The Molecular Actions of Medroxyprogesterone Acetate on Androgen Receptor Signalling and the Promotion of Breast Cancer

A thesis submitted to the University of Adelaide in fulfilment of the requirements for the degree Doctorate of Philosophy

by

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Abstract

The Women's Health Initiative (WHI) clinical trial was the first randomised, double blind, placebo-controlled disease prevention trial to demonstrate epidemiological evidence of a casual link between the use of combined hormone replacement therapy (cHRT) comprising conjugated equine estrogens (CEE) and the synthetic progestin medroxyprogesterone acetate (MPA) and increased breast cancer risk in post-menopausal women. Since the first WHI report in 2002, other observational studies have demonstrated that it is the addition of the synthetic progestin in the cHRT that is associated with an increase in breast cancer risk.

The focus of this thesis was to investigate the following hypothesis formulated in the Dame Roma Mitchell Cancer Research Laboratory, which proposed that MPA possesses antagonistic actions on androgen receptor (AR)-signalling and thereby can disrupt the protective effect of androgens in the breast, thus leading to an increased risk of breast cancer. Androgens have been associated with a growth restrictive role in breast tissue in both humans and animals and are now emerging as key hormonal pathways involved in the pathogenesis of breast cancer. The objectives of this thesis were firstly to determine the relationship between the use of cHRT containing MPA and breast cancer incidence in Australian women, and secondly to perform biological studies to investigate the effect of AR-action in breast epithelial cells. Initial findings described in this thesis led to the identification of a positive association between the use of cHRT preparations containing MPA and breast cancer incidence in Australian women. Subsequent biological based studies were undertaken with non-malignant breast tissues samples from pre- and post-

menopausal women in an *ex vivo* breast explant tissue culture experimental model and the oestrogen receptor (ER), progesterone receptor (PR) and AR positive ZR-75-1 breast cancer cell line to investigate the actions of MPA on AR-signalling and cancer-related intracellular signalling pathways. Collectively these studies demonstrated that the actions of MPA can impede the anti-proliferative actions of DHT in both human post-menopausal non-malignant and malignant breast epithelial cells via AR-mediated actions. Furthermore, the combined actions of DHT and MPA were also shown to de-regulate cancer-related intracellular pathways compared to individual hormone treatments. The findings described in this thesis provide novel results indicating that MPA may promote the development of breast cancer in post-menopausal women taking cHRT via AR-mediated actions and that use of this form of hormone therapy remains a major public health concern.

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 Aleksandra Monica Ochnik 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.

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Aleksandra Monica Ochnik

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List of Abbreviations

3β-HSD	3β-hydroxysteroid dehydrogenase
17β-HSD	17β-hydroxysteroid dehydrogenase
A	androstenedione
AAT	androgen ablation therapy
AB	alveolar buds
ACTH	adreno-corticotrophic hormone
AGMA	Australian Government Medicare Australia
AIHW	Australian Institute Health and Welfare
AIS	androgen insensitivity syndrome
ANOVA	analysis of variance
AR	androgen receptor
ARE	androgen response element
BALB	albino laboratory-bred strain of the *mus musculus*
BEK1	fibroblast growth factor receptor 2
Bic	bicalutamide
Bis	bisulphate
BMI	body mass index
Brd-U	bromodeoxyuridine
BRCA1	breast cancer 1, early onset
BRCA2	breast cancer 2, early onset
BSA	bovine serum albumin
C1orf116	chromosome 1 open reading frame 116
CBG	corticosteroid binding protein
CEE	conjugated equine estrogen
ChIA-PET	chromatin interaction analysis by paired-end tag sequencing
ChIP	chromatin immunoprecitation
CHA	chlormadinone acetate
cHRT	combined hormone replacement therapy
CI	confidence interval

CK	cytokeratin
COC	combined oral contraceptives
CPA	cyproterone acetate
CpG	cytosine and guanine linked by a phosphodiester bond
CT	calibration threshold
CYP11A	gene for cholesterol side chain cleavage cytochrome
CYP17	gene for 17α-hydroxylase p450c17
CYP19	gene for P450 aromatase
DAB	3, 3'-diaminobenzidine
DBD	DNA binding domain
DCC	dextran coated charcoal-stripped
DCIS	ductal carcinoma *in-situ*
DD-Ct	delta-delta-Ct
DE	dienogest
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulphate
DHT	5α - dihydrotestosterone
DMBA	dimethylbez [a]-anthracene
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DP	drospirenone
DPX	Di-n-butylPhthalate in xylene
DRMCRL	Dame Roma Mitchell Cancer Research Laboratory
DTT	Dithiothreitol
E_1	oestrone
E_2	17β-oestradiol
E_3	oestriol
ECL	enhanced chemilumiescence
EDTA	ethylenediamine tetra-acetic acid
EG	etonogesterol
EGF	epidermal growth factor

EGFR	epidermal growth factor receptor
EtOH	ethanol
ER	oestrogen receptor
ERα	oestrogen receptor alpha
ERβ	oestrogen receptor beta
ERE	oestrogen response element
ERT	estrogen replacement therapy
FCS	foetal calf serum
FDA	Food and Drug Administration
FGFR2	fibroblast growth factor receptor 2
FKBP5	FK-506 binding protein 51
FSH	follicle stimulating hormone
GD	gestodene
GFP	green fluorescent protein
GnRH	gonadotrophin releasing hormone
GO	gene ontology
GR	glucocorticoid receptor
GST	glutathione sepharose transferase
GWAS	genome wide association studies
HBECs	human breast epithelial cells
hCG	human chronic gonadotrophin
HC	hormonal contraceptives
H&E	haematoxylin and eosin
HER2	human epidermal growth factor receptor 2
HPG	hypothalamic pituitary gonadal-axis
HRE	hormone response element
HRP	horseradish peroxidase
HRT	hormone replacement therapy
HS	histoscore
hsp	heat shock protein
HT	hormone therapy

HZ	hazard ratio
IDC	invasive ductal carcinoma
ILC	invasive lobular carcinoma
IGF	insulin growth factor
IgG	immunoglobulin
IMVS	Institute of Medical and Veterinary Science
IPA	Ingenuity Pathway Analysis
IUD	intrauterine device
KITL	kit-ligand
LBD	ligand binding domain
LCIS	lobular carcinoma *in-situ*
LCM	laser capture micro-dissection
LH	luteinising hormone
LHRH	luteinising hormone releasing hormone
LNG	levonorgesterol
LOH	loss of heterozygosity
MG	medrogestone
MIOD	mean integrated optical density
MOD	mean optical density
MPA	medroxyprogesterone acetate
MR	mineralocorticoid receptor
MWS	Millions Women Study
N/C	amino and carboxy terminal domain
NDP	Nanozoomer digital pathology
NETA	norethisterone acetate
NG	norgestimate
NGA	nomegesterol acetate
NLS	nuclear localization sequence
NR	nuclear receptor
NDRG1	n-myc downstream regulated 1
NTD	amino-terminal transactivation domain

OHF	hydroxyflutamide
OLR1	oxidised low density lipoprotein, (lectin-like) receptor 1
Org2058	synthetic progestagenic compound
PAGE	polyacrylamide gel electrophoresis
PBS	Pharmaceutical Benefits Scheme or phosphate buffered saline
PBSS	Pharmaceutical Benefits Scheme Statistics
PCNA	proliferating cell nuclear antigen
PCOS	polycystic ovary syndrome
Pg	progesterone
PG	promegestone
PSA	prostate specific antigen
PR	progesterone receptor
PRA	progesterone receptor isoform A
PRB	progesterone receptor isoform B
PRF	phenol-red free
POC	progestin only contraceptive
P/S	penicillin and streptomycin
qRT-PCR	quantitative real-time polymerase chain reaction
R5020	promegesterone
RAR	retinoic acid receptor
RBA	relative binding affinity
RGS2	regulator of G-protein signalling 2
RIPA	Radio-Immunoprecipitation Assay
RPE	rating of perceived exertion
RPMI	Roswell Park Memorial Institute
RO	reverse osmosis
RNA	ribonucleic acid
RQI	Rice Quantum Institute
RR	relative risk
RT	reverse transcription
RU58668	steroidal anti-oestrogen

RXR	retinoid X receptor
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SERM	selective oestrogen receptor modulator
SHBG	sex hormone binding globulin
siRNA	small interfering RNA
SNPs	single nucleotide polymorphisms
SRD5A1	gene for 5α-reductase type 1
SRD5A1	gene for 5α-reductase type 2
STDEV	standard deviation
T	testosterone
TBST	tris-buffered saline and Tween 20
TEB	terminal end buds
TEMED	Tetramethylethylenediamine
TG	trimegestone
TDLU	terminal ductal lobular unit
TIB	tibolone
TM	transcriptional machinery
TPR	tetratricopeptide repeat
TR	thyroid receptor
UDP-GlcDH	uridine diphosphoglucose dehydrogenase
U-PMTVC	under light microscopy
VDR	vitamin D receptor
VIA	video image analysis
WHI	Women's Health Initiative

Units:

ºC	degrees Celsius
Da	Daltons(s)
g	grams or relative centrifugal force

kDa	kilo Dalton
kB	kilo bases
L	litre
min	minutes
M	molar (moles per litre)
mA	milliampere
mL	millilitre
nM	nanomolar
µL	microlitre
µM	micromolar
µm	microns
rpm	revolutions per minute
sec	seconds
U	units
V	volts

Chemical Formulas:

Cl	chloride
CO_2	carbon dioxide
H^3	hydrogen
HCL	hydrochloride
NaCl	sodium chloride

Country and State:

ADL	Adelaide
AU	Australia
CA	California
DE	Germany
KS	Kansas
JP	Japan
MEL	Melbourne
MO	Missouri

NSW	New South Wales
NY	New York
SA	South Australia
SYD	Sydney
USA	United States of America
UK	United Kingdom
VIC	Victoria
WA	Washington

Publications Arising From This Thesis

Articles in preparation:

Ochnik AM, *Moore NLM, Jankovic-Karasoulos T, Cops EJ, Birrell SN, Bianco-Miotto T, Butler LM, Jindal S, Ricciardelli C, Tilley WD and Hickey TE.* Medroxyprogesterone acetate impedes androgen receptor function and inhibits the anti-proliferative effect of 5α-dihydrotestosterone in normal and malignant breast epithelial cells derived from post-menopausal women. ***(Submission to Cancer Research)***

Ochnik AM, *Briggs N, Hickey TE and Tilley WD.* An association between the use of MPA-containing cHRT preparations and increased breast cancer incidence rates in Australian women. (***Submission to Medical Journal of Australia)***

Cops EJ, **Ochnik AM,** Jankovic-Karasoulos T, Birrell SN, Jindal S, Thomas M, Bianco-Miotto T, Butler LM, Tilley WD and Hickey TE. *Androgen receptor expression predominates over estrogen receptor alpha in normal human breast epithelia but not in estrogen sensitive breast tumours.(**Submission to Breast Cancer Research)***

Abstracts published in the proceedings of scientific meetings:

Moore NL, Hanson AR, ***Ochnik AM,*** *Buchanan G, Butler LM, Hickey TE and Tilley WD.* Opposing actions of androgen and the progestin medroxyprogesterone acetate on endogenous androgen receptor signalling in human breast cancer cells: ***5th International PACRIM Breast and Prostate Cancer Meeting, (2011), Byron Bay, QLD, Australia, May 4th-7th, 2011.***

Ochnik AM, *Jankovic-Karasoulos T, Cops EJ, Birrell SN, Butler LM, Jindal S, Bianco-Miotto T, Thomas M, Tilley WD and Hickey TE.* Medroxyprogesterone acetate impairs androgen receptor function and inhibits the anti-proliferative effect of 5α-dihydrotestosterone in non-malignant breast tissue derived from post-menopausal women. ***American Association of Cancer Research 101st Annual Scientific Meeting, Washington DC, USA, April 22nd-24th, 2010.***

Hickey TE, Cops EJ, Jankovic-Karasoulos T, ***Ochnik AM****, Jindal S, Birrell SN, Moore NL, and Tilley WD.* The synthetic progestin medroxyprogesterone acetate increases human breast epithelial proliferation by disrupting androgen receptor signalling. ***Gordon Breast Cancer Conference Salve Regina University, NewPort RI, California, USA, June 14th-19th, 2009.***

Ochnik AM*, Jankovic-Karasoulos T, Cops EJ, Birrell SN, Bianco-Miotto T, Butler LM, Jindal S, Ricciardelli C, Tilley WD and Hickey TE.* The synthetic progestin medroxyprogesterone acetate increases human breast epithelial proliferation by disrupting androgen receptor signalling. ***The Endocrine Society of Australia Annual***

Scientific Meeting, Adelaide Convention Centre, Adelaide, South Australia, Australia, August 23rd-26th, 2009.

Ochnik AM*, Jankovic-Karasoulos T, Cops EJ, Birrell SN, Bianco-Miotto T, Butler LM, Jindal S, Ricciardelli C, Tilley WD and Hickey TE.* Increased breast epithelial cell proliferation by the synthetic progestin medroxyprogesterone acetate is associated with disruption of AR signalling in breast tissue: A potential mechanism of carcinogenesis associated with hormone replacement therapy. ***The Australian Society of Medical Research Annual Scientific Meeting, Adelaide Convention Centre, Adelaide, SA, Australia, June 2009.***

Ochnik AM, *Hickey TE, Bianco-Miotto T, Butler LM and Tilley WD.* Disruption of Androgen Receptor Signalling In The Breast By the Synthetic Progestin Medroxyprogesterone Acetate: A Potential Mechanism Of Carcinogenesis Associated With Hormone Replacement Therapy. ***The Australian Society of Medical Research Annual Scientific Meeting, Adelaide Entertainment Centre, Adelaide, SA, Australia June 2008.***

Hickey TE, ***Ochnik AM,*** *Bianco-Miotto T, Butler LM and Tilley WD.* Androgen regulates KITL expression in normal breast epithelium and an androgen receptor positive breast cancer cell line. ***The Era of Hope Annual Scientific Meeting, US Department of Defense, Baltimore Convention Centre, USA, June 25th-28th, 2008.***

Hickey TE, ***Ochnik AM****, Bianco-Miotto T, Butler LM and Tilley WD.* Functional Androgen Signalling in an Explant Model of Normal Human Breast Tissue. ***The Society for the Study of Reproduction 41st Annual Scientific Meeting, Kailua-Kona, Hawaii, USA, May 25th-30th, 2008.***

Chapter 1

Introduction

1.1 - Background

Breast tissue is highly and dynamically regulated by sex steroid hormonal activity and is predominantly stimulated by ovarian hormones to progress through various developmental changes during the female reproductive life. The current understanding of these endogenous hormonally driven biological processes have further provided a knowledge basis into key aspects associated with breast carcinogenesis. This literature review provides an overview of breast biology and details the mechanistic actions of sex steroid hormones in normal tissue and their potential role in breast cancer initiation, development and progression which have enabled human intervention by preventative lifestyle changes, detection and treatment of breast cancer. The information gained from the years of work in the area of hormone mediated breast carcinogenesis has further provided a foundation for hypothesis driven investigation into the pivotal driving forces in the pathogenesis of breast cancer.

1.2 - Embryogenesis, development and differentiation of the female breast

1.2.1 - Organogenesis of breast tissue during embryogenesis

The normal human breast tissue progresses through developmental, differentiation and structural changes during embryogenesis, puberty, pregnancy, lactation and menopause. Development of the breast during embryogenesis is divided into ten progressive stages (i.e. mammary ridge, milk hill, mammary disc, lobule type, cone, budding, indentation, branching, canalization and end vesicle stages), which occur at various embryo-foetal

stages defined by the length of the embryo (5mm-10cm) and specific weeks during gestation (Russo, Tay et al. 1982). In a newborn, the breast is comprised of the nipple attached to an undeveloped ductal network. The breast structure remains in this undeveloped state until puberty when subsequent breast developmental events occur that are distinct from those that happen during embryogenesis (Russo, Tay et al. 1982).

1.2.2 - Puberty

Pubertal breast development is stimulated by the sex steroid hormones oestrogen and progesterone, produced predominantly by the ovary and adrenal glands. The combined hormonal actions stimulate an increase of the glandular breast tissue through increased ductal extension, by a process of elongation and branching of the breast ducts. At the end of the breast ducts, the formation of clubbed end structures occurs, which are referred to as terminal end buds (TEB) (Russo and Russo 2004) [Figure 1.1]. At the TEB, new branches and small ductules, commonly referred to as alveolar buds, are produced, which collectively form lobular type 1(virginal lobules) breast structures [Figure 1.1]. The lobular type 1 structures are referred to as terminal ductal lobular units (TDLU) and are the predominant site of breast cancer development (Wellings, Jensen et al. 1975). The breast largely remains in this immature developmental phase until pregnancy and lactation, when further development and differentiation of the breast tissue occurs.

1.2.3 - Development and differentiation of the breast tissue

During terminal breast development associated with full-term pregnancy the breast glandular tissue undergoes dramatic structural, biological and genetic changes,

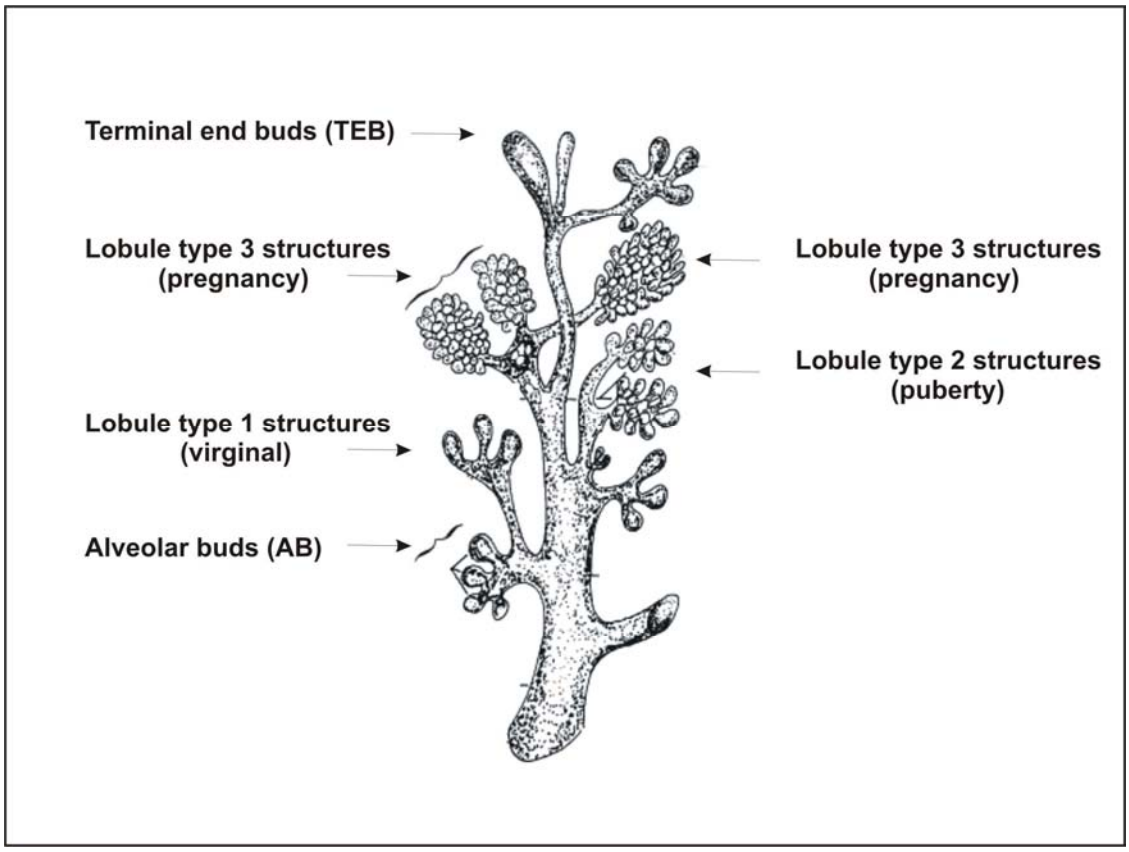

Figure 1.1: Normal anatomy of breast tissue structures during development (puberty and pregnancy). The breast tissue is comprised of the following breast structures: terminal end buds (TEB); alveolar buds (AB); lobule type 1 structures (virginal breast tissue); lobule type 2 structures (pubertal and nulliparous adult breast tissue) and lobule type 3 structures (pregnancy-induced breast tissue) (diagram modified from (Russo and Russo 1998)).

that are associated with hormone-induced differentiation of the breast tissue into maturity. The degree of breast differentiation is directly related to the type of breast structures, which in turn are dictated by the pregnancy status and history of the female (i.e. exposure to exogenous hormones). In nulliparous pre-menopausal women, the breast is comprised predominantly of lobule type 1 structures (65-80%) and undifferentiated TEB (Russo, Rivera et al. 1992; Russo and Russo 1994) [Figure 1.1; 1.2A]. Only a small percentage of lobule type 1 structures in nulliparous women are stimulated to progress to lobule type 2 (10-35%) and 3 structures (0-5%), which occurs during sexual maturity stimulated by normal endogenous hormonal exposure over the menstrual cycle or by exogenous hormone stimulation (hormone contraceptives and endocrine imbalances) (Russo and Russo 1998) [Figure 1.1; 1.2A].

In parous women, during the early stages of pregnancy, maturation of breast development occurs resulting in the formation of new ductules from the alveolar buds, also referred to as alveoli or acini at the TDLU. This developmental process produces the development of lobule type 1 breast structures into lobule type 2 (20%) and 3 structures (70-90%), with a small percentage of lobule type 1 structures (3%) still remaining (Russo, Rivera et al. 1992) [Figure 1.1; 1.2A]. Additional glandular development occurs during the second half of pregnancy, which results in complete differentiation of the breast tissue via the expansion of lobule type 3 structures into lobule type 4 structures that are biologically required for the production and secretion of milk during pregnancy-induced lactation [Figure 1.2A]. The promotion of hormone-induced differentiation in the breast tissue during full-term pregnancy leads to permanent genetic imprinted signatures of gene

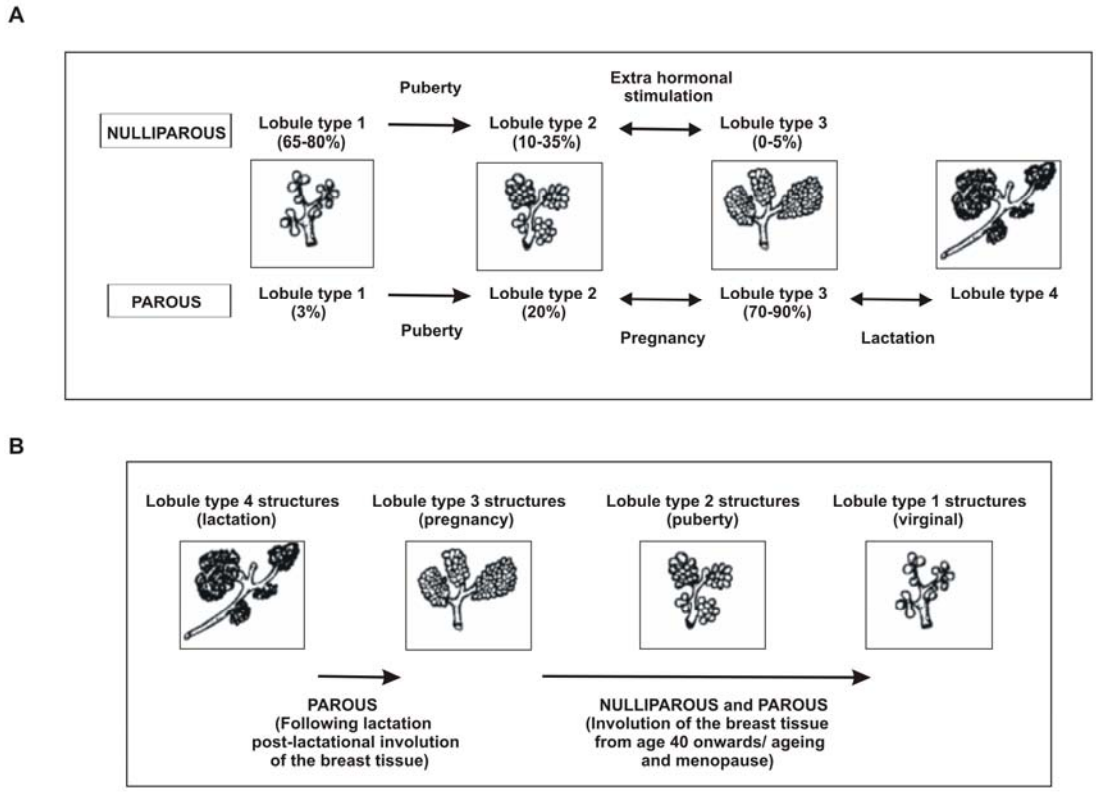

Figure 1.2 (A and B): Developmental stages and structural changes in the human breast tissue during reproductive stages in females. (A) Lobule type structures in the breast tissue in nulliparous and parous women between the ages of 25-40 yrs. The lobule type breast structures differ in composition and percentage depending on the parity status of the female. **(B)** Lobule type structures in the breast tissue following involution by lactation, ageing and menopause (Diagram modified from (Russo and Russo 1998)).

expression profiles in the breast tissue. These findings have been identified following the extraction of RNA from nulliparous and parous human breast epithelium and stroma by laser capture micro dissection (LCM) and Affymetrix gene microarray analysis (Balogh, Heulings et al. 2006; Balogh, Russo et al. 2007). The changes in gene expression during pregnancy-induced differentiation, mediated by pregnancy stimulated maternal hormones are associated with epigenetic changes to the chromatin inducing: heterochromatin reorganisation and hypomethylation or hypermethylation of CpG islands in gene promoters (Devinoy and Rijnkels; Kress, Ballester et al.; Rijnkels, Kabotyanski et al.; Muller, Ivarsson et al. 2004; Russo and Russo 2007).

Post-lactational involution of the breast glandular tissue involves regression of lobule type 4 structures back to lobule type 3 structures via apoptosis of epithelial cells within acini, leading to the elimination of secretory activity and milk production in the breast [Figure 1.2B] (Russo, Rivera et al. 1992). The breast tissue in nulliparous and parous women remain in these biological states induced by differential levels of hormone stimulation until approximately 40 yrs of age, when all lobules begin the process of involution back to lobule type 1 structures in the absence of additional pregnancies [Figure 1.2B] (Hutson, Cowen et al. 1985). Complete involution and regression of the breast tissue occurs over 10-20 years and further changes to the breast tissue occur during the menopausal transition that is reviewed in the subsequent section.

1.2.4 - Menopause

Menopause is a biological process representing the final stages of female reproductive life, which is initiated by a decrease in the production of ovarian sex steroid hormones, and has been reported to occur at the average age of 51 yrs (Treloar 1981; McKinlay, Brambilla et al. 2008). The initiation of menopause is biologically triggered by the complete depletion of ovarian follicles by ovulation or atresia, resulting in cessation of menstruation (WHO 1996). The decline in ovarian follicles occurs throughout the female reproductive life, and is most rapid after the age of 40 in women (Burger 1999). Since developing follicles are a female's major source of sex steroid hormones, the biological reproductive event of menopause leads to the rapid decline in circulating 17β-oestradiol levels which is mirrored by a rapid increase in the production and circulation of pituitary hormones including follicle stimulating hormone (FSH) and luteinising hormone (LH) (Rannevik, Jeppsson et al. 1995; Burger 1999). The menopausal transition occurs over a 4 yr period, during which women are referred to as being peri-menopausal (Burger, Dudley et al. 1995; Burger, Dudley et al. 2002). During the menopausal transition, women are commonly prescribed exogenous hormones in the form of hormone replacement therapy (HRT) to assist with symptoms and disease associated with the decline in ovarian hormones including the following: hot-flushes; excessive sweating; nervousness and irritability; depression; lack of concentration; decline in memory; decreased libido; insomnia and osteoporosis (Lauritzen 1990).

The altered gene expression profiles that occur as a result of epigenetic changes in breast epithelial cells following full-term pregnancy are reported to be permanently maintained

following involution of the breast tissue during the menopausal transition (Russo, Balogh et al. 2006). However, the lobule type 1 breast structures in post-menopausal nulliparous females remain in an immature epigenetic state due to the lack of differentiation induced by pregnancy and lactation related hormones compared to lobule type 1 structures in post-menopausal parous women, which are characterised by a more mature epigenetic signature (Devinoy and Rijnkels; Kress, Ballester et al.; Rijnkels, Kabotyanski et al.; Muller, Ivarsson et al. 2004; Russo and Russo 2007). While the histological characteristics of lobule type 1 breast structures in nulliparous and parous women are essentially identical, biological differences exist in the altered genetic programming in the breast tissue as a consequence of full-term pregnancy in parous women (Russo and Russo 2007). In essence, these differentiation events stimulated by pregnancy are thought to act as a lifetime protective mechanism against breast cancer development in females.

1.2.5 - Terminal differentiation in breast tissue and rates of breast epithelial proliferation

A well established biological characteristic extensively studied in the literature is the reduction of breast tissue proliferation in parous compared to nulliparous women. Breast epithelial cell proliferation is highest in lobule type 1 structures and there is a progressive decline in proliferation in lobule 2 and 3 structures (Russo, Mills et al. 1989). Moreover, reduced breast epithelial cell proliferation specifically within lobule type 1 structures of parous compared to nulliparous post-menopausal women is also reported (Russo, Rivera et al. 1992; Russo, Ao et al. 1999; Taylor, Pearce et al. 2009). A mouse cell line obtained from the BALB/c mouse during mid pregnancy (HC11 mammary cell line) is used

commonly to study differentiation in the mammary gland (Ball, Friis et al. 1988). Reduced breast epithelial cell proliferation by terminal differentiation in the mouse breast epithelial cell line has been demonstrated by the decreased expression of proliferating cell nuclear antigen (PCNA) (a proliferation factor) (Desrivieres, Prinz et al. 2003). Recently reported is the tendency towards decreased expression of the proliferation marker MIB1 in the human breast tissue from parous (1.2%) compared to nulliparous women (2.2%), however this finding was not significant (Taylor, Pearce et al. 2009). These studies illustrate that pregnancy influences the proliferation rates in breast tissue subsequent to complete differentiation of the breast tissue. The importance of these findings is the potential differences that exist in breast tissue subsequent to pregnancy that can alter responsiveness to both endogenous and exogenous hormones, ultimately impacting on the development of breast cancer, reviewed in the following section.

1.2.6 - Differentiation of the breast and breast cancer

Prior to pregnancy, and following puberty the breast tissue remains in an highly undifferentiated biological state that has a high susceptibility to carcinogenic stimulation and subsequent neoplastic transformation, defined as the '*window of susceptibility*' [Figure 1.3] (Russo, Mailo et al. 2005). In contrast, the breast tissue is thought to remain in a protective state to neoplastic transformation following first pregnancy until the initiation of menopause defined as the '*protection window*' [Figure 1.3] (Pike, Krailo et al. 1983; Russo, Rivera et al. 1992). Social aspects related to contemporary life including increased longevity, delayed age of first birth, medical advancements, the reproductive

Figure 1.3: Susceptibility to neoplastic transformation in breast tissue during the female reproductive lifespan. Various stages of breast development are distinctly associated with breast tissue of different structural compositions. The developmental period during which the breast tissue is most susceptible to neoplastic transformation is between menarche and first birth. This '*window of susceptibility'*, is characterised by breast tissue that is comprised predominantly of the most undifferentiated breast structures (lobule type 1 structures). During this time period, the breast tissue is most susceptible to neoplastic transformation (stimulation of pre-malignant lesions) from exposure to endogenous and exogenous hormones. Following pregnancy the breast tissue undergoes complete maturation which results in terminal differentiation of the breast tissue into predominantly lobule type 2 and 3 breast tissue structures. During the time period between first pregnancy and menopause, there is a *'protection window'* when breast cancer risk is at its lowest. During menopause the breast tissue regresses to predominantly lobule type 1 breast tissue structures. The lobule type 1 breast structures in parous women following menopause are thought to retain protective genetic characteristics which occur during full-term pregnancy. Use of hormone replacement therapy (HRT) is commonly administered during the menopausal transition and may be associated with stimulation of pre-existing pre-malignant lesions during this time.

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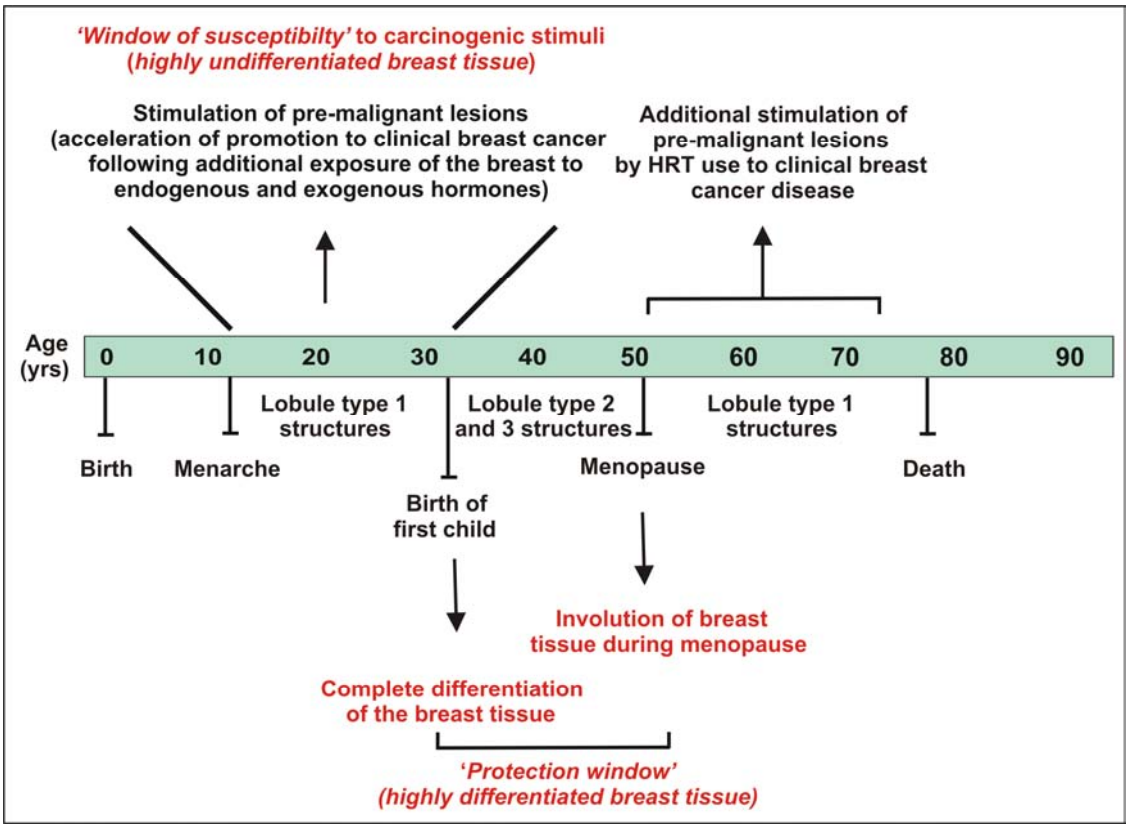

'Window of susceptibilty' to carcinogenic stimuli
(highly undifferentiated breast tissue)
Stimulation of pre-malignant lesions
(acceleration of promotion to clinical breast cancer
following additional exposure of the breast to
endogenous and exogenous hormones)
Additional stimulation of
pre-malignant lesions
by HRT use to clinical breast
cancer disease
Age
(yrs)
0
10
20
30
40
50
60
70
80
90
Lobule type 1
structures
Lobule type 2
and 3 structures
Lobule type 1
structures
Birth
Menarche
Birth of
first child
Menopause
Death
Involution of breast
tissue during menopause
Complete differentiation
of the breast tissue
'Protection window'
(highly differentiated breast tissue)

life-span of females and the natural process of human evolution may lead to a higher rate of breast cancer development by increasing the *'window of susceptibility'* during the pre-menopausal period [Figure 1.3] (Holliday 1997; Kirkwood 1997; Kirkwood 2002; Kirkwood 2008).

The development of clinically detectable breast cancer and other cancers such as lung cancer has been documented to be a biological process that occurs on an average over a 10-20 year period. This has been shown using mathematical theoretical models that calculate the growth kinetics of cancer following pre-initiation events of carcinogenesis (Klawansky and Fox 1984; Semiglazov, Moiseenko et al. 1989). Interestingly, these mathematical models suggest that the proliferation rates of cancer cells is reduced to normal rates following removal of the carcinogenic trigger (Semiglazov, Moiseenko et al. 1989). In light of these findings, it may also be proposed that once a tumour has been fueled by a carcinogen and is clinically detectable by mammography screening, that removal of the carcinogen (i.e. exogenous hormones or tobacco), may not in all cases lead to complete regression of the tumour, but prevents further growth of the pre-existing tumour (Semiglazov, Moiseenko et al. 1989; Zahl, Maehlen et al. 2008). This may explain reports in the literature that report the existence of dormant breast cancer lesions (Almog, Ma et al. 2009), and the natural history of invasive breast cancer where in some cases spontaneous regression of breast tumours can occur (Zahl, Maehlen et al. 2008)

It may be suggested that in order to prevent pre-initiation events related to breast carcinogenesis and subsequent development of long-term development of tumourigenesis

from occurring, an earlier age of first pregnancy to reduce the '*window of susceptibility'* is likely to be beneficial (Russo, Rivera et al. 1992; Russo, Mailo et al. 2005). Additionally, stimulation of pre-malignant lesions formed during the pre-menopausal years of a female may lead to further development into clinical breast cancer following fueling by the exogenous hormones used in HRT during menopause (Horwitz and Sartorius 2008) [Figure 1.3]. Interestingly, in support of this notion, a recent study in Norway has shown an increased breast cancer risk in women with prior history of combined oral contraceptives (COC) use, compared to non COC use who have also taken HRT (Lund, Bakken et al. 2007). This study may give basis to the formation of pre-malignant lesions by COC use in pre-menopausal women, which are further stimulated to clinical breast cancer by use of HRT therapies for menopausal symptoms in peri- and/or post-menopausal women [Figure 1.3].

1.2.7 - Ageing and breast cancer

The breast cancer age-incidence curve highlights the exponential increase in breast cancer with age, that reaches a plateau at 45 yrs and maintains this maximal level during the menopausal years [Figure 1.4] (Pike, Krailo et al. 1983; Klawansky and Fox 1984). This plateau in breast cancer rates is likely to be associated with the rapid decline of ovarian hormone production that occurs during the menopausal transition. The highest rates of breast cancer occur around 45 yrs of age, which on average corresponds to a 25-30 year reproductive time period in females following menarche (Anderson, Dallal et al. 2003).

Importantly, this exponential age-incidence breast cancer curve reflects the association

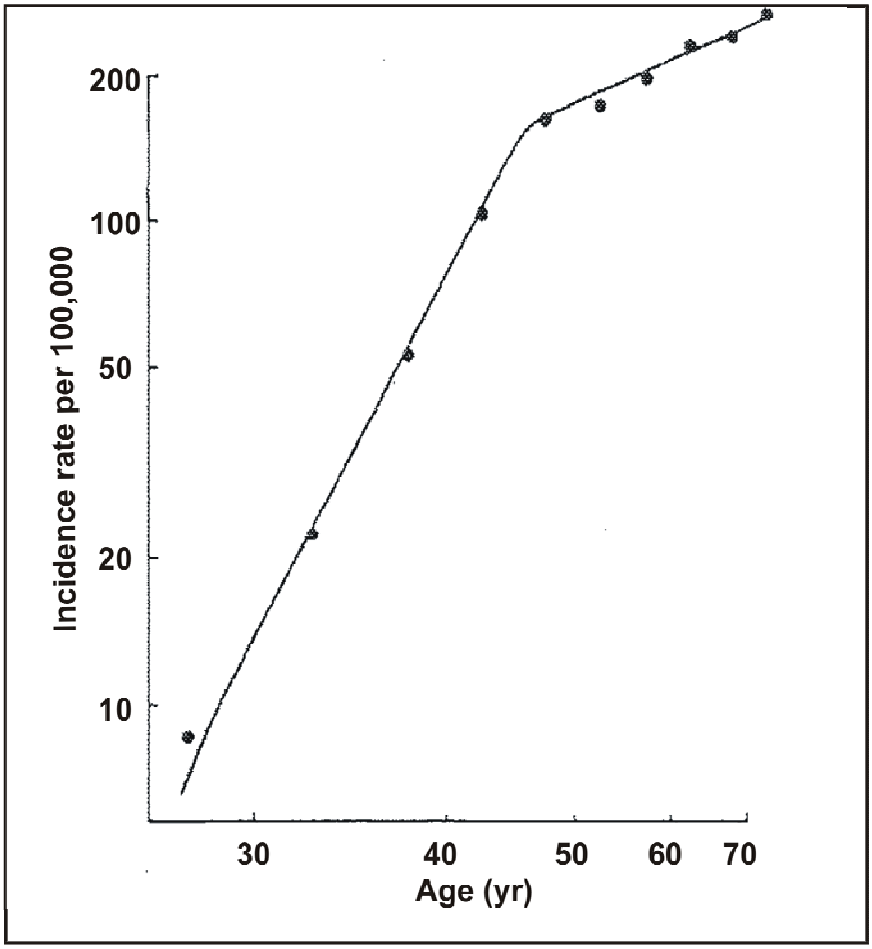


Figure 1.4: Breast cancer and age-incidence exponential curve. Age-specific incidence rates for breast caner in US white females (YNCS 1969-81) and fitted curve calculated from established mathematical model described in the following references (Pike, Krailo et al. 1983; Klawansky and Fox 1984).

between the lifetime cumulative exposure of the breast tissue to endogenous sex hormones leading to a higher rate of breast cancer development in post-menopausal compared to pre-menopausal women (Pike, Krailo et al. 1983). It may also be feasible to speculate that there are intrinsic biological differences in post-menopausal compared to pre-menopausal breast tissue that occur during the menopausal transition that contribute to the increased incidence of breast cancer in post-menopausal women.

1.3 - Normal and malignant human breast tissue

1.3.1 - Anatomy of the human breast

The human breast is a complex glandular secretory organ, which biologically is required for the production and secretion of milk following pregnancy to nourish the offspring. The breast tissue is comprised of specific structural characteristics and cell types. The four major components of breast tissue include: 1) fibrous/stromal connective tissue; 2) glandular (epithelial) tissue; 3) adipose connective; 4) nerve fibers and 5) blood and lymph vessels. Anatomically, the glandular tissue is supported by fibrous connective tissue (comprised of fibroblasts in adipose connective breast tissue) above the pectoral muscle [Figure 1.5]. The breast tissue contains blood and lymph vessels which provide a blood supply and eliminates fluid and wastes into axillary lymph nodes located in the upper chest and arm-pits [Figure 1.5]. Secretion of milk from the lobular-alveolar structures occurs by draining into the mammary ducts, which are connected to the lactiferous sinus and ducts [Figure 1.5]. The mammary lactiferous ducts connect to the

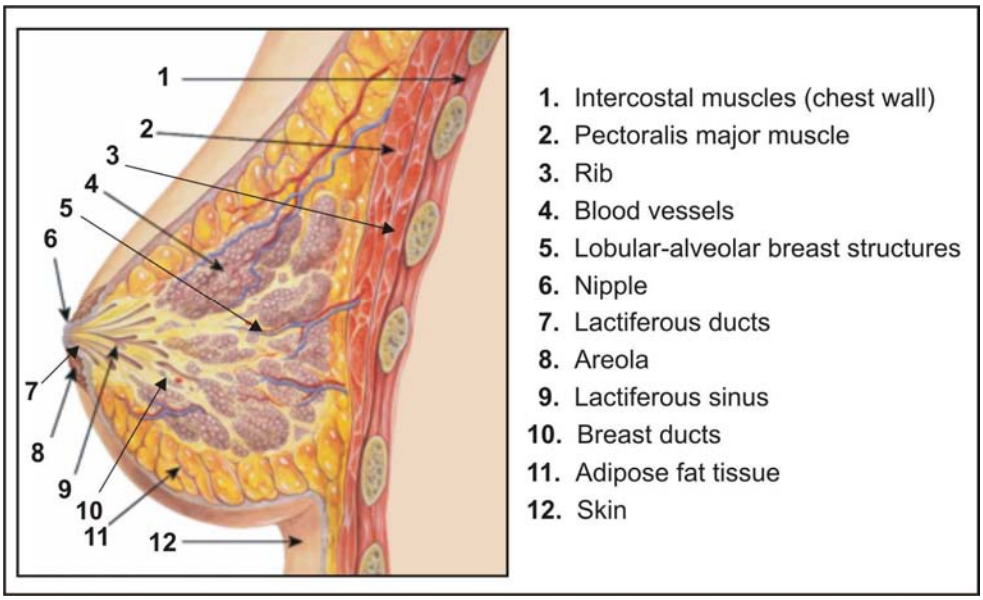


Figure 1.5: Anatomy of the human breast tissue. The human breast is a glandular tissue which acts to produce and secrete milk during pregnancy-induced lactation. The anatomical structure of the breast tissue is comprised of various structures described in the figure (Werner Kahle 2004).

nipple (surrounded by the areola and sebaceous glandular tissue) where breast milk is expelled during lactation [Figure 1.5].

1.3.2 - Cell types in the breast tissue

In the glandular breast tissue, two main types of human breast epithelial cells have been identified, including luminal and myoepithelial cells, thought to be derived from luminal-restricted and myoepithelial-restricted progenitor cells, respectively (Stingl, Eaves et al. 2001; Stingl, Raouf et al. 2005). A third cell type in the breast epithelium has also been described, (basal clear cells) and are thought to act as precursors for myoepithelial cells (Smith, Monaghan et al. 1984). Lining the interior regions of the ducts and alveoli are a single layer of luminal epithelial cells (cuboidal/columnar cells), surrounded by a layer of myoepithelial cells which separate the luminal epithelial cells from the basement membrane (Emerman and Vogl 1986). Around the ducts, the myoepithelial cells form a sheath, whereas in the lobular-alveoli breast structures the myoepithelial cells form more a basket-like structure (Emerman and Vogl 1986). Myoepithelial cells are partly comprised of smooth muscle and thus possess contractile abilities. Basal cells have been identified in both the ducts and lobular-alveoli breast tissue structures, however are predominantly located in the lobular-alveolar structures and are positioned between luminal and myoepithelial cells (Smith, Monaghan et al. 1984). The basal cell types are responsive to the lactation-induced hormone oxytoxin during pregnancy which intiates the basal cells to enlarge and contract during lactation. This promotes the excretion of milk from the alveoli sacs into the breast ducts and into the main lactiferous ducts through to the nipple [Figure 1.5].

The various subtypes of breast cells (i.e. luminal, myoepithelial and basal) are commonly identified by the use of a number of specific markers via immunohistochemistry analysis including the following: cytokeratin (CK)18, CK7, CK8 and CK19 for luminal braest cells, smooth muscle alpha-actin, CK5, CK14, CK17, calponin and p63 for myoepithelial cells and CK5/6, CK14 and CK17 for detection of basal cells (O'Hare, Ormerod et al. 1991).

Recent studies have now identified the existence of stem and progenitor cell types in normal mouse and human breast tissue (Boecker and Buerger 2003; Visvader 2009). The identification of stem and progenitor cell types in the normal breast tissue adds essential information to our current contemporary understanding of the cell types involved in proliferative conditions and malignant transformation in the breast tissue (Dontu, Al-Hajj et al. 2003). Essentially stem cells can undergo differentiation into various committed progenitor subtypes (i.e. luminal or myo-epithelial lineages) which in turn are stimulated into specific breast tissue types (i.e. ductal and alveolar sublineages for luminal cells) (Visvader 2009). The transformation of stem cells into progenitor cells is proposed to occur as a direct result of the hormonal environmental milleu (i.e. puberty or pregnancy). These studies have importantly demonstrated a much more dynamic interplay of the breast tissue cell types based on a highly intricate hierarchical organisation of cell types than initially identified in previous studies. The relevance to the discovery of stem and progenitor cells in the breast to breast cancer disease is discussed in a subsequent section *(refer to section 1.4.3).*

1.3.3 - Non-malignant and malignant conditions of the breast tissue

A number of conditions arise in the breast tissue including inflammatory breast disease, fibrocystic changes, benign lesions (pathologically abnormal lesions which remain non-malignant or progress to invasive breast disease), benign tumours (pathologically abnormal masses of cells which are classified as either non-invasive or invasive) and carcinoma (abnormal masses of cells which divide without control and have the ability to become invasive and metastasise to other tissues). Inflammatory breast diseases include mastitis (staphylococcus aureus and streptococcus infection developed during lactation) and ductal ectasia (blockage of the breast ducts), both resulting in acute and chronic inflammation (Rosa; Cardenosa, Doudna et al. 1994; Wockel, Abou-Dakn et al. 2008; Rosa 2010). Benign breast diseases are common in women and are comprised of a number of different types of conditions. These include non-proliferative changes including cysts (i.e. fibroadenomatoid hyperplasia or fibroadenosis) and fibrosis and the proliferative changes including sclerosing adenoma, epithelial and atypical hyperplasia (Townsend 2003). Benign tumours in the breast include adenosis, phyllode tumours, fibroadenomas and intraductal papillomas, and these are usually painless, well circumscribed, firm and rubbery in texture on palpitation and are movable within the breast itself (Townsend 2003). An increased risk of invasive carcinoma development in the breast tissue has been associated with benign breast diseases that present with proliferative changes such as sclerosing adenoma, epithelial and atypical hyperplasia (Carter, Corle et al. 1988; Jensen, Page et al. 1989; Krieger and Hiatt 1992; Bodian, Perzin et al. 1993; Dupont, Page et al. 1994).

Breast carcinoma occurs in distinct breast tissue anatomical structures and luminal epithelial cell types. Ductal carcinoma *in-situ* (DCIS) is located within the ductal breast structures and lobular carcinoma *in-situ* (LCIS) is confined within the lobular structures of the breast. Both DCIS and LCIS are further classified as low, intermediate or high grade carcinomas based on specific histopathological characteristics. A key diagnostic criteria of non-invasive *in-situ* ductal and lobular carcinoma, is the presence of an intact myoepithelial layer. In comparison, invasive ductal carcinomas (IDC) and invasive lobular carcinoma (ILC) are recognised by the loss of the myoepithelial cell layer and invasion of luminal epithelial cells through the basement membrane into the intra-stromal breast tissue. The most common type of invasive breast carcinoma is IDC (53%) followed by IDC mixed with some ILC (30%), ILC alone (10%), and some other types of cancer (i.e. medullary) (7%). Breast carcinoma is reported to develop in most cases within the intra-lobular ducts (TDLU) breast tissue structures, which are comprised predominantly of the lobule type 1 breast structures (Wellings, Jensen et al. 1975; Russo, Gusterson et al. 1990). In contrast, lobular carcinomas mainly occur in lobular type 2 structures, hyperplasia, fibroadenomas, scerlosing adenomas and cysts mainly occur in lobule type 3 structures, and lactating adenomas in lobule type 4 structures (Russo, Gusterson et al. 1990).

1.3.4 - Gene expression profiles of breast cancer sub-types

Advancements in genomics have provided insights into different gene expression patterns of various breast cancer sub-types, which provide a unique molecular fingerprint. Six different sub-types of breast cancer have been identified based on specific gene

expression profiles, which are luminal A and B, basal-like, claudin-low, HER2/ERBB2 overexpressing and normal-breast-like subtypes (Perou, Sorlie et al. 2000; Sorlie, Perou et al. 2001; van 't Veer, Dai et al. 2002; Sorlie, Tibshirani et al. 2003). Luminal breast cancers account for the highest percentage (70-80%) of diagnosed breast cancers; basal-like cancers occur in 15-20% of cases and are commonly genotyped as triple-negative breast cancers (Sorlie, Perou et al. 2001; van 't Veer, Dai et al. 2002; Sorlie, Tibshirani et al. 2003). Clinical prognosis studies have demonstrated that luminal A breast cancers often have a good prognostic outcome whereas luminal B breast cancers are associated with a poorer prognostic outcome (Sorlie, Perou et al. 2001). Basal and ERBB2+ breast cancers are both associated with a poor prognosis (Sorlie, Tibshirani et al. 2003). Additional genome wide analysis has shown that BRCA mutations are most ofter associated with basal-like tumours and are associated with a poor prognostic outcome (van 't Veer, Dai et al. 2002).

The different classifications of breast cancer subtypes are proposed to reflect different origins of cell type (i.e. stem and progenitor cells), as previously discussed in an earlier section and in turn possess a unique geno-type status (Visvader 2009) [Figure 1.6]. Importantly, the identification of stem and progenitor cells and the classification of breast tumours by genotyping has allowed for specific clonal populations in tumour cells to be linked to a particular cell type (Visvader 2009) [Figure 1.6]. Additionally, patient tailored therapy strategies can now be based directly on the gene expression profiles linked to a particular subtype of breast cancer. Collectively these studies have been highly influential in developing the current understanding of breast cancer and therapeutic intervention

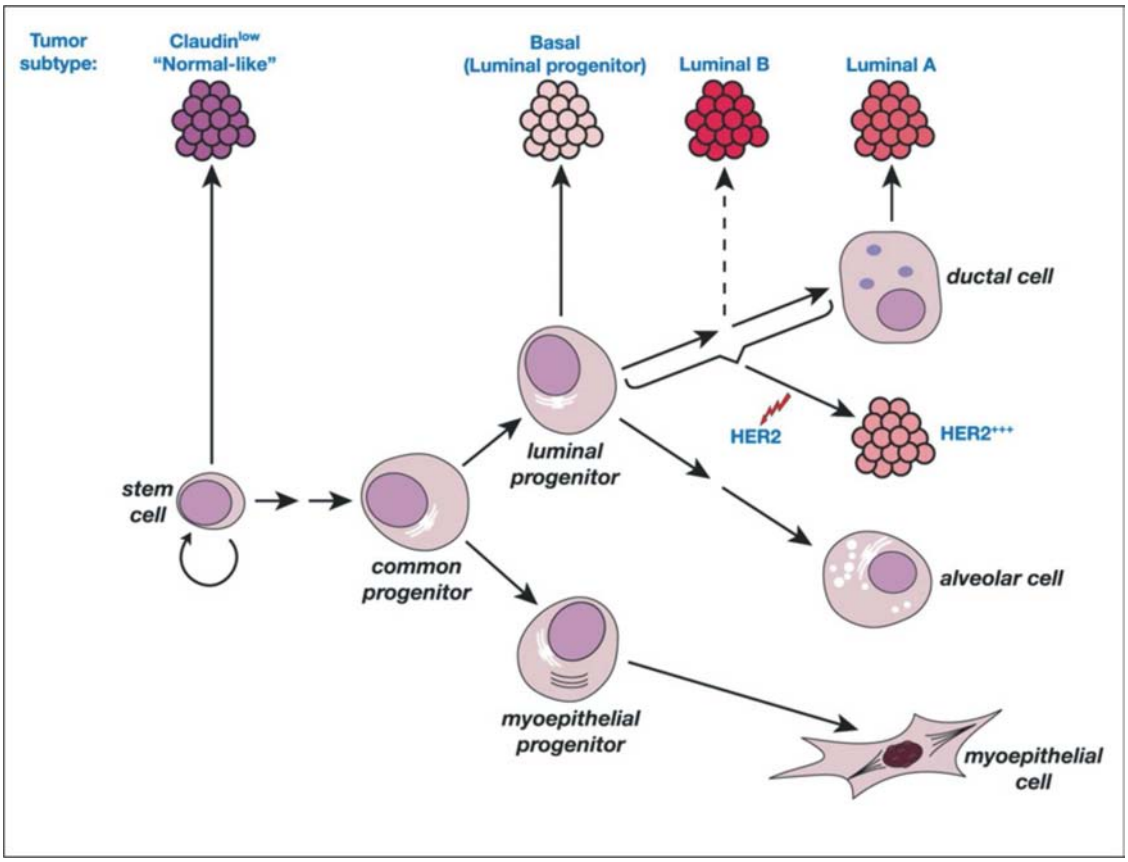


Figure 1.6: Stem and progenitor cell types in breast tissue and relationship with various breast cancer subtypes. A hierachical organisation of breast progenitor cell types from a stem cell in the breast tissue is depicted schematically. These include a common progenitor cell type that can be transformed into either a luminal or myoepithelial progenitor cell. Subsequent transformation can occur whereby the luminal progenitor cells transform into either ductal or myoepithelial cells. The diagram depicts the relationship between between the origin of cell type and a particular sub-type of breast cancer (i.e. luminal A and B (HER2 positive and negative), basal-like, normal-like and claudin-low). The diagram has been adapted from (Visvader 2009).

strategies currently used for breast cancer treatment.

1.3.5 - Breast cancer development and breast epithelial cell proliferation

Breast epithelial cell proliferation occurs mostly in the luminal ductal and lobular epithelial cells and to a smaller degree in myoepithelial cells (Joshi, Smith et al. 1986; Monaghan, Perusinghe et al. 1991; Perusinghe, Monaghan et al. 1992; Monaghan, Clarke et al. 1995). Morphological studies using specific markers for the different breast cell types have shown that breast tumours consist mostly of luminal epithelial cells (Wellings, Jensen et al. 1975; Taylor-Papadimitriou, Stampfer et al. 1989; Santini, Ceccarelli et al. 1996). This suggests excessive breast cell proliferation occurs largely in the breast luminal epithelial cells during the initiation, promotion and development of breast carcinogenesis. During progression, loss of myoepithelial cells and subsequent invasion of the luminal epithelial cells through the basement membrane occurs.

Excessive luminal breast epithelial cell proliferation resulting from oestrogen activity has been proposed to lead to the accumulation of genetic errors during cell replication and subsequent neoplastic transformation (Liehr 1997; Liehr 2000). Studies have shown that breast tumours consist mostly of luminal epithelial cells by the detection of specific cellular characteristics of luminal cells (Wellings, Jensen et al. 1975; Taylor-Papadimitriou, Stampfer et al. 1989; Santini, Ceccarelli et al. 1996). The exact causes leading to neoplastic transformation have been linked to different reproductive events throughout the female's lifespan. These hormonally driven factors associated with the etiology of breast cancer include: degree of exposure of the breast tissue to endogenous and exogenous hormones; age of menarche; age of first birth; number of pregnancies and

age of menopause. The hormone related risk factors are well established in the literature to play a significant contribution to breast carcinogenesis as discussed in the following sections.

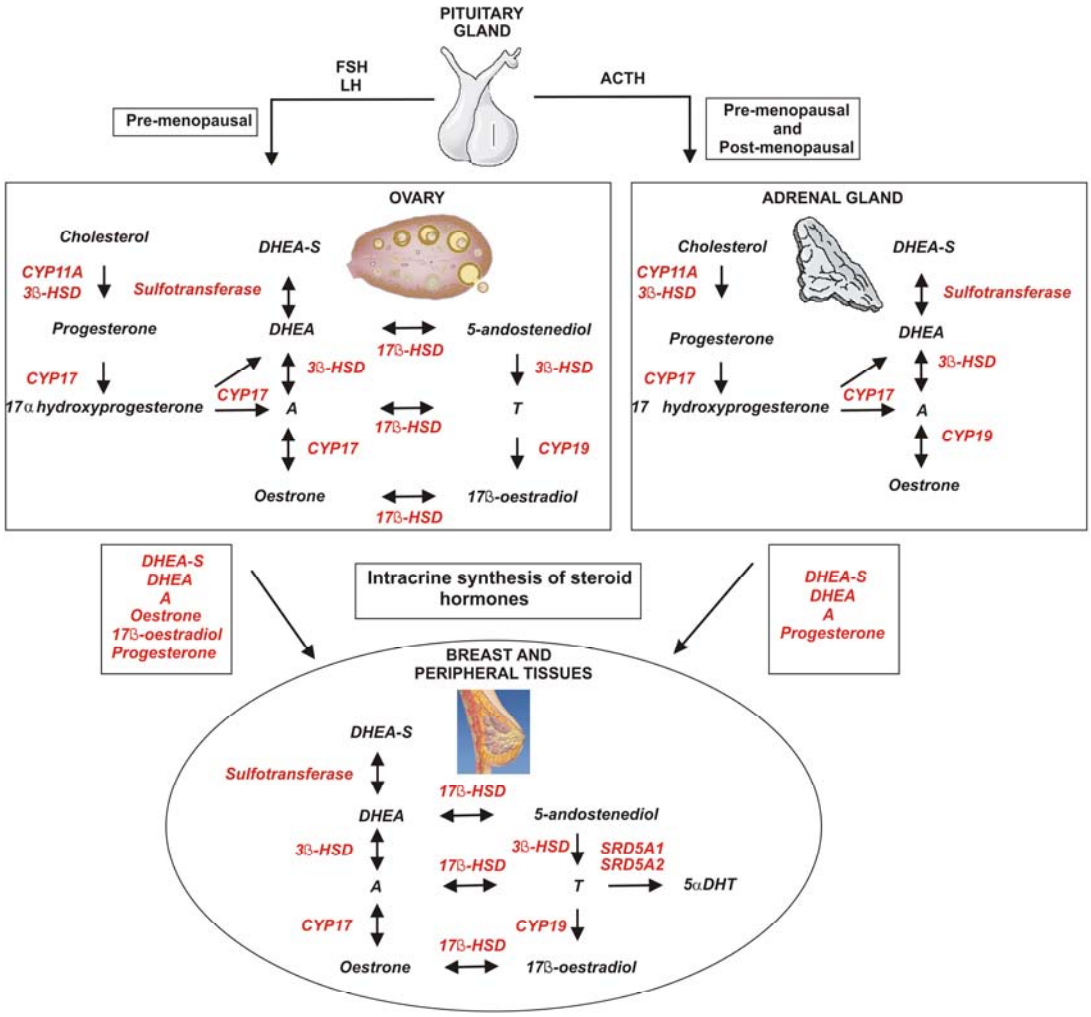
1.4 - Synthesis and activity of sex steroid hormones and receptors in females

1.4.1 - Types and synthesis of sex steroid hormones

The developmental, morphological, proliferative and differentiation events within breast tissue are predominantly regulated by endogenous sex hormones secreted by the ovaries and adrenal glands. Of particular relevance to the thesis are the oestrogens including: (oestrone (E_1)); 17ß-oestradiol (E_2) and oestriol (E_3), progesterone (Pg) including: 17α-hydroxprogesterone (17α-OHP) and androgens including: (dehydroepiandrosterone (DHEA)); DHEA-sulphate (DHEA-S); androstenedione (A); testosterone (T) and 5α-dihydrotestosterone (DHT).

Biosynthesis of sex steroid hormones in pre- and post-menopausal women occurs by the conversion of cholesterol both in the ovary and adrenal gland, the former regulated by the pituitary hormones follicle stimulating hormone (FSH) and luteinising hormone (LH) and the latter by adreno-corticotrophic hormone (ACTH) [Figure 1.7]. The first stages of steroid hormone synthesis occur through a series of reactions involving the enzymes p450scc (encoded by the CYP11A gene), 3β-hydroxysteroid dehydrogenase (3β-HSD) (encoded by the 3β-HSD gene) and p450c-17 (encoded by the CYP17 gene), which produce Pg and 17α-OHP and the precursor androgens DHEA and A [Figure 1.7].

Figure 1.7: Synthesis of endogenous sex hormones in pre- and post-menopausal women. The endogenous oestrogens, progesterone and androgens are synthesised in pre-menopausal women from the ovary following stimulation by the pituitary hormones follicle stimulating hormone (FSH) and luteinising hormone (LH). In pre- and post-menopausal women, active sex hormones are produced from cholesterol in the adrenal gland following stimulation with the pituitary hormone adreno-corticotrophic hormone (ACTH). Intracrine synthesis of sex steroid hormones in peripheral tissue occurs in both pre- and post-menopausal women (Diagram modified from (Henderson 2003)). **Abbreviations: CYP11A** (cholesterol side-chain cleavage enzyme p450scc), **3β-HSD** (enzyme 3β-hydroxysteroid oxioreductase), **CYP17** (17α-hydroxylase p450c17), **17β-HSD** (17β-hydroxysteroid oxidoreductase), **CYP19** (P450-aromatase), **SRD5A1** (5α-reductase type 1), **SRD5A2 (**5α-reductase type 2), **DHT** (5α-dihydrotestosterone) **FSH** (follicle stimulating hormone), **LH** (luteinising hormone) and **ACTH** (adreno-corticotrophic hormone).


PITUITARY GLAND
FSH
LH
ACTH
Pre-menopausal
Pre-menopausal and Post-menopausal
OVARY
Cholesterol
DHEA-S
CYP11A
3ß-HSD
Sulfotransferase
Progesterone
DHEA
17ß-HSD
5-andostenediol
CYP17
3ß-HSD
3ß-HSD
17α hydroxyprogesterone
CYP17
A
17ß-HSD
T
CYP17
CYP19
Oestrone
17ß-HSD
17ß-oestradiol
ADRENAL GLAND
Cholesterol
DHEA-S
CYP11A
3ß-HSD
Sulfotransferase
Progesterone
DHEA
CYP17
3ß-HSD
17 hydroxyprogesterone
CYP17
A
CYP19
Oestrone
DHEA-S
DHEA
A
Oestrone
17ß-oestradiol
Progesterone
Intracrine synthesis of steroid hormones
DHEA-S
DHEA
A
Progesterone
BREAST AND PERIPHERAL TISSUES
DHEA-S
Sulfotransferase
17ß-HSD
DHEA
5-andostenediol
3ß-HSD
3ß-HSD
SRD5A1
SRD5A2
17ß-HSD
A
T
5αDHT
CYP17
CYP19
17ß-HSD
Oestrone
17ß-oestradiol

Conversion of the precursor steroid hormones DHEA and A to 5-androstenediol and T respectively by the enzymes 17β-hydroxysteroid dehydrogenase (encoded by the 17β-HSD gene) and 3β-HSD also occur [Figure 1.7].

Synthesis of the most potent androgen DHT, results from the conversion of T to DHT following reductase type 1 and 2 (encoded by the SRD5A1 and SRD5A2 genes) enzymatic activity [Figure 1.7]. Predominantly, the prostate expresses the more active 5α-reductase type 2 enzyme, whereas the breast tissue expresses the 5α-reductase type I enzyme (Thigpen, Silver et al. 1993; Silver, Wiley et al. 1994; Suzuki, Miki et al. 2007). The synthesis of the most potent oestrogenic-(C_{18}) steroids including E_1 occurs by the conversion of A to E_1 by the enzyme p450c-17 (Simpson 2002). Whereas the androgenic-(C_{19}) steroid, DHT is non-aromatisable, the precursor androgen T can be converted by the process of aromatization to the oestrogenic-(C_{18}) steroid E_2, by the enzyme P450 aromatase (encoded by the CYP19 gene) [Figure 1.7]. A separate conversion can also occur of E_1 to E_2 by the enzyme 17β-hydroxysteroid dehydrogenase [Figure 1.7].

In pre-menopausal women the synthesis of sex steroid hormones occurs predominantly through two main pathways: 1) the production of sex steroid hormones from the ovarian tissue and adrenal glands that enter into the general circulation and 2) synthesis of steroid hormones from circulating precursor hormones (referred to as intracrinology) in peripheral tissues including breast, placenta, endometrium, liver, kidney, bones, skeletal muscle, skin and adipose tissue (Labrie 1991) [Figure 1.7]. The second process of sex hormone synthesis occurs within cell specific types that possess steriodogenic enzyme

activity. Unlike steroids produced by the ovary and adrenal gland, steroids produced in an intracrine manner are not secreted into the circulation. Often the intracrine steroids represent potent hormones such as DHT and E_2. Both ovarian and adrenal gland steroid synthesis pathways contribute to circulating oestrogen and the progesterone serum levels, in pre-menopausal women. However, in post-menopausal women the production of these steroid hormones are synthesised predominantly within the adrenal glands and peripheral tissues, due to cessation of ovarian function during the menopausal transition [Figure 1.7].

Circulating sex steroids undergo transportation into the intracellular compartment by a process of diffusion across the cell plasma membrane. Furthermore, the bioavailability of circulating steroid hormone levels within the peripheral blood is determined by the ratio calculated from free unbound steroids and steroids bound to serum transport proteins. In most cases, unbound steroid hormones are referred to as bioavailable sex steroids and can be utilised by target cells for biological activity and metabolism. The most studied serum steroid binding proteins include the sex hormone binding globulin (SHBG) (binds to E_2, T and DHT), albumin (binds to T) and corticosteroid binding globulin (CBG) (binds to corticosteroids and progesterone) (Westphal 1971; Rosner 1991; Hobbs, Jones et al. 1992). Inactivating metabolism of steroid hormones is essential for the elimination of steroids in the peripheral circulation and occurs predominantly in the liver. The conversion of steroid hormones into inactive forms is achieved primarily by the following processes including 1) reduction, 2) oxidation and 3) conjugation (Henderson 2003).

1.4.2 - Nuclear receptors

A number of intracellular nuclear receptors have been identified and characterised which display unique biological functions involved in embryogenesis, organogenesis and female and male reproduction (Henderson 2003). The sex steroid hormones oestrogens progesterone and androgens act biologically by binding to specific intracellular nuclear receptos which belong to the steroid receptor super-family of nuclear receptors (i.e. oestrogen receptor alpha (ERα), oestrogen receptor beta (ERβ), progesterone receptor ((PR); A, B and C isoforms) and the androgen receptor (AR) (Mangelsdorf, Thummel et al. 1995). They are structurally similar and are comprised of the distinct functional domains including: 1) the amino-terminal domain (NTD), involved in transcriptional activation by binding to transcriptional co-regulators; 2) the DNA binding domain (DBD) (a highly conserved domain among steroid receptors), involved in DNA binding and receptor dimerisation; 3) the hinge region, which provides stability and flexibility to the steroid receptor and contains a nuclear localisation sequence (NLS) comprised predominantly of lysine residues and 4) the carboxyl-terminal ligand binding domain (LBD), which recognises and binds to specific ligands in a hydrophobic ligand binding pocket (Henderson 2003).

The nuclear receptos are divided into four distinct classes, determined by the dimerisation capabilities and ability to recognise specific hormone response elements (HREs) (Denayer, Helsen et al.; Henderson 2003; Denayer, Helsen et al. 2010). The AR, PR, glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) all belong to the class I group and ERα and ERβ belong to class II of nuclear receptors (Henderson 2003;

Denayer, Helsen et al. 2010). Specific characteristics of nuclear receptos within the class I group include the ability to undergo homodimerisation and bind to 3-nucleotide spaced inverted palindromic sequence motifs that are referred to as either classical androgen response elements (AREs) (specific for the AR) or selective AREs (can bind all nuclear receptors in the class I group), respectively (Denayer, Helsen et al. 2010). Class I steroid receptors can recognise similar types of hormone response elements (HREs) due to the highly conserved sequences that comprise the DBD of the individual receptors (De Vos, Claessens et al. 1993; Henderson 2003). The ability of the AR to bind as a monomer to single half-sites in HREs in the chromatin has also been documented (Kemppainen, Langley et al. 1999; Massie, Adryan et al. 2007).

The class II type of steroid receptors, ERα and ERβ bind to a distinct set of HREs and can form homo- and heterodimers, and also heterodimerise with the 9-*cis* retinoid acid receptor (RXR) (Glass 1994; Pace, Taylor et al. 1997). There are also reports that PRA and PRB isoforms can homo- and heterodimerise (Tetel, Jung et al. 1997; Leonhardt, Altmann et al. 1998; Connaghan-Jones, Heneghan et al. 2007). Recognition and binding of nuclear receptos to specific DNA sequences in proximal and distal regions in 5' untranslated regions upstream from target genes, leads to activation and/or repression of genes resulting in biological output of steroid receptor function and activity.

1.4.3 - Nuclear receptor mechanism of action

Nuclear receptors are transcriptional regulators that act dynamically in the cell to either stimulate or repress gene transcription. The maturation of the nuclear receptors occurs in

the cytoplasm where they reside in the absence of ligand as part of a maturation hetero-complex comprised of heat shock proteins (hspP90, hsp70 and hsp40) and other co-chaperones (Faresse, Ruffieux-Daidie et al.; Pratt and Toft 1997; Cheung and Smith 2000; Buchanan, Ricciardelli et al. 2007). The dissociation of the AR, GR and MR from the maturation complex occurs subsequent to ligand binding, followed by dimerisation and phosphorylation events of the receptor and nuclear translocation into the cytoplasm of the cell (Faresse, Ruffieux-Daidie et al.; Henderson 2003). However, in comparison the nuclear receptors such as the ER and PR can dissociate from the maturation heterocomplex in the cytoplasm and undergo nuclear translocation in an unliganded state (Haverinen, Passinen et al. 2001; Dull, Goncharova et al. 2010).

The transcriptional output of nuclear receptor action in the nucleus is complex and is mediated via the intricate interplay between co-regulators and co-transcriptional machinery to form nuclear receptor co-regulatory cistromes. Over the last ten years the understanding of nuclear receptor gene regulation has dramatically advanced. Recent technological advancements in studying steroid receptor action has allowed for extensive chromosomal mapping to be performed via chromatin-immunoprecipitation (ChIP)-on-chip and ChIP-seq studies (Massie, Adryan et al. 2007; Wang, Li et al. 2007; Lupien and Brown 2009). These studies have demonstrated that while AREs can be located in proximal (close proximity of the transcriptional start site i.e. 10-50 base pairs), they are often located in distal genomic regions 100-200 kb upstream of target genes (Massie, Adryan et al. 2007; Wang, Li et al. 2007). A study of a similar nature has recently been performed to map ERα and ERβ chromatin binding sites in a human breast cancer cell

line (Charn 2010). The study identified a high overlap of ERα and ERβ binding sites by oestrogen when the receptors were in isolation (Charn 2010). However, when both oestrogen receptors were present together in the cell there was a substantial reduction in the overlap of chromatin binding sites by oestrogen (Charn 2010).

Technological advancements have led to the identification of numerous binding motifs whereby co-regulators can bind in a non-promoter specific manner (e.g. GATA2 to GATA motifs and Oct-1 to OCT motifs) to form cross-regulatory loops with nuclear receptors at distant chromatin sites (Wang, Li et al. 2007). The pioneer cofactor FoxA1,a forkhead family member, binds directly to FKH motifs in the chromatin and has been widely studied in breast and prostate cancer cell lines (Lupien, Eeckhoute et al. 2008). FoxA1 has been reported to be located at 50-60% of ERα and AR chromatin binding sites and the chromatin mapping of FoxA1 has now demonstrated a highly evolved transcriptional system associated with nuclear receptor action (Lupien, Eeckhoute et al. 2008). Functional studies have linked the genomic actions of FoxA1 as a chromatin re-modelling factor, leading to the unwinding and opening of genomic DNA (Carroll, Liu et al. 2005). Additionally, classical promoter specific co-regulators, including the transcriptional p160 co-activators (i.e. p300, SRC/p160s and CREB-binding protein (CBP)) and co-repressors (i.e. SMRT and N-CoR) that form the basal transcriptional machinery to either repress or promote gene expression via the recruitment of the RNA polymerase II to gene promoter sequences typically located upstream from target genes (Henderson 2003; Wu, Smith et al. 2005) [Figure 1.8]. Nuclear receptors can also interact with chromatin in an indirect manner by a tethering mechanism, for example via

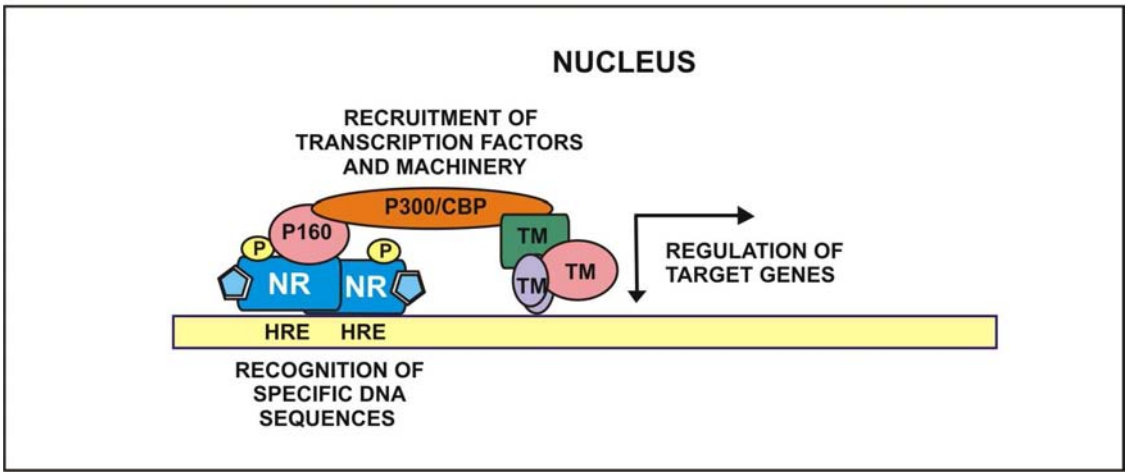


Figure 1.8: Transcriptional regulation of target genes by steroid hormone nuclear receptors. The molecular process of gene transcription occurs following the translocation of nuclear receptors (NR) into the nucleus of various cell types. Dimerisation and post-translational modifications (e.g. phosphorylation) of the NR occur, which subsequently leads to the recognition and binding to specific DNA sequences referred to as hormone response elements (HREs) in proximal and distal genomic sites located in 5' untranslated regions upstream from target genes. The recruitment of transcriptional machinery (TM) (e.g. RNA polymerase II) and transcriptional factors (e.g. p160 co-regulators) allows for NR-mediated gene transcription.

SP1 motifs involving protein-protein interactions mediated by SP1 and AP1 transcription factors (Sanchez, Nguyen et al. 2002). Thus the contemporary understanding of nuclear receptor mediated gene regulation indicates a complex interplay of nuclear receptors and co-regulators in a promoter and non-promoter specific manner to regulate gene expression.

1.5 - Endogenous sex hormones and breast cancer risk

1.5.1 - Endogenous sex hormones, steroid receptors and breast cancer risk

Breast cancer is established worldwide as the most prevalent form of neoplasia (17.9%) in females, accounting for over 1 million cases of the total reported forms of cancer per year (Parkin, Bray et al. 2005; Porter 2009). In the Australian population, 1 in 11 women (risk to age 75 yrs) or 1 in 9 women (risk to age 85 yrs) will develop breast cancer during their lifetime (Australian Cancer Incidence and Mortality (ACIM) book *(http://aihw.gov.au/search/?q=Breast+Cancer+ACIM+book)*). The majority of breast cancers (approximately two-thirds) are detected in post-menopausal women, compared to only a third of all breast cancers in pre-menopausal women (Pike, Krailo et al. 1983). The etiology of breast cancer is complex and is influenced by a number of risk factors including: relative exposure to natural and synthetic hormones (HRT and contraceptives); hereditary factors (breast cancer susceptibility genes BRCA1 and BRCA2), dietary factors; pregnancy and lactation; anthropometric factors; alcohol intake; exercise; socio-economic status; age and environment (exposure to pollutants and carcinogens) (Henderson 2003).

Well established factors associated with the cumulative lifetime exposure of the breast tissue to endogenous hormones that can influence the risk of breast cancer include: age of menarche; parity; age of first pregnancy; multiparity; lactation and age of menopause (Pike, Krailo et al. 1983; Kampert, Whittemore et al. 1988; Pathak and Whittemore 1992; Rosner, Colditz et al. 1994). Complete pregnancy-induced differentiation in the breast tissue is reported to provide protection against breast cancer development by a decrease in ERα and PR expression (Russo, Mills et al. 1989; Russo and Russo 1994; Russo, Ao et al. 1999; Taylor, Pearce et al. 2009). Furthermore, a significant decrease in PRA and a trend towards a decrease in PRB and ERα expression has also been observed in the breast tissue of parous compared to nulliparous women (Taylor, Pearce et al. 2009). Decreased steroid receptor expression following full-term, pregnancy is reported to occur by the silencing of gene expression following methylation of CpG islands in gene promoter regions of differentiated lobule type 2 and 3 structures compared to the undifferentiated lobule type 1 structures of pre-menopausal breast tissue (Russo, Ao et al. 1999; Russo and Russo 2007).

1.5.2 - Summary

Collectively, the introductory sections described in this literature review, have provided an overview of the integral role of sex steroid hormones mediated by steroid receptors in key biological reproductive processes in the normal breast tissue and their involvement in breast cancer. The subsequent sections provide a more detailed account of the involvement of endogenous oestrogen and progesterone hormone action, in non-malignant and malignant breast tissue. This provides a basis for the final sections of the

literature review whereby the role of exogenous hormones used in combined hormone replacement therapy (cHRT) and their associations with breast cancer are discussed which leads into the hypothesis and aims of the research studies in this thesis.

1.6 - Oestrogen and ERα actions in normal and malignant breast tissue

1.6.1 - Role of oestrogen in normal and malignant breast tissue

The growth and proliferation of the breast parenchyma (the key elements of an organ essential to its functioning), is primarily stimulated by the oestrogenic sex hormone E_2, (classified as a breast epithelial cell mitogen) (Henderson, Ross et al. 1982). Excessive proliferation and cell division within the breast tissue is reported to promote accumulation of random genetic errors, leading to pre-malignant changes in the breast tissue (Cohen and Ellwein 1991). The mitogenic action of oestrogens in stimulating proliferation of both normal and malignant breast epithelial cells has been established, as discussed below.

The potential role of oestrogens and stimulation of breast cancer growth was first identified by pioneering studies discovering the reduction of breast tumour growth subsequent to surgical removal of the ovaries (bilateral oophorectomy) (Beatson 1896). Subsequent studies in women presenting with metastatic breast cancer demonstrated tumour regression following surgical removal of the adrenal glands (bilateral adrenalectomy) (Huggins and Bergenstal 1952), and the pituitary gland (hypophysectomy) (Luft and Olivecrona 1953). As previously described, synthesis of sex

steroid hormones in females occurs via the conversion of cholesterol in the ovary and adrenal gland by the stimulation of the pituitary hormones LH and ACTH, respectively *(refer to section 1.4.1).* Therefore, removal of the pituitary gland directly prevents activation of these pathways and ultimately reduces hormone synthesis via the ovary and adrenal gland.

Later studies undertaken using human female non-malignant and malignant breast tissue confirmed that oestrogen possesses stimulatory actions on breast epithelial proliferation. In postmenopausal women topical application of E_2, but not a placebo treatment to the breast tissue prior to surgery increased breast epithelial proliferation, measured by the proliferation marker proliferating nuclear cell antigen (PCNA) (Chang, Lee et al. 1995; Foidart, Colin et al. 1998). An *in vitro* study using normal human breast epithelial cells has documented that oestrogen can induce an increase in proliferation in a dose dependent manner (Malet, Gompel et al. 1988). Lastly, an *in vivo* study reported an increase in DNA replication in 7/10 human breast tumours obtained from women treated with oestrogen and progesterone combined prior to surgery (Dao, Sinha et al. 1982).

A number of *in vivo* studies using normal and malignant human breast tissue specimens and epithelial cells in mouse xenograft studies have demonstrated a link between oestrogens and promotion of breast tissue proliferation. Specifically, an induction of proliferation in MCF-7 xenografted breast tumours in athymic nude mice following treatment with E_2, compared to control mice has been identified (Soule and McGrath 1980; Shafie and Grantham 1981; Welsch, Swim et al. 1981). Several studies using

normal breast cells embedded in matrigel and then xenografted subcutaneously into athymic nude mice have reported that subsequent treatment with oestrogens increased mammary epithelial cell proliferation (McManus and Welsch 1981; McManus and Welsch 1984; Laidlaw, Clarke et al. 1995; Clarke, Howell et al. 1997; Popnikolov, Yang et al. 2001). Additionally, an increase in DNA synthesis within the breast ductal epithelium of human breast tissue transplanted into BALB/athymic nude mice following treatment with oestrogen compared to placebo has been reported (McManus and Welsch 1981). In further support of these findings, dose dependent oestrogen stimulation of human breast epithelial cell proliferation has been demonstrated by measuring the H^3-thymidine labeling index in normal human breast tissues xenografted into athymic nude mice (Laidlaw, Clarke et al. 1995). Collectively, these studies demonstrated that breast tumour growth and survival are dependent on hormonal stimulation however do not definitively provide evidence of the effects of oestrogen specifically mediated by ERα.

1.6.2 - Actions of ERα in normal and malignant breast tissue on breast epithelial proliferation

The most potent oestrogen, 17β-estradiol (E_2), regulates breast tissue development, differentiation and cell proliferation by binding to the sex steroid nuclear receptor ERα (Henderson 2003). Oestrogens predominantly mediate stimulatory effects on breast epithelial proliferation essentially through the actions associated with ERα, and anti-proliferative actions by ERβ (Paruthiyil, Parmar et al. 2004; Strom, Hartman et al. 2004; Hartman, Lindberg et al. 2006). Since an objective of this thesis was aimed at investigating the mechanistic actions of medroxyprogesterone acetate (MPA) used in

combined hormone replacement therapy (cHRT) and the effects on promotion of breast epithelial proliferation, the following sections are focused on the role of ERα in normal and malignant breast tissue.

The actions of ERα and various ERα isoforms, on breast epithelial proliferation has been extensively studied in human normal and breast cancer cells (Dotzlaw, Leygue et al. 1997; Jarvinen, Pelto-Huikko et al. 2000; Bieche, Parfait et al. 2001; Fuqua, Schiff et al. 2003; Carder, Murphy et al. 2005). A separate *in vitro* study has shown using the normal mouse mammary breast cell line (HC11), an increase in proliferation following treatment with a selective ERα agonist (Helguero, Faulds et al. 2005). Moreover, proliferation of ERα positive breast cancer cell lines MCF-7 and T47D is increased in response to E_2 and this is reversed following exposure to the ERα specific anti-oestrogen tamoxifen (Aspinall, Stamp et al. 2004). These studies specifically illustrate the proliferative capacity of E_2 mediated by ERα, in normal and malignant breast tissues.

The contribution of ERα expression to breast cancer development and progression is also highly reported in the literature. Epidemiological studies have shown an increased level of ERα expression in breast cancer specimens from women who reside in high risk, compared to low risk countries based on population studies detailing worldwide rates of breast cancer (Khan, Rogers et al. 1994; Lawson, Field et al. 1999; Lawson, Field et al. 2002). Clinical studies have also identified higher levels of ERα expression in the benign breast epithelium from women diagnosed with breast cancer compared to women without the disease (Khan, Rogers et al. 1994; Khan, Rogers et al. 1998). Moreover, at the early

stages of breast cancer progression, an increase in ERα expression in hyperplastic breast tissue and a further increase in pre-malignant lesions has been reported (Shoker, Jarvis et al. 1999). In conjunction with the earlier literature of this review the biological nature of oestrogens mediated via the ERα is shown to possess the capacity to positively regulate breast tissue proliferation and play an integral role in breast cancer.

1.6.3 - Paracrine actions of oestrogen and ERα in the regulation of breast cell proliferation in normal and malignant breast epithelial cells

It is proposed from the findings in the literature that ERα signalling is required to maintain normal breast epithelial cell proliferation mediated by paracrine-mediated events initiated via growth factor interactions between luminal breast epithelial and stromal breast cells (Clarke, Howell et al. 1997; Mallepell, Krust et al. 2006). Developmental breast tissue studies using mouse tissue recombinants grafted under the subrenal capsule in wild-type or knock-out nude mice have demonstrated the requirement of ERα expression in the stromal breast tissue to stimulate development of the breast epithelium to undergo ductal morphogenesis (Cunha, Young et al. 1997). During pubertal development of the mouse mammary gland it has been shown that predominantly ERα negative breast epithelial cells in the TEB and lining the mammary ducts undergo proliferation, whereas the ERα positive epithelial cells are non-dividing (Zeps, Bentel et al. 1998). Furthermore, studies using human non-malignant breast tissue and rat luminal breast epithelial cells indicate that proliferating cells are negative for ERα, whereas non-proliferating are positive for ERα (Clarke, Howell et al. 1997). Despite these findings, it has been demonstrated *in vivo* that ERα negative mammary epithelium possesses the

capacity to proliferate when in close proximity to ERα positive mammary epithelium (Clarke, Howell et al. 1997; Russo, Ao et al. 1999; Mallepell, Krust et al. 2006). These studies suggest that breast epithelial proliferation occurs in normal cells that do not express ERα. Furthermnore they suggest that that there are paracrine mediated cellular processes in ERα positive breast epithelial cells that act to regulate breast epithelial proliferation in ERα negative adjacent cells.

Interestingly, in benign breast tissue lesions and early pre-malignant stages of breast cancer including hyperplasia and *in situ* disease, there is a shift in the discordance of ERα positive cells and Ki67 expression to an increase in co-localisation of ERα and Ki67 expression within a single breast epithelial cell (Clarke, Howell et al. 1997; Shoker, Jarvis et al. 1999; Shoker, Jarvis et al. 2000). This finding has been supported by another study which reports similar observations in the ERα positive breast cancer cell lines (MCF-7, T47D and ZR-75-1), whereby regulation of cell proliferation in cancerous breast epithelial cells can occur via autocrine regulation (Tan, Zhong et al. 2009). Thus, these studies indicate that there are changes in the biological nature of regulatory cellular processes involved in breast epithelial proliferation in non-malignant compared to malignant breast epithelial cells. As highlighted in the literature, the nature of this change is associated with the gain of autocrine mediated control of breast epithelial proliferation in malignant breast tissue, compared to a predominant paracrine-mediated process in non-malignant breast tissue. The importance of these studies is the requirement for studies investigating alterations to growth factor signalling pathways cell signalling pathways that may be linked to initial early pre-malignant changes in the breast tissue to obtain a

better understanding of the molecular mechanisms associated with the carcinogenic process.

1.6.4 - ERα and Ki67 expression based on age and menopausal status

Changes in ERα and Ki67 expression that occur in the breast tissue during ageing in women, especially with changing menopausal status, provide insights into the effects of endogenous hormones in breast tissue. Increased ERα expression within normal breast tissue in women from 45 yrs onwards has been reported with a plateau in ERα expression occuring after menopause (Shoker, Jarvis et al. 1999; Shoker, Jarvis et al. 2000). Additional findings report an inverse relationship between ERα expression and breast epithelial proliferation by a decrease in the Ki67 proliferation marker in breast tissue obtained from post-menopausal (Ki67 = 0.34%) compared to pre-menopausal (Ki67 = 2.6%) women (Shoker, Jarvis et al. 1999; Shoker, Jarvis et al. 2000). However, it should be noted that there was an 11% increase in dual labelling of ERα and Ki67 in post-menopausal compared to pre-menopausal breast tissue (Shoker, Jarvis et al. 1999). The dual labelling of ERα and Ki67 expression in post-menopausal breast epithelial cells is more similar to that detected in malignant compared to non-malignant cells (Clarke, Howell et al. 1997; Shoker, Jarvis et al. 1999) as reviewed in the previous section.

The expression pattern of ERα also changes during menopause where a contiguous appearance is more evident in post-menopausal breast epithelial cells, compared to a scattered and disordered appearance in pre-menopausal breast epithelial cells (Shoker, Jarvis et al. 1999). This change in pattern of ERα expression is reported to be associated

with the higher levels of ERα expression in post-menopausal breast tissue (Shoker, Jarvis et al. 1999). The molecular basis for this change in ERα expression and the higher concordance of ERα and Ki67 expression following menopause is not known. However, it has been shown that oestrogens can down-regulate ERα expression in breast cancer cells, and that the reduction in ovarian oestrogen production following menopause may lead to the loss of inhibition of ERα expression (Eckert and Katzenellenbogen 1982). Furthermore a higher number of cells which present with dual labeling of both ERα and Ki67 may be related to early pre-neoplastic changes in the breast tissue (Shoker, Jarvis et al. 2000).

The findings discussed above illustrate that there is a dynamic shift in ERα expression and breast tissue proliferation exists in post- compared to pre-menopausal women. Furthermore, these studies highlight that differences between pre-menopausal and post-menopausal breast tissue may contribute to the higher rates of breast cancer detected in post-menopausal women. The changes in these hormonally driven cellular processes based on menopausal status potentially have strong implications for different actions of exogenous hormones used in combined hormone replacement therapy (cHRT) comprised of an oestrogen and synthetic progestin within breast tissue of post-menopausal breast tissue compared to identical hormones used in contraception for pre-menopausal women. The main focus of this thesis therefore was to investigate the role of the synthetic progestin MPA used in cHRT on the promotion of breast cancer. Since synthetic progestins possess strong progestagenic actions in the body, the following sections address the role of progesterone and PR in normal and malignant breast tissue.

1.7 - Progesterone and PR actions in normal and malignant breast tissue

1.7.1 - Rates of proliferation in the endometrium and breast tissue during the menstrual cycle

To unravel the role of progesterone in normal breast tissue, comparisons of the cellular proliferation rates in the endometrium, and breast tissue during the menstrual cycle have been used as guide. During the menstrual cycle, endometrial proliferation is highest in the follicular phase when serum oestrogen levels are maximal and serum progesterone levels are low, which is commonly termed *'the proliferative phase'* (Brenner and West 1975). The luteal phase of the menstrual cycle begins mid phase following ovulation, which is commonly referred to as *'the secretory phase'*. During this stage the endometrium becomes vascularised and oedematous in preparation for implantation of the embryo (Norwitz, Schust et al. 2001). The increased serum levels of progesterone are produced predominantly from the corpus luteum prior to fertilisation during the luteal phase (Norwitz, Schust et al. 2001). The proliferation rates decrease in the endometrium during the luteal phase which coincides with maximal serum levels of both oestrogen and progesterone (Brenner and West 1975). Hence, oestrogen acts to stimulate endometrial proliferation in the follicular phase of the menstrual cycle, whereas progesterone reduces oestrogen-induced proliferation during the luteal phase of the menstrual cycle to promote secretory endometrial conditions receptive for embryonic implantation. It is evident from these findings that progesterone primarily inhibits oestrogen stimulated proliferation within the endometrial tissue.

In direct opposition to what occurs in the endometrium, the highest rates of proliferation in the breast tissue occur during the luteal phase when both oestrogen and progesterone serum levels are maximal (Masters, Drife et al. 1977; Meyer 1977; Going, Anderson et al. 1988; Potten, Watson et al. 1988; Soderqvist, Isaksson et al. 1997). The role of progesterone in the regulation of breast epithelial cell proliferation remains controversial and is documented to act in both a stimulatory and inhibitory manner (Lange, Richer et al. 1999; Lange 2008). However, it has been reported that progesterone acts in a cyclical manner in the breast tissue to regulate proliferation, by initially stimulating a peak in proliferation, followed by an anti-proliferative phase (Lange, Richer et al. 1999; Lange 2008). The basis for this cyclical effect of progesterone in the breast tissue most likely reflects the biological requirement of progesterone in preparing the breast tissue for pregnancy-induced lactation by stimulating lobular-alveolar differentiation, as reviewed in an earlier section (Lydon, DeMayo et al. 1995; Atwood, Hovey et al. 2000). It is also likely that progesterone initially stimulates proliferation as a natural response to ovulation, but in the absence of pregnancy-induced hormones, progesterone activity reverts to an anti-proliferative role as a biological mechanism indicating that induction of further proliferation to promote full differentiation of the breast tissue for lactation is not required (Basuray, Rawlins et al. 1988).

1.7.2 - Role of progesterone in normal breast growth and proliferation

A number of studies have observed the effect of progesterone alone and its actions on oestrogen-induced breast epithelial cell proliferation. However, only a few studies have used natural progesterone compared to the majority, which have used progestagenic

compounds called synthetic progestins that mimic the biological effects of progesterone. The reason for the therapeutic and experimental use of synthetic progestins as opposed to natural progesterone is the rapid metabolism of progesterone *in vitro* and *in vivo* (Horwitz, Pike et al. 1986). However, since synthetic progestins additionally possess non-progestagenic actions it is difficult to compare the actions directly to natural progesterone (Sitruk-Ware 2004).

In vitro studies using cultured normal human breast epithelial cells obtained from reduction mammoplasties reported a dose dependent reduction in proliferation rates by the addition of the synthetic progestin, promegesterone (R5020), in the presence and absence of oestrogen (Gompel, Malet et al. 1986). Additionally, application of oestrogen and natural progesterone topically to the breast prior to the surgical excision of normal breast tissue adjacent to breast lesions has been shown to inhibit breast epithelial proliferation (Chang, Lee et al. 1995; Foidart, Colin et al. 1998). Thus, these studies suggest progesterone exerts an anti-proliferative action in breast tissue.

In contrast to progesterone acting to reduce oestrogen-induced growth of breast epithelial cells studies have shown progesterone can induce mammary gland proliferation, in addition to exerting no effect on mammary gland growth. Rat mammary gland tissue samples grown in organ culture were stimulated by progesterone in the presence of insulin as measured by DNA synthesis and mitotic index (Koyama, Sinha et al. 1972). Moreover, studies *in vivo* show after the implantation of progesterone pellets containing the synthetic progestin R5020 into mice in the presence and absence of oestrogen, an

increase in DNA synthesis within the mammary gland epithelium to a small degree with progesterone above control and progesterone and oestrogen combined above oestrogen alone (Wang, Counterman et al. 1990). However opposing these findings, *in vivo* studies demonstrate no effect of natural progesterone on breast epithelial cell proliferation in non-malignant human breast tissue transplanted into athymic nude mice (McManus and Welsch 1981; Laidlaw, Clarke et al. 1995; Clarke, Howell et al. 1997). These studies give evidence for differences in the actions of natural compared to synthetic progestins in stimulating breast tissue proliferation and give caution to directly comparing the effects between either endogenous and exogenous forms of progesterone on biological activity. Moreover, the findings of these studies indicate the role of progesterone action in regulating breast proliferation may in fact be more complex and likely involves additional factors that contribute to its biological effects.

1.7.3 - Oestrogen induced breast cell proliferation and induction of PR expression

Oestrogen action mediated through the ERα has been shown to increase expression of PR in luminal breast epithelial cells (Horwitz, Costlow et al. 1975; Nardulli, Greene et al. 1988; Kastner, Krust et al. 1990; Clarke, Howell et al. 1997). This up-regulation of PR expression by ERα signalling has been shown to be reversed by the addition of the anti-oestrogen tamoxifen, further demonstrating that PR is an ERα regulated gene (Horwitz, Costlow et al. 1975). Transactivation of the PR gene is directly regulated by ERα binding to two distinct response elements located in the promoter region (Kastner, Krust et al. 1990; Savouret, Bailly et al. 1991; Kraus, Montano et al. 1993; Kraus, Montano et al. 1994), effectively producing two PR isoforms, PRA and PRB (Tung, Mohamed et al.

1993; Vegeto, Shahbaz et al. 1993; Wen, Xu et al. 1994; Shyamala, Yang et al. 1998; Richer, Jacobsen et al. 2002; Mulac-Jericevic, Lydon et al. 2003). The differences in PRB structure compared to PRA is the additional 164 amino acids in the amino terminus of PRB (Vegeto, Shahbaz et al. 1993). Essentially the protein differences between PRA and PRB are dictated by the production of two unique mRNA transcripts that are synthesised from two different initiation codens (Kastner, Krust et al. 1990).

The specific roles of PRA compared to PRB in normal breast proliferation and differentiation and breast cancer development has been the focus of a number of studies. The two PR isoforms, PRA and PRB, are functionally different and elicit distinct effects during breast development (Shyamala 1999; Shyamala, Yang et al. 2000) and on breast cell proliferation (Pike, Spicer et al. 1993; Foidart, Colin et al. 1998; Russo, Ao et al. 1999; Clarke 2006). Differential gene expression by PRA compared to PRB indicates distinct biological roles of the PR isoforms (Tung, Mohamed et al. 1993; Vegeto, Shahbaz et al. 1993; Richer, Jacobsen et al. 2002). The action of PRB has been identified as inducing transcription of progesterone responsive genes compared to PRA, which can act as a suppressor of PRB, GR, AR and MR gene transcription (Vegeto, Shahbaz et al. 1993). Preferential up-regulation of PRB by ERα compared to PRA has also been reported (Graham, Roman et al. 1995; Flototto, Niederacher et al. 2004). Interestingly, in breast cancer an altered ratio of PRA to PRB has been identified compared to non-malignant breast tissues, indicative of a shift in steroid receptor expression (Mote, Bartow et al. 2002).

In the breast tissue, PRB is predominantly expressed compared to PRA and is reported to potentially be the mediator of the effects of progesterone and synthetic progestins (Graham, Roman et al. 1995; Lange 2008). However, in comparison, the endometrial tissue expresses a higher amount of the PRA isoform compared to the PRB isoform (Lange 2008). These studies indicate there are vast differences in the expression of the two PR isoforms in the endometrium and breast tissue which may in part be a basis for the differences in proliferation to endogenous hormones observed in the different tissue types (Lange, Richer et al. 1999; Pike and Ross 2000). The different expression and biological actions of PRA compared to PRB is likely to be due to tissue specific co-factor expression and activity.

1.7.4 - Role of progesterone in breast cancer growth and proliferation

The actions of progesterone in breast cancer is controversial, as it is in normal breast tissue (Lange, Richer et al. 1999; Lange 2008) with *in vivo* studies demonstrating progesterone to exhibit both stimulatory (Koyama, Sinha et al. 1972; Sheffield and Welsch 1988; Wang, Counterman et al. 1990) and inhibitory roles in breast cancer proliferation (Chang, Lee et al. 1995; Foidart, Colin et al. 1998). Additionally a plethora of studies have addressed the role of progesterone in mediating changes to tumour growth. Supporting a role of progesterone in the promotion of breast tumour growth is the subsequent reduction in breast tumour growth following administration of progesterone antagonists in rats (Bakker, Setyono-Han et al. 1990; Michna, Schneider et al. 1990). Additionally, DMBA-induced breast cancer growth in rats has been reported to be increased by progesterone, which was reversed by tamoxifen (Jabara and Harcourt 1971;

Jabara and Anderson 1982; Robinson and Jordan 1987). In PR knock-out mice, reduced tumour growth in DMBA treated mice, was observed compared to WT mice, further suggesting a dependence of mammary gland tumour growth on the presence of PR (Lydon, Ge et al. 1999). Progesterone alone demonstrated no effect on tumour growth in athymic nude mice injected with the MCF-7 breast epithelial cancer cells into the mammary fat pad (Soule and McGrath 1980). However, progesterone in combination with oestrogen showed a synergistic effect on tumour growth above oestrogen alone (Soule and McGrath 1980). The dual dependence of breast cancer cell growth by both oestrogen and progesterone has been shown in ovariectomised August-Copenhagen-Irish (ACI) rats which develop a high rate of oestrogen-driven mammary tumours (Blank, Wong et al. 2008). This study demonstrates progesterone can induce breast tumour growth via combined actions with oestrogen, presumably by an up-regulation of PR by oestrogen. However, increased growth rates of cultured human mammary tumour epithelium by progesterone, compared to no effect in normal epithelial and non-malignant cells has also been described (Klevjer-Anderson and Buehring 1980).

In contrast to the reports detailing the stimulatory actions of progesterone in promoting breast cancer growth are studies outlining the inhibitory actions of progesterone. Increased breast cancer risk in progesterone deficient women has been described (Cowan, Gordis et al. 1981). Additionally, the growth fraction (GF) of cultured human breast adenocarcinoma tissue samples was predominantly inhibited by natural progesterone at low and high doses, although the GF of a few were stimulated (Jones and Russo 1987). In the same study, the effect of progesterone was shown to be independent of ERα or PR

steroid receptors status, suggesting progesterone may be mediating non-specific effects induced through indirect cell signalling pathways (Jones and Russo 1987). Additionally, the expression of PR membrane bound forms have been identified in breast epithelial cells suggesting potential non-genomic mediated actions of progesterone (Zuo, Li et al. 2010).

The regulation of proliferation in normal breast tissue and breast cancer by progesterone and PR is highly relevant to understanding the actions of synthetic progestins used in hormonal therapies (e.g. cHRT). A widespread public health concern has developed regarding the use of synthetic progestins in cHRT following reports of the Women's Health Initiative (WHI) clinical trial preliminary findings in 2002, which indicated an increased breast cancer risk in post-menopausal women taking cHRT compared to a placebo control group (Rossouw, Anderson et al. 2002). Interestingly, the ERT only parallel arm of the WHI trial reported a reduction in breast cancer in women, compared to a placebo control group (Anderson, Limacher et al. 2004). The reduced rate of breast cancer in post-menopausal women taking ERT compared to a placebo is thought to have occurred based on the high numbers of obese post-menopausal women included in the WHI cohort (Anderson, Limacher et al. 2004). The basis for the selection criteria of the cohort of post-menopausal women included in the WHI was primarily designed to investigate the effect of cHRT on cardiovascular disease. Since women of a higher body mass index (BMI) are more susceptible to increased rates of heart disease the trial included a higher rate of obese women (Rossouw, Anderson et al. 2002). Moreover, the high rate of obesity in the WHI cohort was proposed to select women with a higher

susceptibility to metabolic syndrome including insulin resistance and hyperinsulinemia which effectively puts women at a higher risk of breast cancer (Kuhl 2005). It has been proposed that oestrogen acts to reduce insulin levels and thus can reduce the risk of breast cancer in hyperinsulinemic women (Kuhl 2005). Hence a possible explanation for the reduced risk of breast cancer in post-menopausal women in the WHI taking ERT compared to a placebo (Anderson, Limacher et al. 2004). Additionally an antihormone resistance effect has recently been proposed that suggests under certain environmental conditions the breast tissue responds to oestrogen to promote apoptosis rather than proliferation (Jordan and Ford 2011).

Earlier and subsequent studies including the Million Women's study have reported a greater risk in breast cancer in women taking cHRT compared to ERT alone (Magnusson, Baron et al. 1999; Ross, Paganini-Hill et al. 2000; Schairer, Lubin et al. 2000; Beral 2003; Li, Anderson et al. 2003). A critical issue is how does a synthetic progestin such as MPA combined with an oestrogen in cHRT contribute to the development of breast cancer in women. One possibility is that the stimulatory actions of synthetic progestins are mediated via the PR. However, synthetic progestins additionally exert actions via alternative steroid receptors including the AR and GR *(reviewed in subsequent sections).* Additional studies are required to determine the mechanism of action of synthetic progestins and in particular how they promote breast cancer in post-menopausal women.

1.8 - Use of synthetic progestins in cHRT and breast cancer

1.8.1 - Types and pharmacological actions of synthetic progestins

Bioidentical progesterone compounds taken for therapeutic use are derived from soy beans, Mexican yam roots and occasionally animal ovaries (Sitruk-Ware 1999). Orally administered and transdermally applied (topical creams and transdermal patches) of commercially available progesterone are not well absorbed in the gastrointestinal tract and epidermal skin layers, respectively, and exhibit a short half-life due to high rates of rapid metabolism in the liver, compared to synthetic forms (Horwitz, Pike et al. 1986; Chakmakjian and Zachariah 1987). These pharmacological problems associated with using natural progesterone therapeutically have led to the development of synthetic progestins for use in hormonal pharmacological therapies to treat health related conditions requiring supplementation with progesterone.

Synthetic progestins are commonly separated into two distinct types, which are classified by the degree of biochemical structural similarity to the endogenously produced hormones, progesterone or testosterone (Sitruk-Ware 2004). These include the progesterone-derived progestins, such as the 17α-hydoxyprogesterone and 19-nor-progesterone derivatives, and testosterone-derived progestins such as the 19-nor-testosterone derivatives (Sitruk-Ware 2004). Drospirenone is a unique progestin since it is and analogue of spironolactone, yet possesses both similar actions to endogenous progesterone through antimineralcortocid and antiandrogenic activity (Krattenmacher 2000). Each individual type of synthetic progestin possesses unique pharmacological and

biological activity within human tissues mediated in part through the different relative binding affinities (RBA) for various steroid receptors [Table 1] (Sitruk-Ware 2004). These tests were performed using tritriated cognate ligands (e.g. promegestone and metribolone) at a saturating concentration and the relative binding affinity is determined by the ability of unlabelled steroids to reduce the specific binding of the cognate ligand (i.e Bmax) by 50%. Furthermore, synthetic progestins exhibit unique actions in different tissues in the body, due to the varying expression of steroid receptors in various tissue types (Sitruk-Ware 2004). Extensive documentation of the pharmacological properties of synthetic progestins, in addition to their progestagenic actions, include oestrogenic, androgenic and gluco-mineralocorticoid effects (Botella, Porthe-Nibelle et al. 1986; Ghatge, Jacobsen et al. 2005; Menendez, Oza et al. 2005). However, the majority of synthetic progestins are not coupled with estrogenic activity (Africander, Verhoog et al. 2011). The importance of this information is linked to the molecular mechanisms associated to the progestagenic and/or non-progestagenic actions of synthetic progestins via steroid receptor signalling pathways (i.e. PR, AR and/or GR) that may promote breast cancer *(discussed in sections 1.9.4, 9.1.3.2 and 9.1.3.3).*

1.8.2 - Historical use of synthetic progestins used as hormonal pharmacological therapies

Synthetic progestins were originally produced in the early 1960s and used pharmacologically for the treatment of excessive uterine bleeding, endometriosis and endometrial cancer (Rakoff 1959; Kelley and Baker 1961; Kennedy 1963), contraception in the FDA approved birth control pill '*Envoid*' (Andrews and Andrews 1962) and

Table 1: Relative binding affinities to steroid receptors and serum binding globulins of progestogens. The values were compiled from the literature by cross comparisons from a number of studies (Kuhl 2005). As the results of the various *in vitro* experiments are largely dependent on the incubation conditions and biological materials used, the values are inconsistent. They do not necessarily reflect the biological effectiveness. The displacement of a natural derivative of the hormones for each of the steroid receptors (PR (promegestone), AR (metribolone R1881), GR (dexamethasone) and MR (aldosterrone) was measured by the synthetic progestin which indicates relative ligand binding affinities. The natural PR hormone progesterone and androgen DHT, was not used in this study, which may not reflect true relative ligand binding affinities of the compounds (Kuhl 2005). **Abbreviations: PR**, progesterone receptor (promegestone, 100%); **AR**, androgen receptor (metribolone R1881, 100%); **ER**, oestrogen receptor (etsradiol-17β, 100%); **GR**, glucocorticoid receptor (dexamethasone, 100%); **MR**, mineralocorticoid receptor (aldosterone. 100%). **HRT**; hormone replacement therapy, **cHRT**; combined hormone replacement therapy, **COCs**; combined oral contraceptives, **POCs**; progestin-only contraceptives and **IUD**; intra-uterine device.

