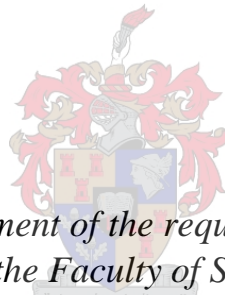


**Investigating the influence of the progesterone receptor isoforms on androgen receptor activity in breast and prostate cancer cell lines**

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*Thesis presented in fulfilment of the requirements for the degree of  
Master in Science in the Faculty of Science at Stellenbosch  
University*

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March 2021

## Declaration

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## Abstract

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Breast cancer growth and survival are primarily dependent on signalling of estrogens via the estrogen receptor (ER), while for prostate cancer it is dependent on androgens acting via the androgen receptor (AR). However, other steroid receptors such as the progesterone receptor (PR) have also been found in both breast and prostate cancer tumours. Results from a previous study in our laboratory, have suggested crosstalk between the AR and both the PR isoforms, PRA and PRB, in both breast and prostate cancer cell lines. This study also showed that, in addition to transactivation on an androgen response element (ARE), the AR could also transactivate an estrogen response element (ERE), which is the binding site for the ER. As the aforementioned study used synthetic ARE- and ERE-containing promoters in reporter assays, the aim of this study was to assess whether similar responses would also be observed on endogenous AR- and ER-regulated genes in the MDA-MB-231 breast cancer cell line. As a proof of concept, a preliminary experiment was also performed in the PC3 prostate cancer cell line. Whether the AR and PRA or PRB occur in a molecular complex was also investigated using co-immunoprecipitation (Co-IP) assays. Realtime quantitative PCR (qPCR) results showed that both PRA and PRB, modulate the activity of the AR on the expression of the endogenous AR-regulated gene, prostate specific antigen (PSA) and on the ER-regulated genes, cathepsin-D (CTSD) and PR. More specifically, both the unliganded and progesterone (P<sub>4</sub>)-liganded PR, further increased the dihydrotestosterone (DHT)-induced expression of the AR-regulated PSA gene in the MDA-MB-231 breast cancer cell line. While neither the unliganded PR isoforms, nor liganded PRB, modulated the AR-mediated downregulation of the ER-regulated CTSD gene by DHT, the P<sub>4</sub>-activated PRA, when expressed in excess, reversed this downregulation. Ligand-activated AR also decreased PR mRNA expression, while both the unliganded and liganded PRA and PRB reversed this decrease. Preliminary results in the PC3 prostate cancer cell line show that ligand activated AR downregulated the expression of the AR-regulated TMPRSS2 gene. Unliganded PRA, expressed at equivalent levels to the AR, lifted this decrease, while when either unliganded or liganded PRA and PRB was present in excess relative to AR, TMPRSS2 mRNA expression was increased. CTSD mRNA expression was increased in MDA-MB-231 breast cancer cells transfected with PRA, suggesting that PRA can activate transcription of an ER-regulated gene. In contrast, PRB decreased expression of an ER-regulated gene, as indicated by the decreased CTSD mRNA expression in MDA-MB-231 cells transfected with PRB. Under the experimental conditions used in this study, we could not show a molecular interaction between the AR and PRA or PRB

in COS-1 or MDA-MB-231 cells. However, collectively, the realtime qPCR results support previous findings with promoter-reporters showing crosstalk between the AR and PR in breast cancer, and contribute to our knowledge of steroid receptor crosstalk in breast cancer.

## Opsomming

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Die groei en oorlewing van borskanker is hoofsaaklik afhanklik van estrogeenseine via die estrogeenreseptor (ER), terwyl prostaatkanker afhanklik is van androgene wat via die androgeenreseptor (AR) funksioneer. Ander steroïedreseptore soos die progesteronreseptor (PR) word egter ook in bors- en prostaatkankergewasse gevind. Resultate van 'n vorige studie in ons laboratorium, het voorgestel dat wisselwerkingsmeganismes tussen die AR en beide die PR isoforme, PRA en PRB, in beide bors- en prostaatkankersellyne voorkom. Hierdie studie het ook getoon dat die AR, benewens transaktivering op 'n androgeenresponselement (ARE), ook 'n estrogeenresponselement (ERE) kan transaktiveer, wat die bindingsplek vir die ER is. Aangesien die voorafgenoemde studie sintetiese ARE- en ERE-bevattende promotors in promotor-rapporteurder toetse gebruik het, was die doel van hierdie studie om te bepaal of soortgelyke response ook op endogene AR- en ER-gereguleerde gene in die MDA-MB-231 borskankersellyn waargeneem sou word. As bewys van die konsep is 'n voorlopige eksperiment ook in die PC3 prostaatkankersellyn uitgevoer. Of AR en PRA of PRB in 'n molekulêre kompleks voorkom, was ook geondersoek met behulp van ko-immunopresipitasie (Co-IP) toetse. Kwantitatiewe intydse PKR (qPCR) resultate het getoon dat beide PRA en PRB die aktiwiteit van die AR op die uitdrukking van die endogene AR-gereguleerde geen, prostaat-spesifieke antigeen (PSA) en op die ER-gereguleerde gene, katepsien-D (CTSD) en PR, moduleer. Meer spesifiek, beide die ligandlose en progesteron ( $P_4$ ) ligand-gebonde PR, het die  $5\alpha$ -dihidrottestosteron (DHT) geïnduseerde uitdrukking van die AR-gereguleerde PSA geen in die MDA-MB-231 borskankersellyn verder verhoog. Alhoewel die ligandlose PR isoforme, en ligand-gebonde PRB, nie die AR-gemedieerde afregulering van die ER-gereguleerde CTSD-geen deur DHT gemoduleer het nie, het die  $P_4$ -geaktiveerde PRA hierdie afregulering omgekeer, wanneer dit in oormaat uitgedruk is. Ligand-geaktiveerde AR het ook die uitdrukking van PR mRNA-uitdrukking verminder, terwyl beide die ligandlose en ligand-gebonde PRA en PRB hierdie afname omgekeer het. Voorlopige resultate in die PC3 prostaatkankersellyn toon dat ligand-geaktiveerde AR die uitdrukking van die AR-gereguleerde TMPRSS2-geen afreguleer. Ligandlose PRA, uitgedruk teen dieselfde vlakke as die AR, lig hierdie afname, terwyl ligandlose en ligand-gebonde PRA en PRB ooruitgedruk teenoor die AR, die TMPRSS2 mRNA uitdrukking opreguleer. CTSD mRNA uitdrukking is verhoog in MDA-MB-231 borskankerselle wat met PRA getransfekteer was, wat daarop dui dat PRA transkripsie van 'n ER-gereguleerde geen kan aktiveer. In teenstelling, verminder PRB

die uitdrukking van 'n ER-gereguleerde geen, soos aangedui deur die verminderde uitdrukking van CTSD mRNA in MDA-MB-231 selle wat met PRB getransfekteer is. Onder die eksperimentele toestande wat in hierdie studie gebruik was, kon ons nie 'n molekulêre interaksie tussen AR en PRA of PRB in COS-1 of MDA-MB-231 selle toon nie. Gesamentlik ondersteun die qPCR resultate egter vorige bevindings met promotor-rapporteerders wat wisselwerking tussen AR en PR in borskanker toon, en dit dra by tot ons kennis van steroïedreseptor wisselwerkingsmeganismes in borskanker.

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## Alphabetical list of abbreviations

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AF	activation function
ANOVA	analysis of variance
AR	androgen receptor
ARE	androgen response element
ARE-luc	androgen response element-luciferase
ATCC	American Type Culture Collection
BPH	benign prostatic hyperplasia
CAF	Central Analytical Facility
cDNA	complementary deoxyribonucleic acid
ChIP	chromatin immunoprecipitation
Co-immunoprecipitation	co-IP
CS-FCS	charcoal-stripped fetal calf serum
CTSD	regulated cathepsin D
CYP17A1	cytochrome P450 17A1
DBD	DNA-binding domain
DHT	5 $\alpha$ -dihydrotestosterone
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
E <sub>2</sub>	17 $\beta$ -estradiol
ECL	enhanced chemiluminescence
EDTA	ethylene-diaminetetra-acetic acid
ER $\alpha$	estrogen receptor alpha
ER $\beta$	estrogen receptor beta
ER	estrogen receptor
ERE	estrogen response element
ERE-luc	estrogen response element-luciferase
EtOH	ethanol
FCS	fetal calf serum
FLIP	Fas-associated death domain-like interleukin-1 $\beta$ - converting enzyme-like inhibitory protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GR	glucocorticoid receptor



GRE	glucocorticoid response element
GFP	green fluorescent protein
GST	Glutathione S-transferase
HRP	horseradish peroxidase
kb	kilobase pairs
kDa	kilodaltons
KLK	kallikrein
LB	Luria Bertani
LBD	ligand-binding domain
Mib	mibolerone
MR	mineralocorticoid receptor
MRE	mineralocorticoid response element
mRNA	messenger ribonucleic acid
NF $\kappa$ B	nuclear factor kappa B
ns	non-significant
NTD	N-terminal transactivation domain
P <sub>4</sub>	progesterone
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PI3K	phosphoinositol-3-kinase
PR	progesterone receptor
PRA	progesterone receptor isoform A
PRB	progesterone receptor isoform B
PRE	progesterone response element
PRE-luc	progesterone response element-luciferase
pS2	trefoil factor 1
PSA	protein-specific antigen
qPCR	quantitative real-time polymerase chain reaction
R1881	methyltrienolone
R5020	promegestone
re-ChIP	sequential chromatin immunoprecipitation
RLU	relative light units
RPMI	Roswell Park Memorial Institute
S	steroid
SDS	sodium dodecyl sulphate

SEM	standard error of the mean
SERM(s)	selective estrogen receptor modulator(s)
siRNA	small interfering RNA
SOC	super optimal broth medium with catabolite repression
Sp1	specificity protein 1
SR	steroid receptor
SRE	steroid response element
TBS	tris buffered saline
TBST	tris buffered saline tween
TE	tris ethylene-diaminetetra-acetic acid
TNBC	triple-negative breast cancer
TMPRSS2	transmembrane protease serine 2
TIF2	transcriptional intermediary factor 2

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# **Chapter 1 Literature review**

## 1.1 Introduction

Breast cancer is the most common type of cancer among the female population worldwide, while prostate cancer accounts for the most new cases of cancer among men (Siegel, Miller and Jemal, 2019). Apart from lung cancer, both breast and prostate cancer have the highest mortality rate of the common forms of cancer in woman and men, respectively (Siegel, Miller and Jemal, 2019). Extensive research has been performed on the possible mechanisms of action driving breast and prostate cancer growth. Considering that both breast and prostate cancer are hormone-dependent cancers, several studies have investigated the role of different steroid hormones acting via their cognate steroid receptors in these malignancies (reviewed in Jordan and Brodie, 2007; Castrellon, 2017; Dai, Heemers and Sharifi, 2017; Groner and Brown, 2017; Stuchbery *et al.*, 2017; Africander and Storbeck, 2018).

It is well established that the natural estrogen  $17\beta$ -estradiol ( $E_2$ ) and the estrogen receptor alpha ( $ER\alpha$ ) subtype are the chief drivers of breast cancer development and progression (Beatson, 1896; Couse and Korach, 1999; Brisken and O'Malley, 2010), and that prostate cancer is driven by androgens acting via the androgen receptor (AR) (Culig and Santer, 2014; Tan *et al.*, 2015). Interestingly, it has also been suggested that  $ER\alpha$  has an oncogenic role in prostate cancer, as studies have found that  $ER\alpha$  is upregulated during the carcinogenesis of the prostatic epithelium, and its expression increased in metastatic prostate cancer by androgen deprivation therapy (Bonkhoff *et al.*, 1999; Shiota *et al.*, 2019). In contrast, the ER beta subtype ( $ER\beta$ ), also found in prostate cancer tumours, has been shown to be protective in prostate cancer by downregulating AR signalling (Bonkhoff, 2018). Interestingly, the role of the AR is also actively investigated in breast cancer and its role is dependent on the presence of  $ER\alpha$ . In  $ER\alpha$ -negative breast cancer, the AR promotes tumorigenesis (Agoff *et al.*, 2003; Rahim and O'Regan, 2017), and it has been suggested that this is due to the AR mimicking the proliferative role of the  $ER\alpha$  (Doane *et al.*, 2006; Peters *et al.*, 2009). While in ER-positive breast cancer, a study showed that the AR may indirectly decrease breast cancer cell proliferation by promoting  $ER\beta$  expression (Rizza *et al.*, 2014), which is known to inhibit the activity of  $ER\alpha$  (Covaleda *et al.*, 2008; Powell *et al.*, 2012). The study by Rizza and co-workers showed increased  $ER\beta$  mRNA as well as protein expression in the presence of the potent natural androgen  $5\alpha$ -dihydrotestosterone (DHT) as well as the synthetic androgen mibolerone (mib) in both the ER-positive MCF-7 and ZR75 breast cancer cell lines. Another study showed that the AR can modulate the activity of  $ER\alpha$  by competing with  $ER\alpha$  for binding to ER-binding sites or DNA

in close proximity to ER-binding sites in ER target genes (Peters *et al.*, 2009; Need *et al.*, 2012).

In addition to the AR and ER subtypes, other members of the steroid receptor family such as the progesterone receptor (PR) are also expressed in breast cancer and prostate cancer tumours. In breast cancer, there is renewed interest in the role of the PR and its natural ligand, progesterone (P<sub>4</sub>), as some studies suggest that it can have beneficial effects, while others report detrimental effects on breast cancer development (reviewed in Kunc, Biernat and Senkus-Konefka, 2018; Jorns, 2019; Wu *et al.*, 2019, 2020; Testa, Castelli and Pelosi, 2020). The PR has been established as a target gene of the ER $\alpha$  (Horwitz *et al.*, 1978; Peters *et al.*, 2009; Dunbier *et al.*, 2010), and its role in breast cancer is dependent on the expression of the two functional PR isoforms, PRA and PRB. In fact, a high expression of PRA relative to PRB has been shown to correlate with worse prognosis and tumour progression in breast cancer patients (Bamberger *et al.*, 2000; Mote *et al.*, 2002; Hopp *et al.*, 2004; Diep *et al.*, 2015; McFall *et al.*, 2018). Although the PR is also expressed in prostate cancer tumours (Grindstad *et al.*, 2015; Chen *et al.*, 2017), its precise role in prostate cancer development and/or progression is not clear. However, at least one study has found that high levels of PRB expression is indicative of a poor prognosis (Grindstad *et al.*, 2018).

A number of studies have shown crosstalk between specific steroid receptors in both breast and prostate cancer. For example, crosstalk between the PR and ER $\alpha$  in breast cancer, indicating that the PR can modulate the transcriptional activity of ER $\alpha$  by reprogramming ER $\alpha$  chromatin binding, resulting in an improved clinical outcome in patients with ER positive tumours (Mohammed *et al.*, 2015; Singhal *et al.*, 2016). Whether the PR does play a role in prostate cancer, either on its own or via crosstalk with the AR, is still unknown. In fact, studies examining putative crosstalk between the PR and AR in both breast and prostate cancer are mostly lacking. To our knowledge, the only study providing evidence of crosstalk between these two steroid receptors in both breast and prostate cancer cell lines, is a recent study from our laboratory (Cabral, MSc Thesis, 2018). This study showed that both functional isoforms of the PR, PRA and PRB, whether unliganded or bound to P<sub>4</sub>, could modulate the transactivation function of the AR on synthetic minimal promoters in both breast and prostate cancer cell lines. PRB upregulated AR transactivation on both the classical androgen response element (ARE) as well as the estrogen response element (ERE) in the breast and prostate cancer cells, while PRA decreased AR-mediated transactivation on the classical ARE and increased AR

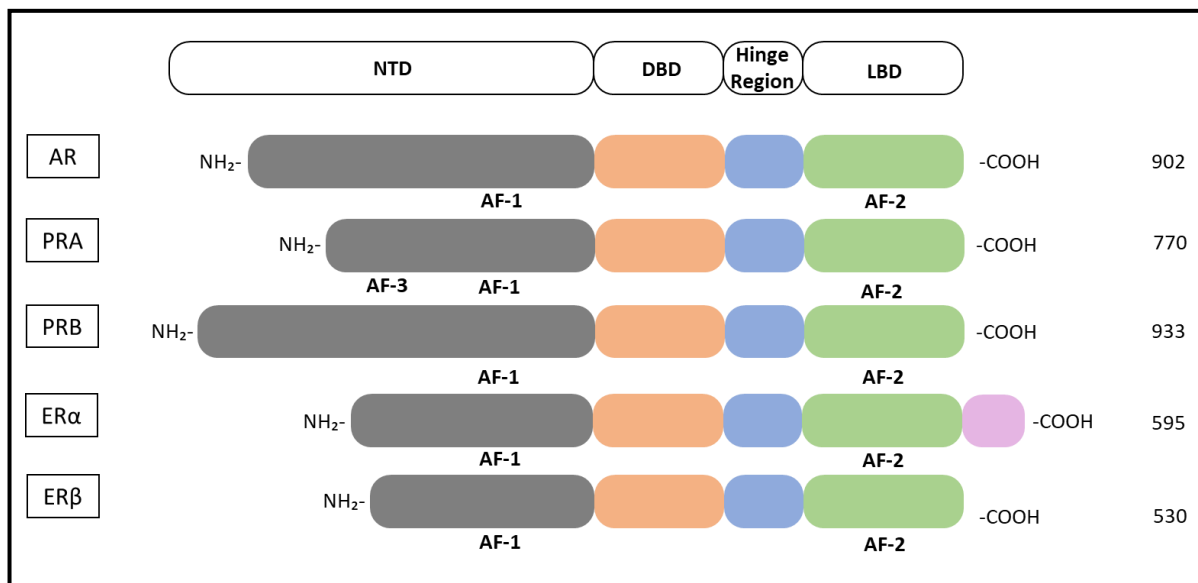
transactivation function on an ERE in both the breast and prostate cancer cells. In fact, the study showed for the first time that co-expression of the PR isoforms and the AR, either at equivalent levels or five times more PR relative to the AR, generally enhanced AR transactivation function. Results from this study also showed that the PR isoforms, like the AR, can transactivate via an ERE in both the breast and prostate cancer cell lines. In the following sections, the general mechanism of action of steroid receptors will be discussed as well as the roles that androgens and the AR, and progestogens and the PR, play in breast and prostate cancer development.

## 1.2 General mechanism of action of steroid receptors

The AR, PR, and other steroid receptors including the ER, glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) are ligand activated transcription factors which belong to the nuclear hormone receptor family (Lange et al., 2007; De Angelis et al., 2013; Bain et al., 2015). PRA and PRB are transcribed from two promoters of a single gene (Kastner *et al.*, 1990b), with PRA being a truncated form of PRB, lacking the additional 164 amino acids at the amino terminal (Björnström and Sjöberg, 2005; Rojas *et al.*, 2017). The ER exists as the two distinct subtypes ER $\alpha$  and ER $\beta$ , which are coded for by two different genes. Although the literature suggests that isoforms transcribed from the same gene are also found for the AR (Xu and Qiu, 2016), not much is known about these isoforms (Guo et al., 2009; Hillebrand et al., 2018).

Steroid receptors have similar structures and consist of several functional domains: a variable N-terminal transactivation domain (NTD), a central highly conserved DNA binding domain (DBD), a hinge region and a moderately conserved C-terminal ligand binding domain (LBD) (Green *et al.*, 1988; Hollenberg and Evans, 1988; Choudhry, Ball and McEwan, 2006). The NTD contains an activation function 1 (AF-1) domain which contributes to the ligand-independent activation of steroid receptors (Lavery and McEwan, 2005), while an AF-2 domain located in the LBD facilitates ligand-dependent effects (Jenster *et al.*, 1991; Baretino, Vivanco Ruiz and Stunnenberg, 1994). The structures for the AR, PR isoforms and ER subtypes are shown in Figure 1.1. An additional activation domain, AF-3 (Sartorius *et al.*, 1994), is found in the NTD of PRB which facilitates the binding of specific co-activators to PRB only (Giangrande, Pollio and McDonnell, 1997), and results in PRB being more transcriptionally active than PRA in the presence of a ligand (Faivre and Lange, 2007; Lanari *et al.*, 2012).



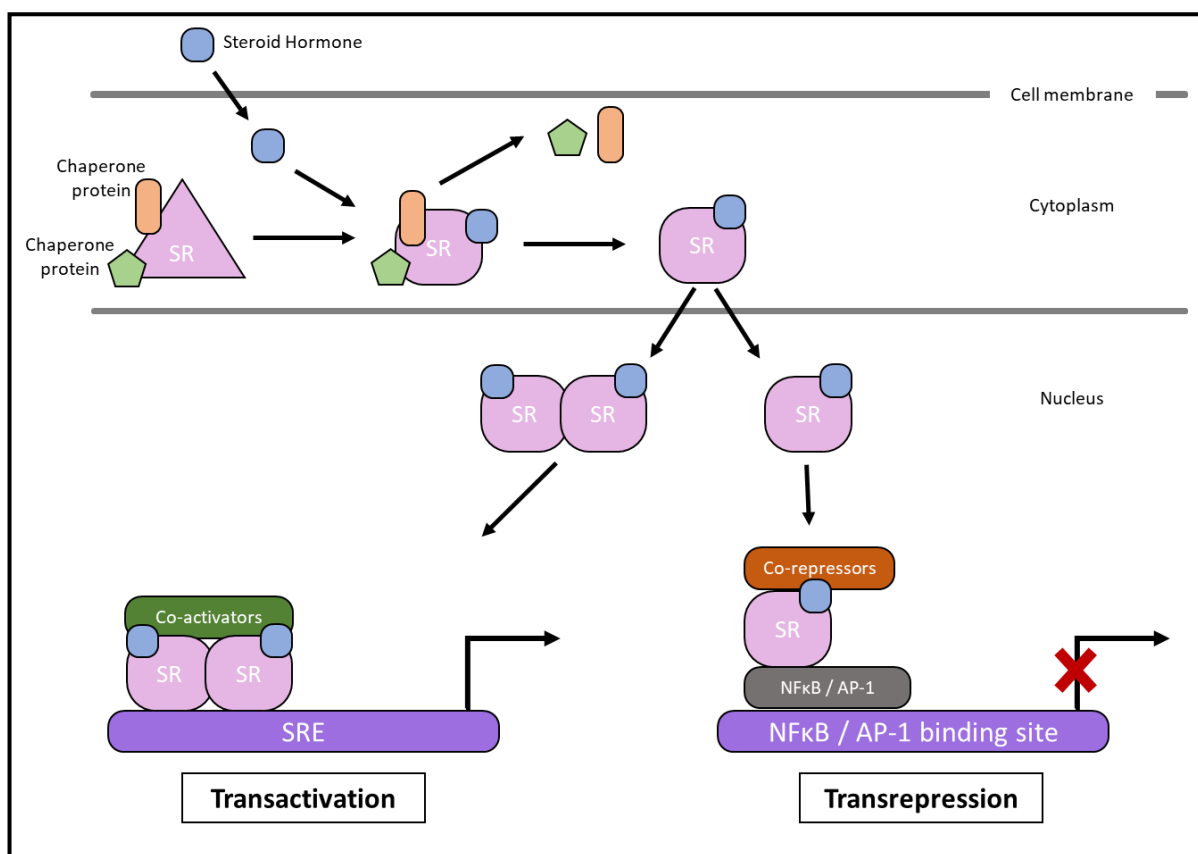


**Figure 1.1: Schematic illustration of the general structure of steroid receptors indicating the different functional domains.** Steroid receptors contain a variable N-terminal transactivation domain (NTD) (grey), with a constitutively active activation function 1 (AF-1) transactivation domain. The highly conserved DNA binding domain (DBD) (orange) lies adjacent to the hinge region (blue), which borders on the moderately conserved ligand binding domain (LBD) (green) located at the C-terminal of the receptor. The LBD contains a ligand-dependent activation function 2 (AF-2) transactivation domain and the additional AF-3 domain in the case of PR-B. ER $\alpha$ , contains an additional carboxy-terminal domain (purple). Numbers to the right of the structures represent the size of the receptor in amino acid residues. Adapted from (Africander, Verhoog and Hapgood, 2011).

