Targeting the androgen receptor in breast cancer

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Targeting the Androgen Receptor in Breast Cancer

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Kee Ming Chia

A thesis in fulfilment of the requirements for the degree of

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Abstract

Estrogen receptor positive (ER+) breast cancer constitutes 70% of all breast cancers and anti-ER therapies such as aromatase inhibitors and tamoxifen represent the main therapeutic strategies in the treatment of this disease. Unfortunately, up to 30% of all primary ER+ tumours will ultimately develop endocrine-resistance and progress on ER-targeted therapies resulting in disease-related morbidity. As a result, there is an urgent medical need for novel therapeutic strategies capable of managing endocrine-resistant breast cancer.

Androgen receptor (AR) is expressed in up to 90% of ER+ breast cancers. AR functions as a tumour suppressor in primary ER+ breast cancer and high AR positivity is strongly associated with a favourable patient outcome in the ER+ setting. However, the role of AR in endocrine-resistant breast tumours is highly controversial with data supporting both oncogenic and tumour suppressive functions reported in the literature.

Here I have used different modulatory approaches on *in vitro* and *in vivo* preclinical models to dissect the functions of AR and determine the best approach to target AR in endocrine-resistant breast cancer. I use an siRNA-mediated approach to knock down AR in cell line models and discover that the basal expression of AR contributes to endocrine-resistance and that loss of AR restores classical ER signalling and reverses endocrine-resistance. However, inhibiting the transcriptional activity of AR with enzalutamide does not recapitulate this effect, suggesting that it is the non-canonical activity of AR which contributes to endocrine-resistance. In contrast, I show that activation of AR by either 5-α dihydrotestosterone (DHT) or selective AR modulator enobosarm *in vitro* and in patient derived (PDX) models of endocrine-resistance results in significant growth suppression. Mechanistically, this growth-inhibitory effect of AR activation is associated with downregulation of ER signalling. Moreover, I identify AR-regulated genes from the global gene expression of an ER+AR+ endocrine-resistant PDX model treated with DHT and establish a highly prognostic AR gene signature based on primary ER+ patients in the METABRIC dataset. This suggests that activity of AR is tumour-suppressive independent of endocrine-sensitivity. In summary, I demonstrate that activation, not antagonism, is the optimal AR-targeted therapeutic strategy in the management of endocrine-resistant breast cancer.

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Presentations

Poster

- International PacRim Meeting, Adelaide Australia 2019
  (Best Poster in the Junior Researcher category)
- San Antonio Breast Cancer Symposium, Texas USA 2018
- Gordon Research Conference (Hormone-Dependent Cancers), Maine USA 2017
  (Poster Prize)
- Lorne Cancer Conference in Victoria, Australia 2016
- NSW Translational Breast Cancer Research Symposium, NSW Australia 2016
  (Poster Prize)

Oral

- Cancer Research UK (CRUK), University of Cambridge 2018
- Faculty meeting at Dame Roma Mitchell Cancer Research Laboratories (University of Adelaide), South Australia 2018
- Dana-Farber Cancer Research Institute, Boston USA 2017
- Memorial-Sloan Kettering Cancer Centre, New York USA 2017

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Frequently used abbreviations

Aromatase inhibitor	AI
5α-dihydrotestosterone	DHT
Abiraterone acetate	Abi
Androgen receptor	AR
B-cell lymphoma 2	BCL2
Bicalutamide	Bic
Chemokine C-X-C Motif Ligand 8	CXCL8
Enobosarm	Eno
Enzalutamide	Enz
Epidermal growth factor receptor	EGFR
Estrogen receptor	ER
Fulvestrant	Ful
Human epidermal growth factor receptor	HER2
Immunohistochemistry	IHC
Long-term estrogen deprived	LTED
Mammary intraductal	MIND
Patient-derived xenograft	PDX
Phosphoinositide 3-kinase	PI3K
Progesterone receptor	PR
Rapid immunoprecipitation mass spectrometry of endogenous protein	RIME
SEC14 Like Lipid Binding 2	SEC14L2
Selective AR degrader	SARD
Selective AR modulator	SARM
Selective ER degrader	SERD
siRNA	si
Tamoxifen	Tam
Tamoxifen resistant	TamR
Triple negative breast cancer	TNBC

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CHAPTER 1

1. Literature review

1.1. Heterogeneity of breast cancer

Breast cancer is the most common cancer in females, affecting approximately one in eight women. It is a highly heterogeneous disease comprising of several molecular subtypes as defined by their global gene expression profiles. These subtypes include the luminal A, luminal B, epidermal growth factor receptor-2 (HER2) and basal subtypes (Perou et al., 2000; Sorlie et al., 2003). The luminal A and B subtypes are estrogen receptor-α (ER) positive tumours and are characterized by expression of genes involved in steroid receptor signalling such as *progesterone receptor* (*PGR*), *GATA-3* and *X-box binding protein 1* (*XBP-1*). Luminal B tumours have relatively higher levels of proliferation-related genes, such as *aurora kinase B* (*AURKB*) and *MKI67*, and lower levels of hormone-signalling related genes, such as *PGR*, relative to luminal A tumours (Prat et al., 2013; Sorlie et al., 2001). HER2 subtype tumours are characterized by high expression of genes in the *ERBB2* amplicon at 17q22.24 which includes *ERBB2* and *growth factor receptor-bound protein 7* (*GRB7*). These tumours also express luminal genes such as *ESR1* and *PGR* at a low to intermediate level. Basal subtype tumours, which do not express ER, PR and HER2, have high expression levels of proliferation genes such as *MKI-67* and basal-type *keratin-5* and *-14*. Importantly, these subtypes are associated with different prognosis with the luminal A subtype having the best prognosis compared to the other subtypes (Sorlie et al., 2001; Sorlie et al., 2003).

Clinically, the classification of breast cancers is based on the expression of the hormone receptors estrogen receptor (ER) and progesterone receptor (PR) by immunohistochemistry and epidermal growth factor receptor-2 (HER2) gene amplification by in situ hybridisation. The expression of these three markers divides breast cancers into several broad clinical subtypes and serves as a proxy for the various molecular subtypes. The clinical subtypes of breast cancer include a) ER+PR+ which can be either HER2+ or HER2-, b) ER-PR-HER2+ and c) ER-PR-HER2-, also known as triple negative breast cancer (TNBC) and which overlap with the basal subtype

tumours. The expression of ER and HER2, in particular, serves as both prognostic and predictive markers.

Androgen receptor (AR) is a ligand-dependent transcription factor which has been implicated in various malignancies such as prostate cancer, ovarian teratocarcinoma and breast cancer (Chia et al., 2015; Chung et al., 2014) . It is expressed across all subtypes of breast cancer, including 90% of ER-positive breast cancers. While clinical trials with AR directed therapies are currently ongoing, AR is not currently routinely used clinically as a prognostic or predictive biomarker.

1.2. Estrogen receptor positive breast cancer

ER-positive (ER+) breast cancers constitute 75% of all breast cancers and are characterized by the ER signaling pathway as a key driver of growth. Notably, ER exists in two isoforms; ERα and ERβ with contrasting effects in ER+ cancer cells (Greene et al., 1986; Mosselman et al., 1996). The two ER isoforms share a high degree of homology structurally and can be activated by the same ligands (Paech et al., 1997). Importantly, ligand-induced activation of these receptors by 17β-estradiol (E2) results in the binding of common DNA sites on the chromatin (Paech et al., 1997). The similarity in binding sites between these receptors has rendered ERβ as a natural partial antagonist of ERα as it can inhibit a subset of genes regulated by ERα. The biology underlying the growth promoting effect of ERα signalling in breast cancer is well-established and is the focus of this thesis. The oncogenic ERα isoform will be referred to as ER in the subsequent sections.

1.2.1. Structure of ER

ER is a member of the nuclear hormone receptor superfamily which also includes other hormone receptors including the androgen receptor (AR) (Tsai & O'Malley, 1994). Members of this superfamily are structurally similar and are organized into different modules with specific functions. They typically possess two activation domains; a ligand-independent activation function 1 (AF-1) and a ligand-dependent activation function 2 (AF-2) where the ligand-binding domain (LBD) is situated. AF-1 is regulated by growth factor signalling and is located in the N-terminus of the protein whereas AF-2 is regulated by the binding of ligands such as E2 (Kumar et al., 1987; Tora et al.,

1989; Tzukerman et al., 1994; Webster et al., 1988). Importantly, activation of both domains is required for the complete agonistic action of estrogen (Kraus et al., 1995). ER also has domains required for DNA binding and nuclear localization and the general structure and functions of ER are summarized in **Fig. 1.1**. The ligand-binding domain comprises 12 α-helices (h1-h12) and in the absence of a ligand, where ER is transcriptionally inactive, this domain is bound to heat shock proteins (HSPs) such HSP-90 (Smith & Toft, 2008). Binding to E2 induces a conformation change in the helix where it results in the loss of binding affinity to HSPs, leading to dimerization of ER and creation of a hydrophobic groove permissible for the binding of co-activators.
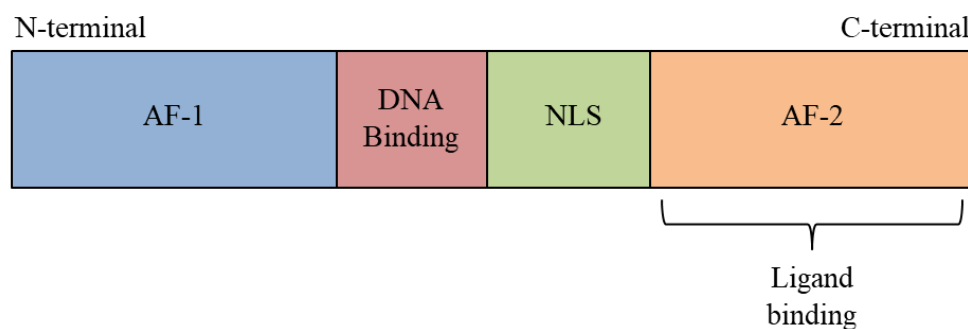
Figure 1.1. Functional domains of ER.
The structure of ER is comprised of several modules including the activation function-1 (AF-1), DNA binding module, nuclear localization signal (NLS) module and the activation function-2 (AF-2) where ligand binding occurs.

1.2.2. Mechanism of ER transcriptional activation

Activation of ER post ligand-binding induces receptor phosphorylation, dimerization and nuclear translocation leading to activation of transcription of target genes. The process of transcriptional activation can occur via two mechanisms; the first mechanism involves the binding of ligand-bound ER to ER-responsive elements (EREs), which are DNA sequences located in the regulatory regions of ER-target genes, where ER subsequently recruits co-activator complexes necessary for the transcriptional regulation of these genes. Co-activators reported to bind to ER include the steroid receptor coactivator (SRC)-family of proteins such as SRC-1, amplified in breast cancer 1 (AIB1), androgen receptor-associated protein 70 (ARA70) and p300 (Anzick et al.,

1997; Chakravarti et al., 1996; Lanzino et al., 2005; Onate et al., 1995). Notably, the interaction of ER with FOXA1, a pioneer factor critical for making the condensed DNA permissive for ER binding, is critical in the transcriptional activity of ER (Cirillo et al., 2002; Hurtado et al., 2011). The second mechanism by which ER can activate gene transcription involves association of ligand-bound ER with other transcription factors such as the specificity protein-1 (SP-1) and activator protein-1 (AP-1) complexes (McKenna & O'Malley, 2002), where this mechanism of ER allows for the transcriptional regulation of genes in an ERE-independent manner.