Table 1: Relative binding affinities to steroid receptors and serum binding globulins of progestogens

Type of Compound	Steroid receptor relative ligand binding affinity					
Natural Progesterone	**PR**	**AR**	**ER**	**GR**	**MR**	**Application**
Progesterone (Prog)	**50**	0	0	**10**	100	**cHRT:** Oral Micronised Progesterone (Prometrium) ┼ **POC:** Progering ┼┼
Progesterone derived synthetic progestins						
17α-hydroxyprogesterones	**PR**	**AR**	**ER**	**GR**	**MR**	**Application**
Chlormadinone acetate (CHA)	67	5	0	8	0	**COC:** Yasmin ┼┼┼
Cyproterone acetate (CPA)	90	6	0	6	8	**COC:** Diane-35 Δ
Medroxyprogesterone acetate (MPA)	115	5	0	29	160	**POCs:**Depo Provera Δ Δ **cHRT** :Premia ΔΔ Δ
19-nor progesterones	**PR**	**AR**	**ER**	**GR**	**MR**	**Application**
Medrogestone (MG)	**?**	?	?	**?**	?	**cHRT:** Prothil (5mg) ¥
Dydrogesterone (DG)	75	?	?	?	?	**cHRT:** Femoston 2/10¥¥ **POC:** Duphaston ¥¥¥
Nestorone (NS)	136	0	0	38	?	**Contraceptive:** Nuvaring Ω
Nomegesterol acetate (NGA)	125	42	0	6	0	**cHRT:** Megace Ω Ω
Trimegestone (TG)	330	1	0	9	120	**cHRT:** Totelle Ciclico Ω Ω Ω
Promegestone (PG)	100	0	0	5	53	**HRT:** Surgestone ₣
Testosterone derived synthetic progestins						
19-nor testosterones (Estranes)	**PR**	**AR**	**ER**	**GR**	**MR**	**Application**
Norethisterone acetate (NETA)	75	15	0	0	0	**cHRT**: Kliovance*, Kliogest**, Trisequens***, Primulot N **** **COCs:** Norimin-1 28 Day/Brevinor-1^ Brevinor-1^^, Norminin 28 Day Day/Brevinor^^^ Improvil 28#Synphasic##, Norinyl-1/28### Norinyl-1#### **POCs:** Micronor>, Locilan 28 Day>>, Noriday 28 Day>>>
Levonorgesterol (LNG)	150	45	0	1	75	**HRT:** Mirena **(IUD)** < **COCs:** Microgynon 50 ED<<, Microgynon 30 ED<<<, Levlen ED<<<<
19-nor testosterones (Gonanes)	**PR**	**AR**	**ER**	**GR**	**MR**	**Application**
Gestodene (GD)	90	85	0	27	290	**COC :** Gynera פ
Norgestimate (NG)	15	0	0	1	0	**COC :** Tri-Previfem פ פ
Dienogest (DE)	5	10	0	1	0	**COC:** Jeanine פ פ פ
Etonogesterol (EG)	150	20	0	14	0	**POCs:**Implanon ჯ **Contraceptives:** Nuvaring ჯ ჯ
Δ4-Tibolone (TIB)	90	35	1	0	2	**HRT:** Livial ل
Drospirenone (DP)	35	65	0	6	230	**COC:** Yasminiq ل ل

Table 1: Product HRT, COC and POC Drug Information

┼	Prometrium (Oral progesterone) – 100mg oral micronized progesterone (Solvay Pharmaceuticals)
┼┼	Progering (Prog-only vaginal ring) – 10mg daily of natural progesterone/per 3 months (Silesia)
┼┼┼	Yasmin (COC) – 3.0mg drospirenone + 0.03mg ethinyloestradiol (Schering AG)
Δ	Diane 35 (COC) – 2mg cyproterone acetate + 0.035ug ethinylo estradiol
ΔΔ	Depo-Provera (POC) – 150mg MPA/3months (Pfizer – US and Australia)
ΔΔΔ	Premia/Premarin (cHRT) – 2.5mg MPA + 0.625mg conjugated-equine oestrogen (Wyeth and Solvay Pharmaceuticals)
¥	Prothil (Oral synthetic progestin) – 5mg medrogesterone (Wyeth and Solvay Pharmaceuticals)
¥¥	Femostan 2/10 (cHRT) – 2mg oestradiol + 10mg dydrogesterone (Solvay Pharmaceuticals)
¥¥¥	Duphaston (POC) – 10mg dydrogesterone (Solvay Pharmaceuticals)
Ω	Nuvaring (Vaginal ring contraceptive) – 11.7mg etonogesterol/0.2mg released daily + 2.7mg ethinyl oestradiol/0.015mg release daily (Schering-Plough Pty Limited)
Ω Ω	Megace (POC) – 5mg nomegestrol acetate (Bristol Myers)
Ω Ω Ω	Totell Ciclico (cHRT) – 1mg 0estradiol hemihydrate + 0.25mg trimesterone
Ғ	Sugestone (HRT) – 0.125, 0.25 and 0.5mg promegestone (Sanofi-Aventis)
*	Kliovance (cHRT) – 1-2mg noresthisterone acetate + 1mg oestradiol
**	Kliogest (cHRT) – 1-2mg noresthisterone acetate + 2mg oestradiol
***	Trisquens (cHRT) – 1mg noresthisterone acetate + 2mg oestradiol
****	Primulot N (HRT) – 5mg norethisterone (Schering Pty Ltd)
^	Normin-1 28 Day/Brevinor-1(COC) – 1mg noresthisterone acetate + 35ug ethinyloestradiol (21 tablets) + 7 inert tablets (Kenral Division of Pharmacia Australia Pty Ltd)
^^	Brevinor 1 (COC) - 1mg noresthisterone acetate + 35ug ethinyl 0estradiol (Pharmacia Australia Pty Ltd)
^^^	Norinyl 28 Day/Brevinor (COC) – 1mg noresthisterone acetate + 50ug mestronal (21 tablets) + 7 inert tablets (Pharmacia Australia Pty Ltd)
^^^^	Normin-1 28 Day (COC) – 500ug noresthisterone acetate + 35ug ethinyl oestradio (21 tablets) + 7 inert tablets (Kenral Division of Pharmacia Australia Pty Ltd)
^^^^^^	Brevinor 1 (COC) – 500ug noresthisterone acetate + 35ug ethinyloestradiol (Pharmacia Australia Pty Ltd)
#	Improvil 28 (COC) – 500ug noresthisterone acetate + 35ug ethinyloestradiol (12 tablets) + 1ug noresthisterone acteate + 35ug ethinylestradiol (9 tablets) (Kenral Division of Pharmacia Australia Pty Ltd)
##	Synphasic (COC) – 500ug noresthisterone acetate + 35ug ethinyloestradiol (12 tablets) + 1ug noresthisterone acteate + 35ug ethinylestradiol (9 tablets) (Pharmacia Australia Pty Ltd)
###	Norinyl-1/28 (COC) – 1mg norethisterone acetate + 50ug mestronal + 7 inert tablets (Pharmacia Australia Pty Ltd)
####	Norinyl-1 (COC) – 1mg norethisterone acetate + 50ug mestronal (Pharmacia Australia Pty Ltd)
>	Micronor (POC) – 350ug norethisterone acetate (Janssen-Cilag Pty Ltd)
>>	Locilan 28 Day (POC) – 350 ug norethisterone acetate (Kenral Division of Pharmacia Australia Pty Ltd)
>>>	Noriday 28 Day (POC) – 350ug noresthisterone acetate (Pharmacia Australia Pty Ltd)
<	Mirena (IUD) – 52mg levonorgesterol/20mcg released daily
<<	Microgynon 50 ED (COC) – 125ug levonorgesterol + 50ug ethinyloestradiol (Schering Pty Ltd)
<<<	Microgynon 30 ED (COC) – 150ug levonorgesterol + 30ug ethinyloestradiol (Schering Pty Ltd)
<<<<	Levlen ED (COC) - 150ug levonorgesterol + 30ug ethinyloestradiol (Schering AG)
ɒ	Gynera (COC) – 0.075mg gestodene + 0.02 or 0.03mg ethinyloestradiol (Bayer Schering Pharm)
ɒ ɒ	Tri-Previfem (COC) – 0.035mg ethinyloestradial + 0.18, 0.215 or 0.25mg norgestimate (Andrx Pharmaceuticals)
ɒ ɒ ɒ	Jeanie (COC) – 30ug ethinyloestradiol + 2mg dienogest (Bayer Scheing Pharm)
₠	Implanon(POC) – 68mg etonogesterol (Organon)
₠ ₠	Nuvaring (vaginal ring contraceptive) – 0.120mg etonogesterol + 0.015mg ethinyloestradiol (Organon
ڸ	Livial (POC) – 2.5mg tibolone (Organon)
ڸ ڸ	Yasminq (COC) – 3mg drospirenone + 0.02mg ethinyloestradiol (Bayer Schering Pharm)

treatment of advanced breast cancer (Crowley and Macdonald 1962). In the 1940's, HRT consisted primarily of a conjugated equine estrogen (CEE) extracted from the urine of pregnant mares and containing E_1 and E_3 as the two most predominant oestrogenic components (Stefanick 2005). This form of HRT was commercially available by the pharmaceutical company Wyeth-Ayrest Pharmaceuticals (Philadelphia, PA, USA) in 1942, and marketed as Premarin for administration to peri- and post-menopausal women for the alleviation of menopausal symptoms associated with the decline in ovarian production of oestrogen (Kenemans 1999; Schindler 2006).

In 1975, subsequent to the wide-spread use of Premarin, there were reports confirming an increased rate of endometrial cancer in post-menopausal women taking Premarin for HRT (Smith, Prentice et al. 1975; Ziel and Finkle 1975). This led to a dual prescription of the synthetic progestin, medroxyprogesterone acetate (MPA) (Provera), already approved by the United States of America (USA) Food and Drug Administration (FDA) for other endometrial related conditions and the CEE (Premarin) for HRT during the early 1980s in the USA. Official approval by the USA FDA for distribution of a single drug formulation containing both MPA and CEE as combined hormone replacement therapy (cHRT) (CEE 0.625 mg + MPA 2.5 mg marketed by Wyeth Pharmaceuticals as Prempro) occurred in mid 1990's (Stefanick 2005). Following the marketing of Prempro, there were reports of over 15 million women taking this cHRT combination per year during 1995 to July 2002, and it was documented as the most frequently prescribed pharmaceutical in the USA during this time (Hersh, Stefanick et al. 2004; Wysowski and Governale 2005).

1.8.3 - Use of MPA in cHRT and contraception and relation to breast cancer

Whilst there are a number of different types of synthetic progestins, MPA has been implicated as the most widely used in cHRT in the USA (Hersh, Stefanick et al. 2004). MPA is most commonly used in cHRT since it presents with the most potent progestagenic activity, required to protect the endometrium from the stimulatory actions of oestrogen and is not heavily metabolised in the body, (Sitruk-Ware 2004; Ghatge, Jacobsen et al. 2005). MPA is classified in the progesterone-derived synthetic progestin sub-group, which possesses ligand binding affinity for the PR, AR, glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (Bentel, Birrell et al. 1999; Africander, Verhoog et al. 2011). The biochemical structure of MPA closely resembles that of T and DHT, as well as progesterone, enabling ligand binding to their respective cognate nuclear receptors, AR and PR, respectively [Table 1].

The findings in the WHI clinical trial in 2002 were the first to show that the use of cHRT (CEE 0.625 mg and MPA 2.5 mg) was associated with an increased risk of breast cancer (hazard ratio (HR), 1.26; (nominal 95% confidence intervals [CIs]) 1.00-1.59), compared to a placebo control group (Rossouw, Anderson et al. 2002), whereas the ERT alone parallel arm of the study was associated with a reduced risk (HR, 0.77; 95% CI, 0.57-1.01) (Anderson, Limacher et al. 2004). A HR of 1.26 reflects a 1.26-fold increase in breast cancer incidence above the normal population risk estimate (i.e. 1 in 10 normal population risk equates to 1.26 in 10 population risk estimate). Earlier *in*-vivo studies demonstrated long-term use of MPA (75 mg/kg every 3 months) can promote mammary nodules in beagle bitches (Frank, Kirton et al. 1979; Concannon, Altszuler et al. 1980).

Furthermore, a number of *in vivo* studies in mice also support a carcinogenic effect of MPA. Administration of MPA (40mg MPA depot /every 2 months) to virgin female BALB/c mice results in increased mammary tumour growth compared to untreated mice (Molinolo, Lanari et al. 1987). Lastly, ovariectomised mice treated with MPA (40 mg silastic implants) had an increased development of mammary tumous compared to untreated mice, and subsequent removal of the MPA pellet was associated with reduced tumour growth (Kordon, Lanari et al. 1990).

In addition to cHRT, MPA is used in the progestogen-only contraceptive, Depo-Provera; There are approximately 20 million users of Depo-Provera worldwide (Affandi 2002). Depo-Provera is administered at the dose of 150 mg via an intramuscular injection (1-7 nM/serum level of MPA) every 3 months (Hiroi, Stanczyk et al. 1975; Mathrubutham and Fotherby 1981). Depo-Provera was not approved for use in the USA by the FDA until the early 1990s due to the reports from *in vivo* studies of an '*increased appearance of mammary nodules'* (excessive mammary tissue proliferation) following administration with depot MPA (75mg/kg) in beagle bitches, as previously described (Frank, Kirton et al. 1979; Concannon, Altszuler et al. 1980; Rosenfield, Maine et al. 1983). Additionally, there was an increase in mammary gland adenocarcinoma in male cats given Depo-Provera as an anti-androgenic treatment to reduce intercat aggression, and fibroepithelial breast hyperplasia developed in felines given Depo-Provera for contraception (Jacobs, Hoppe et al.; Loretti, Ilha et al. 2005). Interestingly, an increased risk of breast cancer has been reported in women taking Depo-Provera compared to never-users between the age of 25-34 yrs (Schindler 2006), and use before the age of 25 yrs for a period of 2-6 yrs

(WHO 1991; Skegg, Noonan et al. 1995; Skegg, Paul et al. 1996; Shapiro, Rosenberg et al. 2000). While these studies do not indicate the parity status of women included in the cohort, based on the higher risk in younger women, they may indicate a greater risk in nulliparous women. Thus, it may be postulated from previous studies that MPA in the form of Depo-Provera induces neoplasia preferentially in undifferentiated breast tissue and should therefore be restricted in use to parous women only (WHO 1991; Skegg, Noonan et al. 1995; Skegg, Paul et al. 1996; Shapiro, Rosenberg et al. 2000; Russo, Mailo et al. 2005). This notion has been suggested to correlate with the '*window of susceptibility'* hypothesis described in an earlier section, which proposes that undifferentiated breast tissue in women between menarche and first pregnancy is most susceptible to neoplastic transformation *(refer to section 1.2.6)* (Russo, Mailo et al. 2005). Based on the literature, the public health implications linked to the use of MPA in cHRT or contraception are concerning, especially given that MPA has the potential to induce hyperplastic conditions in the breast and promote mammary tumour growth. Furthermore, in 2005 the World Health Organisation (WHO) officially declared MPA as a carcinogenic agent in the breast tissue of humans and animals (WHO 2005).

1.8.4 - Biological effects of synthetic progestins and breast cancer

The anti-proliferative and anti-oestrogenic actions of synthetic progestins within the endometrium has been clearly established in the literature (Lindahl and Willen 1994; 1995; Pike, Peters et al. 1997). However, the biological effects of synthetic progestins in breast tissue currently are not well understood. It is suggested that synthetic progestins act differently to natural progesterone in the breast tissue (Pike, Spicer et al. 1993). This

notion is supported by the findings in the E3N-EPIC French cohort, where an increased risk of breast cancer was detected in women taking cHRT including synthetic progestins (RR; 1.4 (95% CI; 1.2-1.4)) compared to no risk of breast cancer in women taking cHRT consisting of the natural form of progesterone, oral micronized progesterone ((OMP) RR; 0.9 (95% CI; 0.7-1.2)) (Kincl, Ciaccio et al. 1978; Maxson and Hargrove 1985; de Lignieres, de Vathaire et al. 2002; Fournier, Berrino et al. 2005).

A number of studies in addition to the WHI clinical trial have reported an increased relative risk of developing breast cancer in women taking cHRT containing MPA and other synthetic progestins compared to oestrogen alone (Persson, Weiderpass et al. 1999; Ross, Paganini-Hill et al. 2000; Schairer, Lubin et al. 2000; Chen, Weiss et al. 2002; Porch, Lee et al. 2002; Rossouw, Anderson et al. 2002; Bliss and Gray 2003; Olsson, Ingvar et al. 2003; Beral, Bull et al. 2005). A major consequence of cHRT is to increase ER^+PR^+ invasive breast cancers and a higher rate of lobular breast cancer (Chen, Weiss et al. 2002; Newcomb, Titus-Ernstoff et al. 2002; Ereman, Prebil et al. 2010).

1.8.5 - Change in cHRT prescription rates and breast cancer incidence rates following the WHI clinical trial

The use of different cHRT formulations, in conjunction with changes in breast cancer incidence rates, predominantly after the WHI trial, has been extensively documented. There was a substantial reduction in the prescription rates of cHRT following the reports of the WHI clinical trial in 2002 in the USA, Australia and Europe (Haas, Kaplan et al. 2004; Hersh, Stefanick et al. 2004; Clarke and Glaser 2007; Glass, Lacey et al. 2007;

Hausauer, Keegan et al. 2007; Kerlikowske, Miglioretti et al. 2007; Ravdin, Cronin et al. 2007; Canfell, Banks et al. 2008), in part due to the widespread public fear following the initial WHI trial media reports in 2002 (Lebow and Arkin 1993; Paine, Stocks et al. 2004). A number of studies published in the USA and Australia have demonstrated an association between the reduction in cHRT prescription rates and breast cancer incidence rates (Ravdin, Cronin et al. 2007; Robbins and Clarke 2007; Canfell, Banks et al. 2008), consistent with the findings in the WHI trial linking cHRT use and increased breast cancer incidence (Rossouw, Anderson et al. 2002). Furthermore, since a similar effect has been reported not only in the USA but also in Australia, the relationship between cHRT and breast cancer incidence is likely to not be associated with a specific environmental stimulus.

Recently, law-suits have been filed against the Pharmaceutical company Wyeth who makes the cHRT preparation Premia (CEE 0.625 mg + MPA 2.5 mg) by women who are claiming that the use of cHRT led to the development of their breast cancer (Twombly 2007). These law-suits have massive public health implications, similar to those filed against tobacco manufacturers who marketed cigarettes without adequate warnings to the public of the increased risk of lung cancer associated with smoking (Douglas, Davis et al. 2006). A population based study was undertaken in this thesis to further investigate from published Australian based epidemiological findings the changes in the prevalence of cHRT therapies containing MPA prescribed in Australia and association to changes in prevalence of breast cancer incidence rates before and after cessation of the cHRT arm of the WHI trial in 2002 (Canfell, Banks et al. 2008). This study was aimed at highlighting

the public health concern for Australian post-menopausal women in regards to the use of MPA in cHRT formulations in order to support implementation of changes to the legislation of MPA by the Australian Government for use its use in cHRT. This study aimed to provide statistical evidence using Australian population data for an association between the use of cHRT containing MPA and breast cancer incidence as a follow-on from an earlier Australian population based study (Canfell, Banks et al. 2008).

The final section of this literature review is aimed at providing relevant background on the role of androgens in normal and malignant breast tissue. This background is central to the main hypothesis of this thesis that the anti-androgenic activity of MPA in cHRT can abrogate AR-signalling in the breast and thereby negate the protective effects of androgens, thus leading to increased risk of developing breast cancer in post-menopausal women taking cHRT. The antagonistic actions of MPA on DHT-induced AR siganlling in breast cancer cells has previously been postulated as being a critical aspect of MPA induced breast cancer risk (Birrell, Butler et al. 2007).

1.9 - Androgens and AR actions in normal and malignant breast tissue

1.9.1 - Androgens and the AR-signalling axis in female breast tissue

The characterisation and biological understanding of androgenic activity and the AR-signalling axis has largely developed from studies examining the actions of androgens in male reproductive physiology and prostate cancer. Androgenic hormones play an essential role in male reproductive biology, being critical for the development and

maintenance of the male urogenital organs including the prostate, epididymis, testis and seminal vesicles (Patrao, Silva et al. 2009). The requirement of androgens in the development and survival of the prostate gland was first discovered in 1970s in a study which demonstrated that surgical castration of men resulted in a reduction of tumour growth in advanced prostate cancer (Huggins and Hodges 1972).

In addition to the prostate gland, androgens and AR-signalling in females is now emerging as an essential pathway in normal breast physiology and breast carcinogenesis (Yeh, Hu et al. 2003; Dimitrakakis and Bondy 2009). Androgens are produced in females and are reported to circulate in the serum at approximately two-thirds of the levels detected in males (Labrie, Belanger et al. 1997; Labrie, Luu-The et al. 2003). Androgens mediated by the AR-signalling axis have been reported to play a protective role in female breast tissue by suppressing ERα-stimulated breast epithelial proliferation. Moreover, AR has been shown to be an independent prognostic factor for overall survival of women with $ER\alpha^{+}$ PR^{+} breast cancer (Gonzalez-Angulo, Stemke-Hale et al. 2009; Peters, Buchanan et al. 2009; Castellano, Allia et al. 2010).

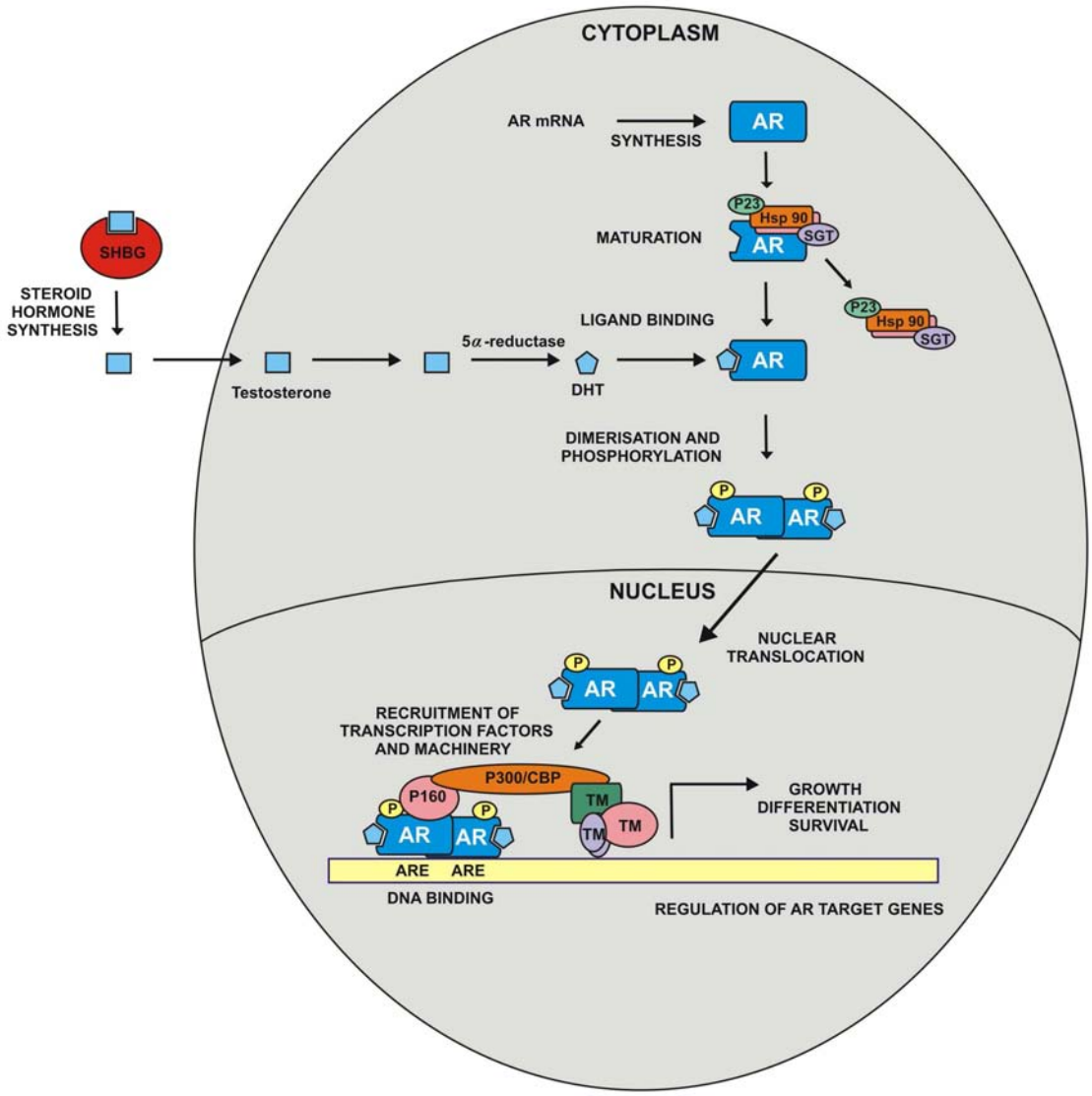
1.9.2 - AR gene and protein structure

In the late 1960s, the AR was isolated from the cytoplasm of the rat prostate (Mainwaring 1969). The human AR was subsequently cloned in the late 1980s (Chang, Kokontis et al. 1988; Trapman, Klaassen et al. 1988; Brinkmann, Faber et al. 1989; Tilley, Marcelli et al. 1989) and shown to be localisation to the long arm of the X chromosome at Xq11-12 region, resulting in 2 alleles in females, yet one is inactivated and one allele in males

(Brown, Goss et al. 1989). Cloning and sequencing of the human AR gene revealed a structure consisting of eight exons of approximately 917 amino acids, encoding a protein of molecular weight 98.9 kDa (Brinkmann, Faber et al. 1989; Tilley, Marcelli et al. 1989). In the cytoplasm, the AR is part of a hetero-complex comprised of heat shock proteins (hspP90, hsp70 and hsp40) and other co-chaperones (Pratt and Toft 1997; Cheung and Smith 2000; Buchanan, Ricciardelli et al. 2007) [Figure 1.9]. Subsequent to ligand binding, the AR dissociates from the maturation complex, leading to dimerisation and phosphorylation of the receptor, nuclear translocation, co-factor recruitment and regulation of AR target gene expression (Henderson 2003) [Figure 1.9].

Key functional sub-domains of the AR include the polyglutamine (poly-Q) and polyglycine (poly-G) repeats located in the NTD, that are comprised of 9-39 CAG and 14-27 GGN microsatellite repeat regions, respectively (Henderson 2003). Differences in the length of the microsatellite regions between individuals, has been associated with aberrant AR function (i.e. CAG repeat regions greater than 40), specifically in a rare condition in men commonly referred to a Kennedy's disease and in a minor role in androgen insensitivity syndrome (AIS) (Ris-Stalpers, Kuiper et al. 1990; La Spada, Roling et al. 1992). Moreover, a longer poly-Q repeat region in the AR has been shown to be associated with reduced AR transactivation and has been associated with breast cancer in women over 40 yrs of age, compared to a shorter poly-Q repeat region which is protective against breast cancer (Elhaji, Gottlieb et al. 2001; Giguere, Dewailly et al. 2001). Interestingly, reduced AR transactivation has been shown to be associated with a longer compared to a shorter poly-Q CAG repeat region in prostate cancer cells

Figure 1.9: AR-signalling axis. Diagram depicts key cellular events associated with the AR-signalling axis including steroid hormone and AR synthesis. Stages of AR maturation are depicted by the activity of chaperone proteins including p23, p60, Hop, FK506-binding immunophillins (FK-506 binding protein (FKBP) 51 and 52) and tetriocopeptide repeat (SGT) proteins. Subsequent cellular activities are depicted which include the following: ligand binding; dimerisation and phosphorylation; nuclear translocation; DNA binding to AREs; recruitment of transcription factors (e.g. p160 coregulators) and transcriptional machinery (TM) (eg RNA polymerase) and the transcriptional regulation of AR target genes leading to expression of genes involved in growth, differentiation and survival intracellular AR-signalling pathways (diagram modified from (Henderson 2003)).


CYTOPLASM
AR mRNA
SYNTHESIS
AR
P23
Hsp 90
SGT
MATURATION
SHBG
STEROID HORMONE SYNTHESIS
LIGAND BINDING
5α-reductase
Testosterone
DHT
DIMERISATION AND PHOSPHORYLATION
P
NUCLEUS
NUCLEAR TRANSLOCATION
RECRUITMENT OF TRANSCRIPTION FACTORS AND MACHINERY
P300/CBP
P160
TM
GROWTH DIFFERENTIATION SURVIVAL
ARE ARE
DNA BINDING
REGULATION OF AR TARGET GENES

potentially due to a reduction of the N-terminal/C-terminal (N/C) interaction (a key dimerisation event required for normal AR function, stability and transactivation) (Tut, Ghadessy et al. 1997; Beilin, Ball et al. 2000; Buchanan, Yang et al. 2004). However, no definitive studies have been performed to validate that a change in the AR poly-Q CAG repeat region can alter breast cancer risk. In relation to the research studies described in this thesis, women taking cHRT who express a shorter poly-Q CAG repeat region have been reported to be at a higher risk of breast cancer than those who express a longer poly-Q CAG repeat regions (Abbas S, Beckmann L et al. 2010). This study suggests that women who express an AR with higher transactivation potential are more susceptible to the carcinogenic actions of cHRT.

1.9.3 - Androgens and AR-signalling in normal breast tissue

Androgens have been shown in *in vivo* studies to suppress basal and oestrogen-stimulated breast epithelial proliferation in normal breast tissue (Dimitrakakis and Bondy 2009). For instance, treatment of ovariectomised rhesus monkeys with a combination of T and E_2 led to a 40% reduction in E_2-stimulated mammary epithelial cell proliferation (Zhou, Ng et al. 2000). Furthermore, androgens (either T or DHT) have been shown to inhibit oestrogen-stimulated breast epithelial proliferation in ovariectomised mice, which corresponds also with a reduction in the expression of the ER-regulated gene, PR (Casey and Wilson 1984). Another study has shown that treatment of female monkeys with flutamide (an AR antagonist) results in increased mammary epithelial proliferation (Dimitrakakis, Zhou et al. 2003). The excessive secretion of androgen by adrenal tumours or from adrenal hyperplasia has been reported to result in suppression of breast

development in females (Sakuma, Yamaguchi et al. 1994; Summers, Herold et al. 1996; New 2004). Moreover, a reduction in breast epithelial cell proliferation has been observed in female athletes taking anabolic-androgenic steroids, and in female to male trans-sexuals taking testosterone (Korkia and Stimson 1997; Slagter, Gooren et al. 2006). Lastly, breast epithelial proliferation was reduced in rats administered methyltestosterone and an oral contraceptive (comprised of levonorgesterol plus ethinyl oestradiol) for treatment of hypoandrogenemia compared to administration of the oral contraceptive alone (Jayo, Register et al. 2000).

The fluctuations in levels of A and T during the menstrual cycle corresponds directly with changes in the rates of breast epithelial proliferation (Abraham 1974; Soules, Clifton et al. 1987). The levels of these androgens are lowest in the early follicular phase of the menstrual cycle, but increase during the late follicular phase, which coincides with low rates of breast epithelial proliferation (Abraham 1974; Soules, Clifton et al. 1987). A gradual decline in androgens occurs during the early, mid and late luteal phase, corresponding with the highest rates of proliferation in breast tissue (Abraham 1974; Soules, Clifton et al. 1987; Massafra, De Felice et al. 1999). Based on these studies the roles of androgens is pivotal to maintaing a balance to regulating breast epithelial proliferation.

1.9.4 - Androgens and AR-signalling in malignant breast tissue

In breast cancer, androgens have been implicated as having a protective role against the development and progression of the disease (Dimitrakakis, Zhou et al. 2002;

Dimitrakakis and Bondy 2009). In particular, a plethora of studies have demonstrated that endogenous and exogenous androgens can suppress basal and oestrogen-stimulated proliferation of the AR^+ MCF-7, ZR-75-1 and T47D breast cancer cell lines, but not AR^- MDA-MB-231 and BT-20 malignant breast epithelial cells lines. This effect of androgens on breast cancer cell proliferation in vitro can be reversed by an anti-androgen (Simard, Dauvois et al. 1990; de Launoit, Dauvois et al. 1991; Szelei, Jimenez et al. 1997; Ando, De Amicis et al. 2002; Ortmann, Prifti et al. 2002; Aspinall, Stamp et al. 2004; Greeve, Allan et al. 2004). Treatment of human breast cancer cells isolated from primary tumours with testosterone has been shown to result in reduced DNA synthesis (Burstein, Kjellberg et al. 1971). Additionally, the growth of ZR-75-1 xenograft tumours and carcinogen-induced mouse mammary tumours is inhibited by administration of androgens (Huggins, Briziarelli et al. 1959; Teller, Kaufman et al. 1969; Zava and McGuire 1977).

Clinical studies have highlighted that the AR is an independent prognostic factor for overall survival in ER^+ PR^+ breast cancers (Gonzalez-Angulo, Stemke-Hale et al. 2009; Peters, Buchanan et al. 2009; Castellano, Allia et al. 2010). Collectively these studies highlight that the inhibitory actions of androgens mediated via the AR-signalling axis in normal and malignant breast tissues results in protection from breast cancer due to a reduction of oestrogen-stimulated malignant breast epithelial cell proliferation and tumour growth. Following the identification of specific gene signatures associated with luminal and basal subtypes of breast cancer (Perou, Sorlie et al. 2000; Sorlie, Perou et al. 2001; van 't Veer, Dai et al. 2002), subsequent analyses identified ER- breast tumours with high levels of AR expression and molecular apocrine features (Farmer, Bonnefoi et

al. 2005). These aspects of AR-signalling in breast tissue provide a basis for investigating potential mechanisms associated with MPA used in cHRT and breast cancer development.

1.9.5 - Androgenic effects of MPA

While the detrimental carcinogenic effects of MPA in breast tissue have been described in the literature, the mechanistic basis for MPA promoting breast cancer risk remain unknown. As previously overviewed, synthetic progestins differ in their biological activities based on their relative ligand binding affinities for different steroid hormone receptors (Sitruk-Ware 2004). In particular, MPA possesses a high ligand binding affinity for the PR, similar to natural progesterone, in addition to high affinity binding for the AR (Bentel, Birrell et al. 1999; Sitruk-Ware 2004). In support of the androgenic actions of MPA, a similar pool of genes has been shown to be regulated by both DHT and MPA in the T47D breast cancer cell line (Ghatge, Jacobsen et al. 2005). However, as expected by the different biochemical structures of the compounds, there is a portion of genes which are distinctly regulated by the either DHT or MPA (Ghatge, Jacobsen et al. 2005). Whether theses differences in gene regulation by the two hormone treatments are mediated by the action of MPA specifically through the PR, GR and/or AR at this stage is still speculative (Ghatge, Jacobsen et al. 2005; Birrell, Butler et al. 2007; Horwitz and Sartorius 2008).

Studies published in the Dame Roma Mitchell Cancer Research Laboratory (DRMCRL) have demonstrated, using a mammalian two-hybrid assay, that MPA can act in a both an

agonist manner on its own and an antagonistic manner in the presence of DHT by inhibiting DHT-induced AR protein N-terminal/C-terminal (N/C) interaction (a key dimerisation event required for normal AR function, stability and transactivation) [Figure 1.10] (Birrell, Butler et al. 2007; Centenera, Harris et al. 2008). Furthermore, these findings have been reflected in another study which reported that MPA (i.e. 1 - 500 nM) can act as an antagonist of the DHT-induced AR N/C interaction (Kemppainen, Langley et al. 1999). These molecular actions of MPA on DHT-induced AR-signalling are not induced by natural progesterone [Figure 1.10] (Birrell, Butler et al. 2007). Thus, given these finding it may be feasible that the antagonistic actions of MPA on DHT-induced AR signalling are not PR-mediated. In support of this notion are additional molecular modelling studies indicating that MPA alters the positioning of amino acid phenylalanine 875 in the AR, which is located in the ligand binding domain (LBD), compared to the native ligand DHT (Birrell, Butler et al. 2007). The altered conformation of the AR induced by MPA compared to DHT, is thought to lead to the disruption in the AR N/C interaction (Kemppainen, Langley et al. 1999; He and Wilson 2002; Birrell, Butler et al. 2007).

It should be noted that MPA in isolation is unable to form an AR N/C interaction at low doses (i.e. 1 nM) and can only induce a weak interaction at higher doses (i.e. 10 -100 nM) [Figure 1.9] (Birrell, Butler et al. 2007). The androgenic actions of MPA via the AR-signalling axis has been reported in women diagnosed with advanced breast cancer, whereby low AR expression is associated with reduced response to MPA treatment. Interestingly, MPA has been suggested to exhibit anti-androgenic activity in men and has

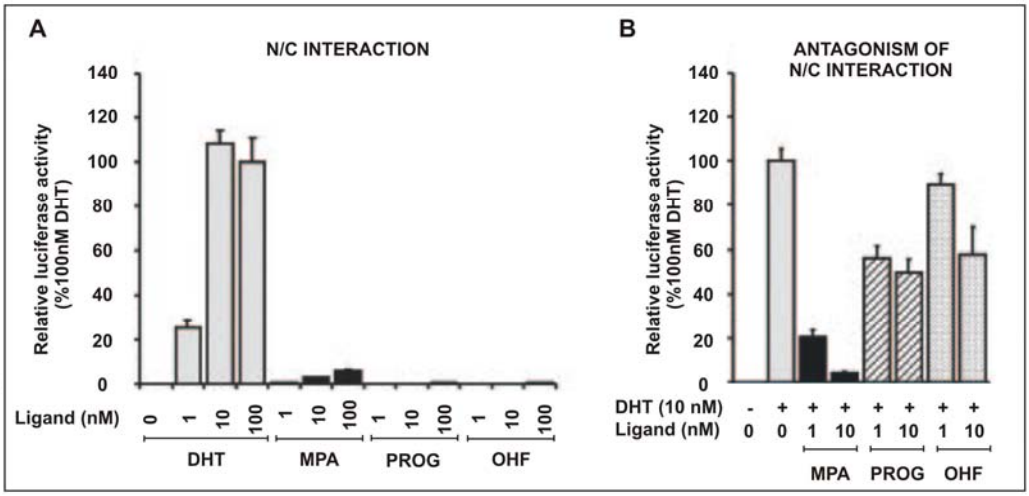


Figure 1.10 (A and B): MPA abrogates DHT-induced interaction of the N-terminal/C-terminal (N/C) of the AR protein. An *in vitro* mammalian two-hybrid N/C interaction assay using prostate cancer cell line PC-3 was used to demonstrate that treatment with 1 - 10 nM MPA results in an abrogation of the N/C interaction induced by 10 nM DHT. In contrast, treatment with 1 - 10 nM progesterone (PROG) or the AR antagonist hydroxyflutamide (OHF) resulted in a less dramatic inhibition of the AR N/C interaction induced by 10 nM DHT. **(A)** In the presence of 1 - 100 nM DHT, an AR N/C interaction was detected, which remained low by MPA alone and absent by PROG or OHF treatments in isolation. **(B)** In the presence of 1 and 10 nM MPA abrogation of DHT-induced AR N/C interaction was markedly higher, compared to treatments of PROG or OHF in isolation (Birrell, Butler et al. 2007).

also been used since the 1960s for the treatment of male sex offenders and a range of behavioral and aggression related conditions (Pinta 1978; Cooper 1986; Cooper 1987). The anti-androgenic potential of MPA described has important implications for the hypothesis to be tested in this thesis that the anti-androgenic activity of MPA in cHRT can abrogate AR-signalling in the breast and thereby negate the protective effects of androgens, thus leading to increased risk of developing breast cancer in post-menopausal women taking cHRT.

1.10 - Summary and conclusions

Human breast tissue is subject to a series of developmental changes throughout female reproductive life. The biological processes associated with the developmental changes of the breast tissue which occur during puberty, pregnancy and menopause, largely mediated by endogenous sex steroid hormones, are dynamic and illustrate the high degree of hormonal responsiveness of the breast tissue. Additionally, the cumulative exposure of breast tissue to endogenous and exogenous hormones is shown to play a major role in determining the risk of breast cancer development. In particular, the use of exogenous hormones in pharmacological hormonal therapies including cHRT and contraception are concerning based on the literature discussed in this review in terms of breast cancer development.

1.11 - Thesis Objectives

Currently, millions of women in the USA and Australia take synthetic hormonal pharmaceuticals in the form of cHRT and COCs; the former are the most highly prescribed medicines worldwide (Hersh, Stefanick et al. 2004). The initial findings in the WHI clinical trial reported in 2002 identified an association between the use of cHRT containing MPA and increased breast cancer risk in post-menopausal women. This has provided the most convincing evidence in humans of a potential public health concern over use of cHRT and breast cancer development. The findings in the WHI clinical trial have been widely debated by the medical and scientific communities (Klaiber, Vogel et al. 2005; Mastorakos, Sakkas et al. 2006). Although, the WHI clinical trial was the first randomised double blind, placebo-controlled study to show a relationship between cHRT use and increased breast cancer incidence, there were a number of population based case studies which predicted this association (Persson, Weiderpass et al. 1999; Ross, Paganini-Hill et al. 2000; Schairer, Lubin et al. 2000; Chen, Weiss et al. 2002; Fernandez, Gallus et al. 2003).

Over the years, other literature has supported a relationship between cHRT use and breast cancer (Beral, Banks et al. 2002; Bliss and Gray 2003; Daling, Malone et al. 2003; Olsson, Ingvar et al. 2003; Opatrny, Dell'Aniello et al. 2008). The identification of molecular mechanisms by which synthetic progestins, in particular MPA, the most commonly used synthetic progestin in cHRT (Hersh, Stefanick et al. 2004) acts to increase breast cancer risk are essential to substantiate epidemiological/observational

findings (Rossouw, Anderson et al. 2002; Beral 2003). Moreover, the use of cHRT preparations containing MPA and their relationship to breast cancer incidence in Australian women has not been well documented. Therefore, this thesis aimed to provide both observational and biological evidence of an association between the actions of MPA in the breast, via AR-mediated molecular mechanisms and the promotion of breast cancer.

1.12 - Thesis Hypothesis and Aims

Thesis Hypothesis 1: Increased use of cHRT containing MPA is associated with increased breast cancer incidence rates in Australian women.

All aims described below (Aims 1-3) were undertaken before and after publication of the cHRT arm of the WHI clinical trial cHRT in 2002.

Aim 1: To investigate the types of annual cHRT containing MPA prescription rates available on the PBS in Australian women using established Australian based Government registries.

Aim 2: To determine the annual breast cancer incidence rates in Australian women using an established Australian breast cancer registry.

Aim 3: To determine the association between annual prescription rate changes in cHRT containing MPA and breast cancer incidence rates in Australian women.

Thesis Hypothesis 2: The anti-androgenic activity of MPA in cHRT can abrogate AR-signalling in the breast and thereby negate the protective effects of androgens, thus leading to increased risk of developing breast cancer in post-menopausal women taking cHRT.

Aim 1: To characterise steroid receptor expression (AR, ERα and PR) in non-malignant pre- and post-menopausal human breast tissues, in addition to non-associated and associated with malignancy.

Aim 2: To establish that the AR-signalling axis is functional in non-malignant post-menopausal breast tissues using an *ex vivo* breast explant tissue culture model.

Aim 3: To determine the effect of MPA on breast epithelial cell proliferation and survival in human pre- and post-menopausal non-malignant breast epithelial cells.

Aim 4: To determine the effect of DHT and MPA alone and in combination on AR, ERα and PR expression in non-malignant pre- and post-menopausal breast epithelial cells.

Aim 5: To investigate the mechanism(s) involved in MPA impeding DHT-induced AR-signalling in the ERα and PR and AR positive ZR-75-1 human breast cancer cell line.

Aim 6: To determine whether DHT and MPA alone and/or in combination acts via AR- and/or PR-mediated pathways known to be involved in the development and progression of breast cancer.

Chapter 2

General Materials and Methods

2.1 - Materials

2.1.1 - Chemicals, solutions and general materials and reagents

- ***AGFA (Mortsel, Belgium)*:** film developer (parts A and B), rapid fixer
- ***Ajax-Finechem Pty Ltd (Sydney, New South Wales (NSW), Australia (AU))*:** acetone, citric acid, ethanol, formaldehyde, methanol, xylene
- ***Amersham Biosciences (Buckinghamshire, United Kingdom (UK))*:** enhanced chemilumiescence (ECL)^{TM} chemiluminescence detection kit, Hybond^{TM}-C Extra nitrocellulose transfer membrane, hyperfilm ECL^{TM}, dextran 70
- ***Asia Pacific Specialty Chemicals (Seven Hills, NSW, AU)*:** hydrogen peroxide, charcoal
- ***Australia Biostain (Traralgon, Victora (VIC), AU)*:** Lillie-Mayer haematoxylin
- ***Baxter Healthcare (Old Toongabbie, NSW, AU)*:** water
- ***BDH laboratory supplies (Kilsyth, VIC, AU):*** Dimethyl Sulfoxide (DMSO)
- ***Bio-Rad Laboratories (Hercules, California (CA), USA)*:** Acrylamide/bis solution 29:1, 40%, Acrylamide/bis solution 37.5:1 40%
- ***Chemsupply (Gilman, South Australia (SA), AU)*:** Di-n-butylPhthalate in xylene (DPX) mounting medium
- ***CSL Biosciences (Parkville, VIC, AU)*:** streptomycin sulphate (5000 mg/ml) and penicillin (5000 U/ml)
- ***Cytosystems (Castle Hill, NSW, AU)*:** MultiSer^{TM} foetal calf serum (FCS)
- ***DAKO (Botany, NSW, AU)*:** fluorescent mounting medium, horseradish peroxidise (HRP)-conjugated streptavidin

- ***Diploma (Melbourne (MEL), VIC, AU)*:** skim milk powder
- ***Fronine Laboratory Supplies (Riverstone, NSW, AU)*:** 10% neutral buffered formalin
- ***Hopkin and Williams (Chadwell Health, Essex, UK)*:** trypan blue
- ***Invitrogen (Carlsbad, CA, USA)*:** Avidin-Biotin blocking kit, SeeBlue Plus 2^{TM} pre-stained protein standard, F12 Nutrient Mixture (HAM) + L-Glutamine, collagenase type II
- ***Spongostan; Johnson & Johnson (Skipton, UK)*:** dental gelatine sponges, cell isolation media
- ***JRH Biosciences (Lenexa, Kansas (KS), USA)*:** phenol red free RPMI medium 1640 (containing L-glutamine), RPMI medium 1640 (containing 2.05 mM L-glutamine), 10x trypsin and ethylenediamine tetra-acetic acid (EDTA), FCS
- ***Lomb Scientific (Taren Point, NSW, AU)*:** tissue embedding cassette

  Merk (Kilsyth, VIC, Australia): chloroform
- ***Menzel-Glaser Braunscweig (Germany (DE))*:** superfrost glass microscopic slides
- ***Nalgene Nunc International (Rochester, New York (NY), USA)*:** cell scrapers (23 mm)
- ***Packard (Mount Waverly, Australian Capital Territory, AU)*:** optical plates
- ***Roche Applied Science (Castle Hill, NSW, AU)*:** complete protease inhibitor cocktail tablets, glycogen
- ***SAFC Biosciences (KS, USA)*:** foetal bovine serum

- ***Sigma-Aldrich (St Louis, Missouri (MO), USA)*:** Accustain Eosin Y solution, ammonium persulphate (APS), bovine serum albumin (BSA), bromophenol blue, 3,3′-Diaminobenzidine tetrahydrochloride (DAB), goat, horse and rabbit serum, sodium dodecyl sulphate (SDS), Tris base, Tris-chloride (Cl), Tris-hydrochloride (HCL), 1% Triton X-100, Tween 20, EDTA, glycerol, insulin solution from bovine pancreas (10 mg/ml), phosphate buffered solution (PBS) 10x, Tetramethylethylenediamine (TEMED), β-mercaptoethanol
- ***Whatman International (Kent, UK)*:** Whatman filter paper

2.1.2 - Antibodies

- ***Chemicon International (Temecula, CA, USA):*** mouse monoclonal KITLG (clone# AB198P)
- ***DAKO (Glostrup, Denmark)*:** mouse monoclonal Ki67 (clone# M7240), polyclonal rabbit prostate specific antigen (PSA) (clone# A0562), monoclonal mouse bcl-2 oncoprotein (clone# M0087) polyclonal rabbit anti-mouse HRP conjugated immunoglobulin (IgG) (clone# P0161), polyclonal goat anti-rabbit HRP conjugated IgG (clone# P0448) and polyclonal goat anti-mouse biotinylated IgG (clone# E0433), polyclonal goat anti-rabbit biotinylated IgG (clone# E0432) and streptavidin/HRP (clone# P0387)
- ***Nova-castra/Leica micro-systems (Northryde, New South Wales (NSW), Australia)*:** mouse monoclonal PR (NCL-L-PGR-AB clone# 1A6)
- ***Santa Cruz Biotechnology (CA, USA)*:** rabbit polyclonal AR (clone# N20), rabbit polyclonal FKBP51 (clone# H-100), rabbit polyclonal BEK1 (i.e. fibroblast

growth factor receptor 2 (FGFR2)) (clone# C-17), rabbit polyclonal hsp-90 (clone# H-114), rabbit polyclonal uridine diphosphoglucose dehydrogenase (UDP-GlcDH) (clone# H-300) and mouse monoclonal ERα (clone# M7047)

- ***Vector Laboratories (Burlingame, CA)*:** polyclonal horse anti-goat HRP conjugated IgG (Clone # BA-9500)

2.1.3 - Cell line

***American Type Culture Collection (Rockville, USA)*:**

- ***ZR-75-1 breast epithelial cancer cell line*:** Derived from a 54 yr old Caucasian female presenting with ductal carcinoma and pleural effusion. The epithelial cells were of ductal epithelium origin and positive for ERα, AR and PR.

2.1.4 - Hormones and AR antagonists

- ***AstraZeneca Pharmaceutical (Cheshire, UK)*:** Bicalutamide (Casodex)
- ***Sigma (St Louis, MO, USA)*:** 5α-dihydrotestosterone (5α-DHT), 17β-oestradiol (E_2), medroxyprogesterone acetate (MPA)

2.1.5 - Equipment

- ***Alpha Innotech Corporation (San Leandro, CA, USA)*:** AlphaImager 2200 gel documentation system
- ***Amersham Biosciences (Buckinghamshire, UK)*:** transfer tank for protein electrotransfer
- ***BioRad (Hercules, CA, USA)*:** Bio-Rad iCycler thermal cycler

- ***Clyde-Apac (Woodville, SA, AU)*:** biological safety cabinet
- ***Eppendorf (Hamburg, DE)*:** Eppendorf centrifuge 5415R and 5810, micro-centrifuges
- ***Leica (Hamburg, DE)*:** Microtome (model# RM2235)
- ***Hamamatsu (Hamamatsu City, Shizuoka, Japan (JP))*:** Nanozoomer virtual microscopy system (Nanozoomer 2.0-HT ultra high resolution slide scanner)
- ***Olympus America Inc (Centre Valley, Virginia, USA)*:** Olympus Bx50 microscope with Olympus under light microscopy (U-PMTVC) solid state camera
- ***Sakura Tissue-Tek VIP (Torrance, CA, USA)*:** Automated tissue processor and tissue embedder

2.1.6 - Software

- ***Alpha Innotech Corporation (San Leandro, CA, USA)*:** AlphaEase FC version 3.1.2
- ***Bio-Rad (Hercules, CA, USA)*:** Bio-Rad IQ5 optical system software version 1.0
- ***SlidePath (Ireland, UK)*:** Distiller Server software, NDP Viewer Software
- ***Leading Edge (Adelaide (ADL), SA, AU)*:** VideoPro 32 video image analysis software
- ***Hamamatsu (Hamamatsu City, Shizuoka, JP)*:** Nanozoomer digital pathology software
- ***Microsoft Office (Redmond, Washington (WA), USA)*:** Microsoft Word, Excel, PowerPoint

- ***Nanodrop Technologies (Wilmington, Delaware, USA)*:** NanoDrop (ND) 1000 3.3
- ***Olympus America Inc. (Melville, NY, USA)*:** DP Controller 1.2.1.108 and DP Manager version 1.2.1.107
- ***SPSS Inc. (Chicago, Illinois, USA)*:** SPSS statistical analysis software version 13

2.2 - Buffers and Solutions

All solutions were stored at room temperature unless otherwise stated:

***10% APS (ammonium persulphate)*:**

APS	1 g
Reverse osmosis (RO) water	10 ml
(stored at -20ºC)	

***Citrate buffer*:**

Citric acid	1.05 g
RO water	500 ml
pH (measure of acidity) to 6.5	

***DAB solution*:**

Tris buffer	105 ml
Isopac of DAB	100 mg

***Dextran coated charcoal in Tris/EDTA buffer*:**

0.5% charcoal	5 g
55 nM dextran	0.5 g
20% glycerol	100 ml

Made up to 1 L with Tris/EDTA buffer and allowed to mix overnight by rotation

***Freezing Mix*:**

DMSO	40%
FBS	40%
RPMI 1640 medium	20%

***Lysis buffer (for mycoplasma detection)*:**

1x TE	
Tween 20	0.5%
Noidet P40	0.5%
Chelex 100	0.25%

Immediately prior to use 0.4 mg/ml proteinase K was added

***6x protein loading dye*:**

4x Tris-Cl/SDS	7 ml
Glycerol	3 ml
SDS	1 g
Dithiothreitol (DTT)	0.93 g
Bromophenol blue	1.2 mg

***Radio-Immunoprecipitation Assay (RIPA) lysis buffer*:**

Tris (pH 7.4)	10 mM
Sodium Chloride (NaCl)	150 mM
EDTA	1 mM
Triton X-100	1%

A complete protease inhibitor cocktail pellet was dissolved into the lysis buffer prior to use.

***10x running buffer*:**

Tris	75.75 g
Glycine	360 g
SDS	25 g
RO water	2.5 L

***1x running buffer*:**

10x running buffer	250 ml
RO water	2.25 L

***20% SDS*:**

SDS	20 g
RO water	100 ml

***Separating gel (7.5%)*:**

RO water	4.3 ml
1 M Tris pH 8.8	3.75 ml
Acrylamide/ Bisulphate (Bis)	1.9 ml
20% SDS	50 µl
10% APS	50 µl
TEMED	5 µl

***Separating gel (12.5%)*:**

RO water	3.2 ml
1 M Tris pH 8.8	3.75 ml
Acrylamide/Bis	3 ml
20% SDS	50 µl
10% APS	50 µl
TEMED	5 µl

***Stacking gel*:**

RO water	3.7 ml
1 M Tris pH 8.8	625 µl
Acrylamide/Bis	500 µl
20% SDS	25 µl
10% APS	25 µl
TEMED	5 µl

***10x transfer buffer*:**

25nM Tris	75.75 g
Glycine	360 g
RO water	2.5 L
(stored at 4ºC)	

***1x transfer buffer*:**

Methanol	800 ml
10x Transfer buffer	400 ml
RO water	2.8 L
(stored at 4ºC)	

***1M Tris pH 8.8*:**

Tris	60.55 g
RO water	500 ml
pH to 8.8	

***1M Tris pH 6.8*:**

Tris	12.11g
RO water	100 ml
pH to 6.8	

***10x Tris buffered saline (TBS)*:**

0.5 M Tris	75.75 g
1.5 M NaCl	109.5 g
RO water	2.5 L
pH to 7.4	

***1x TBS*:**
10x TBS 250 ml
RO water 2.25 L
(stored at 4ºC)

***1x TBS + Tween 20 (TBST)*:**
10x TBS 250 ml
RO water 2.25 L
Tween 20 5 ml
(stored at 4ºC)

***4x Tris-Cl/SDS*:**
Tris/Cl 3.025 g
SDS 0.2 g
Made up Tris-Cl with RO water and pH to 6.8 prior to addition of SDS
Following addition of SDS made up to 50 ml with RO water

***Trypan blue*:**
0.01% trypan blue dissolved in sterile saline

2.3 - Methods

2.3.1 - Ethical approvals, breast surgery and transportation of breast tissue samples

In this study, human breast tissue was obtained with informed consent from pre- and post-menopausal women undergoing breast surgery for a prophylactic mastectomy, excision of benign breast disease, localised or invasive breast cancer at the Burnside Private Hospital (Adelaide, South Australia) under approval from the Human Ethics Committee of the University of Adelaide, South Australia (approval number: #H-065-2005). A non-malignant breast tissue sample was excised from breast tissue surrounding the tumour (either adjacent (peri-tumoural) or distal) to the tumour margin. The breast tissue excised adjacent to malignant disease was located at a minimum distance of 4 cm away from the malignant lesion. Breast tissue was also obtained from women undergoing partial or bilateral prophylactic mastectomy for high familial risk of developing breast

cancer. Histopathology assessment was performed by a pathologist specialising in breast pathology to verify non-pathological and pathological status of the breast tissue samples *(refer to Acknowledgements).* All women who participated in this study provided written consent prior to surgery.

2.3.2 - Patient characteristics

Patient clinical information was obtained from Burnside Hospital clinical reports following appropriate approval of ethical protocols. Clinical details relevant to this study included age, lesion pathology, excision location of breast tissue sample with respect to location of tumour, quadrant of the breast tissue where the benign or malignant lesion was located, type of breast surgery, age of menopause (where appropriate), history of hysterectomy or oophorectomy, HRT and contraceptive pill use and parity. Patient clinical information required for this study that was unavailable from the hospital clinical records was entered in tables within this thesis as not recorded (NR) or not applicable (NA).

In this study, non-malignant (histologically normal) breast tissue was obtained from twelve post-menopausal women of age ranged from 51-81 yrs (median 61 yrs) [Table 1 and 2]. All of the following patient information is included in Table 1 or 2. The reason for breast surgery differed among patients and included the following: non-malignant disease (i.e. prophylactic mastectomy (n=1)), benign disease (i.e. cysts, fibrocystic changes and ductal ectasia (n=4)) and malignant disease (i.e. partial or bilateral mastectomy and excision of malignant lesion (n=7)). Use of HRT also differed between patients and

included previous use of HRT (ERT only (n=3)) and no prior history of HRT use (n=9). Previous use of the contraceptive pill also differed between patients and included previous use of the contraceptive pill (n=5), no prior history of the contraceptive pill (n=4) and NR (n=3). The post-menopausal women included in this study had different parity status divided into parous (n=11) and NR (n=1). Prior hysterectomy (n=4) and/or oophorectomy (n=1), neither (n=5) and NR (n=3) was also recorded. The age of menopause ranged from 44-55 yrs (median age 48 yrs) (n=5), and NR (n=7).

Histologically non-malignant breast tissue was also obtained from six pre-menopausal women in this study [Table 3]. All the following patient information is described in Table 3. The women ranged in age from 28-47 yrs (median age 46 yrs). Of the pre-menopausal women represented within this study, breast surgery was performed for benign breast disease (n=3), prophylactic mastectomy (n=1) and malignant breast disease (n=2). Previous use of the contraceptive pill was only recorded in one patient. Parity status of the pre-menopausal women was as recorded as parous (n=3) and nulliparous (n=3) women.

A separate grouping of the breast tissues was undertaken with respect to the proximity (distal or adjacent) to benign or malignant breast disease. Of the post-menopausal breast tissue samples used in this study, the location with respect to the lesion was either adjacent to benign (n=5) or malignant disease (n=5) and distal to malignant breast disease (n=2) [Table 1]. Similarly, the following was recorded for the pre-menopausal breast tissue samples either adjacent to benign (n=4) or malignant disease (n=1), and distal to

Table 1: Post-menopausal patient characteristics

Patient #	Age	Quadrant	Breast Surgery and Pathology Details	Location of Non-Malignant Breast Tissue Samples to Malignant Disease
#1 †	58	NR	BRCA1 positive, prophylactic mastectomy	NA
#2	60	Left nipple	No malignancy. Ductal ectasia	NA
#3	54	L,U,O	Fibrocystic changes with apocrine lined cysts	NA
#4	68	LL,C	Grade III infiltrating ductal ER/PR negative malignancy. Partial mastectomy and sentinel node biopsy	Adjacent
#5	81	R,L,I	Ductal carcinoma in situ of macro papillary and cribriform type, intermediate nuclear grade. No evidence of invasive malignancy. Partial mastectomy and sentinel node biopsy	Adjacent
#6	63	R,U,O	Cyst only with haemorrhage and inflammation	NA
#7	57	Left nipple	Duct ectasia and fibrocystic change	NA
#8	73	R,L,C (nipple)	Infiltrating carcinoma of no special type. Mastectomy	Distal
#9	62	R,U,C	Bilateral mastectomy: Right: Focal duct carcinoma in situ. Left: Prophylactic (fibrocystic change)	Distal
#10	72	R,U,I	Infiltrating carcinoma no special type with lobular involvement. Right breast resection, sentinel node biopsy and axillary fat	Adjacent
#11	51	L,U,O	Infiltrating carcinoma no special type. Partial mastectomy and axillary dissection	Adjacent
#12	54	R,L,I	Partial mastectomy and sentinel node biopsy. Tubular carcinoma	Adjacent

† = BRCA mutated

Abbreviations: L = Left or Lower, **U** = Upper, **O** = Outer, **LL** = Lower Left, **R** = Right, **I** = Inner, **C =** Centre**, N** = No, **Y** = Yes

NR = Not recorded

NA = Not applicable

Table 2 (continued): Post-menopausal patient characteristics

Patient #	Age of Menopause	Hysterectomy	Oophorectomy	HRT/Duration	Contraceptive Pill (CP)/Duration	Parity
#1 †	NR	NR	NR	N	NR	2
#2	NR	NR	NR	N	NR	2
#3	47	Y	N	N	CP/10 yrs	2
#4	48	N	N	N	CP/4-5 yrs	1
#5	44	N	N	N	N	1
#6	55	Y	N	Y (ERT)	CP	2
#7	NR	Y	N	Y (Tibolone, Vaginal cream and ERT)	CP	3
#8	NR	Y	Y	Y (ERT)	N	2
#9	NR	NR	NR	N	NR	NR
#10	50	N	N	N	N	2
#11	NR	N	N	N	N	3
#12	NR	N	N	N	CP (stopped taking in 1990)	3

Table 3: Pre-menopausal patient characteristics

Patient #	Age	Quadrant	Breast Surgery and Pathology Details	Location of Non-Malignant Breast Tissue Samples to Malignant Disease	Contraceptive Pill (CP)/Duration	Parity
#1	45	R,I,C	Benign fibroadenoma surgery	NA	CP/10 yrs	2
#2	30	NR	Benign disease	NA	NR	0
#3	47	L,O,M	Lobular carcinoma grade 2. 2/5 lymph nodes involved. Had a mastectomy and axillary clearance ER/PR positive. HER2 negative (RE only +1)	Distal	NR	4
#4	36	R,U,I Nipple	Benign fibroadenoma surgery	NA	N	0
#5	49	L,L,I	Infiltrating ductal carcinoma, no special type. Medial partial mastectomy and sentinel node biopsy	Adjacent	NR	2
#6	47	NR	Prophylactic bilateral mastectomy - no cancer (significant family history)	NA	NR	0

Abbreviations: L = Left, **U** = Upper, **O** = Outer, **LL** = Lower Left, **R** = Right, **I** = Inner, **C =** Centre**, M** = Middle, **N** = No, **Y** = Yes

NR = Not recorded

NA = Not applicable

malignant disease (n=1) [Table 3]. The non-malignant post-menopausal breast tissue samples obtained from patients diagnosed with malignancy (n=7) used in this study were either infiltrating carcinoma (n=4) (one patient had prior history of ERT use), DCIS (n=2) or tubular carcinoma (n=1) (a rare pathological type of breast cancer) [Table 1]. In comparison, the non-malignant pre-menopausal breast tissue samples obtained from women diagnosed with malignancy (n=2) were either lobular carcinoma or infiltrating ductal carcinoma [Table 3].

2.3.3 - Transportation and preparation of breast tissue samples for culture

Breast tissue was transported to the laboratory in sterile containers on ice and cultured within 1-2 hrs following breast surgery. The breast tissue was prepared for culture by placing specimens into 10 cm sterile petri dishes containing 5 ml of phenol-red free (PRF)-RPMI media supplemented with 10 µg/ml insulin, 10 µg/ml hydrocortisone, 100x anti-mycotic antibiotic (5 ml of stock solution per 500 ml of media) and 100 nM L-glutamine (5 ml of stock solution per 500 ml of media), which aided in washing the excess blood from the samples. Dissection of the breast tissue samples and excess adipose tissue was removed using sterile tweezers, forceps and scalpel. The remaining white fibrous connective tissue, which is comprised predominantly of the glandular tissue structures (i.e. breast epithelial cells in both breast lobules and ducts), was subsequently diced into small pieces (i.e. 3 mm^3) used for culture.

2.3.4 - Ex vivo breast explant tissue culture method

In this study human pre- (n=6) and post-menopausal (n=12) non-malignant breast explant tissue was used to further develop an *ex vivo* culture model with modifications of a method originally developed in 1959 (Trowell 1959; Zhuang, Saaristo et al. 2003). The patient numbers used in this study were based on similar previous published literature using similar *ex vivo* breast explant tissue culture models to study the effects of endogenous and exogenous hormones on aspects of breast tissue biology (Zhuang, Saaristo et al. 2003; Eigeliene, Harkonen et al. 2006; Eigeliene, Harkonen et al. 2008). In accordance with a recent study that only included only three breast tissue samples per treatment group and reached statistical significance in various comparisons between hormone treatments (Eigeliene, Harkonen et al. 2008), the patient numbers used in this study are relatively high in comparison. Culturing of breast tissue samples was undertaken following dissection into (3 mm^3) pieces and placing 2-3 pieces of tissue onto pre-soaked gelatine sponges in 24-well tissue culture plates [Figure 2.1]. Pre-soaked gelatine sponges were placed into individual wells of 24-well culture dishes containing 500 μl of PRF-RPMI breast explant tissue culture media supplementated with 10% steroid-deplete dextran-coated charcoal-stripped foetal bovine serum (DCC-FCS) in a 5% CO_2 enriched atmosphere at 37ºC for 15-30 min was undertaken prior to the addition breast tissue samples.

A sample of non-cultured, non-malignant breast tissue adjacent to that used for culture was collected for analysis of steroid receptor expression (AR, ERα and PR) prior to culture. The remaining breast tissue was used in culture by an initial pre-treatment period

Diagrammatic Representation of the *ex vivo* Breast Explant Tissue Culture Model

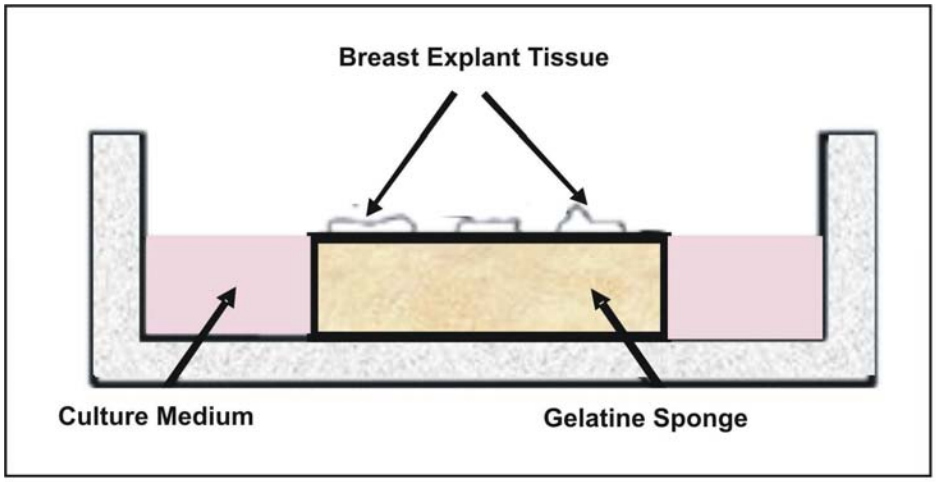


Figure 2.1: Human *ex vivo* breast explant culture model. Human female breast tissue was obtained from pre- (n=6) or post-menopausal (n=12) women undergoing surgery for benign or malignant disease. The breast tissue samples dissected into 3 mm^3 pieces were placed on pre-soaked 1 cm^3 gelatine sponges (2-3 pieces per sponge) in 24-well plates and pre-treated in phenol red free (PRF)-RPMI 1640 media supplemented with 10% dextran-charcoal stripped-foetal calf serum (DCC-FCS) for 36 hrs. The medium was aspirated and replaced with PRF-RPMI-1640 media supplemented with 10% DCC-FCS and cultured under the following conditions: i.e. 0.1% ethanol, 1 nM DHT, 1 nM MPA, 1 nM DHT plus 1 nM MPA, 1 μM bicalutamide (Bic) or 1 nM DHT plus 1 μM Bic for 24 and 48 hrs. The breast tissues are cultured in incubators in a 5% CO_2 enriched atmosphere at 37ºC.

for 36 hrs in steroid-deplete media, prior to the addition of hormone treatments. After this pre-treatment period, the media was removed and the tissue was cultured for 24 or 48 hrs in fresh media containing the following treatments: vehicle control (0.1% ethanol); 1 nM DHT; 1 nM MPA and/or 1 μM bicalutamide (Bic) (an AR antagonist). Both the non-cultured and cultured breast tissue samples were inserted into plastic embedding cassettes fixed in 4% formalin for 8-16 hrs. Tissues were subsequently impregnated with paraffin wax using an automated tissue processor and embedded in additional paraffin wax to form a paraffin block.

The time-points used in this study were based on several similar previously published papers that identified hormone responses on steroid receptor expression using similar *ex vivo* breast explant tissue models following addition of endogenous and exogenous hormones (i.e. E_2 and MPA) at both 24 and 48 hrs (Zhuang, Saaristo et al. 2003; Eigeliene, Harkonen et al. 2008). Furthermore, some preliminary experiments using longer time-points of 7 days were undertaken as a part of this project in the DRMCRL, to implement future studies for longer-term culture studies *(data not included in this thesis).*

The dose of MPA chosen for the experimental studies in this thesis was based on concentrations of MPA (i.e. 1 nM) that correlate more with serum levels in women taking cHRT (Svensson, Johnson et al. 1994), compared to other similar studies that have used higher doses of MPA (i.e. 10, 100 and 1000 nM) (Zhuang, Saaristo et al. 2003; Eigeliene, Harkonen et al. 2006; Eigeliene, Harkonen et al. 2008). Additionally, the dose of DHT (i.e. 1 nM), was used in studies in this thesis to obtain comparable dose effects on AR-

signalling by the endogenous hormone, compared to MPA, as per doses used *ex vivo* using human breast cancer cell lines sufficient to detect a change in breast epithelial proliferation mediated via AR-signalling (Birrell, Bentel et al. 1995). The AR antagonist Bic was used at a thousand fold excess (i.e. 1 μM) in this thesis to allow for abrogation of DHT-induced AR-mediated effects. High doses of 100 nM MPA were also included in separate experiments in the presence and absence of 1 nM DHT to investigate potential differences between low and high doses of MPA on AR-signalling in human breast tissue *(majority of data not included in this thesis).* A diagrammatic representation of the experimental protocol designed for studies performed in this thesis is depicted in Figure 2.2.

2.3.5 - Haematoxylin and eosin staining

Haematoxylin and eosin (H&E) staining was completed on all human breast tissue samples before and after culture. Paraffin-embedded human breast tissues were cut at 2 μm in thickness using a microtome and placed in a water-bath (30-40ºC) for 1-2 min to un-crease small folds in the paraffin. The tissue sections were then placed onto ultra superfrost glass slides and left to air-dry for 2-3 min before transferring to a heating platform (50-60ºC) for 1-2 hrs to melt the paraffin wax and assist in adherence of the tissue sections to the glass slides. The tissues were de-waxed in 3 x 5 min washes of xylene, and dehydrated in 3 x 5 min washes of 100% ethanol. Tissue sections were then washed for 5 min in running tap water and placed in diluted (1/10) Lillie Mayer's haematoxylin for 10 min. Excess haematoxylin was removed from the tissues by several dips in acid-alcohol, and subsequently rinsed in running tap water for 5 min. To counter

Diagrammatic Representation of *ex vivo* Breast Expant Tissue Experimental Protocol

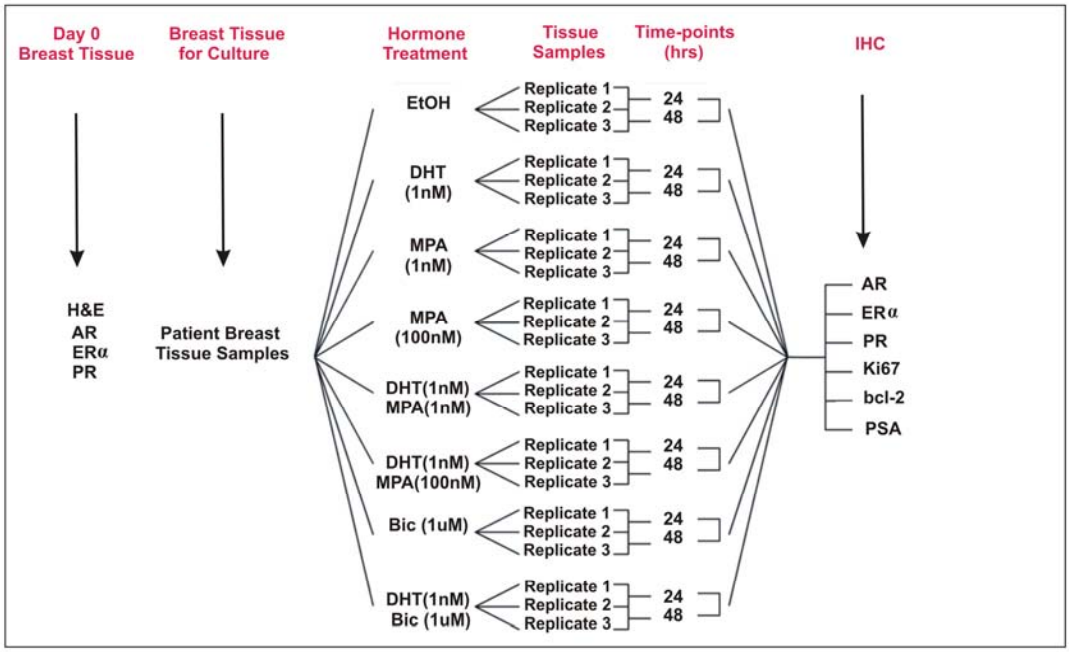


Figure 2.2: *Ex vivo* breast explant tissue culture experimental protocol. The diagram depicts the various stages of the experimental plan using the *ex vivo* breast explant tissue experimental model. The day of collection of non-cultured breast tissue samples are designated Day 0, where a 4% formalin fixed paraffin embedded piece of tissue was used for H&E staining and immunostaining with antibodies for AR, ERα and PR to determine relative levels prior to culture. The remaining breast tissue sample was used for culturing (as described in Figure 2.1). Following culturing of the breast tissue samples for 24 and 48 hrs, immunohistochemistry was preformed on 4% formalin fixed paraffin embedded breast tissue samples using the following antibodies: AR, ERα, PR, Ki67, PSA and bcl-2. *(Majority of data not included in this thesis for breast explant tissue samples treated with 100 nM MPA alone and in combination with 1 nM DHT).*

stain the tissue sections, slides were submerged in tap water for 1-2 min, rinsed in tap water and then immersed in concentrated eosin for 10 sec and rinsed in running tap water for 5 min. The tissue sections were dehydrated in 3 x 100% ethanol washes (10 dips) and cleared in 3 x xylene washes (10 dips). The tissue sections were then mounted using glass cover slips (2 x 2 cm) using DPX mounting medium and left to dry overnight before visualisation by light microscope.

2.3.6 - Immunohistochemistry

Immunohistochemistry (IHC) was performed by citrate buffer antigen retrieval to detect proteins using the following antibodies and dilutions from stocks: AR (1:1000); PR (1:400); ERα (1:400); bcl-2 (1:400) and Ki67 (1:400). IHC was also performed for PSA (1:400), which does not require heat-induced antigen retrieval. Prior to completion of IHC on the non-cultured and cultured breast tissue samples, optimisation experiments were undertaken for all antibodies used in IHC experiments in this thesis. These were performed by using serial dilutions from the starting dilution specifications suggested by the pharmaceutical company for the antibodies on three different non-cultured breast tissue samples to determine the most appropriate dilution for IHC studies. Within all IHC studies performed in this thesis, internal control tissue samples were included as follows: a negative control (i.e. no addition of primary antibody) and a positive control (i.e. a breast tissue sample shown to be positive for protein by the primary antibody). All primary antibodies used in this thesis have previously been used in published studies. Additionally, the majority of antibodies used in this thesis have a peptide that can be purchased from the pharmaceutical company which can be used to verify specificity of

the antibody for the protein. Peptide blocking studies for the AR antibody used in this thesis have been shown in other research projects in the DRMCRL to possess specificity for the AR and protein *(data not included in this thesis).*

The following protocol was utilised for detection of these antigens by IHC. Serial tissue sections were cut at 2 μm using a microtome, mounted on Superfrost Plus slides and left to adhere on a heating platform for 2 hrs prior to IHC staining. Tissue sections were dewaxed in xylene 3 x 5 min, cleared by dipping 10 x in 3 separate pots of fresh 100% ethanol and left in running RO water for 30 min. Subsequently, the sections were washed 2 x 5 min in 1 x PBS, endogenous peroxide activity was blocked using by using 0.3% hydrogen peroxide in 1 x PBS for 15 min with lack of exposure to light. Prior to antigen retrieval if required, the sections were washed 2 x 5 min in 1 x PBS, followed by microwave antigen retrieval in 1M citrate buffer (pH 6.5) for 15 min at boiling temperature. The sections were cooled at room temperature for 30 min, followed by 2 x 5 min washes in 1 x PBS. Blocking of tissue sections was performed by adding 5% of goat serum diluted in 1 x PBS from the species the secondary biotinylated antibody was raised in for 30 min at room temperature in a humid chamber. The blocking solution was tapped off the sections and the primary antibody was hybridised to tissue sections at the appropriate dilution in blocking serum overnight at 4ºC.

The following day, the sections were washed 2 x 5 min in 1 x PBS, and the secondary biotinylated antibody was added in blocking solution at a dilution of 1:400 for 60 min at 4ºC. A wash step of 2 x 5 min washes in 1 x PBS was again performed, and the tissues

were incubated in streptavidin-HRP complex diluted in 1 x PBS for 60 min at 4ºC. All hybridisation steps were completed in a humid chamber, to prevent the sections from drying out during the incubation period. The sections were again washed for 2 x 5 min in 1 x PBS, before the addition of a 1:1 mixture of 1mg/ml DAB and 0.1% hydrogen peroxide for 6 min at 4ºC, in a fume hood, for detection of the biotinylated secondary antibody. To remove excess solution, the sections were washed in running tap water for 2 min. Counterstaining of the sections was performed by incubation in a 1/10 dilution of 1/10 Lillie Mayer's haematoxylin for 25 sec. Again, the sections were washed in tap water for 2 min to remove excess counterstain. Lastly, the sections were dehydrated in fresh 100% ethanol and xylene, by 10 dips in each and mounted on glass coverslips, adhered with DPX.

2.3.7 - Video image analysis

Percent positivity and intensity of positive immunostaining within breast epithelial cells was quantified using an automated colour video image analysis (VIA) system, as previously described (Tilley, Lim-Tio et al. 1994). Glandular tissue areas were captured per individual breast tissue sample (i.e. luminal and myoepithelial breast cells, contained within both acinar and ductal breast tissue structures). Prior to quantification of immunoreactivity, the stromal areas of the captured breast tissue images were edited from the image to allow for measurement of the glandular breast epithelial cellular areas only [Figure 2.3]. Following histopathology assessment of the breast explant tissues, any areas of abnormal appearance were eliminated from the VIA quantification analysis [Figure 2.3]. An intensity threshold was set prior to the VIA to allow for specific detection of

Diagrammatic Representation of Video Image Analysis (VIA)

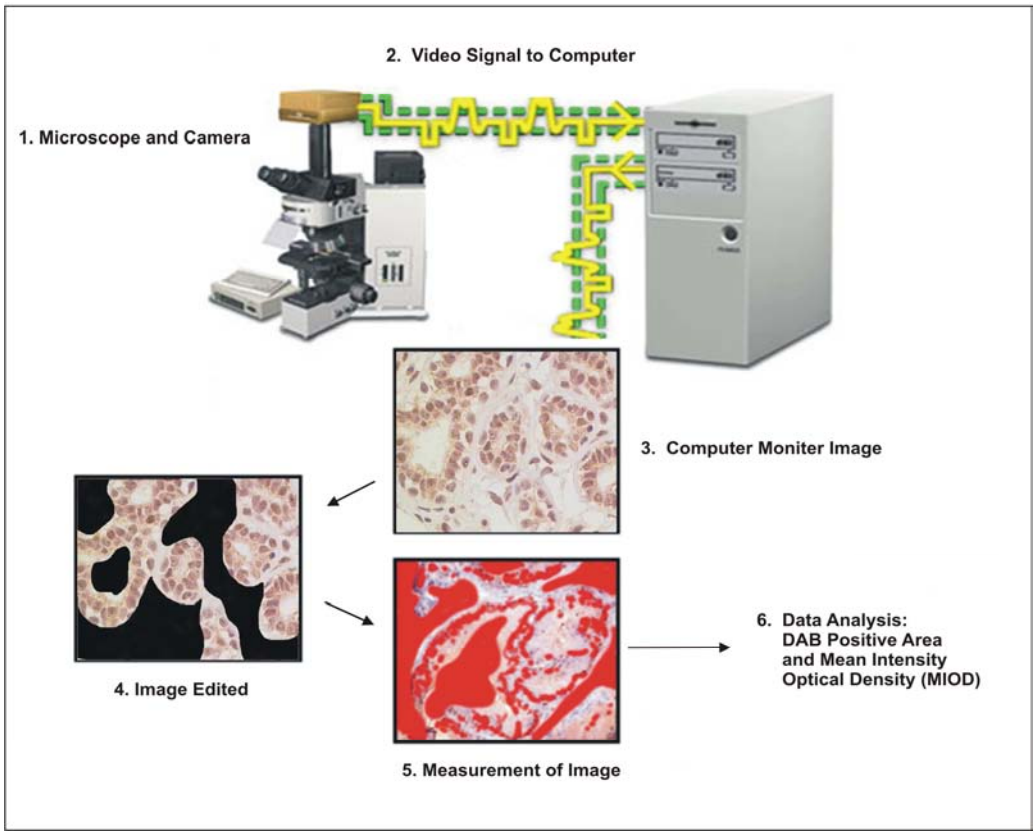


Figure 2.3: Quantification of immunostaining in breast explant tissue using VIA. Immunostaining of Day 0 and cultured pre- or post-menopausal breast explant tissue was quantified using VIA. VIA quantification entails the following: (1) capturing immunostained images using a microscope attached to a camera; (2) transfer of captured images to a computer; (3) visualisation of the images on a computer monitor; (4) editing the images using VIA analysis software and (5) measuring the edited images using VIA analysis software including the quantification parameters DAB positive area (% positivity) and mean integrated optical density (MIOD).

positively immunostained areas in the breast epithelial cells. The various output parameters which were obtained by VIA that were used in subsequent analysis included the following: screen and total area; total DAB stained area; integrated optical density (IOD); DAB density; % total area and % total DAB stained area. These output parameters were used to calculate the measurement of immunoreactivity for all antigens using the VIA quantitative measurements including: 1) mean integrated optical density (MIOD) (i.e. derived from the following formula, IOD/total area x 100), which depicts the intensity of DAB staining in the glandular tissue and 2) positive area of DAB immunostaining (% positivity) (i.e. derived from the following formula: DAB area/total area x 100), which reflects the amount of positive DAB staining in the glandular tissue irrespective of DAB intensity levels.

2.3.8 - Distiller Server software analysis

One limitation of VIA is the inability to perform qualitative and quantitative assessment of immunohistochemical staining within a large number of slides in an expedient manner. A new high-throughput automated slide scanner was trialled as a tool to overcome this limitation in this thesis. This instrument, a Nanozoomer 2.0-HT slide scanner (Hamamatsu), captures ultra-high resolution images of 210 glass slides automatically in a single batch. These images are analysed using Distiller Server software to quantify immunostaining. The Nanozoomer virtual microscopy system possesses a number of advantages over traditional image analysis using VIA with respect to analysis, including increased speed in completion of image analysis, objectivity and accuracy of measurement in regards to the intensity of immunoreactive areas within the tissues and

measurement of the number of positive individual epithelial cells. This process is described in the following sub-sections 2.3.8.1 and 2.3.8.2.

2.3.8.1 - Construction of a nuclear algorithm

Different algorithms can be developed using the Distiller Server software for image analysis that are specifically designed for the analysis of membranes, nuclei or positive pixels (i.e. detection of both cytoplasmic and nuclear staining). An algorithm to measure Ki67 and AR positive nuclear immunostaining in a subset of cultured breast explant tissue samples was designed using parameters aimed at eliminating the detection of stromal cells. These parameters were based on the differentiation of round epithelial cells and long elongated stromal cells. However, since the parameters used for construction of the nuclear algorithm were unlikely to remove all stromal cells from the analysis, additional editing was applied to the tissue sections prior to image analysis by placing annotated boxes included in the Distiller Software around the epithelial cells within the whole tissue section, to eliminate stromal areas surrounding the glandular epithelial cells. Other input parameters applied to the nuclear algorithm included: nuclear area low and high threshold (range from 0 - 1000, which represent the area of the nucleus in pixels); nuclear density low and high threshold (range from 0 - 100, low to high density); % of stained pixels in a nucleus cut-off (range 0 - 100); strong/moderate staining intensity cut-off (range 0 - 255, zero to maximum intensity); moderate/weak staining intensity cut-off (range 0 - 255 zero to maximum intensity); type of staining (1 = immunohistochemisty and 2 = haematoxylin and eosin), and strength of staining (range 1 - 10, contrast level

between the nuclei and remaining tissue). A standard colour definition file to detect DAB staining included in the Distiller software was applied to the nuclear algorithm.

2.3.8.2 - Submitting images for high throughput image analysis

After creation of the nuclear algorithms and application of a colour definition file to detect DAB staining, high throughput image analysis on a subset of cultured breast explant tissues was performed. A number of output parameters are obtained following application of the nuclear algorithm to the tissue images that included the % accepted nuclei and percentages within each staining intensity category (i.e. weak, moderate and strong nuclei). A histoscore was calculated that represents the intensity of immunostaining within all breast epithelial cells of cultured tissue samples for the measurement of AR immunostaining using the following formula: histoscore = (% weak x 1) + (% moderate x 2) + (% strong x 3). For the purpose of the measurement of Ki67 % positivity, in the cultured breast explant tissues, the total % accepted nuclei which included weak, moderate and strong was used in subsequent analysis described in the following section.

2.3.9 - Comparison of VIA and Distiller Server Software analysis

At the commencement of this project, the Distiller Software was being implemented in the Hanson Institute and DRMCRL for image analysis. Due to a number of technical problems during the course of the implementation of both the Distiller Server and Software for image analysis, only a subset of post-menopausal breast explant tissue samples cultured as previously described under control conditions (i.e. ethanol), or

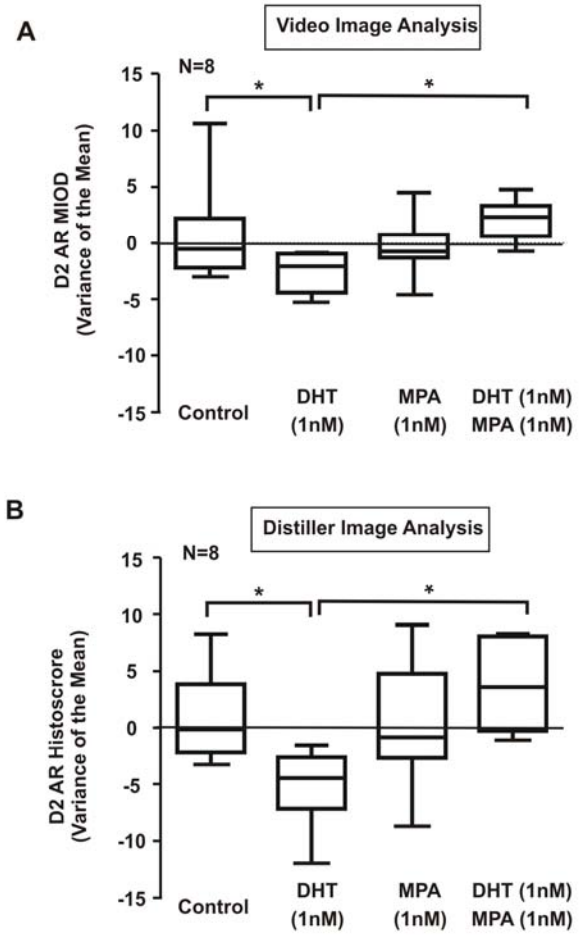


Figure 2.4 (A-B): Comparison of Ki67 immunostaining quantification by VIA or by Distiller Server Software. VIA quantification was compared with similar quantification analysis preformed using Distiller Server Software analysis on post-menopausal breast explant tissues (n=8) cultured for 48 hrs (D2). Breast explant tissues were cultured in 0.1% ethanol (control), or treated with 1 nM DHT or 1 nM MPA alone and combined. Quantification of Ki67 immunostaining was performed on breast explant tissues using Ki67 % positivity measurements for **(A)** VIA and **(B)** Distiller Server Analysis. Statistical analysis performed using Wilcoxon matched pairs test, *$p \leq 0.05$. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value.

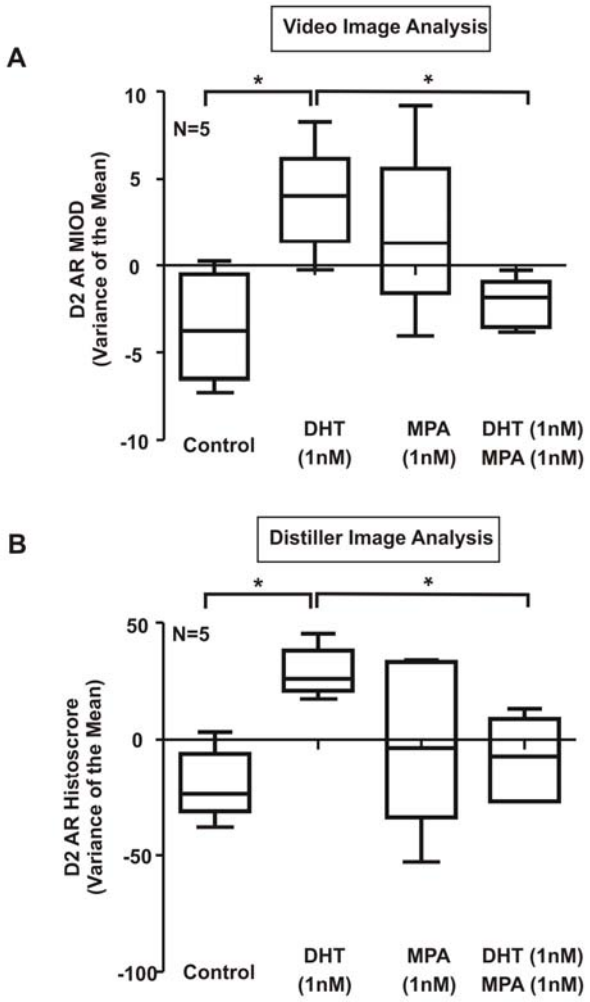


Figure 2.5 (A-B): Comparison of AR immunostaining quantification by VIA or by Distiller Server Software. VIA quantification was compared with similar quantification analysis preformed using Distiller Server Software analysis on cultured post-menopausal breast explant tissues (n=8) for 48 hrs (D2). Breast explant tissues were cultured in 0.1% ethanol (control), or treated with 1 nM DHT or 1 nM MPA alone and combined. Quantification of AR immunostaining was performed on breast explant tissues using AR % positivity measurements for **(A)** VIA and **(B)** Distiller Server Software. Statistical analysis performed using Wilcoxon matched pairs test, *$p \leq 0.05$. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value

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treated with 1 nM DHT or 1 nM MPA alone and combined cultured in hormone treatments previously described for 48 hrs were used in the quantification of Ki67 (n=8) and AR (n=5) immunostaining. A comparative analysis of VIA compared to the Distiller Software was subsequently performed. Both forms of image analysis resulted in similar measurements for both Ki67 (n=8) [Figure 2.4A and B] and AR (n=5) [Figure 2.5A and B] in the cultured tissue samples.

The quantification results of the Ki67 immunostaining presented with less variation in the spread of the data, for the VIA analysis compared to the Distiller Software analysis [Figure 2.4A and B]. The basis for the differences in the range of the quantitative measurements between the two types of analysis may be associated with the nature of the positivity measurements and analysis used in VIA compared to Distiller Software. Distiller Software analysis is performed on heavily annotated tissue samples which effectively leads to a greater number of individual measurements, compared to VIA which does not require as many annotations due to the difference in editing tools. As a result, this may lead to a greater spread in the variation in the data using Distiller Software analysis compared to VIA. Despite these differences, the effect of the hormone treatments on either AR or Ki67 expression had similar statistical significance using either VIA or Distiller Software analysis.

2.3.10 - Statistical analysis

A Wilcoxon-signed rank test (a non-parametric statistical test to compare two related samples) was used to determine statistical differences between % positivity or MIOD

VIA measurements in hormone treated breast explant tissue samples for 24 and 48 hrs following IHC for AR, ERα, PR, bcl-2 and PSA using SPSS statistical analysis software version 13. A separate analysis was undertaken using a Wlicoxon-signed rank test to determine statistical differences in hormone treated breast explant tissues that were either non-associated with malignancy (i.e. either distal to malignant or adjacent to benign breast tissue), or adjacent to malignancy at 48 hrs following IHC for proteins described above using SPSS statistical analysis software version 13. To account for biological variation between hormone treatments in each individual patient a correction for the variance of the mean among all hormone-treated samples per patient was performed. This correction was undertaken by calculating the average of all hormone treated samples (i.e. vehicle control (0.1% ethanol), 1 nM DHT, 1 nM MPA, 1 μM Bic alone or in combinations), per patient at each time-point (i.e. 24 and 48 hrs) and the variation of each hormone treated sample from the overall mean was calculated. The standard error of the mean (SEM) was further calculated on these variances between individual patient treatment samples. The data was depicted using box and whisker plots, which represent the maximum and minimum values, the upper and lower quartiles and the median value, using GraphPad Prism version 13.0. Inclusion of the VIA quantification raw data for both % positivity and MIOD (i.e. median, average +/- SEM) for Ki67, bcl-2, AR, PR and ERα expression for all hormone treatments (i.e. vehicle control (0.1% EtOH), DHT 1nM, MPA 1nM, DHT 1nM + MPA 1nM, Bic 1 μM and DHT (1nM + Bic 1 μM) is referenced in Appendix 2 of this thesis. A comparison of Ki67 % positivity between pre- and post-menopausal cultured breast tissue samples at 48 hrs was undertaken by a Mann Whitney U statistical test (a non-parametric test to compare two-independent samples), using

SPSS statistical analysis software version 13. Statistical significance was accepted in all statistical analyses at $p>0.05$. Power calculations to validate adequate sample sizes in reaching statistical significance was additionally performed and commented on in some sections of this thesis when statistical significance was not reached *(http://www.dssrearch.com/ toolkit/spcalc/power_a2.asp).* A 5% Ki67 positivity cut-off point was applied to some data for comparative analysis between pre- and post-menopausal base-line levels in cultured breast explant tissue samples, (i.e. >5% and ≤5% Ki67). Statistical significance for all tests was accepted at $p\leq0.05$.

2.3.11 - Maintenance of cell line

The cell line used in this study was maintained in RPMI medium 1640 supplemented with 10% FCS (ZR-75-1), in addition to penicillin and streptomycin, in incubators at 37ºC in a 5% CO_2 enriched atmosphere.

2.3.11.1 - Charcoal stripping of foetal calf serum

FCS was thawed in a 37 ºC water bath overnight. A solution of dextran-coated charcoal (DCC) was centrifuged for 30 min at 4,000 rpm and the supernatant aspirated off the charcoal pellet. Endogenous steroid was stripped from the FCS by adding 50 ml FCS to the charcoal pellet and rotating the FCS for 2 hrs at room temperature. The FCS and charcoal solution was then centrifuged at 4000 rpm for 30 min and the supernatant transferred to a fresh tube, to remove the charcoal pellet, and the incubation and centrifugation was repeated. The charcoal-stripped FCS solution was filter-sterilised and stored at -20ºC. The steroid content of the media following charcoal-stripping is performed routinely at the Institute of Medical and Veterinary Science (IMVS).

2.3.12 - Preparation of steroid stocks and steroid hormone antagonists

The steroid hormones DHT, MPA, E_2 and the AR antagonist Bic were dissolved in 100% ethanol to a concentration of 10^{-2} M (DHT and E_2) and 10^{-3} M (MPA and Bic) and stored in glass vials at -20ºC.

Chapter 3

Combined hormone replacement therapy pharmaceuticals containing MPA and their association with breast cancer incidence in Australian women

3.1 - Introduction

The main focus of this chapter was to determine whether an association between breast cancer incidence, and the use cHRT preparations containing MPA (prescribed in individual prescriptions (i.e. Premarin and Provera) or as a single prescription (i.e. Premia) was evident in Australian women. Studies were undertaken using data obtained from publically available databases consisting of breast cancer incidence and cHRT prescription rates in Australian women that were statistically analysed by Poisson and Binomial analysis to determine the changes and association of the two factors either before or after the WHI clinical trial findings were reported in 2002 (Rossouw, Anderson et al. 2002).

The Women's Health Initiative (WHI) clinical trial was the first randomised, double blind, placebo-controlled disease prevention trial funded by the USA National Institutes of Health (NIH), to determine the effect of daily use of cHRT (comprised of a combination of 0.625 mg conjugated-equine oestrogen (CEE) and 2.5 mg of the synthetic progestin MPA, compared to a placebo control on coronary heart disease in 16,608 enrolled post-menopausal women aged between 50-79 yrs over a period of 8.5 yrs (Rossouw, Anderson et al. 2002). An ERT parallel arm of the WHI trial was also undertaken to investigate the effects of ERT on coronary heart disease in 10,739 post-menopausal women aged 50-79 yrs, with prior hysterectomy, compared to women taking a placebo (Anderson, Limacher et al. 2004). The release of the WHI trial cHRT arm findings were published in 2002 (Rossouw, Anderson et al. 2002), whereas the parallel

ERT arm was published at a latter date in 2004 (Anderson, Limacher et al. 2004).

The cHRT arm of the WHI trial was stopped early after 5.2 yrs, due to the identification of an increased risk of invasive breast cancer (Hazard Ratio (HR), 1.26; 95% Confidence Interval (CI), 1.00-1.59), compared to women who received a placebo (Rossouw, Anderson et al. 2002). Recent re-analysis of the WHI trial has identified an even greater risk of breast cancer in adherent women taking cHRT prior to cessation of the trial (HR, 1.62; 95% CI, 1.10 to 2.39), followed by a rapid decline subsequent to cessation of the trial, compared to adherent and non-adherent women combined (HR, 1.26; 95% CI, 0.73-2.20) (Chlebowski, Kuller et al. 2009). Surprisingly, the ERT only parallel arm of the WHI trial which ended after approximately 6.8 yrs, revealed a 23% reduction in the risk of invasive breast cancer (HR, 0.77; 95% CI, 0.57-1.01) (Anderson, Limacher et al. 2004). Subsequent, re-analysis of the WHI ERT only trial, demonstrates that there is no significant change in breast cancer risk in post-menopausal women taking ERT once corrected for the initiation time of ERT following menopause (Prentice, Chlebowski et al. 2008). Moreover, there are a plethora of studies published subsequent to the WHI trial supporting the notion that post-menopausal women taking cHRT have an increased risk of invasive breast cancer compared to post-menopausal women taking ERT, never users and placebo groups (Magnusson, Baron et al. 1999; Ross, Paganini-Hill et al. 2000; Schairer, Lubin et al. 2000; Beral, Banks et al. 2002; Li, Malone et al. 2003). Specifically, these findings emphasise that the increased risk of invasive breast cancer observed in post-menopausal women taking cHRT in the WHI trial is associated with the addition of MPA. Although other synthetic progestins are included in cHRT, MPA is the

most commonly used in cHRT, particularly in the USA (Hersh, Stefanick et al. 2004).

An association between cHRT use and breast cancer risk has been further substantiated using databases that have compiled cHRT prescription rates and invasive breast cancer incidence rates in the female population of the USA and Australia (Haas, Kaplan et al. 2004; Hersh, Stefanick et al. 2004; Clarke and Glaser 2007; Glass, Lacey et al. 2007; Hausauer, Keegan et al. 2007; Kerlikowske, Miglioretti et al. 2007; Ravdin, Cronin et al. 2007; Canfell, Banks et al. 2008). These retrospective-based studies have provided additional support to the epidemiological findings published in the WHI trial indicating a relationship between the use of cHRT in post-menopausal women and increased breast cancer incidence. To date, the changes in cHRT prescription rates in the Australian population have only been observationally reported, and speculated to be associated with direct changes to breast cancer incidence (MacLennan, Wilson et al. 2002; Canfell, Banks et al. 2008; Main and Robinson 2008). Moreover, no study in Australia has investigated the statistical prescription rate changes of cHRT preparations containing MPA both as a direct comparison before and after cessation of the cHRT arm of the WHI trial in 2002 and determined whether a direct association with cHRT use and breast cancer exists. Hence, the hypothesis of the studies described in this chapter, that the use of cHRT preparations containing MPA is associated with increased breast cancer incidence rates in Australian women.

The intention of the studies described in this chapter was therefore to investigate the public health associated risk of breast cancer in Australian women specifically with use

of MPA in cHRT, based upon a similar study published in the USA (Robbins and Clarke 2007) and to further investigate findings reported in an Australian epidemiological study published in 2008 (Canfell, Banks et al. 2008). Moreover, this chapter was aimed at emphasising the public health related concerns associated with MPA use in cHRT and breast cancer development. The epidemiological based studies described in this chapter have provided a foundation for additional molecular based studies undertaken in this thesis aimed at investigating a mechanistic basis associated with MPA and breast cancer development.

3.2 - Methods

3.2.1 - Software

- ***SAS Software (Institute Inc, Cary, NC, USA):*** SAS Version 9.2 for statistical analyses

3.2.2 - Australian female population and age-standardised breast cancer incidence

The incidence of age-standardised breast cancer for the Australian female population was obtained from the Australian Institute of Health and Welfare (AIHW) *(http://aihw.gov.au/)*, which produce the publically-available database included in the Australian Cancer Incidence and Mortality (ACIM) book *(http://aihw.gov.au/search/?q=Breast+Cancer+ACIM+book*). This information was separated into 5 yr age-groups and tabulated for Australian females aged between 1 to 85+ yrs. For the purpose of this study, females aged ≥50 yrs were classified as post-

menopausal, as per previous published Australian data of a similar nature (Canfell, Banks et al. 2008). This sub-grouping of women based on menopausal status is most likely to be based on the reported average age of menopause (i.e. 51 yrs) (Treloar 1981; McKinlay, Brambilla et al. 2008).

3.2.3 - HRT formulations available on the Pharmaceutical Benefits Scheme (PBS) in Australia

Listings of data describing all oral HRT preparations available in Australia on the PBS was accessed via the Australian Government Department of Health and Ageing publically available database (called the Schedule of Pharmaceutical Benefits) *(http://www.pbs.gov.au/pbs/home)*. The PBS listings of HRT preparations used in this thesis were updated as of April 2009 and include individual and combined formulations of natural and synthetic oestrogens and synthetic progestins. All transdermal ERT and cHRT patches and vaginal oestrogen preparations were excluded from the listings analysed in this thesis, since the rates of prescription use were relatively low compared to oral HRT preparations. The information provided on each individual type of HRT preparation and hormone-based contraceptive on the Schedule of Pharmaceutical Benefits database included: item code; name; manner of administration; form; dose; brand name; manufacturer; maximum quantity; number of repeats; price premium; dispensed price for maximum quantity and price to consumer and information sheets.

3.2.4 - Number of HRT preparation prescriptions available on the PBS in Australia during 1992 - 2009

The number of prescriptions of the various types of HRT preparations was drawn via the Australian Government Medicare Australia (AGMA), Pharmaceutical Benefits Scheme Statistics (PBSS)) database *(https://www.medicareaustralia.gov.au/statistics/pbs_item.shtml)*. Information relating to the prescription rates of each individual type of HRT preparation was obtained using the item code derived from the Australian Government Department of Health and Ageing (Schedule of Pharmaceutical Benefits) database. The total number of prescriptions dispensed yearly by the PBS from the earliest available year of 1992 to the latest year of 2008 was obtained from the PBSS. Information relating to the number of prescriptions for each State and Territory in Australia was available but not included in the analysis, due to lack of matched breast cancer incidences rates for each State and Territory available on the AIHW database.

3.2.5 - HRT preparation use and breast cancer incidence prevalence estimates in Australian women

Changes in the prescription rates of cHRT preparations containing MPA and age-standardised breast cancer incidence rates in Australian women aged (≥50 or 50-74 yrs) during the time period 1992-2001 (before publication of the cHRT arm of the WHI trial in 2002), and 2001-2004 (following publication of the cHRT arm of the WHI trial in 2002), was undertaken by prevalence tests from data obtained from the AGMA PBSS and AIHW databases. The sub-group analysis of the two age-groups was aimed at demonstrating a greater effect in women aged 50-74 yrs, compared to all women aged ≥

50 yrs since it has been reported that Australian women in the former age-group have the highest rates of HRT use (Keating, Cleary et al. 1999; MacLennan, Wilson et al. 2002). An association between changes in the prevalence of cHRT preparations containing MPA prescription rates and age-standardised breast cancer incidence rates during the two time periods was also undertaken to demonstrate a potential relationship between the two factors.

The time periods used in this study to investigate the association between the use of MPA-containing cHRT preparations and breast cancer incidence rates were selected based on previous literature. These included a study which used a 2001-2004 time period to study regional changes in cHRT use within Californian counties following the publication of the WHI trial in 2002 in addition to an Australian study which used a 1996-2001 time period to investigate changes in cHRT use in Australian women prior to the reports of the cHRT arm of the WHI trial in 2002 (Canfell, Banks et al. 2008). The inclusion of the 2001 year in both time periods was based on identifying the change in cHRT use prior to publication of the cHRT arm of the WHI trial in 2002 (i.e. year of 2001 when prescription rates were at their peak level) and the relative change after the publication of the WHI trial in 2002 [Figure 3.1].

The inclusion of prescription data commencing from 1992, compared to another similar Australian based study which started from 1996 (Canfell, Banks et al. 2008) allowed for the combined effect of both prescriptive types of cHRT preparations containing MPA, i.e. from 1992 onwards, 5 mg MPA, distributed as Provera (item #2323G) and 0.625 mg

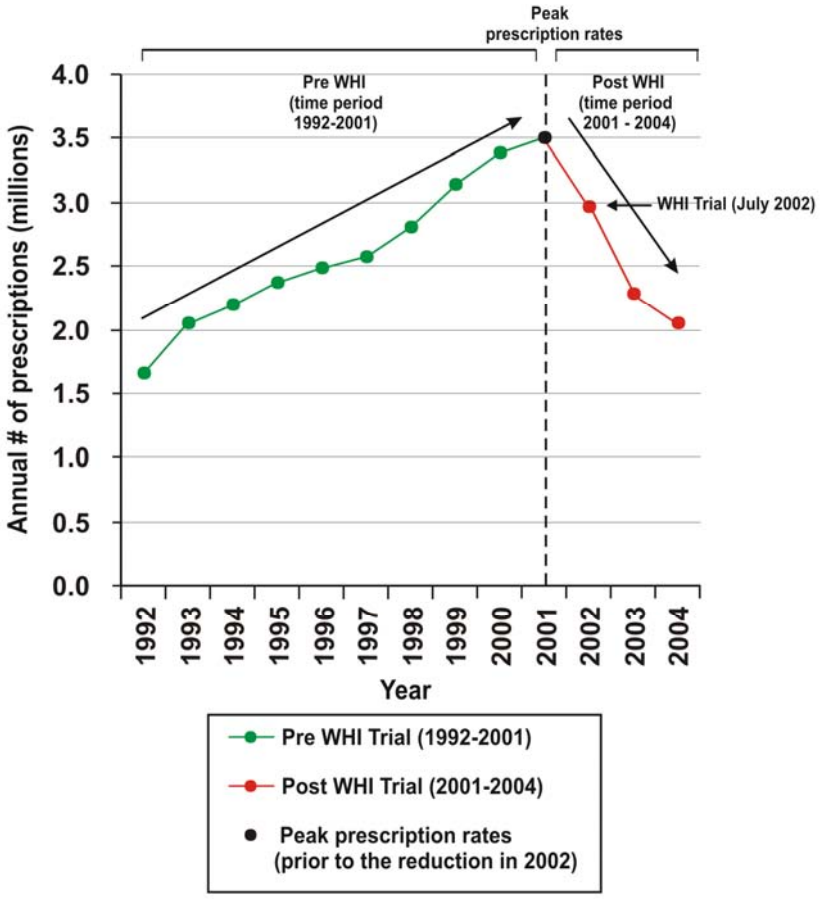


Figure 3.1: Representative model used to investigate changes in cHRT containing MPA prescription rates before and after cessation of the cHRT arm of the WHI clinical trial published in 2002. To investigate changes in the prescription rates of cHRT preparations containing MPA available in Australia on the PBS, the two time periods were used (i.e. before 1992-2001 and after 2001-2004 cessation of the WHI trial in 2002). The cHRT arm of the WHI trial was published in July 2002, which followed a dramatic decline in cHRT prescriptions. Therefore in order to determine the changes in cHRT prescription rates before and after, the relative change before (1992-2001) and after (2001-2004) cessation of the WHI trial using the calculated difference from the peak prescription rates observed in the year 2001. These time periods were based on previous literature of a similar nature (Robbins and Clarke 2007; Canfell, Banks et al. 2008).

CEE, distributed as Premarin (item #1734G) were prescribed separately, and from 1997 onwards, 0.625 mg CEE + 5 mg MPA (item #8169Y) or 0.625 mg CEE + 2.5 mg MPA (item # 8168X) were prescribed together as part of Premia.

3.2.6 - Statistical analysis

All statistical analysis in this chapter was performed by the statistician Dr Nancy Briggs located at the Data Management and Analysis Centre, Discipline of Public Health, University of Adelaide. The statistical models used in this analysis predicted age-standardised breast cancer incidence rates and cHRT preparations containing MPA prescription rates by year and age-group using Poisson regression tests. Subsequent analysis was performed to predict changes in prevalence of both age-standardised breast cancer incidence and prescription rates of cHRT using binomial regression tests. Binomial regression analysis was also used to determine associations between age-standardised breast cancer incidence rates and prescription prevalence between the two time periods by a change to the R^2 value which reflects how much of the change in breast cancer incidence can be accounted for by a change in prescription prevalence (Robbins and Clarke 2007). This was calculated using the following predictive regression model: breast cancer predicted by year period, breast cancer incidence, age group and prescription prevalence (main effect only) following addition of the interaction between to the two factors: prescription prevalence and year period. Final statistical tests were undertaken to determine the number of breast cancer cases potentially attributed to by cHRT use per time period in Australian women aged ≥50 and 50-74 yrs using Poisson regression tests. All statistical analyses were considered significant if p-value <0.05.

3.3 - Results

3.3.1 - Changes in breast cancer incidence rates in Australian women before and after publication of the cHRT arm of the WHI clinical trial in 2002

To determine the changes in age-standardised breast cancer incidence in Australian women during time periods prior and subsequent to publication of the WHI trial in 2002, statistical analysis was performed using female population data obtained from the AIHW database. An increase in breast cancer incidence rates was evident in women of all age groups between 50-79 yrs during the time period 1992-2001 [Table 1]. This increase was most dramatic in women aged between 60-64 yrs (i.e. 64% increase) [Table 1]. All age groups were associated with a decrease in breast cancer incidence during the time period 2001-2004, ranging from 1-9% [Table 1]. Interestingly, similar to the increase in breast cancer incidence rates, the decrease was most pronounced in women aged 60-64 yrs (i.e. 9% decrease) [Table 1]. Importantly, these findings illustrate that there may be common driving forces (i.e. use of HRT) underlying the changes in breast cancer incidence in Australian women [Table 1]. Findings of this nature have to date not been published in either Australia or the USA.

On average, there was a 28% increase of breast cancer incidence in Australian women ≥50 yrs during 1992-2001, and a 35% increase in women aged 50-74 yrs [Table 1]. Conversely, during 2001-2004, the incidence of breast cancer decreased by 4.5% and 6.2% in women aged >50 and 50-74 yrs, respectively that supports a similar previous

Table 1: Change in breast cancer incidence rates in Australian women before and after publication of the cHRT arm of the WHI clinical trial in 2002.

Table 1: % Change in age-standardised breast cancer incidence per 100,000 Australian women[1]		
Age-groups (yrs)	**Pre WHI 1992-2001**	**Post WHI 2001-2004**
50-54	26% ↑	5% ↓
55-59	39% ↑	6% ↓
60-64	64% ↑	9% ↓
65-69	27% ↑	1% ↓
70-74	23% ↑	5% ↓
75-79	9% ↑	5% ↓
80-84	9% ↓	4% ↓
85+	NC	1% ↓
Average (50+ yrs)	28% ↑	4.5% ↓
Average (50-74 yrs)	35% ↑	6.2% ↓

[1] Age-standardised breast cancer incidence rates were obtained from the Australian Institute of Health and Welfare (AIHW) publically available database. The fold change in age-standardised breast cancer incidence pre- (1992-2001) and post- (2001-2004) the WHI clinical trial in 2002 was calculated as a percentage. NC = No change.

study (Canfell, Banks et al. 2008) [Table 1]. However during the two time periods the changein breast cancer incidence was markedly higher in Australian women before compared to after the publication of the WHI trial in 2002. These findings have not to date been reported in the current literature.

Prevalence tests indicated a significant increase in age-standardised breast cancer incidence in Australian women aged both ≥50 and 50-74 yrs during the time period 1992-2001 ($p<0.001$) [Table 2A]. The age-standardised breast cancer incidence rates per 100,000 women aged 50 and 50-74 yrs increased during the time period 1992-2001, resulting in an 11% increase (489.8 to 546.6) and a 34% increase (233.5 to 315), respectively ($p<0.001$) [Table 2A]. Conversely, the age-standardised breast cancer incidence rates per 100,000 women aged 50 and 50-74 yrs decreased during the time period 2001-2004, resulting in a 3% decrease (546.6 to 531.2) and a 5% decrease (315.0 to 299.7) respectively, yet did not reach significance [Table 2A]. However, statistical analysis comparing the change in age-standardised breast cancer incidence in Australian women aged ≥50 and 50-74 yrs between the two time periods before and after publication of the cHRT arm of the WHI trial, revealed a significant difference ($p>0.001$) [Table 2A]. Subsequent analysis demonstrated an additional 1,623 and 2,159 estimated breast cancer cases, during the time period 1992-2001 and a reduction of 358 and 342 estimated breast cancer cases, respectively during the time period 2001-2004, in the whole Australian female population aged ≥ 50 yrs and 50-74 yrs, respectively [Table 2B].

Table 2 (A and B): (A) Age-standardised breast cancer incidence (BCI) rates per 100,000 Australian women aged ≥50 and 50-74 yrs and **(B)** estimated total change in the number of breast cancer cases (BC), before and after publication of the cHRT arm of the WHI trial in 2002.

Table 2A: Age-standardised breast cancer incidence rates per 100,000 Australian women[1]					
Age group (yrs)	**Pre WHI**		**Absolute change in prevalence (ACP)**	**95% CI**	**p-value**
	1992	**2001**			
≥ 50	489.8	546.6	+ 50.0	33.7 to 66.2	<0.001
50-74	235.5	315.9	+ 72.0	51.7 to 92.3	<0.001
	Post WHI				
	2001	**2004**			
≥ 50	546.6	531.2	-11.8	-28.6 to -5.1	0.18
50-74	315.9	299.7	-15.0	-36.4 to 6.4	0.18
	ACP Pre WHI 1992 – 2001	**ACP Post WHI 2001- 2004**	**Difference in ACP between time periods**		
≥ 50	50.0	-11.8	+ 61.7	32.8 to 90.7	<0.001
50-74	72.0	-15.0	+ 87.0	50.4 to 123.6	<0.001

Using values from the Table 2A

Table 2B: Estimated number of breast cancer cases before and after the publication of cHRT arm of the WHI clinical trial in Australian women aged ≥ 50 and 50-74 yrs[1]							
Age group (yrs)	**1992 BCI**	**2001 BCI**	**2001 Pop^n**	**Absolute prevalence**	**Change 1992 - 2001 (per 100,000 women)**	**Estimated change in BC cases 1992-2001**	
						Total	**95% CI**
≥ 50	489.8	546.6	2911052	15912.6	50.0	1623.4	(1094.5 to 2152.2)
50-74	235.5	315.9	2233939	7057.0	72.0	2158.8	(1550.0 to 2767.2)
	2001 BCI	**2004 BCI**	**2004 Pop^n**	**Absolute prevalence**	**Change 2001 - 2004 (per 100,00 women)**	**Estimated change in BC cases 2001-2004**	
						Total	**95% CI**
≥ 50	546.6	531.2	3134800	16652.4	-11.8	-358.4	(-871.7 to 155.0)
50-74	315.9	299.7	2407907	7215.7	-15.0	-341.9	(-830.7 to 147.0)

[1]The incidence of age-standardised breast cancer for the Australian female population was obtained from the Australian Institute of Health and Welfare (AIHW) (http://aihw.gov.au/), which produce the publically-available database included in the Australian Cancer Incidence and Mortality (ACIM) book *(http://aihw.gov.au/search/?q= Breast+Cancer+ACIM+book*). The statistical analysis performed was comprised of Poisson and Binomial regression prevalence tests ($p<0.05$). **Abbreviations:** Population = Pop^n.