Although ligand-independent effects have been reported for the PR (Jacobsen *et al.*, 2005) and GR (Oseid *et al.*, 2020), steroid receptors are generally inactive in the absence of a ligand, and found in the cytoplasm bound to the chaperone proteins such as heat shock proteins Hsp90 and Hsp70 (Pratt and Toft, 2003; Gougelet *et al.*, 2007). Generally, the AR is primarily expressed in the cytoplasm, while ER $\alpha$ , ER $\beta$  and PRA, are found in the nucleus (Griekspoor *et al.*, 2007). PRB is found in both the cytoplasm and nucleus. As shown in Figure 1.2, upon ligand binding, the steroid receptor undergoes a conformational change, allowing it to dissociate from the chaperone proteins and translocate to the nucleus of the cell (Pratt *et al.*, 2004; Griekspoor *et al.*, 2007). Once inside the nucleus, the receptor generally binds as a dimer to specific DNA sequences called steroid response elements (SRE) within the promoter region of target genes, thereby activating gene transcription (transactivation) (Hayashi *et al.*, 2004; Perissi and Rosenfeld, 2005; Lu *et al.*, 2006). Most steroid receptors can bind to the same SREs. For example, the PR binds to an SRE called the progesterone response element (PRE) sequence

and has the sequence GGTACAnnnTGTTCT, which can also serve as a SRE for the AR, GR and MR. When the latter receptors are bound, the SRE is referred to as the androgen response element (ARE), glucocorticoid response element (GRE) or mineralocorticoid response element (MRE), respectively (Beato, 1989; Schauwaers *et al.*, 2007; Africander, Storbeck and Hapgood, 2014). The AR also binds to a so-called AR-selective ARE which is a direct repeat sequence of the consensus monomer TGTTCT (Claessens *et al.*, 1996b). This ARE is termed selective ARE, as the AR, but not the GR, can transactivate via this binding motif (Claessens *et al.*, 1996b). Notably, the ARE that can be bound by the AR, PR, GR and MR, will be referred to as the classical ARE in this thesis. The ER binds to unique sequences AGGTCAgagTGACCT called the estrogen response element (ERE). Interestingly, the AR is recruited along with ER $\alpha$  to the promoter of the estrogen responsive Cathepsin D (CTSD) gene, but only in the presence of both cognate ligands for these receptors, namely DHT and E<sub>2</sub>, respectively (Peters *et al.*, 2009). In contrast, AR and ER $\alpha$  are co-recruited to the estrogen responsive PR gene in the absence of ligand, with increased recruitment observed in the presence of E<sub>2</sub> but not DHT.

Results from our laboratory showed that the AR and both PR isoforms could activate a synthetic minimal promoter containing an ERE in both the breast and prostate cancer cell lines (Cabral, MSc Thesis, 2018). In addition to transactivation, the ligand activated steroid receptors can repress the transcription of target genes (transrepression) by binding as a monomer to another DNA-bound transcription factor, such as nuclear factor kappa B (NF- $\kappa$ B) and activator protein 1 (AP-1) (Zhong *et al.*, 2002; Hayashi *et al.*, 2004; Siggers *et al.*, 2012; Smale, 2012). The conformation of the steroid receptor upon ligand binding determines its transcriptional activity, as this change determines which cofactors are recruited to the promoters of target genes. Generally, co-activators are recruited during transactivation, while co-repressors are recruited during transrepression. However, this thesis will only be focusing on the transactivation of gene expression via the AR and whether it is influenced by PRA and/or PRB.



**Figure 1.2: A diagram of the classical mechanism of action of steroid receptors.** A lipophilic steroid hormone (blue) diffuses across the cell membrane and binds to an intracellular steroid receptor (SR). This triggers a conformational change in the SR and causes the chaperone proteins, bound to the SR, to dissociate. The ligand-bound steroid receptor translocates to the nucleus where it can activate transcription (transactivation) by directly binding as a dimer to a steroid response element (SRE) which is followed by the recruitment of co-activators. Alternatively, the SR can repress gene expression (transrepression) by tethering as a monomer to other transcription factors bound to the DNA such as, NFκB or AP-1, allowing for the recruitment of co-repressors. Adapted from (Africander, Verhoog and Hapgood, 2011).

### 1.3 The role of androgens and the AR in breast and prostate cancer biology

#### 1.3.1 Androgens and the AR in prostate cancer

Androgens acting via the AR, are key players in the development and maintenance of the male sexual phenotype (McPhaul *et al.*, 1991; Quigley *et al.*, 1995; Brinkmann, 2001). The natural androgen, testosterone, can be converted by the enzyme steroid 5 $\alpha$ -reductase to the more potent AR ligand, DHT, which is responsible for some primary as well as secondary male

characteristics (Baulieu, Lasnitzki and Robel, 1968; Wilson, 1999; Yamada *et al.*, 2006). The activated AR is also well known to be the key molecular driver of prostate cancer (Culig and Santer, 2014; Tan *et al.*, 2015). As heterogeneity exists in prostate cancer, its diagnosis involves a variety of factors that affects prognosis and risk (Litwin and Tan, 2017). This includes assessing prostate-specific antigen (PSA) levels (Cooperberg *et al.*, 2005; Stephenson *et al.*, 2009; Mohler *et al.*, 2016). This protein serves as a determinant for prostate cancer risk and disease monitoring, as it is known that the AR regulates the expression of the kallikrein-related peptidase 3 (KLK3) gene, which encodes the PSA protein. The expression level of this gene thus reflects the activity of the AR and its response to testosterone or other androgens (Kim and Coetzee, 2004; Hoffman, 2011).

The development and progression of prostate cancer is dependent upon the ratio of the rate of cell proliferation, to the rate of cell death (apoptosis) (Denmeade, Lin and Isaacs, 1996). Androgens acting via the AR are capable of both stimulating cell proliferation and differentiation, as well as inhibiting the rate of apoptosis (Cunha *et al.*, 1987; Waltregny *et al.*, 2001). This role of the AR in mediating cell proliferation and apoptosis has been shown in many cell lines (Lu, Tsai and Tsai, 1997; Cornforth *et al.*, 2008). The mechanism behind this action of the AR facilitating cell proliferation lies in its regulation of the expression of specific target genes. An example of this is AR regulation of the expression of the cyclin-dependent kinases, CDK2 and CDK4, in the LNCaP cell line (Lu, Tsai and Tsai, 1997). CDKs have been implicated in cancer development due to their role in dysregulating cell proliferation (Tadesse *et al.*, 2020). CDK2 is a known regulator of the cell cycle, and together with CDK4 initiates the transcription of genes required for cell cycle progression (Tadesse *et al.*, 2020). Lu and co-workers found that the CDK2 and CDK4 genes were upregulated, and CDK2 kinase activity increased, within hours of androgen treatment with the synthetic AR agonist methyltrienolone (R1881) (Lu, Tsai and Tsai, 1997). The same study found that R1881 stimulation downregulated expression of the CDK inhibitor p16 gene, a tumour suppressor gene that encodes a specific inhibitor of CDK4 (Meng and El-Deiry, 2002), which would in effect further promote proliferation. Besides facilitating proliferation, the anti-apoptotic actions of the AR can also be described mechanistically. For example, AR activation with R1881 is required for the upregulation of Fas-associated death domain-like interleukin-1 $\beta$ -converting enzyme-like inhibitory protein (FLIP), a protein known to be an inhibitor of apoptosis and part of the phosphoinositol-3-kinase (PI3K) and Akt signalling pathway (Irmeler *et al.*, 1997; Cornforth *et al.*, 2008). Cornforth and co-workers showed that the AR binds to the Forkhead box O3a

(FOXO3a) protein, a transcription factor which binds to a FOXO3a binding site in the promoter region of the FLIP gene (Cornforth *et al.*, 2008).

The first-line treatment for men diagnosed with prostate cancer involves androgen deprivation therapy, aimed at lowering the amount of testosterone being produced by physical or chemical castration, or inhibition of the activity of the AR (Heinlein and Chang, 2004). The AR and the androgen-signalling pathway can be targeted using AR antagonists (Tan *et al.*, 2015). These drugs can be steroidal or nonsteroidal in their chemical structure and can be provided as treatments conducted via monotherapy, thus using a single drug for the treatment, or using novel combinations. Examples of AR antagonists include the oral nonsteroidal competitive AR inhibitors bicalutamide and enzalutamide. These drugs are typically used for the treatment of castration-resistant prostate cancer, and in the case of enzalutamide, also for castration-sensitive prostate cancer (Fradet, 2004; See and Tyrrell, 2006; Penson *et al.*, 2016; Shore *et al.*, 2016). Resistance to bicalutamide treatment is known to occur as studies have found that AR mutations, as well as coactivators enhancing AR transactivation such as transcriptional intermediary factor 2 (TIF2) (Gregory *et al.*, 2001; Ye *et al.*, 2005), play a role in the mechanism of resistance to treatment (Bohl *et al.*, 2005; Feng *et al.*, 2009). Castration-resistant prostate cancer occurs when the expression levels of certain steroidogenic enzymes is altered in such a way that tumours can derive androgens from an alternative source besides the testes, namely circulating adrenal androgen precursors (reviewed in Barnard *et al.*, 2020). Thus, the tumour is able to locally produce DHT, which continues to activate the AR. Another mechanism by which castration-resistant prostate cancer can develop is due to increased levels of AR expression or mutations that cause the AR to be resistant to AR antagonists. Clinical studies have found that the expression of the AR gene was upregulated in 30% to 50% of castration resistant prostate cancer patients (Visakorpi *et al.*, 1995; LaTulippe *et al.*, 2002). The high levels of AR expression lower the effectiveness of androgen deprivation therapy, as even with the low levels of remaining androgens, the upregulated AR enables prostate cancer to progress (Visakorpi *et al.*, 1995). Abiraterone acetate, an inhibitor of the key enzyme required for androgen biosynthesis, cytochrome P450 17 $\alpha$ -hydroxylase/ 17,20-lyase (CYP17A1), has been used in conjunction with an appropriate corticosteroid hormone as another treatment for prostate cancer. By inhibiting androgen biosynthesis, multiple studies have shown that abiraterone acetate elicits significant anti-tumour activity in castration-resistant prostate cancer by reducing PSA levels and increasing overall patient survival (De Bono *et al.*, 2011; Ryan *et al.*, 2013).

Various studies have observed the role the two ER subtypes play in prostate cancer, importantly noting the distinct roles ER $\alpha$  and ER $\beta$  play when co-expressed in this cancer. As seen in breast cancer, ER $\alpha$  has been shown to stimulate prostate cancer cell proliferation, while ER $\beta$  inhibits ER $\alpha$ -mediated cell proliferation (vom Saal et al., 1997; McLachlan et al., 1998; Strauss et al., 1998; Prins et al., 2001a, 2001b, 2006, 2007; Attia and Ederveen, 2012). Estrogen therapy, in the form of high dose diethylstilbestrol (DES), was central to androgen deprivation therapy in the treatment of prostate cancer for over half a century (Huggins and Hodges, 1941). This approach has however been replaced with novel therapeutic agents in recent years, due to estrogen therapy being associated with side effects such as cardiovascular toxicity (reviewed in Reis, Zani and García-Perdomo, 2018). Interestingly, selective estrogen receptor modulators (SERM) known to block the actions of estrogens and used as a line of therapy in ER positive breast cancer, have also been utilized as a therapeutic target in prostate cancer (Bergan et al., 1999; Shazer et al., 2006). One study showed that upon treatment with the SERM tamoxifen, 20% of castration-resistant prostate cancer patients showed tumour regression (Bergan et al., 1999). Another study found that 27% of patients with androgen-independent prostate cancer showed tumour regression after treatment with the SERM raloxifene (Shazer et al., 2006).

### ***1.3.2 Androgens and the AR in breast cancer***

Androgens and the AR do not only play a role in prostate cancer development but also in breast cancer development. The AR is expressed in most breast cancer tumours, occurring in up to 77% of metastatic and approximately 86% of local cancers (Collins *et al.*, 2011), and is often co-expressed along with the ER (Kuenen-Boumeester et al. 1996, Moinfar et al. 2003, Castellano et al. 2010). Breast cancer is an heterogeneous disease with many different histological subtypes, namely luminal, normal-like, basal or triple negative breast cancer (TNBC) and human transmembrane epidermal growth factor type II receptor (HER2) overexpression (Cheang *et al.*, 2009; Curtis *et al.*, 2012; Koboldt *et al.*, 2012; Dai *et al.*, 2015). The subtypes are distinguished from one another based on the presence of ER and PR, Luminal A and B (Yanagawa *et al.*, 2012; Dai *et al.*, 2015; Gao and Swain, 2018), or by their absence, in the case of TNBC (Bidard *et al.*, 2007). Other factors including the presence of HER2 as well as the levels of the pro-proliferative marker Ki67 also play a role (Gerdes *et al.*, 1984, 1987; Dowsett *et al.*, 2011).

ER positive breast cancers are typically treated with ER targeted therapies which include inhibitors of ER function as well as inhibitors of cytochrome P450 aromatase (CYP19A1), the enzyme responsible for producing estrogens from androgens (Platet *et al.*, 2004; Zhao and Ramaswamy, 2014). While anti-estrogenic drug treatment is standard practice, resistance to therapy is common, possibly stemming from compensatory mechanisms which aid the reactivation of ER signalling (Toy *et al.*, 2013). As mentioned earlier, SERMs are another line of therapy of ER positive breast cancer which block the actions of estrogens. Interestingly, a clinical study found that in ER positive tumours, a high ratio of AR expression compared to ER indicated a greater than four-fold increased risk for failure of treatment with a SERM, specifically tamoxifen (Cochrane *et al.*, 2014). TNBC on the other hand, is currently treated using non-specific chemotherapy (Bidard *et al.*, 2007). This usually results in poor outcomes for patients (Gucalp *et al.*, 2013; Traina *et al.*, 2015; Bonnefoi *et al.*, 2016), whereas the development of targeted therapies could provide better and more effective treatment. While TNBC cancer lacks expression of the PR and ER, some cases may have AR expression (Rampurwala, Wisinski and O'Regan, 2016). The AR was found to be expressed in about 50% of TNBC cases (Gucalp *et al.*, 2013; Traina *et al.*, 2015; Bonnefoi *et al.*, 2016). Indeed, targeting the AR using bicalutamide, as well as enzalutamide, antiandrogen therapy in a subset of AR positive TNBC patients has yielded some promising results (Gucalp *et al.*, 2013; Traina *et al.*, 2015; Bonnefoi *et al.*, 2016). For example, inhibition of AR activity by bicalutamide caused anti-tumour activity in patients with metastatic AR positive and ER/PR negative breast cancer (Gucalp *et al.*, 2011), and stabilized disease progression in metastatic ER/PR negative breast cancer if the AR was present (Traina, AC and Giri, 2009). Enzalutamide has also been investigated as a safe and effective treatment for patients with advanced AR positive TNBC (Gucalp and Traina, 2017). Interestingly, seviteronel, a new oral nonsteroidal CYP17A1 inhibitor that functions by inhibiting the biosynthesis of androgens and therefore also estrogens, has been developed for the treatment of prostate cancer, as well AR positive TNBC and ER positive breast cancer due to its ability to lower production of both androgens and downstream estrogens (Bardia *et al.*, 2016).

The literature on the role of the AR in breast cancer is contradictory, with some studies suggesting a poor prognosis, while others suggest a favourable prognosis (reviewed in Salvi, Bonafè and Bravaccini, 2020). Clinical studies have found the AR presence in breast cancer tumours to be associated with lower malignancy (Ogawa *et al.*, 2008; Collins *et al.*, 2011). These effects are however dependent on the breast cancer subtype and whether the ER is

expressed or not (Hu *et al.*, 2011; Lehmann *et al.*, 2016). Evidence on the prognostic significance of AR in TNBC is contradicting. An experimental study indicated that the AR facilitated cell proliferation in an ER independent manner in the MDA-MB-453 TNBC breast cancer cell line (Doane *et al.*, 2006), suggesting the AR takes on the proliferative role of the ER $\alpha$  in TNBC. Another study found that the AR, upon stimulation with the synthetic androgen R1881, induced cell migration and invasion in MDA-MB-231 and MDA-MB-453 breast cancer cells (Giovannelli *et al.*, 2019). This study found that a complex formation between the AR, the Src protein and a PI3K subunit to be the driving mechanism behind the cell migration and invasiveness. A clinical study observed that TNBC with high AR expression was less lethal and slower growing than TNBC lacking the AR, however it also displayed the poorest response to neoadjuvant chemotherapy (Lehmann *et al.*, 2016). Another clinical study found that tumours from patients diagnosed with TNBC which were AR positive had better overall survival and disease free survival compared to TNBC tumours that were AR negative (Zuo *et al.*, 2018). Furthermore, the investigators observed that increased AR expression in the tumours was positively correlated with increased disease-free survival. These results are supported by another clinical study which observed that ER negative breast cancer tumours with AR expression had significantly better disease-free survival than when no AR was present (Agoff *et al.*, 2003).

The literature provides conflicting information regarding whether the role of the AR in ER positive breast cancer is associated with good or poor prognosis. As discussed in the introduction, DHT-induced AR inhibits the effects of ER $\alpha$ -mediated cell proliferation, by antagonizing the activity of endogenous ER $\alpha$  in the T47D breast cancer cell line (Peters *et al.*, 2009), and upregulates the activity of ER $\beta$  once activated by the synthetic androgen mib, to decrease ER $\alpha$ -mediated breast cancer cell proliferation in the MCF-7 ER positive breast cancer cell line (Rizza *et al.*, 2014). Another study also showed that DHT had an inhibitory effect on ER $\alpha$ -mediated cell proliferation in MCF-7 cells (Macedo *et al.*, 2006). However, in other preclinical breast cancer studies, which utilized the T47D and MCF-7 ER positive cell lines, the AR has been indicated to play a role in driving resistance to aromatase inhibitor therapy aimed to stop estrogen production in ER positive cancer (De Amicis *et al.*, 2010; Fujii *et al.*, 2014; Rechoum *et al.*, 2014).

Taken together, the AR has a complex role in breast cancer. Considering the high expression of the AR in ER positive breast cancer (Collins *et al.*, 2011), it is paramount to understand the



mechanism by which it elicits its actions and whether these would be altered when expressed with other steroid receptors such the PR.

## **1.4 Role of progestogens and the PR in breast and prostate cancer biology**

### ***1.4.1 Role of progestogens and the PR in breast cancer***

Both the ER and PR have been implicated as drivers of the malignant transformation of breast tissue and induction of breast cancer (Carroll *et al.*, 2017). While the role of the ER appears more definite, the role of the PR in breast cancer remains controversial (Briskin, 2013; Yip and Rhodes, 2014). The majority of breast cancer cases show co-expression of the ER and PR, with about 75% of breast cancer cases expressing the ER and 67% also express the PR (Stierer *et al.*, 1993; Rhodes *et al.*, 2000). The SEER (Surveillance, Epidemiology, and End Results) database revealed that the overall survival, breast cancer-specific survival and disease-free survival were significantly better for patients with tumours positive for both ER and PR, than for patients expressing the ER but lacking PR expression (Dunnwald, Rossing and Li, 2007). This database provides information regarding U.S. cancer statistics such as diagnosis, treatment and survival. These results are supported by a study which found that patients positive for either the ER or PR individually had lower rates of survival and higher risk of resurgence than patient positive for both the ER and PR (Wu *et al.*, no date). The expression of the PR gene is directly regulated by ER $\alpha$  in response to E<sub>2</sub> (Kastner *et al.*, 1990a; Quadros and Wagner, 2008). The PR has thus been used as a bio-marker for ER positive breast cancer as its function is dependent on the normal structure and function of ER (Horwitz and McGuire, 1978; Yu, Leung and Gao, 1981).

While transcribed from the same gene, the two PR isoforms, PRA and PRB, differ greatly in their physiological roles and they have variable effects in cancer development and progression (Diep *et al.*, 2015; Rojas *et al.*, 2017; McFall *et al.*, 2018). Many studies fail to address the isoform specific activities of PRA and PRB, and their consequential roles in cancer progression, by simply referring to these receptor isoforms collectively as PR. This is imperative as these isoforms can activate similar, but also distinct gene sets (Sartorius *et al.*, 1994; Hopp *et al.*, 2004; Kariagina, 2008). While in healthy breast tissue PRA and PRB are expressed at similar levels, the ratio of the PR isoform expression becomes dysregulated in breast cancer, with higher PRA levels relative to PRB (Mote *et al.*, 2002; Rojas *et al.*, 2017;

Lamb *et al.*, 2018). These higher levels of PRA have mostly been shown to correlate with worse prognosis and tumour progression, as well as recurrence of cancer after tamoxifen treatment (Bamberger *et al.*, 2000; Mote *et al.*, 2002; Hopp *et al.*, 2004). However, at least one study has found that when observing the ratio of PRA to PRB expression, patients with lower PRA levels relative to PRB levels, had a worse prognosis (Rojas *et al.*, 2017). The median ratio of PRA to PRB expression in breast cancer patients was 1.2, with 52.3% being PRA predominant, and 23.8% PRB predominant with the rest being at equimolar concentration. When compared with the PRA predominant patients, the PRB predominant patients had larger tumours with worse histological grade. On a molecular level, and in the presence of a ligand, PRB typically acts as a more potent transcription factor on target genes than PRA (Elizalde and Proietti, 2012), whereas PRA is more transcriptionally active than PRB in the absence of ligand. In addition, PRA functions as a transcriptional repressor of P<sub>4</sub>-responsive promoters in many cell lines, and has also been found to function as a repressor of the transcriptional activity of the ER, GR, AR and MR (Giangrande and McDonnell, 1999). These observations support findings from our laboratory which showed that the presence of PRA decreases AR-mediated transactivation on the classical ARE (Cabral, MSc Thesis, 2018).

The PR influences breast cancer prognosis via a variety of mechanisms. It has been shown to induce invasiveness in breast cancer cell lines in response to its natural ligand P<sub>4</sub>, as well as synthetic progestogens (progestins) (Carnevale *et al.*, 2007; Diaz *et al.*, 2012). Diaz and co-workers found that the PR can regulate the expression of key target genes involved in the blood coagulation cascade, as well as cell migration in the ER positive ZR-75 and T47D cell lines (Diaz *et al.*, 2012). Specifically, the PR regulates the expression of protease-activated receptors (PARs) known to be involved in the blood coagulation cascade as well as cell migration (Yang *et al.*, 2009; Diaz *et al.*, 2012). Another study observed that progestins acting via the PR were able to induce growth of different breast cancer lines and induce cell migration by modulating the activity of matrix metalloproteinases and the urokinase-type plasminogen activator via the MAPK and PI3K/Akt pathways (Carnevale *et al.*, 2007). In addition to this, the study found that the unliganded PR caused decreased cell proliferation. When considering isoform specific actions of the PR, PRB in particular has been associated with increased cell metastasis in breast cancer (Ibrahim *et al.*, 2008). When acting via PRB, progestins have been shown to modulate growth factor signalling pathways in MCF-7 breast cancer cells by shifting the balance in the phosphorylation state of insulin receptor substrate (IRS)-1 to IRS-2 (Ibrahim *et al.*, 2008). This shift to IRS-2 activation allows for type 1 insulin growth factor receptor signalling via IRS-2,

which is associated with enhanced cell migration (Jackson, White and Yee, 1998; Ibrahim *et al.*, 2008). PRB has also been shown to decrease the expression of the E-cadherin, a protein involved in cell adhesion, in T47D cells to increase cell invasiveness (Kariagina *et al.*, 2013). Interestingly, Wargon and co-workers showed the proliferative action of PR in mice with higher PRA to PRB ratios (Wargon *et al.*, 2015a, 2015b, 2011, 2009). The overexpression of PRA has been associated with a more aggressive phenotype in breast cancer cells due to its hormone-independent gene regulation (Jacobsen *et al.*, 2005). This includes PRA regulating cell adhesion to the extracellular matrix, as well as cell migration and survival. These results suggest that PRA, as well as PRB, are involved in promoting an aggressive breast cancer phenotype.

In contrast to these findings, other studies have provided evidence of the favourable effect of the PR in breast cancer prognosis. P<sub>4</sub> has been shown to inhibit cell proliferation in the MDA-MB-231 breast cancer cell line co-transfected with PRA and PRB (Lin *et al.* 1999). These anti-proliferative effects were also observed in the T47D breast cancer cell line which endogenously expresses both PRA and PRB (Formby & Wiley 1998). In addition, recent studies have suggested that the PR is a binding partner of ER $\alpha$ , thereby modifying the activity of ER $\alpha$  and resulting in a good prognosis for patients with ER positive tumours (Mohammed *et al.*, 2015; Singhal *et al.*, 2016; Trabert *et al.*, 2019).