Ligand-bound ER has also been reported to have non-genomic activity where it can activate signalling molecules located near the plasma membrane. Pathways which can be activated in this manner include insulin growth factor receptor 1 (IGFR-1), epidermal growth factor receptor (EGFR) and HER2, leading to activation of downstream signalling nodes PI3K/Akt and MAPK (reviewed in (Bjornstrom & Sjoberg, 2005)). This non-genomic activity of activated ER can also feed into the genomic activity of ER through phosphorylation and activation of transcription factors such as the AP-1 complex (Karin, 1995).

Ultimately, estrogen-induced activation of ER leads to the proliferation of ER+ breast cancers. The mechanisms by which proliferation can be achieved include induction of cell cycle promoters such as cyclin D1 and c-Myc in parallel with redistribution of CDK inhibitor p21 (Hermeking et al., 2000; Planas-Silva & Weinberg, 1997; Prall et al., 1998; Prall et al., 1997; Sabbah et al., 1999). Cyclin D1, which is a direct transcriptional target of ER, binds to and activates CDK4 and CDK6 which subsequently phosphorylate retinoblastoma protein (Rb) (Prall et al., 1997; Sabbah et al., 1999). Phosphorylation of Rb alleviates its inhibitory action on E2F, a family of transcription factors critical for cell cycle progression, and thereby allowing E2F to induce expression of cyclin E and cyclin A and initiating S-phase entry (Dyson, 1998). As part of promoting cell cycle progression, ligand-bound ER can also induce the expression of c-Myc, via binding to a distal enhancer upstream of the gene in a process requiring the assistance of AP-1, to further promote S-phase entry through CDK4 upregulation (Hermeking et al., 2000; C. Wang et al., 2011).

1.2.3. Anti-estrogen therapies

The importance of ER signalling in ER+ tumours is demonstrated by the effectiveness of anti-ER therapies, which have improved the survival of patients with ER+ breast cancer (EBCTCG, 2005; Goss et al., 2003; Howell, 2005; Mouridsen et al., 2001). There are different classes of anti-estrogen therapies with distinct mechanisms of action and these include the selective estrogen receptor modulators (SERMs), selective estrogen receptor degraders (SERDs) and aromatase inhibitors (AIs). Tamoxifen is the most commonly used SERM and functions by preventing the E2-dependent activation of the AF2 domain. Binding of tamoxifen to the ligand binding domain of ER induces a confirmation change in ER and this restricts its access to co-activators required for AF2 domain driven transcriptional activity (Riggs & Hartmann, 2003). Notably, binding of tamoxifen does not prevent dimerization and nuclear translocation of ER. Furthermore, it is also reported to behave like an agonist and can induce the expression of genes implicated in cell-cycle progresssion (Hodges et al., 2003). Clinically, treatment of ER+ disease with five years of adjuvant tamoxifen halves the rate of recurrence and reduce mortality by a third in the first 15 years (Davies et al., 2011).

Aromatase inhibitors reduce peripheral biosynthesis of estrogen which is the major source of estrogen in postmenopausal women where ovarian functions have ceased. This is achieved through inhibition of the enzyme aromatase (*CYP19*) which catalyzes the biosynthesis of estradiol from adrenal testosterone precursors, leading to the antagonism of ligand-activated ER activity (Simpson, 2003). Expression of aromatase can be found peripherally in muscles, in the stroma of adipose tissues in the breast (Santen et al., 1994; Sasano et al., 1994) and in breast cancer cells (Sasano et al., 2005; Z. Zhang et al., 2002). Clinically-used aromatase inhibitors can be classified into either non-steroidal inhibitors, which include anastrozole and letrozole, or steroid inhibitors such as exemestane. Anastrozole and letrozole are competitive inhibitors of aromatase and they bind to both the catalytic and substrate binding sites of the enzyme thereby resulting in a complete shutdown of the enzyme (Furet et al., 1993; Lang et al., 1993). In contrast, exemestane inhibits aromatase by binding covalently and irreversibly to the substrate binding site of the enyzme (Brodie et al., 1981).

In postmenopausal women with ER+ breast cancer, the efficacy of tamoxifen and anastrozole was first evaluated in the Anastrazole, Tamoxifen Alone or in Combination

(ATAC) trial where they reported that the 3-year disease-free survival (DFS) in patients who received anastrazole was significantly higher than in patients who received tamoxifen (Hazard ratio (HR) 0.78; 95% confidence interval (CI), 0.65-0.93; p=0.005) (Baum et al., 2002). An updated 10-year analysis of the ATAC trial reported that the rate of disease recurrence remained significantly lower for patients on anastrozole compared to those on tamoxifen (HR 0.81; 95% CI 0.67-0.98; p=0.03) (Cuzick et al., 2010). However, overall survival was not significantly dissimilar between the two arms. A similar observation was reported when the efficacy of letrozole and tamoxifen was compared, with patients who received letrozole reporting a significantly higher DFS relative to patients who received tamoxifen over a 5-year follow up (HR 0.81; 95% CI 0.70-0.93; p=0.003) (Thurlimann et al., 2005). An updated analysis of this trial at a median follow up of 71 months reported an insignificant increase in overall survival in patients who received letrozle compared to tamoxifen (HR 0.87; 95% CI 0.75-1.02; p=0.08) similar to the ATAC trial (Mouridsen et al., 2009). Finally, a recent phase 3 study reported that the efficacy of anastrozole, letrozole and exemestane in early stage ER+ breast cancer patients were comparable over a 5-year peroid (De Placido et al., 2018). The superiority of aromatase inhibitors over tamoxifen establishes aromatase inhibitors as the first line standard of care in postmenopausal breast cancer patients with early stage breast cancer.

Another class of ER antagonist is the selective ER downregulators (SERDs) and fulvestrant is the first generation drug of its kind currently in clinical use (Osborne et al., 2004). Fulvestrant has a more complete inhibitiory effect on the transactivation of ER compared to tamoxifen as binding of fulvestrant to the ligand-binding domain of ER prevents receptor dimerization and nuclear translocation leading to increased degradation of ER (Wakeling et al., 1991). Due to this "pure anti-estrogenic" effect, fulvestrant treatment does not lead to some of the side-effects associated with tamoxifen, such as endometrial hyperplasia (Fisher et al., 1994). The clinical efficacy of fulvestrant was first explored in patients with advanced ER+ cancers who have progressed on tamoxifen and two phase 3 studies have demonstrated that 250 mg fulvestrant had a similar efficacy and an acceptable side-effect profile relative to anastrozole (Howell et al., 2002; Osborne et al., 2002). In the study undertaken by Osborne and colleagues, the clinical benefit rate of 42.2% achieved with fulvestrant was insignificantly higher than the 36.1% achieved with anastrozole (95% CI -4.00% to

16.41%; p=0.26) (Osborne et al., 2002). These studies resulted in the registration of fulvestrant for the treatment of patients with advanced ER+ breast cancer who had progressed on tamoxifen.

In the subsequent phase 3 Comparison of Faslodex in Recurrent or Metastatic Breast Cancer (CONFIRM) trial, 500 mg was demonstrated to be more effiacious and it significantly increases progression-free survival (HR 0.80; CI 0.68-0.94; p=0.006) relative to the initially-approved dose of 250 mg fulvestrant (Di Leo et al., 2010). Importantly, no dose-dependent side effects associated with this higher dose were observed. These studies led to the approval of 500 mg fulvestant as the standard dose. A follow-up analysis further affirmed the superiority of the higher dose as it was associated with an extension of the median overall survival by 4.1-months (HR 0.81; 95% CI 0.69- 0.96; p=0.02) when compared to the lower dose (Di Leo et al., 2014). These studies led to the approval of 500 mg fulvestant as the standard dose for the treatment of recurrent breast cancer which had previously progressed on an endocrine-therapy.

The efficacy of fulvestrant in postmenopausal women with ER+ advanced breast cancer who had not received prior endocrine therapy was first evaluated in a phase 2 trial known as the The Fulvestrant First-Line Study (FIRST). Patients in this study who received fulvestrant had a 34% reduction in risk of progression relative to the comparative anastrozole arm (HR 0.66; 95% CI 0.47-0.92; p=0.01) (Robertson et al., 2012). Follow-up analysis of the overall survival of these patients demonstrated a significant extension of OS in the fulvestrant arm (HR 0.70; 95% CI 0.50-0.98; p=0.04) (Ellis et al., 2015). The superiority of fulvestrant over anastrozole in postmenopausal women with endocrine therapy-naïve breast cancer was further affirmed in a phase 3 Fulvestrant and Anastrozole Compared in Hormonal Therapy Naïve Advanced Breast Cancer (FALCON) trial where PFS was significantly longer in patients who received fulvestrant as compared to anastrozole (HR 0.797; 95% CI 0.6637-0.999; p-0.0486). These studies indicate that fulvestrant should be the preferred choice of treatment option over an aromatase inhibitor for the treatment of endocrine-therapy naïve ER+ breast cancer.

1.3.   Mechanisms of endocrine therapy-resistance in ER+ breast cancer

While endocrine therapies are highly effective for ER+ breast cancer and have dramatically improved overall survival, a significant proportion of patients will relapse during or after the 5-10 year of adjuvant therapy, develop distant metastasis, resulting in morbidity and mortality. Furthermore, ER+ disease has a long natural history and recurrence of this disease can occur many years after initial diagnosis (E. Lim et al., 2012) and ~15% of patients within 5 years and 30% of patients within 15 years will subsequently suffer from disease relapse (Dowsett et al., 2010; EBCTCG, 2005). The propensity of ER+ disease to develop late recurrence is demonstrated by the higher annualized hazard rate of recurrence in ER+ tumours relative to ER- tumours past the 5-year mark (recurrence rate at 15 to 20 years; ER+, 28% vs ER-, 1.2%; $p<0.001$) (Colleoni et al., 2016; Pan et al., 2017).

The failure of ER+ tumours to respond to endocrine therapy can be attributed to either intrinsic resistance, where tumours do not respond to endocrine therapies at all, or acquired resistance, where tumours gradually adapt to and overcome the growth inhibition imposed by the endocrine therapy and regrow in its presence. At present, there are no established biomarkers that accurately predict for response to endocrine therapy. However, efforts are underway to identify tumours which are likely to be intrinsically resistant to endocrine therapies and this involves assessing the response of ER+ tumours to neoadjuvant endocrine therapy where patients are typically treated for 2-4 weeks prior to surgery. This investigational approach known as the pre-operative endocrine therapy prognostic index (PEPI) is based on a scoring system incorporating pathological staging, expression level of ER and degree of Ki-67 positivity and tumours with a low PEPI score of 0 are reported to have a low risk of relapse of 3% over a median follow-up of 5 years (Ellis et al., 2008). Observations from these studies are awaiting validation in the larger phase 3 ALTERNATE trial (NCT01953588). Overall, disease recurrence in patients who have received endocrine therapies develops in a significant proportion of patients and this presents a critical clinical problem.

