. DHT is rapidly metabolised to 3α-diol, 3β-diol and their glucuronidated derivatives (Figure 1.1 (b)), which have less potent androgenic activity than DHT (Rochefort and Garcia, 1983; Roy *et al*, 1992). The level of glucuronidated DHT metabolites has been shown to increase in culture medium from MCF-7 cells upon exposure to E_2 (Roy *et al*, 1992). Furthermore, 3β-diol can act via the ER to stimulate proliferation of MCF-7 cells (Hackenberg *et al*, 1993c). These studies therefore suggest that DHT metabolites may bind to the ER and activate pathways that stimulate breast cancer cell proliferation, and that this process may be stimulated by E_2. This apparent counteraction of the inhibitory effects of DHT by its metabolites may explain the weaker inhibition by DHT in T-47D cells compared with mibolerone, which is not metabolised to any significant degree. Although fresh DHT was added to cells every third day during these assays, more frequent, perhaps daily, replenishment may be required to maintain a maximal inhibitory effect. In contrast, addition of fresh mibolerone to cells every third day during these assays may result in its accumulation to levels which cannot be readily antagonised by a 1000-fold molar excess of bicalutamide. Less frequent supplementation of cells with fresh mibolerone in future experiments may resolve this issue.

While antagonism between mibolerone and estrogens has not previously been described, inhibition of E_2-induced breast cancer cell proliferation by DHT has been observed in other studies. More potent inhibitory effects of DHT on E_2-stimulated breast cancer cell proliferation were observed in previous studies (Poulin *et al*, 1988; Reese *et al*, 1988; Labrie *et al*, 1990b; Ando *et al*, 2002) compared to those described in this chapter. The differences in inhibition by DHT observed in the various studies may

be attributed to mechanics of the assay itself, such as differences in cell seeding density, serum conditions and the duration of the assay. Variation in endogenous levels of receptors, cofactors or steroid metabolising enzymes may also determine cell-specific responses to androgens and estrogens.

The inhibitory effects of androgens on breast cancer cells observed in these studies are also in agreement with the reported effects of androgens on estrogen-stimulated cell proliferation in *in vivo* models. DHT increases regression of ZR-75-1 xenografts growing in the presence and absence of exogenous E_2 in ovariectomised athymic nude mice, an effect that is clearly additive to the inhibitory effect of the anti-estrogen EM170 (Dauvois *et al*, 1991). E_2-stimulated growth of DMBA-induced mammary tumours in rats is also inhibited by DHT, and these effects are opposed by co-treatment with the AR antagonist flutamide (Dauvois *et al*, 1989). In addition, clinical studies show that androgens, such as testosterone propionate and fluoxymesterone, are effective both alone and in combination with anti-estrogenic drugs for the treatment of advanced breast cancer (Manni *et al*, 1981; Tormey *et al*, 1983; Ingle *et al*, 1991).

Previous studies have suggested that the ER may mediate the inhibitory effects of androgens on estrogen-stimulated breast cancer cell proliferation. Androgens can bind with low affinity to the ER (K_d > 100nM) (Rochefort and Garcia, 1976) and induce estrogenic responses such as nuclear translocation of the ER and expression of estrogen responsive genes (Ruh *et al*, 1975; Rochefort and Garcia, 1976; Zava and McGuire, 1978; Adams *et al*, 1981; Poulin and Labrie, 1986; Markiewicz and Gurpide, 1997). Although results from the current study imply that 100nM DHT does not activate the endogenous ER in T-47D cells, it is possible that androgens bind to the ER without inducing ER activity. High concentrations of androgens may thereby competitively

exclude binding of estrogens, which could contribute to reducing their stimulatory effects. Another potential ER-mediated mechanism for the inhibition of E_2 action by androgens may be through the reduction of E_2 bioavailability. Androgens have been shown to stimulate conversion of E_2 to estrone (E_1) by enhancing the reductive capacity of 17β-hydroxy-steroid dehydrogenase (Couture *et al*, 1993), thereby decreasing cellular concentrations of the most potent estrogen, E_2.

Observation that the effects of DHT on E_2-stimulated proliferation of T-47D cells are blocked by the AR antagonist bicalutamide provides evidence that the inhibitory effects of DHT are mediated by a mechanism involving the AR, a hypothesis which is supported by a number of previous studies. The AR antagonist hydroxyflutamide (OHF) can completely block the inhibition of E_2-stimulated proliferation by maximally effective concentrations of DHT (Poulin *et al*, 1988; Ando *et al*, 2002), while the concentration of androgens required to maximally inhibit E_2-stimulated proliferation (0.1-1nM) is in agreement with the binding affinity for the AR in breast cancer cell lines (Poulin *et al*, 1988; Bentel *et al*, 1999). This concentration is lower than that required for stimulation of ER activity (Rochefort and Garcia, 1976), suggesting that the inhibitory effects of androgens in these studies are not mediated by the ER. The inhibitory effects of DHT are also additive to those induced by maximally effective concentrations of the anti-estrogen LY156758 in ZR-75-1 cells (Poulin *et al*, 1988; Labrie *et al*, 1990b). As ER activity is completely blocked by LY156758 in these experiments, the effects of DHT do not appear to be mediated through interaction with the ER. These results are also consistent with clinical observations that combination therapy with androgens and anti-estrogens is more effective than anti-estrogens alone (Manni *et al*, 1981; Tormey *et al*, 1983; Ingle *et al*, 1991). These studies therefore provide compelling evidence that the inhibitory effects of physiological concentrations

of DHT are mediated by the AR. In contrast, abrogation of the inhibitory effects of mibolerone by bicalutamide was not observed in the current studies. Co-culture with other AR antagonists (OHF and nilutamide) also failed to block the inhibitory effects of mibolerone at 1000-fold molar excesses in T-47D cells (Ms Elisa Cops, Dame Roma Mitchell Cancer Research Laboratories, unpublished observations). This finding could indicate that inhibition of E_2-stimulated proliferation by mibolerone occurs, at least in part, through non-AR mediated mechanisms.

Studies comparing the kinetics of ligand binding and AR stabilisation by DHT and mibolerone provide little insight into potential differences in their mechanisms of action. DHT and mibolerone have been shown to have comparable binding affinity and dissociation rates for the wild type AR (Marcelli *et al*, 1994; Kemppainen *et al*, 1999). Furthermore, DHT and mibolerone induce N/C interactions, DNA binding and transcriptional activity of the AR, and stabilise the ligand-AR complex at similar concentrations (Kemppainen *et al*, 1999). However mibolerone is approximately 10-fold more effective at competing with R1881 for binding to the AR than DHT (Kemppainen *et al*, 1999). If mibolerone is also a more effective competitor than DHT for AR binding in the presence of bicalutamide, the ability of bicalutamide to antagonise AR activity induced by mibolerone may be diminished. However this is unlikely in the presence of large excesses of the AR antagonist. The use of antisense oligonucleotides directed at the AR may be a better strategy than receptor antagonists for dissecting the contribution of AR action in T-47D cells treated with mibolerone and DHT. AR antisense oligonucleotides destabilise AR mRNA and suppress translation of AR transcripts, resulting in reduced cellular AR levels (Birrell *et al*, 1995a; Eder *et al*, 2000). This alternative approach for specifically blocking the activity of the AR is less

likely to be influenced by competition between agonists and antagonists for binding to the AR.

Another potential explanation for why mibolerone action is not abrogated by AR antagonists is its ability to act through the PR. Binding of mibolerone to the PR has been demonstrated in various human and animal cell types (Bannister *et al*, 1985; Murthy *et al*, 1986). The T-47D cell line expresses high levels of PR (Horwitz *et al*, 1978) and stimulation of alkaline phosphatase activity in T-47D cells by nanomolar concentrations of mibolerone is blocked by anti-progestins but not by the anti-androgen OHF (Markiewicz and Gurpide, 1997). This suggests that inhibition of E_2-stimulated cell proliferation by mibolerone may occur through cross-talk between ER and PR. Inclusion of anti-progestins such as RU-486 in future experiments may indicate the relative contributions of AR and PR to the inhibition of E_2-stimulated T-47D cell proliferation by mibolerone.

In summary, these studies show that androgens inhibit the stimulatory effect of E_2 on breast cancer cell proliferation. This suggests that interactions between the estrogen and androgen signalling pathways may control the growth of breast cancer cells. However, as the AR antagonist bicalutamide blocked the inhibitory effect of DHT, but not mibolerone, the precise role of the AR in mediating these interactions remains unresolved. Studies in the following chapter were performed to determine whether androgens inhibit E_2-induced breast cancer cell proliferation, at least in part, by AR mediated inhibition of ER signalling.

CHAPTER 4

CROSS-TALK BETWEEN ANDROGEN AND ESTROGEN SIGNALLING PATHWAYS

4.1 – Introduction

Results from experiments using the T-47D cell line, presented in Chapter 3, are consistent with previously reported *in vitro* and *in vivo* inhibitory effects of androgens on E_2-stimulated breast cancer cell proliferation (Poulin *et al*, 1988; Reese *et al*, 1988; Dauvois *et al*, 1989; Labrie *et al*, 1990b; Prins and Woodham, 1995; Zhou *et al*, 2000; Ando *et al*, 2002). The mechanisms associated with this antagonism between androgens and estrogens are yet to be characterised. AR antagonists have previously been shown to abrogate the inhibitory effect of androgens on E_2-stimulated growth (Poulin *et al*, 1988; Ando *et al*, 2002, and Chapter 3), suggesting that the AR is an important mediator of these effects. The overall objective of this chapter was to further investigate the molecular mechanisms by which androgens may modulate estrogen signalling pathways in breast cancer cells and to further characterise the specific role of the AR in mediating these effects.

Coupled with data on the effects of androgens and estrogens on cell proliferation, there is considerable evidence that androgens inhibit induction of estrogen responsive genes in breast cancer cells. Well characterised estrogen responsive genes include the tumour marker pS2 (Masiakowski *et al*, 1982; Brown *et al*, 1984), the lysosomal endoprotease cathepsin D (Westley and May, 1987) and the progesterone receptor (PR) (Li and Li, 1978; MacIndoe and Etre, 1981; Berkenstam *et al*, 1989; Ree *et al*, 1989; Poulin *et al*, 1989b; Liberato *et al*, 1993). The induction of pS2 protein levels by E_2 (0.1nM) in ZR-75-1 cells is completely antagonised by DHT (1nM), and this inhibitory effect is comparable to that observed with the pure anti-estrogen EM-139 (100nM) (Lapointe *et al*, 1999). In contrast, studies in MCF-7 cells have shown that DHT does not alter the levels of E_2-induced pS2 mRNA (el Tanani and Green, 1995). pS2 expression does not

appear to be modulated by the androgens DHT, testosterone or androstenedione in the absence of E_2, although at high concentrations (100nM) and over long time periods (greater than 4 days), testosterone does induce pS2 mRNA in MCF-7 cells, most likely as a result of aromatic conversion to E_2 (el Tanani and Green, 1995; Burak, Jr. *et al*, 1997). Consistent with changes in pS2 expression, treatment of ZR-75-1 cells with DHT (1-10nM) also suppresses both basal and 1nM E_2-induced pro-cathepsin D mRNA expression by up to 50% (Simard *et al*, 1989; Labrie *et al*, 1990b). Furthermore, PR levels induced by E_2 in MCF-7 and ZR-75-1 cells are suppressed by testosterone and DHT in a dose dependent manner, with complete inhibition observed at concentrations greater than 10nM (MacIndoe and Etre, 1980; MacIndoe and Etre, 1981; Shapiro and Lippman, 1985; Poulin *et al*, 1989b). Importantly, the inhibitory effects of DHT on E_2-induced PR expression have been shown to be blocked by the AR antagonists cyproterone, flutamide and SCH-16423 (MacIndoe and Etre, 1980; MacIndoe and Etre, 1981). This suggests that DHT inhibits E_2-induced PR expression via a mechanism involving the AR. Basal PR protein expression in T-47D and MCF-7 cells is also inhibited by up to 50% with DHT (10-100nM) or testosterone (100-1000nM) alone (MacIndoe and Etre, 1980; Shapiro and Lippman, 1985; Liberato *et al*, 1993).

In the studies outlined above, and those discussed in Chapter 3, androgens and estrogens have both been shown to have independent and opposing effects on estrogen regulated gene expression and cell proliferation. It is currently unclear whether the antagonism between androgens and estrogens results simply from counteraction of their individual effects or whether AR activation following ligand binding permits direct inhibition of estrogen signalling pathways in breast cancer cells.

Contemporary studies investigating androgen-estrogen antagonism have focussed on the direct alterations of ER activity that are mediated via cross-talk with the AR. Reporter gene assays have been used to study the direct effect of AR signalling on ER transactivation activity. Endogenous ER activity induced by E_2 (100nM) in MCF-7 cells is reduced approximately 50% by co-transfection of an expression construct for the full length wild type AR (Ando *et al*, 2002). Interestingly, overexpression of wild type AR, even in the absence of DHT, was sufficient to reduce ER activity in these studies and co-treatment with DHT (100nM) in the presence of E_2 did not enhance the inhibitory effect of the exogenous or endogenous AR. This suggests a ligand independent mechanism for inhibition of ER activity by AR. In contrast, in CV-1 cells transfected with AR and ERα expression constructs, a 74% inhibition of 10nM E_2-induced ERα activity is observed in the presence of mibolerone (3nM) however in the absence of mibolerone the AR had no significant effect on ERα activity (Panet-Raymond *et al*, 2000). This result suggests that inhibition of ERα activity by the AR requires androgens. Measurement of endogenous *versus* exogenous ER activity and the use of different cell lines may have lead to conflicting results in these studies. These inconsistencies highlight the need for further investigation of the functional interactions between AR and ER activity in breast cancer cells.

The T-47D breast cancer cell line, which was used as an experimental model in Chapter 3 to demonstrate antagonism of estrogens and androgens on cell proliferation, was also used in studies described in this chapter to further define the mechanisms associated with inhibition of estrogen signalling pathways by androgens in breast cancer cells. The specific aims of this chapter were to investigate the effects of E_2 and DHT on the expression of the endogenous PR gene and on endogenous ER activity in T-47D cells.

Furthermore, the specific role of the AR in mediating the effects of androgens was investigated using AR antagonists or by overexpression of the AR.

4.2 – Methods

4.2.1 – Reporter gene assay

Transactivation activity of ER and AR in response to different combinations of E_2, DHT and receptor antagonists was measured using luciferase reporter gene assays. Plasmid DNA was prepared as described in Section 2.3.4. T-47D or PC-3 cells were seeded in 96 well plates at a density of 1-2 x 10^4 cells/well in phenol red free RPMI 1640 medium containing 5% CSS and allowed to attach overnight at 37°C. Cells were transiently transfected with reporter plasmids (100ng/well) and receptor expression constructs as indicated using Lipofectamine™ 2000 (Section 2.3.6). When experiments using increasing concentrations of AR expression constructs were performed, the molar amount of expression vector transfected per well was balanced by addition of empty pcDNA3.1(+) plasmid, and the nanogram amount of plasmid DNA transfected per well was kept constant by addition of pBluescript, which lacks promoter elements that may squelch transcription factors. After transfection for 5hrs, cells were treated with steroids (DHT or E_2) and/or receptor antagonists (bicalutamide, OHF or OHT) as indicated at 37°C for 24-48hrs. Cells were lysed and luciferase activity was measured using the Luciferase Assay System and a Top Count plate reading luminometer. A minimum of 3 replicate wells of each treatment were measured and results are expressed as the mean RLU ± SEM.

4.2.2 – Immunoblot analysis

Expression of PR, an estrogen regulated gene, in response to different combinations of E_2, DHT and receptor antagonists was measured by immunoblotting. T-47D cells were seeded in 10cm petri dishes at a density of 4 x 10^6 cells/dish in phenol red free RPMI 1640 medium containing 5% CSS and allowed to attach at 37°C. After 72hrs, medium was replaced with phenol red free RPMI containing 5% CSS and steroids and receptor antagonists as indicated for up to 48hrs. Treatments were replenished after 24hrs. Cells were washed twice with 10mL ice cold saline and then harvested in 400µL RIPA buffer (containing protease and phosphatase inhibitors) using a cell scraper. Cells were homogenised with a 21-gauge needle and centrifuged at 10000rpm for 10mins at 4°C to pellet cell debris. The supernatant was retained and protein concentration was estimated using a Bio-Rad protein assay kit, according to the manufacturer's instructions. Samples were kept on ice throughout the procedure to minimise protein degradation, and stored at -70°C. 20µg of each lysate was electrophoresed on a 7.5% SDS-PAGE gel followed by wet transfer to nitrocellulose membrane as described in Section 2.3.14. PR and actin (which was used as a loading control) were detected using specific primary antibodies: hPRa6 and hPRa7 combined (which recognise the A and B forms of the PR) with each diluted at 1/400, and anti-actin (I-19) diluted at 1/250. This was followed by incubation with the appropriate HRP-conjugated secondary antibody (anti-mouse for PR, anti-sheep/goat for actin) and detection using ECL. Band intensity was quantitated using the AlphaImager 2000 gel documentation system.

4.3 – Results

4.3.1 – Measurement of endogenous ER activity in T-47D cells

In T-47D cells transiently transfected with an ERE-tk-luc estrogen responsive reporter construct, a dose dependent increase in ER activity was observed with increasing concentrations of E_2 relative to the vehicle treated control (Figure 4.1). A maximal 20-fold increase in ER activity was attained using 1nM E_2.

4.3.2 – Measurement of endogenous AR activity in T-47D cells

Transactivation activity of the endogenous AR in T-47D cells was initially tested using three different androgen responsive luciferase reporter constructs (see Appendix 1). DHT (10nM) induced a 2.4-fold increase in AR transactivation activity in cells transfected with the tk81-PB_3-luc construct and a 120-fold increase in cells transfected with the PSA_{630} (promoter + enhancer)-luc construct relative to controls (Figure 4.2 (a) and (b)). No significant difference in AR transactivation activity was observed between DHT treated cells and vehicle treated controls with the PSA_{630} (promoter)-luc construct, which lacks the enhancer sequence (Figure 4.2 (c)). This is consistent with the low levels of androgen responsiveness the PSA promoter sequence confers in the absence of the upstream enhancer element in LNCaP prostate cancer cells (Cleutjens *et al*, 1996; Schuur *et al*, 1996; Cleutjens *et al*, 1997; Zhang *et al*, 1997c).

In T-47D cells transfected with the PSA_{630} (promoter + enhancer)-luc reporter construct, which of the three androgen responsive reporters tested above demonstrated the highest level of AR transactivation activity in response to DHT, a dose dependent increase in AR activity was observed with increasing concentrations of DHT (Figure 4.3). A maximal 200-fold induction of endogenous AR activity was attained with 100nM DHT.

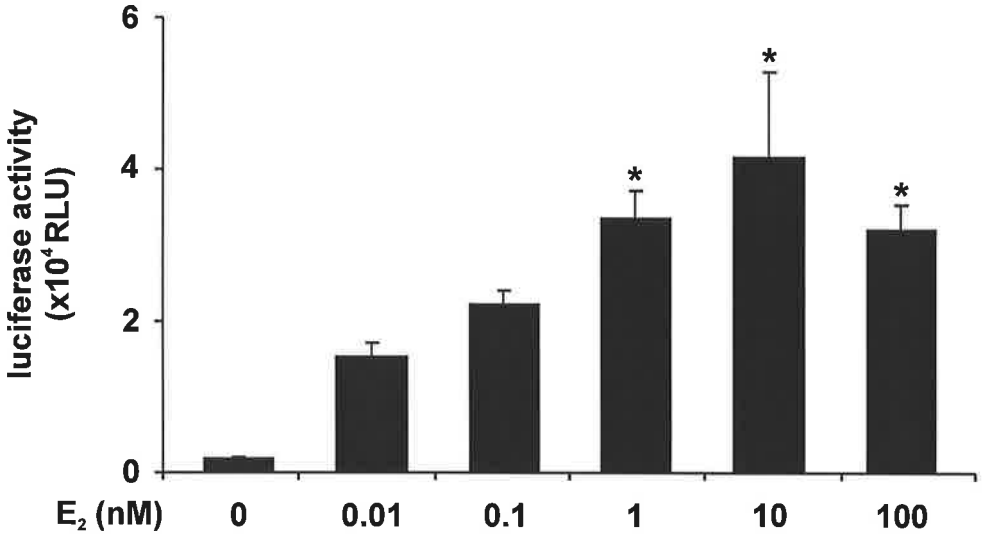


Figure 4.1: Transactivation activity of the endogenous ER in the T-47D breast cancer cell line. Cells were seeded in 96 well plates (2 x 10^4 cells/well) and transfected with 100ng/well of the ERE-tk-luc reporter plasmid using Lipofectamine 2000. Five hours after transfection, cells were treated with phenol red free RPMI medium containing 5% CSS and increasing concentrations of E_2 (0.01-100nM) or vehicle (0.1% ethanol) as indicated. After 48hrs, the medium was removed and cell lysates were assayed for luciferase reporter gene activity using the Luciferase Assay System. Each bar represents the mean +/- SEM of 4 replicate wells. * ANOVA; $p < 0.006$: E_2 *versus* control.

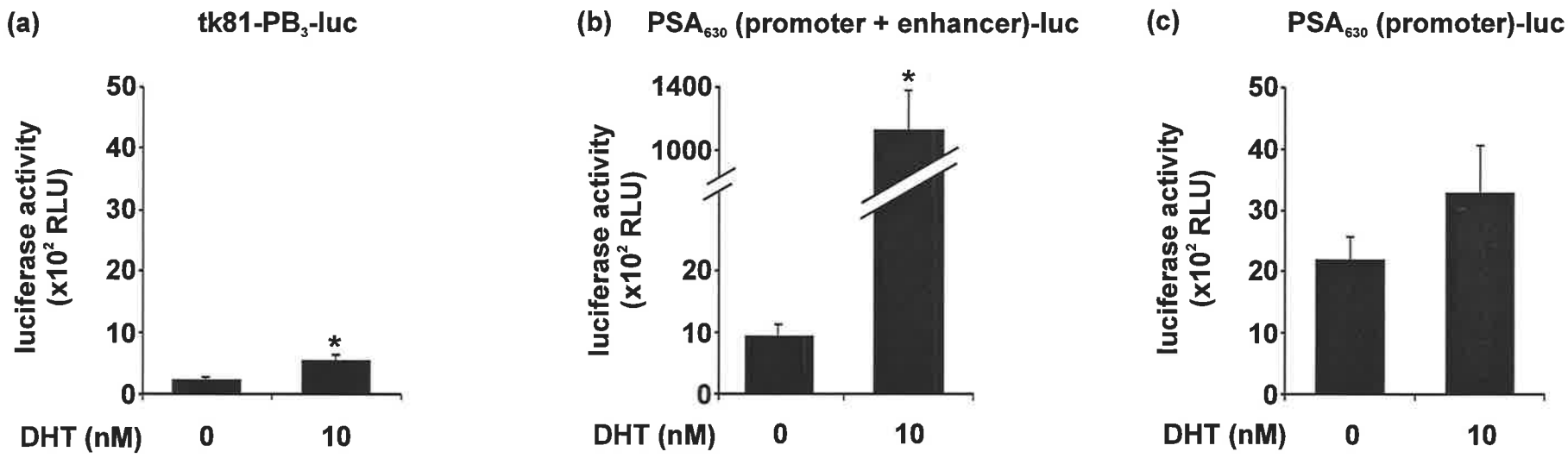


Figure 4.2: Transactivation activity of the endogenous AR at different androgen responsive promoters in the T-47D breast cancer cell line. Cells were seeded in 96 well plates (2 x 10^4 cells/well) and transfected with 100ng/well of the *(a)* tk81-PB_3-luc, *(b)* PSA_{630} (promoter + enhancer)-luc or *(c)* PSA_{630} (promoter)-luc reporter plasmids using Lipofectamine 2000. Five hours after transfection, cells were treated with phenol red free RPMI medium containing 5% CSS and DHT (10nM) or vehicle (0.1% ethanol) as indicated. After 48hrs, the medium was removed and cell lysates were assayed for luciferase reporter gene activity using the Luciferase Assay System. Each bar represents the mean +/- SEM of a minimum of 3 replicate wells. * t-test; $p < 0.03$: DHT *versus* control.

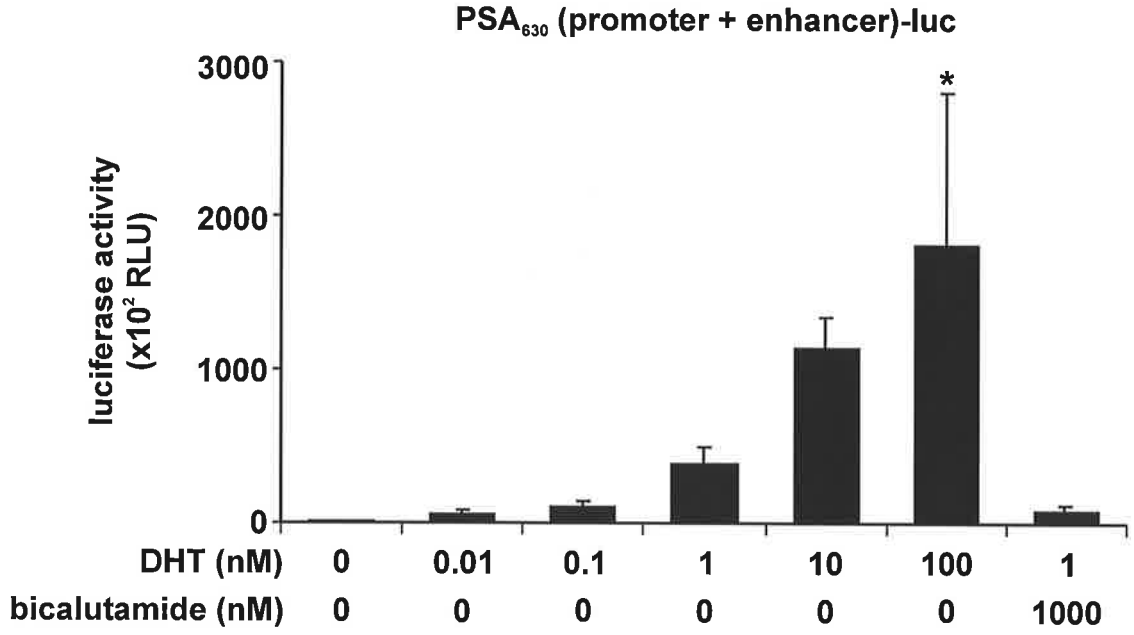


Figure 4.3: Transactivation activity of the endogenous AR in the T-47D breast cancer cell line in response to increasing concentrations of DHT. Cells were seeded in 96 well plates (2×10^4 cells/well) and transfected with 100ng/well of the PSA_{630} (promoter + enhancer)-luc reporter plasmid using Lipofectamine 2000. Five hours after transfection, cells were treated with phenol red free RPMI medium containing 5% CSS and increasing concentrations of DHT (0.01-100nM) or vehicle (0.1% ethanol), in the presence or absence of bicalutamide (1μM)as indicated. After 48hrs, the medium was removed and cell lysates were assayed for luciferase reporter gene activity using the Luciferase Assay System. Each bar represents the mean +/- SEM of a minimum of 3 replicate wells. * ANOVA; $p = 0.041$: DHT *versus* control.

A 1000-fold molar excess of the AR antagonist bicalutamide partially blocked AR transactivation by 1nM DHT, although this did not reach significance.

4.3.3 – Modulation of PR expression by E_2 and DHT

Having demonstrated that the T-47D breast cancer cell line contains functional ER and AR, the effect of DHT and E_2 on expression of the PR, an endogenous estrogen responsive gene, was examined using immunoblot analysis. Treatment with E_2 (1nM) resulted in a 2.1-fold increase in PR levels relative to the vehicle treated control (Figure 4.4). Co-treatment with E_2 (1nM) and either OHT (1μM) or DHT (1nM) completely blocked the stimulatory effect of E_2, with PR levels reduced to below the level of the control. This is consistent with previous studies showing complete inhibition of E_2-induced PR expression by DHT in breast cancer cell lines (MacIndoe and Etre, 1980; MacIndoe and Etre, 1981; Shapiro and Lippman, 1985; Poulin *et al*, 1989b) and suggests that DHT has comparable efficacy to the anti-estrogen for blocking gene expression induced by E_2. The inhibitory effect of DHT was almost completely antagonised by a 1000-fold molar excess of bicalutamide, with PR levels increasing to 85% of that observed in E_2 treated cells (Figure 4.4). While treatment with DHT (1nM) alone reduced PR levels by 65% relative to the control, this inhibition was not antagonised by bicalutamide.

4.3.4 – Modulation of endogenous ER activity by DHT

In T-47D cells transiently transfected with an ERE-tk-luc estrogen responsive reporter construct, treatment with DHT inhibited ER activity induced by E_2 (1nM) (Figure 4.5). This is consistent with results described above, where DHT also inhibited induction of an endogenous gene by E_2. However, the inhibitory effect observed with DHT was not as strong in these reporter gene assays, with a maximal 57% inhibition of ER activity

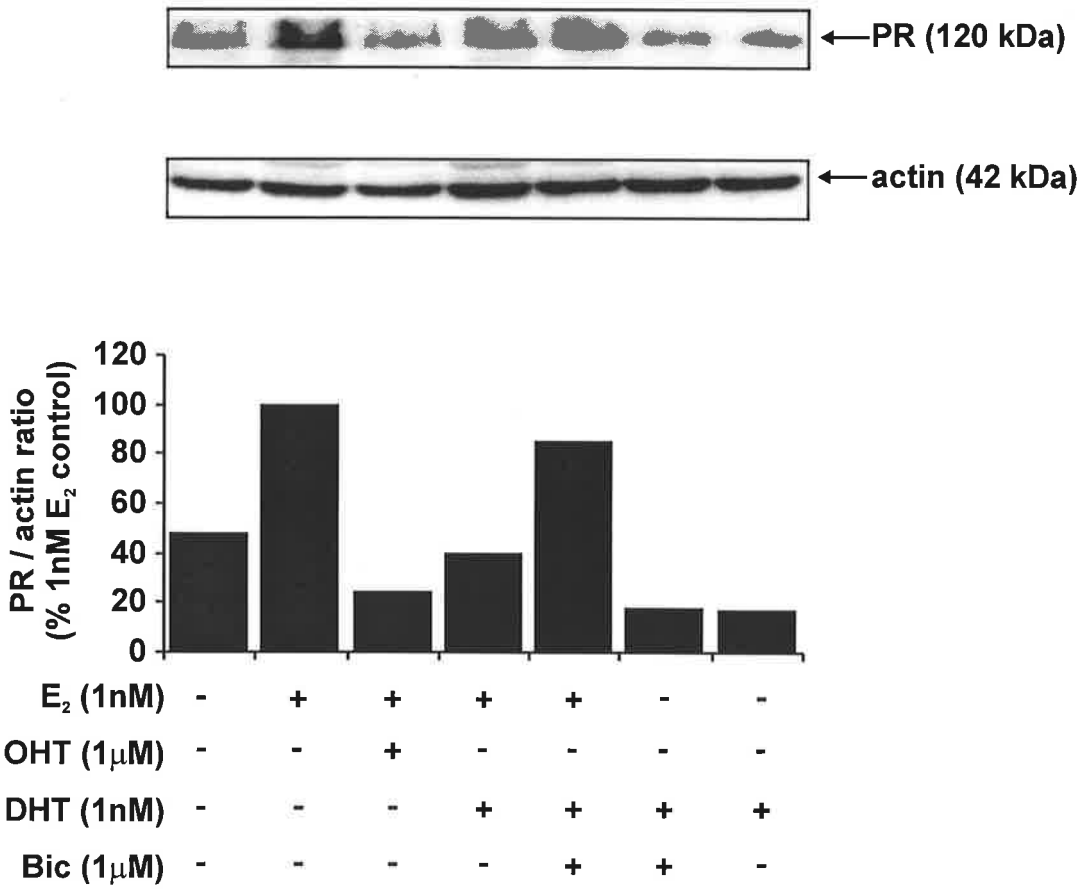


Figure 4.4: Expression of the estrogen responsive gene, PR, in T-47D breast cancer cells treated with E_2 and DHT. Cells were seeded in 10cm petri dishes (5×10^5 cells/dish) and allowed to attach for 72hrs. The medium was removed and replaced with phenol red free RPMI containing steroids and receptor antagonists as indicated. After 48hrs, cell lysates were prepared and protein content was measured using a protein assay kit. 20μg of each lysate was electrophoresed on a 7.5% SDS PAGE gel and transferred to nitrocellulose membrane. PR (upper panel) and actin (centre panel) were detected by immunoblotting using specific primary antibodies as described in the text. PR protein levels were measured by densitometric analysis and corrected for actin (lower panel).

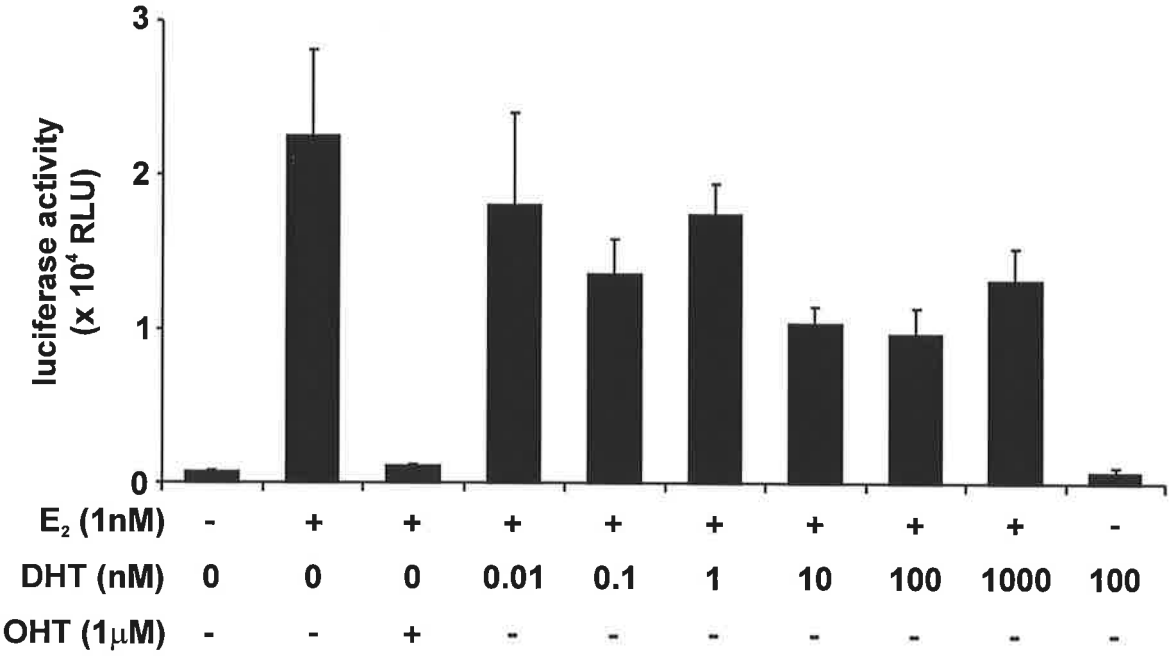


Figure 4.5: Effect of DHT on endogenous ER activity in T-47D breast cancer cells. Cells were seeded in 96 well plates (2 x 10^4 cells/well) and transfected with 100ng/well of the ERE-tk-luc reporter construct using Lipofectamine 2000. Five hours after transfection, cells were treated with phenol red free RPMI medium containing 5% CSS and vehicle (0.1% ethanol) or E_2 (1nM) alone or in combination with OHT (1μM) or increasing concentrations of DHT (0.01-1000nM) as indicated. After 24hrs, the medium was removed and cell lysates were assayed for luciferase reporter gene activity using the Luciferase Assay System. Each bar represents the mean +/- SEM of 4 replicate wells.

observed with 100nM DHT. 1μM DHT was slightly less effective at inhibiting ER activity than 100nM DHT, possibly due to the activation of ER at high DHT concentrations (Ruh *et al*, 1975; Rochefort and Garcia, 1976; Zava and McGuire, 1978; Adams *et al*, 1981; Poulin and Labrie, 1986; Markiewicz and Gurpide, 1997). 100nM DHT had no significant effect on ER activity in the absence of E_2

The contribution of the AR to inhibition of ER activity by DHT was assessed using the AR antagonists bicalutamide and OHF. Results from a representative experiment using OHF are shown in Figure 4.6. In this experiment, a significant 42% reduction in E_2-induced ER activity was observed with 10nM DHT. However this inhibition by DHT was not blocked by a 100-fold molar excess of OHF. Rather, the antagonist slightly enhanced the inhibitory effect of DHT, although the difference was not statistically significant. The inability of OHF or bicalutamide to block the inhibitory effects of DHT was noticed in numerous experiments of this type, even when cells were pre-treated with antagonist for 1hr before addition of DHT (data not shown).

4.3.5 – Inhibition of transfected ERα activity by transfected AR

Experiments performed by Dr Grant Buchanan in our research group examined the effect of AR on E_2-induced ERα activity, using a transient transfection assay in PC-3 prostate cancer cells. Cells were transiently transfected with the ERE-tk-luc reporter construct, the HEGO/pSG5 ERα expression construct and either the pCMV3.1-AR:wt or the pcDNA-AR(NTD/DBD) expression constructs, which encode the full length AR or the constitutively active amino terminal transactivation and DNA binding domains (NTD/DBD, amino acids 1-646) of the AR respectively.

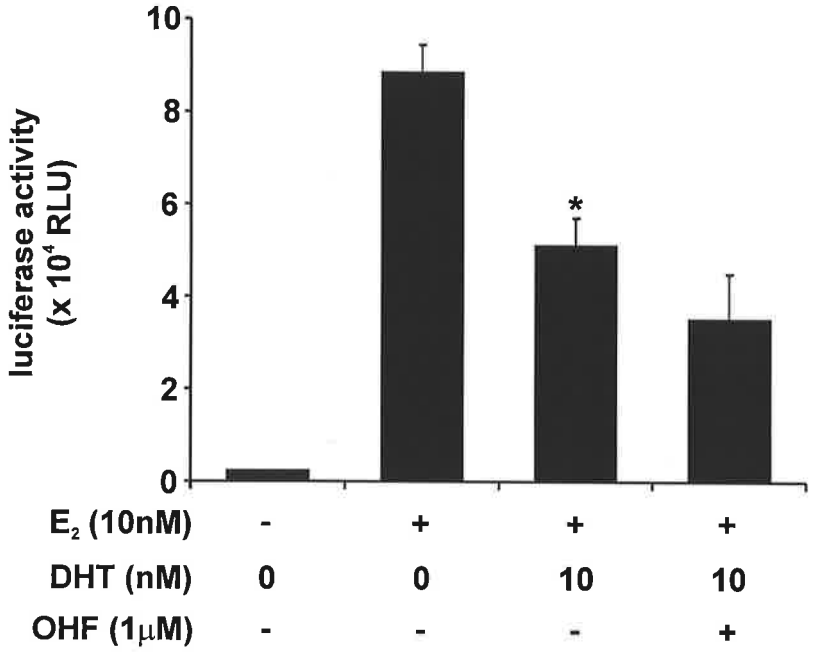


Figure 4.6: Effect of DHT and hydroxyflutamide (OHF) on endogenous ER activity in T-47D breast cancer cells. Cells were seeded in 96 well plates (2 x 10^4 cells/well) and transfected with 100ng/well of the ERE-tk-luc reporter construct using Lipofectamine 2000. Five hours after transfection, cells were treated with phenol red free RPMI medium containing 5% CSS and vehicle (0.1% ethanol), E_2 (10nM) alone or in combination with DHT (10nM), with or without the AR antagonist OHF (1μM) as indicated. After 48hrs, the medium was removed and cell lysates were assayed for luciferase reporter gene activity using the Luciferase Assay System. Each bar represents the mean +/- SEM of 4 replicate wells. * ANOVA; $p = 0.007$: DHT (10nM) +E_2 (10nM) *versus* E_2 (10nM).

4.3.5.1 – Effect of the full length AR

In PC-3 cells transiently transfected with the HEGO/pSG5 ERα expression construct and the ERE-tk-luc reporter construct, co-transfection of increasing amounts of the pCMV3.1-AR:wt expression construct modulated activation of ERα by E_2 (Figure 4.7). Although there was variation in the levels of absolute ERα activity observed in the presence of E_2 alone in this experiment with transfection of different amounts of the AR expression construct, there was no significant difference observed in the fold-induction by E_2 between these groups, which ranged from 3.7 to 6.9-fold. Addition of DHT decreased both absolute ERα activity and fold-induction of ERα activity by E_2, with significant 39-52% inhibition observed with transfection of greater than 2.5ng/well of the full length AR expression construct. These results suggest that the full length AR inhibits E_2-induced ERα activity in the presence of DHT.

4.3.5.2 – Effect of the AR-NTD

In PC-3 cells transiently transfected with the HEGO/pSG5 ERα expression construct and the ERE-tk-luc reporter construct, co-transfection of increasing amounts of the pcDNA-AR(NTD/DBD) expression construct also significantly inhibited ERα activity (Figure 4.8). The AR-NTD is constitutively active (Simental *et al*, 1991; Jenster *et al*, 1991; Ikonen *et al*, 1997), eliminating the requirement for treatment with DHT in these experiments. An 8.0-fold increase in ERα activity was observed with E_2 (1nM) in the absence of exogenous AR-NTD. Increasing amounts of the AR-NTD resulted in a dose dependent decrease in E_2-induced ERα activity, with significant inhibition observed at 2.5, 5 and 10ng/well of the pcDNA-AR(NTD/DBD) construct. A maximal 81% inhibition of ERα activity was attained with 10ng/well of the AR-NTD. Additionally, a dose dependent inhibition of basal ERα activity in the absence of E_2 was

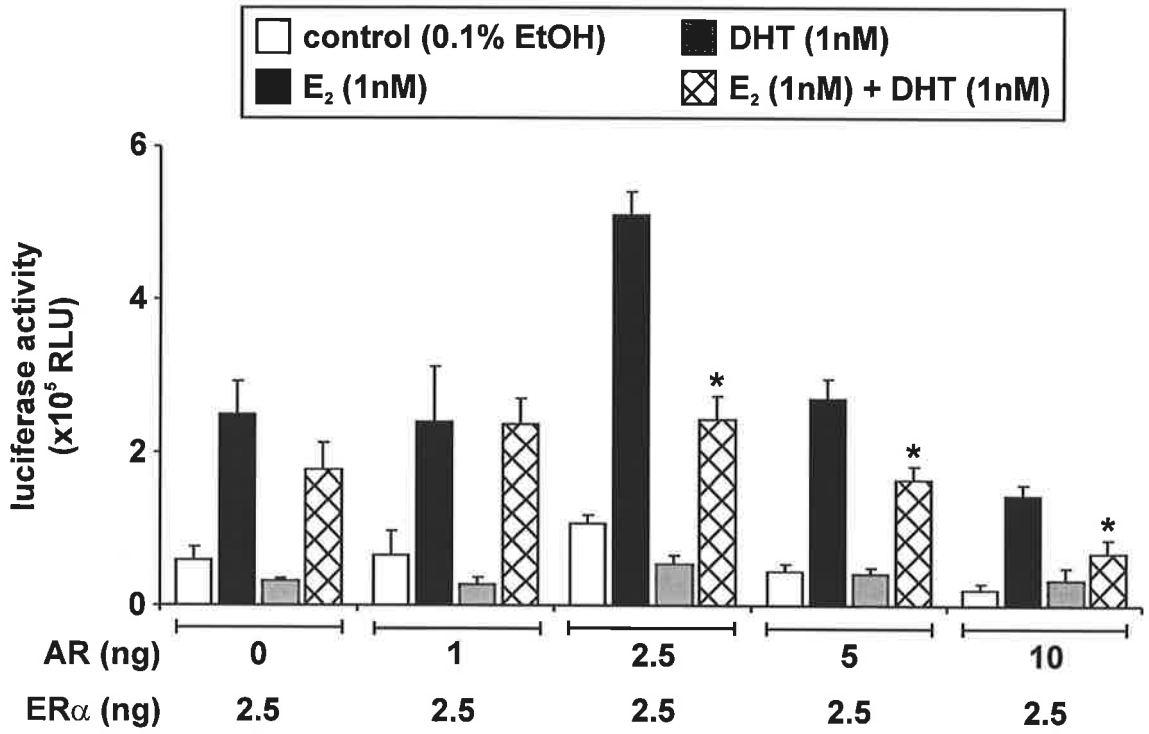


Figure 4.7: Effect of the full length AR on ERα activity. PC-3 cells, seeded in 96 well plates (1.5 x 10^4 cells/well) were transfected with 2.5ng/well of the HEGO/pSG5 ERα expression construct, 100ng/well of the ERE-tk-luc reporter plasmid and increasing amounts (0-10ng/well) of the pCMV3.1-AR:wt expression construct using Lipofectamine 2000. Five hours after transfection, cells were treated with phenol red free RPMI medium containing 5% CSS and E_2 (1nM) in the presence or absence of DHT (1nM) as indicated. After 36hrs, the medium was removed and cell lysates were assayed for luciferase reporter gene activity using the Luciferase Assay System. Each bar represents the mean +/- SEM of a minimum of 3 replicate wells. * t-test; $p < 0.013$: DHT (1nM) + E_2 (1nM) *versus* E_2 (1nM). This experiment was performed by Dr Grant Buchanan.

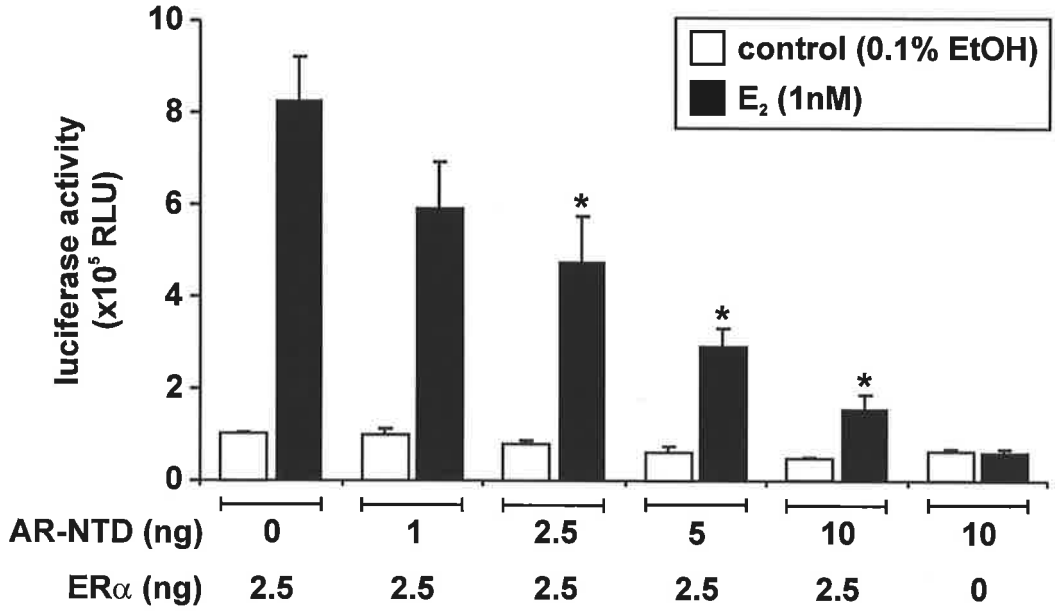


Figure 4.8: Effect of the constitutively active AR amino terminal transactivation domain (NTD, amino acids 1-646) on ERα activity. PC-3 cells, seeded in 96 well plates (1 x 10^4 cells/well) were transfected with 2.5ng/well of the HEGO/pSG5 ERα expression construct, 100ng/well of the ERE-tk-luc reporter plasmid and increasing amounts (0-10ng/well) of the pcDNA-AR(NTD/DBD) expression construct using Lipofectamine 2000. Five hours after transfection, cells were treated with phenol red free RPMI medium containing 5% CSS and E_2 (1nM) or vehicle (0.1% ethanol) as indicated. After 36hrs, the medium was removed and cell lysates were assayed for luciferase reporter gene activity using the Luciferase Assay System. Each bar represents the mean +/- SEM of a minimum of 3 replicate wells. * ANOVA; $p < 0.04$: E_2 + AR-NTD *versus* E_2 alone. This experiment was performed by Dr Grant Buchanan.

observed with increasing amounts of the AR-NTD, although this inhibition did not reach significance. E_2 did not induce reporter gene activity in cells transfected with the AR-NTD only (data not shown).

4.3.6 – Inhibition endogenous ER activity by transfected AR

The effect of the AR-NTD on activity of the endogenous ER in the T-47D breast cancer cell line is shown in Figure 4.9. A 16-fold increase in ER activity was observed with E_2 (1nM) in the absence of exogenous AR-NTD. Co-transfection of increasing amounts of the pcDNA-AR(NTD/DBD) expression construct in the presence of E_2 resulted in a dose dependent decrease in ER activity, with significant inhibition observed at 40-100ng/well of the pcDNA-AR(NTD/DBD) construct. A maximal 63% inhibition of ER activity was attained with 100ng/well of the pcDNA-AR(NTD/DBD) expression construct. A dose dependent inhibition of basal ER activity was also observed with increasing amounts of the AR-NTD, although this was not statistically significant.

4.4 – Discussion

The studies presented in this chapter provide further evidence for a functional interaction between androgen and estrogen signalling pathways in breast cancer cells which results in inhibition of ER activity. This is supported by experiments showing that DHT suppresses E_2-induced endogenous PR expression and that DHT can inhibit E_2-induced activity of the endogenous ER in T-47D cells. Furthermore, antagonism of estrogen signalling pathways by androgens appears to be mediated, at least in part, by the AR, as the AR antagonist bicalutamide can block the inhibitory effects of DHT on E_2-induced PR expression and overexpression of the AR, or its constitutively active NTD, can inhibit E_2-induced ER activity in a dose dependent manner. Inhibition of

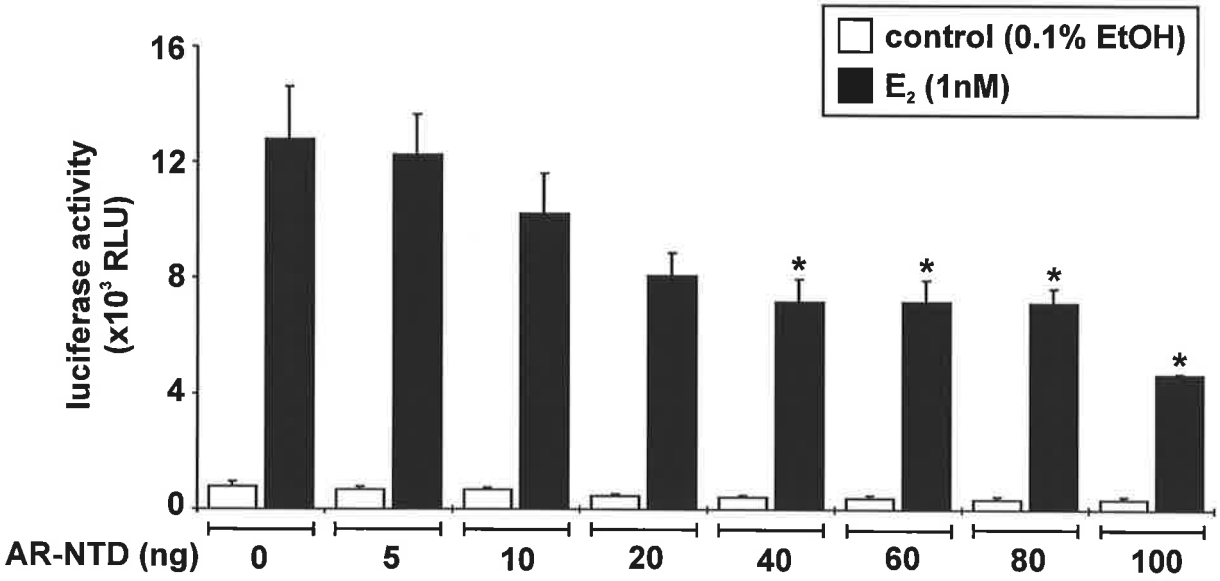


Figure 4.9: Effect of the constitutively active AR amino terminal transactivation domain (NTD, amino acids 1-646) on endogenous ERα activity in T-47D breast cancer cells. Cells were seeded in 96 well plates (2 x 10^4 cells/well) and transfected with 100ng/well of the ERE-tk-luc reporter plasmid and increasing amounts (0-100ng/well) of the pcDNA-AR(NTD/DBD) expression construct using Lipofectamine 2000. Five hours after transfection, cells were treated with phenol red free RPMI medium containing 5% CSS and E_2 (1nM) or vehicle (0.1% ethanol) as indicated. After 24hrs, the medium was removed and cell lysates were assayed for luciferase reporter gene activity using the Luciferase Assay System. Each bar represents the mean +/- SEM of a minimum of 3 replicate wells. * ANOVA; $p < 0.025$: E_2 + AR-NTD *versus* E_2 alone.

estrogen signalling pathways by androgens, or the AR, may be one mechanism by which androgens inhibit E_2-stimulated breast cancer cell proliferation.

A more potent inhibitory effect of DHT on E_2-induced endogenous PR expression was observed compared to the effect that DHT had on E_2-induced endogenous ER activity, as measured by reporter gene assays. Reporter gene assays, which are a measure of ER activity, suggest that DHT can directly, although incompletely, inhibit E_2-induced ER activity but that DHT has no effect on ER activity in the absence of E_2. On the other hand, inhibition of E_2-induced PR expression by DHT may be mediated by two independent mechanisms: inhibition of ER activity (as observed in reporter gene assays) as well as inhibition by the androgen signalling pathway. The latter concept is supported by observations that DHT can downregulate basal PR expression in the absence of E_2. The additive effects of these independent mechanisms may have contributed to the more potent inhibitory effect of DHT observed with measurement of endogenous PR expression.

Complete inhibition of endogenous ER activity, either by overexpression of the AR-NTD or by activation of the endogenous AR with DHT, was not observed in experiments performed in T-47D cells. Furthermore, the anti-estrogen OHT had more potent anti-estrogenic effects than DHT. This is consistent with previous studies which have also demonstrated incomplete inhibition of ER activity by androgens or the AR. In CV-1 cells transiently transfected with full length AR and ERα expression constructs, mibolerone inhibited E_2-induced ERα activity by 74% (MacIndoe and Etre, 1981). Similarly, in MCF-7 cells, E_2-induced endogenous ER activity was not appreciably inhibited by the endogenous AR in the presence of DHT, while transfection of a wild type AR expression construct into these cells only inhibited ER activity by

approximately 50% (Ando *et al*, 2002). Interestingly, experiments performed by Ando *et al* (2002) demonstrated that overexpression of the full length AR was sufficient for inhibiting E_2-induced ER activity and treatment with DHT did not elicit further inhibition. This suggests that AR expression alone, rather than the level of AR activity induced by DHT, may be associated with the degree of inhibition.

AR antagonists were used in order to ascertain the specific role of the AR in the inhibition of ER activity. An AR mediated mechanism of inhibition is supported by experiments which showed that bicalutamide almost completely blocked the inhibitory effect of DHT on E_2-induced endogenous PR protein levels in T-47D cells. In contrast, reporter gene assays showed that the AR antagonists OHF and bicalutamide failed to block the inhibitory effect of DHT on ER activity, suggesting that inhibition by DHT is not mediated by the AR. However, binding of AR antagonists may also potentially enable the AR to interact with the ER and inhibit its activity. Bicalutamide and OHF are non-steroidal anti-androgens which prevent assembly of an active transcription complex and expression of androgen responsive genes (Fenton *et al*, 1997; Kemppainen *et al*, 1999; Shang *et al*, 2002). However bicalutamide and OHF have been shown to induce nuclear translocation of the AR and chromatin immunoprecipitation assays have demonstrated that the bicalutamide-AR complex binds to androgen response elements (Jenster *et al*, 1993; Poukka *et al*, 2000; Shang *et al*, 2002; Masiello *et al*, 2002). This suggests that the antagonist-bound AR may assume a conformation that not only translocates to the nucleus but that also potentially retains the ability to interact with ERα, thereby inhibiting its activity. This is further supported by observations that ER, to some extent, can inhibit R5020-induced PR activity in the presence of the anti-estrogen OHT, although the degree of inhibition is not as great as that observed in the presence of E_2 (Meyer *et al*, 1989).

A previous study using yeast and mammalian two-hybrid assays has shown a direct interaction between ERα and AR, which is associated with inhibition of ERα activity (Panet-Raymond *et al*, 2000). These authors demonstrated an interaction between the LBD of ERα and the full length AR. However the dependence of this interaction on ligand is unclear, with yeast two-hybrid assays demonstrating a ligand independent interaction and mammalian two-hybrid assays showing increased interaction in the presence of both mibolerone and E_2 (Panet-Raymond *et al*, 2000). Whereas the ERα-LBD did not interact with the AR-LBD, an interaction between the ERα-LBD and the AR-NTD was detected, which suggests that the site for interaction is situated in the NTD of the AR. The current study, which has shown inhibition of ER activity by the AR-NTD, is consistent with this observation. Similar interactions may potentially occur between endogenous ER and AR in breast cancer cells. Investigation of the interactions between endogenous ER and AR, using co-immunoprecipitation experiments on lysates from T-47D cells exposed to different combinations of E_2 and DHT, may enable more precise definition of the mechanisms associated with interference between ER and AR in breast cancer cells. These experiments would importantly address whether this interaction is dependent on ligand, which has not been definitively shown in previous studies (Panet-Raymond *et al*, 2000).

The interaction between the AR-NTD and the ERα-LBD, demonstrated by Panet-Raymond *et al* (2000), may be analogous to N/C interactions that occur in the AR following agonist binding (Section 1.3.4.1). An AR N/C interaction is considered essential for optimal ligand dependent activation of the AR (Ikonen *et al*, 1997; Berrevoets *et al*, 1998; He *et al*, 1999; He *et al*, 2000). Two LxxLL-like pentapeptide motifs within the AR-NTD (^{23}FQNLF^{27} and ^{432}WHTLF^{436}) mediate the interaction with the AR-LBD (He *et al*, 2000). As the LBDs of AR and ERα are highly homologous,

these motifs within the AR-NTD are strong candidates for mediating heterodimeric interaction with the ERα-LBD.

The AR-ER heterodimeric interactions may inhibit ER activity by altering its ability to bind E_2 and/or estrogen response elements (EREs) in the regulatory region of target genes. This is supported by previous studies showing that the retinoic X receptor (RXR) exhibits reduced ligand binding capacity and transactivation activity upon interaction between its LBD and the retinoic acid receptor (RAR) LBD (Forman *et al*, 1995). There is little evidence in the literature to support an effect on DNA binding. In fact, previous studies suggest that heterodimer formation may not reduce DNA binding affinity. The two isoforms of ER, ERα and ERβ (Section 1.1.3), interact with each other (Ogawa *et al*, 1998), however ERα/ERβ heterodimers are still capable of binding DNA with comparable affinity to homodimers (Pace *et al*, 1997). Furthermore, ER binding to estrogen response elements in the PR gene does not appear to be modified in the presence of PR, c-Jun or RARα, which each inhibit E_2-induced ER activity at this element (Savouret *et al*, 1994). Interestingly, formation of heterodimers between RXR and RAR enhances binding of RXR to its response element (Kliewer *et al*, 1992). In one study, an AR with a single amino acid substitution in the DBD (C574R) was unable to inhibit endogenous ER activity in MCF-7 cells (Ando *et al*, 2002). This mutation has previously been shown to impair transactivation activity and binding of the AR to the androgen responsive mouse mammary tumour virus long terminal repeat promoter (Zoppi *et al*, 1992). This may suggest that binding of the AR to AREs is required for inhibition of ER activity. Alternatively, this AR-DBD mutation may alter the stability or structure of the AR rendering it unable to interact with the ER. Chromatin immunoprecipitation or DNA mobility shift assays may enable further characterisation of changes in the DNA binding ability of the ER in the presence or absence of AR.