An observational finding indicated that the absolute change in prevalence (ACP) during the two time periods before and after publication of the cHRT arm of the WHI trial was greater for women aged 50-74 yrs (i.e. +72 (1992-2001) and -15 (2001-2004)), compared to ≥ 50 yrs (i.e. +50 (1992-2001) and -11.8 (2001-2004)), respectively [Table 2A]. No statistical analysis was performed on this data since it was statistically in-valid to include the same population data in two individual sub-groups. These findings demonstrate that the change in age-standardised breast cancer incidence is potentially greater in women aged 50-74 yrs compared to ≥50 yrs. Moreover, the 50-74 yr age-group has been previously reported to present with the highest rates of cHRT use in Australian women (MacLennan, Wilson et al. 2002).

3.3.2 - Prescription rates of oral HRT preparations available on the PBS in Australia before and after publication of the cHRT arm of the WHI clinical trial in 2002

To determine the change in prescription rates of cHRT preparations containing MPA that reflected time periods before and after publication of the WHI trial in 2002, data available on the PBS in Australia was obtained from the publically available on-line database (*Source:* Australian Government Department of Health and Ageing publically available database/Schedule of Pharmaceutical Benefits *(http://www.pbs.gov.au/pbs/home)*. The HRT prescription types were separated into four different subgroups consisting of 21 oral HRT preparations [Table 3]. These included the following subgroups: 1) conjugated equine oestrogens (CEE); 2) non-conjugated oestrogens; 3) synthetic progestins and 4) cHRT preparations (a synthetic progestin plus an oestrogen i.e. conjugated or non-conjugated formulations) [Table 3].

Table 3: Hormone replacement therapy (HRT) preparations prescribed in Australia on the Pharmaceutical Benefits Scheme (PBS) from listings last updated April 2010.

Table 3: Pharmaceutical listing of oral hormone replacement therapy (HRT) preparations in Australia[1]		
	HRT types	
Item ID	**Group (1): Conjugated Equine Oestrogens (CEE)**	**Pharmaceutical Name**
1733F	Oestradiol-conjugated (0.3 mg)	Premarin
1734G	Oestradiol-conjugated (0.625 mg)	Premarin
	Group (2): Non-conjugated Oestrogens	
1778N	Generol (1.25 mg)	Ogen/Generol
1777M	Generol (0.625 mg)	Ogen/Generol
1771F	Oestriol (0.5 mg)	Ovestin
8274L	Oestradiol (2 mg)	Zumenon
1664N	Oestradiol Valerate (2 mg)	Progynova
1663M	Oestradiol Valerate (1 mg)	Progynova
	Group (3): Synthetic Progestins	
2323G	MPA (5 mg)	Ralovera/Provera/Medroxyhexal
2321E	MPA (10 mg) (2 x 30 tablets)	Ralovera/Provera
2722G	MPA (10 mg) (2 x 100 tablets)	Ralovera/Provera
2993M	NETA (5 mg)	Primulot
1350C	Dydrogesterone (10 mg)	Duphaston
	Group (4): Combined Hormone Replacement Therapy (cHRT) Formulations	
8168X	CEE (0.625 mg) + MPA (2.5 mg)	Premia 2.5
8169Y	CEE (0.625 mg) + MPA (5 mg)	Premia 5
8244X	Oestradiol (2 mg) + Dydrogesterone (10 mg)	Femoston 2/10
8353P	Oestradiol (1 mg) + NETA (0.5 mg)	Kliovance
8081H	Oestradiol (2 mg) + NETA (2 mg-1 mg)	Kliogest
1764W	Oestradiol (2 mg) + NETA (1 mg)	Trisequens

[1] Listings of all hormone-based contraceptives available in Australia on the PBS was accessed via the Australian Government Department of Health and Ageing publically available database (called the Schedule of Pharmaceutical Benefits) (*http://www.pbs.gov.au/pbs/home)*. **Abbreviations:** Medroxyprogesterone Acetate (MPA); Conjugated Equine Oestrogen (CEE) and Noresthisterone Acetate (NETA).

Conjugated equine estrogens, predominantly used in the cHRT formulation Premarin, are obtained from mare's urine and are a mixture of sodium salts of estrogen sulfates that include the following major forms: estrone; equilin and equilenin. They are commonly referred to as conjugated (i.e. a chemical compound that has been formed by the joining of two or more compounds) since most forms are attached to a sulfate by a double bond (i.e. hydrophilic side group). Estrone sulfate is converted in the female body into estradiol, however the actions of equilin in comparison to estrogen are not well known. For treatment of climacteric symptoms associated with menopause, post-menopausal women who retain an intact endometrium are prescribed either a CEE (group 1) or a non-conjugated oestrogen (group 2) in conjunction with a synthetic progestin (group 3), or a single cHRT formulation (group 4) [Table 3]. Post-menopausal women who have undergone a hysterectomy and who therefore do not require the protective effects of a synthetic progestin in the endometrium from the unopposed actions of oestrogen, are administered a CEE (group 1) or a non-conjugated oestrogen (group 2) for the alleviation of symptoms associated with menopause [Table 3].

The total number of HRT prescriptions obtained from the AGMA and PBSS databases listed in sub-groups 1-4 from 1992 to the end of 2008 has been depicted graphically [Table 3 and Figures 3.2 - 3.7]. However, the changes in HRT prescription rates described in the subsequent sections are only included up to the year 2004 so that a direct comparison to the changes in breast cancer incidence rates, which were only available on the AIHW database at the commencement of this study up to the year 2004, could be

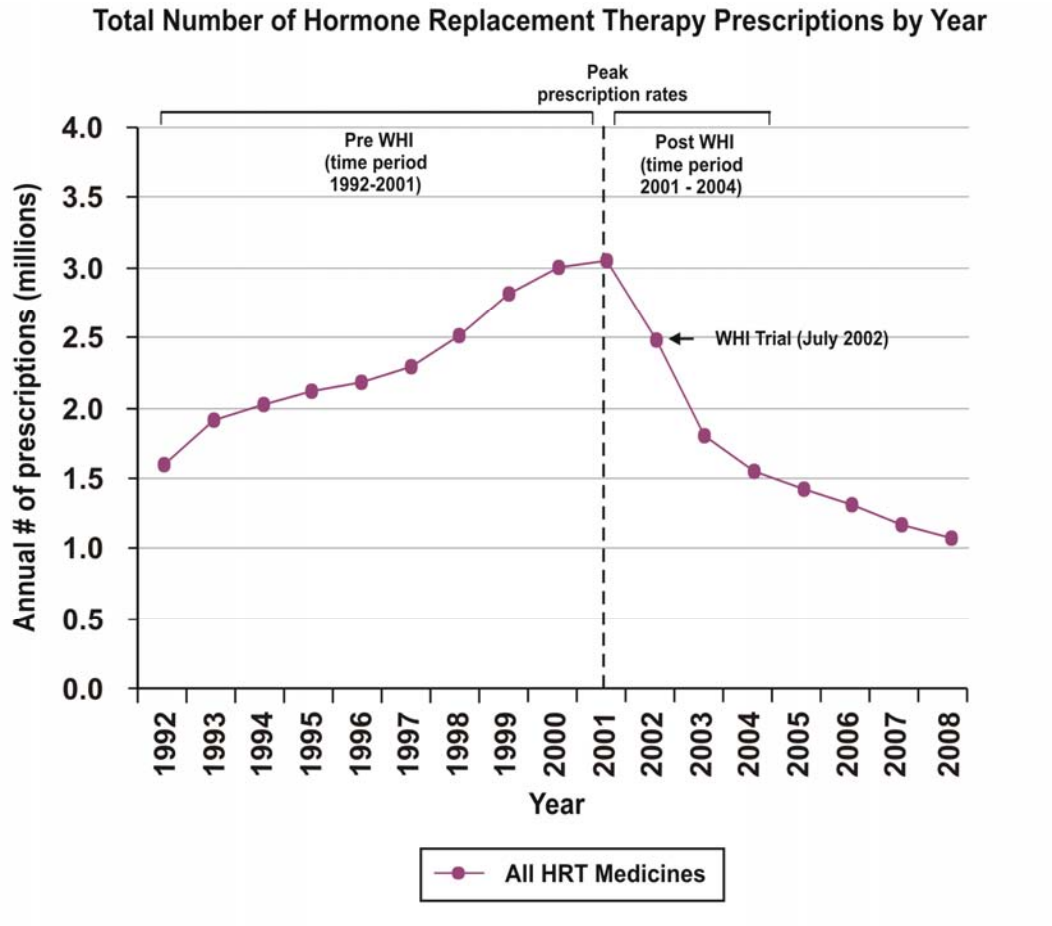


Figure 3.2: Use of all HRT preparations in Australia. Total number of all HRT prescriptions combined available on the PBS in Australia represented at yearly intervals between January 1992 and December 2008. The graph depicts the two different time periods used to compare changes in prescription rates of all HRT preparations combined in Australia (i.e. 1992-2001 before and 2001-2004 after publication of the cHRT arm of the WHI clinical trial in 2002 (Rossouw, Anderson et al. 2002), based on changes either side of the peak prescription rates in the year 2001. The number of prescriptions of the various types of HRT preparations was drawn via the Australian Government Medicare Australia (AGMA), Pharmaceutical Benefits Scheme Statistics (PBSS) database (https:// */www.medicareaustralia.gov.au/statistics/pbs/item.shtml).*

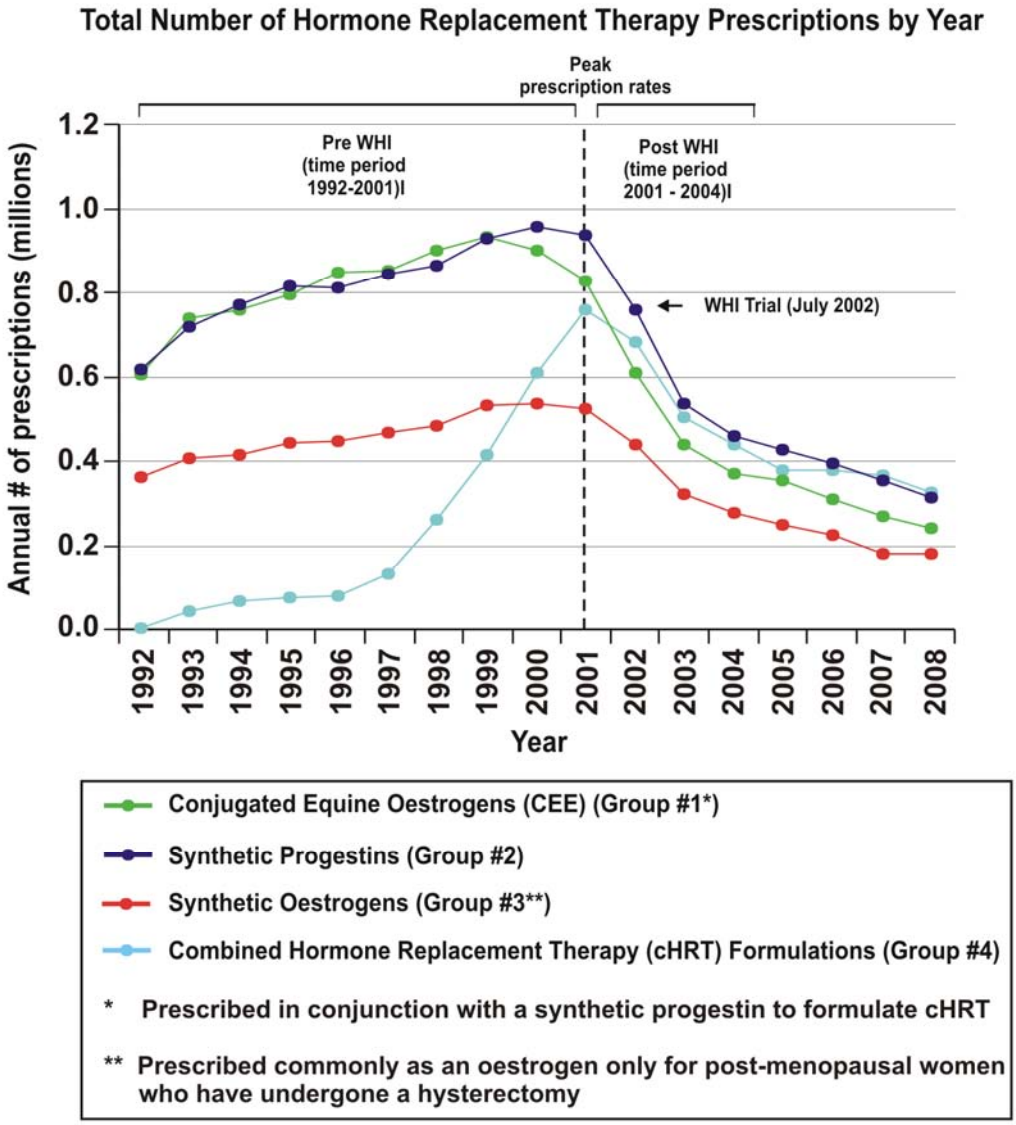


Figure 3.3: Use of HRT preparations in Australia. Total number of prescriptions of HRT preparations combined available on the PBS in Australia represented at yearly intervals between January 1992 and December 2008. The graph depicts the two different time periods used to compare changes in prescription rates of HRT preparations in Australia (i.e. 1992-2001 before and 2001-2004 after publication of the cHRT arm of the WHI clinical trial in 2002, based on changes either side of the peak prescription rates in the year 2001. The number of prescriptions of the various types of HRT preparations was drawn via the Australian Government Medicare Australia (AGMA), Pharmaceutical Benefits Scheme Statistics (PBSS) database *(https://www.medicareaustralia.gov.au/statistics/pbs/item.shtml)*.

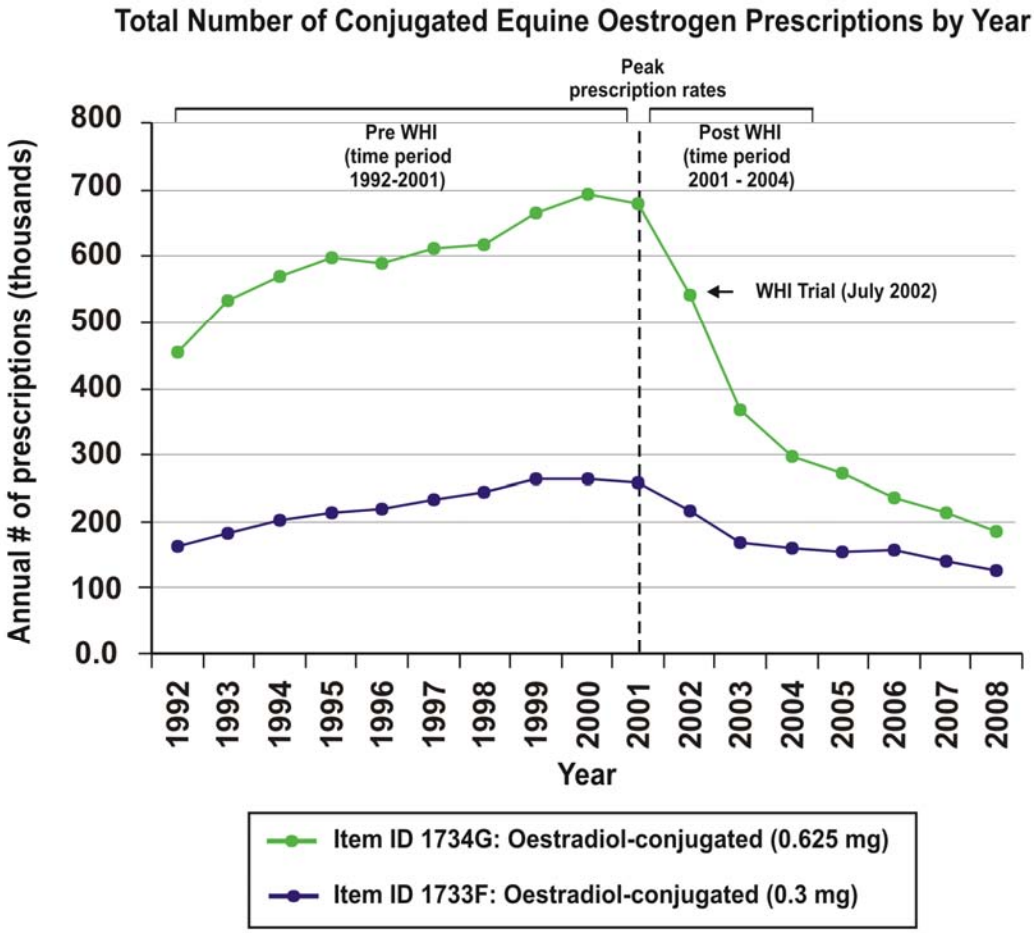


Figure 3.4: Use of CEE in Australia (Group #1). Total number of conjugated equine oestrogens (CEE) prescriptions available on the PBS in Australia represented at yearly intervals between January 1992 and December 2008. The graph depicts the two different time periods used to compare changes in prescription rates of conjugated oestrogens in Australia (i.e. 1992-2001 before and 2001-2004 after publication of the cHRT arm of the WHI clinical trial in 2002, based on changes either side of the peak prescription rates in the year 2001. The number of prescriptions of the various types of HRT preparations was drawn via the Australian Government Medicare Australia (AGMA), Pharmaceutical Benefits Scheme Statistics (PBSS) database *(https://www.medicareaustralia.gov.au/statistics/pbs/item.shtml)*.

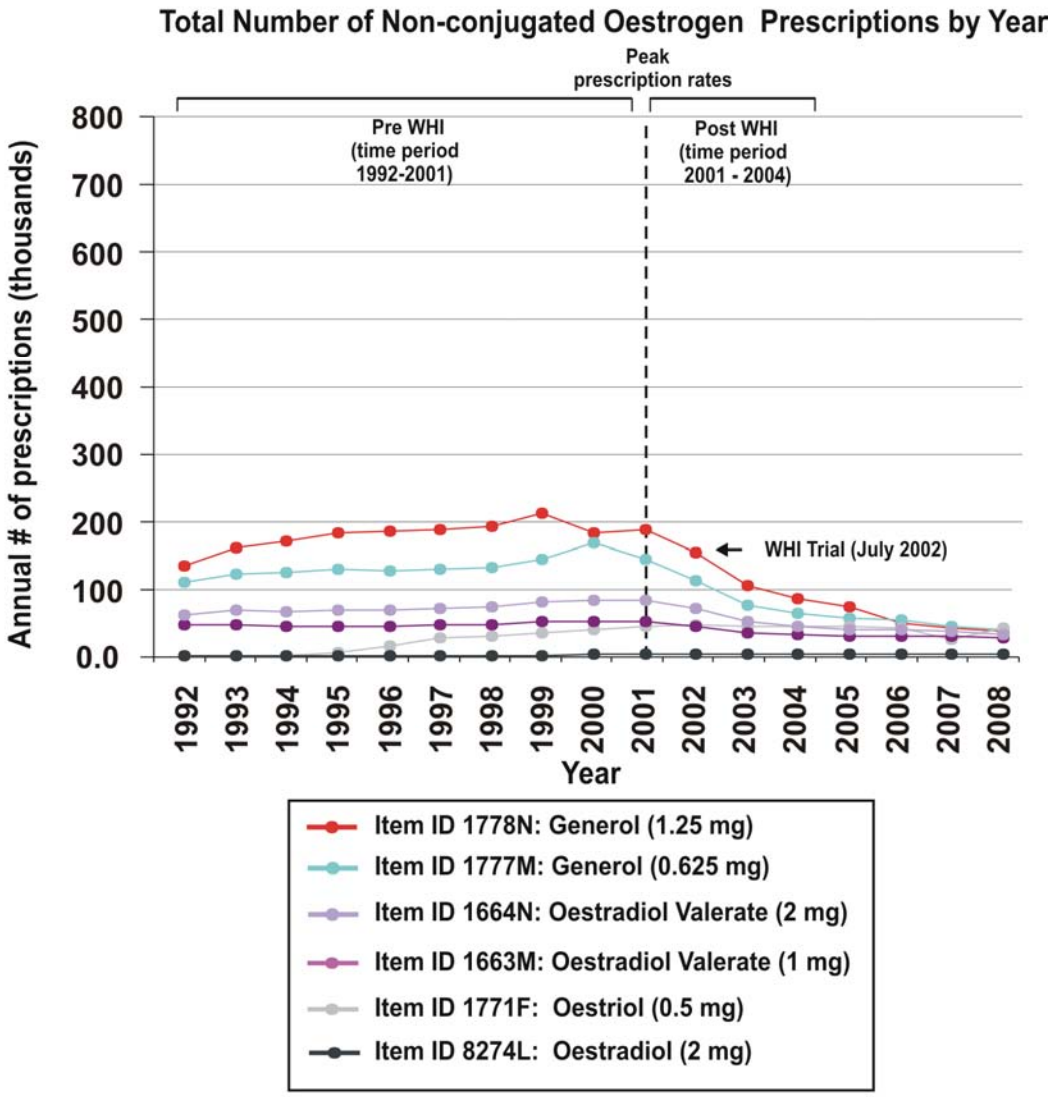


Figure 3.5: Use of non-conjugated oestrogens in Australia (Group #2). Total number of oestrogen prescriptions available on the PBS in Australia represented at yearly intervals between January 1992 and December 2008. The graph depicts the two different time periods used to compare changes in prescription rates of non-conjugated oestrogens in Australia (i.e. 1992-2001 before and 2001-2004 after publication of the cHRT arm of the WHI clinical trial in 2002, based on changes either side of the peak prescription rates in the year 2001. The number of prescriptions of the various types of HRT preparations was drawn via the Australian Government Medicare Australia (AGMA), Pharmaceutical Benefits Scheme Statistics (PBSS) database *(https://www.medicareaustralia.gov.au/statistics/pbs/item.shtml)*.

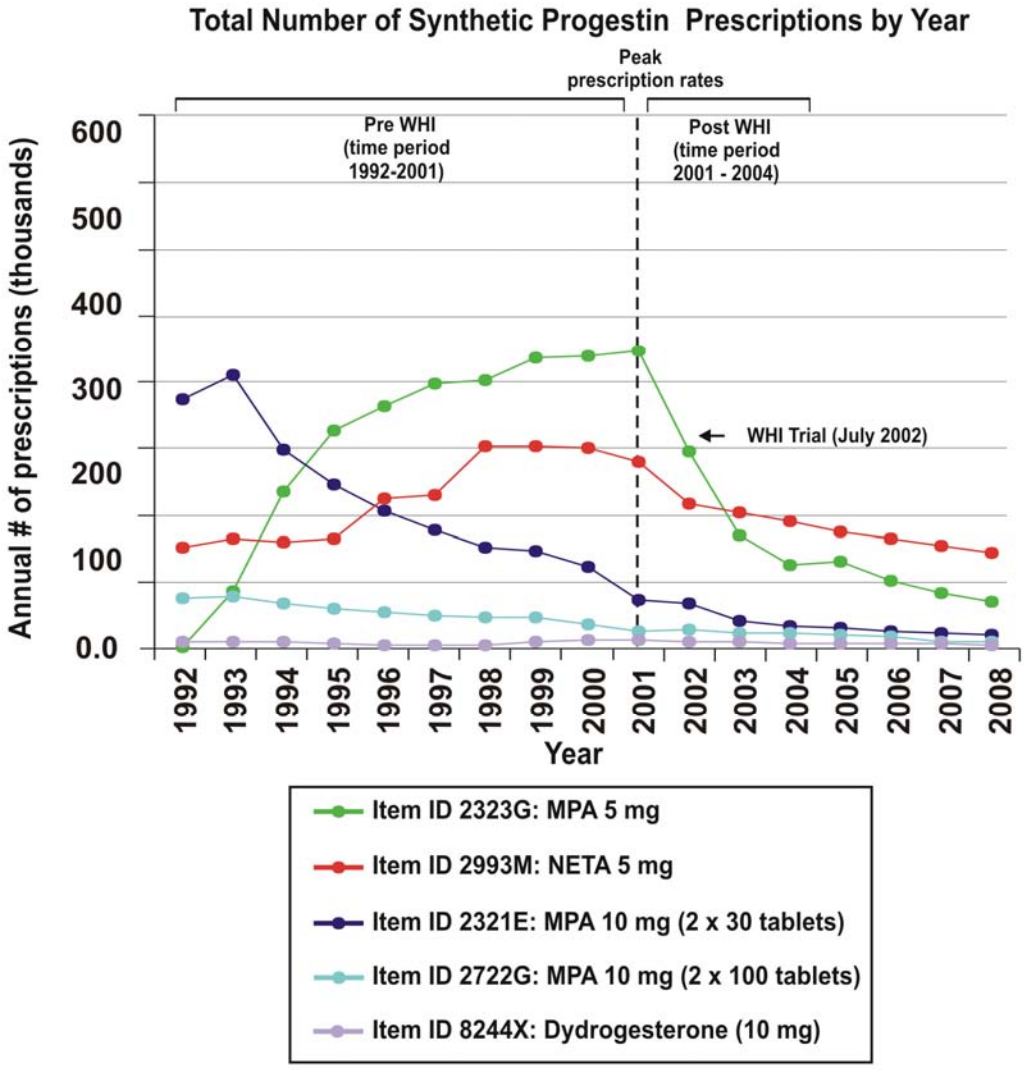


Figure 3.6: Use of synthetic progestins in Australia (Group #3). Total number of synthetic progestin prescriptions available on the PBS in Australia represented at yearly intervals between January 1992 and December 2008. The graph depicts the two different time periods used to compare changes in prescription rates of synthetic progestins in Australia (i.e. 1992-2001 before and 2001-2004 after publication of the cHRT arm of the WHI clinical trial in 2002, based on changes either side of the peak prescription rates in the year 2001. The number of prescriptions of the various types of HRT preparations was drawn via the Australian Government Medicare Australia (AGMA), Pharmaceutical Benefits Scheme Statistics (PBSS) database *(https://www.medicareaustralia.gov.au/statistics/pbs/item.shtml)*.

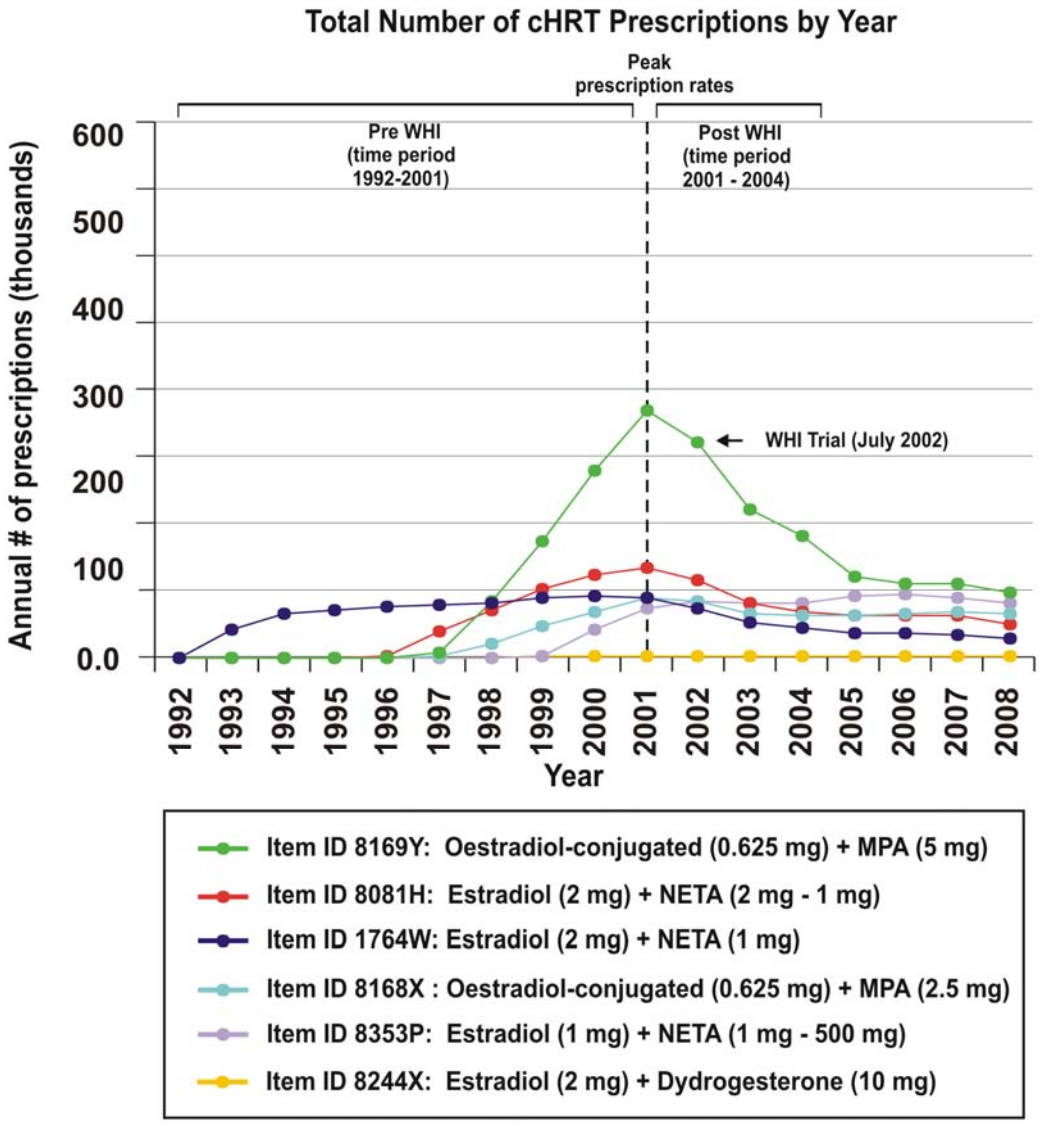


Figure 3.7: Use of cHRT preparations in Australia (Group #4). Total number of cHRT prescriptions available on the PBS in Australia represented at yearly intervals between January 1992 and December 2008. The graph depicts the two different time periods used to compare changes in prescription rates of cHRT preparations in Australia (i.e. 1992-2001 before and 2001-2004 after publication of the cHRT arm of the WHI clinical trial in 2002, based on changes either side of the peak prescription rates in the year 2001. The number of prescriptions of the various types of HRT preparations was drawn via the Australian Government Medicare Australia (AGMA), Pharmaceutical Benefits Scheme Statistics (PBSS) database *(https://www.medicareaustralia.gov.au/statistics/pbs/item.shtml)*.

determined. In support of the Australian literature a total of 34,192,309 HRT prescriptions were filled during 1992-2004, which reached peak levels of 3,042,824 in the year 2001 [Figure 3.2] (MacLennan, Wilson et al. 2002).

Prior to the publication of the WHI trial in 2002 during the time period 1992-2001 an increase in the number of prescriptions for each type of HRT preparation listed in sub-groups 1-4 was observed, i.e. 52% for CEE (group 1), 44% for non-conjugated oestrogens (group 2), 37% for synthetic progestins (group 3) and 490% for cHRT preparations (group 4) [Figure 3.3]. In contrast, during the time period 2001-2004 subsequent to the WHI trial in 2002, a decrease in the number of prescriptions for the same sub-groups was observed, i.e. 74% for CEE (group 1), 63% for non-conjugated oestrogens (group 2), 90% for synthetic progestins (group 3) and 70% for cHRT preparations (group 4) [Figure 3.3].

Changes in the number of each individual prescription type of HRT preparation listed in groups 1-4 during the time periods 1992-2001 and 2001-2004 has been tabulated (*refer to Appendix 2: Tables 1-2*). The most commonly prescribed HRT preparation during 1992-2004 in each group were as follows: group 1, 0.625 mg oestradiol-conjugated (item #1734G; 6,923,962 prescriptions) [Figure 3.4]; group 2, 1.25 mg oestrogen (item #1778N; 2,100,272 prescriptions) [Figure 3.5]; group 3, 5 mg MPA (item #2323G; 3,577,387 prescriptions) [Figure 3.6] and group 4, 0.625 mg CEE + 5 mg MPA (item #8169; 1,456,219 prescriptions) [Figure 3.7]. The availability of cHRT as a single prescription (i.e. 0.625 mg CEE plus 2.5 mg or 5.0 mg MPA; trade name in Australia,

Premia 2.5 or 5, respectively) was not available to the Australian population until 1997 [Figure 3.7]. Prior to 1997, cHRT was prescribed using two separate prescriptions (i.e. 0.3 or 0.625 mg CEE) (trade name in Australia, Premarin) and 5 mg MPA (trade name in Australia, Provera) [Figure 3.6]. The prescription of the single forms (i.e. Premarin and Provera) in addition to dual forms of cHRT containing MPA (i.e. Premia) subsequent to 1997 continued at high rates [Figure 3.6].

3.3.3 - Changes in the use of cHRT preparations containing MPA before and after publication of cHRT arm findings of the WHI clinical trial in 2002 by the Australian female population

Prevalence changes of cHRT containing MPA in Australian women aged >50 and 50-74 yrs, was investigated to determine statistical changes before and after publication of the cHRT arm of the WHI trial. The cHRT formulation 0.625 mg CEE + 2.5 mg MPA (item #8168X) was combined with the 0.625 mg CEE + 5 mg MPA (item #8169Y) formulation in this analysis, since it is the only other cHRT consisting of both a CEE and MPA that was available on the PBS at the time of this study. In addition, prevalence changes of the single prescriptions of 5 mg MPA (item #2323G) and 0.625 mg CEE (item #1734G) were used in separate statistical tests.

Prior to publication of the WHI trial in 2002 during the time period 1992-2001 there was a significant increase in the prescription rates of cHRT containing MPA (single and combined) in women aged ≥50 and 50-74 yrs, i.e. prescriptions increased for the single

Tables 4 and 5: Use of 5 mg MPA (item #2323G) and 0.625 mg oestrogen-conjugated (item #1734G) per 100,000 Australian women before and after publication of the cHRT arm of the WHI clinical trial in 2002.

Table 4: Use of 5 mg MPA per 100,000 Australian women[1]					
Age group (yrs)	Pre WHI		Absolute change in prevalence (ACP)	95% CI	p-value
	1993	2001			
≥ 50	3,576	15,292	11,716	11,668 to 11,763	<.0001
50-74	4,574	19,928	15,353	15,293 to 15,414	<.0001
	Post WHI				
	2001	2004			
≥ 50	15,292	3,903	-11,389	-11,435 to -11,342	<.0001
50-74	19,928	5,082	-14,845	-14,905 to -14,786	<.0001
	Pre WHI	Post WHI	Difference in ACP between time periods		
	1993 – 2001	2001- 2004			
≥ 50	11,716	-11,389	23,105	23,016 to 23,194	<.0001
50-74	15,353	-14,845	30,199	30,086 to 30,311	<.0001

Table 5: Use of 0.625 mg oestrogen-conjugated per 100,000 Australian women[1]					
Age group (yrs)	Pre WHI		Absolute change in prevalence (ACP)	95% CI	p-value
	1992	2001			
≥ 50	19,773	24,569	3,519	3,448 to 3,590	<.0001
50-74	25,219	31,954	5,133	5,045 to 5,220	<.0001
	Post WHI				
	2001	2004			
≥ 50	30,352	12,356	-13,801	-18,069 to -17,922	<.0001
50-74	23,292	9,491	-17,966	-13,003 to -12,900	<.0001
	Pre WHI	Post WHI	Difference in ACP between time periods		
	1992 – 2001	2001- 2004			
≥ 50	-910	-12,952	12,041	11,935 to 12,147	<.0001
50-74	5,133	-17,996	23,129	22,986 to 23,271	<.0001

[1] The number of prescriptions of the various types of HRT preparations was drawn via the Australian Government Medicare Australia (AGMA), Pharmaceutical Benefits Scheme Statistics (PBSS) database *(https://www.medicareaustralia.gov.au/statistics/pbs_item.shtml)*. The statistical analysis performed was comprised of Poisson and Binomial regression prevalence tests ($p<0.05$).

Table 6: Use of the cHRT preparations 0.625 mg CEE + 2.5 mg MPA (item #8169Y) and 0.625 mg CEE + 5 mg MPA (item #8168X) per 100,000 Australian women before and after publication of the cHRT arm of the WHI clinical trial in 2002.

Table 6: Use of CHRT containing MPA per 100,000 Australian women[1]					
Age group (yrs)	Pre WHI		Absolute change in prevalence (ACP)	95% CI	p-value
	1997	2001			
≥ 50	418	15,713	15,294	1,5251 to 15,336	<.0001
50-74	541	20,475	19,934	19,880 to 19,988	<.0001
	Post WHI				
	2001	2004			
≥ 50	15,713	7,801	-6,403	-6,456 to -6,350	<.0001
50-74	20,475	10,156	-8,346	-8,413 to -8,279	<.0001
	Pre WHI	Post WHI	Difference in ACP between time periods		
	1997 – 2001	2001- 2004			
≥ 50	15,294	-6,403	21,697	21,607 to 21,787	<.0001
50-74	19,934	-8,346	28,281	28,166 to 28,395	<.0001

[1] The number of prescriptions of the various types of HRT preparations was drawn via the Australian Government Medicare Australia (AGMA), Pharmaceutical Benefits Scheme Statistics (PBSS) database *(https://www.medicareaustralia.gov.au/statistics/pbs_item.shtml)*. The statistical analysis performed was comprised of both Poisson and Binomial regression prevalence tests ($p<0.05$).

cHRT preparations including Provera, 5 mg MPA (item #2323G) (p>0.0001) [Table 4] and Premarin, 0.625 mg CEE (item #1734G) (p>0.0001) [Table 5] in addition to the combined cHRT preparations including Premia CEE + 2.5 mg MPA (item #8169Y) and CEE + 5 mg MPA (item #8168X) combined (p>0.0001) [Table 6]. Following publication of the WHI trial in 2002 during the time period 2001-2004 there was a significant decrease in the prescription rates of the same cHRT individual and combined preparations for women aged ≥50 and 50-74 yrs (p<0.0001) [Tables 4-6]. An observational finding, albeit not substantiated by statistical analysis, due to the invalid statistical comparison in overlap of the same age-groups, indicated that there was a greater change in cHRT containing MPA use in women before and after publication of the WHI cHRT arm findings aged 50-74 yrs compared to women aged ≥50 yrs [Tables 4-6]. Similar findings described in earlier sections of this chapter were also observed for the change in age-standardised breast cancer incidence *(refer to section 3.2.2)*. Importantly, these findings illustrate the potential for a greater positive relationship between breast cancer incidence rates and the use of cHRT comprised of a CEE and MPA particularly in Australian women aged 50-74 yrs.

3.3.4 - Relationship between prevalence changes in breast cancer incidence and cHRT containing MPA prescription rates before and after publication of the cHRT arm of the WHI clinical trial

Given previous findings demonstrating a significant change in prevalence of both age-standardised breast cancer incidence and prescription prevalence of cHRT preparations containing MPA in Australian women between time periods before and after publication

of the cHRT arm of the WHI trial in 2002, subsequent analysis was performed to elucidate whether an association between the two was evident. Findings indicated that there was a significant association in breast cancer incidence rates and the individual cHRT prescriptions including MPA 5 mg (item #2323G) and oestrogen-conjugated 0.625 mg (item #1734G) by a significant increase in the R^2 value in Australian women aged ≥ 50 yrs and 50-74 yrs ($p<0.05$) [Table 7]. A separate association between the use of the single cHRT containing MPA preparations comprised of either CEE 0.625 mg or 2.5 mg or 5 mg MPA (item #8169 and 8168X) was detected also by an increase in R^2 value in women aged 50-74 yrs ($p<0.05$), yet not in women aged ≥ 50 yrs [Table 7]. In conjunction with previous findings in this chapter, these findings give evidence to a relationship between breast cancer incidence and use of cHRT containing MPA, particularly in Australian women aged 50-74 yrs.

3.4 - Discussion

This is the first study in Australia to report statistical changes and differences between breast cancer incidence and the use of cHRT containing MPA before and after publication of the cHRT arm of the WHI clinical trial in 2002. Additionally, the data described in this chapter includes the most comprehensive listings to date of all HRT preparations available on the PBS and prescription rates before and after cessation of the WHI trial in 2002 Australia (effective from April 2009). The main findings in this chapter demonstrate by prevalence statistical tests, that MPA specifically used in cHRT is positively associated to breast cancer incidence rates in post-menopausal Australian

Table 7: Relationship between changes in age-standardised breast cancer incidence rates in Australian women and cHRT containing MPA prescription rates before and after publication of the cHRT arm of the WHI clinical trial in 2002.

Table 7: Association between changes in age-standardised breast cancer incidence and cHRT prescription prevalence between the time periods 1997-2001 and 2001-2004			
Age-group (yrs)	5mg MPA (item #2323G)		
	$^1R^2$	Change in $^2R^2$	p-value of $^2R^2$ change
≥ 50	0.89	0.22	p=0.04
50-74	0.84	0.19	p=0.016
	0.625 mg Oestrogen-conjugated (item #1734G)		
	$^1R^2$	Change in $^2R^2$	p-value of $^2R^2$ change
≥ 50	0.89	0.18	p=0.036
50-74	0.78	0.16	p=0.041
	cHRT preparations containing MPA 0.625 mg CEE + MPA 2.5 mg (item #8169Y) + 0.625 mg CEE + MPA 5 mg (item #8168X)		
	$^1R^2$	Change in $^2R^2$	p-value of $^2R^2$ change
≥ 50	0.87	0.006	p=0.647
50-74	0.85	0.09	p=0.049

$^1R^2$: Calculated using regression analysis fit to the regression model 2, i.e. breast cancer predicted by year period, breast cancer incidence, age group, prescription prevalence and prescription prevalence*year period (i.e. interaction between to the two factors).

^{2}Change in R^2: Calculated from the difference in the R^2 value obtained from the regression model 2 (as above) compared to regression model 1, i.e. breast cancer predicted by year period, age group and prescription prevalence (main effect only) in the absence of the interaction between the two factors. A statistical change in the R^2 value from in regression model 2 compared to 1 indicates an interaction between breast cancer incidence and prescription prevalence based between the two year periods (i.e. 1997-2001 and 2001-2004). The statistical analysis performed was comprised of Poisson and Binomial regression prevalence tests ($p<0.05$).

women, at potentially higher rates in women aged 50-74 yrs. Moreover, the findings in this chapter emphasise the public health concern of the use of the carcinogenic synthetic progestin MPA in cHRT, for the treatment of menopause in Australian women.

Novel findings in this chapter have identified a significant difference in age-standardised breast cancer incidence between time periods prior to and subsequent to the publication of the WHI trial in 2002 in Australian women aged ≥50 and potentially a greater effect in women aged 50-74 yrs. In comparison, other similar studies have focused mainly on the changes in age-standardised breast cancer incidence following the release of the findings of the WHI trial in the USA (Ereman, Prebil et al.; Marshall, Clarke et al.; Key and Pike 1988; Glass, Lacey et al. 2007; Hausauer, Keegan et al. 2007; Jemal, Ward et al. 2007; Keegan, Chang et al. 2007; Ravdin, Cronin et al. 2007) Australia (Canfell, Banks et al. 2008) and Europe (Fontenoy, Leux et al.; Renard, Vankrunkelsven et al.; Sharpe, McClements et al.; Katalinic and Rawal 2008; Seradour, Allemand et al. 2009). The basis for the predominant focus on the changes in cHRT use following publication of the WHI trial in 2002 was primarily associated with the rapid decline in use of cHRT in women following media reports of the potential link between cHRT use and breast cancer (Lebow and Arkin 1993; Paine, Stocks et al. 2004). The importance of undertaking studies as reported in this chapter allows for the cumulative exposure of the breast tissue to cHRT over a longer time period (i.e. 9 yrs), compared to the shorter time period following cessation of use (i.e. 3 yrs).

The WHI trial has indicated that cHRT driven breast cancers only become clinically detectable by mammography screening after 5 yrs (Rossouw, Anderson et al. 2002; Beral 2003). Nonetheless, epidemiological studies have shown a reduction in breast cancer rates following publication of cHRT arm of the WHI trial use after only 2 years to normal population breast cancer risk levels (Chlebowski, Kuller et al. 2009). Despite these findings, a greater change in breast cancer incidence rates was detected during the exposure period of the breast tissue to cHRT in this chapter, compared to cessation of cHRT use subsequent to publication of the 2002 WHI trial. This change in breast cancer incidence was mirrored by a 110% increase during 1992-2001 and 53% reduction during 2001-2004 in all HRT prescriptions combined in the Australian population *(data not shown).* These findings emphasise the importance of investigating the effects of cHRT during the exposure period when breast cancers may be stimulated, in addition removal of cHRT. It has been demonstrated in the USA that the reduction in breast cancer following the WHI trial is directly linked to the reduction in cHRT use (Robbins and Clarke 2007), and now in Australian women from the findings reported in this chapter.

The lack of a statistical decrease in age-standardised breast cancer incidence following the WHI clinical trial in this chapter, compared to a previous Australian study is potentially linked to the different source of data used to age-standardise the data (Canfell, Banks et al. 2008). It is likely that the age-standardised breast cancer incidence rates used in the other similar Australian study are tabulated into individual yearly rates, which would substantially reduce to the standard deviation (Canfell, Banks et al. 2008) compared to the AIHW population data which only tabulates breast cancer incidence

rates into 5-yearly bands (i.e. 50-74 and 75-79 yrs). However the specific details of the grouping of the breast cancer incidence rates of Australian women based on age was not included in the methodology of the associated study (Canfell, Banks et al. 2008). This potential difference between the two Australian population databases was not known until after completion of the statistical analysis in this study. Nonetheless, a similar decrease in breast cancer incidence of 5% was observed in Australian women aged 50-74 yrs during 2001-2004 in this study, which mirrors a similar 6.5% change to that reported in the Australian study (Canfell, Banks et al. 2008). Whereas the other similar Australian study identified a reduction in breast cancer and a 40% reduction HRT use in concession cardholders during 2001 to the end of 2003, they did state that these findings do not necessarily establish a casual connection between the two factors (Canfell, Banks et al. 2008). Novel findings described in this chapter have identified for the first time a positive association between the rates of breast cancer incidence and cHRT use in Australian women, whereas this notion has previously only been speculated to be casually linked.

In the Australian population, a significant decline in HRT prescriptions of 55.4% was observed in the first 12 months following publication of the cHRT arm of the WHI clinical trial in 2002 (Main and Robinson 2008). Furthermore, the use of cHRT in Australian women has been predicted to account 1066 extra breast cancer cases in 2001 (Coombs, Taylor et al. 2005). Based on these findings it has been suggested that a 40% reduction in prescription rates of HRT in Australian women during 2001 to 2003 would lead to 430 fewer breast cancer cases (Canfell, Banks et al. 2008). These reports are similar to the findings in this chapter that report a reduction of 358 and 341 breast cancer

casers in Australian women aged ≥50 and 50-74 yrs, respectively during the same time period. Currently, the literature consists of only one other Australian study, that reported a significant increase in the number of HRT prescriptions between 1991-2000 in Australian women aged >50 yrs (MacLennan, Wilson et al. 2002). However, as per the majority of studies, the contribution of specific synthetic progestins, i.e. MPA, are not emphasised in the findings either before or after publication of WHI trial in 2002. The main focus on MPA in this study was aimed at highlighting the documented carcinogenic effects specifically of MPA in human and animals (WHO 2005), to give a basis to subsequent studies in this thesis that were aimed at identifying a molecular basis to the biological actions of MPA and increased breast cancer development.

MPA is most commonly prescribed in cHRT within the USA (Hersh, Stefanick et al. 2004), and shown also in this study within Australia. Since it has the highest reported rates of use it may suggest that contribution to the change in breast cancer incidence may also be greater. In this chapter, the total number cHRT preparations containing MPA prescribed to the Australian female population during 1992-2004 equates to a total of approximately 9.1 million single prescriptions. In comparison, the cHRT formulation containing NETA contributed to approximately 4.1 total million single prescriptions during the same time period. Based on the total number of oral HRT prescriptions in Australia during the same time period of approximately 27.5 million, in support of previous findings (Coombs and Boyages 2005), the cHRT preparations containing MPA account for the greatest contribution, of approximately one third of the total number of HRT prescriptions in Australia. This emphasises the importance of identifying a

molecular link specifically between the effects of the MPA and the development of breast cancer. Nevertheless, the findings in this chapter do not suggest that it is only the carcinogenic activity of MPA that can increase breast cancer incidence, since the data shows that other cHRT preparations comprised of different synthetic progestins also follow a similar pattern of change. Furthermore the prescription rates of cHRT containing NETA prepartations, compared to MPA prescription rates are still significantly high and it should be noted that both formulations of cHRT have been linked to increased breast cancer rates in British post-menopausal women in the Million Women's Study (MWS) (Beral 2003).

It has been proposed that changes in breast mammography screening rates may account for the increased detection of breast cancer cases in women taking cHRT during and subsequent to the release of the findings of the WHI trial in 2002 (McDonough 2002; Mastorakos, Sakkas et al. 2006). Breast mammography screening is well established in the literature to contribute to the increase in detection of breast cancers, in addition to regular self checks and general practitioner visits (Bennett, McCaffrey et al. 1990). Mammography screening was implemented in Australia in 1991, however high rates of screening in post-menopausal women did not occur until 1999 in Australian women aged 50-69 yrs, (Coombs, Taylor et al. 2005). Hence, breast mammography screening rates are likely to not contribute to a high degree to the breast cancer incidence rates reported in this chapter during 1992-1999. Moreover, a number of reports have identified similar numbers of breast mammography screens in both placebo and treatment groups for

participants of the WHI clinical trial during and following cessation of the study in 2002 (Chlebowski, Hendrix et al. 2003; Chlebowski, Kuller et al. 2009).

The association between increased mammography screening and breast cancer incidence rates have also been disputed in Australia (Kerlikowske, Miglioretti et al. 2007; Canfell, Banks et al. 2008), the USA (Robbins and Clarke 2007) and Europe (Fontenoy, Leux et al. 2010). Furthermore, a Norwegian-based population study that included a cohort of women aged 50-64 yrs demonstrated a similar rate of breast cancer incidence between 1999-2004 in women taking HRT who were undergoing mammography screening compared to a separate group of women also taking HRT who were not undergoing mammography screening (Kumle 2008). Additionally, the latter study reported a similar incidence of breast cancer in non-users in the presence and absence of mammography screening (Kumle 2008). Collectively, these findings oppose the argument that differences in the rates of mammography screening are the causative influence of changes in breast cancer incidence in the general population in women involved in the WHI trial.

It may also be interesting to speculate that increased rates of mammography screening may result in a higher detection rate of cHRT-driven breast cancers. This proposal is supported by data obtained using the California Teachers cohort, and women located in particular Californian counties who are reported to present with some of the highest use of cHRT, incidence of invasive breast cancers and mammography screening rates (Marshall, Clarke et al.; Bernstein, Allen et al. 2002; Clarke, Glaser et al. 2002; Robbins and Clarke 2007). An emerging concept of cHRT-driven breast cancer growth is the

potential activation of pre-existing dormant breast cancer cells (Almog; Dietel, Lewis et al. 2005; Horwitz and Sartorius 2008; Almog, Ma et al. 2009). The biological actions of cHRT have been reported in the literature to promote the development of pre-malignant lesions, which may alternatively undergo spontaneous regression in the absence of exogenous hormone stimulation (Dietel, Lewis et al. 2005; Zahl, Maehlen et al. 2008). Thus, the greater rates of mammography screens may in fact be adding to an increased detection of small, clinically detectable cHRT-driven breast cancer tumours in addition to non cHRT-related breast cancers. In contrast, it has been suggested that cHRT-driven breast cancers may be difficult to detect by routine mammography as a result of the following: 1) increase in breast tissue density in women taking cHRT (Daling, Malone et al. 2002; Newcomb, Titus-Ernstoff et al. 2002) and 2) a higher rate of lobular type breast cancer in post-menopausal women taking cHRT, that are difficult to detect by current mammography screening techniques (Stomper, Van Voorhis et al. 1990; Marugg, van der Mooren et al. 1997; Greendale, Reboussin et al. 2003).

In summary, the findings in this chapter report: 1) statistical analysis confirming proof-of-principle from other established international studies of a positive association between the prevalence change in cHRT preparations containing MPA and age-standardised breast cancer incidence rates in Australian women and 2) the use of the synthetic progestin MPA, contributes to the highest percentage of all cHRT preparations available on the PBS in Australia. In conclusion these findings emphasise the high public health-related concern associated with the use of the synthetic progestin MPA in cHRT within the Australia female population.

Chapter 4

Characterisation of steroid receptor expression in pre-menopausal and post-menopausal human breast tissue

4.1 - Introduction

The actions of the ovarian hormones oestrogen and progesterone that are mediated through the ERα and PR respectively, in normal human breast tissue biology and cancer are well documented in the current literature (Henderson 2003). The contributory role of AR-signalling in human breast tissue has also been emerging over the years to play a significant role to maintain normal breast hormone homeostasis, by acting in a protective manner to reduce basal and oestrogen induced breast epithelial proliferation (Birrell, Bentel et al. 1995; Zhou, Ng et al. 2000).

In subsequent chapters of this thesis, experimental studies have been undertaken to investigate the effects of MPA on breast epithelial cell proliferation, survival and AR-signalling in both pre- and post-menopausal non-malignant human breast tissue samples using an established *ex vivo* breast explant tissue culture model (Zhuang, Saaristo et al. 2003). The use of both pre- and post-menopausal breast tissue for the studies undertaken in this thesis was based on the literature reporting that the use of the progestogen-only contraceptive (POC) Depo-Provera comprised of MPA can result in increased rates of breast cancer in women < 35 yrs of age (Skegg, Noonan et al. 1995; Skegg, Paul et al. 1996; Shapiro, Rosenberg et al. 2000) in addition to cHRT containing MPA in post-menopausal women (Rossouw, Anderson et al. 2002; Beral 2003). Furthermore, Depo-Provera is the highest prescribed POC in Australian women (approximately 2 million prescriptions in Australia from 1992-2009) *(data not shown)*. Thus, in order to accurately assess the effects of MPA on DHT-induced AR-signalling, breast epithelial cell

proliferation and survival, it was essential to undertake studies to determine potential similarities and differences between the steroid receptor expression profiles in pre- and post-menopausal breast tissue that may influence hormonal responsiveness.

To date, the relative differences in AR, ERα and PR expression within and between pre- and post-menopausal non-malignant breast tissue are not well known. Since two-thirds of breast cancers arise in post- compared to pre-menopausal women (Pike, Krailo et al. 1983) and androgens act via the AR to reduce ERα-driven oestrogen stimulated breast epithelial proliferation (Henderson, Ross et al. 1982; Aspinall, Stamp et al. 2004), it was important to elucidate the differences and similarities in steroid receptor expression in pre- compared to post-menopausal breast tissue. Therefore, the main focus of the studies undertaken in this chapter were to better characterise AR expression compared to ERα and PR and to determine a comparative ratio analysis (AR:ERα, ERα:PR and AR:PR) within and between non-malignant pre- and post-menopausal breast tissue samples. Furthermore, since the breast tissue samples used in this study were obtained from pre- and post-menopausal women diagnosed with either non-malignant or malignant disease, a separate comparative analysis of the steroid receptor expression profiles was additionally undertaken of breast tissues that were non-associated and associated with malignancy. The hypothesis of this chapter was that a different steroid receptor expression profile (AR, ERα and PR) exists between human non-malignant pre- and post-menopausal breast tissues, and between non-malignant breast tissues associated and non-associated with malignancy leading to altered hormonal responsiveness.

4.2 - Methods

Refer to Chapter 2 - General Materials and Methods for details on Materials *(section 2.1),* Buffers and Solutions *(section 2.2)* and Methods *(sections 2.31 - 2.33 and 2.35 - 2.37)* included in this chapter.

4.2.1 - Statistical analysis

Statistical analysis was performed to determine differences in the base-line level of steroid receptor expression (AR, ERα and PR) between non-cultured non-malignant pre- and post-menopausal breast explant tissues. In addition, statistical analysis of the differences in AR, ERα and PR expression within the post-menopausal breast tissue samples following division into two sub-groups was completed (i.e. non-associated and associated with malignancy). Statistical analysis was performed using the non-parametric Mann Whitney U paired test (Graph Pad Prism Version 5.02). Statistical significance in all analysis was set at a p-value <0.05.

4.3 - Results

4.3.1 - Steroid receptor immunoreactivity (AR, ERα and PR) within and between non-cultured, non-malignant pre- and post-menopausal human breast tissues

In order to determine and compare the relative base-line levels of AR, ERα and PR within and between pre- and post-menopausal non-malignant breast epithelial cells, immunostaining and VIA for quantification of steroid receptor immunoreactivity was

performed on separate pieces of non-cultured breast explant tissue samples [Figure 4.1]. Immunoreactivity for AR in both pre- and post-menopausal breast explant tissue samples was evident in the nuclear and cytoplasm cellular compartments of the breast epithelium (luminal and myoepithelial cells), and in the nuclear compartment of the breast stromal cells (fibroblasts) [Figure 4.1]. In contrast, ERα and PR immunoreactivity was detected only in the breast epithelium (luminal epithelial cells only) and was localised to the nuclear region [Figure 4.1].

AR immunoreactivity was markedly higher compared to ERα and PR in both non-cultured pre- and post-menopausal breast epithelial cells [Figure 4.2A-D]. Overall, a similar ERα and PR expression level was observed in either pre- or post-menopausal breast epithelial cells [Figure 4.2A-D]. These findings are only reported as observational and no statistical tests were performed to compare the immunoreactivity of the different steroid receptor proteins within pre- and post-menopausal breast tissue samples, due to potential differences in the affinities and specificities of the antibodies used in this study (i.e. AR vs. ERα and ERα vs. PR). No statistical difference of AR, ERα or PR immunoreactivity (i.e. AR vs. AR and ERα vs. ERα) was identified in either pre- or post-menopausal breast epithelial cells [Figure 4.2A-D]. The lack of significance in the change of steroid receptor expression between the two breast tissue types may be based on the low sample patient numbers used in this study.

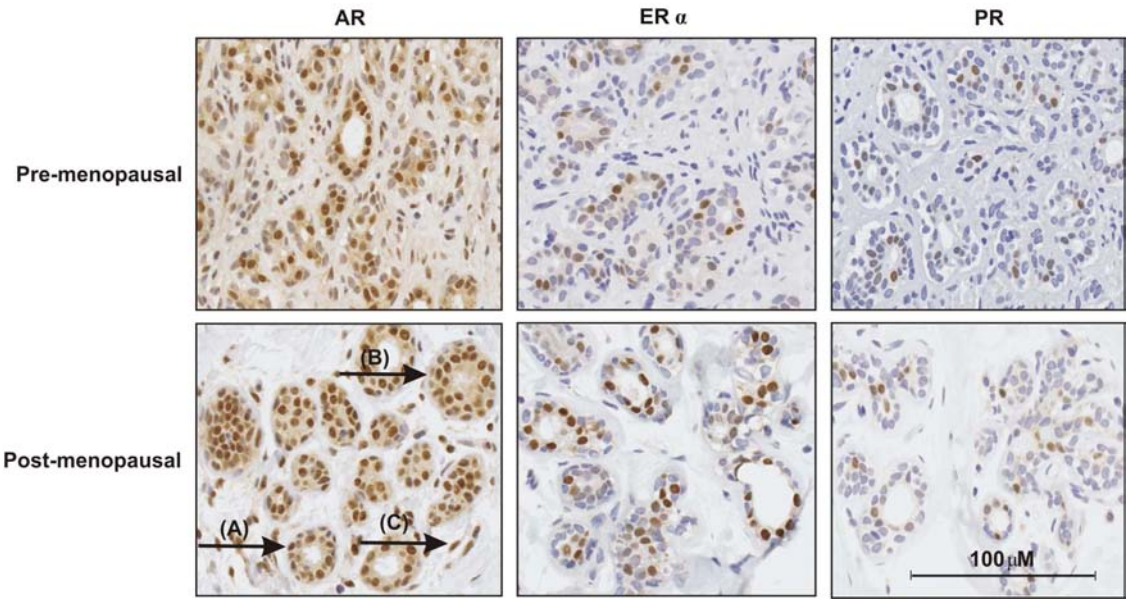


Figure 4.1: AR, ERα and PR immunoreactivity in non-cultured, non-malignant human breast tissues. Representative images of AR, ERα and PR immunoreactivity in non-cultured, non-malignant pre- and post-menopausal breast tissue samples. AR is detected in both the cytoplasm and nucleus of the breast epithelial cells and in nucleus of stromal cells, whereas ERα and PR are predominantly detected only in the nucleus of breast epithelial cells. Arrows on images depict: (A) luminal breast epithelial cells; (B) myoepithelial cells and (C) stromal cells. Images were digitally scanned and taken using a 40x objective microscopic lens.

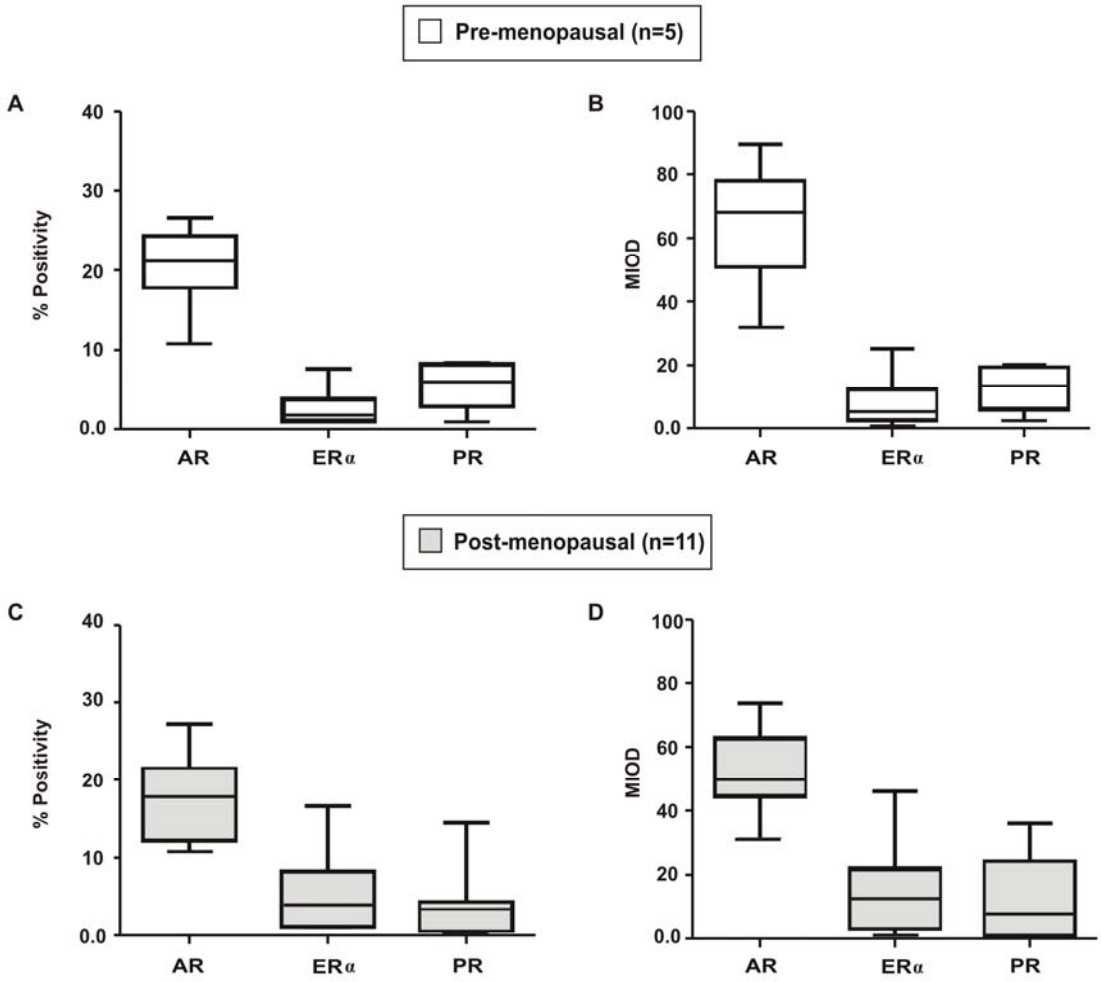


Figure 4.2 (A-D): Higher proportion of AR expressing breast epithelial cells, compared to ERα and PR in non-cultured, non-malignant breast tissues. Comparisons between relative base-line expression levels of AR, ERα and PR were determined in non-cultured **(A and B)** pre- (n=5) and **(C and D)** post-menopausal (n=11) patient breast tissues. Immunostaining of AR, ERα and PR expression levels were quantified using **(A and C)** DAB positive area (% positivity) and **(B and D)** mean integrated optical density (MIOD) VIA measurements. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median. One data patient sample was excluded from the pre- and post-menopausal patient groups due to the lack of glands within some tissue sections.

4.3.2 - Comparative analysis in the ratios of AR, ERα and PR immunoreactivity in pre- and post-menopausal non-cultured, non-malignant human breast tissues

To gain some further insight into potential differences between AR, ERα and PR expression between pre- and post-menopausal patient groups, additional analysis was undertaken in this section to compare the following steroid receptor ratios including: 1) AR:ERα; 2) ERα:PR and 3) AR:PR. Observational studies were only performed in this section based on the different affinities of antibodies of the steroid receptor proteins. An approximate 4-fold higher AR:ERα ratio expression was observed in pre- compared to post-menopausal breast epithelial cells for both VIA measurements [Figure 4.3A-D]. In contrast, the ERα:PR expression ratio was approximately 2-fold higher in post- compared to pre-menopausal breast tissue, whilst the AR:PR expression ratio remained similar [Figure 4.3A-D]. These findings suggest that there may be differences in the steroid receptor expression profiles based on menopausal status. However since no statistical tests were performed in this studies described in this section, the findings can only be speculative.

4.3.3 - ERα expression is increased in non-malignant breast tissue of post-menopausal women diagnosed with malignancy, compared to non-malignant disease

The breast tissue patient samples used in this analysis were obtained from women undergoing surgery for benign or malignant breast disease, in addition to a partial or bilateral prophylactic mastectomy. During the surgical procedure, the breast tissue samples were obtained under specified surgical guidelines, and were either adjacent or distal to benign or malignant breast tissue *(refer to chapter 2, section 2.3.2).* Analysis of

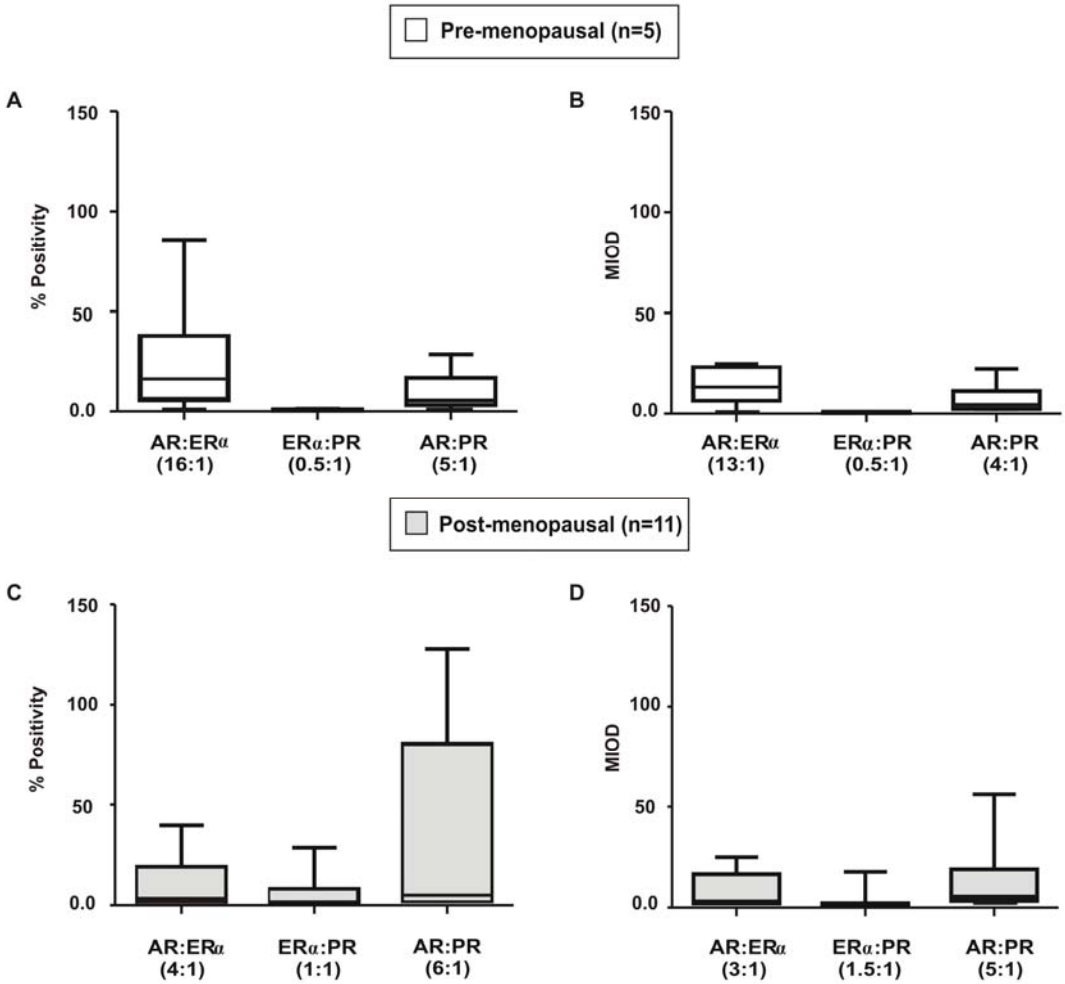


Figure 4.3 (A-D): Differences in the AR, ERα and PR ratios within pre- or post-menopausal non-cultured, non-malignant human breast tissues. Differences in the ratios of AR, ERα and PR immunoreactivity were determined in non-cultured **(A and B)** pre-menopausal (n=5) and **(C and D)** post-menopausal (n=11) breast tissue samples. Immunostaining of AR, ERα and PR expression levels were quantified using **(A and C)** DAB positive area (% positivity) and **(B and D)** mean integrated optical density (MIOD) VIA measurements. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median. One data patient sample was excluded from both the pre- and post-menopausal patient groups due to the lack of glands within some tissue sections.

AR, ERα and PR immunoreactivity was specifically undertaken to determine any potential differences in steroid hormone responsiveness, in breast tissue associated with non-malignant compared to malignant disease. The specific types of non-malignant and malignant disease have been previously described *(refer to chapter 2, section 2.3.2).* Non-malignant post-menopausal patient samples presented with significantly higher ERα immunoreactivity in breast tissue from women diagnosed with malignant (n=5), compared to non-malignant disease (n=6) ($p \leq 0.05$) [Figure 4.4A and B]. No statistical analysis was performed on the pre-menopausal breast tissue samples separated into the same sub-groups since there was only one breast tissue sample from a patient diagnosed with malignant disease [Figure 4.5 A and B].

4.4 - Discussion

The main findings described in this chapter using non-cultured, non-malignant human female breast tissues provided observational evidence of: 1) a markedly higher AR expression compared to ERα and PR in both pre- and post-menopausal breast tissues and 2) a reduction in the AR:ERα and increase in the ERα:PR expression ratio in post- compared to pre-menopausal breast tissues. Additionally a significant increase in ERα expression in post-menopausal breast tissue obtained from women diagnosed with malignant disease, compared to non-malignant disease was identified.

It was important for the subsequent studies undertaken in this thesis to investigate the effect of MPA on breast epithelial proliferation, survival and AR-signalling in human

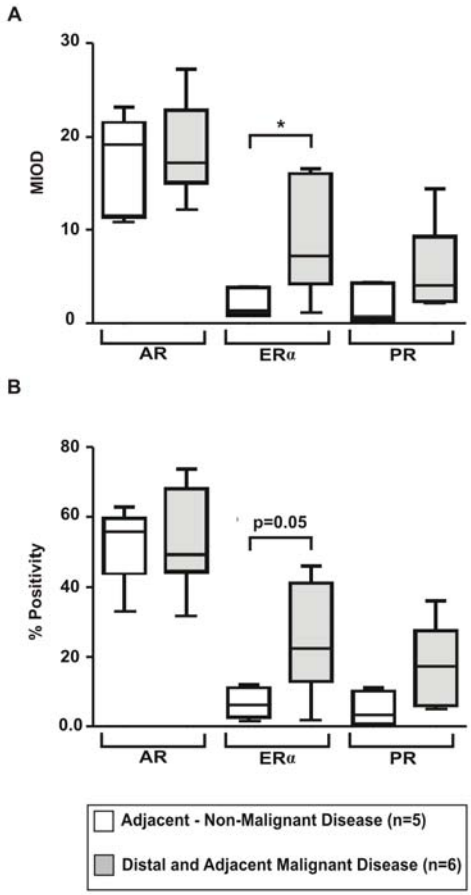


Figure 4.4 (A and B): Differences in AR, ERα and PR immunoreactivity in non-cultured, non-malignant post-menopausal breast tissue from women diagnosed with either non-malignant or malignant disease. The expression of AR, ERα and PR immunoreactivity were compared in non-cultured, non-malignant breast epithelial cells in post-menopausal breast tissues obtained from women diagnosed with either non-malignant (n=5) or malignant (n=6) breast disease. Immunohistochemical quantification was performed using **(A)** mean integrated optical density (MIOD) and **(B)** DAB positive area (% positivity) VIA measurements. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. Statistical analysis was performed using non-parametric Mann Whitney test; *p-value <0.05. One data patient sample was excluded from the analysis due to the lack of glands within tissue section. DAB positive area (% positivity) and **(B and D)** mean integrated optical density (MIOD).

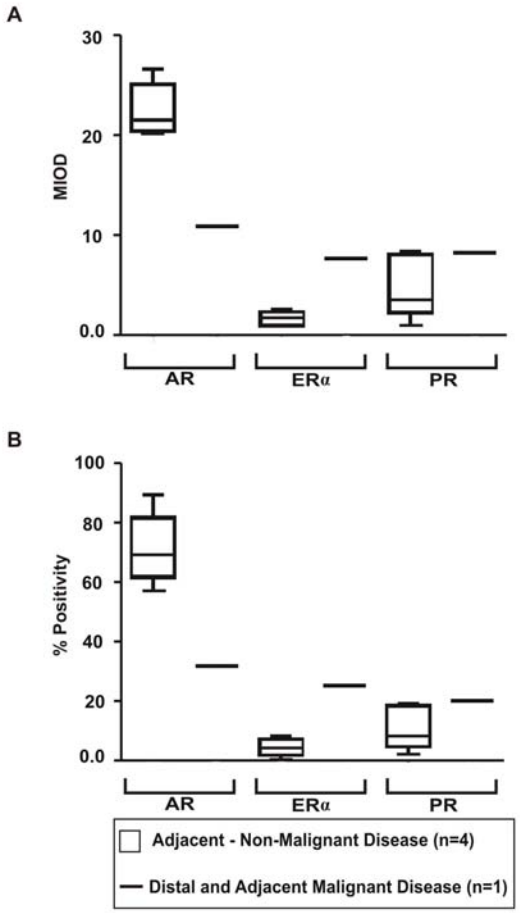


Figure 4.5 (A and B): Differences in AR, ERα and PR immunoreactivity in non-cultured, non-malignant pre-menopausal breast tissue from women diagnosed with either non-malignant or malignant disease. The expression of AR, ERα and PR immunoreactivity in non-cultured, non-malignant breast epithelial cells in post-menopausal breast tissues obtained from women diagnosed with either non-malignant (n=4) or malignant (n=1) breast disease. Immunohistochemical quantification was performed using **(A)** mean integrated optical density (MIOD) and **(B)** DAB positive area (% positivity) VIA measurements. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. One data patient sample was excluded from the patient groups due to the lack of glands within tissue section.

non-malignant breast tissue samples that possessed normal steroid receptor (AR, ERα and PR) expression. This is based on well-established findings in the literature demonstrating that the activity of ERα and PR are involved in paracrine signalling within breast epithelial and stromal cells to regulate the development, proliferation, apoptosis and differentiation of the breast, as well as the initiation and progression of breast cancer (Cunha, Young et al. 1997; Humphreys, Lydon et al. 1997; Cunha, Cooke et al. 2004). In addition, the AR is expressed at a high frequency in both normal and malignant breast tissues (Dimitrakakis and Bondy 2009), and can act to regulate a number of processes involved in breast tissue biology (Dimitrakakis and Bondy 2009). Immunohistochemical analysis of pre- and post-menopausal non-cultured, non-malignant breast tissue samples included in this study indicated that all the tissues were positive for AR, ERα and PR protein.

The biological roles of ERα and PR in human normal and malignant breast tissue have been investigated extensively in the literature (Henderson 2003). Additionally, AR expression is also emerging as playing a pivotal role in human breast cancer (Dimitrakakis and Bondy 2009). This has been reflected in several clinical studies that have shown that the AR acts as an independent prognostic factor in breast cancer by an improved overall survival (Gonzalez-Angulo, Stemke-Hale et al. 2009; Peters, Buchanan et al. 2009; Castellano, Allia et al. 2010). Moreover, the potential importance of AR expression in breast cancer has been highlighted by studies reporting a much higher percentage of breast cancers that express the AR (70-90%), compared to ERα (55-80%) and PRA and PRB isoforms (45-65%) (Lea, Kvinnsland et al. 1989; Kuenen-Boumeester,

Van der Kwast et al. 1992; Soreide, Lea et al. 1992; Isola 1993; Moinfar, Okcu et al. 2003; Riva, Dainese et al. 2005; Rosa, Caldeira et al. 2008).

Findings described in this chapter that have previously been observed in malignant breast tissue have reported markedly higher expression of AR, than either ERα or PR in both normal pre- and post-menopausal breast tissues. Since MPA can bind with high affinity to the AR, in addition to the PR and GR (Sitruk-Ware 2004), the levels of AR expression are essential to investigate in relation to the understanding of the molecular basis associated with the actions of MPA and the development of breast cancer in post-menopausal women. Furthermore, based on the findings in this chapter demonstrating that a high proportion of pre- and post-menopausal breast epithelial cells express the AR, it is likely that some of the carcinogenic effects by MPA may occur directly via AR-mediated actions. However, this does not suggest that all of the carcinogenic actions by MPA are mediated via AR-signalling and additional effects in the breast tissue are likely to be promoted via PR- and/or GR-mediated actions as discussed in a subsequent section of this thesis *(refer to Chapter 9, section 9.1.3).* The GR is reported to be expressed predominantly in the myo-epithelial breast cells in both normal and malignant breast epithelial cells (Lien, Lu et al. 2006). Additionally, the expression of the GR has been shown to be dramatically reduced in over 98% of malignant breast lesions, compared to normal breast tissue (Lien, Lu et al. 2006). The use of glucocorticoids for the treatment of breast cancer are often used clinically, yet they are associated with acquired resistance to chemotherapy (Vaidya, Baldassarre et al. 2010). These findings suggest that the actions of the GR do play an important role in breast cancer and the effects of MPA via GR-

mediated mechanisms leading to the increased development of breast cancer are also important to consider, yet were outside the scope of this thesis.

In all breast tissues AR, ERα and PR ranged in expression, which is to be expected based on the inherent heterogeneity that exists within the female population. The few studies that have examined steroid receptor protein levels in non-malignant breast tissue were obtained from breast reduction mammoplasties, and from women diagnosed with benign or malignant breast disease (Kuttenn, Fournier et al. 1981; Petersen, Hoyer et al. 1987; Ricketts, Turnbull et al. 1991; Walker 1992; Li, Han et al. 2010). Even though these studies are informative, breast reduction mammoplasties are often associated with hyperplasia and other pathological characteristics linked to increased carcinoma risk (Ishag, Bashinsky et al. 2003; Ambaye, MacLennan et al. 2009) and may therefore exhibit different biological characteristics that can regulate breast epithelial proliferation. Whilst this notion may be true, breast tissues from these women in the population should not be excluded from research studies since they to are still susceptible to carcinogenic insults (Ambaye, MacLennan et al. 2009). Moreover there may be common molecular changes that are linked to the development of breast cancer, irrespective of breast size.

This study is the first to observationally report a reduction of the AR:ERα ratio in conjunction with an increased ERα:PR expression ratio in post- compared to pre-menopausal non-malignant breast epithelial cells. The change in steroid receptor ratio expression may be associated with the decline in serum sex steroid hormone production during the menopausal transistion. Furthermone based on these findings one could

speculate that post-menopausal women are more susceptible to breast cancer development than pre-menopausal women as a result of altered steroid receptor mediated hormonal homeostasis. The mechanisms underlying these biological changes may be insightful into future preventative breast cancer therapeutics that can mimic and maintain the molecular patterns that exist within pre-menopausal breast tissue during the menopausal transition. Moreover, in light of the published findings from the DRMCRL, reporting that MPA can act as an antagonist of DHT-induced AR-signalling (Birrell, Butler et al. 2007), the effect of MPA on AR-signalling could be of higher detriment in post-menopausal compared to pre-menopausal breast tissue. This notion is based on the lower AR expression relative to ERα expression that already exists.

In support of the findings reported in this chapter previous studies have shown that ERα expression increases with age and reaches maximal levels in non-malignant breast tissue during menopause (Walker, Price-Thomas et al. 1991; Walker, McClelland et al. 1992; Shoker, Jarvis et al. 1999; Walker and Martin 2007; Jarzabek, Koda et al. 2009). Additionally, a significant increase in ERα protein has been reported in breast lobules in contrast to no change in the breast ducts of post- compared to pre-menopausal women (Walker, Price-Thomas et al. 1991). The change in ERα expression based on menopausal status may be driven by the higher rate of adipose aromatase expression (Misso, Jang et al. 2005). Furthermore, increased circulating levels of bio-available testosterone in post-menopausal women as a result of a decline in SHBG may provide a higher level of substrate for aromatase conversion to E_2 (Burger, Dudley et al. 2000). Alternatively, the ERα protein levels that increase with age may be a compensatory mechanism that occurs

as a physiological adaptation to reduced circulating oestrogen levels during the menopause transition (Lea, Kvinnsland et al. 1989). In addition to the reduction in oestrogen serum levels that occur during menopause, the androgenic hormones including DHEA, A and T have also been shown to reduce in ageing women (Labrie, Belanger et al. 1997; Labrie, Luu-The et al. 2003). This reduction in androgens may potentially lead to a reduction in AR expression, by a loss in AR stabilisation and/or increased protein degradation.

Intriguingly, unreported findings in the literature that were detected in this study indicated an increase in ERα protein in non-malignant breast tissues obtained from women diagnosed with malignant disease in post-menopausal breast tissues. Since serum oestrogens have been reported to increase in breast cancer and are implicated as a driving-force of breast cancer development in women (Henderson 2003), an increase in ERα expression in breast tissue obtained from women diagnosed with malignant disease may be expected. The rationale for this statement is based on the studies that have reported a higher expression of ERα in benign breast tissue from women diagnosed with breast cancer compared to no disease (Khan, Rogers et al. 1994). The driving stimulus that leads to an increase in serum oestrogen levels and thus an increase in ERα expression or vice versa is mechanistically unknown. The molecular basis associated with the increase of ERα expression in non-cultured non-malignant breast tissue samples from women diagnosed with malignant breast tissue may be initiated by the actions of paracrine mediated effects of malignant cancerous breast epithelial cells. To clarify further on this notion, it is possible that there is increased activation of ERα in non-

malignant breast epithelial cells via the paracrine mediated actions of tyrosine growth factor intracellular signalling pathways from neighbouring cancerous breast epithelial cells. In particular the insulin growth factor (IGF)/PI3K signalling pathway has been reported at a much higher frequency in breast cancer and has been shown to be involved in the regulation of ERα (Lee, Weng et al. 1997; Fagan and Yee 2008; Yee 2009). Based on the literature findings and the data in this chapter, it is possible that subtle biological and/or molecular changes are involved in a shift in both serum hormone and steroid receptor expression during the development of breast cancer and further studies are required to investigate this notion.

In summary, the findings in this chapter suggest that: 1) the predominance of steroid receptor expression may vary in human pre- and post-menopausal breast tissue; 2) the menopausal transition leads to different steroid receptor expression profiles and 3) that non-malignant breast tissue from women diagnosed with malignant disease may respond differently to steroid hormones compared to non-malignant breast tissue non-associated with malignant disease. These initial studies have provided an understanding of the different capacities of hormonal responsiveness in breast tissue types based on menopausal status and association with malignancy that allows for a more accurate account of the susceptibility to carcinogenesis, mediated by alterations to steroid receptor expression and/or activity.

Chapter 5

Development of an *ex vivo* breast explant tissue culture model to study the effects of MPA on DHT-induced AR-signalling

5.1 - Introduction

The main focus of the studies described in this chapter were to further develop an *ex vivo* breast explant tissue culture model that maintains functional AR-signalling in order to investigate potential AR-mediated carcinogenic effects of MPA in human breast epithelial cells. The use of an *ex vivo* breast explant tissue culture model was considered the optimal experimental approach to undertake these studies based on the ability to examine the effects of MPA in a cellular environment that is closely representative of normal human tissue biology after considering the use of other experimental models discussed below.

In vitro two-dimensional (2D) cell culture models using human malignant and non-malignant breast epithelial cell lines are common experimental approaches used to investigate molecular mechanisms linked to breast cancer (Ethier 1996). *In vitro* three-dimensional (3D) co-culture models have also been developed to allow for breast epithelium and stromal cells to be grown together using either collagen or Matrigel (Dhimolea, Maffini et al.; Shekhar, Werdell et al. 2001). Alternatively *in vivo* approaches using animal models (i.e. carcinogen-induced (e.g. DMBA), xenograft or observational studies) are also common experimental models widely used to study human breast cancer (Russo, Gusterson et al. 1990). These include *in vivo* xenograft models whereby transplantation of non-malignant human breast tissue suspended in Matrigel is surgically placed into the fat pads of mouse mammary glands (Sheffield and Welsch 1988), and non-malignant human epithelial cells suspended in Matrigel or human breast tissue explants are implanted under the skin of athymic nude mice (McManus and Welsch

1981; McManus and Welsch 1984; Laidlaw, Clarke et al. 1995; Clarke, Howell et al. 1997). Additionally, cynomlogus macaques, which are reported to have a similar anatomical mammary gland structure and reproduction to humans are often used in *in vivo* experimental studies (Cline, Soderqvist et al. 1996; Cline, Register et al. 2002; Isaksson, Wang et al. 2003; Stute, Wood et al. 2004).

The *in vitro* and *in vivo* experimental models described above have contributed significantly to studying breast cancer. However, there are a number of limitations in the application of these experimental models to study human breast cancer as follows. The restrictions of using non-malignant and malignant *in vitro* (2D) cell culture models include: 1) the lack of stromal-epithelial interactions (Clarke, Dickson et al. 1992; Cunha, Young et al. 1997; Cunha, Cooke et al. 2004); 2) a high variation in steroid receptor expression profiles (Ethier 1996) and 3) the high rate of *in vitro* senescence, loss of steroid receptor expression and the high rates of spontaneous immortalisation (i.e. cells that no longer undergo cell death) that occurs after short-term culture (Whitescarver, Rechier et al. 1968; Ham and McKeehan 1978; Russo, Mills et al. 1989; Wolman, Mohamed et al. 1994). Moreover, the limiting factor associated with the use of *in vitro* 3D co-culture models is the differences in structural composition of breast epithelial and stromal cells that are formed in Matrigel or collagen, compared to normal human breast tissue structures (Dhimolea, Maffini et al. 2010). In 3D co-culture models, the formation of ductal breast structures occurs predominantly in the upper layers of the collagen gel, whereas the acini breast structures form predominantly in the middle and lower layers of the collagen gel. This is considerably different from the structural architecture of breast

tissue that is observed in normal human breast tissue whereby the distribution of breast structures is more uniform (Dhimolea, Maffini et al. 2010). The use of *in vivo* animal model experimental systems includes other circulating endogenous hormonal pathways produced from the pituitary, adrenal and ovarian glands that prevents specific hormonal effects to be studied (Labrie, Luu-The et al. 2003). Additionally, there are vast differences in rodent mammary gland developmental biology, structural composition and function compared to human mammary glands (Russo, Gusterson et al. 1990).

The main benefits of using an *ex vivo* human breast explant tissue culture model to undertake the aims and objectives of the studies outlined in this thesis which cannot be obtained from the alternative experimental approaches described above are as follows: 1) the model allows for the maintenance of a normal human breast tissue structure and consequently normal epithelial-stromal paracrine-mediated interactions in a 3D environment, which are a requirement in normal breast biology for functional paracrine signalling (McGrath 1983; Clarke, Dickson et al. 1992; Cunha, Young et al. 1997; Silberstein 2001; Cunha, Cooke et al. 2004); 2) provides an experimental system whereby specific hormonal effects can be studied in the absence of other confounding actions by endogenous hormones within human breast tissue and 3) allows for the inherent biological heterogeneity that exists in the female population to be integrated into the experimental findings.

The use of an *ex vivo* breast explant culture system has been previously used as an experimental model to study the effects of natural and synthetic hormones predominantly

on ERα and PR expression and signalling (Zhuang, Saaristo et al. 2003; Eigeliene, Harkonen et al. 2006; Eigeliene, Harkonen et al. 2008). However, the use of this experimental approach has not previously been used to study AR-signalling in human non-malignant breast epithelial cells. Thus, the main focus of the studies undertaken in this chapter were aimed at developing an *ex vivo* breast explant tissue culture model in a steroid hormone-deprived environment to study DHT-induced AR-mediated effects of MPA. In order to implement this experimental model in subsequent studies in this thesis, studies were undertaken in this chapter to demonstrate maintenance of functional AR-signalling, post culture in a steroid hormone-deprived environment that included the following: 1) preservation of steroid receptor expression; 2) addition of an exogenous hormone was able to modulate AR expression at a protein level and 3) preservation of a functional AR-signalling axis by the modulation of AR expression and the well-characterised AR target gene PSA by DHT and/or the addition of the potent AR antagonist Bic. Hence, the hypothesis of the studies described in this chapter was that functional AR-signalling is maintained in cultured human breast tissue samples allowing for the investigation of DHT-induced AR-mediated effects of MPA associated with the development of breast cancer.

5.2 - Methods

Refer to Chapter 2 - General Materials and Methods for details on Materials *(section 2.1),* Buffers and Solutions *(section 2.2)* and Methods *(sections 2.3.1 - 2.3.7 and 2.3.10)* included in this chapter.

5.2.1 - Histopathology assessment of breast explant tissues

H&E staining of breast explant tissues was used to perform histopathology assessment on non-cultured and cultured tissue samples. Digital images of the tissue sections stained by H&E were generated using a tissue section scanner, which allowed for a high power image to be viewed on a computer using NDP software. The non-malignant status of the breast explant tissues (normal acini and ductal breast structures) was confirmed by a qualified pathologist (Dr. Shalini Jindal, IMVS, Adelaide, SA) using standard histopathological criteria. This histopathological assessment was undertaken on breast tissue sections using NDP software at a 40x magnification in all glandular areas of the tissue section.

5.2.2 - Statistical analysis

The statistical analysis using non-parametric tests were performed to determine relative differences in AR, ERα and PR expression in non-cultured compared to cultured breast tissue samples included the following; Mann Whitney U paired test, Kruskal Wallis one way analysis of variance (ANOVA) or paired t-test (Graph Pad Prism Version 5.02). Statistical significance in the analysis was set at p-value <0.05.

5.3 - Results

5.3.1 - Histopathological assessment of non-cultured and cultured, non-malignant breast explant tissues

The focus of this thesis was aimed primarily at investigating the effects of MPA in non-malignant breast tissues to understand the molecular basis associated with increased breast cancer development in post-menopausal women taking cHRT. Therefore, in order to undertake subsequent studies, initially the non-malignant status of all breast tissue samples included in the analysis was verified. All the non-cultured and cultured breast tissue samples included in this study were verified as histologically non-malignant [Figure 5.1; 5.2; 5.3]. No visible change in the histopathological status of the acinar and/or ductal breast tissue structures in cultured compared to non-cultured breast tissue samples was observed [Figure 5.1; 5.2; 5.3]. However, the acinar breast tissue structures displayed different characteristics based on menopausal status [Figure 5.2; 5.3]. These difference are based on the higher percentage of lobule type 1 breast structures in post-menopausal compared to pre-menopausal breast tissue following the decline in ovarian sex steroid hormone production and the high rate of regression via involution back to a premature biological state during the menopausal transition (Treloar 1981; Russo and Russo 2004) [Figure 5.3]. In comparison, the pre-menopausal breast tissues included in this study were obtained predominantly from parous women, whereby the breast tissue is in a highly differentiated state and has attained a higher percentage of lobule type 2 and 3 breast tissue structures, thus appearing more condensed and tightly packed (Russo and Russo 2004) [Figure 5.2].

5.3.2 - Changes in AR, ERα and PR immunoreactivity in cultured compared to non-cultured, non-malignant human breast explant tissues

Immunostaining was performed to determine the effect of culturing pre- and post-menopausal breast explant tissues in media supplemented with 10% DCC-FCS under control conditions, i.e. 0.1% ethanol on AR expression by measuring and comparing base-line AR immunoreactivity in non-cultured (day 0) and cultured (24 or 48 hrs) breast tissue samples [Figure 5.4]. Quantification of AR immunostaining was undertaken using MIOD and % positivity VIA measurements. In both pre- and post-menopausal breast explant tissue samples a decrease in AR immunoreactivity following 24 or 48 hrs of culture compared to day 0 was observed for both VIA measurements ($p<0.001$) [Figure 5.5A and B]. A decrease in AR immunoreactivity was observed at 48 hrs compared to 24 hrs in cultured pre-menopausal breast tissue sample ($p<0.01$), yet was not detected in post-menopausal tissue samples [Figure 5.5A and B]. A significant reduction of AR % positivity in non-cultured post- compared to pre-menopausal breast tissues was also identified in the analysis ($p<0.05$) [Figure 5.5B]. The detection of a significant reduction of AR expression in non-cultured post- compared to pre-menopausal breast tissues in this chapter was not similarly identified in the previous chapter. The difference in this finding is most likely based on the higher number of individual groups (i.e. 6) included in the analysis and the use of an ANOVA test in this chapter compared to the use of a Mann Whitney U paired test in the previous chapter since there were only two test groups *(refer to chapter 4 - section 4.3.1)*.

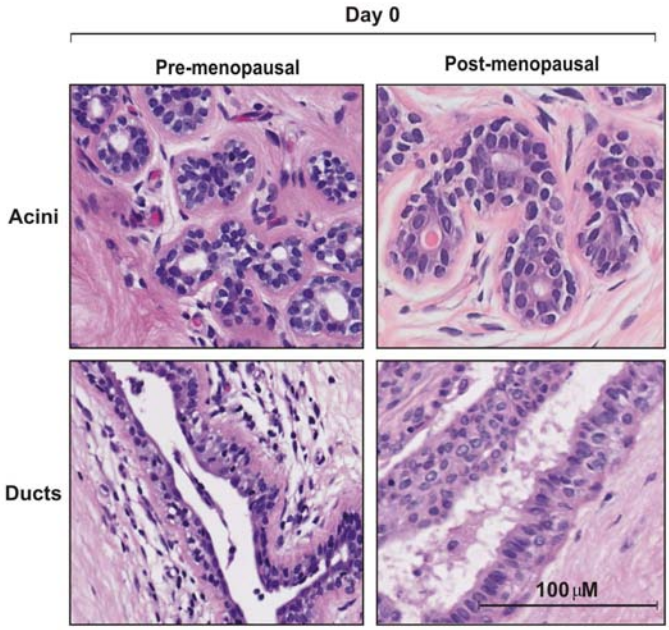


Figure 5.1: Histopathology assessment of non-cultured human pre- and post-menopausal breast explant tissue. Representative images of H&E staining in human breast explant tissue (acini and ductal breast structures) in non-cultured (Day 0) pre- and post-menopausal breast tissues. H&E staining was performed on 4% paraformaldehyde-fixed paraffin embedded, non-cultured breast tissue samples. Histopathological assessment of breast acini and ductal structures was performed by the qualified pathologist Dr. Shalini Jindal (IMVS, Adelaide, SA). Images were digitally scanned and taken using a 40x objective microscopic lens.

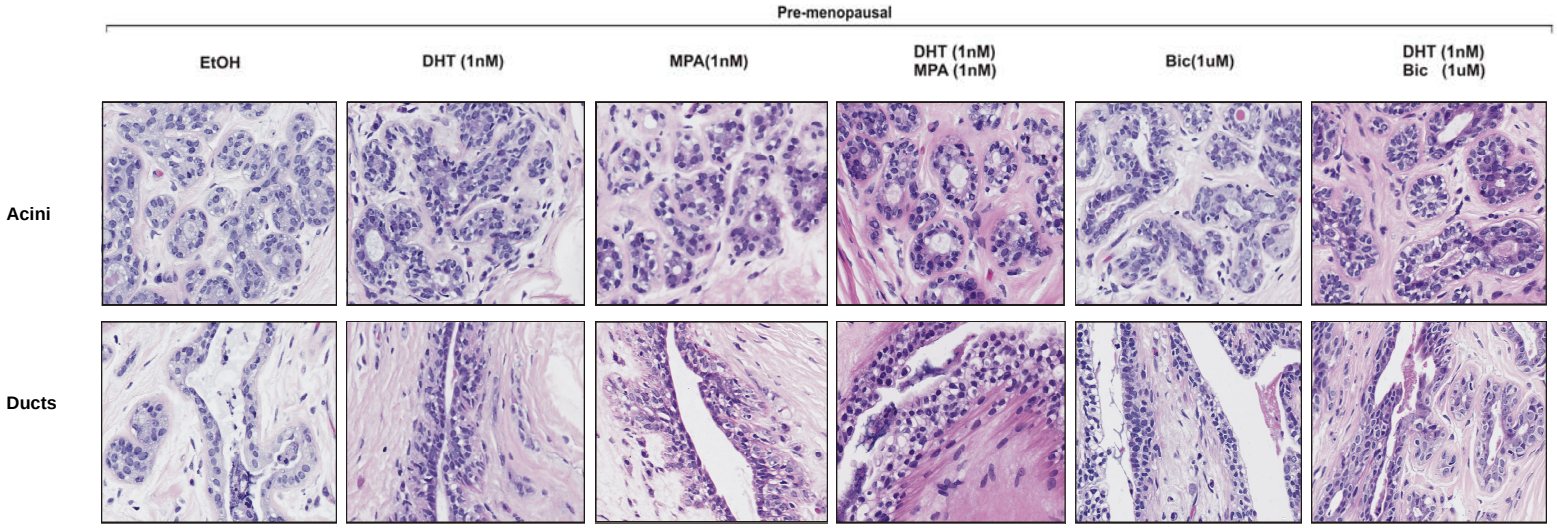


Figure 5.2: Histopathology assessment of cultured human pre-menopausal breast explant tissue following hormone treatments. Representative images of H&E staining in human breast explant tissue (acini and ductal breast structures) following culture in hormone treatments described below for 48 hrs following an initial pre-treatment period for 36 hrs. H&E staining was performed on 4% paraformaldehyde-fixed paraffin embedded pre-menopausal breast tissue, and breast tissue cultured in explant media supplemented with 10% DCC-FCS under control conditions, i.e. 0.1% ethanol, or treated with 1 nM DHT, 1 nM MPA, 1 nM DHT and 1 nM MPA combined, 1 μM Bic, or 1 nM DHT and 1 μM Bic combined, for 24 or 48 hrs. Histopathological assessment of breast acini and ductal structures was performed by the qualified pathologist Dr. Shalini Jindal (IMVS, Adelaide, SA) (images not shown for 24 hrs). Images were digitally scanned and taken using a 40x objective microscopic lens.

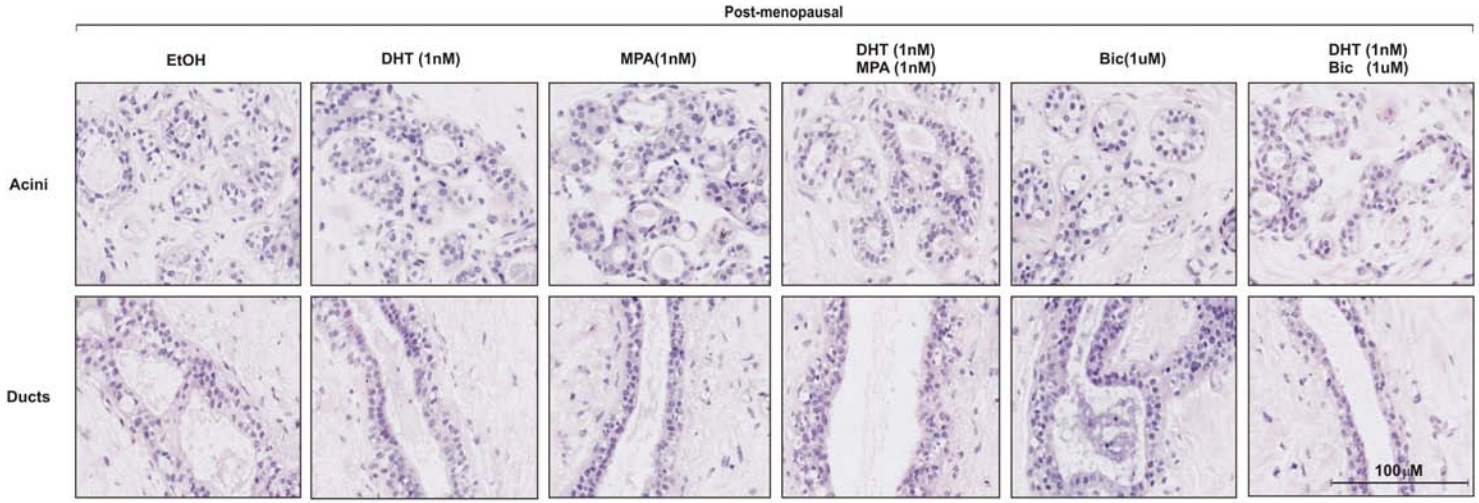


Figure 5.3: Histopathology assessment of cultured human post-menopausal breast explant tissue following hormone treatments. Representative images of H&E staining in human breast explant tissue (acini and ductal breast structures) following culture in hormone treatments described below for 48 hrs following an initial pre-treatment period for 36 hrs. H&E staining was performed on 4% paraformaldehyde-fixed paraffin embedded post-menopausal breast tissue, and breast tissue cultured in explant media supplemented with 10% DCC-FCS under control conditions, i.e. 0.1% ethanol, or treated with 1 nM DHT, 1 nM MPA, 1 nM DHT and 1 nM MPA combined, 1 µM Bic, or 1 nM DHT and 1 µM Bic combined, for 24 or 48 hrs. Histopathological assessment of breast acini and ductal structures was performed by the qualified pathologist Dr. Shalini Jindal (IMVS, Adelaide, SA) (images not shown for 24 hrs). Images were digitally scanned and taken using a 40x objective microscopic lens.

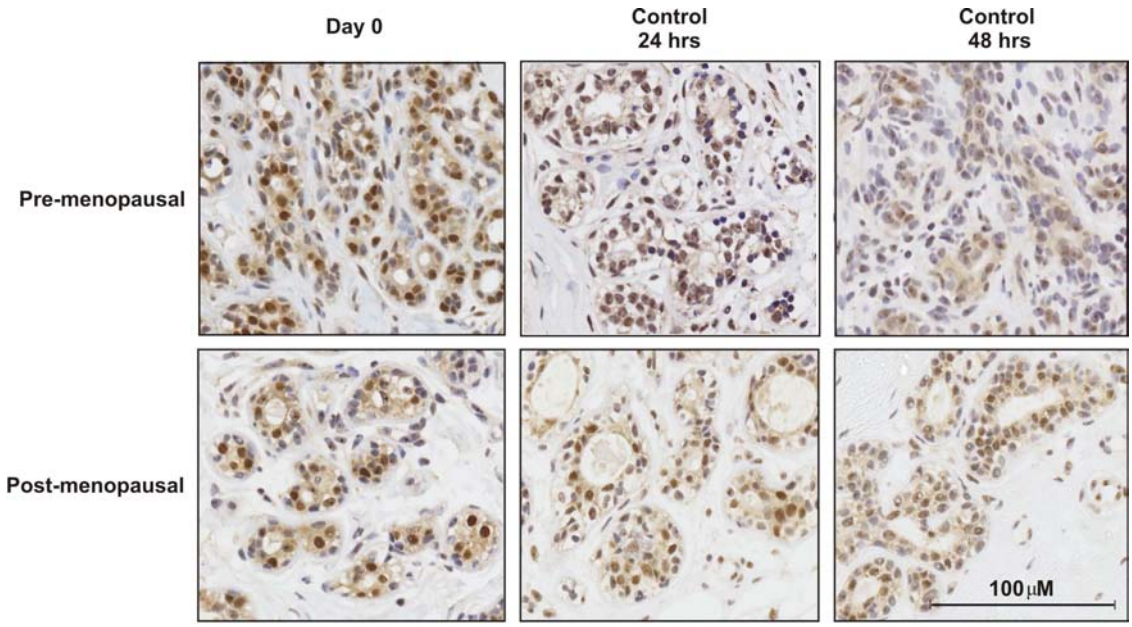


Figure 5.4: AR immunoreactivity in non-cultured and cultured, non-malignant pre- and post-menopausal human breast tissue. Representative images of AR immunostaining in non-cultured or cultured pre- and post-menopausal breast explant tissues following an initial pre-treatment period for 36 hrs and subsequently cultured for 24 and 48 hrs in media supplemented with 10% DCC-FCS under control conditions, i.e. 0.1% ethanol. Images were digitally scanned and taken using a 40x objective microscopic lens.

Analysis was also undertaken in this study to compare ERα and PR expression changes in cultured compared to non-cultured post-menopausal breast tissues. The focus of these studies was to further determine whether a similar reduction in ERα and PR immunoreactivity was observed as reported for the AR using the same experimental conditions described above. ERα and PR immunoreactivity significantly declined following culture for 48 hrs compared to non-cultured post-menopausal breast tissue samples ($p<0.05$) [Figure 5.6A and B].

5.3.3 - Examination of a functional AR-signalling axis using an ex vivo breast explant tissue model

Experiments were performed to determine whether AR immunoreactivity could be modulated in pre- and post-menopausal breast explant tissue samples following an initial 36 hr pre-treatment culture period and subsequent treatment in 1 nM DHT for 24 and 48 hrs in steroid hormone-deprived environmental conditions. Immunostaining of AR protein in breast explant tissue samples was undertaken following culture under control conditions, i.e. 0.1% ethanol, and after treatment with 1 nM DHT for 48 hrs [Figure 5.7A]. An increase in AR immunoreactivity was detected in pre- and post-menopausal breast explant tissues following treatment with 1 nM DHT, compared to 0.1% ethanol, at 48 hrs for both MIOD and % positivity VIA measurements ($p<0.05$) [Figure 5.7B and C]. The fold increase was slightly more pronounced in pre- versus post-menopausal breast tissue samples [Figure 5.7B and C]. Interestingly, AR immunoreativity also increased in pre-menopausal breast tissues after treatment with 1 nM DHT for 24 hrs for both VIA measurements (approximate 1.5-fold increase, $p<0.05$), yet this effect was not

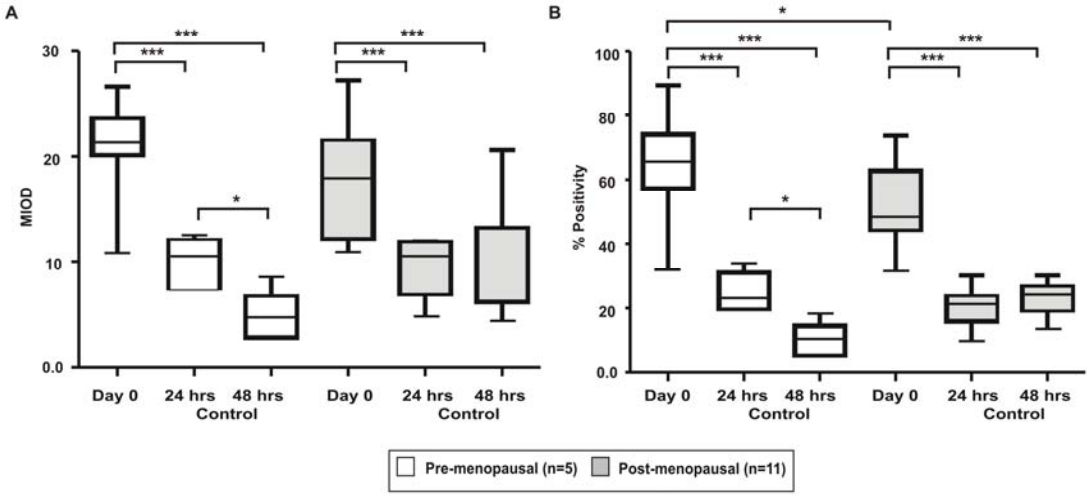


Figure 5.5 (A and B): Reduction in AR immunoreactivity subsequent to culture in steroid-deprived conditions, in non-malignant pre- and post-menopausal breast explant tissues. AR immunoreactivity was quantified in pre- (n=5) and post-menopausal (n=11) breast explant tissues before (day 0) and after an initial pre-treatment period for 36 hrs and subsequently cultured for 24 and 48 hrs in media containing 10% DCC-FCS under control conditions, i.e. 0.1% ethanol (control). AR immunoreactivity in breast epithelial cells located in the breast ducts and lobules was quantified using **(A)** mean integrated optical density (MIOD) and **(B)** DAB positive area (% positivity) VIA measurements. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. Statistical analysis was performed within and between patient groups using the non-parametric statistical tests Kruskal-Wallis one way ANOVA for comparison between all groups and Mann Whitney tests for individual comparisons; * p-value <0.05, ** p<0.01 and *** p<0.001. One data patient sample was excluded from the pre- and post-menopausal patient groups due to the lack of glands within some tissue sections.

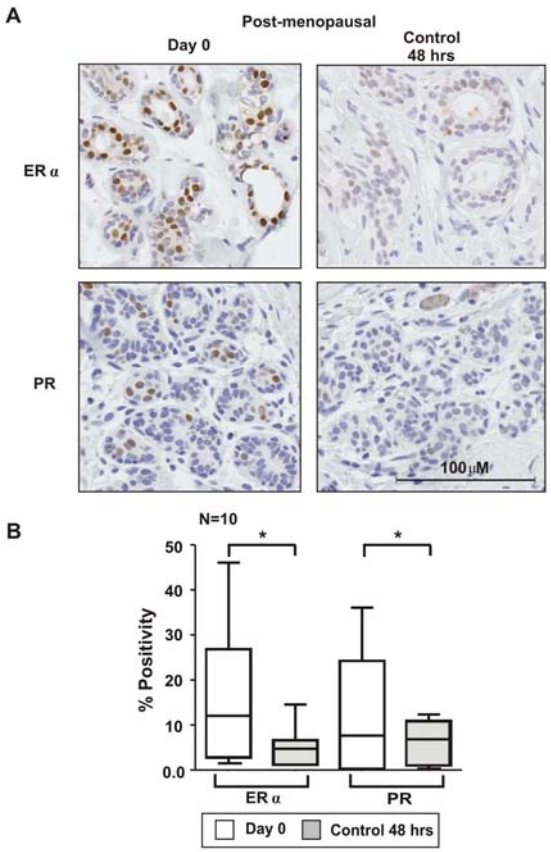
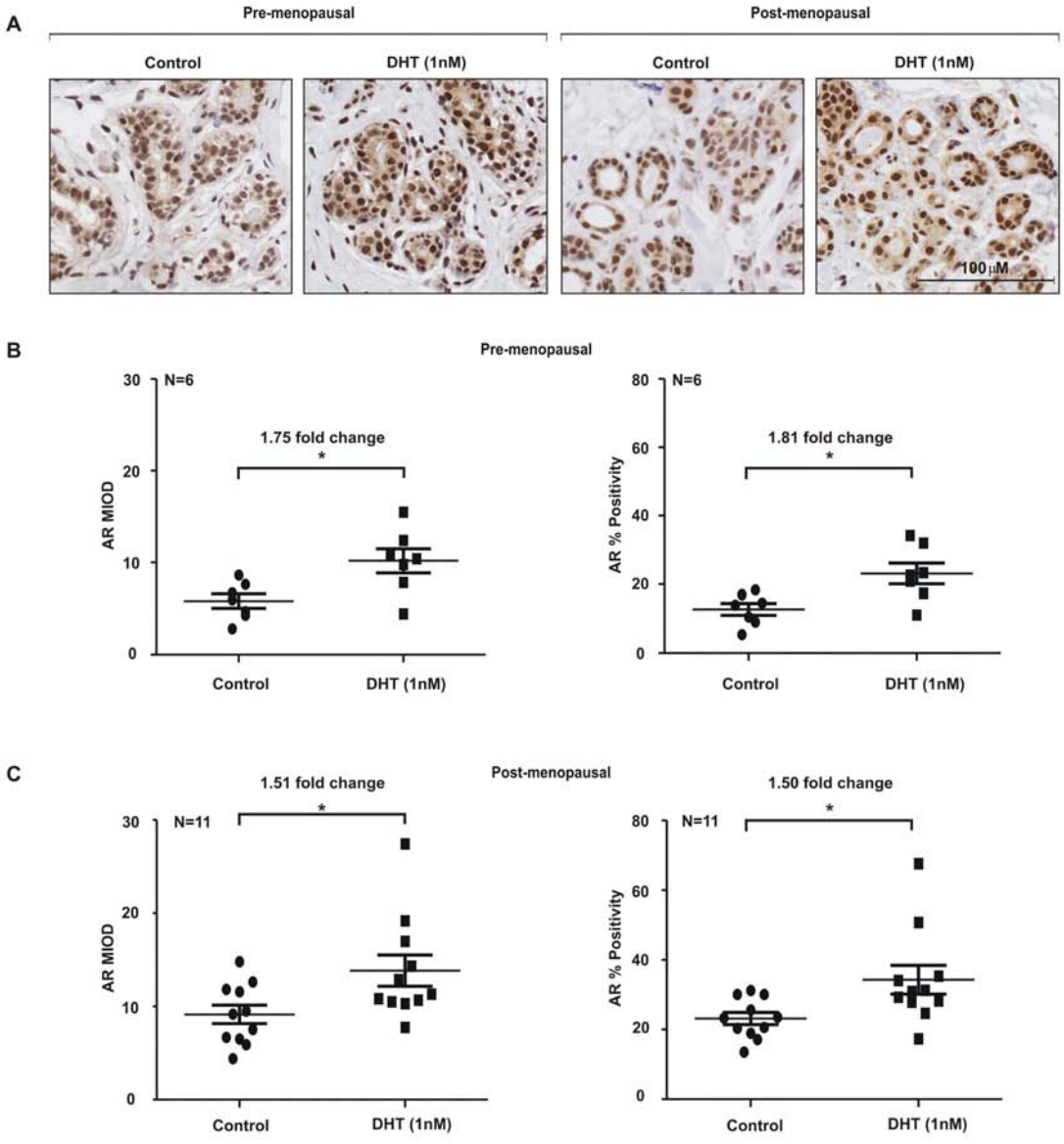


Figure 5.6 (A and B): Decreased ERα and PR immunoreactivity following culture in steroid-hormone deprived conditions in non-malignant, post-menopausal breast explant tissues. (A) Representative images of ERα and PR immunostaining in post-menopausal breast explant tissues (n=10) following an initial pre-treatment period for 36 hrs and subsequently cultured for 48 hrs in media containing 10% DCC-FCS under control conditions, i.e. 0.1% ethanol. Images were digitally scanned and taken using a 40x objective microscopic lens. **(B)** ERα and PR immunoreactivity in breast epithelial cells located in the breast ducts and lobules was quantified using the VIA mean integrated optical density (MIOD) and DAB positive area (% positivity) measurements *(data not shown for VIA MIOD measurement)*. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. Statistical analysis was performed using non-parametric t-test; *p-value <0.05. Two data patient samples were excluded due to the lack of glands within some tissue sections.