Given the high prevalence of patients who develop endocrine-resistance, understanding the mechanisms of resistance is critical in order to identify means to overcome it. Furthermore, endocrine-resistance is a multi-faceted process involving aberrations at the

molecular, epigenetic and genomic levels (Osborne et al., 2003; Stone et al., 2015; N. Turner et al., 2010) and are summarized in the following sections.

1.3.1. Increased activity of growth-factor signalling

Historically, cell line models which have acquired resistance to either tamoxifen (TamR) or long-term estrogen deprivation (LTED) have been utilized to identify mechanisms of resistance associated with tamoxifen and aromatase-inhibitor respectively (Ma et al., 2015; Musgrove & Sutherland, 2009). Commonly-reported mechanisms which can contribute to the acquisition of resistance in these resistant cell line models include increased growth factor signalling pertaining to HER2, insulin-like growth factor receptor 1 (IGFR-1) and fibroblast growth factor receptor (FGFR-1) (Martin et al., 2003; Shou et al., 2004; Stephen et al., 2001; N. Turner et al., 2010; Y. Zhang et al., 2011). These upstream signalling pathways often converge onto either the MAPK or PI3K signalling nodes and hyper-activation of these signalling nodes has been demonstrated to result in activation of ER signalling in an estrogen-independent manner which underlies endocrine resistance (T. W. Miller et al., 2011; Y. Zhang et al., 2011). In support, activation of these signalling nodes using epidermal growth factor (EGF) has been demonstrated to elicit a distinct program of ER binding and activity which is associated with endocrine-resistance (Lupien et al., 2010).

1.3.2. Aberrant activation of pioneer factor or transcription factor

Aberrant activation of pioneer factors or transcription factors can also result in endocrine-resistance. The overexpression of FOXA1 in MCF7 cells was reported to alter ER chromatin binding leading to a gene expression profile that was associated with endocrine-resistance (Fu et al., 2016). Notably, interleukin-8 (IL-8) was identified as a key FOXA1- and ER-regulated gene which mediated tamoxifen resistance in these FOXA1-overexpressing MCF7 cells. Increased activity of transcription factor AP-1 has also recently been reported to contribute to endocrine-resistance in a manner that also involved the reprogramming of ER signalling (Malorni et al., 2016). In this study, they demonstrated that blockade of this transcriptional factor via inducible dominant-negative (DN) expression of c-Jun, which together with c-Fos constitutes the AP-1 complex, was able to confer sensitivity of tumours to tamoxifen *in vivo*.

1.3.3. Polymorphism in CYP2D6 and loss of ER expression

Tamoxifen is metabolized in the liver to its highly potent active metabolites 4-hydroxytamoxifen and endoxifen by cytochrome P450 enzymes CYP2D6 and CYP3A4 respectively (Desta et al., 2004). Hence, aberrant metabolism of tamoxifen can therefore reduce its efficacy. However, there is controversy concerning the polymorphism of this enzyme and clinical outcome in patients who received tamoxifen. Early studies have demonstrated that in patients who received tamoxifen, those who harboured polymorphisms in *CYP2D6* had worse DFS compared to wild-type *CYP2D6* (Goetz et al., 2005; Xu et al., 2008). These observations were validated in a subsequent retrospective study where they reported that patients with two alleles associated with poor metabolism of tamoxifen were more likely to experience disease recurrence than patients who had two alleles associated with extensive metabolism of tamoxifen (Goetz et al., 2013). However, these observations did not accord with the analyses of a subset of patients enrolled in the ATAC and Breast International Group 1-98 (BIG 1-98) prospective studies (Rae et al., 2012; Regan et al., 2012). The lack of significance between *CYP2D6* polymorphism and efficacy of tamoxifen in these two trials has been attributed to the poor sample representation used in these studies (Goetz et al., 2016). Overall, there is some evidence to suggest that polymorphism in *CYP2D6* is associated with poor response to tamoxifen.

Considering that ER is the target of tamoxifen in ER+ tumours, the loss of ER expression, resulting in a loss of therapeutic target for tamoxifen, can also contribute to the acquisition of tamoxifen resistance. In a study undertaken by Gutierrez et al, ER loss was reported in 17% of resistant tumours when 36 pairs of pre-and post-tamoxifen-treated tumours were assessed (Gutierrez et al., 2005).

1.3.4. Epigenetic, genetic and genomic changes related to ER signalling

Advancements in sequencing technologies have also led to the identification of acquired changes at the epigenetic and genetic levels in endocrine-resistant breast cancers. At the epigenetic level, hypermethylation of DNA at estrogen-responsive elements was identified in tumours which relapsed shortly after 5 years of adjuvant endocrine therapy relative to those in patients who had experienced relapse-free survival of more than 14 years (Stone et al., 2015). Hypermethylation of these estrogen-responsive elements was demonstrated to be associated with reduced binding of ER and

this was identified in the *NCOR2* gene which encodes for a key co-regulatory protein important for the classical activity of ER. Hence, it is postulated that hypermethylation of estrogen enhancers can result in "shutting down" of classical ER signalling and diminishing the efficacy of ER-targeted therapies.

Mutations in ER have emerged as a major mechanism of resistance in patients treated with an aromatase inhibitor. These mutations have been identified in the range of 11-39% of endocrine-resistant breast cancers, particularly in patients who had been exposed to an aromatase inhibitor (Chandarlapaty et al., 2016; Fribbens et al., 2016; Jeselsohn et al., 2015; Niu et al., 2015; Schiavon et al., 2015; Spoerke et al., 2016). These mutations are often "missense" mutations located in the ligand-binding domain with Y537S and D538G being the most frequently occurring mutations (Reinert et al., 2017). Functionally, these mutations render the ER protein constitutively active via stabilization of the AF-2 domain and ligand-independent recruitment of co-factors (Fanning et al., 2016). As a consequence of changes to the conformation of the ligand-binding domain, these mutations also reduce sensitivity of ER to anti-ER targeted therapies such as tamoxifen and fulvestrant (Bahreini et al., 2017; Harrod et al., 2017). The identification of naturally-occurring *ESR1* mutations (Y537C and Y537S) in long-term estrogen deprived cell line models, but not in tamoxifen-resistant cell lines, have recently been reported (Martin et al., 2017).

Genomic structural rearrangement of ESR1 has also been identified in endocrine-resistant ER+ breast cancer. The discovery of an *ESR1*-Coiled-Coil Domain Containing 170 (*CCDC170)* fusion protein was first reported in an aggressive subset (8%) of primary breast cancer which was also enriched in the luminal B subtype (Veeraraghavan et al., 2014). The presence of *ESR1-CCDC170* fusion protein renders these cells more aggressive and less sensitive to endocrine therapy and these phenotypical changes are likely a consequence of increased ERK and Akt activity (Veeraraghavan et al., 2014).

Subsequent studies have identified up to 11 other unique *ESR1*-fusion proteins in endocrine-resistant breast cancers (Hartmaier et al., 2018; Lei et al., 2018; Li et al., 2013). In the study reported by Lei and colleagues, they identified that 3 out of 25 (12%) of endocrine therapy resistant tumours had an *ESR1*-fusion with one harbouring a *ESR1*-YES-associated protein 1 (YAP1) protein and the other an *ESR1*-protocadherin 11 X-Linked (PCDH11X). In the study undertaken by Hartmaier and colleagues, 9 other

unique *ESR1*-fusion proteins including *ESR1*-disabled-2 (DAB2) and *ESR1-SOX9* were discovered. A common feature of these *ESR1*-fusion proteins is that the breakpoint occurs between exon 6 and 7 and the resultant fusion proteins lack the ligand binding domain, which often leads to hyperactivity of ER (Hartmaier et al., 2018; Lei et al., 2018). Further characterization of the *ESR1*-YAP1 and *ESR1*-PCDH11X fusion proteins indicated that these fusion proteins induced a transcriptomic program that is associated with metastases and were also relatively resistant to fulvestrant (Lei et al., 2018).

Amplification of *CYP19A1* (aromatase) and *ESR1* has also been identified in endocrine-resistant tumours. These events were postulated to be acquired during the acquisition of endocrine-resistance given their low frequency of incidence (0.006% for *CYP19A1* and 0.0018% for *ESR1*) in the TCGA dataset. In the resistant tumours, *CYP19A1* and *ESR1* were amplified in 32% and 21% of AI-resistant patients respectively. Further, more than half of the tumours with *CYP19A1* amplification also had *ESR1* amplification and the high frequency of co-occurrence suggests that it could underlie the acquisition of endocrine-resistance in these tumours. Given that *in vitro* AI-resistant cells have been reported to increase cholesterol biosynthesis through epigenetic reprogramming (Nguyen et al., 2015) and that testosterone is derived from cholesterol (Waterman & Keeney, 1992), it has been postulated that the high level of aromatase in these tumours can overcome AR-imposed systemic estrogen-deprivation by increasing biosynthesis of estrogen from endogenous testosterone precursors, leading to autocrine activation of ER (Magnani et al., 2017).

1.3.5. Genomic alterations in transcriptions factors, MAPK pathway and CCND1

In order to identify genomic aberrations enriched in endocrine-resistant breast tumours, large-scale genomic sequencing studies of sample from patients who have progressed on prior endocrine therapy have been undertaken to elucidate the "genomic landscape" of these resistant tumours (Giltnane et al., 2017; Razavi et al., 2018). In the study performed by Razavi and colleagues, they sequenced ~1,900 tumours and identified genetic mutations in proteins leading to the activation of the MAPK signalling and activating mutations in ER transcription coregulators, such as *MYC, CTCF* and *FOXA1,* in tumours which have progressed on endocrine therapies. The hyperactivation of the MAPK signalling pathway was inferred by the discovery of 1) activating mutations in

ERRB2, 2) loss-of-function mutations in neurofibromin (*NF1*) which is a natural inhibitor of MAPK kinase signalling pathway (Lau et al., 2000) and 3) amplification of *EGFR*. Importantly, these events leading to activation of MAPK signalling or ER transcriptional coregulators appeared to be mutually exclusive to the occurrence of hotspot *ESR1* mutations and were present in 22% of all endocrine-resistant breast cancers. The response of tumours harbouring alterations in the MAPK pathway to subsequent hormonal therapies such as an aromatase inhibitor and fulvestrant was significantly diminished as compared to tumours without known alterations in *ESR1*, MAPK and ER co-regulators.

In the study undertaken by Giltnane and colleagues (Giltnane et al., 2017), they demonstrated that clinically-relevant genomic aberrations can be detected in tumours treated with neoadjuvant aromatase inhibitor and they investigated 153 samples from 143 patients. 21% of these samples did not demonstrate reduction in Ki-67 from baseline and the mean Ki-67 index of these samples was 24.6%. Exome sequencing of these tumours identified frequent amplifications of *FGFR1* (*8p11-12*) and *CCND1* (*11q13*) in 35% and 45% of tumours evaluated respectively where amplifications of *FGFR1* and *CCND1* was identified in 9% and 20% of endocrine-sensitive tumours. Importantly, co-amplification of *FGFR1* and *CCND1* were found in 30% of these tumours and had an enrichment of cell cycle genes above and beyond the level associated with amplification of either gene alone. The amplification of *CCND1* in ER+ tumours has previously been reported to identify a subset with poor prognosis (Roy et al., 2010).