#### ***1.4.2 Role of progestogens and the PR in prostate cancer***

While the PR has been shown to be expressed in prostate cancer tumours (Grindstad *et al.*, 2015; Chen *et al.*, 2017), its role in prostate cancer development is not as clearly established as that of the AR, as studies present conflicting findings regarding whether the PR prohibits or aids cancer development and progression (Grindstad *et al.*, 2015; Chen *et al.*, 2017). Some studies have found that the PR has increased expression in prostate cancer tumours (Bonkhoff *et al.*, 2001), with high levels of PRB expression in tumours being associated with a poorer prognosis (Grindstad *et al.*, 2015, 2018). However, the relative expression levels of PRB compared to PRA are not known. Another study observed that the PR expressed in prostate stroma cells has the ability to control and inhibit cell proliferation by interfering with cell cycle phases (Yu *et al.*, 2013). The same researchers found that the PR could regulate and inhibit the secretion of cytokines, such as stromal cell-derived factor 1 (SDF-1) and interleukin 6 (IL-6), by stromal cells, (Yu *et al.*, 2014, 2015). As these cytokines are known to enhance prostate

tumour progression when elevated levels are present, the authors suggest that the PR has a role in inhibiting cancer progression. The PR was also found to suppress the differentiation of the stromal cell phenotype that occurs during prostate cancer tumour development (Yu *et al.*, 2015). This work is supported by other studies which have found that the PR could inhibit prostate gland enlargement, also known as benign prostatic hyperplasia (BPH), decrease the differentiation of cells into the cancerous phenotype and inhibit prostate cancer progression (Piasecka *et al.*, 2015; Chen *et al.*, 2017). The expression of PR was found to be increased after ADT (Chen *et al.*, 2017), suggesting that androgens acting via the AR may be downregulating PR expression. These findings support a possible protective role of the PR in prostate cancer pathogenesis. However, the PR has also been associated with negative effects in prostate cancer. Detchokul and co-workers showed that PR expression and activity was significantly upregulated in LNCaP cells in response to DHT, and that oncogenesis was maintained by the PR regulating various AR-target genes. This study also implicated the PR as a possible mediator of resistance to treatment in castration-resistant prostate cancer patients. (Detchokul *et al.*, 2015). Taken together, these findings demonstrate the complex role of the PR in regulating prostate cancer pathogenesis. Understanding the contribution of the individual isoforms, PRA and PRB, may aid in the understanding of this complexity.

### **1.5 AR and PR crosstalk in breast and prostate cancer**

Recent studies have revealed an interplay between steroid receptors in breast cancer (Wu *et al.*, 2019; Bardou *et al.*, 2003; Peters *et al.*, 2009; Pan, Kocherginsky and Conzen, 2011; Brisken, 2013; Rizza *et al.*, 2014; Verde *et al.*, 2018). For example, Guilianelli and co-workers observed that an association between PR and ER $\alpha$  on target gene promoters in the T47D cell line, as well as in human breast cancer samples, was essential for cell proliferation induced by the progestin medroxyprogesterone acetate (MPA) (Giulianelli *et al.*, 2012). Furthermore mechanisms facilitating the anti-proliferative role of the AR in ER-positive breast tumours have been demonstrated by the action of the AR inhibiting the activity of ER $\alpha$  (Goldenberg *et al.*, 1975; Szelei *et al.*, 1997; Panet-Raymond *et al.*, 2000; Peters *et al.*, 2009; Need *et al.*, 2012) as well as to regulate ER $\beta$  expression (Rizza *et al.*, 2014). Crosstalk between steroid receptors, other than the ERs, in prostate cancer has received little attention. At least two studies have indicated that ER $\beta$  inhibits AR-mediated prostate cancer cell proliferation, in response to either androgen metabolites (Grubisha and DeFranco, 2013), or the estrogenic metabolite of DHT, 3 $\beta$ -androstanediol (Muthusamy *et al.*, 2011).

Although studies considering crosstalk between the AR and PR are mostly lacking, there is evidence that such crosstalk may in fact occur. For example, it has been shown that Kallikrein 4 (KLK4), a protease known to activate pro-PSA (Takayama *et al.*, 2001; Clements *et al.*, 2004; Paliouras, Borgono and Diamandis, 2007), is only expressed in the presence of the PR and AR, in the T47D breast cancer cells and LNCaP prostate cancer cells, respectively (Lai *et al.*, 2009). Previously studies have confirmed the endogenous expression of both the PR and AR in the T47D cell line, and although Storbeck and co-workers showed that the PR is not endogenously expressed in the LNCaP cell line (Barnard, PhD thesis, 2019), Detchokul and co-workers did detect its presence in the LNCaP cell line (Horwitz *et al.*, 2008; Detchokul *et al.*, 2015). Notably, the AR expressed in the LNCaP cell line contains a single point mutation in its ligand binding domain, which directly affects both binding specificity and the induction of target gene expression (Veldscholte *et al.*, 1992). In this study, KLK4 expression was upregulated by P<sub>4</sub> in the T47D breast cancer cell line, as well as by R1881 in the LNCaP prostate cancer cell line (Lai *et al.*, 2009). These results suggest that P<sub>4</sub> and androgens play an active role in influencing KLK4 gene expression, and support previous findings of KLK4 mRNA upregulation in response to P<sub>4</sub> in breast cancer cells, and androgens in prostate cancer cells (Korkmaz *et al.*, 2001; Lai *et al.*, 2009; Bardia *et al.*, 2016). The KLK4 gene promoter, like the PSA gene, contains both a PRE and a selective ARE sequence within its promoter region (Nelson *et al.*, 1999; Lai *et al.*, 2009). Lia and co-workers found that P<sub>4</sub> upregulated KLK4 gene expression via the PR binding directly to the PRE in the T47D cell line (Lai *et al.*, 2009). However, in the LNCaP cell line, the AR did not directly interact with the promoter of the KLK4 gene, containing a PRE or a selective ARE. Lia and co-workers thus suggested that the AR regulates KLK4 gene expression by tethering to AR co-regulators.

As mentioned, a recent study conducted in the Africander laboratory showed that AR-mediated transactivation via synthetic minimal promoters containing either the classical ARE, a selective ARE or the ERE, were generally increased by the PR isoforms in both MDA-MB-231 breast cancer cells and PC3 prostate cancer cell lines (Cabral, MSc Thesis, 2018). In addition, it was shown for the first time that the increase in AR function by the PR isoforms, was further enhanced in the presence of excess PRA or PRB relative to the AR. An exception to this was for PRA, which did not affect AR function by PRA on a classical ARE. This study also provided novel evidence of the PR isoform's ability to mimic the activity of the AR on the selective ARE and ERE in both the breast and prostate cancer cell lines. The limitation of the

aforementioned study however, was that only reporter constructs containing specific *cis*-regulating elements were used. Considering that endogenous genes are far more complex than simple synthetic promoters, and can contain multiple *cis*-regulating elements or binding sites to which the PR and AR could bind (Choi, 2007), it is important to investigate whether the PR isoforms would also regulate AR function on androgen and estrogen responsive endogenous genes in both breast and prostate cancer. Moreover, whether there is in fact a molecular interaction between the AR and PR should also be evaluated.

## 1.6 Conclusion

Evidence in the literature suggest crosstalk between steroid receptors in breast cancer (Wu *et al.*, 2019; Bardou *et al.*, 2003; Peters *et al.*, 2009; Pan, Kocherginsky and Conzen, 2011; Brisken, 2013; Rizza *et al.*, 2014; Verde *et al.*, 2018). When considering the striking similarity in mechanism between breast and prostate cancer, it is likely that similar crosstalk mechanisms are also seen in prostate cancer. While the AR is well known to be the key molecular driver of prostate cancer (Culig and Santer, 2014; Tan *et al.*, 2015), its role in breast cancer is largely dependent on the presence of ER $\alpha$ , with AR presence considered protective in ER positive breast cancer (Doane *et al.*, 2006; Macedo *et al.*, 2006; Peters *et al.*, 2009; Rizza *et al.*, 2014; Giovannelli *et al.*, 2019) and harmful in TNBC (Doane *et al.*, 2006; Giovannelli *et al.*, 2019). For the PR, its role in breast cancer is dependent upon the absence or presence of a ligand, as well as which specific ligand the PR is bound to (Mote *et al.*, 2002; Hopp *et al.*, 2004). A further complexity is that PRA and PRB have been shown to have very different effects in breast cancer development and progression (Diep *et al.*, 2015; Rojas *et al.*, 2017; McFall *et al.*, 2018). Studies observing the role of the PR, as well as the isoform specific activities of either PRA or PRB in prostate cancer, are scarce. Some studies have indicated that the PR is upregulated in prostate cancer tumours (Bonkhoff *et al.*, 2001; Thomas, Pang and Dong, 2014; Grindstad *et al.*, 2015, 2018), while others show that the PR inhibits cell proliferation by interfering with cell cycle phases (Yu *et al.*, 2013, 2014, 2015). It is clear that the AR and PR are co-expressed in both breast and prostate cancer tumours, and that they play important, yet complex roles in breast cancer biology. Recent results from a study using reporter assays suggest crosstalk existing between the AR and PR isoforms in both breast and prostate cancer (Cabral, MSc Thesis, 2018). Identifying crosstalk mechanisms occurring in breast and prostate cancer is paramount to our understanding of the role that these steroid receptors play in the

development and progression of these cancers. Greater insight into this area of research could aid in the development of more effective therapies for treatment of both breast and prostate cancer.

### **1.7 Aims of the study**

Evidence from our laboratory using minimal promoters suggests that crosstalk occurs between the AR and PR in both breast and prostate cancer, and that the PR, like the AR, can transactivate via an ERE. The main aim of this study was to investigate whether these effects could be mimicked on the complex promoters of physiologically relevant endogenous genes implicated in breast and/or prostate cancer development. The MDA-MB-231 and T47D breast cancer lines, and the PC3 prostate cancer cell line, were utilized to specifically investigate whether unliganded and/or liganded PR isoforms can modulate AR transactivation on endogenous AR and ER regulated genes. To support the hypothesis of crosstalk, it was also investigated whether the AR and either PRA or PRB occur in a molecular complex.

Hypothesis:

The AR occurs in a molecular complex with PRA and/or PRB, resulting in the PR isoforms modulating the transcriptional activity of the AR on endogenous AR and ER regulated genes.

The specific aims were:

1. To validate the results by Cabral (Cabral, MSc Thesis, 2018) on the synthetic minimal promoter containing the classical ARE.
2. To investigate whether the PR isoforms, unbound or bound to its cognate ligand, can modulate the transactivation function of the AR, on an endogenous classical ARE containing gene or on endogenous ER regulated genes.
3. To evaluate whether PRA and PRB can activate endogenous ER regulated genes.
4. To determine whether the PR and AR can occur in a molecular complex.

## **Chapter 2 Materials and Methods**



## 2.1 Test compounds

The test compounds utilised in this study included the natural androgen  $5\alpha$ -androstane- $17\beta$ -ol-3-one ( $5\alpha$ -dihydrotestosterone; DHT), the synthetic androgen  $7\alpha,17\alpha$ -dimethyl-19-nortestosterone (mibolerone; mib), the natural progestogen 4-pregnene-3,20-dione (progesterone;  $P_4$ ),  $17\beta$ -estra-1,3,5(10)-etriene-3,17 diol ( $17\beta$ -estradiol;  $E_2$ ), as well as the synthetic progestogen (progestin) 17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione (promegestone; R5020). All the above-mentioned test compounds were purchased from Sigma-Aldrich, South Africa. A 1  $\mu$ M stock solution of all the test compounds was prepared using absolute ethanol and stored at  $-20^\circ\text{C}$  in light-protective vials. For all experiments, the test compounds were diluted 1000 times in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, South Africa) resulting in a final ethanol concentration of 0.1% (v/v) or 0.2% (v/v) depending on whether one or two compounds were used for treatment. A solvent control of 0.1% (v/v) or 0.2% (v/v) ethanol (EtOH) was thus included for all experiments.

## 2.2 Mammalian tissue culture

The triple negative human breast cancer cell line, MDA-MB-231, was generously provided by Prof. A. Edkins (Rhodes University, South Africa), and was maintained in DMEM containing phenol red and 4.5 g/L glucose (Sigma-Aldrich, South Africa), supplemented with 10% fetal calf serum (FCS) (ThermoFischer Scientific, South Africa), as well as 1% penicillin/streptomycin (penstrep) (Sigma-Aldrich, South Africa) and 2 mM L-glutamine (Sigma-Aldrich, South Africa). The T47D human breast cancer cell line was obtained from Prof. I. Parker (University of Cape Town, South Africa), while the COS-1 African monkey kidney fibroblast cell line and HEK-293 human embryonic kidney cells were obtained from the American Type Culture Collection (ATCC, Virginia, USA). The T47D, COS-1 and HEK-293 cell lines were maintained in DMEM containing phenol red and 4.5 g/L glucose supplemented with 10% FCS and 1% penstrep. The PC3 human prostate cancer cell line, was kindly gifted by Prof. A. Swart (Stellenbosch University, South Africa), was maintained in Roswell Park Memorial Institute (RPMI) medium (Sigma-Aldrich, South Africa) containing 4.5 g/L glucose (Sigma-Aldrich, South Africa) and was supplemented with 10% FCS and 1% penstrep. All cell lines were maintained at  $37^\circ\text{C}$  in an atmosphere of 90% humidity and 5%  $\text{CO}_2$  in 75  $\text{cm}^2$  culture flasks (Bio-Smart Scientific, South Africa). All experiments were conducted within 30 passages after thawing the cell lines, and only cells shown to be mycoplasma negative were used for experiments (Addendum).

## 2.3 Plasmid DNA

### 2.3.1 Expression vectors

The cDNA expression vector expressing human AR (pSG5-hAR) was obtained from Prof. H. Klocker (Department of Urology, Medical University, Innsbruck, Austria) (Shatkina *et al.*, 2003), while the human PRA (pSG5-hPR-A) and human PRB (pSG5-hPR-B) expression vectors were provided by Prof. E. Kalkhoven (University Medical Centre, Utrecht, Netherlands) (Kalkhoven *et al.*, 1996). The pSG5 expression vector, which did not contain a cDNA insert, was provided by Prof. G. Mellgren (University of Bergen, Norway) and was used as a control to ensure that the total DNA concentration remained constant in all experiments. The PRE luciferase promoter-reporter construct (pTAT-PRE-E1b-luc) driven by the E1b promoter and containing two copies of the TAT-PRE was a gift from Prof. G. Jenster (Erasmus University of Rotterdam, Rotterdam, Netherlands) (Jenster *et al.*, 1997). Both the AR and PR isoforms can bind to the pTAT-PRE-E1b-luc construct; thus it will be referred to as the classical ARE onwards.

### 2.3.2. Plasmid DNA preparation

Plasmid DNA was extracted from the *E.coli* cells transformed with the respective plasmids using the NucleoBond® Xtra Maxi Plasmid Preparation kit (Macherey-Nagel, USA) as per the manufacturer's instructions. To accomplish this, a single colony of the transformed cells was inoculated into 5 mL LB broth (1% w/v NaCl, 1% w/v tryptone, 0.5% w/v yeast extract, pH 7.5) containing 50 µg/mL ampicillin to create a starter culture, and incubated at 37°C for 8 hours at 300 rpm. The 5 mL starter culture was then transferred to 250 mL LB broth containing 50 µg/mL ampicillin and incubated at 37°C for 16 hours at 300 rpm. The cell culture OD<sub>600</sub> was then measured to ensure that the cells were in the exponential growth phase at an OD reading between 1.2 to 1.6, before cells were pelleted by centrifugation at 6000 x g for 10 minutes at 4°C. All the following reagents used were provided in the NucleoBond® Xtra Maxi Plasmid Preparation kit (Macherey-Nagel, USA), with the exception of isopropanol (Sigma-Aldrich, South Africa). The cell pellets were resuspended by pipetting the cells up and down in 12 mL resuspension buffer containing RNase A. Thereafter, the cells were lysed using 12 mL lysis buffer and the suspension inverted 5 times, and then incubated for 5 minutes at room temperature. After incubation, 12 mL neutralization buffer was added to the solution which was then inverted 15 times. The Nucleobond® Xtra Maxi column was prepared and fitted with a filter insert and equilibrated by adding 25 mL equilibration buffer in order to purify the plasmid DNA. The cell lysate was then applied to the column filter and allowed to empty by gravitational flow. Thereafter,

15 mL of the equilibration buffer was added to the filter fitted column and allowed to flow through the column filter by gravitational flow. The filter was then removed, and the column washed with 25 mL wash buffer. The plasmid DNA was eluted by adding 15 mL elution buffer to the column. The eluate was collected in a 50 mL centrifuge tube and 10.5 mL isopropanol was added at room temperature. The plasmid DNA was precipitated by centrifugation at 15 000 x g for 30 minutes at 4°C. The supernatant was discarded and the pellet washed with 14 mL 70% (v/v) EtOH, followed by centrifugation at 15 000 x g for 5 minutes at room temperature. The DNA pellet was set aside to dry at room temperature before resuspension in 1 mL Tris-EDTA (TE) buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0).

### **2.3.3 Restriction enzyme digest and agarose gel electrophoresis**

The concentration and purity of the isolated plasmid DNA were determined using a NanoDrop™ MicroVolume spectrophotometer (ThermoFisher Scientific, South Africa) which was available at the Stellenbosch University Central Analytical Facility (CAF). To confirm that the correct plasmids were purified and to establish the size and integrity of the plasmid DNA, undigested DNA and restriction enzyme digests of the DNA were separated on an agarose gel by electrophoresis. For digestion of the plasmids, 200 ng of the plasmid DNA was incubated with 0.2 U of the specific enzyme (Promega, USA) and an appropriate concentration of the respective buffer supplied with the enzyme, at 37°C for 1 hour. All plasmid DNA samples were diluted to a final concentration of 10 ng/μL in 6X orange DNA loading dye (ThermoFisher Scientific, South Africa) and loaded onto a 1% agarose gel prior to gel electrophoresis at 100 V.

### **2.4 Luciferase promoter-reporter assays**

MDA-MB 231 cells were maintained as described in Section 2.2 and seeded into 10 cm dishes (WhiteSci, South Africa) at a density of  $2 \times 10^6$  cells per dish in DMEM supplemented with 10% FCS and 1% penstrep. After 24 hours, the cells were transiently transfected with 900 ng of the empty vector or steroid receptor expression vector, and 9 000 ng of the classical ARE promoter-reporter construct. This was done in the following combinations: pSG5 or pSG5-AR, in the absence or presence of either pSG5-PR-A or pSG5-PR-B using XtremeGENE HP DNA transfection reagent as per the manufacturer's instructions. After a 24-hour incubation period, the transfected cells were re-seeded into 96-well plates using phenol red-free DMEM supplemented with 10% CS-FCS and 1% penstrep at a density of  $1 \times 10^4$  cells per well. The following day, the cells were treated for 24 hours with either 0.1% (v/v) EtOH or 1 nM of ligand prepared in supplemented phenol-red free DMEM.

After the incubation period, the cells were washed with 200  $\mu\text{L}$  ice cold 1x PBS and incubated in 25  $\mu\text{L}$  passive lysis buffer (0.2% v/v Triton, 10% v/v glycerol, 2.8% v/v Tris-phosphate-EDTA, 1.44 mM EDTA) for 15 minutes at room temperature with agitation. The cell lysates were then stored at  $-20^{\circ}\text{C}$  until use. Luciferase activity of the thawed cell lysates (5  $\mu\text{L}$ ) was measured in relative light units (RLU) utilizing the Promega luciferase assay system (Anatech, South Africa) along with the Veritas microplate luminometer (Turner Biosystems, USA). The RLU values obtained were normalised to the total protein concentration (mg/mL) determined using 5  $\mu\text{L}$  lysate and the Bradford protein determination assay (Bradford, 1976).

## 2.5 Western blot analysis

MDA-MB-231 and T47D cells were maintained as described in Section 2.2. The confluent cells were then washed with 1x PBS before being lysed with 100  $\mu\text{L}$  passive lysis buffer. To pellet the cell debris, the samples were centrifuged for 10 minutes at 12 000 x g at  $4^{\circ}\text{C}$ , and the protein lysate transferred to a sterile tube. Positive controls were prepared by maintaining COS-1 and HEK293 cells as described in Section 2.2 and seeding these cells into 12-well plates at a density of  $1 \times 10^5$  cells per well. The COS-1 cells were transiently transfected with 250 ng of an AR expression vector, while the HEK293 cells were similarly transiently transfected with PRA or PRB expression vectors, using the XtremeGENE HP DNA transfection reagent as per the manufacturer's instructions. Negative controls were prepared by seeding un-transfected COS-1 cells into 12-well plates at a density of  $1 \times 10^5$  cells per well. All cells were grown to confluence, washed with 1x PBS and then lysed with 80  $\mu\text{L}$  of 2x Laemmli SDS-sample buffer (100 mM Tris-HCl, 20% v/v glycerol, 5% w/v SDS, 0.1% w/v bromophenol blue, 2% v/v  $\beta$ -mercaptoethanol).

The cell lysates were boiled at  $95^{\circ}\text{C}$  and separated using electrophoresis on a 10% SDS polyacrylamide gel at 75 V for 15 minutes, followed by 150 V for 1 hour in SDS-PAGE running buffer (35 mM SDS, 250 mM Tris-HCl, 1.92 M glycine). The Color Protein standard ladder was used as a reference standard (New England BioLabs Inc., USA). Following electrophoresis, proteins were transferred to nitrocellulose membranes (AEC-Amersham, South Africa) in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 10% v/v methanol) using the BioRad transfer unit (Bio-Rad, South Africa) for 1.5 hours at 180 mA. The membranes were then blocked for 2 hours using 10% fat free milk powder made up in Tris buffered saline with 0.1% (v/v) Tween (TBST). After the incubation, the membranes were washed with TBST and then probed with either anti-AR (Santa Cruz Biotechnology, South Africa), anti-PR (SMM Instruments, South Africa) or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, South Africa) primary antibodies

using dilutions shown in Table 2.1, for 16 hours at 4°C. GAPDH was utilized as a loading control. Following the probe, membranes were washed again and incubated with horse radish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology, South Africa) using dilutions shown in Table 2.1, for 90 minutes. The blots were visualised using ECL Western blotting substrate (Biorad, South Africa) on a MyECL Imager (ThermoFischer Scientific, South Africa). The images were quantified using the myImageAnalysis software version 2.0 (ThermoFisher Scientific, South Africa), and the steroid receptor protein levels were normalised to GAPDH protein levels. The details of the primary and secondary antibodies used in this study are summarised in Table 2.1.

**Table 2.1 Details of Primary and secondary antibodies used for western blotting.**

Protein	Primary antibody	Dilution	Secondary antibody	Dilution
AR	AR (441)	1:1000	Rabbit anti-mouse	1:3000
PRA / PRB	PR (PGR-312-L-CE)	1:1000	Rabbit anti-mouse	1:2000
GAPDH	GAPDH (0411)	1:1000	Rabbit anti-mouse	1:1000

## 2.6 Co-immunoprecipitation (co-IP) assay

MDA-MB-231 cells were maintained as described in Section 2.2 and seeded into 10 cm dishes at a density of  $2 \times 10^6$  cells per dish. The next day, cells were transiently transfected with 900 ng pSG5-AR, along with either pSG5-PR-A or pSG5-PR-B using XtremeGENE HP DNA transfection reagent (Sigma-Aldrich, South Africa) as per the manufacturer's instructions. After 24 hours, the transfected cells were re-seeded into 12 well culture plates at a density of  $1 \times 10^5$  cells per well and incubated for a further 24 hours, before treatment for 1 hour with 0.2% EtOH or 1 nM of ligand in supplemented DMEM. The cells were then washed with 500  $\mu$ L ice cold 1x PBS and harvested in 1 mL Radio immunoprecipitation assay (RIPA) buffer (50 mM Tris, 150 mM NaCl, 1% nondidet P-40 substitute, 1% sodium deoxycolate, 0.1% SDS) containing protease inhibitors, before centrifugation at 15 000 x g for 10 minutes at 4°C. The supernatant was collected and 100  $\mu$ L stored as the input control at 20°C. Protein A/G PLUS beads (Santa Cruz Biotechnology, South Africa) were pre-blocked with salmon sperm DNA and 20  $\mu$ L of the blocked beads were incubated with 57  $\mu$ g/mL of the anti-AR antibody, the anti-PR antibody or IgG control on a rotating wheel at 4°C overnight (Santa Cruz Biotechnology, South Africa). The immunoprecipitation (IP) samples were made by adding 500  $\mu$ L of the cell lysate to the protein A/G PLUS beads, followed by incubation on a rotating wheel overnight at 4°C. The next day, the samples were centrifuged at 1 000 x g for 1 minute at 4°C, whereafter the supernatant was discarded. The pellet was washed twice with ice cold 1x PBS, whereafter 40  $\mu$ L Laemmli buffer

was added. Samples were boiled at 90°C and centrifuged at 15 000 x g for 5 minutes at room temperature. Laemmli buffer (20µL) was also added to the input samples before boiling. Input samples and IP samples were separated by SDS-PAGE and proteins visualized by western blot analysis as described in Section 2.5.