Figure 5.7 (A-C): Increased AR immunoreactivity following treatment with DHT in cultured, non-malignant pre- and post-menopausal breast explant tissues. (A) Representative images of AR immunostaining in pre- and post-menopausal breast explant tissues following an initial pre-treatment period for 36 hrs and subsequently cultured in media containing 10% DCC-FCS under control conditions, i.e. 0.1% ethanol or treated with 1 nM DHT for 48 hrs. Images were digitally scanned and taken using a 40x objective microscopic lens. AR immunostaining was quantified using the VIA mean integrated optical density (MIOD) and DAB positive area (% positivity) measurements in **(B)** pre- (n=6) and **(C)** post-menopausal (n=11) breast explant tissue samples. Data is presented as individual AR MIOD and % positivity VIA measurements (i.e. average of all fields captured per tissue section), and the median value. Statistical analysis was performed using Wilcoxon matched pairs test; *$p<0.05$. One data patient post-menopausal patient sample was excluded from the pre- and post-menopausal patient groups due to the lack of glands within some tissue sections.


A
Pre-menopausal
Post-menopausal
Control
DHT (1nM)
Control
DHT (1nM)
100 μM
B
Pre-menopausal
N=6
1.75 fold change
*
AR MIOD
30
20
10
0
Control
DHT (1nM)
N=6
1.81 fold change
*
AR % Positivity
80
60
40
20
0
Control
DHT (1nM)
C
Post-menopausal
1.51 fold change
*
N=11
AR MIOD
30
20
10
0
Control
DHT (1nM)
1.50 fold change
*
N=11
AR % Positivity
80
60
40
20
0
Control
DHT (1nM)

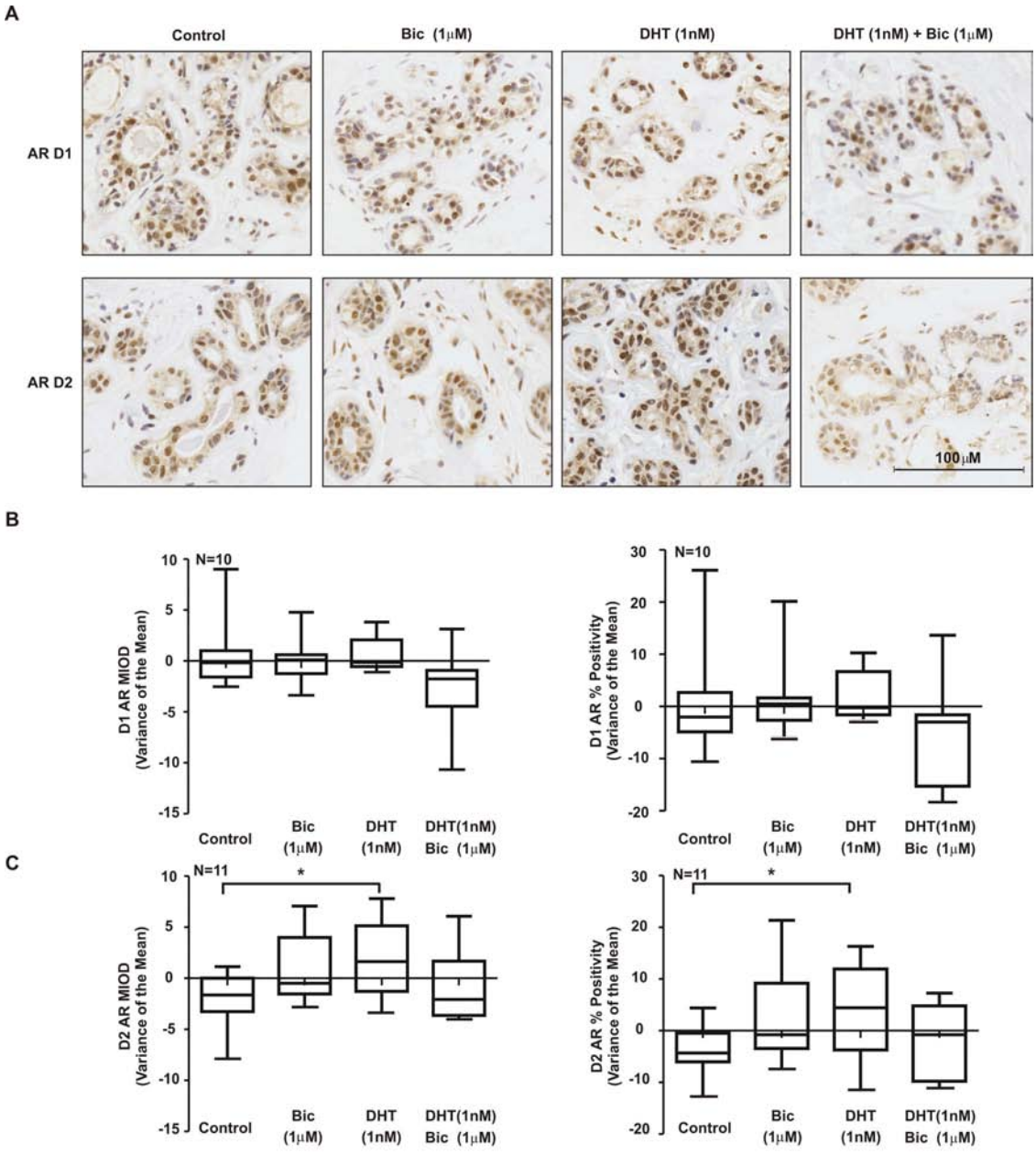
observed in post-menopausal breast tissues at the same time-point *(data not shown)*. This effect may be associated with a higher stabilisation of the AR following treatment with DHT in pre- compared to post-menopausal breast tissues.

Given these findings indicating the potential ability of DHT to modulate AR expression in cultured breast tissues, further studies were designed with the aim of investigating whether a functional AR-signalling axis was preserved using the *ex vivo* breast explant tissue experimental model. Immunostaining was used to determine the baseline levels of AR protein in the post-menopausal breast explant tissue samples cultured in 0.1% ethanol, or treated with 1 nM DHT and/or 1 μM Bic for 24 or 48 hrs [Figure 5.8A]. Following 24 hrs of culture with 1 nM DHT and/or 1 μM Bic, no change in AR immunoreactivity was detected, compared to control tissue [Figure 5.8B]. However, following 48 hrs of treatment with 1 nM DHT, AR immunoreactivity increased above control tissue expression levels which was impeded by the addition of 1 μM Bic ($p<0.05$) [Figure 5.8C].

5.3.4 - An impaired AR-signalling axis in non-malignant post-menopausal breast explant tissues that are in close proximity to malignant breast disease

A significant increase in ERα expression in post-menopausal non-malignant breast tissue samples associated with malignant disease compared to non-malignant breast tissue was detected in a previous chapter *(refer to chapter 4 - section 4.3.3)*. Therefore, based on these findings demonstrating differences in steroid receptor expression within post-menopausal non-malignant breast tissues associated with malignancy, further statistical

Figure 5.8 (A-C): Preservation of AR-signalling in cultured, non-malignant post-menopausal breast explant tissues. (A) Representative images of AR immunostaining in post-menopausal breast explant tissues were initially pre-treated for 36 hrs and subsequently cultured at 24 (D1) and 48 hrs (D2) in media containing 10% DCC-FCS under control conditions, i.e. 0.1% ethanol, or treated with 1 μM Bic and/or 1 nM DHT. Images were digitally scanned and taken using a 40x objective microscopic lens. Quantification of AR immunostaining is presented as normalised data using the VIA mean integrated optical density (MIOD) and DAB positive area (% positivity) measurements at **(B)** 24 hrs (D1) and **(C)** 48 hrs (D2). Statistical analysis was performed using Wilcoxon matched pairs test; * $p<0.05$. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. Two samples were excluded from D1, and one sample from D2 time-point due to lack of glandular tissue in some tissue sections. (*Refer to Appendix 2 - Table 1A for VIA quantification raw data).*


A
Control
Bic (1μM)
DHT (1nM)
DHT (1nM) + Bic (1μM)
AR D1
AR D2
100μM
B
N=10
D1 AR MIOD (Variance of the Mean)
D1 AR % Positivity (Variance of the Mean)
Control
Bic (1μM)
DHT (1nM)
DHT(1nM) Bic (1μM)
C
N=11
*
D2 AR MIOD (Variance of the Mean)
D2 AR % Positivity (Variance of the Mean)

analysis in this chapter was aimed at investigating whether functional AR-signalling was evident in post-menopausal breast explant tissues non-associated (i.e. either distal to malignant or adjacent to benign breast tissue) (n=6) or adjacent to malignant breast disease (n=5). In post-menopausal breast explant tissues non-associated with malignant breast disease, AR immunoreactivity was significantly increased by 1 nM DHT treatment, compared to 0.1% ethanol which was significantly reversed by 1 μM Bic ($p<0.05$) [Figure 5.9A]. In contrast no change in AR immunoreactivity was detected in breast explant tissue adjacent to malignant breast tissue [Figure 5.9B].

To investigate the effects of AR-signalling in cultured post-menopausal breast explant tissues based on association with malignancy further, the well-characterised androgen regulated gene PSA in the breast (Magklara, Grass et al. 2000; Paliouras and Diamandis 2008) and prostate (Schuur, Henderson et al. 1996) was used as a read-out of active DHT-induced AR-signalling. In all the post-menopausal breast tissue included in this study, the immunoreactivity levels of PSA was detected at very low and even non-detectable expression levels in the absence of hormone in some tissue samples. The immunoreactivity of PSA in post-menopausal breast tissues was predominantly expressed in the cytoplasm of breast epithelial cells in control, 1 μM Bic and/or 1 μM DHT treated breast tissues [Figure 5.10A]. However, in the presence of 1 nM DHT, PSA immunoreactivity was detected in the breast epithelial luminal and myoepithelial nuclear and cytoplasm cellular compartments, in addition to the stromal tissue and fibroblast cells [Figure 5.10A]. An increase in PSA MIOD was detected following treatment with 1 nM DHT compared to vehicle control, in breast tissues non-associated with malignant disease

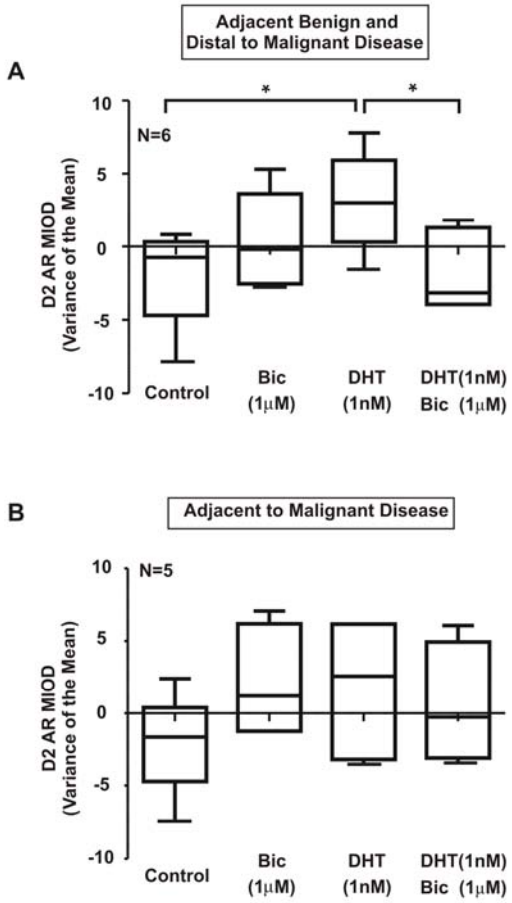


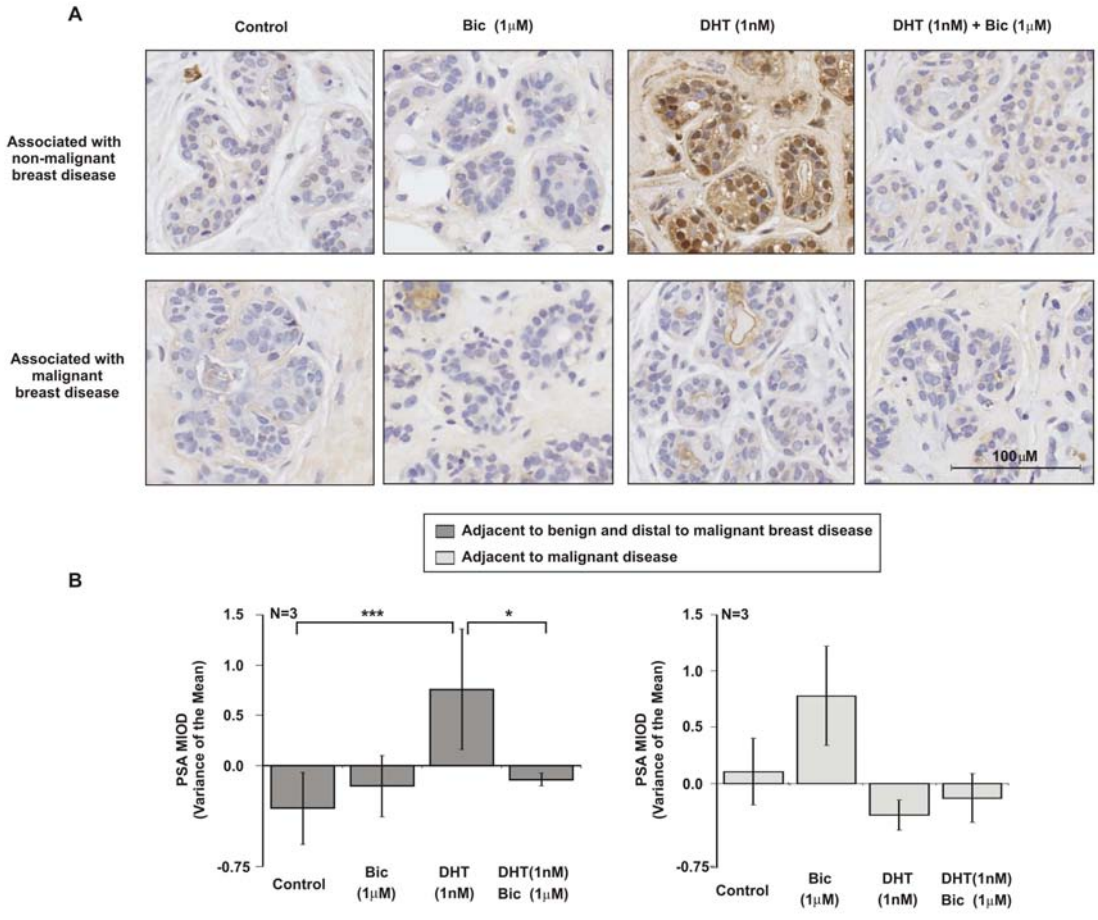
Figure 5.9 (A and B): Impaired AR-signalling in cultured, non-malignant post-menopausal breast tissue adjacent to malignant breast tissue. Post-menopausal breast explant tissues were initially pre-treated for 36 hrs and subsequently cultured for 48 hrs (D2) in media containing 10% DCC-FCS under control conditions, i.e. 0.1% ethanol, or treated with 1 μM Bic and/or 1 nM DHT. Quantification of AR immunostaining is presented as normalised data of AR using the VIA mean integrated optical density (MIOD) measurement in breast explant tissues **(A)** non-associated (n=6) or **(B)** adjacent to malignant breast tissue (n=5). Statistical analysis was performed using Wilcoxon matched pairs test; * $p<0.05$. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. Similar data observed for VIA % positivity measurement *(data not shown).* (*Refer to Appendix 2 - Table 1B for VIA quantification raw data).*

(n=3) ($p<0.001$) [Figure 5.10B]. Furthermore, a reversal in this induction by DHT was detected following treatment with 1 μM Bic ($p<0.05$) in the same tissue samples [Figure 5.10B]. In contrast, PSA MIOD remained similar to vehicle control following treatment with 1 nM DHT, in breast tissues adjacent to malignant breast tissue, and neither 1 μM Bic alone or combined with 1 nM DHT treatments resulted in any differences in PSA expression (n=3) [Figure 5.10B]. These findings in conjunction with the AR expression data demonstrate that non-malignant breast tissue in close proximity to malignant disease exhibits an impaired AR-signalling axis and should be considered as possessing different responsiveness to natural and synthetic hormones.

5.4 - Discussion

The objective of this chapter was to establish an *ex vivo* non-malignant breast explant tissue culture model that possesses functional AR-signalling characteristics. The findings described in this study indicated that non-malignant breast explant tissues cultured in steroid hormone-deprived conditions led to the following: 1) a greater reduction in AR expression in pre- compared to post-menopausal breast tissues; 2) a reduction in ERα and PR expression in post-menopausal breast tissues; 3) preservation and modulation of an intact DHT-induced AR-signalling axis in tissues from women non-associated with malignancy and 4) that functional DHT-induced AR-signalling was impaired in non-malignant post-menopausal breast tissue in close proximity to malignancy.

Figure 5.10 (A and B): De-regulation of the AR regulated gene PSA, in post-menopausal breast explant tissue adjacent to malignant breast disease. (A) Representative examples of PSA immunostaining in post-menopausal breast explant tissues non-associated or adjacent to malignant breast disease following an initial pre-treated period for 36 hrs, and subsequently cultured for 24 (D1) and 48 hrs (D2) in culture media containing 10% DCC-FCS under control conditions, i.e. 0.1% ethanol, or treated with 1 μM Bic and/or 1 nM DHT. Images were digitally scanned and taken using a 40x objective microscopic lens. **(B)** Quantification of PSA immunostaining is presented as normalised data of PSA using the VIA mean integrated optical density (MIOD) measurement for cultured post-menopausal breast explant tissue not associated (n=3) and adjacent to and malignant breast disease (n=3). Statistical analysis was performed using non-parametric Mann Whitney paired test using patient raw data; * $p<0.05$; $p<0.001$. Similar data observed for VIA DAB positive area (% positivity) measurement, *(data not shown).* Some tissues were excluded from the analysis due to low to absent PSA immunoreactivity, and lack of glandular areas in some tissue sections. (*Refer to Appendix 2 - Table 2 for VIA quantification raw data).*


A
Control
Bic (1μM)
DHT (1nM)
DHT (1nM) + Bic (1μM)
Associated with non-malignant breast disease
Associated with malignant breast disease
100μM
Adjacent to benign and distal to malignant breast disease
Adjacent to malignant disease
B
PSA MIOD (Variance of the Mean)
N=3
***
*
1.5
1.0
0.5
0.0
-0.75
Control
Bic (1μM)
DHT (1nM)
DHT(1nM) Bic (1μM)

The breast tissues included in this thesis were all verified as histologically non-malignant. Moreover, cultured and non-cultured breast tissues possessed similar breast tissue acinar and ductal breast structural characteristics, indicating no detriment of culture on the histopathological status of the breast tissue samples. These findings are in accordance with a previous study that reported no change in morphology of non-malignant breast tissue structural integrity following short-term culture for 48 hrs (Eigeliene, Harkonen et al. 2006). Longer-term breast explant culture studies have also demonstrated no detriment to breast tissue morphology in the absence of additional hormone (i.e. similar ductal and acini breast tissue structures were observed in non-cultured breast tissue samples), after 21 days in steroid hormone deprived media (Eigeliene, Harkonen et al. 2006), and up to 2 months in steroid hormone supplemented medium (Zhuang, Saaristo et al. 2003). These studies in conjunction with the findings in this chapter indicate that culture of breast tissue is not susceptible to any histopathological changes that may alter the effects of hormone treatment.

The ability of DHT to modulate AR expression and signalling using the *ex vivo* breast explant tissue model has been developed in this chapter. The model has been utilised by other groups to demonstrate normal responsiveness to exogenous hormones. It has been shown previously that oestrogen and progesterone combined promote mammary development using an *ex vivo* breast explant tissue model as evidenced by lobuloalveolar development in pre-menopausal breast explant tissues cultured in steroid hormone-deprived media for up to 5 days (Ceriani, Contesso et al. 1972). Moreover, analysis of the histology and morphology of breast tissue structures (i.e. ducts and acini) following

culture in E_2 and/or MPA for 14-21 days has shown increased thickness of acinar and ductal wall thickness and E_2 stimulated hyperplasia 14-21 days compared to control tissue, yet no evident changes at 7 days (Eigeliene, Harkonen et al. 2006). In conjunction with the findings in this chapter, these studies demonstrate that cultured breast explant tissue samples are biologically responsive to both exogenous hormones and are therefore suitable for experimental studies investigating aspects of hormonally driven breast carcinogenesis.

An important focus of this study was to further validate whether the *ex vivo* breast explant tissue model maintain low levels of expression of steroid receptor protein (AR, ERα and PR) in hormone-deprived culture conditions since it may be postulated that complete loss may abrogate the potential for modulation following hormone treatment. Following culture AR, ERα and PR immunoreactivity was significantly reduced compared to non-cultured breast tissue as expected by the lack of steroid hormone in culture medium. The decline in steroid receptor expression subsequent to culture in steroid-hormone deprived conditions described in this chapter, are supported by previous studies of a similar nature. These include reports of reduced steroid receptor immunoreactivity (ERα and PR) in breast explant tissue following culture in media supplemented with 10% FCS for 7 days, and a weaker but detectable immunoreactivity in 10% DCC-FCS compared to control culture conditions (Zhuang, Saaristo et al. 2003). A slight reduction in ERα and ERβ immunostaining in breast explant tissues cultured for 21 days in media supplemented with 10% DCC-FCS has also been reported (Eigeliene, Harkonen et al. 2006). However,

no study has investigated the change to AR expression following culture in hormone-free conditions using an *ex vivo* breast explant tissue culture model.

Interestingly, in this study pre-menopausal breast tissues were more susceptible to a reduction in AR expression compared to post-menopausal breast tissues subsequent to culture in steroid-hormone deprived conditions for 48 hrs. This biological effect has not been previously reported, but may be based on the change in steroid synthesis following menopause leading to a differences in sex steroid hormone synthesis and metabolism, stability and turnover rates of the AR (van den Brandt, Spiegelman et al. 2000; Key, Appleby et al. 2002; Key, Appleby et al. 2003; Baglietto, English et al. 2009). Additional findings in this chapter demonstrating an earlier AR induction response following treatment with DHT in pre- compared to post-menopausal breast tissues indicate that there are differences in hormone responsiveness between the two tissue types. This finding is potentially based on increased AR stabilisation in the presence of DHT in pre-menopausal breast tissue and does suggest that the menopausal transition leads to altered steroid receptor expression.

The molecular basis underlying an impaired AR-signalling axis in non-malignant breast tissue in close proximity to malignant breast tissue is difficult to elucidate based on the limited amount of literature examining the nature of the AR-signalling axis in breast cancer. Nonetheless recent studies published in the DRMCRL and others using *in vivo* studies in breast cancer cells have demonstrated that the AR can suppress ERα transcriptional activity, by binding directly to consensus ERE located in proximal regions

upstream from ER target genes (Peters, Buchanan et al. 2009). In addition, androgens can suppress the ER signalling axis and decrease the expression of the ER-regulated gene PR in MCF-7 cells, which is reversed by an anti-androgen (MacIndoe and Etre 1980). These findings suggest that androgens do possess anti-oestrogenic effects in breast epithelial cells. Furthermore, in light of previous literature and findings included in this thesis that have identified an increase in ERα expression in non-malignant breast tissue obtained from women diagnosed with malignancy *(refer to chapter 4, section 4.3.3)*, it may be speculated that an impaired AR-signalling axis in non-malignant breast tissue associated with malignant breast tissue may be leading to a loss of the inhibitory actions of the AR on ERα expression, thus resulting in the increase of ERα expression. These findings may also provide insight into pre-initiation events which occur in the development of breast cancer and aid in the advancement of further studies investigating the nature of the AR-signalling axis in pre-malignant and malignant breast tissue.

The impaired AR-signalling in breast tissue within close proximity to malignant breast tissue identified in this chapter, was also shown to translate through to an alteration in DHT-induced AR-signalling of the androgen regulated gene PSA (Cleutjens, van der Korput et al. 1997). Interestingly, studies have reported a high frequency of single point gene mutations in both the PSA gene enhancer and promoter exon regions within breast cancer cell lines (Majumdar and Diamandis 1999), in addition to decreased PSA secretion in nipple aspirate fluid in women diagnosed with invasive breast cancer (Sauter, Daly et al. 1996; Sauter, Klein et al. 2004). The implications of the findings in this study in conjunction with others, may suggest that a decrease in the regulation of the PSA gene is

affected in a similar pattern as reported in breast cancer in normal breast tissue adjacent to malignant disease.

Previous studies have identified a significant increase in ERα and PR expressing breast epithelial cells in pre- and post-menopausal breast tissue adjacent to proliferating breast lesions (Jacquemier, Hassoun et al. 1990). These changes in ERα and PR expression are speculated to occur via autocrine and paracrine mediated signalling via the secretion of growth factors (i.e. IGF-1, epidermal growth factor (EGF) and KITL) from malignant breast epithelial cells that effectively initiate intracellular signalling changes within and to neighbouring breast epithelial cells (Lippman, Dickson et al. 1986). It is possible that autocrine and paracrine intracellular AR-signalling pathways are also altered in malignant breast disease, leading to secondary changes to the AR-signalling axis in neighbouring non-malignant breast epithelial cells. At present the literature is very limited in reporting whether malignant breast tissue can induce pre-neoplastic changes in non-malignant neighbouring breast tissue. Furthermore, the initial cell signalling changes that lead to the development of breast carcinogenesis are not well known.

In summary, the studies reported in this chapter have provided an experimental platform using the *ex vivo* breast explant tissue culture model suitable for use in subsequent experimental studies described in this thesis investigating the actions of MPA on DHT-induced AR-signalling.

Chapter 6

The effect of the synthetic progestin MPA on breast epithelial cell proliferation and survival

6.1 - Introduction

In vitro studies undertaken in the DRMCRL and other laboratories have demonstrated that MPA can act in an antognistic manner on DHT-induced AR signalling (Kemppainen, Langley et al. 1999; He, Bowen et al. 2001; Birrell, Butler et al. 2007). Additionally a recent *in vivo* study has demonstrated that cHRT containing MPA decreases AR expression in mammary glands of ovariectomised females primates (Hofling, Ma et al. 2009). In light of these studies it was hypothesised in this chapter that the anti-androgenic actions of MPA can act via AR-mediated actions to promote breast cancer by increasing breast epithelial proliferation and cell survival in post-menopausal women taking cHRT. Therefore the aim of this chapter was to determine the effects of MPA on breast epithelial cell proliferation and survival, alone and combined with the potent androgen DHT, in human non-malignant pre- and post-menopausal breast tissue samples using an *ex vivo* breast explant tissue culture model.

Despite the population-based findings including the WHI and Million Women's clinical studies, identifying a link between long-term $\geq$ 5 yrs use of cHRT and increased invasive breast cancer in post-menopausal women, compared to either a placebo or ERT alone, the associated molecular mechanisms have not been identified (Rossouw, Anderson et al. 2002; Beral 2003). Nonetheless, an increase in mammographic density, which is also a strong determinant of breast cancer risk (Boyd, Rommens et al. 2005) has been reported in post-menopausal women taking cHRT compared to ERT (Stomper, Van Voorhis et al. 1990; Marugg, van der Mooren et al. 1997; Greendale, Reboussin et al. 1999; Greendale,

Reboussin et al. 2003). Furthermore, *in vivo* studies demonstrate that treatment of female non-human primates with cHRT results in increased ductal and breast epithelial cell proliferation, and overall mammary gland size, compared to those treated with E_2 alone or untreated primates (Cline, Soderqvist et al. 1996; Cline, Soderqvist et al. 1998; Isaksson, Wang et al. 2003; Wood, Register et al. 2007). Additionally, breast epithelial cell proliferation and mammographic density has also been shown to increase in the terminal ductal lobular unit of post-menopausal women taking cHRT compared to ERT alone (Hofseth, Raafat et al. 1999). Similar findings of a relationship between increased breast epithelial proliferation and use of combined oral contraceptives has also been reported (Pike, Spicer et al. 1993). Lastly, a reduction in Ki67 expression, of a median decrease of 31.4%, has been detected in ER-positive, but not ER-negative breast tumours within post-menopausal women following prior surgery cessation of cHRT (Prasad, Boland et al. 2003). Collectively, these studies highlight that cHRT can act to stimulate increased breast epithelial cell proliferation, above oestrogen alone.

The development of breast cancer can be driven by alterations to intracellular signalling pathways (i.e. oncogenes, tumour suppressors, DNA damage and/or gene mutations) that lead to uncontrolled breast epithelial cell proliferation (Brenner and Aldaz 1997; Liehr 1997). A number of biomarkers are used to measure rates of breast tissue proliferation in malignancy which include the following: thymidine incorporation (Meyer, Rao et al. 1977); bromodeoxyuridine uptake (Brd-U) (Thor, Liu et al. 1999); proliferating cell nuclear antigen (PCNA) (Haerslev, Jacobsen et al. 1996); s-phase fractions and mitotic index (Olszewski, Darzynkiewicz et al. 1981) and Ki67 (Gerdes, Lemke et al. 1984).

Androgens acting via the AR-signalling axis are thought to play a protective role in the development of breast cancer by inhibiting basal and oestrogen-stimulated breast epithelial cell proliferation. These biological actions of androgens in the breast tissue have been demonstrated *in vitro* using breast cancer cell lines (Poulin, Baker et al. 1988; Hackenberg, Luttchens et al. 1991; Birrell, Bentel et al. 1995; Szelei, Jimenez et al. 1997; Ando, De Amicis et al. 2002) and *in vivo* studies using primates treated with AR agonists and inhibitors (Zhou, Ng et al. 2000; Dimitrakakis, Zhou et al. 2003). Clinically, it has been strongly suggested that androgens also have anti-proliferative actions in human breast tissue (Somboonporn and Davis 2004; Dimitrakakis and Bondy 2009; Peters, Buchanan et al. 2009). Collectively, these studies indicate that the suppression of breast epithelial cell proliferation by androgen action in breast tissue is mediated by AR-signalling. Although this hypothesis has been widely implicated in the literature from both clinical and non-human primate studies (Dimitrakakis and Bondy 2009), the anti-proliferative effects of androgens in human non-malignant pre- and post-menopausal breast tissue has not been definitively proven. In order to further investigate the effects of MPA and/or DHT in human breast tissues on changes to breast epithelial proliferation studies were undertaken in this chapter by measuring changes to the proliferation biomarker Ki67.

It has been proposed that the most commonly used synthetic progestin in cHRT, MPA, may act in part to promote mammary cancer by increased breast epithelial proliferation and by the protection against DNA-damage-induced cell death (Schramek, Leibbrandt et al. 2010). The initiation of programmed cell death (apoptosis) can be inhibited by the

activation of pro-survival and anti-apoptotic factors that are common to the bcl-2 family (Hengartner 2000). Bcl-2 is a proto-oncogene which was first identified in follicular non-Hodgkin lymphomas and is predominantly involved in suppressing cell death and increasing cell survival (Hockenbery, Nunez et al. 1990; van Slooten, van de Vijver et al. 1998). *In vitro* studies using the ZR-75-1 breast cancer cell line have reported a reduction of E_2 stimulated bcl-2 mRNA and protein expression following treatment with DHT (Lapointe, Fournier et al. 1999). This effect was subsequently reversed by the addition of the anti-androgen hydroxyflutamide, demonstrating a specific AR-mediated effect (Lapointe, Fournier et al. 1999).

Subsequent studies following the establishment of a functional AR-signalling axis using a *ex vivo* breast explant tissue culture model in this thesis were undertaken in this chapter to determine the effects of DHT and/or MPA on potential AR driven biological processes including breast epithelial proliferation and survival. Thus the hypothesis of this chapter was that the anti-androgenic actions of MPA can antagonise the inhibitory effects of DHT on breast epithelial proliferation and survival via AR-mediated actions that may lead to breast cancer.

6.2 - Methods

Refer to Chapter 2 - General Materials and Methods for details on Materials *(section 2.1),* Buffers and Solutions *(section 2.2)* and Methods *(sections 2.3.1 - 2.3.7 and 2.3.10)* included in this chapter.

6.3 - Results

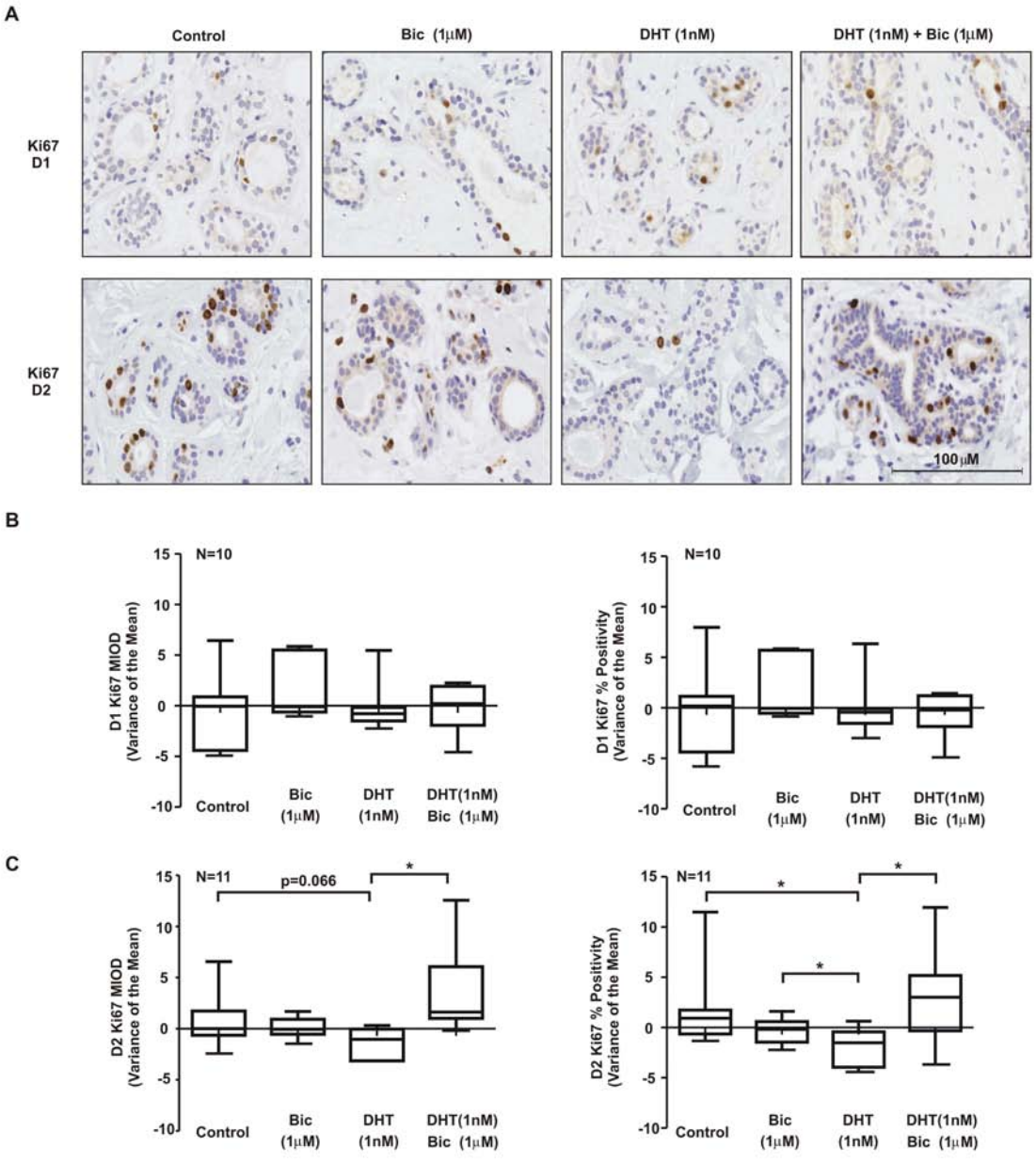
6.3.1 - Inhibition of breast epithelial cell proliferation by DHT-induced AR-signalling in post-menopausal breast explant tissue

Experiments were performed to determine whether DHT was anti-proliferative in human non-malignant post-menopausal breast explant tissues. In this study post-menopausal breast explant tissues (n=12) were cultured under conditions previously described *(refer to chapter 2, sections 2.3.2 and 2.3.4*). The expression of Ki67 was predominantly located in the nucleus of breast epithelial cells [Figure 6.1A]. At 24 hrs, no change in Ki67 expression between hormone treatments in cultured post-menopausal breast explant tissue was detected [Figure 6.1B]. At 48 hrs treatment with DHT resulted in a significant decrease in Ki67 % positivity ($p<0.05$) and Ki67 MIOD trended towards a decrease (albeit not significantly, $p=0.066$) compared to control samples [Figure 6.1C]. Additionally, a significant decrease in Ki67 % positivity was observed following treatment with DHT compared to Bic alone ($p<0.05$) [Figure 6.1C]. Co-treatment with DHT and Bic resulted in a significant reversal in Ki67 expression at 48 hrs compared to DHT alone for both VIA measurements ($p<0.05$) [Figure 6.1C]. These results specifically demonstrate that the anti-proliferative effects of DHT in post-menopausal breast tissue are mediated via the AR.

6.3.2 - Reversal of DHT-induced inhibition of breast epithelial proliferation by MPA in post-menopausal breast explant tissues

Subsequent experiments were undertaken to investigate the effect of MPA on DHT-

Figure 6.1 (A-C): DHT-induced inhibition of breast epithelial cell proliferation mediated by the AR-signalling axis in post-menopausal breast explant tissues. (A) Representative examples of Ki67 immunostaining in post-menopausal breast explant tissues initially pre-treated for 36 hrs, and subsequently cultured for 24 hrs (D1) (n=10) and 48 hrs (D2) (n=11) in media containing 10% DCC-FCS under control conditions, i.e. 0.1% ethanol, or treated with 1 nM DHT and/or 1 μM Bic. Images were digitally scanned and taken using a 40x objective microscopic lens. Quantification of Ki67 immunostaining was performed on breast explant tissues using VIA. Data in graphs represent Ki67 expression for mean integrated optical density (MIOD) and DAB positive area (% positivity) measurements at **(B)** 24 hrs (D1) and **(C)** 48 hrs (D2). *$p<0.05$, Wilcoxon matched pairs test. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. Two samples were excluded from the D1 time-point, and one sample from D2 time-point due to lack of glandular tissue in some tissue sections. (*Refer to Appendix 2 - Table 3A for VIA quantification raw data).*

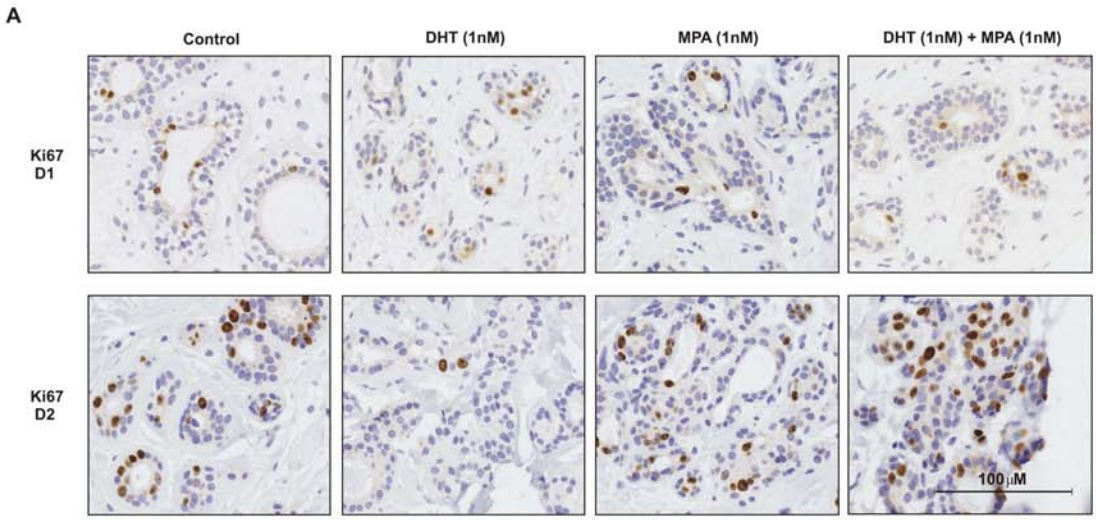

A
Control
Bic (1μM)
DHT (1nM)
DHT (1nM) + Bic (1μM)
Ki67 D1
Ki67 D2
100μM
B
N=10
D1 Ki67 MIOD (Variance of the Mean)
D1 Ki67 % Positivity (Variance of the Mean)
15
10
5
0
-5
-10
Control
Bic (1μM)
DHT (1nM)
DHT(1nM) Bic (1μM)
C
N=11
p=0.066
*
D2 Ki67 MIOD (Variance of the Mean)
D2 Ki67 % Positivity (Variance of the Mean)

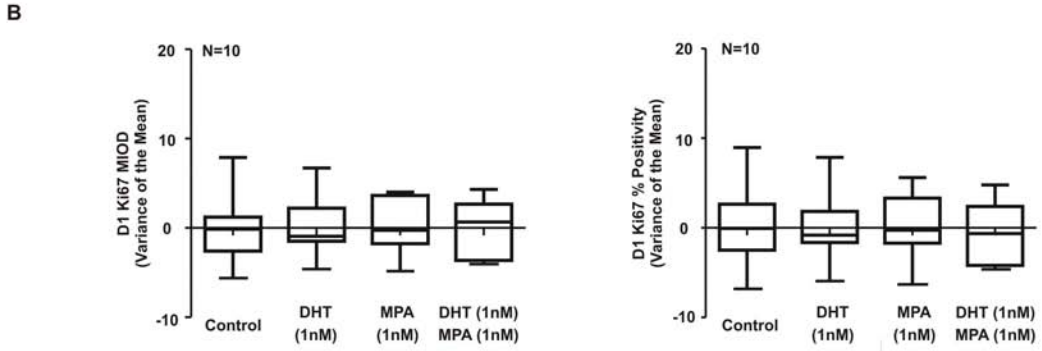
induced, AR-mediated inhibition of breast epithelial cell proliferation. Following 24 hrs of treatment, Ki67 expression remained similar for all hormone treatments for both VIA measurements [Figure 6.2A and B]. The treatment effects of DHT on Ki67 expression have been described in the previous section *(refer to section 6.3.1)*. Additionally in this section a significant reduction in Ki67 % positivity at 48 hrs following DHT treatment compared to MPA alone was observed ($p<0.05$) [Figure 6.2C]. However treatment of breast explant tissues with MPA alone resulted in no significant change in Ki67 expression by either VIA measurements at 48 hrs [Figure 6.2C]. Co-treatment of DHT and MPA led to a significant increase in Ki67 immunoreactivity ($p<0.05$ (MIOD) and $p<0.01$ (% positivity)) compared to either DHT or MPA as individual treatments at 48 hrs [Figure 6.2C]. Importantly, these findings demonstrate that the Ki67 % positivity values (i.e. median value >5% and average value > 10%) *(data not shown)* in post-menopausal breast tissue samples cultured by DHT and MPA combined, verge closer to Ki67 expression levels reported in breast cancer, compared to normal breast tissue samples (Bottini, Berruti et al. 2001).

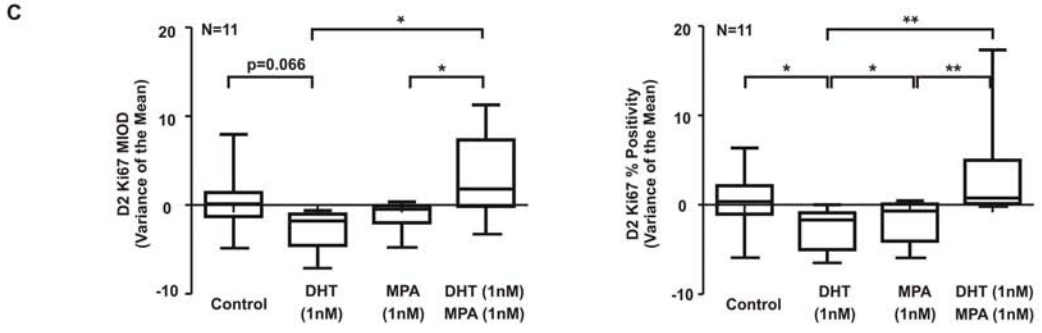
6.3.3 - Promotion of breast epithelial proliferation by the combined actions of DHT and MPA compared to DHT alone in post-menopausal breast tissues irrespective of association to malignancy

Previous findings in earlier chapters identified a significant increase in ERα steroid receptor expression between post-menopausal non-malignant breast tissue samples that were obtained from women diagnosed with malignancy compared to non-

Figure 6.2 (A-C): Reversal of DHT-induced inhibition of breast epithelial proliferation by the co-treatment of DHT and MPA. (A) Representative examples of Ki67 immunostaining in post-menopausal breast explant tissues initially pre-treated for 36 hrs, and subsequently cultured for 24 hrs (D1) (n=10) and 48 hrs (D2) (n=11) in media containing 10% DCC-FCS under control conditions, i.e. 0.1% ethanol, or treated with 1 nM DHT and/or 1 nM MPA. Images were digitally scanned and taken using a 40x objective microscopic lens. Quantification of Ki67 immunostaining was performed on breast explant tissues using VIA. Data in graphs represent Ki67 expression for mean integrated optical density (MIOD) and DAB positive area (% positivity) measurements at **(B)** 24 hrs (D1) and **(C)** 48 hrs (D2). *$p<0.05$, **$p<0.01$ Wilcoxon matched pairs test. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. Two samples were excluded from D1, and one sample from D2 time-point due to lack of glandular tissue in some tissue sections. (*Refer to Appendix 2 - Table 3A for VIA quantification raw data).*


A
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) + MPA (1nM)
Ki67 D1
Ki67 D2
100 μM


B
N=10
D1 Ki67 MIOD (Variance of the Mean)
D1 Ki67 % Positivity (Variance of the Mean)
20
10
0
-10
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) MPA (1nM)


C
N=11
p=0.066
*
**
D2 Ki67 MIOD (Variance of the Mean)
D2 Ki67 % Positivity (Variance of the Mean)
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) MPA (1nM)

malignant disease *(refer to chapter 4 - section 2.3.2).* Furthermore, results described previously in this thesis demonstrate a potentially impaired AR-signalling axis in non-post-menopausal non-malignant breast tissue samples that were adjacent to malignant breast tissue *(refer to chapter 5 - sections 5.3.3 and 5.3.4).* Given these findings, additional analysis was undertaken to investigate the effect of DHT and MPA alone and combined on breast epithelial cell proliferation in breast tissue adjacent compared to non-associated with cancer (i.e. adjacent to benign or distal to malignant disease).

At 48 hrs, Ki67 % positivity trended towards inhibition (albeit not significantly, $p=0.068$) following treatment with DHT in post-menopausal breast tissue samples that were non-associated with malignancy (n=6) compared to vehicle control [Figure 6.3A]. In contrast, DHT treatment had no effect on Ki67 immunoreactivity in breast tissue samples adjacent to malignant disease (n=5), compared to vehicle control [Figure 6.3B]. MPA treatment also had no effect on Ki67 % positivity in any of the post-menopausal breast tissues studied, irrespective of whether the tissue was adjacent to malignancy or not [Figure 6.3A and B]. However, breast tissues adjacent and non-associated with malignancy exhibited significantly higher Ki67 immunoreactivity following treatment with DHT and MPA combined compared to DHT alone ($p<0.05$) [Figure 6.3A and B]. Additionally, co-treatment by DHT and MPA resulted in a significant increase in Ki67 immunoreactivity compared to MPA as an individual treatment in breast tissue non-associated with malignancy ($p<0.05$) [Figure 6.3A].

6.3.4 - DHT and/or MPA have no effect on breast epithelial cell proliferation in pre-menopausal breast explant tissue

Experiments were similarly undertaken to determine the effect of MPA on breast epithelial cell proliferation in pre-menopausal breast tissue samples (n=6) in the presence and absence of DHT. As observed in post-menopausal breast tissue patient samples, Ki67 was predominantly expressed in the nucleus of the breast epithelial cells in pre-menopausal breast tissue samples [Figure 6.4A] and no significant change in Ki67 immunoreactivity between hormone treatments was detected at 24 hrs (n=5) [Figure 6.4B]. In contrast to that observed for the post-menopausal breast tissue samples used in this study, Ki67 immunoreactivity in pre-menopausal breast tissue samples was not inhibited by DHT treatment at 48 hrs, compared to vehicle control (n=6) [Figure 6.4C]. Moreover, DHT and MPA co-treatment resulted in similar Ki67 levels compared to DHT and MPA as individual treatments [Figure 6.4C]. The mean and STDEV values of Ki67 % positivity for the control pre- menopausal breast tissue samples was 1.42 ± 1.37, and in DHT treated samples was 2.46 ± 2.44 at 48 hrs *(data not shown)*. Power calculations indicate that a sample size of 20 breast tissues are required to detect a statistical difference between DHT treated samples and vehicle control. Lastly, in this study, the majority of pre-menopausal breast tissue samples were non-associated with malignant breast disease (n=5). Therefore, further comparative analysis of the differences in Ki67 expression in pre-menopausal breast tissue samples cultured in hormone treatments previously described, either adjacent or non-associated with malignancy was not undertaken in this study due to the low numbers of breast tissue samples associated with malignant disease (n=1).

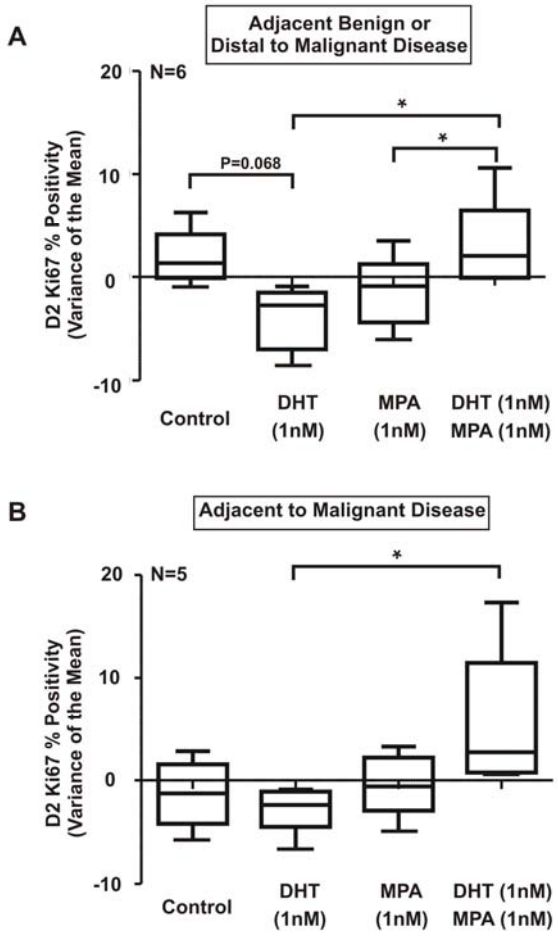
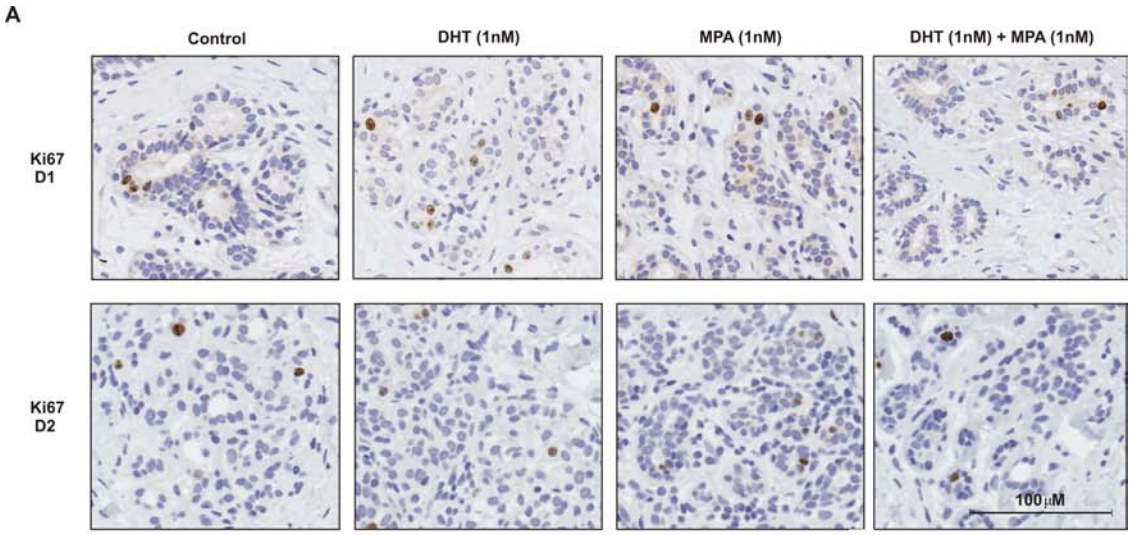
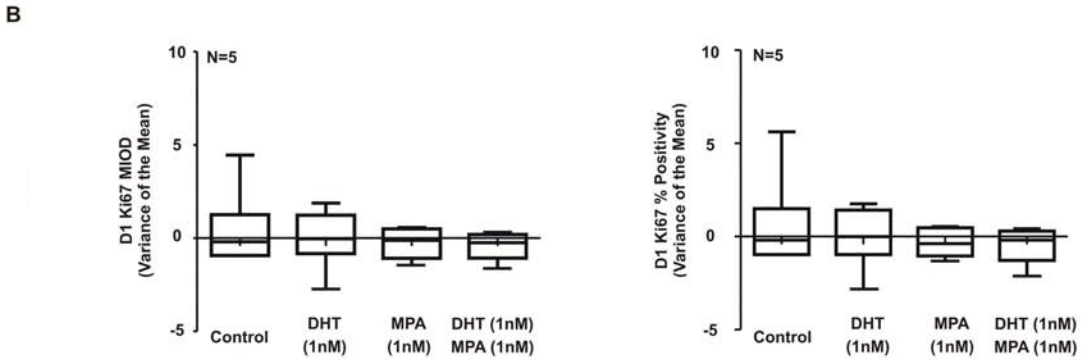
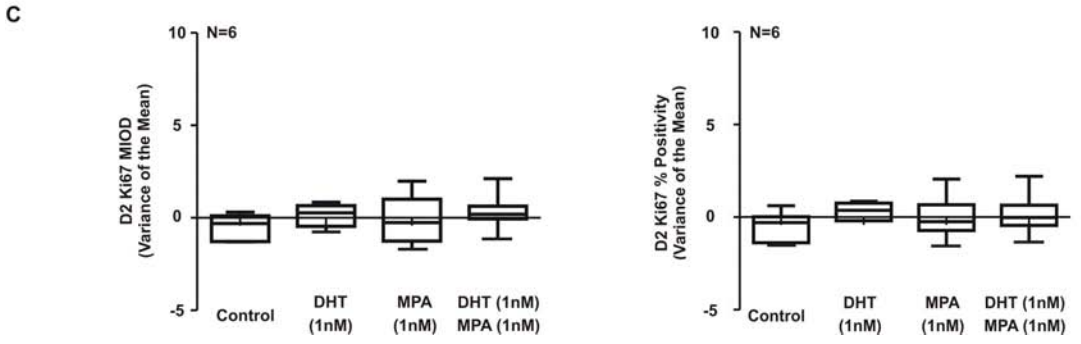


Figure 6.3 (A-B): Stimulation of breast epithelial proliferation by the combined actions of DHT and MPA in post-menopausal breast adjacent and not adjacent to malignant disease. Post-menopausal breast explant samples were sub-divided into the following groups: **(A)** adjacent to benign and distal to malignant breast disease (n=6) and **(B)** adjacent to malignant breast disease (n=5). Quantification of Ki67 immunostaining was performed on breast explant tissues using VIA mean integrated optical density (MIOD) and DAB positive area (% positivity) measurements at 48 hrs (D2) post treatment. *$p<0.05$, Wilcoxon matched pairs test. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. Similar data was observed for Ki67 MIOD measurements *(data not shown)*. One sample was excluded due to lack of glandular tissue in some tissue sections. (*Refer to Appendix 2 - Table 3B for VIA quantification raw data).*

Figure 6.4 (A-C): No effect of DHT and MPA alone or combined on breast epithelial cell proliferation in pre-menopausal breast explant tissue. (A) Representative examples of Ki67 immunostaining in pre-menopausal breast explant tissues pre-treated for 36 hrs and subsequently cultured for 24 hrs (D1) (n=5) and 48 hrs (D2) (n=6) in media containing 10% DCC-FCS supplemented under control conditions, i.e. 0.1% ethanol, or treated with 1 nM DHT and/or 1 nM MPA. Images were digitally scanned and taken using a 40x objective microscopic lens. Quantification of Ki67 immunostaining was performed on breast explant tissues using VIA mean integrated optical density (MIOD) and DAB positive area (% positivity) measurements at **(B)** 24 hrs (D1) and **(C)** 48 hrs (D2). Statistical analysis performed using Wilcoxon matched pairs test, significance accepted $p<0.05$. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. Two samples were excluded from D1, and one sample from D2 time-point due to lack of glandular tissue in some tissue sections. (*Refer to Appendix 2 - Table 4 for VIA quantification raw data).*


A
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) + MPA (1nM)
Ki67 D1
Ki67 D2
100 μM


B
N=5
D1 Ki67 MIOD (Variance of the Mean)
D1 Ki67 % Positivity (Variance of the Mean)
10
5
0
-5
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) MPA (1nM)


C
N=6
D2 Ki67 MIOD (Variance of the Mean)
D2 Ki67 % Positivity (Variance of the Mean)
10
5
0
-5
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) MPA (1nM)

6.3.5 - Comparative analysis of baseline Ki67 immunoreactivity in pre- compared to post-menopausal breast explant tissue following culture

Given previous findings demonstrating an inhibitory effect of DHT on Ki67 immunoreactivity only in post-menopausal, yet not in pre-menopausal breast tissues, additional analysis was performed to compare the base-line immunoreactivity levels of Ki67 following culture in vehicle control (i.e. 0.1% ethanol) for 48 hrs in pre- compared to post-menopausal breast tissues.

In this study the base-line Ki67 % positivity in pre-menopausal explant tissue samples (n=6) following culture ranged from 0.15 - 3.62 (median value, 1.16 and average ± SEM, 1.62 ± 0.80) and in post-menopausal breast tissue samples ranged from 0.43 - 18.28 (median value, 2.67 and average ± SEM, 5.19 ± 1.81) (n=11) [Figure 6.5]. A trend towards significantly higher Ki67 % positivity was found in post- compared to pre-menopausal breast explant tissue samples under control conditions, (i.e. 0.1% ethanol) (albeit, not significantly p=0.07) [Figure 6.5].

The 5% Ki67 cut-off (i.e. ≤5% and >5%) applied to the data in this section was based on the average Ki67 % positivity in the cultured post-menopausal breast tissue samples (i.e. 5.19), as previously described [Figure 6.5]. Observational findings indicated that in three post-menopausal breast tissues, under the same experimental conditions, Ki67 % positivity was >5% (i.e. 18.28, 11.14 and 8.4) [Figure 6.5]. Of these patient samples, only one was located adjacent to malignant disease and the other two were obtained from post-menopausal women diagnosed with benign breast disease. However, the small

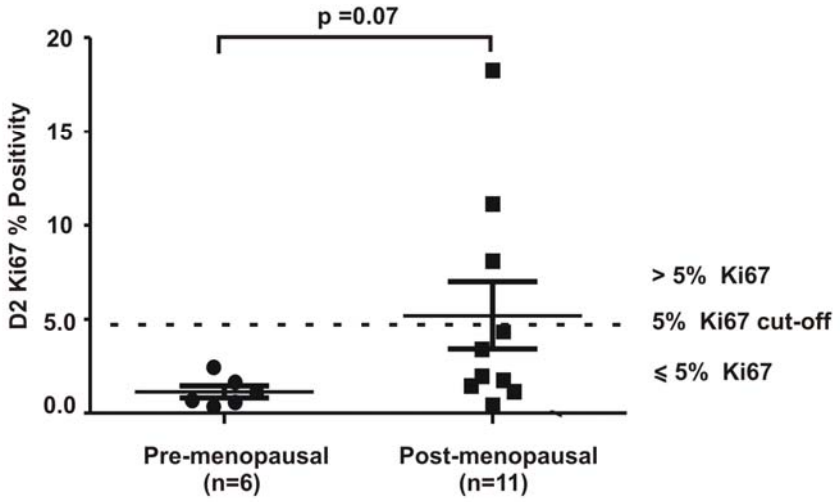


Figure 6.5: Comparative analysis of Ki67 base-line expression in cultured pre- and post-menopausal breast epithelial cells. Ki67 immunoreactivity in pre- (n=6) and post-menopausal (n=10) breast explant tissues pre-treated for 36 hrs and subsequently cultured for 48 hrs under control conditions, i.e. 0.1% ethanol. Statistical analysis performed using non-parametric Mann Whitney test, significance accepted $p<0.05$. Similar data was observed for Ki67 mean integrated optical density (MIOD) *(data not shown).* The data has been sub-divided into samples which presented with >5% Ki67 or ≤5% Ki67 positive epithelial cells represented by a dashed line. Scatter plot represents each individual patient sample the upper and lower quartiles and the mean values. One data point at the D2 time-point was excluded from the post-menopausal patient group in the analysis due to a lack of glands in the control tissue.

numbers (i.e. n=3) of post-menopausal breast tissues used in this comparison make it difficult to determine the significance of these findings. In contrast, Ki67 immunoreactivity in all the pre-menopausal breast tissues (n=6) was ≤ 5% Ki67 positivity [Figure 6.5]. Thus, the lower base-line level of Ki67 % positivity in pre- compared to post-menopausal patient samples subsequent to culture in steroid-hormone deprived culture conditions may have comprised the detection of an inhibitory effect of DHT, compared to control tissue samples in pre-menopausal tissue samples.

6.3.6 - No effect of DHT and/or MPA on breast epithelial cell survival in pre- and post-menopausal breast explant tissue

Additional experiments were undertaken to determine whether MPA alone or in combination with DHT, also affected breast epithelial cell survival as detected for proliferation. In order to study this aim, immunohistochemistry was undertaken using an antibody to detect the bcl-2 protein in pre- and post-menopausal breast explant tissue following hormone treatment. Immunostaining for bcl-2 was localised in both pre- and post-menopausal breast tissue patient samples in the cytoplasm of breast epithelial cells [Figure 6.6A; 6.7A; 6.8A]. In this study no significant change in bcl-2 expression was identified in either pre- or post-menopausal breast explant tissue samples by any hormone treatment at 24 or 48 hrs [Figures 6.6B and C; 6.7B and C; 6.8B and C]. Power calculations were completed to determine the number of samples required to detect changes in bcl-2 expression following DHT and/or MPA treatment. These results suggested that approximately 62 post-menopausal and 974 pre-menopausal breast tissue samples are required at the 48 hr time-point to detect a change between the hormone

Figure 6.6 (A-C): No effect of the DHT-induced AR-signalling on a marker of breast epithelial cell survival in post-menopausal breast explant tissue. (A) Representative examples of bcl-2 immunostaining in post-menopausal breast explant tissues initially pre-treated for 36 hrs and subsequently cultured for 24 hrs (D1) (n=10) and 48 hrs (D2) (n=11) in media containing 10% DCC-FCS under control conditions, i.e. 0.1% ethanol, or treated with 1 nM DHT and/or 1 μM Bic. Images were digitally scanned and taken using a 40x objective microscopic lens. Quantification of bcl-2 immunostaining was performed on breast explant tissues using VIA. Data in graphs represent bcl-2 expression for mean integrated optical density (MIOD) and DAB positive area (% positivity) measurements at **(B)** 24 hrs (D1) and **(C)** 48 hrs (D2). Statistical analysis performed using Wilcoxon matched pairs test, significance accepted $p<0.05$. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. Two samples were excluded from D1, and one sample from D2 time-point due to lack of glandular tissue in some tissue sections. (*Refer to Appendix 2 - Table 5 for VIA quantification raw data).*

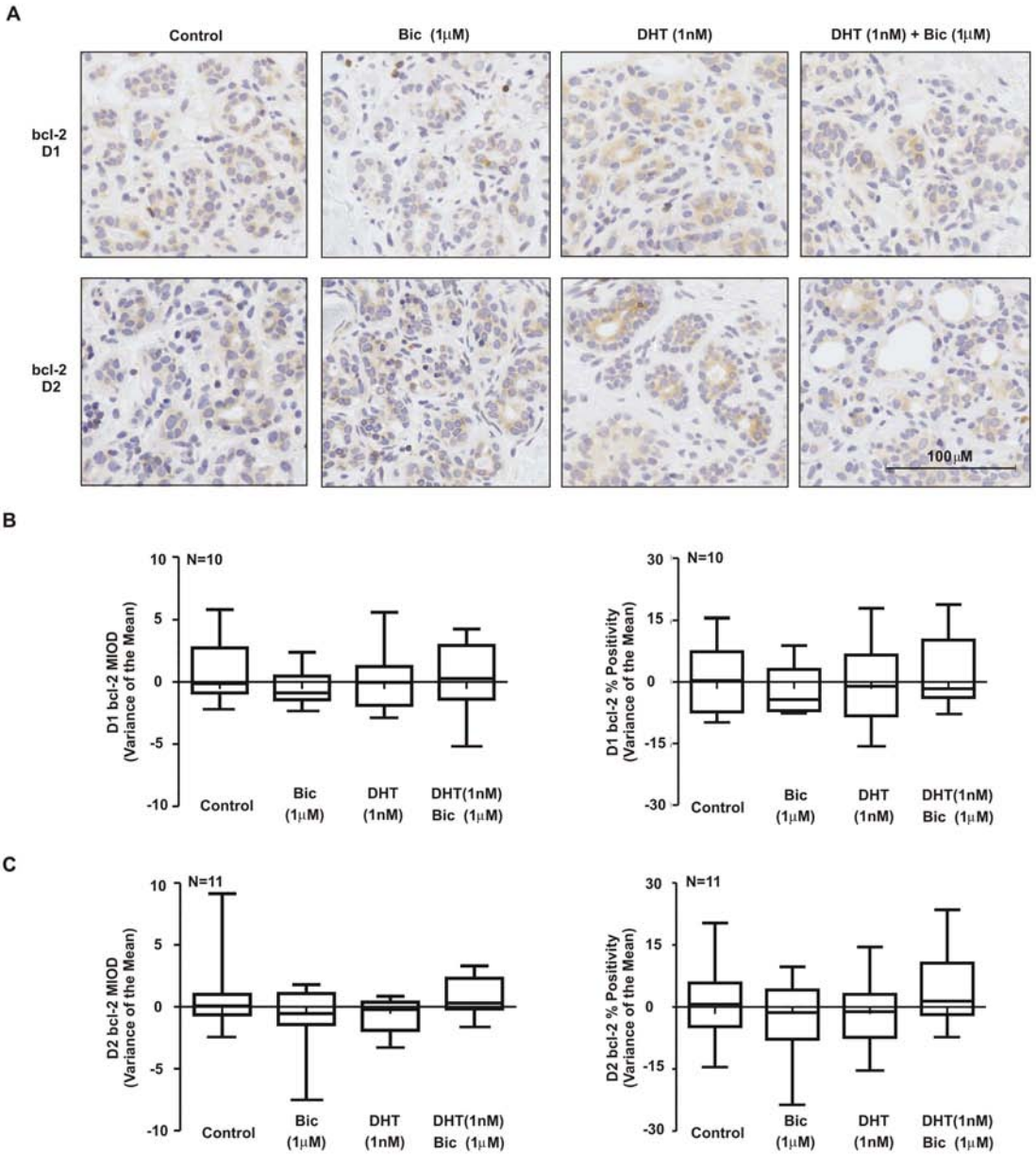

A
Control
Bic (1μM)
DHT (1nM)
DHT (1nM) + Bic (1μM)
bcl-2 D1
bcl-2 D2
100μM
B
N=10
D1 bcl-2 MIOD (Variance of the Mean)
D1 bcl-2 % Positivity (Variance of the Mean)
Control
Bic (1μM)
DHT (1nM)
DHT(1nM) Bic (1μM)
C
N=11
D2 bcl-2 MIOD (Variance of the Mean)
D2 bcl-2 % Positivity (Variance of the Mean)

Figure 6.7 (A-C): No effect of DHT and/or MPA on a marker of breast epithelial cell survival in post-menopausal breast explant tissue. (A) Representative examples of bcl-2 immunostaining in post-menopausal breast explant tissues initially pre-treated for 36 hrs and subsequently cultured for 24 hrs (D1) (n=10) and 48 hrs (D2) (n=11) in media containing 10% DCC-FCS under control conditions, i.e. 0.1% ethanol, or treated with 1 nM DHT and/or 1 nM MPA. Images were digitally scanned and taken using a 40x objective microscopic lens. Quantification of bcl-2 immunostaining was performed on breast explant tissues using VIA. Data in graphs represent Ki67 expression for mean integrated optical density (MIOD) and DAB positive area (% positivity) measurements at **(B)** 24 hrs (D1) and **(C)** 48 hrs (D2). Statistical analysis performed using Wilcoxon matched pairs test, significance accepted $p<0.05$. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. Two samples were excluded from D1, and one sample from D2 time-point due to lack of glandular tissue in some tissue sections. (*Refer to Appendix 2 - Table 5 for VIA quantification raw data).*

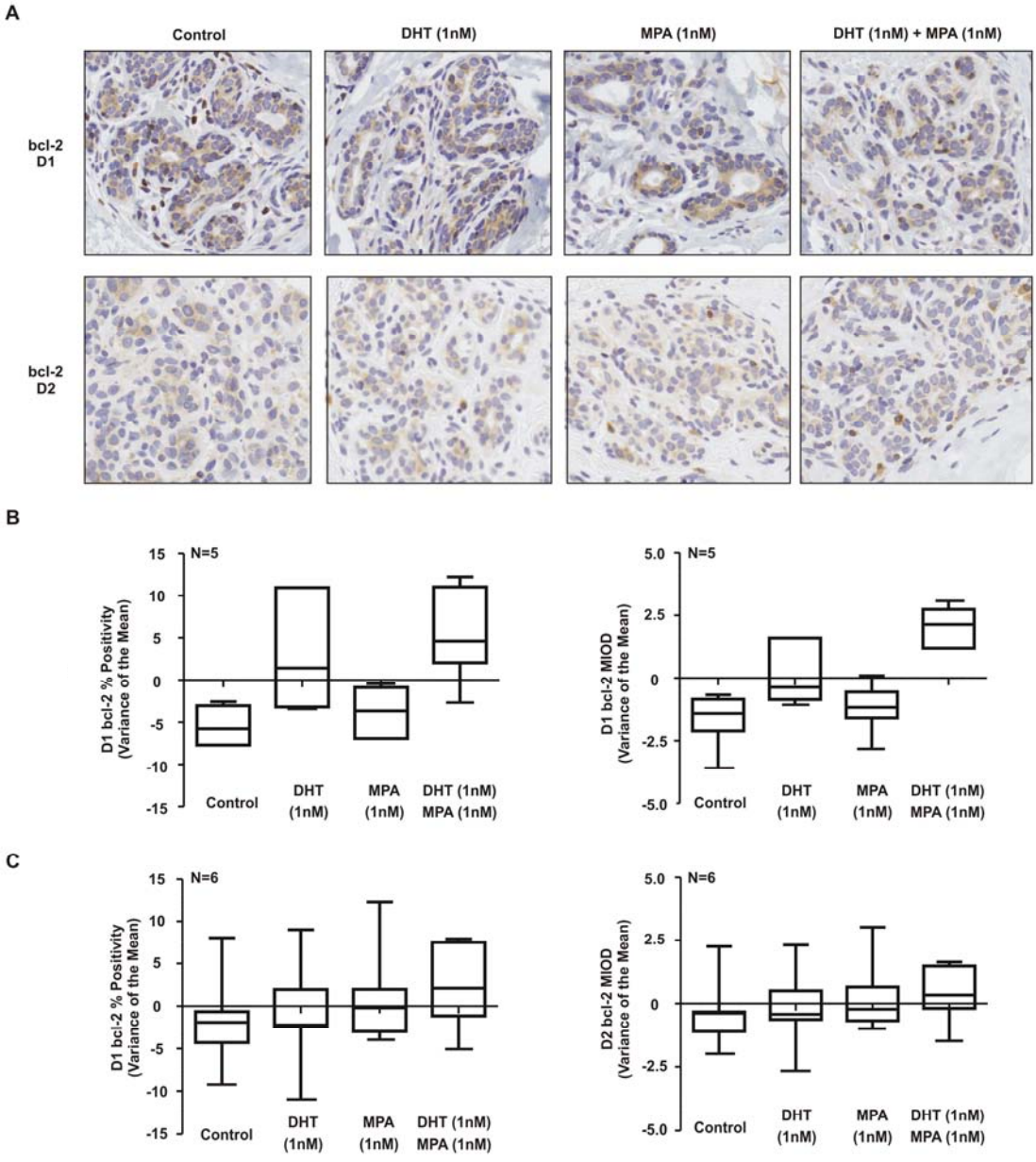
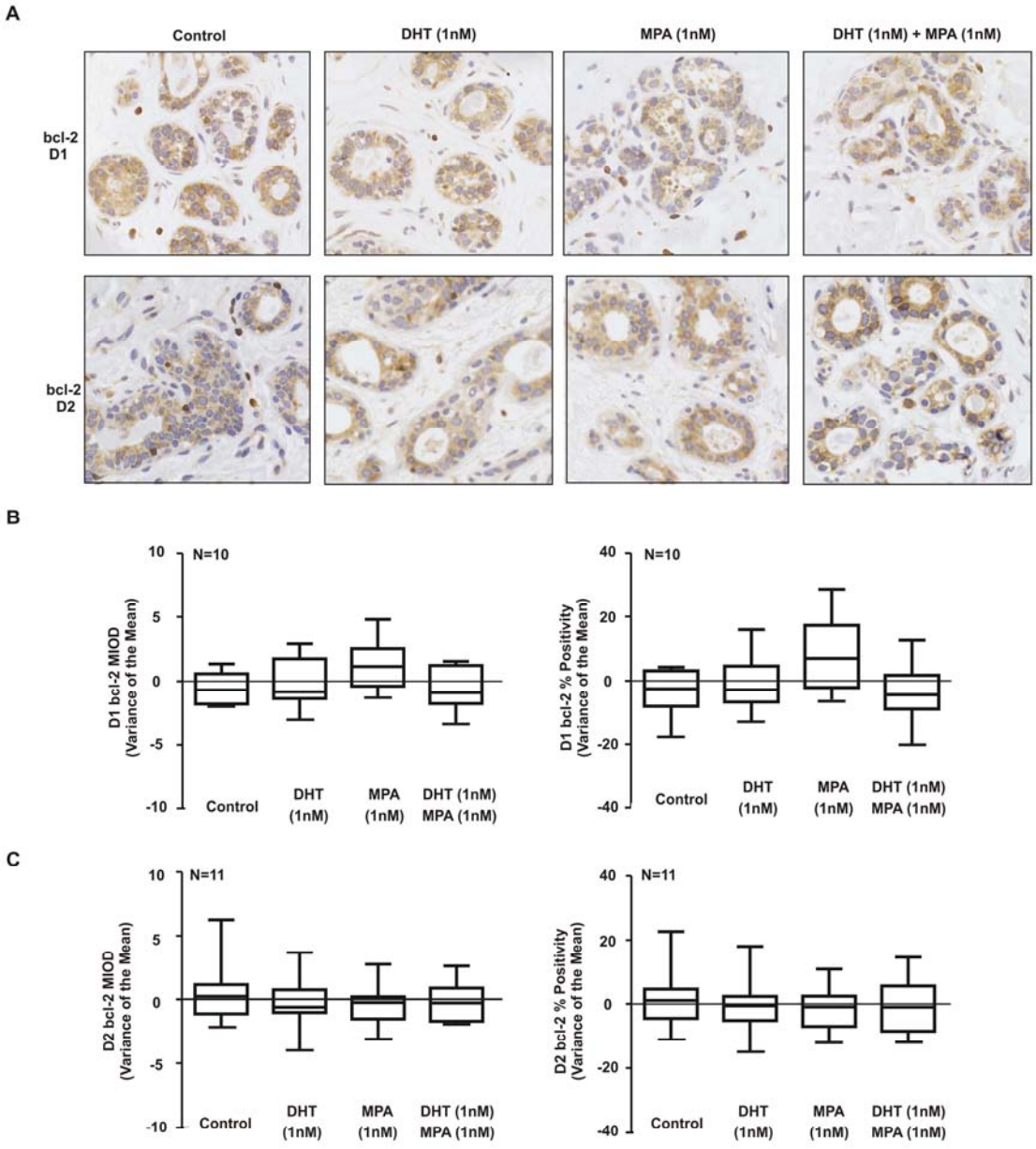

A
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) + MPA (1nM)
bcl-2 D1
bcl-2 D2
B
N=5
D1 bcl-2 % Positivity (Variance of the Mean)
15
10
5
0
-5
-10
-15
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) MPA (1nM)
N=5
D1 bcl-2 MIOD (Variance of the Mean)
5.0
2.5
0
-2.5
-5.0
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) MPA (1nM)
C
N=6
D1 bcl-2 % Positivity (Variance of the Mean)
15
10
5
0
-5
-10
-15
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) MPA (1nM)
N=6
D2 bcl-2 MIOD (Variance of the Mean)
5.0
2.5
0
-2.5
-5.0
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) MPA (1nM)

Figure 6.8 (A-C): The marker of breast epithelial survival, bcl-2 remains unaffected by DHT and/or MPA in pre-menopausal breast tissue. (A) Representative examples of bcl-2 immunostaining in pre-menopausal breast explant tissues initially pre-treated for 36 hrs and subsequently cultured for 24 hrs (D1) (n=5) and 48 hrs (D2) (n=6) in media containing 10% DCC-FCS under control conditions, i.e. 0.1% ethanol, or treated with 1 nM DHT and/or 1 nM MPA. Images were digitally scanned and taken using a 40x objective microscopic lens. Quantification of bcl-2 immunostaining was performed on breast explant tissues using VIA. Data in graphs represent bcl-2 expression for mean integrated optical density (MIOD) and DAB positive area (% positivity) measurements at **(B)** 24 hrs (D1) and **(C)** 48 hrs (D2). Statistical analysis performed using Wilcoxon matched pairs test, significance accepted $p<0.05$. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. Two samples were excluded from D1, and one sample from D2 time-point due to lack of glandular tissue in some tissue sections. (*Refer to Appendix 2 - Table 6 for VIA quantification raw data).*


A
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) + MPA (1nM)
bcl-2 D1
bcl-2 D2
B
N=10
D1 bcl-2 MIOD (Variance of the Mean)
10
5
0
-5
-10
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) MPA (1nM)
N=10
D1 bcl-2 % Positivity (Variance of the Mean)
40
20
0
-20
-40
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) MPA (1nM)
C
N=11
D2 bcl-2 MIOD (Variance of the Mean)
10
5
0
-5
-10
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) MPA (1nM)
N=11
D2 bcl-2 % Positivity (Variance of the Mean)
40
20
0
-20
-40
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) MPA (1nM)

treatments. Thus, it is likely that an effect of DHT and/or MPA treatmen may be detected on bcl-2 expression with an increased sample size of breast tissue samples. However, from these power calculations it would suggest that 15-fold additional pre-menopausal breast tissue samples are required to reach statistical significance in response to DHT and/or MPA compared to post-menopausal women.

6.4 - Discussion

This is the first unpublished study to demonstrate the modulation of the breast epithelial proliferation marker Ki67 in response to androgenic stimulation in human post-menopausal breast tissue using an *ex vivo* breast explant tissue model. The main key findings in this chapter provide evidence that DHT can inhibit breast epithelial cell proliferation in human non-malignant post-menopausal breast tissue via AR-mediated mechanisms. Moreover, the results described in this chapter demonstrate that DHT and MPA, when combined, can act to increase breast epithelial cell proliferation compared to the anti-proliferative actions of DHT alone in post-menopausal breast tissues. The mechanism(s) underlying increased breast rates of breast proliferation, that may lead to breast cancer development in post-menopausal women taking cHRT are therefore likely to result from the combined cellular activity of DHT and MPA, rather than MPA alone. Furthermore, the data in this chapter suggests that the combined action of DHT and MPA that lead to these effects can occur in breast epithelial cells that possess either a functional or impaired AR-signalling axis.

The findings reported in this chapter are in agreement with previous literature demonstrating that androgens have an inhibitory effect on the growth of human and monkey breast tissue *in vivo* (Jayo, Register et al. 2000; Zhou, Ng et al. 2000; Dimitrakakis, Zhou et al. 2003), as well as in some AR positive breast cancer cell lines, and that this effect is mediated directly through the AR-signalling axis (Poulin, Baker et al. 1988; Labrie, Poulin et al. 1990; Birrell, Bentel et al. 1995). Moreover, a plethora of experimental and clinical data illustrate that androgens are protective in breast cancer by acting as tumour suppressors. These studies include studies that have identified the AR as an independent prognostic factor by an overall improved survival outcome (i.e. disease-free survival), in women diagnosed with ERα positive breast cancers who have high AR expression compared to women with low AR expression (Bryan, Mercer et al. 1984; Kuenen-Boumeester, Van der Kwast et al. 1996; Agoff, Swanson et al. 2003; Schippinger, Regitnig et al. 2006; Gonzalez, Corte et al. 2008; Peters, Buchanan et al. 2009; Castellano, Allia et al. 2010; Hu, Dawood et al. 2010). The expression level of the AR has also been reported to be higher in breast tumours of a lower clinical grade and is associated with lower proliferation rates (Isola 1993; Kuenen-Boumeester, Van der Kwast et al. 1996; Hanley, Wang et al. 2008). In contrast, the incidence of node-positive breast cancer is significantly higher in primary breast lesions which express low levels of AR (Soreide, Lea et al. 1992). These studies collectively highlight the protective actions of AR expression in breast tissue, and emphasise the detriment to the inhibition of its actions.

Previous breast explant tissue culture studies have reported that high doses of MPA (100 nM) can increase Ki67 and proliferating cell nuclear antigen (PCNA) expression in peri- and post-menopausal breast explant tissue samples (Eigeliene, Harkonen et al. 2006; Eigeliene, Harkonen et al. 2008), yet interestingly not in pre-menopausal tissues breast epithelial cells (Eigeliene, Harkonen et al. 2006). In contrast, the lower doses of MPA (1 nM) used in this study, which correlate more closely with serum levels of MPA in women taking cHRT (Svensson, Johnson et al. 1994) did not increase Ki67 expression in either pre- or post-menopausal breast explant tissues. These findings illustrate that there are biological differences in the actions of MPA at low and high doses, and emphasise that studies using high doses of MPA (i.e. 100 nM) should not be used as a reflection of the biological actions of lower doses of MPA used in cHRT. These differences may be associated with additional effects of MPA mediated through other steroid receptors including PR and GR at the higher dose of 100 nM MPA, which are not as abundant at the lower dose of 1 nM MPA (Sitruk-Ware 2004). Furthermore, this study demonstrates previously unreported findings indicating that the combination of DHT and MPA, compared to MPA alone can reverse the anti-proliferative effects of DHT, in post-menopausal non-malignant breast explant tissue samples.

The % positivity of Ki67 is routinely used clinically as a predictor of response to breast cancer therapeutics (i.e. relapse-free survival) (Clarke, Laidlaw et al. 1993; Bottini, Berruti et al. 2001; Assersohn, Salter et al. 2003; Dowsett, Ebbs et al. 2005), in addition to a prognostic indictor of long-term outcome in women diagnosed with breast cancer (Weikel, Beck et al. 1991; Rudas, Gnant et al. 1994; Billgren, Tani et al. 2002; Dowsett,

Smith et al. 2007). Ki67 is expressed at high levels during mitosis, and is used only as a marker of proliferation (van Dierendonck, Keijzer et al. 1989; Urruticoechea, Smith et al. 2005). The relative expression levels of Ki67 in human normal breast epithelial cells, has been reported in the literature. These findings indicate that normal breast tissue exhibits <3% Ki67 positivity (Clarke, Howell et al. 1997; Harper-Wynne, Ross et al. 2002; de Lima, Facina et al. 2003; Dowsett, Ebbs et al. 2005), whereas a much greater Ki67 % positivity is reported in breast cancer tissue samples (i.e. >5% Ki67 positivity) (Bottini, Berruti et al. 2001). Interestingly, findings in this chapter have demonstrated that Ki67 % positivity in cultured post-menopausal breast explant tissues is more similar to breast cancer, than normal breast tissue subsequent to the co-treatment with DHT and MPA. Importantly, these findings illustrate that the cellular actions of the combined hormone treatment possesses the ability to stimulate breast epithelial proliferation rates to similar levels detected in breast cancer and thus may attain the capacity to promote carcinogenesis. A noted limitation of the data included in this chapter is the use of only one biomarker (i.e. Ki67) as an indicator of changes to breast epithelial proliferation following DHT and/or MPA treatment. Thus, additional studies are required using agents that incorporate into the DNA and be used to detect active cell division (i.e. bromodeoxyuridine (BrdU) and/or carboxyfluorescein diacetate, succinimidyl ester (CFSE)) which have not been undertaken in the research studies included in this thesis.

Within patient post-menopausal tissue samples adjacent to malignant breast tissue, previously shown in an earlier chapter to possess an impaired AR-signalling *(refer to chapter 5 - sections 5.3.3 and 5.3.4)*, findings in this chapter also demonstrate the

impairment of anti-proliferative effects of DHT in the same tissue samples. Despite these findings, breast epithelial cell proliferation was similarly increased by the combination of DHT and MPA, compared to DHT treatment alone, in breast tissues adjacent and non-associated with malignancy. Thus, this is the first study to report that the synthetic progestin MPA, when used in conjunction with DHT, can increase breast epithelial proliferation compared to DHT alone in breast epithelial cells exhibiting a normal or impaired AR-signalling axis. The ability of MPA to promote the growth of pre-malignant lesions in post-menopausal women taking cHRT has been described in the literature (Dietel, Lewis et al. 2005). Moreover, whether impaired AR-signalling is reflective of pre-malignant changes in normal breast tissue has not to date been investigated. In light of the findings in this chapter, it may be intriguing to speculate that if a relationship does exist between impaired AR-signalling and pre-malignant breast disease, then MPA in combination with DHT may feasibly attain the ability to accelerate rates of proliferation in both normal and pre-malignant breast tissue.

The majority of pre- and post-menopausal breast tissue samples used in this study was obtained from parous women, compared to only a few nulliparous women. Interestingly, Ki67 expression was not affected by DHT treatment alone or in conjunction with MPA in pre-menopausal breast tissues, compared to post-menopausal breast tissues which showed responses to both hormone treatments. These findings suggest that there are differences in hormone stimulated breast epithelial proliferation responsiveness based on the menopausal status of the female independent of parity status. The literature has reported a lower proliferation rate in parous compared to nulliparous women (Russo, Mills et al.

1989; Russo, Rivera et al. 1992; Taylor, Pearce et al. 2009). Complete pregnancy-induced differentiation in the breast tissue is also reported to provide protection against breast cancer development by a decrease in steroid receptor expression (Russo, Mills et al. 1989; Russo and Russo 1994; Russo, Ao et al. 1999; Taylor, Pearce et al. 2009). This biological effect is reported to result from the methylation of gene promoter regions, in the lobule type 1, 2 and 3 structures following breast tissue differentiation during pregnancy (Russo, Ao et al. 1999; Russo and Russo 2007). A significant decrease in PRA expression and trend toward a decrease in PRB and ERα expression has additionally been observed in breast tissue of parous compared to nulliparous women (Taylor, Pearce et al. 2009). However, no study has investigated changes to AR expression subsequent to pregnancy in pre- and post-menopausal women. Therefore, it may be postulated based on the findings in this chapter and published findings that epigenetic changes including the methylation of gene promoters that occur following full-term pregnancy which are thought to lead to reduced steroid expression in the breast lobule structures and potentially reduced rates of breast epithelial proliferation are not in fact permanent and are reversed during menopause (Russo and Russo 2007; Devinoy and Rijnkels 2010; Rijnkels, Kabotyanski et al. 2010).

A potential relationship between the change in breast epithelial proliferation by the combined actions of DHT and MPA, mediated by the AR-signalling axis has been illustrated in this chapter. However, in contrast to a previous study, DHT did not affect expression of the androgen regulated pro-survival factor bcl-2 (Lapointe, Fournier et al. 1999) in either non-malignant pre- or post-menopausal breast explant tissues. Additional

findings in this study have also indicated that there was no increase in the expression of bcl-2 by the combined treatment of DHT and MPA compared to DHT alone as detected for Ki67 in the post-menopausal breast explant tissues samples. Despite the findings in this chapter reporting DHT has no affect on bcl-2 expression in non-malignant breast tissue, high bcl-2 expression levels have been identified during the early luteal phase of the menstrual cycle, during which time the rates of breast epithelial proliferation are maximal and oestrogen levels are high (Sabourin, Martin et al. 1994; Ferrieres, Cuny et al. 1997; Massafra, De Felice et al. 1999). Furthermore, bcl-2 declines in expression at the end of the luteal phase of the menstrual cycle when breast tissue proliferation rates and oestrogen levels are low and a high rate of breast epithelial sloughing via apoptosis occurs (Sabourin, Martin et al. 1994). These studies suggest that bcl-2 expression is cyclically hormonally regulated in the breast tissue, and expression rates correspond positively to breast epithelial proliferation.

The lack of an association between the changes in bcl-2 and Ki67 expression following hormone treatment observed in this chapter may be associated with the following: 1) low sample size; 2) low base-line levels of Ki67 and/or bcl-2 in cultured breast tissue and 3) no oestrogen supplementation in the *ex vivo* breast explant tissue model experimental conditions. The latter is based on the fact that bcl-2 in malignant breast tissue is well documented in the literature to be stimulated by oestrogens, as follows. Treatment of MCF-7 cells with E_2 has been shown to result in an increase in bcl-2 mRNA and protein expression, which can be reversed by the anti-oestrogens 4-hydroxytamoxifen and RU58668 (Kandouz, Siromachkova et al. 1996). Moreover, using chromatin

immunoprecipitation and electromobility shift assays in MCF-7 breast cancer cells, binding of oestrogen to ERα has been shown to result in activation of oestrogen response elements located in the bcl-2 P1 promoter and enhancer regions (Perillo, Sasso et al. 2000; Perillo, Ombra et al. 2008). Future studies require the addition of oestrogen and an anti-oestrogen (i.e. tamoxifen) to the *ex vivo* breast explant tissue model experimental conditions used in this thesis to allow for the contribution of oestrogen to regulate bcl-2 expression to be determined. This experimental approach may also provide better experimental conditions to investigate the effect of DHT and/or MPA alone and on changes to cell survival.

In summary, it has been identified that DHT-induced inhibition of breast epithelial cell proliferation in post-menopausal breast tissue samples can be reversed by a combination of DHT and MPA potentially via AR-mediated actions. Since cHRT use has been shown to result in increased rates of ER and PR positive breast cancers (Chen, Hankinson et al. 2004; Li, Malone et al. 2008; Marshall, Clarke et al. 2010), these findings indicate that the actions of cHRT in promoting breast cancer are likely to be mediated via steroid receptor actions. Subsequent studies therefore been undertaken in the final chapters of this thesis to further examine the contribution of an AR-mediated effect specifically by the cellular actions of DHT and MPA combined that can initiate the development of breast cancer.

Chapter 7

MPA impedes DHT-induced AR expression in non-malignant human breast epithelial cells

7.1 - Introduction

Recent findings from the DRMCRL and others have demonstrated *in vitro* that a key dimerisation event required for functional DHT-induced AR-signalling is abrogated by the synthetic progestin MPA (Kemppainen, Langley et al. 1999; He, Bowen et al. 2001; Birrell, Butler et al. 2007). In addition, an *in vivo* study has shown that treatment with cHRT containing MPA results in a marked reduction in AR expression in the mammary glands of ovariectomised rhesus monkeys (Hofling, Ma et al. 2009). Based on these findings, studies were undertaken in this chapter to determine the effect of MPA on DHT-induced AR transactivation and the receptor stabilisation capacity in human non-malignant pre- and post-menopausal breast tissue using an *ex vivo* breast explant tissue model.

Evidence suggests that a low-dose of MPA (i.e. 1 nM) inhibits DHT-induced AR-signalling, via abrogation of the N/C AR interaction and can induce AR-signalling in isolation that does not require the N/C AR interaction (Kemppainen, Langley et al. 1999; Birrell, Butler et al. 2007). In comparison high-doses of MPA (i.e. 10 - 100 nM) can induce a weak N/C AR interaction leading to functional AR-signalling effects, and 10 nM MPA, and potentially 100 nM MPA can also inhibit the DHT-induced AR N/C interaction *(refer to chapter 1 - section 1.9.4)* (Kemppainen, Langley et al. 1999; Birrell, Butler et al. 2007). The inhibition of DHT-induced AR-signalling by progesterone, which does not bind to the AR yet binds to the PR (Africander, Verhoog et al. 2011) is much weaker than that observed by MPA, indicating that disruption of DHT-induced AR-

signalling by MPA is potentially distinct to PR-mediated actions (Birrell, Butler et al. 2007). Molecular modelling studies indicate that MPA-bound AR alters the positioning of amino acid phenylalanine 875 located in the LBD of the AR, compared to the orientation of the residues the AR adopts bound to DHT (Birrell, Butler et al. 2007). This alteration in the spatial orientation of phenylalanine 875 residue in the LBD of the AR by MPA may disrupt DHT-induced AR dimerisation required for the normal function, receptor stability and AR-signalling via the altered conformation of the AR (Kemppainen, Langley et al. 1999; He and Wilson 2002; Buchanan, Yang et al. 2004; Birrell, Butler et al. 2007). These findings provide a potential mechanistic basis associated with the abrogation of DHT-induced AR-signalling by MPA.

In this thesis, DHT-induced inhibition of breast epithelial proliferation in post-menopausal breast explant tissue was shown to be reversed by MPA *(refer to chapter 6 - section 6.3.2)*. Additional studies demonstrated that the anti-proliferative actions of DHT in post-menopausal breast explant tissues are mediated directly through the AR-signalling axis, via the addition of the potent AR antagonist, Bic *(refer to chapter 6 - section 6.3.1)*. It is therefore hypothesised in this chapter that anti-androgenic AR-mediated carcinogenic actions of MPA in normal and malignant breast epithelial cells may in part contribute to the development of breast cancer in post-menopausal women taking cHRT containing MPA. Since MPA possesses strong ligand binding affinity for both the AR and PR (Bentel, Birrell et al. 1999; Sitruk-Ware 2004) and both steroid receptors have been implicated in playing a role in normal and breast cancer biology (Anderson 2002; Dimitrakakis and Bondy 2009(Ricketts, Turnbull et al. 1991; Kuenen-Boumeester, Van

der Kwast et al. 1992; Kuenen-Boumeester, Van der Kwast et al. 1996; Li, Han et al. 2010) the carcinogenic actions of MPA via AR and/or PR-mediated effects were important to the research studies included in this thesis. Furthermore, the actions of MPA in post-menopausal women taking cHRT have also been heavily reported in the literature to potentially occur via PR-mediated actions (Hyder, Murthy et al. 1998; Eigeliene, Harkonen et al. 2008; Horwitz and Sartorius 2008) as discussed in detail in chapter 9.

In light of previous findings in this thesis demonstrating that DHT and MPA combined can lead to an increase in breast epithelial proliferation, and published reports that AR can inhibit ERα activity in breast cancer cells (Panet-Raymond, Gottlieb et al. 2000; Peters, Buchanan et al. 2009) and androgens can inhibit oestrogen-stimulated breast epithelial proliferation in non-malignant and malignant breast epithelial cells *in vivo* and *in vitro* (Labrie, Poulin et al. 1990; Dimitrakakis, Zhou et al. 2003) studies were also undertaken in this chapter to investigate changes in ERα expression by DHT and/or MPA. This is also based on the literature which indicates that oestrogens and the actions mediated via its cognate receptor ERα act to stimulate breast epithelial proliferation (Aspinall, Stamp et al. 2004; Helguero, Faulds et al. 2005). Furthermore, oestrogen driven ERα-mediated cellular actions are implicated as pivotal driving forces in the promotion and development of breast cancer which has led to the development of targeted hormonal based breast cancer therapeutic treatments (Henderson 2003).

The aims of this chapter were to determine the effects of DHT and/or MPA on the steady state proteins levels of AR, PR and ERα in human non-malignant pre- and post-

menopausal breast tissues, using the *ex vivo* breast explant tissue culture model. Thus, the hypothesis of this chapter was that the anti-androgenic actions of MPA can lead to an increase in breast epithelial proliferation by interfering with DHT-induced AR-signalling.

7.2 - Methods

Refer to Chapter 2 - General Materials and Methods for details on Materials *(section 2.1),* Buffers and Solutions *(section 2.2)* and Methods *(sections 2.31 - 2.34, 2.35 - 2.37 and 2.3.10)* included in this chapter.

7.3 - Results

7.3.1 - MPA inhibits the steady state protein levels of AR induced by DHT in pre- and post-menopausal breast explant tissues

To determine the effect of MPA on DHT-induced AR immunoreactivity in breast explant tissues, pre- and post-menopausal breast explant tissue samples were treated with DHT and/or MPA for 24 and 48 hrs and subsequently immunostained to detect AR protein [Figure 7.1A; 7.2A]. Earlier, in this thesis, treatment with DHT was found to significantly increase AR immunoreactivity in cultured post-menopausal breast explant tissues compared to vehicle control at 48 hrs ($p<0.05$) [Figure 7.1C]. This effect was also observed in pre-menopausal breast explant tissue samples at both 24 and 48 hrs, ($p<0.05$) [Figure 7.2B and C] *(refer to chapter 5 - section 5.3.3).*

Figure 7.1 (A-C): MPA impedes DHT-induced AR immunoreactivity in post-menopausal breast explant tissue. (A) Representative example of AR immunostaining in a post-menopausal breast explant tissue following an initial pre-treatment for 36 hrs and subsequently cultured at 24 hrs (D1) (n=10) and 48 hrs (D2) (n=11) in media containing 10% DCC-FCS under control conditions, i.e. 0.1% ethanol, or treated with 1 nM DHT and/or 1 nM MPA. Images were digitally scanned and taken using a 40x objective microscopic lens. Quantification of AR immunostaining was performed on breast explant tissues using VIA measurements mean integrated optical density (MIOD) and DAB positive area (% positivity). Data in graphs represent AR expression for MIOD and % positivity measurements at **(B)** 24 hrs (D1) and **(C)** 48 hrs (D2). Statistical analysis was performed using Wilcoxon matched pairs test; *$p<0.05$. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. Two samples excluded from the D1, and one sample from D2 time-point due to lack of glandular tissue in some tissue sections. (*Refer to Appendix 2 - Table 1A for VIA quantification raw data).*

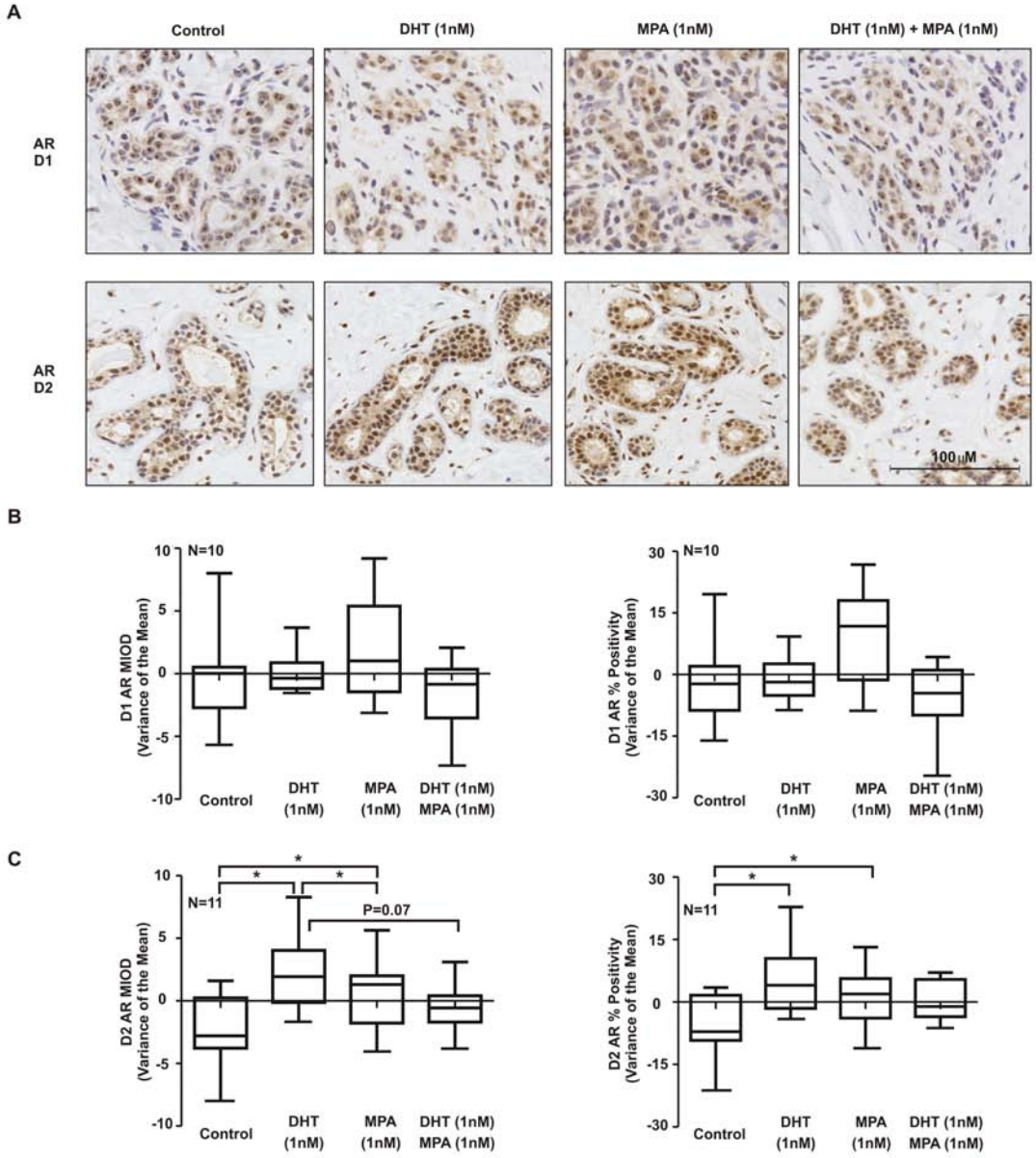
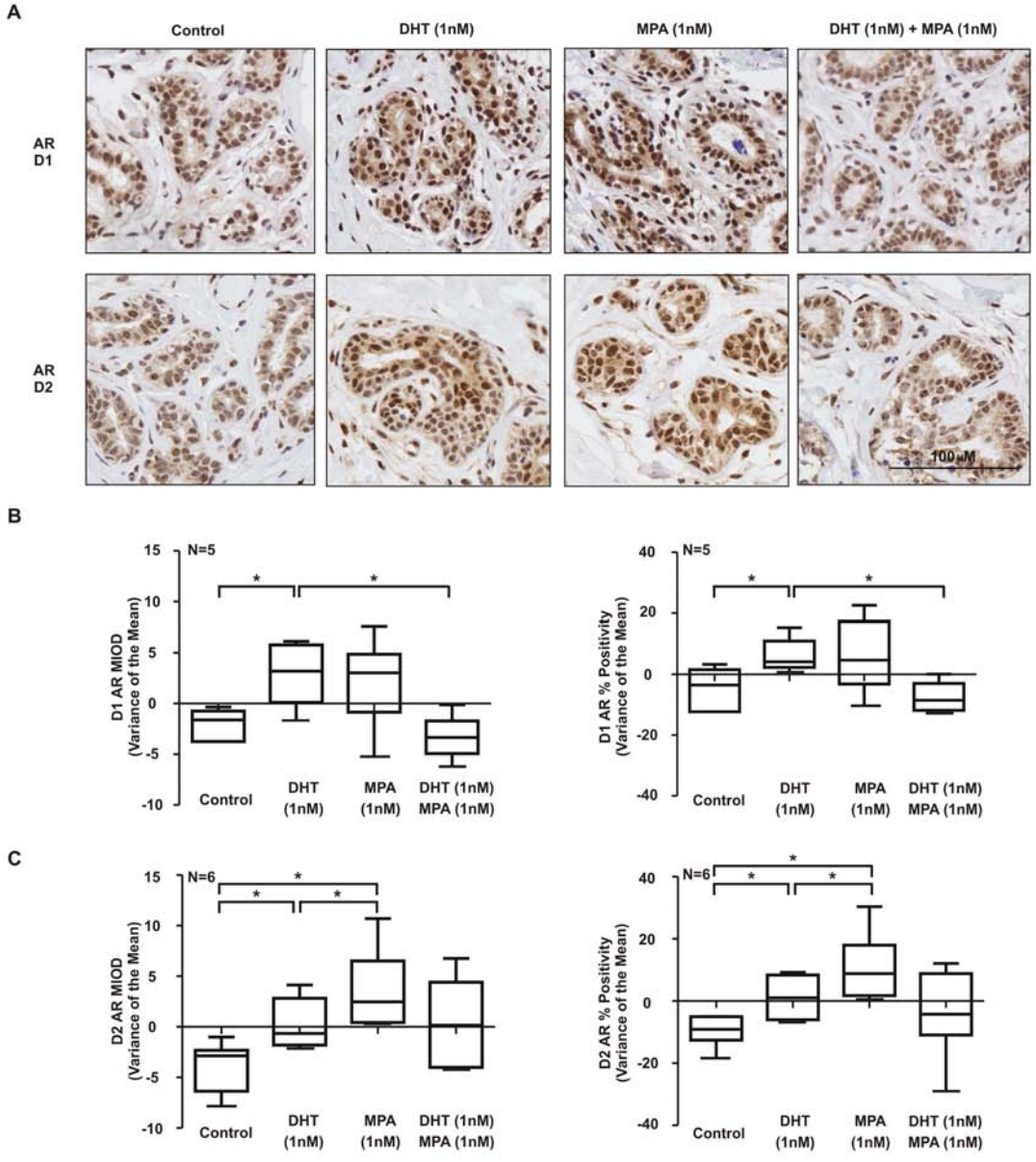

A
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) + MPA (1nM)
AR D1
AR D2
100μM
B
N=10
D1 AR MIOD (Variance of the Mean)
D1 AR % Positivity (Variance of the Mean)
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) MPA (1nM)
C
N=11
P=0.07
D2 AR MIOD (Variance of the Mean)
D2 AR % Positivity (Variance of the Mean)

Figure 7.2 (A-C): MPA abrogates DHT-induced AR immunoreactivity in pre-menopausal breast explant tissue. (A) Representative example of AR immunostaining in a pre-menopausal breast explant tissue following and initial pre-treatment for 36 hrs and subsequently cultured at 24 hrs (D1) (n=5) and 48 hrs (D2) (n=6) in media containing 10% DCC-FCS under control conditions, i.e. 0.1% ethanol, or treated with 1 nM DHT and/or 1 nM MPA. Images were digitally scanned and taken using a 40x objective microscopic lens. Quantification of AR immunostaining was performed on breast explant tissues using VIA measurements MIOD and % positivity. Data in graphs represent AR expression for mean integrated optical density (MIOD) and DAB positive area (% positivity) measurements at **(B)** 24 hrs (D1) and **(C)** 48 hrs (D2). Statistical analysis was performed using Wilcoxon matched pairs test; *$p<0.05$. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. One sample excluded from the D1 time-point due to lack of glandular tissue in some tissue sections. (*Refer to Appendix 2 - Table 7 for VIA quantification raw data).*


A
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) + MPA (1nM)
AR D1
AR D2
100 µM
B
N=5
D1 AR MIOD (Variance of the Mean)
D1 AR % Positivity (Variance of the Mean)
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) MPA (1nM)
C
N=6
D2 AR MIOD (Variance of the Mean)
D2 AR % Positivity (Variance of the Mean)

Studies in this chapter have shown treatment with MPA has no effect on AR immunoreactivity in either patient group at 24 hrs [Figure 7.1B; 7.2B]. However, MPA treatment significantly increased AR immunoreactivity (measured by both VIA measurements) in both pre- and post-menopausal breast explant tissues at 48 hrs ($p<0.05$) [Figure 7.1C; 7.2C]. Additional findings indicate a significantly higher induction of AR protein by MPA compared to DHT in pre-menopausal breast tissue cultured for 48 hrs, by both VIA measurements ($p<0.05$) [Figure 7.2C]. In contrast, this effect of MPA was not observed in post-menopausal breast tissues at either time-points [Figure 7.1B and C]. Similar findings demonstrating that MPA can impede DHT-induced AR immunoreactivity (i.e. AR expression remained similar to controls) after 48 hrs was detected in both pre- and post-menopausal tissues [Figure 7.1C; 7.2C]. Interestingly, a complete abrogation of DHT-induced AR immunoreactivity was also detected by MPA for both VIA measurements in pre-menopausal breast tissues at 24 hrs ($p<0.05$) [Figure 7.2B], and a trend towards this effect was detected in post-menopausal breast tissues using the MIOD VIA measurement only at 48 hrs (albeit not significantly, $p=0.07$) [Figure 7.1C]. Since MPA is used in both cHRT and the POC Depo-Provera and have both been implicated in increased rates of breast cancer (Shapiro, Rosenberg et al. 2000; Rossouw, Anderson et al. 2002), the findings in this chapter may give insights into AR-mediated related actions of MPA and breast cancer in both pre- or post-menopausal women. Further studies are required to investigate whether these changes to AR protein expression by the hormone combination reported in this chapter can also lead to changes in AR function at a transcriptional and/or translational level.

7.3.2 - MPA exhibits unique activity on DHT-induced AR immunoreactivity in post-menopausal breast explant tissue adjacent compared to non-associated with malignant breast tissue

As previously described in this thesis, an impaired AR-signalling axis was evident in post-menopausal breast explant tissue adjacent, compared to non-associated with malignant breast tissue (i.e. either distal to malignant or adjacent to benign breast tissue) at 48 hrs *(refer to chapter 5 - sections 5.3.3 and 5.3.4).* Additional studies were therefore undertaken in this chapter to determine the effect of DHT and/or MPA on post-menopausal breast explant tissue adjacent and non-associated with malignant breast disease at the 48 hr time-point only, since no effect of hormone treatment was observed in post-menopausal breast tissue at 24 hrs. In breast explant tissue samples non-associated with malignant breast disease, DHT treatment resulted in a significant increase in AR immunoreactivity, compared to MPA and control treatments ($p<0.05$) [Figure 7.3A]. This effect was impeded by MPA (i.e. AR immunoreactivity levels following treatment with DHT and MPA combined remained similar to levels detected in control) and was verging towards abrogation of DHT-induced AR immunoreactivity (albeit not significantly, $p=0.07$) [Figure 7.3A]. In contrast, no change in AR immunoreactivity following treatment with DHT was detected in breast explant tissues adjacent to malignant disease [Figure 7.3B]. However, MPA treatment resulted in a significant increase in AR immunoreactivity ($p<0.05$), which also impeded and verged towards abrogation by the hormone combination (albeit not significantly, $p=0.07$) [Figure 7.3B].