In summary, there are diverse mechanisms by which resistance to endocrine therapies can be developed. These mechanisms can be broadly divided into either intrinsic or acquired mode of resistance. Phenomena such as polymorphism of *CYP2D6 or* genomic aberrations such as amplification of *CCND1* or *FGFR1* which exists prior to treatment can be considered as intrinsic resistance mechanisms. The other changes which are selected for by treatment, such as the occurrence of *ESR1* mutations, are more likely to be acquired mechanisms of resistance. This diversity in resistance mechanisms can be attributed to the different mechanisms of ER-directed therapies and possible outgrowth of different clones (Hole et al., 2015; Patani et al., 2014). However, observations from

these studies suggest that ER signalling still plays a crucial role in the majority of these endocrine-resistant tumours.

1.4. Current therapies for endocrine-resistant breast cancer

Advancement in the understanding of mechanisms underlying endocrine-resistance has led to the development of novel agents in the clinical management of these resistant tumours. Agents that have been approved for clinical use include inhibitors of the mammalian target of rapamycin (mTOR) signalling pathway and cyclin-dependent kinase 4 and 6 (CDK4/6). MAPK and PI3K/Akt signalling nodes are effectors of growth factor signalling which have often been reported to be hyper-activated in endocrine-resistant breast cancer (Schiff et al., 2004). The mTOR signalling pathway, is downstream of these major signalling nodes (Dibble & Cantley, 2015; Saini et al., 2013) and has downstream regulatory functions on proliferation and metabolism (Ricoult & Manning, 2013; Zoncu et al., 2011). In the context of endocrine-resistance, inhibition of mTOR was associated with reversal of tamoxifen-resistance (deGraffenried et al., 2004) and estrogen-independent cell proliferation (Miller et al., 2010). Everolimus is a derivative of rapamycin and inhibits the mTOR pathway by covalently binding to and inhibiting the mTOR complex 1 (mTORC1) (reviewed in(Efeyan & Sabatini, 2010)). Everolimus has demonstrated efficacy in combination with tamoxifen in the phase 2 TamRad (Tamoxifen Plus Everolimus) clinical trial. Patients enrolled in this study have had prior exposure to aromatase inhibitors and the combination of everolimus and tamoxifen significantly extended PFS compared to tamoxifen alone in these patients (PFS 8.6 vs 4.5 months; HR 0.54; 95% CI 0.36-0.81) (Bachelot et al., 2012). In the phase 3 Bolero-2 clinical trial, the addition of everolimus to exemestane as a second-line therapy increased the median PFS of patients with advanced ER+ breast cancer who had progressed on non-steroidal aromatase inhibitors to 10.6 months compared to 4.1 months with exemestane alone (HR 0.36; 95% CI 0.27-0.47; $p<0.001$) (Baselga et al., 2012). This combination has been approved by the Food and Drug Administration (FDA) in the USA and the Pharmaceutical Benefits Scheme (PBS) in Australia as a treatment strategy for advanced ER+ breast cancer patients who have progressed on prior non-steroid aromatase inhibitor treatment.

CDK4 and 6 are critical regulators of the cell cycle where they govern the rate-limiting S-phase entry. Biologically, these molecules are downstream of the MAPK, PI3K/Akt

and ER signalling pathways and mediate the proliferative consequences of these signalling pathways (Prall et al., 1997; Sabbah et al., 1999; Sherr, 1996). Mitogenic stimulation of these pathways results in the hetero-dimerization of CDK4/6 with cyclin D. This activated CDK4/6 complex phosphorylates Rb and relieves Rb-imposed inhibition on E2F family of transcription factors leading to the commitment of a transcriptional program required for S-phase. Inhibitors of CDK4/6 have been developed to inhibit the active sites of these proteins and hence reduce the growth promoting activity of these kinases (reviewed in (Asghar et al., 2015)). Pharmacological inhibition of CDK4/6 has been demonstrated to be particularly effective against preclinical models of endocrine-sensitive and -resistant breast cancer (Finn et al., 2009; Haricharan et al., 2017). Palbociclib (Ibrance®, Pfizer) (Fry et al., 2004), ribociclib (Kisqali®, Norvatis) (Infante et al., 2016) and abemaciclib (Verzenio®, Eli Lilly) (Patnaik et al., 2016) are CDK4/6 inhibitors which are currently used clinically for the management of breast cancer.

Palbociclib is the first CDK4/6 inhibitor that was approved by the FDA in 2015 for the management of endocrine-resistant breast cancers, based on results from the PALOMA trials. In the phase 2 PALOMA-1 trial, the median progression-free survival of patients with advanced breast cancer treated with the combination of palbociclib with letrozole in the 1st line metastatic setting was 20.2 months compared to 10.2 months with letrozole alone (HR 0.488; 95% CI 0.319-0.748; p=0.004) (Finn et al., 2015). In the follow-up phase 3 PALOMA-2 trial, they similarly reported an extension of median PFS (24.8 vs 14.5 months; HR 0.58; 95% CI 0.46-0.72; p<0.001) (Finn et al., 2016). In the subsequent phase 3 PALOMA-3 trial, the combination of palbociclib and fulvestrant as second-line therapy increased the median PFS of patients with advanced breast cancer who had progressed on an endocrine therapy by 4.8 months compared to fulvestrant alone (PFS 9.5 months vs 4.6 months; HR 0.46; 95% CI 0.36-0.59; p<0.0001) (Cristofanilli et al., 2016).

The efficacy of ribociclib was assessed in the Monaleesa trials. In the phase 3 Monaleesa-2 trial, patients with advanced ER+ breast cancer who received ribociclib and letrozole as first-line treatment had a median PFS of 25.3 months as compared to 10 months with letrozole alone (HR 0.568; 95% CI 0.457-704; p<0.0001) (Hortobagyi et al., 2016; Hortobagyi et al., 2018). This led to the approval from FDA for the treatment

use of ribociclib in combination with an AI as a first-line therapy in advanced ER+ breast cancer. The efficacy of ribociclib/fulvestrant combination as a first- and second-line treatment in advanced ER+ breast was assessed in the Monaleesa-3 trial (Slamon et al., 2018). In this study, the combination therapy as a first- or second-line treatment significantly extended the median PFS of patients as compared to fulvestrant alone (HR 0.593; 95% CI 0.48 – 0.732; p<0.001).

The impact of abemaciclib was evaluated in the Monarch trials. In the phase 2 Monarch 1 trial, the abemaciclib as a monotherapy was associated with a clinical benefit rate (complete response, partial response and stable disease $\geq$ 6 months) of 42.4% in heavily pre-treated patients with advanced ER+ breast cancer (Dickler et al., 2017). In the phase 3 Monarch 2 trial, the abemaciclib and fulvestrant combination significantly extended median PFS of patients with ER+ breast cancer, who had progressed on prior neoadjuvant, adjuvant endocrine therapy or first-line endocrine-therapy for metastatic disease as compared to fulvestrant alone (PFS 16.4 vs 9.3 months; HR 0.553; 95% CI 0.449-0.681; p<0.001) (Sledge et al., 2017). In the Monarch 3 trial, the abemaciclib/fulvestrant combination as a first-line therapy significantly prolonged median PFS of patients with advanced ER+ breast cancer as compared to fulvestrant alone (HR 0.53; 95% CI 0.41-0.72; p=0.000021) (Goetz et al., 2017).

However, patients with advanced breast cancer will ultimately progress on these targeted therapies. The median duration of progression free survival with first line treatment with CDK4/6 inhibitor and endocrine-therapy combination is ~25 months (Finn et al., 2015; Hortobagyi et al., 2018) and the median duration of progression free survival for this combination in the second line is 9.5 months (Cristofanilli et al., 2016). Similarly, the median duration of PFS for the combination of endocrine therapy and everolimus in the second line in advanced ER+ breast cancer was 10.6 months (Baselga et al., 2012). Importantly, the addition of palbociclib to fulvestrant in the PALOMA-3 trial has yet to report an improvement in the overall survival of patients (Turner et al., 2018). While these new therapy combinations represent a significant leap in the management of ER+ breast cancers, unfortunately almost all patients will still ultimately relapse from their disease as resistance develops. Hence, new therapeutic strategies are necessary for the treatment of advanced ER+ breast cancers.

1.5. Novel therapies currently being evaluated for endocrine-resistant breast cancer

1.5.1. Oral selective estrogen receptor downregulators

The efficacy of fulvestrant, which has the most potent inhibitory effect on ER amongst currently-used ER antagonists, is limited by poor pharmacokinetics and is administered intramuscularly which affects its bioavailability (Robertson, 2007; Robertson et al., 2004). Improved versions of SERDs which can be administered orally have now been developed and these include AZD9496 (AstraZeneca) (Weir et al., 2016) and GDC-0810 (Genentech) (Lai et al., 2015). Both AZD9496 and GDC-0810 have been demonstrated to suppress preclinical models of endocrine-resistant breast cancers harbouring wild-type or *ESR1* mutations. A phase 1 trial with AZD9496 on patients with heavily pre-treated advanced breast cancer was recently completed and reported that this agent was well-tolerated and that there was evidence of stabilization of disease in these patients (Hamilton et al., 2018). These oral SERDs are now also being evaluated in combination with targeted therapies such as CDK4/6 inhibitors.

1.5.2. Inhibitors of PI3K

Class I PI3K enzymes are heterodimers composed of a catalytic subunit (p110α, β or δ) and a regulatory subunit (p85). Hotspot mutations in *PI3KCA* which encodes for the catalytic subunit p110α and which leads to the hyper-activation of the PI3K/Akt (Zhao & Vogt, 2010), is observed in over a third of ER+ breast cancers (Giltnane et al., 2017). Pan or specific inhibitors of the catalytic subunits of PI3K have been developed and these include the pan inhibitor BKM120 (Novartis) and PI3K p110α-specific BYL719 (Norvartis). The efficacy of these agents in breast cancer is currently being evaluated. Specific inhibition of PI3K p110α with BYL719 has been reported to enhance ER signalling and this effect is postulated to underlie the increased tumour suppression of ER+*PI3KCA*[mut] models *in vivo* observed with the combination of BYL719 and fulvestrant (Bosch et al., 2015). The efficacy of this combination is currently being assessed in ER+ patients who have progressed on an aromatase inhibitor in the SOLAR-1 trial (NCT02437318). The clinical efficacy of BKM120 in combination with fulvestrant is also currently being evaluated in patients with advanced breast cancer who have progressed on an aromatase inhibitor (NCT01610284).

1.5.3. Inhibitor of histone deacetylase

Entinostat is a histone deacetylase inhibitor which has been granted "breakthrough designation" status by the FDA for the treatment of advanced breast cancer. The acetylation state of histones is dynamic and is regulated by histone acetyl-transferases (HATs) and histone deacetylases (HDACs). HATs mediate the addition of acetyl groups to the lysine residues located in the histone tails and this process opens up the chromatin and allows access for transcription factors leading to increased transcription. On the other hand, HDACs have opposing functions as they mediate the removal of acetyl groups thereby resulting in a condensed chromatin state which is associated with transcriptional silencing. HDACs are divided into Classes 1-4 and entinostat is a specific inhibitor of Class I and IV HDACs (reviewed in (Connolly et al., 2017)). A preclinical study with entinostat reported that this agent restored the sensitivity of a letrozole-resistant MCF7 xenograft to letrozole through the downregulation of HER2 expression (Sabnis et al., 2013). Clinically, the efficacy of entinostat in combination with exemestane was evaluated in the phase 2 Encore trial which enrolled patients who have progressed on a non-steroidal aromatase inhibitor. The combination of entinostat and exemestane was associated with a significant extension of PFS (4.3 vs 2.3 months; HR 0.73; 95% CI 0.50-1.07; p = 0.06) and an increase in overall survival as compared to exemestane alone (28.1 vs 19.8 months; HR 0.59; 95% CI, 0.36-0.97; p = 0.036) (Yardley et al., 2013). A phase 3 trial (E2112) is now currently underway to validate the results of the phase 2 trial (NCT02115282) (Yeruva et al., 2018).