Alternatively, interactions between AR and ER may lead to decreased ER activity by altering its ability to recruit coactivators, which mediate interactions between nuclear receptors and the basal transcriptional machinery to activate expression of target genes (Section 1.3.7). Interaction with the ^{23}FQNLF^{27} and ^{432}WHTLF^{436} motifs in the AR-NTD may competitively exclude binding of LxxLL-containing coactivator molecules to the LBD of ERα. Alternatively, reduced ER activity may occur through sequestering of common cofactor molecules to activated AR. This hypothesis is supported by experiments which show that overexpression of coactivators such as steroid receptor coactivator 1 (SRC1) and CREB-binding protein (CBP) can relieve transcriptional interference by steroid receptors (Aarnisalo *et al*, 1998; Vasudevan *et al*, 2001). The differential effects of interactions with thyroid hormone receptor (TR), RAR and RXR on ER activity in different cell lines (Lee *et al*, 1998) also suggest that changes in interactions with cofactor molecules, which have cell specific expression profiles (Rosenfeld and Glass, 2001; Aranda and Pascual, 2001; Magklara *et al*, 2002; McKenna and O'Malley, 2002), may be integral to the alterations in receptor activity. It is currently unknown whether heterodimeric interactions between steroid receptors alter recruitment of specific coactivator molecules.

In the current studies, complete inhibition of endogenous ER activity by DHT or overexpression of the AR-NTD in T-47D cells was not observed. This may relate to expression of ERβ. ERβ mRNA has been detected in T-47D as well as other breast cancer cell lines (Dotzlaw *et al*, 1997; Vladusic *et al*, 2000; Vienonen *et al*, 2003). Both ERα and ERβ bind E_2 with comparable high affinity and activate transcription of estrogen responsive reporter genes, including those regulated by the estrogen responsive element used in the current studies (the palindromic ERE from the *Xenopus laevis* vitellogenin A2 gene, see Appendix 1) (Kuiper *et al*, 1996; Mosselman *et al*, 1996; Pace

et al, 1997). Thus ERβ, in addition to ERα, may be contributing to the activity of endogenous ER measured in T-47D cells. However the inhibition of ER activity by androgen signalling may be specific for ERα. Importantly, in mammalian two-hybrid assays performed in the presence and absence of E_2 and mibolerone, the full length AR did not interact with the ERβ-LBD, while in cells transiently transfected with full length AR and ERβ, the E_2-induced transactivation activity of ERβ was not significantly inhibited by mibolerone (Panet-Raymond *et al*, 2000). This may imply that in cell types where both forms of ER are expressed, only E_2-induced ERα activity is overcome by androgens and the residual ER activity may be attributed to ERβ.

In addition, the degree of inhibition of ER activity may be related to the expression level of AR relative to ERα and ERβ. The AR-NTD was more effective at inhibiting ER activity in PC-3 cells than in T-47D cells with respect to both the amount of AR-NTD required for maximum inhibition and the degree of maximum inhibition observed. These cell lines have been shown to express both ERα and ERβ (Carruba *et al*, 1994; Dotzlaw *et al*, 1997; Vladusic *et al*, 2000; Lau *et al*, 2000; Vienonen *et al*, 2003). Furthermore, expression of the ERE-tk-luc reporter is detected upon stimulation with E_2 in both PC-3 (Dr Paul Lambert, Dame Roma Mitchell Cancer Research Laboratories, personal communication) and T-47D (this study) cells without transfection of an ER expression construct. However, the relative expression levels of ERα and ERβ in these cell lines have not previously been defined. If PC-3 cells express lower levels of ERβ than T-47D cells this may potentially enable more effective inhibition of ER activity by the AR-NTD in this cell line. In T-47D cells, the relative levels of ER and AR have been previously examined by Scatchard analysis (Liberato *et al*, 1993). This study demonstrated that there are approximately 15000 AR binding sites per cell and approximately 7600 ER binding sites per cell. As E_2 binds to both ERα and ERβ with

comparable affinity (Kuiper *et al*, 1996), the ER binding sites estimated by this method can be expected to include those for both ERα and ERβ. This apparent 2-fold excess of AR relative to ER in T-47D cells may not be sufficient to enable complete inhibition of ER activity via interaction between endogenous receptors, regardless of the level of endogenous AR activity induced by DHT. This suggests that complete inhibition of ER activity may only be attained by exogenous expression of a significant excess of AR (or the NTD), to levels not normally observed in cell lines or *in vivo*.

Androgens have been shown to inhibit the expression of several well characterised estrogen responsive genes, including pS2, cathepsin D and the PR (see Section 4.1). However antagonism of these genes may occur in a cell specific manner, as E_2-induced pS2 expression is inhibited by DHT in ZR-75-1 cells (Lapointe *et al*, 1999) but not in MCF-7 cells (el Tanani and Green, 1995). In addition, transcriptional interference between steroid receptors may occur in a promoter sequence-specific manner (Scott *et al*, 1997). These results suggest that the anti-estrogenic action of androgens does not affect all aspects of estrogen signalling and may further support the role of cell-specific or promoter-specific cofactors in the modulation of ER activity. Examination of the androgenic antagonism of a larger spectrum of estrogen regulated genes may provide insight into whether androgens are general or specific inhibitors of estrogen action.

Clinical studies have shown that androgens such as testosterone propionate and fluoxymesterone acetate are effective for the treatment of advanced breast cancer (Fels, 1944; The Cooperative Breast Cancer Group, 1961; Manni *et al*, 1981; Tormey *et al*, 1983; Ingle *et al*, 1991), however the masculinising side effects of these drugs have limited their use. Alternative strategies utilising the AR for manipulation of estrogen signalling pathways, which promote the growth of breast cancer cells, may also be

clinically effective. These studies show that the AR-NTD is capable of inhibiting ER activity in breast cancer cells, potentially through direct interaction between the AR-NTD and the ERα-LBD. More precise mapping of the interaction sites between AR and ER may facilitate development of AR peptides which are transcriptionally inactive but still capable of interacting with the ER and inhibiting its activity. AR based therapies which do not activate the androgen signalling mechanisms that lead to masculinising side effects may be a suitable therapeutic approach for the inhibition of ER-mediated breast cancer growth (see Section 9.2.2.1).

Overall, these studies provide evidence that estrogen signalling pathways in breast cancer cells are inhibited by cross-talk with androgen signalling pathways. This appears to involve direct inhibition of ERα activity by the AR, which may be mediated by physical interactions between the ERα-LBD and the AR-NTD, which have previously been identified using yeast and mammalian two-hybrid assays (Panet-Raymond *et al*, 2000). Further studies will confirm whether these interactions also occur between endogenous ERα and AR in breast cancer cells. Cross-talk between androgen and estrogen signalling pathways may be one mechanism by which androgens inhibit E2-stimulated proliferation. These studies imply that the balance between androgen and estrogen signalling pathways is therefore likely to be critical in the regulation of breast cancer cell proliferation.

In addition to their inhibitory effects on estrogen-stimulated proliferation of breast cancer cells, androgens also have inhibitory effects that are independent of the estrogen signalling pathway (Section 1.2.4.1). Studies presented in the following chapters investigate the mechanisms by which classical (eg DHT) and non-classical (eg the synthetic progestin MPA) AR agonists may directly inhibit breast cancer cells, with

particular focus on the induction of AR activity and the expression of androgen responsive genes.

CHAPTER 5

ANDROGEN RECEPTOR FUNCTION IN THE MDA-MB-453 CELL LINE

5.1 – Introduction

AR positive cell lines, such as T-47D, ZR-75-1 and MCF-7, have been widely used to study AR function in breast cancer cells. However evaluation of androgen signalling in these cell lines is complex as they also express ER and PR, which may be able to mediate the effects of androgens, especially at high concentrations (Zava and McGuire, 1978; Poulin and Labrie, 1986; Markiewicz and Gurpide, 1997; Maggiolini *et al*, 1999), or interfere with AR activity (Simard *et al*, 1989; Simard *et al*, 1990; Kumar *et al*, 1994; Yu *et al*, 1994b; Panet-Raymond *et al*, 2000). The MDA-MB-453 breast cancer cell line expresses high levels of AR but undetectable levels of ER and PR (Hall *et al*, 1992; Hall *et al*, 1994), making it a potentially valuable model in which to investigate androgen action. Furthermore, the AR positive, but ER and PR negative phenotype reflects that of approximately 25% of metastatic breast cancers (Lea *et al*, 1989; Moinfar *et al*, 2003).

It has previously been shown that AR mRNA and protein expression in MDA-MB-453 is higher than in T-47D, ZR-75-1 and MCF-7 breast cancer cell lines, and comparable to the expression level observed in the LNCaP prostate cancer cell line (Hall *et al*, 1992; Hall *et al*, 1994; Birrell *et al*, 1995a). Cytosols from MDA-MB-453 cells bind with high affinity to both DHT and the synthetic progestin MPA, which has both progestagenic and androgenic properties (Sections 1.2.3, 1.2.4.1, 1.3.1), and these ligands induce transcription of androgen responsive endogenous genes and exogenous reporter genes in MDA-MB-453 cells (Hall *et al*, 1994; Bentel *et al*, 1999; Hartig *et al*, 2002; Wilson *et al*, 2002). The transcriptional effects of both DHT and MPA are blocked by the AR antagonists nilutamide and OHF in these cells (Bentel *et al*, 1999; Hartig *et al*, 2002).

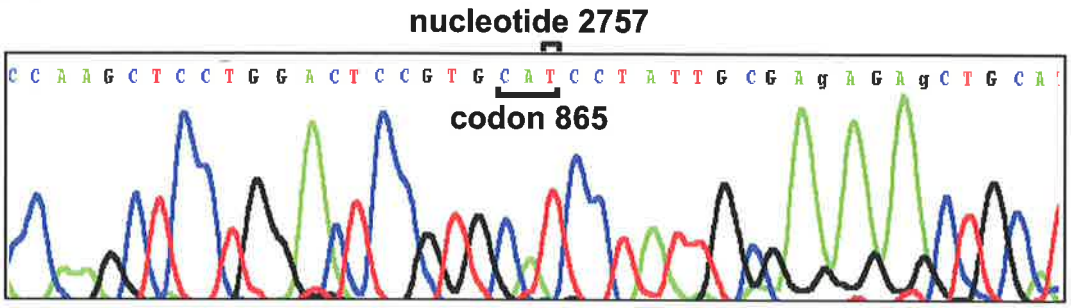
Interestingly, DHT and the synthetic androgen mibolerone stimulate proliferation of MDA-MB-453 cells whereas MPA inhibits proliferation (Hall *et al*, 1994; Birrell *et al*, 1995a; Bentel *et al*, 1999). These proliferative responses have been independently confirmed in numerous experiments in our laboratory (unpublished data). How DHT and MPA, both acting through the AR in MDA-MB-453 cells, can induce divergent proliferative effects is not known. Maximal stimulation of MDA-MB-453 cell proliferation was observed with a dose of 0.1nM DHT (approximately 22% increase in cell number relative to untreated controls), while 100nM MPA was required for an inhibitory effect (approximately 30% decrease in cell number). The relatively high dose of MPA required for inhibition is consistent with that required for inhibition of other breast cancer cell lines and xenografts and is equivalent to the dose used for the treatment of advanced breast cancers (Sutherland *et al*, 1988; Poulin *et al*, 1989a; Santen *et al*, 1990; Hackenberg *et al*, 1993a; Bentel *et al*, 1999). The inhibitory effect of MPA on MDA-MB-453 cell proliferation is in line with its effect on other breast cancer cell lines. While DHT and mibolerone stimulate MDA-MB-453 cells, they both inhibit proliferation of AR positive breast cancer cell lines such as T-47D and ZR-75-1 (Labrie *et al*, 1990a; de Launoit *et al*, 1991; Birrell *et al*, 1995a; Ortmann *et al*, 2002; and Chapter 3).

As the AR mediates the effects of both DHT and MPA in MDA-MB-453 cells, differences in AR function in response to these ligands may potentially account for their opposing proliferative effects. One feature of AR function that is mediated differently by DHT and MPA is induction of the N/C interaction. This interaction, which may occur in an intra- or inter-molecular fashion, is induced by binding of classical AR agonists but not binding of AR antagonists, and is considered essential for high level AR activation (Kemppainen *et al*, 1999). Agonist binding induces repositioning of

helix 12 of the LBD across the ligand binding pocket, exposing the AF2 surface which interacts with two LxxLL like motifs in the NTD (^{23}FQNLF^{27} and ^{32}WHTLF^{436}) (Ikonen *et al*, 1997; Berrevoets *et al*, 1998; He *et al*, 1999; He *et al*, 2000). The AR N/C interaction induces a conformation which is capable of binding DNA and recruiting the appropriate cofactors and transcription factors necessary for activation of gene transcription (He *et al*, 2000). The use of mammalian two-hybrid systems has shown that these interactions are strongly induced by DHT and other androgenic agonists such as R1881, mibolerone and testosterone (Langley *et al*, 1995; Kemppainen *et al*, 1999). However, MPA does not induce an N/C interaction and actually inhibits the DHT induced N/C interaction more effectively than the AR antagonist OHF (Kemppainen *et al*, 1999). This suggests that induction of AR activity by MPA may be mediated by a mechanism distinct from classical AR agonists.

Aberrant activity arising from structural variation in the AR may also provide a potential rationale for the divergent proliferative effects of DHT and MPA in MDA-MB-453 breast cancer cells. A missense nucleotide substitution in the endogenous AR in the MDA-MB-453 cell line has been recently identified in this laboratory (unpublished data). DNA sequencing of the full length AR cDNA synthesised from MDA-MB-453 cells revealed a G-T transversion at nucleotide 2757 (codon 865) (Figure 5.1), which results in the substitution of a glutamine residue with histidine. The codon 865 AR gene mutation in the MDA-MB-453 cell line has also been recently entered on the National Center for Biotechnology Information nucleotide sequence database (accession number AF162704), but the functional consequences of this AR mutation have not been reported. While previous studies indicate that the endogenous AR in MDA-MB-453 cells can respond to DHT and MPA, they do not

(a) MDA-MB-453



(b) T-47D

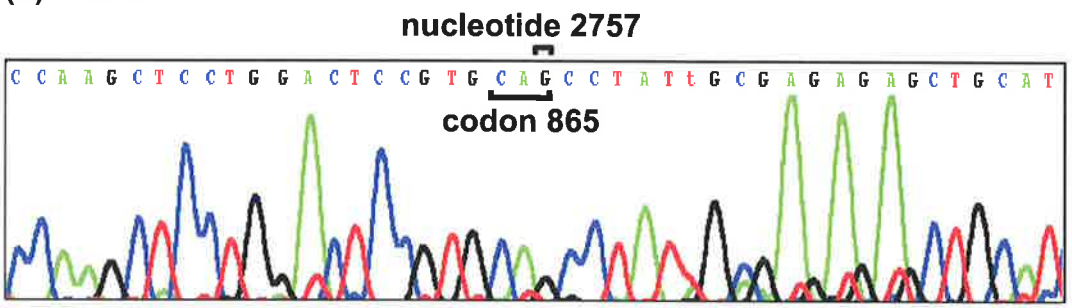


Figure 5.1: Sequencing of AR exon 8 cDNA in breast cancer cell lines. Total RNA was extracted from the *(a)* MDA-MB-453 and *(b)* T-47D cell lines and reverse transcribed using random hexamers. Exon 8 of the AR was amplified by PCR using ARCS3 and S primers. Sequencing of the exon 8 PCR fragment from the MDA-MB-453 cell line revealed a G-T transversion at nucleotide position 2757, resulting in the substitution of a glutamine residue for histidine at amino acid 865. AR sequence was wild type in the T-47D cell line. PCR products were prepared for DNA sequencing by Ms Coralie Lockwood.

indicate whether AR function is altered by this Q865H mutation compared to wtAR. Characterisation of the activity of the AR-Q865H variant is required in order to validate the suitability of this cell line as an *in vitro* model for androgen action in breast cancer. Any deviation in the activity of the AR-Q865H variant in response to DHT and MPA may also provide insight into how these ligands induce divergent proliferative effects in this cell line.

The specific aims of this chapter were to investigate AR function in MDA-MB-453 cells in response to DHT and MPA and, in particular, determine whether functional differences between wtAR and the AR-Q865H variant could explain the divergent proliferative effects of DHT and MPA in these cells.

5.2 – Methods

5.2.1 – Sequencing of AR cDNA

RNA was extracted from 1 x 10^6 MDA-MB-453 cells and reverse transcribed using random hexamers as described in Sections 2.3.7 and 2.3.10. The entire AR cDNA was amplified by PCR (Section 2.3.11) using seven primer pairs which generated overlapping fragments sized between 412 and 638bp (Table 5.1). PCR products were purified and sequenced in both sense and antisense orientations by automated sequencing using the original PCR primers (Section 2.3.13).

5.2.2 – Site directed mutagenesis

The G-T base substitution was introduced into the pCMV3.1-AR(ms-):wt expression construct using the "megaprimer" method of site directed mutagenesis as described in Sarkar and Sommer (1990) and Buchanan *et al* (2001b). All PCR reactions were

Table 5.1: Primers* used and expected sizes of PCR products generated for amplification of AR cDNA.

Primer set	Region amplified	Sense primer	Antisense primer	Product size (bp)
1	NTD	N111	N222	470
2	NTD	N122	N2330	500
3	NTD	144	244	449
4	NTD/DBD	N155	Hind III AS	412
5	NTD/DBD/LBD	N166	x4AS	628
6	LBD	ARCS1	ARCAS3	566
7	LBD	ARCS3	S	638

* Primer sequences are indicated in Appendix 2.

performed using the proof-reading DyNAzyme EXT™ DNA polymerase (Finnzymes) according to the manufacturer's instructions with the following temperature profile: initial denaturation at 94°C for 3mins followed by 25 cycles of 94°C for 30secs, 65°C for 1min, 72°C for 1min and a final extension step of 72°C for 20mins. A 662bp fragment of pCMV3.1-AR(ms-):wt, spanning the 3' portion of the AR gene, was amplified using a mutagenic sense primer spanning codon 865 (primer Q865H, see Appendix 2) and an antisense primer annealing to the vector backbone (Xba I antisense). The PCR reaction was run on a 1% agarose-TAE gel and the product of correct size was excised and purified using the QIAquick gel extraction kit. The purified PCR product was used as a megaprimer in combination with an upstream sense primer spanning the Bst BI restriction site in the AR (primer K718E) in a second PCR reaction, performed as described above. The resulting 1070bp PCR product was purified using the QIAquick gel extraction kit and digested with 10U Xba I at 37°C for 3hrs in the appropriate buffer. DNA was further purified using the QIAquick PCR purification kit and a second digestion was performed with 20U Bst BI at 65°C for 3hrs in the appropriate buffer. The resulting 761bp fragment was electrophoresed on a 1% agarose-TAE gel, purified using the QIAquick gel extraction kit and ligated into reciprocal sites of pCMV3.1-AR(ms-):wt using 50ng vector, a 3 fold molar excess of insert (~15ng), 1x ligase buffer and 4U T4 DNA ligase, with incubation at 14°C overnight. Competent INVαF' cells were combined with 2μL ligation reaction, incubated on ice for 30mins, heat shocked at 42°C for 30secs and rested on ice for a further 2mins. Cells were then recovered in 250μL luria broth at 37°C for 1hr and plated onto LB agar plates containing 100μg/mL ampicillin. Plates were incubated overnight at 37°C, colonies were selected for analysis and plasmid DNA was isolated as described in Section 2.3.4. The presence of the correct base substitution and the sequence integrity across the entire length of the cloned fragment was confirmed by

automated DNA sequencing of both DNA strands using ARCS3 and AR917 primers as described in Section 2.3.13. The resulting plasmid is referred to as pCMV3.1-AR(ms-):Q865H.

5.2.3 – Transient transfection of mammalian cells using DEAE Dextran

Plasmid DNA was prepared as described in Section 2.3.4. Cos-1 cells were seeded in 10cm petri dishes at a density of 1 x 10^6 cells/dish in phenol red free DMEM containing 5% CSS and 4mM L-glutamine and allowed to attach for approximately 72hrs at 37°C. Cells were washed with PBS pH 7.4 then overlaid with 8mL PBS pH 7.8 containing diethylaminoethyl (DEAE) Dextran (0.5mg/mL) and 2μg plasmid DNA. After incubation at 37°C for 30mins, the transfection mix was removed and replaced with 8mL phenol red free DMEM containing 5% CSS, 4mM L-glutamine and 100μM chloroquine, and cells were incubated for a further 4hrs at 37°C. Medium was then replaced with phenol red free DMEM containing 5% CSS and 4mM L-glutamine and transfected cells were incubated at 37°C for 48hrs.

5.2.4 – Preparation of cell cytosols

Transfected cells were washed with 5mL ice cold cytosol buffer containing fresh DTT (1mM) then harvested in 400μL cytosol buffer + DTT using a cell scraper. Remaining cells were harvested in an additional 200μL cytosol buffer + DTT and cytosols were pooled and homogenised with a 21-gauge needle. Cell debris was pelleted by centrifugation at 15000rpm for 30mins at 4°C and the cytosol fraction (supernatant) was transferred to a fresh tube. Tubes were maintained on ice at all stages to prevent protein degradation. Protein concentration was determined using a Bio-Rad protein assay kit, according to the manufacturer's protocol.

5.2.5 – Radioligand binding assay

A dextran coated charcoal saturation assay was used to measure binding of tritiated steroids to cytosol extracts of transfected cells. Bulk solutions containing increasing amounts of labelled steroid with (vials 1'-5') or without (vials 1-5) added unlabelled steroid were prepared as specified in Appendix 3.

Total binding was measured by co-incubation of equal volumes (50μL) of cytosol extract with increasing concentrations of ^{3}H-labelled bulk solution (ie from vials 1-5) overnight at 4°C. The following day, unbound steroid was removed by the addition of an equal volume (100μL) of dextran coated charcoal followed by incubation at 4°C for 20mins. Samples were centrifuged at 1400rpm for 20mins at 4°C to pellet the charcoal-steroid complexes. The total amount of bound tritiated steroid was determined by combining 100μL each supernatant with 2mL scintillation fluid and counting using a liquid scintillation counter. Non-specific binding was determined in the same manner as described above however, with the inclusion of a 250-fold molar excess of unlabelled steroid (ie using bulk solutions 1'-5').

Scatchard analysis was performed to calculate the dissociation constant (K_d) of ligand binding to wtAR and the AR-Q865H variant. Specific binding (B*), bound steroid (BS) and free steroid (F) were calculated for each bulk solution using Formula 5.1 and a scatter plot incorporating B* *versus* BS/F values, each expressed as the mean of a minimum of four replicate experiments, was graphed. The slope of the linear regression line was used to calculate the K_d value (Formula 5.2). As variation in the K_d value is dictated by variation in the slope of the linear regression line, the SEM for each K_d value was determined using the REG procedure, as recommended by Ms Kristyn Willson (Statistician, Department of Public Health, University of Adelaide, SA).

Formula 5.1:

$$B^* = \frac{BS \times \text{conversion factor} \times 20\ \text{fmoles/mg cytosol protein}}{\text{cytosol protein concentration (mg/ml)} \times 2}$$

where BS = dpm for total ^{3}H-ligand binding (labelled steroid only)
– dpm for non-specific ^{3}H-ligand binding (labelled + unlabelled steroid)

conversion factor = 1/(specific activity of tritiated steroid stock x 2.22)

specific activity ^{3}H-DHT stock = 111Ci/mmol

specific activity ^{3}H-MPA stock = 49.6 Ci/mmol

Formula 5.2:

$$K_d = \frac{-P \times 10^{-12}}{2a}\ mol/L$$

where P = cytosol protein concentration

a = slope of linear regression line from scatchard plot

Statistically significant differences between K_d values, which are dictated by differences between the slopes of regression lines, were determined using the Wald test, also recommended by Ms Kristyn Willson.

5.2.6 – Temperature stability assay

Cytosol extracts were mixed in equal volumes with a single concentration (6nM) of ^{3}H-labelled steroid in the presence or absence of a 250-fold molar excess of unlabelled steroid and incubated overnight at 4°C. 100µL aliquots were transferred to a microtitre plate and incubated at 37°C for various times (0.5 to 4hrs). At completion of the time course, unbound steroid was removed by addition of 100µL/well of dextran coated charcoal and centrifugation followed by scintillation counting as above. B* at each time point was calculated as a percentage of the 0hr control.

5.2.7 – Reporter gene assay

Plasmid DNA was prepared as described in Section 2.3.4. Cells (MDA-MB-453 or PC-3) were seeded in 96 well plates at a density of 2 x 10^4 cells/well in phenol red free DMEM containing 5% CSS and 4mM L-glutamine and allowed to attach overnight at 37°C. Cells were transiently transfected with 100ng/well reporter plasmid (tk81-PB_3-luc or PSA_{630} (promoter + enhancer)-luc as indicated) and 10ng/well AR expression construct (pCMV3.1-AR(ms-):wt or pCMV3.1-AR(ms-):Q865H) if required using Lipofectamine™ 2000 as described in Section 2.3.6. 1ng/well of the transfection control plasmid pRL-tk was also included in some experiments. Cells were then treated with vehicle (0.1% ethanol) or varying concentrations of DHT or MPA (0.01-100nM) as indicated for 36-48hrs. Cell lysates were harvested and assayed for luciferase activity using a Packard TopCount. In assays where the pRL-tk transfection control plasmid was included, luciferase activity from the firefly and renilla luciferase reporters was

measured using the Dual-Luciferase Reporter Assay System and the TopCount Luminometer. 4-8 replicate wells of each treatment were assayed and results are expressed as the mean RLU ± SEM.

5.2.8 – Mammalian two-hybrid assay

Interactions between the AR-LBD and the AR-NTD (ie the N/C interaction) were determined using a mammalian two-hybrid system. In this system, parental vectors encoding the activation domain (AD) of the yeast VP16 protein (pVP16) and the GAL4-DBD (pM) are fused in frame to the AR-NTD and AR-LBD respectively. When co-transfected into mammalian cells (Cos-1) with a GAL4 responsive reporter (pGK1), the DNA bound GAL4-DBD-AR:LBD chimera recruits the VP16AD-AR:NTD fusion protein in an agonist dependent manner (N/C interaction), enabling transcription of the luciferase reporter by the VP16AD. Luciferase activity is a semi-quantitative determinant of the strength of the N/C interaction. This system has previously been utilised to assess the agonist potential of ligands and to characterise the function of AR mutants (Langley *et al*, 1998; Kemppainen *et al*, 1999; James *et al*, 2002; Buchanan, 2002).

Plasmid DNA was prepared as described in Section 2.3.4. Cos-1 cells were seeded in 96 well plates at a density of 1.5 x 10^4cells/well in phenol red free DMEM containing 5% CSS and 4mM L-glutamine and allowed to attach overnight at 37°C. Cells were transiently transfected with 50ng/well pVP16AD-AR:NTD, 50ng/well pM-AR:LBD(wt) or pM-AR:LBD(Q865H) and 50ng/well of the pGK1 reporter construct using Lipofectamine™ 2000 as described in Section 2.3.6. Cells were treated with increasing concentrations of DHT (0.1-100nM) or MPA (1-1000nM) or vehicle (0.1% ethanol) and lysates were assayed for luciferase activity, as described above.

Cos-1 cells transfected with the pM-53 and pVP16-T constructs, which encode the known interacting partners p53 and the SV40 large T antigen, served as a positive control. Cos-1 cells transfected with either parental (pM and pVP16) vectors, or with the pM-53 or pVP16-T vectors, alone or in combination with their AR-NTD or AR-LBD (wt or Q865H) fusion partners, served as negative controls. Four replicate wells of each treatment were assayed and results are expressed as the mean RLU ± SEM.

5.2.9 – Immunoblot analysis

AR protein levels in transfected Cos-1 cell cytosols were measured using immunoblot analysis. Extracts (15μg) were combined with 6x protein loading dye and electrophoresed on a 6% SDS PAGE gel as described in Section 2.3.14. Following semi-dry transfer of proteins to PVDF membrane, AR was detected using the U407 primary antibody (1/200 dilution) followed by the HRP-conjugated sheep anti-rabbit IgG (1/2000 dilution). Immunoreactive bands were detected with ECL™ western blotting detection reagents as described in Section 2.3.14. The intensity of immunoreactive bands was measured using an Imaging Densitometer. Equal protein loading was confirmed by staining the membrane in Coomassie Blue and the staining intensity across the length of each lane was measured by densitometric analysis.

5.2.10 – Molecular modelling

Molecular models of the AR-LBD were generated based on the crystal structure coordinates of the human PR-LBD complexed with progesterone (Williams and Sigler, 1998). This method of homology modelling has been used previously to accurately predict the structure of the AR-LBD, which shares a 54% sequence identity with the PR-LBD (Matias *et al*, 2000). DHT and MPA were docked into the AR model by

superimposing the AR-LBD model onto the crystal structure of the PR-LBD bound to progesterone. Energy minimised structures for DHT and MPA were constructed using ISIS/Draw and Sculpt 3 (Surles *et al*, 1994) and aligned with progesterone by a Maximal Common Substructure Search (Kubinyi *et al*, 1998). Electrostatic and van der Waals interactions of DHT and MPA with the AR-LBD were minimised locally using Monte Carlo Minimisation (Abagyan and Argos, 1992). van der Waals interactions were modelled using a modified Lennard-Jones function between atoms within 6Å of each other and electrostatic interactions were modelled with a distance-dependent dielectric between atoms within 10Å. The surface of the ligand binding pocket was calculated using a 1.4Å water molecule as a probe and transparencies were rendered using the ray tracing program POV-RAY 3 (http://www.povray.org, 2003). Ribbon diagrams were constructed using the MOLSCRIPT/Raster3D program (Kraulis, 1991; Merritt and Murray, 1994). Structural modelling was performed by Dr Jonathan Harris (Queensland University of Technology, Brisbane, Queensland, Australia).

5.3 – Results

5.3.1 – Sequence analysis of AR mRNA in the MDA-MB-453 cell line

Sequence analysis of the entire coding region of the AR in MDA-MB-453 cells identified a single nucleotide substitution, G-T, at position 2757 in exon 8. Figure 5.1 (a) and (b) show the partial nucleotide sequence of the sense strand of exon 8 in MDA-MB-453 (T at nucleotide 2757) and T-47D (A at nucleotide 2757 is wild type) breast cancer cells respectively. The nucleotide substitution in exon 8 of the AR gene in MDA-MB-453 cells was confirmed by sequencing in the antisense orientation (data not shown). This G-T transversion results in the substitution of a glutamine residue for histidine at amino acid 865 in the AR-LBD (the AR-Q865H variant). No other

sequence alterations were observed in the coding region of the AR gene in the MDA-MB-453 cell line (data not shown).

5.3.2 – Construction of the AR-Q865H variant expression plasmid

DNA sequence analysis of the reconstructed AR-Q865H variant expression plasmid confirmed the presence of the G-T transversion at nucleotide 2757 (data not shown). No other sequence alterations were detected across the Bst BI–Xba I insert fragment. AR expression levels were measured by immunoblot analysis of lysates from Cos-1 cells transiently transfected with the pCMV3.1-AR(ms-):wt or pCMV3.1-AR(ms-):Q865H expression constructs. Expression levels of the wtAR and the AR-Q865H variant were very similar (Figure 5.2 (a)-(c)), suggesting that the amino acid substitution at position 865 does not appreciably affect receptor expression level or stability.

5.3.3 – Comparison of wtAR *versus* AR-Q865H function

Four aspects of AR function – ligand binding, receptor stability, transactivation activity and induction of the N/C interaction – were investigated in cells transiently transfected with either the pCMV3.1-AR(ms-):wt or pCMV3.1-AR(ms-):Q865H expression constructs.

5.3.3.1 – Ligand binding

The binding affinities of wtAR and the AR-Q865H variant for DHT and MPA were measured in cytosols from Cos-1 cells transiently transfected using the DEAE Dextran method. K_d values calculated from scatchard analyses are shown in Table 5.2. The K_d for DHT binding to the wtAR was significantly lower than the K_d for MPA binding to the wtAR, implying that the wtAR has greater affinity for DHT than for MPA. This has

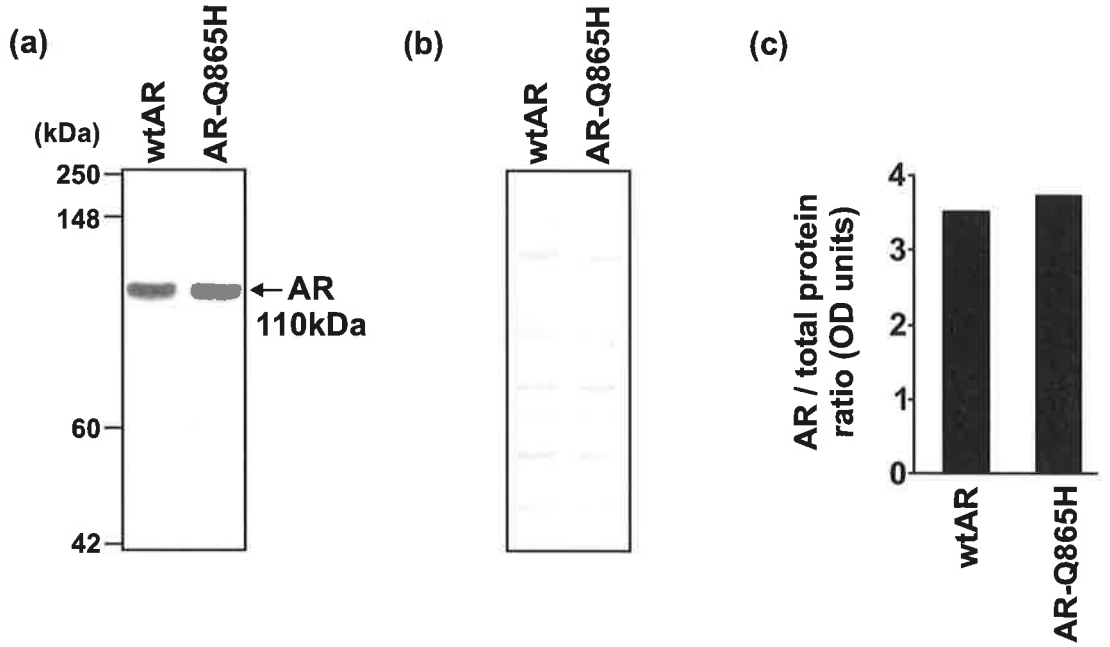


Figure 5.2: Expression of wtAR and AR-Q865H in transiently transfected Cos-1 cells. *(a)* Equal amounts (15μg) of cytosol extracts were electrophoresed on a 6% SDS PAGE gel and transferred to PVDF membrane. The membrane was immunoblotted with an antibody directed at the amino terminus of the AR (U407) as described in the text. *(b)* Following ECL detection, the membrane was stained with Coomassie Blue to confirm equal protein loading. *(c)* The intensity of immunoreactive bands was measured by densitometric analysis and corrected for total protein, as determined by the intensity of Coomassie blue stain across the length of each lane.

Table 5.2: K_d values for DHT and MPA binding to wtAR and AR-Q865H in Cos-1 cells and the endogenous AR-Q865H in MDA-MB-453 cells. Values were calculated from Scatchard plots combining a minimum of 4 replicate experiments.

Cell type	**AR**	**K_d (nM)**	
		DHT	MPA
Cos-1	wtAR	0.22 (±0.04) *#	0.47 (±0.04)
Cos-1	AR-Q865H	0.64 (±0.13)	0.48 (±0.06)
MDA-MB-453	endogenous AR-Q865H	0.13 (±0.03) §	1.23 (±0.20)

* Wald test; $p < 0.0001$: DHT *versus* MPA (wtAR, Cos-1 cells).
# Wald test; $p = 0.003$: wtAR *versus* AR-Q865H (DHT, Cos-1 cells).
§ Wald test; $p < 0.0001$: DHT *versus* MPA (endogenous AR-Q865H, MDA-MB-453 cells).

been observed in previous studies (Hackenberg *et al*, 1993a; Kemppainen *et al*, 1999). There was no significant difference in the K_d values for DHT and MPA binding to the AR-Q865H variant. While the K_d value for DHT binding to the wtAR was reduced compared to the AR-Q865H variant, there was no significant difference in the K_d values for MPA binding to the wtAR and AR-Q865H variant. These results suggest that the Q865H mutation may diminish AR binding affinity for DHT without altering affinity for MPA.

5.3.3.2 – Receptor stability

The stability of DHT-AR and MPA-AR complexes at 37°C were compared in cytosols from Cos-1 cells transiently transfected with expression constructs for the wtAR or the AR-Q865H variant. Consistent with the relative binding affinities demonstrated above, both wtAR and the AR-Q865H variant appeared more stable at 37°C when bound to DHT compared to MPA (Figure 5.3 (a)-(d)). Importantly, the wtAR was more stable than the AR-Q865H variant when incubated with either ligand. DHT bound to wtAR showed the greatest stability, with a half-life of approximately 90mins at 37°C (Figure 5.3 (a)). The half-life of other complexes tested was estimated at 45mins for DHT-AR-Q865H (Figure 5.3 (b)), 30mins for MPA-wtAR (Figure 5.3 (c)) and 20mins for MPA-AR-Q865H (Figure 5.3 (d)). Specific binding remaining was reduced to less than 10% of control levels within 4hrs for all samples.

5.3.3.3 – Transactivation activity

In initial experiments investigating AR transactivation activity in transiently transfected PC-3 and MDA-MB-453 cells, the pRL-tk renilla luciferase reporter was included as a control for transfection efficiency. As there was no significant difference in the renilla luciferase activity observed across all 96 transfected wells in multiple plates tested (data

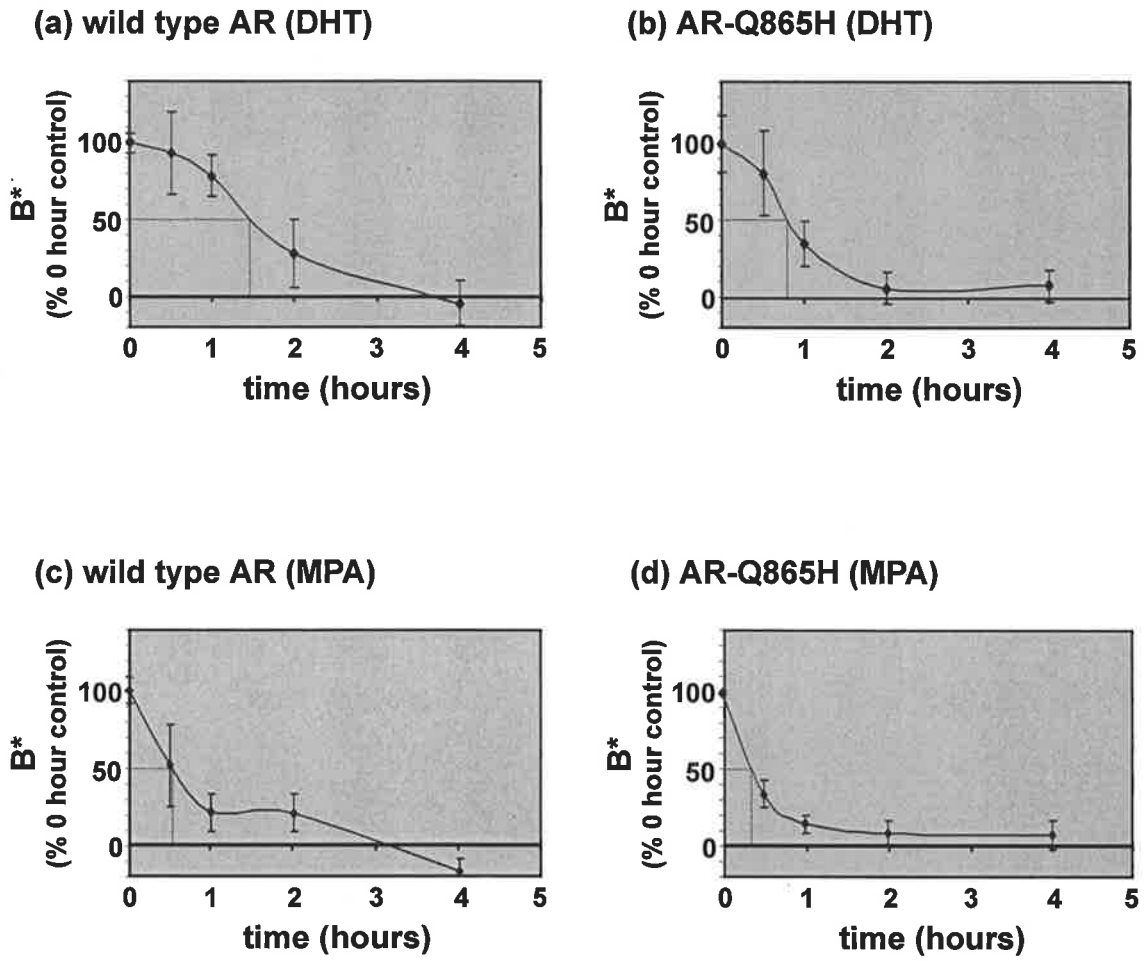


Figure 5.3: Stability of wild type and variant ligand-AR complexes at 37°C. Cos-1 cells were transiently transfected with wild type AR (*(a)* and *(c)*) or the AR-Q865H variant (*(b)* and *(d)*) expression constructs using the DEAE-dextran method. Cytosol extracts from transfected cells were incubated with ^{3}H-DHT (6nM, *(a)* and *(b)*) or ^{3}H-MPA (6nM, *(c)* and *(d)*) in the presence or absence of a 250-fold molar excess of the corresponding unlabelled steroid overnight at 4°C. The following day, complexes were incubated at 37°C for the indicated times. Values for specific ligand binding remaining (B*), expressed as the percentage of the 0 hour control value, are the mean (+/- SEM) of a minimum of 6 replicate experiments. Dotted lines denote the time taken for B* to fall by 50%.

not shown), indicating that transfection efficiency was uniform, the pRL-tk reporter was omitted from subsequent transfections.

DHT and MPA-induced activities of the wtAR and the AR-Q865H variant on the tk81-PB_3-luc androgen responsive reporter were compared in transiently transfected PC-3 prostate cancer and MDA-MB-453 breast cancer cells. In PC-3 cells, a dose dependent increase in both wtAR and AR-Q865H activity was observed with increasing concentrations of DHT (Figure 5.4 (a)) and MPA (Figure 5.4 (b)). There was no significant difference in the maximal DHT-induced activity observed between wtAR and the AR-Q865H variant, although a significant difference in wtAR and AR-Q865H activity was observed with 0.1nM DHT. Maximal activity induced by MPA was significantly greater for the wtAR, suggesting that the variant AR-Q865H has reduced sensitivity to activation by MPA in PC-3 cells.

In contrast to PC-3 cells, the activity of the wtAR in MDA-MB-453 cells in response to both DHT (Figure 5.5 (a)) and MPA (Figure 5.5 (b)) was significantly higher than that of the AR-Q865H variant for all concentrations of ligand. In addition, wtAR activity was 10-fold greater than AR-Q865H activity in the absence of ligand. Furthermore, at ligand concentrations greater than 0.1nM, both the wtAR and the AR-Q865H were more sensitive to activation by DHT than to equal concentrations of MPA in MDA-MB-453 cells. In MDA-MB-453 cells, the endogenous AR-Q865H variant could be expected to contribute to the observed AR activity. However endogenous AR activity is much lower than that of transfected AR (see Section 5.3.4.3) and is not likely to significantly affect these experiments.

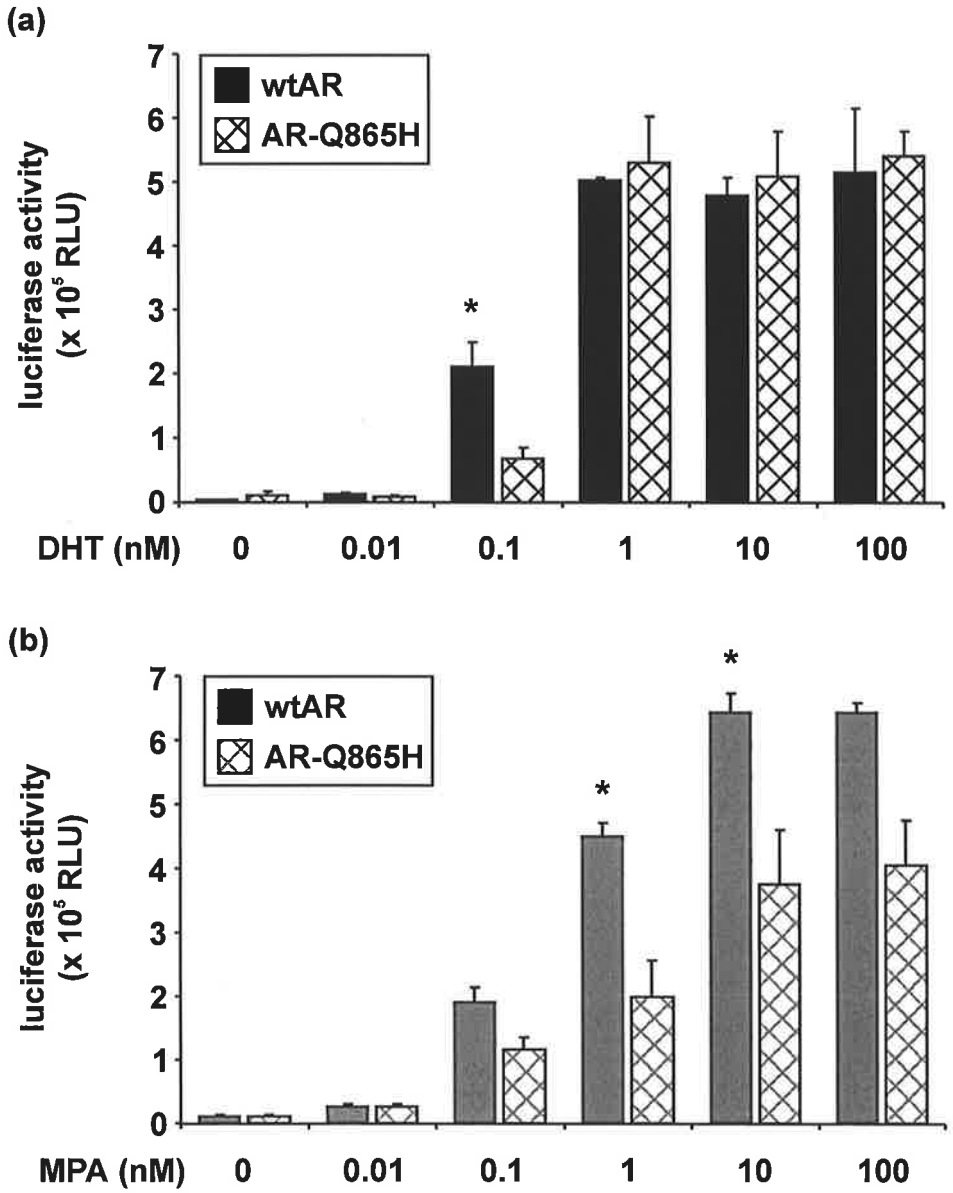


Figure 5.4: Comparison of wtAR and AR-Q865H transactivation activity in the PC-3 prostate cancer cell line. Cells (2 x 10^4 cells/well) were transiently transfected with wtAR (solid bars) or AR-Q865H (hatched bars) expression constructs (10ng/well) and the tk81-PB_3-luc reporter construct (100ng/well) using Lipofectamine 2000. Five hours after transfection, cells were treated with phenol red free DMEM containing 5% CSS, 4mM L-glutamine and increasing concentrations (0.01-100nM) of *(a)* DHT or *(b)* MPA or vehicle (0.1% ethanol) as indicated. After 48hrs, the medium was removed and cell lysates were assayed for luciferase reporter gene activity using the Luciferase Assay System. Each bar represents the mean +/- SEM of a minimum of 3 replicate wells. * t-test; $p < 0.025$: wtAR *versus* AR-Q865H.

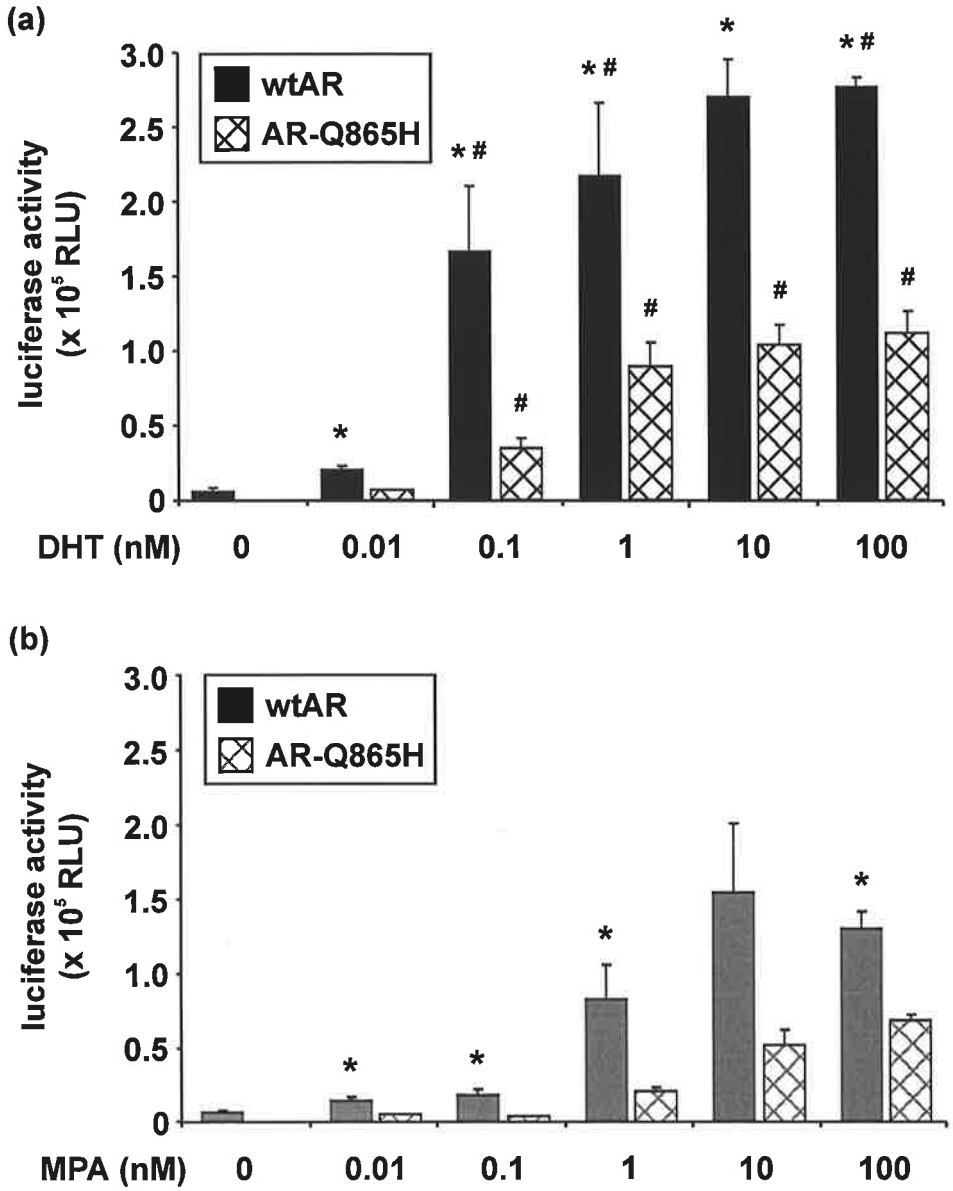


Figure 5.5: Comparison of wtAR and AR-Q865H transactivation activity in the MDA-MB-453 breast cancer cell line. Cells (2 x 10^4 cells/well) were transiently transfected with wtAR (solid bars) or AR-Q865H (hatched bars) expression constructs (10ng/well) and the tk81-PB_3-luc reporter construct (100ng/well) using Lipofectamine 2000. Five hours after transfection, cells were treated with phenol red free DMEM containing 5% CSS, 4mM L-glutamine and increasing concentrations (0.01-100nM) of *(a)* DHT or *(b)* MPA or vehicle (0.1% ethanol) as indicated. After 48hrs, the medium was removed and cell lysates were assayed for luciferase reporter gene activity using the Luciferase Assay System. Each bar represents the mean +/- SEM of a minimum of 3 replicate wells. * t-test; $p < 0.05$: wtAR *versus* AR-Q865H. # t-test; $p < 0.05$: DHT *versus* MPA.

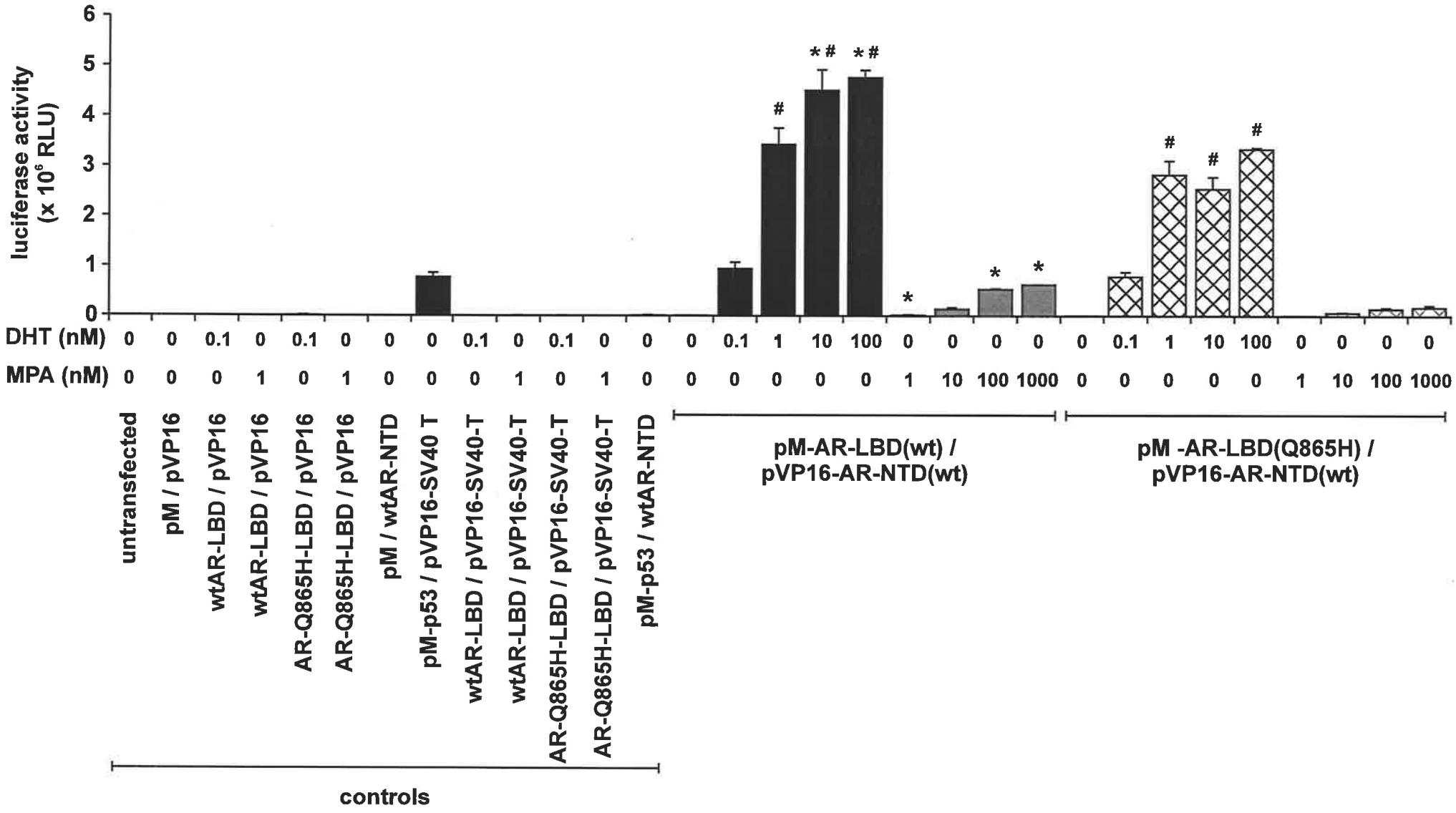
Whereas 10nM MPA was required for maximal activity of wtAR and AR-Q865H in both PC-3 and MDA-MB-453 cells, only 0.1-1nM DHT was required for maximal AR activity in both cell lines. These results are in agreement with previous studies showing that MPA has weaker agonist activity than DHT (Kemppainen *et al*, 1999).

5.3.3.4 – N/C interactions

In cells transfected with parental (pM and pVP16AD) vectors alone or in combination with their AR-NTD or AR-LBD fusion vector partners, no induction of reporter gene activity by DHT or MPA was observed, indicating the specificity of the interaction between the AR-NTD and the AR-LBD. The mammalian two-hybrid system was validated in cells transfected with the pM-53 and pVP16-T constructs (the positive control), where luciferase activity was approximately 700-fold higher than in cells transfected with parental vectors alone (Figure 5.6).

Luciferase activity induced by DHT was significantly higher than that induced by equal concentrations of MPA (Figure 5.6). These results are in agreement with previous studies showing that N/C interactions induced by MPA are relatively weak and that a higher concentration of MPA is required to stabilise the AR (Kemppainen *et al*, 1999). This effect was also observed for the AR-Q865H variant. However the DHT-induced N/C interaction for the AR-Q865H was 15-40% lower than for the wtAR, suggesting that the amino acid substitution at position 865 may compromise the ability of the AR-LBD to interact with the NTD in the presence of DHT. Interestingly, this mutation almost completely abolished MPA-induced N/C interactions for the AR-Q865H variant. These results could provide a rationale for reduced stability of the AR-Q865H variant at 37°C compared to wtAR in the presence of DHT or MPA (Figure 5.3 compare (a) with (b), and (c) with (d)).

Figure 5.6: Induction of the AR N/C interaction by DHT and MPA. Cos-1 cells (1.5×10^4 cells/well) were transiently transfected with the pVP16AD-AR:NTD and the pM-AR:LBD(wt) (solid bars) or pM-AR:LBD(Q865H) (hatched bars) expression constructs and the pGK1 reporter using Lipofectamine 2000. Cos-1 cells transfected with the pM-p53 and pVP16-SV40-T constructs, which are known to interact, served as a positive control. Cos-1 cells transfected with either parental (pM and pVP16) vectors, or with the pM-p53 or pVP16-SV40-T vectors, alone or in combination with their AR-NTD or AR-LBD(wt or Q865H) fusion partners, served as negative controls. Five hours after transfection, cells were treated with phenol red free DMEM containing 5% CSS, 4mM L-glutamine and increasing concentrations of DHT (0.1-100nM) or MPA (1-1000nM) or vehicle (0.1% ethanol) as indicated. After 48hrs, the medium was removed and cell lysates were assayed for luciferase reporter gene activity using the Luciferase Assay System. Each bar represents the mean +/- SEM of a minimum of 3 replicate wells. * t-test; $p < 0.006$: LBD(wt) *versus* LBD(865). # t-test; $p = 0.0$: DHT *versus* MPA. This experiment was performed by Dr Grant Buchanan.


luciferase activity (x 10^6 RLU)
6
5
4
3
2
1
0
DHT (nM) 0 0 0.1 0 0.1 0 0 0 0.1 0 0.1 0 0 0 0.1 1 10 100 0 0 0 0 0 0.1 1 10 100 0 0 0 0
MPA (nM) 0 0 0 1 0 1 0 0 0 1 0 1 0 0 0 0 0 0 1 10 100 1000 0 0 0 0 0 1 10 100 1000
untransfected
pM / pVP16
wtAR-LBD / pVP16
wtAR-LBD / pVP16
AR-Q865H-LBD / pVP16
AR-Q865H-LBD / pVP16
pM / wtAR-NTD
pM-p53 / pVP16-SV40 T
wtAR-LBD / pVP16-SV40-T
wtAR-LBD / pVP16-SV40-T
AR-Q865H-LBD / pVP16-SV40-T
AR-Q865H-LBD / pVP16-SV40-T
pM-p53 / wtAR-NTD
controls
pM-AR-LBD(wt) / pVP16-AR-NTD(wt)
pM -AR-LBD(Q865H) / pVP16-AR-NTD(wt)

5.3.4 – Endogenous AR function in MDA-MB-453 cells

Ligand binding, temperature stability of ligand-AR complexes and transactivation activity of the endogenous variant AR in MDA-MB-453 cells in response to DHT and MPA were also examined.