## 2.7 Total RNA isolation

MDA-MB-231 cells were maintained as described in Section 2.2 and seeded into 10 cm dishes at  $2 \times 10^6$  cells per dish for 24 hours. The cells were transiently transfected with 900 ng of the expression vector for the AR (pSG5-hAR) in the absence and presence of either 900 ng (1:1) or 4 500ng (1:5) of pSG5-hPR-A or pSG5-hPR-B using the X-TremeGENE HP DNA transfection reagent according to the manufacturer's protocol. The pSG5 empty vector was used in the absence of the PRA or PRB expression vectors to maintain a constant amount of transfected DNA. The following day, the transfected cells were re-seeded into 12 well culture plates at a density of  $1 \times 10^5$  cells per well and treated for 24 hours with 0.2% EtOH and 1 nM of ligand in supplemented DMEM. The cells were then lysed with 400 µL Tri-reagent (Sigma-Aldrich, South Africa). Lysates were then transferred to 1.5 mL microcentrifuge tubes and incubated for 5 minutes at room temperature and stored at -80°C to aid the lysing process. Following thawing of the lysates, 80 µL chloroform (Sigma-Aldrich, South Africa) was added to each sample, followed by vortexing for 15 seconds and incubation at room temperature for 3 minutes. The samples were subsequently centrifuged at 14 000 rpm for 20 minutes at 4°C to separate the solution into three different phases, firstly a clear aqueous phase which contains RNA, then a white interphase containing DNA, and lastly a pink organic phase containing protein. The RNA-containing aqueous phase was transferred to a sterile 1.5 mL microcentrifuge tube to which the same volume of ice-cold isopropanol was added. Samples were vortexed for 5 seconds and incubated for 15 minutes at room temperature, before centrifugation at 14 000 rpm for 15 minutes at 4°C. The supernatant was subsequently discarded, and the RNA pellets washed with 500 µL 75% (v/v) EtOH in diethyl pyrocarbonate (DEPC)-treated water. Samples were vortexed for 1 minute, centrifuged at 8 000 rpm for 10 minutes at 4°C and the supernatant was discarded. The RNA pellets were left to air dry on ice and subsequently dissolved in 15 µL DEPC-treated water. The RNA concentration, in addition to the A260/A280 ratios, were measured using a NanoDrop™ MicroVolume spectrophotometer (ThermoFisher Scientific, South Africa). The samples in a 10 mM Tris buffer were all pure as shown by the A260/A280 ratios between 1.9 and 2.1 (Sambrook, Fritsch and Maniatis, 1989). The RNA integrity was assessed by the presence of intact 18S and 28S ribosomal RNA subunits using denaturing agarose gel electrophoresis (Addendum). All RNA samples were stored at -80°C.

## 2.8 cDNA synthesis

The total RNA was subsequently used to synthesise cDNA by reverse transcription of the RNA using the Promega ImProm-II™ Reverse Transcription System cDNA synthesis kit (Anatech, South Africa) according to the manufacturer's instructions. More specifically, 0.5 µg total RNA, 30 ng/µL oligo (dT)<sub>15</sub> primer as well PCR-grade water (where necessary) was added to a final volume of 2.5 µL in thin-walled PCR tubes. The template-primer mixture was allowed to incubate for 5 minutes at 70°C to denature the RNA secondary structures and then placed on ice for 5 minutes. A volume of 7.5 µL master mix containing the rest of the components required for the reverse transcription process was added to each thin-walled PCR tube containing the denatured RNA. The master mix contained: 3.5 µL PCR-grade water, 2 µL Im-Prom-II™ 5x reaction buffer, 0.75 µL MgCl<sub>2</sub> (final concentration of 1.5 mM), 0.5 µL deoxynucleotide triphosphate (dNTP) mix (final concentration of 0.5 mM for each nucleotide), 0.25 µL recombinant RNasin ribonuclease inhibitor (10 U per reaction), and 0.5 µL ImProm-II reverse transcriptase (80 U per reaction). The samples were subsequently incubated for 5 minutes at 25°C to allow the primers to anneal, followed by incubation at 45°C for 1 hour to allow for extension. Finally, the samples were incubated for 15 minutes at 70°C to inactivate the Im-Prom-II™ reverse transcriptase enzyme and the cDNA samples stored at -20°C.

## 2.9 Realtime quantitative PCR (qPCR)

Realtime qPCR was performed using the KAPA SYBR FAST® qPCR master mix (Sigma-Aldrich, South Africa) and the Lightcycler 96 system (Roche Life Science, South Africa) according to the manufacturer's instructions. The mRNA expression levels of prostate specific antigen (PSA), trefoil factor 1 (pS2), PR, cathepsin D (CTSD) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Biotech Industries, South Africa) were determined using the primers described in Table 2.2. GAPDH was used as the reference gene. The reaction mixture per well of the PCR 96 well plate was prepared using 1 µL cDNA or PCR-grade water (negative control), 5 µL KAPA SYBR FAST® qPCR master mix, forward and reverse primers (final concentration of 0.5 µM each) and PCR-grade water to a final volume of 10 µL. To activate the Taq polymerase, the PCR plates were pre-incubated in the LightCycler 96 system for 5 minutes at 95°C. Further details regarding the three PCR amplification steps are described in Table 2.2. Quantification cycle (C<sub>q</sub>) values were used to calculate the relative transcript levels of PSA, pS2 and GAPDH using the method described by Pfaffl (Pfaffl, 2001). The primer efficiency of each primer set was determined using a cDNA dilution series to generate standard curves (Addendum). The efficiencies were 1.94, 1.91, 1.94, 1.96, 1.86 and 2.12 for PSA, pS2, CTSD, PR, TMPRSS2 and GAPDH, respectively.

**Table 2.2. Primer sequences and thermal cycling conditions of the genes investigated using qPCR**

Gene	Primers (5' – 3')	Strand	Amplicon length (bp)	Denaturation	Annealing	Elongation	Number of cycles	Reference
PSA	AGGCCTTCCCTGTACACCAA	Forward	133	95°C for 10 s	60°C for 10 s	72°C for 10 s	45	(Masoodi <i>et al.</i> , 2017)
	GTCTTGGCCTGGTCATTTCC	Reverse						
CTSD	GCGAGTACATGATCCCCTGT	Forward	89	95°C for 15 s	58°C for 30 s	72°C for 20 s	40	(Kocanova <i>et al.</i> , 2010)
	CTCTGGGGACAGCTTGTAGC	Reverse						
PR	CTTAATCAACTAGGCGAGAG	Forward	122	95°C for 15 s	58°C for 30 s	72°C for 20 s	40	(Kocanova <i>et al.</i> , 2010)
	AAGTCATCCAAGAATACTG	Reverse						
pS2	ATACCATCGACGTCCCTCCA	Forward	147	95°C for 30 s	62°C for 60 s	72°C for 90 s	40	(Chatagnon <i>et al.</i> , 2010)
	AAGCGTGTCTGAGGTGTCCG	Reverse						
TMPRSS2	CTGCCAAGGTGCTTCTC	Forward	150	95°C for 10 s	60°C for 20 s	72°C for 2 s	45	(Kumagai <i>et al.</i> , 2013)
	TTAGCCGTCTGCCCTC	Reverse						
GAPDH	TGAACGGGAAGCTCACTGG	Forward	307	95°C for 10 s	59°C for 10 s	72°C for 12 s	35	(Ishibashi <i>et al.</i> , 2003)
	TCCACCACCCTGTTGCTGTA	Reverse						



## 2.10 siRNA transfection

The T47D cells were maintained as described in Section 2.2 and seeded into 6 cm dishes (WhiteSci, South Africa) at a density of  $8 \times 10^5$  cells in DMEM supplemented with 10% FCS and 1% penstrep. The next day, cells were transiently transfected with 10 nM of either validated non-silencing scrambled sequence control (NSC) siRNA (Qiagen, USA), validated siRNA directed against PRB only (a combination of two target-specific siRNAs targeting only PRB) (Hardy *et al.*, 2008) or validated siRNA directed against both the human PR isoforms (a combination of four target-specific siRNAs, Qiagen, USA) (Chen, Hardy and Mendelson, 2011) using Dharmafect transfection reagent (Dharmacon, USA), according to the manufacturer's instructions. After a 48 hour incubation period, the transfected cells were seeded into a 12-well plate (Bio-Smart Scientific, South Africa) at  $1 \times 10^5$  cells per well using phenol red-free DMEM supplemented with 10% charcoal-stripped FCS (CS-FCS) and 1% penstrep. On the following day, the cells were treated with either 0.1% (v/v) EtOH or 1 nM of ligand prepared in unsupplemented phenol-red free DMEM for 24 hours prior to harvesting RNA (Section 2.7) for realtime qPCR (Section 2.9). Western blot analysis was used to confirm reductions in protein levels as described in Section 2.6.

## 2. 11 Statistical Analysis

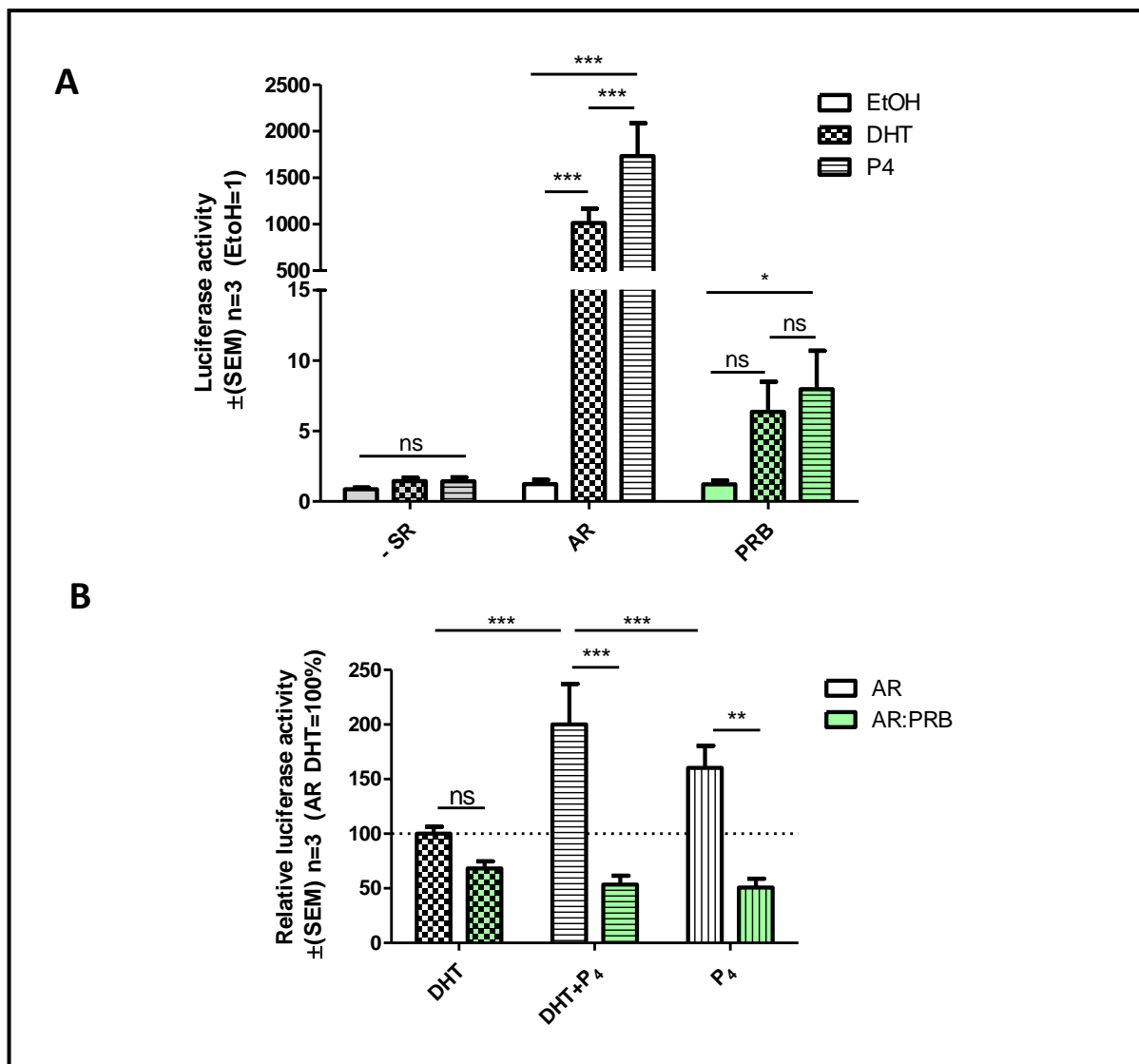
Visual representation, data manipulation as well as statistical analysis was conducted using the GraphPad Prism® v 5.00 software package, for Windows (GraphPad Software, USA). Student's t-test and one-way analysis of variance (ANOVA) or two-way ANOVA with Bonferroni's (Compares all pairs of columns) post-test was used. The error bars denote the standard error of the mean (SEM) of three independent experiments. Statistically significant differences will be represented by \*, \*\* and \*\*\* indicating  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$ , and  $p > 0.05$ , and ns indicating no statistical significance), respectively.

## **Chapter 3 Results**

### 3.1 Unliganded and liganded PRB downregulates AR transactivation activity on the synthetic classical ARE.

In the first part of the study, some of the previous experiments showing probable crosstalk between the AR and the PR (Cabral, MSc Thesis, 2018) were repeated in order to confirm the findings. The MDA-MB-231 breast cancer cell line was utilised as the model cell line, as previous reports indicate that it lacks expression of the ER, PR and AR (Holliday and Speirs, 2011; Narayanan *et al.*, 2014). The absence of these steroid receptors were also previously confirmed in the MDA-MB-231 cell line used in our laboratory (Ndlovu, MSc thesis, 2015; Cabral, MSc Thesis, 2018). The cells were transiently transfected with a PRE luciferase (PRE-luc) promoter-reporter construct and either an AR expression vector, a PRB expression vector or both the AR and PR expression vectors. Cells were treated with the potent natural androgen, DHT, or P<sub>4</sub>, the natural PR ligand, or both (Figure 3.1). Notably, the PRE-luc is referred to as a classical ARE-luc in this thesis, as both the AR and PR can bind to it.

The results in Figure 3.1A indicate that the AR, upon activation with DHT, caused a significant increase in transactivation on the classical ARE. This result is indicative of the reporter assay working and that the ligand is functional. Surprisingly, treatment with P<sub>4</sub> caused increased transactivation to a significantly greater extent than DHT treatment. This finding is in contrast to previous reports showing very weak AR agonist activity for P<sub>4</sub> on the classical ARE (Africander *et al.*, 2014). When the cells transfected with the AR were treated with both DHT and P<sub>4</sub>, AR transactivation function was further increased. As expected, the PR ligand P<sub>4</sub> activated PRB on the classical PRE/ARE, and DHT appeared to be a PRB agonist for transactivation. Although the DHT response was not statistically significant in this study, Cabral has previously shown the response of DHT via PRB to be statistically significant. When PRB and the AR were co-expressed in the MDA-MB-231 cells, and the cells were treated with both DHT and P<sub>4</sub>, the transcriptional activity was decreased (Figure 3.1B). Although it appears that there was also a decrease in the absence of PR ligand, this response was not statistically significant. Surprisingly, these results contradict the previous findings showing that both unliganded and liganded PRB upregulate the transactivation activity of the AR (Cabral, MSc Thesis, 2018).



**Figure 3.1: Co-expression of PRB with the AR results in a decrease of AR transactivation function on a classical ARE when the PR is liganded.** (A) MDA-MB-231 cells were transiently transfected with 3000 ng of the pTAT-PRE-E1b-luc reporter construct and either 300 ng of the empty pSG5 vector (no steroid receptor transfected (-SR); grey); or 300 ng pSG5-hAR (white), or 300 ng pSG5-hPR-B (green). (B) MDA-MB-231 cells were transiently transfected with 300 ng pSG5-hAR (white) in the absence and presence of 300 ng pSG5-hPR-B (green). The next day, all cells were treated with 0.2% v/v EtOH or 1 nM ligand(s) for 24 hours. Luciferase activity was measured and normalized to total protein concentration. (A) Luciferase activity is shown where EtOH treatment for each transfection condition was set as 1 and all other responses were set relative to this. (B) Relative luciferase activity is shown, where the fold induction at 1 nM DHT via the AR was set as 100% and other responses were set relative to this. The results indicate the average of three independent experiments, each performed in triplicate ( $\pm$ SEM). Statistical analysis was performed using column statistics and a two-way ANOVA with a Bonferroni (comparing each column to all other columns) post-test. Statistically significant differences are indicated by \*\* or \*\*\*, to indicate  $p < 0.01$  or  $p < 0.001$ ; no statistical significance (ns) indicates  $p > 0.05$ .

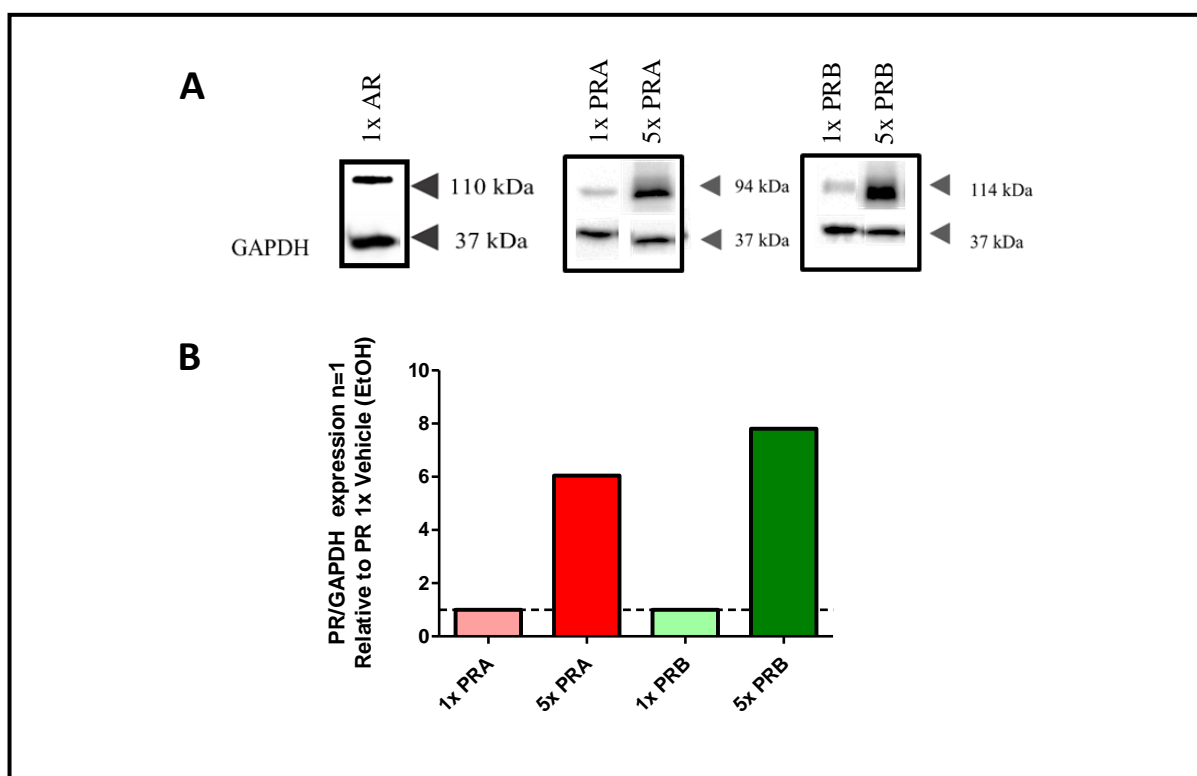
### 3.2 The PR isoforms modulate AR transactivation of the endogenous PSA gene.

Even though the results by Cabral showing upregulation of AR activity in the presence of the PR isoforms (Cabral, MSc thesis, 2018) could not be reproduced, the result showing decreased AR transactivation activity via PRB still suggests probable crosstalk between the AR and PR. Thus, we next wanted to establish whether the PR would also modulate AR function on the endogenous expression of PSA, a well-known classical ARE-containing gene (Lai *et al.*, 2009). Notably, the collective term “we” and “our” is often used in the thesis. However, all the experimental work was performed by the candidate. It is known that PSA is expressed in the MDA-MB-231 breast cancer cell line and that it is upregulated in breast, as well as prostate, cancer (Catalona *et al.*, 1994; Ferguson *et al.*, 1996; Cuzick *et al.*, 2011). The MDA-MB-231 cells were again used as the model cell line and transiently transfected with the AR expression vector in the absence and presence of expression vectors for PRA or PRB at equimolar (1x) or excess (5x) concentrations relative to the AR. The cells were then treated for 24 hours with DHT in the absence and presence of P<sub>4</sub>.

Western blot analysis was performed to confirm the expression levels of the respective receptors following the transfection of the plasmids, and the representative blots and quantifications are shown in Figure 3.2. The results confirm the expression of both the AR and PR isoforms transiently transfected into the MDA-MB-231 cells. Interestingly, transfection of the same amount of DNA (1x) for the AR, PRA and PRB into the cells appeared to result in a higher level of AR expression in the cells than either that of PRA or PRB. It is unlikely that differences in expression levels are due to the promoter-reporter constructs, as the cDNA for the AR, PRA or PRB were all cloned into the pSG5 expression vector. This could however be a reflection of variable sensitivity between different antibodies used to probe for the AR or PR, rather than an indicator of more AR relative to the PR isoforms. Transfecting 5x more PRA and PRB DNA relative to the AR resulted in 6-fold higher PRA and 8-fold higher PRB (Figure 3.3). However, as this is the result of a single experiment, we will continue referring to the excess PR as 5x PRA or 5x PRB in this thesis.

As expected, the realtime qPCR results in Figure 3.3B show an increase (2-fold) in the mRNA expression of PSA in response to DHT in MDA-MB-231 cells transfected with the AR. This response is consistent with an AR-mediated response as it was not seen in cells lacking the AR

(Figure 3.3A). Notably, although PSA mRNA expression appeared to be further upregulated in the presence of both  $P_4$  and DHT, this response was not statistically significant (Figure 3.3B). In MDA-MB-231 cells co-transfected with equivalent levels of AR and PRA or PRB, DHT stimulated AR transactivation function was significantly upregulated by unliganded PRA (Figure 3.3C) and unliganded PRB (Figure 3.3D). Interestingly, although it appears that PSA mRNA expression in the presence of 5x PRA or 5x PRB relative to the AR, was upregulated, this response was not statistically significant.



**Figure 3.2: Expression of AR and PR isoforms protein levels in the MDA-MB-231 breast cancer cell line following transient transfections.** Whole cell extracts were prepared from the MDA-MB-231 breast cancer cell line transfected with either 300 ng (1x) or 1500 ng (5x) pSG5-hPR-A or pSG5-hPR-B. Protein lysates were analysed by western blotting using antibodies specific to PRA, PRB, and GAPDH (loading control). Western blots were quantified using myImageAnalysis software. Protein expression of 1x PRA (pink) or 1x PRB (light green) was set as 1 and the expression of 5x PRA (red) or 5xPRB (dark green), set relative to the respective 1x.

Figure 3.4A indicates that in the presence of both the AR and PRA, as well as both the AR ligand (DHT) and PR ligand ( $P_4$ ), the presence of equivalent levels of (1x) PRA, but not excess (5x) PRA, caused a significant decrease in PSA expression, while it appears that a decrease was also observed with 1x PRB (Figure 3.4B), this response was not statistically significant.