1.6. Nuclear receptor crosstalk in breast cancer

It is now well-established that hormone receptors do not act independently of one another in the regulation of their target genes. A hormone receptor, when activated, requires other hormone receptors and transcription factors for the coordination of transcriptional gene regulation. In support, conclusions drawn from a study which comprehensively mapped the genomic binding of 33 proteins reported that a nuclear receptor binds to an average of eleven other transcription factors (Kittler et al., 2013). Of particular interest is the ER which has been demonstrated to participate in a crosstalk with other nuclear receptors such as glucocorticoid receptor (GR) (Karmakar et al., 2013), retinoic acid receptor-α (RARα) (Hua et al., 2009), PR (Mohammed et al., 2015) and AR (Lanzino et al., 2005). Interestingly, co-activation of any of these receptors, in tandem with ER activation with their respective cognate ligands, is reported to be

associated with inhibition of growth via modulation of ER signalling in ER+ breast cancer (Hua et al., 2009; Karmakar et al., 2013; Lanzino et al., 2005; Mohammed et al., 2015).

There are several mechanisms which could underpin this crosstalk between ER and other nuclear receptors; the first is the high degree of overlap in DNA binding sites between the nuclear receptors as in the case of GR (Karmakar et al., 2013) and RARα (Hua et al., 2009) with ER. This leads to competition for EREs which are critical for ER-mediated signalling. Activated GR binding at EREs has also been demonstrated to displace steroid receptor coactivator-3 (SRC-3) from these sites (Karmakar et al., 2013). Another plausible mechanism contributing to this nuclear receptor crosstalk is the reliance on similar co-activators. The transcriptional activation of AR and ER requires common co-activators such as ARA70 and FOXA1 (Lanzino et al., 2005; J. L. Robinson et al., 2011) and hence it is possible that activation of AR signalling can reduce the activation of ER signalling through competition for the same pool of co-factors. Collectively, these mechanisms belong to a phenomenon known as "transcriptional interference" which is loosely defined as the "direct negative impact of one transcriptional activity on a second transcriptional activity *in cis* "(Shearwin et al., 2005).

Physical interaction between these nuclear receptors, as exemplified by the interaction between ER and PR (Mohammed et al., 2015), is another mechanism underpinning this crosstalk. The ability to detect previously unidentified binding partners of ER was pioneered by the establishment of a technique known as rapid immunoprecipitation mass spectrometry of endogenous protein (RIME) (Mohammed et al., 2013). This technique involves cross-linking proteins and DNA, pulling down proteins which physically interact with ER via immunoprecipitation and then identifying these proteins using mass spectrometry. Using this technique, it was demonstrated that progesterone-activated PR can physically bind to ER leading to a redistribution of ER chromatin binding to sites associated with a lower proliferative state (Mohammed et al., 2015). This process of modulating ER activity indirectly via the activation of another hormone receptor is sometimes referred to as "reprogramming".

A summary illustrating possible mechanisms by which ER activity can be impinged by the activation of other nuclear receptor is illustrated in **Fig. 1.2**. In this thesis, we sought

to assess the efficacy of inhibiting ER transcriptional activity via the modulation of AR activity. Given that the biology of AR signalling is most well-established in the aetiology of prostate cancer, the following section will provide an overview of the role and signalling mechanisms of AR in driving progression of prostate cancer as well as the repertoire of AR antagonists used for the management of this disease.
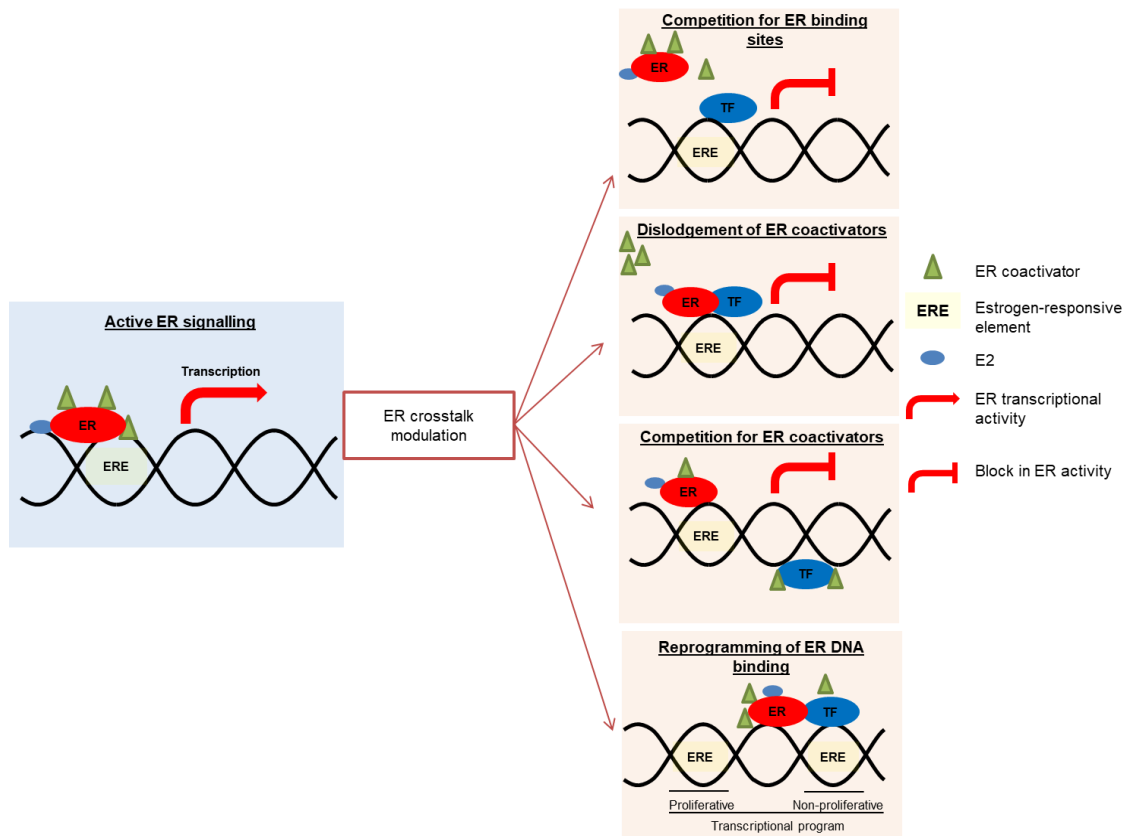
Figure 1.2. ER crosstalk with other hormone receptors.

E2-bound ER binding to coactivators and estrogen-response elements (ERE) is critical for initiation of transcription of ER-target genes. ER crosstalk with other hormone receptors can impinge on its transactivation, via competition for EREs, dislodgement of coactivators, competition for cofactors and reprogramming of ER chromatin binding, leading to ablated ER signalling. Adapted from (Elgene Lim et al., 2012)

1.7. The Androgen Receptor

The *AR* gene is situated on the X chromosome at Xq11-Xq12 (Brown et al., 1989). The AR protein is 110 kDa, comprising of 989 amino acids, and is encoded by eight exons within the gene. AR is a ligand-dependent transcription factor and belongs to the same steroid hormone superfamily as the ER (Tsai & O'Malley, 1994). Binding of AR to its ligands such as testosterone or its most potent active metabolite 5 α-dihydrotestosterone (DHT), induces the transcriptional activity of AR in a manner similar to ER as previously described. The specific DNA sequences recognized by ligand-bound AR are known as the androgen response elements (AREs) (Janne et al., 1993). Interaction of AR with co-regulators is also necessary for its transcriptional activation and the C terminus has been reported to be essential for this interaction (Glass et al., 1997). Co-factors critical for the transcriptional activity of AR include steroid receptor co-activator-1 (SRC-1), steroid receptor coactivator-3 (SRC-3) and androgen receptor associated protein 70 (ARA70) (Culig & Santer, 2012). The AR protein has a polymorphic polyglutamine (CAG) tract within its N-terminal domain that has been reported to influence the level of basal AR activity with shorter repeats associated with higher AR transactivation activity (Choong et al., 1996).

While the ligand-induced transcriptional activity of AR represents the predominant and more well-established mechanism of AR signalling, alternative mechanisms of AR activation commonly referred to as "non-genomic" or "non-canonical" activity of AR have also been reported (Liao et al., 2013). This class of AR signalling has been implicated in prostate cancer (L. Gao et al., 2013) and ovarian teratocarcinoma (Chung et al., 2014). This non-canonical activity of AR typically has very quick onset upon exposure to androgens (Peterziel et al., 1999) and can induce the activation of pro-survival kinase signalling such as the MAPK and PI3K/Akt signalling pathways (Horowitz et al., 2004; Mebratu & Tesfaigzi, 2009). Numerous mechanisms by which AR can activate these signalling pathways have been reported. These include the physical interaction between AR and Src homology-3 (SH3) domain of Src, which releases the auto-inhibitory effect of Src homology-2 (Sh2) and Sh3 domains leading to the activation of Src and downstream MAPK signalling (Unni et al., 2004), and the direct activation of the PI3K pathway by AR through physical interaction with p85α, the regulatory subunit of PI3K (M. Sun et al., 2016). Importantly, non-genomic AR activity has been reported to be unaffected by anti-androgens, such as hydroxyflutamide and

bicalutamide, which were designed to inhibit the transcriptional activity of AR (Peterziel et al., 1999).

Functionally, AR is involved in diverse androgenic and anabolic physiological processes and aberrations in AR signalling have been reported to lead to a range of clinical disorders such as prostate cancer, muscular atrophy, androgen insensitivity syndrome and cachexia. A range of pharmacological modulators have been developed to rectify the AR signalling aberration in these diseases.

1.7.1. AR antagonists

AR signalling is critical to the aetiology and progression of prostate cancer and inhibition of this pathway is a mainstay in the management of this disease. Pharmacological inhibitors of AR can be broadly classified as either direct AR antagonists or androgen-deprivation therapy. Examples of clinically-used AR antagonists include bicalutamide (Casodex[®], AstraZeneca) and enzalutamide (Xtandi[®], Pfizer) which represent the 1st and 2rd generation anti-androgens respectively. The binding of these agents to AR in the ligand-binding domain in the carboxyl terminus is reported to induce a conformational change which makes it inaccessible for coactivators and hence preventing its transcriptional activation (Helsen et al., 2014; Osguthorpe & Hagler, 2011). Enzalutamide is a superior clinical AR antagonist relative to bicalutamide as it has a relatively higher AR-binding affinity and inhibits multiple steps in the transcriptional activation of AR including nuclear translocation and DNA-binding (Tran et al., 2009). Furthermore, while bicalutamide has reported agonistic effect on AR leading to transcriptional activation of AR-target genes such as prostate-specific antigen (PSA), enzalutamide has no such reported effect (Tran et al., 2009). Apalutamide (Erleada[®], Janssen) is an improved version of enzalutamide with demonstrated equipotency *in vitro* but a superior efficacy *in vivo* in prostate cancer xenograft models compared to enzalutamide (Clegg et al., 2012). Abiraterone acetate (Zytiga[®], Janssen) on the other hand, is an androgen-deprivation therapy as it potently inhibits the liver enzyme CYP17 which is critical for the downstream bioconversion of both androgens and estrogens from progesterone precursors (Chan et al., 1996). A phase 1 clinical trial assessing the safety of abiraterone acetate on advanced prostate cancer patients has demonstrated reductions in both androgens and estrogens in these patients (Attard et al., 2008).