5.3.4.1 – Ligand binding

The affinity of the endogenous AR for DHT and MPA was examined in MDA-MB-453 cell cytosol fractions. K_d values calculated from scatchard analyses are shown in Table 5.2. The K_d value for DHT binding to the endogenous AR-Q865H variant in MDA-MB-453 cells was significantly reduced in comparison to that observed for MPA, implying a greater affinity for DHT.

5.3.4.2 – Temperature stability

The endogenous AR-Q865H was more stable at 37°C when bound to DHT than when bound to MPA (Figure 5.7). Half-life of the DHT-AR complex was estimated at 50mins, with specific binding remaining reduced to 0% after 2hrs. The MPA-AR complex, in comparison, was rapidly degraded with an approximate half-life of 15mins and specific binding reduced to 0% after 30mins.

5.3.4.3 – Transactivation activity

The activity of the endogenous variant AR in MDA-MB-453 cells was tested using two different androgen responsive luciferase reporters – tk81-PB_3-luc and PSA_{630} (promoter + enhancer)-luc (Figure 5.8). In MDA-MB-453 cells transfected with the tk81-PB_3-luc reporter, a dose dependent increase in AR activity was observed with increasing concentrations of DHT, with maximal induction attained with 1nM DHT (Figure 5.8 (a), black bars). In contrast, MPA demonstrated little ability to activate the

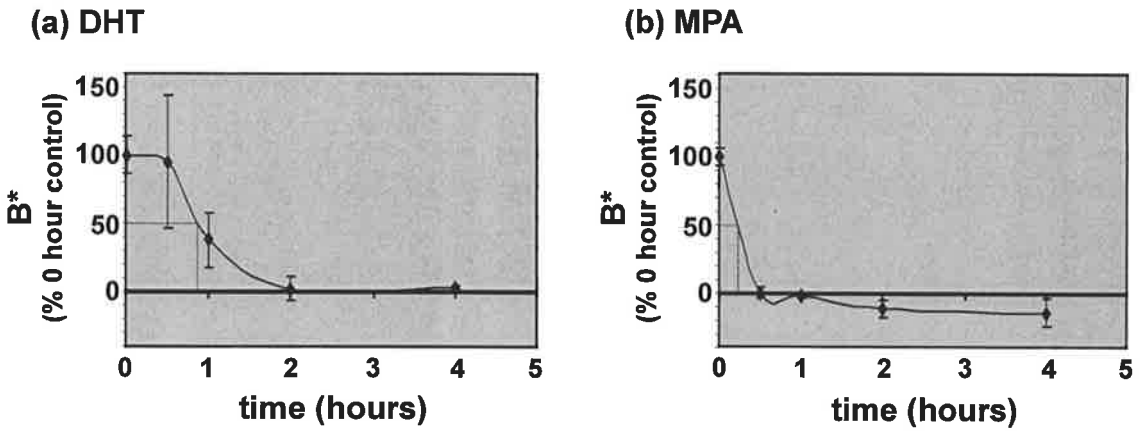


Figure 5.7: Stability of endogenous ligand-AR complexes in cytosol extracts from MDA-MB-453 breast cancer cells at 37°C. Cytosol extracts were incubated with *(a)* ^{3}H-DHT (6nM) or *(b)* ^{3}H-MPA (6nM) in the presence or absence of a 250-fold molar excess of unlabelled steroid overnight at 4°C. The following day, complexes were incubated at 37°C for the indicated times. Values for specific ligand binding remaining (B*), expressed as the percentage of the 0 hour control value, are the mean (+/- SEM) of 4 replicate experiments. Dotted lines denote the time taken for B* to fall by 50%.

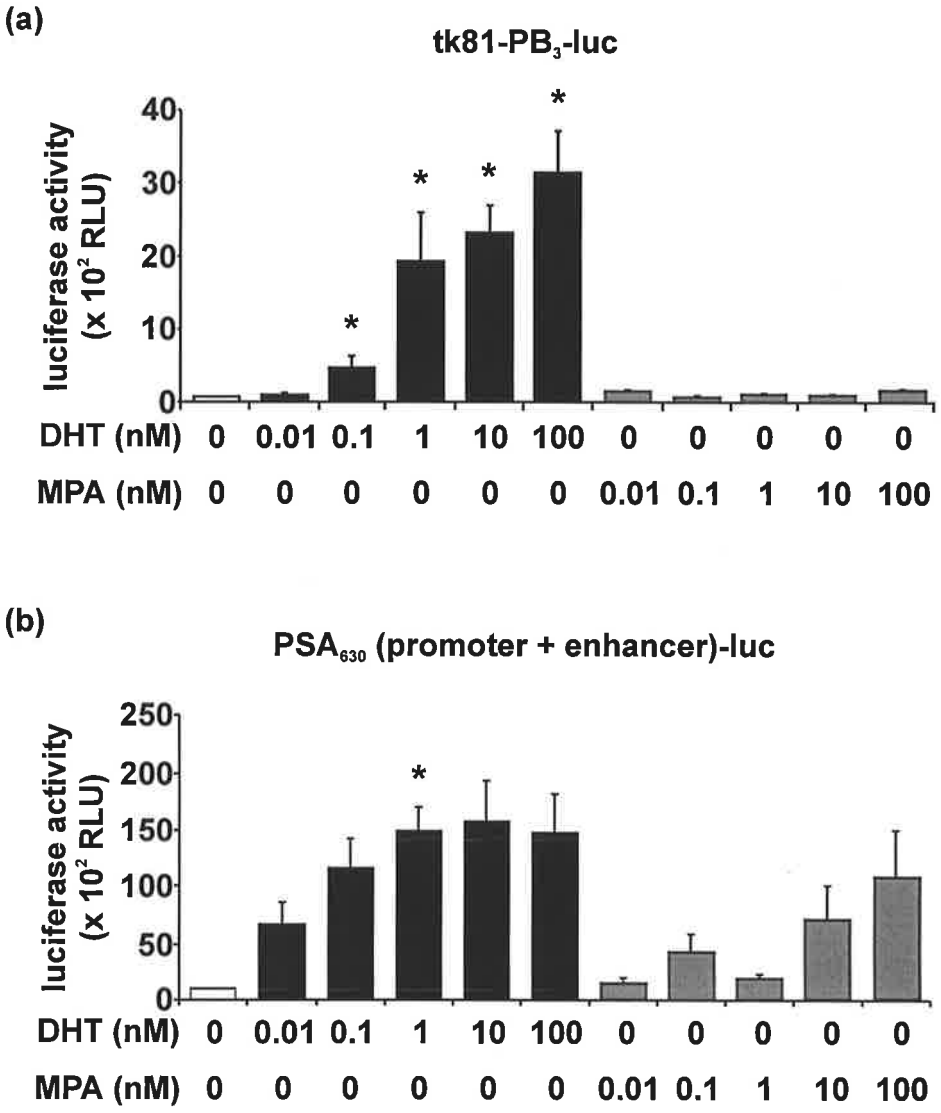


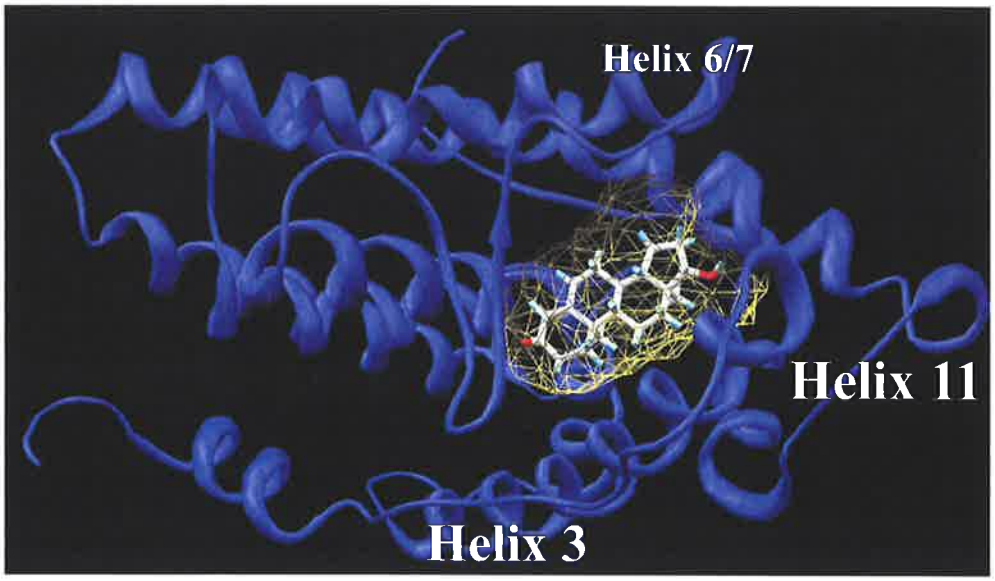
Figure 5.8: Transactivation activity of the endogenous AR-Q865H in the MDA-MB-453 breast cancer cell line. Cells were seeded in 96 well plates (2×10^4 cells/well) and transfected with 100ng/well of the *(a)* tk81-PB_3-luc or *(b)* PSA_{630} (promoter + enhancer)-luc reporter plasmids using Lipofectamine 2000. Five hours after transfection, cells were treated with phenol red free-DMEM containing 5% CSS, 4mM L-glutamine and increasing concentrations (0.01-100nM) of DHT (black bars) or MPA (grey bars) or vehicle (0.1% ethanol) as indicated. After 48hrs, the medium was removed and cell lysates were assayed for luciferase reporter gene activity using the Luciferase Assay System. Each bar represents the mean +/- SEM of a minimum of 3 replicate wells. * ANOVA; $p < 0.002$: DHT *versus* MPA.

AR at the PB_3 promoter (Figure 5.8 (a), grey bars). In cells transfected with the PSA_{630} (promoter + enhancer)-luc reporter, there was no significant difference between the maximal activity induced by DHT and MPA (Figure 5.8 (b)). In addition, a higher level of AR activity was measured at the PSA promoter and enhancer compared to that observed at the PB_3 promoter in the presence and absence of DHT and MPA.

5.3.5 – Molecular modelling

To investigate possible structural variations that may contribute to the observed differences in wtAR and AR-Q865H activity when bound by DHT and MPA, a homology modelling protocol was used to generate models of the AR-LBD. Figure 5.9 shows little variation in the shape of the ligand binding pocket of the wtAR when bound to either DHT or MPA. Moreover, the Q865H mutation does not appear to markedly alter the disposition of either ligand in the ligand binding pocket (Figure 5.10). These results suggest that the mutation does not invoke any major structural changes within the receptor. More detailed analysis of the holo AR-LBD model revealed that the glutamine residue at position 865 in the wtAR potentially forms a hydrogen bond with helix 8 (Figure 5.11). In contrast, the aromatic ring of the histidine residue in the AR-Q865H variant may alter this, forming a new hydrogen bond with β-strand 4 in the carboxy-terminal extension of the AR, which comprises the terminal 10 amino acids of the receptor. A hydrogen bond at this position could modify ligand induced folding of the adjacent helix 12 over the ligand binding pocket. As helix 12 constitutes a major portion of the AF2 surface (Section 1.3.6), altered hydrogen bond interactions could have implications for the N/C interaction, receptor stability and cofactor recruitment, providing a potential explanation for the observed variations between wtAR and AR-Q865H activity.

(a) DHT



(b) MPA

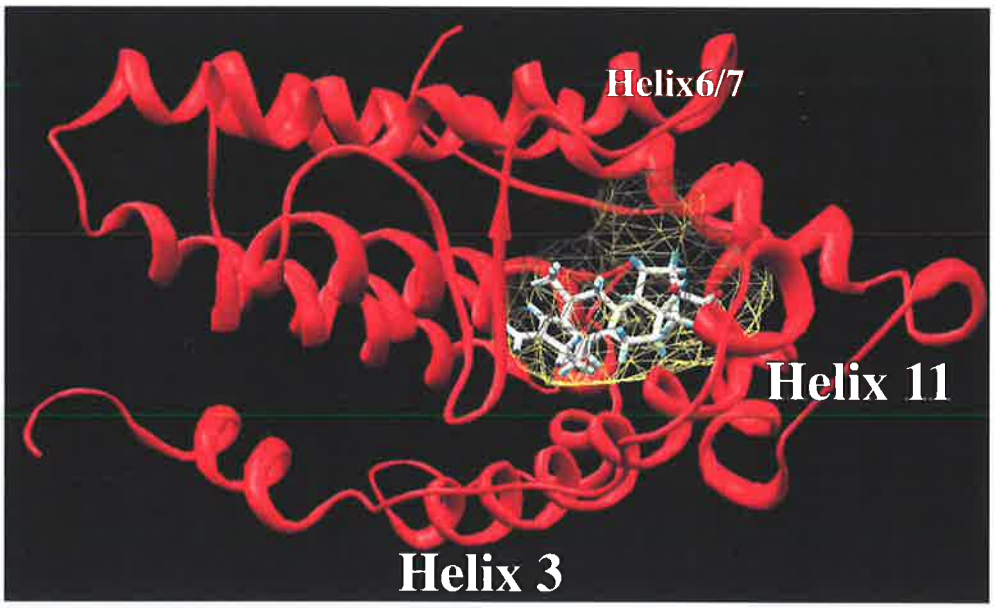


Figure 5.9: Homology modelling of the wtAR-LBD bound to *(a)* DHT and *(b)* MPA. Ribbon diagrams showing α-helical and β-sheet structures of the AR-LBD were generated using the MOLSCRIPT/Raster3D program. Ligand, shown in white, is docked in the ligand binding pocket with the surface of the pocket depicted as a yellow lattice. These models were generated by Dr Jonathan Harris (Queensland University of Technology).

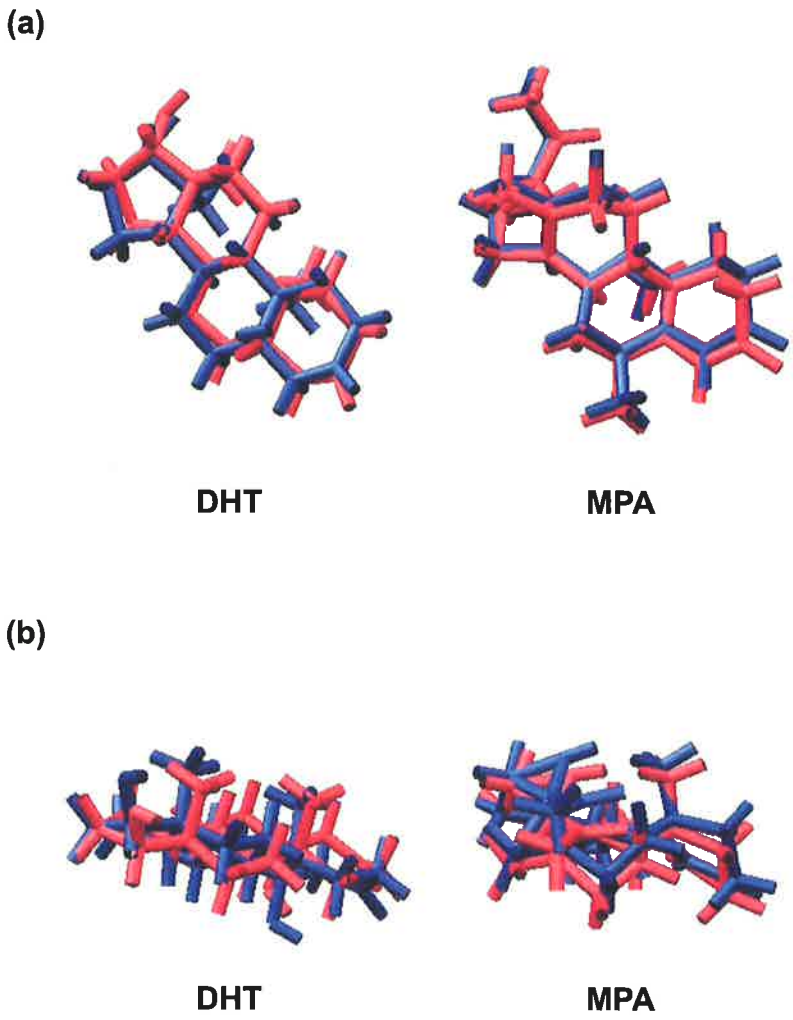


Figure 5.10: Homology modelling of the AR bound to DHT (left panel) or MPA (right panel) was performed to distinguish differences in the disposition of ligand in the ligand binding pocket of wild type and variant receptors. Diagrams indicate the predicted position of ligand in the wtAR (blue) overlaid with the predicted position of ligand in the AR-Q865H variant (red). *(a)* side view, *(b)* top view. These models were generated by Dr Jonathan Harris (Queensland University of Technology).

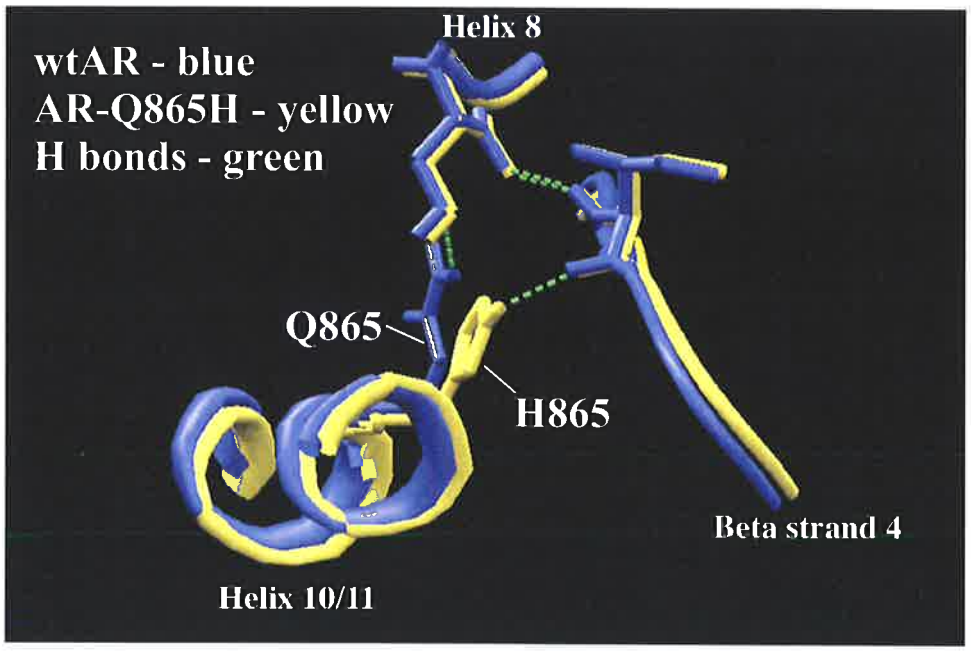


Figure 5.11: Homology modelling of the holo wtAR (blue) and AR-Q865H (yellow) AF2 region. Diagrams showing the α-helical and β-sheet backbone of the AR-LBD were generated using the MOLSCRIPT/Raster3D program. Important amino acid side chains are depicted in stick form and predicted hydrogen bonds are shown as green dotted lines. Wild type (Q) and variant (H) residues at position 865 are indicated. These models were generated by Dr Jonathan Harris (Queensland University of Technology).

5.4 – Discussion

These studies have demonstrated that the AR-Q865H variant identified in the MDA-MB-453 breast cancer cell line exhibits predominantly reduced transactivation activity compared to the wtAR in response to both the native ligand, DHT, and the synthetic progestin, MPA. However this effect appears to be dependent on cell context, as quantitative differences in activity of the variant AR were observed in PC-3 prostate cancer cells compared with MDA-MB-453 breast cancer cells. Furthermore, the functional effect of the Q865H mutation may also be dependent on ligand and promoter context as DHT and MPA differed in their ability to activate the endogenous AR in MDA-MB-453 cells at two different androgen responsive promoters.

While the current studies clearly demonstrate that the AR-Q865H variant has altered activity in comparison to the wtAR, it remains unclear whether the mutation contributes to the divergent proliferative effects of DHT and MPA in MDA-MB-453 cells. The inhibitory effect of MPA on MDA-MB-453 cells is consistent with its effect on other breast cancer cell lines which express wtAR (Sutherland *et al*, 1988; Poulin *et al*, 1989a; Hackenberg *et al*, 1993a; Bentel *et al*, 1999). However in contrast to its inhibitory effect on most AR positive breast cancer cell lines (Poulin *et al*, 1988; Labrie *et al*, 1990a; Hackenberg *et al*, 1991; de Launoit *et al*, 1991; Birrell *et al*, 1995a; Ortmann *et al*, 2002), DHT stimulates the proliferation of MDA-MB-453 cells (Birrell *et al*, 1995a). The unusual proliferative response of MDA-MB-453 cells to DHT may result from aberrant DHT-induced activity of the endogenous AR-Q865H variant.

The Q865H mutation may alter AR activity in a cell specific manner. This is supported by observations that DHT-induced activity of the variant AR was significantly

diminished in comparison to the wtAR in MDA-MB-453 cells but not in PC-3 cells. One possible way this could be regulated is at the level of cofactor molecules, which exhibit tissue specific and cell type specific variation in expression profiles (Rosenfeld and Glass, 2001; Aranda and Pascual, 2001; Magklara *et al*, 2002; McKenna and O'Malley, 2002). Molecular modelling predicts that the AR-Q865H variant may have an altered ability to recruit cofactors as a result of altered hydrogen bonding within the AF2 region. Indeed, studies performed in our laboratory have demonstrated that the AR-Q865H variant has an altered ability to interact with the corepressor SMRT (Section 1.3.7). In the mammalian two-hybrid system, the LBD of the AR-Q865H variant exhibited increased recruitment of SMRT in the presence of DHT compared to the wtAR, while MPA-induced recruitment of SMRT to the AR-Q865H LBD was diminished compared to the wtAR (Buchanan, 2002). These studies suggest that the observed variation in AR-Q865H activity between PC-3 and MDA-MB-453 cells may reflect a fundamental difference between breast and prostate cell lines, which is likely to result, at least in part, from tissue-specific variations in AR cofactor expression profiles.

In contrast to the differences in DHT-induced AR-Q865H activity between breast and prostate cell lines, the MPA-induced activity of the AR-Q865H variant at the PB_3 promoter was partially diminished in comparison to wtAR in both PC-3 and MDA-MB-453 cells. Interestingly, the endogenous AR-Q865H variant in MDA-MB-453 cells transiently transfected with the same reporter construct showed no activation in response to MPA. It is currently unclear how transfected and endogenous variant ARs can have different activities in experiments where the promoter, ligand concentration and cell line remain constant. One possible explanation is that the level of variant AR in transfected cells is likely to be significantly higher than the endogenous AR level in MDA-MB-453 cells. AR overexpression potentially squelches factors that

prevent activation of the endogenous AR-Q865H by MPA. Decreasing the amount of AR expression construct used in transient transfections may enable more precise determination of the functional consequences of the Q865H mutation as receptors are likely to be expressed at a more natural level. The observed differences in endogenous and exogenous AR activity in these experiments suggest that the levels of exogenous gene expression, transcription factor squelching and non-chromatin integrated reporter genes should be considered when interpreting results from transiently transfected cells.

In MDA-MB-453 cells, promoter specific differences in the activity of the endogenous AR-Q865H variant were observed in response to DHT and MPA. This suggests that DHT and MPA may differentially regulate the expression of AR target genes in a promoter specific manner in MDA-MB-453 cells. Given that MPA does not induce N/C interactions for the AR-Q865H variant, the MPA-AR complex may potentially be recruited to different promoters than the DHT-AR complex in MDA-MB-453 cells. Genes regulated differently in response to DHT and MPA may be critical in mediating the divergent proliferative effects of these ligands in MDA-MB-453 cells. Identification of these genes would provide valuable information regarding the mechanisms leading to the opposite proliferative effect of DHT and MPA on MDA-MB-453 cells. This is investigated further in Chapter 6.

A number of studies have identified alterations in AR sequence in patients suffering from complete or partial androgen insensitivity syndrome as well as in prostate cancers, including cell lines and xenografts and clinical specimens of various stages of progression. Published AR mutations are listed at http://www.mcgill.ca/androgendb/ (Gottlieb *et al*, 1998 and references therein). Relatively few mutations in the AR gene have been identified in breast tumours. Single stranded conformational polymorphism

and sequence analysis of 54 primary breast cancers from Japanese women found no evidence of somatic mutations in exons 2-8 of the AR (Shan *et al*, 2000). Likewise, no AR gene mutations were identified in cohorts of male breast cancers (Hiort *et al*, 1996; Haraldsson *et al*, 1998; Kwiatkowska *et al*, 2001; Syrjakoski *et al*, 2003) with the exception of two germline mutations found in breast cancer patients with partial androgen insensitivity syndrome (Wooster *et al*, 1992; Lobaccaro *et al*, 1993). These mutations, at codons 605 (arginine to glutamine) and 606 (arginine to lysine), lie in the second zinc finger of the DNA binding domain. In comparison to wtAR, these variants have reduced DNA binding ability *in vitro* and reduced transactivation capacity (Poujol *et al*, 1997; Aarnisalo *et al*, 1999).

AR mutations identified in prostate cancers predominantly result in receptors with increased transactivation capacity and/or broadened ligand binding specificity (Veldscholte *et al*, 1990; Veldscholte *et al*, 1992; Culig *et al*, 1993; Suzuki *et al*, 1993; Gaddipati *et al*, 1994; Taplin *et al*, 1995; Ėlo *et al*, 1995; Suzuki *et al*, 1996; Tan *et al*, 1997; Taplin *et al*, 1999; Matias *et al*, 2000; Sack *et al*, 2001; Buchanan *et al*, 2001b), characteristics which may provide a growth advantage for prostate cells, which are generally stimulated by androgen signalling pathways (Buchanan *et al*, 2001a). In contrast, androgen signalling pathways predominantly inhibit proliferation of breast cancer cells (Section 1.2.4). Given the reduced activity exhibited by the AR-Q865H variant relative to wtAR in these studies, it may be hypothesised that breast cancer associated AR mutations confer loss of function which diminish the inhibitory effects of androgen signalling pathways on breast cancer cell growth. This hypothesis is further supported by previous studies which show reduced transactivation capacity of the only other breast cancer associated AR variants reported, R605Q and R606K, relative to wtAR (Poujol *et al*, 1997; Aarnisalo *et al*, 1999).

In prostate cancer, the frequency of AR gene mutations is low in primary disease (approximately 5%) but increases to a maximum of approximately 50% in metastatic cancers following androgen ablation therapy (Newmark *et al*, 1992; Culig *et al*, 1993; Suzuki *et al*, 1993; Castagnaro *et al*, 1993; Gaddipati *et al*, 1994; Taplin *et al*, 1995; Elo *et al*, 1995; Tilley *et al*, 1996; Suzuki *et al*, 1996; Watanabe *et al*, 1997; Taplin *et al*, 1999; Wallen *et al*, 1999; Marcelli *et al*, 2000). Absence of a selective pressure provided by androgen ablation therapy has been proposed for the comparatively lower frequency of AR gene mutations identified in early stage disease (Taplin *et al*, 1999). Similarly, breast tumours may not accrue mutations in the AR gene in the absence of an appropriate selective pressure, such as hormonal therapies targeting androgen signalling pathways. This could, in part, explain why previous studies have failed to identify significant numbers of somatic AR mutations in breast cancers. The only breast cancer associated AR gene mutations that have previously been reported, R605Q and R606K, are germline mutations identified in male breast cancer patients with partial androgen insensitivity syndrome (Wooster *et al*, 1992; Lobaccaro *et al*, 1993). Although these mutations result in receptors with reduced transactivation activity, which may confer a growth advantage to breast cancer cells (Poujol *et al*, 1997; Aarnisalo *et al*, 1999), these mutations did not arise to combat selective pressures. It is currently unknown whether the AR-Q865H mutation in the MDA-MB-453 breast cancer cell line arose due to selective pressures applied *in vivo* or following establishment as a cell line. However, the AR-Q865H variant may have an altered response to DHT, providing a potential mechanism by which MDA-MB-453 cells have developed a selective growth advantage.

As the therapeutic effects of MPA are mediated at least in part by the AR (Birrell *et al*, 1995b), treatment of breast cancers with MPA could potentially provide an appropriate

selective pressure for the outgrowth of cells containing AR mutations. Studies in our laboratory have investigated the AR in a cohort of female breast cancer patients who received MPA as a second line therapy following relapse with tamoxifen (Yang *et al*, manuscript in preparation). Of 41 tumours which did not respond to MPA therapy, a subset showed strong immunoreactivity with an antibody directed at the amino terminus of the AR but lacked detectable radioligand binding, suggesting the presence of AR gene mutations in the LBD. Subsequent screening of genomic DNA isolated from these tumours identified mutations in the *AR* gene in five of these tumours: four missense mutations, at codons 640 (E640K) and 641 (G641E) in the hinge region and 778 (M778T) and 805 (M805V) in the ligand binding domain, as well as one silent mutation in codon 856. Functional analysis has demonstrated that the AR-M778T and AR-M805V variants are inactive in response to DHT and MPA, consistent with the loss of function phenotypes of other breast cancer associated AR mutations. Outgrowth of cells containing these mutations could have led to resistance to MPA therapy in these patients.

The AR gene is located on the X chromosome and therefore one allele is expressed in female somatic cells while the other is repressed through X inactivation which occurs during embryonic development. AR gene mutations have primarily been identified in prostate cancers, which contain one copy of the AR gene, by sequencing of PCR amplified genomic DNA isolated from archival tumour samples. When performed on breast cancer specimens, these techniques do not distinguish whether the mutation lies on the active or inactive X chromosome. Further analysis, perhaps investigating DNA methylation patterns, is required to determine if the mutant AR gene is expressed in breast cancer cells. In the present study, identification of the AR mutation in codon 865

in RNA isolated from the MDA-MB-453 breast cancer cell line indicates that the mutant gene is expressed in these cells and not silenced by X inactivation.

In summary, studies performed in PC-3 and MDA-MB-453 cells transiently transfected with wild type or variant AR have demonstrated that the amino acid substitution at position 865 in the ligand binding domain results in a receptor with predominantly reduced DHT-and MPA-induced transactivation capacity in comparison to wtAR. However promoter specific differences in activity of the endogenous AR-Q865H variant were observed in response to DHT and MPA in MDA-MB-453 cells. The promoter specific effects of DHT and MPA provide a potential mechanism for the divergent proliferative effects of these ligands on MDA-MB-453 cells. This possibility is investigated further in Chapter 6, where gene expression profiles in DHT and MPA treated MDA-MB-453 cells are compared in order to identify genes that are differentially regulated by these ligands. Studies that further characterise AR function in MDA-MB-453 cells are required to identify the precise mechanisms contributing to the divergent proliferative effects of DHT and MPA. In particular, investigation of the role of coactivators and corepressors in regulating AR activity and mechanisms that lead to promoter specific gene expression will provide valuable information on androgen action in breast cancer cells. Reduced activity of the AR-Q865H variant compared to wtAR may provide a growth advantage for MDA-MB-453 breast cancer cells, perhaps by altering the response to DHT such that cell proliferation is stimulated. It therefore appears that the MDA-MB-453 cell line is a valuable *in vitro* model in which to study the effects of AR gene mutations on breast cancer growth. Similar to MDA-MB-453, the MFM-223 breast cancer cell line has been reported to express high levels of AR and undetectable levels of ER and PR, however its proliferation is inhibited by both DHT and MPA (Hackenberg *et al*, 1991; Hackenberg *et al*, 1993a), a response more typical

of other AR positive breast cancer cell lines such as T-47D and ZR-75-1. Therefore comparison of the effects of DHT and MPA in MDA-MB-453 and MFM-223 cells may further enhance our understanding of mechanisms regulating androgen action in breast cancer cells.

CHAPTER 6

GENE EXPRESSION PROFILES IN BREAST CANCER CELLS: IDENTIFICATION OF ANDROGEN REGULATED GENES

6.1 – Introduction

The potent androgen DHT stimulates proliferation of MDA-MB-453 breast cancer cells whereas the synthetic progestin MPA inhibits proliferation (Birrell *et al*, 1995a; Bentel *et al*, 1999). How DHT and MPA, both acting through the AR in MDA-MB-453 cells, can induce divergent proliferative effects is not fully understood. One possible explanation, inferred from studies performed in Chapter 5, is that a structural alteration (ie the Q865H amino acid substitution) in the AR-LBD in MDA-MB-453 cells may have different functional consequences depending on the nature of the ligand bound to the receptor. While both DHT and MPA induced a lower level of transactivation activity of the AR-Q865H variant compared to the wtAR, experiments using different androgen responsive reporter constructs demonstrated that DHT and MPA differentially activate the endogenous AR variant in MDA-MB-453 cells in a promoter specific manner. This has led to the hypothesis that DHT-AR and MPA-AR regulate different sets of target genes, thereby providing a potential mechanism for how these two ligands can induce divergent proliferative effects on MDA-MB-453 cells.

A relatively small number of AR target genes, which are modulated by androgens or by MPA, have been identified and characterised in breast cancer cells. These genes provide limited insight into how androgens control proliferation. As discussed in Section 1.3.2, PSA is the most well characterised androgen regulated gene, and its expression is induced by androgens (such as DHT, testosterone and mibolerone) in certain breast cancer cell lines (Yu *et al*, 1994b; Zarghami *et al*, 1997; Hsieh *et al*, 1997; Magklara *et al*, 2000). Although induction of PSA expression by androgens in the T-47D and MFM-223 breast cancer cell lines is associated with inhibition of proliferation (Hackenberg *et al*, 1991; Birrell *et al*, 1995a; Magklara *et al*, 2000), PSA is

not induced in all breast cancer cell lines (eg ZR-75-1) which are inhibited by androgens (Magklara *et al*, 2000). Additionally, PSA is induced by androgens in LNCaP prostate cancer cells which are growth stimulated by androgens (Young *et al*, 1991; Henttu *et al*, 1992; Jia *et al*, 2003). Therefore the relationship between PSA expression and cell proliferation is unclear. Induction of gross cystic disease fluid proteins (GCDFP-15, GCDFP-24/Apolipoprotein D and GCDFP-44) by DHT in breast cancer cell lines has also been extensively studied (Murphy *et al*, 1987; Chalbos *et al*, 1987; Dumont *et al*, 1989; Simard *et al*, 1990; Labrie *et al*, 1990b; Simard *et al*, 1992; Haagensen *et al*, 1992; Hall *et al*, 1994; Loos *et al*, 1999; Lapointe *et al*, 1999). GCDFPs have a potential role in mediating the proliferative effects of steroids (eg androgens and E_2) and other growth factors (such as retinoic acid and interleukin-1), as GCDFP expression has been shown to be inversely associated with proliferation in some breast cancer cell lines treated with these agents, such as T-47D and ZR-75-1 (Simard *et al*, 1990; Labrie *et al*, 1990a; Simard *et al*, 1992; Blais *et al*, 1994; Lopez-Boado *et al*, 1994). Other genes which are induced by androgens (DHT, testosterone, mibolerone or R1881) in breast cancer cells include hK2 (a kallikrein related to PSA) (Hsieh *et al*, 1997; Magklara *et al*, 2000), fatty acid synthase (Hall *et al*, 1994), spot 14 (Ormandy *et al*, 1992; Heemers *et al*, 2000), prolactin receptor (Ormandy *et al*, 1992; Hall *et al*, 1994), L-plastin (Lin *et al*, 2000b), uridine diphosphoglucose dehydrogenase (Lapointe and Labrie, 1999), mucin 1 (Mitchell *et al*, 2002), normal epithelial cell specific-1 (NES-1 (Luo *et al*, 2000)), vascular endothelial growth factor (VEGF, (Ruohola *et al*, 1999)) and pepsinogen C (Balbin and Lopez-Otin, 1996). Although these genes may be associated with proliferative effects of androgens, they do not have clear roles in regulating cell proliferation. Interestingly, expression of bcl-2, a gene which inhibits apoptosis (Reed, 1997), is repressed by DHT in ZR-75-1 breast cancer cells (Lapointe *et al*, 1999; Kandouz *et al*, 1999). Therefore, although studies performed in this thesis have

demonstrated that androgens do not induce breast cancer cell death (Chapter 3), bcl-2 may potentially be involved in mediating androgen-induced inhibition of breast cancer cell proliferation (at least in ZR-75-1 cells). PSA, prolactin receptor and mucin 1 have also been shown to be induced by MPA in breast cancer cell lines (Ormandy *et al*, 1992; Zarghami *et al*, 1997; Mitchell *et al*, 2002). However as DHT and MPA do not differentially regulate these genes, they may not be important in mediating the divergent proliferative effects of these ligands on MDA-MB-453 cells.

It is possible that androgens regulate the expression of other genes which may have direct proliferative effects on breast cancer cells. Identification and characterisation of additional specific genes, or gene families, that are regulated by the AR and are essential for the growth and maintenance of breast cancer cells, is essential for a better understanding of the mechanisms by which androgens, and MPA, modulate breast cancer cell behaviour. In this chapter, gene expression profiles were compared in MDA-MB-453 cells treated with DHT or MPA in order to differentiate between genes expressed in growth-stimulated and growth-inhibited cells. cDNA arrays spotted with predominantly known genes were used for this study, providing an opportunity to identify novel AR target genes which, by virtue of their known function, may mediate the divergent proliferative effects of DHT and MPA in MDA-MB-453 cells. Previous studies in our laboratory have shown that 1nM DHT maximally stimulates proliferation of MDA-MB-453 cells relative to untreated cells (Birrell *et al*, 1995a) while 100nM MPA inhibits proliferation of MDA-MB-453 cells (Bentel *et al*, 1999). These concentrations of DHT and MPA are also sufficient to induce expression of MMTV-CAT (Bentel *et al*, 1999) and PSA_{630} (promoter + enhancer)-luc (Chapter 5) androgen responsive reporter genes in transiently transfected MDA-MB-453 cells.

Therefore, in this chapter, gene expression was examined by cDNA microarray analysis in MDA-MB-453 cells treated with 1nM DHT and 100nM MPA to identify candidate genes which may mediate the divergent proliferative effects of these ligands. Interestingly, 1nM MPA has no effect on proliferation of MDA-MB-453 cells although this concentration does activate expression of the MMTV-CAT androgen responsive reporter gene (Bentel *et al*, 1999). Consequently, gene expression profiles were also examined in MDA-MB-453 cells treated with 1nM MPA in these studies in order to identify potential candidate genes which are modulated by low concentrations of MPA but which may not contribute to a proliferative effect.

6.2 – Methods

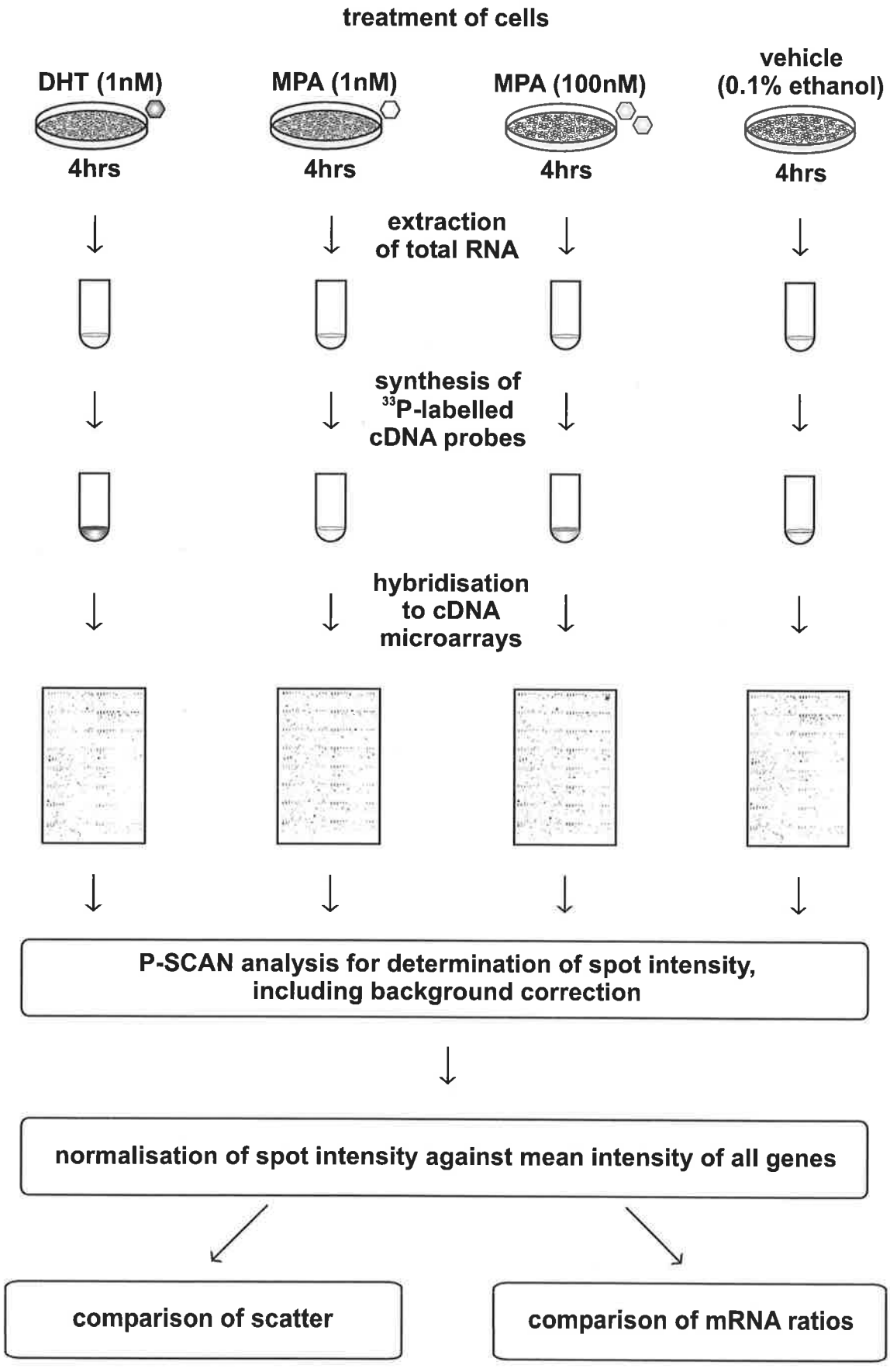
6.2.1 – Cell culture

MDA-MB-453 cells were seeded in 10cm petri dishes at a density of 4 x 10^6 cells/dish in phenol red free DMEM containing 5% CSS and 4mM glutamine. After 72 hours, medium was replaced with DMEM containing 5% CSS, 4mM glutamine and either 1nM DHT, 1nM MPA, 100nM MPA or vehicle (0.1% ethanol). Cells were harvested by trypsinisation after four and 24hrs of treatment, and collected by centrifugation at 2000rpm for 5mins at 4°C. Cell pellets were stored at -70°C.

6.2.2 – Preparation of [α-^{33}P] dCTP-labelled probes for cDNA microarray analysis

cDNA microarray analysis was performed using RNA extracted from MDA-MB-453 cells treated with 1nM DHT, 1nM MPA, 100nM MPA or vehicle for 4hrs. The design of cDNA microarray experiments performed in these studies is illustrated schematically in Figure 6.1.

Figure 6.1: Design of cDNA microarray experiments. MDA-MB-453 cells were seeded in 10cm petri dishes (4 x 10^6 cells/dish) in phenol red free DMEM containing 5% CSS and 4mM L-glutamine. After 72hrs cells were treated with 1nM DHT, 1nM MPA, 100nM MPA or vehicle (0.1% ethanol) for 4hrs. Total RNA, extracted from cell pellets was used to synthesise ^{33}P-labelled cDNA probes, which were subsequently hybridised to Human GeneFilters "Named Genes" cDNA microarrays (Research Genetics). Images were scanned using a PhosphorImager. The intensity of each spot on each array was quantified using P-SCAN software, with automatic background correction, and normalised against the mean intensity for all genes on the array. Normalised intensity values were compared between arrays to determine mRNA ratios for each gene. Following extraction of data, membranes were stripped and rehybridised.


treatment of cells
DHT (1nM)
MPA (1nM)
MPA (100nM)
vehicle (0.1% ethanol)
4hrs
4hrs
4hrs
4hrs
extraction of total RNA
synthesis of ^{33}P-labelled cDNA probes
hybridisation to cDNA microarrays
P-SCAN analysis for determination of spot intensity, including background correction
normalisation of spot intensity against mean intensity of all genes
comparison of scatter
comparison of mRNA ratios

Total RNA was extracted from cell pellets using the RNeasy Mini Kit (Section 2.3.7) and RNA integrity was determined by electrophoresis on an agarose-formaldehyde gel as described in Section 2.3.8. [α-^{33}P] dCTP labelled cDNA probes were synthesised from total RNA in a reverse transcription reaction as described in Section 2.3.10, but with the following modifications: 5µg total RNA was used with 2µg oligo(dT)$_{12-18}$ primer and 100µCi (3000Ci/mmol) [α-^{33}P] dCTP was incorporated into cDNA products. Unincorporated nucleotides were removed using Bio-Spin 6 chromatography columns (Bio-Rad) according to the manufacturer's instructions. The specific activity of each cDNA probe was determined by scintillation counting.

6.2.3 – cDNA array hybridisation

Research Genetics Human GeneFilters™ ("Named Genes") cDNA microarrays were used for this study. These consist of 5760 elements arrayed onto a 5 x 7cm nylon membrane (Figure 6.2). Of these elements, 4127 represented known genes and the remainder comprised total genomic DNA and blank spots. This array was chosen because it is spotted with cDNA fragments of genes with known sequence and function, eliminating the need for further characterisation required with cDNA arrays spotted with expressed sequence tags (ESTs). A complete list of the genes included on this array can be found at ftp://ftp.resgen.com/pub/genefilters/GF211_update_ugb146_011702.txt. Spots contained 10ng of approximately 1000bp of the 3' end of each gene, cross linked to the membrane. Total genomic DNA spots were used as positive controls and for alignment of the membranes during data analysis, therefore it was not necessary to treat RNA samples with DNase I prior to probe synthesis. Blank spots were used as negative controls.

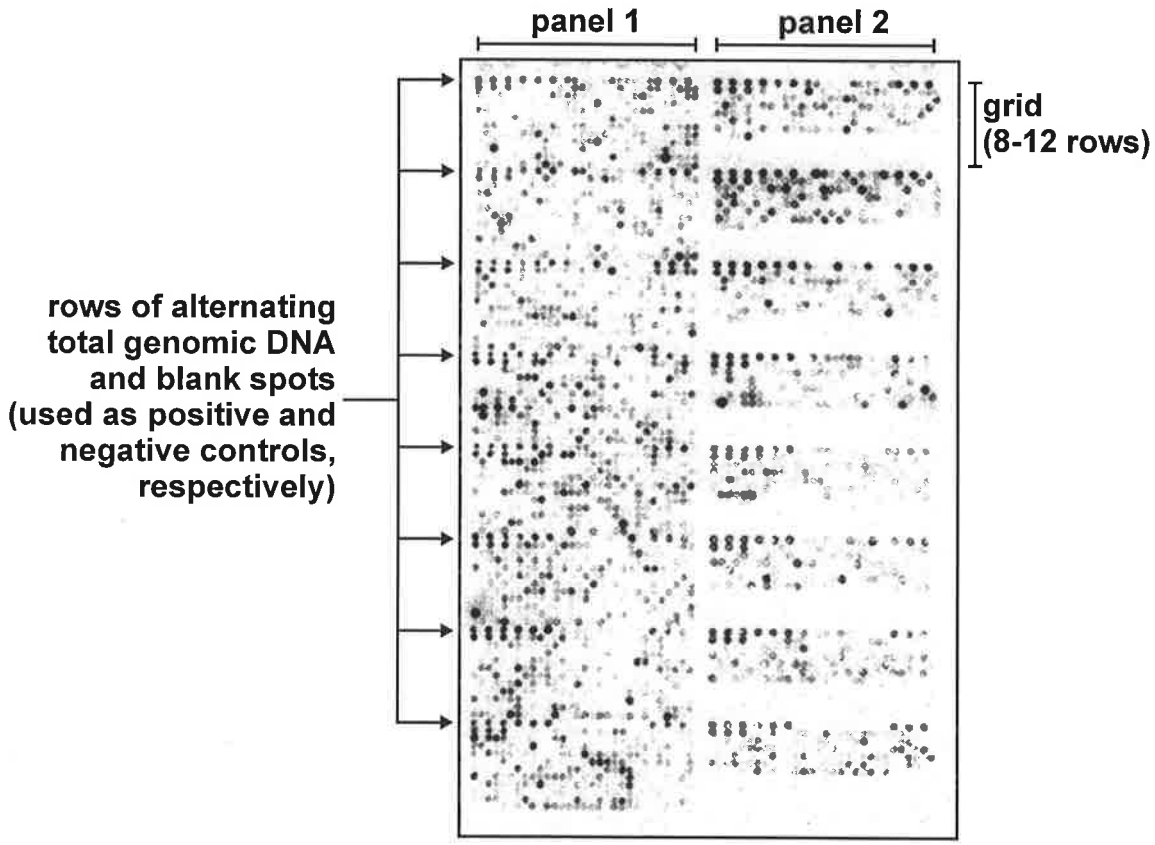


Figure 6.2: Image of a cDNA array hybridised with ^{33}P-labelled cDNA probes from MDA-MB-453 cells. Each array is divided into 16 sub-arrays arranged in two panels of eight grids each. Each sub-array contains 360 spaces arranged in 30 columns and 12 rows, with sections of the top two rows of each sub-array containing alternating total genomic DNA spots and blank spaces (indicated by arrows), which are used as positive and negative controls, respectively, and for orientation of the membrane during quantitation. Each space in panel 1 is occupied however sub-arrays in panel 2 are incomplete. The total number of spots is comprised of 192 total genomic DNA, 1441 blank and 4127 unique cDNAs for known genes. Actual size is 5 x 7 cm.

Prior to use, microarray membranes were washed with boiling 0.5% SDS for 5mins with gentle agitation. Membranes were then pre-hybridised in roller bottles with 5mL MicroHyb hybridisation solution, 5μg Cot-1 DNA (denatured by heating at 95°C for 5mins) and 5μg poly dA at 42°C for 2hrs with rotation. Purified cDNA probes were denatured at 95°C for 5mins and then transferred directly into the prehybridisation solution. The entire volume of each probe was added to hybridisations to ensure comparison of the same amounts of RNA. Hybridisation was performed at 42°C overnight with rotation. The following day, membranes were washed twice with 2x SSC, 1% SDS at 50°C for 20mins and once with 0.5x SSC, 1% SDS at 55°C for 15mins. Membranes were covered in plastic wrap and exposed to a PhosphorImager screen for 72hrs. Images were scanned using a PhosphorImager SI at a resolution of 50μm and visualised using ImageQuaNT software. Following exposure, membranes were stripped by washing in boiling 0.5% SDS for 1hr. The efficiency of the stripping procedure was confirmed by overnight exposure of the membranes to a PhosphorImager screen. No signal was detectable following stripping using this procedure.

6.2.4 – Microarray data analysis

6.2.4.1 - Determination of spot intensity

Digital images (in tiff format) of each hybridised array were analysed using the Peak quantification with Statistical Comparative Analysis (P-SCAN) macro running in MATLAB (http://abs.cit.nih.gov/pscan, 2002; Carlisle *et al*, 2000). This involved construction of a grid over the array image, where lines intersected over the centre of each spot. Pixel intensities within a 300μm radius of this point of intersection were integrated. Data output from P-SCAN was automatically corrected for background, which was determined by measuring the intensity of 384 spot-sized regions in the margins of each membrane. Spots with an integrated intensity greater than the mean

plus two standard deviations of background were considered to be significantly above background.

6.2.4.2 – Normalisation

To allow comparison of spot intensities between hybridisations, each image was normalised. Previous studies using Research Genetics microarrays have used a variety of methods for normalisation. These include normalising against the mean intensity of all genes on the array (Carlisle *et al*, 2000; McCormick *et al*, 2001), against the mean intensity of the total genomic DNA control spots (Walker and Rigley, 2000) or against the intensity of known housekeeping genes (Rep *et al*, 2000). These alternative methods of normalisation were attempted with the current data. Although the final number of differentially expressed genes varied, candidate genes showing large changes in gene expression were similar when normalised against all genes or against the total genomic DNA controls. In contrast, correction against the intensity for β-actin generated data which differed considerably from that generated using other normalisation methods. Therefore, in the current study, data for each array was normalised by dividing the raw intensity for each spot (corrected for background) by a value equal to the mean spot intensity of all genes on that array. This method of normalisation was chosen as it samples genes across the entire array and therefore may be expected to be more robust than other methods which sample fewer numbers of genes for normalisation.

6.2.4.3 – Criteria for identification of candidate differentially expressed genes

Changes in mRNA levels were determined by pairwise comparisons between hybridisations and induction or repression of mRNA levels was determined by the ratio of normalised intensity values between hybridisations. Calculations were performed

using Excel. Genes were identified as being differentially expressed if they showed a treatment:control intensity ratio greater than 3.0 (ie induced) or less than 0.33 (ie repressed). In order to minimise the inclusion of unreliable data from genes which are expressed at very low levels, genes which were not significantly above background in both hybridisations were not considered.

In these experiments, the data presented is based on one hybridisation for each treatment. Most previous studies have used duplicate or triplicate hybridisations for each cell type, which reduces the incidence of false positive results. Duplicate hybridisations were initially performed in the current experiments for each treatment. However differences in the signal:background ratios were detected, leading to considerable difficulty in accurately comparing duplicate hybridisations, even following normalisation. Therefore, although potentially leading to an overestimation of the number of differentially expressed genes detected, only one hybridisation of each treatment was analysed in order to prevent distortion of the data.

6.3 – Results

6.3.1 – cDNA array hybridisation

An overview of the experimental design used for comparison of gene expression profiles in MDA-MB-453 cells treated with DHT (1nM), MPA (1nM, 100nM) or vehicle (0.1% ethanol) is illustrated schematically in Figure 6.1. Electrophoresis of RNA on an agarose-MOPS gel confirmed high integrity of each RNA sample used for the synthesis of ^{33}P-labelled cDNA probes (Figure 6.3). Hybridisation of cDNA probes to microarrays yielded signals of high intensity for positive control total genomic DNA spots and low signals for negative control blank spots. A representative hybridisation is

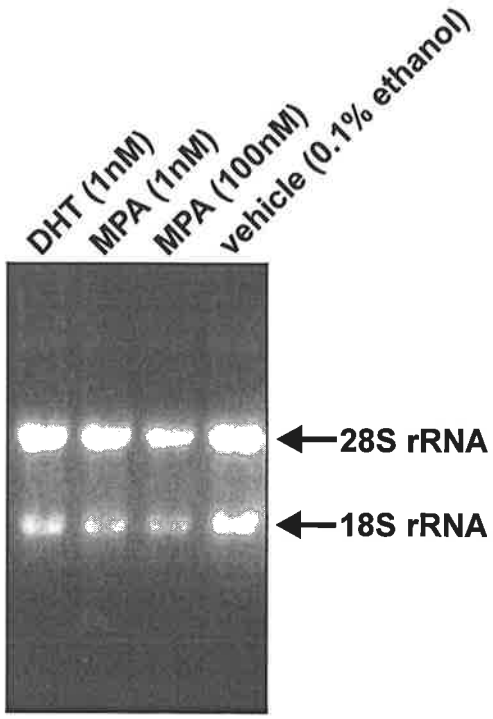


Figure 6.3: Integrity of total cellular RNA isolated from the MDA-MB-453 cell line. RNA was extracted from cells treated with DHT (1nM, lane 1), MPA (1nM, lane 2; 100nM, lane 3) or vehicle (0.1% ethanol, lane 4) for 4hrs. 2μL (2-4μg) RNA was electrophoresed on a 1% agarose-MOPS gel and visualised by ethidium bromide staining. The 28S and 18S rRNA bands are indicated by arrows.

shown in Figure 6.2. Intensity values for each spot on each array were calculated by P-SCAN software, corrected for background and normalised for comparison between hybridisations. These values were used for all subsequent analyses.

6.3.2 – Expression of previously identified androgen regulated genes in MDA-MB-453 cells

A subset of genes that have previously been demonstrated to be regulated by androgens in breast cancer cells were included in this particular cDNA array. For comparative purposes, the changes in mRNA levels of these genes in MDA-MB-453 cells treated with 1nM DHT, 1nM MPA and 100nM MPA were examined. Messenger RNA levels for some of these genes were found to be modulated (greater than 3-fold or less than 0.33-fold) in response to DHT or MPA relative to controls (Table 6.1). The induction of GCDFP-24, fatty acid synthase and prolactin receptor observed in MDA-MB-453 cells is consistent with results from previous studies (Table 6.1). PSA mRNA levels were not induced by DHT or MPA in MDA-MB-453 cells, an observation in agreement with previous studies which have demonstrated that PSA is not induced by androgens in this cell line (unpublished observations, and Magklara *et al*, 2000). Other genes which have previously been shown to be regulated by androgens in prostate cells, such as α-tubulin (Lin *et al*, 2000a), insulin-like growth factor binding proteins 3 and 5 (IGFBP3, IGFBP5, (Nickerson *et al*, 1998; Peterziel *et al*, 1999; Sugimoto *et al*, 1999; Sivashanmugam *et al*, 1999; Gregory *et al*, 1999; Miyake *et al*, 2000; Yeh *et al*, 2000; Lin *et al*, 2000a) and matrix metalloproteinase 1 (MMP-1, (Schneikert *et al*, 1996)), also demonstrated altered mRNA levels in response to DHT and/or MPA compared to control cells (Table 6.1). IGFBP3 and MMP-1 mRNA levels were induced by DHT while IGFBP5 mRNA levels were inhibited by DHT and MPA. Interestingly, mRNA levels for α-tubulin were decreased by DHT but induced by MPA (100nM). These

Table 6.1: Previously identified androgen regulated genes showing treatment:control mRNA ratios greater than 3.0, or less than 0.33, in response to DHT and/or MPA in MDA-MB-453 cells.

Gene	Accession number	Treatment	treatment:control in MDA-MB-453 cells	Up/down regulated* by androgens in previous studies (cell type)	References
GCDFP-24	AA457084	MPA (100nM)	3.0	↑ (breast: T-47D, ZR-75-1, MDA-MB-453)	Simard *et al*, 1990; Haagensen *et al*, 1992; Simard *et al*, 1992
Fatty acid synthase	H50323	MPA (100nM)	3.0	↑ (breast: MDA-MB-453)	Hall *et al*, 1994
Prolactin receptor	R63647	DHT (1nM) MPA (1nM) MPA (100nM)	3.0 6.3 10.7	↑ (breast: MDA-MB-453, MCF-7)	Ormandy *et al*, 1992; Hall *et al*, 1994
α-tubulin	AA626698	DHT (1nM) MPA (100nM)	0.33 4.0	↑ (prostate: CWR22 model)	Gregory *et al*, 1998
IGFBP3	AA598601	DHT (1nM)	7.5	↓ (rat ventral prostate)	Nickerson and Pollak, 1999; Bruyninx *et al*, 2000
IGFBP5	T52830	DHT (1nM) MPA (1nM) MPA (100nM)	0.31 0.32 0.22	↑ (prostate: CWR22 model) ↓ (rat ventral prostate)	Gregory *et al*, 1998; Nickerson *et al*, 1998; Gregory *et al*, 1999; Nickerson and Pollak, 1999; Bruyninx *et al*, 2000
MMP-1	AA143331	DHT (1nM)	12.5	↓ (prostate: LNCaP)	Schneikert *et al*, 1996

* ↑, expression induced; ↓, expression repressed

results are a reassuring indication that the experimental system used for these studies was appropriate for identification of androgen regulated genes.