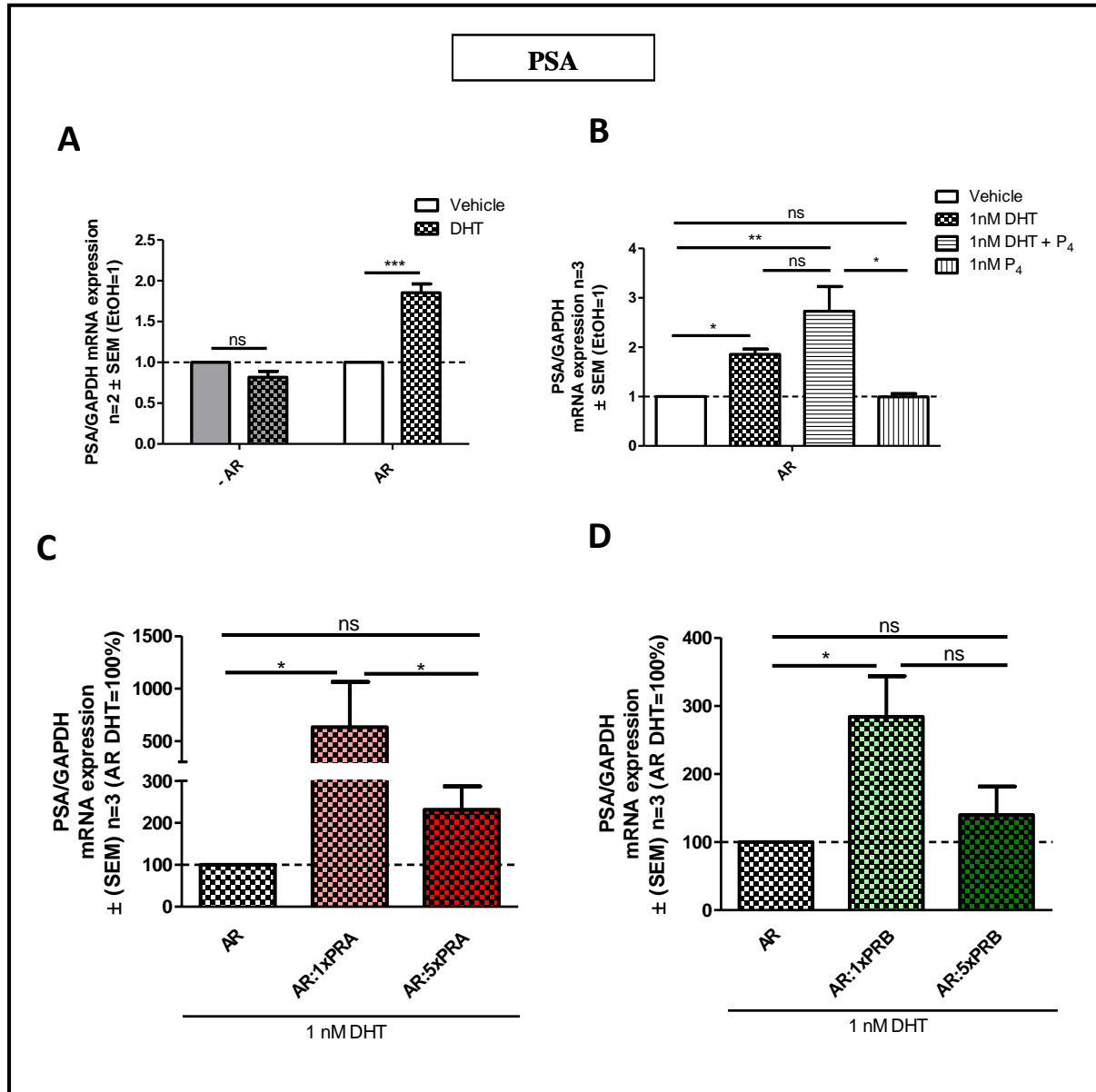


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**Figure 3.3: Equivalent levels of unliganded PRA and PRB relative to that of the AR, significantly upregulate AR transactivation of an endogenous ARE containing gene in the MDA-MB-231 cell line.** The human MDA-MB-231 breast cancer cell line was transiently transfected with (A) 300 ng empty pSG5 vector (-AR; grey), or (A and B) pSG5AR (white), or (C and D) 300 ng pSG5AR in the absence and presence of either 300 ng (1x) or 1500 ng (5x) of (C) pSG5PR-A (red) or (D) pSG5PR-B (green). The next day, all cells were treated with 1 nM ligand(s) for 24 hours. Total RNA was isolated, cDNA synthesised and realtime qPCR performed to determine the mRNA expression levels of the endogenous PSA gene. GAPDH was used as the reference gene. (A-B) The ratio of PSA mRNA expression/GAPDH mRNA of samples treated with EtOH were calculated relative to the vehicle of each transfection condition set as 1 and all other responses were set relative to this. (C and D) The fold response of AR with DHT treatment was set as 100% and all other responses were set relative to this. The results indicate the average of three independent experiments ( $\pm$ SEM). Statistical analysis was performed using column statistics and a one-way ANOVA with a Bonferroni (comparing each column to all other columns) post-test. Statistically significant differences are shown by \*, \*\* or \*\*\*, to indicate  $p < 0.05$ ,  $p < 0.01$  or  $p < 0.001$ ; no statistical significance (ns) indicates  $p > 0.05$ .

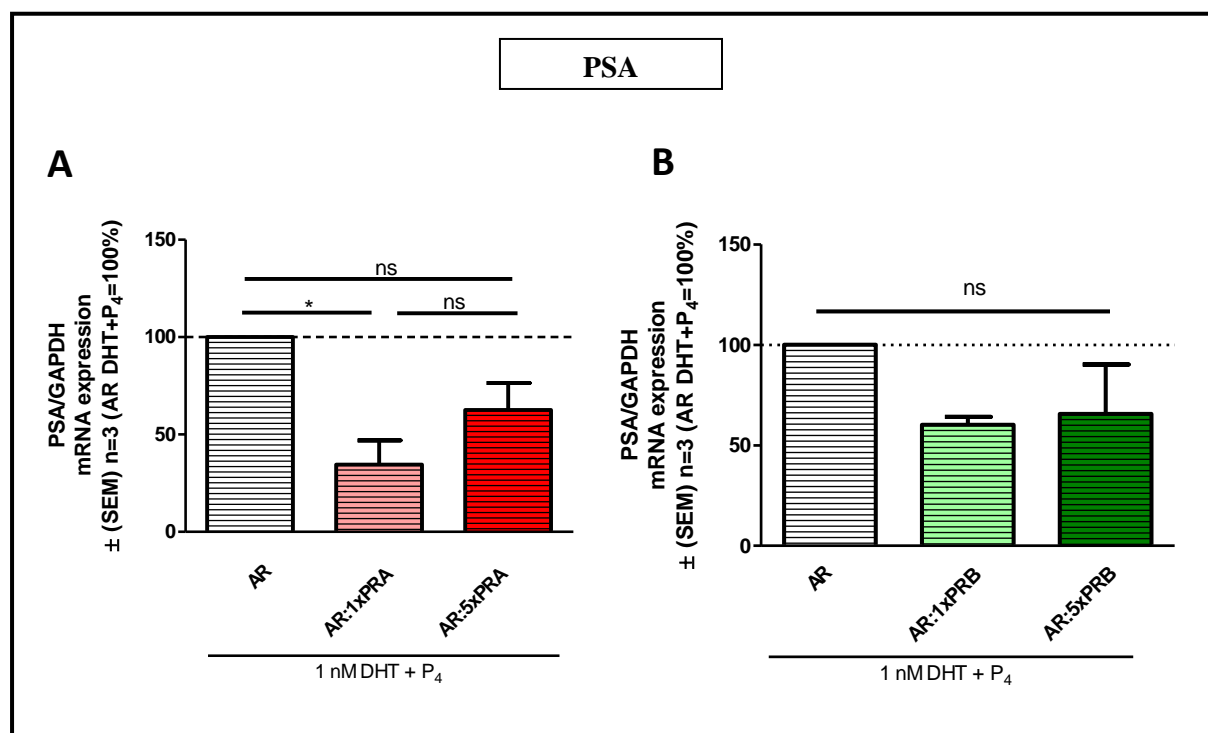


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**Figure 3.4: Liganded PRA and PRB downregulate AR transactivation on an endogenous ARE containing gene in the MDA-MB-231 cell line.** The human MDA-MB-231 breast cancer cell line was transiently transfected with 300 ng of pSG5AR (white) in the absence and presence of either 300 ng (1x) or 1500 ng (5x) of (A) pSG5PR-A (red) or (B) pSG5PR-B (green) and treated for 24 hours with 1 nM DHT in combination with P<sub>4</sub>. Total RNA was isolated, cDNA synthesised and realtime qPCR performed to determine the mRNA expression levels of the endogenous PSA gene. GAPDH was used as the reference gene. Fold mRNA expression at 1 nM DHT and P<sub>4</sub> via AR only was set as 100% and all other responses set relative to this. The results indicate the average of three independent experiments ( $\pm$ SEM). Statistical analysis was performed using column statistics and a one-way ANOVA with a Bonferroni (comparing each column to all other columns) post-test. Statistically significant differences are shown by \*, \*\* or \*\*\*, to indicate  $p < 0.05$ ,  $p < 0.01$  or  $p < 0.001$ ; no statistical significance (ns) indicates  $p > 0.05$ .

### **3.3 The AR-regulated TMPRSS2 gene is downregulated in the PC3 prostate cancer cell line transfected with the AR, but upregulated in cells transfected with both the AR and excess PRA or PRB.**

Considering that it was previously shown that PRB upregulates AR activity via a synthetic ARE-containing minimal promoter in the PC3 prostate cancer cell line (Cabral, MSc Thesis, 2018), while PRA had no statistically significantly effect, we next assessed whether the PR would also modulate AR function on the expression of an endogenous AR-regulated gene in the PC3 cells. Although we assessed effects on the PSA gene for breast cancer, we could not investigate PSA here, as PSA is not endogenously expressed in the PC3 cell line (Tai *et al.*, 2011). We thus evaluated effects on the AR-regulated TMPRSS2 gene, as it is known that TMPRSS2 gene expression is elevated in prostate cancer (Afar *et al.*, 2001), and is expressed in the PC3 cell line (Lin *et al.*, 1999). Previous reports have also shown that the PC3 cell line does not endogenously express the PR isoforms or the AR (Nowakowska *et al.*, 2016), and this was confirmed in the PC3 cell line used in our laboratory (Cabral, MSc Thesis, 2018). The PC3 cells were thus transiently transfected with the AR expression vector in the absence and presence of expression vectors for PRA or PRB at equivalent (1x) or excess (5x) concentrations relative to that of the AR. The cells were then treated for 24 hours with DHT in the absence and presence of P<sub>4</sub>.

Surprisingly, the realtime qPCR results in Figure 3.5B show a decrease in the mRNA expression of TMPRSS2 in response to DHT in PC3 prostate cancer cells transfected with the

AR. This response is consistent with an AR-mediated response as it was not seen in the cells lacking the AR (Figure 3.5A). The results in Figure 3.5B show that P<sub>4</sub> also downregulated TMPRSS2 mRNA, while the downregulation of TMPRSS2 mRNA expression by DHT, was not modulated in the presence of both DHT and P<sub>4</sub>. In PC3 cells co-transfected with AR and PRA or PRB at equivalent levels, the decrease in TMPRSS2 expression by DHT via the AR, was abolished in the presence of unliganded PRA (Figure 3.5C), but not PRB (Figure 3.5D). Interestingly, TMPRSS2 mRNA expression was increased in the presence of the AR and 5x excess PRA (Figure 3.5C) or PRB (Figure 3.5D), relative to the AR. In Figure 3.6 it appears that the TMPRSS2 gene was also downregulated when the AR is co-expressed with either (1x) PRA (Figure 3.6A) or PRB (Figure 3.6B), in the presence of DHT and P<sub>4</sub>. However, when an excess (5x) of either of the PR isoforms was expressed relative to the AR, an increase in TMPRSS2 mRNA expression was observed in the presence of both DHT and P<sub>4</sub>. This increase in TMPRSS2 expression in the presence of excess PRA or PRB appeared to be independent of the PR ligand, P<sub>4</sub>, as similar increases are observed in the absence (Figure 3.5C and D) and presence (Figure 3.6), of P<sub>4</sub>.

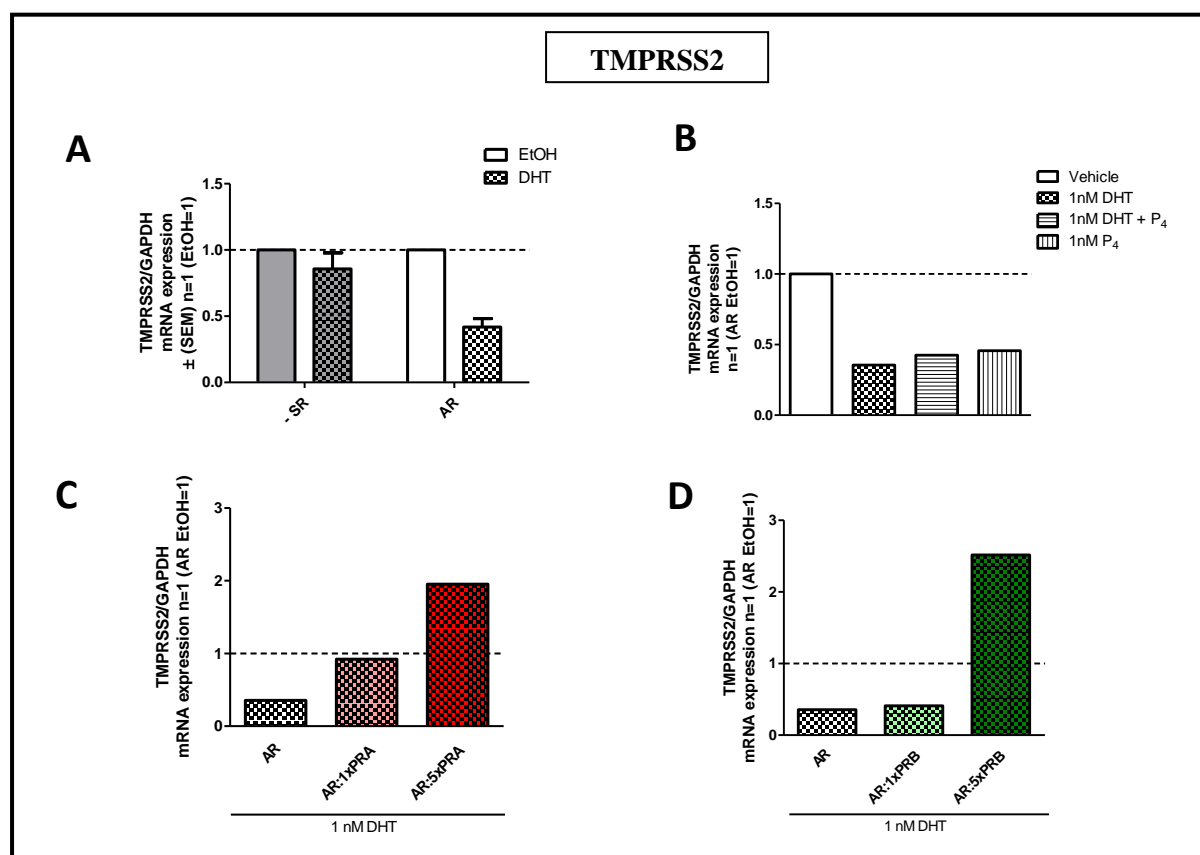
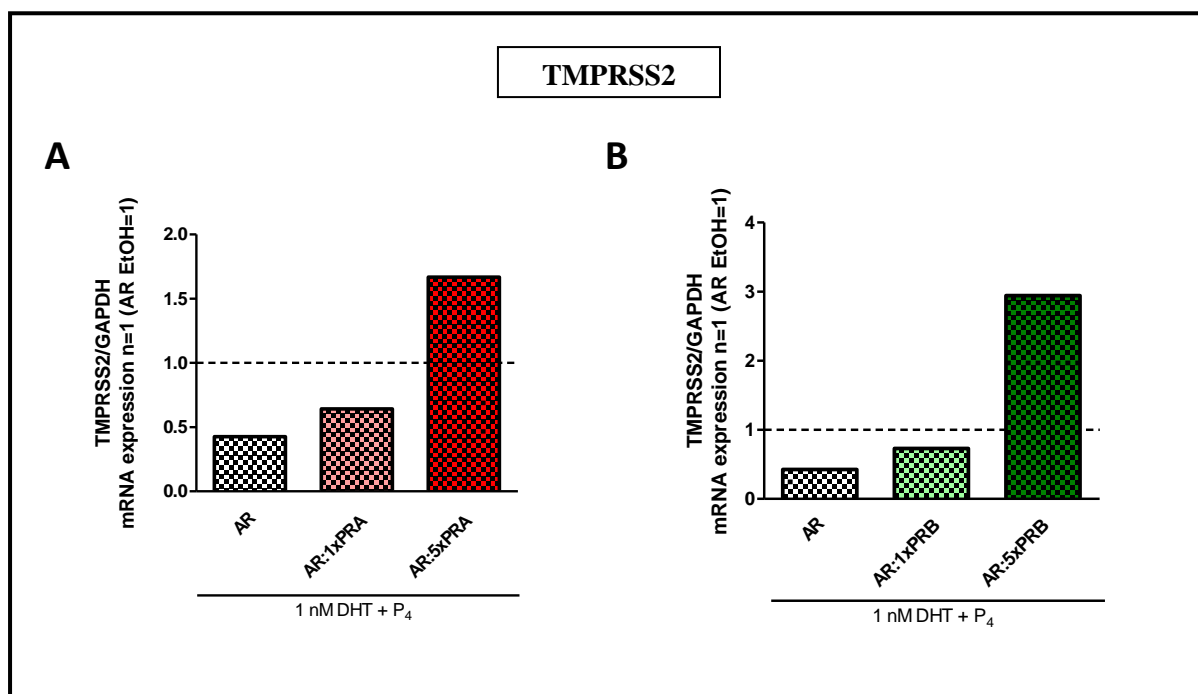


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**Figure 3.5:** In the presence of both the AR and excess (5x) unliganded PRA and PRB, TMPRSS2 mRNA expression is increased in the PC3 prostate cancer cell line. The human PC3 prostate cancer cell line was transiently transfected with (A) 300 ng empty pSG5 vector (-AR; grey), or (A and B) pSG5AR (white), or (C and D) 300 ng pSG5AR in the absence and presence of either 300 ng (1x) or 1500 ng (5x) of (C) pSG5PR-A (red) or (D) pSG5PR-B (green). The next day, the cells were treated with 1 nM ligand(s) for 24 hours. Total RNA was isolated, cDNA synthesised and realtime qPCR performed to determine the mRNA expression levels of the endogenous TMPRSS2 gene. GAPDH was used as the reference gene. (A and B) The ratio of TMPRSS2 mRNA expression/GAPDH mRNA of samples treated with EtOH were calculated relative to the vehicle set as 1, (C and D) and is represented by the dotted line. The results shown are from a single experiment.



**Figure 3.6:** TMPRSS2 mRNA expression is increased in the presence of the AR and excess (5x) liganded PRA and PRB in the PC3 prostate cancer cell line. The human PC3 prostate cancer cell line was transiently transfected with 300 ng of pSG5AR (white) in the absence and presence of either 300 ng (1x) or 1500 ng (5x) (A) pSG5PR-A (red) or (B) pSG5PR-B (green) and treated for 24 hours with 1 nM DHT in combination with P<sub>4</sub>. Total RNA was isolated, cDNA synthesised and realtime qPCR performed to determine the mRNA expression levels of the endogenous TMPRSS2 gene. GAPDH was used as the reference gene. The ratio of TMPRSS2 mRNA expression/GAPDH mRNA of samples treated with EtOH were calculated relative to the vehicle set as 1, and represented by the dotted line. The results shown are from a single experiment.

### 3.4 AR-mediated downregulation on the ER-regulated CTSD gene by DHT is not significantly modulated by the presence of either PRA or PRB, while expression of the ER-regulated PR gene is increased in the presence of both the AR and PR isoforms

It is known that the AR can modulate the activity of ER $\alpha$  by competing for binding to ER-binding sites in ER target genes (Peters et al., 2009). This study by Peters and co-workers found that the AR could bind to the ERE within ER-regulated genes. Results from the study by Cabral are in agreement with the former study, in that it was shown that DHT-activated AR can activate a synthetic minimal promoter containing an ERE (Cabral, MSc Thesis, 2018). We thus investigated AR regulation of the endogenous ER-regulated cathepsin D (CTSD) and PR genes. These two genes are known to be overexpressed in women with breast cancer (Chatagnon *et al.*, 2010; Ishibashi *et al.*, 2017), and both genes are endogenously expressed in the MDA-MB-231 cell line (Ndlovu, 2015; Ashraf *et al.*, 2019).

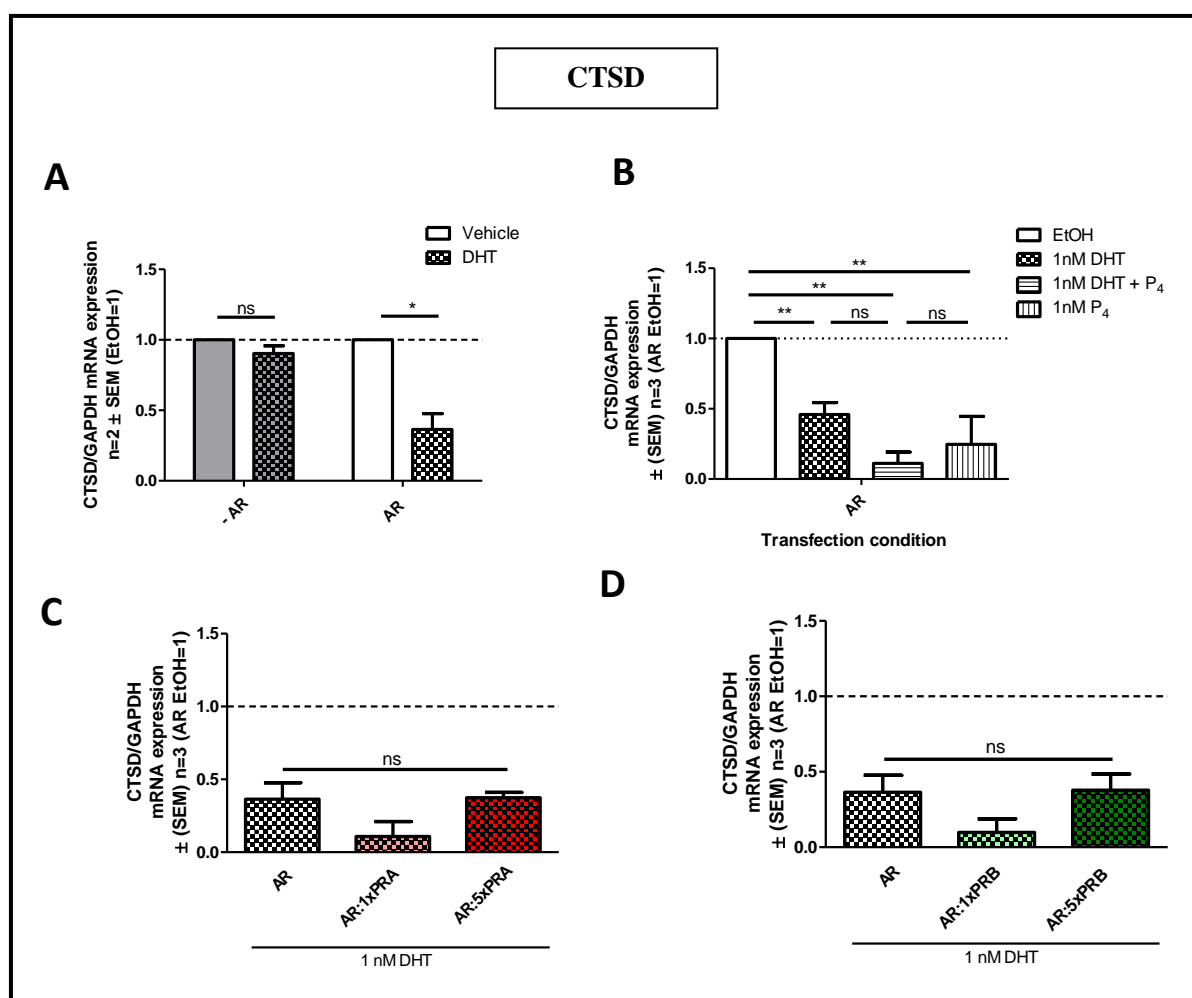


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**Figure 3.7: Unliganded PRA and PRB do not affect DHT-induced suppression of the CTSD gene mediated by the AR in the MDA-MB-231 cells co-expressing the AR and PRA or PRB.** The human MDA-MB-231 breast cancer cell line was transiently transfected with (A) 300 ng empty pSG5 vector (-AR; grey), or (A and B) pSG5AR (white), or (C and D) 300 ng pSG5AR in the absence and presence of either 300 ng (1x) or 1500 ng (5x) of (C) pSG5PR-A (red) or (D) pSG5PR-B (green). The next day, the cells were treated with 1 nM ligand(s) for 24 hours. Total RNA was isolated, cDNA synthesised and realtime qPCR performed to determine the mRNA expression levels of the endogenous CTSD gene. GAPDH was used as the reference gene. (A and B) The ratio of CTSD mRNA expression/GAPDH mRNA of the treated samples were calculated relative to EtOH set as 1, (C and D) represented by the dotted line. The results indicate the average of three independent experiments ( $\pm$ SEM). Statistical analysis was performed using column statistics and a one-way ANOVA with a Bonferroni (comparing each column to all other columns) post-test. Statistically significant differences are shown by \*, \*\* or \*\*\*, to indicate  $p < 0.05$ ,  $p < 0.01$  or  $p < 0.001$ ; no statistical significance (ns) indicates  $p > 0.05$ .