Persistent AR signalling in the presence of AR inhibitors occur can either in the form of truncated AR variants or mutations in *AR* (Wadosky & Koochekpour, 2017; Watson et al., 2015). The AR-variant 7 (AR-v7) which lacks the ligand-binding domain, results in constitutive activation of AR whereas the emergence of *AR* mutations such as the F876L mutation renders enzalutamide and apalutamide to behave like an agonist (Joseph et al., 2013; Korpal et al., 2013; Lu & Luo, 2013) and these events contribute to resistance against AR-targeted therapies in castration-resistant prostate cancer (Wadosky & Koochekpour, 2017; Watson et al., 2015). This has led to the development of a new generation of AR-degraders such as UT-155 and AR-PROteolysis TArgeting Chimera (AR PROTAC) which can degrade AR. Binding of UT-155 to the amino-terminus of AR induces proteosomal degradation of AR (Ponnusamy et al., 2017). This agent is ~3X more potent at suppressing AR transcriptional activity in *AR*-wild type LNCaP cells as compared to enzalutamide and can degrade AR variants, such as the AR-v7 variant, and reduce the growth of AR-v7 expressing prostate cancer cells. The AR PROTAC is a heterobifunctional compound with one end recognizing the ligand-binding domain of AR and the other end recognizing E3 ligase. Binding of this compound to AR brings it into close proximity with an E3 ubiquitin ligase which results in increased ubiquitination and subsequent proteosomal degradation (reviewed in (Toure & Crews, 2016)). AR PROTAC can degrade full length AR and AR mutants such as the F876L and it has increased efficacy over enzalutamide in relation to the suppression of AR transcriptional activity, cell proliferation and induction of apoptosis of prostate cancer cells (Salami et al., 2018). These novel agents are currently being developed for the treatment of castration-resistant prostate cancer.

1.7.2. Selective AR modulators (SARMs)

Given the established role of AR in anabolism, there are several clinical conditions related to muscular-wasting disorders where increased AR signalling may be beneficial. These conditions include sarcopenia and cancer cachexia. Sarcopenia is defined as a decline in skeletal muscle mass and strength as a consequence of ageing and cancer cachexia is atrophy of muscular mass associated with cancer. In both of these clinical conditions, increased AR activity-induced anabolic effect leading to elevations in muscular strength is beneficial (Dalton et al., 2013) and this has led to the development of nonsteroidal SARMs which activate the AR signalling pathway in a tissue-specific manner. These agents allow for the activation of a spectrum of AR activity related to its

anabolic activity and not its undesirable androgenic activity and have high selectivity for muscular over prostatic tissues (Mohler et al., 2009; Negro-Vilar, 1999). SARMs which are in development include enobosarm (GTx®)(J. Kim et al., 2005), BMS-564929 (Bristol-Myers Squibb) (Ostrowski et al., 2007), LGD-0433 (Viking Therapeutics) and RAD140 (Radius Health) (C. P. Miller et al., 2011). These agents have high bioavailability and can be administered orally. Enobosarm is the most clinically-advanced SARM and two phase 3 clinical trials for the treatment of cachexia in patients with lung cancer have been completed (NCT01355497; 01355484).

The exact mechanisms underlying the tissue-specificity of SARMs are unclear but there is evidence to suggest that variation in the recruitment of the co-regulator repertoire in the different tissues could contribute to the tissue-specificity of SARMs. Dalton and colleagues identified that while DHT-bound AR recruited co-activator SRC-1 in a prostate cancer cell line (LNCaP), SARM-bound AR recruited both SRC-1 and co-repressor NCoR, which may account for the blunting of AR transactivation in prostatic tissues exposed to enobosarm (Narayanan et al., 2018). Furthermore, they also identified that SRC-1 was able to mediate transactivation of AR in a mouse muscle cell line (C2C12) when exposed to enobosarm but it failed to mediate DHT-induced AR transactivation in these cells. Overall, these observations suggest that a differential requirement for coactivators may underlie the tissue-specificity observed with SARMs.

In summary, there is a plethora of AR modulators developed with different mechanisms and these are summarized in **Fig. 1.3**. Some of these agents have already been trialled in breast cancer and details of these trials will be provided in the subsequent sections where we review the role of AR signalling in breast cancer.
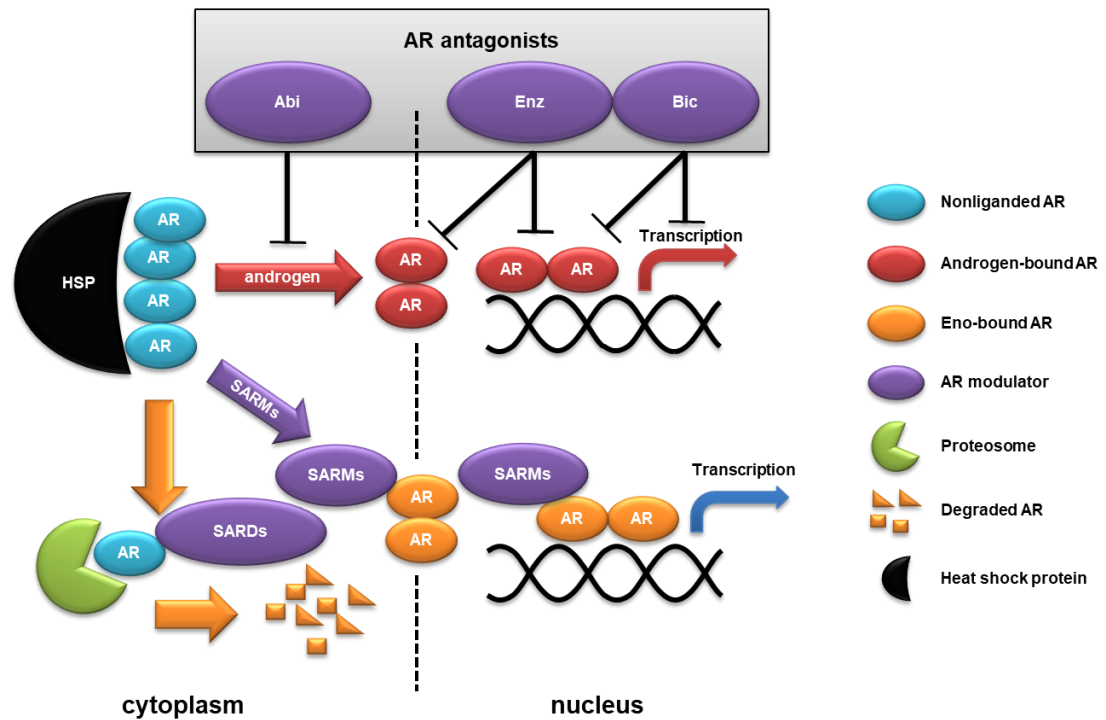
Figure 1.3. Mechanisms of AR modulators.

This schematic illustrates the mechanisms associated with the different modulators of AR. Non-ligand bound AR is chaperoned by heat shock proteins (HSPs) in the cytoplasm. Binding of androgen to AR leads to dissociation from HSPs, homo-dimerization, nuclear translocation, DNA binding and consequently initiation of transcription. AR antagonists such as bicalutamide (Bic) antagonize AR signalling via inhibiting the transcriptional activity of AR whereas enzalutamide (Enz) inhibits AR activity by blocking nuclear translocation and binding of activated AR to the chromatin. Abiraterone acetate (Abi) antagonizes AR signalling through the inhibition of CYP17A1 enzyme which is critical for the biosynthesis of androgens. Selective AR degraders (SARDs) such as UT-155 and AR PROTAC induce the proteosomal degradation of AR. Selective AR modulators (SARMs) such as enobosarm and RAD140 induce transcriptional activation of AR differently from androgens.

1.8. AR in breast cancer

AR is expressed across the major clinical subtypes of breast cancer. It is most highly expressed in the ER+ subtype where ~90% of these tumours demonstrate AR positivity (Collins et al., 2011; Hu et al., 2011; Niemeier et al., 2010; Qi et al., 2012). AR has also been reported to be expressed in ~60-75% of ER-/HER2+ tumours (Micello et al., 2010; Niemeier et al., 2010) and 10-53% in TNBC (Lehmann et al., 2011; Micello et al., 2010; Safarpour et al., 2014). The effect of AR signalling in breast cancer is complex and its signalling consequences are reported to be subtype-dependent. Ligand induced AR activation has been shown to promote proliferation of ER- breast cancer. In contrast, it is associated with growth suppression in ER+ breast cancer (Birrell, Bentel, et al., 1995; Chia et al., 2011; Cops et al., 2008; Lapointe et al., 1999; Ni et al., 2011; J. L. Robinson et al., 2011). The role of AR signalling in endocrine-resistant ER+ breast cancer is controversial and opposing growth-inhibitory and growth-promoting roles of AR have been reported (Birrell, Roder, et al., 1995; De Amicis et al., 2010). A summary of the biology and consequences of AR signalling across the different subtypes of treatment-naive breast cancer will be provided in the following sections.

1.8.1. AR signalling in ER-HER2+ breast cancer

This molecular apocrine subtype of breast cancer is characterized by the ER-AR+HER2+ phenotype, and where AR signalling has been demonstrated to have a prominent role in oncogenesis (Doane et al., 2006; Farmer et al., 2005). These tumours were named molecular apocrine breast cancer due to their histopathological resemblance to the apocrine carcinomas, which also have strong AR expression (Farmer et al., 2005; Sapino et al., 2001). This finding has resulted in a growing interest in targeting AR in this breast cancer subtype.