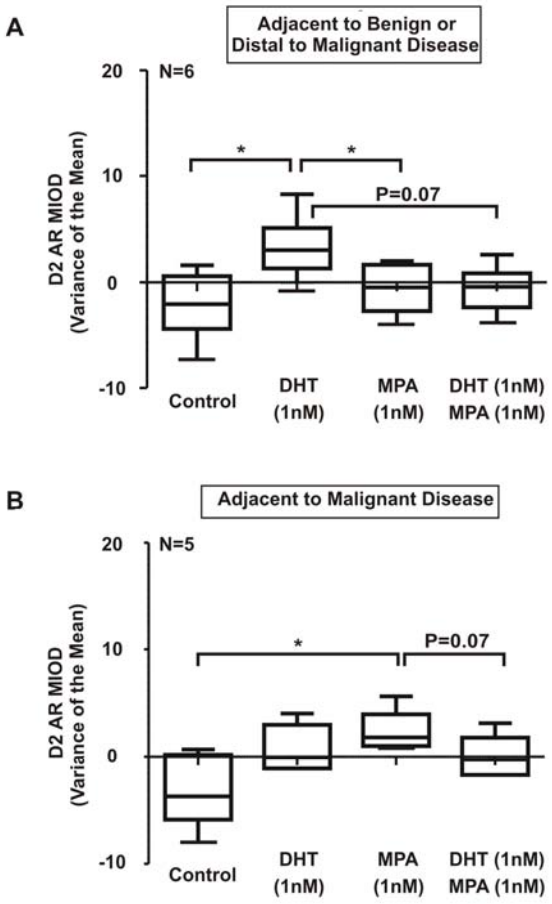


Figure 7.3 (A and B): MPA-induces AR immunoreactivity in post-menopausal non-malignant breast explant tissues adjacent to malignant breast tissue. Post-menopausal breast explant tissue patient samples were sub-divided into groups, i.e. **(A)** adjacent to benign and distal to malignant breast disease (n=6) and **(B)** adjacent to malignant breast disease (n=5). AR immunostaining was measured by VIA and expressed as mean integrated optical density (MIOD) and DAB positive area (% positivity) at **(B)** 24 hrs (D1) and **(C)** 48 hrs (D2). Statistical analysis performed using Wilcoxon matched pairs test; * $p<0.05$. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. Similar data was observed for AR % positivity VIA measurements *(data not shown).* One sample excluded from the D2 time-point due to lack of glandular tissue in some tissue sections. (*Refer to Appendix 2 - Table 1B for VIA quantification raw data).*

7.3.3 - DHT and MPA combined tend towards increased ERα immunoreactivity in post-menopausal breast explant tissue non-associated with malignant breast tissue

Based on the reported findings in an earlier chapter of this thesis indicating that MPA can reverse the anti-proliferative actions of DHT *(refer to chapter 6 - sections 6.3.1 and 6.3.2)*, further studies were undertaken to investigate the effect of treatment with DHT and/or MPA on ERα expression in cultured post-menopausal breast explant tissues. Immunostaining for ERα was shown to be predominantly located in the nucleus of breast epithelial cells [Figure 7.4A]. Since previous findings in this thesis have detected statistical effects of hormone treatment on both AR and Ki67 expression in cultured post-menopausal breast tissue samples at 48 hrs *(refer to chapter 6 and 7)*, the effect of DHT and/or MPA on ERα expression was analysed only at this time-point. Hormone treatments had no effect on ERα immunoreactivity in breast explant tissues after 48 hrs [Figure 7.4B]. However in post-menopausal breast tissue non-associated with malignant disease, treatment with DHT and MPA combined tended towards an increase in ERα immunoreactivity compared to treatment with DHT alone (albeit not significantly, p=0.07) [Figure 7.5A]. In post-menopausal breast explant tissues adjacent to malignancy DHT and/or MPA treatment resulted in similar levels of ERα immunoreactivity [Figure 7.5B].

7.3.4 - DHT and/or MPA have no effect on PR immunoreactivity in post-menopausal breast explant tissues irrespective of association to malignant breast tissue

Data in this chapter demonstrates that MPA can impede DHT-induced AR immunoreactivity in post-menopausal breast explant tissues and can abrogate this effect

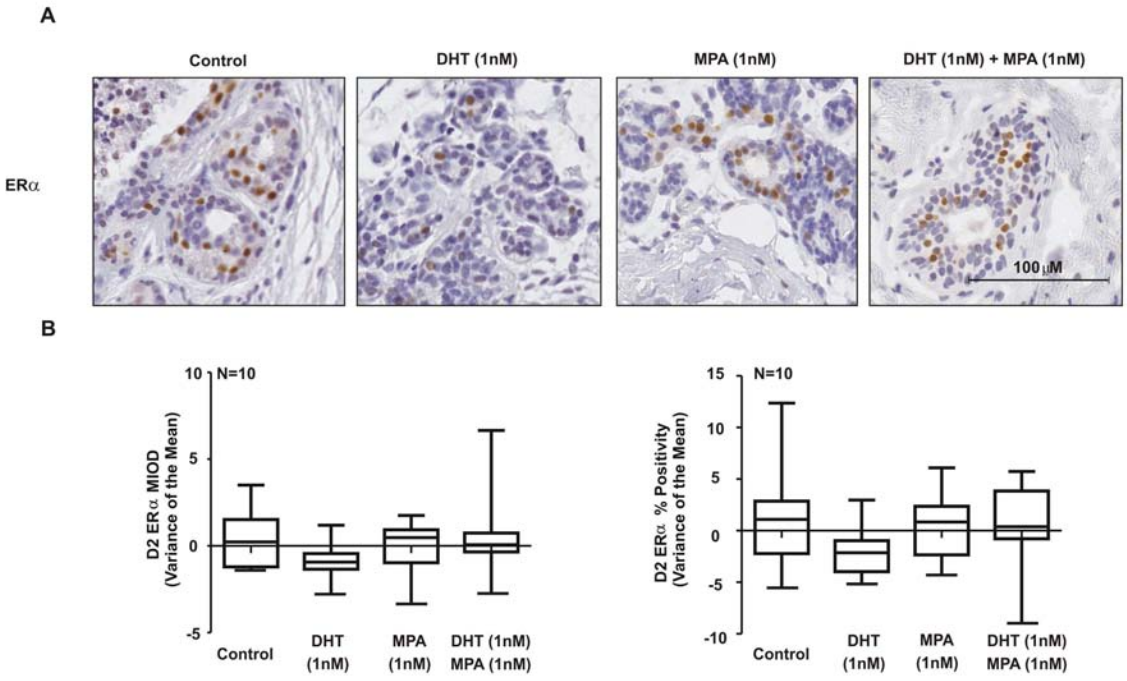


Figure 7.4 (A and B): Treatment with DHT and/or MPA has no effect on ERα expression in post-menopausal breast explant tissue. (A) Representative example of ERα immunostaining in a post-menopausal breast explant tissue following an initial pre-treatment for 36 hrs and subsequently cultured for 48 hrs (D2) (n=10) in media containing 10% DCC-FCS under control conditions, i.e. 0.1% ethanol, or treated with 1 nM DHT and/or 1 nM MPA. Images were digitally scanned and taken using a 40x objective microscopic lens. Quantification of ERα immunostaining was performed on breast explant tissues using VIA measurements mean integrated optical density (MIOD) and DAB positive area (% positivity). Data in graphs represent ERα expression for MIOD and % positivity measurements at **(B)** 48 hrs (D2). Statistical analysis performed using Wilcoxon matched pairs test, significance accepted $p<0.05$. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. Two samples excluded from the D2 time-point due to lack of glandular tissue in some tissue sections. (*Refer to Appendix 2 - Table 8A for VIA quantification raw data).*

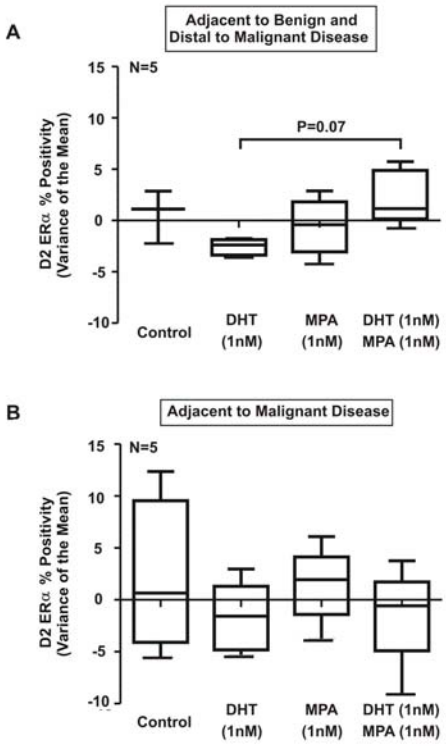


Figure 7.5 (A and B): ERα immunoreactivity tends towards an increase following co-treatment with DHT and MPA in post-menopausal breast explant tissue adjacent with malignant breast disease. Post-menopausal breast explant tissue patient samples cultured for 48 hrs (D2) were sub-divided into groups, i.e. (A) adjacent to benign and distal to malignant breast disease (n=5) and (B) adjacent to malignant breast disease (n=5). ERα immunostaining was measured by VIA and expressed as % positivity. Data in graphs represent AR expression for mean integrated optical density (MIOD) and DAB positive area (% positivity) measurements. Statistical analysis performed using Wilcoxon matched pairs test, significance accepted $p<0.05$. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. Similar data was observed for ERα MIOD VIA measurement *(data not shown).* Two samples excluded from the D2 time-point due to lack of glandular tissue in some tissue sections. (*Refer to Appendix 2 - Table 8B for VIA quantification raw data).*

in pre-menopausal breast explant tissues (*refer to section 7.3.1)*. Therefore, additional studies were undertaken to determine whether the reduction in of DHT-induced AR immunoreactivity by MPA also led to changes in PR steady state protein levels. These studies were performed by immunostaining on cultured post-menopausal breast explant tissues using experimental conditions previously described. Similarly to that observed for ERα, PR immunoreactivity was predominantly located in the nucleus of post-menopausal breast epithelial cells [Figure 7.6A]. Treatment of post-menopausal breast explant tissues with DHT and/or MPA for 48 hrs had no effect on PR immunoreactivity [Figure 7.6B]. This lack of effect on PR was observed irrespective of whether the post-menopausal breast tissues were adjacent or non-associated with malignant breast tissue [Figure 7.7A and B]. However, at 48 hrs, PR expression was significantly reduced by the treatment of MPA at a higher dose (i.e. 100 nM), compared to vehicle control [Figure 7.8].

7.4 - Discussion

The most striking findings reported in this chapter have identified a potential basis mediated by MPA, underpinned by aberrant AR function that may lead to the reversal of the anti-proliferative actions of DHT in post-menopausal breast tissues. This effect been shown to occur in isolation to changes to steady state PR protein levels.

Interestingly, the effects of DHT and/or MPA were more pronounced at the 24 hr time-point in pre- compared to post-menopausal breast tissue. A biological understanding to

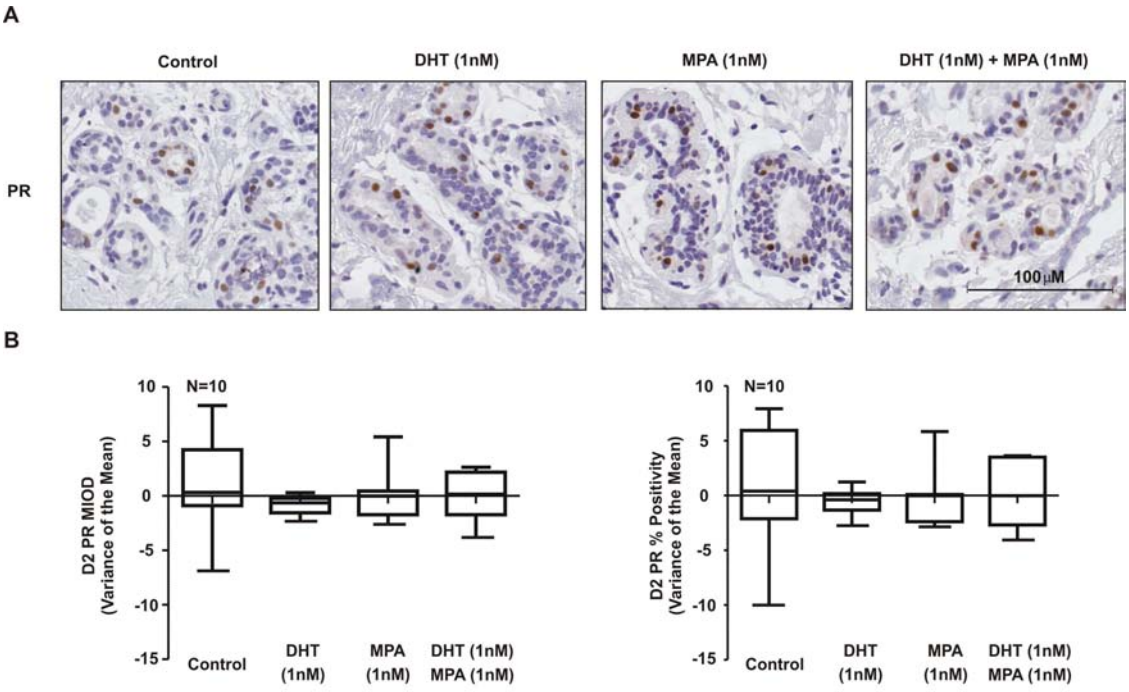


Figure 7.6 (A and B): Treatment with DHT and/or MPA has no effect on PR expression in post-menopausal breast explant tissue. (A) Representative example of PR immunostaining in a post-menopausal breast explant tissue following an initial pre-treatment for 36 hrs and subsequently cultured for 48 hrs (D2) (n=10) in media containing 10% DCC-FCS under control conditions, i.e. 0.1% ethanol, or treated with 1 nM DHT and/or 1 nM MPA. Images were digitally scanned and taken using a 40x objective microscopic lens. Quantification of PR immunostaining was performed on breast explant tissues using VIA measurements MIOD and % positivity. Data in graphs represent PR expression for mean integrated optical density (MIOD) and DAB positive area (% positivity) measurements at **(B)** 48 hrs (D2). Statistical analysis performed using Wilcoxon matched pairs test, significance accepted $p<0.05$. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. Two samples excluded from the D2 time-point due to lack of glandular tissue in some tissue sections. (*Refer to Appendix 2 - Table 9A for VIA quantification raw data).*

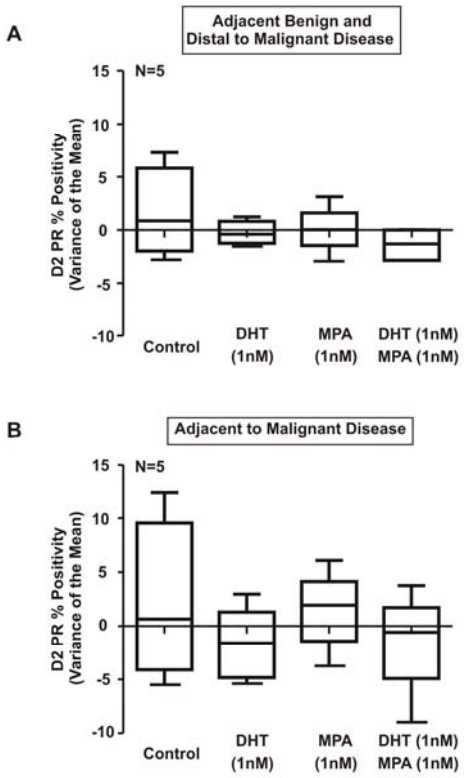


Figure 7.7 (A and B): No effect of DHT and/or MPA on PR immunoreactivity in post-menopausal breast explant tissue irrespective or association with malignancy. Post-menopausal breast explant tissue patient samples were sub-divided into groups, i.e. **(A)** adjacent to benign and distal to malignant breast disease (n=5) and **(B)** adjacent to malignant breast disease (n=5). PR immunostaining was measured by VIA and expressed as DAB positive area (% positivity). Statistical analysis performed using Wilcoxon matched pairs test, significance accepted $p<0.05$. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. Similar data was observed for PR mean integrated optical density (MIOD) VIA measurement *(data not shown).* Two samples excluded from D2 time-point due to lack of glandular tissue in some tissue sections. (*Refer to Appendix 2 - Table 9B for VIA quantification raw data).*

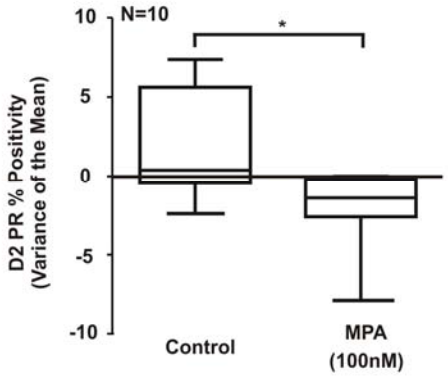


Figure 7.8: Reduction in PR immunoreactivity in post-menopausal breast explant tissues by a high dose of MPA. Post-menopausal breast explant tissue were initially pre-treatment for 36 hrs and subsequently cultured for 48 hrs (D2) (n=10) in media containing 10% DCC-FCS under control conditions, i.e. 0.1% ethanol, or treated with 100 nM MPA. Quantification of PR immunostaining was performed on breast explant tissues using VIA measurements mean integrated optical density (MIOD) and DAB positive area (% positivity). Data in graphs represent PR expression for % positivity measurements 48 hrs (D2). Statistical analysis performed using Wilcoxon matched pairs test, significance accepted $p<0.05$. Box and whisker plots represent the maximum and minimum values, the upper and lower quartiles and the median value. Similar data was observed for PR MIOD VIA measurement *(data not shown).* Two samples excluded from the D2 time-point due to lack of glandular tissue in some tissue sections. (*Refer to Appendix 2 - Table 9A for VIA quantification raw data).*

the difference in the breast tissue responsiveness to hormones based on menopausal status may be associated with alteration in steroid hormone metabolism, receptor stability and turn-over rates. Moreover, the lower AR expression in post- compared to pre-menopausal breast tissues in non-cultured, non-malignant breast tissue samples that has been discussed in an earlier chapter *(refer to chapter 5 - section 5.3.2)* suggests that there are potential differences in AR expression based on menopausal status.

An unexpected finding reported in this chapter suggested that the AR is responsive to the synthetic androgenic hormone MPA, compared to non-responsive to the natural cognate ligand DHT in non-malignant breast tissue within close proximity to malignancy. This data supports previous findings in this thesis demonstrating that post-menopausal breast tissue adjacent to malignant breast tissue harbours an impaired DHT-induced AR-signalling axis, compared to breast tissue non-associated with malignant breast tissue. Intriguingly, in the non-malignant breast tissue samples associated with malignancy, the co-treatment of DHT and MPA acted in an anti-androgenic manner similar to non-malignant breast tissue non-associated to malignancy. The molecular basis for the difference in hormone responsiveness in non-malignant breast tissues within close proximity to malignancy is unknown and requires further investigation.

In this chapter findings indicate that ERα protein may be induced (albeit not significantly, $p=0.07$) by the co-treatment of DHT and MPA, compared to DHT in post-menopausal non-malignant breast tissues samples non-associated with malignant breast tissue. Interestingly, the AR is reported to inhibit ERα expression and activity in breast cancer

cells (Panet-Raymond, Gottlieb et al. 2000; Cops, Bianco-Miotto et al. 2008; Peters, Buchanan et al. 2009). Based on this literature, it may be interesting to speculate that the anti-androgenic actions of MPA on DHT-induced AR expression may also lead to the loss of ERα inhibition by DHT. A molecular basis associated with the different effects of the co-treatment on ERα expression in non-malignant breast tissue adjacent to malignant breast disease, compared to non-associated is difficult to interpret based on the current literature.

The data described in this chapter gives support to the notion that MPA can impede DHT-induced AR-signalling independent of changes to PR protein in human post-menopausal non-malignant breast epithelial cells. Despite these findings, it must be considered that DHT and/or MPA treatment may result in changes to PR-signalling which was not examined in this study. Hence, additional studies investigating changes in the expression of PR-regulated genes by the hormone treatments included in this thesis are required to gain further insight into the changes to PR-signalling. However, data in this chapter has shown that high doses of MPA (i.e. 100 nM) decreased PR protein compared to control treatment in post-menopausal non-malignant breast tissue samples. These findings are important in demonstrating that the post-menopausal breast tissues used in this study were responsive to MPA at higher doses, in support of the current literature (Eigeliene, Harkonen et al. 2006; Cops, Bianco-Miotto et al. 2008). Additionally, the decreased response of PR protein by MPA, illustrate that MPA at low and high doses potentially possess vast differences in the actions of sex nuclear receptors. This notion is also reflected in earlier findings in this thesis described in chapter 6 indicating that MPA at

low doses of 1 nM did not effect Ki67 expression, whereas high doses of MPA (i.e. 100 nM) has been reported to stimulate breast epithelial proliferation in non-malignant post-menopausal breast tissues (Eigeliene, Harkonen et al. 2006).

The effect of DHT on ERα expression in human breast epithelial cells is not well documented. However, 10 - 100 nM DHT treatment of the T-47D breast cancer cell line leads to a reduction in the E_2 regulated gene PR (Cops, Bianco-Miotto et al. 2008). It may be speculated that since AR can inhibit ER expression and function in breast cancer epithelial cells (Panet-Raymond, Gottlieb et al. 2000; Peters, Buchanan et al. 2009), that this may also translate through to similar effects in human non-malignant breast epithelial cells, whereby ERα steady state protein levels are reduced. However, in this study, no change in ERα expression or activity via changes in the ERα-regulated gene PR (Horwitz, Costlow et al. 1975; Kastner, Krust et al. 1990) was detected following treatment with DHT, as previously reported (Cops, Bianco-Miotto et al. 2008; Peters, Buchanan et al. 2009). Similarly no change in PR expression was observed by the co-treatment, compared to DHT, in breast tissues non-associated with malignancy despite a tendency towards increased ERα protein levels in the same tissues. The lack of oestrogen supplementation in the experimental conditions and/or the use of non-malignant human breast tissues in this study compared to others which have used malignant breast cell lines may have compromised the findings. Furthermore, the low base-line protein expression levels of ERα and PR in cultured breast tissue samples subsequent to hormone deprivation *(refer to chapter 5 - section 5.3.2)* may have prevented the detection of reduced expression by DHT and/or MPA in this study. Future studies which include

supplementation of oestrogen in the experimental conditions used for the *ex vivo* breast explant tissue culture model used in this thesis may provide for the following to be detected: 1) an inhibitory effect of DHT on ERα expression and 2) a more dramatic increase in ERα following treatment with DHT and MPA combined, compared to DHT alone.

The mechanisms by which androgens contribute to reduced oestrogen stimulated breast epithelial cell proliferation have been reported in the literature to potentially be associated with, reduced ERα expression and activity (Poulin, Simard et al. 1989; Panet-Raymond, Gottlieb et al. 2000; Zhou, Ng et al. 2000; Dimitrakakis, Zhou et al. 2003), decreased PR activity (MacIndoe and Etre 1980; MacIndoe and Etre 1981), increased apoptosis (Kandouz, Lombet et al. 1999; Lapointe, Fournier et al. 1999), increased G1 cell cycle arrest (Szelei, Jimenez et al. 1997; Ando, De Amicis et al. 2002; Greeve, Allan et al. 2004), increased activation of ERβ (Asano, Maruyama et al. 2003; Dimitrakakis, Zhou et al. 2003) and/or increased conversion of 17β-oestradiol to oestrone (E_1) (Couture, Theriault et al. 1993). In addition, androgens can act indirectly on breast tissue by negative feedback on the hypothalamic-pituitary axis, which can reduce LH stimulation of steroid receptor synthesis in the ovary and adrenal gland (Yen 1977; Glidewell-Kenney, Hurley et al. 2007). Thus, a relationship between increased ERα expression and breast epithelial proliferation by the anti-androgenic actions of the hormone combination *(refer to chapter 6 - section 6.3.2)* may not be unexpected findings. In light of a study published in the DRMCRL it would be interesting to examine whether the AR can maintain binding to oestrogen response elements in the presence of DHT and MPA

combined, since AR bound to DHT can and it is thought that this effect potentially leads to the reduction in ERα expression by DHT alone (Peters, Buchanan et al. 2009).

In summary, this chapter in conjunction with findings described in chapter 6 has provided novel evidence of a potential relationship between the loss of DHT-induced inhibition of breast epithelial proliferation by DHT and MPA combined in post-menopausal breast epithelial cells by the reduction in AR steady state protein levels. An *'endocrine disruption hypothesis'* has been developed by the DRMCRL whereby DHT-induced AR-signalling is abrogated by the MPA used in cHRT (Birrell, Butler et al. 2007). This hypothesis has now been further developed by the findings in this study suggesting that MPA can impede DHT-induced AR-signalling and may subsequently lead to the altered regulation of breast epithelial proliferation [Figure 7.9]. Thus, further studies have been undertaken in the final chapter of this thesis to delineate potential actions of MPA that may lead to an increase in breast cancer promotion in post-menopausal taking cHRT. These final studies are aimed at investigating AR-mediated changes by MPA, to AR-signalling and key intracellular cancer-related pathways that may be modified by the hormone combination.

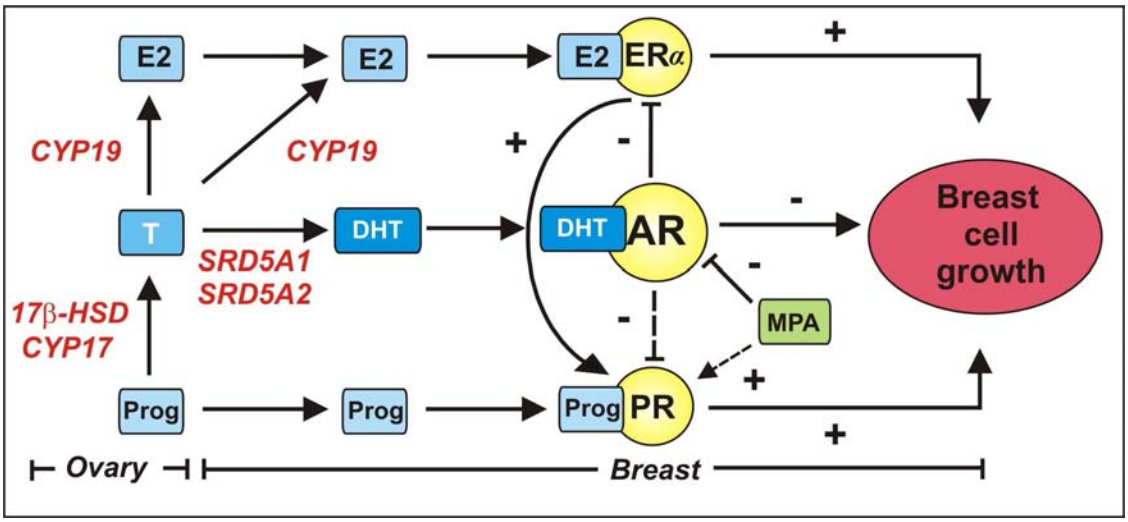


Figure 7.9: Endocrine disruption hypothesis in breast tissue. Diagram depicts a mechanistic basis associated with the synthetic progestin MPA and loss of normal hormonal homeostasis in human breast tissue. The proposed model represents abrogation of DHT-induced AR-signalling by MPA in breast epithelial cells, leading to an increase in breast epithelial proliferation by the loss of inhibition of ERα-stimulated activity.

Chapter 8

MPA abrogates DHT-induced AR-signalling and regulates unique intracellular pathways in malignant breast epithelial cells

8.1 - Introduction

Studies described in earlier chapters 5 and 6 of this thesis have revealed that MPA prevents DHT from inhibiting normal breast epithelial cell proliferation, and that MPA potentially inhibits DHT signalling via AR-mediated actions. However, the molecular mechanism(s) underlying the actions of DHT and MPA combined, in promoting these changes are unknown. Therefore the aims of this chapter were to validate these changes in breast epithelial proliferation by the combined actions of DHT and MPA and to investigate such mechanisms in malignant breast epithelial cells by: 1) determining the effect of DHT and/or MPA on the proliferation of malignant breast epithelial cells in the presence and absence of E_2; 2) examining the effect of MPA on DHT-induced AR gene expression and 3) identifying the intracellular AR-signalling pathways and genes underlying the interplay between DHT and MPA activity that may lead to the promotion and/or development of breast cancer development.

The experimental studies in previous chapters have been undertaken using normal human breast tissue samples, whereas the studies in this chapter have primarily been performed using the AR, ERα and PR positive malignant breast epithelial cell line, ZR-75-1. The reason for using a malignant cell line rather than human breast tissue was based on the ability to undertake studies to investigate the effects of DHT and MPA combined on global gene regulation by Affymetrix gene microarray, which is associated with a number of technical difficulties using breast explant tissue. These include: 1) high heterogeneity of biological responses between human patient tissue samples; 2) acquiring large

concentrations of high quality RNA for gene microarray studies and 3) the high experimental cost due to a high number of biological replicates required for accurate representation of biological heterogeneity in the female population. The use of a normal breast epithelial cell line was also eliminated as a feasible option to undertake Affymetrix gene microarray studies in this study due to various issues stated in an earlier chapter, namely the lack of steroid receptors in these models *(refer to chapter 4, section 4.1)*.

The findings described in chapters 5-7 indicate potential differences in the combined actions of DHT and MPA in non-malignant breast tissue in close proximity to malignancy, which may have already undergone pre-malignant changes as a consequence of altered paracrine signalling events from neighbouring malignant cells (Clarke, Howell et al. 1997; Mallepell, Krust et al. 2006). Moreover, the promotion of breast cancer in post-menopausal women taking cHRT has been reported to occur by the stimulation of occult pre-existing, pre-malignant in the breast tissue (Horwitz and Sartorius 2008). In light of these findings, the use of the ZR-75-1 malignant breast epithelial cell line is also useful as an experimental approach in this chapter to determine whether the effects of DHT and MPA combined already described in this thesis can potentially occur also in breast epithelial cells that have already undergone carcinogenic transformation.

The ZR-75-1 cell line has been widely used to investigate the effect of various natural and synthetic hormones on the growth of malignant breast epithelial cells (Poulin, Baker et al. 1988; Poulin, Baker et al. 1989; Labrie, Poulin et al. 1990; Birrell, Bentel et al. 1995). An inhibitory action of DHT on basal and E_2 stimulated proliferation of ZR-75-1

cells has previously been reported in the literature (Poulin, Baker et al. 1988; Poulin, Simard et al. 1989; Labrie, Poulin et al. 1990; Labrie, Simard et al. 1990; de Launoit, Dauvois et al. 1991; Birrell, Bentel et al. 1995; Landrieu, Smet et al. 2006). The inhibitory effect of DHT on proliferation may be mediated by either of the following effects: 1) reduction of ER expression (Poulin, Simard et al. 1989); 2) growth arrest in the G0 - G2 phase of the cell cycle (de Launoit, Dauvois et al. 1991) and 3) increased metabolism of E_2 to E_1 caused by changes to the expression of the steroid synthesis hormone 17βHSD (Couture, Theriault et al. 1993). MPA can also inhibit basal and E_2 ZR-75-1 breast epithelial at low (0.01 μM) and high doses (0.1 - 1 μM), but it is more effective in the high dose range (Labrie, Poulin et al. 1990; Poulin, Baker et al. 1991). Despite these findings, the comparative effects of DHT and MPA alone and combined on proliferation in the ZR-75-1 and other malignant breast epithelial cell lines in the absence and presence of E_2, is relatively unknown, hence the experimental studies in this chapter.

A number of androgen-regulated genes implicated in breast cancer have also been identified using the ZR-75-1 cell line, including bcl-2 (a pro-survival factor) (Lapointe, Fournier et al. 1999) and UDP-GlcDH (Lapointe and Labrie 1999). However, the majority of androgen regulated genes have been identified in malignant breast and non-breast epithelial cell lines, including PSA (Hall, Clements et al. 1998; Sauter, Tichansky et al. 2002; Sauter, Lininger et al. 2004; Narita, Raica et al. 2005), gross cystic fluid disease protein-15 (GCFDP-15) (Dumont, Dauvois et al. 1989; Blais, Sugimoto et al. 1994; Blais, Sugimoto et al. 1995), GCFDP-24 (which corresponds to apolipoprotein-D (apo-D) (Simard, Dauvois et al. 1990; Hall, Aspinall et al. 1996), GCFDP-44

(Haagensen, Stewart et al. 1992), L-Plastin (Lin, Lau et al. 2000), and MYC (Bieche, Parfait et al. 2001). Compared to classic androgen-responsive tissues such as the prostate gland (Singh, Manickam et al. 2006; Morgenbesser, McLaren et al. 2007; Wang, Wang et al. 2007), the global transcriptional response to androgens in human breast tissue has not been described.

Emerging evidence for the importance of the AR-signalling axis in maintaining a homeostatic balance of proliferation in breast epithelial cells and AR expression as a prognostic indicator of breast cancer overall survival rates (Dimitrakakis and Bondy 2009; Peters, Buchanan et al. 2009) necessitates a more comprehensive identification of androgen regulated genes and determination of key intracellular AR-signalling changes associated with the initiation, development and progression of breast cancer. Thus, the aim of this chapter was to identify key changes to AR-signalling by MPA that may lead to the onset of carcinogenesis formulating the hypothesis that MPA promotes breast cancer by altering DHT-induced AR-signalling and by de-regulating intracellular signalling cancer-related pathways in breast epithelial cells.

8.2 - Methods

Details of general cell culture procedures, compositions of buffers and reagents used in experimental studies in this chapter and companies which supplied equipment, reagents and general materials have been previously described in Chapter 2 - General Materials and Methods *(sections 2.1 and 2.2)*, unless otherwise stated.

8.2.1 - Chemicals, solutions, general materials and reagents

- ***Affymetrix (Santa Clara, CA, USA)*:** UniGem human cDNA Affymetrix Array (28,600 cDNA probes) Human Genechip Gene 1.0 ST Arrays
- ***Ambion Inc Applied Biosystems (MEL, VIC, AU)*:** Ultra pure water, RNase free water, Turbo DNAfree kit (10x Turbo DNase, Turbo DNase free and DNase inactivation reagent), glycogen
- ***BioRad (Sydney (SYD), NSW, Australia)*:** iScript reaction mix, iQ SYBR green supermix
- ***Invitrogen (Carlsbad, CA, USA)*:** trizol
- ***Sigma (St Louis, MO, USA)*:** chloroform, ethanol and isopropanol
- ***Qiagen (MEL, VIC, Australia)*:** RNeasy kit (RNeasy column, RW1 buffer, RPE buffer)

8.2.2 - Equipment

- ***BioRad (SYD, NSW, AU)*:** CFX384 Real-Time PCR detection system and Experion System
- ***ThermoSceintific (Wilmington, DE, USA)*:** Nanodrop 2000c Spectrophotometer

8.2.3 - Software

- ***BioRad (SYD, NSW, AU)*:** CFX, Experion
- ***geNorm Software*:** Microsoft Excel 2002
- ***GraphPad Software (San Diego, CA, USA)*:** GraphPad Prism5.02
- ***Microsoft Office (Redmond, WA, USA)*:** Microsoft Office Excel 2003

- ***Nanodrop Technologies (Wilmington, DE, USA)*:** NanoDrop ND 1000 3.3
- ***Partek Genomics Suite (ADL, SA, AU)*:** Version 6.5

8.2.4 - Growth curves

8.2.4.1 - Plating of cells

ZR-75-1 cells were maintained and grown in phenol red free (PRF)-RPMI media supplemented with 10% FCS and the antibiotics penicillin and streptomycin (P/S) prior to plating for growth assays. The cells were washed in sterile 1x PBS to remove excess culture media, trypsinised at 37ºC for 5 min, transferred to a sterile tube containing culture media and centrifuged at 1,500 rpm for 5 min to pellet cells. Cell number was obtained using a haemocytometer by a standardised protocol. ZR-75-1 cells were seeded in 24-well culture plates in 1 ml of PRF-RPMI media supplemented with 10% dextran coated charcoal stripped foetal calf serum DCC-FCS and P/S at a density of 5.0×10^5 cells/ml for short-term growth assays (i.e. 8 days), and 8.0×10^4 cells/ml for long-term growth assays (i.e. 15 days). The time-points used for the growth assays performed in this chapter were derived form similar experimental studies using the ZR-75-1 to study the effects of exogenous hormones on cell growth (Labrie, Poulin et al. 1990; Poulin, Baker et al. 1991; Birrell, Bentel et al. 1995). The cells were incubated in a 5% CO_2 incubator at 37ºC for 48 hrs after cell plating to allow for cellular attachment prior to treatment with hormones.

8.2.4.2 - Hormone treatment of cells

Treatments of ZR-75-1 cells were prepared in PRF-RPMI media supplemented with 10% DCC-FCS and P/S. For short-term growth assays (i.e. 8 days), ZR-75-1 cells were treated as follows: control (0.1% ethanol), 1 nM DHT, 1 nM MPA, 1 nM DHT plus 1 nM MPA. All treatments were performed in the presence or absence of 1 nM E_2. For long-term growth assays (i.e. 15 days) ZR-75-1 cells were exposed to the same treatments as short-term growth assays, with the following additional treatments: 1 nM DHT plus 1 µM Bic alone or combined. In the long-term experiments, a lower plating density was required to allow for a longer duration of cell growth without reaching confluence. During the short and long-term growth assays, treatments were replenished every 2 days by a half media change and addition of new hormone, containing 2 x the concentration. Three replicates per hormone treatment at each time-point were included in each experiment, and the data was represented as the mean ± standard error of the mean (SEM) of each set of three replicates. These experiments were performed two times for validation of results.

8.2.4.3 - Counting of viable and dead cells

ZR-75-1 cells were treated as described and viable and dead cells were counted on the first day of treatment, and every 2 days after that in short-term growth assays, and every 3 days after that in long-term growth assays. Counting of cells was undertaken by pipetting all of the culture media of each corresponding well into a 25 ml conical tube to allow collection of unattached cells. 1 ml of 1 x PBS was then added to each well (to wash away excess media), followed by an incubation for 5 min at 37ºC in 500 µl of 1 x trypsin and EDTA, for detachment of cells from the culture plate. 500 µl of PRF-RPMI media

supplemented with 10% DCC-FCS was added to the wells of each plate to collect any remaining cells, and subsequently transferred to the corresponding 25 ml conical tubes (this step was repeated two times). The cells were centrifuged at 1,500 rpm for 5 min and the supernatant aspirated off and re-suspended in 50 µl of medium. This cell suspension was mixed with 50 µl of trypan blue in a 96-well plate and 20 µl was loaded onto a haemocytometer for cell counting. At least 100 cells were counted in 8-15 large squares (each comprised of 16 smaller squares) of the haemocytometer using a microscopic lens of 20x magnification. Total cell numbers were calculated using the following formula: (total number of cells/total number of squares)*dilution factor. All hormone treatment triplicates per time-point remained as individual samples for counting.

8.2.5 - Immunoblot Analysis

8.2.5.1 - Preparation of protein lysates

ZR-75-1 cells were plated in 6-well plates at a density of $5x10^5$ cells per well in phenol red RPMI media supplemented with 10% FCS and P/S and left to adhere for 24 hrs at 37ºC. Subsequently, the plating media was aspirated off and the cells were washed twice in 2 ml of sterile 1 x PBS to remove all phenol red media and FCS from the cells and dish. A steroid hormone deprivation time period of 48 hrs was further performed by the addition of 2 ml PRF-RPMI media supplemented with 10% DCC-FCS and P/S prior to hormone treatments. Following this pre-treatment, the media was removed and the following treatments were added in the same media conditions described containing control (0.1% ethanol) 1 nM DHT, 1 nM MPA and/or 1 µM Bic and 1 nM DHT plus 1 nM MPA for 1, 2 and 8 days. Treatments were replenished every 2 days by a full media

change. At the designated time-points, the culture dishes were placed on ice, the media was aspirated off and the cells were washed twice with 2 ml ice cold 1 x PBS. RIPA buffer was added to the culture dishes and the cells dislodged using a cell scraper. The cells were then transferred to a chilled 1.5 ml Eppendorf tube, lysed using a 21-gauge needle connected to a 1 ml syringe (8-10 times) and centrifuged at 8,000 rpm at 4ºC for 10 min to remove cell debris. Protein lysates were transferred to a new chilled 1.5 ml Eppendorf tube. Protein concentration was determined by Bradford assay as follows: 2 µl of protein lysate was added to 198 µl of Bradford assay reagent and quantified using a standard curve generated from known standards of bovine serum antigen (BSA) (1, 2, 4, 6 and 8 ng/ml). The concentration of protein samples was measured using a spectrophotometer at 595 nm.

8.2.5.2 - Gel electrophoresis

20 µg of protein from the ZR-75-1 lysates representing each hormone treatment at each designated time-point was loaded onto SDS PAGE gels. The protein lysates were prepared for gel electrophoresis by adding the appropriate amount in RO water to make a final 20 µl sample volume. A 6 x loading dye reagent was subsequently added to the protein samples and the protein denatured for 5 min at 95ºC. The samples were re-collected by briefly spinning in a table-top centrifuge. 20 µg of a standard protein marker for protein size determination and the protein samples were loaded onto each SDS PAGE gel. The protein lysates were stacked by electrophoresis (15 mA) through an 8.5% SDS stacking gel for 15-20 min then separated by electrophoresis (25 mA) through an 8.5% or 15% separating SDS gel for 15 min. Both stages of gel electrophoresis associated with

stacking and separating of the protein lysates was undertaken in 1 x running buffer. The separated proteins were then transferred onto a nitrocellulose membrane for 1.5 hrs at 250 mA in chilled 1 x transfer buffer.

8.2.5.3 - Protein detection

Following transfer of the protein samples onto a nitrocellulose membrane, a ponceau stain was performed to confirm the transfer of protein. The membranes were incubated in ponceau stain for 5-10 sec and then washed 3 times with RO water on a table top rotator. Prior to the addition of the primary antibody, the nitrocellulose membranes were blocked in 3% skim milk in 1 x TBST by gentle agitation at room temperature for 1.5 hrs. The primary antibody at the appropriate dilution was added in a mixture of 5 ml 1.5% skim milk prepared in 1 x TBST and left overnight at 4ºC to probe for specific proteins [Table 1]. Following incubation with the primary antibody, the nitrocellulose membranes were washed 3 x in 1 x TBST for 10 min by gentle agitation at room temperature on a table top rotator. A secondary antibody at a 1:1000 dilution [Table 1] was added to 5 ml 1.5% skim milk mixture prepared in 1 x TBST for 1 hr at room temperature by gentle agitation on a table-top rotator and washed for 3 x in 1 x TBST for 10 min. Antibody detection of protein was undertaken using a chemiluminescent kit (ECLTM). The two chemiluminescent reagents were mixed together in a 1:1 dilution and incubated with the membranes for 1 min at room temperature. Specific proteins were visualised by exposing the membranes to hyperfilm ECLTM in a dark room. The hyperfilm was developed and fixed using developer and fixer solutions at optimised exposure times.

Table 1 - Primary and secondary antibody dilutions used for immunoblot analysis

Primary Antibody	Dilution	Secondary Antibody	Dilution
AR	1/1000	**Goat –Anti-Rabbit**	1/1000
PR	1/500	**Rabbit – Anti-Mouse**	1/1000
FKBP5	1/1000	**Goat – Anti-Rabbit**	1/1000
KITL	1/500	**Rabbit – Anti-Goat**	1/1000
UDP-GlcDH	1/500	**Goat – Anti-Rabbit**	1/1000
BEK-1 (FGFR2)	1/200	**Goat - Anti-Rabbit**	1/1000
HSP-90	1/1000	**Goat – Anti-Rabbit**	1/1000

8.2.6 - Preparation of ZR-75-1 cDNA for Affymetrix gene microarray and qRT-PCR analysis

Completion of technical work associated with experiments described in sections 8.2.5 - 8.2.6 were undertaken by Dr Nicole Moore and Andrienne Hanson in the DRMCRL. All the analysis of the Affymetrix gene microarray data and qRT-PCR included in this chapter was performed by the candidate unless otherwise stated.

8.2.6.1 - Cell plating and hormone treatments

ZR-75-1 cells were plated at a density of $5x10^5$ cells per well in 6-well culture plates in PRF-RPMI media supplemented with 10% DCC-FCS and P/S. The cells were left to adhere for 48 hrs at 37ºC prior to treatment. The following treatments were added to the cells in quadruplicate: control (0.1% ethanol), 1 nM DHT, 1 nM MPA, 1 nM DHT and 1 nM MPA combined. The cells were further incubated for 6 hrs and then harvested prior to the extraction of RNA as individual quadruplicate samples per treatment.

8.2.6.2 - RNA extraction

ZR-75-1 cells were washed with 1 x PBS before adding 500 µl of trizol reagent. The cells were then homogenised by pipetting 6 times through a 1 ml syringe attached to a 25-gauge needle. RNA was extracted by the addition of 100 µl chloroform and vigorous shaking for 15 sec and subsequent incubation on ice for 15 min. The trizol-chloroform solution was centrifuged at 6,500 rpm for 30 min at 4ºC, and the upper layer (200 µl only) was transferred to a fresh 1.5 ml Eppendorf tube. 200 µl of 70% ethanol (diluted in RNase free H_20) was added and each sample was transferred into an RNeasy column and centrifuged twice at 6,500 rpm for 1 min at room temperature. The elute was re-added to

the RNeasy column, and centrifuged again at 6,500 rpm for 1 min at room temperature, according to manufacturer's protocol. The column was washed through with 700 µl RW1 buffer, 500 µl RPE buffer (wash completed twice) and centrifuged at 6,500 rpm for 1 min at room temperature. Following these washes, the column was spun briefly to remove residual buffer and placed in a fresh 1.5 ml Eppendorf tube. The RNA was eluted from the RNeasy column by adding 30 µl RNase-free water, allowing the RNA to incubate for 1 min at room temperature and centrifuging at 6,500 rpm for 1 min. 1 µl of the 30 µl of RNA elute was used for a concentration measurement using nanodrop spectrophotometer and the remainder sample stored at -80ºC.

8.2.6.3 - DNase treatment

RNA stored at -80ºC was thawed on ice for 15-20 min and 1 µg was pipetted in 1.5 ml Eppendorf tube for DNase treatment. To each sample a DNase master mix was added which was comprised of 5 ul 10 x Turbo DNase and 1 ul TurboDNase free, and incubated at 37 ºC for 30 min. 2 ul of DNase inactivation reagent was added, and mixed well by gently pipetting. The samples were centrifuged at 10,000 rpm at 4ºC for 1.5 min, and subsequently transferred to ice. The RNA was transferred to a fresh 1.5 ml Eppendorf tube on ice, 2 µl of glycogen was added and gently flicked several times. The RNA was precipitated by adding 50 µl of 75% isopropanol and incubating at -80ºC overnight. The RNA samples were then centrifuged at 15,000 rpm for 20 min at 4ºC. The supernatant was removed and the RNA pellet was washed in 1 ml 75% ethanol and centrifuged at 15,000 rpm for 15 min at 4ºC. The remaining supernatant was removed and each tube centrifuged for 1 min to remove residual ethanol. The RNA pellet was left to air-dry at

room temperature for 5 min. The RNA was resuspended by the addition of 10 µl of 1 x TE and incubation at 55ºC for 10 min with gentle two gentle flicks during the incubation. The concentration of 1 µl of RNA was determined using a nanodrop spectrophotometer and the remainder of the DNase-treated RNA was stored at -80ºC.

8.2.6.4 - Preparation of cDNA (reverse transcription (RT) - PCR))

200 ng of DNase-treated RNA was aliquoted into 1.5 ml Eppendorf tubes chilled on ice and made up to a 15 µl volume with RNase-free water. A RT master mix was prepared which was comprised of 4 µl 5 x iScript reaction mix and 1 µl iScript or 1 µl RNase free water for control reactions (RT-control), per sample. Either RT master mix was added to each RNA sample and further centrifuged briefly at room temperature to collect the sample. An additional control was used which included 4 µl 5 x iScript reaction mix and 1 µl iScript reaction mix in the absence of the RNA sample. The RNA was incubated at room temperature, 42ºC and 85ºC for 5, 30 and 5 min, respectively. The cDNA samples were placed at -20ºC for long-term and 4ºC for short-term, storage.

8.2.6.5 - Quantitative real-time polymerase chain reaction (qRT-PCR)

All qRT-PCR reactions were diluted 1/5 (20 µl cDNA sample into 80 µl of RNase free water) and each treatment consisted of quadruplicate biological replicates. A master mix was prepared from 2 x iQ SYBR Green Supermix, Ultra Pure Water and both of the forward and reverse primers at a starting concentration of 5 pmol/µl for either AR, FGFR2, OLR1, C1ORF116, PR, FKBP5, pS2, L19 and HMBS (primers were designed

Table 2 - Oligonucleotide primers for qRT-PCR

Primer	Ref Seq Accession Number	Forward primer 5' - 3'	Reverse primer 3' - 5'
AR	NM_000044	CCTGGCTTCCGCAACTTACAC	GGACTTGTGCATGCGGTACTCA
FGFR2	NM_000141	**No available sequence from Qiagen**	**No available sequence from Qiagen**
OLR1	NM_002543	GCACAGCTGATCTGGACTTCAT	GACAGCGCCTCGGACTCTAA
C1ORF116	NM_023938	CATCTCTGCCCTTTGAAACAAAA	GGGCATCACCCGAAACAAG
PR	NM_003225	CGCGCTCTACCCTGCACTC	TGAATCCGGCCTCAGGTAGTT
FKBP5	NM_004117	AAAAGGCCAAGGAGCACAAC	TTGAGGAGGGGCCGAGTTC
pS2	NM_003225	TTGTGGTTTTCCTGGTGTCA	GCAGATCCCTGCAGAAGTGT
L19	NM_000981	TGCCAGTGGAAAAATCAGCCA	CAAAGCAAATCTCGACACCTTG
HMBS	NM_000190	CCACACACAGCCTACTTTCCAA	TTTCTTCCGCCGTTGCA

All primers apart from FGFR2 ordered by Qiagen were prepared by GeneWorks (http://www.geneworks.com.au/default.aspx?p=1) (Thebarton, SA, Australia).

by Dr Nicole Moore from or obtained from published studies and were designed by GeneWorks, Thebarton, SA, Australia (*http://www.geneworks.com.au/Default.aspx?p=1*) [Table 2]. The FGFR2 primer was ordered from Qiagen in the absence of a primer sequence [Table 2]. 2 ul of each dilute cDNA standard and test sample was added to 10 ul of master mix. The standard controls used in the qRT-PCR analysis were prepared from the samples which showed the highest RNA expression following hormone treatment from the microarray analysis (i.e. 1 nM DHT for C1ORF116 and FGFR2). All RT standard controls were diluted 1/10, 1/100, 1/1,000 and 1/10,000 in RNase free H_20, and prepared as triplicate technical replicates. A standard curve was generated using CT values from these standard controls. Each qRT-PCR analysis was performed using the CFX384 Real-Time PCR detection system and analysis performed using CFX software. The qRT-PCR conditions were 3 min 95ºC (1 x) (denaturation step), followed by 40 cycles of 15 sec 95ºC (denaturation step), 15 sec 55ºC (primer annealing step) and 30 sec at 72ºC (extension step), and lastly 1 cycle each for 1 min at 95ºC and 55ºC for complete extension of cDNA products. To confirm that a single PCR product was generated, a melt analysis was performed by heating the PCR products from 60-95°C and holding for 10 seconds at 0.5°C increments. GeNorm software was used to determine the most stable reference genes amplified in the experiment to assess the most suitable gene for further correction against each target gene. A correction of the amount of cDNA for each target gene was performed against each reference genes using CFX software, which allows for correction against multiple reference genes (i.e. L19 and HMBS).

8.2.7 - Affymetrix gene microarray analysis

8.2.7.1 - Experion analysis

50-500 ng of RNA was used to assess the integrity of the RNA samples extracted from the hormone-treated ZR-75-1 cells using Experion analysis by the visualisation of the18S and 28S rRNA bands. A calculation of the integrity of the RNA is obtained from a RQI value of up to 10, with a value ≥ 7 considered suitable for gene microarray analysis.

8.2.7.2 - Affymetrix gene microarray

Quadruplicate biological replicates of 300 ng of RNA per hormone treatment (i.e. vehicle control (0.1% ethanol), 1 nM DHT, 1 nM MPA and 1 nM DHT plus 1 nM MPA), extracted by trizol, and cleaned in RNeasy columns as previously described, was sent to the Adelaide Microarray Centre (Adelaide, SA). Subsequent reverse transcription was performed by the Adelaide Microarray Centre to make the labelled cDNA probes from the RNA test samples, and gene expression was determined by hybridisation of the cDNA probes to the Human cDNA Affymetrix Array (Affymetrix Human Genechip Gene 1.0 ST Arrays) (28,000 cDNA probes). The raw data was expressed as individual values per sample and the average was calculated over the quadruplicate replicates.

8.2.7.3 - Gene expression pathway analysis

Identification of gene expression pathways significantly enriched by DHT and/or MPA treatment in the ZR-75-1 cells was achieved using Gene Ontology (GO) pathway analysis *(http://genecodis.dacya.ucm.es/analysis/)* and Ingenuity Pathway Analysis (IPA) *(http://analysis.ingenuity.com/pa).* The GO pathway analysis was undertaken using

significantly enriched gene pathways associated with biological processes. A core component gene pathway analysis was undertaken using IPA, and the percent of induced and repressed genes in significantly enriched gene ontology (GO) groups most relevant to the this study (i.e. growth and proliferation, cancer, cell cycle, apoptosis and gene expression) was determined following treatment with DHT and/or MPA ($p<0.05$). Candidate genes were selected for validation following GO pathway analysis and IPA from independently generated ZR-75-1 cDNA samples by qRT-PCR as previously described *(refer to section 8.2.5).* A statistical comparison was undertaken to determine the number of genes regulated by DHT and MPA combined which were abrogated compared to DHT alone, independent and dependent of MPA regulation. Additionally, these statistical comparisons were performed to determine the number of genes which were uniquely regulated by the hormone combination compared to DHT or MPA alone.

8.2.8 - Statistical analysis

Statistical analysis was performed on growth assays on the final day of counting for short- (i.e. 8 days) and long-term (i.e. 15 days) experimental studies using the non-parametric Kruskal-Wallis one-way analysis of variance (ANOVA) and Dunnet post-hoc test (GraphPad Prism 5.02 software). Statistical significance was set at p-value <0.05. Significantly regulated genes by DHT and MPA alone and combined above control treatments by Affymetrix microarray gene analysis were identified using the non-parametric Kruskal-Wallis one-way ANOVA on the average of quadruplicate replicates and statistical significance was set at p-value <0.05 (performed by the Adelaide Microarray Centre using Partek Genomic Suite, Version 5.2). All genes were included as

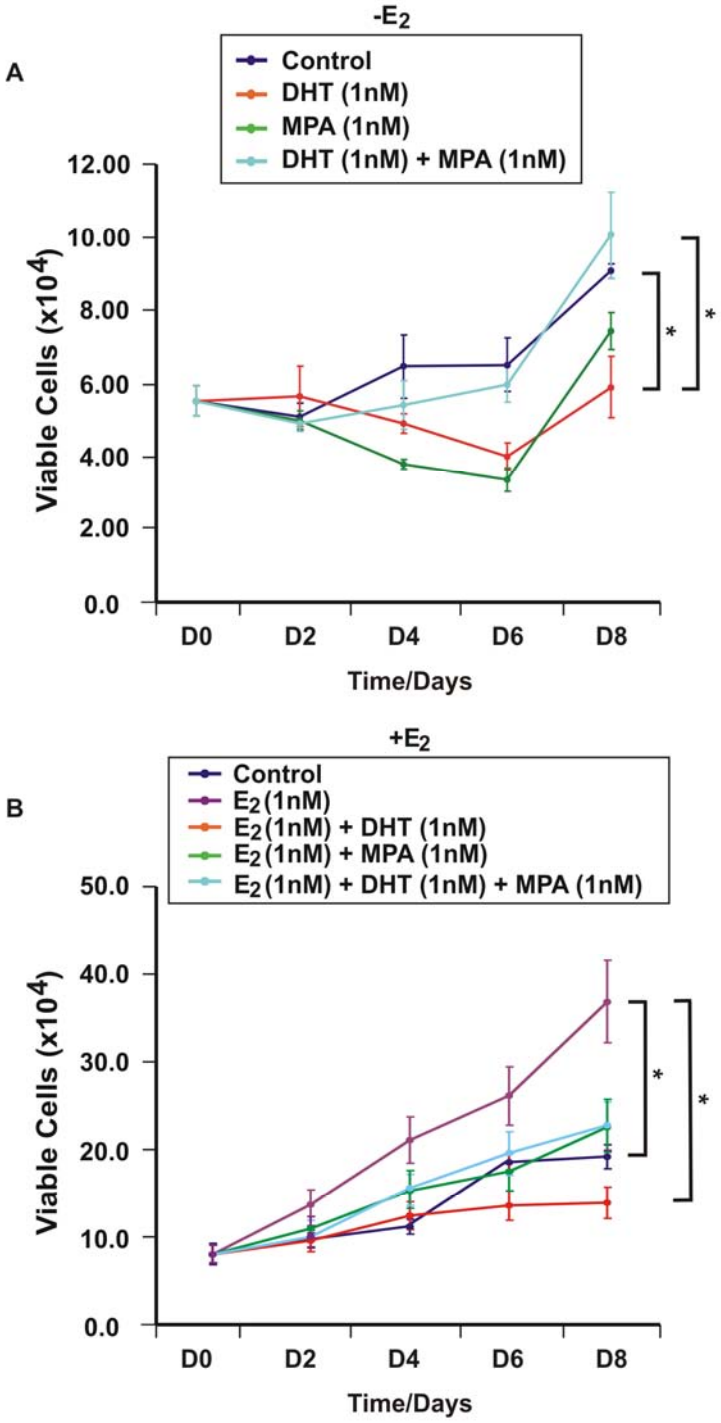
significant if step-up $p<0.05$, despite individual fold changes (i.e. majority of genes presented with a fold change <1.5). Additional statistical analysis of gene expression differences between hormone treatments from the microarray analysis was performed using the raw data intensity values in side comparison by t-test analysis (Microsoft Office Excel 2003). Multiple testing corrections was performed by a Bonferroni Step-down (Holm) correction (B&H) (i.e. corrections which adjust p-values to eliminate false positives) ($p<0.05$), completed by Dr Nicole Moore and Dr Luke Selth (Microsoft Office Excel 2003). Differences in mRNA expression levels between hormone treatment groups detected by qRT-PCR was determined by non-parametric Kruskal-Wallis one-way ANOVA (GraphPad Prism 5.02 software) with statistical significance set at p-value <0.05.

8.3 - Results

8.3.1 - MPA prevents DHT from inhibiting the growth of ZR-75-1 breast cancer epithelial cells

Previous findings in this thesis indicated that the combined action of DHT and MPA reversed the inhibitory effects of non-malignant post-menopausal breast epithelial proliferation by DHT alone, while MPA alone had no effect *(refer to chapter 6 - sections 6.3.1 and 6.3.2).* Preliminary short-term growth assays (i.e. duration of 8 days) demonstrated that DHT significantly decreased basal and E_2-stimulated ZR-75-1 growth ($p<0.05$) [Figure 8.1A and B]. The reduction in ZR-75-1 growth caused by DHT was

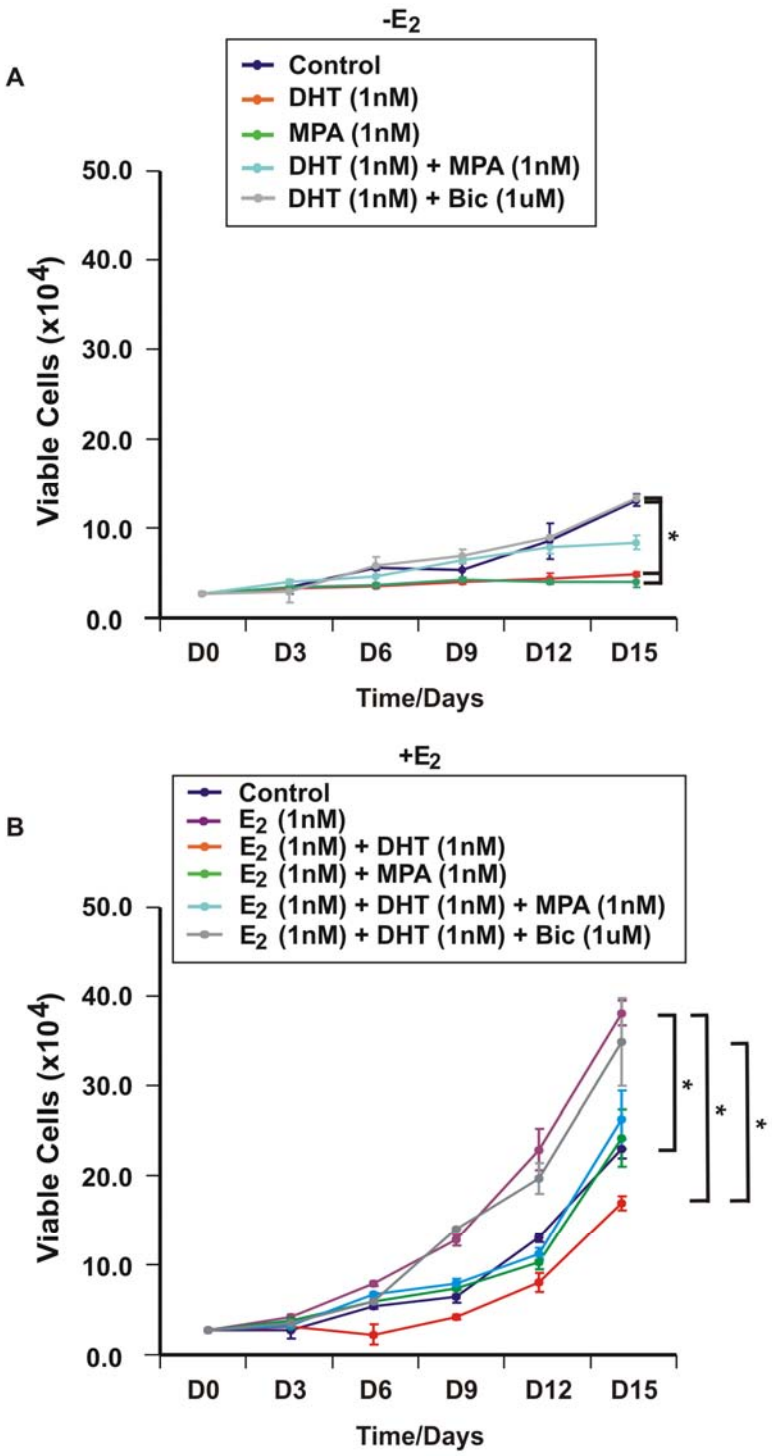
Figure 8.1 (A and B): MPA impedes DHT-induced inhibition of ZR-75-1 breast epithelial cell proliferation in the presence and absence of E_2. ZR-75-1 breast cancer epithelial cells were grown in PRF-RPMI media supplemented with 10% DCC-FCS and penicillin and streptomycin (P/S) under control conditions (i.e. 01% ethanol) or treated with 1 nM DHT and/or 1 nM MPA **(A)** $-E_2$ and **(B)** $+E_2$ for 8 days (and media replenished every 2 days). Viable and dead cells (trypan blue detection) were counted using a haemocytometer. Results are presented as the mean +/- SEM of three separate wells and graphs are representative of two individual experiments. Statistical analysis was performed using non-parametric one-way ANOVA and a Dunnet post-hoc test; * $p \leq 0.05$. Statistical comparisons included **(A)** 1 nM DHT vs. control, 1 nM MPA vs. control and **(B)** 1 nM E_2 vs. control and 1 nM E_2 vs. 1 nM E_2 plus 1 nM DHT.


A
-E2
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) + MPA (1nM)
Viable Cells (x10^4)
12.00
10.00
8.00
6.00
4.00
2.00
0.0
D0
D2
D4
D6
D8
Time/Days
*
*
B
+E2
Control
E2 (1nM)
E2 (1nM) + DHT (1nM)
E2 (1nM) + MPA (1nM)
E2 (1nM) + DHT (1nM) + MPA (1nM)
Viable Cells (x10^4)
50.0
40.0
30.0
20.0
10.0
0.0
D0
D2
D4
D6
D8
Time/Days
*
*

reversed by MPA in the absence of E_2 ($p<0.05$) [Figure 8.1A] and in the presence of E_2 remained similar to the control treatment [Figure 8.1B]. MPA also led to a reduction in basal and E_2-stimulated ZR-75-1 growth at 8 days, however showed no significant difference compared to control or E_2 treatment, respectively [Figure 8.1A and B]. No change in the number of dead cells was observed in response to the different hormone treatments during the 8 day culture duration *(data not shown)*.

Long-term growth assays (i.e. duration of 15 days) were subsequently performed to determine whether the ability of MPA to prevent DHT from inhibiting growth was more pronounced over a longer duration of time and could lead to complete statistical reversal in the presence of E_2 as detected under basal conditions in the short-term growth assays. In these experiments the role of AR was additionally investigated by treating the cells with a potent AR antagonist, Bic. Similar to the short-term assays (8 days), DHT caused a significant reduction in basal and E_2-stimulated growth at 15 days ($p<0.05$) and this effect was impeded by MPA under both experimental conditions [Figure 8.2A and B]. MPA alone also significantly inhibited basal growth at 15 days in the absence of E_2, which was not observed in the short-term growth assay and was expected based on similar reports in the literature (Labrie, Poulin et al. 1990; Poulin, Baker et al. 1991) ($p<0.05$) [Figure 8.1A; 8.2A]. Although a similar reduction in E_2-stimulated growth of ZR-75-1 by MPA was observed, it did not reach significance, as observed in the short-term growth assay [Figure 8.1B; 8.2B]. Previous studies have also shown an inhibition of E_2 stimulated breast epithelial proliferation by 1 nM MPA, yet a more dramatic effect is detected at higher doses of MPA (i.e. 0.1 - 1 μM). Since no statistical analysis was

Figure 8.2 (A and B): Loss of DHT-induced inhibition of ZR-75-1 growth by MPA in the presence and absence of E_2 via an AR-mediated effect. ZR-75-1 breast cancer epithelial cells were grown in RPF-RPMI supplemented with 10% DCC-FCS and P/S under control conditions (i.e. 0.1% ethanol), 1 nM DHT and/or 1 nM MPA and co-treatment of 1nM DHT and 1μM Bic **(A)** -E_2 **(B)** or +E_2 for 15 days. Viable and dead cells (trypan blue detection) were counted using a haemocytometer media replenished every 3 days and hormone treatments replenished every 2 days. Results are presented as the mean +/- SEM of three separate wells and graphs are representative of two individual experiments. Statistical analysis was performed using non-parametric one-way ANOVA and a Dunnet post-hoc test; *$p \leq 0.05$. Statistical comparisons included **(A)** 1 nM DHT vs. control, 1 nM MPA vs. control and 1 nM DHT plus 1 μM Bic vs. 1 nM DHT or 1 nM MPA and **(B)** 1 nM E_2 vs. control, 1 nM E_2 vs. 1 nM E_2 plus 1 nM DHT and 1 nM E_2 plus 1 nM DHT vs. 1 nM E_2, 1 nM DHT plus 1 μM Bic.


A
-E2
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) + MPA (1nM)
DHT (1nM) + Bic (1uM)
Viable Cells (x10^4)
50.0
40.0
30.0
20.0
10.0
0.0
D0
D3
D6
D9
D12
D15
Time/Days
*
B
+E2
Control
E2 (1nM)
E2 (1nM) + DHT (1nM)
E2 (1nM) + MPA (1nM)
E2 (1nM) + DHT (1nM) + MPA (1nM)
E2 (1nM) + DHT (1nM) + Bic (1uM)
Viable Cells (x10^4)
50.0
40.0
30.0
20.0
10.0
0.0
D0
D3
D6
D9
D12
D15
Time/Days
*
*
*

performed on the comparable studies it is difficult to compare the findings in this chapter with the published findings (Labrie, Poulin et al. 1990; Poulin, Baker et al. 1991). Inhibition of DHT mediated basal and E_2-stimulated growth were both reversed by the co-treatment of DHT and Bic ($p<0.05$) [Figure 8.2A and B]. In support of the literature, these findings suggest that the inhibition of ZR-75-1 proliferation by DHT alone is mediated by the AR-signalling axis (Poulin, Baker et al. 1988; Birrell, Bentel et al. 1995). Furthermore, it suggests that the impeding effect of MPA on the anti-proliferative actions of DHT, are anti-androgenic AR-mediated effects as previously reported (Birrell, Butler et al. 2007).

8.3.2 - Abrogation of DHT-induced AR protein expression by MPA can occur irrespective of changes to PR protein expression in ZR-75-1 breast cancer cells

Based on previous findings in the literature and current findings reporting that the inhibition of ZR-75-1 growth by DHT requires the AR (Poulin, Baker et al. 1988; Birrell, Bentel et al. 1995) [Figure 8.2], it is possible that MPA affects the levels of this factor. To investigate this hypothesis further, immunoblot analysis was performed using protein lysates extracted from hormone-treated ZR-75-1 cells. Immunoblot analysis revealed that DHT increased the levels of the AR and FKBP5 (an AR-regulated gene in the prostate) (Amler, Agus et al. 2000) after 24 and 48 hrs of treatment, whereas MPA alone had no effect [Figure 8.3]. The combination of DHT and MPA resulted in AR and FKBP5 protein levels that were similar to that of DHT alone at both time-points [Figure 8.3]. The addition of 1 µM Bic reduced DHT-induced AR and FKBP5 protein levels at 48 hrs but had a minimal effect at 24 hrs [Figure 8.3]. These findings suggest that a

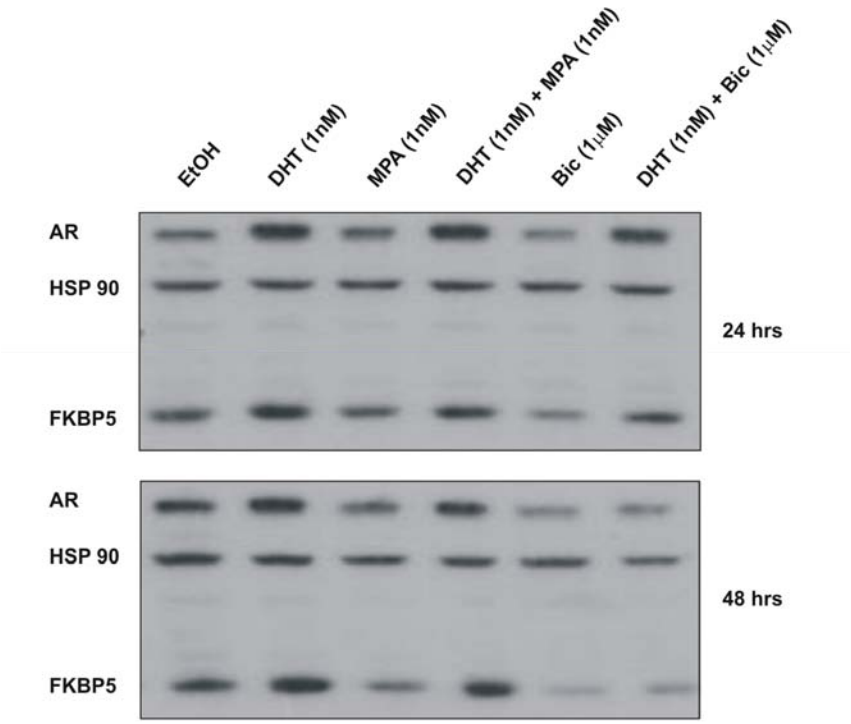


Figure 8.3: No effect of DHT and MPA combined on DHT-induced AR-signalling in ZR-75-1 malignant breast epithelial cells at 24 and 48 hrs. Protein lysates were prepared from ZR-75-1 breast cancer epithelial cells cultured in RPMI-PRF media supplemented with 10% DCC-FCS and P/S under control conditions (i.e. 0.1% ethanol) or treated with 1 nM DHT and/or 1 nM MPA, 1 μM Bic alone a combined with 1 nM DHT for 24 and 48 hrs. Protein lysates were run on SDS-PAGE gels, transferred to nitrocellulose membranes and probed for AR and FKBP5. For each immunoblot HSP-90 was used as a loading control. Each immunoblot image is representative of two separate experiments.

longer duration of hormone treatment may be required to detect changes to the DHT-regulated AR-signalling by the combined treatment of DHT and MPA at a protein level.

To determine whether this was in fact the case, further studies were performed using protein lysates from ZR-75-1 cells cultured under the same experimental conditions but treated with hormone for a longer duration (i.e. 8 days). This time-point was chosen to allow direct comparison with the findings observed in the short-term growth assays. Immunoblot analysis of these protein lysates showed that DHT and MPA alone increased AR protein compared to control treatment at day 8 [Figure 8.4]. In contrast to earlier endpoints, treatment with DHT and MPA combined for 8 days, resulted in a dramatic reduction in AR protein levels to lower than that observed in the control treatment [Figure 8.4]. Immunoblot analysis further demonstrated that co-treatment with DHT and Bic resulted in similar AR protein levels in ZR-75-1 cells compared to control treatment [Figure 8.4]. Very low to absent protein expression levels of both PR isoforms A and B were detected in all of the lysates, due to culture in steroid hormone free conditions for 48 hours prior to hormone treatment, compared to ZR-75-1 cells cultured in the presence of 10% FCS [Figure 8.4]. Subsequent studies have shown a marked reduction in the protein expression of both PR isoforms A and B and FKBP5 expression (a PR regulated gene in the breast and androgen regulated gene in prostate) (Kester, van der Leede et al. 1997; Amler, Agus et al. 2000) by immunoblot analysis in ZR-75-1 cells, following culture in steroid hormone free conditions for 8 days [Figure 8.4]. These findings suggest that the reduction in AR protein levels by DHT and MPA combined occurs via MPA actions mediated by the AR, irrespective to changes in PR protein expression.

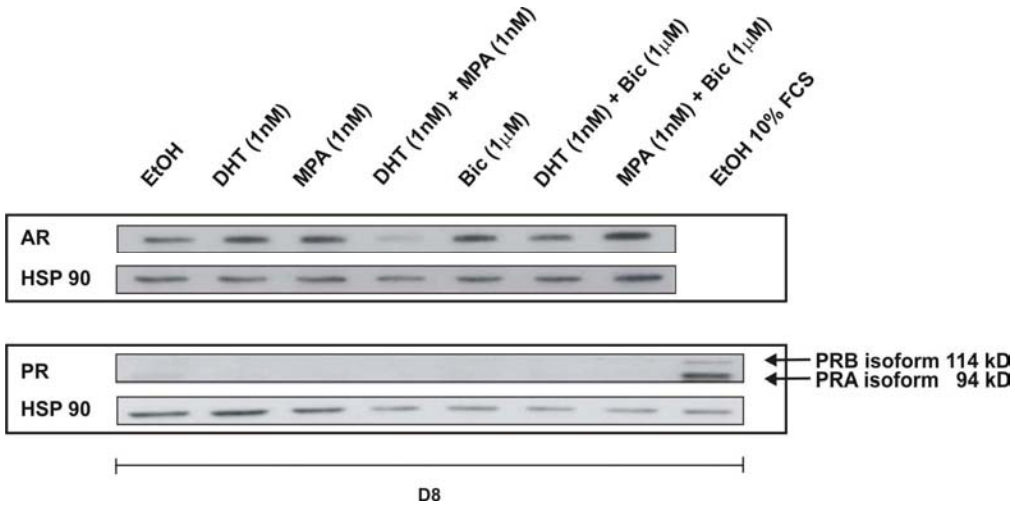


Figure 8.4: Abrogation of DHT-induced AR expression irrespective to changes in PR expression by DHT and MPA combined in ZR-75-1 malignant breast epithelial cells at 8 days. Protein lysates were prepared from ZR-75-1 breast cancer epithelial cells cultured in RPMI-PRF media supplemented with 10% DCC-FCS and P/S under control conditions (i.e. 0.1% ethanol) or treated with 1 nM DHT and/or 1 nM MPA, 1 μM Bic alone and combined with either 1 nM DHT or 1 nM MPA for 8 days. Protein lysates were run on SDS-PAGE gels, transferred to nitrocellulose membranes and probed for AR and PR. For each immunoblot HSP-90 was used as a loading control. The positive control represents ZR-75-1 cells cultured in 10% FCS and P/S for 48 hrs.

8.3.3 - MPA impedes DHT-induced AR-signalling in ZR-75-1 breast cancer cells

In light of the findings demonstrating the effect of long-term combined DHT and MPA treatment on breast epithelial proliferation and the reduction in DHT-induced AR protein levels, additional studies were undertaken to determine whether these changes also affected expression of known AR-target genes. Immunoblot analysis was performed using the same lysates from 8 days with antibodies to detect proteins that have been shown to be androgen regulated as follows: FKBP5; UDP-GlcDH (Lapointe and Labrie 1999) and KITL a putative androgen regulated gene (Shiina, Matsumoto et al. 2006). All proteins were increased in response to DHT treatment, and FKBP5, UDP-GlcDH and KITL were all shown to be directly AR-regulated by the reversal in induction subsequent to co-treatment with DHT and Bic [Figure 8.5].

Both FKBP5 and UDP-GlcDH were induced by MPA alone in a manner that was reversed by treatment with Bic, whereas KITL remained non-responsive to MPA treatment and was similar in expression to control treatments [Figure 8.5]. These findings indicate that both FKBP5 and UPD-GlcDH can be regulated by DHT and MPA in isolation via AR-mediated mechanisms, whereas the KITL1 isoform can only be regulated by DHT in an AR-mediated manner [Figure 8.5]. Thus, in support of a published study using a different breast cancer cell line, DHT and MPA do overlap in the regulation of some androgen regulated genes, but also have distinct effects (Ghatge, Jacobsen et al. 2005). The KITL protein is a transmembrane protein which exists as two different isoforms and both can produce soluble forms. These isoforms include KITL1 (248 aa) (approximately 45kD full length and 31 kD soluble) and KITL2 (220 aa)

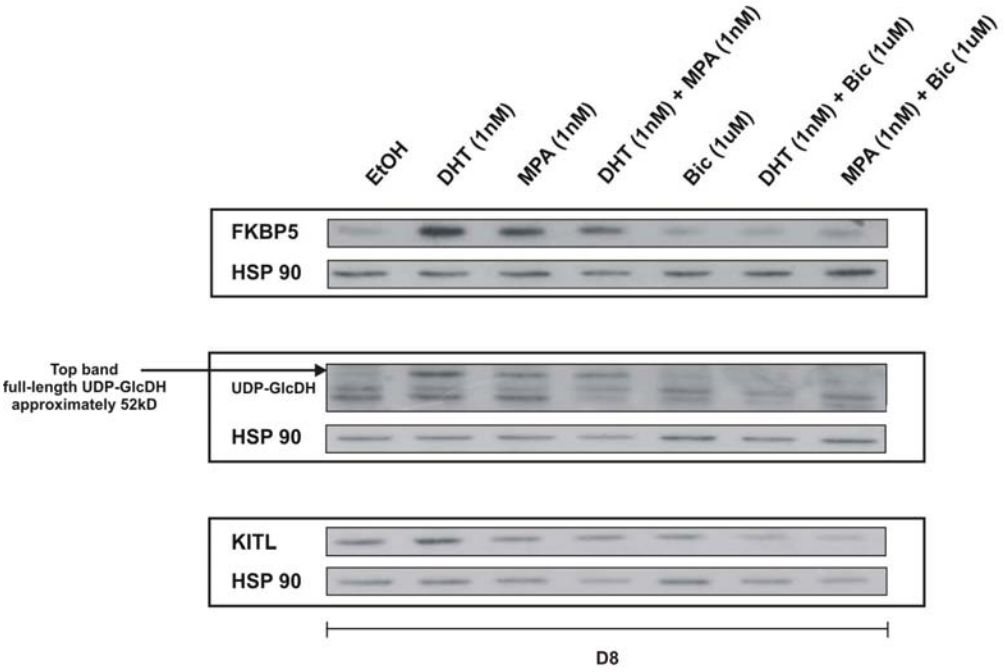


Figure 8.5: De-regulation of the DHT-induced AR-signalling axis by DHT and MPA combined in ZR-75-1 malignant breast epithelial cells at 8 days. Protein lysates were prepared from ZR-75-1 breast cancer epithelial cells cultured in RPMI-PRF media supplemented with 10% DCC-FCS and P/S under control conditions (i.e. 0.1% ethanol) or treated with 1 nM DHT and/or 1 nM MPA and 1 μM Bic alone and combined with either 1 nM DHT or 1 nM MPA for 8 days. Protein lysates were run on SDS-PAGE gels, transferred to nitrocellulose membranes and probed for FKBP5, UDP-GlcDH and KITL. For each immunoblot HSP-90 was used as a loading control.

(approximately 32 kD full length and 23 kD soluble form) (Thomas, Ismail et al. 2008). Interestingly, only the KITL1 isoform was detected in the ZR-75-1 cells, compared to the ovarian KGN granulosa tumour cell line which expressed both isoforms *(data not shown).* Importantly, the protein expression of all three AR-regulated genes was reduced by DHT and MPA co-treatment compared to DHT treatment alone [Figure 8.5]. These findings are in accordance with the proposed hypothesis of this chapter that MPA acts to impede DHT-induced AR signalling and thereby antagonise its growth-inhibitory function in breast epithelial cells [Figure 8.5].

8.3.4 - Treatment of ZR-75-1 breast cancer epithelial cells with DHT and/or MPA results in differential gene expression profiles

Based on the findings reported in chapters 5-7 it is highly feasible that the combined treatment of DHT and MPA activates intracellular signalling pathways that can lead to differential gene regulation, distinct from those activated alone. To identify intracellular AR-signalling pathways differentially regulated by DHT and/or MPA, ZR-75-1 cells were subjected to gene expression profiling analyses using an Affymetrix gene microarray following treatments with either hormone alone or in the combination. Interestingly, relative to vehicle controls, fewer genes were regulated by DHT alone (439) compared to either MPA alone (858) or the combination of both hormones (1494) [Table 3]. A subset of 231 genes were shown to overlap and to be similarly regulated by each hormone treatment (i.e. DHT and MPA alone or combined) [Figure 8.6]. A comparable number of genes were uniquely regulated by DHT (114) and MPA (136) alone [Figure 8.6]. Interestingly however, the combined hormone treatment resulted in a

5-fold increase in regulated genes (690), compared to treatments in isolation [Figure 8.6]. Similar findings were observed for the number of genes induced [Figure 8.7A] or repressed [Figure 8.7B] by each hormone treatment. Two genes regulated by DHT and MPA alone were inversely regulated of the total thirteen genes [Figure 8.6], and not included in the gene numbers depicted in the Venn diagrams separated into induced and repressed genes [Figure 8.7A and B] (i.e. HEY2 and FAM49A) *(refer to Appendix 4 - Table 4 and 9).* Additionally, four genes inversely regulated by DHT alone, compared to DHT and MPA combined were identified and were also not included in the separate induced and repressed gene numbers depicted in the Venn diagrams [Figure 8.7A and B] (i.e. SLC6A6, 20194, NFX1 and TBC1D8) *(refer to Appendix 3 - Table 5).*

8.3.5 - Gene Ontology pathway analysis of genes regulated by DHT and MPA alone in the combination in ZR-75-1 breast cancer epithelial cells by Gene Ontology pathway analysis

The results outlined in section 8.3.4 suggest that DHT and MPA alone and combined can regulate a number of similar genes. However, despite the overlap in the regulation of similar genes, the results indicate DHT and MPA combined also leads to the regulation of a unique pool of genes, which are not regulated by either treatment alone. These findings indicate the actions of the genes uniquely regulated by each hormone treatment (i.e. DHT and MPA alone and combined), are likely to elicit unique biological actions compared to the genes which are similarly regulated by either sets of hormone treatments. To investigate this idea in more detail, gene sets uniquely regulated by DHT and/or MPA were examined using gene expression pathway analysis to identify enriched gene

ontology (GO) pathways and biological processes. DHT treatment led to altered gene expression pathways regulating apoptosis, induction of apoptosis by extracellular processes, protein amino acid phosphorylation, activation of the JUN kinase pathway, organ morphogenesis, blood circulation, regulation of cell migration and peptidyl serine activation ($p<0.05$) [Figure 8.8A]. No enriched gene pathways were identified using genes regulated by MPA alone ($p<0.05$). Furthermore, the combination of DHT and MPA altered a distinct set of gene expression pathways compared to DHT alone, with enrichment of genes involved in multi-cellular organismal development, metabolic processes, cell cycle, cell division, regulation of transcription from the RNA polymerase II promoter and mitosis ($p<0.05$) [Figure 8.8B]. Consistent with the findings observed in the cell growth assays, these additional findings indicate that the combined treatment of DHT and MPA is likely to promote cell growth compared to DHT alone *(refer to section 8.3.1).*

The apoptosis pathways altered by DHT alone were not significantly affected by the combined hormone treatment [Figure 8.8A-B]. However, an increase in dead cells following treatment with DHT was not detected in the growth assays performed in this thesis *(data not shown)*. The differences between these findings may relate to the ability of DHT to induce G1 cell cycle arrest via the induction of apoptosis cell signalling pathways, observed in the ZR-75-1 cell line after 24-30 hrs (de Launoit, Dauvois et al. 1991), yet no measurable cell death.

Table 3: Total number of regulated genes in ZR-75-1 gene microarray following treatment with DHT and MPA alone and combined

Table 3: Total number of regulated genes identified in ZR-75-1 gene microarray[1]			
Gene regulation	**Hormone Treatment**		
	DHT (1nM)	**MPA (1nM)**	**DHT (1nM) MPA (1nM)**
Induced	201	554	869
Repressed	238	304	625
Total number of significantly regulated genes	439	858	1494

[1]All genes were significantly different compared to control treatment using non-parametric Kruskal Wallis one-way ANOVA, $p<0.05$. *(Refer to Appendix 4 - Tables 1-10 for complete gene reference list).* Technical work for the Affymetrix gene microarray for this figure was performed by Dr Nicole Moore and Andrienne Hanson in the DRMCRL.

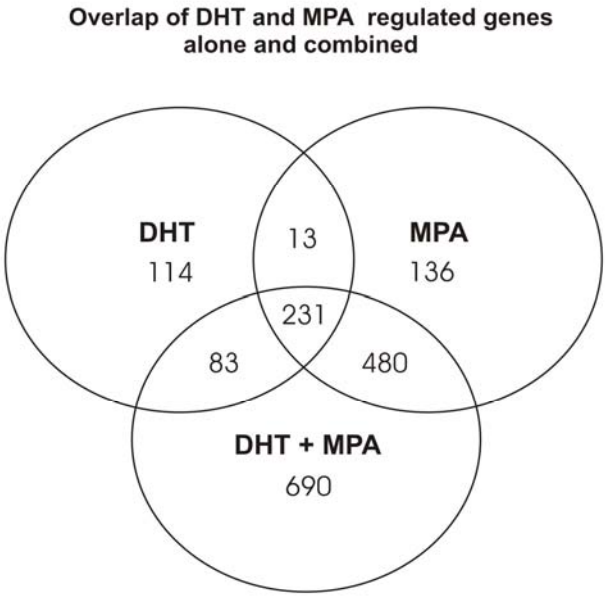


Figure 8.6 (A and B): Genes regulated by DHT and MPA are distinct and different to those regulated by the hormone combination. Genes that were induced and repressed by treatment with DHT and MPA alone and combined were identified from the ZR-75-1 Affymetrix gene microarray. All genes were significantly different compared to control treatment using non-parametric Kruskal Wallis one-way ANOVA, $p<0.05$. The overlap of the regulated genes was tabulated and depicted in a Venn diagram. (*Refer to Appendix 4 - Tables 1-10 for complete gene reference lists).* Technical work for the Affymetrix gene microarray for this figure was performed by Dr Nicole Moore and Andrienne Hanson in the DRMCRL.

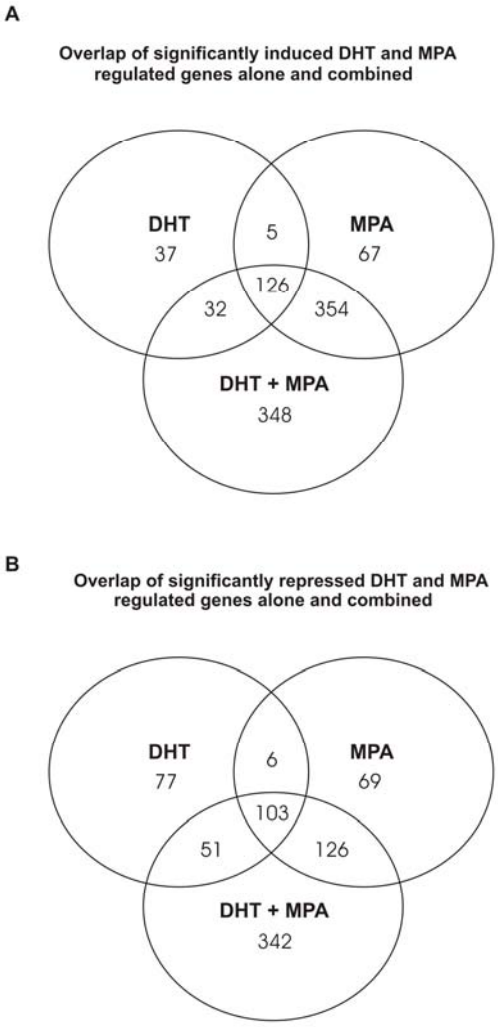


Figure 8.7 (A and B): Genes regulated by DHT and MPA are distinct and different to those regulated by the hormone combination. Genes that were induced or repressed by treatment with DHT and MPA alone and combined were identified from the ZR-75-1 Affymetrix gene microarray. All genes were significantly different compared to control treatment using non-parametric Kruskal Wallis one-way ANOVA, $p<0.05$. The overlap of the regulated genes was tabulated and depicted in Venn diagrams for **(A)** induced and **(B)** repressed genes. (*Refer to Appendix 4 - Tables 1-10 for complete gene reference lists).* Technical work for the Affymetrix gene microarray for this figure was performed by Dr Nicole Moore and Andrienne Hanson in the DRMCRL.

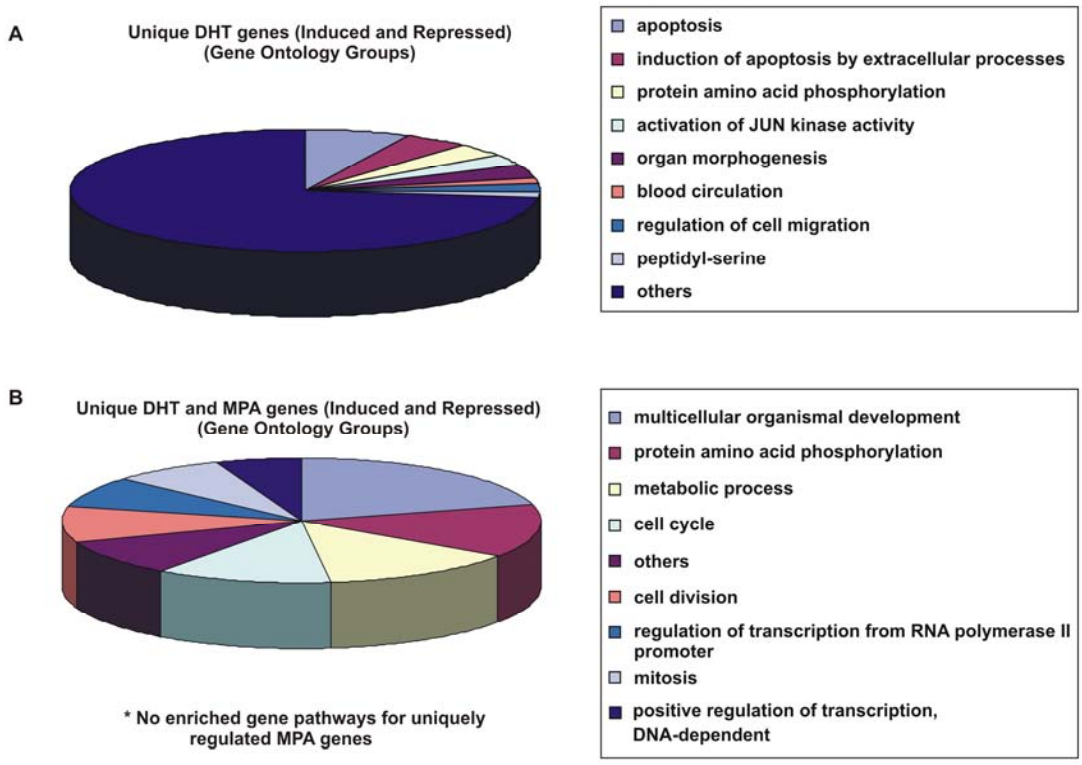
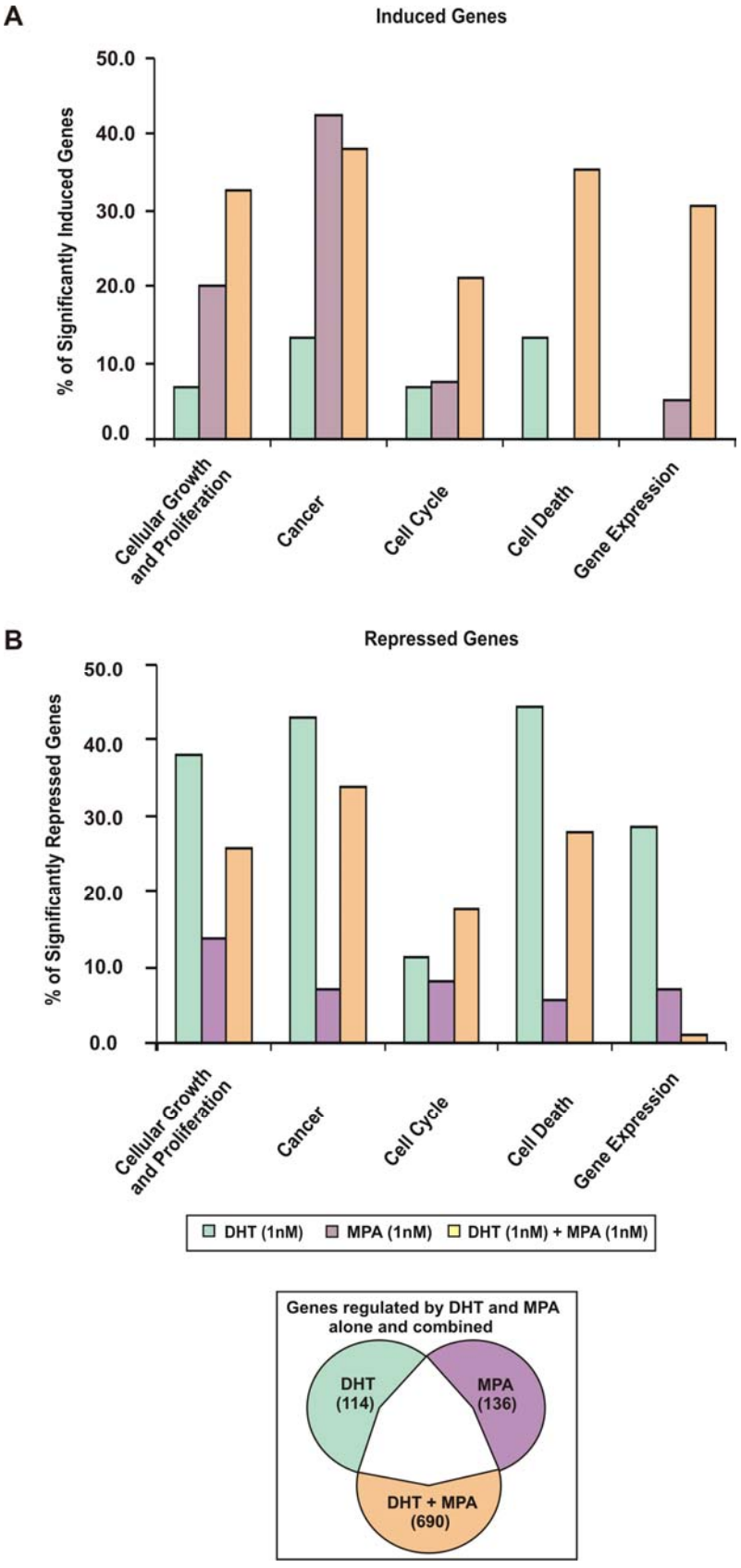


Figure 8.8 (A and B): ZR-75-1 Affymetrix gene microarray enriched pathways in response to DHT and MPA alone and combined using Gene Ontology (GO) pathway analysis. GO pathway analysis was performed on regulated unique genes following treatment with **(A)** 1 nM DHT and **(B)** 1 nM DHT and 1 nM MPA combined. No enriched gene pathways were identified in this GO pathway analysis using genes regulated by MPA only. All significantly regulated genes included in this analysis were identified by a p-value <0.05. Technical work for the Affymetrix gene microarray for this figure was performed by Dr Nicole Moore and Andrienne Hanson in the DRMCRL.

8.3.6 - Enriched gene expression pathways regulated by DHT and MPA alone and combined in ZR-75-1 breast cancer epithelial cells by Ingenuity Pathway Analysis

To develop these findings obtained by GO pathway analysis further, additional analysis was undertaken using Ingenuity Pathway Analysis (IPA). The IPA software uses literature linked gene network and pathway databases that provide more detailed and sophisticated information regarding the relationships between gene sets and intracellular signalling pathways. IPA was performed on gene sets uniquely induced or repressed by DHT and/or MPA. Interestingly, IPA revealed enrichment ($p<0.05$) of a greater percentage of genes associated with pathways and functional groups involved in growth, proliferation and survival, cell cycle, cell death, cancer and gene expression in the gene set uniquely induced by DHT and MPA combined, compared to individual treatments [Figure 8.9A]. Despite the induction in genes associated with cell death by the combination of DHT and MPA compared to DHT alone, the majority of genes in this treatment group were involved with induction of cell growth, survival and viability. Conversely, combined treatment with DHT and MPA down-regulated more genes associated with cellular growth and proliferation, cancer, cell death and gene expression than individual treatments ($p<0.05$) [Figure 8.9B]. Full gene reference lists associated with each biological functional gene pathway can be referenced in Appendix 3 of this thesis. Collectively, these analyses reveal that the hormone combination leads to altered regulation in cancer-related intracellular AR-signalling pathways which are likely to play a pivotal role in the carcinogenic process.

Figure 8.9 (A and B): ZR-75-1 Affymetrix gene microarray enriched pathways in response to DHT and MPA alone and combined by Ingenuity pathway analysis (IPA). IPA analysis was performed using regulated genes by DHT and MPA alone and combined. Graphs depict percent change in genes enriched in functional groups for **(A)** induced and **(B)** repressed genes. All genes were significantly different compared to control treatment using non-parametric Kruskal Wallis one-way ANOVA, $p<0.05$. IPA analysis was performed using corrections for fold change and p-values of individual genes from the one-way ANOVA. The unique genes included in the analysis are depicted in the Venn diagram, and the colours match the data depicted in the graphs. Technical work for the Affymetrix gene microarray for this figure was performed by Dr Nicole Moore and Andrienne Hanson in the DRMCRL.


A
Induced Genes
% of Significantly Induced Genes
50.0
40.0
30.0
20.0
10.0
0.0
Cellular Growth and Proliferation
Cancer
Cell Cycle
Cell Death
Gene Expression
B
Repressed Genes
% of Significantly Repressed Genes
50.0
40.0
30.0
20.0
10.0
0.0
Cellular Growth and Proliferation
Cancer
Cell Cycle
Cell Death
Gene Expression
DHT (1nM)
MPA (1nM)
DHT (1nM) + MPA (1nM)
Genes regulated by DHT and MPA alone and combined
DHT (114)
MPA (136)
DHT + MPA (690)

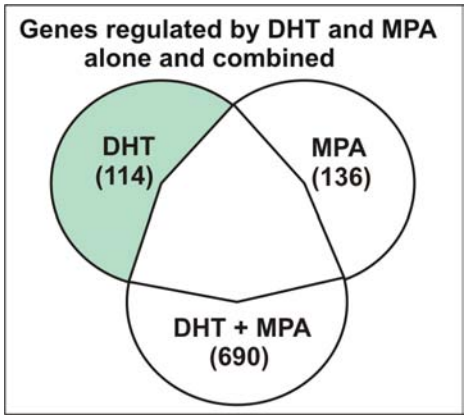
8.3.7 - Identification of candidate genes related to the de-regulation of DHT-induced AR-signalling by co-treatment

The findings in this chapter have demonstrated an alteration in key cancer-related intracellular AR-signalling pathways by the hormone combination of DHT and MPA compared to treatments in isolation. To further identify key genes within these pathways which were affected by the hormone combination, a t-test comparison on the raw data and Bonferroni Step-down (Holm) correction (B&H) obtained from the micro-array analysis was performed to identify significant changes between hormone treatments. This analysis was specifically aimed at identifying uniquely regulated genes which were most significantly affected by the hormone combination either by: 1) abrogation of DHT-induced gene regulation; 2) abrogation of MPA-induced gene regulation and 3) genes regulated uniquely by the hormone combination.

Firstly, of the 114 genes uniquely regulated by DHT, 32% (i.e. 36 genes) were abrogated by the hormone combination ($p<0.05$) (i.e. 41% induced and 27% repressed, 15 and 21 genes respectively) [Table 4]. Secondly, of 136 genes uniquely regulated by MPA, 13% (i.e. 17 genes) were significantly abrogated by the hormone combination ($p<0.05$) (i.e. 13% induced and 12% repressed, 9 and 8 genes respectively) [Table 5]. These findings demonstrate a higher percentage of DHT uniquely regulated genes (i.e. 32%) [Table 4] compared to MPA uniquely regulated genes (i.e. 13%) [Table 5] are abrogated by either MPA or DHT alone. Moreover, the data suggests that uniquely induced DHT-regulated genes (i.e. 41%) may be affected to a greater degree than repressed (i.e. 27%), by the addition of MPA [Table 4]. This effect was not evident to the same degree for genes

Table 4: Uniquely DHT-regulated genes abrogated by MPA

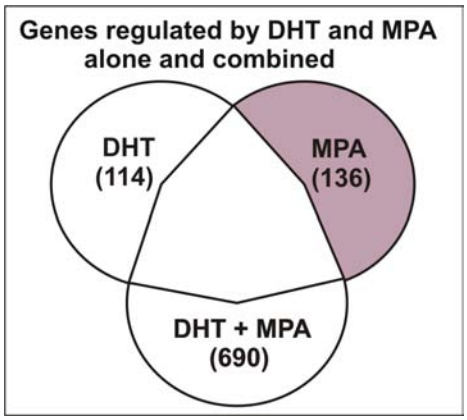
Table 4: Total number of uniquely regulated DHT genes abrogated by MPA[1]						
	DHT - Induced		DHT - Repressed		All genes (Induced and Repressed by DHT)	
	Total	Total DHT Vs DHT + MPA (p<0.05)	Total	Total DHT Vs DHT + MPA (p<0.05)	Total	Total DHT Vs DHT + MPA (p<0.05)
Number of regulated genes	37	15 (41%)	77	21 (27%)	114	36 (32%)



[1]Uniquely regulated DHT genes (i.e. 114 genes) included in the analysis included in Table 8.4, are depicted in the Venn diagram in blue. *(Refer to Appendix 4 - Tables 1 and 2 for complete gene reference lists).* Technical work for the Affymetrix gene microarray for this figure was performed by Dr Nicole Moore and Andrienne Hanson in the DRMCRL.

Table 5: Uniquely MPA regulated genes abrogated by DHT

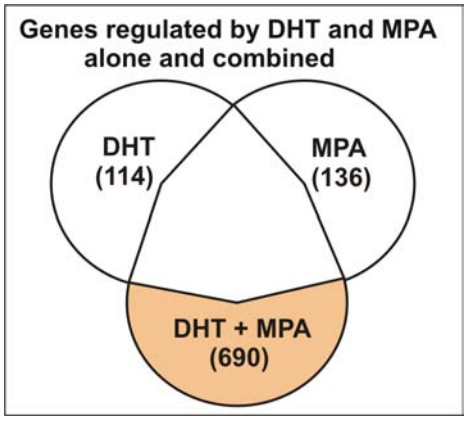
Table 5: Total number of uniquely regulated MPA genes abrogated by DHT[1]						
	MPA - Induced		MPA - Repressed		All genes (Induced and Repressed by MPA)	
	Total	Total MPA Vs DHT + MPA (p<0.05)	Total	Total # MPA Vs DHT + MPA (p<0.05)	Total	Total # MPA Vs DHT + MPA (p<0.05)
Number of regulated genes	67	9 (13%)	69	8 (12%)	136	17 (13%)



[1]Uniquely regulated MPA genes (i.e. 136 genes) included in the analysis included in Table 8.4, are depicted in the Venn diagram in purple. (*Refer to Appendix 4 - Tables 3 and 4 for complete gene reference lists).* Technical work for the Affymetrix gene microarray for this figure was performed by Dr Nicole Moore and Andrienne Hanson in the DRMCRL.

Table 6: Uniquely DHT and MPA combined regulated genes different compared to DHT alone

Table 6: Total number of uniquely regulated DHT and MPA combined genes abrogated by DHT[1]						
	DHT and MPA Induced		DHT and MPA Repressed		All genes (Induced and Repressed by DHT and MPA)	
	Total	Total DHT + MPA Vs DHT ($p<0.05$)	Total	Total DHT + MPA Vs DHT ($p<0.05$)	Total	Total DHT + MPA Vs DHT ($p<0.05$)
Number of regulated genes	348	179 (51%)	342	82 (24%)	690	261 (38%)



[1]Uniquely regulated DHT + MPA genes (i.e. 690 genes) included in the analysis outlined in Table 8.6, are depicted in the Venn diagram in yellow. (*Refer to Appendix 4 - Tables 5 and 6 for complete gene reference lists.* Technical work for the Affymetrix gene microarray for this figure was performed by Dr Nicole Moore and Andrienne Hanson in the DRMCRL.

uniquely regulated by MPA [Table 5], suggesting a more specific antagonistic effect of MPA on DHT-induced gene regulation, rather than the reverse.

Thirdly, of the 690 uniquely regulated genes by DHT and MPA combined, 38% (i.e. 261 genes) were different from DHT alone (i.e. 51% induced and 24% repressed, 179 and 82 genes respectively) ($p<0.05$) [Table 6]. This finding in particular illustrates that a large percentage of unique genes regulated specifically by the hormone combination are significantly different compared to DHT alone. Collectively these findings suggest that the antagonistic actions of MPA on DHT-regulated gene expression and the induction of a unique sub-set of genes by the co-treatment may both play a critical role in the promotion and/or development of breast cancer.

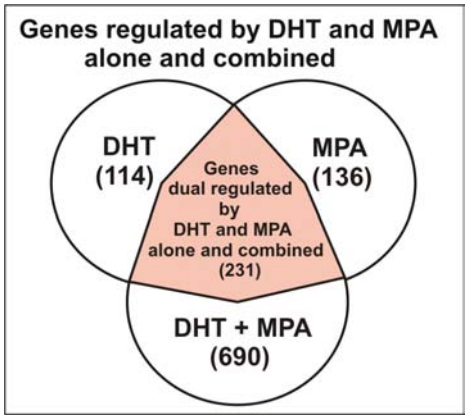
Lastly, of the 229 genes dual regulated by either DHT and/or MPA, 45% (i.e. 108 genes) were differentially expressed following treatment by the hormone combination, compared to DHT alone ($p<0.05$) (i.e. 63% induced and 25% repressed, 79 and 29 genes respectively) [Table 7]. Interestingly, ≥ 99% of these differentially regulated genes were shown to be enhanced by the hormone combination, rather than suppressed compared to DHT alone ($p<0.05$) [Table 7]. These findings indicate that the genes in this particular sub-set may be dual regulated via PR-signalling in the presence of MPA and via AR-signalling in the presence of DHT, which therefore produces an additive effect of gene expression when the hormones are combined, compared to the individual treatments. An example of a gene in this sub-set which has been reported to be regulated in this manner is FKBP5 (i.e. PR-regulated in breast epithelial cells and AR-regulated in the prostate)

(Kester, van der Leede et al. 1997; Amler, Agus et al. 2000; Hubler, Denny et al. 2003). In this study, PR mRNA expression was down-regulated by both DHT and MPA alone ($p<0.001$) [Figure 8.10A]. The dual treatment of DHT and MPA led to a further decrease in PR mRNA expression compared to either DHT ($p<0.01$) or MPA treatment alone ($p<0.05$) [Figure 8.10A]. This is likely to be an additive effect of the two hormones in isolation. Furthermore, mRNA expression of FKBP5 in the same samples was induced by both DHT and MPA alone, compared to control ($p<0.05$) [Figure 8.10B]. An additive effect of DHT and MPA combined on FKBP5 mRNA expression was detected compared to both DHT and MPA alone ($p<0.05$) [Figure 8.10B] (i.e. the same effect was not observed in the immunoblot analysis, due to the low to absent levels of PR following long-term culture in hormone deprived conditions, discussed further in the following section).

The detection of PR protein after 6 hrs of hormone treatment was confirmed by immunoblot analysis, suggesting that the actions of PR-signalling mediated via MPA may also contribute to gene regulation in the microarray studies described in this chapter *(data not shown).* Furthermore, in conjunction with earlier findings, it should be noted that in the absence of PR protein, FKBP5 steady state protein levels detected by immunoblot analysis were abrogated by the hormone combination, rather than enhanced *(refer to sections 8.3.2 and 8.3.3).* The differences in these findings is likely to be associated with the longer duration of culture in hormone deprived media (i.e. 8 days compared to 6 hrs) in the immunoblot analysis compared to the gene microarray studies, which may lead to the reduction in PR steady state protein expression level over time.

Table 7: Total number of genes similarly regulated by DHT and/or MPA which were abrogated or enhanced compared to DHT alone

Table 7: Total number of dual regulated genes by DHT and/or MPA abrogated by DHT[1]				
	Total	**Total number DHT Vs DHT + MPA ($p<0.05$)**	**Total number abrogated by DHT + MPA**	**Total number enhanced by DHT + MPA**
Total number of genes	**229**	**108 (45%)**	**1(<1%)**	**197 (>99%)**
Total number of genes (induced)	**126**	**80 (63%)**	**1(1%)**	**79 (99%)**
Total number of genes (repressed)	**103**	**28 (25%)**	**0(0%)**	**29 (100%)**



[1]Genes dual regulated by DHT and/or MPA (i.e. 231 genes) included in the analysis included in Table 8.7, are depicted in the Venn diagram in orange. Refer to Appendix 4 Tables 7 and 8 for complete gene reference lists. Two genes were excluded from the induced and repressed gene sub-total since they were inversely regulated by hormone treatments (i.e. TBC1D8 and SLC6A6, *refer Appendix 3 - Table 5)* (*Refer to Appendix 4 - Tables 7 and 8 for complete gene reference lists).* Technical work for the Affymetrix gene microarray for this figure was performed by Dr Nicole Moore and Andrienne Hanson in the DRMCRL.

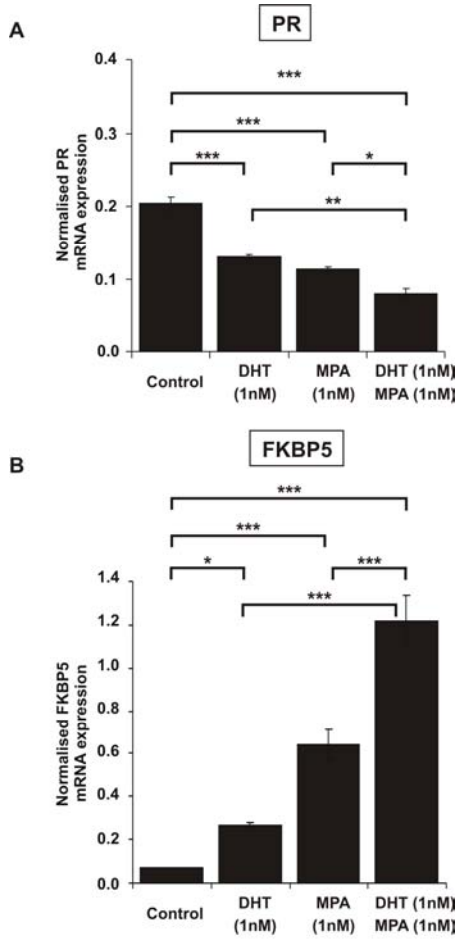


Figure 8.10: Effect of DHT and MPA alone and combined on PR and the PR regulated gene FKBP5 mRNA expression levels in ZR-75-1 cells. Quantitative real-time PCR analysis was performed on mRNA obtained from ZR-75-1 cells grown under control conditions (i.e. 0.1% ethanol) or treated with 1 nM DHT and/or 1 nM MPA for 6 hrs with primers designed for **(A)** PR and **(B)** FKBP5. Statistical analysis was performed using Kruskal-Wallis non-parametric one-way ANOVA; *$p<0.05$, **$p<0.01$, ***$p<0.001$. Other statistical comparisons were identified in the ANOVA not representated on the graph. Results are presented as the mean +/- SEM of three individual replicates and are representative of two individual experiments. Normalisation was performed to L19 and HMBS. Completion of experiments and technical work required for this figure, were undertaken by Dr Nicole Moore and Andrienne Hanson in the DRMCRL.

It is also possible that the different sample lots of DCC-FCS used in the experimental studies (i.e. potentially not all the endogenous steroid hormone was stripped from the serum samples during charcoal stripping), contributed the different results. Importantly, these findings highlight the necessity of separating genes into different categories based on hormone regulation (i.e. DHT and MPA alone or combined uniquely regulated genes, in addition to genes dual regulated by all hormone treatments), to accurately delineate the effects of MPA on DHT-induced AR-signalling, independently of PR. In addition, the findings demonstrate that the genes regulated by DHT and MPA alone or combined are different, depending on the presence of PR. Moreover it is intriguing to speculate that the biological actions of DHT and MPA combined may exert differential effects on gene regulation in breast tissue of women with either low or high expression levels of PR. Other gene comparisons have been additionally performed which include genes regulated by both DHT and MPA alone, yet not the hormone combination *(refer to Appendix 4 - Table 9)* and genes regulated by DHT alone in combination with MPA or MPA alone in combination with DHT *(refer to Appendix 4 - Table 10).*

8.3.8 - Identification of candidate genes associated with de-regulation of DHT-induced AR-signalling by DHT and MPA combined

Key candidate genes which were enriched in each of the GO groups included in the IPA were identified. These candidate genes included a subset of representative examples which reflected an abrogation of induced DHT-regulated gene activity by MPA ($p<0.05$) *(refer to Appendix 3 - Tables 1 and 2).* The de-regulation of both induced and repressed genes lends support to the tenet that MPA abrogates DHT-induced AR-signalling, by

impeding both its stimulatory and inhibitory actions on gene regulation. Additionally, another subset of genes were identified which are representative examples reflecting a unique induction and repression of gene regulation specifically by the hormone combination ($p<0.05$) *(refer to Appendix 3 - Tables 3 and 4).* Lastly, a subset of genes were identified which were inversely regulated by the hormone combination compared to treatments in isolation ($p<0.05$) *(refer to Appendix 3 - Table 5).* These findings in particular are instrumental in illustrating the ability of the same gene to be inversely regulated by DHT alone compared to the combined hormone treatment. Moreover, it highlights the ability of DHT alone and combined to act distinctly in modulating transcriptional responses.