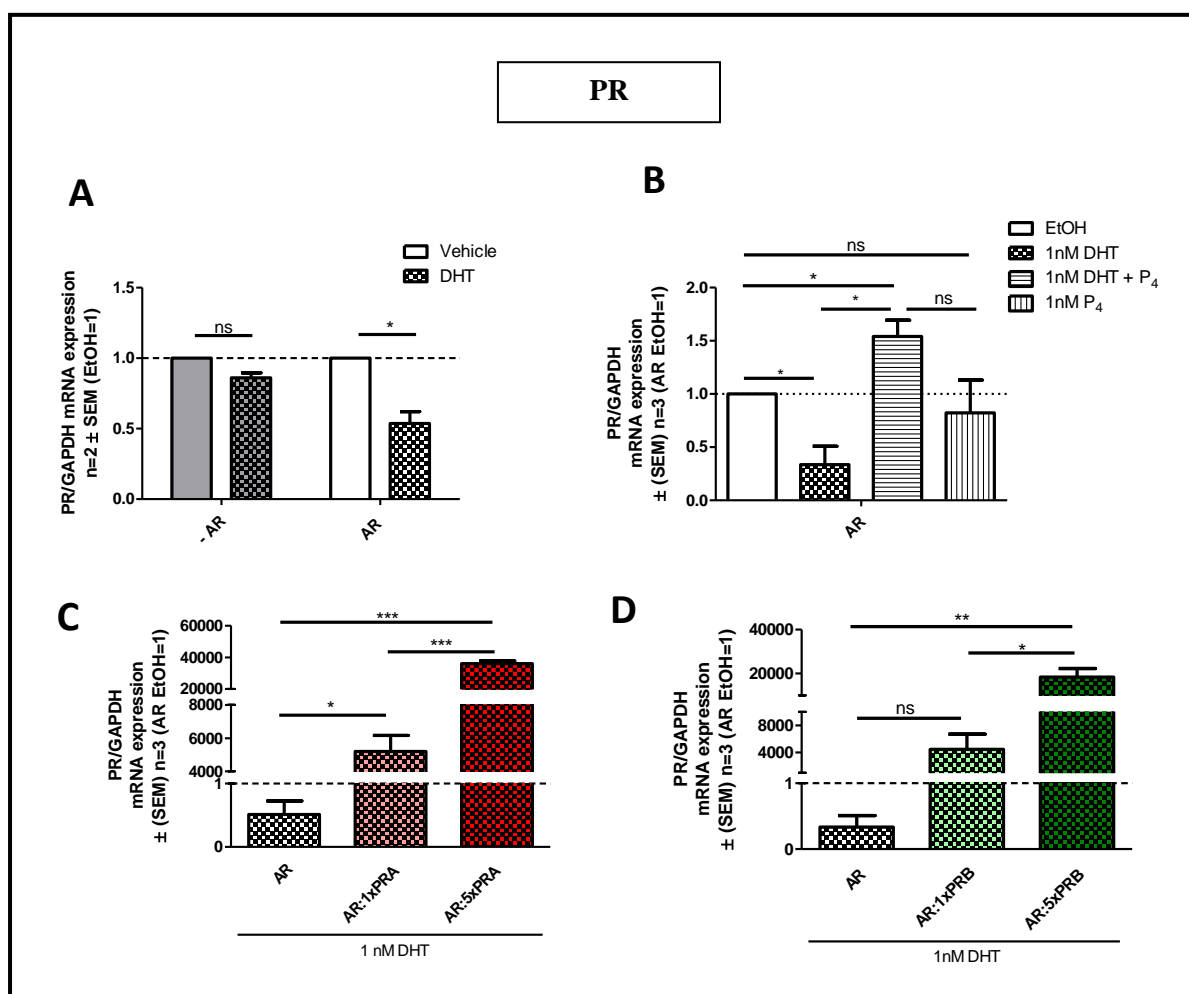
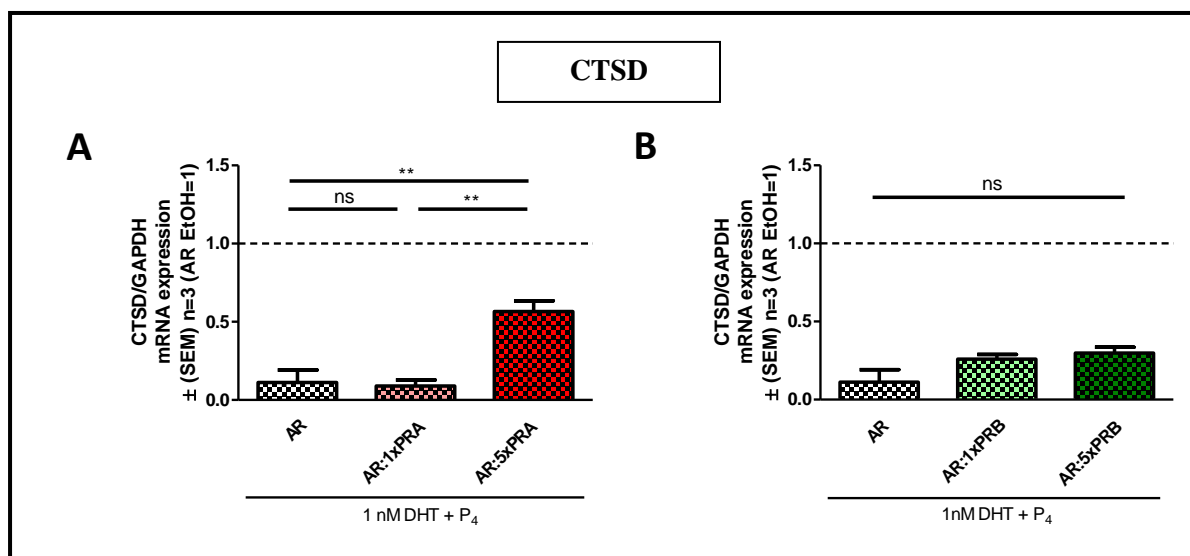


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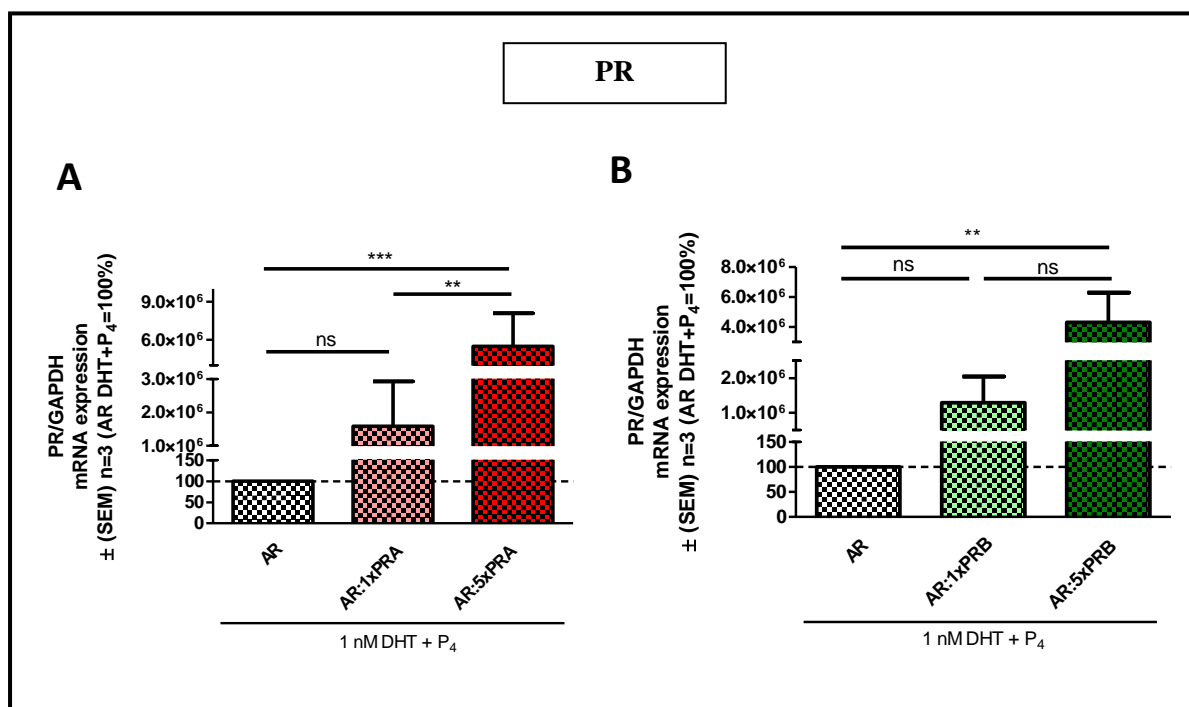
**Figure 3.8: Unliganded PRA and PRB abolished DHT-induced suppression of the PR gene mediated by the AR in MDA-MB-231 cells co-expressing the AR and PRA or PRB.** The human MDA-MB-231 breast cancer cell line was transiently transfected with (A) 300 ng empty pSG5 vector (-AR; grey), or (A and B) pSG5AR (white), or (C and D) 300 ng pSG5AR in the absence and presence of either 300 ng (1x) or 1500 ng (5x) of (C) pSG5PR-A (red) or (D) pSG5PR-B (green). The next day, the cells were treated with 1 nM ligand(s) for 24 hours. Total RNA was isolated, cDNA synthesised and realtime qPCR performed to determine the mRNA expression levels of the endogenous PR gene. GAPDH was used as the reference gene. (A and B) The ratio of PR mRNA expression/GAPDH mRNA of the treated samples were calculated relative to EtOH set as 1, (C and D) represented by the dotted line. The results indicate the average of three independent experiments ( $\pm$ SEM). Statistical analysis was performed using column statistics and a one-way ANOVA with a Bonferroni (comparing each column to all other columns) post-test. Statistically significant differences are shown by \*, \*\* or \*\*\*, to indicate  $p < 0.05$ ,  $p < 0.01$  or  $p < 0.001$ ; no statistical significance (ns) indicates  $p > 0.05$ .

Surprisingly, the results show that the mRNA expression of both CTSD (Figure 3.7B) and the PR (Figure 3.8B) was significantly downregulated by DHT in MDA-MB-231 cells transfected with the AR. Again, this is consistent with a response requiring the AR, as no effect was observed for DHT in the absence of transfected AR (Figure 3.7A and 3.8A). Although it appeared that CTSD expression was further decreased in the presence of both DHT and P<sub>4</sub>, this response was not statistically significantly different to the response with only DHT (Figure 3.7B). In contrast, the PR gene was upregulated in the presence of both DHT and P<sub>4</sub> (Figure 3.8B), suggesting that P<sub>4</sub> antagonized the DHT-induced repression of the PR gene mediated by the AR. It has previously been shown that P<sub>4</sub> is an AR antagonist (Africander *et al.*, 2014). Our results show gene-specific regulation of ER-regulated genes by both DHT and P<sub>4</sub> in MDA-MB-231 cells transfected with the AR. Co-expressing either PRA or PRB with the AR, did not significantly modulate the observed downregulation of CTSD mRNA expression by DHT is (Figure 3.7C and D). Notably, although it appeared that co-expression of PRA or PRB, at equivalent levels to that of the AR, resulted in a further downregulation in CTSD expression, this response was not statistically significant. In the presence of both DHT and P<sub>4</sub>, and a 5-fold excess PRA relative to the AR, the AR-mediated downregulation of CTSD expression by DHT was significantly lifted, albeit that the gene was still downregulated (Figure 3.9A). A similar response was not observed in the presence of either equivalent or (5x) excess expression levels of PRB relative to the AR (Figure 3.9B).



**Figure 3.9: Excess (5x) liganded PRA increases, while PRB does not alter, the transactivation of the endogenous ER-regulated CTSD gene in the MDA-MB-231 cell line.** The human MDA-MB-231 breast cancer cell line was transiently transfected with **(A and B)** 300 ng of pSG5AR (white) in the absence and presence of either 300 ng (1x) or 1500 ng (5x) of **(A)** pSG5PR-A (red) or **(B)** pSG5PR-B (green) and treated for 24 hours with 1 nM DHT in combination with P<sub>4</sub>. Total RNA was isolated, cDNA synthesised and realtime qPCR performed to determine the mRNA expression levels of the endogenous CTSD gene. GAPDH was used as the reference gene. The ratio of CTSD mRNA expression/GAPDH mRNA of the treated samples were calculated relative to EtOH set as 1, represented by the dotted line. The results indicate the average of three independent experiments ( $\pm$ SEM). Statistical analysis was performed using column statistics and a one-way ANOVA with a Bonferroni (comparing each column to all other columns) post-test. Statistically significant differences are shown by \*, \*\* or \*\*\*, to indicate  $p < 0.05$ ,  $p < 0.01$  or  $p < 0.001$ ; no statistical significance (ns) indicates  $p > 0.05$ .

In Figure 3.8C, the addition of unliganded PRA, both at equivalent and excess concentrations relative to the AR, lifted the AR-mediated downregulation of the PR gene, and resulted in the upregulation of PR mRNA expression. This effect was also observed for unliganded PRB, but was only statistically significant when PRB was expressed in excess of PRB relative to the AR (Figure 3.8D). In the presence of the AR and 5x excess PRA or PRB, as well as both DHT and P<sub>4</sub>, PR mRNA expression was significantly upregulated (Figure 3.10). Although this upregulation also appeared to occur at equivalent levels of PRA and PRB relative to the AR, these responses were not statistically significant.



**Figure 3.10: Excess (5x) liganded PRA and PRB upregulated AR transactivation on the endogenous ER-regulated PR gene in the MDA-MB-231 cell line.** The human MDA-MB-231 breast cancer cell line was transiently transfected with 300 ng of pSG5AR (white) in the absence and presence of either 300 ng (1x) or 1500 ng (5x) of (A) pSG5PR-A (red) or (B) pSG5PR-B (green) and treated for 24 hours with 1 nM DHT in combination with P<sub>4</sub>. Total RNA was isolated, cDNA synthesised and realtime qPCR performed to determine the mRNA expression levels of the endogenous PR gene. GAPDH was used as the reference gene. The fold mRNA expression at 1 nM DHT and P<sub>4</sub> in the presence of AR only was set as 100%, and all other responses were set relative to this. The results indicate the average of three independent experiments (±SEM). Statistical analysis was performed using column statistics and a one-way ANOVA with a Bonferroni (comparing each column to all other columns) post-test. Statistically significant differences are shown by \*, \*\* or \*\*\*, to indicate p<0.05, p<0.01 or p<0.001; no statistical significance (ns) indicates p>0.05.



### **3.5 Whether the PR isoforms can modulate the regulation of the endogenous pS2 gene by DHT in the T47D cell line could not be determined.**

Next, we evaluated whether the AR could transactivate another ER-regulated gene, namely trefoil factor 1 (pS2). Here the human T47D breast cancer cells were used as they are known to endogenously express pS2 (Patterson *et al.*, 2015; Chottanapund *et al.*, 2017) and multiple steroid receptors, including the AR, both PR isoforms, the ER subtypes, GR and the MR (Nardulli and Katzenellenbogen, 1988; Sartorius *et al.*, 1994; Jacobsen and Horwitz, 2012). To delineate effects that require the PR isoforms, T47D cells were transiently transfected with either non-silencing scrambled sequence control (NSC) siRNA, siRNA directed against PRB only (Chen, Hardy and Mendelson, 2011) or siRNA directed against both the human PR isoforms. The cells were subsequently treated with either 1 nM E<sub>2</sub>, P<sub>4</sub>, the non-metabolizable synthetic androgen mib, the natural androgen DHT, or a combination of either DHT or mib with P<sub>4</sub> for 24 hours. E<sub>2</sub> was used as a positive control as it is known to upregulate pS2 expression via the ER (Amiry *et al.*, 2009), while mib was used as it is reportedly an AR-specific agonist (Miki *et al.*, 1988). Western blot analysis confirmed the successful reductions in PRB protein levels with siPRB, and reduction in both PRA and PRB with sitotalPR (Figure 3.11A-C). Interestingly, siPRB also appeared to partially downregulate PRA expression. The results in Figure 3.11D show that E<sub>2</sub> upregulated pS2 expression in the untransfected T47D cells, indicating that the primers and realtime qPCR assay were working. However, the response in cells transfected with NSC, albeit also an upregulation, was significantly different to that in the untransfected T47D cells. Similar differences were seen between the untransfected T47D cells and those transfected with NSC in response to Mib and DHT, but not P<sub>4</sub> (Figure 3.11D). We did not continue with the analysis of responses when the PR was silenced.

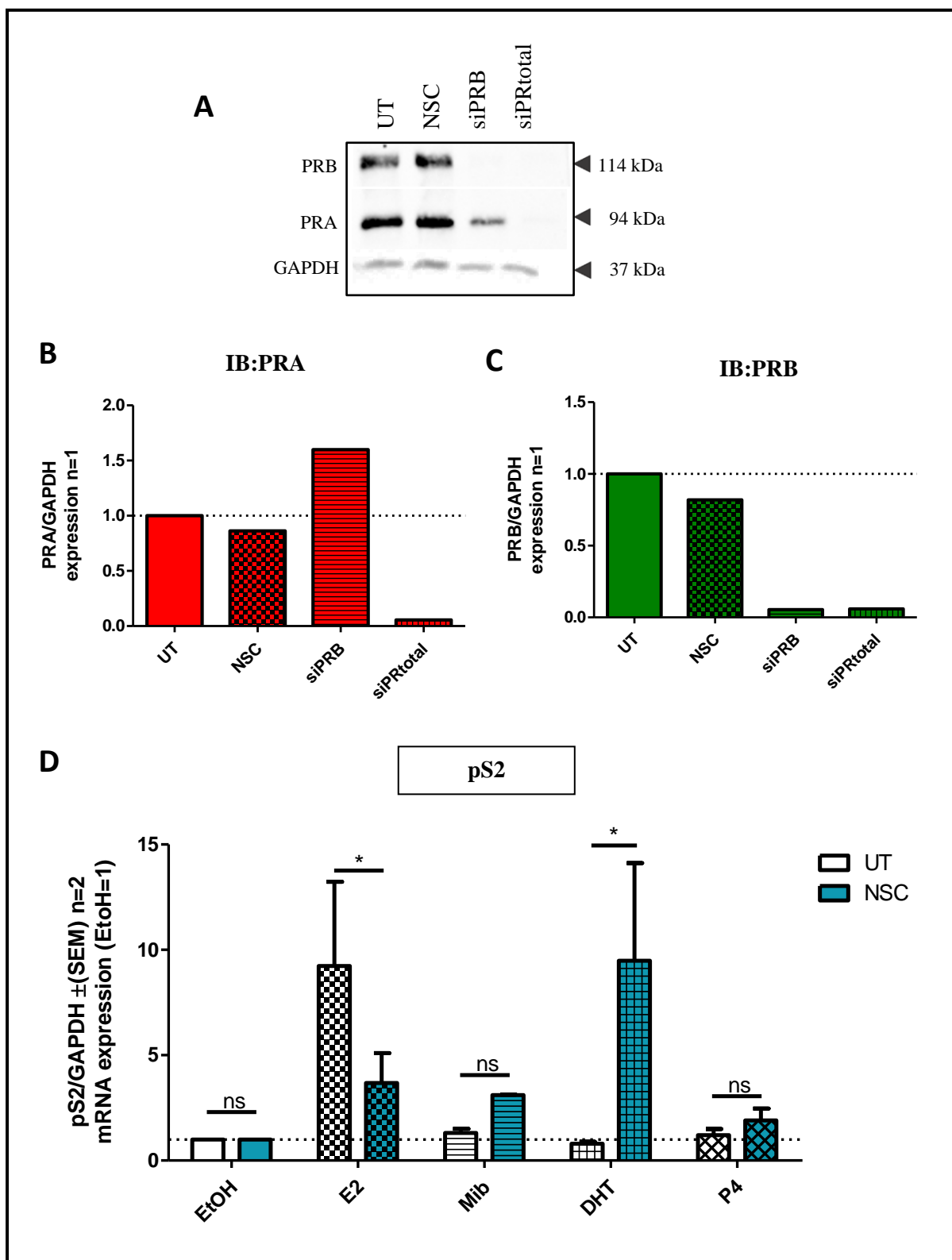


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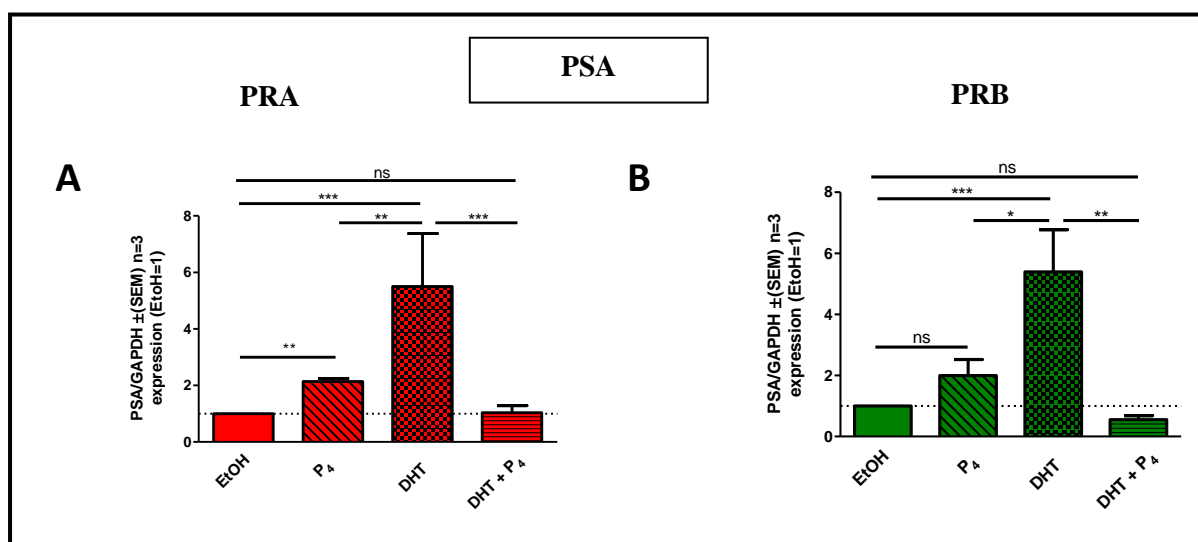
**Figure 3.11: NSC siRNA influenced responses on pS2 gene expression on T47D cells.** Untransfected (UT) T47D breast cancer cells or T47D cells transfected with 10 nM of either non-silencing scrambled sequence control (NSC) siRNA, siRNA directed against PRB only (siPRB) or siRNA directed against both the human PR isoforms (siPRtotal) were treated with 0.2% v/v EtOH or 1 nM ligand(s) for 24 hours. For verification of PR-A/B or knockdown western blotting was performed using antibodies specific for PR and GAPDH. (A) A representative blot is shown. Western blots were quantified using myImageAnalysis software. Protein expression of (B) PRA (red) or (C) PRB (green) in untransfected T47D cells was set as 1 and all siRNA transfected cells were set relative to this. (D) Total RNA was isolated, cDNA synthesised and realtime qPCR performed to determine the mRNA expression levels of the endogenous pS2 gene. GAPDH was used as the reference gene. The EtOH of each transfection condition was set as 1 and the relative mRNA expression in the treated samples set relative to this. Results shown are the averages of at least two independent experiments. Statistical analysis was performed using column statistics and a one-way ANOVA with a Bonferroni (comparing each column to all other columns) post-test. Statistically significant differences are shown by \*, \*\* or \*\*\*, to indicate  $p < 0.05$ ,  $p < 0.01$  or  $p < 0.001$ ; no statistical significance (ns) indicates  $p > 0.05$ .

### 3.6 The PR isoforms can regulate the expression of the ER- and AR-regulated endogenous CTSD and PSA genes, respectively.

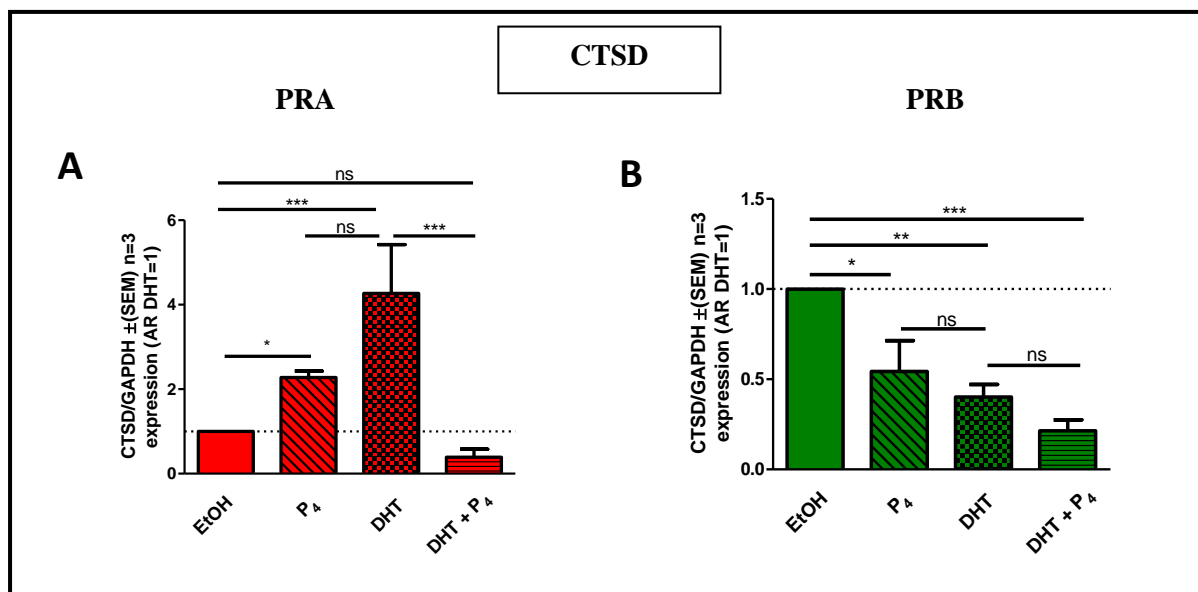
Having shown that the effects on ER- and AR-regulated genes can be modulated when the AR and PRA or PRB are co-expressed, we wanted to determine whether the PR isoforms can directly regulate the mRNA expression of these genes. As discussed previously the structure of the PR DBD is about 88% homologous to that of the AR (Beato, 1989; Gao *et al.*, 2005). Thus, it is expected that the PR isoforms would regulate PSA mRNA expression. Interestingly, results by Cabral using a synthetic minimal promoter, showed an upregulation of the classical ARE-containing promoter in cells expressing liganded PRA and PRB, this response was however non-significant (Cabral, MSc Thesis, 2018).