MDA-MB-453 has been extensively used as a representative molecular apocrine cell line, where AR activation with DHT consistently enhanced cell proliferation. A network of positive feedback loops, leading to the amplification of AR and HER2 signalling pathways, involving CREB1, WNT/beta-catenin and c-Myc have been identified (Chia et al., 2011; Naderi & Hughes-Davies, 2008; Ni et al., 2011; J. L. Robinson et al., 2011). There is functional evidence of cross regulation between AR and HER2 signalling pathways, where inhibition of HER2 abrogated the effects of AR activation-induced growth and inhibition of AR abolished HER2-activated induced growth (Naderi

& Hughes-Davies, 2008). Subsequent work demonstrated a positive feedback loop involving a direct transcriptional upregulation of *HER2* by AR, which results in activated transcription of AR through its downstream mediators involving ERK and CREB1 (Chia et al., 2011). AR signalling can also indirectly feed into HER2 signalling via increasing the transcription of *HER3* which forms a potent HER2 heterodimer. Activated AR induced Wnt7B, a ligand of the Wnt signalling pathway, and can activate β-catenin leading to its nuclear translocation and subsequent activation of *HER3* transcription (Ni et al., 2011). Furthermore, HER2/HER3 heterodimer-induced PI3K signalling can lead to the activation of c-Myc, which is also a direct target of AR, which in turn can augment the AR signalling network and establish an elaborate network of positive regulatory loops with AR as a central node (Ni et al., 2013). These signalling pathways are illustrated in **Fig. 1.4**.
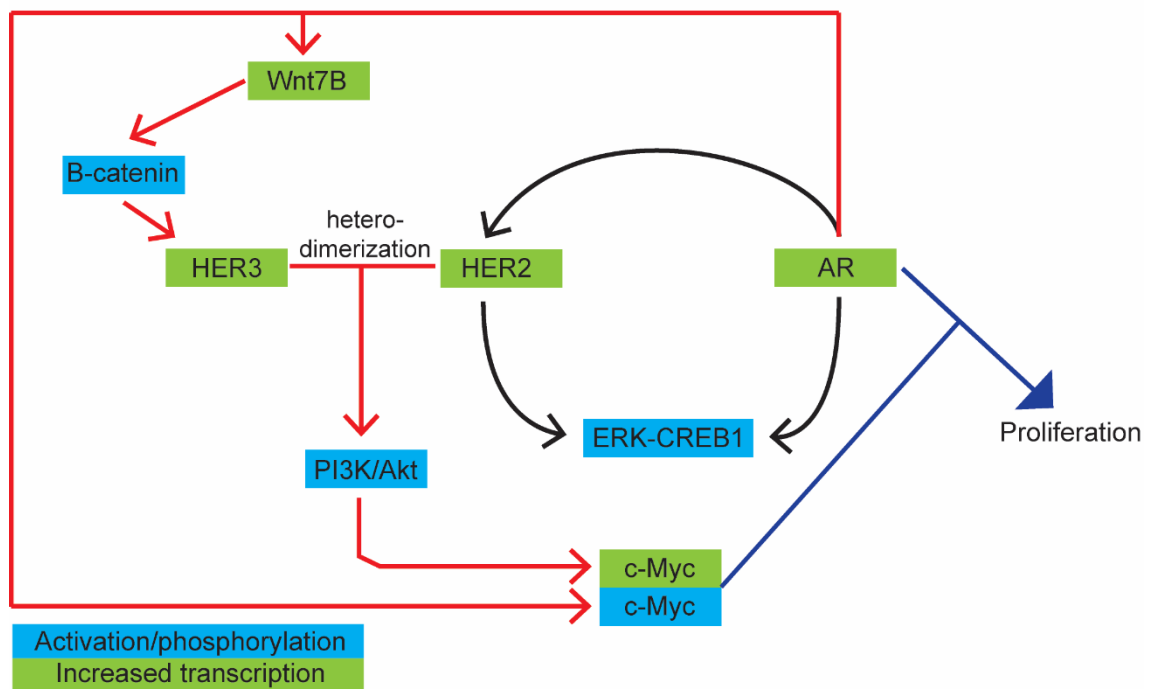
Figure 1.4. Mechanisms of AR-induced growth in molecular apocrine breast cancer.
AR can promote proliferation of ER-HER2+ breast cancer via a series of regulatory loops. These include the increased transcription of HER2 and Wnt7B which leads to the activation of ERK-CREB1 and Wnt7B/β-catenin signalling pathways respectively. Activation of ERK-CREB1 further increases the transcription of AR where activation of Wnt7B/β-catenin increases HER3 transcription which promotes the activation of HER2 signalling. Activated HER2 signalling leads to downstream activity of PI3K/Akt which induces c-Myc activation leading to the amplification of the growth-promoting effect of AR. AR further accentuates this process by directly increasing the transcription of c-Myc.

More recently, contemporary techniques such as chromatin-immunoprecipitation followed by massive parallel sequencing (ChIP-seq) of AR in MDA-MB-453 cells has uncovered a significant overlap between AR binding sites and FOXA1 (Ni et al., 2011; J. L. Robinson et al., 2011). FOXA1 is a pioneer factor which plays a crucial role in mediating ER signalling in ER+ breast cancer (Hurtado et al., 2011). AR similarly utilizes FOXA1 to maintain a gene signature similar to that present in the ER+ breast cancer in the ER- MDA-MB-453 cells.

Preclinical studies using AR antagonists flutamide and bicalutamide have been shown to reduce cell growth in both *in vitro* and *in vivo* molecular apocrine models (Naderi et al., 2011; Ni et al., 2011). Given that the HER2 signalling pathway is the major oncogenic signalling pathway in this subset of breast cancer and anti-HER2 therapies currently the standard of care for these cancers, two studies have demonstrated that enzalutamide further increased the growth-inhibitory effect of HER2 antibody trastuzumab in this subset of breast cancer (Gordon et al., 2017; He et al., 2017). Experimental combination of co-targeting AR and MEK, which is a downstream signalling mediator of HER2 signalling pathway, was also highly effective in reducing tumour growth as compared to its respective monotherapies and this justifies the evaluation of co-targeting AR and HER2 in clinical trials (Naderi et al., 2011). A phase II trial is currently underway to evaluate the efficacy of enzalutamide in combination with trastuzumab in patients with advanced ER-/HER2-amplified/AR+ breast cancer (NCT02091960).

1.8.2. AR signalling in TNBC

AR is expressed in 10% to 53% of TNBC (Ellis et al., 2011; Lehmann et al., 2011; Mouridsen et al., 2003; Piccart et al., 2014). The variability in the frequency of AR expression in these studies can be attributed to the differences in the definition of AR positivity and the small study cohorts. A meta-analysis of 587 TNBCs from 21 breast cancer data sets has led to the identification of a subset of TNBC with a steroid response gene signature, which has been termed the luminal androgen receptor (LAR) subtype (Lehmann et al., 2011). The discovery of the LAR subtype is in agreement with the discovery of inclusion of the ER-/AR+ molecular apocrine breast cancer subtype which also includes non-HER2 amplified ER- breast cancers (Doane et al., 2006; Farmer et al., 2005; Lehmann et al., 2011). The LAR subtype cell lines SUM185PE and CAL-148

were highly sensitive to AR antagonist bicalutamide *in vitro* and *in vivo* (Lehmann et al., 2011). A significant association between activating PIK3CA mutations and AR expression has been identified in the LAR subtype, and an additive growth suppressive effect was observed LAR cell line models treated with a combination of AR and PI3K inhibitors *in vitro* and *in vivo* (Lehmann et al., 2014).

In contrast to the effect of AR antagonists on preclinical LAR models, the effect of DHT is less well understood. Some studies have demonstrated an anti-proliferative response with DHT treatment in MFM223 cells, a cell line sometimes classified as a LAR subtype (Hackenberg et al., 1991; Hickey et al., 2012). There is controversy if this cell line is a true LAR TNBC subtype as it was established from the pleural effusion of a post-menopausal patient who had an ER+/PR+/AR+ cancer (Hackenberg et al., 1991), and ER and PR expression was lost in the later passages. It is intriguing that DHT activation of AR can similarly induce a growth-suppressive effect in the absence of ER expression, and therefore independent of ER signalling.

In summary, AR signalling has functional effects in the LAR TNBC subtype and appears to drive the "luminal" phenotype. Importantly, AR signalling has found to be oncogenic (Lehmann et al., 2011; Lehmann et al., 2014), and coupled with the lack of currently available targeted therapies for TNBC, has provided a strong impetus to evaluate AR-targeted therapies in this subtype. A phase 2 trial of AR antagonist bicalutamide in patients with advanced AR+ (>10%) TNBC demonstrated a 6-month clinical benefit rate of ~20% (95% CI 0.07-0.39) (Gucalp et al., 2013) whereas a more recent phase 2 trial with enzalutamide in a similar patient cohort was associated with a higher clinical benefit rate of 33% at 4 months (Traina et al., 2018). The clinical response achieved with enzalutamide in particular AR+ TNBC is promising and warrants further development of this agent in this subtype of breast cancer and three trials are currently underway as shown in **Table 1.1**.

Therapy	Phase	Breast Cancer Subtype	Sponsor	NCT	Enrolment
Enza plus Taxol	2b	Primary AR+ TNBCs	M.D. Anderson Cancer Centre	NCT02689427	Sep 2016 - Sep 2020
Enza	2	Primary AR+ TNBCs	Memoria Sloan Kettering Cancer Centre	NCT02750358	May 2016 - May 2019
Enza plus BYL719	1	Advanced AR+ PTEN+ TNBCs	M.D. Anderson Cancer Centre	NCT03207529	Sep 2018 - Sep 2020

Table 1.1. Clinical trials assessing enzalutamide (Enza) in TNBCs.

1.9. AR signalling in endocrine-sensitive ER+ breast cancer

AR is expressed in approximately 90% of all endocrine-sensitive ER+ breast cancers (Collins et al., 2011; Niemeier et al., 2010; Qi et al., 2012). High AR expression in ER+ breast cancer is reported to be a good prognostic marker as highly AR positive ER+ breast cancers are often lower grade and have a better clinical outcome (Aleskandarany et al., 2016; Gonzalez et al., 2008; Jiang et al., 2016; Vera-Badillo et al., 2014). Moreover, high AR expression has also been reported to be predictive of endocrine therapy responsiveness (Park et al., 2012) and associated with a response to aromatase inhibitor in post-menopausal women (Macedo et al., 2006). In the latter study, it has been postulated that part of the clinical efficacy of aromatase inhibitor, which reduces the peripheral conversion of androgen precursors to estradiol, can be attributed to the consequential elevation in androgen levels and AR signalling. Collectively, results from these studies are suggestive of a growth suppressive role for AR in ER+ breast cancers.

A majority of the *in vitro* studies examining the effect of androgens on the proliferation of ER+AR+ breast cancers have utilized MCF7, T47D and ZR-75-1 cells. ZR-75-1 cells have the highest level of AR expression followed by T47D and MCF7 (Birrell, Bentel, et al., 1995) and studies with ZR-75-1 and T47D cell lines have consistently demonstrated a growth antagonistic effect associated with activated AR signalling (Birrell, Bentel, et al., 1995; Cops et al., 2008; Lapointe et al., 1999). Both proliferative and anti-proliferative effects of AR activation have been observed in MCF7 cells (Birrell, Bentel, et al., 1995; Greeve et al., 2004; Hackenberg et al., 1988; Macedo et al., 2006) and this disparity in response to androgens can be attributed to the intrinsic heterogeneity of MCF7 cells (Cassanelli et al., 1995), source of cells, differences in growth media and passage numbers which can potentially affect AR expression levels and hence response to androgen stimulation.

Interestingly, in the study conducted by Di Monaco et al they demonstrated a similar growth inhibitory effect with AR activation by DHT and with AR antagonism by hydroxyflutamide, an active AR antagonist metabolite (Di Monaco et al., 1995). Hydroxyflutamide is a well-established AR antagonist but has also been reported to function as an AR agonist by promoting AR binding to the androgen responsive elements and activating transcription (Wong et al., 1995). This balance between the agonistic and antagonistic effects of hydroxyflutamide was postulated to be dictated by

the level of natural competing ligands with an absence of competing ligands rendering hydroxyflutamide an agonist. Therefore, it is plausible that in this study hydroxyflutamide acted in an agonistic manner, similarly to DHT, leading to AR activation and subsequent suppression of growth in these MCF7 cells.