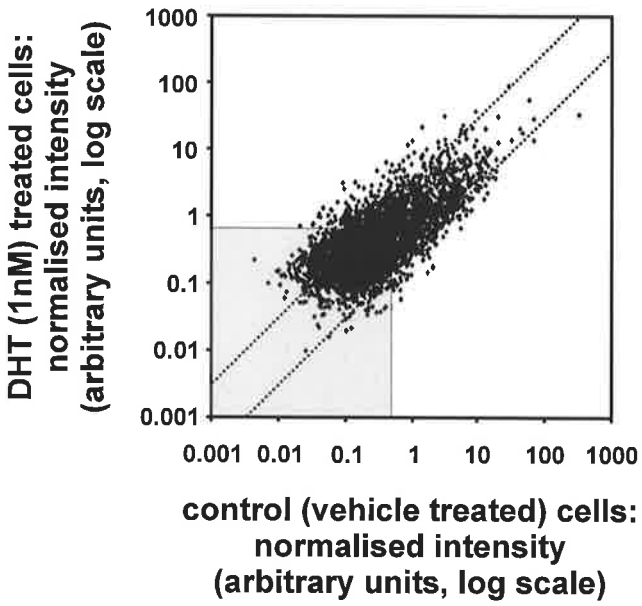
6.3.3 – Identification of novel candidate genes induced or repressed by DHT or MPA in MDA-MB-453 cells

Scatter plots, representing one point for the normalised intensity value of each gene, were used to visualise differences in global gene expression profiles between two hybridisations (Figure 6.4). Indicated on these plots are dashed lines representing 3-fold limits – points lying above the upper line represent genes with mRNA levels increased at least 3-fold in treated compared to control cells while points lying below the lower line represent genes with mRNA levels that are reduced (to 0.33-fold or less) by treatment compared to the control. The grey area covers spots that were not significantly above background intensity in both hybridisations.

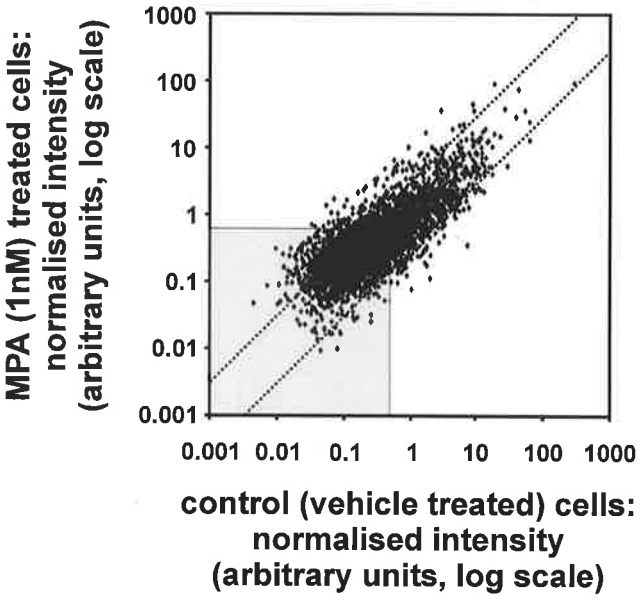
Figure 6.4 (a) shows a scatter plot comparing gene expression in control and DHT (1nM) treated MDA-MB-453 cells. The majority of genes have similar mRNA levels in control and DHT treated cells, represented by points lying between the 3-fold limit lines. A total of 340 genes (equating to 8.2% of the total number of unique cDNAs on the array) showed at least 3-fold increased mRNA levels following treatment with 1nM DHT for 4hrs, while mRNA levels for 167 genes (4.0%) were reduced to less than 0.33-fold by 1nM DHT. The 25 genes showing the highest DHT(1nM):control ratio and the 25 genes showing the lowest DHT(1nM):control ratio are listed in Table 6.2 and Table 6.3 respectively. These genes were grouped according to their function, as derived from information on the UniGene database (accessed from the National Center for Biotechnology Information website at http://www.ncbi.nlm.nih.gov/). The majority of genes fitted into eight categories: (1) proliferation and cell cycle regulation;

Figure 6.4: Comparison of gene expression in treated *versus* untreated control MDA-MB-453 cells, visualised using scatter plots. Arrays were hybridised with probes synthesised from MDA-MB-453 cells treated with 1nM DHT, 1nM MPA, 100nM MPA or vehicle (0.1% ethanol, control) for 4 hours. Intensity of spots on each array was determined using P-SCAN and normalised against the mean intensity of all genes. One point for each gene was plotted on the scatter plots for *(a)* DHT (1nM) *versus* control, *(b)* MPA (1nM) *versus* control, *(c)* MPA (100nM) *versus* control. Dashed lines represent threefold limits: points lying above the upper line represent genes with mRNA levels at least 3-fold higher in treated cells compared to the control while points lying below the lower line represent genes with mRNA levels that are reduced (to 0.33-fold or less) by treatment compared to the control. The grey area covers spots that were not greater than two standard deviations above the mean background intensity in both hybridisations.

(a)


DHT (1nM) treated cells:
normalised intensity
(arbitrary units, log scale)
1000
100
10
1
0.1
0.01
0.001
0.001 0.01 0.1 1 10 100 1000
control (vehicle treated) cells:
normalised intensity
(arbitrary units, log scale)

(b)


MPA (1nM) treated cells:
normalised intensity
(arbitrary units, log scale)
1000
100
10
1
0.1
0.01
0.001
0.001 0.01 0.1 1 10 100 1000
control (vehicle treated) cells:
normalised intensity
(arbitrary units, log scale)

(c)

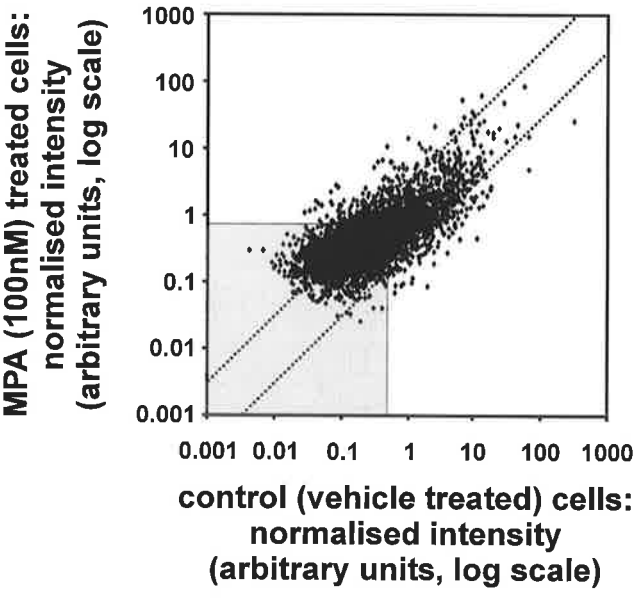

MPA (100nM) treated cells:
normalised intensity
(arbitrary units, log scale)
1000
100
10
1
0.1
0.01
0.001
0.001 0.01 0.1 1 10 100 1000
control (vehicle treated) cells:
normalised intensity
(arbitrary units, log scale)

Table 6.2: Candidate DHT regulated genes[#] that increase in MDA-MB-453 cells treated with 1nM DHT compared to untreated control cells.

Gene	Accession number	DHT(1nM):control
Proliferation, cell cycle regulation		
40 kDa protein kinase related to rat ERK2	W45690	35.6
V-ski avian sarcoma viral oncogene homolog	T50498	24.1
Deoxyhypusine synthase (clone 13a)	R37766	15.8
Vasopressin-activated calcium mobilizing putative receptor protein (VACM-1)	AA086475	14.4
Apoptosis		
40 kDa protein kinase related to rat ERK2	W45690	35.6
IAP homolog C (MIHC)	AA002126	20.4
Vasopressin-activated calcium mobilizing putative receptor protein (VACM-1)	AA086475	14.4
Signal transduction pathways, transcription and translation		
40 kDa protein kinase related to rat ERK2	W45690	35.6
Gu protein	AA465386	29.7
GS3955	AA458653	27.7
X-arrestin	H86518	25.7
V-ski avian sarcoma viral oncogene homolog	T50498	24.1
FKBP54 (54 kDa progesterone receptor-associated immunophilin)	W86653	24.1
Recepin	R53998	22.4
Nmi	AA279762	21.8
IAP homolog C (MIHC)	AA002126	20.4
Myo-inositol monophosphatase 2	R42685	17.2
Mannosyl(beta-1,4-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase	H47026	16.6
A28-RGS14p	AA453774	14.8
Vasopressin-activated calcium mobilizing putative receptor protein (VACM-1)	AA086475	14.4
Dr1-associated corepressor (DRAP1)	AA406285	14.3
Carbohydrate, lipid, steroid or protein synthesis/metabolism		
VHL binding protein-1 (VBP-1)	AA426341	18.3
Lactate dehydrogenase-A (LDH-A, EC 1.1.1.27)	H05914	16.3
Deoxyhypusine synthase (clone 13a)	R37766	15.8
Cytoskeleton/structure		
Grancalcin	R44739	14.7
Other		
KIAA1007	T64885	34.7
Peroxisome biogenesis disorder protein 1 (PEX1)	AA598527	27.4
MaxiK potassium channel beta subunit	AA029299	23.8
ART4	N20349	23.5
Antisecretory factor-1	AA450227	18.9
HS1 binding protein HAX-1, nuclear gene encoding mitochondrial protein,	R76263	16.3
Procholecystokinin precursor	N30191	14.1

[#] 25 from a total of 340 genes that showed at least 3-fold greater mRNA level in DHT (1nM) treated *versus* control cells. Genes are grouped based on function, with some genes listed under more than one category.

Table 6.3: Candidate DHT regulated genes[##] that decrease in MDA-MB-453 cells treated with 1nM DHT compared to untreated control cells.

Gene	Accession number	DHT(1nM):control
Proliferation, cell cycle regulation		
Serine/threonine protein kinase (clone C-2k, CDK9)	H45967	0.086
LDL-receptor related protein	AA464566	0.164
Annexin XIII	AA884167	0.169
Differentiation		
Annexin XIII	AA884167	0.169
Cell adhesion, motility and invasion		
Galectin-2	AA872397	0.164
CD58 antigen, (lymphocyte function-associated antigen 3)	AA136271	0.164
DNA repair		
Replication protein A (E coli RecA homolog, RAD51 homolog)	AA873056	0.179
Signal transduction pathways, transcription and translation		
Serine/threonine protein kinase (clone C-2k)	H45967	0.086
Glucocorticoid receptor alpha {alternative products}	N66871	0.088
SWI/SNF complex 60 KDa subunit (BAF60b)	AA478436	0.112
Nuclear factor I-B2 (NFIB2)	W87528	0.122
Eukaryotic translation initiation factor 4E	AA194246	0.139
Serine/threonine protein kinase receptor R1 precursor	AA136882	0.139
Partial C1 mRNA	H70464	0.143
TAR RNA binding protein (TRBP)	AA436409	0.154
E2F transcription factor 5, p130-binding	AA455521	0.159
HuR RNA binding protein (HuR)	W72322	0.159
Glutathione S-transferase theta 1	H99813	0.164
LDL-receptor related protein	AA464566	0.164
CD58 antigen, (lymphocyte function-associated antigen 3)	AA136271	0.164
Annexin XIII	AA884167	0.169
cAMP responsive element binding protein beta subunit (CREBPA)	R22790	0.175
Carbohydrate, lipid, steroid or protein synthesis/metabolism		
Cytochrome P450, subfamily XVII (steroid 17-alpha-hydroxylase), adrenal hyperplasia	R16838	0.087
Dodecenoyl-Coenzyme A delta isomerase (3,2 trans-enoyl-Coenzyme A isomerase)	AA405800	0.123
Galectin-2	AA872397	0.164
LDL-receptor related protein	AA464566	0.164
Preprocarboxypeptidase A2 (proCPA2)	AA844831	0.169
Cytochrome P450, subfamily XXI (steroid 21-hydroxylase, congenital adrenal hyperplasia)	T58430	0.172
Cytoskeleton/structure		
Collagen, type IX, alpha 3	AA496735	0.088
Partial C1 mRNA	H70464	0.143
Other		
HES1 protein	AA034250	0.159
Autoantigen pericentriol material 1 (PCM-1)	AA164440	0.161
RP3 mRNA	AA398011	0.164

[##] 25 from a total of 167 genes that showed less than 0.33-fold lower mRNA level in DHT (1nM) treated *versus* control cells. Genes are grouped based on function, with some genes listed under more than one category.

(2) apoptosis; (3) differentiation; (4) cell adhesion, motility and invasion; (5) DNA repair; (6) signal transduction pathways, transcription and translation; (7) carbohydrate, lipid, steroid or protein metabolism; and (8) cytoskeleton/structural proteins. Among genes which showed the greatest induction by DHT were the 40 kDa protein kinase related to rat ERK2 (a serine/threonine kinase which has a role in regulating cell cycle and apoptosis), the v-ski avian sarcoma viral oncogene homolog (which has a role in regulating cell proliferation) and IAP homolog C (an inhibitor of apoptosis). DHT also strongly reduced mRNA levels for genes involved in cell proliferation (eg cyclin dependent kinase 9, CDK9) as well as two members of the P450 family of enzymes which regulate steroid hormone biosynthesis (17α-hydroxylase and 21-hydroxylase).

A scatter plot comparing mRNA levels in control and 1nM MPA treated MDA-MB-453 cells is shown in Figure 6.4 (b). mRNA levels for 255 genes (6.2%) were increased by 1nM MPA while mRNA levels for 139 genes (3.4%) were reduced by 1nM MPA. Table 6.4 lists the 25 genes with the greatest MPA(1nM):control ratio, while Table 6.5 lists the 25 genes with the lowest MPA(1nM):control ratio, each grouped according to their function. Although 1nM MPA does not have a significant effect on proliferation of MDA-MB-453 cells (Bentel *et al*, 1999), this concentration does result in altered mRNA levels for some genes involved in proliferation and apoptosis, such as angiopoietin-2, ionizing radiation resistance conferring protein and CDK9 (which was also downregulated by DHT).

Increasing the concentration of MPA from 1nM to 100nM resulted in a slightly greater number of genes with mRNA levels increased (270 genes, 6.5%) or decreased (191 genes, 4.6%) relative to the untreated control (Figure 6.4 (c)). Table 6.6 and Table 6.7 list the 25 genes with the highest and lowest MPA(100nM):control ratios respectively,

Table 6.4: Candidate MPA regulated genes[$] that increase in MDA-MB-453 cells treated with 1nM MPA compared to untreated control cells.

Gene	Accession number	MPA(1nM):control
Proliferation, cell cycle regulation		
Angiopoietin-2	AA125872	19.2
Hs-cul-3	N25141	10.3
Hepatoma-derived growth factor	AA453749	10.2
Apoptosis		
Ionizing radiation resistance conferring protein	R43325	11.7
Pig8 (PIG8)	AA702548	10.6
Hs-cul-3	N25141	10.3
DNA repair		
Histone H2A.X	H95392	14.0
Signal transduction pathways, transcription and translation		
Zinc finger protein 76	AA626012	25.5
Angiopoietin-2	AA125872	19.2
Histone H2A.X	H95392	14.0
Eukaryotic translation initiation factor 3 (eIF-3) p36 subunit	AA936783	12.4
RNA polymerase II transcription factor SIII p18 subunit	AA630017	12.3
B4-2 protein	AA669637	11.8
Hepatoma-derived growth factor	AA453749	10.2
Carbohydrate, lipid, steroid or protein synthesis/metabolism		
NADH dehydrogenase	AA111999	26.7
NRD convertase	AA630302	17.4
L-serine dehydratase	T71363	14.7
Glutamine-fructose-6-phosphate transaminase	AA478571	13.2
Eukaryotic translation initiation factor 3 (eIF-3) p36 subunit	AA936783	12.4
Glucose-6-phosphatase	T98887	12.4
Xanthine dehydrogenase	R09503	11.4
Alpha NAC	AA664241	11.2
Apolipoprotein C-III	N53169	10.7
Cytoskeleton/structure		
p63 mRNA for transmembrane protein	AA598787	15.9
P80-coilin	W88740	14.1
Plastin 1 (I isoform)	AA017379	13.4
Other		
Zinc finger protein 9 (a cellular retroviral nucleic acid binding protein)	N69908	13.9
dbpB-like protein	AA599175	12.7
Multispanning membrane protein	AA127685	11.9
Uncoupling protein 2 (mitochondrial, proton carrier)	H61243	11.8

[$] 25 from a total of 255 genes that showed at least 3-fold greater mRNA level in MPA (1nM) treated *versus* control cells. Genes are grouped based on function, with some genes listed under more than one category.

Table 6.5: Candidate MPA regulated genes[$$] that decrease in MDA-MB-453 cells treated with 1nM MPA compared to untreated control cells.

Gene	Accession number	MPA(1nM):control
Proliferation, cell cycle regulation		
Serine/threonine protein kinase (clone C-2k, CDK9)	H45967	0.120
Insulin-like growth factor binding protein 6	AA478724	0.192
Cell adhesion, motility and invasion		
CD58 antigen, (lymphocyte function-associated antigen 3)	AA136271	0.172
Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	AA666269	0.179
DNA repair		
ATP-dependent helicase II, 86 kDa subunit	AA775355	0.189
Signal transduction pathways, transcription and translation		
Serine/threonine protein kinase (clone C-2k)	H45967	0.120
Transcription factor SL1	R32478	0.125
Steroid hormone receptor ERR1	AA098896	0.172
Calmodulin	W44860	0.175
Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	AA666269	0.179
Protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform (calcineurin A alpha)	AA682631	0.189
Ataxin-2 related protein	AA029964	0.192
Insulin-like growth factor binding protein 6	AA478724	0.192
Histone H2A.2	AA047260	0.192
Hematopoietic progenitor kinase (HPK1)	T50313	0.196
Leukaemia virus receptor 1 (GLVR1)	W46972	0.200
Carbohydrate, lipid, steroid or protein synthesis/metabolism		
Cytochrome P450, subfamily XVII (steroid 17-alpha-hydroxylase), adrenal hyperplasia	R16838	0.057
N-benzoyl-L-tyrosyl-p-amino-benzoic acid hydrolase alpha subunit (PPH alpha)	AA454113	0.077
Apolipoprotein C-IV	T71887	0.133
Oxidoreductase (HHCMA56)	AA128041	0.154
60S ribosomal protein L30	AA775364	0.189
Other		
Autoantigen pericentriol material 1 (PCM-1)	AA164440	0.050
Two P-domain K+ channel TWIK-1	N62620	0.099
Heterochromatin protein p25	AA448667	0.141
Ubiquitin carrier protein (E2-EPF)	AA464729	0.143
Coch-5B2 mRNA	R60995	0.154
Biliverdin-IXalpha reductase	AA192419	0.179
Voltage-gated calcium channel beta subunit	R36947	0.196

[$$] 25 from a total of 139 genes that showed less than 0.33-fold lower mRNA level in MPA (1nM) treated *versus* control cells. Genes are grouped based on function, with some genes listed under more than one category.

Table 6.6: Candidate MPA regulated genes[†] that increase in MDA-MB-453 cells treated with 100nM MPA compared to untreated control cells.

Gene	Accession number	MPA(100nM):control
Proliferation, cell cycle regulation		
RCL	AA132086	41.4
Mitotic feedback control protein Madp2 homolog	AA481076	35.0
Cyclin D2	H84154	16.4
OS-9 precursor	AA418104	15.7
Deoxyhypusine synthase (clone 13a)	R37766	13.6
Apoptosis		
40 kDa protein kinase related to rat ERK2	W45690	14.8
Cell adhesion, motility and invasion		
Capping protein alpha	AA083228	16.7
Signal transduction pathways, transcription and translation		
GABA-BR1a (hGB1a) receptor	N70841	34.7
Transcription factor hGATA-6	H77652	33.8
ATPase, DNA-binding protein (HIP116)	AA459407	30.7
Plasminogen activator inhibitor, type I	N54794	28.6
Histone H3.3	AA608514	23.4
Orphan G protein-coupled receptor (RDC1)	N53172	18.6
Transcriptional activator (Croc 4)	H05655	18.2
Adenosine receptor A3	AA863086	16.8
GS3955	AA458653	15.5
40 kDa protein kinase related to rat ERK2	W45690	14.8
(Hin-3)/HIV1 promoter region chimeric	AA479741	14.6
Carbohydrate, lipid, steroid or protein synthesis/metabolism		
Argininosuccinate synthetase	AA676466	23.5
Deoxyhypusine synthase (clone 13a)	R37766	13.6
Cytoskeleton/structure		
Incomplete cDNA for a mutated allele of a myosin class I, myh-1c	AA047778	31.9
Capping protein alpha	AA083228	16.7
Alpha-centractin	R40850	12.1
Other		
Fln29	N21170	40.1
ATP-binding cassette protein mRNA 06B09 clone	R83876	36.1
BET3	AA429882	20.9
Autoantigen (RCD-8)	AA481276	17.4
Cleavage stimulation factor, 3' pre-RNA, subunit 1, 50kD	W72816	12.3

[†] 25 from a total of 270 genes that showed at least 3-fold greater mRNA level in MPA (100nM) treated *versus* control cells. Genes are grouped based on function, with some genes listed under more than one category.

Table 6.7: Candidate MPA regulated genes[††] that decrease in MDA-MB-453 cells treated with 100nM MPA compared to untreated control cells.

Gene	Accession number	MPA(100nM):control
Proliferation, cell cycle regulation		
Breast cancer 1, early onset (BRCA1)	H90415	0.074
Friend leukaemia virus integration 1	N50806	0.116
Differentiation		
Breakpoint cluster region protein BCR	AA419342	0.104
Cell adhesion, motility and invasion		
G protein-coupled receptor (STRL22)	N57964	0.043
Small inducible cytokine A5 (RANTES)	AA486072	0.133
DNA repair		
Breast cancer 1, early onset	H90415	0.074
Signal transduction pathways, transcription and translation		
G protein-coupled receptor (STRL22)	N57964	0.043
Putative protein phosphatase 2C	AA706929	0.045
Breast cancer 1, early onset	H90415	0.074
cAMP response element binding protein	H12320	0.081
SWI/SNF complex 60 KDa subunit (BAF60b)	AA478436	0.088
Histone H1D	T66815	0.088
MXI1	AA705886	0.102
Breakpoint cluster region protein BCR	AA419342	0.104
TATA-binding protein associated factor 30 kDa subunit (tafII30)	AA235706	0.108
Friend leukaemia virus integration 1	N50806	0.116
TAR RNA binding protein (TRBP)	AA436409	0.116
Replication factor C (activator 1) 1 (145kD)	H73714	0.116
Signal transducer and activator of transcription 5A	AA280647	0.118
Protein phosphatase PP2A, 65 kDa regulatory subunit, alpha isoform	AA427433	0.120
Ataxin-2 related protein	AA029964	0.120
Small inducible cytokine A5 (RANTES)	AA486072	0.133
Nuclear factor I-B2 (NFIB2)	W87528	0.141
Carbohydrate, lipid, steroid or protein synthesis/metabolism		
Carbonyl reductase	AA280846	0.072
Glutamate-cysteine ligase (gamma-glutamylcysteine synthetase)	W96179	0.137
Cytoskeleton/structure		
Kinesin-like spindle protein HKSP	AA504625	0.122
Other		
Testis specific protein 1 (probe H4-1 p3-1)	AA868278	0.080
Clone 22 mRNA, alternative splice variant alpha-1	AA489633	0.122
ATPase, Cu++ transporting, beta polypeptide (Wilson disease)	N26536	0.125
GTP-binding protein	AA456291	0.127
Pancreatic polypeptide	AA844998	0.133

[††] 25 from a total of 191 genes that showed less than 0.33-fold lower mRNA level in MPA (100nM) treated *versus* control cells. Genes are grouped based on function, with some genes listed under more than one category.

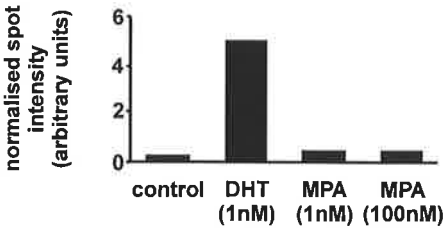
each grouped according to their function. 100nM MPA markedly increased mRNA levels for genes involved in cell proliferation, in particular RCL and mitotic feedback control protein Madp2 homolog, which were increased 41.4 and 30.5-fold respectively. 100nM MPA also strongly inhibited mRNA levels for genes involved in proliferation (such as the breast cancer susceptibility gene BRCA1) and adhesion, motility and invasion (eg the G-protein coupled receptor STRL22). Generally, 100nM MPA induced more significant changes in mRNA levels relative to the control than was observed with 1nM MPA. For example, the highest and lowest MPA(1nM):control ratios were 26.7 (NADH dehydrogenase) and 0.05 (PCM-1) respectively while the highest and lowest MPA(100nM):control ratios were 41.4 (RCL) and 0.043 (STRL22) respectively.

Messenger RNA levels of the majority of genes included in Tables 6.2-6.7 were increased or decreased by either DHT or MPA only, and examples of these genes are shown in Figure 6.5. Messenger RNA levels for myo-inositol monophosphatase 2 and calcineurin B were increased more than 3-fold by DHT but not by MPA (Figure 6.5 (a)). mRNA for ionising radiation resistance conferring protein was increased by 1nM MPA and 100nM MPA, but not by DHT, while adenosine receptor A3 was induced by 100nM MPA only (Figure 6.5 (b)). Conversely, serine/threonine protein kinase receptor R1 precursor and partial C1 mRNA were downregulated to less than 0.33 of the control level by DHT only (Figure 6.5 (c)) while carbonyl reductase and BRCA1 were downregulated by 100nM MPA only (Figure 6.5 (d)). These results suggest that DHT and MPA have the ability to differentially regulate certain genes. Interestingly, two genes identified as being differentially regulated by DHT and MPA showed opposite responses to these ligands relative to the untreated control. Messenger RNA for testis specific protein 1 (a cysteine rich secretory protein) was increased by DHT but repressed by MPA relative to control and mRNA for α-tubulin (a component

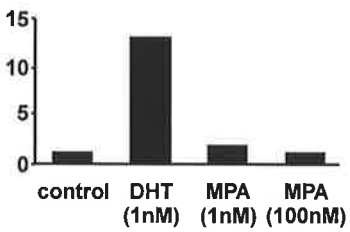
Figure 6.5: Examples of candidate genes showing different responses to DHT and MPA in MDA-MB-453 cells. Arrays were hybridised with probes synthesised from MDA-MB-453 cells treated with 1nM DHT, 1nM MPA, 100nM MPA or vehicle (0.1% ethanol, control) for 4 hours. Intensity of spots on each array was determined using P-SCAN and normalised against the mean intensity of all genes. *(a)* Genes showing at least 3-fold increase in mRNA level in response to DHT only. *(b)* Genes showing at least 3-fold increase in mRNA level in response to MPA only. *(c)* Genes showing less than 0.33-fold lower mRNA level in response to DHT only. *(d)* Genes showing less than 0.33-fold lower mRNA level in response to MPA only. *(e, over page)* Genes divergently regulated by DHT and MPA. *(f, over page)* Genes showing at least 3-fold increase in mRNA level in response to DHT and MPA. *(g, over page)* Genes showing less than 0.33-fold lower mRNA level in response to DHT and MPA.

(a) mRNA increased by DHT only

myo-inositol monophosphatase 2

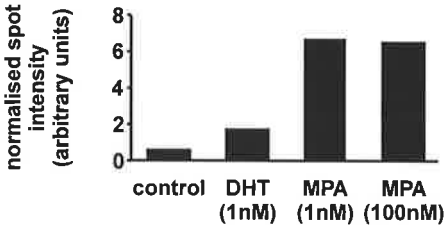


calcineurin B

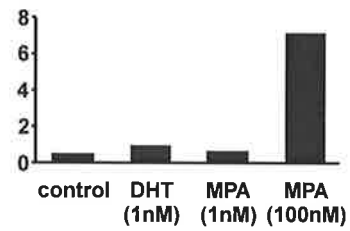


(b) mRNA increased by MPA only

ionizing radiation resistance conferring protein

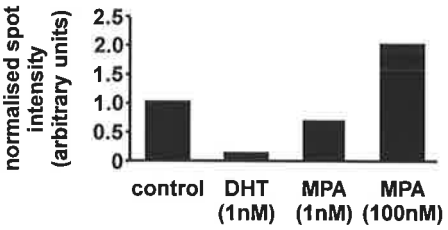


adenosine receptor A3

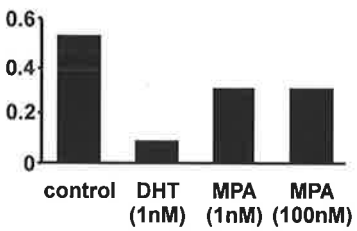


(c) mRNA decreased by DHT only

serine/threonine protein kinase receptor R1 precursor

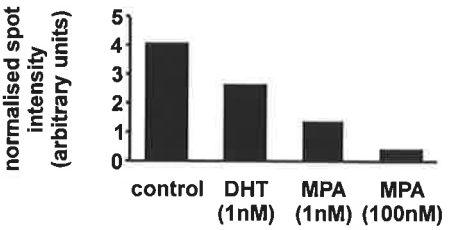


partial C1 mRNA

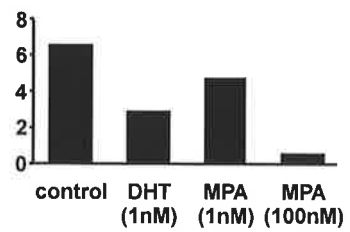


(d) mRNA decreased by MPA only

Carbonyl reductase

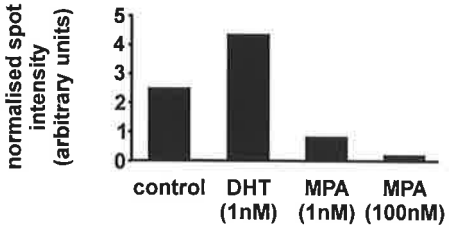


BRCA1

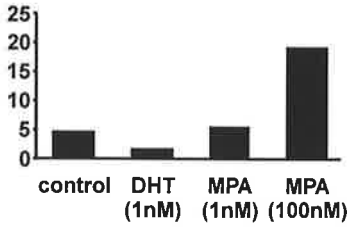


(e) divergent response to DHT and MPA

testis specific protein 1 (probe H4-1 p3-1)

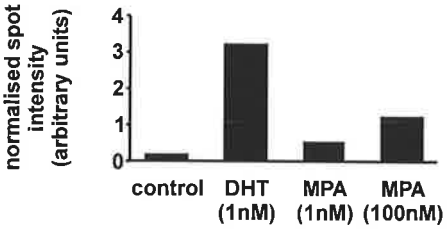


tubulin alpha-4 chain

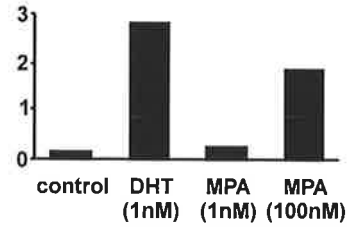


(f) mRNA increased by DHT and MPA

40 kDa protein kinase related to rat ERK2

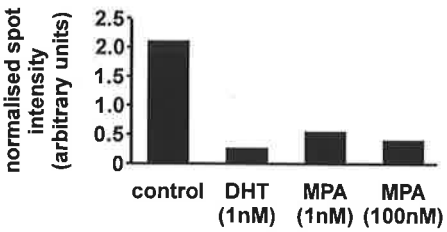


VHL binding protein-1

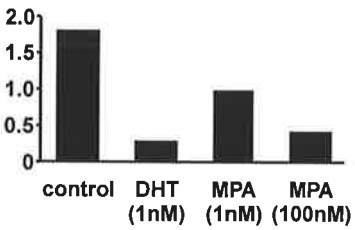


(g) mRNA decreased by DHT and MPA

glucocorticoid receptor alpha



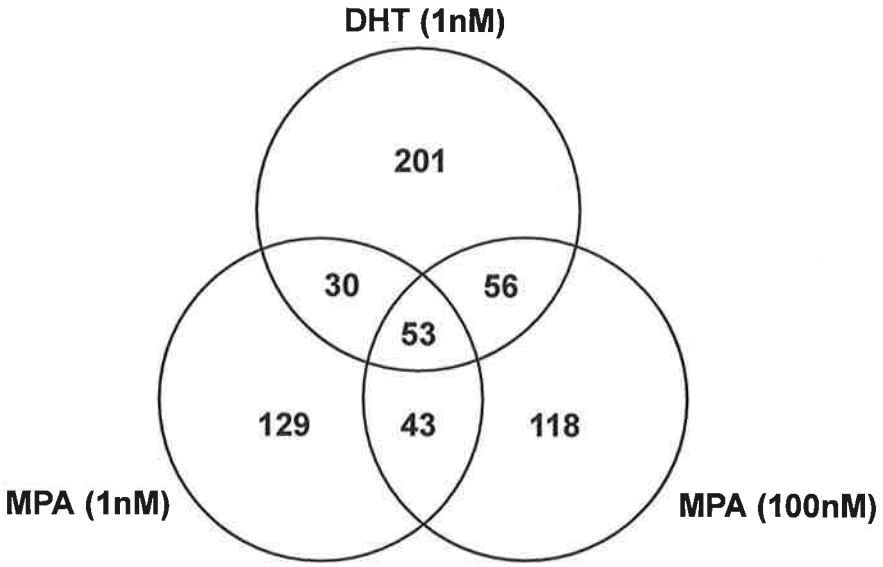
eukaryotic translation initiation factor 4E



of the cytoskeleton) was repressed by DHT and induced by MPA (Figure 6.5 (e)). Testis specific protein 1 is a member of the CRISP family of cysteine rich secretory proteins and other members of this family have been shown to be regulated by androgens in monkey epididymus (Sivashanmugam *et al*, 1999), while α-tubulin is induced by androgens in prostate cancer cells (Gregory *et al*, 1998). These two genes are examples of extreme differential regulation by DHT and MPA and suggest that these ligands can induce divergent effects on the expression of certain genes in MDA-MB-453 cells in addition to their divergent effects on proliferation. A subset of genes were also increased or repressed by both DHT and by MPA. Genes with mRNA levels induced by DHT and MPA include 40 kDa protein kinase related to rat ERK2 and VHL binding protein-1 (Figure 6.5 (f)) as well as GS3955 and deoxyhypusine synthase (data not shown). Genes which were repressed by both DHT and MPA include glucocorticoid receptor α and eukaryotic translation initiation factor 4E (Figure 6.5 (g)) as well as 17α-hydroxylase, PCM-1, SWI/SNF complex 60 KDa subunit (BAF60b), nuclear factor I-B2 and TAR RNA binding protein (data not shown). Interestingly, cDNA for deoxyhypusine synthase was spotted onto the cDNA array at two locations and greater than 3-fold increase in mRNA levels following DHT and MPA treatment, relative to the untreated control, was observed when measuring the intensity of both spots. Consistent detection of differential expression was also observed for other genes which were spotted more than once on the array, such as heat shock protein 90 and lactate dehydrogenase-A and α-centractin. These results are further indication of the robustness of data obtained in these experiments.

A summary of the total numbers of genes up and downregulated by each treatment is illustrated using Venn diagrams in Figure 6.6. Fifty-three genes were upregulated more than 3-fold by 1nM DHT, 1nM MPA and 100nM MPA relative to the control, while 24

(a) Genes upregulated



(b) Genes downregulated

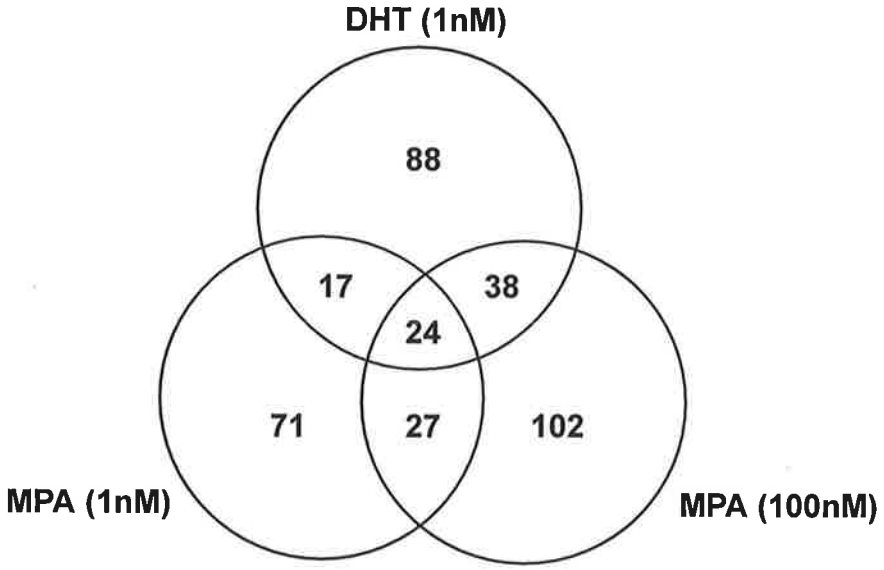


Figure 6.6: Summary of genes induced and repressed by DHT and MPA in MDA-MB-453 breast cancer cells, represented using Venn diagrams. Numbers represent the total numbers of genes in each category. *(a)* Genes with mRNA levels induced greater than 3-fold compared to untreated control by 1nM DHT and/or 1nM MPA and/or 100nM MPA. *(b)* Genes with mRNA levels reduced to less then 0.33-fold by 1nM DHT and/or 1nM MPA and/or 100nM MPA.

genes were downregulated to less than 0.33-fold by all treatments. Whereas some genes were up- or downregulated by two of the three treatments, the majority of genes were modulated by only one of the treatments.

6.3.4 – Candidate genes associated with DHT-mediated stimulation and MPA-mediated inhibition of MDA-MB-453 cell proliferation

The gene expression profiles of MDA-MB-453 cells treated with 1nM DHT and 100nM MPA were directly compared, without comparison to untreated controls, in order to identify genes that are differentially expressed between growth stimulated and growth inhibited MDA-MB-453 cells. A scatter plot comparing global gene expression in MDA-MB-453 cells treated with 1nM DHT and 100nM MPA is shown in Figure 6.7. A total of 112 genes (2.7%) show more than 3-fold greater expression in cells treated with 1nM DHT while 182 genes (4.4%) show more than 3-fold greater expression in cells treated with 100nM MPA. The 25 genes showing the highest DHT:MPA ratio are indicated in Table 6.8 and the genes showing the highest MPA:DHT ratio are indicated in Table 6.9. More genes involved in signal transduction pathways and transcription, cell proliferation and cell cycle regulation and inhibition of apoptosis exhibited increased steady state mRNA levels in cells treated with DHT compared to cells treated with MPA. Of particular interest is the higher mRNA levels of three oncogenes (v-ski, v-myb and v-raf) in DHT treated cells compared to MPA treated cells (Table 6.8). In contrast, fewer genes involved in proliferation, apoptosis and adhesion were increased in cells treated with MPA, however a greater number of cytoskeleton/structural genes, such as keratin 10 and myosin class I, were increased in cells treated with 100nM MPA compared to 1nM DHT.

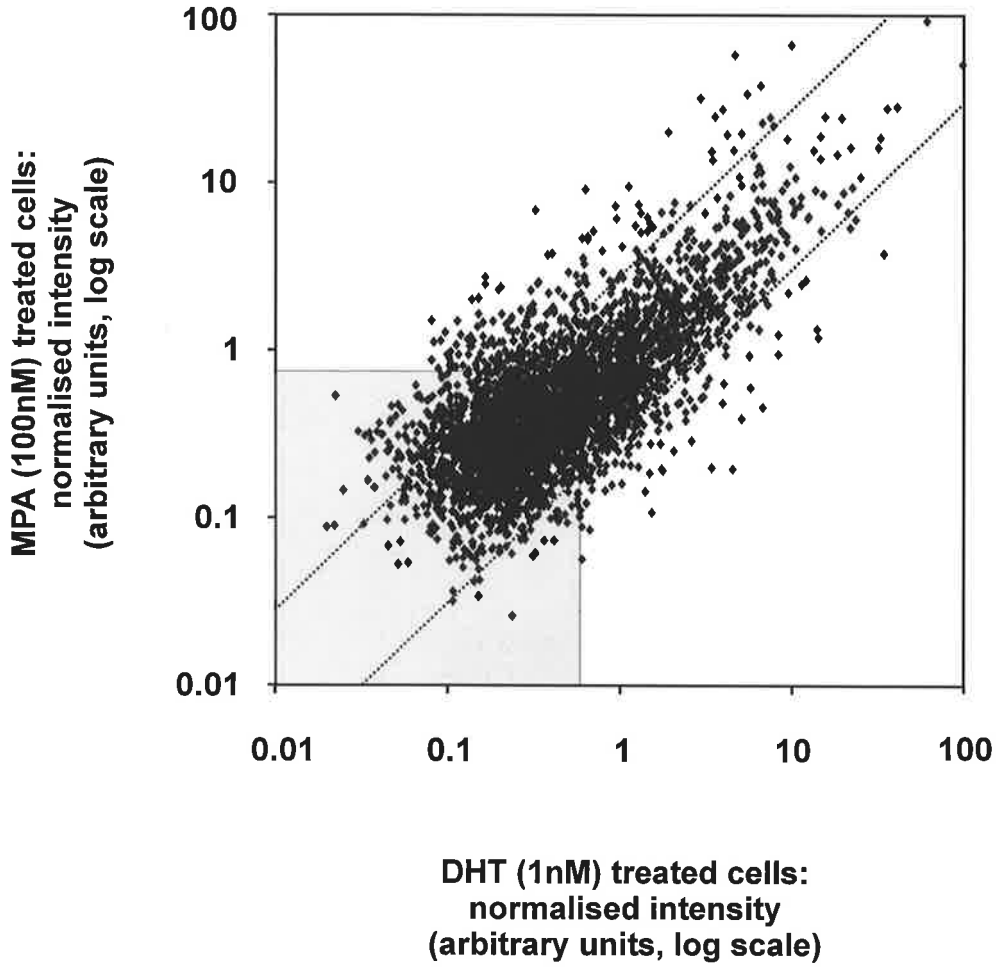


Figure 6.7: Comparison of gene expression in MDA-MB-453 breast cancer cells treated with 1nM DHT or 100nM MPA for 4hrs, visualised using a scatter plot. Intensity of spots on each array was determined using P-SCAN and normalised against the mean intensity of all genes. One point for each gene was plotted on the scatter plot. Dashed lines represent threefold limits: points lying above the upper line represent genes with mRNA levels at least 3-fold higher in MPA treated cells compared to DHT treated cells while points lying below the lower line represent genes with mRNA levels at least 3-fold higher in DHT treated cells compared to MPA treated cells. The grey area covers spots that were not greater than two standard deviations above the mean background intensity in both hybridisations.

Table 6.8: Candidate genes showing the highest DHT(1nM):MPA(100nM) ratios[§] in MDA-MB-453 cells.

Gene	Accession number	DHT(1nM):MPA(100nM)
Proliferation, cell cycle regulation		
V-ski avian sarcoma viral oncogene homolog	T50498	16.76
Protein tyrosine kinase (Syk)	R59598	5.69
Breast cancer 1, early onset (BRCA1)	H90415	5.77
Histone deacetylase 3 (HDAC3)	H79779	5.53
V-myb avian myeloblastosis viral oncogene homolog-like 2	AA457034	5.52
Apoptosis		
V-raf murine sarcoma viral oncogene homolog B1	W88566	13.72
Histone deacetylase 3 (HDAC3)	H79779	5.53
V-myb avian myeloblastosis viral oncogene homolog-like 2	AA457034	5.52
Differentiation		
HS1 binding protein HAX-1 mRNA, nuclear gene encoding mitochondrial protein	R76263	14.32
Cell adhesion, motility and invasion		
Matrix metalloproteinase 1 (interstitial collagenase)	AA143331	9.43
G protein-coupled receptor (STRL22)	N57964	8.07
Matrix metalloproteinase 12 (macrophage elastase)	R92994	7.78
DNA repair		
Breast cancer 1, early onset (BRCA1)	H90415	5.77
Signal transduction pathways, transcription and translation		
V-ski avian sarcoma viral oncogene homolog	T50498	16.76
V-raf murine sarcoma viral oncogene homolog B1	W88566	13.72
Myo-inositol monophosphatase 2	R42685	12.74
AP-3 complex delta subunit	AA630776	9.35
G protein-coupled receptor (STRL22)	N57964	8.07
Mannosyl(beta-1,4-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase	H47026	7.93
Phosphatidylinositol 4-kinase	R40460	6.55
Platelet-type phosphofructokinase	R38433	6.13
IL-13Rα	AA411324	6.12
Breast cancer 1, early onset	H90415	5.77
Protein tyrosine kinase (Syk)	R59598	5.69
Histone deacetylase 3 (HDAC3)	H79779	5.53
V-myb avian myeloblastosis viral oncogene homolog-like 2	AA457034	5.52
Carbohydrate, lipid, steroid or protein synthesis/metabolism		
Carbonyl reductase	AA280846	8.73
Glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2)	AA487521	8.55
N-acetylglucosaminyltransferase I	AA775378	6.16
Platelet-type phosphofructokinase	R38433	6.13
Cytoskeleton/structure		
Glial fibrillary acidic protein	AA069414	10.23
Tropomyosin alpha chain (skeletal muscle)	W58092	8.65
Other		
Testis specific protein 1 (probe H4-1 p3-1)	AA868278	22.13
Calcineurin B	AA457092	11.52
Immunoglobulin-related 14.1 protein precursor	W73790	8.83
Signal recognition particle 9 kD protein	R43360	8.77

[§] 25 from a total of 112 genes that showed a DHT(1nM):MPA(100nM) ratio greater than 3.0. Genes are grouped based on function, with some genes listed under more than one category.

Table 6.9: Candidate genes showing the highest MPA(100nM):DHT(1nM) ratios[§§] in MDA-MB-453 cells. .

Gene	Accession number	100nM MPA:1nM DHT
Proliferation, cell cycle regulation		
Tax1-binding protein TXBP181	AA718910	13.5
Cyclin-dependent kinase 6	H73724	10.9
MADER	X70991	9.8
Apoptosis		
Prostaglandin E receptor 3 (subtype EP3)	AA406362	16.6
Differentiation		
Keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris)	AA479882	15.1
Cell adhesion, motility and invasion		
Osteonidogen	AA479199	11.1
DNA repair		
DNA repair protein XRCC4	R39148	11.8
Signal transduction pathways, transcription and translation		
Prostaglandin E receptor 3 (subtype EP3)	AA406362	16.6
Serine/threonine-protein kinase receptor R1 precursor	AA136882	13.7
Rab11	AA025059	13.5
Orphan G protein-coupled receptor (RDC1)	N53172	12.4
Cyclin-dependent kinase 6	H73724	10.9
Regulator of G protein signalling 10	AA709036	9.6
Glutathione S-transferase theta 1	H99813	9.3
Carbohydrate, lipid, steroid or protein synthesis/metabolism		
HU-K4	U60644	18.8
Dihydrofolate reductase	R00884	12.8
60S ribosomal protein L18	AA775874	11.1
Carbonic anhydrase IV	AA855158	9.6
Cytoskeleton/structure		
Keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris)	AA479882	15.1
Myosin class I, myh-1c	AA047778	14.6
Rab11	AA025059	13.5
Tubulin alpha-4 chain	AA180742	10.5
Myosin-IC	AA029956	9.7
Other		
SMT3B protein	AA775415	21.3
Gamma crystallin A	AA780079	14.6
T-cell surface glycoprotein CD8 beta.3 chain precursor	AA293671	13.3
p76	AA479100	11.8
ATP-binding cassette protein mRNA 06B09 clone	R83876	10.5
Annexin IV (placental anticoagulant protein II)	AA419015	9.6

[§§] 25 from a total of 182 genes that showed a MPA(100nM):DHT(1nM) ratio greater than 3.0. Genes are grouped based on function, with some genes listed under more than one category.

6.4 – Discussion

This study has been the first to use cDNA microarrays to investigate expression profiles of AR target genes in breast cancer cells. The findings of the microarray study suggest that DHT and MPA can regulate different sets of target genes in the MDA-MB-453 breast cancer cell line, providing a potential mechanism for the observed divergent proliferative effects induced by these ligands on MDA-MB-453 cells. Furthermore, as MDA-MB-453 cells express AR without detectable levels of ER and PR, and the proliferative effects of DHT and MPA are mediated by the AR in this cell line (Birrell *et al*, 1995a; Bentel *et al*, 1999), the genes identified are likely candidates for direct regulation by the AR in breast cancer cells.

Comparison of gene expression profiles between DHT or MPA treated MDA-MB-453 cells and vehicle treated control cells enabled identification of candidate AR target genes. This included a number of previously defined androgen regulated genes as well as many novel androgen regulated candidate genes. The majority of genes that have previously been identified as androgen regulated in breast cancer cells are stimulated by androgens. However in the current experiments, comparable numbers of genes showed mRNA levels either greater than 3-fold or less than 0.33-fold in treated cells relative to untreated controls, suggesting that, in addition to stimulation, repression of gene expression is a potentially important aspect of AR action (at least in MDA-MB-453 cells). While many studies have investigated the mechanisms of AR-induced gene expression, using either endogenous or transfected reporter genes, the mechanisms associated with repression of gene expression by the AR are less well defined. Further studies to better define the mechanism of AR repression of gene expression are necessary considering that the AR appears to downregulate a substantial number of

genes with potential roles in cell proliferation, differentiation and invasion, which may facilitate breast cancer growth.

A number of previously identified androgen regulated genes were included on the cDNA arrays used in the current study. Messenger RNA levels for GCDFP-24, fatty acid synthase and prolactin receptor were each upregulated by DHT and/or MPA in MDA-MB-453 cells, which is in agreement with previous studies showing that they are induced in response to androgens in breast cancer cells (Simard *et al*, 1990; Ormandy *et al*, 1992; Simard *et al*, 1992; Haagensen *et al*, 1992; Hall *et al*, 1994; Lapointe *et al*, 1999). Tpx1, a component of the sperm tail, is a member of the cysteine-rich secretory protein (CRISP) family which are induced in castrated monkeys in response to testosterone (Sivashanmugam *et al*, 1999). Consistent with this observation, Tpx1 mRNA levels were stimulated by 1nM DHT, although only slightly, while 100nM MPA markedly repressed Tpx1. The expression of this gene in MDA-MB-453 cells therefore reflects the proliferative response to DHT and MPA, and may be significant in the regulation of cell proliferation. Conversely, α-tubulin, a critical regulator of microtubule polymerisation and cytoskeleton function, was downregulated by DHT but upregulated by MPA. The repression of α-tubulin by DHT in MDA-MB-453 breast cancer cells contrasts with the stimulation observed in response to testosterone in the CWR22 prostate cancer model (Lin *et al*, 2000a). IGFBP3 and IGFBP5 have roles in regulating apoptosis. In this study, IGFBP5 mRNA levels were reduced by DHT and MPA, an observation that is in agreement with previous studies demonstrating that IGFBP5 is induced following androgen ablation in the rat ventral prostate (Gregory *et al*, 1998; Nickerson *et al*, 1998; Gregory *et al*, 1999; Nickerson and Pollak, 1999; Bruyninx *et al*, 2000) and decreased in response to growth inhibitory doses of MPA in T-47D breast cancer cells (Coutts *et al*, 1994).

In contrast, IGFBP3 mRNA levels were induced by DHT, which is contrary to the increase observed in rat ventral prostate following androgen ablation (Nickerson *et al*, 1998; Bruyninx *et al*, 2000). MMP-1, which can positively regulate cell invasion and degradation of the extracellular matrix, was upregulated by DHT in MDA-MB-453 cells which also conflicts with the observed repression of MMP-1 expression by DHT in LNCaP prostate cancer cells (Schneikert *et al*, 1996). The apparent differential regulation of α-tubulin, IGFBP3 and MMP-1 expression in breast and prostate cell lines may be explained by tissue specific variations in cofactor molecules required for regulation of these genes by the AR.

While DHT and MPA each had qualitatively similar effects on the mRNA levels of a subset of genes, the majority of candidates were differentially regulated by these treatments. Examination of the genes which are differentially expressed between MDA-MB-453 cells treated with 1nM DHT and 100nM MPA revealed groups of genes with particular functions that may mediate the divergent proliferative effects of these ligands. Interestingly, cells treated with 1nM DHT showed relatively higher steady state mRNA levels for more genes associated with cell proliferation and the inhibition of apoptosis than cells treated with 100nM MPA. These observations strengthen the hypothesis that DHT and MPA can differentially regulate genes that mediate their opposing proliferative effects *in vitro*.

A number of genes with roles in cell proliferation, apoptosis, cell adhesion, mobility and invasion, DNA repair and differentiation were modulated by DHT and/or MPA in MDA-MB-453 cells. These genes are novel candidates for regulation by the AR in breast cancer cells and may potentially influence tumour development, growth and progression *in vit*ro and *in vivo*. One particularly interesting candidate gene is the breast

cancer susceptibility gene BRCA1, which was differentially downregulated by 100nM MPA but not by 1nM MPA or 1nM DHT. Inherited mutations in *BRCA1*, a tumour suppressor with established roles in DNA repair, transcriptional activation and regulation of the cell cycle (see Section 7.1), predispose to breast and ovarian cancer development (Hall *et al*, 1990; Miki *et al*, 1994) (see Section 1.1.2.1). As BRCA1 has been clearly established as a critical regulator of breast cancer, and regulation of BRCA1 expression by androgen signalling pathways has not previously been reported, BRCA1 expression in response to DHT and MPA was studied in further detail in Chapter 7. Other candidate AR target genes identified in this study are also worthy of further investigation. The v-ski, v-myc and v-raf oncogenes (which each showed greater mRNA levels in DHT compared to MPA treated cells), the protein kinase ERK2 (stimulated by DHT) and the mitotic feedback control protein Madp2 homolog (which may protect against malignancy and is stimulated by MPA) each exhibited differential regulation in response to DHT and MPA and may therefore be important in mediating the divergent proliferative effects of these ligands in MDA-MB-453 cells. Furthermore, two genes (testis specific protein 1 and α-tubulin) identified as differentially regulated showed a divergent response to DHT and MPA compared to untreated cells. Although the function of these genes in regulating cell proliferation is unknown, mRNA levels for testis specific protein 1 correlated with the proliferative effects of DHT and MPA while mRNA levels for α-tubulin were inversely correlated with proliferation, suggesting that they may be important in mediating the divergent proliferative effects of DHT and MPA in MDA-MB-453 cells.

Results from previous studies may potentially explain how DHT and MPA can differentially regulate the expression of target genes through the AR. DHT and MPA differ in their ability to induce an N/C interaction of the AR (Kemppainen *et al*, 1999

and Section 5.3.3.4), suggesting that specific conformations of DHT-AR and MPA-AR complexes lead to fundamental differences in the mechanisms by which these ligands regulate AR activity. Ligand-specific structural conformations of the AR may firstly be able to recognise AREs in a sequence-specific manner. This hypothesis is supported by results from Section 5.3.4.3, where DHT and MPA differed in their ability to activate AR activity at the probasin and PSA promoters in MDA-MB-453 cells. Similarly, previous studies have demonstrated that DHT and testosterone can differentially regulate expression of certain genes, such as TDD5, FAR17a, interleukin-4, interleukin-5 and γ-interferon (Araneo *et al*, 1991; Hisaoka *et al*, 1991; Seki *et al*, 1991; Lin and Chang, 1997). The differential regulation of gene expression by testosterone and DHT may occur through sequence-specific recognition of AREs by testosterone-AR and DHT-AR complexes, and ARE sequences conferring quantitatively different levels of AR activation by testosterone and DHT have been identified (Hsiao *et al*, 2000). These studies suggest that ARE sequence is important in conferring ligand specificity and may be one possible mechanism by which the AR can regulate the expression of different genes in response to DHT and MPA. Secondly, conformation of the AR induced by DHT and MPA may differentially modulate interactions between the AR and other critical cofactors or transcription factors necessary for AR activity. This hypothesis is supported, at least in MDA-MB-453 cells, by experiments performed in our laboratory showing that DHT and MPA exhibit marked differences in their ability to induce an interaction between the AR-Q865H variant and the corepressor SMRT (Buchanan, 2002).

Interestingly, 1nM MPA and 100nM MPA differentially regulated the expression of many genes. Although both concentrations of MPA activate transcription of the MMTV-CAT androgen responsive reporter gene, 100nM MPA inhibits proliferation of

MDA-MB-453 cells, while 1nM MPA has no proliferative effect (Bentel *et al*, 1999). In the current experiments, 100nM MPA resulted in differential expression of more genes than was observed with 1nM MPA. Furthermore, many genes, such as those indicated in Figure 6.5, showed a dose dependent change in mRNA levels in 1nM and 100nM MPA treated cells relative to the controls. However, there were also qualitative differences between the genes induced and repressed by these concentrations of MPA, which suggests that 1nM MPA and 100nM MPA may have considerably different mechanisms of action in MDA-MB-453 cells. This may be related to the dose-dependent induction of the N/C interaction by MPA. Results from Section 5.3.3.4 show that 100nM MPA induces a detectable AR N/C interaction (although this is significantly weaker than the interaction induced by the strong AR agonist DHT) while 1nM MPA does not. This suggests that, at a concentration of 100nM, MPA acts in an agonist manner and induces an N/C interaction which allows the AR to regulate transcription of target genes. In contrast, at a concentration of 1nM, MPA may instead regulate gene expression via an alternative mechanism that does not require an AR N/C interaction.

Several protein kinases, transcription factors and components of intracellular signalling cascades were also identified as candidate AR target genes in these studies. This includes some genes which are involved in steroid hormone signalling pathways, such as FKBP54 (a progesterone receptor associated immunophilin which, along with heat shock proteins and p23 forms part of the receptor maturation complex, see Section 1.3.2), glucocorticoid receptor-α and estrogen related receptor 1 (ERR1) as well as 17α-hydroxylase and 21-hydroxylase (which catalyse reactions in steroid hormone biosynthesis pathways, see Figure 1.1 (a)). These results suggest that the AR can control gene expression directly or indirectly via regulating the expression of a complex

array of transcription factors and signalling molecules which can themselves regulate gene expression.

Further studies are required to confirm the differential expression of candidate AR target genes identified in this study. This may be accomplished by measuring mRNA levels for these genes using either RNase protection assays or real time RT-PCR, in combination with immunoblotting experiments to demonstrate that the modulation of mRNA levels of individual genes is associated with a corresponding change in protein level. The use of tissue microarrays will also potentially provide valuable information on the expression of these genes in breast cancers of various phenotypes and stages of progression. Furthermore, as the current cDNA array experiments have only detected changes in steady-state mRNA levels, studies using the mRNA synthesis inhibitor actinomycin D will indicate whether DHT or MPA modulate specific genes by affecting mRNA stability while the direct effect of these ligands on gene transcription could be investigated using nuclear run on assays. Although the candidate genes identified are likely to be regulated by the AR, additional experiments using AR antagonists are required to confirm this while experiments using protein synthesis inhibitors, in combination with chromatin immunoprecipitation and/or DNA mobility shift assays, will determine whether the AR directly regulates these genes via binding to regulatory elements in the promoter or whether other regulatory factors are required. Finally, over expression of candidate genes, or inhibition of their expression using either short interfering RNA (siRNA) or antisense oligonucleotides, are required to determine the effect of these genes on cell proliferation, and importantly, to determine whether specific candidate genes can mediate the divergent proliferative effects of DHT and MPA on MDA-MB-453 breast cancer cells.

The experiments described in this chapter have been the first to examine expression of large numbers of genes in response to the potent androgen, DHT, and the synthetic progestin, MPA, in breast cancer cells. The results suggest that DHT and MPA can regulate different groups of target genes, which provides a potential mechanism for how these ligands induce divergent proliferative effects on MDA-MB-453 cells. These experiments have identified a number of previously defined and novel candidate AR target genes that are regulated by DHT and MPA in breast cancer cells. Definition of the mechanisms by which the AR regulates expression of these genes in response to DHT and MPA, and the functional effects of these genes, is critical for a greater understanding of androgen action in breast cancer cells. In the following chapter, regulation of the expression of one candidate gene identified in these cDNA array studies, BRCA1, in response to DHT and MPA has been further characterised and the potential implications for alterations in BRCA1 expression on cell proliferation are also discussed. BRCA1 was selected for further investigation due to its known role in breast tumourigenesis. Furthermore, because its expression was differentially regulated by DHT and MPA in MDA-MB-453 cells, it has the potential to be important in mediating the divergent proliferative effects of these two ligands.