8.3.9 - Antagonism and enhancement of AR gene expression by combined DHT and MPA treatment occurs in the absence of changes to AR mRNA expression

As reported from the micro-array gene analysis, a high proportion of genes were found to be abrogated or impeded by the hormone combination compared to DHT alone. Additionally, a large number of genes were identified to be regulated by the unique actions of the hormone combination. To advance our understanding of the molecular actions of MPA on inhibition and enhancement of DHT-induced AR-signalling, qRT-PCR analysis of AR mRNA derived from hormone treated ZR-75-1 cells was performed to determine whether the effect of the hormone combination was mediated by changes to AR mRNA expression (i.e. transcriptional changes). qRT-PCR revealed that DHT or MPA alone caused a comparable reduction in AR mRNA, compared to control treatment ($p<0.05$) [Figure 8.11]. However, the AR mRNA expression after the combined treatment

with DHT and MPA was not different to treatment with DHT alone ($p<0.05$) [Figure 8.11]. These results are in support of the changes in AR mRNA expression identified in the micro-array studies (refer to Appendix 4 - Table 3). Based on these and previous findings demonstrating that MPA can act to abrogate and enhance DHT-induced AR-signalling, it could be speculated that the effect of the hormone combination in altering gene regulation is mediated via altered AR protein function and not AR expression.

8.3.10 - Validation of candidate genes from the ZR-75-1 Affymetrix gene microarray analysis

Changes in the expression of a number of candidate genes identified in the ZR-75-1 Affymetrix gene microarray were validated by qRT-PCR. Genes were validated by qRT-PCR that were induced by DHT ($p<0.001$), but unaffected by MPA alone and reversed by the combination (e.g. OLR1 and C1ORF116) ($p<0.01$) [Figure 8.12A and B]. A potentially important candidate gene, FGFR2 based on the literature, was identified from the microarray gene analysis. FGFR2 was shown to be induced by DHT alone, but unaffected by MPA alone and abrogated by the hormone combination in the microarray analysis *(refer to Appendix 3, Table 3)*. These changes were validated by qRT-PCR [Figure 8.13A] and also by immunoblot [Figure 8.13B] using ZR-75-1 protein lysates used in previously in this chapter *(refer to sections 8.2.4).* The reverse effect was observed in the microarray analysis for the FGFR1 gene where DHT and MPA combined led to an increase in mRNA expression compared to control treatment ($p<0.05$), whereas

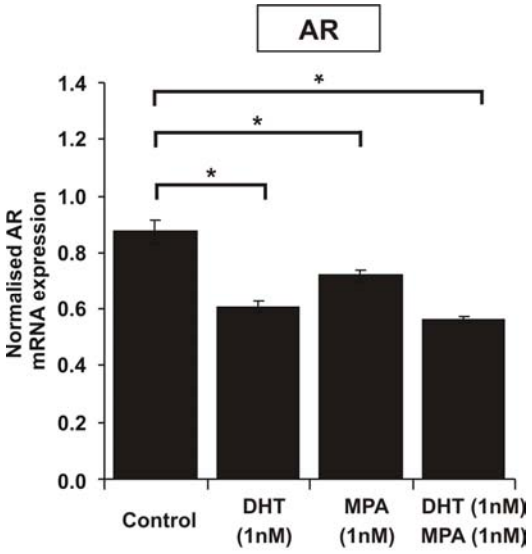


Figure 8.11: Effect of DHT and MPA alone and combined on AR mRNA expression levels in ZR-75-1 cells. qRT-PCR was performed on mRNA obtained from ZR-75-1 cells under control conditions (i.e. 0.1% ethanol) or treated with 1 nM DHT and/or 1 nM MPA for 6 hrs using primers designed for AR. Statistical analysis was performed using Kruskal-Wallis non-parametric one-way ANOVA; *$p<0.05$. Other statistical comparisons were identified in the ANOVA not representated on the graph Results are presented as the mean +/- SEM of three individual replicates and are representative of two individual experiments. Normalisation was performed to L19 and HMBS. Completion of experiments and technical work for the Affymetrix gene microarray required for this figure were performed by Dr Nicole Moore and Andrienne Hanson in the DRMCRL.

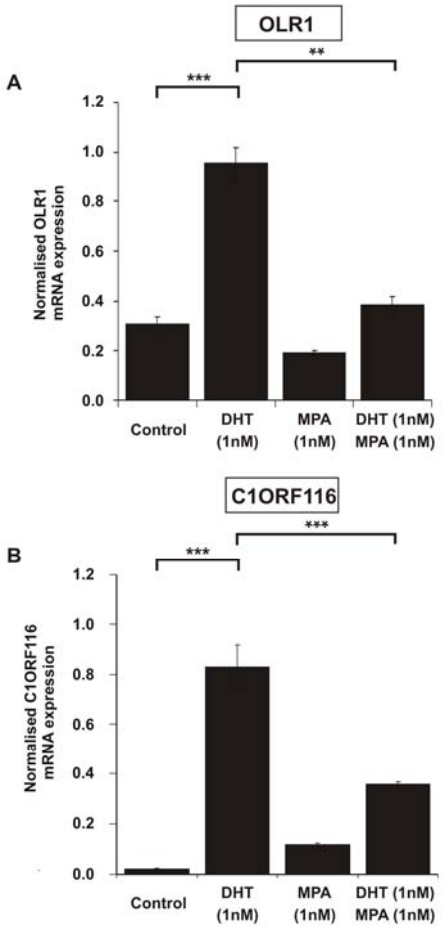
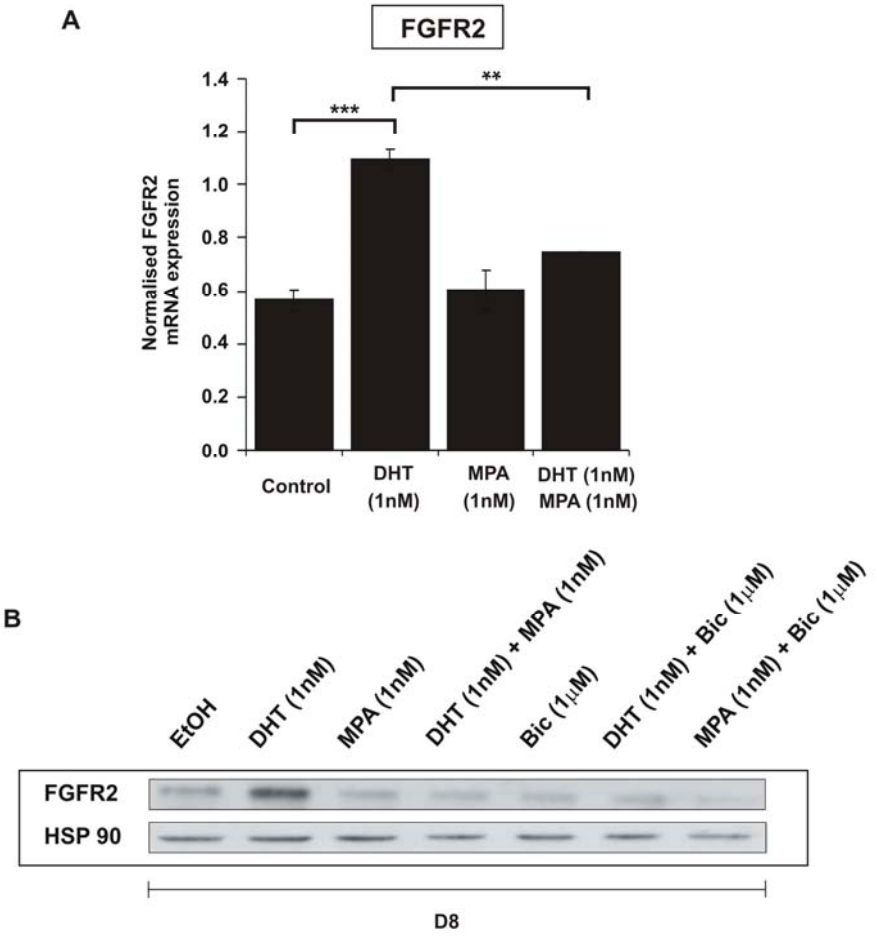


Figure 8.12 (A and B): Validation of candidate genes identified from ZR-75-1 Affymetrix gene microarray by qRT-PCR. Quantitative real-time PCR analysis was performed on mRNA obtained from ZR-75-1 cells grown under control conditions (i.e. 0.1% ethanol) or treated with 1 nM DHT and/or 1 nM MPA for 6 hrs with primers designed for **(A)** ORF1 and **(B)** C1ORF116. Statistical analysis was performed using Kruskal-Wallis non-parametric one-way ANOVA; **p<0.01, *** p<0.001. Other statistical comparisons were identified in the ANOVA not representated on the graph. Results are presented as the mean +/- SEM of three individual replicates and are representative of two individual experiments. Normalisation was performed to L19 and HMBS. Completion of experiments and technical work for the Affymetrix gene microarray required for this figure were performed by Dr Nicole Moore and Andrienne Hanson in the DRMCRL.

Figure 8.13 (A and B): Identification of the candidate gene FGFR2 demonstrating abrogation of DHT-induced AR-signalling in ZR-75-1 cells. (A) Quantitative real-time PCR analysis was performed on mRNA obtained from ZR-75-1 cells grown under control conditions (i.e. 0.1% ethanol) or treated with 1 nM DHT and/or 1 nM MPA for 6 hrs with primers designed for FGFR2. Statistical analysis was performed using Kruskal-Wallis non-parametric one-way ANOVA; **$p<0.01$, ***$p<0.001$. Other statistical comparisons were identified in the ANOVA not representated on the graph. Results are presented as the mean +/- SEM of three individual replicates and are representative of two individual experiments. Normalisation was performed to L19 and HMBS. Completion of experiments and technical work for the Affymetrix gene microarray required for this figure were performed by Dr Nicole Moore and Andrienne Hanson in the DRMCRL. **(B)** Protein lysates were prepared from ZR-75-1 breast cancer epithelial cells cultured in RPMI-PRF media supplemented with 10% DCC-FCS and P/S under control conditions (i.e. 0.1% ethanol) or treated with 1 nM DHT and/or 1 nM MPA, 1 μM Bic alone and combined with either 1 nM DHT or 1 nM MPA for 8 days. Protein lysates were run on SDS-PAGE gels, transferred to nitrocellulose membranes and probed for FGFR2. For each immunoblot HSP-90 was used as a loading control.


A
FGFR2
Normalised FGFR2 mRNA expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
**
Control
DHT (1nM)
MPA (1nM)
DHT (1nM) MPA (1nM)
B
EtOH
DHT (1nM)
MPA (1nM)
DHT (1nM) + MPA (1nM)
Bic (1μM)
DHT (1nM) + Bic (1μM)
MPA (1nM) + Bic (1μM)
FGFR2
HSP 90
D8

DHT and MPA as individual treatments remained similar to vehicle control *(refer to Appendix 3 - Table 3).* It should be noted that the hormone stimulated changes in the expression of the FGFR1 gene detected by microarray analysis were not validated by qRT-PCR in this thesis.

8.3.11 - The combined actions of DHT and MPA reverse the inhibitory effects of DHT and MPA alone on the oestrogen regulated gene, pS2 in ZR-75-1 cells

Lastly, given previous findings described in chapter 7 suggesting that the dual treatment of DHT and MPA may lead to an increase in ERα protein expression in non-malignant breast explant tissue samples, additional analysis to determine the effect of DHT and/or DHT and/or MPA on the oestrogen regulated gene pS2 was undertaken (Roberts, Wallace et al. 1988; Martin, Ribieras et al. 1997). Subsequent to treatment with either DHT or MPA alone, pS2 mRNA expression was reduced compared to control ($p<0.01$) [Figure 8.14]. Interestingly, following co-treatment with DHT and MPA, pS2 mRNA expression was increased compared to individual treatments ($p<0.01$) [Figure 8.14]. Additional experiments demonstrated an increase in pS2 mRNA expression compared to control following treatment with 1 nM E_2 ($p<0.001$) *(data not shown).* These findings may indicate that the combined actions of DHT and MPA can reverse the inhibitory effects of DHT on some E_2 regulated genes and warrants future investigation.

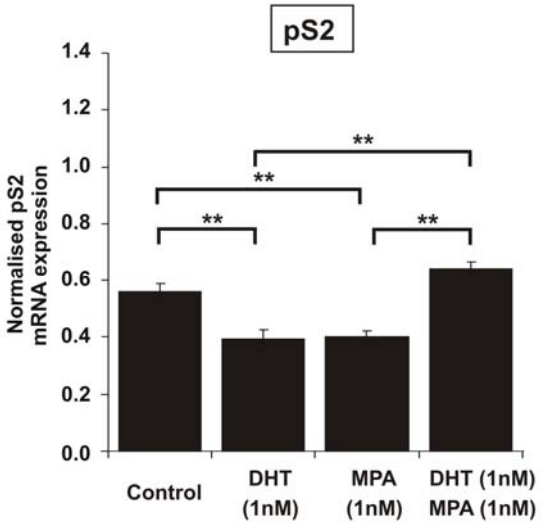


Figure 8.14: Effect of DHT and/or MPA on the ERα regulated gene PS2 mRNA expression in ZR-75-1 cells. qRT-PCR was performed on mRNA obtained from ZR-75-1 cells under control conditions (i.e. 0.1% ethanol) or treated with 1 nM DHT and/or 1 nM MPA for 6 hrs using primers designed for pS2. Statistical analysis was performed using Kruskal-Wallis non-parametric one-way ANOVA; **$p<0.01$. Other statistical comparisons were identified in the ANOVA not representated on the graph. Results are presented as the mean +/- SEM of three individual replicates and are representative of two individual experiments. Normalisation was performed to L19 and HMBS. Completion of experiments and technical work for the Affymetrix gene microarray required for this figure were performed by Dr Nicole Moore and Andrienne Hanson in the DRMCRL.

8.4 - Discussion

The data reported in this chapter identifies, in part, the genomic mechanism(s) by which the synthetic progestin MPA may promote breast cancer. The main findings have shown that inhibition of basal and E_2-stimulated DHT-induced breast epithelial proliferation by MPA occurs in the ZR-75-1 breast cancer cell line. Furthermore, DHT-induced AR-expression and signalling was impeded by MPA also in the ZR-75-1 cells. Using a genome-wide profile by gene microarray studies allowed for the identification of a number of androgen regulated genes which were shown to be abrogated by MPA and uniquely regulated by the combination of DHT and MPA, compared to DHT alone. Data described in this chapter using GO pathway analysis has provided evidence of de-regulation in cancer-related intracellular pathways by the hormone combination, compared to treatments in isolation. Thus, this chapter have provided insight into the potential AR-mediated molecular mechanism(s) associated with the increased incidence of breast cancer in post-menopausal women who take cHRT.

Findings in this chapter have shown that both DHT and MPA are able to suppress basal epithelial proliferation as previously reported (Birrell, Bentel et al. 1995), yet novel findings described in this chapter have shown that MPA is not as effective as DHT at reducing E_2-stimulated breast epithelial proliferation at low doses of 1 nM. Irrespective of these findings, co-treatment with DHT and MPA impedes DHT-induced inhibition of growth in the presence and absence of E_2. Moreover, these findings give insights into the effects of DHT and MPA combined to occur in post-menopausal women with both low

and high circulating serum levels of E_2. A prior study has reported a similar loss of DHT-inhibition of proliferation by MPA in the AR positive, ERα and PR negative breast cancer epithelial cell line MFM-223 (Hackenberg, Hawighorst et al. 1993) in the absence of E_2. However, the latter study did not include any statistical analysis, and was in a breast cancer epithelial cell line, which abnormally expressed only AR, in the absence of ERα and PR. Moreover, clinical studies indicate that the protective actions of AR are evident in ERα positive, yet not in ERα negative breast cancer (Peters, Buchanan et al. 2009). Interestingly, MPA is shown to promote development of ERα positive breast cancers (Saxena, Lee et al. 2010), thus, the detrimental effects of MPA on AR-signalling potentially leading to the promotion of breast cancer are likely to involve ERα as depicted in working hypothesis in chapter 7 *(refer to Figure 7.9).*

The relevance of the ability of MPA to impede DHT-induced inhibition of basal and E_2-stimulated proliferation to the current literature may provide further insight into the mechanistic nature of the dual actions of DHT and MPA and the promotion of breast cancer in post-menopausal women taking cHRT (Magnusson, Baron et al. 1999; Saxena, Lee et al. 2010). A population-based case-control large scale epidemiological study performed in Sweden reported that 3,345 post-menopausal women diagnosed with invasive breast cancer aged 50-74 yrs who took cHRT for >60 months and had a low body mass index (BMI), i.e. <27 kg/m^2 and increase in breast cancer risk (HR, 2.27; 95% CI, 1.46 to 3.53), compared to women who had a high BMI, i.e. >27 kg/m^2 and a lower breast cancer risk (HR, 1.48; 95% CI, 0.95 to 2.31) (Magnusson, Baron et al. 1999). Similar findings have been reported in another USA population-based case-controlled

epidemiological study in the California Teachers Study cohort including 2,857 women diagnosed with invasive breast cancer over a 9.8 year follow-up period (Saxena, Lee et al. 2010).

The biological basis for the increase in breast cancer risk for post-menopausal women of low BMI taking cHRT has not been comprehensively addressed. However, obesity is a strong determinant of the development of breast cancer in post-menopausal women (Pike, Wu et al. 2007). Authors suggest that an increase in adipose breast tissue and in turn steroid hormone synthesis (i.e. increased intracrine production of oestrogen in the breast tissue) is the driving carcinogen in women of a high BMI (Key and Pike 1988; van den Brandt, Spiegelman et al. 2000; Key, Appleby et al. 2003; Baglietto, English et al. 2009). It is therefore possible that post-menopausal women with a low BMI may have a lower intracrine oestrogen synthesis of steroid hormones and may harbour a relatively larger number of oestrogen non-stimulated occult pre-existing breast cancer lesions (Horwitz and Sartorius 2008). Thus, exposure of the breast tissue to cHRT may lead to a greater promotion in growth of these pre-malignant lesions to malignant breast cancer in the absence of high circulating oestrogen serum levels, compared to women of a higher BMI with potentially higher circulating oestrogen serum levels.

Emerging mechanistic effects of MPA acting to alter DHT-induced AR-signalling and gene expression has been revealed by changes to gene regulation in this chapter. It has been demonstrated that changes in gene regulation by the hormone combination can occur as early as 6 hrs in the absence of altered AR mRNA expression, compared to

individual treatments. A reduction in AR mRNA expression by DHT in prostate cancer cells has also been previously identified, suggesting these mRNA changes may be associated with active AR-signalling (Shen, Lin et al. 1996). However, long-term exposure of the breast epithelial cells to DHT and MPA combined leads to the loss of AR protein. Mechanistically the data described in this chapter suggests that the reduction in AR protein by the hormone combination may occur as a result of series of intracellular AR-mediated signalling events leading to either of the following: 1) de-stabilisation of the AR and/or 2) increased proteosomal degradation of the AR. These events may be induced by: 1) altered conformation of the AR in the presence of DHT and MPA; 2) increased nuclear export of the AR and/or 3) increased glucuronidation of sex steroid hormones involved in the regulation and degradation pathways of nuclear steroid receptor.

It has previously been reported that transcriptional programs regulated by MPA alone overlap with genes similarly regulated by DHT, in an engineered PR-negative, AR-positive human breast epithelial cell line, referred to as T47D-Y-AR (Ghatge, Jacobsen et al. 2005). These findings support the current study whereby a high percentage of identical genes can be regulated by both DHT and MPA as individual treatments. Additionally these findings may lead to the assumption that the biological effects of either hormone would be similar in the breast tissue. However, as demonstrated from the data in this thesis, there are also a large percentage of genes that are uniquely regulated by DHT and MPA as individual hormone treatments. Moreover, DHT alone can inhibit breast epithelial proliferation, whereas MPA in isolation does not result in the same effect.

It is also assumed from the literature that DHT in part regulates AR-target genes following an intra- or intermolecular N/C interaction (Kemppainen, Langley et al. 1999; Centenera, Harris et al. 2008). However, dimerisation of the AR can also occur through residues located in the LBD and DBD (Centenera, Harris et al. 2008). These different dimerisation capabilities of the AR may provide an understanding to how MPA can potentially regulate a similar set of DHT-regulated genes in the absence of an N/C interaction (Kemppainen, Langley et al. 1999; Birrell, Butler et al. 2007). This leads to the notion that if MPA can regulate a similar set of DHT-regulated AR-target genes in the absence of an N/C interaction, that despite an abrogation of DHT-induced N/C interaction by MPA, the hormone combination may similarly regulate a distinct and/or unique set of AR-modulated genes independent of an N/C interaction. From studies undertaken in the DRMCRL, loss of the DHT-induced N/C interaction of the AR by MPA may be a fundamental molecular basis for abrogation of AR activity (Birrell, Butler et al. 2007). Interestingly, it has recently been highlighted in a review that synthetic progestins which have partial agonist activity (i.e. can induce some transactivational activity, yet is not maximal on its own), also exhibit antagonistic activity of the receptors in the presence of its native ligand (i.e. DHT for AR) (Africander, Verhoog et al. 2011). These findings are congruent with the results described in this thesis, whereby MPA can act as an agonist in isolation on DHT, yet in the presence of DHT, acts as an antagonist.

A number of candidate genes which may be used as potential biomarkers to detect increased breast cancer in women taking cHRT were identified in this study. The most interesting and potentially relevant candidate gene identified from the study which

demonstrates abrogation of DHT-induced AR-signalling by MPA was FGFR2, a member of the fibroblast growth factor receptor family. The tyrosine kinase FGFR2 has been implicated in breast cancer by acting in an anti-proliferative manner (Grose and Dickson 2005) and a reduction of FGFR2 expression in sebaceous skin cells following anti-androgen treatment demonstrates androgen regulation of this gene (Melnik, Schmitz et al. 2009). Interestingly, a number of single nucleotide polymorphisms (SNPs) in the FGFR2 gene have been identified that are classed as low-penetrance susceptibility loci for breast cancer risk (Easton, Pooley et al. 2007; Hunter, Kraft et al. 2007; Liang, Chen et al. 2008). Moreover, of high relevance to this study are findings reporting that post-menopausal women who take cHRT and who harbour a SNP in the FGFR2 gene suffer a higher incidence of breast cancer than women who take ERT (Prentice, Huang et al.; Prentice, Huang et al. 2009). Recent studies have also found that FGFR2 immunoreactivity and mRNA expression is decreased in human breast carcinoma samples that possess an increased number of SNPs in the FGFR2 gene compared to normal breast tissue samples (Zhu, Asa et al.; Luqmani, Graham et al. 1992). Moreover, post-menopausal women are at a higher risk of developing ERα and PR positive breast cancers in conjunction with either FGFR2 gene SNPs (Garcia-Closas, Hall et al. 2008; Liang, Chen et al. 2008; Rebbeck, DeMichele et al. 2009) or cHRT (Saxena, Lee et al. 2010).

The mechanism underlying the suppression of proliferation by FGFR2 has been linked to an increase in FGFR1 expression and activity in breast cancer (Penault-Llorca, Bertucci et al. 1995). In support of these findings, the combination of DHT and MPA caused a

significant decrease in FGFR2 mRNA and protein expression compared to DHT alone. Moreover, FGFR1 mRNA was increase by the hormone combination, and was unaffected by either treatment of DHT and MPA in isolation. Collectively, these findings support the notion that the increased risk in development of breast cancer in women who take cHRT and who harbour SNPs in the FGFR2 gene is dependent on steroid receptor activity.

In conclusion, the most interesting findings in this chapter suggest that the mechanism(s) whereby the combined action of DHT and MPA that may be associated with breast cancer include both the inhibition of DHT-induced AR-signalling and the unique regulation of gene expression. Changes to this gene expression in the breast tissue potentially leads to an increase in breast epithelial proliferation via alteration to intracellular signalling pathways primarily associated with cellular growth and proliferation, cancer, cell cycle, cell death and gene expression. These findings support the view and the hypothesis of this thesis that MPA is carcinogenic via an anti-androgenic AR-mediated action and therefore should not be used in cHRT for the general public.

Chapter 9

General Discussion

9.1 - General Discussion

9.1.1 - Major findings of this thesis

Initial studies in this thesis have provided evidence of an association between the use of cHRT preparations containing MPA and age-standardised breast cancer incidence rates in Australian women aged ≥ 50 yrs. Specifically, these findings have demonstrated that breast cancer incidence rates and cHRT containing MPA in Australian women increase prior to the publication of the cHRT arm of the WHI trial and both decline subsequently. Moreover, studies in this thesis have shown that this effect is potentially greater in post-menopausal Australian women aged between 50-74 yrs, compared to all women ≥ 50 yrs. This data has provided supporting evidence for ongoing public health related risks associated with the use of MPA in cHRT, which has been implicated as a breast carcinogen, for treatment of menopausal symptoms in post-menopausal Australian. Collectively, these findings are supportive of trends that have been identified in both the USA and Europe, and illustrates that MPA is more commonly prescribed than other synthetic progestins for cHRT in Australia, and is therefore of a high concern.

The most striking findings reported in this thesis have provided the first documented experimental evidence of a potential AR-mediated biological mechanism by which MPA may contribute to the development of breast cancer. Using the *ex vivo* breast explant tissue model, I have demonstrated that MPA impedes DHT-induced AR-signalling in post-menopausal non-malignant human breast epithelial cells. Importantly, these studies have established a potential biological link between the effects of MPA promoting

increased breast epithelial proliferation in post-menopausal women taking cHRT by the disruption of the DHT-induced AR-signalling. Moreover, these studies substantiate earlier speculations derived from *in vitro* studies undertaken in the DRMCRL implicating MPA as an AR antagonist in the presence of the native ligand, DHT (Birrell, Butler et al. 2007).

Follow on studies in this thesis using the malignant breast cancer cell line, ZR-75-1, substantiated that the AR-mediated effects of MPA observed in non-malignant cells are also evident in malignant cells. These findings are important since cHRT is thought to promote breast carcinogenesis by stimulating pre-existing pre-malignant breast lesions. Furthermore, the experimental studies conducted using the ZR-75-1 cell line enabled confirmation of an antagonist effect of MPA on AR-signalling in the presence of DHT and further identified that the combined actions of DHT and MPA can lead to the regulation of a unique subset of genes. These findings have provided some insight into a mechanistic basis for the loss of inhibition of breast epithelial proliferation by the hormone combination observed in both post-menopausal non-malignant breast tissue and malignant breast cells at the genomic level. Furthermore, the identification of a number of candidate genes and altered cancer-related intracellular AR-signalling pathways by the hormone combination provide important information which can be utilised as a foundation for future studies to investigate mechanism(s) associated with the actions of MPA and breast cancer development, as addressed in subsequent sections.

Intriguing findings described in this thesis which were not the main focus of the

hypothesis suggest that there are intrinsic molecular based differences which exist in the biological nature of pre- compared to post-menopausal breast tissue that alters responsiveness to natural and synthetic hormones. A similar effect of treatment by DHT and/or MPA was observed on AR expression in both tissue types. However, in post-menopausal breast tissues, DHT led to an inhibition in breast epithelial proliferation and this effect was reversed by MPA, a hormonal response that was not detected similarly in pre-menopausal breast tissue. These findings suggest that there are potential key molecular based changes in post- compared to pre-menopausal breast tissue which prevent the anti-proliferative actions of DHT on breast epithelial proliferation. It has been shown that pregnancy leads to a reduction in breast cancer potentially by inducing lower proliferation rates by the process of breast differentiation *(refer to chapter 1 - sections 1.2.5 and 1.2.6)*. However, to date it has not been shown that the breast tissue reverts back to a hormonally responsive proliferative state following menopause in parous women. Additionally, the detection of reduced AR expression and the reduced AR:ERα ratio in post- compared to pre-menopausal breast tissue suggests that the menopausal transition does lead to alterations in steroid receptor expression which may be instrumental to further understanding the molecular basis associated with higher rates of breast cancer in post- compared to pre-menopausal women. Moreover, these findings suggest that parous pre-menopausal women may not be at a risk of breast cancer following exposure to MPA used in the POC Depo-Provera.

Performance of similar studies described in this thesis using nulliparous pre-menopausal breast tissue samples would be extremely informative in further investigating the

potential carcinogenic effects of MPA in younger women which have been reported to be at a higher risk of breast cancer following use of the MPA based POC, Depo-Provera (Skegg, Noonan et al. 1995; Skegg, Paul et al. 1996). However, it should be noted that the lower base-line levels of Ki67 % positivity in pre- compared post-menopausal cultured non-malignant breast tissue samples in vehicle control may have comprised the effects of DHT and/or MPA on breast epithelial proliferation in the studies undertaken in this thesis. It is clear from the studies described in this thesis that more research is required to investigate the cellular and genomic based changes that occur during menopause in parous women that potentially can lead to breast tissue that is of a higher susceptibility to carcinogenic insults. The use of this knowledge may act to implement therapeutic intervention during menopause to maintain the protective nature which exists in parous pre-menopausal breast tissue.

The studies in this thesis have indicated that a functional AR-signalling axis by DHT exists and is maintained in cultured human post-menopausal breast explant tissue using the *ex vivo* breast explant tissue model. In addition to the benefit these scientific studies have provided to investigate the actions of MPA on AR-signalling in human breast tissue samples, they have additionally provided a foundation for the use of this experimental approach for future studies to study aspects of AR-signalling in relation to normal breast biology and carcinogenesis. No other study has demonstrated the existence of any type of functional steroid receptor signalling axis following the addition of specific receptor antagonists (e.g. tamoxifen or RU486) using the *ex vivo* breast explant tissue model. Thus, the studies included in this thesis have lead to experimental approaches that can

applied to study the effects of newly developed targeted cancer therapies aimed at modulating steroid receptor expression using human malignant breast tissue samples. Studies of this type have been implemented in the DRMCRL and are currently in progression.

The studies in this thesis have also provided some interesting findings indicating that there are alterations to the AR-signalling axis and increased ERα expression in non-malignant breast tissue in close proximity to malignancy, which may in fact reflect early cellular changes that occur during initiation stages of breast cancer. Future *ex vivo* breast explant tissue model studies utilising these findings would be greatly beneficial to study specific changes to steroid receptor signalling and responsiveness to natural and synthetic hormones in non-malignant, pre-malignant and invasive breast cancer tissue samples. Furthermore, since MPA is thought to promote breast cancer in pre-existing malignant lesions in the breast tissue, additional studies using pre-malignant and malignant breast tissues in the *ex vivo* breast explant tissue model would be useful in determining the effects of MPA on AR-signalling in breast tissue that have already undergone carcinogenic transformation, compared to non-malignant breast tissue.

Some experimental limitations that exist when using human breast tissue samples in the *ex vivo* culture model described in this thesis are as follows: 1) the high rates of biological heterogeneity that exists in women within the population, thus requiring sufficient numbers to detect a robust biological effect (i.e. bcl-2 changes) and 2) the difficulty in studying hormone effects at a gene expression level by changes in RNA

expression, due to the difficulty in obtaining high quality, non-degraded RNA for experimental studies and variability in stromal to epithelial ratio between tissue samples. Despite these limitations, the use of primary human non-malignant and malignant breast tissue is highly attractive for scientific studies, based on the closer biological representation to human biology, compared to cell lines and animal experimental models and there is hope for the use of these models to be utilised as a more common practice for scientific endeavours. Since there is high inherent heterogeneity in female breast tissue biological hormonal responsiveness within the population the studies in this thesis are quite significant based on relatively low patient numbers. Moreover other studies using similar patient numbers have also identified biological responses to hormonal treatment in breast tissues using an *ex vivo* breast explant culture experimental system (Zhuang, Saaristo et al. 2003; Eigeliene, Harkonen et al. 2008). Validation of the findings in this thesis could be completed by the use of human breast biopsies from post-menopausal women taking cHRT compared to a control group with no prior history of cHRT use. In summary, this thesis has provided epidemiological evidence and biological data relating to the health implications associated with the use of MPA in cHRT and has provided compelling findings indicating potential anti-androgenic AR-mediated effects of MPA leading to an increased promotion of breast cancer.

9.1.2 - Specific effects of MPA in the absence of E_2

Whether the increased promotion of breast cancer in post-menopausal women taking cHRT, compared to ERT, is linked specifically to the actions of the synthetic progestin or the combined effects of both the synthetic oestrogen and progestin in cHRT has not been

reported in the literature to date. The lack of E_2 supplementation in the experimental studies performed in this thesis may in fact appear to be a limiting factor when interpreting the data in terms of the molecular basis associated with cHRT use and breast cancer. However, data included in this thesis has demonstrated that there are unique actions of MPA in combination with DHT compared to individual treatments involved in the promotion of breast cancer that may occur independently of the synthetic oestrogen used in cHRT. Given this proposed hypothesis, some explanation to the findings in the literature, which report that post-menopausal women of a low BMI, who typically have a lower percentage of body fat (i.e. lower production of oestrogen in adipose tissue) are associated with higher rates of breast cancer following use of cHRT (Magnusson, Baron et al. 1999; Pike, Wu et al. 2007). The basis for these finding may be linked to a higher percentage of pre-existing, oestrogen non-stimulated occult breast cancer lesions in women of a lower BMI, compared to a higher BMI. The steroid receptor expression profile of these types of occult breast cancer lesions in women of this physiological status however is not known and requires further investigation. It is feasible to suggest that the addition of cHRT leads to the acceleration of growth within oestrogen non-stimulated pre-existing breast cancer lesions more often in women of a low BMI by the combined actions of DHT and MPA based on the findings of this study.

A previous breast explant tissue culture study has also shown by the measurement of the proliferation markers Ki67 and PCNA, that 10 nM E_2 stimulates breast epithelial proliferation to a greater degree compared to either 100 nM MPA alone or combined with E_2 at 7, 14 and 21 days of hormone treatment in peritumoural tissues of women aged 49-

64 yrs (Eigeliene, Harkonen et al. 2006). The higher dose of MPA used in the latter study makes it difficult to compare the findings with the lower dose used in the studies in this thesis (i.e. 1 nM MPA). However, based on the literature findings reporting that the combined actions of E_2 and MPA does not result in increased effects on breast epithelial proliferation, compared to individual treatments, it would suggest that there is no additional detrimental effect linked to combined actions of the two hormones compared to individual effects. Moreover, since E_2 was associated with a greater effect than MPA alone on promoting breast epithelial proliferation, the findings may lead to conclusions that use of ERT is associated with a greater risk of breast cancer compared to cHRT, which has not been substantiated by epidemiological studies (Rossouw, Anderson et al. 2002; Beral 2003). Thus, in light of the literature, the finding in this thesis have led to intriguing new mechanisms associated with the promotion of breast cancer in women taking cHRT by the combinatorial actions of DHT and MPA, independent of E_2.

9.1.3 - Mechanisms associated with MPA and breast cancer in the current literature by PR and GR-mediated mechanisms

9.1.3.1 - Contribution of PR-mediated actions of MPA and breast cancer

It is shown in the literature that cHRT can lead to clinically detectable breast cancers following 2-5 yrs of use (Rossouw, Anderson et al. 2002; Beral 2003). Moreover, a higher rate of breast cancers has been detected in women taking cHRT with prior history of breast cancer (Holmberg, Iversen et al. 2008). Therefore, it has been suggested that MPA is acting on occult, dormant breast disease and/or pre-existing, pre-invasive malignant breast lesions that are non-detectable by mammography screening prior to the

commencement of cHRT (Horwitz and Sartorius 2008). These effects of MPA could feasibly occur since it has been shown in autopsy based studies that women greater than the age of 40 yrs exhibit a high rate of invasive breast cancer (median prevalence, 1-3%) and a greater amount of non-invasive breast cancer (i.e. DCIS, median prevalence, 8.9%) than women < 40 yrs (Pollei, Mettler et al. 1987; Horwitz and Sartorius 2008).

Studies investigating the role of MPA in cHRT and breast cancer development via PR-mediated actions have formulated hypothesised theories that MPA acts on stem cells in pre-existing occult breast disease. This notion has been supported by a study demonstrating that MPA acts via PR within differentiated T47D ER^+ PR^+ $CK5^-$ breast cells to reactivate CDK5 in neighboring ER^- PR^- tumourigenic stem cells, in small, undetectable occult disease (Horwitz and Sartorius 2008). Interestingly, in the latter study, proliferation was unaffected by MPA treatment but subsequent treatment with oestrogen led to an increase in differentiated ER^+, PR^+, $CDK5^-$ breast cancer cells (Horwitz and Sartorius 2008). However, this study did not use a PR antagonist to demonstrate that the effects of MPA are conclusively PR mediated, and T47D cells do express AR, so the findings of this study are not definitive. Mechanistically, MPA has been implicated as a driver of breast cancer development by the stimulation of the vascular epidermal growth factor (VEGF) and angiogenesis, via PR mediated actions (Hyder, Murthy et al. 1998; Mueller, Vigne et al. 2003). A separate study also reported activation of the PR-B regulated gene, cyclin-D1 (a positive regulator of the cell cycle) and stimulation of breast epithelial proliferation, through non-genomic mechanisms in the T47D cell line (Saitoh, Ohmichi et al. 2005). In support of the latter findings

demonstrating cyclin-D1 is regulated via PRB, post-menopausal breast explant cultured tissues by E_2 alone and combined with MPA have been shown to be associated with an increase in cyclin-D1 and Ki67 protein, via IHC detection (Eigeliene, Harkonen et al. 2008). Despite these findings, cyclin-D1 has also been reported to be negatively regulated via the AR in MCF-7 breast cancer cells following treatment with DHT, thus interference with DHT-induced AR-signalling by MPA may also potentially lead to increased cyclin-D1 expression independent of PR-mediated actions (Lanzino, Sisci et al. 2010). Lastly, a recent study has identified increased RANKL expression and breast epithelial proliferation in mammary gland tissue following subcutaneous administration of MPA in mice (Schramek, Leibbrandt et al. 2010). This study identified by *in vivo* and *in vitro* experiments up-regulation of RANKL expression by MPA, leading to activation of its receptor RANK and a subsequent increase in cyclin-D1 expression and cell survival or reduction in apoptosis (Schramek, Leibbrandt et al. 2010). Furthermore, a delayed progression of DMBA carcinogen-induced breast tumours was reported in this study in RANK knock-out female mice in conjunction with MPA treatment (Schramek, Leibbrandt et al. 2010).

It has further been demonstrated in a separate study that MPA is acting by PR-mediated effects to stimulate RANKL expression (Beleut, Rajaram et al. 2010). This is shown *in vivo* where reduced progesterone-induced breast epithelial proliferation is observed in conjunction with the blocking agent OPG of RANKL to its receptor RANK (Beleut, Rajaram et al. 2010). Although the findings from the described literature propose that MPA acts by PR-mediated mechanisms to promote breast cancer, MPA also exhibits high

ligand binding affinity for both the AR and GR (Sitruk-Ware 2004), and the lack of specific receptor antagonists for PR in any of the described studies, makes it difficult to form definite conclusions. Interestingly, in this thesis, findings indicated that the disruption of DHT-induced AR-signalling by MPA potentially leading to increased breast epithelial proliferation can occur independent to changes in PR protein in cultured, non-malignant post-menopausal breast tissue samples and in ZR-75-1 malignant breast cancer cells expressing absent to low expression levels of PR. Whilst these findings have been documented in this thesis, it does not suggest there are not PR-mediated signalling effects by the actions of MPA that can occur also in conjunction with AR-mediated signalling effects to promote breast cancer.

9.1.3.2 - Contribution of GR-mediated actions of MPA and breast cancer

In this section MPA actions via GR-mediated effects that may lead to breast cancer actions that have been described in the literature are discussed. Firstly, it should be noted that MPA has higher ligand binding affinity for the GR, compared to the native GR ligand cortisol, yet MPA has a higher dissociation rate compared to cortisol of the GR *(refer to Appendix 1 - Table 1)* (Africander, Verhoog et al. 2011). Additionally similar and distinct gene expression profiles have been identified in the T47D breast cancer cells by Affymetrix oligionucleotide gene microarray following treatment with either glucocorticoids or progestins (Wan and Nordeen 2003). Progesterone also possesses partial agonist activity on the GR, yet is not associated with an increase in breast cancer in women taking the cHRT medicine, Prometrium (de Lignieres, de Vathaire et al. 2002; Fournier, Berrino et al. 2008). In comparison, two other synthetic progestins, NETA and

LNG, that are also commonly used in cHRT have also been shown to increase breast cancer risk, yet do not in fact possess any ligand binding affinity for GR (Beral 2003; Africander, Verhoog et al. 2011). These findings may suggest that the actions of synthetic progestins associated with breast cancer are not in fact mediated by the GR. However, it should also be considered that different synthetic progestins may exhibit unique effects that can promote breast cancer by similar, yet distinct steroid receptor mediated effects.

The contribution of GR-mediated MPA effects in the promotion of breast cancer has not been as extensively reported in the literature as compared to PR, since MPA is more often associated with its progestagenic activity (Sitruk-Ware 2004). However, the few studies that have been published in the subject area indicate that the actions of oestrogen and MPA combined mediate both proliferative and anti-apoptotic actions in normal luminal human breast epithelial cells and the breast cancer cells (MCF-7 and T47D), via GR-mediated actions, using a glucocorticoid antagonist (Courtin, Communal et al. 2011). In contrast, E_2 and progesterone combined displayed anti-proliferative and differentiation actions under the same experimental conditions (Courtin, Communal et al. 2011). Conversely, an increase in the metastasis tumour suppressor gene, Nm23-H1, via GR-mediated actions binding to a GRE in the promoter region of the gene, in the MDA-MB-231 (AR, ERα and PR negative) and MDA-MB-453 (AR^+ and ERα, PR negative) breast cancer cell lines, following MPA treatment has been identified (Ouatas, Halverson et al. 2003). Overall, the limited studies that have been published describing the actions of MPA by GR-mediated effects and breast cancer render it difficult to form conclusive interpretations. Moreover, they do not provide compelling evidence that MPA, or other

synthetic progestins commonly used in cHRT that are also linked to increased breast cancer (i.e. NETA and LNG), are acting specifically to promote breast cancer only through GR-driven signalling pathways in women taking cHRT.

In line with the described studies, it may be feasible to form the hypothesis that MPA acts in a combinatorial manner by the interplay of effects mediated through the AR, PR and/or GR. The concept of steroid receptor competition by MPA for AR, PR and GR compared to their native ligands is also likely to be influenced by the relative baseline steroid receptor expression levels that exist in the breast tissue prior to commencement of cHRT in post-menopausal women. It may also be possible that the high heterogeneity of steroid receptor expression among women in the population influences the biological effects of a particular type of synthetic progestin used in cHRT in certain sub-groups of women.

9.1.4 - Postulated mechanistic actions of MPA leading to increased rates of breast cancer

To further understand the molecular events associated with the combined actions of DHT and MPA by AR-mediated effects in isolation or in combination with the actions of MPA via PR and/or GR, the following sections address potential mechanism(s). Speculatively, steroid receptor ligand binding including AR bound to DHT and MPA bound to either, AR, PR and/or GR may allow for: 1) alternative dimerisation events (i.e. homo- and/or heterodimerisation); 2) differential recognition of HREs and/or 3) differential recruitment of co-transcriptional machinery leading to altered gene regulation. It has been reported in the literature that ligand binding of MPA to the AR does in fact lead to an altered

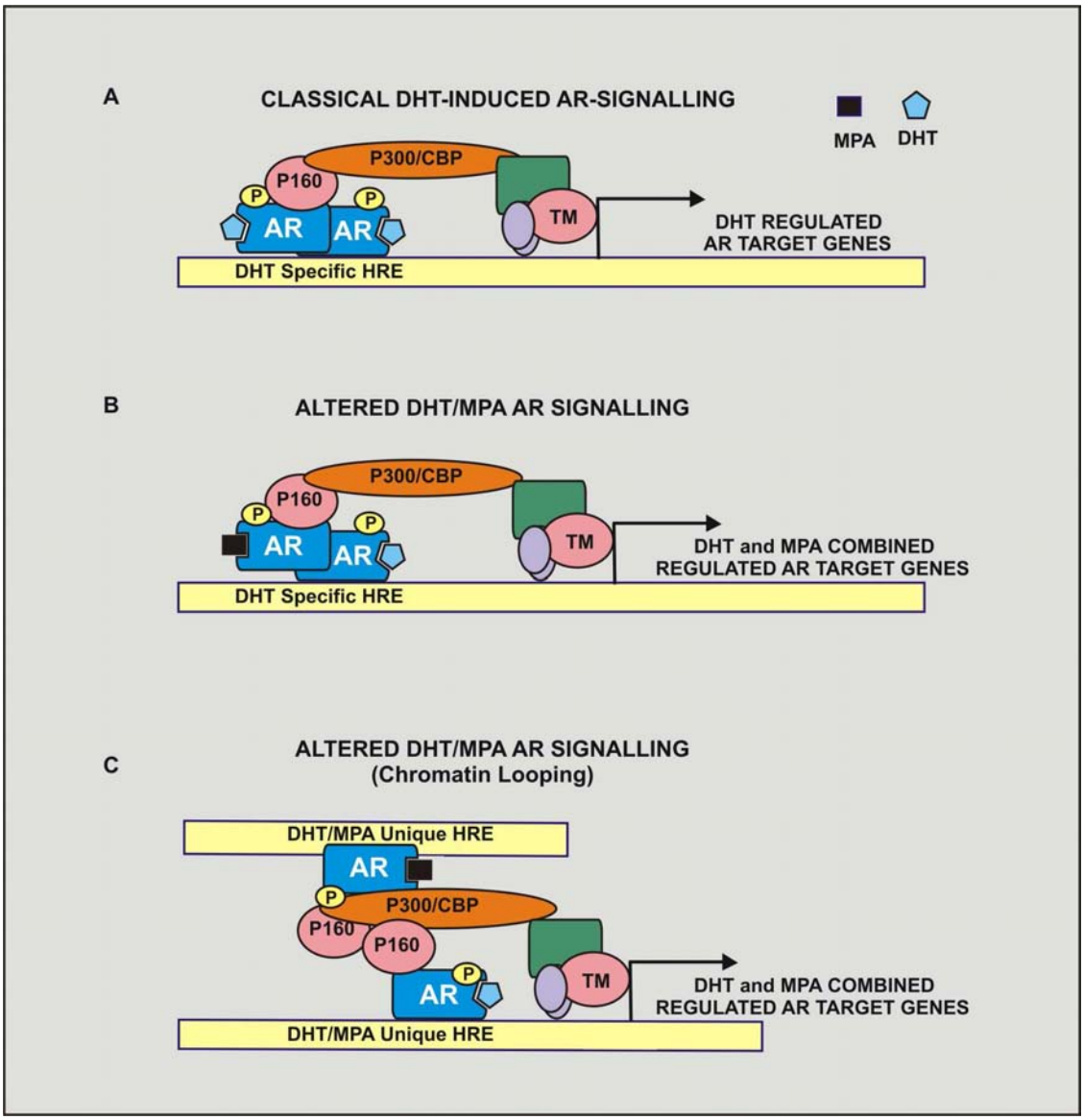
conformation of the AR (i.e. altered positioning of the amino acid phenylalanine located at residue 875 in the LBD of the AR), compared to the conformation induced by the native ligand DHT (Birrell, Butler et al. 2007). This change in conformational structure of the AR by MPA has been further proposed to contribute to and/or cause the loss of antagonism of DHT-induced AR N/C dimerisation (i.e. amino and carboxy terminal interactions) (Birrell, Butler et al. 2007).

The nature of the 3-nucleotide spaced partial palindromic sequence that comprises the ARE binding motifs, dictates the conformational orientation of the AR on the DNA (Verrijdt, Haelens et al. 2003). On classical AREs that are comprised of inverted repeats, the AR forms a head-to-head conformation, in contrast to selective AREs that are comprised of direct repeats, whereby the AR binds in a head-to-tail conformation (Verrijdt, Haelens et al. 2003). In relation to the data described in this thesis reporting differential genomic effects of DHT and MPA combined, compared to individual treatments, it is possible that altered dimerisation and/or recognition of either or both classical and selective AR response elements may be a mechanistic basis. Moreover, since abrogation or impediment by MPA was only evident on a subset of DHT-regulated genes, it is likely that the different binding motifs that comprise either classical or selective AREs can influence that effects of the hormone combination (Verrijdt, Haelens et al. 2003; Denayer, Helsen et al. 2010). Furthermore, findings in this thesis indicating that the AR can maintain regulation of a subset of DHT-target genes in the presence of MPA may be based on the whether the AR forms a head-to-tail conformation, rather than a head-to-head conformation.

Currently it is not known whether AR-DHT and AR-MPA monomers can maintain aspects of classical AR dimerisation as reported for DHT [Figure 9.1A and B] (Centenera, Harris et al. 2008). In addition, the binding of AR-DHT and AR-MPA monomers in the absence of dimerisation, may potentially lead to long-range chromatin looping events to modulate gene regulation [Figure 9.1C]. The ability and potential differences of an AR monomer bound to DHT or MPA to dimerise with other nuclear steroid receptors, including PR or GR, in a classical or non-classical manner also remains unknown. The ability of ERα and ERβ to heterodimerise has been demonstrated in the literature using an *in vitro* GST pull-down technique (Ogawa, Inoue et al. 1998). Studies have also demonstrated the ability of different PR isoforms to heterodimerise (Tetel, Jung et al. 1997; Leonhardt, Altmann et al. 1998), in addition to the ability of class III nuclear receptors, including the thyroid hormone receptor, retinoic acid receptor and vitamin D receptor to form heterodimers with the retinoid X receptor (Verrijdt, Haelens et al. 2003). Further investigation as to whether AR bound to DHT can form a heterodimer with PR and/or GR in the presence of MPA would be beneficial to further understanding the molecular mechanism associated with the combined actions of DHT and MPA that lead to differential gene regulation.

Given findings in this thesis, it is possible that there are two distinct mechanisms occurring mediated by the combined actions of DHT and MPA that lead to altered AR-signalling, either independently or in conjunction with PR and/or GR, which include the following: 1) the altered conformation of the AR by MPA, leading to the loss of DHT-induced N/C interaction (abrogation or impediment of AR-signalling) and 2) the

Figure 9.1 (A-C): Potential mechanistic action of a differential effect of DHT and MPA combined on gene regulation compared to DHT alone. Differential gene regulation meditated by AR activity may occur in the presence of DHT and MPA combined compared to DHT alone by a number of hypothetical scenarios. **(A)** Classical DHT-induced AR-targeted gene regulation occurs by the dimerisation of the AR, recognition and bindings to the HREs in proximal and distal regions upstream from AR gene promoters, recruitment of co-regulators and transcriptional machinery. Alternative AR signaling events which may occur in the presence of DHT and MPA combined leading to abrogation of DHT-induced AR signaling and the regulation of unique genes include **(B)** dimerisation of the AR via classical and/or non-classical events at distinct HREs and the loss of recognition to classical DHT-induced AR response elements and **(C)** binding of AR as monomers by either DHT or MPA to gene promoter half-sites located in proximal and distal regions of the genome, creating platforms for molecular interactions via long-range chromatin looping events.


A
CLASSICAL DHT-INDUCED AR-SIGNALLING
MPA
DHT
P300/CBP
P160
P
P
AR
AR
TM
DHT REGULATED
AR TARGET GENES
DHT Specific HRE
B
ALTERED DHT/MPA AR SIGNALLING
P300/CBP
P160
P
P
AR
AR
TM
DHT and MPA COMBINED
REGULATED AR TARGET GENES
DHT Specific HRE
C
ALTERED DHT/MPA AR SIGNALLING
(Chromatin Looping)
DHT/MPA Unique HRE
AR
P
P300/CBP
P160
P160
P
AR
TM
DHT and MPA COMBINED
REGULATED AR TARGET GENES
DHT/MPA Unique HRE

acquisition of alternative recognition of genome wide HREs by the AR, PR and/or GR leading to differential gene regulation. The following sections address specific future experimental studies which are required to further investigate these speculative mechanistic actions of the co-treatment acting to promote altered AR-signalling.

9.1.5 - Future studies

9.1.5.1 - Genome wide profiling

To investigate further the mechanistic nature of the combined effects of DHT and MPA on de-regulation of DHT-induced AR-signalling and the distinct regulation of a unique subset of genes, additional studies are required. To determine whether these effects by the hormone combination, compared to treatments alone, are mediated by altered binding to specific HREs, genome-wide profiling by ChIP-on-chip and/or ChIP-seq assays would provide for a global genomic-based investigation of potential changes to the AR cistrome by the co-treatment of DHT and MPA compared to individual treatments. Firstly, these types of studies would provide comprehensive information of specific changes to differential binding to HREs at a chromatin level. Secondly, they would allow for the identification of specific DNA sequences that the AR can bind to in the presence of DHT and MPA alone and combined in a comparative analysis.

Recently using the more advanced chromatin interaction technique, called chromatin interaction analysis by paired-end tag sequencing (ChiA-PET), global genome analysis of ERα chromatin interactions have identified the ability of ER to mechanistically regulate genes leading to either activation or repression of genes by long-range chromatin

interactions (Fullwood, Liu et al. 2009). Similar long-range interactions by other nuclear steroid receptors, including AR, PR and GR, are therefore also likely to occur within the cell. Studies using the ChiA-PET technique would allow for further investigation into whether long-range chromatin interactions of an AR-monomer with another AR-, PR- or GR-monomer can occur in the presence of DHT and MPA combined.

9.1.5.2 - Altered dimerisation and/or conformation of the AR by the co-treatment of DHT and MPA

The mechanistic basis for the altered gene regulation by the hormone combination may be initiated by the altered ability of the AR, PR and/or GR to homo- and/or heterodimerise on the chromatin, as discussed previously, compared to native ligands of the nuclear receptos in isolation. Since the effects observed in this thesis have demonstrated that MPA can impede and in some instances abrogate DHT-induced AR-signalling and increase breast epithelial proliferation, dimerisation studies could be undertaken using co-immunopreciptation, non-denaturing PAGE and/or Fluorescence resonance energy transfer (FRET)-based experimental techniques to detect the following protein-protein interactions: 1) AR bound to DHT and PR bound to MPA and/or 2) AR bound to DHT and GR bound to MPA, compared to the native ligands of PR and GR, progesterone and cortisol, respectively.

There are a number of technical difficulties in determining protein-protein interactions between AR bound to DHT and AR bound to MPA using these experimental techniques. Co-immunopreciptation assays to demonstrate AR dimerisation by DHT and MPA

combined, compared to individual treatments, is confounded by the pull-down of both AR bound to DHT and AR bound to MPA using a specific AR antibody, yet specific dimerisation partnerships of the AR can not be determined. In order to undertake these studies it may be possible to perform co-immunoprecipitation studies using tagged ligands, and pulling down for specific ligands, rather than for the using antibodies to pull down for the steroid receptor. The use of non-denaturing PAGE gels may be useful since they maintain native protein conformation, and protein-protein interactions in the cell. However, similarities in the size of AR homodimers differentially bound by ligand is likely to interfere with the differentiation of various forms (i.e. AR homodimers by DHT only, AR homodimers by MPA only and AR homodimers by DHT and MPA combined). Another alternative experimental approach may be to use FRET-based techniques which allow for the detection of receptors in close proximity to each other to be determined using labeled flurophores by fluorescent microscopy. However, this technique is still associated with confounding issues which include the inability to determine whether two different AR bound to either DHT or MPA forming a homodimer or just located in close proximity to each other in the cell.

9.1.5.3 - Co-contribution of PR and GR in alternative actions of DHT and MPA combined, compared to individual treatments

To delineate whether the effects reported in this thesis by the hormone combination occur specifically by alternative actions of the AR, independently of PR and/or GR, siRNA knock-down studies and/or steroid receptor antagonists studies followed by hormone treatment and subsequent qRT-PCR and immunoblot analysis of specific candidate genes

in breast explant tissue and breast cancer cell lines are required. Additionally, it has been illustrated in the work presented in this thesis, that there may be a considerable number of genes which can be dual regulated by AR via treatment with DHT or MPA-mediated effects and PR by MPA-mediated effects (i.e. FKBP5) *(refer to Chapter 8 - section 8.3.4).* As a result, the effect of the hormone combination on the expression of these types of genes may be confounded by the presence of PR. It would also be worthwhile undertaking these types of studies using the receptor antagonists including, RU486 (a dual GR and PR antagonist) and Org31710 (a pure PR antagonist) to differentiate between GR and PR contributions. These studies are relevant to both non-malignant primary tissues and the malignant breast cell line, ZR-75-1, since studies investigating the role of GR and PR in normal breast tissue and the ZR-75-1 cells has been documented in the literature (Poulin, Baker et al. 1991; Lien, Lu et al. 2006; Lange 2008; Moutsatsou and Papavassiliou 2008).

Lastly, it may be useful to undertake studies using breast cancer cell lines that present with different steroid receptor profiles, to underpin specific effects of DHT and MPA on AR activity, independent of confounding effects from other steroid receptors (i.e. MDA-MD-453 cells express AR and GR) (Wilson, Bobseine et al. 2002). These studies may be informative in relation to potential effects of DHT and MPA on pre-existing malignant lesions which possess different steroid receptor expression profiles. Interestingly, some breast cancer cells including MCF-7 and the MD-453 cells that only express AR have been shown to be growth stimulated, rather than inhibited, by androgens (Birrell, Bentel et al. 1995). Additionally, the use of AR antagonists is currently in Phase II trial to treat

ER^-PR^- advanced breast cancer *(Memorial Sloan-Kettering Cancer Centre/NY, USA - A Phase II study of bicalutamide for the treatment of androgen-receptor-positive, estrogen- and progesterone-receptor negative metastatic breast cancer).*

9.1.5.4 - Detection of AR protein from cultured breast explant tissue by immunoblot analysis

In order to validate the experimental findings reporting an abrogation in DHT-induced AR-signalling by MPA, preliminary experiments were performed to detect protein obtained from hormone-treated breast explant tissue by immunoblot analysis. The studies were aimed at detecting changes to AR protein specifically in breast epithelial cells, by an alternative means to IHC. However, due to high degradation of the protein during the protein extraction process, more optimisation experiments are required to progress with these studies *(data not shown).* A number of experimental optimisations that are essential include: 1) a shorter digestion period and addition of hormone to prevent protein degradation during digestion; 2) increased concentration of collagenase and 3) increased mincing of breast explant tissue samples prior to enzymatic digestion to allow for a shorter digestion time. The elimination of stromal cells from the protein sample is optimal in undertaking these experiments since the effects of DHT and/or MPA on AR-signalling and breast epithelial proliferation was specifically identified in the breast epithelial cells in both non-malignant and malignant breast cells in the studies undertaken in this thesis. However, it would be worthwhile undertaking these immunoblot analyses also using liquid nitrogen snap frozen pieces of cultured breast tissues via homogenisation using a tissue disruptor or by a mortal and pestle.

9.1.5.5 - Changes to steroid hormone synthesis and metabolism

The studies in this thesis have provided preliminary evidence that there is a reduction in AR protein following long-term exposure (i.e. 8 days) of ZR-75-1 breast cancer cells, to DHT and MPA combined, compared to DHT alone. Thus, additional experiments investigating changes to hormone synthesis and metabolism (i.e. expression of steroid synthesis and metabolising enzymes) would also be informative in identifying a mechanistic basis associated with the co-treatment of DHT and MPA and the promotion of breast cancer. Previous studies have shown that MPA is used for psychological behavioral conditions including aggression, sexual-related conditions (i.e. paedophilia), by its anti-gonadotrophic and anti-androgenic actions in male primates and men, by the decrease in LH production in the pituitary gland (Cordoba and Chapel 1983; Kiersch 1990; Michael, Bonsall et al. 1991). Interestingly, in male primates, treatment with MPA to suppress sexual activity led to a marked reduction in both DHT and T via the reduction in LH production from the pituitary gland, although circulating oestradiol levels were unaffected within the brain tissue (Michael, Bonsall et al. 1991). Furthermore, T and DHT levels were reduced in the male reproductive tissues, including seminal vesicles, prostate and penis (Michael, Bonsall et al. 1991).

In a separate study, treatment with MPA to male primates and men led to a reduction in circulating T levels, similar to levels in females and castrate levels (Gordon, Southren et al. 1970; Cordoba and Chapel 1983; Michael, Bonsall et al. 1991). Similar actions of progesterone are not evident, indicating that these biological effects of MPA may be distinct from those PR-mediated, and in fact may be AR-mediated (Rothchild 1965).

Furthermore, the differences in effects of MPA compared to progesterone may be associated with the high rates of progesterone metabolism, whereas MPA is not metabolised (Africander, Verhoog et al. 2011). Nonetheless, progesterone also does possess anti-androgenic activity by reducing 5α-reductase activity, by competitive binding to the enzyme (Africander, Verhoog et al. 2011). Moreover, it should be considered that the reduction in circulating levels of DHT by progesterone does not eliminate the effects of T via the AR, which may regulate similar DHT-regulated genes but to a lower extent based on the lower ligand binding affinity, and regulate a sub-set of unique genes. Additionally, whereas MPA is reported to possess ligand binding affinity for the AR, progesterone possess low to no ligand binding affinity for the AR (Kuhl 2005). The anti-androgenic effects of progesterone occur by steroid hormone production that are likely to be a part of a naturally evolved negative feedback pathway in human physiology, whereas the effects of MPA reported in this chapter are proposed to occur by alteration in AR protein activity. Thus, the anti-androgenic actions of MPA and progesterone are potentially vastly different and comparisons should be avoided.

9.1.6 - Summary and conclusion

The findings in this thesis have provided previously unreported biological evidence of a molecular mechanistic basis of MPA in conjunction with DHT associated with the promotion of breast cancer, mediated potentially by the AR. Importantly, the findings described in this thesis have indicated a combinatorial effect of DHT and MPA in stimulating alternative biological actions in breast epithelial cells, compared to the hormones in isolation. In light of the current scientific knowledge and understanding of

hormonally regulated normal breast tissue biology and breast cancer, the findings in this thesis give further insight into the complexity of hormonal cellular effects, and how changes to the structure of an endogenous hormone can lead to altered cell biology. Additionally, these studies have given further evidence to support the concept of a protective role for AR in breast cancer, and have given insight into key molecular pathways that may be de-regulated in the carcinogenic process.

The biological actions and specificity of native ligands for sex nuclear receptos is finely tuned to allow for an orchestrated interplay within various hormone responsive tissue organs in the body. These biological responses are required and have therefore evolved to allow for human and animal reproduction, and the continuation of life. However, based on the requirement of hormones for reproduction, a brake and acceleration type biological system is required, to allow for hormonally responsive cells to respond accordingly to reproductive signals. Thus, disruption of an evolved balanced biological system which involves an orchestrated interplay of cross-talk between different sex steroid nuclear receptor by the addition of synthetic hormones or chemicals that can act as endocrine disruptors (Shanle and Xu; Soto and Sonnenschein; Hess-Wilson and Knudsen 2006), leading to the loss of a naturally evolved biological system are likely to detrimental to normal hormonal homeostasis.

In order to treat specific hormonal health related conditions using synthetic compounds that mimic endogenous hormones in an identical manner is difficult based on differential ligand binding to alternative steroid receptors of synthetic compounds. The potent

progestagenic actions of synthetic progestins are beneficial and effective in the reduction of oestrogen induced endometrial proliferation, thus acting to reduce rates of endometrial cancer in post-menopausal women taking cHRT (Pike, Peters et al. 1997). However, the non-progestagenic actions can be detrimental and interfere with AR-mediated actions in the presence of its native ligand DHT in breast tissue. Whether similar anti-androgenic AR-mediated actions of MPA can also occur in other hormonally responsive tissues (i.e. bone and endometrium), as shown in this thesis in breast tissue requires further investigation. However, it could be speculated from the findings in this thesis that in some cases, the anti-androgenic actions of MPA may be beneficial, for instance when increased AR-signalling is detrimental (i.e. treatment of prostate cancer) (Tomic, Ljungberg et al. 1988; Anderstrom, Eddeland et al. 1995; Di Leo, Bajetta et al. 1995).

This thesis highlights the complexity of hormonal activity in breast tissue, and the ongoing requirement of studies examining the mechanistic basis linked to the carcinogenic actions of synthetic progestins used frequently in cHRT. From these studies, improved practices in the use of cHRT and administration of alternative formulations that are not associated with a risk of breast cancer can be implemented. The findings in this thesis may suggest that other synthetic progestins that also possess androgenic activity may promote similar carcinogenic actions as MPA and are therefore not safe for use in cHRT. The development of highly sophisticated screening methods that can detect occult early stage pre-malignant breast lesions, currently undetectable by mammography screening techniques, are required to screen women prior to use of cHRT to eliminate potentially high risk women in the population.

In conclusion, this thesis has now provided novel findings associated with the actions of MPA in conjunction with DHT and the promotion of breast cancer involving the AR-signalling axis. The findings in this thesis have provided a framework for future studies to gain an improved understanding of the mechanistic nature associated with MPA and other synthetic progestins used in cHRT that can lead to breast cancer and insights into potential alterations to the AR-signalling axis that may occur in early stages of malignancy. Additionally, the studies in this thesis have highlighted potential changes to androgen responsiveness in regulating breast epithelial proliferation in parous pre- compared to post-menopausal breast tissue and changes to AR expression that may underpin mechanisms associated with an increased frequency of breast cancer in post-menopausal women. In final conclusion the AR-signalling axis is fundamentally essential for maintaining normal breast hormonal homeostasis and alteration to its activity by MPA may be highly detrimental to promoting breast carcinogenesis.

Appendix 1

Appendix 1 - Table 1: Prescription rates in conjugated and non-conjugated oestrogens prescribed Australia before and after the cHRT arm findings of the WHI clinical trial (effective of April 2009).

Changes in prescription rates of conjugated and non-conjugated oestrogens available in Australia on the PBS before and after the cHRT arm of the WHI clinical trial[1]						
		Pre WHI		Post WHI		Pre and Post WHI
Item ID	HRT Preparations	# of prescriptions	% change	# of prescriptions	% change	# of prescriptions
	CEE	**1992-2001**		**2001-2004**		**1992-2004**
1733F	Oestradiol-conjugated (0.3 mg)	162,170 - 257,623	59% ↑	257,623 - 169,080	52% ↓	2,627,993
1734G	Oestradiol-conjugated(0.625 mg)	454,309 - 678,067	50% ↑	678,067 - 367,138	85% ↓	6,923,962
	Non-conjugated Oestrogens	**1992-2001**		**2001-2004**		**1992-2004**
1778N	Generol (1.25 mg)	136,618 - 191,819	40% ↑	191,819 - 107,962	78% ↓	2,100,272
1777M	Generol (0.625 mg)	112,488 - 145,560	30% ↑	145,560 - 77,480	88% ↓	1,547,073
1664N	Oestradiol Valerate (2 mg)	63,239 - 83,894	32% ↑	83,894 - 52,313	60% ↓	858,848
1663M	Oestradiol Valerate(1 mg)	47,346 - 51,849	9% ↑	51,849 - 35,430	46% ↓	560,320
		1995-2001		**2001-2004**		**1992-2004**
1771F	Oestriol (0.5 mg)	3,739 - 4,768	>1000%↑	45,768 - 44,046	3% ↓	282,778
		1998-2001		**2001-2004**		**1992-2004**
8274L	Oestradiol (2 mg)	49 - 2,235	>1000%↑	2,235 - 2,217	<1%↓	9,011

[1]Total number of different types of conjugated and non-conjugated oestrogen prescriptions available on the PBS in Australia represented at yearly intervals between January 1992 and December 2004. The graph depicts the two different time periods used to compare changes in prescription rates of conjugated and non-conjugated oestrogen prescriptions in Australia (i.e. 1992-2001 before and 2001-2004 after the WHI clinical trial in July 2002 (Rossouw, Anderson et al. 2002). The number of prescriptions of the various types of HRT preparations was drawn via the Australian Government Medicare Australia (AGMA), Pharmaceutical Benefits Scheme Statistics (PBSS) database *(https://www.medicareaustralia.gov.au/statistics/pbs_item.shtml)*.

Appendix 1 - Table 2: Prescription rates of synthetic progestins available on the PBS in Australia before and after the cHRT arm findings of the WHI clinical trial (effective of April 2009).

Changes in prescription rates of synthetic progestins available in Australia on the PBS before and after the cHRT arm of the WHI clinical trial[1]						
		Pre WHI		Post WHI		Pre and Post WHI
		# of prescriptions	% change	# of prescriptions	% change	# of prescriptions
Item ID	Synthetic Progestins	1993-2001		2001-2004		1993-2004
2322G	MPA 5 mg (2 x 30 tablets)	83,632 - 437,504	132% ↑	43,7504 - 122,373	166% ↓	3,577,387
		1992-2001		2001-2004		1993-2004
2321E	MPA 10 mg (2 x 30 tablets)	373,150 - 70,724	428% ↓	70,724 - 39,943	77% ↓	2,291,116
2722G	MPA 10 mg (2 x 100 tablets)	73,181 - 23,087	217% ↓	23,087 - 20,964	10% ↓	569,138
2993M	NETA (5 mg)	149,384 - 278,397	86% ↑	278,397 - 201,066	38% ↓	2,680,363
1350C	Dydrogesterone (10 mg)	9,031 - 10,890	20% ↑	10,890 - 6,936	57% ↓	82,577

[1]Total number of different types of synthetic progestin prescriptions available on the PBS in Australia represented at yearly intervals between January 1992 and December 2004. The graph depicts the two different time periods used to compare changes in prescription rates of synthetic progestin prescriptions in Australia (i.e. 1992-2001 before and 2001-2004 after the WHI clinical trial in July 2002 (Rossouw, Anderson et al. 2002). The number of prescriptions of the various types of synthetic progestin was drawn via the Australian Government Medicare Australia (AGMA), Pharmaceutical Benefits Scheme Statistics (PBSS) database *(https://www.medicareaustralia.gov.au/statistics/pbs_item.shtml).*

Appendix 1 - Table 3: Prescription rates of cHRT preparations containing MPA available on the PBS in Australia before and after the cHRT arm findings of the WHI clinical trial (effective of April 2009).

Changes in prescription rates of MPA-containing cHRT preparations containing MPA in Australia on the PBS before and after the cHRT arm of the WHI clinical trial[1]						
		Pre WHI		Post WHI		Pre and Post WHI
		# of prescriptions	% change	# of prescriptions	% change	# of prescriptions
Item ID	cHRT Formulations MPA-comprised	1997-2001		2001-2004		1993-2004
8169Y	Oestradiol conjugated (0.625 mg) + MPA (5 mg)	8,529 - 367,469	43% ↑	367,469 - 220,290	67% ↓	1,456,219
8168X	Oestradiol conjugated (0.625 mg) + MPA (2.5 mg)	2,314 - 89,952	38% ↑	89,952 - 64,706	40% ↓	378,650
	Dydrogesterone-comprised	**1998 - 2001**		**2001- 2004**		**1998-2004**
8244X	Oestradiol (2 mg) + Dydrogesterone(10mg)	3 - 3,666	>1000% ↑	2,666 - 2,560	4% ↓	14,018
	NETA-comprised	**1996- 2001**		**2001- 2004**		**1992-2004**
8081H	Oestradiol (2 mg) + NETA (2 mg - 1 mg)	2,100 - 134,282	>1000% ↑	134,282 - 80,294	67% ↓	663,304
		1992- 2001		**2001- 2004**		**1992-2004**
1764W	Oestradiol (2 mg) + NETA (1 mg)	496 - 89,601	>1000% ↑	89,601 - 51,798	72% ↓	558,447
		1999- 2001		**2001- 2004**		**1999-2004**
8353P	Oestradiol (1mg) + NETA (0.5mg)	2,033 - 72,811	> 1000% ↑	72,811 - 82,272	12% ↑	282,736

[1]Total number of different types of cHRT prescriptions available on the PBS in Australia represented at yearly intervals between January 1992 and December 2004. The graph depicts the two different time periods used to compare changes in prescription rates of cHRT prescriptions in Australia (i.e. 1992-2001 before and 2001-2004 after the WHI clinical trial in July 2002 (Rossouw, Anderson et al. 2002). The number of prescriptions of the various types of cHRT was drawn via the Australian Government Medicare Australia (AGMA), Pharmaceutical Benefits Scheme Statistics (PBSS) database *(https://www.medicareaustralia.gov.au/statistics/pbs_item.shtml).*

Appendix 2

Appendix 2 - Table 1 (A and B): (A) Raw data following VIA quantification of AR immunoreactivity in all cultured post-menopausal breast explant tissues and **(B)** non-malignant tissues either non-associated or adjacent to malignant tissue.

Table 1A: Quantification of AR immunoreactivity in human post-menopausal breast explant tissues cultured for 24 hrs (D1) (n=10) and 48 hrs (D2) (n=11)[1]						
VIA	**Control**	**1 μM Bic**	**1 nM DHT**	**1 μM Bic + 1 nM DHT**	**1 nM MPA**	**1 nM DHT + 1 nM MPA**
MIOD (D1)	**10.59 (10.57 ± 4.81)**	**8.55 (7.89 ± 2.89)**	**12.06 (11.04 ± 3.09)**	**7.02 (7.35 ± 5.34)**	**13.69 (13.24 ± 5.46)**	**9.55 (9.49 ± 3.10)**
% positivity (D1)	**23.59 (25.84 ± 13.88)**	**24.55 (24.11 ± 8.13)**	**26.18 (26.80 ± 9.16)**	**20.07 (25.09 ± 12.56)**	**40.58 (37.51 ± 18.38)**	**25.23 (22.66 ± 6.17)**
MIOD (D2)	**9.18 (9.15 ± 3.26)**	**9.82 (11.34 ± 5.19)**	**11.34 (13.85 ± 5.57)**	**10.34 (9.82 ± 1.64)**	**13.68 (12.43 ± 5.31)**	**11.35 (11.16 ± 2.98)**
% positivity (D2)	**23.4 (24.08 ± 6.87)**	**33.68 (30.87 ± 13.98)**	**31.30 (35.20 ± 13.62)**	**29.35 (25.26 ± 12.19)**	**34.35 (30.65 ± 12.28)**	**26.32 (27.97 ± 7.14)**
Table 1B: Quantification of AR immunoreactivity in human cultured for 48 hrs post-menopausal breast non-associated (NA) (n=6) and adjacent (A) (n=5) to malignancy[1]						
VIA	**Control**	**1 μM Bic**	**1 nM DHT**	**1 μM Bic + 1 nM DHT**	**1 nM MPA**	**1 nM DHT + 1 nM MPA**
MIOD (NA)	**8.52 (9.35 ± 3.44)**	**8.17 (9.23 ± 3.94)**	**12.12 (14.86 ± 7.24)**	**5.49 (7.15 ± 4.19)**	**8.97 (10.90 ± 6.73)**	**10.22 (10.90 ± 3.89)**
% positivity (NA)	**22.01 (23.24 ± 2.70)**	**19.54 (24.28 ± 11.40)**	**33.34 (36.78 ± 16.71)**	**12.74 (19.14 ± 12.02)**	**30.64 (30.97 ± 14.75)**	**33.41 (31.90 ± 13.46)**
MIOD (A)	**9.18 (8.90 ± 3.40)**	**14.17 (14.69 ± 5.44)**	**10.84 (12.63 ± 2.92)**	**13.71 (13.16 ± 4.66)**	**14.22 (14.27 ± 2.47)**	**11.35 (11.46 ± 1.75)**
% positivity (A)	**30.11 (25.08 ± 7.80)**	**36.78 (38.85 ± 13.97)**	**29.27 (33.31 ± 10.33)**	**32.89 (32.91 ± 7.94)**	**28.47 (33.36 ± 15.26)**	**40.78 (36.04 ± 8.21)**

[1] AR immunoreactivity in non-malignant human post-menopausal breast explant from cultured breast tissues obtained from all patients combined was determined using the VIA % positivity and MIOD measurements at 24 (D1) (n=10) and 48 hrs (D2) (n=11) or either non-associated (NA) (n=6) or adjacent (A) (n=5) to malignant disease at 48 hrs (D2). Data is represented as median values (average ± STDEV) of all fields captured per post-menopausal patient tissues (n=12). Two data patient sample was excluded from the D1 time-point and one from the D2 time-point d due to the lack of glands within the tissue samples.

Appendix 2 - Table 2: Raw data following VIA quantification of PSA immunoreactivity in cultured non-malignant post-menopausal breast explant tissues either non-associated or adjacent to malignant tissue.

Table 2: Quantification of PSA immunoreactivity in human post-menopausal breast non-associated (NA) (n=3) and associated (A) (n=3) with malignancy explant tissues cultured for 48 hrs (D2)[1]				
VIA	**Control**	**1 µM Bic**	**1 nM DHT**	**1 µM Bic + 1 nM DHT**
MIOD (NA)	**0.28 (0.23 ± 0.17)**	**0.38 (0.45 ± 0.14)**	**0.62 (1.41 ± 1.50)**	**0.23 (0.52 ± 0.57)**
% positivity (NA)	**1.42 (1.72 ± 1.24)**	**1.82 (3.08 ± 2.73)**	**3.40 (4.27 ± 3.31)**	**1.18 (2.03 ± 2.41)**
MIOD (A)	**0.28 (0.94 ± 1.29)**	**0.66 (0.48 ± 0.73)**	**0.78 (0.55 ± 0.73)**	**0.43 (0.43 ± 0.16)**
% positivity (A)	**6.70 (7.78 ± 2.76)**	**2.92 (4.67 ± 0.27)**	**5.14 (4.53 ± 3.70)**	**7.93 (4.65 ± 7.38)**

[1] PSA immunoreactivity in non-malignant human post-menopausal breast explant from cultured breast tissues either non-associated (n=3) or associated (n=3) with malignant disease was determined using the VIA % positivity and MIOD measurements at 48 hrs. Data is represented as median values (average ± STDEV) of all fields captured per post-menopausal patient tissues. One data patient sample was excluded from the D2 time-point due to the lack of glands within the tissue samples and three patient samples were not included in the analysis due to low to absent PSA immunoreactivity.

Appendix 2 - Table 3 (A and B): (A) Raw data following VIA quantification of Ki67 immunoreactivity in all cultured post-menopausal breast explant tissues and **(B)** non-malignant tissues either non-associated or adjacent to malignant tissue.

Table 3A: Quantification of Ki67 immunoreactivity in human post-menopausal breast explant tissues cultured for 24 hrs (D1) and 48 hrs (D2) (n=12)[1]						
VIA	**Control**	**1 μM Bic**	**1 nM DHT**	**1 μM Bic + 1 nM DHT**	**1 nM MPA**	**1 nM DHT + 1 nM MPA**
MIOD (D1)	**2.67 (4.33 ± 5.01)**	**3.73 (6.68 ± 7.28)**	**2.35 (4.96 ± 5.41)**	**4.83 (5.85 ± 25.25)**	**4.26 (4.61 ± 3.00)**	**4.23 (4.80 ± 2.35)**
% positivity (D1)	**2.81 (4.81 ± 5.84)**	**5.44 (7.73 ± 8.49)**	**2.33 (4.85 ± 6.27)**	**3.48 (5.70 ± 6.54)**	**4.27 (4.85 ± 3.92)**	**3.21 (4.21 ± 3.24)**
MIOD (D2)	**2.56 (4.57 ± 5.05)**	**3.70 (4.23 ± 2.90)**	**2.08 (2.44 ± 1.13)**	**4.94 (4.59 ± 3.47)**	**2.36 (3.70 ± 3.15)**	**5.05 (7.96 ± 6.53)**
% positivity (D2)	**2.67 (5.19 ± 5.72)**	**4.14 (4.21 ± 3.37)**	**1.99 (2.73 ± 2.67)**	**3.20 (4.95 ± 3.76)**	**2.92 (4.48 ± 5.06)**	**5.79 (10.06 ± 8.23)**
Table 3B: Quantification of Ki67 immunoreactivity in human cultured for 48 hrs post-menopausal breast non-associated (NA) (n=6) and adjacent (A) (n=5) to malignancy[1]						
VIA	**Control**	**1 μM Bic**	**1 nM DHT**	**1 μM Bic + 1 nM DHT**	**1 nM MPA**	**1 nM DHT + 1 nM MPA**
MIOD (NA)	**3.31 (6.24 ± 5.58)**	**3.62 (4.60 ± 3.56)**	**1.67 (2.71 ± 3.27)**	**1.48 (3.48 ± 3.18)**	**1.93 (3.10 ± 6.75)**	**5.49 (8.21 ± 8.64)**
% positivity (NA)	**6.44 (8.15 ± 8.12)**	**2.87 (4.30 ± 4.75)**	**1.74 (3.03 ± 3.85)**	**5.51 (4.97 ± 3.84)**	**3.31 (6.24 ± 5.58)**	**5.05 (6.20 ± 7.53)**
MIOD (A)	**1.41 (2.90 ± 2.66)**	**4.41 (3.77 ± 2.23)**	**1.89 (1.79 ± 0.60)**	**6.17 (5.98 ± 3.73)**	**4.29 (3.21 ± 1.67)**	**4.40 (7.48 ± 5.92)**
% positivity (A)	**1.96 (3.19 ± 3.11)**	**5.24 (4.43 ± 2.65)**	**1.55 (1.79 ± 0.84)**	**5.41 (5.93 ± 4.04)**	**2.92 (3.10 ± 1.65)**	**5.79 (9.94 ± 8.72)**

[1] Ki67 immunoreactivity in non-malignant human post-menopausal breast explant from cultured breast tissues obtained from all patients combined was determined using the VIA % positivity and MIOD measurements at 24 (D1) (n=10) and 48 hrs (D2) (n=11) or either non-associated (NA) (n=6) or adjacent (A) (n=5) to malignant disease at 48 hrs (D2). Data is represented as median values (average ± STDEV) of all fields captured per post-menopausal patient tissues (n=12). Two data patient sample was excluded from the D1 time-point and one from the D2 time-point due to the lack of glands within the tissue samples.

Appendix 2 - Table 4: Raw data following VIA quantification of Ki67 immunoreactivity in cultured pre-menopausal breast explant tissues.

Table 4: Quantification of Ki67 immunoreactivity in human pre-menopausal breast explant tissues cultured for 24 hrs (D1) (n=5) and 48 hrs (D2) (n=6))[1]				
VIA	**Control**	**1 nM DHT**	**1 nM MPA**	**1 nM DHT + 1 nM MPA**
MIOD (D1)	**1.41 (2.85 ± 3.22)**	**1.52 (2.45 ± 2.28)**	**1.69 (2.19 ± 1.63)**	**2.04 (2.35 ± 1.70)**
% positivity (D1)	**1.07 (2.92 ± 3.86)**	**1.55 (2.33 ± 2.22)**	**1.55 (2.02 ± 1.54)**	**1.94 (2.16 ± 1.61)**
MIOD (D2)	**1.10 (1.42 ± 1.37)**	**1.20 (2.46 ± 2.44)**	**1.17 (2.14 ± 2.52)**	**1.32 (2.03 ± 2.55)**
% positivity (D2)	**1.22 (1.62 ± 1.42)**	**1.23 (2.49 ± 2.41)**	**1.33 (2.20 ± 2.44)**	**1.62 (2.13 ± 2.58)**

[1] Ki67 immunoreactivity in non-malignant human pre-menopausal breast explant from cultured breast tissues obtained from all patients combined was determined using the VIA % positivity and MIOD measurements at 24 (D1) (n=5) and 48 hrs (D2) (n=6). Data is represented as median values (average ± STDEV) of all fields captured per post-menopausal patient tissues (n=6). Statistical analysis was performed using non-parametric Wilcoxon paired matched test. Statistical significance accepted $p<0.05$. One data patient sample was excluded from the D1 time-point due to the lack of glands within the tissue samples.

Appendix 2 - Table 5: Raw data following VIA quantification of bcl-2 immunoreactivity in cultured post-menopausal breast explant tissues and **(B)** non-malignant tissues either non-associated or adjacent to malignant tissue.

Table 5: Quantification of bcl-2 immunoreactivity in human post-menopausal breast explant tissues cultured for 24 hrs (D1) (n=10) and 48 hrs (D2) (n=11)[1]						
VIA	**Control**	**1 µM Bic**	**1 nM DHT**	**1 µM Bic + 1 nM DHT**	**1 nM MPA**	**1 nM DHT + 1 nM MPA**
MIOD (D1)	**3.29 (3.14 ± 2.16)**	**2.86 (2.97 ± 2.31)**	**3.94 (3.57 ± 1.77)**	**3.37 (4.36 ± 3.43)**	**4.46 (4.87 ± 3.00)**	**2.85 (3.13 ± 2.86)**
% positivity (D1)	**13.31 (14.81 ± 14.41)**	**7.15 (12.73 ± 12.79)**	**17.95 (17.18 ± 9.24)**	**13.74 (16.98 ± 18.49)**	**22.87 (26.50 ± 17.33)**	**16.24 (14.12 ± 12.71)**
MIOD (D2)	**2.52 (3.83 ± 3.67)**	**2.24 (3.04 ± 2.79)**	**1.80 (2.92 ± 2.37)**	**4.75 (4.81 ± 3.43)**	**3.28 (2.87 ± 1.69)**	**2.98 (2.87 ± 1.90)**
% positivity (D2)	**11.44 (17.29 ± 19.33)**	**13.20 (15.70 ± 17.81)**	**11.17 (15.64 ± 13.27)**	**19.87 (23.14 ± 17.27)**	**15.82 (14.32 ± 9.75)**	**12.41 (15.25 ± 16.45)**

[1] Bcl-2 immunoreactivity in non-malignant human post-menopausal breast explant from cultured breast tissues obtained from all patients combined was determined using the VIA % positivity and MIOD measurements at 24 (D1) (n=10) and 48 hrs (D2) (n=11). Data is represented as median values (average ± STDEV) of all fields captured per post-menopausal patient tissues (n=12). Two data patient sample was excluded from the D1 time-point and one from the D2 time-point due to the lack of glands within the tissue samples.

Appendix 2 - Table 6: Raw data following VIA quantification of bcl-2 immunoreactivity in cultured pre-menopausal breast explant tissues.

Table 6: Quantification of bcl-2 immunoreactivity in human pre-menopausal breast explant tissues cultured for 24 hrs (D1) (n=5) and 48 hrs (D2) (n=6)[1]				
VIA	**Control**	**1 nM DHT**	**1 nM MPA**	**1 nM DHT + 1 nM MPA**
MIOD (D1)	**0.73 (1.29 ± 1.32)**	**3.52 (2.78 ± 1.65)**	**1.01 (1.51 ± 1.37)**	**3.88 (4.74 ± 1.74)**
% positivity (D1)	**1.75 (3.29 ± 3.40)**	**11.51 (11.23 ± 8.41)**	**2.51 (4.44 ± 5.84)**	**13.04 (13.71 ± 9.14)**
MIOD (D2)	**0.73 (1.33 ± 1.40)**	**0.70 (1.43 ± 1.28)**	**1.52 (1.91 ± 1.94)**	**1.46 (1.98 ± 1.34)**
% positivity (D2)	**2.65 (4.08 ± 4.97)**	**1.68 (4.59 ± 4.77)**	**6.37 (6.80 ± 8.04)**	**6.22 (7.59 ± 6.18)**

[1] Bcl-2 immunoreactivity in non-malignant human pre-menopausal breast explant from cultured breast tissues obtained from all patients combined was determined using the VIA % positivity and MIOD measurements at 24 (D1) (n=5) and 48 hrs (D2) (n=6). Data is represented as median values (average ± STDEV) of all fields captured per post-menopausal patient tissues (n=6). Statistical analysis was performed using non-parametric Wilcoxon paired matched test. Statistical significance accepted $p<0.05$. One data patient sample was excluded from the D1 time-point due to the lack of glands within the tissue samples.

Appendix 2 - Table 7: Raw data following VIA quantification of AR immunoreactivity in cultured non-malignant pre-menopausal breast explant tissues

Table 7: Quantification of AR immunoreactivity in human pre-menopausal breast explant tissues cultured for 24 hrs (D1) (n=5) and 48 hrs (D2) (n=6)[1]				
VIA	**Control**	**1 nM DHT**	**1 nM MPA**	**1 nM DHT + 1 nM MPA**
MIOD (D1)	**10.51 (10.48 ± 1.94)**	**16.03 (15.65 ± 3.14)**	**16.38 (14.95 ± 4.90)**	**9.98 (9.49 ± 1.77)**
% positivity (D1)	**23.72 (25.09 ± 15.71)**	**34.13 (35.80 ± 5.82)**	**34.75 (35.75 ± 14.96)**	**21.30 (22.12 ± 3.55)**
MIOD (D2)	**5.98 (5.84 ± 2.02)**	**10.42 (10.18 ± 3.46)**	**12.92 (13.81 ± 5.76)**	**8.53 (9.66 ± 5.69)**
% positivity (D2)	**14.02 (12.74 ± 4.59)**	**22.70 (23.14 ± 8.00)**	**30.94 (34.16 ± 15.49)**	**19.40 (19.77 ± 8.76)**

[1] AR immunoreactivity in non-malignant human pre-menopausal breast explant from cultured breast tissues obtained from all patients combined was determined using the VIA % positivity and MIOD measurements at 24 (D1) (n=5) and 48 hrs (D2) (n=6). Data is represented as median values (average ± STDEV) of all fields captured per post-menopausal patient tissues (n=6). Statistical analysis was performed using non-parametric Wilcoxon paired matched test. Statistical significance accepted $p<0.05$. One data patient sample was excluded from the D1 time-point due to the lack of glands within the tissue samples.

Appendix 2 - Table 8 (A and B): **(A)** Raw data following VIA quantification of ERα immunoreactivity in all cultured post-menopausal breast explant tissues or **(B)** non-associated or adjacent to malignant breast tissue.

Table 8A: Quantification of ERα immunoreactivity in human post-menopausal explant tissues cultured for 48 hrs (D2) (n=10)[1]				
VIA	**Control**	**1 nM DHT**	**1 nM MPA**	**1 nM DHT + 1 nM MPA**
MIOD	**2.71 (3.21 ± 2.19)**	**1.30 (1.73 ± 1.43)**	**1.56 (2.32 ± 2.21)**	**1.91 (3.14 ± 3.16)**
% positivity	**6.22 (8.81 ± 7.87**	**2.59 (4.51 ± 4.57**	**4.23 (6.60 ± 7.60)**	**3.53 (6.57 ± 6.59)**
Table 8B: Quantification of ERα immunoreactivity in human cultured for 48 hrs post-menopausal breast non-associated (NA) (n=5) and adjacent (A) (n=5) to malignancy[1]				
VIA	**Control**	**1 nM DHT**	**1 nM MPA**	**1 nM DHT + 1 nM MPA**
MIOD (NA)	**2.71 (2.34 ± 1.57)**	**1.13 (1.04 ± 0.47)**	**1.18 (1.46 ± 1.03)**	**2.43 (3.72 ± 4.15)**
% positivity (NA)	**5.23 (4.38 ± 2.79)**	**2.06 (1.82 ± 1.17)**	**2.82 (3.33 ± 2.59)**	**4.56 (6.14 ± 4.32)**
MIOD (A)	**3.29 (3.86 ± 2.57)**	**2.17 (2.29 ± 1.75)**	**1.66 (3.18 ± 2.84)**	**1.64 (2.56 ± 2.12)**
% positivity (A)	**9.79 (12.13 ± 9.18)**	**7.92 (7.66 ± 5.26)**	**6.3 (9.87 ± 9.82)**	**3.30 (6.99 ± 8.86)**

[1] ERα immunoreactivity in all non-malignant human breast explants from cultured post-menopausal breast tissue samples (n=10) or sub-grouped into tissue either non-associated (n=6) or adjacent (n=5) to malignant disease using the VIA MIOD and % positivity measurements at 48 hrs (D2). Data is represented as median values (average ± STDEV) of all fields captured per post-menopausal patient tissues. Two data patient samples were excluded from the D2 time-point due to a lack of glandular tissue in some tissue sections.

Appendix 2 - Table 9 (A and B): **(A)** Raw data following VIA quantification of PR immunoreactivity in all cultured post-menopausal breast explant tissues or **(B)** non-associated or adjacent to malignant breast tissue.

Table 9A: Quantification of PR immunoreactivity in human post-menopausal explant tissues cultured for 48 hrs (D2) (n=10)[1]					
VIA	**Control**	**1 nM DHT**	**1 nM MPA**	**1 nM DHT + 1 nM MPA**	**100 nM MPA**
MIOD	**5.02 (4.84 ± 4.22)**	**1.61 (3.04 ± 3.45)**	**2.33 (4.21 ± 5.51)**	**1.94 (3.20 ± 4.18)**	**0.42 (0.99 ± 1.17)**
% positivity	**6.83 (5.83 ± 4.88)**	**2.10 (4.24 ± 5.29)**	**1.47 (4.32 ± 6.85)**	**2.51 (4.41 ± 6.02)**	**1.02 (1.39 ± 1.45)**

Table 9B: Table 7.2: Quantification of PR immunoreactivity in human cultured for 48 hrs post-menopausal breast non-associated (NA) (n=5) and adjacent (A) (n=5) to malignancy[1]				
VIA	**Control**	**1 nM DHT**	**1 nM MPA**	**1 nM DHT + 1 nM MPA**
MIOD (NA)	**4.85 (4.29 ± 2.78)**	**2.25 (2.52 ± 1.31)**	**3.99 (3.75 ± 3.99)**	**2.19 (2.03 ± 1.89)**
% positivity (NA)	**6.76 (6.07 ± 5.25)**	**2.49 (3.41 ± 2.20)**	**0.87 (2.73 ± 2.96)**	**2.82 (2.77 ± 2.68)**
MIOD (A)	**5.22 (5.17 ± 5.20)**	**0.95 (3.45 ± 4.70)**	**1.73 (4.67 ± 7.33)**	**1.69 (4.36 ± 5.69)**
% positivity (A)	**6.90 (5.68 ± 5.28)**	**1.44 (4.91 ± 7.15)**	**1.75 (5.91 ± 9.52)**	**2.19 (6.05 ± 8.23)**

[1] PR immunoreactivity in all non-malignant human breast explants from cultured post-menopausal breast tissue samples (n=10) or sub-grouped into tissue either non-associated (n=6) or adjacent (n=5) to malignant disease using the VIA MIOD and % positivity measurements at 48 hrs (D2). Data is represented as median values (average ± STDEV) of all fields captured per post-menopausal patient tissues. Two data patient samples were excluded from the D2 time-point due to a lack of glandular tissue in some tissue sections.

Appendix 3

Appendix 3 - Table 1: Representative examples of candidate genes induced by DHT and abrogated by DHT and MPA combined

Genes induced by DHT and DHT and MPA combined, abrogated by MPA	Fold Induction			Functional Group
	DHT	MPA	DHT + MPA	
Chromosome 1 open reading frame 116 ***(c1ORF116)*** ***(Referred to also as specifically androgen- regulated gene protein (SARG))***	8.69*	1.95	5.18***	Unknown (Steketee, Ziel-van der Made et al. 2004)
Oxidised low density lipoprotein, (lectin-like) receptor 1 ***(OLR1)***	2.60*	-1.06	1.23***	Cell death (Parlato, Romagnoli et al.)
Genes uniquely induced by DHT alone, abrogated by MPA	**Fold Induction**			**Functional Group**
	DHT	**MPA**	**DHT + MPA**	
Cell Death				
Cytochrome P450, family 2, subfamily B, polypeptide 6 ***(CYP2B6)***	1.24*	1.10	-1.01**	Cell death (Thum and Borlak 2008)
Cellular Growth and Proliferation, Cell Cycle and Cancer				
Fibroblast growth factor receptor 2 ***(FGFR2)***	1.49*	-1.04	1.17**	Inhibitor of proliferation (Grose and Dickson 2005)
Others				
Transmembrane protein 45B ***(TMEM45B)***	1.17*	1.00	-1.06**	Unknown
Defensin, beta 130 ***(DEFB130)***	1.35*	1.26	1.12**	Unknown
Glycerol kinase 5 (putative) ***(GK5)***	1.16*	1.05	1.00**	Unknown
ST6 beta-galactosamide alpha-2,6-sialyltranferase 1 ***(ST6GAL1)***	1.17*	-1.19	-1.05**	Unknown
Fibronectin leucine rich transmembrane protein *3****(FLRT3)***	1.21*	1.01	-1.03**	Unknown
Zinc finger protein 385B ***(ZNF385B)***	1.35*	-1.01	1.08**	Inflammatory Disease

* $p<0.05$ DHT compared to control
** $p<0.05$ DHT + MPA compared to DHT
*** $p<0.05$ DHT + MPA compared to DHT and control

Appendix 3 - Table 2: Representative examples of candidate genes repressed by DHT and abrogated or impeded by DHT and MPA combined

Genes uniquely repressed by DHT alone, abrogated or impeded by MPA	Fold Induction			Functional Group
	DHT	MPA	DHT + MPA	
Cell Death				
Baculoviral IAP repeat-containing 6 ***(BIRC6)***	-1.18*	-1.10	-1.07	Inhibitor of apoptosis (Qiu, Markant et al. 2004)
Receptor-interacting serine-threonine kinase 1 ***(RIPK1)***	-1.17*	-1.07	-1.07	Inhibitor of apoptosis (Cheung, Mahoney et al. 2009)
Gene Expression				
Mitogen-activated protein kinase kinase kinase 15 ***(MAP3K1)***	-1.11*	-1.04	1.01**	Regulator of transcription(Schlesinger, Bonvin et al. 2002)
Janus kinase 1 ***(JAK1)***	-1.13*	-1.02	-1.01	Regulator of transcription (Shang, Baumrucker et al. 1999)
Basic helix-loop-helix family, member e41 (***BHLHE41)***	-1.23*	-1.02	1.06**	Regulator of transcription
TNF receptor-associated factor 5 ***(TRAF 5)***	-1.42*	-1.13	-1.10	Regulator of transcription (Au and Yeh 2007)
Cellular Growth and Proliferation				
TIMP metallopeptidase inhibitor *2* ***(TIMP2)***	-1.16*	-1.04	-1.06*	Positive regulator of proliferation (D'Alessio, Ferrari et al. 2008)
Forkhead box A3 ***(FOXA3)***	-1.24*	-1.01	1.03**	Positive regulator of proliferation(Li, Du et al. 2007)
SMAD family member 6 ***(SMAD6)***	-1.30*	-1.10	-1.00	Positive regulator of proliferation
Cell Cycle				
LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransfe ***(LFNG)***	-1.35*	1.06	-1.08**	Cell division
A kinase (PRKA) anchor protein 8 ***(AKAP8)***	-1.16*	1.01	1.02	Cell division process (Steen, Cubizolles et al. 2000)
Cancer				
Dual specificity phosphatase 4 ***(DUSP4)***	-1.24*	1.02	1.08**	Tumourigenesis
Angiomotin ***(AMOT)***	-1.17*	1.10	1.12**	Tumourigenesis (Almog, Ma et al. 2009)
IQ motif containing GTPase activating protein 1 ***(IQGAP1)***	-1.08*	1.02	-1.01**	Tumourigenesis (Jadeski, Mataraza et al. 2008)
T-box 3 (***TBX3)***	-1.26*	-1.09	-1.04**	Tumourigenesis (Yarosh, Barrientos et al. 2008)

* $p<0.05$ DHT compared to control
** $p<0.05$ DHT + MPA compared to DHT

Appendix 3 - Table 3: Representative examples of candidate genes uniquely induced by DHT and MPA combined

Genes uniquely induced by DHT and MPA combined	Fold Induction			Functional Group
	DHT	MPA	DHT + MPA	
Cell Death				
Cysteine-rich, angiogenic inducer, 61 ***(CYR61)***	1.02**	-1.04	1.48*	Regulator of cell growth, positive gene expression
Fibroblast growth factor receptor 1 ***(FGFR1)***	1.07	1.06	1.14*	Proliferation, outgrowth, positive gene expression and regulator of cell cycle
Gene Expression				
Nuclear factor I/A **(*NFIA*)**	-1.01**	-1.09	1.22*	Regulation of transcription
TNFAIP3 interacting protein 2 (***TNFAIP3)***	1.02**	1.02	1.49*	Regulation of transcription
Cellular Growth and Proliferation				
Chromosome 6 open reading frame 81 (***C6orf81)***	1.04**	-1.07	1.26*	Promotion of growth and proliferation
Forkhead box O3B pseudogene (***FOXO3B)***	-1.11**	1.02	1.17*	Promotion of growth and proliferation
Ubiquitin-conjugating enzyme E2D 3 (***UBE2D3)***	-1.00**	1.01	1.06*	Promotion of growth and proliferation
Cell Cycle				
Zinc finger homeobox 3 ***(ZFHX3)***	-1.00**	1.15	1.29*	Cell cycle progression
Inhibitor of DNA binding 3 ***(ID3)***	1.08	1.13	1.24*	Cell cycle progression
Ring finger protein 167 ***(RNF147)***	-1.03**	-1.02	1.14*	Cell cycle progression
Cancer				
Growth factor receptor-bound protein 7 (***GRB7)***	-1.09**	-1.04	1.31*	Cancer tumourigenesis
SID1 transmembrane family, member 2 (***SIDT2)***	-1.00**	-1.02	1.25*	Cancer tumourigenesis
tumour necrosis factor receptor superfamily ***(TNFRSF1A)***	1.00**	1.10	1.19*	Cancer tumourigenesis

* $p<0.05$ DHT + MPA compared to control
** $p<0.05$ DHT compared to DHT + MPA

Appendix 3 - Table 4: Representative examples of candidate genes uniquely repressed by DHT and MPA combined

Genes uniquely repressed by DHT and MPA combined	Fold Induction			Functional Group
	DHT	MPA	DHT + MPA	
Cell Death				
Tumour necrosis factor receptor superfamily, member 11a ***(TNFAF11A)***	-1.11**	-1.19	-1.35*	Apoptosis
Coagulation factor VII ***(F7)***	-1.07**	-1.14	-1.25*	Apoptosis
Gene Expression				
General transcription factor IIH, polypeptide 1 ***(GTF2H1)***	-1.01**	-1.06	-1.13*	Repression of gene transcription
Cellular Growth and Proliferation				
Growth differentiation factor 15 ***(GDF15)***	1.05**	-1.17	-1.24*	Arrest in Cell Proliferation
Cell Cycle				
Ribonucleotide reductase M1 ***(RRM1)***	-1.01**	1.03	-1.15*	Arrest in cell cycle
Aurora kinase A ***(AURKA)***	-1.00**	-1.11	-1.19*	Arrest in cell cycle
Cancer				
PMS2 postmeiotic segregation increased 2 ***(PMS2)***	-1.09**	-1.06	-1.16*	Latency of Tumourigenesis

* $p<0.05$ DHT + MPA compared to control

** $p<0.05$ DHT compared to DHT + MPA

Appendix 3 - Table 5: Overlap of genes inversely regulated by DHT alone and in combination with MPA

Overlap of genes inversely regulated by hormone treatments	Fold Induction			Functional Group
	DHT	MPA	DHT + MPA	
TBC1 domain family, member 8 (with GRAM domain) ***(TBC1D8)***	-1.21**	1.67*	2.32*	Metabolism (Hsu, Zillikens et al.)
20194	1.12**	-1.02	-1.15*	Unknown
Nuclear transcription factor, X-box binding 1 ***(NFX1)***	-1.10**	1.04	1.11*	Transcriptional repressor
Solute carrier family 6 ***(SLC6A6)***	-1.17**	2.17*	2.32*	Cell survival, detoxification (Ito, Fujio et al. 2006)

* $p<0.05$ DHT, MPA or DHT + MPA compared to control
** $p<0.05$ DHT compared to DHT + MPA and control

Appendix 4

Appendix 4 - Tables 1-10: Microarray gene lists

All hormone treatments 1 nM DHT and 1 nM MPA

Tables 1 (induced) and 2 (repressed) - Unique DHT regulated genes:
* Control Vs DHT ($p<0.05$)
* * DHT Vs DHT and MPA combined ($p<0.05$)

Tables 3 (induced) and 4 (repressed) - Unique MPA regulated genes:
* Control Vs MPA ($p<0.05$)
* * MPA Vs DHT and MPA combined ($p<0.05$)

Tables 5 (induced) and 6 (repressed) - Unique DHT and MPA regulated genes:
* DHT and MPA combined Vs control ($p<0.05$)
* * DHT and MPA combined Vs DHT ($p<0.05$)

Table 7 (induced) and 8 (repressed) - Genes regulated by all hormone treatments DHT and MPA alone and combined:
Note: All genes regulated by DHT and MPA alone and combined Table vii and viii were significantly different from control in Table vii and viii ($p<0.05$)
* DHT and MPA combined Vs DHT alone ($p<0.05$)

Table 9 - Genes significantly regulated (induced or repressed) by DHT and MPA alone and combined:
* Control Vs DHT or Control Vs MPA ($p<0.05$)
** DHT and MPA combined Vs DHT alone ($p<0.05$)
*** DHT and MPA combined Vs DHT and MPA alone ($p<0.05$)

Table 10 - Select genes significantly regulated by DHT and MPA combined compared to DHT or MPA alone:
* Control Vs DHT or Control Vs MPA ($p<0.05$)
** DHT and MPA combined Vs DHT alone or MPA alone ($p<0.05$)

Appendix 4 -Table 1: Unique DHT induced genes

Unique DHT induced genes		Accession Number	Fold Change		
Gene Symbol	Gene Name		1nM DHT	1nM MPA	1nM DHT 1nM MPA
FGFR2	fibroblast growth factor receptor 2	NM_000141	1.49*	-1.04	1.17**
HS3ST3B1	heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1	NM_006041	1.41	1.29	1.29
ZNF385Bα	zinc finger protein 385B	NM_152520	1.36*	-1.01	1.09**
DEFB130	defensin, beta 130	NM_001037804	1.35*	1.27	1.13**
Unknown	ncrna:snoRNA chromosome	ENST00000391310	1.35*	1.22	1.00**
RP5-1022P6.6	Homo sapiens hypothetical LOC149837	NR_015406	1.34*	-1.01	-1.01**
MXD4	MAX dimerization protein 4	NM_006454	1.34*	1.21	-1.03**
Unknown	Transcript ID: 7898405		1.32*	1.25	1.06
PFKFB2	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	NM_006212	1.29*	1.06	1.22
Unknown	Transcript ID: 8147746		1.28*	1.07	1.13
Unknown	ncrna:misc_RNA chromosome:GRCh37	ENST00000364694	1.27*	-1.06	-1.09**
C6orf145	chromosome 6 open reading frame 145	NM_183373	1.27*	1.11	1.21
UGT2B15	UDP glucuronosyltransferase 2 family, polypeptide B15	NM_001076	1.26*	1.11	1.14
ANUBL1	AN1, ubiquitin-like, homolog (Xenopus laevis)	NM_174890	1.25*	1.06	1.14
PPIAL4B	peptidylprolyl isomerase A (cyclophilin A)-like 4B	NM_001143883	1.25*	1.12	-1.08**
CNNM1	cyclin M1	NM_020348	1.24	-1.01	1.11
CYP2B6	cytochrome P450, family 2, subfamily B, polypeptide 6	NM_000767	1.24*	1.10	-1.01**
PRPS1	phosphoribosyl pyrophosphate synthetase 1-like 1	NM_175886	1.22*	-1.02	-1.01
LOC283767	golgi autoantigen, golgin subfamily a-like	NM_001001413	1.22*	1.10	1.07
FLRT3	fibronectin leucine rich transmembrane protein 3	NM_198391	1.22*	-1.02	-1.04**
BANK1	B-cell scaffold protein with ankyrin repeats 1	NM_017935	1.20*	1.07	1.16
Unknown	ncrna:misc_RNA chromosome	ENST00000411163	1.19*	1.12	1.07
OR13A1	olfactory receptor, family 13, subfamily A, member 1	NM_001004297	1.19*	1.12	1.07
HGD	distal-less homeobox 2	NM_004405	1.19*	-1.01	1.09
TMEM45B	transmembrane protein 45B	NM_138788	1.18*	1.00	-1.06**
Unknown	ncrna:misc_RNA chromosome	ENST00000365448	1.18*	1.08	1.12
ST6GAL1	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1	NM_173216	1.17*	1.13	-1.05**
CD1A	CD1a molecule	NM_001763	1.17*	1.03	-1.06**
GK5	glycerol kinase 5 (putative)	NM_001039547	1.16*	1.06	1.00**
C1orf158	chromosome 1 open reading frame 158	BC029894	1.16*	1.04	1.03
APOBEC4	apolipoprotein B mRNA editing enzyme, catalytic polypeptide	NM_203454	1.14*	1.04	-1.02
MEP1A	meprin A, alpha (PABA peptide hydrolase)	NM_005588	1.14*	1.04	1.08
TMEM62	transmembrane protein 62	NM_024956	1.14*	1.08	1.10
GPR18	G protein-coupled receptor 182	NM_007264	1.11*	1.01	1.04

Appendix 4 - Table 2: Unique DHT repressed genes

Unique DHT repressed genes		Accession Number	Fold Change		
Gene Symbol	Gene Name		1nM DHT	1nM MPA	1nM DHT 1nM MPA
IARS	isoleucyl-tRNA synthetase	NM_013417	-1.08*	-1.05	-1.05
MYOF	myoferlin	NM_013451	-1.08*	-1.01	-1.06
IQGAP1	IQ motif containing GTPase activating protein 1	NM_003870	-1.09*	1.02	-1.02**
CMTM4	CKLF-like MARVEL transmembrane domain containing 4	NM_181521	-1.10*	-1.02	-1.06
AATF	apoptosis antagonizing transcription factor	NM_012138	-1.10*	-1.00	-1.05
SPTBN5	spectrin, beta, non-erythrocytic 5	NM_016642	-1.11*	-1.09	-1.07
GFPT1	glutamine-fructose-6-phosphate transaminase 1	NM_002056	-1.12*	-1.07	-1.05
SLC35F2	solute carrier family 35, member F2	NM_017515	-1.12*	-1.06	-1.18
MAN1A2	mannosidase, alpha, class 1A, member 2	NM_006699	-1.12*	-1.10	-1.07
MAP3K1	mitogen-activated protein kinase kinase kinase 11	NM_002419	-1.12*	1.07	1.01**
SMG1	SMG1 homolog, phosphatidylinositol 3-kinase-related kinase	NM_015092	-1.12*	-1.10	-1.06
KIAA1467	KIAA1467	NM_020853	-1.13*	-1.04	-1.08
RAB27B	RAB27B, member RAS oncogene family	NM_004163	-1.13*	-1.05	-1.06
FEM1C	fem-1 homolog c (C. elegans)	NM_020177	-1.13*	1.01	-1.08
HGSNAT	heparan-alpha-glucosaminide N-acetyltransferase	NM_152419	-1.13*	-1.07	-1.08
KLHL20	kelch-like 20 (Drosophila)	NM_014458	-1.13*	-1.05	-1.07
JAK1	Janus kinase 1	NM_002227	-1.14*	-1.03	-1.01
DSP	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide	NM_005730	-1.15*	1.03	1.02**
STK35	serine/threonine kinase 35	NM_080836	-1.15*	-1.07	1.00**
TNFAIP2	tumour necrosis factor, alpha-induced protein 2	NM_006291	-1.15*	-1.09	-1.12
NOP56	NOP56 ribonucleoprotein homolog (yeast)	NR_027700	-1.15*	-1.08	-1.12
RAVER2	ribonucleoprotein, PTB-binding 2	NM_018211	-1.16*	1.01	-1.03
FAM20B	family with sequence similarity 20, member B	NM_014864	-1.16*	-1.05	-1.08
DNAJC13	DnaJ (Hsp40) homolog, subfamily C, member 13	NM_005858	-1.16*	-1.14	-1.10
AKAP8	A kinase (PRKA) anchor protein 8	NM_005858	-1.17*	1.01	1.02**
TIMP2	TIMP metallopeptidase inhibitor 2	NM_003255	-1.17*	-1.05	-1.07**
C8orf79	chromosome 8 open reading frame 79	NM_020844	-1.17*	-1.11	-1.07
ADCY9	adenylate cyclase 9	NM_001116	-1.17*	-1.02	-1.07
SAPS2	SAPS domain family, member 2	NM_014678	-1.17*	-1.06	-1.08
TMEM106A	transmembrane protein 106A	NM_145041	-1.17*	-1.11	-1.03
AMOT	angiomotin like 2	NM_016201	-1.18*	1.11	1.12**
RIPK1	receptor (TNFRSF)-interacting serine-threonine kinase 1	NM_003804	-1.18*	-1.07	-1.08
BIRC6	baculoviral IAP repeat-containing 6	NM_016252	-1.18*	-1.11	-1.08
ZBTB38	zinc finger and BTB domain containing 38	NM_001080412	-1.18*	-1.13	-1.13
SLC11A2	solute carrier family 11	NM_000617	-1.19*	-1.09	-1.15
PRKAA1	protein kinase, AMP-activated, alpha 1 catalytic subunit	NM_206907	-1.19*	-1.14	-1.08
SOS2	son of sevenless homolog 2 (Drosophila)	NM_006939	-1.20*	-1.09	-1.10
UTP20	UTP20, small subunit (SSU)	NM_014503	-1.20*	-1.12	-1.13