MDA-MB-231 cells were thus transiently transfected with a PRA expression vector (pSG5-hPR-A) or a PRB expression vector (pSG5-hPR-B) and treated for 24 hours with 1 nM P<sub>4</sub> or a combination of P<sub>4</sub> and DHT. Following this, qPCR analysis was performed for the PSA (Figure 3.12), and CTSD gene (Figure 3.13), to assess the relative mRNA expression. PRA, once activated by P<sub>4</sub>, caused an increase in PSA transactivation (Figure 3.12). Interestingly, treatment with DHT enhanced PSA mRNA expression in MDA-MB-231 cells transfected with either PRA or PRB, while combination treatment of DHT and P<sub>4</sub> did not affect PSA expression.

A significant increase in expression of CTSD is shown in MDA-MB-231 cells transfected with PRA, in response to both P<sub>4</sub> and DHT (Figure 3.13). This corresponds with the findings of our laboratory using a synthetic minimal promoter, which showed induction of the classical ERE-containing promoter in cells expressing liganded PRA, albeit that this induction was not statistically significant (Cabral, MSc Thesis, 2018). Interestingly, combination treatment with P<sub>4</sub> and DHT diminished the increase in CTSD expression seen in response to the individual treatment with the ligands. In contrast, CTSD mRNA expression was downregulated by both P<sub>4</sub> and DHT in MDA-MB-231 cells transfected with PRB. Although it appeared that expression was further downregulated in the presence of both P<sub>4</sub> and DHT, this response was not statistically significant. These results contradict those found using the synthetic minimal ERE containing promoter which showed ERE induction in cells expressing and overexpressing the PRB (Cabral, MSc Thesis, 2018).



**Figure 3.12: PR-A and PR-B both cause transactivation of the endogenous PSA gene in a ligand-specific manner.** Human MDA-MB-231 cells were transiently transfected with either 900 ng of (A) PRA (red) or (B) PRB (green) and treated for 24 hours with 0.2% v/v EtOH or 1 nM ligand(s). Total RNA was isolated, cDNA synthesised and realtime qPCR analysis was performed to quantify the mRNA expression levels of the endogenous PSA gene in the cells. GAPDH was used as the reference gene. The ratio of PSA mRNA expression/GAPDH mRNA of the treated samples were calculated relative to EtOH set as 1. The results indicate the average of three independent experiments ( $\pm$ SEM). Statistical analysis was performed using column statistics and a student's t-test and a one-way ANOVA with a Bonferroni (comparing each column to all other columns) post-test. Statistically significant differences are indicated by \*\* or \*\*\*, to indicate  $p < 0.01$  or  $p < 0.001$ ; no statistical significance (ns) to indicate  $p > 0.05$ .



**Figure 3.13: PR-A and PR-B regulate the expression of the endogenous CTSD gene, in a ligand-specific manner in the MDAMB- 231 cell line.** Human MDA-MB-231 cells were transiently transfected with either 900 ng of (A) PRA (red) or (B) PRB (green) and treated for 24 hours with 0.2% v/v EtOH or 1 nM ligand(s). Total RNA was isolated, cDNA synthesised and realtime qPCR analysis was performed to quantify the mRNA expression levels of the endogenous CTSD gene in the cells. GAPDH was used as the reference gene. The ratio of CTSD mRNA expression/GAPDH mRNA of the treated samples were calculated relative to EtOH set as 1. The results indicate the average of three independent experiments ( $\pm$ SEM). Statistical analysis was performed using column statistics and a one-way ANOVA with a Bonferroni (comparing each column to all other columns) post-test. Statistically significant differences are indicated by \*\* or \*\*\*, to indicate  $p < 0.01$  or  $p < 0.001$ ; no statistical significance (ns) to indicate  $p > 0.05$ .

### 3.7 Results of whether the PR isoforms and the AR occur in a molecular complex is inconclusive.

In order to gain a better understanding of the mechanism underlying the modulation of AR function by the PR isoforms, we performed a co-immunoprecipitation (Co-IP) assay to ascertain whether the AR and PR isoforms can occur in a molecular complex. MDA-MB-231 breast cancer cells were transiently transfected with an AR expression vector together with either a PRA or PRB expression vector, and treated with either 1nM DHT or P<sub>4</sub>, or a both. This was followed by immunoprecipitation (IP) using antibodies specific to the AR and both PR isoforms, and western blot analysis to probe for the presence of PR or the AR. However, the

blots repeatedly failed to show the presence either the primary, AR or PRA/B, or the secondary, PRA/B or AR, protein of interest in the IP and input samples, respectively. After numerous attempts, we thus utilized the COS-1 monkey kidney cell line employing the same experimental design and performing immunoprecipitation using antibodies specific to the AR (Figure 3.14) and both PR isoforms (Figure 3.15).

The results show that the AR was present in the input samples (Figure 3.14A and B), but that PRA (Figure 3.14A) or PRB (Figure 3.14B), could not be detected in the input samples. The subsequent absence of PRA and PRB in the AR IP samples is thus to be expected. Similarly, in the PR pulldown, we can see that the primary protein of interest, namely PRA (Figure 3.15A) or PRB (Figure 3.15B), was present in the input samples. The blots probing for the secondary protein of interest, namely the AR, show that the AR was absent from the input samples, and thus also absent from the immunoprecipitated samples.

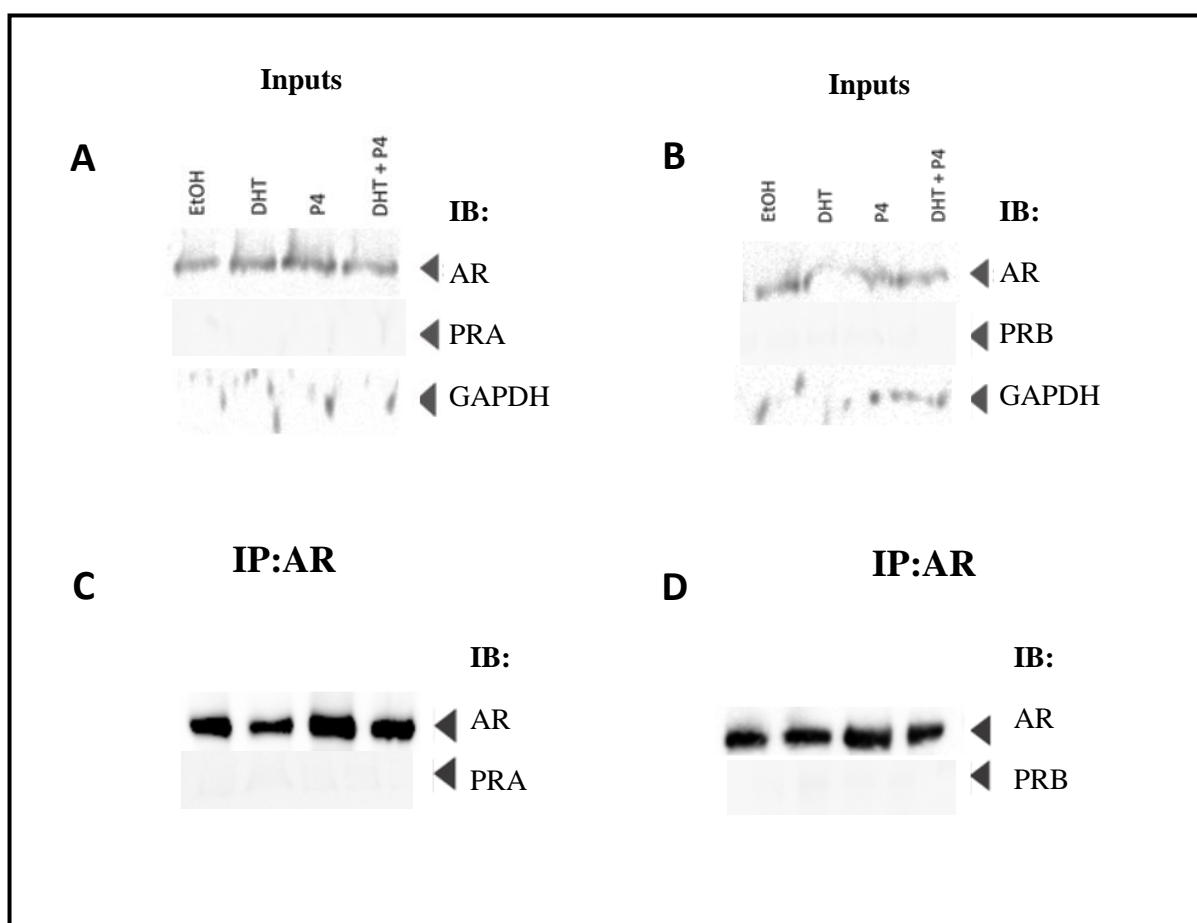
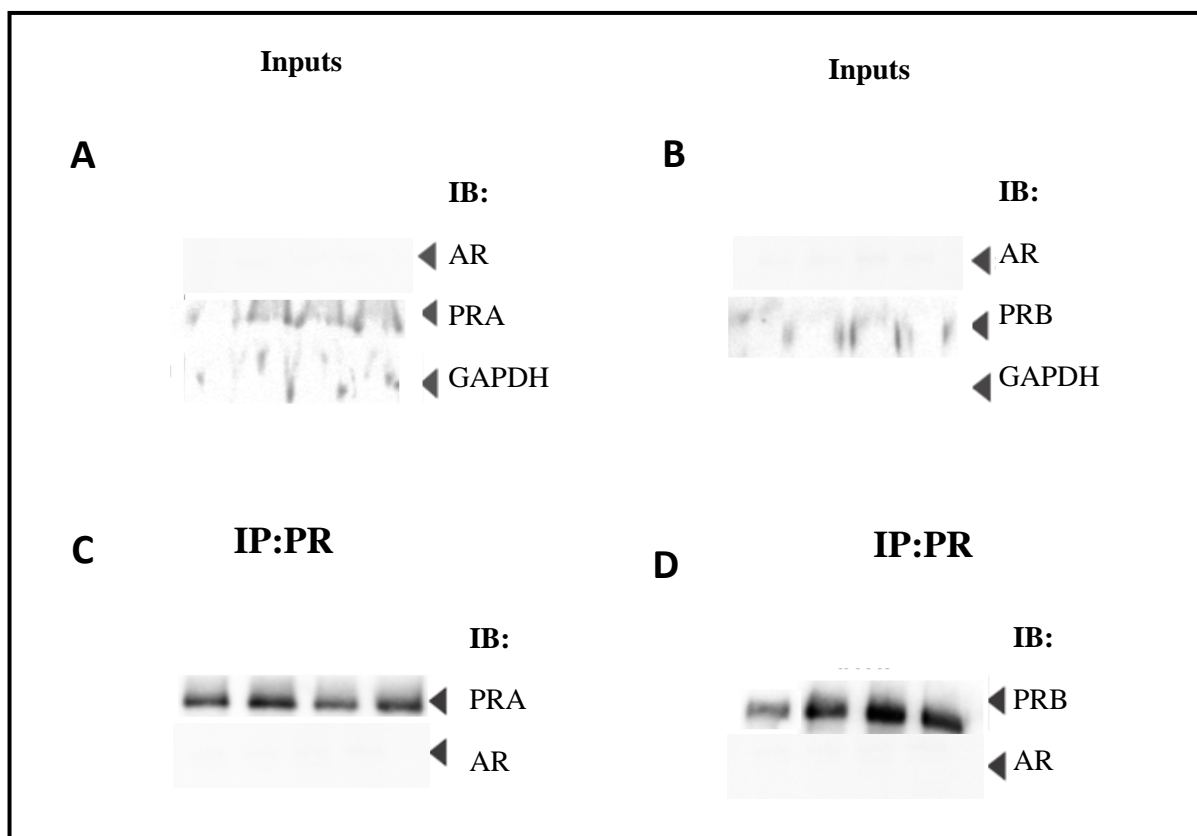


Figure 3.14 on next page.

**Figure 3.14: AR is present in the input samples, but PRA and PRB are not detected in the input samples in the COS-1 cell line transfected with AR and PRA/PRB.** The COS-1 monkey kidney cells were transiently transfected with 900 ng of (A-D) pSG5-hAR and 900 ng of either the (A and C) pSG5-hPR-A or (B and D) pSG5-hPR-B expression vectors and treated for 1 hour with 1 nM DHT or P<sub>4</sub> or both, and immunoprecipitated (IP) with primary antibodies specific to the AR. This was followed by probing for the presence of the AR and PR isoforms with immunoblotting (IB). Representative western blots are shown.



**Figure 3.15: PRA and PRB are present in the input samples, but AR is not detected in the input samples in the COS-1 cell line transfected with AR and PRA/PRB.** The COS-1 monkey kidney cells were transiently transfected with 900 ng of (A-D) pSG5-hAR and 900 ng of either the (A and C) pSG5-hPR-A or (B and D) pSG5-hPR-B expression vectors and treated for 1 hour with 1 nM DHT or P<sub>4</sub> or both, and immunoprecipitated (IP) with primary antibodies specific to the PR. This was followed by probing for the presence of the AR and PR isoforms with immunoblotting (IB). Representative western blots are shown.

## **Chapter 4 Discussion**



## 4.1 Introduction

Both breast and prostate cancer are hormone-dependent cancers, and numerous studies have investigated the role of steroid hormones acting via their cognate steroid receptors in these malignancies (reviewed in Castellon, 2017; Africander and Storbeck, 2018). It is well established that  $E_2$  and  $ER\alpha$  are the chief drivers of breast cancer development and progression (Beatson, 1896; Couse and Korach, 1999; Brisken and O'Malley, 2010), while prostate cancer is driven by androgens acting via the AR (Culig and Santer, 2014; Tan *et al.*, 2015). However, as the AR is also expressed in the majority of breast cancer tumours (Collins *et al.*, 2011), it is being actively investigated as a drug target in breast cancer. The action of the AR is dependent on the presence of  $ER\alpha$ , promoting tumorigenesis in  $ER\alpha$ -negative breast cancer (Agoff *et al.*, 2003; Doane *et al.*, 2006; Peters *et al.*, 2009; Rahim and O'Regan, 2017), while decreasing breast cancer cell proliferation in  $ER\alpha$ -positive breast cancer (Peters *et al.*, 2009; Rizza *et al.*, 2014). Although the PR is also expressed in breast and prostate cancer tumours (Grindstad *et al.*, 2015; Carroll *et al.*, 2017), its expression was previously used as an indication of ER functionality in breast cancer (Horwitz *et al.*, 1978; Dunbier *et al.*, 2010). However, the PR can also contribute to breast cancer (Diep *et al.*, 2015; Rojas *et al.*, 2017; McFall *et al.*, 2018), with its specific role in breast cancer dependent on the expression of the two functional PR isoforms, PRA and PRB. Although the PR is also expressed in prostate cancer tumours (Grindstad *et al.*, 2015; Chen *et al.*, 2017), its precise role in prostate cancer development and/or progression is not clear. However, at least one study has found that high levels of PRB expression is indicative of a poor prognosis (Grindstad *et al.*, 2018).

While a number of studies have shown crosstalk between specific steroid receptors in both breast and prostate cancer, studies specifically examining putative crosstalk between the PR and AR in both breast and prostate cancer are mostly lacking. To our knowledge, the only study providing evidence of a possible crosstalk between these two steroid receptors, in both breast and prostate cancer cell lines, is a recent study from our laboratory (Cabral, MSc Thesis, 2018). The aforementioned study examined whether AR transactivation function was modulated by the presence of PRB or PRA, at different densities, on synthetic minimal promoters. Given that results on minimal promoters are not always mimicked on endogenous genes (Ndlovu, 2015), we investigated whether similar effects would also occur on endogenous AR- and ER-regulated genes. In this study, we thus investigated whether AR function is modulated by the presence of unliganded and liganded PRA or PRB, on both AR- and ER-regulated genes in the MDA-

MB-231 cell line transiently transfected with the AR in the absence and presence of the respective PR isoform. These effects were also confirmed in the PC3 prostate cancer cell line. Lastly, we aimed to demonstrate that the AR can occur in a molecular complex with PRA or PRB.

#### **4.2 Validating results on the synthetic minimal promoter containing the classical ARE**

Before investigating effects on endogenously expressed genes, at least one of the previous experiments by Cabral was repeated in order to confirm the findings of a probable crosstalk between the AR and the PR (Cabral, MSc Thesis, 2018). A promoter reporter assay was thus performed in the MDA-MB-231 breast cancer cell line transiently transfected with the classical ARE and the AR in the absence and presence of PRB. The results in Figure 3.1B do not agree with the previous findings by Cabral that both unliganded and liganded PRB upregulate the transactivation activity of the AR on the classical ARE (Cabral, MSc Thesis, 2018). Instead, results in this study shows a downregulation of AR transactivation function. Considering that the same cell line and transfection conditions were used by Cabral and the current study, the contrasting results were unexpected. One possible explanation could be the use of different passages of the MDA-MB-231 cell line. Although both used cells within 35 passages from thawing for experiments, the exact passage numbers are not known. It is known that cell lines lose their ability to effectively differentiate after a certain number of passages, to the point that cell cultures no longer maintain key gene functions and consistent morphology (Langelier *et al.*, 1993; Briske-Anderson, Finley and Newman, 1997; Chang-Liu and Woloschak, 1997; Esquenet *et al.*, 1997; Yu, Cook and Sinko, 1997; Sambuy *et al.*, 2005). This could lead to results that may not be accurate or reproducible (Wenger *et al.*, 2004). For example, it has been shown that the expression of a green fluorescent protein (GFP) reporter gene varies when transfected into the MCF7 breast cancer cell line at different passage numbers (Hughes *et al.*, 2007). Similarly, the LNCaP prostate cancer cell line displays differing proliferative responses in response to the synthetic androgen R1881, as well as different secretion rates of PSA, at different passage numbers (Esquenet *et al.*, 1997). Even though the exact response shown by Cabral was not confirmed, the results in Figure 3.1 still allude to crosstalk between the AR and PR. A more likely explanation for the contrasting results, however, is that there may have been differences in the transfection efficiencies between the experiments performed in this study, and those performed by Cabral (Cabral, MSc Thesis, 2018). It is known that the transfection efficiency can be influenced by a variety of factors, including the confluency of the cell line at

the time of transfection, the ratio of transfection reagent to DNA, as well as the absence or presence of serum (Rahimi *et al.*, 2018).

#### **4.3 PR isoforms, at equivalent and excess concentrations relative to that of the AR, influence AR transactivation on the endogenous PSA gene**

To answer the question of whether the PR isoforms also modulate AR function on endogenous genes, the MDA-MB-231 triple negative human breast adenocarcinoma cancer cell line was again utilised as the model cell line as it lacks expression of the ER and PR, as well as the AR (Ferguson, Lapidus and Davidson, 1998; Liu *et al.*, 2017). However, the GR is expressed in these cells, and as previous observations have indicated that the GR, PR, MR, and AR may all recognize the same DNA elements (Claessens *et al.*, 1996a), the GR could also induce PSA expression. To exclude this possibility, our experimental design compared responses in MDA-MB-231 cells transfected with the respective steroid receptors to responses in cells transfected with the psg5 empty vector. The results from Figure 3.3A indicate that in cells that have not been transfected with the AR or PR isoforms, there is no effect on PSA mRNA expression levels upon treatment with DHT.

We firstly confirmed the expression of the AR and PR isoforms in the MDA-MB-231 cell line following transfection (Figure 3.2). The results show that transfecting in 5x more DNA of the pSG5hPR-A and pSG5hPR-B expression vectors resulted in a greater than 5x expression of PRA, as well as PRB. This effect is however unlikely due to the vector utilized, as the cDNA for the AR and PR isoforms were all cloned into the pSG5 expression vector. Notably, this is the result of a single experiment, and would need to be repeated in order to confirm the expression levels of the steroid receptors. PSA, containing a PRE/classical ARE sequence within its promoter, is expressed in the MDA-MB-231 cell line and is known to be upregulated in breast, as well as prostate cancer (Catalona *et al.*, 1994; Ferguson *et al.*, 1996; Cuzick *et al.*, 2011). In fact, multiple clinical studies have shown that PSA levels are significantly higher in women with breast cancer than in healthy women (Margot H. Black *et al.*, 2000; Mashkoor, Al-Asadi and Al-Naama, 2013). Results from qPCR analysis indicate PSA gene expression is increased in the presence of the AR and DHT (Figure 3.3B), while unliganded PRA or PRB, caused a significant increase in the transactivation activity of the AR (Figure 3.3C and D). These results are in contrast to the decrease in AR only activity shown on the synthetic minimal ARE-containing promoter (Figure 3.1), but in agreement with the upregulation shown by

Cabral. A similar upregulation of AR function was not observed when the PR isoforms were overexpressed relative to the AR in the MDA-MB-231 cells.

#### **4.4 Preliminary results show that the AR-regulated TMPRSS2 gene is upregulated in the PC3 prostate cancer cells co-expressing the AR and excess PRA or PRB**

To answer the question of whether the PR isoforms also modulate AR function within prostate cancer, we assessed the mRNA expression of an endogenous AR-regulated gene within the PC3 prostate cancer cell line. This cell line was utilised as it lacks endogenous expression of the AR and the PR isoforms (Nowakowska *et al.*, 2016). Although there are reports that the PC3 cells express ER $\beta$  (Kim *et al.*, 2002), our laboratory did not detect ER $\beta$  expression in these cells (Cabral, MSc, Thesis, 2018). Nonetheless, we compared responses between PC3 cells transfected with the AR and PRA or PRB to responses in cells transfected with the pSG5 empty vector. The results from Figure 3.5A indicate that in cells that have not been transfected with the AR or PR isoforms, there was no effect on TMPRSS2 mRNA expression levels upon treatment with DHT, suggesting that responses observed in cells transfected with the AR in the absence and presence of the PR isoforms, was indeed mediated by the respective steroid receptors.

Results from qPCR indicating that the expression of the TMPRSS2 gene was decreased in the presence of the AR and DHT (Figure 3.5B), are not consistent with previous reports indicating an increase in expression (Wang *et al.*, 2007). Nonetheless, we continued the investigation on whether the response would be modulated in the presence of the PR isoforms. The results showed that adding unliganded PRA, expressed at equivalent levels to AR, lifted the AR-mediated decrease in TMRPSS2 mRNA expression. Unliganded and liganded PRA and PRB, only when present at an excess (5x) concentration relative to the AR, appeared to increase the expression of TMPRSS2 gene (Figure 3.5C, D and 3.6). In the presence of both DHT and P<sub>4</sub>, activated PRA and PRB, in an excess concentration relative to the AR, upregulated TMRPSS2 mRNA expression (Figure 3.6). This result is in agreement with the reporter assay results from our group showing increased transactivation on the classical ARE containing promoter at excess (5x) PRB expression relative to the AR in PC3 cells (Cabral, MSc Thesis, 2018). In contrast to our results showing a similar response on the TMPRSS2 gene for excess PRA and PRB, Cabral did not show increased transactivation in the presence of excess PRA. However, no definitive conclusions can be drawn from our findings on the TMPRSS2 gene in the PC3

cells, as it is the result of a single experiment. This experiment would thus need to be repeated, and future work should also include investigating other AR-regulated genes, such as FKPB5, which is known to be overexpressed in prostate cancer (Ni *et al.*, 2010), and is also expressed in the PC3 prostate cancer cell line (Jividen *et al.*, 2018).

#### **4.5. Neither of the two PR isoforms significantly modulate AR-mediated downregulation of the CTSD gene by DHT, while the presence of both the AR and PR isoforms results in increased PR gene expression**

Expression of the ER-regulated CTSD and PR genes was also investigated with the use of qPCR in the MDA-MB-231 cell line transfected with the AR, in the absence and presence of either PRA or PRB. Results indicate that DHT-activated AR downregulated the mRNA expression of both the CTSD and PR genes (Figure 3.7B and 3.8B). Furthermore, the suppressive effect on CTSD was enhanced in the presence of P<sub>4</sub>. Further research is needed to understand the mechanism underlying these effects. There was no statistically significant modulation of CTSD expression observed when either of the unliganded PR isoforms, or P<sub>4</sub>-activated PRB (Figure 3.9B), are co-expressed with the AR (Figure 3.7C and D). However, in the presence of P<sub>4</sub> and a 5x excess concentration of PRA relative to the AR, a significant increase in CTSD expression was seen (Figure 3.9A). This effect was however not sufficient to lift the AR-mediated downregulation of CTSD mRNA expression. For the PR gene, however, the addition of either PR isoforms in the presence of the AR caused an upregulation of PR gene expression, thereby lifting the AR-mediated downregulation of the PR gene expression (Figure 3.8C and D). Gene specific effects have previously been observed for the expression of the CTSD and PR genes, as it was shown that the AR can be recruited to specific EREs with the promoter region of these two genes (Peters *et al.*, 2009).