CHAPTER 7

REGULATION OF BRCA1 EXPRESSION

7.1 – Introduction

Results from the cDNA array analysis presented in Chapter 6 suggest that the breast cancer susceptibility gene, BRCA1, is regulated by the AR in MDA-MB-453 cells. Interestingly, BRCA1 mRNA levels were not modulated by DHT but were markedly downregulated by high doses of MPA (Figure 7.1), consistent with the dose required for inhibition of proliferation of MDA-MB-453 cells (Bentel *et al*, 1999). The mechanisms regulating expression of BRCA1 have been extensively studied however there are currently no reports demonstrating modulation of BRCA1 expression by androgen signalling pathways. Given the critical role that androgens and BRCA1 play in the pathogenesis of breast cancer, characterisation of their functional associations may enable a greater understanding of the mechanisms by which androgens exert their proliferative effects.

Inherited mutations in the *BRCA1* gene, which is located on chromosome 17q21, are associated with approximately 45% of familial breast cancers (Miki *et al*, 1994; Rosen *et al*, 2003), and the risk of developing breast cancer by age 70 is estimated at 85-90% for women carrying *BRCA1* mutations (Ford *et al*, 1994; Wooster *et al*, 1995; Easton *et al*, 1995). The majority of *BRCA1* mutations are frameshift mutations, which result in a truncated protein, or point mutations in critical functional domains such as the amino terminal ring domain or the carboxy terminal transcription activation domain (Kennedy *et al*, 2002; Rosen *et al*, 2003). Wild type BRCA1, a 220kDa phosphoprotein, is a tumour suppressor and loss of function through mutation leads to breakdown in processes that maintain cell integrity, such as transcription regulation and DNA repair (Gowen *et al*, 1998; Irminger-Finger *et al*, 1999; Welcsh *et al*, 2000; Monteiro, 2000; Scully and Livingston, 2000; Hilakivi-Clarke, 2000; Venkitaraman, 2002; Rosen *et al*,

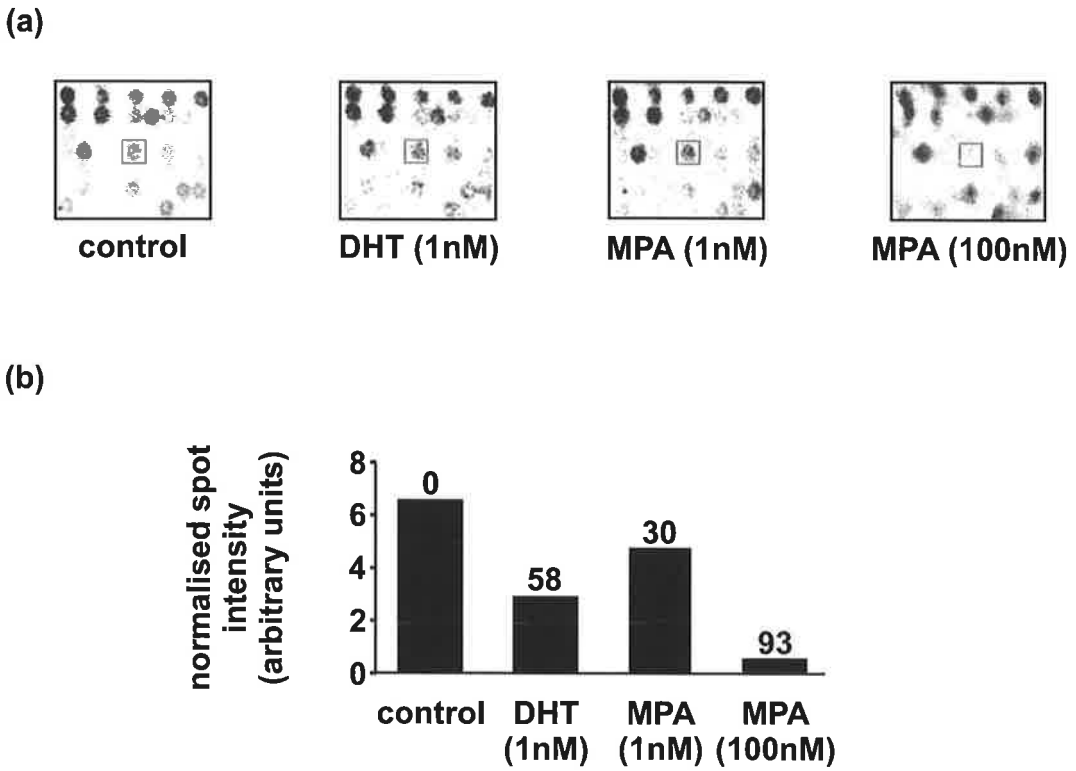


Figure 7.1: BRCA1 mRNA levels in MDA-MB-453 breast cancer cells, analysed by cDNA microarray analysis. Arrays were hybridised with probes synthesised from MDA-MB-453 cells treated with 1nM DHT, 1nM MPA, 100nM MPA or vehicle (0.1% ethanol, control) for 4 hours. *(a)* Regions of cDNA microarray images corresponding to BRCA1. The relevant spots on the cDNA array are surrounded by coloured boxes. *(b)* The normalised spot intensity for BRCA1 on each array is represented graphically. Numbers above bars represent BRCA1 mRNA levels expressed as the percent reduction compared to untreated control.

2003). BRCA1 also has roles in the regulation of cell proliferation, the heat shock response, ubiquitination and apoptosis (Shao *et al*, 1996; Ruffner *et al*, 2001; Venkitaraman, 2002; Rosen *et al*, 2003; Xian *et al*, 2003).

While *BRCA1* mutations are associated with hereditary breast cancer, mutations are rare in sporadic cancers (Futreal *et al*, 1994; Papa *et al*, 1998; van der Looij *et al*, 2000). Instead, alterations in BRCA1 expression levels may contribute to sporadic tumourigenesis, as levels of BRCA1 mRNA and protein are often reduced in sporadic tumours compared to normal breast epithelium (Magdinier *et al*, 1998; Taylor *et al*, 1998; Ozcelik *et al*, 1998; Wilson *et al*, 1999; Yoshikawa *et al*, 1999; Lee *et al*, 1999b; Yang *et al*, 2001). Decreased BRCA1 expression has been reported upon progression from *in situ* to invasive breast cancer (Thompson *et al*, 1995) and low levels of BRCA1 expression have been associated with distant metastases (Seery *et al*, 1999). This decrease in expression can arise from hypermethylation of the BRCA1 promoter (Dobrovic and Simpfendorfer, 1997; Rice *et al*, 1998; Mancini *et al*, 1998; Catteau *et al*, 1999; Rice *et al*, 2000; Esteller *et al*, 2000; Magdinier *et al*, 2000) or in response to various agents that have been reported to alter BRCA1 expression. BRCA1 expression is induced in breast cancer cells or non-malignant breast epithelial cells in response to the chemopreventive agent indole-3-carbinol (Meng *et al*, 2000a; Meng *et al*, 2000b), 1α,25-dihydroxyvitamin D_3 (Campbell *et al*, 2000), prolactin (Rajan *et al*, 1996; Favy *et al*, 1999), progesterone (Gudas *et al*, 1995; Rajan *et al*, 1997) and ethanol (Fan *et al*, 2000). Downregulation of BRCA1 expression has been observed in breast cancer cells in response to DNA damaging agents such as UV light, adriamycin and camptothecin (Andres *et al*, 1998), the carcinogen benzo(a)pyrene (Jeffy *et al*, 1999; Jeffy *et al*, 2002) and in non-malignant breast epithelial cells in response to transforming growth factor-β (Gudas *et al*, 1996).

BRCA1 closely interacts with estrogen signalling pathways in breast cancer cells. This is believed to be one reason why germline mutations in *BRCA1* are almost exclusively associated with hormone dependent cancers such as breast (and ovarian) cancer. BRCA1 expression is induced by estrogens in the breast cancer cell lines MCF-7, ZR-75-1 and T-47D and in mammary epithelial cells of ovariectomised mice, and experiments performed with the anti-estrogens OHT and ICI 182780 suggest that this effect is mediated via a mechanism involving the ER (Gudas *et al*, 1995; Marquis *et al*, 1995; Spillman and Bowcock, 1996; Rajan *et al*, 1997; Marks *et al*, 1997; Romagnolo *et al*, 1998; Chambon *et al*, 2003). This induction does not appear to be directly mediated by binding of the activated ER to DNA, as promoter regions of BRCA1 that contain putative EREs are not responsive to E_2 in reporter gene assays performed in breast cancer cell lines (Marks *et al*, 1997). Abolition of BRCA1 induction by E_2 with the protein synthesis inhibitor cycloheximide (Spillman and Bowcock, 1996; Marks *et al*, 1997), and the delayed response compared to the directly estrogen regulated gene pS2 (Gudas *et al*, 1995; Romagnolo *et al*, 1998), further support an indirect response of BRCA1 to E_2 requiring the action of intermediary factors. Further evidence for a role for BRCA1 in estrogen signalling pathways stems from studies showing that BRCA1 can modulate the activity of ERα. BRCA1 represses the activity of ERα via a direct interaction between the LBD of ERα and the amino terminus of BRCA1 (Fan *et al*, 1999; Zheng *et al*, 2001; Fan *et al*, 2001a). Additionally, it has been recently shown that this inhibitory effect of BRCA1 is mediated by the ERα coactivators, CBP and p300 (Fan *et al*, 2002). It is proposed that this repression of ERα activity restricts the stimulatory effects of estrogens on proliferation of cells expressing wild type BRCA1.

There are two lines of evidence that suggest a close association between BRCA1 and androgen signalling pathways. First, BRCA1 enhances DHT-dependent AR

transcriptional activity and can synergistically increase AR coactivation by GRIP1, SRC1a, AIB1, ARA70, ARA55 and CBP (Yeh *et al*, 2000; Park *et al*, 2000b). These effects are mediated via direct interactions between two contact sites in the carboxy terminal of BRCA1 (amino acids 758-1064 and 1314-1863) and sites in the amino (specifically AF1) and carboxy terminal regions of the AR (Yeh *et al*, 2000; Park *et al*, 2000b). BRCA1 also enhances DHT-dependent stimulation of $p21^{(WAF1/CIP1)}$ (Yeh *et al*, 2000), a gene associated with the induction of apoptosis (Sekiguchi and Hunter, 1998; Fotedar *et al*, 1999). This also provides a potential link between androgen signalling pathways, BRCA1 and mechanisms regulating cell survival. Second, the penetrance of inherited mutations in *BRCA1* may be modulated by the length of the polymorphic AR polyQ tract (Rebbeck *et al*, 1999). This study demonstrated that women carrying *BRCA1* mutations are at increased risk of developing breast cancer, and at an earlier age, if they have at least one AR allele with a polyQ length greater than 28 repeats compared to women with shorter polyQ alleles. Furthermore, all women in the cohort with one AR allele of at least 29 repeats developed breast cancer. The reduced activity of ARs with increasing polyQ lengths (Chamberlain *et al*, 1994; Kazemi-Esfarjani *et al*, 1995; Tut *et al*, 1997; Beilin *et al*, 2000), together with the reduction in AR activity, or loss of coactivation, in the absence of wild type BRCA1 (Yeh *et al*, 2000), indicates that the risk of breast cancer development in *BRCA1* mutation carriers is enhanced when the protective effects of androgen signalling pathways are lost.

These studies support a critical role for BRCA1 in androgen and estrogen signalling pathways in breast cancer cells. Whereas the expression of BRCA1 in response to estrogens has been well characterised in previous studies, little is known about the effect of androgens on BRCA1 expression. Further characterisation of the mechanisms regulating BRCA1 expression may lead to a better understanding of the role it plays in

breast tumourigenesis, particularly within the context of androgen signalling. Results from cDNA array analysis presented in Chapter 6 provided preliminary evidence that the androgen signalling pathway can modulate BRCA1 expression. The aims of this chapter were therefore to further investigate the expression of BRCA1 mRNA in response to DHT and MPA in breast cancer cells. The specific role of the AR in mediating the effects of DHT and MPA on BRCA1 expression was also examined using the AR antagonist bicalutamide.

7.2 – Methods

7.2.1 – Quantitation of BRCA1 mRNA levels by RNase protection assay

In order to confirm the downregulation of BRCA1 mRNA levels by 100nM MPA that was observed in the cDNA array study, RNase protection assays were performed on RNA samples previously extracted from MDA-MB-453 cells treated with 1nM DHT, 1nM MPA, 100nM MPA or vehicle (0.1% ethanol) for either 4hrs or 24hrs (as described in Sections 6.2.1 and 6.2.2). These assays were performed using digoxygenin-labelled RNA probes, as has previously been used in our laboratory (Sakko, 2001).

7.2.1.1 – Preparation of DIG-labelled riboprobes

Total RNA was reverse transcribed using Superscript II reverse transcriptase as described in Section 2.3.10. Probe templates were generated by PCR (Section 2.3.11), with BRCA1 amplified using the BRCA1 sense and BRCA1 antisense + SP6 primers and 18S rRNA amplified using the 18S rRNA sense and 18S rRNA antisense + SP6 primers (Appendix 2). SP6 promoter sites were linked to the 5' end of antisense primers to enable generation of antisense riboprobes directly from the PCR product

without the need for cloning. Following amplification, probe templates were purified using the QIAquick PCR purification kit and an aliquot was submitted for automated DNA sequencing to confirm the specificity of each product. Because the 18S rRNA primers generated a product of similar size to BRCA1, the size of the 18S rRNA probe template was reduced by restriction enzyme digestion. 200µL purified 18S rRNA PCR product was incubated in a 300µL reaction with 8U Nar I in the presence of the appropriate buffer at 37°C overnight, yielding a 360bp fragment retaining the SP6 promoter site. The digestion reaction was electrophoresed on a 2% agarose-TAE gel and the 360bp product was excised and purified using the QIAquick gel extraction kit.

Digoxygenin (DIG)-labelled RNA probes were synthesised in *in vitro* transcription reactions from 100-200ng of the PCR product template using the DIG RNA labelling kit (Roche) according to the manufacturer's instructions. RNA probes of the appropriate size (BRCA1: 448bp, 18S rRNA: 360bp) were separated from shorter, degraded or incomplete fragments by electrophoresis on a 6% denaturing PAGE gel in 1x TBE buffer, as described in the RPA II kit (Ambion). The gel was then stained in 0.5µg/mL ethidium bromide (diluted in DEPC-H_2O) for 10mins and RNA bands were visualised under 365nm UV light. Full length RNA probes were excised from the gel with a sterile scalpel blade and RNA was eluted by soaking the gel slice in DEPC-H_2O for 5mins and overnight incubation at 37°C in 350µL probe elution buffer (from the RPA II kit). RNasin (100U) was added and probes were stored in aliquots at -70°C.

The yield of DIG-labelled RNA was estimated by comparison to a control riboprobe of known concentration, as described in the DIG RNA labelling kit. Control and test probes were serially diluted in RNA dilution buffer and 1µL of each dilution was spotted on to a strip of nylon membrane. RNA was fixed to the nylon by crosslinking in

a Stratalinker 1800 UV cross linker and DIG-labelled RNA was detected as described below (Section 7.2.1.2).

7.2.1.2 – Detection of DIG-labelled RNA

Digoxygenin-labelled RNA was detected using an anti-digoxygenin-AP antibody and reagents from the DIG Wash and Block buffer set. The membrane was washed in 1x washing buffer for 2mins with gentle shaking followed by a 30min incubation in 1x blocking solution. The membrane was probed with anti-digoxygenin-AP antibody (diluted 1/10000 in 1x blocking solution) for 30mins at room temperature then incubated twice in 1x washing buffer for 15mins and once in 1x detection buffer for 5mins. Signal was detected by incubating the membrane with disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro) tricyclo $\{3.3.1.1^{3,7}]$decan}-4-yl) phenyl phosphate (CSPD, diluted 1/100 in 1x detection buffer) for 5mins at room temperature in the dark, followed by removal of CSPD and further incubation at 37°C for 10mins in the dark. The membrane was exposed to Hyperfilm™ ECL™ for 15mins and films were developed manually as described in Section 2.3.14.

7.2.1.3 – RNase protection assay

DIG-labelled probes were used with the RPA II kit (Ambion), as described in the manufacturer's protocol. Briefly, 5μg each total RNA sample was combined with 1μL purified DIG-labelled 18S rRNA probe (diluted 1/50) and 3μL purified DIG-labelled BRCA1 probe. Probes and RNAs were co-precipitated by addition of 2μL 5M ammonium acetate and 50μL 100% ethanol and incubation at -20°C for 15mins. Tubes were centrifuged at 13000rpm for 15mins at 4°C and pellets were air-dried for 5mins at room temperature followed by resuspension in 20μL hybridisation buffer. Tubes were vortexed for 10secs then RNA was denatured at 95°C for 5mins and

allowed to hybridise at 42°C overnight. Single stranded RNA was digested with 200µL RNase A / RNase T1 (diluted 1/50 in RNase digestion buffer) at 37°C for 1hr. RNA was combined with 300µL RNase inactivation/precipitation solution and 2.2µL GlycoBlue and precipitated at -20°C for 30mins. After centrifugation at 13000rpm for 15mins at 4°C, pellets were resuspended in 8µL gel loading buffer and tubes were heated at 95°C for 5mins. Four µL of each reaction was electrophoresed on a 6% denaturing PAGE gel in 1x TBE buffer for 90mins at 200V. Controls included hybridisations of each probe to yeast RNA with and without subsequent treatment with RNase to determine the specificity of hybridisation and the integrity of the probes respectively.

RNA fragments were transferred to nylon membrane using a semi-dry transfer apparatus as described for protein transfer in Section 2.3.14, except for the use of 0.2x TBE in place of transfer buffer. RNA was then cross linked to the membrane in a Stratalinker. RNA was detected by probing the membrane with anti-digoxygenin-AP antibody (diluted 1/10000), followed by development using CSPD and exposure to Hyperfilm™ ECL™ as described above (Section 7.2.1.2). Band intensities for BRCA1 and 18S rRNA, which was used as an internal control, were measured using a scanning densitometer and Quantity One software. Mean optical density values for background intensity were measured in each lane and subtracted from mean optical density values for bands. Results are expressed as the ratio of BRCA1 intensity to 18S rRNA intensity, after correction for background.

7.2.2 – Quantitation of BRCA1 mRNA levels by real-time RT-PCR

To further investigate the expression of BRCA1 in response to DHT and MPA, fresh RNA samples were prepared from MDA-MB-453 and T-47D breast cancer cells. In

these experiments, mRNA levels were analysed using the more sensitive technique of real-time RT-PCR.

7.2.2.1 - RNA extraction and preparation of cDNA for time course experiments

MDA-MB-453 and T-47D breast cancer cells were seeded at a density of 7 x 10^5 cells/well in six well plates in phenol red free RPMI 1640 medium containing 5% CSS. After 48-72hrs medium was replaced with phenol red free RPMI 1640 medium containing 5% CSS and DHT (1nM), MPA (100nM) or vehicle (0.1% ethanol) in the presence and absence of bicalutamide (5μM) as indicated. Treatments were replenished every 24hrs. Cells were harvested by trypsinisation and collected by centrifugation at 2000rpm for 5mins at 4°C. Cells were kept on ice throughout the harvesting procedure. Total RNA was extracted from cell pellets using the RNeasy mini kit as described in Section 2.3.7 and an aliquot of each RNA sample (approximately 15μL) was treated with DNase I and ethanol precipitated (Section 2.3.9). Following resuspension of DNase I-treated RNA in 5μL RNase free H_2O, 1μL of each RNA (approximately 500ng) was reverse transcribed to cDNA as described in Section 2.3.10.

7.2.2.2 – Confirmation of DNA degradation

β-actin was amplified by PCR for each cDNA sample, including negative controls in which reverse transcriptase was omitted, using β-actin sense and antisense primers (Appendix 2) and 0.5μL of cDNA template as described in Section 2.3.11. These primers anneal on either side of the 111bp intron 4 of the β-actin gene, and therefore detect a 202bp product amplified from cDNA and a 314bp product amplified from any residual genomic DNA (which includes the intron). Reactions were denatured at 95°C for 5mins followed by 35 cycles of 95°C for 1min, 60°C for 1min, 72°C for 1min and a

final extension at 72°C for 20mins. Five μL of each product was electrophoresed on a 2% agarose-TAE gel and visualised by ethidium bromide staining.

7.2.2.3 – Real time PCR using SYBR Green I incorporation

Reverse transcribed samples were diluted 1/50 with sterile H_2O and 8μL was used as a template for real time PCR. The reaction contained 5pmol each of sense and antisense primers and 10μL SYBR green 2x PCR reaction mix in a 20μL volume. BRCA1 cDNA was amplified using BRCA1 5349 sense and BRCA1 5523 antisense primers (Appendix 2), which anneal to exons 20 and 22 respectively of BRCA1 cDNA. These primers are different to those used to generate templates for riboprobe synthesis (Section 7.2.1.1) and were designed to amplify a smaller fragment of the BRCA1 cDNA (174bp) in order to achieve optimal amplification in the real time PCR experimental system (Dr Julianne Henry, Flinders Medical Centre, SA, personal communication). β-actin, which was used as an internal control in these experiments, was amplified using β-actin sense and β-actin antisense primers (Appendix 2), which generate a 202bp product. The specificity of each of these primer sets was confirmed by DNA sequencing (Section 2.3.13). A negative control reaction lacking cDNA template was included for each primer set in each real-time PCR run. Reactions were run on the Rotor-Gene 3000 real time thermal cycler with initial template denaturation at 95°C for 10mins, followed by 25-55 cycles of denaturation at 95°C for 15secs, annealing at 60°C for 15secs and extension at 72°C for 30secs. Incorporation of SYBR green I intercalating dye into double stranded DNA during the reaction was measured at the end of the extension step of each cycle and plotted against cycle number by Rotor-Gene software.

Prior to analysis of all cDNA samples, reaction efficiencies for BRCA1 and β-actin were determined using a standard curve, where serial 5-fold dilutions (1/5, 1/25, 1/125, 1/625 and 1/3125) of a single cDNA sample were set up in triplicate PCR reactions as described above. A standard curve was plotted by Rotor-Gene software with the log of the starting quantity (in relative units based on the dilution factor) against C_T value. Reaction efficiency (E) was calculated using Formula 7.1. When the reaction is 100% efficient and accumulated PCR products double each cycle, E will have a value of 2.0. Standard curve runs were performed twice for both BRCA1 and β-actin to confirm that reaction efficiency did not vary significantly between runs. These runs also served to confirm optimal amplification conditions for each primer set.

C_T values were determined from amplification curves for each reaction by Rotor-Gene software, with the threshold set in the exponential phase of amplification. Relative mRNA levels (R) were calculated using Formula 7.2, as described by Pfaffl (2001).

Thermal cycling was followed by a melt profile to indicate whether the PCR reaction amplified specific and/or non-specific products. This is dependent on DNA fragments with different sequences having different melting temperatures. DNA was subjected to increasing temperatures from 72°C to 95°C, rising by one degree at a time and holding for 2secs at each degree. Loss of SYBR green I incorporation was measured at each temperature as the products denatured into single strands, and the rate of change of SYBR green I fluorescence was plotted against temperature by Rotor-Gene software, allowing evaluation of the composition of PCR products from each reaction. Amplification of single PCR products was then confirmed by electrophoresis of samples on a 1-2% agarose-TAE gel and ethidium bromide staining.

Formula 7.1:

$$E = 10^{-slope}$$

where slope is the slope of the standard curve (given by Rotor-Gene software)

Formula 7.2:

$$R = \frac{E\ (BRCA1)^{\Delta CT\ (reference - sample)}}{E\ (\beta\text{-}actin)^{\Delta CT\ (reference - sample)}}$$

where E = reaction efficiency for a given primer set, and

$\Delta C_T = C_T$ for the reference sample – C_T for a given sample

7.3.1 – Confirmation of BRCA1 downregulation by RNase protection assay

In the cDNA array study, BRCA1 mRNA levels were markedly reduced in MDA-MB-453 cells following treatment with 100nM MPA for 4hrs (Figure 7.1). In order to confirm this observation, an RNase protection assay was used to examine BRCA1 mRNA levels in MDA-MB-453 cells treated with 1nM DHT, 1nM MPA, 100nM MPA and vehicle (0.1% ethanol) for 4hrs (ie the same RNA samples used for the cDNA array study) and 24hrs. The reduction of BRCA1 mRNA levels by 100nM MPA at 4hrs relative to the control, observed with the array analysis, was not observed using RNase protection assay. Instead, BRCA1 mRNA was markedly reduced by 100nM MPA after 24hrs of treatment. Densitometric analysis of band intensity indicated that BRCA1 mRNA levels, following correction for 18S rRNA, were reduced by 96% compared to control after 24hrs (Figure 7.2), which is comparable to the 93% reduction in BRCA1 mRNA levels detected with the cDNA arrays (Figure 7.1). BRCA1 mRNA levels detected by protection assay in cells treated with 1nM DHT and 1nM MPA were not markedly changed at 4hrs and 24hrs relative to the control level (Figure 7.2), reflecting the array data where a less marked change in BRCA1 expression was observed with these treatments (Figure 7.1). Differences in the sensitivity of radioactive and DIG experimental systems may explain the qualitative differences in BRCA1 mRNA levels observed in cDNA array and RNase protection assays. Real-time RT-PCR was used in subsequent experiments in order to increase the sensitivity and accuracy of BRCA1 mRNA detection.

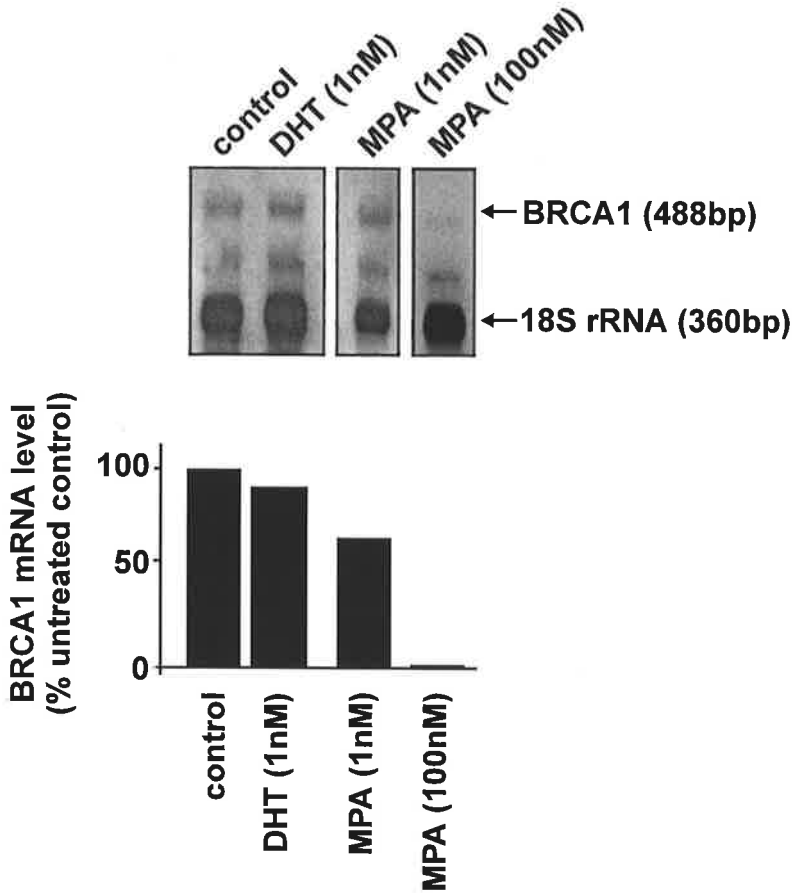


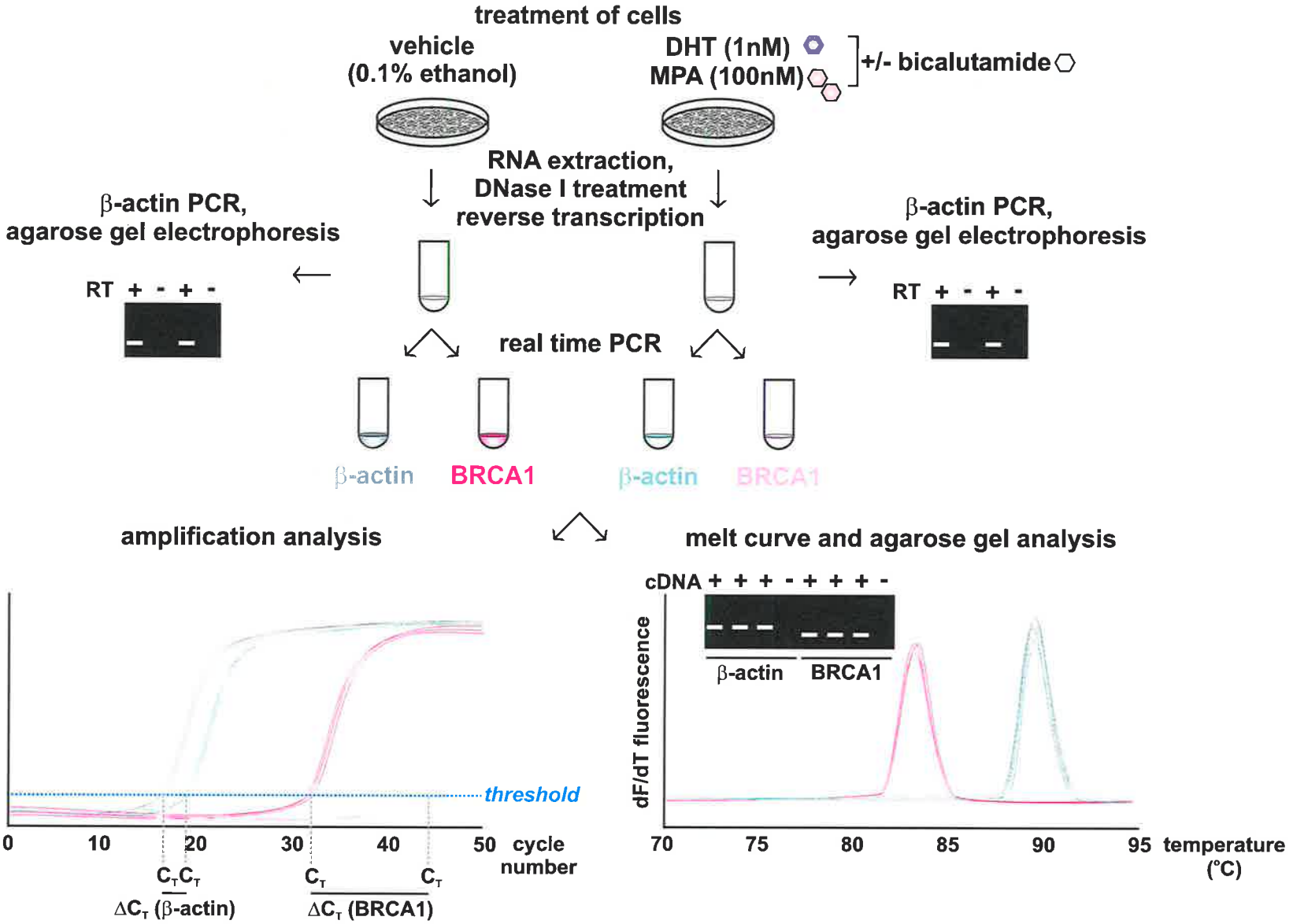
Figure 7.2: Analysis of BRCA1 mRNA levels in MDA-MB-453 breast cancer cells by RNase protection assay. Total RNA extracted from MDA-MB-453 cells treated with DHT (1nM), MPA (1nM or 100nM) or vehicle (0.1% ethanol, control) for 24hrs was hybridised with digoxygenin-labelled RNA probes for 18S rRNA and BRCA1. Single-stranded RNA was digested using RNase A / RNase T1 and protected fragments were visualised by electrophoresis and detection using an anti-digoxygenin-AP antibody (upper panel). Band intensity was measured using a scanning densitometer and is represented graphically as the mean optical density value, corrected for background and normalised for 18S rRNA intensity (lower panel). A control hybridisation to yeast RNA, with inclusion of RNase treatment, yielded no protected fragments (data not shown), indicating the specificity of BRCA1 and 18S rRNA probes. Hybridisation to yeast RNA without subsequent RNase treatment yielded single bands corresponding to undigested probes (data not shown), indicating that there was no significant degradation of probes during the assay.

7.3.2 – Optimisation of experimental system for real time PCR

The design of the experimental system used to quantify relative changes in BRCA1 mRNA levels in response to DHT and MPA is illustrated schematically in Figure 7.3. Following reverse transcription of RNA, all cDNA samples were screened for the absence of residual genomic DNA prior to use in real time PCR reactions. This was carried out by amplification of β-actin cDNA in all reverse transcription reactions performed in the presence or absence of RT. A representative result is shown in Figure 7.4. A 202bp PCR product was detected in all reactions amplified from RT positive reactions, confirming the integrity of cDNA samples. Although primer dimers (less than 100bp) were detected in reactions amplified from RT negative templates, the 314bp product amplified from genomic DNA was not detected (Figure 7.4), indicating that genomic DNA was effectively degraded by treatment with DNase I in these samples. PCR products were also not detected in negative control reactions which contained no cDNA template. Consequently, these negative controls were excluded from analysis in subsequent real time PCR reactions. In the very few cDNA samples in which genomic DNA was detected, DNase I treatment of the corresponding RNA was repeated.

Standard curve runs were initially performed to test the suitability of primers for BRCA1 (BRCA1 5349 sense and BRCA1 5523 antisense) and β-actin (β-actin sense and β-actin antisense) for amplification using real time PCR with SYBR green I incorporation. Due to the non-specific nature of detection using SYBR green I dye, which binds to any double stranded DNA, melt curve analysis and agarose gel electrophoresis of PCR products were also performed to confirm amplification of single homogeneous products. The specificity of amplified products was verified by DNA sequencing (data not shown).

Figure 7.3: Schematic representation of the experimental design for real time RT-PCR analysis of β-actin and BRCA1 mRNA levels. Following treatment of cells with steroid, RNA was extracted using the RNeasy Mini Kit, treated with DNAse I and reverse transcribed. An aliquot of cDNA from reactions performed in the presence and absence of reverse transcriptase was used for amplification of β-actin by PCR to confirm degradation of genomic DNA prior to further analysis. β-actin and BRCA1 cDNA were then amplified in separate real time PCR reactions using specific primers and incorporation of SYBR Green I dye. C_T values for triplicate reactions were determined with Rotor-Gene software and used to calculate relative mRNA levels between samples and controls (Formula 7.2). Amplification of single products was confirmed by melt curve analysis and agarose gel electrophoresis.


treatment of cells
vehicle
(0.1% ethanol)
DHT (1nM)
MPA (100nM)
+/- bicalutamide
RNA extraction,
DNase I treatment
reverse transcription
β-actin PCR,
agarose gel electrophoresis
RT + - + -
β-actin PCR,
agarose gel electrophoresis
RT + - + -
real time PCR
β-actin
BRCA1
β-actin
BRCA1
amplification analysis
melt curve and agarose gel analysis
fluorescence
threshold
0
10
20
30
40
50
cycle
number
C_T C_T
ΔC_T (β-actin)
C_T
C_T
ΔC_T (BRCA1)
cDNA + + + - + + + -
β-actin
BRCA1
dF/dT fluorescence
70
75
80
85
90
95
temperature
(°C)

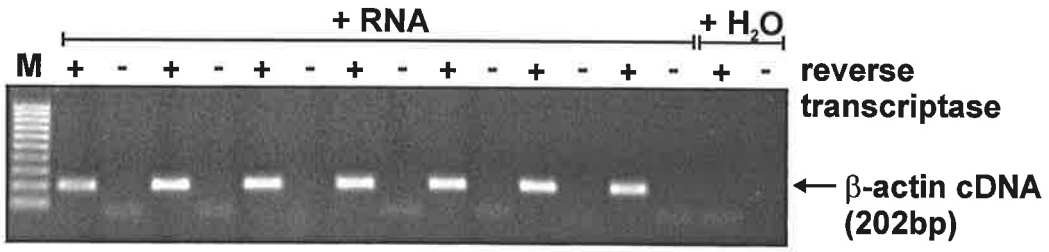


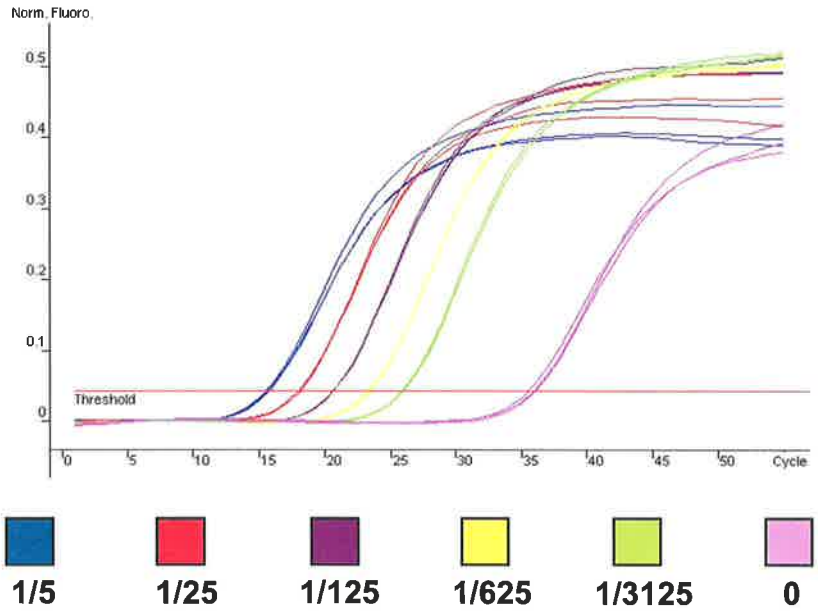
Figure 7.4: Agarose gel electrophoresis of β-actin PCR products amplified from reverse transcription reactions. Reactions were performed using 500ng DNase I treated RNA or H_2O in the presence (+) or absence (-) of reverse transcriptase. PCR reactions were performed using 0.5μL cDNA and the β-actin sense and antisense primers, which bind on either side of the 111bp intron 4. A 202bp PCR product was generated in all reactions containing reverse transcriptase and cDNA. The absence of PCR products in reverse transcription reactions performed without reverse transcriptase confirms successful degradation of genomic DNA by DNase I. M = 100bp DNA molecular weight marker.

Figure 7.5 (a) shows an amplification curve for β-actin amplified from different amounts of cDNA. Standard deviation for triplicate C_T values was less than 0.08 for all samples and the calculated input template amount was within two standard deviations of the expected amount for dilutions between 1/5 and 1/125, within six standard deviations for the 1/625 dilution and within 13 standard deviations for the 1/3125 dilution (Figure 7.5 (b)). Reaction efficiency (E) for this run was calculated at 1.87 (Table 7.1) from the slope of the standard curve (Figure 7.5 (c)). Melt curve analysis, showing a single peak at the same temperature for each PCR product (Figure 7.5 (d)), and agarose gel analysis (Figure 7.5 (e)) demonstrated amplification of homogeneous products of the expected size. The generation of primer dimers in reactions lacking cDNA template accounts for the melting peak at a lower temperature. These results indicate that the β-actin primers generate a product that can be quantitated accurately by real time PCR using SYBR green I dye, with the most precise results obtained with a cDNA template dilution between 1/5 and 1/125.

An amplification curve for BRCA1 primers is shown in Figure 7.6 (a). Standard deviations for triplicate C_T values ranged from 0.12 to 0.75 (Figure 7.6 (b)). The calculated template input amount was within one standard deviation of the expected amount for the 1/25 dilution, within three standard deviations for the 1/5, 1/125 and 1/625 dilutions and within eight standard deviations for the 1/3125 dilution. Reaction efficiency (E) for this run was calculated at 1.89 (Table 7.1) from the slope of the standard curve (Figure 7.6 (c)). The generation of homogeneous products of the same composition was demonstrated by melt curve analysis (Figure 7.6 (d)). This was confirmed by agarose gel analysis which showed a single BRCA1 product of the expected size in each reaction (Figure 7.6 (e)). These results indicated that the BRCA1 primers are also suitable for use in this real time PCR system, however the most

Figure 7.5: Real time PCR data for β-actin amplified from varying amounts of template cDNA. *(a)* Incorporation of SYBR green I into β-actin PCR products. The fluorescence of each reaction, measured at completion of the extension step of each cycle and normalised for background fluorescence, was plotted against cycle number. A threshold was set for each run in the range where amplification was increasing exponentially, and C_T values for each reaction were determined by Rotor-Gene software. Reactions were performed in triplicate using serial dilutions of template cDNA as indicated. *(b)* Relative template amounts for each reaction were determined from C_T values. Calculated relative template amounts, expressed as the mean +/- SEM (shaded bars), were plotted on a logarithmic scale alongside the expected relative template amount (solid bars) for each dilution of template cDNA. *(c)* Standard curve generated by Rotor-Gene software plotting arbitrary concentration of template cDNA against C_T, showing R^2 and reaction efficiency (E) values. *(d, over page)* Melt curve analysis of β-actin PCR products. The rate of change of SYBR green I fluorescence (dF/dT) was plotted against temperature, and peaks for each reaction represent melting points for products generated. *(e, over page)* Five μL of each reaction was electrophoresed on a 2% agarose-TAE gel alongside molecular weight markers (M) to confirm amplification of single bands.

(a)


Norm. Fluoro.
0.5
0.4
0.3
0.2
0.1
0
Threshold
0
5
10
15
20
25
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Cycle
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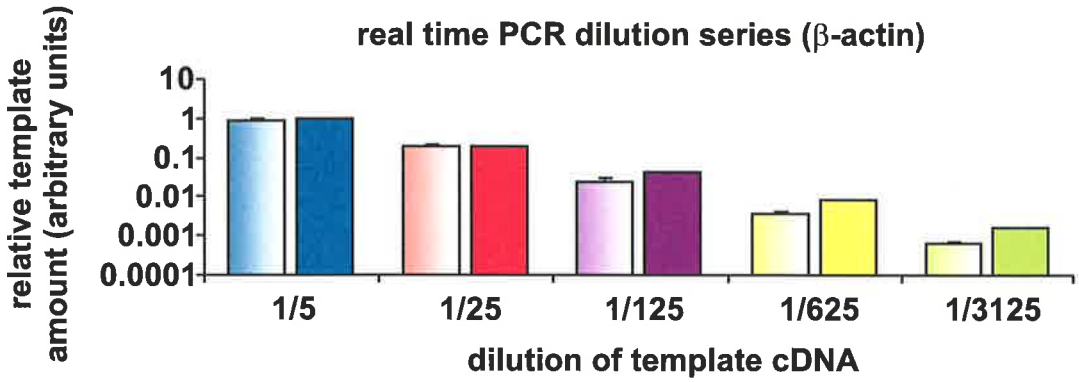
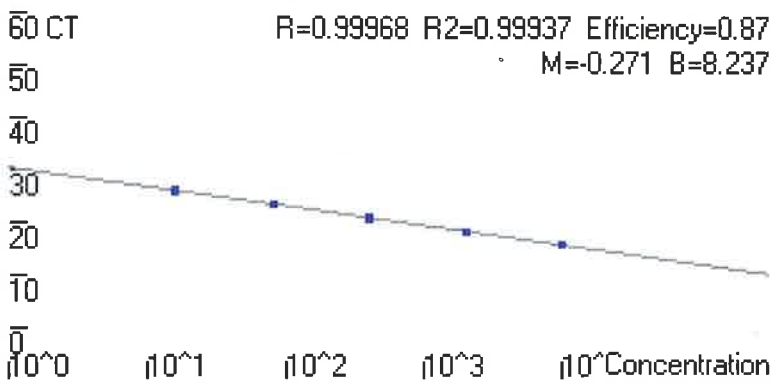
1/25
1/125
1/625
1/3125
0

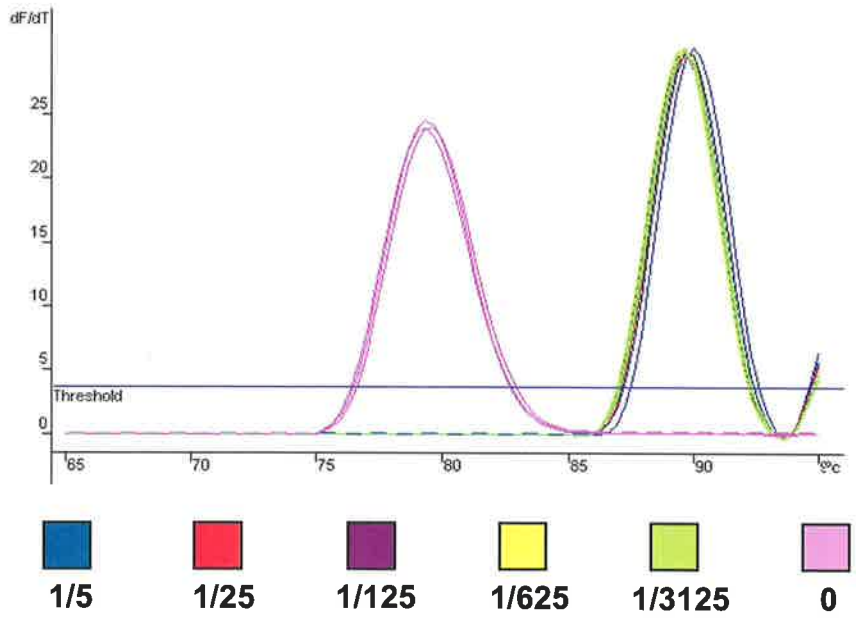
(b)


real time PCR dilution series (β-actin)
relative template amount (arbitrary units)
10
1
0.1
0.01
0.001
0.0001
1/5
1/25
1/125
1/625
1/3125
dilution of template cDNA

(c)


60 CT
50
40
30
20
10
0
R=0.99968 R2=0.99937 Efficiency=0.87
M=-0.271 B=8.237
10^0
10^1
10^2
10^3
10^Concentration

(d)


dF/dT
25
20
15
10
5
Threshold
0
65
70
75
80
85
90
°C
1/5
1/25
1/125
1/625
1/3125
0

(e)


M
1/5
1/25
1/125
1/625
1/3125
H2O
β-actin
202bp

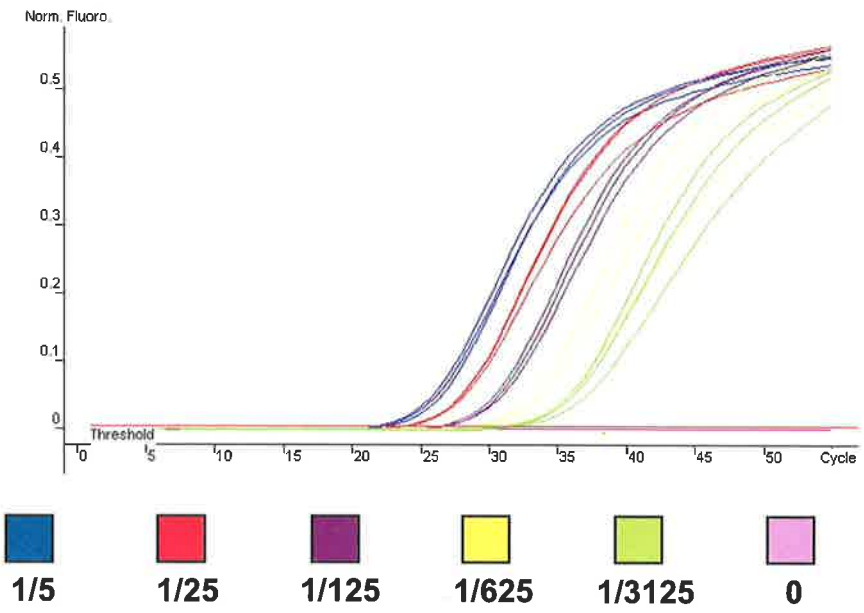
Table 7.1: Calculation of reaction efficiency (E) for β-actin and BRCA1 for two independent standard curve runs.

Gene	**Run 1**			**Run 2**		
	slope*	R^2	E	slope*	R^2	E
β-actin	-0.271	0.99936	1.87	-0.267	0.99818	1.85
BRCA1	-0.277	0.88409	1.89	-0.275	0.97602	1.88

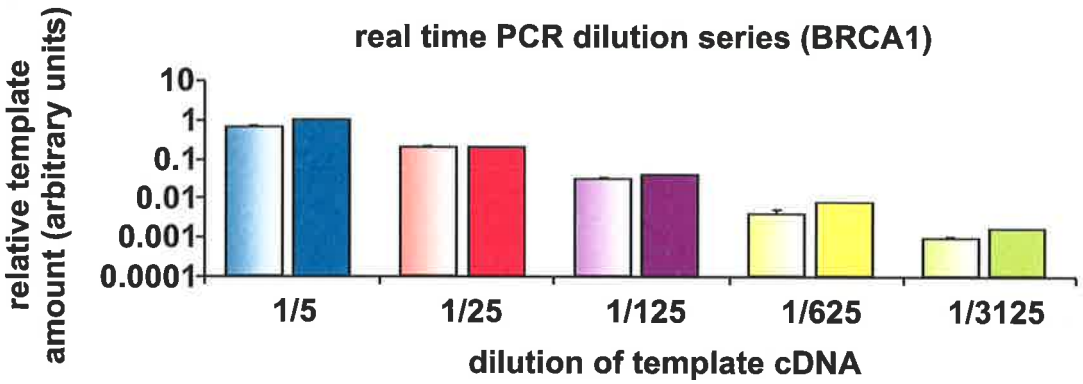
* Slope and R^2 values were determined by Rotor-Gene software and E was calculated using formula 7.1.

Figure 7.6: Real time PCR data for BRCA1 amplified from varying amounts of template cDNA. *(a)* Incorporation of SYBR green I into BRCA1 PCR products. The fluorescence of each reaction, measured at completion of the extension step of each cycle and normalised for background fluorescence, was plotted against cycle number. A threshold was set for each run in the range where amplification was increasing exponentially, and C_T values for each reaction were determined by Rotor-Gene software. Reactions were performed in triplicate using serial dilutions of template cDNA as indicated. *(b)* Relative template amounts for each reaction were determined from C_T values. Calculated relative template amounts, expressed as the mean +/- SEM (shaded bars), were plotted on a logarithmic scale alongside the expected relative template amount (solid bars) for each dilution of template cDNA. *(c)* Standard curve generated by Rotor-Gene software plotting arbitrary concentration of template cDNA against C_T, showing R^2 and reaction efficiency (E) values. *(d, over page)* Melt curve analysis of BRCA1 PCR products. The rate of change of SYBR green I fluorescence (dF/dT) was plotted against temperature, and peaks for each reaction represent melting points for products generated. *(e, over page)* Five μL of each reaction was electrophoresed on a 2% agarose-TAE gel alongside molecular weight markers (M) to confirm amplification of single bands.

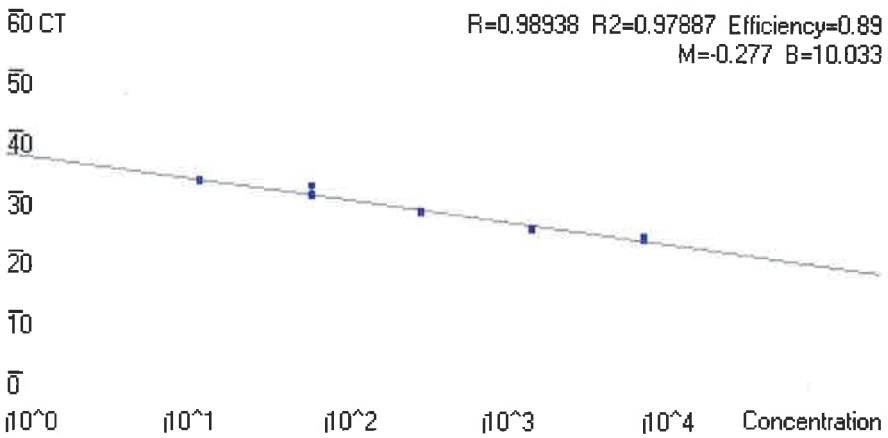
(a)


Norm. Fluoro.
0.5
0.4
0.3
0.2
0.1
0
Threshold
0
5
10
15
20
25
30
35
40
45
50
Cycle
1/5
1/25
1/125
1/625
1/3125
0

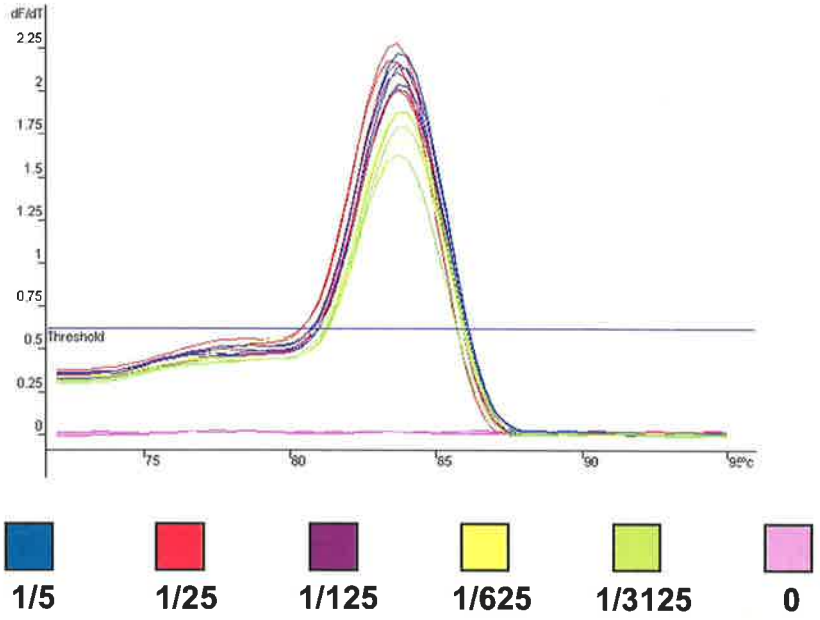
(b)


real time PCR dilution series (BRCA1)
relative template amount (arbitrary units)
10
1
0.1
0.01
0.001
0.0001
1/5
1/25
1/125
1/625
1/3125
dilution of template cDNA

(c)


60 CT
R=0.98938 R2=0.97887 Efficiency=0.89
M=-0.277 B=10.033
50
40
30
20
10
0
10^0
10^1
10^2
10^3
10^4
Concentration

(d)


dF/dT
2.25
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
Threshold
75
80
85
90
95°c
1/5
1/25
1/125
1/625
1/3125
0

(e)


M
1/5
1/25
1/125
1/625
1/3125
H2O
BRCA1
174bp

accurate results are obtained with dilution of cDNA of approximately 1/25 (all templates were diluted at 1/50). E values for β-actin and BRCA1 amplification, calculated in two independent standard curve runs for each primer set (Figure 7.5 (c) and 7.6 (c)), were consistent (Table 7.1), suggesting that the two genes have approximately equal amplification rates.

7.3.3 – Modulation of BRCA1 mRNA levels by DHT

Using the experimental system optimised above, BRCA1 mRNA levels were measured in time course experiments where cells were treated with DHT (1nM) for 0, 1, 4, 12, 24 and 48hrs. Although slight variations were detected, there was no significant difference in BRCA1 mRNA levels observed between any of the time points in MDA-MB-453 cells treated with DHT (Figure 7.7). Similarly, no significant modulation of BRCA1 mRNA levels by DHT was observed in T-47D cells (data not shown).

7.3.4 – Modulation of BRCA1 mRNA levels by MPA

Treatment of MDA-MB-453 cells with MPA (100nM) significantly reduced BRCA1 mRNA by 72% relative to control levels within 1hr, by 94% after 4hrs and by 92% at 12hrs (Figure 7.8). At 24hrs and 48hrs, BRCA1 mRNA levels were reduced by 50% and 13% respectively, which were not significantly different from control levels, indicating that modulation of BRCA1 mRNA by MPA was transient. This inhibitory effect of MPA was specific for MDA-MB-453 cells as BRCA1 mRNA levels did not significantly change over 48hrs of treatment with 100nM MPA in T-47D cells (data not shown).

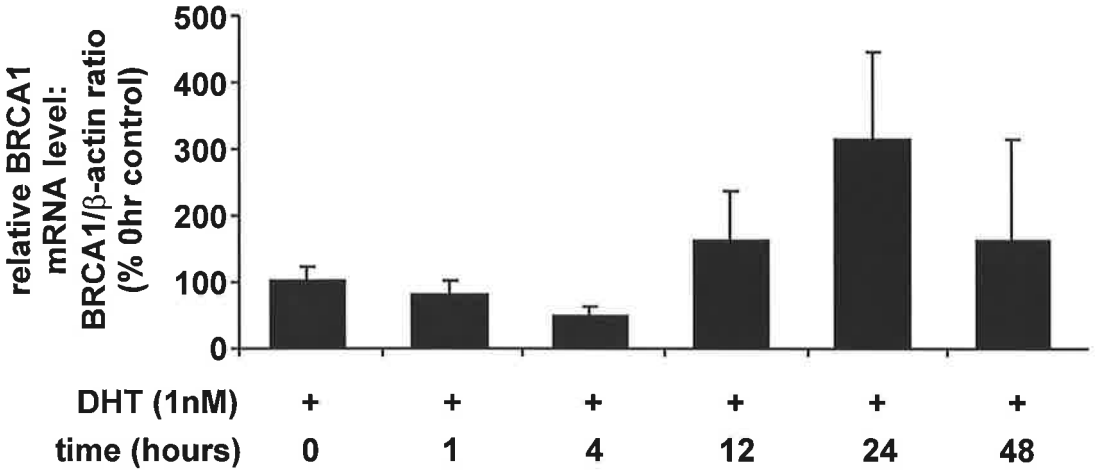


Figure 7.7: Effect of DHT on BRCA1 mRNA levels in MDA-MB-453 breast cancer cells. Cells were seeded in 6 well plates (7×10^5 cells/well) in phenol red free RPMI medium containing 5% CSS and treated with DHT (1nM) for 0-48hrs. Total RNA was extracted and reverse transcribed using random hexamers. BRCA1 and β-actin mRNA were amplified in separate real-time PCR reactions incorporating SYBR green I dye and relative expression levels (E) were calculated. BRCA1 mRNA was corrected for β-actin and is expressed as relative levels (% 0hr control) calculated as the mean +/- SEM of a minimum of 3 replicate reactions.

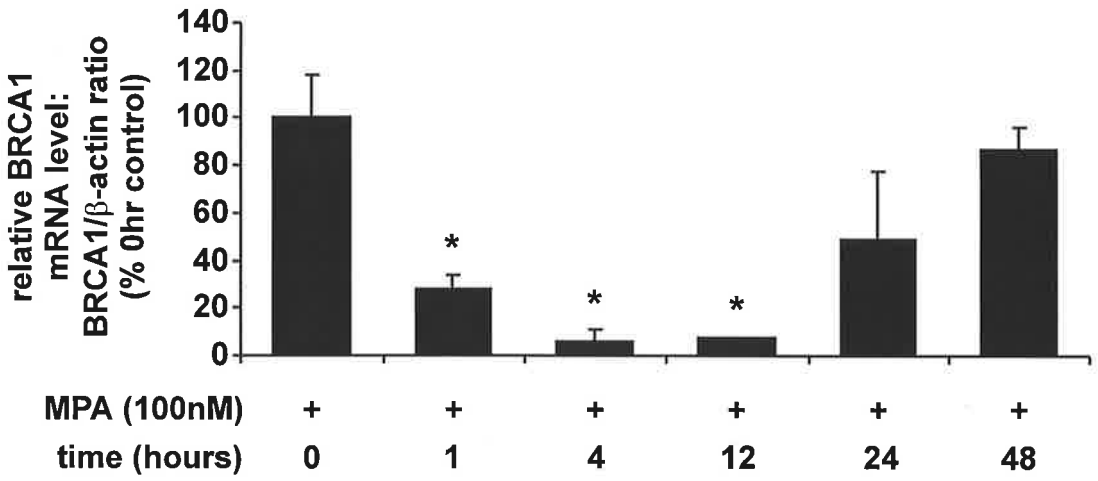


Figure 7.8: Effect of MPA on BRCA1 mRNA levels in MDA-MB-453 breast cancer cells. Cells were seeded in 6 well plates (7×10^5 cells/well) in phenol red free RPMI medium containing 5% CSS and treated with MPA (100nM) for 0-48hrs. Total RNA was extracted and reverse transcribed using random hexamers. BRCA1 and β-actin mRNA were amplified in separate real-time PCR reactions incorporating SYBR green I dye and relative expression levels (E) were calculated. BRCA1 mRNA was corrected for β-actin and is expressed as relative levels (% 0hr control) calculated as the mean +/- SEM of a minimum of 3 replicate reactions. * ANOVA; $p < 0.05$: 100nM MPA 1-12hrs *versus* 0hr control.