Gene Symbol	Unique DHT repressed genes Gene Name	Accession Number	Fold Change 1nM DHT	 1nM MPA	 1nM DHT 1nM MPA
SLC4A8	solute carrier family 4, sodium bicarbonate cotransporter	NM_001039960	-1.20*	1.00	-1.03**
PSMD5	proteasome (prosome, macropain) 26S subunit, non-ATPase, 5	NM_005047	-1.20*	-1.06	-1.11
GUCY1B3	guanylate cyclase 1, soluble, beta 3	NM_000857	-1.20*	-1.09	-1.14
MATN3	matrilin 3	NM_002381	-1.20*	-1.03	-1.12**
MAP3K9	mitogen-activated protein kinase kinase kinase 9	NM_033141	-1.20*	-1.11	-1.05
PDZRN3	PDZ domain containing ring finger 3	NM_015009	-1.20*	-1.05	-1.11
ANK3	pantothenate kinase 3	NM_024594	-1.21*	-1.01	-1.07
DLL1	delta-like 1 (Drosophila)	NM_005618	-1.21*	-1.13	-1.17
BCL2L11	BCL2-like 11 (apoptosis facilitator)	NM_138621	-1.21*	-1.06	-1.07
ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	NM_000392	-1.21*	-1.11	-1.08**
MAP3K5	mitogen-activated protein kinase kinase kinase 5	NM_005923	-1.21*	-1.12	-1.12
MYB	cyclin D binding myb-like transcription factor 1	NR_024549	-1.21*	-1.10	-1.19
SNTB1	syntrophin, beta 1 (dystrophin-associated protein A1	NM_021021	-1.21*	-1.11	-1.08
GPR157	G protein-coupled receptor 157	NM_024980	-1.21*	-1.07	-1.11**
ARHGAP21	Rho GTPase activating protein 21	NM_020824	-1.21*	-1.08	-1.12
KCNH1	potassium voltage-gated channel, subfamily H (eag-related)	NM_172362	-1.22*	-1.16	-1.19
PDZD2	PDZ domain containing 2	NM_178140	-1.22*	1.02	1.05**
ATP13A5	ATPase type 13A5	NM_198505	-1.23*	1.01	-1.08
OAS1	2',5'-oligoadenylate synthetase 1, 40	NM_016816	-1.23*	-1.01	-1.09
DTX4	deltex homolog 4 (Drosophila)	NM_015177	-1.23*	-1.09	-1.07
BHLHE41	basic helix-loop-helix family, member e41	NM_030762	-1.24*	-1.02	1.07**
BMF	Bcl2 modifying factor	NM_001003940	-1.24*	1.09	-1.01**
DUSP4	dual specificity phosphatase 4	NM_001394	-1.24*	1.03	1.08**
FOXA3	forkhead box A3	NM_004497	-1.24*	-1.02	1.04**
MEIS1	Meis homeobox 1	NM_002398	-1.25*	-1.10	-1.12
ARHGEF3	Rho guanine nucleotide exchange factor (GEF) 3	NM_001128615	-1.25*	-1.21	-1.19
ARL4D	ADP-ribosylation factor-like 4D	NM_001661	-1.25*	1.01	-1.16
TBX3	T-box 3	NM_016569	-1.26*	-1.10	-1.05**
HPX	hemopexin	NM_000613	-1.28*	-1.18	1.02**
PLCE1	phospholipase C, epsilon 1	NM_016341	-1.28*	1.00	-1.16
GPR81	G protein-coupled receptor 81	NM_032554	-1.28*	1.11	1.05**
KLF8	Kruppel-like factor 8	NM_007250	-1.28*	-1.13	-1.10
SIDT1	SID1 transmembrane family, member 1	NM_017699	-1.29*	-1.10	-1.14
SMAD6	SMAD family member 6	NR_027654	-1.30*	-1.10	-1.12
GPD1L	glycerol-3-phosphate dehydrogenase 1-like	NM_015141	-1.32*	1.04	-1.04
unknown	Homo sapiens PNAS-138 mRNA, complete cds	AF277175	-1.33*	-1.00	-1.04
LFNG	LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	NM_001040167	-1.35*	1.06	-1.08**

Gene Symbol	Unique DHT repressed genes Gene Name	Accession Number	Fold Change 1nM DHT	 1nM MPA	 1nM DHT 1nM MPA
RNF39	ring finger protein 39	NM_025236	-1.38*	1.03	-1.08**
TRAF5	TNF receptor-associated factor 5	NM_145759	-1.42*	-1.14	-1.11

Appendix 4 - Table 3: Unique MPA induced genes

Gene Symbol	Unique MPA induced genes Gene Name	Accession Number	Fold Change 1nM DHT	 1nM MPA	 1nM DHT 1nM MPA
GAGE12C	G antigen 12C	NM_001098408	1.51	1.76*	-1.06*
STYK1	serine/threonine/tyrosine kinase 1	NM_018423	1.07	1.48*	1.35*
SSTR2	somatostatin receptor 2	NM_001050	1.20	1.42*	1.11**
CITED4	Cbp/p300-interacting transactivator	NM_133467	-1.09	1.40*	1.02*
ROPN1L	ropporin 1-like	NM_031916	1.04	1.38*	1.15**
unknown	ncrna:misc_RNA chromosome	ENST00000384271	1.19	1.34*	1.16*
KGFLP1	keratinocyte growth factor-like protein 1	NR_003674	1.15	1.31*	-1.01*
RPS9	Homo sapiens ribosomal protein S9 (RPS9), mRNA	NM_001013.3	1.22	1.30*	1.06
ACOT7	acyl-CoA thioesterase 7	NM_007274	1.08	1.28*	1.20
GPR110	G protein-coupled receptor 110	NM_153840	-1.08**	1.28*	-1.01**
GNG5	guanine nucleotide binding protein (G protein), gamma 5	NM_005274	1.10	1.28*	1.16
RPS6KA5	ribosomal protein S6 kinase, 90kDa, polypeptide 5	NM_004755	1.08	1.26*	1.21
ANXA1	annexin A10	NM_007193	-1.09	1.25*	1.18
IFNA4	interferon, alpha 4	NM_021068	1.18	1.25*	1.01
SERPINA5	serpin peptidase inhibitor, clade A	NM_000624	1.01	1.25*	1.13
FAM49A	family with sequence similarity 49, member A	NM_030797	-1.55	1.25*	1.08
IFNA17	interferon, alpha 17	NM_021268	1.21	1.24*	-1.02
CHMP7	CHMP family, member 7	NM_152272	-1.04	1.24*	1.16
USP17L6P	ubiquitin specific peptidase 17-like 6 (pseudogene)	NR_027279	1.20	1.23*	-1.11**
ANKRD43	ankyrin repeat domain 43	NM_175873	1.10	1.23*	1.09
WNT4	wingless-type MMTV integration site family, member 4	NM_030761	1.00	1.23*	1.15
AKAP6	A kinase (PRKA) anchor protein 6	NM_004274	-1.05	1.23*	1.15
unknown	ncrna:snRNA chromosome	ENST00000362511	1.20	1.23*	1.02
INPP5J	inositol polyphosphate-5-phosphatase J	NM_001002837	-1.18	1.22*	1.08
B3GNT9	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase	NM_033309	1.03	1.22*	1.13
NEDD9	neural precursor cell expressed, developmentally	NM_001142393	1.02	1.22*	1.10
ADAM32	ADAM metallopeptidase domain 32	NM_145004	1.11	1.21*	1.06
OSBPL9	oxysterol binding protein-like 9	NM_024586	-1.05	1.21*	1.13
NXPH3	neurexophilin 3	NM_007225	-1.05	1.21*	1.03**
CLTB	clathrin, light chain (Lcb)	NM_007097	1.02	1.20*	1.06
PLEKHG6	pleckstrin homology domain containing, family G	NM_018173	-1.02	1.20*	1.10
ZNF436	zinc finger protein 436	NM_001077195	1.02	1.20*	1.12
RASAL1	RAS protein activator like 1 (GAP1 like)	NM_004658	-1.02	1.19*	1.09
ETHE1	ethylmalonic encephalopathy 1	NM_014297	1.06	1.18*	1.10

Gene Symbol	Unique MPA induced genes Gene Name	Accession Number	Fold Change 1nM DHT	 1nM MPA	 1nM DHT 1nM MPA
TNFRSF12A	tumour necrosis factor receptor	NM_016639	-1.00	1.18*	1.09
LPAR4	lysophosphatidic acid receptor 4	NM_005296	1.11	1.18*	1.10
IL1RAPL2	interleukin 1 receptor accessory protein-like 2	NM_017416	1.08	1.18*	1.14
LOC401620	Homo sapiens similar to LINE-1 reverse transcriptase	NM_001013688.1	1.07	1.17*	-1.02**
unknown	ncrna:snoRNA chromosome	ENST00000459357	1.07	1.17*	1.01
NAGK	N-acetylglucosamine kinase	NM_017567	1.07	1.17*	1.13
LLGL2	lethal giant larvae homolog 2 (Drosophila)	NM_001031803	-1.02	1.17*	1.14
unknown	ncrna:snRNA chromosome	ENST00000362515	1.10	1.17*	1.08
OR2W3	olfactory receptor, family 2, subfamily W, member 3	NM_001001957	1.15	1.16*	1.13
ACSM1	acyl-CoA synthetase medium-chain family member 1	NM_052956	1.09	1.16*	1.10
SYT10	synaptotagmin X	NM_198992	1.12	1.16*	1.03
ACSS1	acyl-CoA synthetase short-chain family member 1	NM_032501	-1.01	1.15*	1.12
unknown			1.06	1.15*	1.07
SLCO1B1	solute carrier organic anion transporter family,	NM_006446	1.06	1.15*	1.05
unknown			1.05	1.14*	-1.07**
SDC1	Homo sapiens syndecan 1 (SDC1), transcript variant 1,	NM_001006946	-1.03	1.14*	1.05
ACADM	acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain	NM_000016	-1.03	1.13*	1.11
SLC39A11	solute carrier family 39 (metal ion transporter)	NM_001159770	1.09	1.13*	1.03
SLC6A15	solute carrier family 6 (neutral amino acid transporter)	NM_182767	1.06	1.13*	1.04
MRPL24	mitochondrial ribosomal protein L24	NM_145729	1.05	1.13*	-1.03**
TMEM109	transmembrane protein 109	NM_024092	1.04	1.13*	1.10
CRK	Cdc2-related kinase, arginine/serine-rich	NM_016507	-1.01	1.12*	1.06
FAM45A	family with sequence similarity 45, member A	NM_207009	1.02	1.12*	1.08
MSTO1	misato homolog 1 (Drosophila)	NM_018116	1.02	1.12*	1.08
PICALM	phosphatidylinositol binding clathrin assembly protein	NM_007166	-1.02	1.11*	1.08
PLEKHB2	pleckstrin homology domain containing, family B	NM_017958	1.04	1.10*	1.06
DNAH8	dynein, axonemal, heavy chain 8	NM_001371	1.05	1.10*	1.07
LRP2BP	LRP2 binding protein	NM_018409	1.09	1.10*	-1.00**
SERINC5	serine incorporator 5	NM_178276	1.03	1.10*	1.07
PLP2	proteolipid protein 2 (colonic epithelium-enriched)	NM_002668	1.03	1.10*	1.06
DGCR8	DiGeorge syndrome critical region gene 8	NM_022720	-1.07	1.09*	1.06
CSMD3	CUB and Sushi multiple domains 3	NM_198124	1.07	1.08*	-1.01
FLNB	filamin B, beta	NM_001457	-1.05	1.08*	1.01
GLI3	GLI family zinc finger 3	NM_000168	1.16	-1.56*	-1.15**

Appendix 4 - Table 4: Unique MPA repressed genes

Gene Symbol	Unique MPA repressed genes Gene Name	Accession Number	Fold Change 1nM DHT	 1nM MPA	 1nM DHT 1nM MPA
TNFRSF12A	tumour necrosis factor receptor superfamily, member 12A	NM_016639	-1.00	1.18*	1.09
NUFIP2	nuclear fragile X mental retardation protein interacting	NM_020772	-1.04	-1.08*	1.02**
FAM3A	family with sequence similarity 3, member A	NM_021806	-1.01	-1.10*	1.01**
IPO9	importin 9	NM_018085	-1.04	-1.10*	-1.05
LRTOMT	leucine rich transmembrane and 0-methyltransferase domain	NM_001145309	-1.01	-1.11*	-1.03
NPIPL3	nuclear pore complex interacting protein-like 3	NM_130464	-1.03	-1.11*	1.06**
ARMCX5	armadillo repeat containing, X-linked 5	NM_022838	-1.07	-1.12*	-1.08
KIAA0556	KIAA0556	NM_015202	-1.04	-1.12*	-1.04
MEGF9	multiple EGF-like-domains 9	NM_001080497	-1.09	-1.12*	-1.04**
SLC48A1	solute carrier family 48 (heme transporter), member 1	NM_017842	-1.11	-1.12*	-1.10
ALOX12P2	arachidonate 12-lipoxygenase pseudogene 2	NR_002710	-1.07	-1.12*	1.00
NCOA2	nuclear receptor coactivator 2	NM_006540	-1.08	-1.12*	-1.07
WASH5P	WAS protein family homolog 5 pseudogene	NR_024540	1.01	-1.12*	1.07
ASH1L	ash1 (absent, small, or homeotic)-like (Drosophila)	NM_018489	-1.09	-1.12*	-1.05
NPIP	nuclear pore complex interacting protein	NM_006985	-1.05	-1.13*	1.00
PPT2	palmitoyl-protein thioesterase 2	NM_005155	-1.08	-1.13*	-1.07
BRAF	canopy 3 homolog (zebrafish)	NM_006586	-1.07	-1.13*	-1.10
PRKCH	protein kinase C, eta	NM_006255	-1.04	-1.13*	-1.10
TMEM63A	transmembrane protein 63A	NM_014698	-1.07	-1.13*	-1.08
SLC16A2	solute carrier family 16, member 2	NM_006517	-1.04	-1.14*	-1.05
ARFGEF1	ADP-ribosylation factor guanine nucleotide-exchange factor	NM_006421	-1.07	-1.14*	-1.12
KPNB1	karyopherin (importin) beta 1	NM_002265	-1.08	-1.15*	-1.08
FEZF1	FEZ family zinc finger 1	NM_001024613	-1.11	-1.15*	-1.09
EPB41L1	erythrocyte membrane protein band 4.1-like 1	NM_012156	-1.05	-1.15*	-1.11
unknown	ncrna:misc_RNA chromosome:	ENST00000363702	-1.05	-1.15*	-1.03
CARD8	caspase recruitment domain family, member 8	NM_014959	-1.08	-1.15*	-1.03
PRSS8	transmembrane protease, serine 8 homolog (mouse) pseudogene	NR_026864	-1.07	-1.16*	-1.07
KIAA0907	KIAA0907	BC062637	-1.07	-1.16*	1.00
NELF	nasal embryonic LHRH factor	NM_001130969	-1.11	-1.16*	-1.12
R3HDM2	R3H domain containing 2	NM_014925	-1.05	-1.17*	-1.00
LIG3	ligase III, DNA, ATP-dependent	NM_013975	-1.04	-1.17*	-1.01**
OBSL1	obscurin-like 1	NM_015311	-1.05	-1.17*	-1.10
unknown	Homo sapiens cDNA FLJ34189 fis, clone FCBBF3017535	AK091508	-1.01	-1.17*	-1.11
ULK4	unc-51-like kinase 4 (C. elegans)	NM_017886	-1.12	-1.17*	-1.13
ALS2CL	ALS2 C-terminal like	NM_147129	-1.04	-1.17*	-1.12
DGAT2	diacylglycerol O-acyltransferase 2-like 6	NM_198512	-1.14	-1.17*	-1.12
DMXL2	Dmx-like 2	NM_015263	-1.15	-1.17*	-1.10
TNFRSF12A	tumour necrosis factor receptor	NM_016639	-1.00	1.18*	1.09

Gene Symbol	Unique MPA repressed genes Gene Name	Accession Number	Fold Change 1nM DHT	 1nM MPA	 1nM DHT 1nM MPA
KCTD7	potassium channel tetramerisation domain containing 7	NM_153033	-1.10	-1.18*	-1.05**
C17orf58	chromosome 17 open reading frame 58	NM_181656	-1.01	-1.18*	-1.13
ZFAT	zinc finger and AT hook domain containing	NM_020863	-1.13	-1.18*	-1.06
KIAA2026	KIAA2026	NM_001017969	-1.08	-1.19*	-1.11
SPATA6	spermatogenesis associated 6	NM_019073	-1.13	-1.19*	-1.08
PIP5K1B	phosphatidylinositol-4-phosphate 5-kinase, type I, beta	NM_003558	-1.15	-1.19*	-1.15
DENND2D	DENN/MADD domain containing 2D	NM_024901	-1.14	-1.19*	-1.10**
MMP16	matrix metallopeptidase 16 (membrane-inserted)	AL136588	-1.12	-1.20*	-1.08
WT1	Wilms tumour 1	NM_024424	-1.07	-1.20*	-1.11
WNT7A	wingless-type MMTV integration site family, member 7A	NM_004625	-1.05	-1.20*	-1.09
MUM1	melanoma associated antigen (mutated) 1-like 1	NM_152423	-1.17	-1.21*	-1.14
TRMU	tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase	NM_018006	-1.11	-1.21*	-1.12
ETNK1	ethanolamine kinase 1	NM_018638	-1.15	-1.21*	-1.17
REV3L	REV3-like, catalytic subunit of DNA polymerase zeta	NM_002912	-1.21	-1.22*	-1.17
MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastom	NM_005378	-1.19	-1.22*	-1.16
ZMAT1	zinc finger, matrin type 1	NM_001011657	-1.14	-1.23*	-1.19
LCA5	Leber congenital amaurosis 5-like	NM_152505	-1.20	-1.23*	-1.19
ZCCHC2	zinc finger, CCHC domain containing 24	NM_153367	-1.08	-1.24*	-1.05
HSPA4L	heat shock 70kDa protein 4-like	NM_014278	-1.14	-1.25*	-1.14
Unknown			-1.09	-1.26*	-1.09
FAM190A	family with sequence similarity 190, member A	NM_001145065	-1.05	-1.27*	-1.19
LRRN1	leucine rich repeat neuronal 1	NM_020873	-1.17	-1.28*	-1.23
SLITRK6	SLIT and NTRK-like family, member 6	NM_032229	1.10	-1.29*	-1.17
PLD1	zona pellucida-like domain containing 1	NM_175056	-1.10	-1.30*	-1.10
CRYBG3	beta-gamma crystallin domain containing 3	NM_153605	-1.17	-1.30*	-1.13
SLC7A11	solute carrier family 7, (cationic amino acid transporter)	NM_014331	1.17	-1.31*	-1.19
TTC6	tetratricopeptide repeat domain 6	BC103915	-1.15	-1.33*	-1.27
CREB3L1	cAMP responsive element binding protein 3-like 1	NM_052854	-1.22	-1.34*	-1.04
GALNT10	UDP-N-acetyl-alpha-D-galactosamine:	NM_198321	-1.23	-1.37*	-1.13
HEY2	hairy/enhancer-of-split related with YRPW motif 2	NM_012259	1.31	-1.43*	-1.21
CYP1A2	cytochrome P450, family 1, subfamily A, polypeptide 2	NM_000761	1.04	-1.45*	-1.22
C8orf73	chromosome 8 open reading frame 73	NM_001100878	-1.04	-1.49*	-1.22
LCA5	Leber congenital amaurosis 5-like	NM_152505	-1.20	-1.23*	-1.19
ZCCHC2	zinc finger, CCHC domain containing 24	NM_153367	-1.08	-1.24*	-1.05
HSPA4L	heat shock 70kDa protein 4-like	NM_014278	-1.14	-1.25*	-1.14
unknown			-1.09	-1.26*	-1.09
FAM190A	family with sequence similarity 190, member A	NM_001145065	-1.05	-1.27*	-1.19
TNFRSF12A	tumour necrosis factor receptor	NM_016639	-1.00	1.18*	1.09
LRRN1	leucine rich repeat neuronal 1 /	NM_020873	-1.17	-1.28*	-1.23

Gene Symbol	Unique MPA repressed genes Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1nM MPA
SLITRK6	SLIT and NTRK-like family, member 6	NM_032229	1.10	-1.29*	-1.17
PLD1	zona pellucida-like domain containing 1	NM_175056	-1.10	-1.30*	-1.10
CRYBG3	beta-gamma crystallin domain containing 3	NM_153605	-1.17	-1.30*	-1.13
SLC7A11	solute carrier family 7, (cationic amino acid transporter)	NM_014331	1.17	-1.31*	-1.19
TTC6	tetratricopeptide repeat domain 6	BC103915	-1.15	-1.33*	-1.27
CREB3L1	cAMP responsive element binding protein 3-like 1	NM_052854	-1.22	-1.34*	-1.04
GALNT10	UDP-N-acetyl-alpha-D-galactosamine:	NM_198321	-1.23	-1.37*	-1.13
HEY2	hairy/enhancer-of-split related with YRPW motif 2	NM_012259	1.31	-1.43*	-1.21
CYP1A2	cytochrome P450, family 1, subfamily A, polypeptide 2	NM_000761	1.04	-1.45*	-1.22
C8orf73	chromosome 8 open reading frame 73	NM_001100878	-1.04	-1.49*	-1.22

Appendix 4 - Table 5: Unique DHT and MPA combined induced genes

Gene Symbol	Unique DHT and MPA combined induced genes Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1nM MPA
FLJ31713	homo sapiens cDNA FLJ31713 fis, clone NT2RI2006487	AK056275	1.06**	1.20	1.71*
LIFR	leukemia inhibitory factor receptor alpha	NM_002310	1.26	1.29	1.58*
C9orf106	chromosome 9 open reading frame 106	NM_001012715	1.10**	1.19	1.55*
unknown			1.05**	1.12	1.54*
ABCC12	ATP-binding cassette, sub-family C (CFTR/MRP), member 12	NM_033226	1.21	1.11	1.53*
CYP4Z2P	cytochrome P450, family 4, subfamily Z, polypeptide 2 psuedogene	NR_002788	1.36	1.40	1.53*
NCRNA00052	non-protein coding RNA 52	NR_026869	1.13	1.30	1.52*
RP11-49G10.8	breast cancer and salivary gland expression gene	NR_026760	1.28	1.36	1.51*
unknown			1.13	1.27	1.50*
TMEM150A	transmembrane protein 150A	NM_001031738	1.11**	1.08	1.50*
TNFAIP3	TNFAIP3 interacting protein 2	NM_024309	1.02**	1.18	1.49*
CYR61	cysteine-rich, angiogenic inducer, 61	NM_001554	1.03**	1.29	1.49*
unknown	ncrna:snRNA chromosome:	ENST00000362794	1.06	1.18	1.48*
CCDC85A	coiled-coil domain containing 85A	NM_001080433	1.14**	1.21	1.44*
OXER1	oxoeicosanoid (OXE) receptor 1	NM_148962	1.30	1.22	1.43*
hsa-mir-200c	MI0000650 Homo sapiens miR-200c		1.06**	1.19	1.42*
TRIM52	tripartite motif-containing 52	NM_032765	1.06	-1.01	1.41*
AUTS2	autism susceptibility candidate 2	NM_015570	1.19**	1.15	1.41*
unknown	ncrna:snoRNA chromosome	ENST00000459149	1.14	1.18	1.40*
unknown	ncrna:snoRNA chromosome:GRCh37	ENST00000410656	1.00**	1.20	1.39*
CHD1L	chromodomain helicase DNA binding protein 1-like	NM_004284	-1.05**	1.25	1.39*
FLJ31713	homo sapiens cDNA FLJ31713 fis, clone NT2RI2006487	AK056275	1.06**	1.20	1.71*
ERO1LB	ERO1-like beta (S. cerevisiae)	NM_019891	1.23	1.18	1.39*
CDKL5	cyclin-dependent kinase-like 5	NM_001037343	1.07**	1.10	1.38*

Gene Symbol	Unique DHT and MPA combined induced genes Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1nM MPA
unknown	ncrna:snRNA chromosome	ENST00000364876	1.09	1.13	1.38*
MPDZ	multiple PDZ domain protein	NM_003829	1.31	-1.07	1.38*
SLC22A5	solute carrier family 22 (organic cation/carnitine transporter	NM_003060	1.12**	1.19	1.37*
SAMD12	sterile alpha motif domain containing 12	NM_207506	1.15	1.28	1.37*
DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	NM_012328	1.07	1.30	1.36*
THRSP	thyroid hormone responsive (SPOT14 homolog, rat)	NM_003251	1.23	1.10	1.36*
PTPRCAP	protein tyrosine phosphatase, receptor type,	NM_005608	1.07**	1.00	1.36*
SPRED1	sprouty-related, EVH1 domain containing 1	NM_152594	1.23	1.07	1.36*
unknown	ncrna:snRNA chromosome:	ENST00000364294	-1.11**	-1.01	1.36*
USP33	ubiquitin specific peptidase 33	NM_015017	1.11	1.18	1.35*
ATP7B	ATPase, Cu++ transporting, beta polypeptide	NM_000053	1.08	1.16	1.34*
FUT3	fucosyltransferase 3 (galactoside 3(4)-L-fucosyltransferase	NM_000149	1.14	1.10	1.34*
VSTM2L	V-set and transmembrane domain containing 2 like	NM_080607	1.08**	1.20	1.34*
DOCK4	dedicator of cytokinesis 4	NM_014705	1.12**	1.15	1.34*
unknown	ncrna:snRNA chromosome:	ENST00000363404	1.02	-1.08	1.34*
HSPA1L	heat shock 70kDa protein 1-like	NM_005527	-1.13**	-1.09	1.33*
RREB1	ras responsive element binding protein 1	NM_001003699	1.14	1.20	1.33*
EFNA5	ephrin-A5	NM_001962	1.00**	1.14	1.33*
unknown	ncrna:misc_RNA chromosome	ENST00000363300	-1.12**	-1.07	1.33*
PPP1R11	protein phosphatase 1, regulatory (inhibitor) subunit 1	NM_021959	1.00**	-1.09	1.33*
PARVA	parvin, alpha	NM_018222	1.04	1.15	1.32*
DGKH	diacylglycerol kinase, eta	NM_178009	1.07	1.25	1.32*
HIST1H3C	histone cluster 1, H3c	NM_003531	1.04**	1.12	1.32*
TTC3	tetratricopeptide repeat domain 35	NM_014673	-1.03**	1.20	1.32*
C9orf75	chromosome 9 open reading frame 75	NM_173691	1.00**	1.17	1.31*
LOC100131860	hypothetical protein LOC100131860	AK097109	-1.10**	-1.11	1.31*
HIST1H4E	histone cluster 1, H4e	NM_003545	1.04**	1.05	1.31*
ANKRD29	ankyrin repeat domain 29	NM_173505	1.03**	1.18	1.31*
HMGXB3	HMG box domain containing 3	NM_014983	1.14	1.14	1.31*
IFRG15	interferon responsive gene 15	NM_022347	1.09	1.19	1.31*
NUDT13	nudix (nucleoside diphosphate linked moiety X)-type motif	NM_015901	1.08	1.07	1.31*
TMEM80	transmembrane protein 80	NM_174940	1.00**	1.17	1.31*
unknown			1.03	1.09	1.31*
GSTM4	glutathione S-transferase mu 4	NM_000850	1.07**	1.14	1.30*
YAP1	Yes-associated protein 1	NM_001130145	1.07**	1.19	1.30*
unknown			1.10	1.19	1.30*
unknown			1.10	1.19	1.30*
MICAL2	microtubule associated monoxygenase,	NM_014632	1.00**	1.12	1.30*
RBMS2	RNA binding motif, single stranded	NM_002898	-1.03	1.17	1.30*
unknown	cdna:pseudogene chromosome	ENST00000442420	1.17	1.23	1.30*

Gene Symbol	Unique DHT and MPA combined induced genes Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1nM MPA
ZFHX3	zinc finger homeobox 3	NM_006885	-1.01**	1.16	1.30*
NBEA	neurobeachin-like 1	NM_001114132	-1.17**	1.21	1.30*
FBXL16	F-box and leucine-rich repeat protein 16	ENST00000397621	1.07**	1.19	1.30*
ELMO1	Homo sapiens engulfment and cell motility 1 (ELMO1)	NM_130442.2	-1.12**	1.10	1.30*
unknown	Homo sapiens cDNA FLJ10246 fis, clone HEMBB1000673	AK001108	1.12	-1.01	1.30*
ANKRD62	ankyrin repeat domain 62	ENST00000314074	1.19	1.01	1.29*
LOC100128868	testin-related protein TRG	AY143171	1.17	1.08	1.29*
MYCBP2	MYC binding protein 2	NM_015057	1.10	1.11	1.29*
NELL2	NEL-like 2 (chicken)	NM_006159	-1.10**	1.25	1.29*
PDE10A	phosphodiesterase 10A	NM_006661	1.11	1.00	1.29*
APCDD1	adenomatosis polyposis coli down-regulated 1	NM_153000	1.16	1.17	1.29*
GORASP1	golgi reassembly stacking protein 1, 65kDa	NM_031899	-1.01**	1.17	1.29*
ARSG	arylsulfatase G	NM_014960	1.01**	1.15	1.29*
unknown			-1.01**	1.06	1.29*
unknown	ncrna:misc_RNA chromosome	ENST00000364793	1.00**	1.17	1.28*
PDPK1	3-phosphoinositide dependent protein kinase-1	NM_002613	-1.01**	1.06	1.28*
LRCH1	leucine-rich repeats and calponin homology (CH) domain	NM_015116	1.12	1.13	1.28*
C2orf67	chromosome 2 open reading frame 67	NM_152519	1.03**	1.09	1.28*
GRAMD3	GRAM domain containing 3	NM_023927	-1.00**	1.20	1.28*
KLF5	Kruppel-like factor 5 (intestinal)	NM_001730	1.23	1.18	1.28*
KCNK1	potassium channel, subfamily K, member 13	NM_022054	1.05**	1.18	1.28*
EPN2	epsin 2	NM_014964	1.20	1.02	1.28*
FARP1	AKR7 family pseudogene	NR_002796	1.04**	1.12	1.28*
C17orf77	chromosome 17 open reading frame 77	NM_152460	1.26	1.26	1.28*
LAMA3	laminin, alpha 3	NM_198129	-1.08**	1.18	1.28*
SRGAP1	SLIT-ROBO Rho GTPase activating protein 1	NM_020762	1.10	1.11	1.28*
PHF8	PHD finger protein 8	NM_015107	1.03**	1.04	1.28*
NAPA	N-ethylmaleimide-sensitive factor attachment protein	NM_003827	1.02**	1.13	1.28*
GDPD3	glycerophosphodiester phosphodiesterase domain	NM_024307	1.17	1.24	1.27*
CREB3L2	cAMP responsive element binding protein 3-like 2	NM_194071	-1.03**	1.10	1.27*
unknown	Homo sapiens cDNA clone IMAGE:4249212	BC034024	1.18	1.20	1.27*
GPR137C	G protein-coupled receptor 137C	NM_001099652	1.21	1.09	1.27*
TOR1AIP2	torsin A interacting protein 2	NM_145034	1.05	1.17	1.27*
SQRDL	sulfide quinone reductase-like (yeast)	NM_021199	1.13	1.17	1.27*
DUSP16	dual specificity phosphatase 16	NM_030640	1.18	1.18	1.27*
MICAL2	microtubule associated monoxygenase, calponin and LIM domain	NM_014632	1.00**	1.12	1.30*
AFMID	arylformamidase	NM_001010982	-1.01**	1.20	1.27*
VEZF1	vascular endothelial zinc finger 1	NM_007146	1.07**	1.16	1.27*
HMX2	H6 family homeobox 2	NM_005519	1.18	1.13	1.27*
C6orf81	chromosome 6 open reading frame 81	BC033033	1.04**	1.12	1.27*

Gene Symbol	Unique DHT and MPA combined induced genes Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1nM MPA
FOXJ2	forkhead box J2	NM_018416	1.03**	1.04	1.26*
TBRG1	transforming growth factor beta regulator 1	NM_032811	1.05**	1.08	1.26*
MFSD6	major facilitator superfamily domain containing 6-like	NM_152599	-1.04**	1.18	1.26*
TFCP2L1	transcription factor CP2-like 1	NM_014553	1.01**	1.21	1.26*
unknown	ncrna:misc_RNA chromosome:	ENST00000384563	1.09	1.13	1.26*
GIPC1	GIPC PDZ domain containing family, member 1	NM_005716	1.00	1.10	1.26*
RBM14	RNA binding motif protein 14	NM_006328	-1.08**	-1.06	1.26*
SESTD1	SEC14 and spectrin domains 1	NM_178123	1.12**	1.11	1.26*
POP1	processing of precursor 1, ribonuclease P/MRP subunit	NM_001145860	1.10	1.20	1.26*
FAM189A2	family with sequence similarity 189, member A2	NM_004816	1.14	1.12	1.26*
UNQ353	GKGM353	AY358648	1.08	-1.02	1.26*
CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	NM_005195	1.06	1.05	1.26*
ZFP36L2	zinc finger protein 36, C3H type-like 2	NM_006887	1.20	1.09	1.26*
MAP7D1	MAP7 domain containing 1	NM_018067	1.09	1.15	1.26*
GRINA	glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein	NM_000837	-1.02**	-1.02	1.26*
ASB13	ankyrin repeat and SOCS box-containing 13	NM_024701	1.06**	1.23	1.25*
RARA	retinoic acid receptor, alpha	NM_000964	1.09	1.07	1.25*
GPR75	G protein-coupled receptor 75	NM_006794	1.03	1.04	1.25*
GRB7	growth factor receptor-bound protein 7	NM_001030002	-1.09**	1.13	1.25*
unknown	Homo sapiens cDNA FLJ13538 fis, clone PLACE1006617	AK023600	1.15	-1.05	1.25*
CNTN6	contactin 6	NM_014461	1.20	1.18	1.25*
GABRQ	gamma-aminobutyric acid (GABA) receptor, theta	NM_018558	1.11**	1.15	1.25*
KIAA1370	KIAA1370	NM_019600	1.22	1.13	1.25*
HOXC13	Homo sapiens homeobox C13 (HOXC13), mRNA.	NM_017410	1.08**	1.00	1.25*
PPP2R4	protein phosphatase 2A activator, regulatory subunit 4	NM_178001	1.04	1.07	1.25*
WEE1	WEE1 homolog (S. pombe)	NM_003390	-1.03**	-1.06	1.25*
TBX6	T-box 6	NM_004608	1.10	1.06	1.25*
ETV1	ets variant 1	NM_004956	1.18	1.19	1.25*
STX18	syntaxin 18	NM_016930	1.08	1.07	1.24*
LRRFIP1	leucine rich repeat (in FLII) interacting protein 1	NM_001137552	1.04	1.21	1.24*
PCDH1	protocadherin 10	NM_032961	1.02**	1.17	1.24*
DNAJB6	DnaJ (Hsp40) homolog, subfamily B, member 6	NM_058246	-1.01**	1.09	1.24*
C20orf95	chromosome 20 open reading frame 95	ENST00000373345	1.22	1.08	1.24*
SNX33	sorting nexin 33	NM_153271	-1.05**	1.06	1.24*
DUSP5	dual specificity phosphatase 5	NR_002834	1.09**	1.12	1.24*
ADAMTSL4	ADAMTS-like 4	NM_019032	1.14	1.08	1.24*
TBC1D16	TBC1 domain family, member 16	NM_019020	1.13	1.05	1.24*
DIO2	deiodinase, iodothyronine, type II	NM_013989	1.12	1.13	1.24*
SAP30	SAP30 binding protein	NM_013260	1.07**	1.09	1.24*

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ACVR1	activin A receptor, type IC	NM_145259	-1.01	1.15	1.23*
ZNRF1	Homo sapiens zinc and ring finger 1 (ZNRF1), mRNA.	NM_032268	1.09**	1.09	1.24*
TBC1D10B	TBC1 domain family, member 10B	NM_015527	-1.06**	1.09	1.23*
BET1	blocked early in transport 1 homolog (S. cerevisiae)	NM_005868	1.04**	1.14	1.23*
MICALCL	MICAL C-terminal like	NM_032867	1.03**	1.05	1.23*
ZNF764	zinc finger protein 764	NM_033410	1.05**	1.01	1.23*
TRIM36	tripartite motif-containing 36	NM_018700	1.10	1.10	1.23*
AACS	acetoacetyl-CoA synthetase	NM_023928	1.15	1.14	1.23*
DUSP8	dual specificity phosphatase 8	NM_004420	-1.08**	-1.06	1.23*
TMTC2	transmembrane and tetratricopeptide repeat containing 2	NM_152588	1.21	1.12	1.23*
NFIA	nuclear factor I/A	NM_00113467	-1.02**	1.17	1.23*
SCAP	S-phase cyclin A-associated protein in the ER	NM_020843	1.04**	1.08	1.22*
SLC38A2	solute carrier family 38, member 2	NM_018976	1.13	1.13	1.22*
DTX3	deltex 3-like (Drosophila)	NM_138287	-1.04**	-1.10	1.22*
GOLGA8B	golgi autoantigen, golgin subfamily a, 8B	NR_027410	-1.02**	-1.04	1.22*
MAPKAPK2	mitogen-activated protein kinase-activated protein kinase	NM_004759	1.02**	1.09	1.22*
AKR1C4	aldo-keto reductase family 1, member C4	NM_001818	1.12	1.18	1.22*
unknown	ncrna:snoRNA chromosome:	ENST00000459610	1.02	1.03	1.22*
unknown	Homo sapiens cDNA FLJ43404 fis, clone OCBBF2017516.	AK125394	1.16	1.10	1.22*
HOXC9	homeobox C	NM_006897	1.06**	1.04	1.22*
CSTF2T	cleavage stimulation factor, 3' pre-RNA, subunit 2,	NM_015235	1.03	1.04	1.22*
CSNK1A1	casein kinase 1, alpha 1-like	NM_145203	-1.01**	1.18	1.22*
unknown	ncrna:snRNA chromosome:	ENST00000384603	-1.03**	1.12	1.22*
LENG9	leukocyte receptor cluster (LRC) member 9	NM_198988	1.01**	-1.10	1.22*
MCL1	germ cell-less homolog 1 (Drosophila)	NM_178439	1.00**	1.14	1.22*
TP53TG1	TP53 target 1 (non-protein coding)	AB007455	1.21	1.11	1.22*
ID3	AT rich interactive domain 3C (BRIGHT-like)	NM_001017363	1.08**	1.13	1.21*
COX18	COX18 cytochrome c oxidase assembly homolog	NM_173827	-1.07**	1.08	1.21*
WEE1	WEE1 homolog	NM_003390	-1.06**	-1.10	1.21*
MAPT	microtubule-associated protein tau	NM_016835	1.02	1.05	1.21*
PLEKHG2	pleckstrin homology domain containing, family G	NM_022835	-1.05**	1.10	1.21*
CDC7	coiled-coil domain containing 75	NM_174931	1.04	1.16	1.21*
ULK2	unc-51-like kinase 2 (C. elegans)	NM_014683	1.05	1.17	1.21*
TACC2	transforming, acidic coiled-coil containing protein 2	NM_206862	1.03	1.05	1.21*
PECI	adenosine deaminase, RNA-specific, B2	NM_018702	1.21	1.01	1.21*
MYO5C	myosin VC	NM_018728	1.08	1.00	1.21*
LRRC16A	leucine rich repeat containing 16A	NM_017640	1.13	1.03	1.21*
TMEM42	transmembrane protein 42	NM_144638	1.15	1.13	1.21*
VASP	Enah/Vasp-like	NM_016337	-1.00**	1.11	1.20*

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MMACHC	methylmalonic aciduria (cobalamin deficiency) cblC type	NM_015506	1.16	1.10	1.20*
ACER1	alkaline ceramidase 1	NM_133492	1.16	1.09	1.20*
GOT1	glutamic-oxaloacetic transaminase 1-like 1	NM_152413	1.03**	1.11	1.20*
DHRS4L2	dehydrogenase/reductase (SDR family) member 4 like 2	NM_198083	1.12	1.12	1.20*
OVOL2	ovo-like 2 (Drosophila)	NM_021220	1.07	1.15	1.20*
unknown			1.06	1.05	1.20*
UAP1	clusterin associated protein 1	NM_015041	1.18	1.04	1.20*
RALBP1	ralA binding protein 1	NM_006788	1.04**	1.13	1.20*
HES1	hairy and enhancer of split 1, (Drosophila)	NM_001007540	1.15	1.06	1.20*
CDH2	cadherin-like 29	NM_022124	1.01	1.11	1.20*
LHFPL5	lipoma HMGIC fusion partner-like 5	NM_182548	1.10	1.06	1.20*
TAT	Homo sapiens N-acetylneuraminate pyruvate lyase 2	NM_138413	1.08**	1.07	1.20*
C15orf39	chromosome 15 open reading frame 39	NM_015492	1.08	1.19	1.20*
IRS2	insulin receptor substrate 2	NM_003749	1.09	1.11	1.20*
RTN4RL1	reticulon 4 receptor-like 1	NM_178568	-1.12**	1.13	1.20*
TBC1D4	TBC1 domain family, member 4	NM_014832	1.06	1.17	1.20*
ZNF518B	zinc finger protein 518B	NM_053042	1.05**	-1.05	1.20*
SGCE	sarcoglycan, epsilon	NM_001099401	1.13	1.09	1.20*
FLJ23834	hypothetical protein FLJ23834	NM_152750	1.09	1.14	1.20*
SFN	stratifin	NM_006142	1.02**	1.16	1.20*
PPP1R13B	protein phosphatase 1, regulatory (inhibitor) subunit 1	NM_015316	-1.05**	1.17	1.20*
CPSF4	cleavage and polyadenylation specific factor 4-like	NM_001129885	1.04**	1.01	1.20*
FZD1	frizzled homolog 1 (Drosophila)	NM_003505	1.05**	1.17	1.19*
AHNAK	AHNAK nucleoprotein	NM_001620	1.06**	1.18	1.19*
unknown			-1.11**	1.01	1.19*
MLPH	melanophilin	NM_024101	1.05	1.06	1.19*
TNFRSF1A	tumour necrosis factor receptor superfamily, member 1A	NM_001065	1.01**	1.11	1.19*
FAR2	free fatty acid receptor 2	NM_005306	1.00**	1.13	1.19*
unknown	ncrna:snRNA chromosome:	ENST00000384258	1.10**	1.11	1.19*
BCL9	B-cell CLL/lymphoma 9	NM_004326	1.08**	1.11	1.19*
MTERFD2	MTERF domain containing 2	NM_182501	-1.00	1.07	1.19*
SMOC2	SPARC related modular calcium binding 2	NM_022138	-1.00**	1.16	1.19*
EGR1	target of EGR1, member 1 (nuclear)	NM_025077	1.11	1.02	1.19*
LOC389832	hypothetical LOC389832	AK124122	-1.10**	1.01	1.19*
ZMYND19	zinc finger, MYND-type containing 19	NM_138462	-1.01**	1.13	1.19*
PSD3	pleckstrin and Sec7 domain containing 3	NM_015310	1.12	1.04	1.19*
SLC19A2	solute carrier family 19 (thiamine transporter)	NM_006996	1.06	1.06	1.18*
IER5L	immediate early response 5-like	NM_203434	1.04**	1.13	1.18*
unknown			1.02**	1.16	1.18*
EPHB4	EPH receptor B4	NM_004444	1.04**	1.08	1.18*
unknown			1.10	1.13	1.18*

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SFXN3	sideroflexin 3	NM_030971	-1.02**	1.12	1.18*
MTMR4	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1,	NM_002494	-1.03**	1.13	1.18*
ZBTB10	zinc finger and BTB domain containing 10	NM_001105539	1.06	1.15	1.18*
TBC1D10A	Homo sapiens TBC1 domain family, member 10A (TBC1D10A)	NM_031937	-1.01**	1.13	1.18*
SPSB1	splA/ryanodine receptor domain and SOCS box containing 1	NM_025106	1.03	1.02	1.18*
HSPB1	heat shock protein family B (small), member 11	NM_016126	1.03**	1.07	1.18*
MAPK1IP1L	mitogen-activated protein kinase 1 interacting protein	NM_144578	-1.01**	1.06	1.18*
ZYG11A	zyg-11 homolog A (C. elegans)	NM_001004339	1.03**	-1.03	1.18*
LENG8	leukocyte receptor cluster (LRC) member 8	NM_052925	-1.03**	1.05	1.18*
ITGAV	integrin, alpha V (vitronectin receptor, alpha polypeptide)	NM_002210	1.12	1.08	1.18*
NBPF10	neuroblastoma breakpoint family, member 10	NM_001039703	1.00**	1.12	1.18*
FOXO3B	forkhead box O3B pseudogene	NR_026718	-1.11**	1.07	1.17*
TFAP2A	transcription factor AP-2 alpha	NM_003220	1.07	-1.03	1.17*
RNF14	ring finger protein 144B	NM_182757	-1.02	1.15	1.17*
VPS26B	vacuolar protein sorting 26 homolog B (S. pombe)	NM_052875	1.03**	1.04	1.17*
ECHDC2	enoyl Coenzyme A hydratase domain containing 2	NM_018281	-1.01**	1.03	1.17*
PRIC285	peroxisomal proliferator-activated receptor A	NM_001037335	1.09	1.14	1.17*
DNAJB6	Homo sapiens DnaJ (Hsp40) homolog, subfamily B, member 6	NM_005494.2	1.02	1.14	1.17*
ERN1	endoplasmic reticulum to nucleus signaling 1	NM_001433	-1.02	1.06	1.17*
SDC2	cold shock domain containing C2, RNA binding	NM_014460	1.13	1.02	1.17*
TSPAN33	tetraspanin 33	NM_178562	1.09	1.10	1.17*
DHRS4	dehydrogenase/reductase (SDR family) member 4	NM_021004	1.10	1.10	1.17*
FZD5	frizzled homolog 5 (Drosophila)	NM_003468	1.03**	1.10	1.17*
SIDT2	SID1 transmembrane family, member 2	NM_001040455	-1.00**	1.10	1.17*
MEF2D	myocyte enhancer factor 2D	NM_005920	-1.04**	1.08	1.17*
TTC32	tetratricopeptide repeat domain 32	NM_001008237	1.03**	1.12	1.17*
KLF6	Kruppel-like factor 6	NM_001300	-1.09**	1.11	1.17*
LRRC26	leucine rich repeat containing 26	NM_001013653	1.01**	1.13	1.17*
SAMD13	sterile alpha motif domain	NM_001010971	1.01**	1.01	1.17*
DAAM1	dishevelled associated activator of morphogenesis 1	NM_014992	-1.06**	-1.02	1.16*
ENAH	Enah/Vasp-like	NM_016337	1.03**	1.04	1.16*
PAQR3	progestin and adipoQ receptor family member III	NM_001040202	1.03**	1.11	1.16*
MYSM1	Myb-like, SWIRM and MPN domains 1	NM_001085487	1.03	1.00	1.16*
KLF7	Kruppel-like factor 7 (ubiquitous)	NM_003709	-1.00**	1.12	1.16*
CCDC85A	coiled-coil domain containing 85A	NM_001080433	1.05**	1.12	1.16*
unknown			1.08	1.01	1.16*
TP53I11	tumour protein p53 inducible protein 11	NM_001076787	-1.00**	1.02	1.16*

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ZDHHC7	zinc finger, DHHC-type containing 7	NM_001145548	-1.02**	1.13	1.16*
DNASE1	deoxyribonuclease I-like 3	NM_004944	1.10	1.09	1.16*
RAB24	RAB24, member RAS oncogene family	NM_001031677	-1.02**	1.05	1.16*
BRD4	bromodomain containing 4	NM_058243	-1.01**	1.04	1.16*
ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 13	NR_003087	-1.01	1.00	1.16*
unknown			1.07**	1.13	1.16*
CYFIP2	cytoplasmic FMR1 interacting protein 2	NM_001037332	1.01**	1.12	1.15*
unknown	ncrna:snRNA chromosome:	ENST00000410144	1.05	1.11	1.15*
RHOBTB2	Rho-related BTB domain containing 2	NM_001160036	1.03	1.09	1.15*
NCOA5	nuclear receptor coactivator 5	NM_020967	1.00	-1.01	1.15*
unknown	ncrna:snRNA chromosome	ENST00000362724	1.03	1.12	1.15*
RNF4	ring finger protein 4	NM_002938	1.08	1.03	1.15*
DYNLL2	dynein, light chain, LC8-type 2	NM_080677	1.04	1.09	1.15*
CAMSAP1L1	calmodulin regulated spectrin-associated protein 1	NM_203459	-1.05**	1.08	1.15*
MAST2	microtubule associated serine/threonine kinase 2	NM_015112	-1.00**	1.01	1.15*
FOXO1	forkhead box O1	NM_002015	-1.01**	1.08	1.15*
MBOAT2	membrane bound O-acyltransferase domain containing 2	NM_138799	-1.03	1.13	1.15*
RNF167	ring finger protein 167	NM_015528	-1.03**	-1.03	1.15*
SLAIN1	SLAIN motif family, member 1	NM_001040153	1.08	1.03	1.15*
TRIO	TRIO and F-actin binding protein	NM_001039141	-1.04	1.11	1.15*
C9orf66	chromosome 9 open reading frame 66	NM_152569	1.16	1.10	1.15*
PLBD1	phospholipase B domain containing 1	NM_024829	1.10	1.10	1.15*
FAM89B	family with sequence similarity 89, member B	NM_001098785	-1.02	1.08	1.15*
CORO1B	coronin, actin binding protein, 1B	NM_020441	1.07	1.02	1.15*
FGFR1	FGFR1 oncogene partner 2	NM_015633	1.07**	1.06	1.15*
ATP7A	ATPase, Cu++ transporting, alpha polypeptide	NM_000052	1.03	1.07	1.15*
AHCY	adenosylhomocysteinase-like 1	NM_006621	1.06	1.04	1.15*
PEX11B	peroxisomal biogenesis factor 11 beta	NM_003846	-1.00**	1.09	1.14*
PAQR5	progestin and adipoQ receptor family member V	NM_001104554	1.03**	1.10	1.14*
ARL8A	ADP-ribosylation factor-like 8A	NM_138795	1.07	1.00	1.14*
NPC1	Niemann-Pick disease, type C1	NM_000271	-1.01**	1.06	1.14*
CMB	carboxymethylenebutenolidase homolog (Pseudomonas)	NM_138809	-1.13**	1.14	1.14*
SEC24D	SEC24 family, member D (S. cerevisiae)	NM_014822	1.03	1.06	1.14*
TMEM8B	transmembrane protein 8B	NM_016446	-1.05**	1.05	1.14*
CLPTM1L	CLPTM1-like	NM_030782	-1.05**	1.10	1.14*
ANP32C	acidic (leucine-rich) nuclear phosphoprotein 32 family	NM_012403	1.01	1.06	1.14*
HYOU1	hypoxia up-regulated 1	NM_006389	-1.07**	1.03	1.14*
RP11-94I2.2	neuroblastoma breakpoint family, member 11-like	NM_183372	1.02	1.09	1.14*
KIAA0195	KIAA0195	NM_014738	-1.03**	1.02	1.13*
KIAA0182	KIAA0182	NM_014615	-1.02**	1.01	1.13*
unknown	ncrna:snRNA chromosome	ENST00000408512	1.07	1.09	1.13*

Gene Symbol	Unique DHT and MPA combined induced genes Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1nM MPA
PIP5K1A	Homo sapiens phosphatidylinositol-4-phosphate 5-kinase	NM_001135638	1.04**	1.07	1.14*
TP53BP2	tumour protein p53 binding protein, 2	NM_005426	-1.05**	1.08	1.13*
unknown	ncrna:misc_RNA chromosome	ENST00000411096	1.12	1.12	1.13*
RP11-94I2.2	neuroblastoma breakpoint family, member 11-like	NM_183372	1.05	1.10	1.13*
SYPL1	synaptophysin-like 1	NM_006754	1.07	1.12	1.13*
ANXA2	annexin A2 pseudogene 2	NR_003573	1.06	1.09	1.13*
MED21	mediator complex subunit 21	NM_004264	1.08	1.12	1.13*
ELOVL7	ELOVL family member 7, elongation of long chain fatty acid	NM_024930	1.01**	1.09	1.13*
RPRD1B	regulation of nuclear pre-mRNA domain containing 1B	NM_021215	-1.03**	1.04	1.13*
PRKACA	protein kinase, cAMP-dependent, catalytic, alpha	NM_002730	-1.06**	-1.03	1.13*
DNM1L	dynamin 1-like	NM_012062	1.01	1.05	1.12*
MYRIP	myosin VIIA and Rab interacting protein	NM_015460	-1.09**	-1.02	1.12*
TM4SF1	transmembrane 4 L six family member 18	NM_138786	1.04**	1.04	1.12*
CEP97	centrosomal protein 97kDa	NM_024548	1.01**	1.09	1.12*
ZNF275	zinc finger protein 275	NM_001080485	-1.05**	1.04	1.12*
RERE	arginine-glutamic acid dipeptide (RE) repeats	NM_012102	1.01**	1.00	1.12*
NFX1	zinc finger, NFX1-type containing 1	NM_021035	-1.10**	1.05	1.12**
FAM129B	family with sequence similarity 129, member B	NM_022833	1.07	1.11	1.11*
HN1	hematological and neurological expressed 1	NM_016185	1.02**	1.07	1.11*
B4GALT5	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase,	NM_004776	1.01**	-1.01	1.11*
STAT3	signal transducer and activator of transcription 3	NM_139276	-1.02**	1.09	1.11*
SPEN	spen homolog, transcriptional regulator (Drosophila)	NM_015001	-1.04**	-1.07	1.11*
OBFC2B	oligonucleotide/oligosaccharide-binding fold containing 2	NM_024068	1.04	-1.02	1.11*
DHCR24	24-dehydrocholesterol reductase	NM_014762	1.07	1.07	1.11*
SNORD56B	small nucleolar RNA, C/D box 56B	NR_001276	1.06	1.10	1.11*
SMARCC2	SWI/SNF related, matrix associated, actin dependent	NM_003075	1.02**	1.00	1.11*
PIAS1	protein inhibitor of activated STAT, 1	NM_016166	1.06	1.04	1.11*
MED13L	mediator complex subunit 13-like	NM_015335	1.02**	1.02	1.11*
ZHX2	zinc fingers and homeoboxes 2	NM_014943	1.03	1.05	1.10*
AGAP5	ArfGAP with GTPase domain, ankyrin repeat and PH domain	NM_001144000	1.04	-1.05	1.10*
CYTSA	cytospin A	NM_015330	1.04	1.05	1.10*
SLC38A1	solute carrier family 38, member 1	NM_030674	1.05	1.08	1.10*
unknown	ncrna:misc_RNA chromosome:	ENST00000362350	1.03	1.04	1.10*
TTLL12	tubulin tyrosine ligase-like family,	NM_015140	1.07	1.07	1.10*
PBOV1	prostate and breast cancer overexpressed 1	NM_021635	1.07	1.03	1.10*
PNPLA4	patatin-like phospholipase domain containing 4	NM_004650	-1.01**	1.00	1.09*
CST3	cystatin C	NM_000099	1.03	1.02	1.10*
ZNF862	zinc finger protein 862	NM_001099220	1.00	1.05	1.09*

Gene Symbol	Unique DHT and MPA combined induced genes Gene Name	Accession Number	Fold Change 1nM DHT	 1nM MPA	 1nM DHT 1nM MPA
NUMB	ligand of numb-protein X 1	NM_001126328	1.03	-1.03	1.10*
SLC2A10	solute carrier family 2 (facilitated glucose transporter)	NM_030777	-1.09	1.09	1.09*
S100A10	S100 calcium binding protein A10	NM_002966	1.00**	1.01	1.09*
MYADM	myeloid-associated differentiation marker-like 2	NM_001145113	1.02**	1.06	1.09*
TAF8	TAF8 RNA polymerase II, TATA box binding protein (TBP)	NM_138572	1.04	1.01	1.09*
SCD	Homo sapiens mesenchymal stem cell protein DSCD28 mRNA	AF242772	1.06	1.05	1.09*
ACTG1	actin, gamma 1	NM_001614	1.02	1.06	1.08*
PPP1R15B	protein phosphatase 1, regulatory (inhibitor) subunit 1	NM_032833	1.04	1.02	1.07*
PTMA	prothymosin, alpha pseudogene 7	AF170294	1.05	1.06	1.07*
unknown	ncrna:misc_RNA chromosome:	ENST00000391033	-1.00**	1.02	1.06*
UBE2D3	Homo sapiens ubiquitin-conjugating enzyme E2D 3	NM_181887.1	1.00**	1.02	1.05*

Appendix 4 - Table 6: Unique DHT and MPA combined repressed genes

Gene Symbol	Unique DHT and MPA combined repressed genes Gene Name	Accession Number	Fold Change 1nM DHT	 1nM MPA	 1nM DHT 1nM MPA
CNOT1	CCR4-NOT transcription complex, subunit 1	NM_016284	-1.06	-1.04	-1.07*
ST6GALNAC2	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1	NM_006456	-1.03	1.04	-1.07*
GNG8	guanine nucleotide binding protein (G protein), gamma 8	NM_033258	-1.03	1.04	-1.07*
CSNK2A1	casein kinase 2, alpha 1 polypeptide	NM_177559	-1.06**	-1.04	-1.08*
MYO1C	myosin IC	NM_001080779	-1.06	-1.03	-1.08*
IMPDH2	IMP (inosine monophosphate) dehydrogenase 2	NM_000884	1.01**	1.00	-1.08*
ROBO1	roundabout, axon guidance receptor, homolog 1	NM_133631	-1.06	-1.06	-1.09*
G3BP1	GTPase activating protein (SH3 domain) binding protein 1	NM_005754	-1.03**	-1.07	-1.09*
EIF4B	eukaryotic translation initiation factor 4B	NM_001417	-1.05	-1.01	-1.10*
MRPL51	mitochondrial ribosomal protein L51	NM_016497	-1.03	-1.04	-1.10*
PRKCD	protein kinase C, delta	NM_006254	-1.08	-1.06	-1.10*
KIF5B	kinesin family member 5B	NM_004521	-1.07	-1.09	-1.10*
KLF11	Kruppel-like factor 11	NM_003597	1.00**	-1.07	-1.10*
SPATA20	spermatogenesis associated 20	NM_022827	-1.08	-1.07	-1.10*
ABLIM1	actin binding LIM protein 1	NM_002313	-1.09	-1.11	-1.10*
OAT	ornithine aminotransferase (gyrate atrophy)	NM_000274	-1.05	-1.04	-1.10*
SLC25A43	solute carrier family 25, member 43	NM_145305	-1.12	-1.07	-1.11*
SLC1A5	solute carrier family 1 (neutral amino acid transporter)	NM_005628	-1.08	-1.09	-1.11*
PGD	hematopoietic prostaglandin D synthase	NM_014485	-1.07	-1.03	-1.11*
KIAA1618	KIAA1618	NM_020954	-1.07	-1.06	-1.11*
HEXIM1	hexamethylene bis-acetamide inducible 1	NM_006460	-1.09	-1.06	-1.11*

Gene Symbol	Unique DHT and MPA combined repressed genes Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1nM MPA
MADD	DENN/MADD domain containing 1C	NM_024898	-1.08	-1.07	-1.11*
ISOC1	isochorismatase domain containing 1	NM_016048	-1.03	-1.09	-1.11*
MRPS27	mitochondrial ribosomal protein S27	NM_015084	-1.05	-1.03	-1.11*
MEPCE	methylphosphate capping enzyme	NM_019606	-1.06	1.00	-1.11*
AP2B1	adaptor-related protein complex 2, beta 1 subunit	NM_001030006	-1.05	-1.07	-1.11*
RSL1D1	ribosomal L1 domain containing 1	NM_015659	1.02**	-1.07	-1.12*
FAM114A1	family with sequence similarity 114, member A1	BC040452	-1.12	-1.11	-1.12*
SNORD33	small nucleolar RNA, C/D box 33	NR_000020	-1.05	-1.01	-1.12*
PCNP	PEST proteolytic signal containing nuclear protein	NM_020357	-1.07	-1.11	-1.12*
CLASP2	cytoplasmic linker associated protein 2	NM_015097	-1.05	-1.05	-1.12*
SLC7A5	solute carrier family 7 (cationic amino acid transporter	NM_003486	-1.04	-1.03	-1.12*
ZMPSTE24	zinc metallopeptidase (STE24 homolog, S. cerevisiae)	NM_005857	-1.06	-1.07	-1.12*
CLINT1	clathrin interactor 1	NM_014666	-1.03**	-1.08	-1.12*
ME1	malic enzyme 1, NADP(+)-dependent, cytosolic	NM_002395	-1.07	-1.11	-1.12*
STARD7	StAR-related lipid transfer (START) domain containing 7	NM_020151	-1.07	-1.03	-1.12*
SRR	SRR1 domain containing	NM_001013694	-1.08	-1.05	-1.12*
C8orf42	chromosome 8 open reading frame 42	NM_175075	-1.10	-1.03	-1.13*
PIK3C2G	phosphoinositide-3-kinase, class 2, gamma polypeptide	NM_004570	-1.11	-1.09	-1.13*
SHOC2	soc-2 suppressor of clear homolog (C. elegans)	NM_007373	-1.08	-1.09	-1.13*
C3orf39	chromosome 3 open reading frame 39	NM_032806	-1.03	-1.01	-1.13*
SLC3A2	solute carrier family 3	NM_001012661	-1.09	-1.01	-1.13*
ELP2	endozepine-like peptide 2 pseudogene	NR_024120	1.01**	-1.06	-1.13*
IPMK	inositol polyphosphate multikinase	NM_152230	-1.12	-1.05	-1.13*
ATP2B4	ATPase, Ca++ transporting, plasma membrane 4	NM_001001396	-1.10	-1.09	-1.13*
ATIC	non-metastatic cells 7, protein	NM_013330	-1.08	-1.09	-1.13*
IGSF9	immunoglobulin superfamily, member 9B	NM_014987	-1.11	-1.06	-1.13*
LGALS9B	lectin, galactoside-binding, soluble, 9B	NM_001042685	1.02**	-1.08	-1.13*
PIGN	phosphatidylinositol glycan anchor biosynthesis, class N	NM_176787	-1.13	-1.09	-1.13*
GTF2H1	general transcription factor IIH, polypeptide 1, 62kDa	NM_005316	-1.02**	-1.07	-1.13*
STRA13	stimulated by retinoic acid 13 homolog	NM_144998	-1.03	-1.01	-1.13*
FAM78A	family with sequence similarity 78, member A	NM_033387	-1.14	-1.05	-1.14*
MAP3K12	mitogen-activated protein kinase kinase kinase 12	NM_006301	-1.11	-1.13	-1.14*
ANLN	anillin, actin binding protein	NM_018685	-1.08	-1.13	-1.14*
TJP3	tight junction protein 3 (zona occludens 3)	NM_014428	-1.09	-1.03	-1.14*
GPATCH2	G patch domain containing 2	NM_018040	-1.06	-1.04	-1.14*
TTC19	tetratricopeptide repeat domain 19	NM_017775	-1.08	1.01	-1.14*
METAP2	methionyl aminopeptidase 2	NM_006838	-1.11	-1.11	-1.14*
unknown	Homo sapiens ubiquitin specific peptidase 12 (USP12), mRNA	NM_182488.1	-1.08	-1.12	-1.14*

Gene Symbol	Unique DHT and MPA combined repressed genes Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1nM MPA
ITPK1	inositol 1,3,4-triphosphate 5/6 kinase	NM_014216	-1.11	-1.08	-1.14*
TM9SF3	transmembrane 9 superfamily member 3	NM_020123	-1.09	-1.03	-1.14*
BTF3L4	basic transcription factor 3-like 4	NM_152265	-1.03**	-1.01	-1.14*
LRRC58	leucine rich repeat containing 58	NM_001099678	-1.05	-1.12	-1.14*
ANXA9	annexin A9	NM_003568	-1.06	-1.11	-1.14*
BTF3L4	basic transcription factor 3-like 4	NM_152265	-1.04	-1.02	-1.14*
FSTL4	follistatin-like 4	NM_015082	-1.08	-1.07	-1.14*
OXCT1	3-oxoacid CoA transferase 1	NM_000436	-1.13	-1.10	-1.15*
ACP2	acid phosphatase 2, lysosomal	NM_001610	-1.06	-1.07	-1.15*
PPT1	palmitoyl-protein thioesterase 1	NM_000310	-1.08	-1.08	-1.15*
TTLL7	tubulin tyrosine ligase-like family, member 7	NM_024686	-1.07	-1.12	-1.15*
POMT1	protein-O-mannosyltransferase 1	NM_001136113	-1.13	-1.04	-1.15*
unknown	ncrna:misc_RNA chromosome:	ENST00000411066	-1.01	-1.07	-1.15*
FAM98C	family with sequence similarity 98, member C	NM_174905	-1.05**	-1.02	-1.15*
MFI2	antigen p97 (melanoma associated)	NM_005929	-1.07	-1.01	-1.15*
BZW2	basic leucine zipper and W2 domains 2	NM_001159767	-1.09	-1.06	-1.15*
ABCB9	ATP-binding cassette, sub-family B (MDR/TAP), member 9	NM_019625	-1.13	-1.08	-1.15*
RSU1	Ras suppressor protein 1	NM_012425	-1.11	-1.12	-1.15*
ABAT	4-aminobutyrate aminotransferase	NM_020686	-1.13	-1.10	-1.15*
PTPLAD1	agmatine ureohydrolase (agmatinase)	NM_016395	-1.10	-1.05	-1.15*
AGMAT	Homo sapiens agmatine ureohydrolase (agmatinase)	NM_024758	1.05**	-1.04	-1.15*
ADAM19	ADAM metallopeptidase domain 19 (meltrin beta)	NM_033274	-1.11	-1.03	-1.15*
RAB6C	RAB6C, member RAS oncogene family	NM_032144	-1.04	-1.15	-1.15*
OCRL	oculocerebrorenal syndrome of Lowe	NM_000276	-1.11	-1.12	-1.15*
GNL3	guanine nucleotide binding protein-like 3 (nucleolar)	NM_206825	-1.14	-1.05	-1.16*
FAM43A	family with sequence similarity 43, member A	NM_153690	-1.02	1.00	-1.16*
EPAS1	endothelial PAS domain protein 1	NM_001430	-1.16	-1.04	-1.16*
XYLT2	xylosyltransferase II	NM_022167	-1.14	-1.03	-1.16*
ASB7†	ankyrin repeat and SOCS box-containing 7	NM_198243	-1.03**	-1.07	-1.16*
HEATR6†	HEAT repeat containing 6	NM_022070	-1.06**	-1.06	-1.16*
NSUN4	NOL1/NOP2/Sun domain family, member 4	NM_199044	-1.08	-1.05	-1.16*
PIGX	phosphatidylinositol glycan anchor biosynthesis, class X	NM_017861	-1.05**	-1.07	-1.16*
CNNM3	cyclin M3	NM_017623	-1.13	-1.07	-1.16*
unknown	ncrna:snoRNA chromosome	ENST00000459178	1.02**	-1.04	-1.16*
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	NM_004827	-1.04	-1.14	-1.16*
ACPP	acid phosphatase, prostate	NM_001099	-1.14	-1.12	-1.16*
unknown			-1.02**	-1.11	-1.16*
CCNG1	cyclin G1	NM_004060	-1.03	-1.11	-1.16*
UGCG	UDP-glucose ceramide glucosyltransferase	NM_003358	-1.10	-1.12	-1.16*
CLCN7	chloride channel 7	NM_001287	-1.07	1.03	-1.16*

Gene Symbol	Unique DHT and MPA combined repressed genes Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1nM MPA
HHAT	hedgehog acyltransferase-like	NR_027753	-1.09	-1.05	-1.16*
unknown	ncrna:snoRNA chromosome	ENST00000458880	1.12**	-1.08	-1.16*
FAM98A	family with sequence similarity 98, member A	NM_015475	-1.13	-1.03	-1.16*
CDH3	cadherin 3, type 1, P-cadherin (placental)	NM_001793	-1.13	-1.09	-1.16*
SLC4A1AP	solute carrier family 4 (anion exchanger),	NM_018158	-1.06	-1.04	-1.16*
PRR19	proline rich 19	NM_199285	-1.08	-1.05	-1.16*
HHIPL2	HHIP-like 2	NM_024746	-1.13	-1.11	-1.16*
PKN2	protein kinase N2	NM_006256	-1.15	-1.13	-1.16*
PYGL	phosphorylase, glycogen, liver	NM_002863	-1.07	1.00	-1.16*
GABPB1	GA binding protein transcription factor, beta subunit 1	NM_005254	-1.12	-1.07	-1.16*
C8orf82	chromosome 8 open reading frame 82	BC073936	-1.01**	-1.08	-1.16*
TMEM116	transmembrane protein 116	NM_138341	-1.04**	-1.10	-1.16*
CCNG2	cyclin G2	NM_004354	-1.06	-1.06	-1.16*
HLA-DQA2	major histocompatibility complex, class II, DQ alpha 2	NM_020056	-1.11	-1.10	-1.17*
BBS2	Bardet-Biedl syndrome 2	NM_031885	-1.04**	-1.06	-1.17*
unknown	ncrna:snoRNA chromosome	ENST00000458788	-1.04**	-1.11	-1.17*
SNORD32A	small nucleolar RNA, C/D box 32A	NR_000021	-1.00**	-1.09	-1.17*
OCLN	occludin	NM_002538	-1.10	-1.14	-1.17*
GUF1	GUF1 GTPase homolog (S. cerevisiae)	NM_021927	-1.09	-1.10	-1.17*
NUF2	NUF2, NDC80 kinetochore complex component, homolog	NM_145697	-1.07	-1.07	-1.17*
unknown			1.05**	-1.04	-1.17*
USP16	dual specificity phosphatase 16	NM_030640	-1.10	-1.04	-1.17*
FTSJD1	FtsJ methyltransferase domain containing 1	NM_018348	-1.11	-1.07	-1.17*
RELL1	RELT-like 1	NM_001085399	-1.13	-1.07	-1.17*
SEH1L	SEH1-like (S. cerevisiae)	NM_031216	-1.09	-1.08	-1.17*
NTN4	contactin 4	NM_175607	-1.14	-1.07	-1.17*
USP2	dual specificity phosphatase 22	NM_020185	-1.15	-1.11	-1.17*
HEATR5A	HEAT repeat containing 5A	NM_015473	-1.11	-1.15	-1.17*
ASPSCR1	alveolar soft part sarcoma chromosome region	NM_024083	-1.15	-1.11	-1.18*
IL17RB	interleukin 17 receptor B	NM_018725	-1.14	-1.09	-1.18*
CHML	choroideremia-like (Rab escort protein	NM_001821	-1.16	-1.14	-1.18*
SPAG16	sperm associated antigen 16	NM_024532	-1.01**	-1.15	-1.18*
GALC	galactosylceramidase	NM_000153	-1.09	-1.06	-1.18*
GOLM1	golgi membrane protein 1	NM_016548	-1.07	-1.10	-1.18*
TBL1XR1	transducin (beta)-like 1 X-linked	NM_024665	-1.07	-1.11	-1.18*
RNF141	ring finger protein 141	NM_016422	-1.07	-1.07	-1.18*
LCN2	chloride channel 2	NM_004366	1.02	-1.15	-1.18*
TMEM47	transmembrane protein 47	NM_031442	-1.08	-1.08	-1.18*
PMS2	RNA binding protein with multiple splicing 2	NM_194272	-1.10**	-1.06	-1.18*
SNORA65	small nucleolar RNA, H/ACA box 65	NR_002449	1.02**	-1.02	-1.18*
HPS3	Hermansky-Pudlak syndrome 3	NM_03238	-1.15	-1.14	-1.18*
SCIN	tenascin XB	NM_019105	-1.15	-1.11	-1.18*

Gene Symbol	Unique DHT and MPA combined repressed genes Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1nM MPA
DEGS1	degenerative spermatocyte homolog 1, lipid desaturase	NM_003676	-1.15**	-1.10	-1.18*
TMX2	thioredoxin-related transmembrane protein 2	NM_015959	-1.04	-1.14	-1.18*
ZNF673	zinc finger family member 673	NM_001129898	-1.06	-1.10	-1.18*
RELB	v-rel reticuloendotheliosis viral oncogene homolog B	NM_006509	-1.03**	-1.13	-1.18*
GPRC5C	G protein-coupled receptor, family C, group 5, member C	NM_022036	-1.11	-1.07	-1.18*
DSC2	desmocollin 2	NM_024422	-1.15	-1.09	-1.18*
NCALD	neurocalcin delta	NM_001040624	-1.14	-1.11	-1.18*
KLHL21	kelch-like 21 (Drosophila)	NM_014851	-1.10	-1.18	-1.18*
ST6GAL1	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1	NM_173216	-1.16	-1.19	-1.18*
CD302	CD302 molecule	NM_014880	-1.06	-1.09	-1.18*
NAT14	N-acetyltransferase 14 (GCN5-related, putative)	NM_020378	-1.03	-1.07	-1.18*
KIAA0323	KIAA0323	NM_015299	1.02**	-1.11	-1.18*
unknown	ncrna:snoRNA chromosome	ENST00000459097	-1.03**	-1.05	-1.18*
TMC7	transmembrane channel-like 7	NM_024847	-1.11	-1.09	-1.19*
NIPAL1	NIPA-like domain containing 1	NM_207330	-1.13	-1.14	-1.19*
TMEM87B	transmembrane protein 87B	NM_032824	-1.11	-1.16	-1.19*
UNC93B1	unc-93 homolog B1 (C. elegans)	NM_030930	-1.09	-1.00	-1.19*
MED22	mediator complex subunit 22	NM_181491	-1.02**	-1.02	-1.19*
CSNK1G2	casein kinase 1, gamma 2	NM_001319	-1.05**	-1.03	-1.19*
KIAA1797	KIAA1797	NM_017794	-1.10	-1.11	-1.19*
ANXA3	annexin A3	NM_005139	-1.11	-1.10	-1.19*
TDRD5	tudor domain containing 5	NM_173533	-1.09	-1.11	-1.19*
NOVA1	neuro-oncological ventral antigen 1	NM_002515	-1.03**	-1.18	-1.19*
HERC1	hect (homologous to the E6-AP (UBE3A) carboxyl terminus)	NM_003922	-1.15	-1.16	-1.19*
CDCA7L	cell division cycle associated 7-like	NM_018719	-1.14	-1.11	-1.19*
SACM1L	SAC1 suppressor of actin mutations 1-like (yeast)	NM_014016	-1.16	-1.08	-1.19*
RFT1	RFT1 homolog (S. cerevisiae)	NM_052859	-1.15	-1.01	-1.19*
ATP6V1C1	ATPase, H+ transporting, lysosomal	NM_001695	-1.17	-1.10	-1.19*
VAMP7	vesicle-associated membrane protein 7	NM_005638	-1.01**	-1.07	-1.19*
CCDC109A	coiled-coil domain containing 109A	NM_138357	-1.16	-1.11	-1.19*
MFSD1	major facilitator superfamily domain containing 11	NM_024311	-1.07**	-1.06	-1.19*
CTTNBP2	cortactin binding protein 2	NM_033427	-1.12	-1.15	-1.19*
NR1D2	nuclear receptor subfamily 1, group D, member 2	NM_005126	-1.15	-1.15	-1.19*
MAP3K7IP1	mitogen-activated protein kinase kinase kinase 7	NM_006116	-1.11	-1.11	-1.19*
SCARA5	scavenger receptor class A, member 5 (putative)	NM_173833	-1.04**	-1.10	-1.19*
CNNM4	cyclin M4	NM_020184	-1.18	-1.10	-1.19*
AURKA	aurora kinase A	NM_198433	-1.01**	-1.12	-1.19*
FAM177B	family with sequence similarity 177, member B	NM_207468	1.10**	-1.14	-1.19*
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	NM_182943	-1.07	-1.06	-1.19*
PRAME	PRAME family member 12 /	NM_001080830	-1.16	-1.07	-1.19*

Gene Symbol	Unique DHT and MPA combined repressed genes Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1nM MPA
ARMCX2	armadillo repeat containing, X-linked 2	NM_177949	-1.01**	-1.16	-1.20*
CMTM8	CKLF-like MARVEL transmembrane domain containing 8	NM_178868	-1.13	-1.17	-1.20*
C1orf107	chromosome 1 open reading frame 107	NM_014388	-1.14	-1.14	-1.20*
SLC35A3	solute carrier family 35 (UDP-N-acetylglucosamine	NM_012243	-1.14	-1.11	-1.20*
SCHIP1	schwannomin interacting protein 1	NM_014575	-1.17	-1.15	-1.20*
TCF7L1	transcription factor 7-like 1 (T-cell specific, HMG-box)	NM_031283	-1.07	-1.11	-1.20*
CPA4	carboxypeptidase A4	NM_016352	-1.18	-1.10	-1.20*
SNORD57	small nucleolar RNA, C/D box 57	NR_002738	1.03**	-1.03	-1.20*
ATP2C2	ATPase, Ca++ transporting, type 2C, member 2	NM_014861	-1.18	-1.10	-1.20*
IRAK2	interleukin-1 receptor-associated kinase 2	NM_001570	-1.09	-1.08	-1.20*
CYB5R1	cytochrome b5 reductase 1	NM_016243	-1.11	-1.10	-1.20*
LOC400965	hypothetical LOC400965	AK098018	-1.04	-1.16	-1.20*
RBM3	RNA binding motif protein 38	NM_017495	-1.04	-1.03	-1.20*
ZNF251	zinc finger protein 251	NM_138367	-1.07	-1.16	-1.20*
ATP10D	ATPase, class V, type 10D	NM_020453	-1.17	-1.16	-1.21*
TMPRSS13	transmembrane protease, serine 13	NM_001077263	-1.02**	-1.13	-1.21*
ITGAL	integrin, alpha L (antigen CD11A (p180),	NM_002209	-1.13	-1.14	-1.21*
ZZZ3	zinc finger, ZZ-type containing 3	NM_015534	-1.12	-1.10	-1.21*
PHEX	phosphate regulating endopeptidase homolog, X-linked	NM_000444	-1.08	-1.08	-1.21*
HMOX1	heme oxygenase (decycling) 1	NM_002133	-1.13	-1.14	-1.21*
ANKRD46	ankyrin repeat domain 46	NM_198401	-1.15	-1.04	-1.21*
TMEM167A	transmembrane protein 167A	NM_174909	-1.10	-1.13	-1.21*
FBXO4	F-box protein 43	NM_001077528	-1.08	-1.19	-1.21*
GATM	glycine amidinotransferase (L-arginine:glycine	NM_001482	-1.12	-1.08	-1.21*
unknown	ncrna:rRNA chromosome:	ENST00000390963	-1.03	-1.13	-1.21*
C20orf54	chromosome 20 open reading frame 54	NM_033409	-1.17	-1.13	-1.21*
ATP13A3	ATPase type 13A3	NM_024524	-1.07	-1.14	-1.21*
DEGS1	degenerative spermatocyte homolog 1, lipid desaturase	NM_003676	-1.07	-1.21	-1.21*
MCTP1	multiple C2 domains, transmembrane 1	NM_024717	-1.09	-1.21	-1.21*
TBC1D8B	TBC1 domain family, member 8B (with GRAM domain)	NM_017752	-1.11	-1.14	-1.21*
LAMB1	laminin, beta 1	NM_002291	-1.14	-1.12	-1.22*
ARHGAP18	Rho GTPase activating protein 18	NM_033515	-1.13	-1.15	-1.22*
GRHL1	grainyhead-like 1 (Drosophila)	NM_198182	-1.16	-1.19	-1.22*
MPP5	membrane protein, palmitoylated 5	NM_022474	-1.13	-1.13	-1.22*
NCAM2	neural cell adhesion molecule 2	NM_004540	-1.13	-1.19	-1.22*
UGT3A2	UDP glycosyltransferase 3 family, polypeptide A2	NM_174914	-1.03**	-1.15	-1.22*
GATSL1	GATS protein-like 1	NM_001145063	-1.06	-1.09	-1.22*
SNORD41	small nucleolar RNA, C/D box 41	NR_002751	-1.01**	-1.12	-1.22*
TPST2	tyrosylprotein sulfotransferase 2	NM_003595	-1.00**	-1.13	-1.22*
TRIM59	tripartite motif-containing 59	NM_173084	-1.13	-1.08	-1.22*
RNF128	ring finger protein 128	NM_024539	-1.10**	-1.06	-1.22*

Gene Symbol	Unique DHT and MPA combined repressed genes Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1nM MPA
ACVR1C	activin A receptor, type IC	NM_145259	-1.12	-1.06	-1.22*
DCAF4	DDB1 and CUL4 associated factor 4-like 1	NM_001029955	-1.20	-1.20	-1.22*
GCHFR	GTP cyclohydrolase I feedback regulator	NM_005258	-1.02**	-1.11	-1.22*
AHCTF1	AT hook containing transcription factor 1	NM_015446	-1.18	-1.14	-1.22*
unknown	ncrna:misc_RNA chromosome	ENST00000410130	-1.06	-1.15	-1.22*
FOLR3	folate receptor 3 (gamma)	NM_000804	-1.03	-1.10	-1.23*
UBR7	ubiquitin protein ligase E3 component n-recognin 7	NM_175748	-1.10	-1.12	-1.23*
IL17RD	interleukin 17 receptor D	NM_017563	-1.13	-1.20	-1.23*
SNORD14C	small nucleolar RNA, C/D box 14C	NR_001453	-1.02	-1.16	-1.23*
NFATC2	nuclear factor of activated T-cells,	NM_032815	-1.19	-1.17	-1.23*
GCNT2	glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	NM_145649	-1.18	-1.18	-1.23*
BBS4	Bardet-Biedl syndrome 4	NM_033028	-1.13	-1.02	-1.23*
SCARNA22	small Cajal body-specific RNA 22	NR_003004	1.05**	-1.16	-1.23*
SH3PXD2A	SH3 and PX domains 2A	NM_014631	-1.23	-1.15	-1.23*
GPR177	protein-coupled receptor 177	NM_024911	-1.11	-1.07	-1.23*
EDN1	endothelin 1	NM_001955	-1.10	-1.17	-1.23*
SNORD36C	small nucleolar RNA, C/D box 36C	NR_000016	-1.15	-1.20	-1.23*
SH2D4A	SH2 domain containing 4A	NM_022071	-1.16	-1.12	-1.24*
WBSCR28	Williams-Beuren syndrome chromosome region 28	NM_182504	-1.12	-1.20	-1.24*
C17orf51	chromosome 17 open reading frame 51	AK096256	1.00**	1.02	-1.24*
SNORD35A	small nucleolar RNA, C/D box 35A	NR_000018	-1.03**	-1.03	-1.24*
GBP1	CGG triplet repeat binding protein 1	NM_001008390	-1.16	-1.16	-1.24*
PLCH1	phospholipase C, eta 1	NM_001130960	-1.24	-1.13	-1.24*
RAPGEF4	Rap guanine nucleotide exchange	NM_007023	-1.11	-1.17	-1.24*
WDR75	WD repeat domain 75	NM_017672	-1.17	-1.10	-1.24*
TRPM7	transient receptor potential cation	NM_017672	-1.18	-1.16	-1.24*
GDF15	growth differentiation factor 15	NM_004864	1.06**	-1.18	-1.24*
TLR5	toll-like receptor 5	NM_003268	-1.15	-1.15	-1.25*
VSNL1	visinin-like 1	NM_003385	-1.22	-1.11	-1.25*
MBLAC2	metallo-beta-lactamase domain containing 2	NM_203406	-1.23	-1.14	-1.25*
CCDC91	coiled-coil domain containing 91	NM_018318	-1.10	-1.14	-1.25*
RHOD	thiosulfate sulfurtransferase (rhodanese)	NM_003312	-1.01**	-1.00	-1.25*
SLCO4C1	solute carrier organic anion transporter family,	NM_180991	-1.12	-1.09	-1.25*
RPS6KA3	ribosomal protein S6 kinase, 90kDa, polypeptide 3	NM_004586	-1.22	-1.24	-1.25*
NT5C2	5'-nucleotidase, cytosolic II	NM_012229	-1.09	-1.16	-1.25*
SRXN1	sulfiredoxin 1 homolog (S. cerevisiae)	NM_080725	-1.11	-1.03	-1.25*
BMPR1B	bone morphogenetic protein receptor, type IB	NM_001203	-1.23	-1.17	-1.25*
TAPT1	transmembrane anterior posterior	NM_153365	-1.21	-1.17	-1.25*
RAP1GAP	RAP1 GTPase activating protein	NM_001145657	-1.26	-1.18	-1.25*
F7	coagulation factor VII	NM_000131	-1.08**	-1.15	-1.25*
BTG2	BTG family, member 2	NM_006763	-1.12	-1.14	-1.26*

Gene Symbol	Unique DHT and MPA combined repressed genes Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1nM MPA
RNU11	RNA, U11 small nuclear	NR_004407	-1.03**	-1.02	-1.26*
NBEAL1	neurobeachin-like 1	NM_001114132	-1.14	-1.17	-1.26*
FAAH2	fatty acid amide hydrolase 2	NM_174912	-1.10	-1.18	-1.26*
TLR3	toll-like receptor 3	NM_003265	-1.15	-1.08	-1.26*
LOC100132167	similar to hCG1993567	BC006438	-1.05**	-1.14	-1.26*
CRYZ	crystallin, zeta (quinone reductase)	NM_001130042	-1.09	-1.17	-1.26*
GNPDA2	glucosamine-6-phosphate deaminase 2	NM_138335	-1.05	-1.08	-1.27*
RHBDL3	rhomboid, veinlet-like 3 (Drosophila)	NM_138328	-1.08	-1.20	-1.27*
SNORD26	small nucleolar RNA, C/D box 26	NR_002564	-1.07	-1.05	-1.27*
ATP10B	ATPase, class V, type 10B	NM_025153	-1.06**	-1.16	-1.28*
ARHGEF6	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6	NM_004840	-1.20	-1.17	-1.28*
MMRN2	multimerin 2	NM_024756	-1.10**	-1.17	-1.28*
SNORD34	small nucleolar RNA, C/D box 34	NR_000019	-1.09	-1.06	-1.28*
CBLN2	cerebellin 2 precursor	NM_182511	-1.05	-1.21	-1.28*
SNORD44	small nucleolar RNA, C/D box 44	NR_002750	-1.19	-1.22	-1.28*
SLC35A1	solute carrier family 35 (CMP-sialic acid transporter)	NM_006416	-1.11	-1.10	-1.29*
CCDC52	coiled-coil domain containing 52	NM_144718	-1.10	-1.15	-1.29*
DRAM1	DNA-damage regulated autophagy modulator 1	NM_018370	-1.18	-1.13	-1.29*
PCDH10	protocadherin 10	NM_032961	-1.12	-1.22	-1.29*
GTDC1	glycosyltransferase-like domain containing 1	NM_001006636	-1.11	-1.19	-1.29*
SNORD59B	small nucleolar RNA, C/D box 59B	NR_003046	-1.27	-1.01	-1.29*
C20orf177	chromosome 20 open reading frame 177	BC054002	-1.18	-1.24	-1.30*
CCL28	chemokine (C-C motif) ligand 28	NM_148672	-1.24	-1.21	-1.30*
unknown	ncrna:snoRNA chromosome	ENST00000459301	-1.02**	-1.12	-1.30*
FMN1	formin 1	NM_001103184	-1.17	-1.28	-1.30*
C9orf3	chromosome 9 open reading frame 3	AF043897	-1.14	-1.30	-1.30*
SYCP2	synaptonemal complex protein 2	NM_014258	-1.30	-1.18	-1.30*
CENPV†	centromere protein V	NM_181716	-1.05**	-1.12	-1.30*
PPIA	peptidylprolyl isomerase A (cyclophilin A)	NM_021130	-1.10	-1.11	-1.30*
ODF2L†	outer dense fiber of sperm tails 2-like	NM_020729	-1.09**	-1.17	-1.30*
PIK3IP1	phosphoinositide-3-kinase interacting protein 1	NM_052880	-1.17	-1.10	-1.30*
HECW2	HECT, C2 and WW domain containing E3 ubiquitin protein ligase	NM_020760	-1.24	-1.26	-1.31*
BAK1	BCL2-antagonist/killer 1	NM_001188	-1.17	-1.30	-1.31*
RNU2-1†	RNA, U2 small nuclear 1	NR_002716	1.03**	-1.09	-1.32*
RUNDC3B†	RUN domain containing 3B	NM_138290	-1.16**	-1.18	-1.32*
DHX35	DEAH (Asp-Glu-Ala-His) box polypeptide 35	NM_021931	-1.20	-1.06	-1.32*
EXPH5	exophilin 5	NM_015065	-1.31	-1.21	-1.32*
KIF20B	kinesin family member 20B	NM_016195	-1.20	-1.15	-1.32*
P2RY6	pyrimidinergic receptor P2Y, G-protein coupled, 6	NM_176796	-1.12	-1.27	-1.32*
BST2†	bone marrow stromal cell antigen 2	NM_004335	-1.09**	-1.13	-1.32*

Gene Symbol	Unique DHT and MPA combined repressed genes Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1nM MPA
TMEM154	transmembrane protein 154	NM_152680	-1.26	-1.20	-1.33*
RGS16††	regulator of G-protein signaling 16	NM_002928	-1.11**	-1.20	-1.33*
unknown †	ncrna:snoRNA chromosome	ENST00000364129	1.03**	-1.07	-1.33*
GPR39†	G protein-coupled receptor 39	NM_001508	-1.15**	-1.12	-1.33*
unknown	ncrna:snoRNA chromosome	ENST00000364953	-1.09	-1.17	-1.33*
SNORD36B	small nucleolar RNA, C/D box 36B	NR_000017	-1.16	-1.19	-1.34*
unknown	ncrna:misc_RNA chromosome	ENST00000410374	-1.15	-1.30	-1.34*
HSU88895†	Human endogenous retrovirus H D1 leader region	U88895	1.12**	1.14	-1.34*
TAS2R19	taste receptor, type 2, member 19	NM_176888	-1.13	-1.29	-1.35*
SNORD74†	small nucleolar RNA, C/D box 74	NR_002579	1.07**	-1.07	-1.35*
CCDC110†	coiled-coil domain containing 110	NM_152775	-1.12**	-1.15	-1.35*
TNFRSF11A†	tumour necrosis factor receptor superfamily, member 11a	NM_003839	-1.11**	-1.20	-1.35*
TDO2	tryptophan 2,3-dioxygenase	NM_005651	-1.27	-1.24	-1.36*
NPR3	natriuretic peptide receptor C/guanylate cyclase C	NM_000908	-1.17	-1.27	-1.36*
NDUFB6†	NADH dehydrogenase subunit 6, Mitochondrion	NC_001807	1.04**	1.09	-1.36*
SNORD82†	small nucleolar RNA, C/D box 82	NR_004398	-1.05**	-1.06	-1.39*
EFNB2	ephrin-B2	NM_004093	-1.27	-1.25	-1.39*
FAM113B	family with sequence similarity 113, member B	BC008360	-1.27	-1.16	-1.40*
SNORD30†	small nucleolar RNA, C/D box 30	NR_002561	-1.11**	-1.03	-1.40*
ZNF624†	zinc finger protein 624	NM_020787	-1.14**	-1.13	-1.40*
CENPE	centromere protein E, 312kDa	NM_001813	-1.18	-1.22	-1.40*
SECTM1†	secreted and transmembrane 1	NM_003004	-1.17**	-1.40	-1.41*
PPP2R3A	protein phosphatase 2 (formerly 2A), regulatory subunit	NM_002718	-1.30	-1.30	-1.44*
unknown†			-1.10**	1.16	-1.45*
unknown	gi|17981852|ref|NC	NC_001807	-1.09	-1.07	-1.45*
MLLT3	myeloid/lymphoid or mixed-lineage leukemia	NM_004529	-1.26	-1.27	-1.46*
MGP	matrix Gla protein	NM_000900	-1.12	-1.27	-1.46*
C6orf211	chromosome 6 open reading frame 211	BC011348	-1.27	-1.36	-1.47*
HAUS6	HAUS augmin-like complex, subunit 6	NM_017645	-1.20	-1.17	-1.47*
SNORA38B†	small nucleolar RNA, H/ACA box 38B (retrotransposed)	NR_003706	1.10**	-1.10	-1.48*
unknown	ncrna:misc_RNA chromosome	ENST00000363651	-1.06	-1.34	-1.49*
unknown†			1.10**	1.21	-1.49*
unknown†			-1.22**	-1.18	-1.55*
VTRNA1-1†	vault RNA 1-1	NR_026703	-1.11**	-1.10	-1.60*
VTRNA1-2†	vault RNA 1-2	NR_026704	1.05**	-1.13	-1.81*
unknown†	ncrna:miRNA chromosome	ENST00000459170	-1.20**	-1.02	-1.81*
unknown	ncrna:misc_RNA chromosome:	ENST00000384467	-1.36	-1.12	-1.83*
unknown†			1.21**	-1.22	-1.89*

Appendix 4 - Table 7: Genes significantly induced by the hormone combination compared to DHT alone (All genes by regulated by DHT and MPA alone or combined were significantly induced compared to control, $p<0.05$)

	DHT and MPA combined induced genes		Fold Change		
Gene Symbol	Gene Name	Accession Number	1nM DHT	1nM MPA	1nM DHT 1nM MPA
C1orf116	chromosome 1 open reading frame 116	NM_023938	7.70	1.9	5.18*
SEC14L2	SEC14-like 2 (S. cerevisiae)	NM_012429	4.52	5.28	7.70*
unknown	ncrna:snRNA chromosome:	ENST00000410983	4.06*	3.59	6.42
CLDN8	claudin 8	NM_199328	4.05	2.97	4.67
UGT2B11	UDP glucuronosyltransferase 2 family, polypeptide B11	NM_001073	3.64	1.67	3.37*
FKBP5	FK506 binding protein 5	NM_001145775	2.61	5.27	5.38*
AQP3	aquaporin 3 (Gill blood group)	NM_004925	2.37	1.39	2.81*
SLC15A2	solute carrier family 15 (H+/peptide transporter)	NM_021082	2.13	2.15	3.12*
EAF2	ELL associated factor 2	NM_018456	2.09	1.94	2.33
STEAP2	six transmembrane epithelial antigen of the prostate 2	NM_152999	1.93	2.80	2.94*
ZNF689	zinc finger protein 689	NM_138447	1.88	2.12	2.53*
unknown	ncrna:snoRNA chromosome	ENST00000458976	1.85	1.56	1.92
DKFZP564O0823	prostatic androgen-repressed message-1	NM_015393	1.78	1.97	3.09*
GREB1	GREB1 protein	NM_014668	1.77	5.43	5.63*
EPGN	epithelial mitogen homolog (mouse)	NM_001013442	1.72	1.97	1.81
SCUBE2	signal peptide, CUB domain, EGF-like 2	NM_020974	1.68	3.67	3.70*
TARP	TCR gamma alternate reading frame protein	NM_30894	1.67	1.33	1.68
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif	NM_139025	1.65	1.73	1.66
THBS1	thrombospondin 1	NM_003246	1.65	2.27	2.31**
FAM105A	family with sequence similarity 105, member A	NM_019018	1.63	3.54	3.19**
SERHL	serine hydrolase-like	NR_027786	1.62	1.37	1.71
SASH1	SAM and SH3 domain containing 1	NM_015278	1.62	1.75	1.94
ANKRD22	ankyrin repeat domain 22	NM_144590	1.62	2.25	2.14
RAB3B	RAB3B, member RAS oncogene family	NM_002867	1.61	2.43	2.57*
CROT	microtubule-associated protein 9	NM_001039580	1.60	1.43	1.58*
SLC26A3	solute carrier family 26, member 3	NM_000111	1.55	1.65	2.03*
FLJ41603	FLJ41603 protein	NM_001001669	1.55	1.86	2.15*
TTC12	tetratricopeptide repeat domain 12	NM_017868	1.54	1.25	1.38*
TBC1D2	TBC1 domain family, member 22B	NM_017772	1.54	2.79	2.73*
WWC1	WW and C2 domain containing 1	NM_001161661	1.49	1.79	2.18*
PISD	phosphatidylserine decarboxylase	NM_014338	1.48	2.33	2.12*
UGDH	UDP-glucose dehydrogenase	NM_003359	1.48	1.59	1.57
TPD52	tumour protein D52-like 1	NM_001003395	1.47	1.56	1.68
WWC3	WWC family member 3	NM_015691	1.47	1.45	1.84*
MIR21	microRNA 21	AY699265	1.46	2.48	3.26*

Gene Symbol	DHT and MPA combined induced genes Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1nM MPA
PMEPA1	prostate transmembrane protein, androgen induced 1	NM_020182	1.46	1.71	1.93*
PER1	period homolog 1 (Drosophila)	NM_002616	1.46	1.94	2.23*
RGNEF	Rho-guanine nucleotide exchange factor	NM_001080479	1.45	1.82	1.81*
PARP9	poly (ADP-ribose) polymerase family, member	NM_031458	1.45	1.61	1.59
PRAGMIN	homolog of rat pragma of Rnd2	NM_001080826	1.43	1.48	1.76*
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer	NM_020529	1.42	1.67	2.05
GNMT	glycine N-methyltransferase	NM_018960	1.42	1.63	1.90*
LEF1	lymphoid enhancer-binding factor 1	NM_016269	1.42	2.00	2.06*
ZNF652	zinc finger protein 652	NM_014897	1.42	1.56	1.73*
MTSS1	metastasis suppressor 1	NM_014751	1.42	1.39	1.83*
SGEF	RasGEF domain family, member 1A	NM_145313	1.40	1.61	1.83*
SOCS2	suppressor of cytokine signaling 2	NM_003877	1.40	1.23	1.47
SLC26A2	solute carrier family 26 (sulfate transporter), member 2	NM_000112	1.39	1.46	1.49
RAB11FIP1	RAB11 family interacting protein 1 (class I)	NM_001002814	1.38	1.60	1.87*
AKR1C3	aldo-keto reductase family 1, member C3	NM_003739	1.38	1.61	1.67*
FAM46C	family with sequence similarity 46, member C	NM_017709	1.38	1.63	1.63*
PTPRJ	protein tyrosine phosphatase, receptor type, J	NM_002843	1.38	1.36	1.39
GPRC5A	G protein-coupled receptor, family C, group 5, member A	NM_003979	1.37	1.40	1.48
CRAT	scratch homolog 2, zinc finger protein (Drosophila)	NM_033129	1.37	1.16	1.41
ADD3	adducin 3 (gamma)	NM_016824	1.36	1.35	1.33*
KRT10	keratin 10	NM_000421	1.35	1.27	1.32*
KRT4	keratin 40	NM_182497	1.33	1.26	1.41
HSD11B2	hydroxysteroid (11-beta) dehydrogenase 2	NM_000196	1.33	1.84	2.18*
NDRG1	N-myc downstream regulated 1	NM_001135242	1.33	3.10	3.27*
TSKU	tsukushin	NM_015516	1.32	1.49	1.53*
RGS2	regulator of G-protein signaling 20	NM_170587	1.32	1.95	2.22*
IL20RA	Homo sapiens interleukin 20 receptor, alpha (IL20RA),	NM_014432	1.31	3.64	3.82*
EHF	ets homologous factor	NM_012153	1.31	1.25	1.35
HPGD	hematopoietic prostaglandin D synthase	NM_014485	1.31	1.68	1.99*
HERC3	hect domain and RLD 3	NM_014606	1.31	1.35	1.33
SLC2A3	solute carrier family 2 (facilitated glucose transporter)	NM_006931	1.31	2.42	3.99*
PPAPDC1A	phosphatidic acid phosphatase type 2 domain	NM_001030059	1.30	1.25	1.33
NEDD4L	neural precursor cell expressed,	NM_001144967	1.30	1.83	1.90*
LMO3	LIM domain only 3 (rhombotin-like 2)	NM_018640	1.30	1.21	1.46
HSPA2	heat shock 70kDa protein 2	NM_021979	1.30	1.34	1.47
TMEM164	transmembrane protein 164	NM_032227	1.28	2.08	2.04*
YPEL3	yippee-like 3 (Drosophila)	NM_031477	1.28	1.20	1.22
GRHL2	grainyhead-like 2 (Drosophila)	NM_024915	1.27	1.32	1.47*

Gene Symbol	DHT and MPA combined induced genes Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1nM MPA
HAAO	3-hydroxyanthranilate 3,4-	NM_012205	1.27	1.28	1.41
CDC42EP3	CDC42 effector protein (Rho GTPase binding) 3	NM_006449	1.27	2.09	1.84*
C10orf141	chromosome 10 open reading frame 141	NM_001039762	1.27	1.92	2.04*
TESK2	testis-specific kinase 2	NM_007170	1.26	1.18	1.30
PARP14	poly (ADP-ribose) polymerase family, member 14	NM_017554	1.25	1.31	1.37
BCL6	B-cell CLL/lymphoma 6	NM_001706	1.25	1.41	1.51*
KRT15	keratin 15	NM_002275	1.25	1.22	1.58*
ZNF703	zinc finger protein 703	NM_025069	1.24	1.47	1.62*
LRIG1	leucine-rich repeats and immunoglobulin-like domains 1	NM_015541	1.24	1.88	2.04*
BCL2L1	BCL2-like 15	NM_001010922	1.24	1.26	1.58*
GSTM3	glutathione S-transferase mu 3 (brain)	NM_000849	1.24	1.52	1.45*
PRR15	proline rich 15	NM_175887	1.24	1.23	1.31
GLUL	glutamate-ammonia ligase (glutamine synthetase)	NM_002065	1.24	1.60	1.62*
PXMP4	peroxisomal membrane protein 4, 24kDa	NM_007238	1.23	1.64	1.75*
SH3PXD2B	SH3 and PX domains 2B	NM_001017995	1.23	1.14	1.39
GPSM2	G-protein signaling modulator 2 (AGS3-like, C. elegans)	NM_013296	1.23	1.67	1.74
BAG3	BCL2-associated athanogene 3	NM_004281	1.23	1.11	1.20
AKR1C1	Homo sapiens aldo-keto reductase family 1, member C1	NM_001353.5	1.23	1.45	1.49
ELL2	elongation factor, RNA polymerase II, 2	NM_012081	1.23	1.30	1.37
RARB	retinoic acid receptor, beta	NM_000965	1.22	1.71	1.84*
CD9	CD96 molecule	NM_198196	1.22	1.27	1.42*
DBI	diazepam binding inhibitor	NM_020548	1.22	1.29	1.51*
MT2A	TRM2 tRNA methyltransferase 2 homolog A	NM_022727	1.21	1.72	1.68*
PNKD	paroxysmal nonkinesigenic dyskinesia	NM_015488	1.20	1.30	1.48*
KRT19	keratin 19 pseudogene 2	AB041269	1.20	1.32	1.35*
SLC16A6	solute carrier family 16, member 6	NM_004694	1.20	1.86	2.02*
TIPARP	TCDD-inducible poly(ADP-ribose) polymerase	NM_015508	1.20	2.07	2.86*
LNX2	ligand of numb-protein X 2	NM_153371	1.19	1.12	1.22
HECTD3	HECT domain containing 3	NM_024602	1.19	1.59	1.79*
ALCAM	activated leukocyte cell adhesion molecule	NM_001627	1.19	1.19	1.27
BIRC3	baculoviral IAP repeat-containing 3	NM_001165	1.19	2.65	2.51*
SULT2B1	sulfotransferase family, cytosolic, 2B, member 1	NM_004605	1.19	1.32	1.41*
BRP44	brain protein 44-like	NM_016098	1.18	1.10	1.23
SGK1	protein kinase-like protein SgK196	NM_032237	1.18	1.64	1.59
SPRED2	sprouty-related, EVH1 domain containing 2	NM_181784	1.18	1.39	1.57*
PRKAB2	protein kinase, AMP-activated, beta 2 non-catalytic subunit	NM_005399	1.17	1.69	1.91*
ZBTB7C	zinc finger and BTB domain containing 7C	NM_001039360	1.17	1.28	1.49*
unknown	ncrna:snRNA chromosome	ENST00000411132	1.16	1.12	1.10*
TES	testis derived transcript	NM_015641	1.17	1.15	1.28*

Gene Symbol	DHT and MPA combined induced genes Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1nM MPA
IRX3	iroquois homeobox 3	NM_024336	1.16	1.17	1.22
MCCC2	methylcrotonoyl-Coenzyme A carboxylase 2 (beta)	NM_022132	1.13	1.25	1.27*
AZGP1	alpha-2-glycoprotein 1, zinc-binding	NM_001185	1.13	1.36	1.42*
PEA15	phosphoprotein enriched in astrocytes 15	NM_003768	1.13	1.17	1.26*
CLMN	calmin (calponin-like, transmembrane)	NM_024734	1.13	1.45	1.50*
EFHD1	EF-hand domain family, member D1	NM_025202	1.12	1.21	1.31*
SLC2A1	Homo sapiens solute carrier family 2	NM_030777	1.12	1.10	1.15
SORD	sorbitol dehydrogenase	NM_003104	1.11	1.12	1.17
FXYD3	FXYD domain containing ion transport regulator 3	NM_021910	1.11	1.10	1.11
EMP2	transmembrane emp24 protein	NM_006858	1.07	1.23	1.22*

Appendix 4 - Table 8: Genes significantly repressed by the hormone combination compared to DHT alone (All genes by DHT and MPA alone or combined were significantly repressed compared to control, $p<0.05$)

Gene Symbol	DHT and MPA Alone and Combined Genes Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1nM MPA
SMA5	glucuronidase, beta pseudogene	AK289851	-1.10	-1.15	-1.15
GUSBL1	glucuronidase, beta-like 1	NR_003504	-1.10	-1.15	-1.12
CDV3	CDV3 homolog (mouse)	NM_001134422	-1.11	-1.14	-1.18
LOC91316	glucuronidase, beta/ immunoglobulin lambda-like polypeptide	NR_024448	-1.11	-1.14	-1.11
STS	megalencephalic leukoencephalopathy with subcortical cysts	NM_015166	-1.13	-1.18	-1.25*
C3orf70	chromosome 3 open reading frame 70	BC137178	-1.13	-1.17	-1.23
SLC12A2	solute carrier family 12	NM_001046	-1.14	-1.13	-1.21
TMTC1	transmembrane and tetratricopeptide repeat containing 1	NM_175861	-1.14	-1.12	-1.15
GLCE	glucuronic acid epimerase	NM_015554	-1.15	-1.20	-1.40*
NVL	nuclear VCP-like	NM_002533	-1.15	-1.24	-1.29
PAK2	p21 protein (Cdc42/Rac)-activated kinase 2	NM_002577	-1.15	-1.21	-1.21
NAIP	NLR family, apoptosis inhibitory protein	NM_004536	-1.15	-1.16	-1.17
unknown			-1.15	-1.16	-1.17
UEVLD	UEV and lactate/malate dehyrogenase domains	NM_001040697	-1.15	-1.14	-1.15
BCAS1	breast carcinoma amplified sequence 1	NM_003657	-1.16	-1.24	-1.31*
PDLIM5	PDZ and LIM domain 5	NM_006457	-1.16	-1.12	-1.13*
NCRNA00085	non-protein coding RNA 85	NR_024330	-1.16	-1.20	-1.13*
GRHL3	grainyhead-like 3 (Drosophila)	NM_198173	-1.16	-1.26	-1.32*
GNA14	guanine nucleotide binding protein (G protein), alpha 14	NM_004297	-1.17	-1.17	-1.17
SECTM1	secreted and transmembrane 1	NM_003004	-1.17	-1.40	-1.41*

Gene Symbol	DHT and MPA Alone and Combined Genes Gene Name	Accession Number	Fold Change 1nM DHT	 1nM MPA	 1nM DHT 1nM MPA
ZSCAN22	zinc finger and SCAN domain containing 22	NM_181846	-1.17	-1.21	-1.16
GCNT1	glucosaminyl (N-acetyl) transferase 1, core 2	NM_001490	-1.17	-1.30	-1.29
LIPH	lipase, member H	NM_139248	-1.17	-1.18	-1.33*
ST3GAL1	ST3 beta-galactoside alpha-2,3-sialyltransferase 1	NM_003033	-1.18	-1.22	-1.36*
GPR37	G protein-coupled receptor 37 like 1	NM_004767	-1.18	-1.19	-1.29
ICOSLG	inducible T-cell co-stimulator ligand	NM_015259	-1.18	-1.24	-1.20
SORL1	sortilin-related receptor	NM_003105	-1.18	-1.18	-1.20
DIP2B	DIP2 disco-interacting protein 2 homolog B (Drosophila)	NM_173602	-1.18	-1.21	-1.25
LRRN2	leucine rich repeat neuronal 2	NM_006338	-1.19	-1.21	-1.17
GLCCI1	glucocorticoid induced transcript 1	NM_138426	-1.19	-1.19	-1.24
USP25	ubiquitin specific peptidase 25	NM_013396	-1.19	-1.18	-1.16*
EPS	EPS8-like 3	NM_139053	-1.19	-1.19	-1.28*
FUT8	fucosyltransferase 8 (alpha (1,6) fucosyltransferase)	NM_178155	-1.19	-1.33	-1.26
PREX1	phosphatidylinositol-3,4,5-trisphosphate-dependent	NM_020820	-1.20	-1.29	-1.37*
CACNA1D	calcium channel, voltage-dependent, L type, alpha 1D subunit	NM_000720	-1.20	-1.25	-1.23
CCNJL	cyclin J-like	NM_024565	-1.20	-1.23	-1.20
RNF144B	ring finger protein 144B	NM_182757	-1.20	-1.16	-1.27
UMODL1	uromodulin-like 1	NM_001004416	-1.20	-1.28	-1.18*
LTBP1	latent transforming growth factor beta binding protein 1	NM_206943	-1.20	-1.17	-1.18*
FREM2	FRAS1 related extracellular matrix protein 2	NM_207361	-1.21	-1.26	-1.32
RERG	RAS-like, estrogen-regulated, growth inhibitor	NM_032918	-1.21	-1.33	-1.59*
SOCS1	suppressor of cytokine signaling 1	NM_003745	-1.21	-1.19	-1.39*
RBL2	retinoblastoma-like 2 (p130)	NM_005611	-1.22	-1.25	-1.25
RABEP1	rabaptin, RAB GTPase binding effector protein 1	NM_004703	-1.22	-1.25	-1.39
HTR1D	5-hydroxytryptamine (serotonin) receptor 1D	NM_000864	-1.23	-1.25	-1.29
UBL3	ubiquitin-like 3	NM_007106	-1.23	-1.29	-1.38
SLC46A3	solute carrier family 46, member 3	NM_181785	-1.24	-1.25	-1.31
AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9	NM_032717	-1.24	-1.33	-1.45*
ROCK2	Rho-associated, coiled-coil containing protein kinase 2	NM_004850	-1.25	-1.29	-1.33
VTCN1	V-set domain containing T cell activation inhibitor 1	NM_024626	-1.25	-1.22	-1.37
SOX9	SRY (sex determining region Y)-box 9	NM_000346	-1.25	-1.26	-1.23*
ITPRIPL2	inositol 1,4,5-triphosphate receptor interacting protein	NM_001034841	-1.25	-1.49	-1.61*
MPP7	membrane protein, palmitoylated 7 (MAGUK p55 subfamily member	NM_173496	-1.26	-1.24	-1.28
PHF20	PHD finger protein 20	NM_016436	-1.26	-1.24	-1.37
MARK1	MAP/microtubule affinity-regulating kinase 1	NM_018650	-1.26	-1.24	-1.23*
AR	androgen receptor	NM_000044	-1.26	-1.39	-1.33
LPIN2	lipin 2	NM_014646	-1.26	-1.26	-1.26
RNF152	ring finger protein 152	NM_173557	-1.26	-1.22	-1.39*

Gene Symbol	DHT and MPA Alone and Combined Genes Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1nM MPA
ZSWIM6	zinc finger, SWIM-type containing 6	NM_020928	-1.27	-1.28	-1.35
SLC16A14	solute carrier family 16, member 14	NM_152527	-1.27	-1.15	-1.31
NRCAM	neuronal cell adhesion molecule	NM_001037132	-1.27	-1.41	-1.44*
ATP8A1	ATPase, aminophospholipid transporter (APLT), class I,	NM_006095	-1.27	-1.23	-1.26*
PPFIBP2	PTPRF interacting protein, binding protein 2	NM_003621	-1.28	-1.31	-1.31
DOCK10	dedicator of cytokinesis 10	NM_014689	-1.28	-1.43	-1.49*
GUCY1A3	guanylate cyclase 1, soluble, alpha 3	NM_000856	-1.28	-1.20	-1.31
DLG2	discs, large homolog 2 (Drosophila)	NM_001364	-1.29	-1.21	-1.33
SOX5	SRY (sex determining region Y)-box 5	NM_152989	-1.29	-1.31	-1.34
PTAFR	platelet-activating factor receptor	NM_000952	-1.30	-1.24	-1.42
PXK	PX domain containing serine/threonine kinase	NM_017771	-1.30	-1.25	-1.21*
PGR	progesterone receptor	NM_000926	-1.30	-1.62	-1.68*
SCYL3	SCY1-like 3 (S. cerevisiae)	NM_181093	-1.31	-1.24	-1.28*
FAM19A2	family with sequence similarity 19	NM_17853	-1.31	-1.35	-1.38
C9orf150	chromosome 9 open reading frame 150	NM_203403	-1.31	-1.28	-1.47*
TRERF1	transcriptional regulating factor 1	NM_033502	-1.32	-1.25	-1.33
TSPAN12	tetraspanin 12	NM_012338	-1.32	-1.26	-1.34
MAP2	microtubule-associated protein 2	NM_002374	-1.32	-1.39	-1.36
TCP11L2	t-complex 11 (mouse)-like 2	NM_152772	-1.33	-1.41	-1.45
CDKN2B	cyclin-dependent kinase inhibitor 2B	NM_078487	-1.33	-1.19	-1.36
FAM83B	family with sequence similarity 83, member B	NM_001010872	-1.33	-1.22	-1.38
POU2F3	POU class 2 homeobox 3	NM_014352	-1.35	-1.26	-1.50
DAB2	disabled homolog 2, mitogen-responsive phosphoprotein	NM_001343	-1.36	-1.28	-1.40
CABLES1	Cdk5 and Abl enzyme substrate 1	NM_138375	-1.37	-1.35	-1.48
CEACAM5	carcinoembryonic antigen-related cell adhesion molecule	NM_004363	-1.37	-1.42	-1.48
FGD6	FYVE, RhoGEF and PH domain containing 6	NM_018351	-1.39	-1.29	-1.39
INSIG2	insulin induced gene 2	NM_016133	-1.41	-1.36	-1.50
BAMBI	BMP and activin membrane-bound inhibitor homolog	NM_012342	-1.42	-1.53	-1.90*
AMIGO2	adhesion molecule with Ig-like domain 2	NM_001143668	-1.42	-1.39	-1.78*
DST	dual serine/threonine and tyrosine protein kinase	NM_015375	-1.42	-1.25	-1.37*
SRGAP3	SLIT-ROBO Rho GTPase activating protein 3	NM_014850	-1.42	-1.32	-1.57
BCL2	BCL2/adenovirus E1B 19kDa interacting protein 1	NM_013979	-1.43	-1.48	-1.70
IL1R1	interleukin 1 receptor, type I	NM_000877	-1.43	-1.56	-1.98*
PDGFD	platelet derived growth factor D	NM_025208	-1.44	-1.58	-1.73
GPR98	G protein-coupled receptor 98	NM_032119	-1.45	-1.36	-1.39*
ESR1	estrogen receptor 1	X74439	-1.46	-1.77	-1.93*
TACR3	tachykinin receptor 3	NM_001059	-1.46	-1.44	-1.61
BMP4	bone morphogenetic protein 4	NM_001202	-1.54	-2.01	-2.43*
unknown	Homo sapiens mRNA; cDNA DKFZp451C2311	AL832451	-1.56	-1.49	-1.66
SOX2	SRY (sex determining region Y)-box 21	NM_007084	-1.74	-2.10	-2.41*

Gene Symbol	DHT and MPA Alone and Combined Genes Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1nM MPA
ITGA5	integrin, alpha 5	NM_002205	-1.81	-1.27	-1.78*
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin)	NM_001143818	-1.88	-2.14	-2.62*

Appendix 4 - Table 9: Genes significantly regulated (induced or repressed) by DHT and MPA alone

Gene Symbol	DHT and MPA Alone Induced Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1 nM MPA
OR4F17†	olfactory receptor, family 4, subfamily F, member 17	NM_001005240	1.55*	1.46*	1.05**
OR11H1†	olfactory receptor, family 11, subfamily H, member 1	NM_001005239	1.40*	1.38*	-1.08**
unknown	24 kDa protein gene	ENST00000341827	1.18*	1.20*	1.03***
DDX60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	NM_017631	1.17*	1.16*	1.12
LOC728212†	hypothetical LOC728212	AK302783	1.17*	1.16*	-1.06***
DDX60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	NM_017631	1.17*	1.16*	1.12
SHANK2	Homo sapiens SH3 and multiple ankyrin repeat domains 2	NM_012309	-1.15*	-1.14*	-1.12
ZNF217	zinc finger protein 217	NM_006526	-1.17*	-1.17*	-1.12
VPS13B	vacuolar protein sorting 13 homolog B (yeast)	NM_017890	-1.17*	-1.17*	-1.12
IGSF3	Homo sapiens immunoglobulin superfamily, member 3	NM_001542	-1.18*	-1.17*	-1.12
SMARCA2	SWI/SNF related, matrix associated,	NM_003070	-1.12*	-1.09*	-1.03
MGC13005	hypothetical LOC84771	NR_024005	-1.22*	-1.19*	-1.16
HEY2†	hairy/enhancer-of-split related with YRPW motif 2	NM_012259	1.31*	-1.43*	-1.21**
FAM49A†	family with sequence similarity 49, member A	NM_030797	-1.55*	1.25*	1.08**

Appendix 3 - Table 10: Select genes significantly regulated by DHT and MPA combined compared to DHT or MPA alone

DHT alone and DHT and MPA combined - Induced Gene Symbol	Gene Name	Accession Number	Fold Change 1nM DHT	1nM MPA	1nM DHT 1 nM MPA
OLR1	Homo sapiens oxidized low density lipoprotein (lectin-like protein)	NM_002543	2.61*	-1.06	1.23**
HIPK2	homeodomain interacting protein kinase 2	NM_022740	1.22*	1.16	1.46**
unknown	ncrna:snoRNA chromosome	ENST00000458880	1.12*	-1.08	-1.16**
DHT alone and DHT and MPA combined - Repressed			**Fold Change**		
Gene Symbol	**Gene Name**	**Accession Number**	**1nM DHT**	**1nM MPA**	**1nM DHT 1 nM MPA**
TSPAN15	tetraspanin 15	NM_012339	-1.09*	-1.05	-1.19**
NFX1	nuclear transcription factor, X-box binding 1	NM_002504	-1.10*	1.05	1.12**
SLC35F2	solute carrier family 35, member F2	NM_017515	-1.12*		-1.18**
RND1	Rho family GTPase 1	NM_014470	-1.15	-1.31*	-1.55**
CASP4	caspase 4, apoptosis-related cysteine	NM_033306	-1.08	-1.14*	-1.36**

MPA alone and DHT and MPA combined - Repressed			Fold Change		
Gene Symbol	**Gene Name**	**Accession Number**	**1nM DHT**	**1nM MPA**	**1nM DHT 1 nM MPA**
ATP13A4	ATPase type 13A4	NM_032279	-1.00	2.12*	3.02**
SPRY4	sprouty homolog 4 (Drosophila)	NM_030964	1.08	1.95*	2.32**
LHFP	lipoma HMGIC fusion partner	NM_005780	1.15	1.89*	2.23**
unknown	Homo sapiens PP10897 mRNA, complete cds	AF370400	1.27	1.88*	2.92**
MUM1L1	melanoma associated antigen (mutated) 1-like 1	NM_152423	1.30	1.81*	2.69**
FMO5	flavin containing monooxygenase 5	NM_001461	1.02	1.65*	2.60**
C14orf162	chromosome 14 open reading frame 162	NR_024630	1.10	1.64*	1.37**
C6orf192	chromosome 6 open reading frame 192	NM_052831	-1.04	1.58*	1.25**
KCNQ3	potassium voltage-gated channel, KQT-like subfamily	NM_004519	1.06	1.57*	1.78**
FHDC1	FH2 domain containing 1	NM_033393	1.17	1.51*	1.86**
MPA alone and DHT and MPA combined - Repressed			**Fold Change**		
Gene Symbol	**Gene Name**	**Accession Number**	**1nM DHT**	**1nM MPA**	**1nM DHT 1 nM MPA**
MTMR9	myotubularin related protein 9	NM_015458	1.17	1.47*	1.72**
unknown	hsa-mir-29c	MI0000735	1.17	1.39*	1.66**
NR4A1	nuclear receptor subfamily 4, group A, member 1	NM_002135	1.06	1.39*	1.58**
TPRA1	transmembrane protein, adipocyte asscociated 1	NM_016372	1.02	1.30*	1.20**
CBX4	chromobox homolog 4 (Pc class homolog, Drosophila)	NM_003655	1.14	1.27*	1.60**
BCL7C	B-cell CLL/lymphoma 7C	NM_004765	-1.02	1.23*	1.15**
CUEDC1	CUE domain containing 1	NM_017949	1.12	1.22*	1.35**
PEG10	paternally expressed 10	NM_015068	1.07	1.20*	1.40**
STEAP4	STEAP family member 4	NM_024636	1.11	1.19*	1.43**
DFNB31	deafness, autosomal recessive 31	NM_015404	1.07	1.18*	1.40**
DNAJB1	DnaJ (Hsp40) homolog, subfamily B, member 12	NM_001002762	1.02	1.15*	1.30**
FAM20C	family with sequence similarity 20, member C	NM_020223	-1.00	1.13*	1.25*8

Bibliography

(1995). "Effects of estrogen or estrogen/progestin regimens on heart disease risk factors in postmenopausal women. The Postmenopausal Estrogen/Progestin Interventions (PEPI) Trial. The Writing Group for the PEPI Trial." Jama **273**(3): 199-208.