The T47D human breast adenocarcinoma cancer cell line was also utilised as an additional breast cancer model cell line as it endogenously expresses the AR and high levels of the PR isoforms, in addition to the ER subtypes and the GR (Nardulli and Katzenellenbogen, 1988; Satorius *et al.*, 1994; Jacobsen and Horwitz, 2012). Here our strategy was to silence the PR, by using siRNA designed to expression of either PRB, or both PRA and PRB. Cells were also transfected with non-silencing scrambled sequence control (NSC) siRNA, which contains a non-targeting siRNA sequence designed as a control and should theoretically not target any steroid receptors, and response between the NSC cells and untransfected cells should be

similar. Results indicate that T47D cells displayed different effects on pS2 gene expression when transfected with the NSC, as compared to untransfected cells (Figure 3.11). Future studies should consider the use of more than one NSC.

Taken together, these results show that the PR isoforms display ligand dependent actions on modulating AR activity on AR- and ER-regulated genes. This is supported by evidence from the literature indicating that the role of the PR in breast cancer is dependent upon the absence or presence of a ligand, as well as which specific ligand the PR is bound to (Mote *et al.*, 2002; Hopp *et al.*, 2004). Liganded PRA and PRB may decrease tumorigenesis in breast cancer by decreasing AR-mediated transactivation of target genes containing the ARE. On the other hand, the effects of PRA and PRB on ER-regulated genes was shown to be gene-specific. Future studies should include assessment of the role of different excess concentrations of the PR isoforms, and not only the 5x excess concentration used by Cabral (Cabral, MSc thesis, 2018), while further studies are also needed to investigate the physiological implications of these results.

#### **4.6. PRA can transactivate endogenous AR- and ER-regulated genes.**

It is known that PRA and PRB can activate similar, and also distinct, gene sets (Graham and Clarke, 2002; Kariagina *et al.*, 2008). When observing the transactivation of the classical ARE-containing PSA gene in the MDA-MB-231 cell line, both activated PRA and PRB (Figure 3.12) increased the expression of the PSA gene. This is not that surprising as the AR and PR are known to bind to similar steroid response elements (Beato, 1989; Schauwaers *et al.*, 2007; Africander, Storbeck and Hapgood, 2014). It is however surprising that there was a greater upregulation of PSA expression in the presence of DHT, than the cognate ligand of the PR. This is in contrast to the findings of the promoter reporter assays on a classical ARE in MDA-MB-231 cells (Cabral MSc, Thesis, 2018). Given that the regulation of an endogenous gene is more complex than regulation of a synthetic promoter (Choi, 2007), it is not completely surprising that effects observed on a synthetic promoter and that on an endogenous gene, are not always similar. Indeed, the promoter of the endogenous PSA gene is known to contain a number of cis-elements, other than the classical ARE, to which the PR isoforms could possibly bind. For example, it is known that the PSA gene contains binding sites for the specificity protein 1 (Sp1) (Sadar *et al.* 1999, Kim and Coetzee 2004), and that both PRA and PRB can interact with Sp1 (You, 2020).

A previous study performed by Peters and co-workers, showed that the AR can modulate the activity of ER $\alpha$  by competing with ER $\alpha$  for binding to ER-binding sites or DNA in close proximity to ER-binding sites in ER target genes (Peters et al., 2009; Need et al., 2012). Peters and co-workers found that the presence of both the AR ligand (DHT) and the PR ligand (P<sub>4</sub>) were necessary for this modulation to occur. Our results show that PRA and PRB differentially regulate the ER-regulated CTSD gene (Figure 3.13). PRA, activated with P<sub>4</sub>, caused a significant increase in the mRNA expression of the CTSD gene. While this also appears to occur on the PR gene, the response was not significant. PRB, in the presence of either DHT or P<sub>4</sub> caused a downregulation of CTSD expression, with this effect being more pronounced in the combination treatment. These results are in contrast to the results by Cabral, which showed that both PRB and PRA induced transactivation on a synthetic minimal promoter containing an the ERE, within the MDA-MB-231 breast cancer cell line. These gene specific effects could possibly be explained by PR interaction with other cis-elements present within the promoter region of an ER-regulated gene, such Sp1 sites within the CTSD gene, which have been identified as targets for the PR to can interact with to influence transcription (Diep *et al.*, 2015).

#### **4.7. Results on whether the AR occurs in a molecular complex with either PRA or PRB are inconclusive.**

Steroid receptors have previously been shown to form multi-protein molecular complexes as a mechanism by which to elicit their effects on gene regulation. For example, the PR has been found in a complex with ER $\alpha$ , which in turn modulates the transcriptional activity of ER $\alpha$  by reprogramming ER $\alpha$  chromatin binding (Mohammed et al., 2015; Singhal et al., 2016). We wanted to establish whether PRA or PRB could form a complex with the AR in an attempt to understand modulation of AR function by the PR isoforms. A continuous problem encountered while performing these assays was very faint luminescent signals on blots after probing for proteins in the input samples. This could be due to the lysate being very dilute, thus resulting in a low concentration of the steroid receptors of interest. We aimed to correct this by decreasing the amount of lysis buffer used to dissolve the cell lysate, as well as using a vacuum concentrator, to increase the concentration of protein in the samples. None of these optimisations were effective. This problem hindered the detection of steroid receptors, as well as the normalisation and quantification of the western blots. Interestingly, the AR and PR have been shown in a molecular complex once before using the same experimental design in the

MDA-MB-231 breast cancer cell line (Addendum; Figure 4 and 5). We could not however reproduce the results that suggested that the AR and PR isoform, whether unliganded or liganded, can indeed occur in a molecular complex.

The same experimental design was utilized in the COS-1 monkey kidney cell line. However, results from the Co-IP assays conducted in this cell line, using an antibody specific for either AR (Figure 3.14A and B) or the PR (Figure 3.15A and B), showed that the primary protein of interest, but not the secondary protein of interest, was successfully pulled down. These results of the experiments are thus inconclusive.

#### **4.8 Conclusions and future work**

To gain further insight into the mechanisms behind gene regulation by AR and PR, a Glutathione S-transferase (GST) fusion protein assay could also be used to probe for an AR-PR interaction and complex formation. In addition, a re-chromatin immunoprecipitation (re-ChIP) assay could be performed to determine whether the AR and PRA or PRB are co-recruited to the promoters of the PSA, TMPRSS2, CTSD, and pS2 genes. When considering the physiological nature of AR and PR crosstalk, in terms of breast and prostate cancer, a proliferation or apoptosis assay could be performed to investigate the effects of the PR isoforms on AR mediated cell survival. In addition, the effects of the AR and PR on metastases can be investigated with the use of migration and invasion assays. All of the aforementioned strategies will better our understanding of the mechanisms underlying AR and PR crosstalk in breast and prostate cancer development and progression. Understanding how these steroid receptors interact in breast and prostate cancer will enable the identification of new and appropriate drug targets for the treatment of these cancers, and the subsequent development of more effective drugs to target cancer growth and spread.



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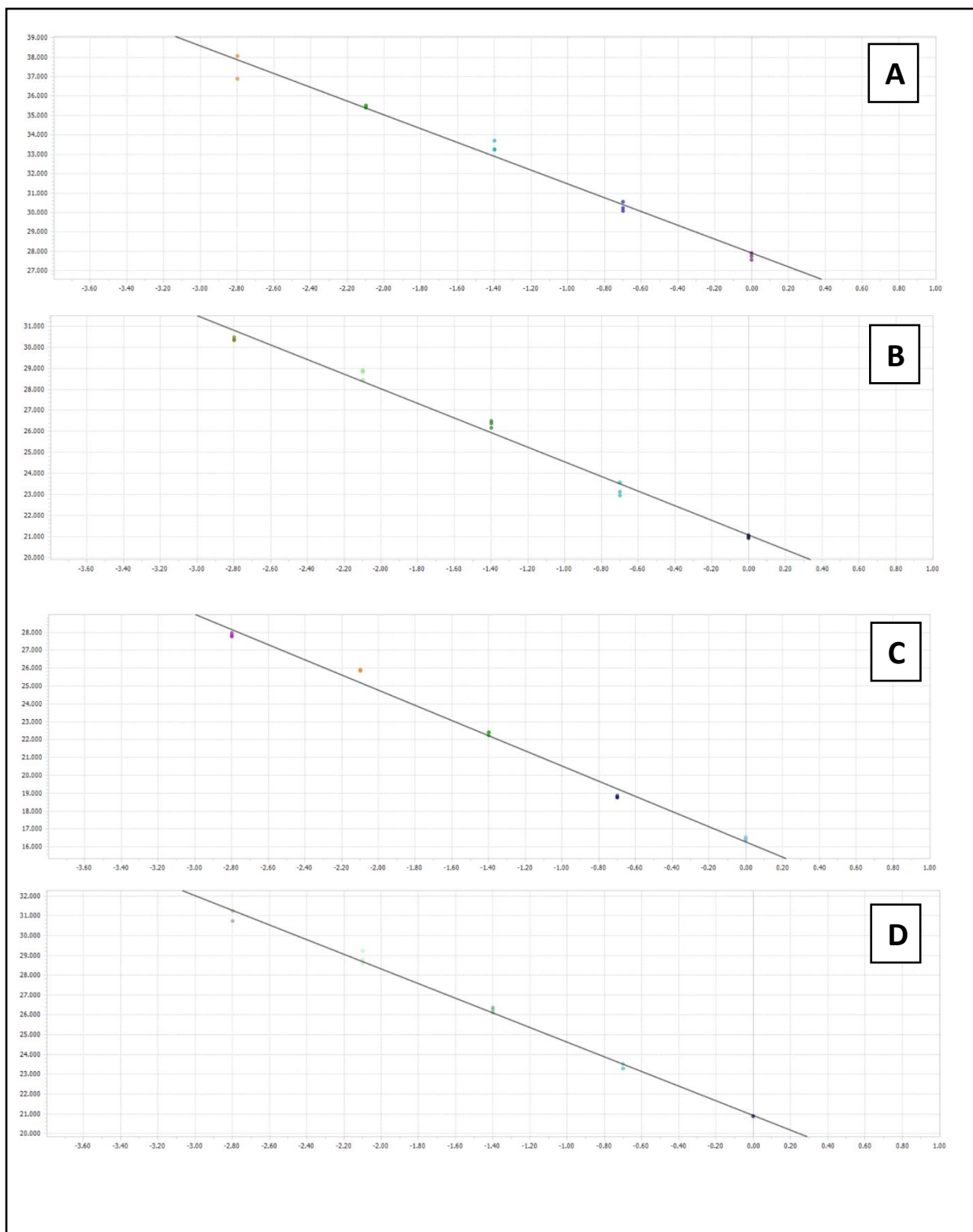
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# **Addendum: Supplementary tables and figures**

**Table 1. Efficiencies for primer pairs used in this study.**

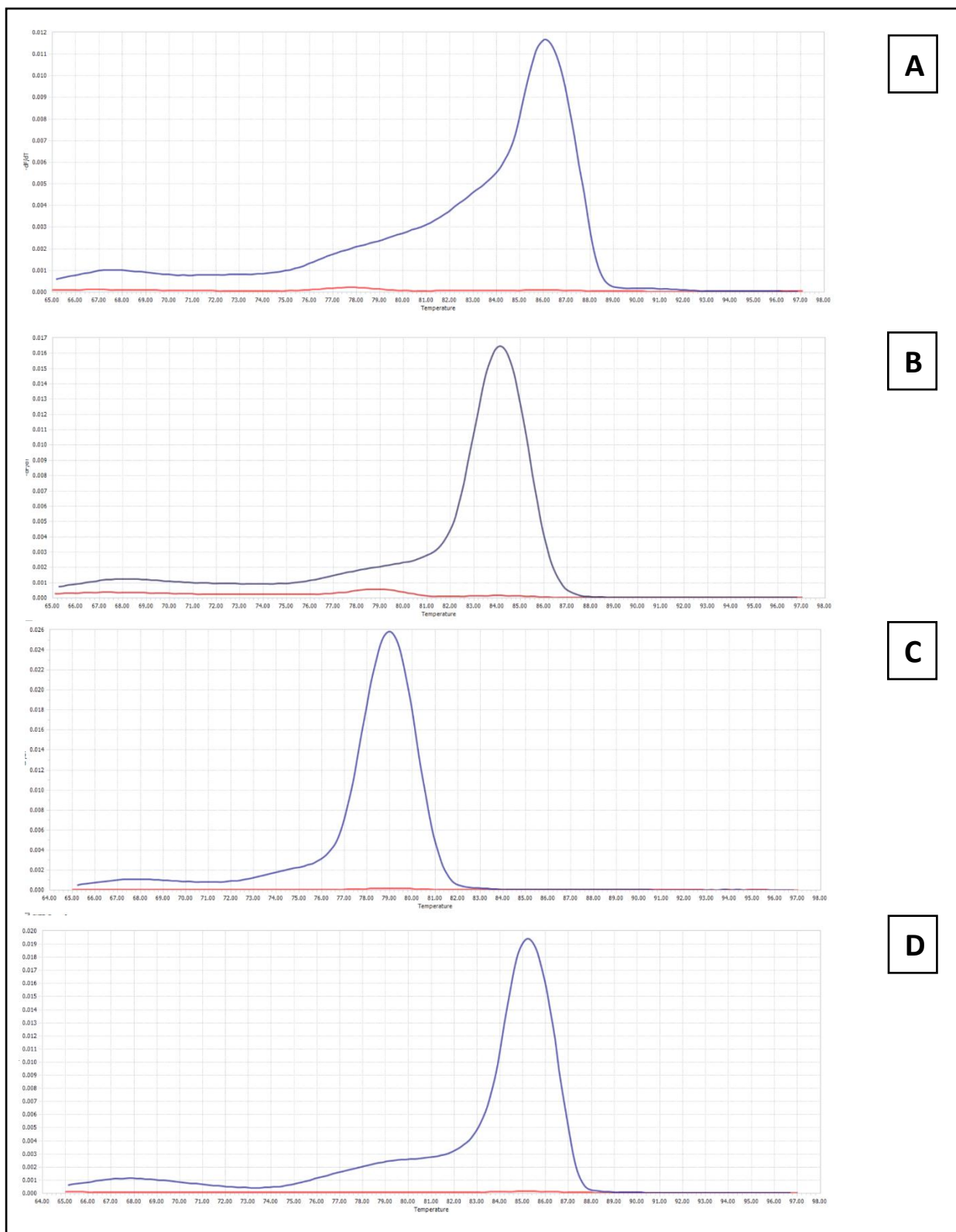
Primer pair	Cell line	Primer efficiency
PSA	MDA-MB-231	1.94*
pS2	T47D	1.91
PR	MDA-MB-231	1.96
CTSD	MDA-MB-231	1.94
TMPRSS2	PC3	1.87
GAPDH	T47D	1.89**
	MDA-MB-231	2.12*

\*(Ndlovu, 2015) \*\*(Brink, 2019)

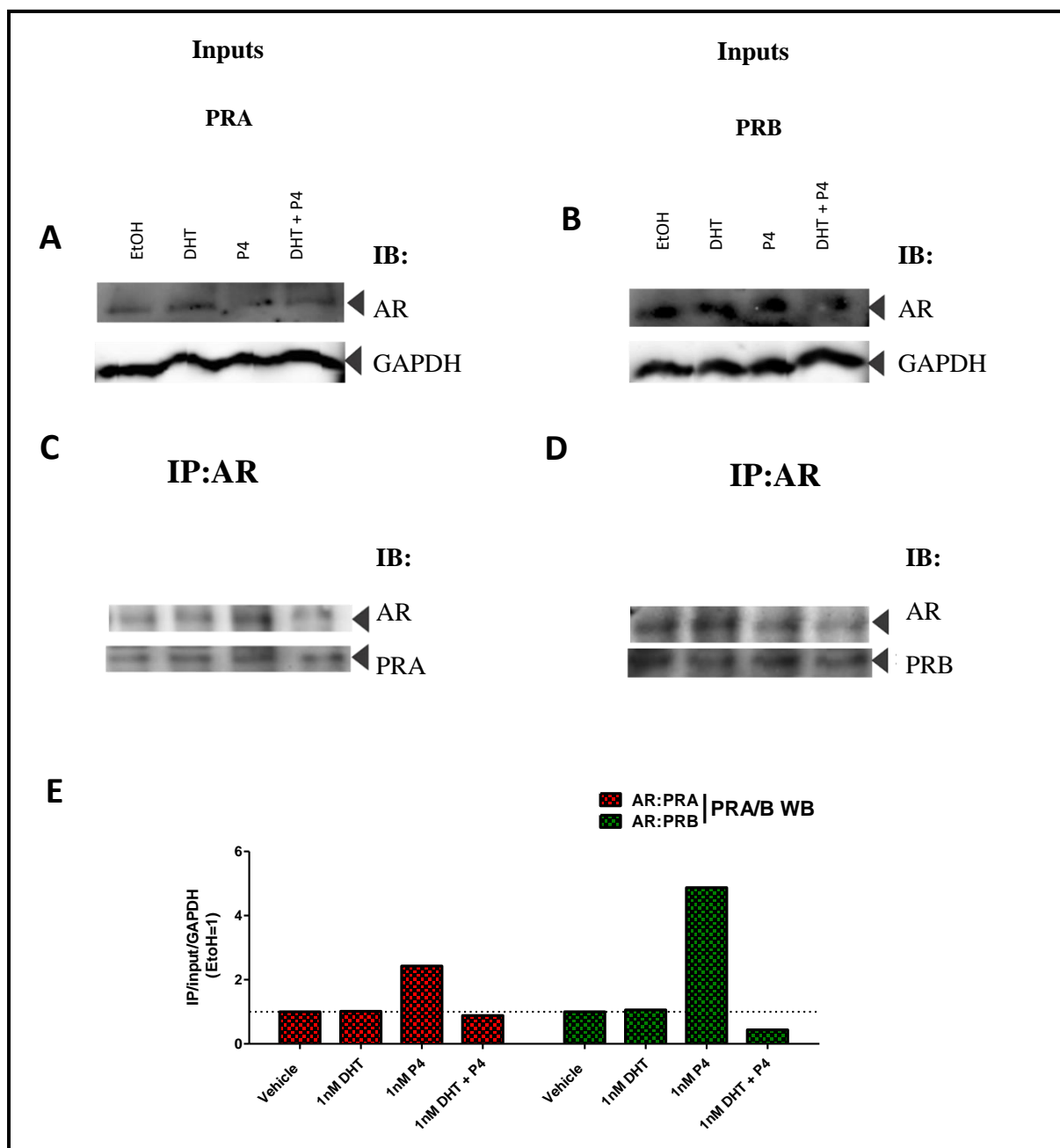


**Figure 2. Representative standard curves for the (A) pS2 primer pair in the T47D cell line, (B) CTSD and (C) PR primer pairs in the MDA-MB-231 cell line and the (D) Tmprss2 primer pair in the PC3 cell line. Standard curves were generated by plotting the Cq value against the log concentration of cDNA. The slope determined from these standard curves was used to calculate the efficiency of the primer pairs used in the respective cell lines.**

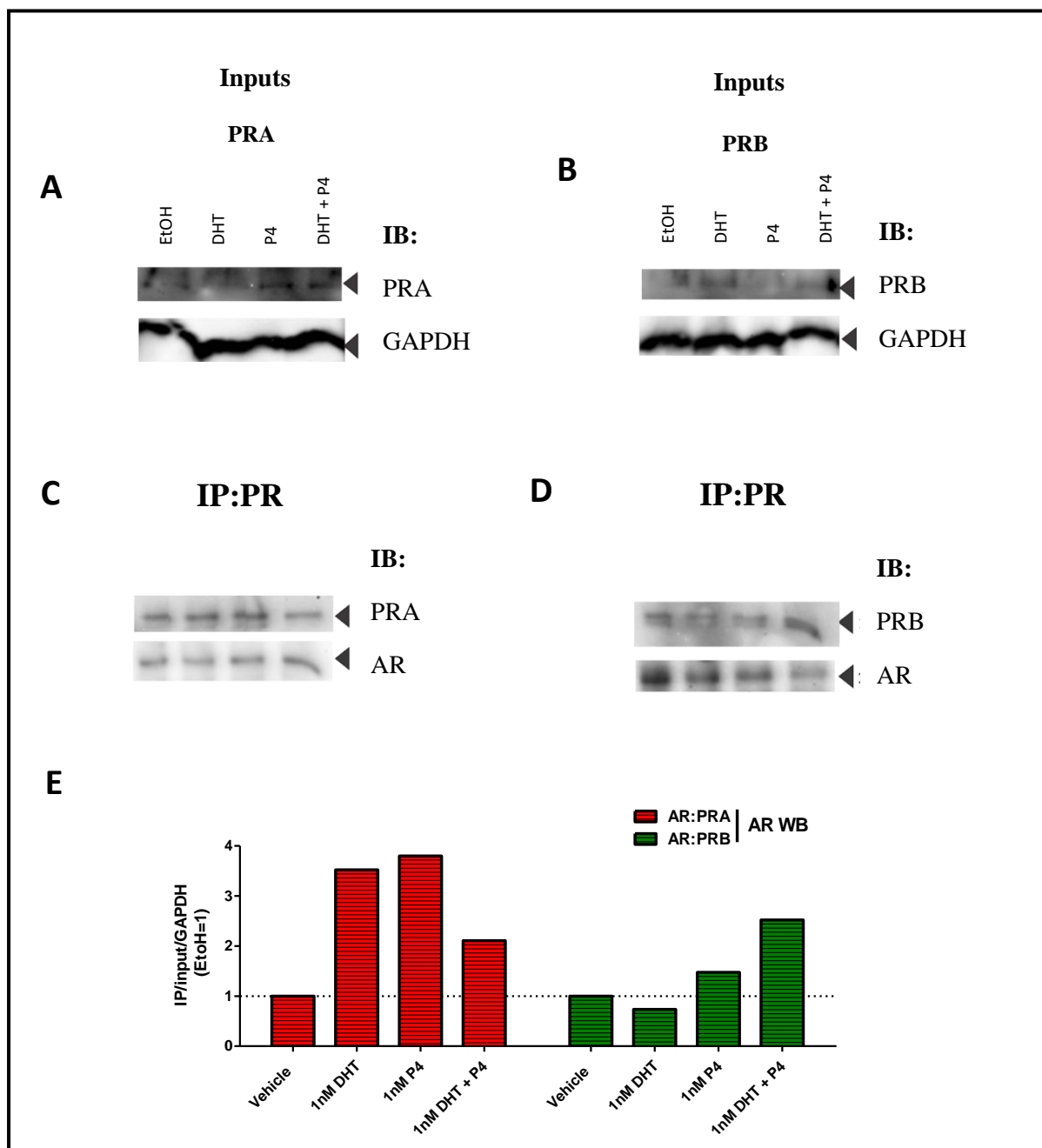




**Figure 3. Representative melting curves for the (A) pS2 primer pair in the T47D cell line, (B) CTSD and (C) PR primer pair in the MDA-MB-231 cell line and the (D) Tmprss2 primer pair in the PC3 cell line. Melting curves were generated using the LightCycler® 96 software. Samples treated with EtOH as the vehicle control (blue) while the negative controls (red) contained no template.**



**Figure 4: Both PR isoforms are found in a molecular complex with the AR.** The human MDA-MB-231 breast cancer cell line was transiently transfected with 900 ng of pSG5-hAR and 900 ng of either the pSG5-hPR-A (red bars) or pSG5-hPR-B (green bars) expression vectors and treated for 1 hour with 1 nM DHT or P<sub>4</sub> or a combination thereof, and immuno-precipitated with primary antibodies specific to the AR. This was followed by probing for the presence of the PR isoforms with immunoblotting. Representative western blots are shown. Quantification of the western blots was performed using ImageJ software. Data shown are normalized to the respective input controls and represented as a fold response relative to the vehicle control which was set as 1.



**Figure 5: Both PR isoforms are found in a molecular complex with the AR.** The human MDA-MB-231 breast cancer cell line was transiently transfected with 900 ng of pSG5-hAR and 900 ng of either the pSG5-hPR-A (red bars) or pSG5-hPR-B (green bars) expression vectors and treated for 1 hour with 1 nM DHT or P<sub>4</sub> or a combination thereof, and immuno-precipitated with primary antibodies specific to the PR. This was followed by probing for the presence of the AR with immunoblotting. Representative western blots are shown. Quantification of the western blots was performed using ImageJ software. Data shown are normalized to the respective input controls and represented as a fold response relative to the vehicle control which was set as 1.