7.3.5 – Blocking of the inhibitory effects of MPA by the AR antagonist bicalutamide

In order to determine whether the inhibitory effect of MPA on BRCA1 mRNA levels was mediated by the AR, MDA-MB-453 cells were treated with MPA (100nM) for 12hrs in the presence or absence of the AR antagonist bicalutamide (5μM). Consistent with the previous findings, treatment with MPA significantly reduced BRCA1 mRNA levels by 83% relative to the control after 12hrs (Figure 7.9). Co-incubation of cells with MPA (100nM) and a 500-fold molar excess of bicalutamide resulted in a significant abrogation of the inhibitory effect of MPA, with BRCA1 mRNA reduced by only 14% relative to the control. Treatment with bicalutamide alone did not significantly change BRCA1 mRNA levels relative to the vehicle treated control.

7.4 – Discussion

The results presented in this chapter provide evidence that MPA downregulates BRCA1 mRNA levels in MDA-MB-453 breast cancer cells, confirming the observation from the cDNA array study described in Chapter 6. Support for a direct role for the AR in regulating the expression of BRCA1 was provided by experiments showing that the AR antagonist bicalutamide can block the inhibitory effects of MPA on BRCA1 expression. In contrast to the inhibitory effect of MPA, DHT did not modulate BRCA1 mRNA levels. This divergent regulation of BRCA1 by DHT and MPA may contribute to the divergent proliferative effects of these ligands on proliferation of MDA-MB-453 cells.

Interestingly, these studies have shown that the androgen signalling axis inhibits BRCA1 expression while previous studies have shown that estrogens stimulate BRCA1 expression (Gudas *et al*, 1995; Marquis *et al*, 1995; Spillman and Bowcock, 1996;

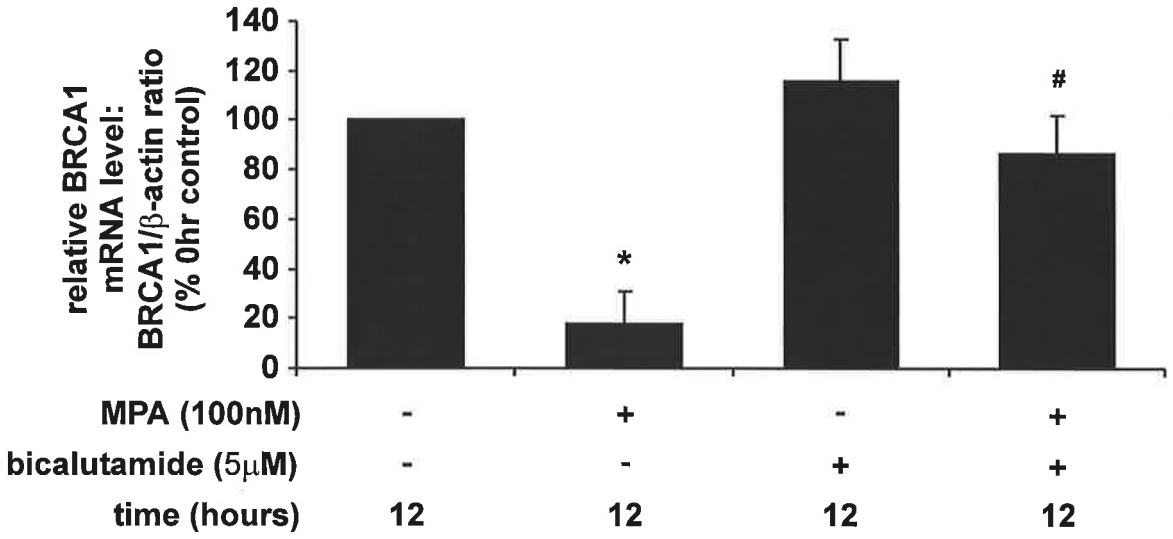


Figure 7.9: Effect of the AR antagonist bicalutamide on the inhibition of BRCA1 mRNA levels by 100nM MPA. MDA-MB-453 cells were seeded in 6 well plates (7×10^5 cells/well) in phenol red free RPMI medium containing 5% CSS. After 72hrs, cells were treated with MPA (100nM) or vehicle (0.1% ethanol) in the presence or absence of bicalutamide (5μM) for 12hrs. Total RNA was extracted and reverse transcribed using random hexamers. BRCA1 and β-actin mRNA were amplified in separate real-time PCR reactions incorporating SYBR green I dye and relative expression levels (E) were calculated. BRCA1 mRNA was corrected for β-actin and is expressed as relative levels (% untreated control) calculated as the mean +/- SEM of a minimum of 3 replicate reactions. * ANOVA; $p = 0.016$: 100nM MPA *versus* untreated control. # ANOVA; $p = 0.029$: 100nM MPA + 5μM bicalutamide *versus* 100nM MPA.

Rajan *et al*, 1997; Marks *et al*, 1997; Romagnolo *et al*, 1998; Chambon *et al*, 2003). Furthermore, BRCA1 has opposing effects on the transcriptional activity of AR and ERα (Fan *et al*, 1999; Yeh *et al*, 2000; Park *et al*, 2000b; Zheng *et al*, 2001; Fan *et al*, 2001a). Together, these studies suggest that the close association between BRCA1 and androgen and estrogen signalling pathways may be critical for the regulation of breast cancer cell growth.

The divergent effects of DHT and MPA on BRCA1 mRNA levels in MDA-MB-453 cells may reflect the fact that MPA, but not DHT, inhibits proliferation of MDA-MB-453 cells (Birrell *et al*, 1995a; Bentel *et al*, 1999). This observation supports an association between reduced BRCA1 expression and reduced cell proliferation rate in response to MPA. Structural alteration in the AR-LBD in MDA-MB-453 cells, resulting from the Q865H amino acid substitution, potentially prevents modulation of BRCA1 mRNA levels (and other genes) by DHT, leading to stimulation of proliferation. Examination of BRCA1 expression in response to androgens in other AR positive cell lines that are growth inhibited by DHT, such as ZR-75-1, would be useful to confirm whether the selective modulation of BRCA1 expression by MPA is unique to MDA-MB-453 cells.

In the current study, BRCA1 mRNA levels were not significantly altered by DHT or MPA in T-47D breast cancer cells. This type of cell specificity has been observed in other studies demonstrating reduced responses of BRCA1 expression to estrogen and prolactin in T-47D cells compared to induction observed in MCF-7 breast cancer cells (Marks *et al*, 1997; Favy *et al*, 1999; Chambon *et al*, 2003). These results are supported by transient transfection of BRCA1 promoter elements linked to reporter genes which also show reduced activity in response to regulatory proteins in T-47D cells in

comparison to MCF-7 cells (Atlas *et al*, 2000). It is possible that receptor expression levels in T-47D cells, or the specific combination of cofactors expressed, are not permissive of significant changes in BRCA1 expression. Alternatively, activation of PR by MPA in these cells may induce BRCA1 expression (Gudas *et al*, 1995) and oppose suppression mediated by the AR. Further studies using a panel of breast cancer cell lines are necessary to assess whether the downregulation of BRCA1 by MPA is a consistent effect in breast cancer cells or specific to MDA-MB-453 cells.

The observed transient repression of BRCA1 by MPA in MDA-MB-453 cells examined over a 48 hour time course may be a result of metabolism of MPA over the duration of the assay. Although cells were supplemented with fresh MPA every 24 hours, significant degradation prior to this may account for the diminished inhibitory effect observed at the 24 hour and 48 hour time points relative to the 1-12 hour time points when the MPA was relatively fresh. It is also possible that the expression levels of BRCA1 were related to natural fluctuations during different phases of the cell cycle – BRCA1 mRNA levels peak during late G1 and early S phase (Gudas *et al*, 1996; Rajan *et al*, 1996; Vaughn *et al*, 1996; Ruffner and Verma, 1997). However without synchronisation of cells in these experiments it is more likely that downregulation of BRCA1 was a consequence of MPA treatment rather due to cyclical variations. Transient induction of BRCA1 by E_2 in the ovarian cancer cell line BG-1 has also been observed (Romagnolo *et al*, 1998) as has temporary induction by progesterone in T-47D cells (Gudas *et al*, 1995). This may be a consequence of rapid feedback responses to changes in BRCA1 expression levels, where downstream molecules continue to exert their effects on cellular behaviour without sustained increases or decreases in BRCA1 levels.

The precise role of BRCA1 in the regulation of breast cancer cell proliferation remains unclear. The tumour suppressor activity of BRCA1 is supported by *in vitro* and animal studies where loss of BRCA1 function results in increased proliferation of malignant breast cells (Thompson *et al*, 1995; Rao *et al*, 1996) and exogenous expression of wild type BRCA1 inhibits proliferation (Holt *et al*, 1996; Fan *et al*, 2001b). Paradoxically, BRCA1 expression appears to be directly correlated with proliferative rate in a number of cell systems. Agents which stimulate breast cancer cell proliferation, such as estrogen, prolactin, epidermal growth factor and insulin-like growth factor, also induce BRCA1 expression (Marks *et al*, 1997; Favy *et al*, 1999). Expression of Brca1, the murine homologue of human BRCA1, in mouse mammary epithelium is high in cells undergoing rapid proliferation associated with developmental stages of puberty and pregnancy (Marquis *et al*, 1995). Conversely, when cell proliferation rates are reduced by growth factors such as transforming growth factor-β, by serum starvation or after reaching confluence, BRCA1 levels are diminished (Gudas *et al*, 1996; Rajan *et al*, 1996). These observations have led to the hypothesis that the changes in BRCA1 expression are due to changes in the cell proliferation rate and are not a direct consequence of the treatment. This theory would also support the function of BRCA1 in a feedback loop where stimulation of proliferation prompts an increase in BRCA1 expression, in turn eliciting suppression of cell growth, or *vice versa*. This is consistent with the MPA-induced inhibition of both BRCA1 mRNA levels and proliferation observed in MDA-MB-453 cells. However BRCA1 was significantly downregulated within one hour of treatment with MPA, suggesting that this effect may be directly mediated by treatment rather than as a consequence of proliferative changes. As there are no mutations in the *BRCA1* gene in MDA-MB-453 cells (unpublished results) it is likely to retain its natural tumour suppressive functions in this cell line. Determination of BRCA1 expression levels in MDA-MB-453 cells cultured in low serum conditions

with or without MPA treatment may ascertain whether the suppression of BRCA1 is a direct consequence of proliferative changes. Furthermore, experiments using either overexpression of BRCA1, or inhibition of BRCA1 expression through the use of short interfering RNA (siRNA) or antisense oligonucleotides, are required to confirm whether the inhibitory effects of MPA on proliferation of MDA-MB-453 cells can be elicited by changes in BRCA1 expression.

The *BRCA1* gene is located on chromosome 17q21 where it lies directly adjacent to the *NBR2* (Next to BRCA1 gene 2) gene (Xu *et al*, 1997a) (Figure 7.10). Examination of the promoter sequence preceding and surrounding the BRCA1 transcription start site, using the GAP sequence alignment program (available at http://www.angis.org.au/new/bioinformatic/ index.html), revealed the presence of two putative AREs. A class I element (AGAACAcgaAGGGCT) is located at position -1226 relative to the transcription start site and a class II element (GGGACAgggGGCCCA) is located at position +149 (Figure 7.10). These AREs differ from the consensus ARE sequences (Section 1.3.5) at the double underlined positions. It is possible that these elements mediate downregulation of BRCA1 transcription through direct binding of the MPA-AR complex, however it is necessary to confirm that these putative AREs can bind AR. This may be accomplished through the use of gel-shift analysis, performed in the presence or absence of an antibody to the AR to detect super-shifted AR-ARE complexes. Alternatively, investigations of other androgen-repressible genes support a model whereby the AR represses transcription through interference of other positive regulatory factors without binding to DNA itself. Repression of the α-subunit of gonadotrophin hormones by androgens is mediated by interference between the AR and proteins binding to cAMP regulatory elements and the α-basal element in the promoter region of the α-subunit gene, rather than by direct binding of the AR to an existing ARE

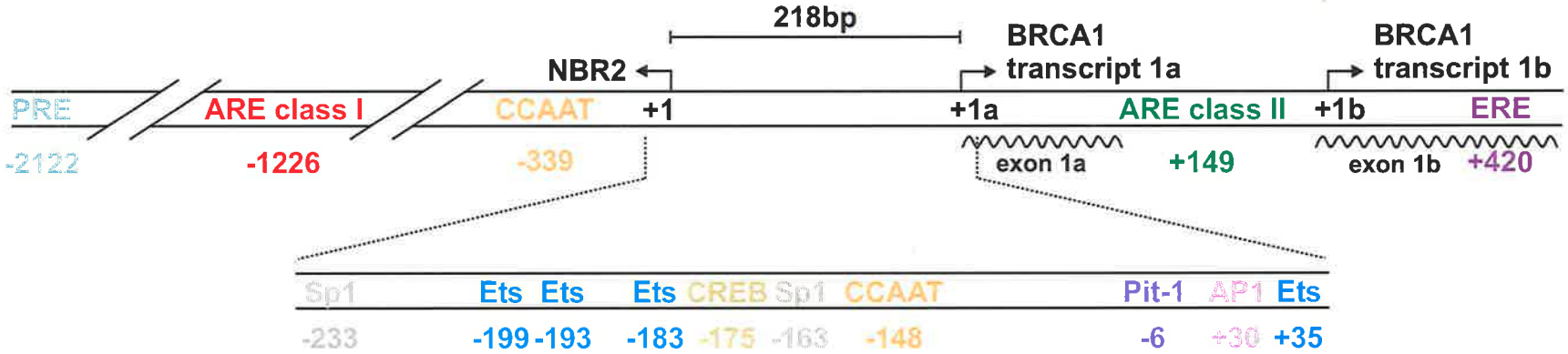


Figure 7.10: Schematic representation of the human BRCA1 promoter region. The region containing Ets, CREB, CCAAT, Pit-1, Sp1 and AP1 sites is a strong bidirectional promoter controlling synthesis of the NBR2 transcript and BRCA1 transcript 1a, which is predominant in the breast. A weaker promoter, positioned between exon 1a and exon 1b, controls synthesis of the BRCA1 transcript 1b which is predominant in the placenta. Additional Sp1 sites are located within exon 1a and 1b and an additional CREB site is located within the region preceding exon 1b (not shown). An alternative ERE is located within exon 1b (+420). A putative progesterone responsive element (PRE) lies at a site distal to the promoter region (-2122). A putative class I ARE is located at position -1226 and a putative class II ARE is located at position +149 in the promoter region preceding exon 1b. Base pair numbering is relative to the transcription start site (+1).

(Heckert *et al*, 1997). Repression of a neurotrophin receptor responsive reporter by androgens is mediated by AR inhibition of c-jun binding to an AP1 site in the vector backbone (Kallio *et al*, 1995). AR also represses induction of a NF-κB responsive reporter gene by RelA (Palvimo *et al*, 1996) through competition for common cofactors such as CBP (Aarnisalo *et al*, 1998). Collagenase I (matrix metalloproteinase I, MMP-1) is downregulated by androgens via a mechanism involving interaction of the AR with ERM, a member of the Ets family of transcription factors which is required for induction of MMP-1 expression (Schneikert *et al*, 1996). Ets proteins can cooperate with the transcription factor c-jun bound at an adjacent AP1 site to induce transcription (Nakae *et al*, 1995) and it has been postulated that the AR may interfere with this interaction (Schneikert *et al*, 1996). The BRCA1 proximal promoter region contains multiple regulatory elements including Ets binding sites, a CCAAT box and putative binding sites for cyclic AMP regulatory element binding protein (CREB), pituitary transcription factor-1 (Pit-1), Sp1 and AP1 (Xu *et al*, 1995; Smith *et al*, 1996; Xu *et al*, 1997b; Atlas *et al*, 2000) (Figure 7.10). Ets and CREB proteins have established roles in the induction of BRCA1 expression (Atlas *et al*, 2000; Atlas *et al*, 2001). The AR, when bound to MPA, therefore has the potential to interfere with the activity of these transcription factors at the BRCA1 promoter, leading to reduced mRNA synthesis. As described in Chapter 6, selective downregulation of BRCA1 mRNA levels in MDA-MB-453 cells by MPA but not by DHT may result from the induction of distinct ligand dependent receptor conformations which recognise AREs in specific contexts. Alternatively, considering the non-classical model for downregulation of gene expression proposed above, the MPA-AR complex may interfere more specifically with transcription factors regulating BRCA1 expression, such as Ets proteins or CREB, than does the DHT-AR complex. Chromatin immunoprecipitation assays may be a suitable

approach for determining whether DHT-AR and MPA-AR differentially interact with other critical regulatory factors at the BRCA1 promoter.

Overall, these studies have provided the first evidence that the androgen signalling pathway can regulate expression of BRCA1. Although putative AREs in the promoter region of the *BRCA1* gene may potentially mediate transcriptional regulation by the AR, the precise mechanisms by which this occurs are yet to be defined. Indeed, the mechanisms associated with the regulation of most androgen responsive genes in breast cancer cells are poorly described. The following chapter investigates the molecular mechanisms associated with the expression of a well characterised androgen responsive gene, prostate specific antigen (PSA), in breast cancer cells, with particular focus on assembly of the AR transcription complex at AREs in the promoter region of the PSA gene.

CHAPTER 8

REGULATION OF

PROSTATE SPECIFIC ANTIGEN EXPRESSION

8.1 – Introduction

Little is known about the mechanisms by which the AR regulates expression of androgen responsive genes in breast cancer cells. One androgen regulated gene which has been extensively studied is prostate specific antigen (PSA). Induction of PSA by androgens in the LNCaP prostate cancer cell line has been well characterised (Young *et al*, 1991; Henttu *et al*, 1992; Wolf *et al*, 1992; Montgomery *et al*, 1992; Jia *et al*, 2003), and has been used as a model to investigate the regulation of gene expression by androgens in prostate cancer cells. However, PSA is not prostate specific and its expression has been detected in a number of other tissues including the breast (Papotti *et al*, 1989; Diamandis *et al*, 1994; Levesque *et al*, 1995). The reported frequency of PSA expression in breast cancers varies considerably, ranging from 15-70% (Diamandis *et al*, 1994; Yu *et al*, 1994a; Wu *et al*, 1995; Yu *et al*, 1995; Ferguson *et al*, 1996; Hall *et al*, 1998), possibly due to different sensitivities of detection procedures such as enzyme immunoassays or immunohistochemistry. These observations imply that PSA is potentially useful as a model gene for investigation of the mechanisms by which androgens regulate gene expression, in the context of breast cancer cells.

PSA is encoded by the KLK3 gene, a member of the kallikrein family of genes which are located on chromosome 19q13.3-13.4 (Riegman *et al*, 1992). The PSA gene is organised into five exons encoding a 33kDa protein. Androgenic regulation of PSA expression is mediated via numerous AREs in the PSA promoter and enhancer. A high affinity, non-consensus ARE, termed ARE I, is located at position -170 of the proximal PSA promoter (Figure 8.1), and confers androgen inducibility (Riegman *et al*, 1991; Cleutjens *et al*, 1996). Other AREs identified in the PSA promoter and distal enhancer synergistically increase AR activity conferred by ARE I. ARE II is located at position

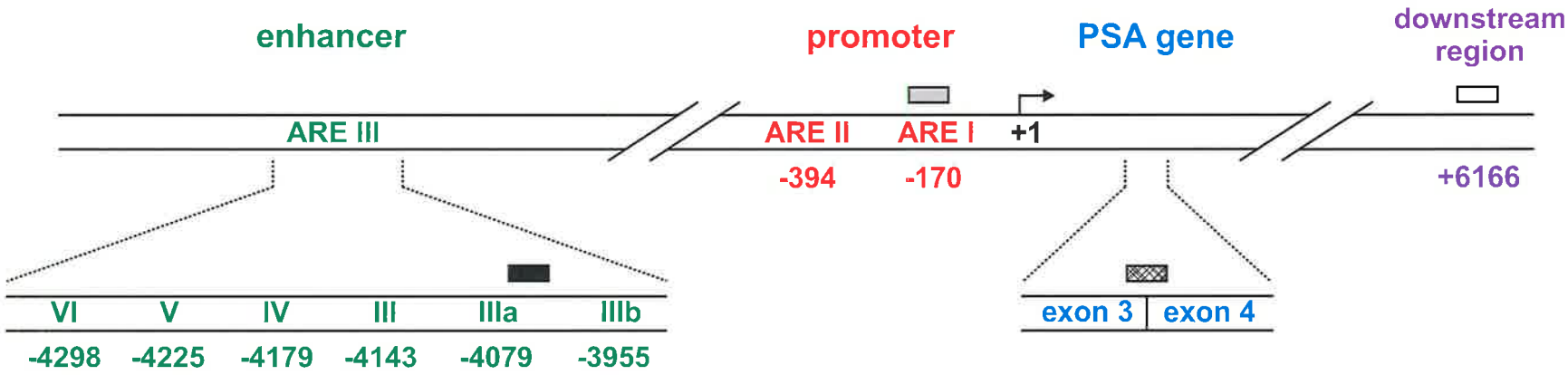


Figure 8.1: Schematic showing regions of the PSA promoter, enhancer and gene. Genomic DNA regions amplified by PCR in ChIP assays are indicated by solid black (PSA enhancer), grey (PSA promoter) and white (downstream irrelevant region) boxes. The PSA mRNA region spanning the splice site between exons 3 and 4, which was amplified by real time RT-PCR, is indicated by the hatched box. Base pair numbering is relative to the transcription start site (+1).

-394 and it binds AR with low affinity but enhances androgen induction of PSA (Riegman *et al*, 1991; Cleutjens *et al*, 1996; Zhang *et al*, 1997a). Androgen responsiveness of ARE I and ARE II is vastly increased by the PSA enhancer, which lies 4.1kb upstream from the transcription start site and contains six non-consensus AREs clustered within a 440bp core region (Schuur *et al*, 1996; Cleutjens *et al*, 1997; Zhang *et al*, 1997c; Huang *et al*, 1999) (Figure 8.1). ARE I and ARE III have the highest affinity for the AR-DBD, although this is still at least 15-fold lower than the consensus ARE sequence (Cleutjens *et al*, 1997; Huang *et al*, 1999). ARE III is active in the absence of the promoter, although it does not induce activity as strongly as ARE I alone (Cleutjens *et al*, 1997; Huang *et al*, 1999; Louie *et al*, 2003).

Recent development of the chromatin immunoprecipitation (ChIP) assay has allowed characterisation of the molecular events occurring at the endogenous PSA promoter and enhancer that lead to the induction of PSA mRNA synthesis in LNCaP cells. Treatment of LNCaP cells with androgen results in rapid binding of AR to the endogenous PSA AREs (Shang *et al*, 2002; Kang *et al*, 2002; Jia *et al*, 2003; Louie *et al*, 2003). AR binding is correlated with the recruitment of coactivators such as GRIP1, AIB1and CBP and subsequent histone acetylation at the same loci. Androgen-induced recruitment of RNA polymerase II is also observed and is synchronised with accumulation of PSA mRNA (Shang *et al*, 2002; Kang *et al*, 2002; Louie *et al*, 2003). Divergent results have been reported, however, when comparing relative recruitment of the transcription complex to the promoter and enhancer. One model suggests that promoter and enhancer elements are brought into close proximity upon formation of the transcription complex, possibly through shared binding of common cofactor molecules. This is supported by experimental data showing androgen-induced recruitment of AR to both promoter and enhancer elements (Shang *et al*, 2002; Kang *et al*, 2002). However, an alternative

model is that the transcription complex, including RNA polymerase, is only recruited to the PSA enhancer and RNA polymerase is subsequently transported to the promoter by moving along the chromatin. This is supported by ChIP assays that show AR and coactivator recruitment to the enhancer only, but detection of histone acetylation and RNA polymerase binding along the entire stretch of chromatin between the promoter and enhancer (Louie *et al*, 2003). Whilst these mechanisms have been characterised in prostate cancer cells, the composition of the AR transcription complex in breast cancer cells, and the mechanisms regulating its assembly, have not previously been described.

In addition to being regulated by androgens, PSA expression can also be modulated through ligand independent activation of the AR by the cytokine interleukin-6 (IL-6). This has been demonstrated by measurement of PSA mRNA or protein levels, or by measurement of a reporter gene linked to the PSA promoter and enhancer, in LNCaP prostate cancer cells. IL-6 has been shown to increase PSA expression in LNCaP cells, although there is considerable variation in the reported magnitude of the stimulatory effect (Hobisch *et al*, 1998; Huang *et al*, 1999; Lin *et al*, 2001; Jia *et al*, 2003; Lee *et al*, 2003). Additionally, IL-6 can modulate PSA expression induced by DHT and the synthetic androgen R1881, although both synergistic (Hobisch *et al*, 1998) and inhibitory (Jia *et al*, 2003) effects of IL-6 have been reported. In studies where IL-6 does stimulate PSA expression, the effect of IL-6 is blocked by AR antagonists, suggesting that the effects of IL-6 are mediated by the AR (Hobisch *et al*, 1998; Ueda *et al*, 2001; Lin *et al*, 2001). Binding of IL-6 to its membrane receptor stimulates intracellular signalling cascades involving kinases such as MAP kinase, JAK, STAT3 PKA, PKC, PI 3'-kinase and Akt, with the balance of these pathways determining the net effect of IL-6 on AR activity (Hobisch *et al*, 1998; Chen *et al*, 2000; Ueda *et al*, 2001; Culig *et al*, 2002; Ueda *et al*, 2002; Lee *et al*, 2003; Yang *et al*, 2003). These

molecules mediate phosphorylation and activation of the AR through direct interaction or cross-talk with the AR-NTD. It has been proposed that variation in the levels of intracellular kinases between different prostate cancer cell sublines may contribute to the observed inconsistencies in the reported effects of IL-6.

Little is known about the effects of IL-6 on AR activity in breast cancer cells, although IL-6 has been shown to inhibit proliferation of MCF-7, ZR-75-1 and T-47D cell lines (Tamm *et al*, 1989; Chen *et al*, 1991; Speirs *et al*, 1993a; Blais *et al*, 1995; Badache and Hynes, 2001), suggesting that it is a potentially important regulator of breast cancer growth. While there is evidence to show that IL-6 activates JAK1, STAT3, MAPK and PI 3'-kinase in T-47D cells (Badache and Hynes, 2001) the effects of these pathways on AR in these cells are not known. Given this evidence that the IL-6 signalling pathway is active in T-47D cells, it has the potential to modulate AR activity. This hypothesis is supported by previous observations that IL-6 can inhibit basal and DHT-induced stimulation of Apolipoprotein D and GCDFP-15 expression in ZR-75-1 breast cancer cells (Blais *et al*, 1995), although it remains unclear whether this activity is mediated by the AR.

Not all AR positive breast cancer cell lines express PSA in response to androgens (Yu *et al*, 1994b; Zarghami *et al*, 1997; Hsieh *et al*, 1997; Magklara *et al*, 2000). These observations suggest that AR expression is required, but not sufficient, for PSA expression in breast cancer cells. The existence of polymorphisms in the PSA promoter region, which may be correlated with PSA expression (Majumdar and Diamandis, 1999; Yang *et al*, 2000), could explain the differences between cell lines. Alternatively, cell specific cofactor expression profiles may be associated with PSA expression. This is supported by observations that the expression of the coactivator SRC1 is correlated with

PSA expression in different breast cancer cell lines (Magklara *et al*, 2002). T-47D has been the most widely used breast cancer cell line for the study of PSA expression. PSA mRNA and protein are induced by DHT, testosterone and mibolerone at concentrations as low as 0.2nM in these cells (Yu *et al*, 1994b; Hsieh *et al*, 1997; Magklara *et al*, 2000). The stimulation of PSA expression by androgens is abrogated by the AR antagonists RU56187, OHF and nilutamide (Zarghami *et al*, 1997), indicating the involvement of the AR. These observations have prompted the use of this cell line as an *in vitro* model for investigation of PSA regulation by androgens in the current study.

In this chapter, PSA has been used as a model gene to further investigate the mechanisms regulating androgen responsive gene expression in breast cancer cells. The specific aims of these studies were to evaluate the effects of DHT on PSA mRNA levels as well as to assess the function of the PSA promoter and enhancer in T-47D breast cancer cells with respect to their relative activity and capacity to recruit the AR transcription complex. Comparison of these events in T-47D breast cancer and LNCaP prostate cancer cells was also performed to gain a greater understanding of the differences and/or similarities between the mechanisms regulating PSA expression in these cell types. Furthermore, the effect of the cytokine IL-6 on PSA expression in T-47D cells was investigated to establish whether ligand independent AR signalling is functional in breast cancer cells.

8.2 – Methods

8.2.1 – Reporter gene assay

Plasmid DNA was prepared as described in Section 2.3.4. T-47D cells were seeded in 96 well plates at a density of 2 x 10^4 cells/well in phenol red free RPMI 1640 medium

containing 5%CSS. The following day, cells were transiently transfected with PSA_{630} (promoter)-luc or PSA_{630} (promoter + enhancer)-luc reporter plasmids (100ng/well) using Lipofectamine™ 2000 as described in Section 2.3.6. After transfection for 5hrs, cells were treated with vehicle (0.1% ethanol), DHT and/or IL-6 at 37°C for 36hrs. For IL-6 treatment, medium was supplemented with 1mg/mL BSA. Cells were lysed and assayed for luciferase activity using the Luciferase Assay System and the MLX or LUMIstar Galaxy plate reading luminometer. Results are expressed as the mean RLU ± SEM of four replicate transfections.

8.2.2 – RNA extraction and DNase I treatment

T-47D and LNCaP cells were seeded at a density of 6.8 x 10^5 cells/well in 6 well plates in phenol red free RPMI 1640 medium containing 5% CSS. After 48hrs medium was replaced with phenol red free RPMI 1640 medium containing 5% CSS and 10nM DHT, or vehicle (0.1% ethanol). Treatments were replenished every 24hrs. Cells were harvested by trypsinisation and collected by centrifugation at 2000rpm for 5mins at 4°C. Cell pellets were immediately lysed by addition of 175µL SV lysis buffer (from the SV Total RNA Isolation System). Lysates were homogenised with a 28-gauge needle and stored at -70°C. Cells were kept on ice throughout the harvesting procedure.

RNA was extracted from homogenised cell lysates using the SV Total RNA Isolation System according to instructions, with inclusion of a DNase I treatment step. Nucleic acid content and purity of each sample was estimated using a spectrophotometer as described in Section 2.3.5. RNA samples were stored at -70°C.

8.2.3 – Real time RT-PCR

The protocol used for real time RT-PCR is illustrated schematically in Figure 8.2. RNA (100-500ng) was reverse transcribed using the TaqMan® Reverse Transcription Reagents kit (Applied Biosystems) according to the manufacturer's instructions. 20μL reactions were performed containing 100-500ng RNA, 2.5μM random hexamers, 8U RNase inhibitor and 25U MultiScribe reverse transcriptase. A control reaction lacking reverse transcriptase was also performed for each RNA sample to verify the absence of genomic DNA contamination. An additional control reaction without RNA template was included to rule out contamination of reagents.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and PSA cDNA were amplified in separate real time PCR reactions containing 5μL cDNA template (diluted 1/5 in sterile H_2O), 12.5μL TaqMan PCR mastermix, 5μL sterile H_2O, 5pmol each of sense and antisense primers and 5pmol of the corresponding dual-labelled probe. PSA sense and antisense primers were used in conjunction with the PSA mRNA probe and GAPDH sense and antisense primers were used in conjunction with the GAPDH mRNA probe (see Appendix 2 for sequences). Exonic primers binding on either side of the junction between exons 3 and 4 were used to amplify PSA mRNA (Figure 8.1). A negative control reaction lacking cDNA template was included for each primer set in each run. Reactions were run in triplicate on the iCycler iQ real time PCR detection system with an initial template denaturation step at 94°C for 10mins followed by 55 cycles of denaturation at 94°C for 15secs and a combined annealing/extension step at 59°C for 1min. All samples to be directly compared in a particular experiment were included in the same PCR run.

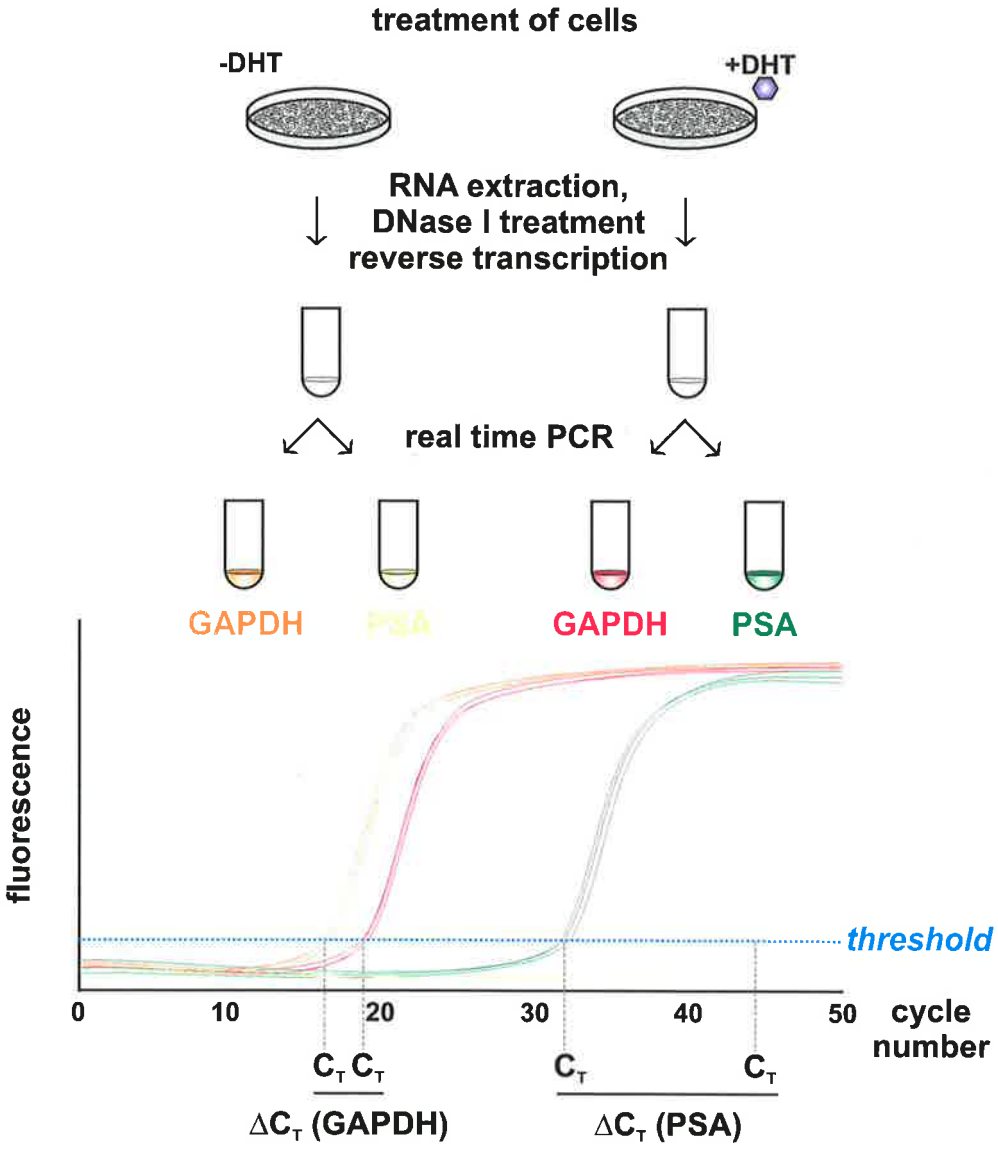


Figure 8.2: Schematic representation of experimental design for real time RT-PCR analysis of PSA and GAPDH mRNA. Following treatment of cells with steroid, RNA was extracted using the SV Total RNA Isolation System, with inclusion of a DNase I digestion step, and reverse transcribed. PSA and GAPDH cDNA were amplified in separate real time PCR reactions using specific primers and dual-labelled probes. C_T values for triplicate reactions were determined with QuickTime software and used to calculate relative mRNA levels (R) between samples and controls.

A standard curve was generated to determine the efficiency of GAPDH and PSA PCR reactions. Serial dilutions of a single cDNA sample (synthesised from RNA isolated from the LNCaP cell line by Dr Li Jia, Norris Comprehensive Cancer Center, University of Southern California) were set up in triplicate PCR reactions as described above. Reaction efficiency (E) was calculated using Formula 8.1[5]. C_T values were determined from amplification curves for each reaction by QuickTime software. Relative mRNA levels (R) were calculated using Formula 8.2, as described by Pfaffl (2001). When triplicate PCRs were performed for each gene for each cDNA, the mean R value for GAPDH was used to correct individual R values for PSA triplicates, giving relative expression values that were expressed as the mean ± SEM.

8.2.4 – ChIP Assay

8.2.4.1 – Preparation of ChIP DNA

The procedure used for ChIP assays is illustrated schematically in Figure 8.3. T-47D or LNCaP cells were seeded in 15cm petri dishes at a density of 7 x 10^6 cells/dish in phenol red free RPMI 1640 medium containing 5%CSS. After 48-72hrs, medium was replaced with phenol red free RPMI 1640 medium containing 5% CSS and DHT (10nM) or vehicle (0.1% ethanol). Protein-DNA complexes were cross-linked by addition of formaldehyde directly into the existing medium to a final concentration of 1% and incubation at room temperature for 10mins. Cells were washed twice with 20mL ice cold PBS and harvested by scraping in 1mL PBS containing 1x protease inhibitors. Tubes were centrifuged at 2000rpm for 5mins at room temperature and cell pellets were lysed in 700µL SDS lysis buffer for 15mins.

[5] Formula 8.1 for calculating reaction efficiency (E) differs from Formula 7.1, used in chapter 7, due to different slope values given by QuickTime software (used for real time PCR analysis in chapter 8) and Rotor-Gene software (used in chapter 7).

Formula 8.1:

$$E = 10^{[-1/slope]}$$

where slope is the slope of the standard curve (given by QuickTime software)

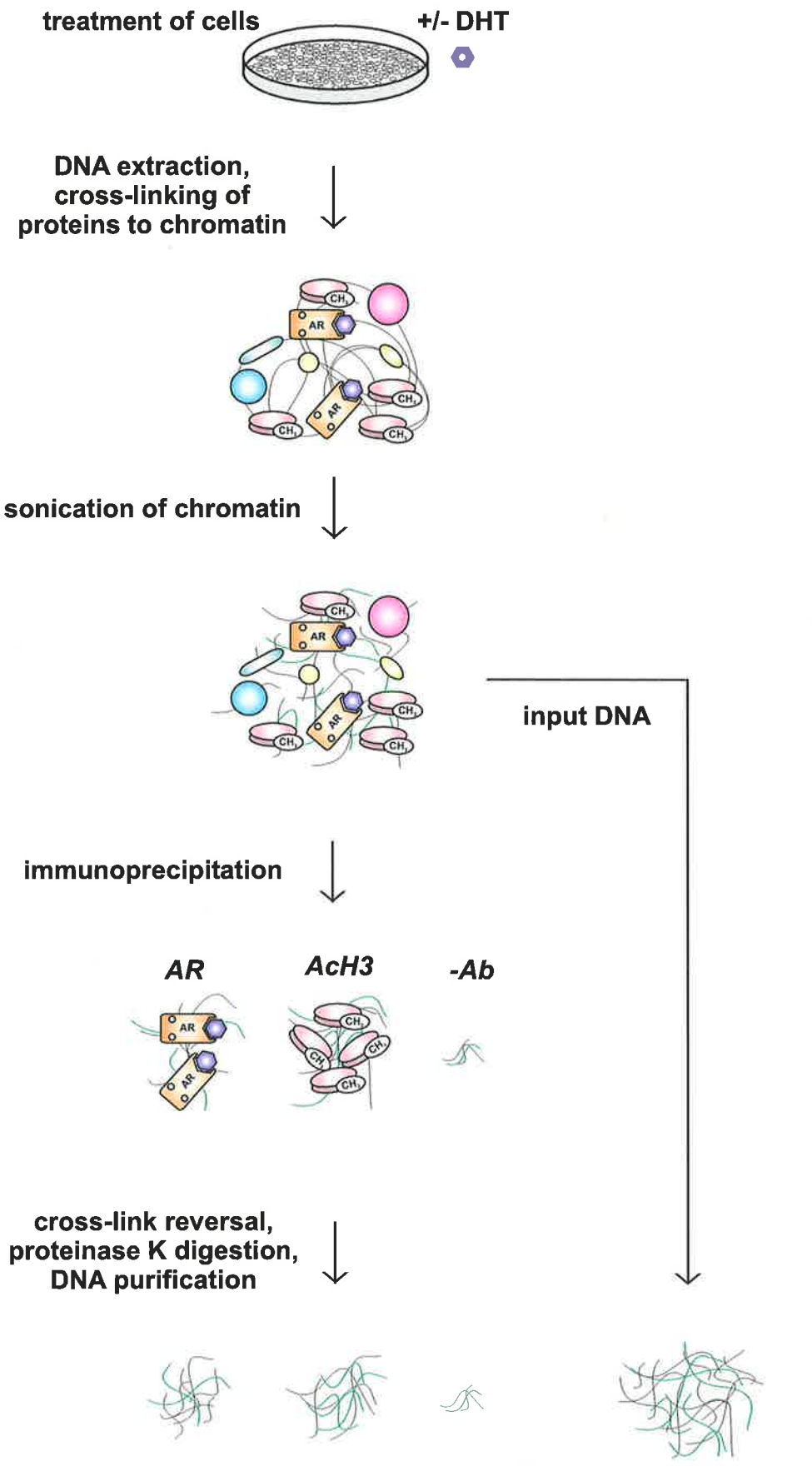
Formula 8.2:

$$R = \frac{E\,(PSA)^{\Delta CT\,(reference - sample)}}{E\,(GAPDH)^{\Delta CT\,(reference - sample)}}$$

where E = reaction efficiency for a given primer/probe set, and

$\Delta C_T = C_T$ for a reference sample – C_T for a given sample

Figure 8.3: The Chromatin ImmunoPrecipitation (ChIP) assay was used to measure AR and AcH3 binding to the PSA promoter and enhancer. Cells were stimulated with steroid for the appropriate time followed by cross-linking of protein complexes to the chromatin using formaldehyde. DNA-protein complexes were extracted and the chromatin sheared into shorter fragments (optimally 100-1000bp) by sonication. Immunoprecipitation with specific antibodies for AR and AcH3 isolated DNA bound to these proteins. Protein-DNA cross-links were reversed by incubation with sodium chloride at 65°C and proteins were digested with proteinase K. DNA fragments were purified by phenol:chloroform extraction and ethanol precipitation and used as templates in real time PCR reactions with primer sets and dual labelled probes targeting various regions of the PSA gene (see Figure 8.1). The use of standards containing known amounts of DNA in real time PCR reactions enabled measurement of the number of copies of ChIP DNA bound to each protein isolated by immunoprecipitation. Input DNA, isolated using the same procedures as ChIP DNA with exclusion of the immunoprecipitation step, was used as a control for variation in DNA amounts isolated from treated cells, while ChIP DNA subjected to immunoprecipitation without antibody (-Ab) was used as a negative control.


treatment of cells
+/- DHT
DNA extraction,
cross-linking of
proteins to chromatin
AR
CH3
sonication of chromatin
input DNA
immunoprecipitation
AR
AcH3
-Ab
cross-link reversal,
proteinase K digestion,
DNA purification
real time PCR analysis,
quantitation of immunoprecipitated DNA,
correction for input DNA

Genomic DNA was sheared into fragments using a sonic dismembrator, with continuous power set at 50%. Each lysate received up to 16 (for T-47D) or 5 (for LNCaP) 5sec sonication bursts with at least 1min on ice between each. Optimum conditions for sonication were determined by subjecting DNA to 1-16 sonication bursts, followed by purification (as described below) and electrophoresis on a 2% agarose-TAE gel. The minimum number of sonications required to shear DNA to 100-1000bp fragments was used for subsequent experiments. Insoluble material was collected by centrifugation at 13000rpm for 15mins at 4°C. Aliquots of soluble chromatin (100µL per immunoprecipitation) were diluted 1/10 with dilution buffer, and pre-cleared with 45µL protein G sepharose slurry and 2µg sheared salmon sperm DNA at 4°C for 1hr with rotation. Beads were collected by centrifugation at 5000rpm for 30secs at 4°C and the supernatant was combined with 4µg AR N-20 or 5µg AcH3 antibody. A negative control containing no antibody (-Ab) was also performed for each sample. Immunoprecipitations were performed at 4°C overnight with rotation.

Protein-DNA complexes were recovered by addition of 45µL protein G sepharose slurry and 2µg sheared salmon sperm DNA at 4°C for 1hr with rotation. Beads were collected by centrifugation at 5000rpm for 30secs at 4°C and washed sequentially with 1mL each of low salt immune complex wash buffer, high salt immune complex wash buffer and lithium chloride immune complex wash buffer, followed by two washes with 1mL 1x TE buffer. Each wash was performed for 5mins at 4°C with rotation. Protein-DNA complexes were eluted from the beads by resuspension in 2 x 250µL elution buffer and incubation for 15mins at room temperature with rotation.

Protein-DNA cross links were reversed by overnight incubation of eluates with 20µL 5M sodium chloride at 65°C. Proteins were then digested in 10mM EDTA,

40mM Tris-HCl pH 6.5 and 20μg proteinase K at 45°C for 1hr. DNA was purified using one extraction with phenol:chloroform:isoamyl alcohol and one extraction with chloroform, followed by precipitation with two volumes 100% ethanol and 20μg glycogen for 2-3hrs at -70°C. DNA pellets were washed with 70% ethanol, air-dried in a laminar flow cabinet designated for PCR and resuspended in 100μL sterile H_2O.

8.2.4.2 – Preparation of input DNA

A 20μL aliquot of each sample of soluble chromatin, representing "input" DNA, was set aside prior to immunoprecipitation. These samples were made up to 500μL with elution buffer and included in the above procedure at the reverse cross linking step. Following precipitation of input DNA, pellets were resuspended in 200μL sterile H_2O.

8.2.4.3 – Real time PCR

ChIP and input DNA samples were prepared as described above. Real time PCRs were performed on three regions of the PSA gene as indicated in Figure 8.1: the PSA enhancer (using PSA ARE III sense and antisense primers and the PSA ARE III probe), the PSA promoter (using the PSA ARE I sense and antisense primers and the PSA ARE I probe) and a downstream irrelevant region (using PSA irrelevant region sense and antisense primers and the PSA irrelevant region probe). Primer and probe sequences are indicated in Appendix 2. Negative controls (without DNA template) and standards were also included in each run. The standards consisted of serial dilutions of a concentrated stock of genomic DNA from LNCaP cells (prepared by Dr Li Jia, University of Southern California). Starting quantity (in copy numbers) was calculated

by QuickTime software for each reaction based on the C_T value and the standard curve. Copy numbers determined for ChIP samples were normalised for input DNA (the mean of triplicate reactions) and expressed as the mean immunoprecipitated DNA/input DNA ratio ± SEM.

8.3 – Results

8.3.1 – Comparative analysis of androgen inducibility of the PSA promoter and enhancer in T-47D cells

A dose dependent increase in endogenous AR activity was observed in T-47D cells transiently transfected with the PSA_{630} (promoter)-luc reporter plasmid, with a significant 3.6-fold induction attained with 100nM DHT (Figure 8.4 (a)). In T-47D cells transiently transfected with the PSA_{630} (promoter + enhancer)-luc reporter plasmid, a considerably higher level of AR activity was observed, with a maximal 550-fold induction attained in response to 100nM DHT (Figure 8.4 (b)). This result is consistent with previous studies which have also observed greater AR activity in T-47D breast cancer and LNCaP prostate cancer cells transfected with similar reporter constructs containing the PSA promoter and enhancer compared the promoter alone (Schuur *et al*, 1996; Cleutjens *et al*, 1997; Zhang *et al*, 1997c).

8.3.2 – Ligand independent induction of PSA promoter and enhancer activity in T-47D cells

In T-47D cells transiently transfected with the PSA_{630} (promoter + enhancer)-luc reporter plasmid, treatment with IL-6 alone induced a dose dependent increase in AR activity, with a maximal 24-fold induction observed at a dose of 50ng/mL (Figure 8.5 (a)). This compares to a 60-fold induction of AR activity induced by 1nM DHT. Both

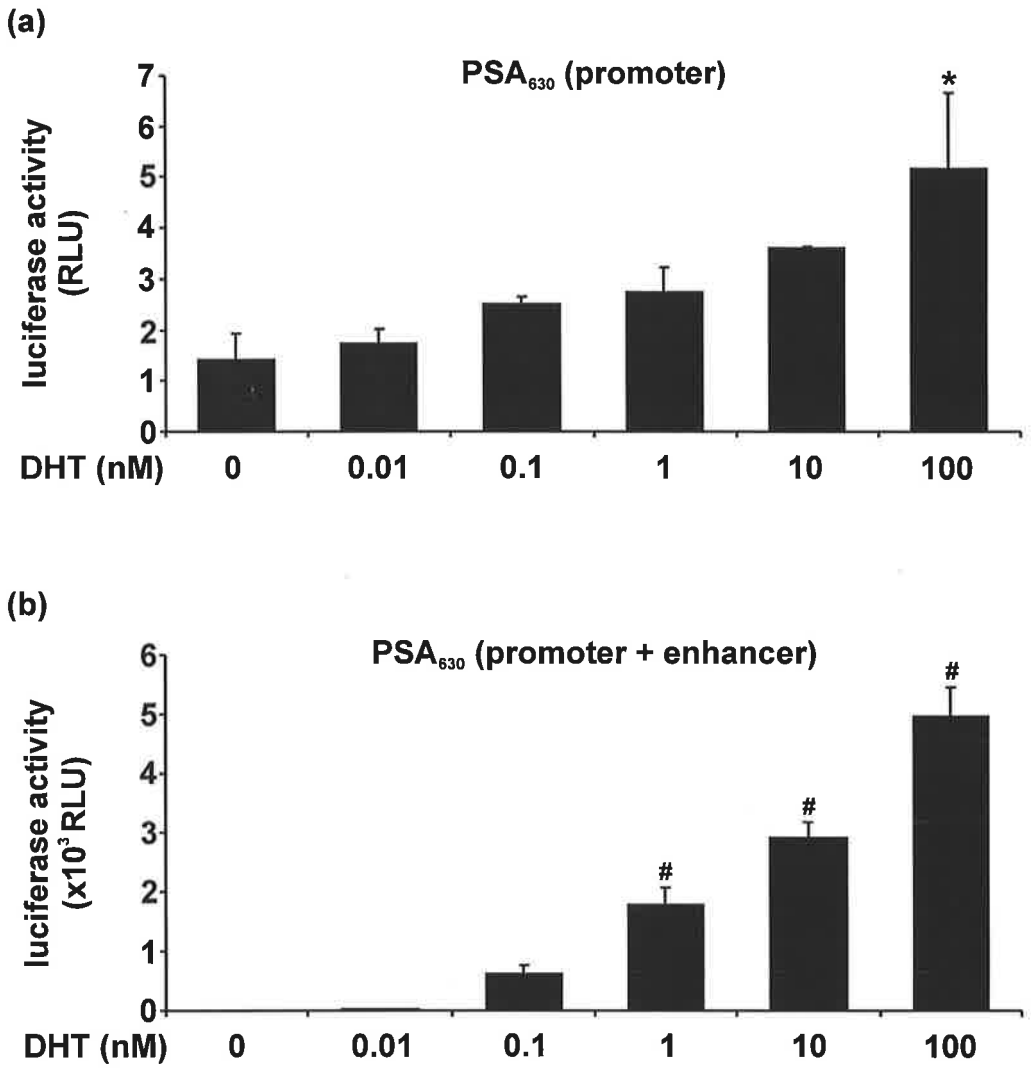


Figure 8.4: Effect of DHT on AR transactivation activity at an exogenous PSA promoter and enhancer in the T-47D breast cancer cell line. Cells were seeded in 96 well plates (2 x 10^4 cells/well) and transiently transfected with 100ng/well of the *(a)* PSA_{630} (promoter)-luc or *(b)* PSA_{630} (promoter + enhancer)-luc reporter plasmids using Lipofectamine 2000. Five hours after transfection, cells were treated with phenol red free RPMI medium containing 5% CSS and increasing concentrations of DHT (0.01-100nM) or vehicle (0.1% ethanol) as indicated. After 36hrs, the medium was removed and cell lysates were assayed for luciferase reporter gene activity using the Luciferase Assay System. Each bar represents the mean +/- SEM of 4 replicate wells. * ANOVA; p = 0.019: DHT (100nM) *versus* control. # ANOVA; $p < 0.002$: DHT *versus* control.

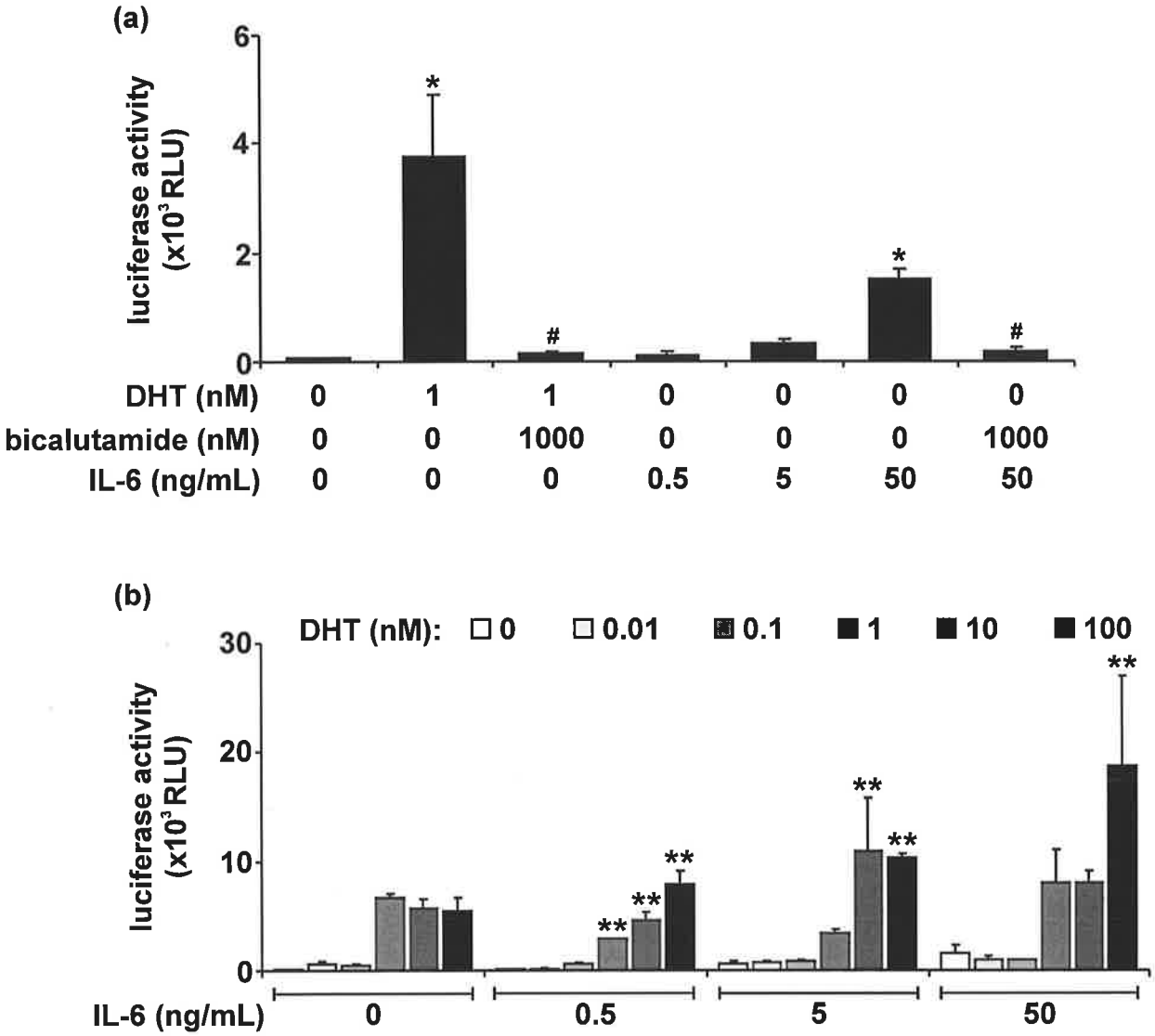


Figure 8.5: Effect of IL-6 and DHT on AR transactivation activity at an exogenous PSA promoter and enhancer in the T-47D breast cancer cell line. Cells were seeded in 96 well plates (2×10^4 cells/well) and transfected with 100ng/well of the PSA_{630} (promoter + enhancer)-luc reporter plasmid using Lipofectamine 2000. Five hours after transfection, cells were treated with phenol red free RPMI medium containing 5% CSS, 1mg/mL BSA and various concentrations of *(a)* DHT or IL-6 alone (+/- bicalutamide) or *(b)* DHT and IL-6 combined. After 36hrs, the medium was removed and cell lysates were assayed for luciferase reporter gene activity using the Luciferase Assay System. Each bar represents the mean +/- SEM of a minimum of 3 replicate wells. * ANOVA; $p = 0.0$: IL-6 (50ng/mL) *versus* control. # ANOVA; $p = 0.0$: IL-6 (50ng/mL) + bicalutamide *versus* IL-6 (50ng/mL). ** ANOVA; $p < 0.05$: DHT + IL-6 *versus* IL-6.

DHT and IL-6 induced AR activity was significantly blocked by co-incubation with bicalutamide (1μM), indicating that the activity induced by both compounds at the PSA promoter + enhancer is mediated by the endogenous AR.

The ability of IL-6 to alter AR activation by DHT was also examined (Figure 8.5 (b)). At concentrations greater than 1nM, DHT had an additive effect on AR activity induced by IL-6. Conversely, IL-6 also had a dose-dependent additive effect on AR activity induced by 100nM DHT, although this did not reach significance.

8.3.3 – Analysis of endogenous PSA mRNA accumulation in T-47D cells stimulated with DHT and/or IL-6

The experimental design for real time RT-PCR analysis of PSA mRNA levels in T-47D cells is illustrated schematically in Figure 8.2. Standard curves were generated for each primer set by running reactions containing serial dilutions of template cDNA (performed by Dr Li Jia, University of Southern California). Reaction efficiency (E) was calculated as 1.95 for GAPDH and 1.9 for PSA in two independent PCR runs. R^2 values were greater than 0.995 for all runs. Real time RT-PCR amplification of GAPDH from reverse transcription negative control reactions performed in the absence of reverse transcriptase indicated that residual genomic DNA levels in RNA preparations were extremely low, accounting for < 0.0001% of available template.

PSA mRNA was undetectable in T-47D cells treated with DHT (10nM) for up to 12hrs, however PSA mRNA was detected at 24hrs, with levels significantly increased at 72hrs (Figure 8.6 (a)). This increase was not due to variation in GAPDH mRNA levels (data not shown).