Abbas S, Beckmann L, et al. (2010). "Polymorphisms in genes of the steroid receptor superfamily modify postmenopausal breast cancer risk associated with menopausal hormone therapy." Int J Cancer **Jun 15**(126(12):): 2935-46.

Abraham, G. E. (1974). "Ovarian and adrenal contribution to peripheral androgens during the menstrual cycle." J Clin Endocrinol Metab **39**(2): 340-6.

Affandi, B. (2002). "Injectable contraceptives: a worldwide perspective." J Fam Plann Reprod Health Care **28**(1): 3-4.

Africander, D., N. Verhoog, et al. (2011). "Molecular mechanisms of steroid receptor-mediated actions by synthetic progestins used in HRT and contraception." Steroids **Mar 15**.

Agoff, S. N., P. E. Swanson, et al. (2003). "Androgen receptor expression in estrogen receptor-negative breast cancer. Immunohistochemical, clinical, and prognostic associations." Am J Clin Pathol **120**(5): 725-31.

Almog, N. "Molecular mechanisms underlying tumor dormancy." Cancer Lett **294**(2): 139-46.

Almog, N., L. Ma, et al. (2009). "Transcriptional switch of dormant tumors to fast-growing angiogenic phenotype." Cancer Res **69**(3): 836-44.

Ambaye, A. B., S. E. MacLennan, et al. (2009). "Carcinoma and atypical hyperplasia in reduction mammaplasty: increased sampling leads to increased detection. A prospective study." Plast Reconstr Surg **124**(5): 1386-92.

Amler, L. C., D. B. Agus, et al. (2000). "Dysregulated expression of androgen-responsive and nonresponsive genes in the androgen-independent prostate cancer xenograft model CWR22-R1." Cancer Res **60**(21): 6134-41.

Anderson, G. L., M. Limacher, et al. (2004). "Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial." Jama **291**(14): 1701-12.

Anderson, S. E., G. E. Dallal, et al. (2003). "Relative weight and race influence average age at menarche: results from two nationally representative surveys of US girls studied 25 years apart." Pediatrics **111**(4 Pt 1): 844-50.

Anderstrom, C., A. Eddeland, et al. (1995). "Epirubicin and medroxyprogesterone acetate versus estramustine phosphate in hormone-resistant prostatic cancer: a prospective randomized study." Eur Urol **27**(4): 301-5.

Ando, S., F. De Amicis, et al. (2002). "Breast cancer: from estrogen to androgen receptor." Mol Cell Endocrinol **193**(1-2): 121-8.

Andrews, W. C. and M. C. Andrews (1962). "The use of progestins for oral contraception." South Med J **55**: 454-6.

Asano, K., S. Maruyama, et al. (2003). "Regulation of estrogen receptor alpha and beta expression by testosterone in the rat prostate gland." Endocr J **50**(3): 281-7.

Aspinall, S. R., S. Stamp, et al. (2004). "The proliferative effects of 5-androstene-3 beta,17 beta-diol and 5 alpha-dihydrotestosterone on cell cycle analysis and cell proliferation in MCF7, T47D and MDAMB231 breast cancer cell lines." J Steroid Biochem Mol Biol **88**(1): 37-51.

Assersohn, L., J. Salter, et al. (2003). "Studies of the potential utility of Ki67 as a predictive molecular marker of clinical response in primary breast cancer." Breast Cancer Res Treat **82**(2): 113-23.

Atwood, C. S., R. C. Hovey, et al. (2000). "Progesterone induces side-branching of the ductal epithelium in the mammary glands of peripubertal mice." J Endocrinol **167**(1): 39-52.

Au, P. Y. and W. C. Yeh (2007). "Physiological roles and mechanisms of signaling by TRAF2 and TRAF5." Adv Exp Med Biol **597**: 32-47.

Baglietto, L., D. R. English, et al. (2009). "Circulating steroid hormone concentrations in postmenopausal women in relation to body size and composition." Breast Cancer Res Treat **115**(1): 171-9.

Bakker, G. H., B. Setyono-Han, et al. (1990). "Treatment of breast cancer with different antiprogestins: preclinical and clinical studies." J Steroid Biochem Mol Biol **37**(6): 789-94.

Ball, R. K., R. R. Friis, et al. (1988). "Prolactin regulation of beta-casein gene expression and of a cytosolic 120-kd protein in a cloned mouse mammary epithelial cell line." Embo J **7**(7): 2089-95.

Balogh, G. A., R. Heulings, et al. (2006). "Genomic signature induced by pregnancy in the human breast." Int J Oncol **28**(2): 399-410.

Balogh, G. A., J. Russo, et al. (2007). "The breast of parous women without cancer has a different genomic profile compared to those with cancer." Int J Oncol **31**(5): 1165-75.

Basuray, R., R. G. Rawlins, et al. (1988). "High progesterone/estradiol ratio in follicular fluid at oocyte aspiration for in vitro fertilization as a predictor of possible pregnancy." Fertil Steril **49**(6): 1007-11.

Beatson, G. T. (1896). "On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases." Lancet **2**: 104-107.

Beilin, J., E. M. Ball, et al. (2000). "Effect of the androgen receptor CAG repeat polymorphism on transcriptional activity: specificity in prostate and non-prostate cell lines." J Mol Endocrinol **25**(1): 85-96.

Beleut, M., R. D. Rajaram, et al. (2010). "Two distinct mechanisms underlie progesterone-induced proliferation in the mammary gland
" Proc Natl Acad Sci U S A **107**(7): 2989-94.

Bennett, I. C., J. F. McCaffrey, et al. (1990). "Changing patterns in the presentation of breast cancer over 25 years." Aust N Z J Surg **60**(9): 665-71.

Bentel, J. M., S. N. Birrell, et al. (1999). "Androgen receptor agonist activity of the synthetic progestin, medroxyprogesterone acetate, in human breast cancer cells." Mol Cell Endocrinol **154**(1-2): 11-20.

Beral, V. (2003). "Breast cancer and hormone-replacement therapy in the Million Women Study." Lancet **362**(9382): 419-27.

Beral, V., E. Banks, et al. (2002). "Evidence from randomised trials on the long-term effects of hormone replacement therapy." Lancet **360**(9337): 942-4.
Beral, V., D. Bull, et al. (2005). "Endometrial cancer and hormone-replacement therapy in the Million Women Study." Lancet **365**(9470): 1543-51.
Bernstein, L., M. Allen, et al. (2002). "High breast cancer incidence rates among California teachers: results from the California Teachers Study (United States)." Cancer Causes Control **13**(7): 625-35.
Bieche, I., B. Parfait, et al. (2001). "Quantification of estrogen receptor alpha and beta expression in sporadic breast cancer." Oncogene **20**(56): 8109-15.
Bieche, I., B. Parfait, et al. (2001). "Quantitation of androgen receptor gene expression in sporadic breast tumors by real-time RT-PCR: evidence that MYC is an AR-regulated gene." Carcinogenesis **22**(9): 1521-6.
Billgren, A. M., E. Tani, et al. (2002). "Prognostic significance of tumor cell proliferation analyzed in fine needle aspirates from primary breast cancer." Breast Cancer Res Treat **71**(2): 161-70.
Birrell, S. N., J. M. Bentel, et al. (1995). "Androgens induce divergent proliferative responses in human breast cancer cell lines." J Steroid Biochem Mol Biol **52**(5): 459-67.
Birrell, S. N., L. M. Butler, et al. (2007). "Disruption of androgen receptor signaling by synthetic progestins may increase risk of developing breast cancer." Faseb J.
Blais, Y., K. Sugimoto, et al. (1994). "Potent stimulatory effect of interleukin-1 alpha on apolipoprotein D and gross cystic disease fluid protein-15 expression in human breast-cancer cells." Int J Cancer **59**(3): 400-7.
Blais, Y., K. Sugimoto, et al. (1995). "Interleukin-6 inhibits the potent stimulatory action of androgens, glucocorticoids and interleukin-1 alpha on apolipoprotein D and GCDFP-15 expression in human breast cancer cells." Int J Cancer **62**(6): 732-7.
Blank, E. W., P. Y. Wong, et al. (2008). "Both ovarian hormones estrogen and progesterone are necessary for hormonal mammary carcinogenesis in ovariectomized ACI rats." Proc Natl Acad Sci U S A **105**(9): 3527-32.
Bliss, J. M. and R. Gray (2003). "Breast cancer and hormone-replacement therapy: the Million Women Study." Lancet **362**(9392): 1328-9; author reply 1330-1.
Bodian, C. A., K. H. Perzin, et al. (1993). "Prognostic significance of benign proliferative breast disease." Cancer **71**(12): 3896-907.
Boecker, W. and H. Buerger (2003). "Evidence of progenitor cells of glandular and myoepithelial cell lineages in the human adult female breast epithelium: a new progenitor (adult stem) cell concept." Cell Prolif **36 Suppl 1**: 73-84.
Botella, J., J. Porthe-Nibelle, et al. (1986). "Interaction of new 19 nor progesterone derivatives with progestagen, mineralocorticoid and glucocorticoid cytosolic receptors." J Pharmacol **17**(4): 699-706.
Bottini, A., A. Berruti, et al. (2001). "Relationship between tumour shrinkage and reduction in Ki67 expression after primary chemotherapy in human breast cancer." Br J Cancer **85**(8): 1106-12.
Boyd, N. F., J. M. Rommens, et al. (2005). "Mammographic breast density as an intermediate phenotype for breast cancer." Lancet Oncol **6**(10): 798-808.
Brenner, A. J. and C. M. Aldaz (1997). "The genetics of sporadic breast cancer." Prog Clin Biol Res **396**: 63-82.

Brenner, R. M. and N. B. West (1975). "Hormonal regulation of the reproductive tract in female mammals." Annu Rev Physiol **37**: 273-302.
Brinkmann, A. O., P. W. Faber, et al. (1989). "The human androgen receptor: domain structure, genomic organization and regulation of expression." J Steroid Biochem **34**(1-6): 307-10.
Brown, C. J., S. J. Goss, et al. (1989). "Androgen receptor locus on the human X chromosome: regional localization to Xq11-12 and description of a DNA polymorphism." Am J Hum Genet **44**(2): 264-9.
Bryan, R. M., R. J. Mercer, et al. (1984). "Androgen receptors in breast cancer." Cancer **54**(11): 2436-40.
Buchanan, G., C. Ricciardelli, et al. (2007). "Control of androgen receptor signaling in prostate cancer by the cochaperone small glutamine rich tetratricopeptide repeat containing protein alpha." Cancer Res **67**(20): 10087-96.
Buchanan, G., M. Yang, et al. (2004). "Structural and functional consequences of glutamine tract variation in the androgen receptor." Hum Mol Genet **13**(16): 1677-92.
Burger, H. G. (1999). "The endocrinology of the menopause." J Steroid Biochem Mol Biol **69**(1-6): 31-5.
Burger, H. G., E. C. Dudley, et al. (2000). "A prospective longitudinal study of serum testosterone, dehydroepiandrosterone sulfate, and sex hormone-binding globulin levels through the menopause transition." J Clin Endocrinol Metab **85**(8): 2832-8.
Burger, H. G., E. C. Dudley, et al. (1995). "The endocrinology of the menopausal transition: a cross-sectional study of a population-based sample." J Clin Endocrinol Metab **80**(12): 3537-45.
Burger, H. G., E. C. Dudley, et al. (2002). "Hormonal changes in the menopause transition." Recent Prog Horm Res **57**: 257-75.
Burstein, N. A., R. N. Kjellberg, et al. (1971). "Human carcinoma of the breast, in vitro: the effect of hormones. A prelininary report." Cancer **27**(5): 1112-6.
Canfell, K., E. Banks, et al. (2008). "Decrease in breast cancer incidence following a rapid fall in use of hormone replacement therapy in Australia." Med J Aust **188**(11): 641-4.
Cardenosa, G., C. Doudna, et al. (1994). "Ductography of the breast: technique and findings." AJR Am J Roentgenol **162**(5): 1081-7.
Carder, P. J., C. E. Murphy, et al. (2005). "A multi-centre investigation towards reaching a consensus on the immunohistochemical detection of ERbeta in archival formalin-fixed paraffin embedded human breast tissue." Breast Cancer Res Treat **92**(3): 287-93.
Carroll, J. S., X. S. Liu, et al. (2005). "Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1." Cell **122**(1): 33-43.
Carter, C. L., D. K. Corle, et al. (1988). "A prospective study of the development of breast cancer in 16,692 women with benign breast disease." Am J Epidemiol **128**(3): 467-77.
Casey, R. W. and J. D. Wilson (1984). "Antiestrogenic action of dihydrotestosterone in mouse breast. Competition with estradiol for binding to the estrogen receptor." J Clin Invest **74**(6): 2272-8.

Castellano, I., E. Allia, et al. (2010). "Androgen receptor expression is a significant prognostic factor in estrogen receptor positive breast cancers." Breast Cancer Res Treat.

Centenera, M. M., J. M. Harris, et al. (2008). "The contribution of different androgen receptor domains to receptor dimerization and signaling." Mol Endocrinol **22**(11): 2373-82.

Ceriani, R. L., G. P. Contesso, et al. (1972). "Hormone requirement for growth and differentiation of the human mammary gland in organ culture." Cancer Res **32**(10): 2190-6.

Chakmakjian, Z. H. and N. Y. Zachariah (1987). "Bioavailability of progesterone with different modes of administration." J Reprod Med **32**(6): 443-8.

Chang, C. S., J. Kokontis, et al. (1988). "Molecular cloning of human and rat complementary DNA encoding androgen receptors." Science **240**(4850): 324-6.

Chang, K. J., T. T. Lee, et al. (1995). "Influences of percutaneous administration of estradiol and progesterone on human breast epithelial cell cycle in vivo." Fertil Steril **63**(4): 785-91.

Chen, C. L., N. S. Weiss, et al. (2002). "Hormone replacement therapy in relation to breast cancer." Jama **287**(6): 734-41.

Chen, W. Y., S. E. Hankinson, et al. (2004). "Association of hormone replacement therapy to estrogen and progesterone receptor status in invasive breast carcinoma." Cancer **101**(7): 1490-500.

Cheung, H. H., D. J. Mahoney, et al. (2009). "Down-regulation of c-FLIP Enhances death of cancer cells by smac mimetic compound." Cancer Res **69**(19): 7729-38.

Cheung, J. and D. F. Smith (2000). "Molecular chaperone interactions with steroid receptors: an update." Mol Endocrinol **14**(7): 939-46.

Chlebowski, R. T., S. L. Hendrix, et al. (2003). "Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative Randomized Trial." Jama **289**(24): 3243-53.

Chlebowski, R. T., L. H. Kuller, et al. (2009). "Breast cancer after use of estrogen plus progestin in postmenopausal women." N Engl J Med **360**(6): 573-87.

Clarke, C. A. and S. L. Glaser (2007). "Declines in breast cancer after the WHI: apparent impact of hormone therapy." Cancer Causes Control **18**(8): 847-52.

Clarke, C. A., S. L. Glaser, et al. (2002). "Breast cancer incidence and mortality trends in an affluent population: Marin County, California, USA, 1990-1999." Breast Cancer Res **4**(6): R13.

Clarke, R., R. B. Dickson, et al. (1992). "Hormonal aspects of breast cancer. Growth factors, drugs and stromal interactions." Crit Rev Oncol Hematol **12**(1): 1-23.

Clarke, R. B. (2006). "Ovarian steroids and the human breast: regulation of stem cells and cell proliferation." Maturitas **54**(4): 327-34.

Clarke, R. B., A. Howell, et al. (1997). "Estrogen sensitivity of normal human breast tissue in vivo and implanted into athymic nude mice: analysis of the relationship between estrogen-induced proliferation and progesterone receptor expression." Breast Cancer Res Treat **45**(2): 121-33.

Clarke, R. B., A. Howell, et al. (1997). "Dissociation between steroid receptor expression and cell proliferation in the human breast." Cancer Res **57**(22): 4987-91.

Clarke, R. B., I. J. Laidlaw, et al. (1993). "Effect of tamoxifen on Ki67 labelling index in human breast tumours and its relationship to oestrogen and progesterone receptor status." Br J Cancer **67**(3): 606-11.
Cleutjens, K. B., H. A. van der Korput, et al. (1997). "An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter." Mol Endocrinol **11**(2): 148-61.
Cline, J. M., T. C. Register, et al. (2002). "Effects of tibolone and hormone replacement therapy on the breast of cynomolgus monkeys." Menopause **9**(6): 422-9.
Cline, J. M., G. Soderqvist, et al. (1996). "Effects of hormone replacement therapy on the mammary gland of surgically postmenopausal cynomolgus macaques." Am J Obstet Gynecol **174**(1 Pt 1): 93-100.
Cline, J. M., G. Soderqvist, et al. (1998). "Effects of conjugated estrogens, medroxyprogesterone acetate, and tamoxifen on the mammary glands of macaques." Breast Cancer Res Treat **48**(3): 221-9.
Cohen, S. M. and L. B. Ellwein (1991). "Genetic errors, cell proliferation, and carcinogenesis." Cancer Res **51**(24): 6493-505.
Concannon, P., N. Altszuler, et al. (1980). "Grwoth hormone, prolactin, and cortisol in dogs developing mammary nodules and an acromegaly-like appearance during treatment with medroxyprogesterone acetate." Endocrinology **106**(4): 1173-7.
Connaghan-Jones, K. D., A. F. Heneghan, et al. (2007). "Thermodynamic analysis of progesterone receptor-promoter interactions reveals a molecular model for isoform-specific function." Proc Natl Acad Sci U S A **104**(7): 2187-92.
Coombs, N. J. and J. Boyages (2005). "Changes in HT prescriptions in Australia since 1992." Aust Fam Physician **34**(8): 697-8.
Coombs, N. J., R. Taylor, et al. (2005). "HRT and breast cancer: impact on population risk and incidence." Eur J Cancer **41**(12): 1775-81.
Cooper, A. J. (1986). "Progestogens in the treatment of male sex offenders: a review." Can J Psychiatry **31**(1): 73-9.
Cooper, A. J. (1987). "Medroxyprogesterone acetate (MPA) treatment of sexual acting out in men suffering from dementia." J Clin Psychiatry **48**(9): 368-70.
Cops, E. J., T. Bianco-Miotto, et al. (2008). "Antiproliferative actions of the synthetic androgen, mibolerone, in breast cancer cells are mediated by both androgen and progesterone receptors." J Steroid Biochem Mol Biol **110**(3-5): 236-43.
Cordoba, O. A. and J. L. Chapel (1983). "Medroxyprogesterone acetate antiandrogen treatment of hypersexuality in a pedophiliac sex offender." Am J Psychiatry **140**(8): 1036-9.
Courtin, A., L. Communal, et al. (2011). "Glucocorticoid receptor activity discriminates between progesterone and medroxyprogesterone acetate effects in breast cells." Breast Cancer Res Treat.
Couture, P., C. Theriault, et al. (1993). "Androgen receptor-mediated stimulation of 17 beta-hydroxysteroid dehydrogenase activity by dihydrotestosterone and medroxyprogesterone acetate in ZR-75-1 human breast cancer cells." Endocrinology **132**(1): 179-85.
Cowan, L. D., L. Gordis, et al. (1981). "Breast cancer incidence in women with a history of progesterone deficiency." Am J Epidemiol **114**(2): 209-17.

Crowley, L. G. and I. Macdonald (1962). "Clinical trial of delautin in the treatment of advanced mammary carcinoma in postmenopausal women." Cancer **15**: 1218-20.
Cunha, G. R., P. S. Cooke, et al. (2004). "Role of stromal-epithelial interactions in hormonal responses." Arch Histol Cytol **67**(5): 417-34.
Cunha, G. R., P. Young, et al. (1997). "Elucidation of a role for stromal steroid hormone receptors in mammary gland growth and development using tissue recombinants." J Mammary Gland Biol Neoplasia **2**(4): 393-402.
D'Alessio, S., G. Ferrari, et al. (2008). "Tissue inhibitor of metalloproteinases-2 binding to membrane-type 1 matrix metalloproteinase induces MAPK activation and cell growth by a non-proteolytic mechanism." J Biol Chem **283**(1): 87-99.
Daling, J. R., K. E. Malone, et al. (2002). "Relation of regimens of combined hormone replacement therapy to lobular, ductal, and other histologic types of breast carcinoma." Cancer **95**(12): 2455-64.
Daling, J. R., K. E. Malone, et al. (2003). "Association of regimens of hormone replacement therapy to prognostic factors among women diagnosed with breast cancer aged 50-64 years." Cancer Epidemiol Biomarkers Prev **12**(11 Pt 1): 1175-81.
Dao, T. L., D. K. Sinha, et al. (1982). "Effect of estrogen and progesterone on cellular replication of human breast tumors." Cancer Res **42**(1): 359-62.
de Launoit, Y., S. Dauvois, et al. (1991). "Inhibition of cell cycle kinetics and proliferation by the androgen 5 alpha-dihydrotestosterone and antiestrogen N,n-butyl-N-methyl-11-[16' alpha-chloro-3',17 beta-dihydroxy-estra-1',3',5'-(10')triene-7' alpha-yl] undecanamide in human breast cancer ZR-75-1 cells." Cancer Res **51**(11): 2797-802.
de Lignieres, B., F. de Vathaire, et al. (2002). "Combined hormone replacement therapy and risk of breast cancer in a French cohort study of 3175 women." Climacteric **5**(4): 332-40.
de Lima, G. R., G. Facina, et al. (2003). "Effects of low dose tamoxifen on normal breast tissue from premenopausal women." Eur J Cancer **39**(7): 891-8.
De Vos, P., F. Claessens, et al. (1993). "Interaction of androgen and glucocorticoid receptor DNA-binding domains with their response elements." Mol Cell Endocrinol **90**(2): R11-6.
Denayer, S., C. Helsen, et al. "The rules of DNA recognition by the androgen receptor." Mol Endocrinol **24**(5): 898-913.
Denayer, S., C. Helsen, et al. (2010). "The rules of DNA recognition by the androgen receptor." Mol Endocrinol **24**(5): 898-913.
Desrivieres, S., T. Prinz, et al. (2003). "Comparative proteomic analysis of proliferating and functionally differentiated mammary epithelial cells." Mol Cell Proteomics **2**(10): 1039-54.
Devinoy, E. and M. Rijnkels "Epigenetics in mammary gland biology and cancer." J Mammary Gland Biol Neoplasia **15**(1): 1-4.
Devinoy, E. and M. Rijnkels (2010). "Epigenetics in mammary gland biology and cancer." J Mammary Gland Biol Neoplasia **15**(1): 1-4.
Dhimolea, E., M. V. Maffini, et al. "The role of collagen reorganization on mammary epithelial morphogenesis in a 3D culture model." Biomaterials **31**(13): 3622-30.

Dhimolea, E., M. V. Maffini, et al. (2010). "The role of collagen reorganization on mammary epithelial morphogenesis in a 3D culture model." Biomaterials **31**(13): 3622-30.
Di Leo, A., E. Bajetta, et al. (1995). "Epirubicin plus medroxyprogesterone as second-line treatment of advanced prostatic cancer. A study by the Italian Trials in Medical Oncology Group." Am J Clin Oncol **18**(3): 239-44.
Dietel, M., M. A. Lewis, et al. (2005). "Hormone replacement therapy: pathobiological aspects of hormone-sensitive cancers in women relevant to epidemiological studies on HRT: a mini-review." Hum Reprod **20**(8): 2052-60.
Dimitrakakis, C. and C. Bondy (2009). "Androgens and the breast." Breast Cancer Res **11**(5): 212.
Dimitrakakis, C., J. Zhou, et al. (2002). "Androgens and mammary growth and neoplasia." Fertil Steril **77 Suppl 4**: S26-33.
Dimitrakakis, C., J. Zhou, et al. (2003). "A physiologic role for testosterone in limiting estrogenic stimulation of the breast." Menopause **10**(4): 292-8.
Dontu, G., M. Al-Hajj, et al. (2003). "Stem cells in normal breast development and breast cancer." Cell Prolif **36 Suppl 1**: 59-72.
Dotzlaw, H., E. Leygue, et al. (1997). "Expression of estrogen receptor-beta in human breast tumors." J Clin Endocrinol Metab **82**(7): 2371-4.
Douglas, C. E., R. M. Davis, et al. (2006). "Epidemiology of the third wave of tobacco litigation in the United States, 1994-2005." Tob Control **15 Suppl 4**: iv9-16.
Dowsett, M., S. R. Ebbs, et al. (2005). "Biomarker changes during neoadjuvant anastrozole, tamoxifen, or the combination: influence of hormonal status and HER-2 in breast cancer--a study from the IMPACT trialists." J Clin Oncol **23**(11): 2477-92.
Dowsett, M., I. E. Smith, et al. (2007). "Prognostic value of Ki67 expression after short-term presurgical endocrine therapy for primary breast cancer." J Natl Cancer Inst **99**(2): 167-70.
Dull, A., E. Goncharova, et al. (2010). "Development of an image analysis screen for estrogen receptor alpha (ERalpha) ligands through measurement of nuclear translocation dynamics." J Steroid Biochem Mol Biol **122**(5): 341-51.
Dumont, M., S. Dauvois, et al. (1989). "Antagonism between estrogens and androgens on GCDFP-15 gene expression in ZR-75-1 cells and correlation between GCDFP-15 and estrogen as well as progesterone receptor expression in human breast cancer." J Steroid Biochem **34**(1-6): 397-402.
Dupont, W. D., D. L. Page, et al. (1994). "Long-term risk of breast cancer in women with fibroadenoma." N Engl J Med **331**(1): 10-5.
Easton, D. F., K. A. Pooley, et al. (2007). "Genome-wide association study identifies novel breast cancer susceptibility loci." Nature **447**(7148): 1087-93.
Eckert, R. L. and B. S. Katzenellenbogen (1982). "Effects of estrogens and antiestrogens on estrogen receptor dynamics and the induction of progesterone receptor in MCF-7 human breast cancer cells." Cancer Res **42**(1): 139-44.
Eigeliene, N., P. Harkonen, et al. (2006). "Effects of estradiol and medroxyprogesterone acetate on morphology, proliferation and apoptosis of human breast tissue in organ cultures." BMC Cancer **6**: 246.

Eigeliene, N., P. Harkonen, et al. (2008). "Effects of estradiol and medroxyprogesterone acetate on expression of the cell cycle proteins cyclin D1, p21 and p27 in cultured human breast tissues." Cell Cycle **7**(1): 71-80.

Elhaji, Y. A., B. Gottlieb, et al. (2001). "The polymorphic CAG repeat of the androgen receptor gene: a potential role in breast cancer in women over 40." Breast Cancer Res Treat **70**(2): 109-16.

Emerman, J. T. and A. W. Vogl (1986). "Cell size and shape changes in the myoepithelium of the mammary gland during differentiation." Anat Rec **216**(3): 405-15.

Ereman, R. R., L. A. Prebil, et al. "Recent trends in hormone therapy utilization and breast cancer incidence rates in the high incidence population of Marin County, California." BMC Public Health **10**: 228.

Ereman, R. R., L. A. Prebil, et al. (2010). "Recent trends in hormone therapy utilization and breast cancer incidence rates in the high incidence population of Marin County, California." BMC Public Health **10**(228).

Ethier, S. P. (1996). "Human breast cancer cell lines as models of growth regulation and disease progression." J Mammary Gland Biol Neoplasia **1**(1): 111-21.

Fagan, D. H. and D. Yee (2008). "Crosstalk between IGF1R and estrogen receptor signaling in breast cancer." J Mammary Gland Biol Neoplasia **13**(4): 423-9.

Faresse, N., D. Ruffieux-Daidie, et al. "Mineralocorticoid receptor degradation is promoted by Hsp90 inhibition and the ubiquitin-protein ligase CHIP." Am J Physiol Renal Physiol **299**(6): F1462-72.

Farmer, P., H. Bonnefoi, et al. (2005). "Identification of molecular apocrine breast tumours by microarray analysis." Oncogene **24**(29): 4660-71.

Fernandez, E., S. Gallus, et al. (2003). "Hormone replacement therapy and cancer risk: a systematic analysis from a network of case-control studies." Int J Cancer **105**(3): 408-12.

Ferrieres, G., M. Cuny, et al. (1997). "Variation of bcl-2 expression in breast ducts and lobules in relation to plasma progesterone levels: overexpression and absence of variation in fibroadenomas." J Pathol **183**(2): 204-11.

Flototto, T., D. Niederacher, et al. (2004). "Molecular mechanism of estrogen receptor (ER)alpha-specific, estradiol-dependent expression of the progesterone receptor (PR) B-isoform." J Steroid Biochem Mol Biol **88**(2): 131-42.

Foidart, J. M., C. Colin, et al. (1998). "Estradiol and progesterone regulate the proliferation of human breast epithelial cells." Fertil Steril **69**(5): 963-9.

Fontenoy, A. M., C. Leux, et al. "Recent trends in breast cancer incidence rates in the Loire-Atlantique, France: a decline since 2003." Cancer Epidemiol **34**(3): 238-43.

Fontenoy, A. M., C. Leux, et al. (2010). "Recent trends in breast cancer incidence rates in the Loire-Atlantique, France: a decline since 2003." Cancer Epidemiol **34**(3): 238-43.

Fournier, A., F. Berrino, et al. (2008). "Unequal risks for breast cancer associated with different hormone replacement therapies: results from the E3N cohort study." Breast Cancer Res Treat **107**(1): 103-11.

Fournier, A., F. Berrino, et al. (2005). "Breast cancer risk in relation to different types of hormone replacement therapy in the E3N-EPIC cohort." Int J Cancer **114**(3): 448-54.

Frank, D. W., K. T. Kirton, et al. (1979). "Mammary tumors and serum hormones in the bitch treated with medroxyprogesterone acetate or progesterone for four years." Fertil Steril **31**(3): 340-6.
Fullwood, M. J., M. H. Liu, et al. (2009). "An oestrogen-receptor-alpha-bound human chromatin interactome." Nature **462**(7269): 58-64.
Fuqua, S. A., R. Schiff, et al. (2003). "Estrogen receptor beta protein in human breast cancer: correlation with clinical tumor parameters." Cancer Res **63**(10): 2434-9.
Garcia-Closas, M., P. Hall, et al. (2008). "Heterogeneity of breast cancer associations with five susceptibility loci by clinical and pathological characteristics." PLoS Genet **4**(4): e1000054.
Gerdes, J., H. Lemke, et al. (1984). "Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67." J Immunol **133**(4): 1710-5.
Ghatge, R. P., B. M. Jacobsen, et al. (2005). "The progestational and androgenic properties of medroxyprogesterone acetate: gene regulatory overlap with dihydrotestosterone in breast cancer cells." Breast Cancer Res **7**(6): R1036-50.
Giguere, Y., E. Dewailly, et al. (2001). "Short polyglutamine tracts in the androgen receptor are protective against breast cancer in the general population." Cancer Res **61**(15): 5869-74.
Glass, A. G., J. V. Lacey, Jr., et al. (2007). "Breast cancer incidence, 1980-2006: combined roles of menopausal hormone therapy, screening mammography, and estrogen receptor status." J Natl Cancer Inst **99**(15): 1152-61.
Glass, C. K. (1994). "Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers." Endocr Rev **15**(3): 391-407.
Glidewell-Kenney, C., L. A. Hurley, et al. (2007). "Nonclassical estrogen receptor alpha signaling mediates negative feedback in the female mouse reproductive axis." Proc Natl Acad Sci U S A **104**(19): 8173-7.
Going, J. J., T. J. Anderson, et al. (1988). "Proliferative and secretory activity in human breast during natural and artificial menstrual cycles." Am J Pathol **130**(1): 193-204.
Gompel, A., C. Malet, et al. (1986). "Progestin effect on cell proliferation and 17 beta-hydroxysteroid dehydrogenase activity in normal human breast cells in culture." J Clin Endocrinol Metab **63**(5): 1174-80.
Gonzalez-Angulo, A. M., K. Stemke-Hale, et al. (2009). "Androgen receptor levels and association with PIK3CA mutations and prognosis in breast cancer." Clin Cancer Res **15**(7): 2472-8.
Gonzalez, L. O., M. D. Corte, et al. (2008). "Androgen receptor expresion in breast cancer: relationship with clinicopathological characteristics of the tumors, prognosis, and expression of metalloproteases and their inhibitors." BMC Cancer **8**: 149.
Gordon, G. G., A. L. Southren, et al. (1970). "Effect of medroxyprogesterone acetate (Provera) on the metabolism and biological activity of testosterone." J Clin Endocrinol Metab **30**(4): 449-56.
Graham, J. D., S. D. Roman, et al. (1995). "Preferential stimulation of human progesterone receptor B expression by estrogen in T-47D human breast cancer cells." J Biol Chem **270**(51): 30693-700.

Greendale, G. A., B. A. Reboussin, et al. (1999). "Effects of estrogen and estrogen-progestin on mammographic parenchymal density. Postmenopausal Estrogen/Progestin Interventions (PEPI) Investigators." Ann Intern Med **130**(4 Pt 1): 262-9.

Greendale, G. A., B. A. Reboussin, et al. (2003). "Postmenopausal hormone therapy and change in mammographic density." J Natl Cancer Inst **95**(1): 30-7.

Greeve, M. A., R. K. Allan, et al. (2004). "Inhibition of MCF-7 breast cancer cell proliferation by 5alpha-dihydrotestosterone; a role for p21(Cip1/Waf1)." J Mol Endocrinol **32**(3): 793-810.

Grose, R. and C. Dickson (2005). "Fibroblast growth factor signaling in tumorigenesis." Cytokine Growth Factor Rev **16**(2): 179-86.

Haagensen, D. E., P. Stewart, et al. (1992). "Secretion of breast gross cystic disease fluid proteins by T47D breast cancer cells in culture--modulation by steroid hormones." Breast Cancer Res Treat **23**(1-2): 77-86.

Haas, J. S., C. P. Kaplan, et al. (2004). "Changes in the use of postmenopausal hormone therapy after the publication of clinical trial results." Ann Intern Med **140**(3): 184-8.

Hackenberg, R., T. Hawighorst, et al. (1993). "Medroxyprogesterone acetate inhibits the proliferation of estrogen- and progesterone-receptor negative MFM-223 human mammary cancer cells via the androgen receptor." Breast Cancer Res Treat **25**(3): 217-24.

Hackenberg, R., S. Luttchens, et al. (1991). "Androgen sensitivity of the new human breast cancer cell line MFM-223." Cancer Res **51**(20): 5722-7.

Haerslev, T., G. K. Jacobsen, et al. (1996). "Correlation of growth fraction by Ki-67 and proliferating cell nuclear antigen (PCNA) immunohistochemistry with histopathological parameters and prognosis in primary breast carcinomas." Breast Cancer Res Treat **37**(2): 101-13.

Hall, R. E., J. O. Aspinall, et al. (1996). "Expression of the androgen receptor and an androgen-responsive protein, apolipoprotein D, in human breast cancer." Br J Cancer **74**(8): 1175-80.

Hall, R. E., J. A. Clements, et al. (1998). "Prostate-specific antigen and gross cystic disease fluid protein-15 are co-expressed in androgen receptor-positive breast tumours." Br J Cancer **78**(3): 360-5.

Ham, R. G. and W. L. McKeehan (1978). "Development of improved media and culture conditions for clonal growth of normal diploid cells." In Vitro **14**(1): 11-22.

Hanley, K., J. Wang, et al. (2008). "Lack of expression of androgen receptor may play a critical role in transformation from in situ to invasive basal subtype of high-grade ductal carcinoma of the breast." Hum Pathol **39**(3): 386-92.

Harper-Wynne, C., G. Ross, et al. (2002). "Effects of the aromatase inhibitor letrozole on normal breast epithelial cell proliferation and metabolic indices in postmenopausal women: a pilot study for breast cancer prevention." Cancer Epidemiol Biomarkers Prev **11**(7): 614-21.

Hartman, J., K. Lindberg, et al. (2006). "Estrogen receptor beta inhibits angiogenesis and growth of T47D breast cancer xenografts." Cancer Res **66**(23): 11207-13.

Hausauer, A. K., T. H. Keegan, et al. (2007). "Recent breast cancer trends among Asian/Pacific Islander, Hispanic, and African-American women in the US: changes by tumor subtype." Breast Cancer Res **9**(6): R90.
Haverinen, M., S. Passinen, et al. (2001). "Heat shock protein 90 and the nuclear transport of progesterone receptor." Cell Stress Chaperones **6**(3): 256-62.
He, B., N. T. Bowen, et al. (2001). "Androgen-induced NH2- and COOH-terminal Interaction Inhibits p160 coactivator recruitment by activation function 2." J Biol Chem **276**(45): 42293-301.
He, B. and E. M. Wilson (2002). "The NH(2)-terminal and carboxyl-terminal interaction in the human androgen receptor." Mol Genet Metab **75**(4): 293-8.
Helguero, L. A., M. H. Faulds, et al. (2005). "Estrogen receptors alfa (ERalpha) and beta (ERbeta) differentially regulate proliferation and apoptosis of the normal murine mammary epithelial cell line HC11." Oncogene **24**(44): 6605-16.
Henderson, B. E., R. K. Ross, et al. (1982). "Endogenous hormones as a major factor in human cancer." Cancer Res **42**(8): 3232-9.
Henderson, B. E. P., B. Ross, R.K. (2003). Hormones, Genes and Cancer.
Hengartner, M. O. (2000). "The biochemistry of apoptosis." Nature **407**(6805): 770-6.
Hersh, A. L., M. L. Stefanick, et al. (2004). "National use of postmenopausal hormone therapy: annual trends and response to recent evidence." Jama **291**(1): 47-53.
Hess-Wilson, J. K. and K. E. Knudsen (2006). "Endocrine disrupting compounds and prostate cancer." Cancer Lett **241**(1): 1-12.
Hiroi, M., F. Z. Stanczyk, et al. (1975). "Radioimmunoassay of serum medroxyprogesterone acetate (Provera) in women following oral and intravaginal administration." Steroids **26**(3): 373-86.
Hobbs, C. J., R. E. Jones, et al. (1992). "The effects of sex hormone binding globulin (SHBG) on testosterone transport into the cerebrospinal fluid." J Steroid Biochem Mol Biol **42**(6): 629-35.
Hockenbery, D., G. Nunez, et al. (1990). "Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death." Nature **348**(6299): 334-6.
Hofling, M., L. Ma, et al. (2009). "Expression of the androgen receptor and syndecan-1 in breast tissue during different hormonal treatments in cynomolgus monkeys." Climacteric **12**(1): 72-9.
Hofseth, L. J., A. M. Raafat, et al. (1999). "Hormone replacement therapy with estrogen or estrogen plus medroxyprogesterone acetate is associated with increased epithelial proliferation in the normal postmenopausal breast." J Clin Endocrinol Metab **84**(12): 4559-65.
Holliday, R. (1997). "Understanding ageing." Philos Trans R Soc Lond B Biol Sci **352**(1363): 1793-7.
Holmberg, L., O. E. Iversen, et al. (2008). "Increased risk of recurrence after hormone replacement therapy in breast cancer survivors." J Natl Cancer Inst **100**(7): 475-82.
Horwitz, K. B., M. E. Costlow, et al. (1975). "MCF-7; a human breast cancer cell line with estrogen, androgen, progesterone, and glucocorticoid receptors." Steroids **26**(6): 785-95.

Horwitz, K. B., A. W. Pike, et al. (1986). "Progesterone metabolism in T47Dco human breast cancer cells--II. Intracellular metabolic path of progesterone and synthetic progestins." J Steroid Biochem **25**(6): 911-6.
Horwitz, K. B. and C. A. Sartorius (2008). "Progestins in hormone replacement therapies reactivate cancer stem cells in women with preexisting breast cancers: a hypothesis." J Clin Endocrinol Metab **93**(9): 3295-8.
Hsu, Y. H., M. C. Zillikens, et al. "An integration of genome-wide association study and gene expression profiling to prioritize the discovery of novel susceptibility Loci for osteoporosis-related traits." PLoS Genet **6**(6): e1000977.
Hu, R., S. Dawood, et al. (2010). "Androgen receptor expression and breast cancer survival in postmenopausal women." Clin Cancer Res **17**(7): 1867-74.
Hubler, T. R., W. B. Denny, et al. (2003). "The FK506-binding immunophilin FKBP51 is transcriptionally regulated by progestin and attenuates progestin responsiveness." Endocrinology **144**(6): 2380-7.
Huggins, C. and D. M. Bergenstal (1952). "Inhibition of human mammary and prostatic cancers by adrenalectomy." Cancer Res **12**(2): 134-41.
Huggins, C., G. Briziarelli, et al. (1959). "Rapid induction of mammary carcinoma in the rat and the influence of hormones on the tumors." J Exp Med **109**(1): 25-42.
Huggins, C. and C. V. Hodges (1972). "Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate." CA Cancer J Clin **22**(4): 232-40.
Humphreys, R. C., J. Lydon, et al. (1997). "Mammary gland development is mediated by both stromal and epithelial progesterone receptors." Mol Endocrinol **11**(6): 801-11.
Hunter, D. J., P. Kraft, et al. (2007). "A genome-wide association study identifies alleles in FGFR2 associated with risk of sporadic postmenopausal breast cancer." Nat Genet **39**(7): 870-4.
Hutson, S. W., P. N. Cowen, et al. (1985). "Morphometric studies of age related changes in normal human breast and their significance for evolution of mammary cancer." J Clin Pathol **38**(3): 281-7.
Hyder, S. M., L. Murthy, et al. (1998). "Progestin regulation of vascular endothelial growth factor in human breast cancer cells." Cancer Res **58**(3): 392-5.
Isaksson, E., H. Wang, et al. (2003). "Effects of long-term HRT and tamoxifen on the expression of progesterone receptors A and B in breast tissue from surgically postmenopausal cynomolgus macaques." Breast Cancer Res Treat **79**(2): 233-9.
Ishag, M. T., D. Y. Bashinsky, et al. (2003). "Pathologic findings in reduction mammaplasty specimens." Am J Clin Pathol **120**(3): 377-80.
Isola, J. J. (1993). "Immunohistochemical demonstration of androgen receptor in breast cancer and its relationship to other prognostic factors." J Pathol **170**(1): 31-5.
Ito, T., Y. Fujio, et al. (2006). "TauT gene expression is regulated by TonEBP and plays a role in cell survival." Adv Exp Med Biol **583**: 91-8.
Jabara, A. G. and P. S. Anderson (1982). "Effects of progesterone on mammary carcinogenesis when various doses of DMBA were applied directly to rat mammae." Pathology **14**(3): 313-6.

Jabara, A. G. and A. G. Harcourt (1971). "Effects of progesterone, ovariectomy and adrenalectomy on mammary tumours induced by 7,12-dimethylbenz(a)anthracene in Sprague-Dawley rats." Pathology **3**(3): 209-14.

Jacobs, T. M., B. R. Hoppe, et al. "Mammary adenocarcinomas in three male cats exposed to medroxyprogesterone acetate (1990-2006)." J Feline Med Surg **12**(2): 169-74.

Jacquemier, J. D., J. Hassoun, et al. (1990). "Distribution of estrogen and progesterone receptors in healthy tissue adjacent to breast lesions at various stages--immunohistochemical study of 107 cases." Breast Cancer Res Treat **15**(2): 109-17.

Jadeski, L., J. M. Mataraza, et al. (2008). "IQGAP1 stimulates proliferation and enhances tumorigenesis of human breast epithelial cells." J Biol Chem **283**(2): 1008-17.

Jarvinen, T. A., M. Pelto-Huikko, et al. (2000). "Estrogen receptor beta is coexpressed with ERalpha and PR and associated with nodal status, grade, and proliferation rate in breast cancer." Am J Pathol **156**(1): 29-35.

Jarzabek, K., M. Koda, et al. (2009). "The significance of the expression of ERRalpha as a potential biomarker in breast cancer." J Steroid Biochem Mol Biol **113**(1-2): 127-33.

Jayo, M. J., T. C. Register, et al. (2000). "Effects of an oral contraceptive combination with or without androgen on mammary tissues: a study in rats." J Soc Gynecol Investig **7**(4): 257-65.

Jemal, A., E. Ward, et al. (2007). "Recent trends in breast cancer incidence rates by age and tumor characteristics among U.S. women." Breast Cancer Res **9**(3): R28.

Jensen, R. A., D. L. Page, et al. (1989). "Invasive breast cancer risk in women with sclerosing adenosis." Cancer **64**(10): 1977-83.

Jones, B. and J. Russo (1987). "Influence of steroid hormones on the growth fraction of human breast carcinomas." Am J Clin Pathol **88**(2): 132-8.

Jordan, V. C. and L. G. Ford (2011). "Paradoxical clinical effect of estrogen on breast cancer risk: a "new" biology of estrogen-induced apoptosis." Cancer Prev Res (Phila) **4**(5): 633-7.

Joshi, K., J. A. Smith, et al. (1986). "Cell proliferation in the human mammary epithelium. Differential contribution by epithelial and myoepithelial cells." Am J Pathol **124**(2): 199-206.

Kampert, J. B., A. S. Whittemore, et al. (1988). "Combined effect of childbearing, menstrual events, and body size on age-specific breast cancer risk." Am J Epidemiol **128**(5): 962-79.

Kandouz, M., A. Lombet, et al. (1999). "Proapoptotic effects of antiestrogens, progestins and androgen in breast cancer cells." J Steroid Biochem Mol Biol **69**(1-6): 463-71.

Kandouz, M., M. Siromachkova, et al. (1996). "Antagonism between estradiol and progestin on Bcl-2 expression in breast-cancer cells." Int J Cancer **68**(1): 120-5.

Kastner, P., A. Krust, et al. (1990). "Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B." Embo J **9**(5): 1603-14.

Katalinic, A. and R. Rawal (2008). "Decline in breast cancer incidence after decrease in utilisation of hormone replacement therapy." Breast Cancer Res Treat **107**(3): 427-30.
Keating, N. L., P. D. Cleary, et al. (1999). "Use of hormone replacement therapy by postmenopausal women in the United States." Ann Intern Med **130**(7): 545-53.
Keegan, T. H., E. T. Chang, et al. (2007). "Recent changes in breast cancer incidence and risk factor prevalence in San Francisco Bay area and California women: 1988 to 2004." Breast Cancer Res **9**(5): R62.
Kelley, R. M. and W. H. Baker (1961). "Progestational agents in the treatment of carcinoma of the endometrium." N Engl J Med **264**: 216-22.
Kemppainen, J. A., E. Langley, et al. (1999). "Distinguishing androgen receptor agonists and antagonists: distinct mechanisms of activation by medroxyprogesterone acetate and dihydrotestosterone." Mol Endocrinol **13**(3): 440-54.
Kenemans, P. (1999). "Menopause, HRT and menopausal symptoms." J Epidemiol Biostat **4**(3): 141-6; discussion 146-53.
Kennedy, B. J. (1963). "A progestogen for treatment of advanced endometrial cancer." Jama **184**: 758-61.
Kerlikowske, K., D. L. Miglioretti, et al. (2007). "Declines in invasive breast cancer and use of postmenopausal hormone therapy in a screening mammography population." J Natl Cancer Inst **99**(17): 1335-9.
Kester, H. A., B. M. van der Leede, et al. (1997). "Novel progesterone target genes identified by an improved differential display technique suggest that progestin-induced growth inhibition of breast cancer cells coincides with enhancement of differentiation." J Biol Chem **272**(26): 16637-43.
Key, T., P. Appleby, et al. (2002). "Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies." J Natl Cancer Inst **94**(8): 606-16.
Key, T. J., P. N. Appleby, et al. (2003). "Body mass index, serum sex hormones, and breast cancer risk in postmenopausal women." J Natl Cancer Inst **95**(16): 1218-26.
Key, T. J. and M. C. Pike (1988). "The role of oestrogens and progestagens in the epidemiology and prevention of breast cancer." Eur J Cancer Clin Oncol **24**(1): 29-43.
Khan, S. A., M. A. Rogers, et al. (1998). "Estrogen receptor expression in benign breast epithelium and breast cancer risk." J Natl Cancer Inst **90**(1): 37-42.
Khan, S. A., M. A. Rogers, et al. (1994). "Estrogen receptor expression of benign breast epithelium and its association with breast cancer." Cancer Res **54**(4): 993-7.
Kiersch, T. A. (1990). "Treatment of sex offenders with Depo-Provera." Bull Am Acad Psychiatry Law **18**(2): 179-87.
Kincl, F. A., L. A. Ciaccio, et al. (1978). "Increasing oral bioavailability of progesterone by formulation." J Steroid Biochem **9**(1): 83-4.
Kirkwood, T. B. (1997). "The origins of human ageing." Philos Trans R Soc Lond B Biol Sci **352**(1363): 1765-72.
Kirkwood, T. B. (2002). "Evolution of ageing." Mech Ageing Dev **123**(7): 737-45.
Kirkwood, T. B. (2008). "Understanding ageing from an evolutionary perspective." J Intern Med **263**(2): 117-27.

Klaiber, E. L., W. Vogel, et al. (2005). "A critique of the Women's Health Initiative hormone therapy study." Fertil Steril **84**(6): 1589-601.
Klawansky, S. and M. S. Fox (1984). "A growth rate distribution model for the age dependence of human cancer incidence: a proposed role for promotion in cancer of the lung and breast." J Theor Biol **111**(3): 531-87.
Klevjer-Anderson, P. and G. C. Buehring (1980). "Effect of hormones on growth rates of malignant and nonmalignant human mammary epithelia in cell culture." In Vitro **16**(6): 491-501.
Kordon, E., C. Lanari, et al. (1990). "Hormone dependence of a mouse mammary tumor line induced in vivo by medroxyprogesterone acetate." Breast Cancer Res Treat **17**(1): 33-43.
Korkia, P. and G. V. Stimson (1997). "Indications of prevalence, practice and effects of anabolic steroid use in Great Britain." Int J Sports Med **18**(7): 557-62.
Koyama, H., D. Sinha, et al. (1972). "Effects of hormones and 7,12-dimethylbenz[a]anthracene on rat mammary tissue grown in organ culture." J Natl Cancer Inst **48**(6): 1671-80.
Krattenmacher, R. (2000). "Drospirenone: pharmacology and pharmacokinetics of a unique progestogen." Contraception **62**(1): 29-38.
Kraus, W. L., M. M. Montano, et al. (1993). "Cloning of the rat progesterone receptor gene 5'-region and identification of two functionally distinct promoters." Mol Endocrinol **7**(12): 1603-16.
Kraus, W. L., M. M. Montano, et al. (1994). "Identification of multiple, widely spaced estrogen-responsive regions in the rat progesterone receptor gene." Mol Endocrinol **8**(8): 952-69.
Kress, C., M. Ballester, et al. "Epigenetic modifications in 3D: nuclear organization of the differentiating mammary epithelial cell." J Mammary Gland Biol Neoplasia **15**(1): 73-83.
Krieger, N. and R. A. Hiatt (1992). "Risk of breast cancer after benign breast diseases. Variation by histologic type, degree of atypia, age at biopsy, and length of follow-up." Am J Epidemiol **135**(6): 619-31.
Kuenen-Boumeester, V., T. H. Van der Kwast, et al. (1996). "The clinical significance of androgen receptors in breast cancer and their relation to histological and cell biological parameters." Eur J Cancer **32A**(9): 1560-5.
Kuenen-Boumeester, V., T. H. Van der Kwast, et al. (1992). "Immunohistochemical determination of androgen receptors in relation to oestrogen and progesterone receptors in female breast cancer." Int J Cancer **52**(4): 581-4.
Kuhl, H. (2005). "Breast cancer risk in the WHI study: the problem of obesity." Maturitas **51**(1): 83-97.
Kuhl, H. (2005). "Pharmacology of estrogens and progestogens: influence of different routes of administration." Climacteric **8 Suppl 1**: 3-63.
Kumle, M. (2008). "Declining breast cancer incidence and decreased HRT use." Lancet **372**(9639): 608-10.
Kuttenn, F., S. Fournier, et al. (1981). "Estradiol and progesterone receptors in human breast fibroadenomas." J Clin Endocrinol Metab **52**(6): 1225-9.

La Spada, A. R., D. B. Roling, et al. (1992). "Meiotic stability and genotype-phenotype correlation of the trinucleotide repeat in X-linked spinal and bulbar muscular atrophy." Nat Genet **2**(4): 301-4.
Labrie, F. (1991). "Intracrinology." Mol Cell Endocrinol **78**(3): C113-8.
Labrie, F., A. Belanger, et al. (1997). "Marked decline in serum concentrations of adrenal C19 sex steroid precursors and conjugated androgen metabolites during aging." J Clin Endocrinol Metab **82**(8): 2396-402.
Labrie, F., V. Luu-The, et al. (2003). "Endocrine and intracrine sources of androgens in women: inhibition of breast cancer and other roles of androgens and their precursor dehydroepiandrosterone." Endocr Rev **24**(2): 152-82.
Labrie, F., R. Poulin, et al. (1990). "Interactions between estrogens, androgens, progestins, and glucocorticoids in ZR-75-1 human breast cancer cells." Ann N Y Acad Sci **595**: 130-48.
Labrie, F., J. Simard, et al. (1990). "Potent antagonism between estrogens and androgens on GCDFP-15 expression and cell growth in the ZR-75-1 human breast cancer cells." Ann N Y Acad Sci **586**: 174-87.
Laidlaw, I. J., R. B. Clarke, et al. (1995). "The proliferation of normal human breast tissue implanted into athymic nude mice is stimulated by estrogen but not progesterone." Endocrinology **136**(1): 164-71.
Landrieu, I., C. Smet, et al. (2006). "Exploring the molecular function of PIN1 by nuclear magnetic resonance." Curr Protein Pept Sci **7**(3): 179-94.
Lange, C. A. (2008). "Challenges to defining a role for progesterone in breast cancer." Steroids **73**(9-10): 914-21.
Lange, C. A., J. K. Richer, et al. (1999). "Hypothesis: Progesterone primes breast cancer cells for cross-talk with proliferative or antiproliferative signals." Mol Endocrinol **13**(6): 829-36.
Lanzino, M., D. Sisci, et al. (2010). "Inhibition of cyclin D1 expression by androgen receptor in breast cancer cells--identification of a novel androgen response element." Nucleic Acids Res **38**(16): 16.
Lapointe, J., A. Fournier, et al. (1999). "Androgens down-regulate bcl-2 protooncogene expression in ZR-75-1 human breast cancer cells." Endocrinology **140**(1): 416-21.
Lapointe, J. and C. Labrie (1999). "Identification and cloning of a novel androgen-responsive gene, uridine diphosphoglucose dehydrogenase, in human breast cancer cells." Endocrinology **140**(10): 4486-93.
Lauritzen, C. (1990). "Clinical use of oestrogens and progestogens." Maturitas **12**(3): 199-214.
Lawson, J. S., A. S. Field, et al. (1999). "Low oestrogen receptor alpha expression in normal breast tissue underlies low breast cancer incidence in Japan." Lancet **354**(9192): 1787-8.
Lawson, J. S., A. S. Field, et al. (2002). "Breast cancer incidence and estrogen receptor alpha in normal mammary tissue--an epidemiologic study among Japanese women in Japan and Hawaii." Int J Cancer **97**(5): 685-7.
Lea, O. A., S. Kvinnsland, et al. (1989). "Improved measurement of androgen receptors in human breast cancer." Cancer Res **49**(24 Pt 1): 7162-7.
Lebow, M. and E. B. Arkin (1993). "Women's health and the mass media: the reporting of risk." Womens Health Issues **3**(4): 181-90.

Lee, A. V., C. N. Weng, et al. (1997). "Activation of estrogen receptor-mediated gene transcription by IGF-I in human breast cancer cells." J Endocrinol **152**(1): 39-47.
Leonhardt, S. A., M. Altmann, et al. (1998). "Agonist and antagonists induce homodimerization and mixed ligand heterodimerization of human progesterone receptors in vivo by a mammalian two-hybrid assay." Mol Endocrinol **12**(12): 1914-30.
Li, C. I., B. O. Anderson, et al. (2003). "Trends in incidence rates of invasive lobular and ductal breast carcinoma." Jama **289**(11): 1421-4.
Li, C. I., K. E. Malone, et al. (2008). "Relationship between menopausal hormone therapy and risk of ductal, lobular, and ductal-lobular breast carcinomas." Cancer Epidemiol Biomarkers Prev **17**(1): 43-50.
Li, C. I., K. E. Malone, et al. (2003). "Relationship between long durations and different regimens of hormone therapy and risk of breast cancer." Jama **289**(24): 3254-63.
Li, S., B. Han, et al. (2010). "Immunocytochemical localization of sex steroid hormone receptors in normal human mammary gland." J Histochem Cytochem **58**(6): 509-15.
Li, Y., H. Du, et al. (2007). "Activation of the signal transducers and activators of the transcription 3 pathway in alveolar epithelial cells induces inflammation and adenocarcinomas in mouse lung." Cancer Res **67**(18): 8494-503.
Liang, J., P. Chen, et al. (2008). "Genetic variants in fibroblast growth factor receptor 2 (FGFR2) contribute to susceptibility of breast cancer in Chinese women." Carcinogenesis **29**(12): 2341-6.
Liehr, J. G. (1997). "Dual role of oestrogens as hormones and pro-carcinogens: tumour initiation by metabolic activation of oestrogens." Eur J Cancer Prev **6**(1): 3-10.
Liehr, J. G. (2000). "Is estradiol a genotoxic mutagenic carcinogen?" Endocr Rev **21**(1): 40-54.
Lien, H. C., Y. S. Lu, et al. (2006). "Differential expression of glucocorticoid receptor in human breast tissues and related neoplasms." J Pathol **209**(3): 317-27.
Lin, C. S., A. Lau, et al. (2000). "Upregulation of L-plastin gene by testosterone in breast and prostate cancer cells: identification of three cooperative androgen receptor-binding sequences." DNA Cell Biol **19**(1): 1-7.
Lindahl, B. and R. Willen (1994). "Spontaneous endometrial hyperplasia. A 5 year follow-up of 82 patients after high-dose gestagen treatment." Anticancer Res **14**(6B): 2831-4.
Lippman, M. E., R. B. Dickson, et al. (1986). "Autocrine and paracrine growth regulation of human breast cancer." J Steroid Biochem **24**(1): 147-54.
Loretti, A. P., M. R. Ilha, et al. (2005). "Clinical, pathological and immunohistochemical study of feline mammary fibroepithelial hyperplasia following a single injection of depot medroxyprogesterone acetate." J Feline Med Surg **7**(1): 43-52.
Luft, R. and H. Olivecrona (1953). "Experiences with hypophysectomy in man." J Neurosurg **10**(3): 301-16.
Lund, E., K. Bakken, et al. (2007). "Hormone replacement therapy and breast cancer in former users of oral contraceptives--The Norwegian Women and Cancer study." Int J Cancer **121**(3): 645-8.
Lupien, M. and M. Brown (2009). "Cistromics of hormone-dependent cancer." Endocr Relat Cancer.

Lupien, M., J. Eeckhoute, et al. (2008). "FoxA1 translates epigenetic signatures into enhancer-driven lineage-specific transcription." Cell **132**(6): 958-70.
Luqmani, Y. A., M. Graham, et al. (1992). "Expression of basic fibroblast growth factor, FGFR1 and FGFR2 in normal and malignant human breast, and comparison with other normal tissues." Br J Cancer **66**(2): 273-80.
Lydon, J. P., F. J. DeMayo, et al. (1995). "Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities." Genes Dev **9**(18): 2266-78.
Lydon, J. P., G. Ge, et al. (1999). "Murine mammary gland carcinogenesis is critically dependent on progesterone receptor function." Cancer Res **59**(17): 4276-84.
MacIndoe, J. H. and L. A. Etre (1980). "Androgens inhibit estrogen action in MCF-7 human breast cancer cells." Life Sci **27**(18): 1643-8.
MacIndoe, J. H. and L. A. Etre (1981). "An antiestrogenic action of androgens in human breast cancer cells." J Clin Endocrinol Metab **53**(4): 836-42.
MacLennan, A. H., D. H. Wilson, et al. (2002). "Hormone replacement therapy use over a decade in an Australian population." Climacteric **5**(4): 351-6.
Magklara, A., L. Grass, et al. (2000). "Differential steroid hormone regulation of human glandular kallikrein (hK2) and prostate-specific antigen (PSA) in breast cancer cell lines." Breast Cancer Res Treat **59**(3): 263-70.
Magnusson, C., J. A. Baron, et al. (1999). "Breast-cancer risk following long-term oestrogen- and oestrogen-progestin-replacement therapy." Int J Cancer **81**(3): 339-44.
Main, P. and M. Robinson (2008). "Changes in utilisation of hormone replacement therapy in Australia following publication of the findings of the Women's Health Initiative." Pharmacoepidemiol Drug Saf **17**(9): 861-8.
Mainwaring, W. I. (1969). "A soluble androgen receptor in the cytoplasm of rat prostate." J Endocrinol **45**(4): 531-41.
Majumdar, S. and E. P. Diamandis (1999). "The promoter and the enhancer region of the KLK 3 (prostate specific antigen) gene is frequently mutated in breast tumours and in breast carcinoma cell lines." Br J Cancer **79**(9-10): 1594-602.
Malet, C., A. Gompel, et al. (1988). "Tamoxifen and hydroxytamoxifen isomers versus estradiol effects on normal human breast cells in culture." Cancer Res **48**(24 Pt 1): 7193-9.
Mallepell, S., A. Krust, et al. (2006). "Paracrine signaling through the epithelial estrogen receptor alpha is required for proliferation and morphogenesis in the mammary gland." Proc Natl Acad Sci U S A **103**(7): 2196-201.
Mangelsdorf, D. J., C. Thummel, et al. (1995). "The nuclear receptor superfamily: the second decade." Cell **83**(6): 835-9.
Marshall, S. F., C. A. Clarke, et al. "Recent breast cancer incidence trends according to hormone therapy use: the California Teachers Study cohort." Breast Cancer Res **12**(1): R4.
Marshall, S. F., C. A. Clarke, et al. (2010). "Recent breast cancer incidence trends according to hormone therapy use: the California Teachers Study cohort." Breast Cancer Res **12**(1): R4.
Martin, V., S. Ribieras, et al. (1997). "Involvement of DNA methylation in the control of the expression of an estrogen-induced breast-cancer-associated protein (pS2) in human breast cancers." J Cell Biochem **65**(1): 95-106.

Marugg, R. C., M. J. van der Mooren, et al. (1997). "Mammographic changes in postmenopausal women on hormonal replacement therapy." Eur Radiol **7**(5): 749-55.
Massafra, C., C. De Felice, et al. (1999). "Androgens and osteocalcin during the menstrual cycle." J Clin Endocrinol Metab **84**(3): 971-4.
Massie, C. E., B. Adryan, et al. (2007). "New androgen receptor genomic targets show an interaction with the ETS1 transcription factor." EMBO Rep **8**(9): 871-8.
Masters, J. R., J. O. Drife, et al. (1977). "Cyclic Variation of DNA synthesis in human breast epithelium." J Natl Cancer Inst **58**(5): 1263-5.
Mastorakos, G., E. G. Sakkas, et al. (2006). "Pitfalls of the WHIs: Women's Health Initiative." Ann N Y Acad Sci **1092**: 331-40.
Mathrubutham, M. and K. Fotherby (1981). "Medroxyprogesterone acetate in human serum." J Steroid Biochem **14**(8): 783-6.
Maxson, W. S. and J. T. Hargrove (1985). "Bioavailability of oral micronized progesterone." Fertil Steril **44**(5): 622-6.
McDonough, P. G. (2002). "The randomized world is not without its imperfections: reflections on the Women's Health Initiative Study." Fertil Steril **78**(5): 951-6.
McKinlay, S. M., D. J. Brambilla, et al. (2008). "The normal menopause transition." Maturitas **61**(1-2): 4-16.
McManus, M. J. and C. W. Welsch (1981). "Hormone-induced ductal DNA synthesis of human breast tissues maintained in the athymic nude mouse." Cancer Res **41**(9 Pt 1): 3300-5.
McManus, M. J. and C. W. Welsch (1984). "The effect of estrogen, progesterone, thyroxine, and human placental lactogen on DNA synthesis of human breast ductal epithelium maintained in athymic nude mice." Cancer **54**(9): 1920-7.
Melnik, B. C., G. Schmitz, et al. (2009). "Anti-acne agents attenuate FGFR2 signal transduction in acne." J Invest Dermatol **129**(8): 1868-77.
Menendez, J. A., B. P. Oza, et al. (2005). "The estrogenic activity of synthetic progestins used in oral contraceptives enhances fatty acid synthase-dependent breast cancer cell proliferation and survival." Int J Oncol **26**(6): 1507-15.
Meyer, J. S. (1977). "Cell proliferation in normal human breast ducts, fibroadenomas, and other ductal hyperplasias measured by nuclear labeling with tritiated thymidine. Effects of menstrual phase, age, and oral contraceptive hormones." Hum Pathol **8**(1): 67-81.
Meyer, J. S., B. R. Rao, et al. (1977). "Low incidence of estrogen receptor in breast carcinomas with rapid rates of cellular replication." Cancer **40**(5): 2290-8.
Michael, R. P., R. W. Bonsall, et al. (1991). "Medroxyprogesterone acetate and the nuclear uptake of testosterone and its metabolites by brain, pituitary gland and genital tract in male cynomolgus monkeys." J Steroid Biochem Mol Biol **38**(1): 49-57.
Michna, H., M. Schneider, et al. (1990). "Progesterone antagonists block the growth of experimental mammary tumors in G0/G1." Breast Cancer Res Treat **17**(2): 155-6.
Misso, M. L., C. Jang, et al. (2005). "Adipose aromatase gene expression is greater in older women and is unaffected by postmenopausal estrogen therapy." Menopause **12**(2): 210-5.

Moinfar, F., M. Okcu, et al. (2003). "Androgen receptors frequently are expressed in breast carcinomas: potential relevance to new therapeutic strategies." Cancer **98**(4): 703-11.
Molinolo, A. A., C. Lanari, et al. (1987). "Mouse mammary tumors induced by medroxyprogesterone acetate: immunohistochemistry and hormonal receptors." J Natl Cancer Inst **79**(6): 1341-50.
Monaghan, P., C. L. Clarke, et al. (1995). "Epidermal growth factor receptor expression on human breast luminal and basal cells in vitro." Epithelial Cell Biol **4**(2): 52-62.
Monaghan, P., N. P. Perusinghe, et al. (1991). "Growth factor stimulation of proliferating cell nuclear antigen (PCNA) in human breast epithelium in organ culture." Cell Biol Int Rep **15**(7): 561-70.
Morgenbesser, S. D., R. P. McLaren, et al. (2007). "Identification of genes potentially involved in the acquisition of androgen-independent and metastatic tumor growth in an autochthonous genetically engineered mouse prostate cancer model." Prostate **67**(1): 83-106.
Mote, P. A., S. Bartow, et al. (2002). "Loss of co-ordinate expression of progesterone receptors A and B is an early event in breast carcinogenesis." Breast Cancer Res Treat **72**(2): 163-72.
Moutsatsou, P. and A. G. Papavassiliou (2008). "The glucocorticoid receptor signalling in breast cancer." J Cell Mol Med **12**(1): 145-63.
Mueller, M. D., J. L. Vigne, et al. (2003). "Progestins activate vascular endothelial growth factor gene transcription in endometrial adenocarcinoma cells." Fertil Steril **79**(2): 386-92.
Mulac-Jericevic, B., J. P. Lydon, et al. (2003). "Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform." Proc Natl Acad Sci U S A **100**(17): 9744-9.
Muller, H. M., L. Ivarsson, et al. (2004). "DNA methylation changes in sera of women in early pregnancy are similar to those in advanced breast cancer patients." Clin Chem **50**(6): 1065-8.
Nardulli, A. M., G. L. Greene, et al. (1988). "Regulation of progesterone receptor messenger ribonucleic acid and protein levels in MCF-7 cells by estradiol: analysis of estrogen's effect on progesterone receptor synthesis and degradation." Endocrinology **122**(3): 935-44.
Narita, D., M. Raica, et al. (2005). "Immunohistochemical localization of prostate-specific antigen in benign and malignant breast conditions." Rom J Morphol Embryol **46**(1): 41-5.
New, M. I. (2004). "An update of congenital adrenal hyperplasia." Ann N Y Acad Sci **1038**: 14-43.
Newcomb, P. A., L. Titus-Ernstoff, et al. (2002). "Postmenopausal estrogen and progestin use in relation to breast cancer risk." Cancer Epidemiol Biomarkers Prev **11**(7): 593-600.
Norwitz, E. R., D. J. Schust, et al. (2001). "Implantation and the survival of early pregnancy." N Engl J Med **345**(19): 1400-8.
O'Hare, M. J., M. G. Ormerod, et al. (1991). "Characterization in vitro of luminal and myoepithelial cells isolated from the human mammary gland by cell sorting." Differentiation **46**(3): 209-21.

Ogawa, S., S. Inoue, et al. (1998). "The complete primary structure of human estrogen receptor beta (hER beta) and its heterodimerization with ER alpha in vivo and in vitro." Biochem Biophys Res Commun **243**(1): 122-6.
Olsson, H. L., C. Ingvar, et al. (2003). "Hormone replacement therapy containing progestins and given continuously increases breast carcinoma risk in Sweden." Cancer **97**(6): 1387-92.
Olszewski, W., Z. Darzynkiewicz, et al. (1981). "Flow cytometry of breast carcinoma: II. Relation of tumor cell cycle distribution to histology and estrogen receptor." Cancer **48**(4): 985-8.
Opatrny, L., S. Dell'Aniello, et al. (2008). "Hormone replacement therapy use and variations in the risk of breast cancer." Bjog **115**(2): 169-75; discussion 175.
Ortmann, J., S. Prifti, et al. (2002). "Testosterone and 5 alpha-dihydrotestosterone inhibit in vitro growth of human breast cancer cell lines." Gynecol Endocrinol **16**(2): 113-20.
Ouatas, T., D. Halverson, et al. (2003). "Dexamethasone and medroxyprogesterone acetate elevate Nm23-H1 metastasis suppressor gene expression in metastatic human breast carcinoma cells: new uses for old compounds." Clin Cancer Res **9**(10 Pt 1): 3763-72.
Pace, P., J. Taylor, et al. (1997). "Human estrogen receptor beta binds DNA in a manner similar to and dimerizes with estrogen receptor alpha." J Biol Chem **272**(41): 25832-8.
Paine, B. J., N. P. Stocks, et al. (2004). "Use and perception of hormone therapy following media reports of the Women's Health Initiative: a survey of Australian WISDOM participants." Climacteric **7**(2): 143-52.
Paliouras, M. and E. P. Diamandis (2008). "Androgens act synergistically to enhance estrogen-induced upregulation of human tissue kallikreins 10, 11, and 14 in breast cancer cells via a membrane bound androgen receptor." Mol Oncol **1**(4): 413-24.
Panet-Raymond, V., B. Gottlieb, et al. (2000). "Interactions between androgen and estrogen receptors and the effects on their transactivational properties." Mol Cell Endocrinol **167**(1-2): 139-50.
Parkin, D. M., F. Bray, et al. (2005). "Global cancer statistics, 2002." CA Cancer J Clin **55**(2): 74-108.
Parlato, S., G. Romagnoli, et al. "LOX-1 as a natural IFN-alpha-mediated signal for apoptotic cell uptake and antigen presentation in dendritic cells." Blood **115**(8): 1554-63.
Paruthiyil, S., H. Parmar, et al. (2004). "Estrogen receptor beta inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest." Cancer Res **64**(1): 423-8.
Pathak, D. R. and A. S. Whittemore (1992). "Combined effects of body size, parity, and menstrual events on breast cancer incidence in seven countries." Am J Epidemiol **135**(2): 153-68.
Patrao, M. T., E. J. Silva, et al. (2009). "Androgens and the male reproductive tract: an overview of classical roles and current perspectives." Arq Bras Endocrinol Metabol **53**(8): 934-45.
Penault-Llorca, F., F. Bertucci, et al. (1995). "Expression of FGF and FGF receptor genes in human breast cancer." Int J Cancer **61**(2): 170-6.

Perillo, B., M. N. Ombra, et al. (2008). "DNA oxidation as triggered by H3K9me2 demethylation drives estrogen-induced gene expression." Science **319**(5860): 202-6.
Perillo, B., A. Sasso, et al. (2000). "17beta-estradiol inhibits apoptosis in MCF-7 cells, inducing bcl-2 expression via two estrogen-responsive elements present in the coding sequence." Mol Cell Biol **20**(8): 2890-901.
Perou, C. M., T. Sorlie, et al. (2000). "Molecular portraits of human breast tumours." Nature **406**(6797): 747-52.
Persson, I., E. Weiderpass, et al. (1999). "Risks of breast and endometrial cancer after estrogen and estrogen-progestin replacement." Cancer Causes Control **10**(4): 253-60.
Perusinghe, N. P., P. Monaghan, et al. (1992). "Effects of growth factors on proliferation on basal and luminal cells in human breast epithelial explants in serum-free culture." In Vitro Cell Dev Biol **28A**(2): 90-6.
Peters, A. A., G. Buchanan, et al. (2009). "Androgen receptor inhibits estrogen receptor-alpha activity and is prognostic in breast cancer." Cancer Res **69**(15): 6131-40.
Petersen, O. W., P. E. Hoyer, et al. (1987). "Frequency and distribution of estrogen receptor-positive cells in normal, nonlactating human breast tissue." Cancer Res **47**(21): 5748-51.
Pike, M. C., M. D. Krailo, et al. (1983). "'Hormonal' risk factors, 'breast tissue age' and the age-incidence of breast cancer." Nature **303**(5920): 767-70.
Pike, M. C., R. K. Peters, et al. (1997). "Estrogen-progestin replacement therapy and endometrial cancer." J Natl Cancer Inst **89**(15): 1110-6.
Pike, M. C. and R. K. Ross (2000). "Progestins and menopause: epidemiological studies of risks of endometrial and breast cancer." Steroids **65**(10-11): 659-64.
Pike, M. C., D. V. Spicer, et al. (1993). "Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk." Epidemiol Rev **15**(1): 17-35.
Pike, M. C., A. H. Wu, et al. (2007). "Estrogens, progestins, and risk of breast cancer." Ernst Schering Found Symp Proc(1): 127-50.
Pinta, E. R. (1978). "Treatment of obsessive homosexual pedophilic fantasies with medroxyprogesterone acetate." Biol Psychiatry **13**(3): 369-73.
Pollei, S. R., F. A. Mettler, Jr., et al. (1987). "Occult breast cancer: prevalence and radiographic detectability." Radiology **163**(2): 459-62.
Popnikolov, N., J. Yang, et al. (2001). "Reconstituted normal human breast in nude mice: effect of host pregnancy environment and human chorionic gonadotropin on proliferation." J Endocrinol **168**(3): 487-96.
Porch, J. V., I. M. Lee, et al. (2002). "Estrogen-progestin replacement therapy and breast cancer risk: the Women's Health Study (United States)." Cancer Causes Control **13**(9): 847-54.
Porter, P. L. (2009). "Global trends in breast cancer incidence and mortality." Salud Publica Mex **51 Suppl 2**: s141-6.
Potten, C. S., R. J. Watson, et al. (1988). "The effect of age and menstrual cycle upon proliferative activity of the normal human breast." Br J Cancer **58**(2): 163-70.
Poulin, R., D. Baker, et al. (1988). "Androgens inhibit basal and estrogen-induced cell proliferation in the ZR-75-1 human breast cancer cell line." Breast Cancer Res Treat **12**(2): 213-25.

Poulin, R., D. Baker, et al. (1989). "Androgen and glucocorticoid receptor-mediated inhibition of cell proliferation by medroxyprogesterone acetate in ZR-75-1 human breast cancer cells." Breast Cancer Res Treat **13**(2): 161-72.
Poulin, R., D. Baker, et al. (1991). "Multiple actions of synthetic 'progestins' on the growth of ZR-75-1 human breast cancer cells: an in vitro model for the simultaneous assay of androgen, progestin, estrogen, and glucocorticoid agonistic and antagonistic activities of steroids." Breast Cancer Res Treat **17**(3): 197-210.
Poulin, R., J. Simard, et al. (1989). "Down-regulation of estrogen receptors by androgens in the ZR-75-1 human breast cancer cell line." Endocrinology **125**(1): 392-9.
Prasad, R., G. P. Boland, et al. (2003). "Short-term biologic response to withdrawal of hormone replacement therapy in patients with invasive breast carcinoma." Cancer **98**(12): 2539-46.
Pratt, W. B. and D. O. Toft (1997). "Steroid receptor interactions with heat shock protein and immunophilin chaperones." Endocr Rev **18**(3): 306-60.
Prentice, R. L., R. T. Chlebowski, et al. (2008). "Conjugated equine estrogens and breast cancer risk in the Women's Health Initiative clinical trial and observational study." Am J Epidemiol **167**(12): 1407-15.
Prentice, R. L., Y. Huang, et al. "Variation in the FGFR2 gene and the effect of a low-fat dietary pattern on invasive breast cancer." Cancer Epidemiol Biomarkers Prev **19**(1): 74-9.
Prentice, R. L., Y. Huang, et al. (2009). "Variation in the FGFR2 gene and the effects of postmenopausal hormone therapy on invasive breast cancer." Cancer Epidemiol Biomarkers Prev **18**(11): 3079-85.
Qiu, X. B., S. L. Markant, et al. (2004). "Nrdp1-mediated degradation of the gigantic IAP, BRUCE, is a novel pathway for triggering apoptosis." Embo J **23**(4): 800-10.
Rakoff, A. E. (1959). "Use of new progestational hormones in gynecology and obstetrics." Fed Proc **18**: 1066-74.
Rannevik, G., S. Jeppsson, et al. (1995). "A longitudinal study of the perimenopausal transition: altered profiles of steroid and pituitary hormones, SHBG and bone mineral density." Maturitas **21**(2): 103-13.
Ravdin, P. M., K. A. Cronin, et al. (2007). "The decrease in breast-cancer incidence in 2003 in the United States." N Engl J Med **356**(16): 1670-4.
Rebbeck, T. R., A. DeMichele, et al. (2009). "Hormone-dependent effects of FGFR2 and MAP3K1 in breast cancer susceptibility in a population-based sample of post-menopausal African-American and European-American women." Carcinogenesis **30**(2): 269-74.
Renard, F., P. Vankrunkelsven, et al. "Decline in breast cancer incidence in the Flemish region of Belgium after a decline in hormonal replacement therapy." Ann Oncol.
Richer, J. K., B. M. Jacobsen, et al. (2002). "Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells." J Biol Chem **277**(7): 5209-18.
Ricketts, D., L. Turnbull, et al. (1991). "Estrogen and progesterone receptors in the normal female breast." Cancer Res **51**(7): 1817-22.
Rijnkels, M., E. Kabotyanski, et al. "The epigenetic landscape of mammary gland development and functional differentiation." J Mammary Gland Biol Neoplasia **15**(1): 85-100.

Rijnkels, M., E. Kabotyanski, et al. (2010). "The epigenetic landscape of mammary gland development and functional differentiation." J Mammary Gland Biol Neoplasia **15**(1): 85-100.
Ris-Stalpers, C., G. G. Kuiper, et al. (1990). "Aberrant splicing of androgen receptor mRNA results in synthesis of a nonfunctional receptor protein in a patient with androgen insensitivity." Proc Natl Acad Sci U S A **87**(20): 7866-70.
Riva, C., E. Dainese, et al. (2005). "Immunohistochemical study of androgen receptors in breast carcinoma. Evidence of their frequent expression in lobular carcinoma." Virchows Arch **447**(4): 695-700.
Robbins, A. S. and C. A. Clarke (2007). "Regional changes in hormone therapy use and breast cancer incidence in California from 2001 to 2004." J Clin Oncol **25**(23): 3437-9.
Roberts, M., J. Wallace, et al. (1988). "The 5' flanking region of the human pS2 gene mediates its transcriptional activation by estrogen in MCF-7 cells." Biochem Biophys Res Commun **151**(1): 306-13.
Robinson, S. P. and V. C. Jordan (1987). "Reversal of the antitumor effects of tamoxifen by progesterone in the 7,12-dimethylbenzanthracene-induced rat mammary carcinoma model." Cancer Res **47**(20): 5386-90.
Rosa, F. E., J. R. Caldeira, et al. (2008). "Evaluation of estrogen receptor alpha and beta and progesterone receptor expression and correlation with clinicopathologic factors and proliferative marker Ki-67 in breast cancers." Hum Pathol **39**(5): 720-30.
Rosa, M. ""Inflammatory" changes in breast: how to provide a better care to our patients." Arch Gynecol Obstet **281**(5): 901-5.
Rosa, M. (2010). ""Inflammatory" changes in breast: how to provide a better care to our patients." Arch Gynecol Obstet **281**(5): 901-5.
Rosenfield, A., D. Maine, et al. (1983). "The Food and Drug Administration and medroxyprogesterone acetate. What are the issues?" Jama **249**(21): 2922-8.
Rosner, B., G. A. Colditz, et al. (1994). "Reproductive risk factors in a prospective study of breast cancer: the Nurses' Health Study." Am J Epidemiol **139**(8): 819-35.
Rosner, W. (1991). "Plasma steroid-binding proteins." Endocrinol Metab Clin North Am **20**(4): 697-720.
Ross, R. K., A. Paganini-Hill, et al. (2000). "Effect of hormone replacement therapy on breast cancer risk: estrogen versus estrogen plus progestin." J Natl Cancer Inst **92**(4): 328-32.
Rossouw, J. E., G. L. Anderson, et al. (2002). "Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial." Jama **288**(3): 321-33.
Rothchild, I. (1965). "Interrelations between progesterone and the ovary, pituitary, and central nervous system in the control of ovulation and the regulation of progesterone secretion." Vitam Horm **23**: 210-327.
Rudas, M., M. F. Gnant, et al. (1994). "Thymidine labeling index and Ki-67 growth fraction in breast cancer: comparison and correlation with prognosis." Breast Cancer Res Treat **32**(2): 165-75.
Russo, I. H. and J. Russo (1998). "Role of hormones in mammary cancer initiation and progression." J Mammary Gland Biol Neoplasia **3**(1): 49-61.

Russo, I. H. and J. Russo (2007). "Primary prevention of breast cancer by hormone-induced differentiation." Recent Results Cancer Res **174**: 111-30.
Russo, J., X. Ao, et al. (1999). "Pattern of distribution of cells positive for estrogen receptor alpha and progesterone receptor in relation to proliferating cells in the mammary gland." Breast Cancer Res Treat **53**(3): 217-27.
Russo, J., G. A. Balogh, et al. (2006). "Molecular basis of pregnancy-induced breast cancer protection." Eur J Cancer Prev **15**(4): 306-42.
Russo, J., B. A. Gusterson, et al. (1990). "Comparative study of human and rat mammary tumorigenesis." Lab Invest **62**(3): 244-78.
Russo, J., D. Mailo, et al. (2005). "Breast differentiation and its implication in cancer prevention." Clin Cancer Res **11**(2 Pt 2): 931s-6s.
Russo, J., M. J. Mills, et al. (1989). "Influence of human breast development on the growth properties of primary cultures." In Vitro Cell Dev Biol **25**(7): 643-9.
Russo, J., R. Rivera, et al. (1992). "Influence of age and parity on the development of the human breast." Breast Cancer Res Treat **23**(3): 211-8.
Russo, J. and I. H. Russo (1994). "Toward a physiological approach to breast cancer prevention." Cancer Epidemiol Biomarkers Prev **3**(4): 353-64.
Russo, J. and I. H. Russo (2004). "Development of the human breast." Maturitas **49**(1): 2-15.
Russo, J., L. K. Tay, et al. (1982). "Differentiation of the mammary gland and susceptibility to carcinogenesis." Breast Cancer Res Treat **2**(1): 5-73.
Sabourin, J. C., A. Martin, et al. (1994). "bcl-2 expression in normal breast tissue during the menstrual cycle." Int J Cancer **59**(1): 1-6.
Saitoh, M., M. Ohmichi, et al. (2005). "Medroxyprogesterone acetate induces cell proliferation through up-regulation of cyclin D1 expression via phosphatidylinositol 3-kinase/Akt/nuclear factor-kappaB cascade in human breast cancer cells." Endocrinology **146**(11): 4917-25.
Sakuma, T., T. Yamaguchi, et al. (1994). "Adrenogenital syndrome caused by an androgen-producing adrenocortical tumor." Intern Med **33**(12): 790-4.
Sanchez, R., D. Nguyen, et al. (2002). "Diversity in the mechanisms of gene regulation by estrogen receptors." Bioessays **24**(3): 244-54.
Santini, D., C. Ceccarelli, et al. (1996). "Differentiation pathways in primary invasive breast carcinoma as suggested by intermediate filament and biopathological marker expression." J Pathol **179**(4): 386-91.
Sauter, E. R., M. Daly, et al. (1996). "Prostate-specific antigen levels in nipple aspirate fluid correlate with breast cancer risk." Cancer Epidemiol Biomarkers Prev **5**(12): 967-70.
Sauter, E. R., G. Klein, et al. (2004). "Prostate-specific antigen expression in nipple aspirate fluid is associated with advanced breast cancer." Cancer Detect Prev **28**(1): 27-31.
Sauter, E. R., J. Lininger, et al. (2004). "Association of kallikrein expression in nipple aspirate fluid with breast cancer risk." Int J Cancer **108**(4): 588-91.
Sauter, E. R., D. S. Tichansky, et al. (2002). "Circulating testosterone and prostate-specific antigen in nipple aspirate fluid and tissue are associated with breast cancer." Environ Health Perspect **110**(3): 241-6.

Savouret, J. F., A. Bailly, et al. (1991). "Characterization of the hormone responsive element involved in the regulation of the progesterone receptor gene." Embo J **10**(7): 1875-83.
Saxena, T., E. Lee, et al. (2010). "Menopausal hormone therapy and subsequent risk of specific invasive breast cancer subtypes in the California Teachers Study." Cancer Epidemiol Biomarkers Prev **19**(9): 2366-78.
Schairer, C., J. Lubin, et al. (2000). "Menopausal estrogen and estrogen-progestin replacement therapy and breast cancer risk." Jama **283**(4): 485-91.
Schindler, A. E. (2006). "Climacteric symptoms and hormones." Gynecol Endocrinol **22**(3): 151-4.
Schippinger, W., P. Regitnig, et al. (2006). "Evaluation of the prognostic significance of androgen receptor expression in metastatic breast cancer." Virchows Arch **449**(1): 24-30.
Schlesinger, T. K., C. Bonvin, et al. (2002). "Apoptosis stimulated by the 91-kDa caspase cleavage MEKK1 fragment requires translocation to soluble cellular compartments." J Biol Chem **277**(12): 10283-91.
Schramek, D., A. Leibbrandt, et al. (2010). "Osteoclast differentiation factor RANKL controls development of progestin-driven mammary cancer." Nature **468**(7320): 98-102.
Schuur, E. R., G. A. Henderson, et al. (1996). "Prostate-specific antigen expression is regulated by an upstream enhancer." J Biol Chem **271**(12): 7043-51.
Semiglazov, V. F., V. M. Moiseenko, et al. (1989). "[Growth kinetics of breast cancer]." Vopr Onkol **35**(3): 288-93.
Seradour, B., H. Allemand, et al. (2009). "Changes by age in breast cancer incidence, mammography screening and hormone therapy use in France from 2000 to 2006." Bull Cancer **96**(4): E1-6.
Shafie, S. M. and F. H. Grantham (1981). "Role of hormones in the growth and regression of human breast cancer cells (MCF-7) transplanted into athymic nude mice." J Natl Cancer Inst **67**(1): 51-6.
Shang, Y., C. R. Baumrucker, et al. (1999). "The induction and activation of STAT1 by all-trans-retinoic acid are mediated by RAR beta signaling pathways in breast cancer cells." Oncogene **18**(48): 6725-32.
Shanle, E. K. and W. Xu "Endocrine disrupting chemicals targeting estrogen receptor signaling: identification and mechanisms of action." Chem Res Toxicol **24**(1): 6-19.
Shapiro, S., L. Rosenberg, et al. (2000). "Risk of breast cancer in relation to the use of injectable progestogen contraceptives and combined estrogen/progestogen contraceptives." Am J Epidemiol **151**(4): 396-403.
Sharpe, K. H., P. McClements, et al. "Reduced risk of oestrogen receptor positive breast cancer among peri- and post-menopausal women in Scotland following a striking decrease in use of hormone replacement therapy." Eur J Cancer **46**(5): 937-43.
Sheffield, L. G. and C. W. Welsch (1988). "Transplantation of human breast epithelia to mammary-gland-free fat-pads of athymic nude mice: influence of mammotrophic hormones on growth of breast epithelia." Int J Cancer **41**(5): 713-9.

Shekhar, M. P., J. Werdell, et al. (2001). "Breast stroma plays a dominant regulatory role in breast epithelial growth and differentiation: implications for tumor development and progression." Cancer Res **61**(4): 1320-6.
Shen, R., M. C. Lin, et al. (1996). "Androgens are not major down-regulators of androgen receptor levels during growth of the immature rat penis." J Steroid Biochem Mol Biol **57**(5-6): 301-13.
Shiina, H., T. Matsumoto, et al. (2006). "Premature ovarian failure in androgen receptor-deficient mice." Proc Natl Acad Sci U S A **103**(1): 224-9.
Shoker, B. S., C. Jarvis, et al. (1999). "Estrogen receptor-positive proliferating cells in the normal and precancerous breast." Am J Pathol **155**(6): 1811-5.
Shoker, B. S., C. Jarvis, et al. (2000). "Abnormal regulation of the oestrogen receptor in benign breast lesions." J Clin Pathol **53**(10): 778-83.
Shoker, B. S., C. Jarvis, et al. (1999). "Oestrogen receptor expression in the normal and pre-cancerous breast." J Pathol **188**(3): 237-44.
Shyamala, G. (1999). "Progesterone signaling and mammary gland morphogenesis." J Mammary Gland Biol Neoplasia **4**(1): 89-104.
Shyamala, G., X. Yang, et al. (2000). "Impact of progesterone receptor on cell-fate decisions during mammary gland development." Proc Natl Acad Sci U S A **97**(7): 3044-9.
Shyamala, G., X. Yang, et al. (1998). "Transgenic mice carrying an imbalance in the native ratio of A to B forms of progesterone receptor exhibit developmental abnormalities in mammary glands." Proc Natl Acad Sci U S A **95**(2): 696-701.
Silver, R. I., E. L. Wiley, et al. (1994). "Expression and regulation of steroid 5 alpha-reductase 2 in prostate disease." J Urol **152**(2 Pt 1): 433-7.
Simard, J., S. Dauvois, et al. (1990). "Regulation of progesterone-binding breast cyst protein GCDFP-24 secretion by estrogens and androgens in human breast cancer cells: a new marker of steroid action in breast cancer." Endocrinology **126**(6): 3223-31.
Simpson, E. R. (2002). "Aromatization of androgens in women: current concepts and findings." Fertil Steril **77 Suppl 4**: S6-10.
Singh, J., P. Manickam, et al. (2006). "Annotation of androgen dependence to human prostate cancer-associated genes by microarray analysis of mouse prostate." Cancer Lett **237**(2): 298-304.
Sitruk-Ware, R. (1999). "Progestins and Antiprogestins in Clinical Practice." Textbook.
Sitruk-Ware, R. (2004). "New progestogens: a review of their effects in perimenopausal and postmenopausal women." Drugs Aging **21**(13): 865-83.
Sitruk-Ware, R. (2004). "Pharmacological profile of progestins." Maturitas **47**(4): 277-83.
Skegg, D. C., E. A. Noonan, et al. (1995). "Depot medroxyprogesterone acetate and breast cancer. A pooled analysis of the World Health Organization and New Zealand studies." Jama **273**(10): 799-804.
Skegg, D. C., C. Paul, et al. (1996). "Progestogen-only oral contraceptives and risk of breast cancer in New Zealand." Cancer Causes Control **7**(5): 513-9.
Slagter, M. H., L. J. Gooren, et al. (2006). "Effects of long-term androgen administration on breast tissue of female-to-male transsexuals." J Histochem Cytochem **54**(8): 905-10.

Smith, C. A., P. Monaghan, et al. (1984). "Basal clear cells of the normal human breast." Virchows Arch A Pathol Anat Histopathol **402**(3): 319-29.
Smith, D. C., R. Prentice, et al. (1975). "Association of exogenous estrogen and endometrial carcinoma." N Engl J Med **293**(23): 1164-7.
Soderqvist, G., E. Isaksson, et al. (1997). "Proliferation of breast epithelial cells in healthy women during the menstrual cycle." Am J Obstet Gynecol **176**(1 Pt 1): 123-8.
Somboonporn, W. and S. R. Davis (2004). "Testosterone effects on the breast: implications for testosterone therapy for women." Endocr Rev **25**(3): 374-88.
Soreide, J. A., O. A. Lea, et al. (1992). "Androgen receptors in operable breast cancer: relation to other steroid hormone receptors, correlations to prognostic factors and predictive value for effect of adjuvant tamoxifen treatment." Eur J Surg Oncol **18**(2): 112-8.
Sorlie, T., C. M. Perou, et al. (2001). "Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications." Proc Natl Acad Sci U S A **98**(19): 10869-74.
Sorlie, T., R. Tibshirani, et al. (2003). "Repeated observation of breast tumor subtypes in independent gene expression data sets." Proc Natl Acad Sci U S A **100**(14): 8418-23.
Soto, A. M. and C. Sonnenschein "Environmental causes of cancer: endocrine disruptors as carcinogens." Nat Rev Endocrinol **6**(7): 363-70.
Soule, H. D. and C. M. McGrath (1980). "Estrogen responsive proliferation of clonal human breast carcinoma cells in athymic mice." Cancer Lett **10**(2): 177-89.
Soules, M. R., D. K. Clifton, et al. (1987). "Gonadotropin-releasing hormone-induced changes in testosterone secretion in normal women." Fertil Steril **48**(3): 423-7.
Steen, R. L., F. Cubizolles, et al. (2000). "A kinase-anchoring protein (AKAP)95 recruits human chromosome-associated protein (hCAP)-D2/Eg7 for chromosome condensation in mitotic extract." J Cell Biol **149**(3): 531-6.
Stefanick, M. L. (2005). "Estrogens and progestins: background and history, trends in use, and guidelines and regimens approved by the US Food and Drug Administration." Am J Med **118 Suppl 12B**: 64-73.
Steketee, K., A. C. Ziel-van der Made, et al. (2004). "A bioinformatics-based functional analysis shows that the specifically androgen-regulated gene SARG contains an active direct repeat androgen response element in the first intron." J Mol Endocrinol **33**(2): 477-91.
Stingl, J., C. J. Eaves, et al. (2001). "Characterization of bipotent mammary epithelial progenitor cells in normal adult human breast tissue." Breast Cancer Res Treat **67**(2): 93-109.
Stingl, J., A. Raouf, et al. (2005). "Epithelial progenitors in the normal human mammary gland." J Mammary Gland Biol Neoplasia **10**(1): 49-59.
Stomper, P. C., B. J. Van Voorhis, et al. (1990). "Mammographic changes associated with postmenopausal hormone replacement therapy: a longitudinal study." Radiology **174**(2): 487-90.
Strom, A., J. Hartman, et al. (2004). "Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D." Proc Natl Acad Sci U S A **101**(6): 1566-71.

Stute, P., C. E. Wood, et al. (2004). "Cyclic changes in the mammary gland of cynomolgus macaques." Fertil Steril **82 Suppl 3**: 1160-70.
Summers, R. H., D. A. Herold, et al. (1996). "Hormonal and genetic analysis of a patient with congenital adrenal hyperplasia." Clin Chem **42**(9): 1483-7.
Suzuki, T., Y. Miki, et al. (2007). "5Alpha-reductase type 1 and aromatase in breast carcinoma as regulators of in situ androgen production." Int J Cancer **120**(2): 285-91.
Svensson, L. O., S. H. Johnson, et al. (1994). "Plasma concentrations of medroxyprogesterone acetate, estradiol and estrone following oral administration of Klimaxil, Trisequence/Provera and Divina. A randomized, single-blind, triple cross-over bioavailability study in menopausal women." Maturitas **18**(3): 229-38.
Szelei, J., J. Jimenez, et al. (1997). "Androgen-induced inhibition of proliferation in human breast cancer MCF7 cells transfected with androgen receptor." Endocrinology **138**(4): 1406-12.
Tan, H., Y. Zhong, et al. (2009). "Autocrine regulation of cell proliferation by estrogen receptor-alpha in estrogen receptor-alpha-positive breast cancer cell lines." BMC Cancer **9**: 31.
Taylor-Papadimitriou, J., M. Stampfer, et al. (1989). "Keratin expression in human mammary epithelial cells cultured from normal and malignant tissue: relation to in vivo phenotypes and influence of medium." J Cell Sci **94 (Pt 3)**: 403-13.
Taylor, D., C. L. Pearce, et al. (2009). "Progesterone and estrogen receptors in pregnant and premenopausal non-pregnant normal human breast." Breast Cancer Res Treat **118**(1): 161-8.
Teller, M. N., R. J. Kaufman, et al. (1969). "Influence of estrogens and endocrine ablation on duration of remission produced by ovariectomy or androgen treatment of 7,12-dimethylbenz(a)anthracene-induced rat mammary tumors." Cancer Res **29**(2): 349-52.
Tetel, M. J., S. Jung, et al. (1997). "Hinge and amino-terminal sequences contribute to solution dimerization of human progesterone receptor." Mol Endocrinol **11**(8): 1114-28.
Thigpen, A. E., R. I. Silver, et al. (1993). "Tissue distribution and ontogeny of steroid 5 alpha-reductase isozyme expression." J Clin Invest **92**(2): 903-10.
Thomas, F. H., R. S. Ismail, et al. (2008). "Kit ligand 2 promotes murine oocyte growth in vitro." Biol Reprod **78**(1): 167-75.
Thor, A. D., S. Liu, et al. (1999). "Comparison of mitotic index, in vitro bromodeoxyuridine labeling, and MIB-1 assays to quantitate proliferation in breast cancer." J Clin Oncol **17**(2): 470-7.
Thum, T. and J. Borlak (2008). "LOX-1 receptor blockade abrogates oxLDL-induced oxidative DNA damage and prevents activation of the transcriptional repressor Oct-1 in human coronary arterial endothelium." J Biol Chem **283**(28): 19456-64.
Tilley, W. D., S. S. Lim-Tio, et al. (1994). "Detection of discrete androgen receptor epitopes in prostate cancer by immunostaining: measurement by color video image analysis." Cancer Res **54**(15): 4096-102.
Tilley, W. D., M. Marcelli, et al. (1989). "Characterization and expression of a cDNA encoding the human androgen receptor." Proc Natl Acad Sci U S A **86**(1): 327-31.

Tomic, R., B. Ljungberg, et al. (1988). "Hormonal effects of high dose medroxyprogesterone acetate treatment in males with renal or prostatic adenocarcinoma." Scand J Urol Nephrol **22**(1): 15-8.
Townsend, C. M. (2003). "Sabiston textbook of surgery: the biological basis of modern surgical practice 18th edition ".
Trapman, J., P. Klaassen, et al. (1988). "Cloning, structure and expression of a cDNA encoding the human androgen receptor." Biochem Biophys Res Commun **153**(1): 241-8.
Treloar, A. E. (1981). "Menstrual cyclicity and the pre-menopause." Maturitas **3**(3-4): 249-64.
Trowell, O. A. (1959). "The culture of mature organs in a synthetic medium." Exp Cell Res **16**(1): 118-47.
Tung, L., M. K. Mohamed, et al. (1993). "Antagonist-occupied human progesterone B-receptors activate transcription without binding to progesterone response elements and are dominantly inhibited by A-receptors." Mol Endocrinol **7**(10): 1256-65.
Tut, T. G., F. J. Ghadessy, et al. (1997). "Long polyglutamine tracts in the androgen receptor are associated with reduced trans-activation, impaired sperm production, and male infertility." J Clin Endocrinol Metab **82**(11): 3777-82.
Twombly, R. (2007). "So far, victories are few as breast cancer patients sue Wyeth over hormone therapy." J Natl Cancer Inst **99**(24): 1828-9, 1835.
Urruticoechea, A., I. E. Smith, et al. (2005). "Proliferation marker Ki-67 in early breast cancer." J Clin Oncol **23**(28): 7212-20.
Vaidya, J. S., G. Baldassarre, et al. (2010). "Role of glucocorticoids in breast cancer." Curr Pharm Des **16**(32).
van 't Veer, L. J., H. Dai, et al. (2002). "Gene expression profiling predicts clinical outcome of breast cancer." Nature **415**(6871): 530-6.
van den Brandt, P. A., D. Spiegelman, et al. (2000). "Pooled analysis of prospective cohort studies on height, weight, and breast cancer risk." Am J Epidemiol **152**(6): 514-27.
van Dierendonck, J. H., R. Keijzer, et al. (1989). "Nuclear distribution of the Ki-67 antigen during the cell cycle: comparison with growth fraction in human breast cancer cells." Cancer Res **49**(11): 2999-3006.
van Slooten, H. J., M. J. van de Vijver, et al. (1998). "Loss of Bcl-2 in invasive breast cancer is associated with high rates of cell death, but also with increased proliferative activity." Br J Cancer **77**(5): 789-96.
Vegeto, E., M. M. Shahbaz, et al. (1993). "Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function." Mol Endocrinol **7**(10): 1244-55.
Verrijdt, G., A. Haelens, et al. (2003). "Selective DNA recognition by the androgen receptor as a mechanism for hormone-specific regulation of gene expression." Mol Genet Metab **78**(3): 175-85.
Visvader, J. E. (2009). "Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis." Genes Dev **23**(22): 2563-77.
Walker, K. J., R. A. McClelland, et al. (1992). "Heterogeneity of oestrogen receptor expression in normal and malignant breast tissue." Eur J Cancer **28**(1): 34-7.

Walker, K. J., J. M. Price-Thomas, et al. (1991). "Influence of the antioestrogen tamoxifen on normal breast tissue." Br J Cancer **64**(4): 764-8.
Walker, R. A., Cowl, J, Dhadly, P.P and Jones, J.L (1992). "Oestrogen receptor, epidermal growth factor receptor and oncoprotein expression in non-involved tissue of cancerous breasts." The Breast **2**: 87-91.
Walker, R. A. and C. V. Martin (2007). "The aged breast." J Pathol **211**(2): 232-40.
Wan, Y. and S. K. Nordeen (2003). "Overlapping but distinct profiles of gene expression elicited by glucocorticoids and progestins." Recent Prog Horm Res **58**: 199-226.
Wang, Q., W. Li, et al. (2007). "A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth." Mol Cell **27**(3): 380-92.
Wang, S., L. J. Counterman, et al. (1990). "Progesterone action in normal mouse mammary gland." Endocrinology **127**(5): 2183-9.
Wang, X. D., B. E. Wang, et al. (2007). "Expression profiling of the mouse prostate after castration and hormone replacement: implication of H-cadherin in prostate tumorigenesis." Differentiation **75**(3): 219-34.
Weikel, W., T. Beck, et al. (1991). "Immunohistochemical evaluation of growth fractions in human breast cancers using monoclonal antibody Ki-67." Breast Cancer Res Treat **18**(3): 149-54.
Wellings, S. R., H. M. Jensen, et al. (1975). "An atlas of subgross pathology of the human breast with special reference to possible precancerous lesions." J Natl Cancer Inst **55**(2): 231-73.
Welsch, C. W., E. L. Swim, et al. (1981). "Estrogen induced growth of human breast cancer cells (MCF-7) in athymic nude mice is enhanced by secretions from a transplantable pituitary tumor." Cancer Lett **14**(3): 309-16.
Wen, D. X., Y. F. Xu, et al. (1994). "The A and B isoforms of the human progesterone receptor operate through distinct signaling pathways within target cells." Mol Cell Biol **14**(12): 8356-64.
Werner Kahle, H. L. a. W. P. (2004). "Color Atlas and Textbook of Human Anatomy: Internal organs (4th revised edition)."
Westphal, U. (1971). "Steroid-protein interactions." Monogr Endocrinol **4**: 1-567.
Whitescarver, J., L. Rechier, et al. (1968). "Problems involved in culturing human breast tissue." Tex Rep Biol Med **26**(4): 613-28.
WHO (1991). "Breast cancer and depot-medroxyprogesterone acetate: a multinational study. WHO Collaborative Study of Neoplasia and Steroid Contraceptives." Lancet **338**(8771): 833-8.
WHO (1996). "Research on the menopause in the 1990s. Report of a WHO Scientific Group." World Health Organ Tech Rep Ser **866**(1-107).
WHO (2005). "IARC Monographs Programme Finds Combined Estrogen-Progestogen Contraceptives (the "pill") and Menopausal Therapy Are Carcinogenic to Humans." **Press Release No. 167**.
Wilson, V. S., K. Bobseine, et al. (2002). "A novel cell line, MDA-kb2, that stably expresses an androgen- and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists." Toxicol Sci **66**(1): 69-81.
Wockel, A., M. Abou-Dakn, et al. (2008). "Inflammatory breast diseases during lactation: health effects on the newborn-a literature review." Mediators Inflamm **2008**: 298760.

Wolman, S. R., A. N. Mohamed, et al. (1994). "Chromosomal markers of immortalization in human breast epithelium." Genes Chromosomes Cancer **10**(1): 59-65.
Wood, C. E., T. C. Register, et al. (2007). "Effects of estradiol with micronized progesterone or medroxyprogesterone acetate on risk markers for breast cancer in postmenopausal monkeys." Breast Cancer Res Treat **101**(2): 125-34.
Wu, R. C., C. L. Smith, et al. (2005). "Transcriptional regulation by steroid receptor coactivator phosphorylation." Endocr Rev **26**(3): 393-9.
Wysowski, D. K. and L. A. Governale (2005). "Use of menopausal hormones in the United States, 1992 through June, 2003." Pharmacoepidemiol Drug Saf **14**(3): 171-6.
Yarosh, W., T. Barrientos, et al. (2008). "TBX3 is overexpressed in breast cancer and represses p14 ARF by interacting with histone deacetylases." Cancer Res **68**(3): 693-9.
Yee, D. (2009). "Targeting the insulin-like growth factor receptor." Clin Adv Hematol Oncol **7**(7): 452-4.
Yeh, S., Y. C. Hu, et al. (2003). "Abnormal mammary gland development and growth retardation in female mice and MCF7 breast cancer cells lacking androgen receptor." J Exp Med **198**(12): 1899-908.
Yen, S. S. (1977). "Regulation of the hypothalamic--pituitary--ovarian axis in women." J Reprod Fertil **51**(1): 181-91.
Zahl, P. H., J. Maehlen, et al. (2008). "The natural history of invasive breast cancers detected by screening mammography." Arch Intern Med **168**(21): 2311-6.
Zava, D. T. and W. L. McGuire (1977). "Estrogen receptors in androgen-induced breast tumor regression." Cancer Res **37**(6): 1608-10.
Zeps, N., J. M. Bentel, et al. (1998). "Estrogen receptor-negative epithelial cells in mouse mammary gland development and growth." Differentiation **62**(5): 221-6.
Zhou, J., S. Ng, et al. (2000). "Testosterone inhibits estrogen-induced mammary epithelial proliferation and suppresses estrogen receptor expression." Faseb J **14**(12): 1725-30.
Zhu, X., S. L. Asa, et al. "Genetic and epigenetic mechanisms down-regulate FGF receptor 2 to induce melanoma-associated antigen A in breast cancer." Am J Pathol **176**(5): 2333-43.
Zhuang, Y. H., R. Saaristo, et al. (2003). "An in vitro long-term culture model for normal human mammary gland: expression and regulation of steroid receptors." Cell Tissue Res **311**(2): 217-26.
Ziel, H. K. and W. D. Finkle (1975). "Increased risk of endometrial carcinoma among users of conjugated estrogens." N Engl J Med **293**(23): 1167-70.
Zuo, L., W. Li, et al. (2010). "Progesterone reverses the mesenchymal phenotypes of basal phenotype breast cancer cells via a membrane progesterone receptor mediated pathway." Breast Cancer Res **12**(3): R34.