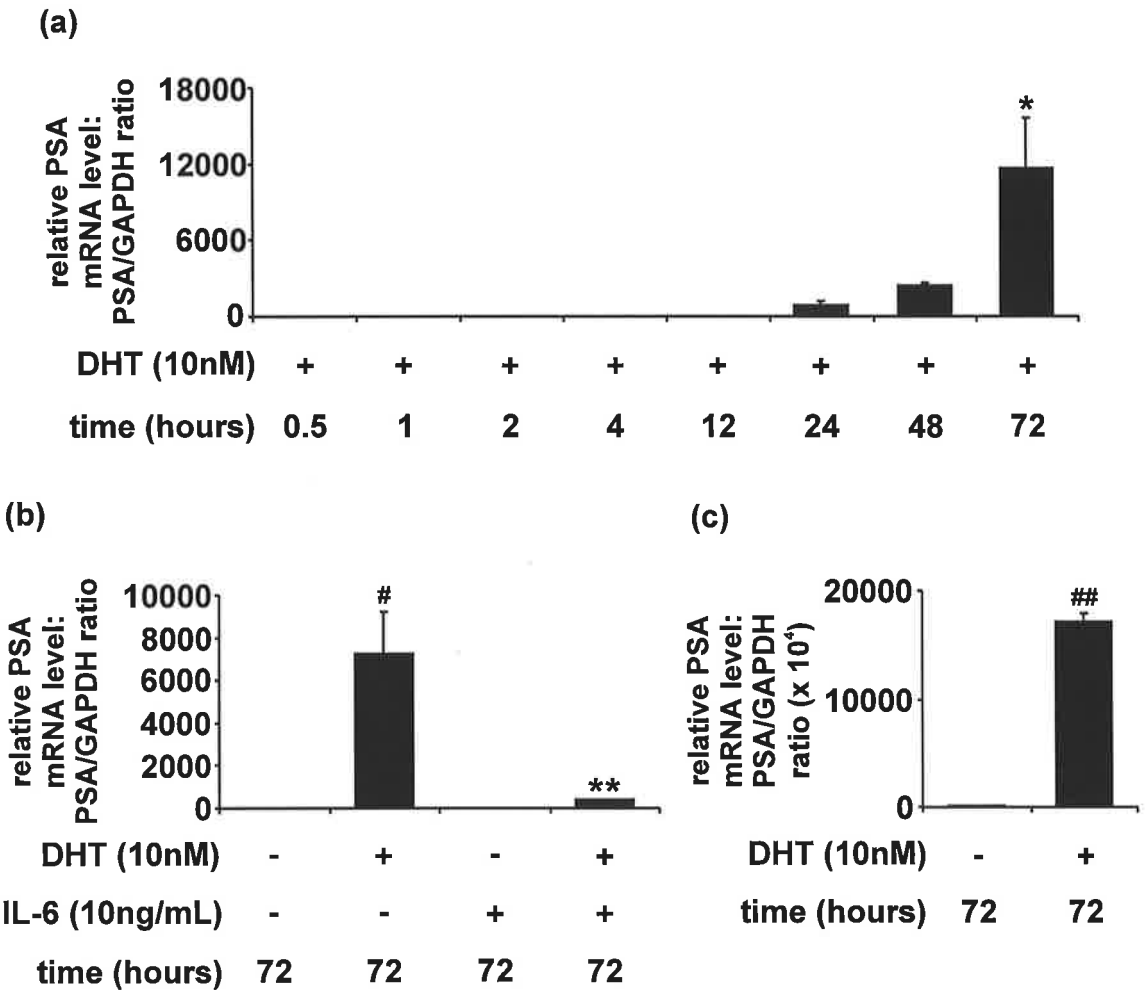


Figure 8.6: Effect of DHT and IL-6 on PSA mRNA levels in T-47D breast cancer and LNCaP prostate cancer cells. Cells were seeded in 6 well plates (7×10^5 cells/well) in phenol red free RPMI medium containing 5% CSS. *(a)* T-47D cells were treated with DHT (10nM) for 0-72hrs. *(b)* T-47D cells were treated with DHT (10nM) and/or IL-6 (10ng/mL) for 72hrs. *(c)* LNCaP cells were treated with DHT (10nM) for 72hrs. *(b)* and *(c)* were performed in parallel. Total RNA was extracted and reverse transcribed using random hexamers and MultiScribe reverse transcriptase. PSA and GAPDH mRNA were amplified in separate real-time PCR reactions incorporating dual labelled probes and relative expression levels (R) were calculated. PSA mRNA was corrected for GAPDH and is expressed as relative levels, calculated as the mean +/- SEM of triplicate reactions. * ANOVA; p = 0.014: DHT 72hrs *versus* DHT 0.5hr. # ANOVA; p = 0.004: DHT *versus* control. ** ANOVA; p = 0.007: DHT + IL-6 *versus* DHT. ## ANOVA; p = 0.0: DHT *versus* control.

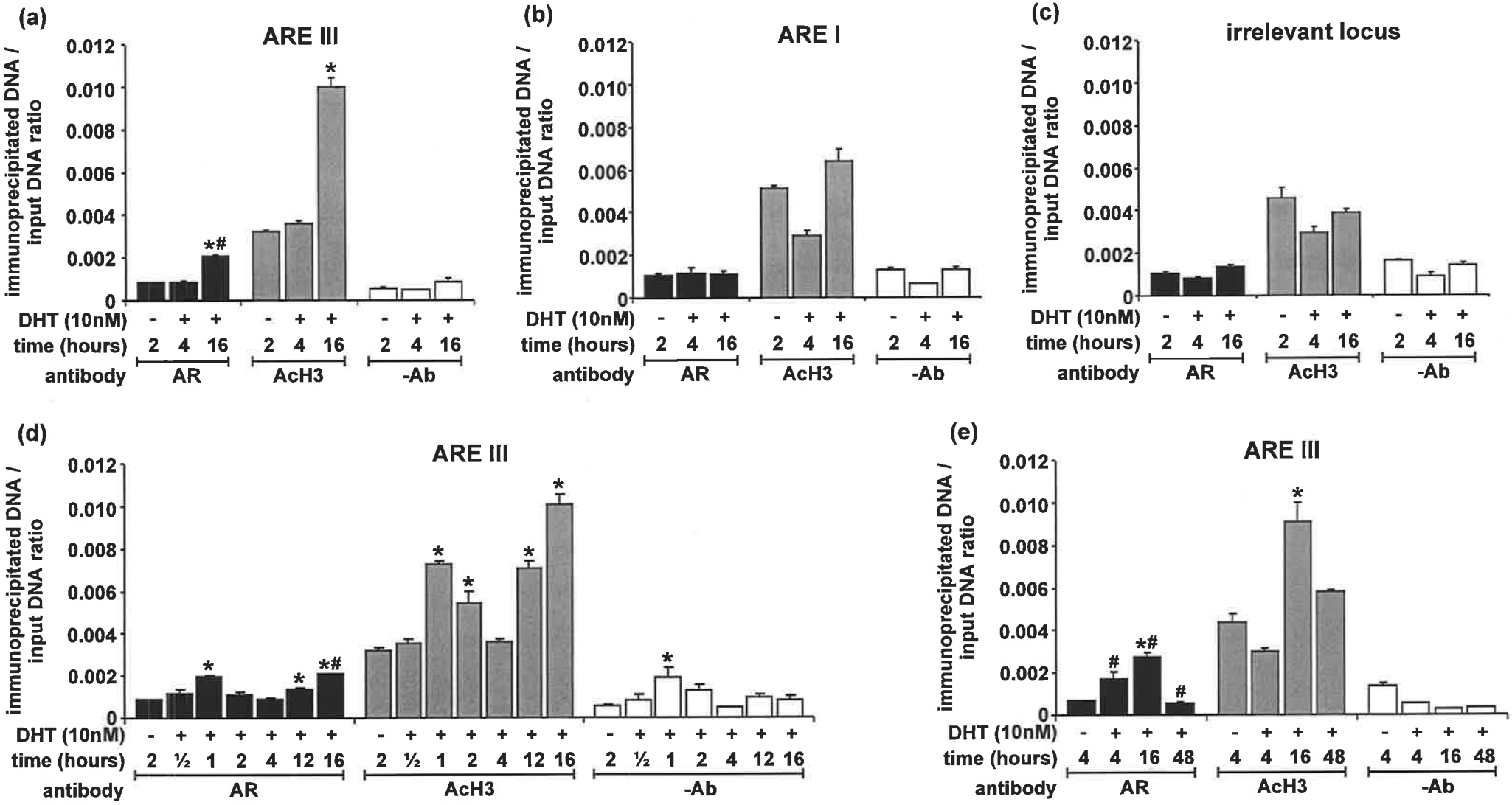
PSA mRNA was not detectable in T-47D cells treated with 10ng/mL IL-6 for up to 72hrs. However, IL-6 significantly inhibited PSA mRNA accumulation induced by 10nM DHT at 72hrs (Figure 8.6 (b)).

PSA mRNA accumulation in breast and prostate cells was compared by parallel treatment of T-47D and LNCaP cells with DHT (10nM) for 72hrs. DHT treatment caused a 120-fold increase in PSA mRNA levels in LNCaP cells (Figure 8.6 (c)). However after 72hrs of DHT treatment, LNCaP cells expressed 26000-fold more PSA than T-47D cells. Basal PSA mRNA levels in LNCaP cells were also markedly higher than basal or DHT-induced PSA in T-47D cells.

8.3.4 – Analysis of AR occupancy and histone acetylation on the PSA promoter and enhancer in T-47D cells

DHT-induced AR occupancy and histone H3 acetylation at different loci of the endogenous PSA gene in T-47D cells were examined using ChIP assays coupled with real time PCR to quantitate immunoprecipitated DNA. The experimental design for ChIP assays is schematically illustrated in Figure 8.3. After 4hrs of DHT (10nM) treatment, there was no change in AR occupancy or histone H3 acetylation at the PSA ARE III compared to the vehicle treated control (Figure 8.7 (a)). However DHT induced a significant 2.5-fold increase in AR occupancy and a significant 3.1-fold increase in histone H3 acetylation at this locus after 16hrs. In contrast, there was no significant increase in AR occupancy or histone H3 acetylation at ARE I over 16hrs of treatment with DHT (Figure 8.7 (b)). The increased AR occupancy observed at the PSA enhancer compared to the promoter may simply be a consequence of the higher density of AREs in the enhancer region. Quantitatively higher levels of AR occupancy at the PSA enhancer compared to the promoter have previously been observed in LNCaP cells

Figure 8.7: Examination of AR occupancy and histone H3 acetylation on the PSA gene in T-47D breast cancer cells. Cells were seeded in 150mm petri dishes (7×10^6 cells) in phenol red free RPMI medium containing 5% CSS. After 72hrs, cells were treated with vehicle (0.1% ethanol) or DHT (10nM) for the indicated times. AR occupancy and histone H3 acetylation at the PSA *(a)* ARE III, *(b)* ARE I, and *(c)* downstream irrelevant region were examined over 16hrs using ChIP analysis. *(d)* AR occupancy and histone H3 acetylation at the PSA ARE III was examined in a more detailed time course experiment. *(e)* AR occupancy and histone H3 acetylation at the PSA ARE III over 48hrs. Values for immunoprecipitated DNA are presented as the mean (corrected for input DNA) +/- SEM of triplicate PCRs. Input DNA was prepared in parallel with ChIP DNA but was not subjected to the immunoprecipitation step. * ANOVA; $p < 0.05$: DHT *versus* control. # ANOVA; $p < 0.05$: AR *versus* -Ab control.


(a)
ARE III
(b)
ARE I
(c)
irrelevant locus
(d)
ARE III
(e)
ARE III
immunoprecipitated DNA / input DNA ratio
0.012
0.010
0.008
0.006
0.004
0.002
0
DHT (10nM)
time (hours)
antibody
AR
AcH3
-Ab
2 4 16
2 ½ 1 2 4 12 16
4 4 16 48

(Jia *et al*, 2003; Louie *et al*, 2003). No increases in AR occupancy or histone H3 acetylation were detected at the PSA irrelevant locus following DHT treatment (Figure 8.7 (c)), as expected for a region of DNA that contains no AREs and does not confer androgen inducibility.

As the ARE III was the only locus tested to show an increase in AR occupancy and histone H3 acetylation in response to DHT, more detailed time course experiments were performed to monitor AR occupancy and histone acetylation at this locus. Figure 8.7 (d) shows that AR occupancy and histone H3 acetylation fluctuated over a 16hr treatment period. This may be accounted for by differences in input DNA, as immunoprecipitated DNA from the –Ab controls fluctuated throughout the time course. As PSA mRNA was not detected in T-47D cells after 16hrs of DHT treatment (Figure 8.6 (a)), the ChIP time course was extended in an independent assay to include a time point that correlates with induction of PSA mRNA. AR occupancy and histone H3 acetylation significantly increased after 16hrs of DHT treatment relative to the untreated control (Figure 8.7 (e)), which is consistent with results from previous assays (Figure 8.7 (a)). However, at 48hrs, both AR occupancy and histone acetylation fell to control levels. These changes were not correlated with variation in the –Ab controls.

Induction of AR occupancy and histone acetylation by DHT at the PSA promoter and enhancer in T-47D cells was not as strong as that reported for LNCaP cells (Jia *et al*, 2003). In order to directly compare these events in breast and prostate cells, ChIP assays on T-47D and LNCaP cells treated with DHT (10nM) for 2hrs were performed in parallel. Figure 8.8 (a) shows no significant increase in AR occupancy or histone H3 acetylation at the PSA ARE III after treatment of T-47D cells with DHT for 2hrs. However immunoprecipitation at this time point was not significantly above –Ab

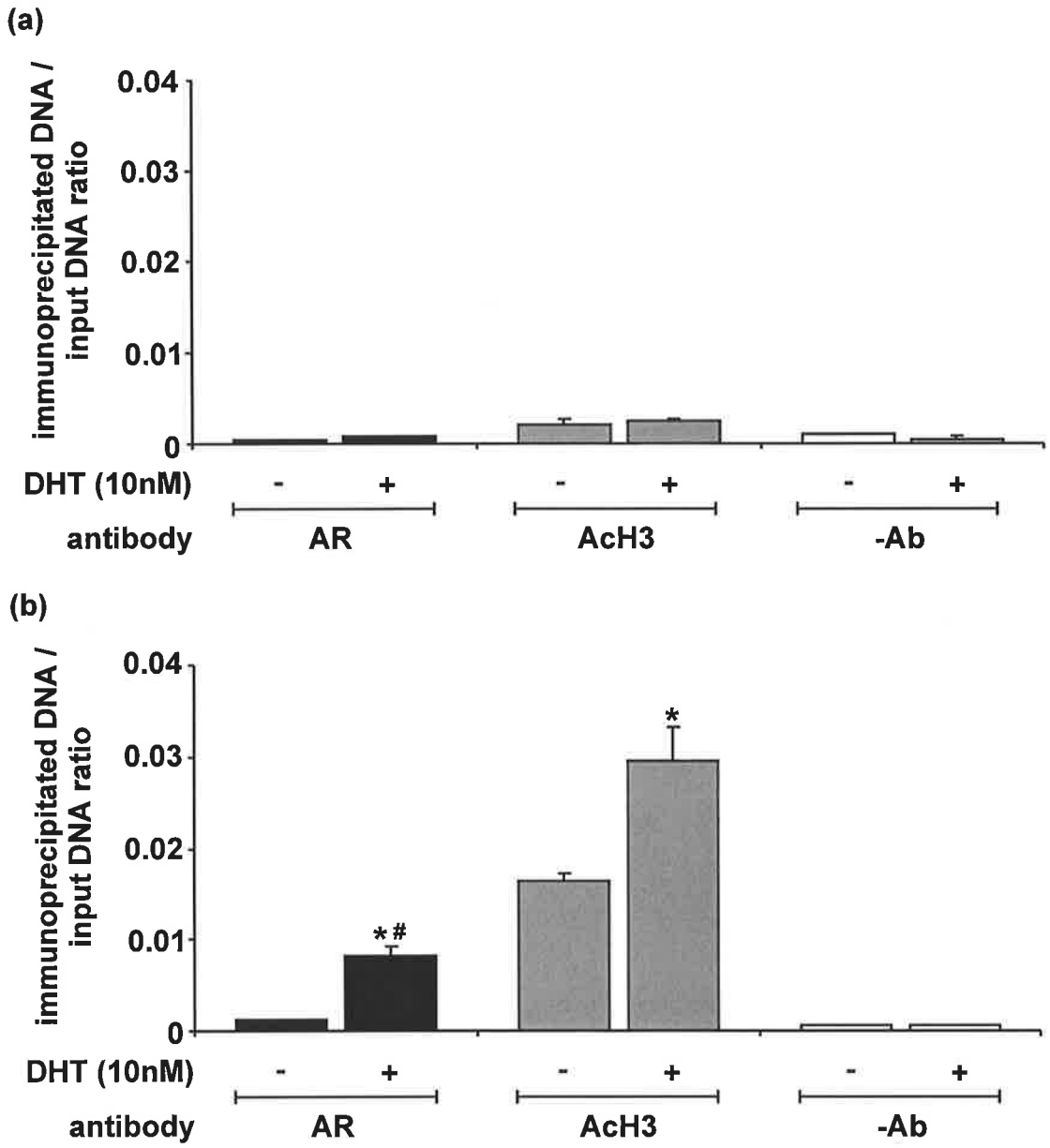


Figure 8.8: Comparison of AR occupancy and histone H3 acetylation at the ARE III in *(a)* T-47D breast cancer and *(b)* LNCaP prostate cancer cells. Cells were seeded in 150mm petri dishes (7 x 10^6 cells) in phenol red free RPMI medium containing 5% CSS. After 72hrs, cells were treated with vehicle (0.1% ethanol, white bars)) or DHT (10nM, black bars) for 2hrs. AR occupancy and histone H3 acetylation at the PSA ARE III were examined by ChIP analysis. Values for immunoprecipitated DNA are presented as the mean (corrected for input DNA) +/- SEM of triplicate PCRs. Input DNA was prepared in parallel with ChIP DNA but was not subjected to the immunoprecipitation step. * ANOVA; $p = 0.0$: DHT *versus* control. # ANOVA; $p = 0.0$: AR *versus* -Ab control.

controls. In contrast, DHT induced a significant 6.5-fold increase in AR occupancy and a 1.8-fold increase in histone H3 acetylation at the same locus in LNCaP cells after 2hrs (Figure 8.8 (b)). Immunoprecipitation was also significantly increased above the –Ab controls in LNCaP cells at this time point. These results suggest that induction of AR occupancy at the PSA ARE III by DHT in LNCaP cells was more rapid and robust than in T-47D cells, and indicate that the low levels of AR occupancy detected at this element in T-47D cells was not due to failure of the assay.

8.4 – Discussion

The results presented in this chapter describe the regulation of PSA expression by androgens in T-47D breast cancer cells with respect to: (i) androgen inducibility of the transiently transfected PSA promoter and enhancer elements, (ii) induction of endogenous PSA mRNA accumulation in response to DHT treatment, and (iii) androgen-induced AR occupancy and histone acetylation at the endogenous PSA promoter and enhancer. This study has been the first to examine these different aspects of PSA gene regulation by androgens in breast cancer cells. Although there was no apparent lag in the time between AR occupancy and histone H3 acetylation in T-47D cells, these events occurred prior to detection of PSA mRNA. These results support a model in which AR binding to DNA and modification of chromatin structure by histone acetylation, occurring predominantly at the enhancer, is a prerequisite for synthesis of PSA mRNA following exposure to DHT.

In T-47D cells, DHT-induced increases in AR occupancy and histone H3 acetylation occurred at the endogenous PSA enhancer but not at the promoter. This result supports the importance of the enhancer for induction of PSA expression. Furthermore, this

result is consistent with the model proposed by Louie (2003), which suggests that the AR transcription complex is only recruited to the PSA enhancer and that RNA polymerase is transported along the chromatin to the promoter, where it initiates transcription. Quantitatively higher levels of AR occupancy at the PSA enhancer compared to the promoter have previously been observed in LNCaP cells (Jia *et al*, 2003; Louie *et al*, 2003). However, increased AR occupancy at the enhancer may be a consequence of the higher density of AREs in this region compared to the promoter.

AR occupancy at the PSA enhancer in T-47D cells may be transient, as ChIP assays demonstrated fluctuation in the amount of DNA occupied by the AR over the first 16hrs of DHT treatment, followed by a fall between 16hrs and 48hrs. Cycling of ER or AR and associated cofactors and transcription factors on and off DNA regulatory elements has been described previously (Shang *et al*, 2000; Kang *et al*, 2002). The proteasome complex, which mediates degradation and turnover of transcription factor molecules, may play an important role in this process as release of the AR from the PSA promoter in LNCaP cells is abolished in the presence of the 26S proteasome inhibitor, MG-132 (Kang *et al*, 2002). It is proposed that release of the AR and its associated cofactors from the DNA allows for continuous monitoring and response to the cellular environment. An important difference between previous studies and the current study is the use of real time PCR to analyse ChIP and input DNA, rather than the traditional method of agarose gel analysis of PCR products. This modification in the protocol enables more accurate quantitation of DNA amounts, and may be more sensitive in detecting subtle changes in protein-DNA binding.

The results from current experiments investigating different aspects of PSA regulation by androgens in T-47D and LNCaP cells, together with results previously published,

imply that the mechanisms regulating PSA expression by androgens are similar in these two cell types, although the extent of PSA induction, and perhaps the mechanisms controlling it, occur to a lesser degree in T-47D cells. There are a number of possible reasons for why this may occur. The most obvious is the difference in AR levels between the two cell lines. Previous studies have shown that T-47D breast cancer cells express considerably less AR than LNCaP prostate cancer cells (Hall *et al*, 1992; Magklara *et al*, 2002). In addition, ligand binding analysis has demonstrated that T-47D cells display comparatively low saturation binding of the synthetic androgen R1881 compared to other breast cancer cell lines, such as BT-474 which expresses similar amounts of AR (Magklara *et al*, 2002). This suggests that T-47D cells may have a reduced sensitivity to stimulation by androgens compared to other breast, and prostate, cancer cell lines. Low AR expression levels in T-47D cells may also explain the difficulty experienced in isolating significantly more ChIP DNA with the AR antibody compared to the –Ab control at some time points of the assay. No such difficulties were encountered in LNCaP cells. Furthermore, the low level of AR occupancy observed at the PSA enhancer in T-47D cells compared to LNCaP cells may also explain, at least in part, the higher concentration of androgen (ie 100nM) required to induce maximal AR activity at the PSA promoter/enhancer in T-47D cells compared to that required in LNCaP cells (ie 1nM, (Schuur *et al*, 1996)).

Transient transfection of luciferase reporter genes linked to either the PSA promoter or PSA promoter/enhancer allowed direct comparison of their androgen inducibility in T-47D breast cancer cells. Whereas minimal androgen induction was observed at the PSA promoter, the enhancer vastly increased AR activity. These results are consistent with those observed previously in LNCaP and T-47D cells transiently transfected with similar reporter constructs, although the magnitude of AR induction differs between

previous and current studies. Cleutjens *et al* (1997) reported greater androgen inducibility in LNCaP cells than that observed in the current study in T-47D cells – a 4-fold induction at the PSA promoter and a 3000-fold induction at the PSA promoter/enhancer in LNCaP cells treated with 1nM R1881. This may be in part attributed to the higher levels of AR expressed in LNCaP cells compared to T-47D cells (Hall *et al*, 1992; Magklara *et al*, 2002). A 1.1-fold induction of AR activity by R1881 at the PSA promoter (Cleutjens *et al*, 1996) and a 17-fold induction at the promoter/enhancer (Cleutjens *et al*, 1997) has also been observed previously in T-47D cells, which is weaker than the induction of AR activity observed at these response elements in the current study. These discrepancies may be attributed to variation in transfection efficiency or the use of different promoter and enhancer sequences in the reporter constructs. Other transcription factors in addition to steroid receptors have been implicated in the regulation of PSA expression, at least in prostate cells (Schuur *et al*, 1996; Yeung *et al*, 2000; Perez-Stable *et al*, 2000; Oettgen *et al*, 2000; Farmer *et al*, 2001). Therefore, inclusion or exclusion of binding sites for additional regulatory factors in the different reporter constructs used may also potentially explain the variation in AR activity observed between the current and previous studies. Furthermore, PSA expression is responsive to progestins (Yu *et al*, 1994b; Zarghami *et al*, 1997; Hsieh *et al*, 1997; Kogan *et al*, 1998; Magklara *et al*, 2000) and the use of R1881, which has been demonstrated to interact with the PR (Zava *et al*, 1979; Asselin *et al*, 1979), by Cleutjens may have contributed to the reduced AR activity observed in comparison to that induced by DHT in the current studies.

Although the magnitude of AR transactivation activity measured at the PSA promoter and enhancer is lower in T-47D breast cancer cells compared to LNCaP prostate cancer cells, the results from reporter gene assays suggest that both the promoter and enhancer

are critical for the regulation of PSA expression by androgens in both cell types. Furthermore, these studies imply that T-47D cells possess the intracellular factors (cofactors, transcription factors etc) required for androgen induction of endogenous PSA expression. It is interesting to note that expression of AR and appropriate intracellular factors may not be sufficient for endogenous PSA expression. AR positive breast cancer cell lines such as ZR-75-1, MDA-MB-453 and MCF-7 do not express detectable amounts of PSA (Hsieh *et al*, 1997; Magklara *et al*, 2000). Of these cell lines, MDA-MB-453 expresses the highest levels of AR, comparable to LNCaP cells (Hall *et al*, 1994). Transient transfection of the PSA_{630} (promoter + enhancer)-luc reporter construct into these cells results in induction of endogenous AR activity by DHT (see Section 5.3.4.3). This suggests that MDA-MB-453 cells, like T-47D and LNCaP, contain the necessary machinery to activate AR activity at the PSA promoter/enhancer. However an additional, as yet unknown mechanism may prevent AR activity at the endogenous PSA promoter, and PSA mRNA synthesis, in MDA-MB-453 cells. This observation highlights the potential shortcomings of assays using transiently transfected reporter genes and has led to the recent use of assays investigating endogenous, chromatin integrated genes. Analysis of PSA mRNA accumulation coupled with ChIP assays may enable a more physiological assessment of AR activity in T-47D breast cancer cells in comparison to transient transfection of androgen responsive reporter genes.

In the current studies, PSA mRNA was not detected in T-47D cells until 24hrs after treatment with DHT (10nM). This is not consistent with the relatively short time required for induction of genes that are directly regulated by androgens. For example, induction of PSA mRNA by DHT occurs within one hour in LNCaP cells (Young *et al*, 1991; Shang *et al*, 2002). In the BT-474 breast cancer cell line, PSA mRNA is

detectable 4hrs after DHT treatment (Magklara *et al*, 2000). While T-47D is used as a model of androgen induction of PSA expression in breast cancer, previous studies have not measured PSA mRNA in these cells within the first 24hrs of exposure to DHT, as was performed in the current study. In one previous study, PSA mRNA, measured using RT-PCR, was just detectable in T-47D cells after exposure to DHT for 28hrs (Hsieh *et al*, 1997) while PSA protein in conditioned medium from androgen stimulated T-47D cells was detectable at 48hrs (Yu *et al*, 1994b). In the current study, ChIP experiments showed that AR occupancy at the PSA enhancer was not significantly increased in T-47D cells until 16hrs after DHT treatment, which may potentially explain why PSA mRNA was not detectable until 24hrs. In contrast, AR occupancy at the PSA enhancer in LNCaP cells was detected within 2hrs of DHT treatment. These results suggest that the induction of PSA expression by androgens in T-47D breast cancer cells is delayed compared to the rapid induction observed in LNCaP prostate cells, which may be a function of the difference in AR levels between these cell lines.

Further studies using ChIP assays will be necessary to define the key coactivators and transcription factors involved in regulating the expression of androgen responsive genes in breast cancer cells. The current studies have demonstrated that investigation of PSA expression in the T-47D cell line is an appropriate model for investigating mechanisms associated with the induction of gene expression by androgens in breast cancer cells. However, the use of other androgen regulated genes, and other AR positive breast cancer cell lines, may further enhance our understanding of the mechanisms regulating androgen responsive genes in breast cancer cells. The BT-474 breast cancer cell line expresses considerably higher levels of PSA than T-47D cells in the presence and absence of androgen (Magklara *et al*, 2000), which may therefore make it a suitable alternative model in which to study composition of the AR transcription complex in

breast cancer cells. Other more highly expressed androgen regulated genes in breast cancer cells may also be suitable alternatives to PSA for use as a model gene. BT-474 cells produce high levels of another androgen-induced kallikrein, hK2 (Magklara *et al*, 2000; Magklara *et al*, 2002). In LNCaP cells, AR and RNA polymerase II are recruited to the hK2 promoter in response to DHT (Kang *et al*, 2002). Together, these observations suggest that hK2 may be appropriate for further *in vivo* study of gene regulation by androgens. Fibroblast growth factor 8 (FGF8) is another well characterised androgen regulated gene. Previous studies using ChIP assays have shown that the promoter region of FGF8 recruits AR in LNCaP cells in response to androgens (Gnanapragasam *et al*, 2002). FGF8 is also regulated by androgens in breast cancer cells, and is correlated with PSA expression levels (Payson *et al*, 1996; Tanaka *et al*, 2002), indicating that it may be another interesting gene to further investigate in ChIP assays.

Ligand independent activation of the AR in breast cancer cells has not previously been investigated. Although there is one report that demonstrates IL-6 inhibition of two androgen regulated genes, GCDFP-24/Apolipoprotein D and GCDFP-15, in ZR-75-1 breast cancer cells, no evidence was provided that this was mediated through the AR (Blais *et al*, 1995). As estrogens can also down-regulate the expression of these genes (Simard *et al*, 1989; Simard *et al*, 1990), and IL-6 can act through the ER (Speirs *et al*, 1993b; Speirs *et al*, 2000), it is possible that the inhibitory effects of IL-6 in that study were mediated by the ER rather than the AR. Because PSA expression is not influenced by estrogen signalling pathways in the absence of DHT (Yu *et al*, 1994b; Zarghami *et al*, 1997; Hsieh *et al*, 1997; Magklara *et al*, 2000), the current results provide evidence that IL-6 and AR signalling pathways interact in T-47D cells. This is further demonstrated by experiments showing that the AR antagonist bicalutamide can block

IL-6 induced AR activity at the PSA promoter/enhancer. Interestingly, IL-6, at least at high concentrations, had a weak additive effect on DHT-induced AR activity at the PSA promoter/enhancer while IL-6 inhibited DHT-induced PSA mRNA levels. These differences in the effect of IL-6 can potentially be explained by the use of assays measuring exogenous reporter genes *versus* endogenous PSA mRNA levels. Discrepancies in the effect of IL-6 on AR activity or PSA mRNA levels in the presence of DHT have also been observed in previous studies using prostate cancer cells (Hobisch *et al*, 1998; Ueda *et al*, 2001; Jia *et al*, 2003). Nevertheless, the current results have demonstrated that IL-6 can modulate AR activity in breast cancer cells. This implies that the inhibitory effect of IL-6 on proliferation of T-47D cells, demonstrated in previous studies (Tamm *et al*, 1989; Chen *et al*, 1991; Badache and Hynes, 2001), may, at least in part, be mediated by the AR.

IL-6 has previously been shown to activate JAK1, STAT3 and MAPK signal transduction pathways in T-47D cells via the IL-6 membrane receptor (Badache and Hynes, 2001). These pathways have also been shown to mediate AR activation by IL-6 in prostate cells, potentially by phosphorylation of the AR at serine residues, while the PI 3'-kinase/Akt pathway has been shown to mediate suppression of AR activity (Hobisch *et al*, 1998; Chen *et al*, 2000; Ueda *et al*, 2001; Lin *et al*, 2001; Culig *et al*, 2002; Ueda *et al*, 2002; Gioeli *et al*, 2002; Lee *et al*, 2003; Yang *et al*, 2003). It is therefore likely that IL-6 activates the AR in breast cancer cells via similar mechanisms to those observed in prostate cancer cells, however further experiments, using either specific inhibitors of these kinase signalling pathways or siRNA knockout of their expression, are required to establish the precise mechanisms by which IL-6 modulates AR signalling pathways in T-47D cells.

While signal transduction pathways may be critical in mediating communication between IL-6 and the AR in breast cancer cells, the downstream effects of this cross talk still require characterisation. One possible downstream effect of IL-6 may be the modification of coactivator and transcription factor recruitment to AREs. Studies performed in LNCaP cells suggest that IL-6 inhibits DHT-induced recruitment of CBP/p300 and histone acetylation at the PSA enhancer, which is consistent with the inhibitory effect of IL-6 on DHT-induced PSA expression (Jia *et al*, 2003). The effect of IL-6 on the composition of the AR transcription complex in breast cancer cells may be further characterised using ChIP assays. An alternative mechanism for the inhibition of DHT-induced PSA expression by IL-6 in T-47D cells may involve the ER. This is supported by previous studies which have demonstrated that IL-6 increases ERα activity in the presence and absence of E_2 (Speirs *et al*, 1993b; Speirs *et al*, 2000). Moreover, as E_2-induced ER activity can inhibit DHT-induced PSA expression in T-47D cells (Yu *et al*, 1994b; Zarghami *et al*, 1997; Hsieh *et al*, 1997), it is possible that ER activity induced by IL-6 may have contributed to the inhibition of DHT-induced PSA expression by IL-6 in the current studies.

In summary, the studies presented in this chapter have investigated the androgen inducibility of transiently transfected PSA promoter and enhancer elements in addition to measuring binding of endogenous AR to the natural, chromatin integrated PSA promoter and enhancer in T-47D breast cancer cells. The results support a model in which AR binding and modification of chromatin structure is a prerequisite for synthesis of PSA mRNA following exposure to DHT. Direct comparisons of T-47D breast cancer and LNCaP prostate cancer cells suggest that PSA expression is controlled by similar mechanisms in both cell types even though PSA mRNA levels are substantially higher in LNCaP cells compared to T-47D. This may be explained by the

variation in AR levels and/or the intracellular milieu of cofactors and transcription factors. Furthermore, preliminary investigations have established that the cytokine IL-6 can modulate AR activity in the presence and absence of DHT in T-47D breast cancer cells. This suggests that the previously reported inhibitory effect of IL-6 on breast cancer cell proliferation (Tamm *et al*, 1989; Chen *et al*, 1991; Speirs *et al*, 1993a; Blais *et al*, 1995; Badache and Hynes, 2001) may be mediated, at least in part, by the AR. Further studies using PSA as a model androgen responsive gene, will more precisely characterise the specific intracellular factors necessary for both ligand dependent and ligand independent induction of gene expression by the AR. These studies will provide greater insight into the mechanisms by which the AR regulates gene expression in breast cancer cells.

CHAPTER 9

GENERAL DISCUSSION

9.1 – Major Findings of this Study

9.1.1 – Mechanisms of AR action in breast cancer cells

The studies presented in this thesis have further characterised the mechanisms involved in androgen signalling and AR function in breast cancer cells. *In vitro* studies confirmed that the AR is functional in breast cancer cell lines, with respect to its ability to activate transcription of androgen responsive reporter genes in response to both the native ligand, DHT, and the synthetic progestin, MPA (Chapters 4 and 5). The findings of this thesis provide further evidence for two mechanisms whereby the AR, following activation, mediates the effects of androgens in breast cancer cells: (i) via cross-talk with estrogen signalling pathways, and (ii) via the direct regulation of androgen responsive genes involved in proliferation, apoptosis, invasion and differentiation.

The potent androgens, DHT and mibolerone, inhibited proliferation of the AR and ER positive T-47D breast cancer cell line when cultured in the presence and in the absence of estrogens (Chapter 3). This was associated with an approximate 60% inhibition of endogenous ER activity by either the full length endogenous AR or by overexpression of the constitutively active AR-NTD (Chapter 4). These results, together with previous studies which have demonstrated inhibition of ERα activity by the AR (Panet-Raymond *et al*, 2000; Ando *et al*, 2002), support the hypothesis that androgens can induce a physical interaction between AR and ER, thereby inhibiting ER activity and the mitogenic effects of estrogen signalling pathways in breast cancer cells. Therefore, the balance between growth inhibitory androgen signalling pathways and growth stimulatory estrogen signalling pathways is likely to be critical in the regulation of breast cancer cell growth.

Another mechanism by which androgens can inhibit breast cancer cells is via the modulation of genes which regulate cell proliferation. The cDNA array study performed in this thesis has demonstrated that the potent natural androgen, DHT, and the synthetic progestin, MPA, which either stimulate or inhibit proliferation of the AR positive but ER and PR negative MDA-MB-453 breast cancer cell line respectively (Birrell *et al*, 1995a; Bentel *et al*, 1999), can alter the expression of different subsets of AR regulated genes involved in proliferation, apoptosis, invasion and differentiation (Chapter 6). This observation is consistent with results from reporter gene assays where the endogenous AR in MDA-MB-453 cells was differentially activated by DHT and MPA in a promoter specific manner (Chapter 5). These results not only suggest that the AR can regulate genes which mediate the proliferative effects of androgens but also that ligand specific gene regulation may provide a potential mechanism for how DHT and MPA induce divergent proliferative effects on MDA-MB-453 cells.

The breast cancer susceptibility gene BRCA1 was identified as a putative AR target gene in the cDNA array screen of MDA-MB-453 cells. Further studies confirmed that expression of BRCA1 was not regulated by DHT, but was markedly downregulated by growth inhibitory doses of MPA, in MDA-MB-453 cells and that the effects of MPA on BRCA1 expression were mediated by the AR (Chapter 7). These findings are potentially significant as this ligand-specific regulation suggests that BRCA1 could be important in mediating the divergent proliferative effects of DHT and MPA in MDA-MB-453 cells. Furthermore, these studies were the first to demonstrate regulation of BRCA1 expression by the AR in breast cancer cells, and provide strong evidence for an association between androgen signalling pathways and the expression of a gene with an established role in breast tumourigenesis.

The well characterised androgen regulated gene, PSA, was used as a model in these studies to further investigate the mechanisms associated with stimulation of gene expression by the AR in breast cancer cells. ChIP and real time PCR analysis demonstrated that induction of PSA expression by DHT in the T-47D breast cancer cell line was associated with an increase in AR occupancy and histone H3 acetylation at AREs in the endogenous PSA gene. These results, together with previous studies performed in prostate cancer cells (Jia *et al*, 2003), suggest that the AR regulates gene expression via similar mechanisms in breast and prostate cancer cells. However, as DHT induces PSA expression in both T-47D breast cancer and LNCaP prostate cancer cells (Chapter 8), which show opposing proliferative responses to physiological concentrations of DHT (Horoszewicz *et al*, 1983; Simard *et al*, 1991; Birrell *et al*, 1995a; Ortmann *et al*, 2002), the expression of PSA may not be important in mediating the proliferative effect of DHT in these cell lines. These experiments provide a basis for further investigating the mechanisms associated with induction, and repression, of target gene expression by the AR in order to further understand the mechanisms by which androgens regulate breast cancer cells.

9.1.2 – Development of a model for interactions between AR, ERα and BRCA1

The work presented in this thesis, together with previously published data, has highlighted a potential association between the AR and two molecules with critical roles in breast cancer – ERα and BRCA1. A model for the interactions between these molecules, and their contributions to breast cancer cell proliferation, is illustrated schematically in Figure 9.1.

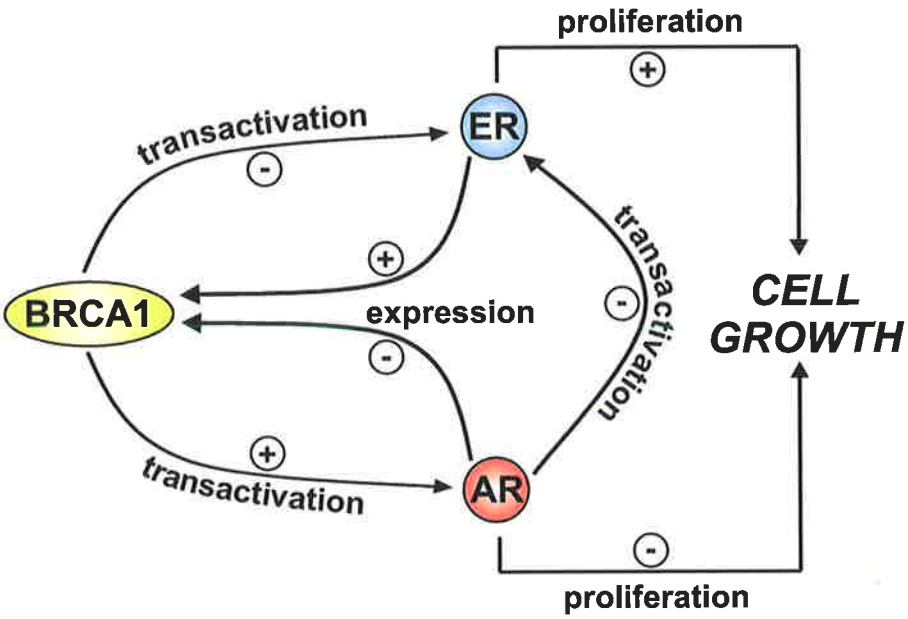


Figure 9.1: A model for interactions between AR, ERα and BRCA1 in breast cancer cells.

9.1.2.1 – Cross-talk between AR and ERα signalling pathways

While ERα signalling stimulates breast cancer cell proliferation (Davidson and Lippman, 1989; Couillard *et al*, 1998; Clemons and Goss, 2001; Ali and Coombes, 2002), AR signalling pathways are predominantly inhibitory (Poulin *et al*, 1988; Simard *et al*, 1990; Labrie *et al*, 1990a; Hackenberg *et al*, 1991; de Launoit *et al*, 1991; Hackenberg *et al*, 1993c; Birrell *et al*, 1995a; Szelei *et al*, 1997; Ortmann *et al*, 2002; Ando *et al*, 2002). Studies in this thesis have shown a functional interaction between AR and ERα which leads to inhibition of ERα activity, estrogen signalling pathways and E_2-stimulated cell proliferation (Chapters 3 and 4, see Figure 9.1). Moreover, previous *in vitro* studies have used yeast and mammalian two-hybrid assays to demonstrate that inhibition of ERα activity by the AR in breast cancer cells is associated with a direct physical interaction between the AR-NTD and the ERα-LBD (Panet-Raymond *et al*, 2000; Ando *et al*, 2002). Further studies using immunoprecipitation experiments on lysates from AR/ER positive breast cancer cells, such as T-47D, will confirm an *in vivo* physical interaction between these molecules.

Additional experiments using transient transfection of expression constructs for smaller fragments of the AR-NTD, performed in breast cancer cells expressing endogenous or exogenous ERα, and subsequent measurement of ER activity using an estrogen responsive reporter will determine which regions of the AR are required for the most effective inhibition of ER activity, while mammalian two-hybrid and GST pulldown assays will establish which regions of the AR and ER physically bind to each other. Candidate regions for mediating this physical interaction are those containing the ^{23}FQNLF^{27} and ^{432}WHTLF^{436} motifs in the AR-NTD and LxxLL-like motifs in the ERα-LBD, which may bind to each other in an interaction analogous to the

intramolecular N/C interaction that occurs in the AR following agonist binding (Sections 1.3.4 and 5.1).

It is postulated that interaction with the AR can inhibit ERα activity in breast cancer cells by inhibiting its ability to bind E_2, to bind to EREs in the regulatory regions of estrogen responsive genes or to recruit essential components of the transcription complex. Radioligand binding assays in cells transiently transfected with ERα and AR expression constructs may be used to characterise potential changes in the ability of ERα to bind E_2 in the presence and absence of AR. Moreover, the use of ChIP and DNA mobility shift assays will determine how the AR may disrupt DNA binding of liganded ER and formation of the ER transcription complex at estrogen responsive genes which mediate stimulation of breast cancer cell proliferation.

It should be noted that the current studies have predominantly investigated the effect of the non-aromatisable androgen, DHT, on AR activity and proliferation of breast cancer cells. However, the ovary predominantly produces testosterone, which is the precursor of DHT and the major physiologically active androgen in the circulation. Testosterone can be converted by 5α-reductase to the more potent metabolite DHT, which enables specific activation of the AR, or by the aromatase complex to E_2, which enables activation of the ER. Although not specifically investigated in this thesis, the latter pathway represents another potentially important mechanism of androgen action *in vivo*, which may not only be regulated by the levels of AR and ER in breast cancer cells but may also be modulated by the relative level and activity of 5α-reductase and aromatase.

9.1.2.2 – Androgen responsive genes in breast cancer cells

The non-aromatisable androgens, DHT and mibolerone, also modulate proliferation of breast cancer cells when cultured in the absence of estrogens (Chapter 3). The studies in this thesis have demonstrated that DHT, and the synthetic progestin MPA, can act via the AR to regulate the expression of genes which may mediate proliferative effects. In the MDA-MB-453 breast cancer cell line, DHT and MPA can alter the expression of different subsets of AR regulated target genes. This suggests that specific gene expression profiles are associated with the divergent proliferative response of MDA-MB-453 cells to DHT and MPA. Furthermore, the microarray experiments identified a number of novel candidate AR target genes potentially involved in mediating the proliferative effects of androgens on breast cancer cells, including BRCA1, the oncogenes v-ski, v-raf and v-myb, the Syk tyrosine kinase, protein kinase related to ERK2, the cell cycle regulator CDK9, histone deacetylase 3, prostaglandin E receptor and Tax-1 binding protein, each of which have established roles in regulating cell proliferation, differentiation or apoptosis. Identification and characterisation of the key genes that mediate the proliferative effects of androgens is vital for a greater understanding of the molecular mechanisms of androgen action in breast cancer cells, and may, in the long term, provide a basis for development of improved diagnostic, therapeutic and/or preventive strategies. Identification of specific genes which mediate anti-proliferative effects of androgens without activating pathways associated with masculinising side effects may assist in achievement of this goal. Further studies using overexpression or siRNA knockout of gene expression will determine which of these genes directly contribute to proliferative outcome. In addition, DHT and MPA modulated the expression of many genes with roles in cell adhesion, motility and invasion, carbohydrate, lipid or protein synthesis and metabolism as well as a number of cytoskeletal/structural genes. Although these genes may not be directly involved in

regulating proliferation, they may be important regulators of breast cancer development and progression *in vivo*. Furthermore, many candidate AR target genes involved in cell signalling cascades and the regulation of transcription were identified, including genes involved in steroid hormone synthesis and signalling. These genes themselves may also mediate regulation of downstream genes that control breast cancer cell behaviour, suggesting that the AR regulates gene expression via complex interactions between multiple signalling pathways.

In addition to identifying androgen regulated genes which mediate the proliferative effects of androgens, characterisation of the mechanisms that regulate their expression will increase our understanding of androgen action in breast cancer cells. Results from chromatin immunoprecipitation assays investigating the induction of PSA by DHT in breast cancer cells support a model whereby DHT induces binding of the AR to AREs in androgen responsive genes, followed by sequential recruitment of essential cofactor molecules and other components of the transcription complex, as has previously been described in prostate cancer cells (Shang *et al*, 2002; Kang *et al*, 2002; Jia *et al*, 2003; Louie *et al*, 2003). However, these classical mechanisms may not regulate expression of all AR target genes. Evident from cDNA array analysis performed in Chapter 6 is that the AR can downregulate expression of many genes, such as BRCA1. Further studies, using DNA mobility shift or ChIP assays, are required to determine whether the AR downregulates expression of genes such as BRCA1 via classical mechanisms, ie by binding to AREs and recruitment of components of the transcriptional machinery, or whether the AR interferes with other transcription factors to repress transcription, as has been described for the gonadotrophin α-subunit (Heckert *et al*, 1997) and matrix metalloproteinase I (Schneikert *et al*, 1996).

9.1.2.3 – Effects of AR and ER on BRCA1 expression

While the current studies have demonstrated that the AR, at least in response to MPA, can downregulate expression of BRCA1 in MDA-MB-453 breast cancer cells, previous studies have demonstrated that estrogens stimulate expression of BRCA1 in MCF-7, ZR-75 and T-47D breast cancer cells (Gudas *et al*, 1995; Marquis *et al*, 1995; Spillman and Bowcock, 1996; Rajan *et al*, 1997; Marks *et al*, 1997; Romagnolo *et al*, 1998; Chambon *et al*, 2003). This opposing effect of ER and AR signalling pathways on BRCA1 expression is consistent with the opposing effects of AR and ER on breast cancer cell proliferation (Figure 9.1), suggesting that BRCA1 may be important in mediating the proliferative effects of androgens and estrogens on breast cancer cells.

9.1.2.4 – Modulation of ERα and AR activity by BRCA1

While AR and ER appear to have opposing effects on the expression of BRCA1, BRCA1 also has opposing effects on the transactivation activity of ERα and AR (Figure 9.1). Previous studies have demonstrated that BRCA1 can inhibit the activity of ERα via a direct interaction between the LBD of ERα and the amino terminus of BRCA1 (Fan *et al*, 1999; Fan *et al*, 2001a). In contrast, BRCA1 is a coactivator of the AR, enhancing its activity via an interaction between the carboxy terminus of BRCA1 and regions in the AR-NTD and AR-LBD (Yeh *et al*, 2000; Park *et al*, 2000b). Considering the model proposed in Figure 9.1, BRCA1 and AR may also cooperate to inhibit estrogen signalling, although this is yet to be demonstrated. Transfection of ER/AR positive T-47D breast cancer cells with increasing amounts of an expression construct for BRCA1 and treatment with different combinations and concentrations of E_2 and DHT will determine whether BRCA1 and AR can synergistically inhibit endogenous ER activity. These regulatory loops controlling ERα and AR activity, with feedback to BRCA1, suggest that BRCA1 is a key modulator of the balance between estrogen and

androgen signalling pathways, which is critical for the control of breast cancer cell proliferation.

9.2 – Future Directions

9.2.1 – Unanswered questions regarding this model for androgen action

9.2.1.1 – DHT versus MPA mediated AR activity

Although the effect of DHT on E_2-induced ER activity and endogenous gene expression has been examined in this thesis, and in previous studies (MacIndoe and Etre, 1980; MacIndoe and Etre, 1981; Shapiro and Lippman, 1985; Simard *et al*, 1989; Poulin *et al*, 1989b; Labrie *et al*, 1990b; Lapointe *et al*, 1999; Panet-Raymond *et al*, 2000; Ando *et al*, 2002), the effect of the synthetic progestin, MPA, on functional interactions between AR, ER and BRCA1 have not been characterised. As MPA was shown in the current study to induce AR activity in breast cancer cells (Chapter 5), it may also have the potential to induce an interaction between AR and ER, thereby inhibiting ER activity. This hypothesis is supported by previous studies showing that MPA can inhibit E_2-induced breast cancer cell proliferation via a mechanism involving the AR (Sutherland *et al*, 1988; Poulin *et al*, 1989a; Hackenberg *et al*, 1990; Dauvois *et al*, 1991). Experiments investigating the effect of different combinations of E_2 and MPA on expression of estrogen responsive endogenous or reporter genes will determine whether MPA can induce a functional interaction between AR and ER. Moreover, mammalian two-hybrid assays or immunoprecipitation experiments, using antibodies specific for AR and ER, will confirm a physical interaction between these receptors. Furthermore, the ability of BRCA1 to coactivate MPA-induced AR transactivation activity is also yet to be established. Transient transfection of an androgen responsive reporter gene and increasing amounts of a BRCA1 expression construct into AR

positive breast cancer cells, followed by treatment with MPA, will verify whether this occurs in breast cancer cells, or whether the effect of BRCA1 is specific for AR activity induced by classical AR agonists.

9.2.1.2 – ER inhibition of AR

Whereas the current studies have investigated the effect of AR on ER activity (Chapter 4), previous studies have demonstrated that ER also has direct inhibitory effects on AR (Kumar *et al*, 1994; Panet-Raymond *et al*, 2000). Furthermore, estrogens can inhibit induction of endogenous androgen responsive genes such as PSA, GCDFP-15 and GCDFP-24 (Yu *et al*, 1994b). This may represent an important mechanism by which estrogens can counteract the inhibitory effects of androgens on breast cancer cell growth. Further studies measuring the expression of androgen responsive endogenous genes and exogenous reporter genes in response to different combinations of androgens and estrogens (analogous to experiments performed in Chapter 4) are required to determine if estrogens inhibit androgen signalling in T-47D breast cancer cells. If this is the case, these studies, taken together with the current observations, may imply that estrogen and androgen signalling pathways actively oppose each other, with the predominant pathway likely to influence breast cancer cell growth.

9.2.1.3 – Ligand independent interactions between AR, ER and BRCA1

Studies performed in prostate cancer cells have shown that growth factors and cytokines, such as HER2/neu and IL-6, can induce ligand independent activity of the AR (Culig *et al*, 1994; Nazareth and Weigel, 1996; Gleave *et al*, 1998; Yeh *et al*, 1999; Craft *et al*, 1999; Sadar, 1999; Sadar and Gleave, 2000; Ueda *et al*, 2001). The current studies were the first to show that the cytokine IL-6 can modulate AR activity, in the presence and absence of DHT, in T-47D breast cancer cells (Chapter 8). This raises the

possibility of whether ligand independent AR activity can also inhibit ER activity and E_2-induced breast cancer cell proliferation as observed for androgens in Chapters 3 and 4. Treatment of AR positive breast cancer cells with IL-6 in the presence or absence of AR antagonists will determine if IL-6 can act through the AR to modulate proliferation. Additionally, examination of the expression of estrogen responsive endogenous or reporter genes in response to E_2 and IL-6 will determine whether IL-6 can inhibit estrogen signalling pathways in breast cancer cells, while co-treatment with AR antagonists in these experiments will determine the specific role of the AR in this inhibition. Finally, immunoprecipitations performed on lysates from AR/ER positive T-47D breast cancer cells treated with E_2 and IL-6 will determine if IL-6 can induce a physical interaction between AR and ER. These studies may lead to alternative methods for modulating androgen signalling pathways in breast cancer cells which circumvent the requirement for androgens.

9.2.1.4 – Effect of AR, ER or BRCA1 variants

Expression of variant AR, ER or BRCA1 has been demonstrated in a small proportion of breast cancers: mutations in the *BRCA1* gene are associated with approximately 2-5% of all breast cancers (Hall *et al*, 1990; Miki *et al*, 1994; Ford *et al*, 1994; Easton *et al*, 1995), mutations in the *ER* gene have been identified at a frequency of up to 10% of breast cancers (Roodi *et al*, 1995; Zhang *et al*, 1997b; Chappell *et al*, 2000) while studies performed in our laboratory have detected *AR* gene mutations in five of 41 (12%) breast tumours which failed to respond to MPA (Yang *et al*, manuscript in preparation) and in the MDA-MB-453 breast cancer cell line (Chapter 5). Although mutations in the *AR*, *ER* and *BRCA1* genes appear to be rare, these variants may have altered ability to interact with each other, potentially disrupting the balance between these pathways and promoting unopposed estrogen stimulated cell proliferation in these

cancers. Previous studies have shown that an AR-DBD variant (AR-R613H) is not coactivated by BRCA1 while the endogenous variant AR (AR-T875A) in the LNCaP cell line displayed a reduced capacity for coactivation by BRCA1 (Yeh *et al*, 2000). These results raise the possibility that mutant ARs in breast cancer cells may have diminished potential for coactivation by BRCA1, perhaps through reduced interactions between these molecules. Ando *et al* (2002) have recently demonstrated that an AR variant with a single amino acid substitution in the DNA binding domain (AR-C574R) is unable to inhibit ERα activity. This suggests that certain mutations may prevent the AR from interacting with ERα and that variant ARs in breast cancer cells may fail to antagonise the mitogenic effects of estrogens. Certain mutations in BRCA1 also render it unable to inhibit ERα activity (Fan *et al*, 2001a) while independently diminishing its ability to coactivate the AR (Yeh *et al*, 2000). It may therefore be hypothesised that *BRCA1* mutations promote tumourigenesis by upsetting the equilibrium between AR and ER signalling pathways in breast cancer cells, leading to unchecked ER activity and unopposed estrogenic stimulation while diminishing the protective effects afforded by androgen signalling pathways. In future studies, transient transfection of expression constructs for breast cancer associated AR, ER and BRCA1 variants into breast cancer cells will determine whether mutations alter the functional and physical interactions between these molecules, and may also enable further definition of the critical domains required for these interactions.

9.2.1.5 – Other proteins

Although not specifically investigated in this thesis, previous studies have provided evidence for a possible role for BRCA2 in this model of interactions between AR and ER. BRCA2 can also enhance ligand dependent AR activity, and appears to act synergistically with BRCA1 and GRIP1 (Shin and Verma, 2003), although the effect of

BRCA2 on ERα activity has not previously been reported. Studies on the expression of BRCA2 have shown that it is coordinately regulated with BRCA1 in response to estrogen as well as other stimuli (Spillman and Bowcock, 1996; Rajan *et al*, 1996; Fan *et al*, 1998; Andres *et al*, 1998), suggesting that BRCA2 is also an AR target gene. The association between AR and BRCA2, and interactions with ERα in breast cancer cells, may therefore warrant further study.

Other proteins, in addition to BRCA1 and BRCA2, are likely to be important in regulating androgen signalling pathways in breast cancer cells. Of these, AR cofactors such as the p160 coactivators or the corepressors NCoR and SMRT, may alter AR activity while proteins whose expression is regulated by the AR may mediate the effects of androgens on breast cancer cell proliferation, apoptosis, differentiation and survival. While BRCA1 and BRCA2 appear to fit into both of these categories, the identification of other key proteins involved in androgen signalling pathways will enable a better understanding of the mechanisms of androgen action in breast cancer cells. The cDNA array studies presented in Chapter 6 have identified a number of candidate AR target genes modulated by DHT and/or MPA, which may mediate the proliferative effects of these ligands. These include the oncogenes v-ski, v-raf and v-myb, the Syk tyrosine kinase, protein kinase related to ERK2, the cell cycle regulator CDK9, histone deacetylase 3, prostaglandin E receptor and Tax-1 binding protein, each of which have established roles in regulating cell proliferation, differentiation or apoptosis. Further study of the regulation of these genes, and the effect of knockout or overexpression of specific genes on breast cancer cell proliferation, may highlight specific candidates which may be targeted to manipulate breast cancer cells. The ChIP assay, which has been used in Chapter 8 to characterise recruitment of the AR to AREs in the PSA gene, is a powerful technique suitable for identification of coactivators, corepressors and

transcription factors required to regulate the expression of androgen responsive genes in breast cancer cells. Cofactors which may be critical for the expression of genes regulating the proliferative effects of androgens in breast cancer cells may potentially be targeted to increase AR action and decrease proliferation of breast cancer cells.

9.2.2 – Development of Novel Breast Cancer Treatments

9.2.2.1 – Potential new therapeutic options for breast cancer

The primary aims of current and future studies are to determine the precise mechanisms by which androgen signalling pathways exert their effects on breast cancer cells, with the ultimate objective being the development of novel treatments for breast cancer which exploit androgen/AR action. These treatments have the potential to complement existing hormonal therapies that target the estrogen signalling axis.

Studies in this thesis, which have demonstrated that the AR-NTD can inhibit ER activity in T-47D breast cancer cells (Chapter 4), may ultimately facilitate the development of new therapies for breast cancer. Precise mapping of the discrete region(s) of the AR which effectively inhibit ER activity in breast cancer cells may enable the design of short AR peptides that can also interact with the ER and inhibit its activity. Stable expression of AR fragments encoding these peptides in breast cancer cells will establish whether they are effective for inhibiting ER activity and E_2-induced proliferation. Structural consideration of the ER may lead to the design of peptide or non-peptide mimetics, molecules which are based on these AR peptides but which may be smaller in size (therefore increasing their ability to enter target cells), have increased stability or have greater potential for inhibiting ER activity than a peptide. Small peptide mimetics for inhibition of the tyrosine kinase receptor HER2/neu have been shown to be effective inhibitors of tumour cell proliferation *in vitro* and in animal models (Park *et al*, 2000a;

Park *et al*, 2003), while numerous chemical inhibitors of HER2/neu are in early stages of clinical trials (Johnston *et al*, 2003). These studies suggest that a small molecule/peptide approach based on AR motifs which interact with and inhibit ER activity in breast cancer cells may be a suitable alternative, or complement, to anti-estrogens or aromatase inhibitors for the treatment of breast cancers that express ER (ie approximately 55-80% of primary breast tumours (Lea *et al*, 1989)).

Peptides, peptide mimetics or non-peptide mimetics based on AR sequences would be a significant improvement on traditional androgenic drugs for breast cancer as they are unlikely to stimulate androgen signalling pathways, thereby preventing masculinising side effects. However, other side effects may develop as inhibition of ER activity may be harmful for tissues and organs which require estrogen signalling pathways for normal maintenance and growth, such as bone, brain, liver and heart (Jordan, 1998; Ali and Coombes, 2002). Stringent assessment of the effects of AR peptides or mimetics on other cell types *in vitro* and *in vivo* is required to prove their value as potential breast cancer therapies. Studies of various anti-estrogens may provide possible guides for the design of AR peptide mimetics which have minimal anti-estrogenic activity in tissues other than the breast. Pure anti-estrogens, such as ICI 164384 and faslodex, inhibit the activity of both the AF1 and AF2 of ERα and are anti-estrogenic in all cell types (Ali and Coombes, 2002). In contrast, partial anti-estrogens, such as tamoxifen, inhibit AF2 only and are anti-estrogenic in cell types where AF2 is the predominant activation function, such as the breast, and estrogenic in other cell types such as the uterus and bone (Ali and Coombes, 2002). Because of these apparent tissue specific effects of anti-estrogens, they are also known as selective ER modulators, or SERMs. Development of AR peptide mimetics which predominantly inhibit AF2 of ERα may therefore afford a certain degree of tissue specificity, circumventing potential

deleterious side effects in some cell types. As previous studies have shown that the AR-NTD interacts with the ERα-LBD (Panet-Raymond *et al*, 2000), it is possible that the AF2 of ERα is predominantly inhibited by this interaction.

Finally, the development of SERMs with increased specificity, such as raloxifene which is anti-estrogenic in the breast and uterus but estrogenic in bone (Ali and Coombes, 2002; Shang and Brown, 2002), raises the possibility of development of selective AR modulators, or SARMs, for breast cancer therapies. Identification of agents that differentially regulate growth inhibitory AR target genes specifically in breast cancer cells, without regulating gene pathways associated with masculinisation or diminishing the beneficial effects of androgens on bone, the cardiovascular system and general well-being, may be effective in the treatment of AR positive breast cancers, which comprise up to 90% of all breast cancers (Lea *et al*, 1989). Furthermore, development of therapies of this nature may be particularly beneficial for tumours which express AR but not ER (approximately 25% of metastatic tumours), and which are therefore unlikely to respond to treatments targeting estrogen signalling pathways. However, development of therapeutically effective SARMs is considered to be more of a longer term goal, and detailed studies investigating the mechanisms of androgen signalling, including identification of the genes which mediate antiproliferative effects of androgens, are essential in order to selectively manipulate androgen signalling pathways specifically in breast cancer cells.

9.2.2.2 – Potential preventive strategies for breast cancer

Although this thesis has investigated the androgen signalling axis in breast cancer cells, very little is currently known about the role of androgens in normal breast epithelial and stromal cells. Epidemiological studies have established potential associations between

endogenous androgens and breast cancer risk, implying that altered androgen signalling pathways may potentially play a role in malignant transformation of breast epithelial cells. Furthermore, AR is expressed in normal ductal epithelium of the breast (Isola, 1993; Wilson and McPhaul, 1996; Selim and Wells, 1999; Zhou *et al*, 2000; Pelletier, 2000; Brys *et al*, 2002; Zhuang *et al*, 2003; Moinfar *et al*, 2003). This suggests that the androgen signalling axis may be a potential target for novel chemopreventive strategies for breast cancer.

Studies in rodent models have demonstrated that androgens can inhibit spontaneous development of breast cancer and inhibit proliferation of mammary epithelial cells (Schwartz, 1979; Purnell, 1980; Pashko *et al*, 1981; Jayo *et al*, 2000). Similarly, in ovariectomised rhesus monkeys, testosterone inhibits the stimulatory effects of E_2 while administration of the AR antagonist flutamide alone increased proliferation of normal mammary epithelial cells (Zhou *et al*, 2000; Dimitrakakis *et al*, 2003). These studies provide evidence that androgens can inhibit proliferation of normal mammary epithelial cells in the presence and absence of estrogens. This may potentially occur by similar mechanisms to those postulated in breast cancer cells, ie via regulating the expression of androgen responsive genes and/or via inhibiting the mitogenic effects of estrogens. However stimulatory effects of androgens on breast epithelial cells have also been reported (Klevjer-Anderson and Buehring, 1980; Welsch, 1985; Baratta *et al*, 2000). The reasons for these discrepancies are currently unclear. This highlights the need for further study to define the precise mechanisms by which androgens regulate mammary epithelial cells.

The proliferative effects of androgens on normal human mammary epithelial cell lines, such as MCF-10, MCF-12, 184A1, 184B5 and Hs 578Bst, have not previously been

investigated. However mammary epithelial cells are embedded in an environment containing stromal cells and fibroblasts which contribute to cell proliferation and differentiation (Levine and Stockdale, 1985; Forsyth, 1991; Gache *et al*, 1998; Robinson *et al*, 1999), making the study of cell lines somewhat artificial. A recent report has described the development of an *in vitro* system to study the growth of normal breast epithelial cells in their natural context (Zhuang *et al*, 2003). This involves culture of mammary tissue on a gelatine sponge submerged in culture medium. Tissues cultured under this system have been used for immunohistochemical studies, which demonstrated that cells maintained morphology and expression of AR, ER and PR for up to two months (Zhuang *et al*, 2003). Use of this system for immunohistochemical analysis of the expression of other proteins may therefore yield important information regarding the effects of androgens on normal breast cells. Culture of cells in the presence or absence of different combinations of androgens and estrogens, followed by immunohistochemical analysis of the Ki-67 antigen, which is specific for proliferating cells (Gerdes *et al*, 1991), may initially determine the effect of these steroids on normal epithelial cell proliferation. The expression of androgen responsive genes in these cells may also be examined using antibodies for PSA, gross cystic disease fluid proteins or for other candidate AR targets identified in the cDNA array study (such as BRCA1). Additionally, antibodies for estrogen responsive genes, including PR, cathepsin D or pS2, may be used to establish the effect of androgens on E_2-induced gene expression and estrogen signalling pathways in normal mammary epithelial cells.

Further study of the role of androgens in normal breast cell physiology may have potential clinical applications. Firstly, definition of the mechanisms associated with the inhibition of breast epithelial cell proliferation by androgens may lead to novel

strategies for the chemoprevention of breast cancer in women at high risk. In particular, demonstration of an association between AR, ER and BRCA1 in normal breast cells, as has been proposed for breast cancer cells (Section 9.1.2), may facilitate the development of drugs which reduce breast cancer risk in BRCA1 mutation carriers.

Secondly, studies on androgens in normal breast cells may lead to improved composition of hormone replacement therapies (HRT). Estrogen replacement, either alone or in combination with progestins (eg MPA, norethisterone, norgestrel), for the relief of menopausal symptoms has been associated with increased risks for breast and endometrial cancer (Colditz *et al*, 1995; Collaborative Group on Hormonal Factors in Breast Cancer, 1997; Magnusson *et al*, 1999; Ross *et al*, 2000; Schairer *et al*, 2000; Persson, 2000; Rossouw *et al*, 2002; Beral *et al*, 2002; Armitage *et al*, 2003; Chlebowski *et al*, 2003; Davison and Davis, 2003a). Some HRT regimens include the synthetic progestin MPA and studies from this thesis have shown that high doses of MPA can reduce expression of BRCA1 (Chapters 6, 7). Although the doses of MPA used in HRT are lower than those shown to alter BRCA1 expression in these studies, it remains possible that combined estrogen-MPA therapy may increase breast cancer risk in some women by modulating BRCA1 expression. HRT, with or without progestins, has also been shown to suppress LH, resulting in a reduction of ovarian androgen production, and increase SHBG, resulting in a decrease in bioavailable androgens (Castelo-Branco *et al*, 1993; Casson *et al*, 1997). HRT may therefore further promote malignant transformation of breast epithelial cells by increasing exposure to estrogens while diminishing the protection provided by androgens. That androgens have anti-proliferative effects on breast epithelium in rodents and monkeys, as described above, suggests that inclusion of androgens in HRT regimens may reduce the mitogenic effects of estrogens in normal breast cells. Androgens are included in some HRT

regimens to improve well-being, quality of life and libido (Sherwin and Gelfand, 1985; Sherwin, 1988; Davis *et al*, 1995; Davis, 1999; Basson, 1999; Shifren *et al*, 2000; Davis and Tran, 2001) and this may confer added protection against breast tumourigenesis. However, further study of the effects of androgens in normal breast epithelial cells will potentially lead to alternative supplements for HRT which exert antiproliferative effects on the breast without causing detrimental side effects associated with androgen replacement (Bardin *et al*, 1991; Davis, 1999; Davis and Tran, 2001; Davison and Davis, 2003b).

9.3 – Conclusions

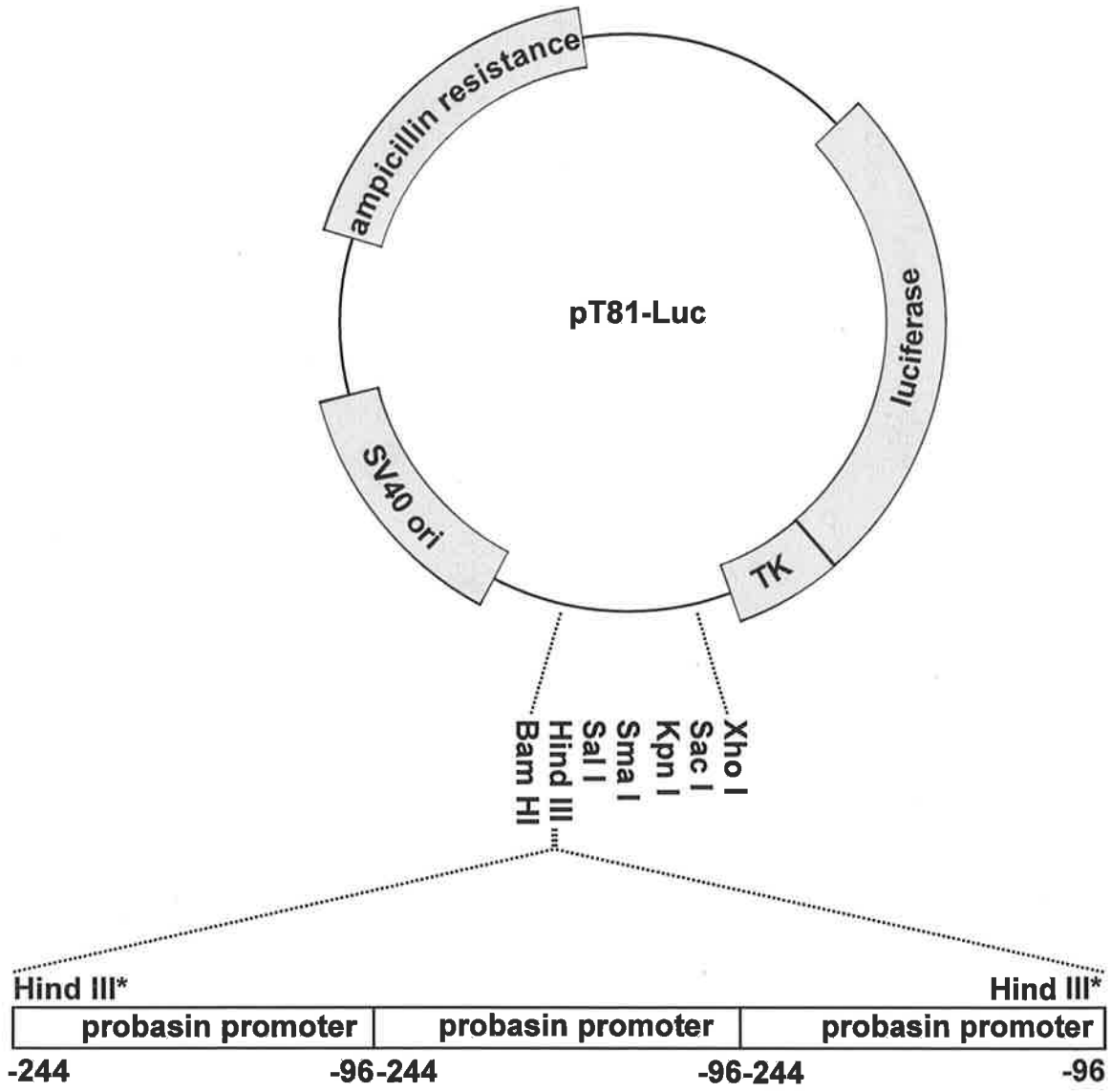
The work completed for this thesis has extended current knowledge on the actions of androgens and the AR in breast cancer cells. Collectively, these studies investigating different aspects of androgen signalling pathways in breast cancer cells can be summarised by a model which proposes that androgen action has both direct and indirect effects on breast cancer cells (Figure 9.1). Direct effects of androgens include the activation and repression of genes that regulate cell proliferation, apoptosis, differentiation and invasion. Indirect effects of androgens include the repression of ERα activity and estrogen-stimulated cell proliferation. Furthermore, the effects of androgens may be modulated by the breast cancer susceptibility gene, BRCA1. Disruption of the balance between AR and ER signalling pathways in breast cancer cells, perhaps resulting from mutations or altered expression in AR, ER or BRCA1, may in part lead to deregulation of breast cancer cell growth. These observations provide a rationale for the development of potential new therapies or preventive strategies for breast cancer which target androgen signalling pathways to correct or avert deregulated

breast cancer cell growth. Further studies are therefore warranted to determine the most effective approaches to achieve this objective.

Appendix 1 – Plasmid Constructs

The composition and construction of plasmid vectors used throughout this thesis is described and illustrated on the following pages.

tk81-PB$_3$-luc contains three copies of the androgen responsive minimal rat probasin promoter sequence (nucleotides -244 to –96) linked in tandem in front of the thymidine kinase enhancer element and firefly (*Photinus pyralis)* luciferase reporter gene of the pT81-luc vector.

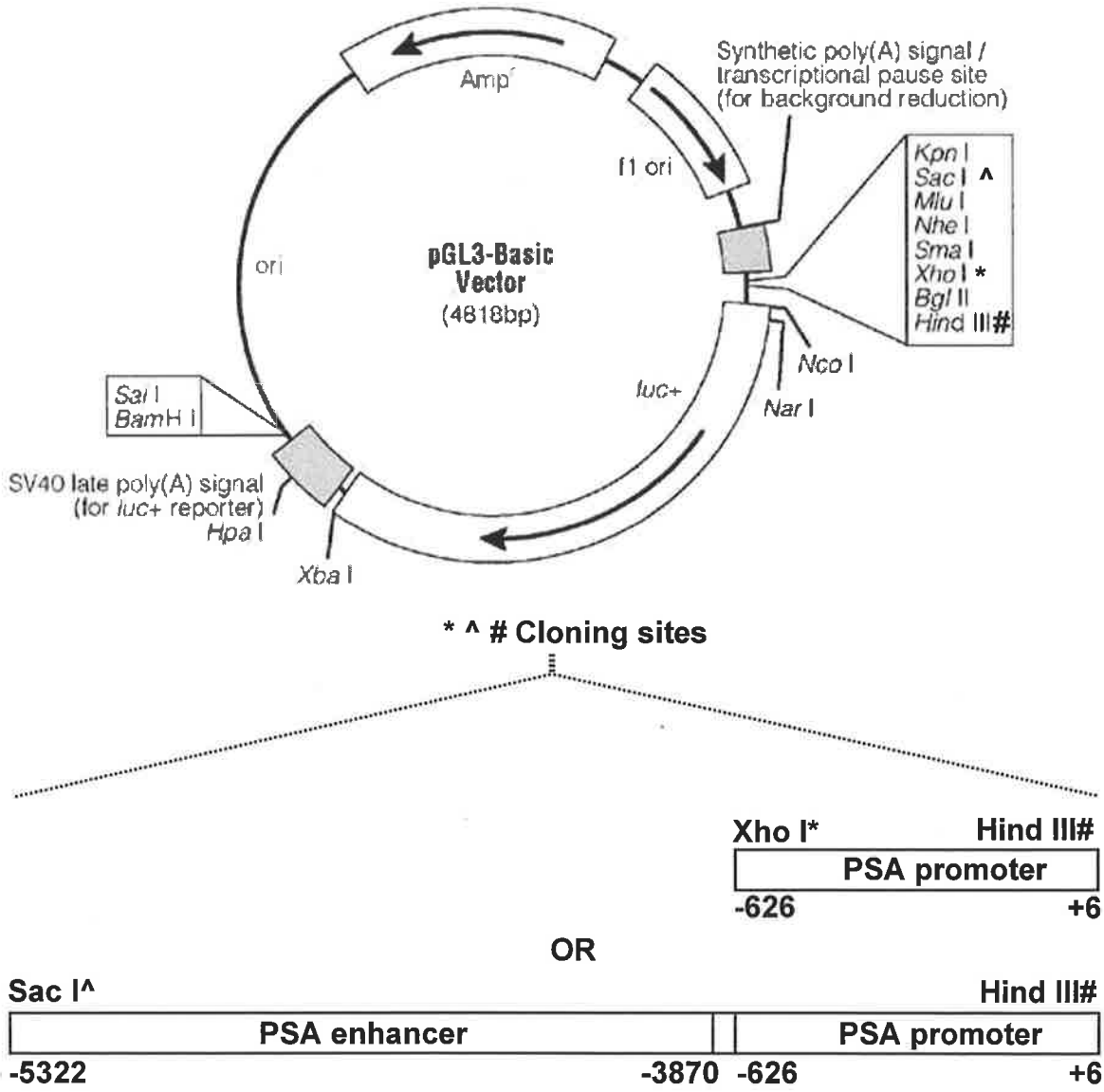


*** Restriction enzyme sites introduced by PCR**

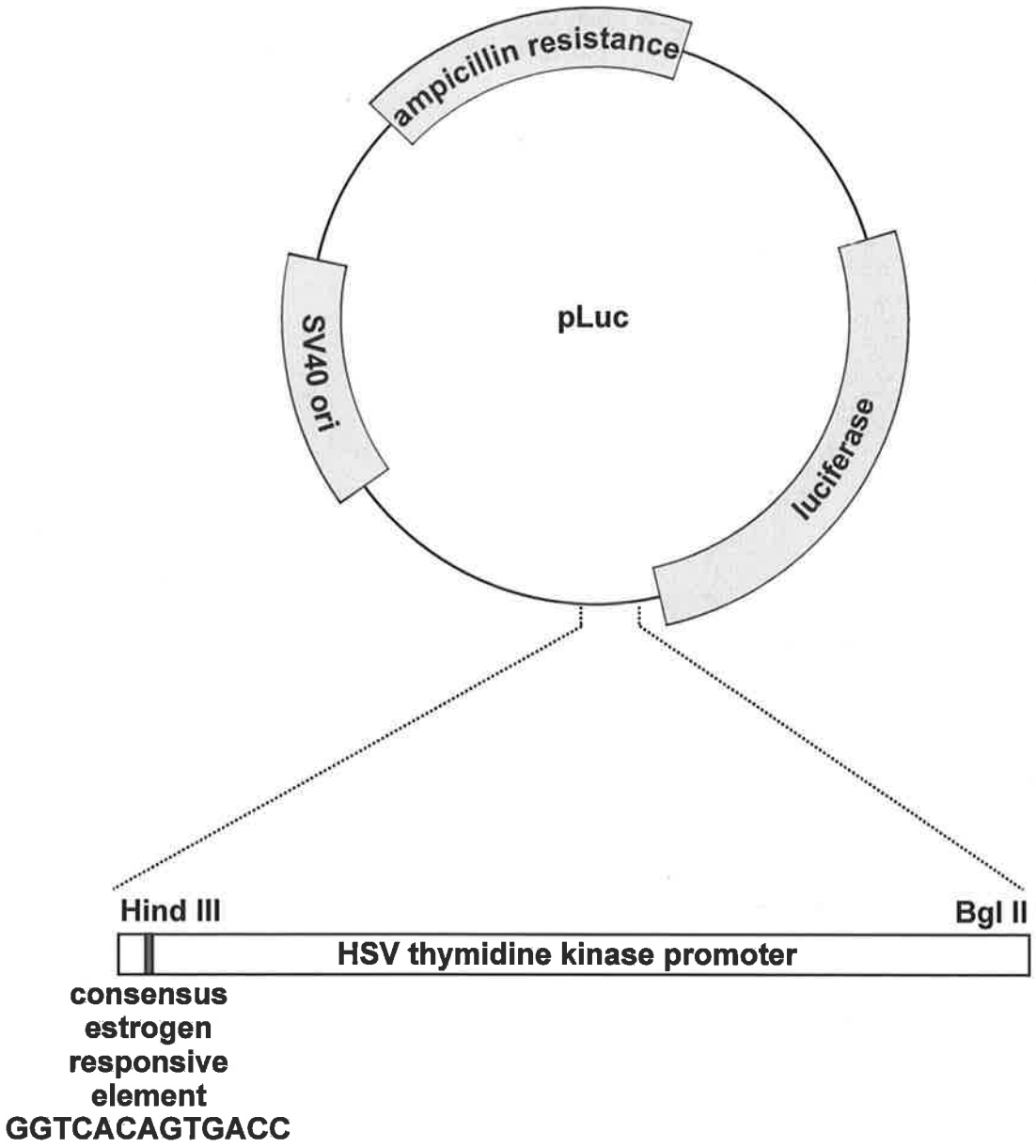
PSA_{630} (promoter)-luc was constructed by ligation of a 633bp Xho I-HindIII fragment (-626 to +6) of the androgen responsive human PSA promoter (from the PSA630-CATSAT vector) into corresponding sites of the pGL3-basic vector.

PSA_{630} (promoter + enhancer)-luc was constructed by ligation of a Sac I-Hind III fragment from the PSE/PSA-CATSAT vector (containing -5322 to -3870 of the human PSA enhancer region and -626 to +6 of the androgen responsive human PSA promoter) into corresponding sites of the pGL3-Basic vector.

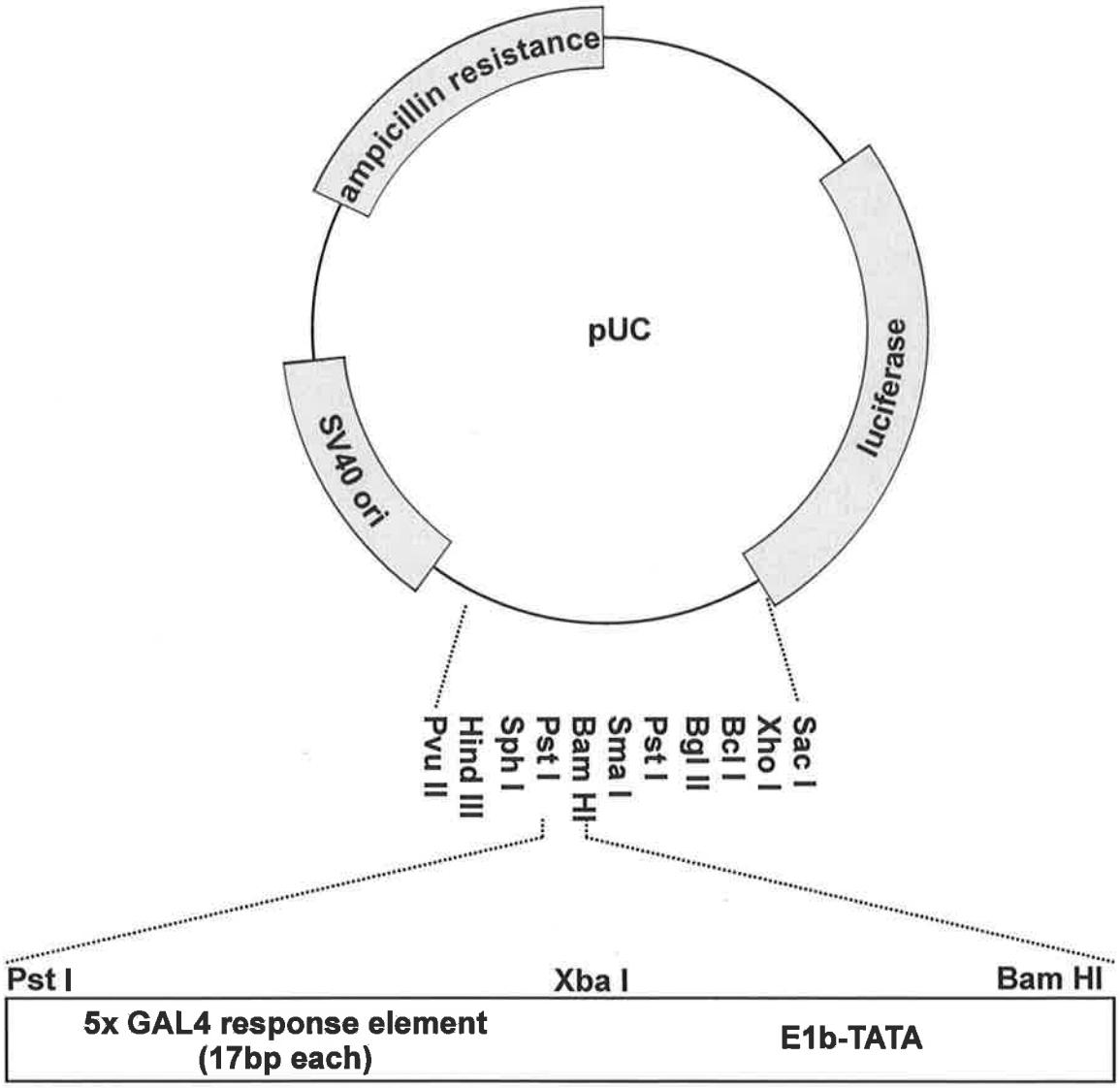
These plasmids were constructed by Dr Miao Yang.



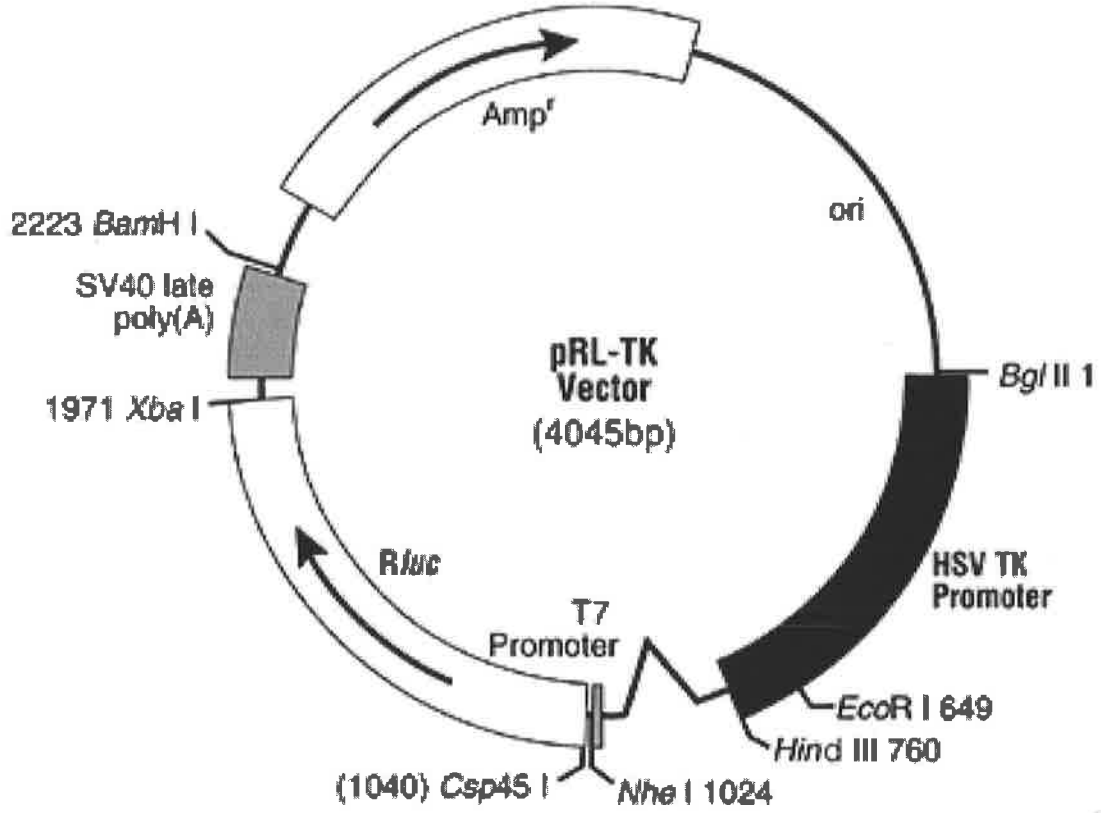
ERE-tk-luc contains a consensus estrogen responsive element isolated from the *Xenopus laevis* vitellogenin A2 gene and the herpes simplex virus thymidine kinase promoter fused to the firefly luciferase reporter gene in the pLuc vector.



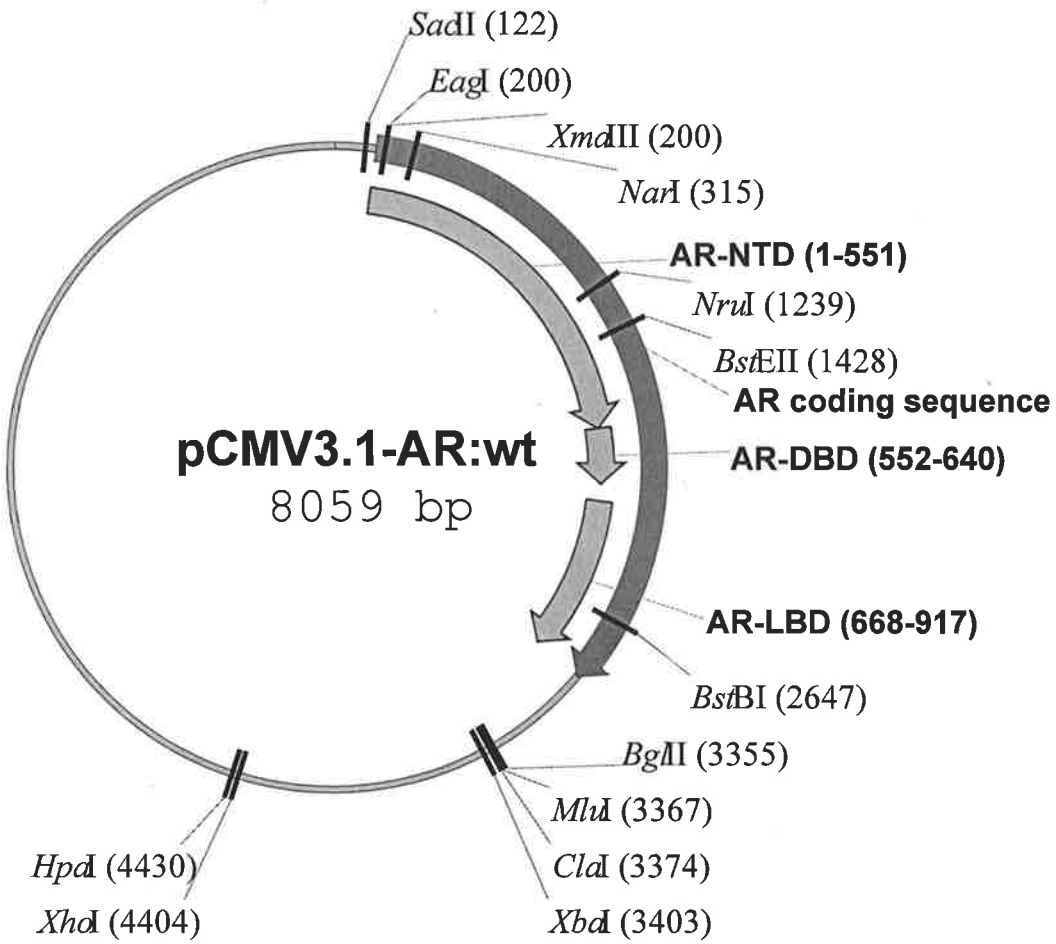
pGK1 contains five copies of the GAL4 response element linked to a minimal adenovirus E1b promoter and the firefly luciferase reporter gene in the pUC vector.



pRL-tk contains a herpes simplex thymidine kinase promoter element fused to the renilla (*Renilla reniformis*) luciferase reporter gene.

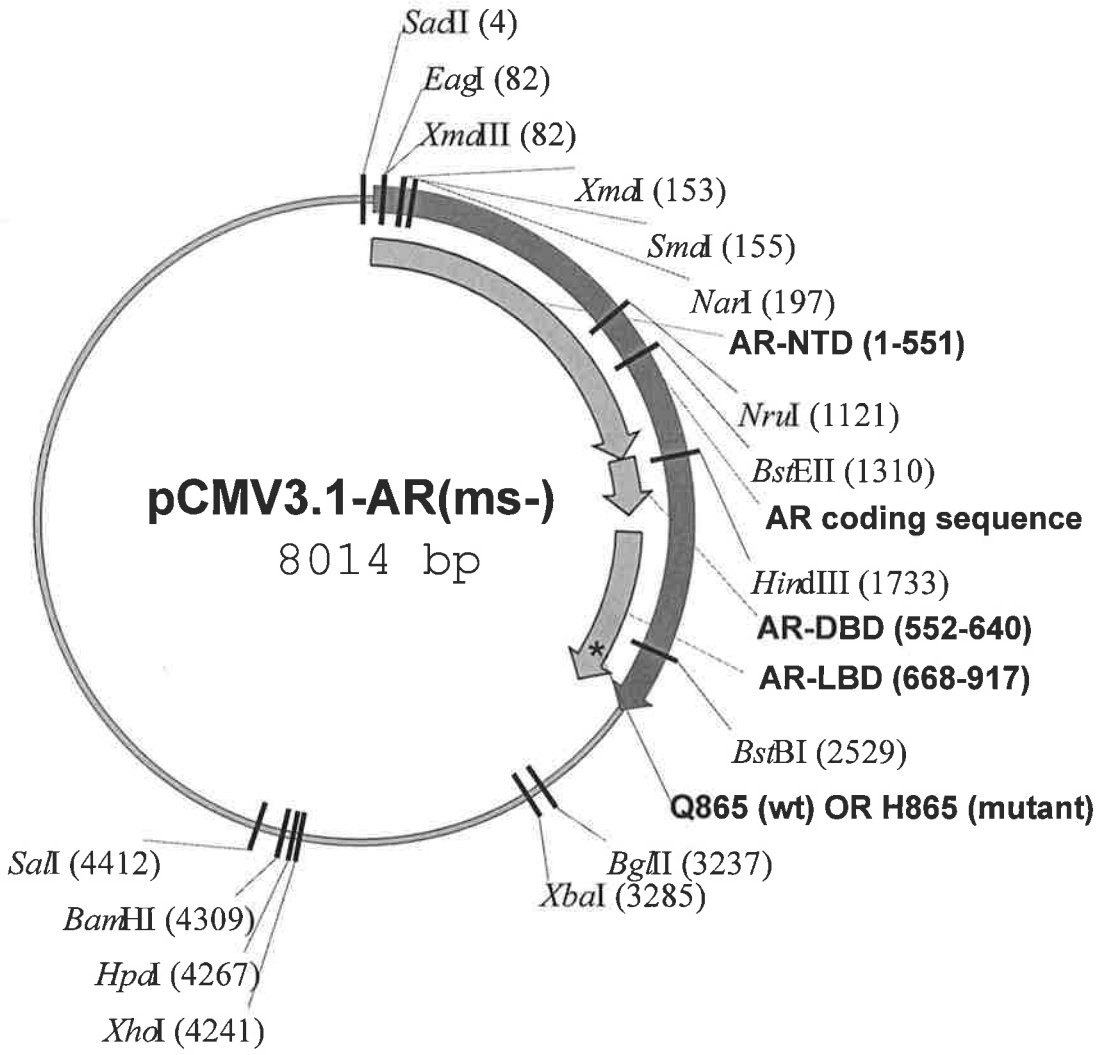


pCMV3.1-AR:wt contains the entire coding region of the wild type human AR cDNA cloned into the Eco RI restriction sites of the pCMV3.1 parental expression vector, allowing expression of the AR cDNA under control of the cytomegalovirus promoter. The AR encoded by this construct contains 21 glutamine residues in the poly-glutamine tract.



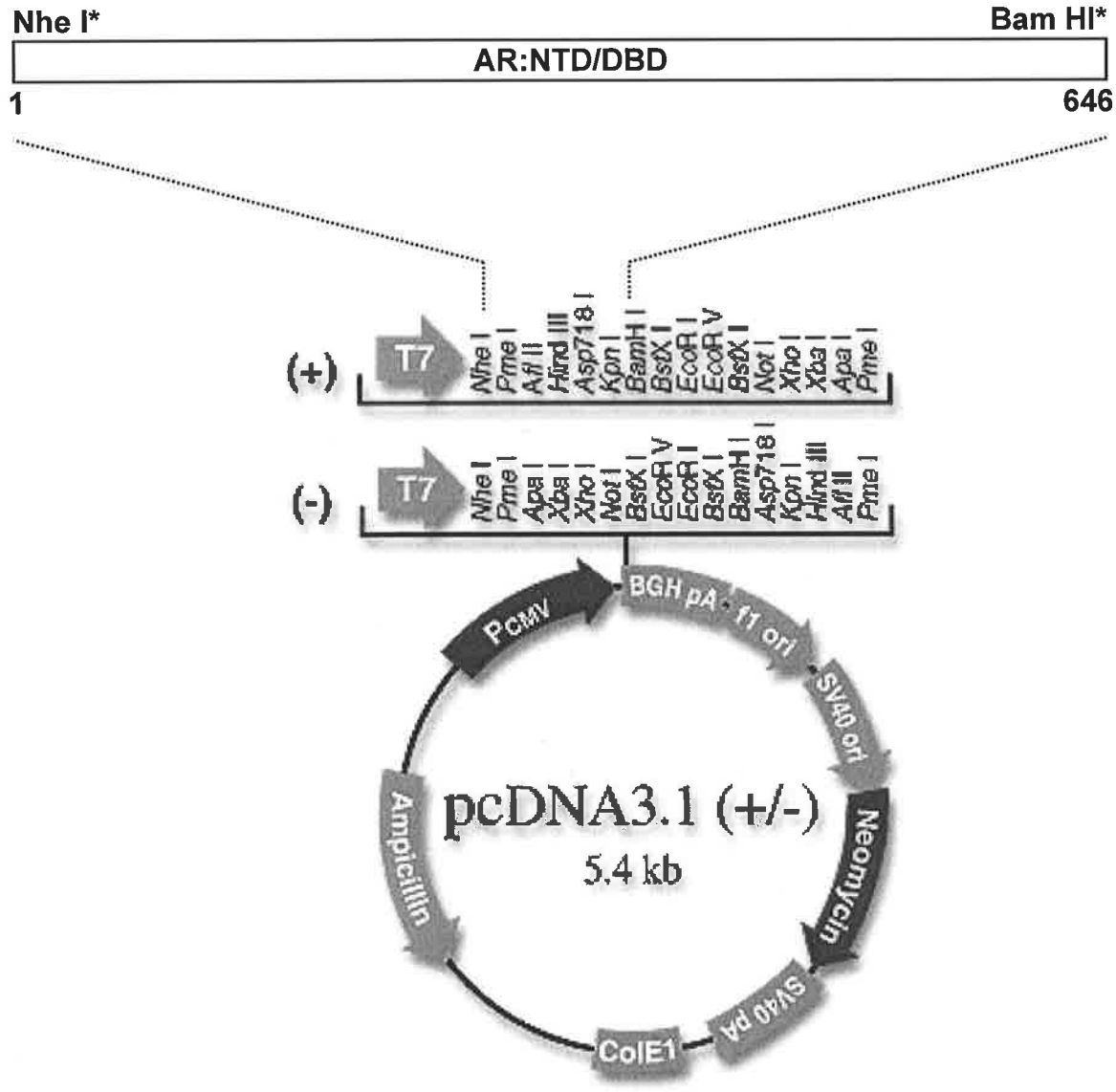
pCMV3.1-AR(ms-):wt was constructed from the pCMV3.1-AR:wt plasmid. Hind III, Kpn I and Sma I restriction sites are located in both the AR coding sequence and in the multiple cloning site of the pCMV3.1 parental expression plasmid. These sites were eliminated from the multiple cloning site to facilitate their use in cloning and mutagenesis of the AR sequence. pCMV3.1-AR:wt was sequentially digested with Sma I and Mlu I. This was followed by end filling with *Pfu* polymerase and blunt end ligation. This plasmid was constructed by Dr Grant Buchanan.

pCMV3.1-AR(ms-):Q865H was constructed from the pCMV3.1-AR(ms-):wt plasmid as described in Section 5.2.2.

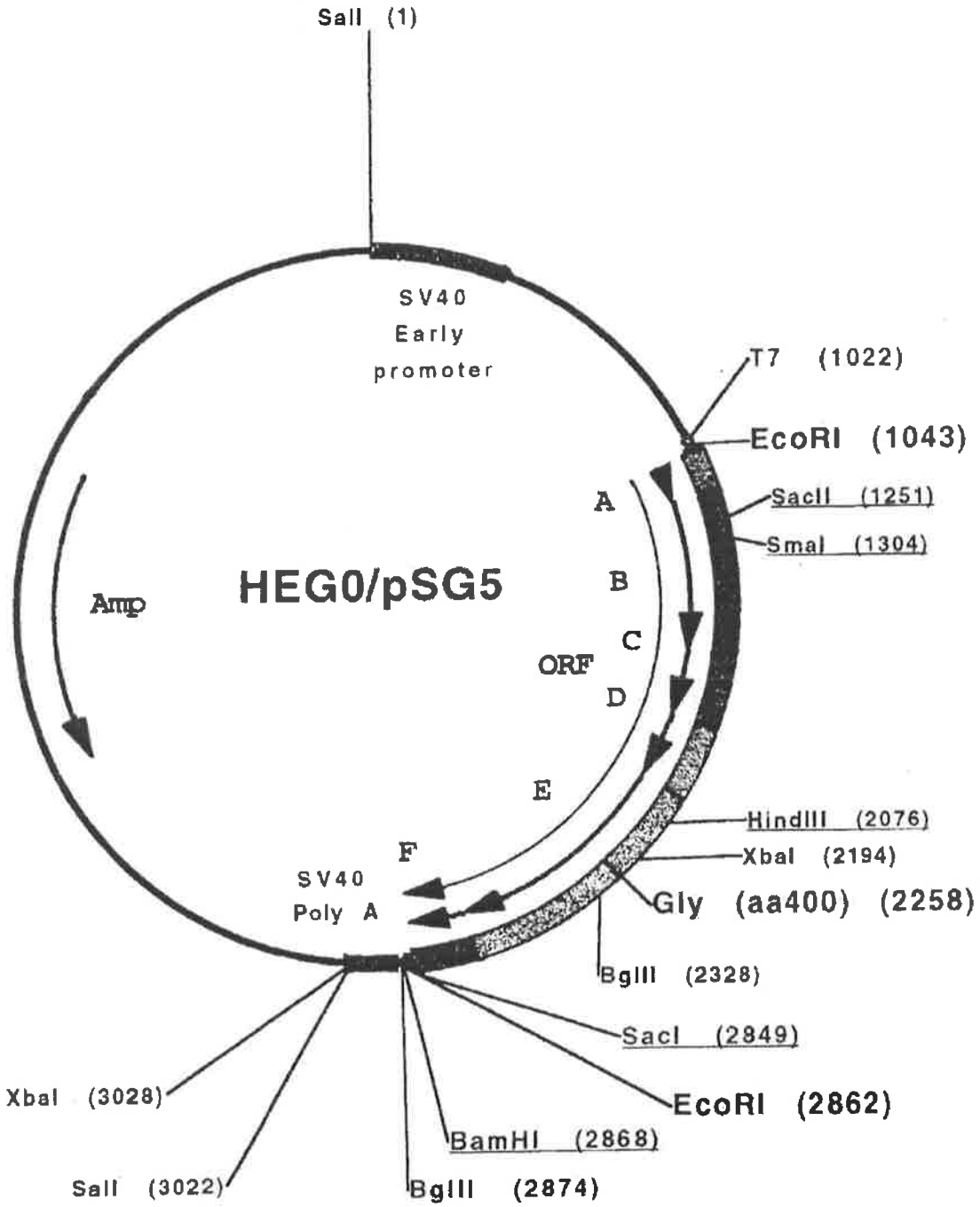


pcDNA-AR(NTD/DBD) contains the human AR cDNA sequence encoding amino acids 1-646 cloned into the pcDNA3.1(+) parental expression vector. The AR encoded by this construct contains 21 glutamine residues in the poly-glutamine tract.

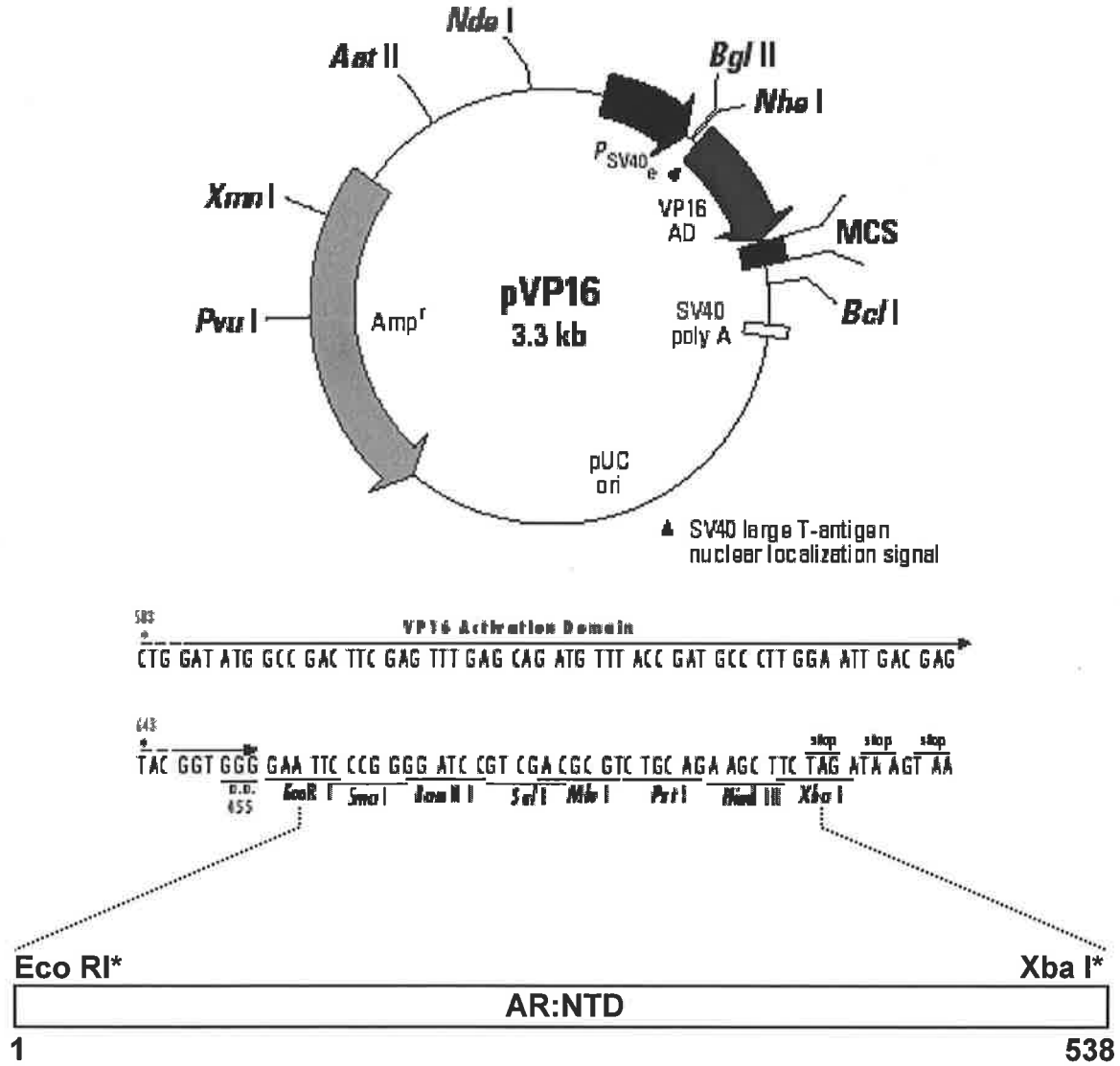
*** Restriction enzyme sites introduced by PCR**



HEGO/pSG5 (ERα) contains the entire coding region of the human ERα cDNA (with amino acid 400 mutated to glycine) cloned into the pSG5 expression vector.



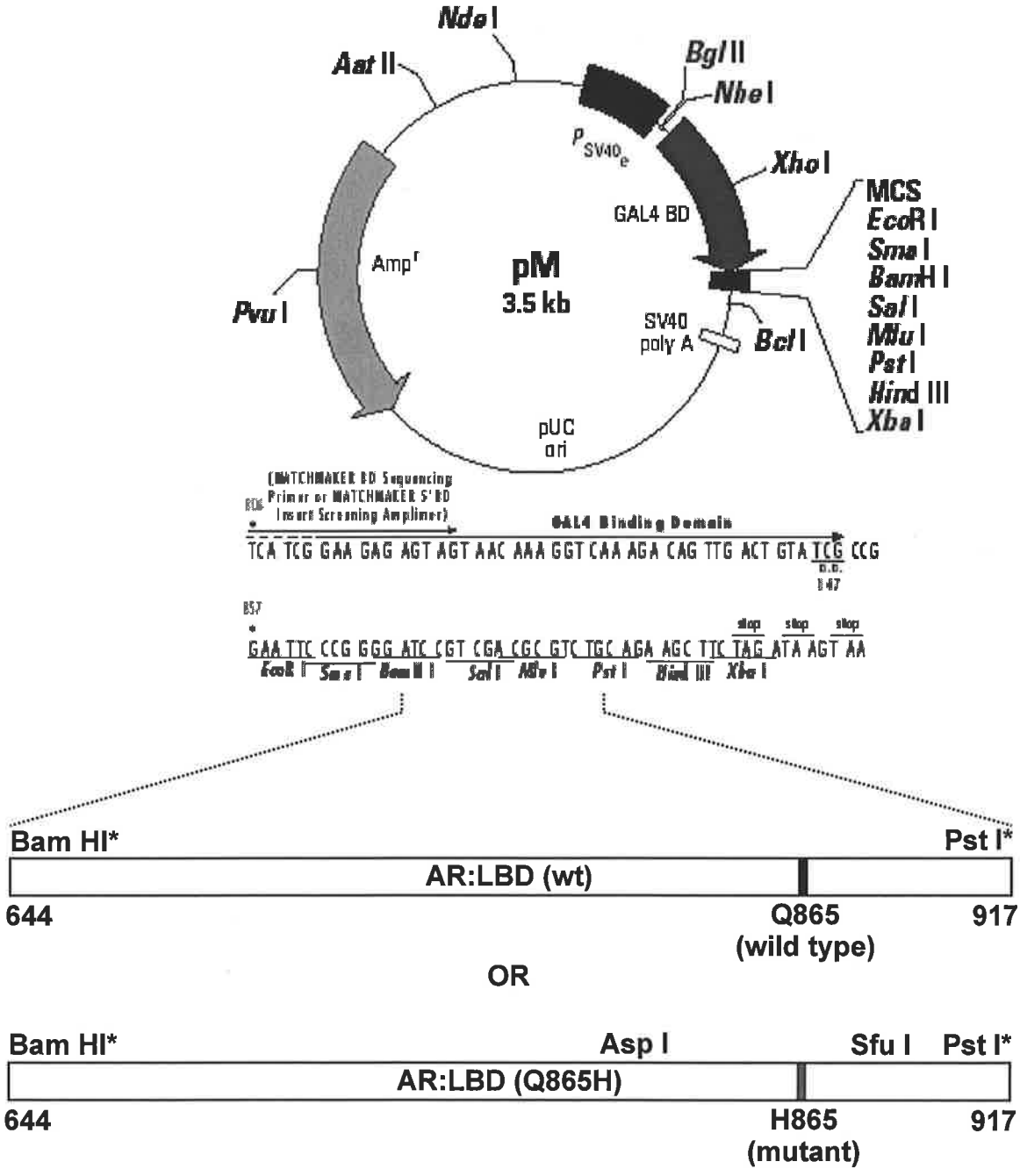
pVP16AD-AR:NTD contains cDNA encoding amino acids 1-538 of the AR cloned into the pVP16 vector. The AR encoded by this construct contains 21 glutamine residues in the poly-glutamine tract.



*** Restriction enzyme sites introduced by PCR**

pM-AR:LBD (wt) contains cDNA encoding amino acids 644-917 of the wtAR cloned into the pM vector.

pM-AR:LBD (Q865H) was constructed by ligation of a 510bp Asp I-Sfu I fragment from the pCMV3.1-AR(ms-):Q865H plasmid into the corresponding restriction enzyme sites in pM-AR:LBD (wt). This plasmid was constructed by Ms Kathleen Saint.



*** Restriction enzyme sites introduced by PCR**

Appendix 2 – Oligonucleotide Primers and Probes

AR primers

N111: ..... 5'-CAGAATCTGTTCCAGAGCGTGC-3'

N222: ..... 5'-GTCTTTAAGGTCAGCGGAGC-3'

N122: ..... 5'-CTGCCCCATCCACGTTGTC-3'

N2330: ..... 5'-CTAGGCTCTCGCCTTCTAGC-3'

144: ..... 5'-CACTGAAGATACTGCTGAGT-3'

244: ..... 5'-CCACCACACGGTCCATACAACT-3'

N155: ..... 5'-CATCCTGGCACACTCTCTTCAC-3'

Hind III AS: ..... 5'-GACACCCAGAAGCTTCATCTCCA-3'

N166: ..... 5'-TAGCCCCCTACGGCTACACT-3'

x4AS: ..... 5'-ACACACTACACCTGGCTCAAT-3'

ARCS1: ..... 5'-TGAAGCAGGGATGACTCTGGG-3'

ARCAS3: ..... 5'-GCTTTCATGCACAGGAATTCC-3'

ARCS3: ..... 5'-TACACGTGGACGACCAGATGG-3'

S: ..... 5'-CAGGCAGAAGACATCTGAAAG-3'

Q865H: ..... 5'-GACTCCGTGCATCCTATTGCG-3' ('Mutant' base underlined)

Xba I antisense: ..... 5'-CCTCTAGAGTCGACCTGCAGG-3'

K718E: ..... 5'-AAGTGGGCCGAGGCCTTG-3'

AR917: ..... 5'-CAGGCAGAAGACATCTGAAAG-3'

Primers for housekeeping genes

18S rRNA sense: ..... 5'-CCATTGGAGGGCAAGTCTGG-3'

18S rRNA antisense + SP6: .. 5'-ATTTAGGTGACACTATAGGAGGTCGGCATCGTTTATGGTC-3'

β-actin sense: ..... 5'-GCCAACACAGTGCTGTCTGG-3'

β-actin antisense: ..... 5'-TACTCCTGCTTGCTGATCCA-3'

GAPDH sense: ..... 5'-GTCATGGGTGTGAACCATGAGA-3'

GAPDH antisense: ..... 5'-GGTCATGAGTCCTTCCACGATAC-3'

BRCA1 primers

BRCA1 sense: ..... 5'-CCCTATAAGCAAGAATCC-3'

BRCA1 antisense + SP6: ..... 5'-ATTTAGGTGACACTATAGGAAGTATGAGCAGCAGCTGGAC-3'

BRCA1 5349 sense: ..... 5'-AGAAACCACCAAGGTCCAAAGC-3'

BRCA1 5523 antisense: ..... 5'-TGCCAAGGGTGAATGATGAAAG-3'

PSA primers

PSA sense: ..... 5'-GGCAGCATTGAACCAGAGGAGTT-3'

PSA antisense: ..... 5'-GCATGAACTTGGTCACCTTCTG-3'

PSA ARE I sense: ..... 5'-CCTAGATGAAGTCTCCATGAGCTACA-3'

PSA ARE I antisense: ..... 5'-GGGAGGGAGAGCTAGCACTTG-3'

PSA ARE III sense: ..... 5'-GCCTGGATCTGAGAGAGATATCAT-3'

PSA ARE III antisense: ..... 5'-ACACCTTTTTTTTTCTGGATTGTTG-3'

PSA irrelevant region sense: ..5'-TCATCATGAATCGCACTGTTAGC-3'

PSA irrelevant region antisense:5'-GCCCAACTGCCTTGGTATACC-3'

Miscellaneous primers

Mycoplasma sense: ..... 5'-ACTCCTACGGGAGGCAGCAGTA-3'

Mycoplasma antisense: ..... 5'-TGCACCATCTGTCACTCTGTTAACCTC-3'

Dual labelled probes

PSA ARE I: ..... 5'-FAM-CAATTACTAGATCACCCTGGATGCACCAGG-BHQ-1-3'

PSA ARE III: ..... 5'-FAM-TGCAAGGATGCCTGCTTTACAAACATCC-BHQ-1-3'

PSA irrelevant region: ..... 5'-FAM-TGAATCATCTGGCACGGCCCAA-BHQ-1-3'

GAPDH mRNA: ..... 5'-FAM-CAGCCTCAAGATCATCAGCAATGCCTC-BHQ-1-3'

PSA mRNA: ..... 5'-FAM-ATGACGTGTGTGCGCAAGTTCACCC-BHQ-1-3'

Appendix 3 – Steroid Bulk Solutions

Bulk solutions for radioligand binding assays were prepared by combination of ^{3}H-labelled steroid and the corresponding unlabelled steroid as described in Tables A3.1 (DHT) and A3.2 (MPA). Open vials were left in a fume hood until the liquid had evaporated and then 10mL cytosol buffer was added to vials 1-5 and 100µL cytosol buffer was added to vials 1'-5'. Steroids were allowed to dissolve at room temperature overnight. The following day, 5mL of solution from "labelled" vials (1-5) were transferred to each of the corresponding "unlabelled" vials (1'-5'), the bulk solutions were mixed and 2 x 50µL aliquots were counted in 2mL of scintillation fluid in a scintillation counter to determine actual concentration.

Table A3.1: Theoretical concentrations and initial volumes used for preparation of ^{3}H-DHT bulk solutions. Actual concentrations of ^{3}H-DHT stock and ^{3}H-DHT bulk solutions were calculated using formula A3.1.

^{3}H-DHT			
Vial number	Theoretical concentration (nM)	µL ^{3}H-DHT (450.5nM)	Actual concentration (nM)
1	0.2	4.4	0.15
2	0.45	10	0.32
3	0.9	20	0.61
4	2.2	49	1.52
5	6.0	133	4.23
^{3}H-DHT + unlabelled DHT			
Vial number	Theoretical concentration (nM), 250x molar excess over ^{3}H-DHT		µL 10^{-4}M unlabelled DHT
1'	50		2.5
2'	112.5		5.6
3'	225		11.5
4'	550		27.5
5'	1500		75

Formula A3.1:

Actual concentration $= \dfrac{\text{dpm (50µL) x 2}}{\text{specific activity x 2.22 x 100}}$

$= \text{dpm (50µL)} \times 0.00008116$

where specific activity (^{3}H-DHT) = 111Ci/mmol

dpm = disintegrations per minute

Table A3.2: Theoretical concentrations and initial volumes used for preparation of ^{3}H-MPA bulk solutions. Actual concentrations of ^{3}H-MPA stock and ^{3}H-MPA bulk solutions were calculated using formula A3.2.

		^{3}H-MPA	
Vial number	Theoretical concentration (nM)	µL ^{3}H-MPA (826.6nM)	Actual concentration (nM)
1	0.2	1.0	0.09
2	0.45	2.2	0.22
3	0.9	4.4	0.40
4	2.2	10.6	0.93
5	6.0	29.0	2.80
		^{3}H-MPA + unlabelled MPA	
Vial number	Theoretical concentration (nM), 250x molar excess over ^{3}H-MPA		µL 10^{-4}M unlabelled MPA
1'	50		1
2'	112.5		2.3
3'	225		4.5
4'	550		11.0
5'	1500		30.0

Formula A3.2:

Actual concentration $= \frac{\text{dpm (50µL) x 2}}{\text{specific activity x 2.22 x 100}}$

$= \text{dpm (50µL)} \times 0.0001816$

where specific activity (^{3}H-MPA) = 49.6Ci/mmol

dpm = disintegrations per minute

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