1.9.1. Mechanisms of AR-induced growth suppression

Hereon, we will refer to ligand-activated AR as AR signalling. Several mechanisms by which AR can inhibit ER signalling have been demonstrated and these mechanisms can be broadly divided into ER-dependent and ER-independent mechanisms. In ER-dependent mechanisms, activated AR signalling with DHT has been reported to downregulate ER transcript and protein levels in ZR-75 cells (Poulin et al., 1989). Increased AR activity has also been demonstrated to directly antagonize the process of ER transcriptional activation. Binding to cofactors is a critical step required for the transcriptional activation of ER and the overlap in binding partners for AR and ER can represent a rate-limiting step for the transcriptional activity of ER. For example, ARA70 is a regulatory cofactor which was found to bind both ER and AR with opposing effects on growth (Lanzino et al., 2005). While association of ARA70 with ER was associated with increased growth, its increased association with AR, through ectopic overexpression of AR in MCF7 cells, was associated with growth reduction and loss of ER expression (Lanzino et al., 2005).

Furthermore, ligand-bound AR has also been reported to bind to estrogen responsive elements located in the promoter of ER-regulated gene *PGR* and abolishing E2-induced PGR expression (Peters et al., 2009). Furthermore, given the high overlap in AR and FOXA1 binding sites in the ER- breast cancers and in FOXA1 and ER binding sites in the ER+ breast cancers it is plausible that activated AR may also oppose the effects of ER at these sites although this has yet to be determined (Hurtado et al., 2011; J. L. Robinson et al., 2011). Given these observations, it is plausible that the activation of ligand-bound AR could induce its growth-suppressive effect through sequestration of ER cofactors and ER chromatin binding sites as previously described.

Interestingly, AR signalling has recently been shown to upregulate ER-β which is another ER isoform (Rizza et al., 2014). Exogenous expression of ER-β in ER+ T47D cell line suppressed E2-induced growth (Strom et al., 2004) and analysis of ER-β and

ER-α binding to the chromatin revealed a significant overlap in binding sites (Grober et al., 2011) which may account for the growth antagonism induced by ER-β.

Anti-proliferative effects of AR signalling have also been shown to be mediated through other mechanisms pertaining to signalling pathways, cell cycle and miRNA. AR has been shown to directly upregulate phosphatase and tension homolog (*PTEN*) which is a well-established tumour suppressor and negative regulator of the oncogenic PI3K-Akt signalling pathway (Y. Wang et al., 2011). AR signalling also reduced cyclin D1, a protein required for G1-S cell cycle progression, through AR binding to the cyclin D1 proximal promoter and recruitment of a multiprotein repressor complex including an atypical orphan nuclear receptor DAX1(Lanzino et al., 2010). In a subsequent study, the same authors demonstrated that DAX1 was a direct transcriptional target of AR and AR induced DAX1 also downregulated the expression of aromatase (Lanzino et al., 2013). Given the importance of aromatase in converting estrogen from its androgenic precursors in postmenopausal women, these results provided a novel mechanism by which AR signalling can oppose E2-driven growth in a postmenopausal setting. Lastly, AR signalling can directly reduce the expression of oncogenic mIR-21 through the recruitment of HDAC3 (Casaburi et al., 2016).

Taken together, these results suggest that there are numerous mechanisms, which predominantly revolve around the downregulation of ER signalling, by which AR signalling can antagonize the growth of ER+ cells. The pertinent question that this thesis addresses is whether AR signalling retains these tumour suppressive effects or if it has taken on an oncogenic role, similar to the AR in ER- breast cancer, as some studies suggest. Herein, a review of the literature regarding the role of AR signalling in endocrine-resistant breast cancers will be presented.

1.10. AR signalling in endocrine-resistant breast cancer

The expression of AR is prevalent in metastatic tumours and up to 90% of these tumours originating from AR+ primary tumours that have retained AR (Grogg et al., 2015). Furthermore, increased AR at the RNA (De Amicis et al., 2010) and at the protein levels (Cimino-Mathews et al., 2012) has been reported in metastatic samples relative to primary tumour samples. Multiple *in vitro* studies have also reported an increase in AR expression with the acquisition of endocrine-resistant cell line models which support clinical observations (Ciupek et al., 2015; Fujii et al., 2014; Rechoum et

al., 2014). However, the functional consequences of AR in endocrine-resistant breast cancer are highly controversial with tumour-suppressive and tumour-promoting functions having been reported. This dichotomous role of AR has confounded the type of AR-targeted therapy that should be used in the treatment of endocrine-resistant breast cancer. Notably, a majority of clinical studies have demonstrated tumour-suppression associated with the activation of AR whereas contemporary cell line studies have associated AR activity as a mechanism for overcoming endocrine-resistance. These studies are discussed in detail in the following sections.

1.10.1.   Clinical efficacy of AR agonists in ER+ endocrine-resistant breast cancer

AR agonists such as testosterone and fluoxymesterone, a synthetic testosterone, have historically been utilized as hormonal therapies in patients with metastatic breast cancer in the 1960s (Beckett & Brennan, 1959; Goldenberg, 1964; Kennedy, 1958). However, these patients were non-selected across the different breast cancer subtypes. In agreement with the tumour suppressive effects of AR activation on endocrine-sensitive tumours as described earlier, clinical response rates of between 21.5-37.5% were observed with these agents (Goldenberg, 1964; Kennedy, 1958). While these AR agonists were modestly effective clinically, the discovery of tamoxifen in the 1970s, which demonstrated a superior side-effect profile and improvement in response rates, resulted in the demise of these AR agonists in the treatment of breast cancer (reviewed in (Rose & Mouridsen, 1988)). The success of tamoxifen in breast cancer has also subsequently led to the development of other classes of ER-targeted drugs such as aromatase inhibitors and ER degraders which now represent the standard of care.

The question which arises from the success of these ER-targeted therapies is whether AR activation remains a feasible therapeutic option for ER+ tumours which have acquired resistance to these ER-directed therapies. In a study undertaken by Ingle and colleagues in 1991, they reported that 40% of postmenopausal patients responded to fluoxymesterone after progressing on tamoxifen (Ingle et al., 1991) which suggests that AR signalling retained its tumour suppressive effects in tamoxifen-resistant tumours. More recent retrospective studies have also similarly demonstrated a tumour suppressive effect of activated AR activity in tumours previously exposed to multiple lines of ER-targeted therapies. A study of 53 postmenopausal patients, who have previously failed a median of 3 lines of endocrine therapies including tamoxifen,

aromatase inhibitors and fulvestrant, demonstrated a remarkable clinical benefit rate of 58.5% when treated with testosterone (Boni et al., 2014).

Another recent retrospective study reported that fluoxymesterone treatment in ER+ patients who have also received a median of 3 lines of hormonal therapies, yielded a clinical benefit rate of 43% (Kono et al., 2016). Furthermore, a clinical benefit rate of 35% was observed when 17 patients, who had progressed on prior adjuvant or salvage endocrine therapy were treated with enobosarm (Overmoyer et al., 2015). On the other hand, inhibiting AR with flutamide treatment did not produce any clinical benefit in 13 ER+ endocrine-resistant patients (Perrault et al., 1988). These clinical studies have not been validated in larger prospective trials, but they do suggest that activation and not inhibition of AR was associated with an anti-proliferative effect in endocrine-resistant tumours. Furthermore, these studies also demonstrated that this effect of AR activation was independent of the type of endocrine-therapy the tumours were previously exposed.

1.10.2. Preclinical studies on the role of AR in endocrine-resistant breast cancer

To study the changes acquired in endocrine-resistance, studies often used matching endocrine-sensitive parental and resistant-derivatives of ER+AR+ cell lines such as MCF7, T47D and ZR-75-1 cells to determine the mechanistic changes acquired in the resistant derivatives (Sweeney et al., 2012). Several approaches to establishing endocrine-resistant cells *in vitro* were commonly used and these include; 1) long term culture of cells in the presence of tamoxifen to generate tamoxifen-resistant derivatives (Selever et al., 2011); 2) long term culture of cells in estrogen-low conditions to establish long-term estrogen-deprived cells (LTED) to mimic patients treated with aromatase inhibitor (Song et al., 2001) and 3) genetic manipulation of cells including ectopic overexpression of AR to induce tamoxifen-resistance (De Amicis et al., 2010). Studies which examined the role of AR using these cell line models are detailed in the following sections.

1.10.3.   Role of AR in cell line models of tamoxifen-resistance

The role of AR in contributing to tamoxifen resistance was first described by De Amicis and colleagues (De Amicis et al., 2010). In this study, they discovered that both AR mRNA and protein levels have been found to be higher in metastatic tumours as compared to their tamoxifen-sensitive counterparts. Furthermore, overexpression of AR in MCF7 increased the sensitivity of these cells to the growth-promoting effect of

heregulin, which induced HER2 activation leading to increased phosphorylation of ERK, and it transformed its response to tamoxifen where tamoxifen now behaved as an ER agonist leading to enhanced colony formation (De Amicis et al., 2010).

More recently, an interaction between AR and EGFR was identified to potentiate the growth effects of tamoxifen on estrogen receptor signalling (Ciupek et al., 2015). These results demonstrate that ER response to tamoxifen can be influenced by the level of AR and a high level of AR conferred tamoxifen as an ER agonist. The finding that patients with a high nuclear AR/ER were 2-3 times more likely to progress on tamoxifen further supports the hypothesis that AR influences the ER response to tamoxifen (Cochrane et al., 2014). Given that tamoxifen induced ER-chromatin binding is similar to estradiol (Hurtado 2011), it remains to be determined how exactly AR influences this interaction.

Interestingly, a recent study has reported higher levels of Prosaposin (PSAP) in both tamoxifen and AI-resistant cell lines (Ali et al., 2015). PSAP is a multi-functional protein necessary for intracellular sphingolipid degradation and possesses extracellular neurotropic properties (O'Brien et al., 1994; Sandhoff & Kolter, 2003). Importantly, it is amplified in castration-resistant prostate cancer and has been identified as an activator of AR (Koochekpour et al., 2005). In line with the discovery of increased PSAP levels, AR levels were identified to be higher in tamoxifen and AI-resistant cell lines (Ali et al., 2015). While the authors have only carried out mechanistic studies on the AI resistant cells, which will be detailed in the subsequent section, it is plausible that AR may have a similar oncogenic role in the tamoxifen-resistant setting where PSAP is high. Furthermore, the authors have also reported a correlation between AR, PSAP and its upstream regulator HOXC11 exclusively in the luminal B breast cancer subtype. The combination of AR and PSAP in this subtype was reported to be highly associated with progression on tamoxifen (HR 2.2; $p<0.006$) compared to PSAP (HR 1.6; $p<0.04$) (Ali et al., 2015). It is important that to note that these studies predominantly utilized genetically-modified cell line models and the clinical relevance of these models remains to be determined.

1.10.4. Role of AR in cell line models of AI-resistance

Several independent studies have implicated AR signalling in the acquisition of AI-resistance, and increased PSA levels in recurrent breast tumours have been demonstrated in a small cohort of 21 patients (Ali et al., 2015; Fujii et al., 2014;

Rechoum et al., 2014). These studies have used different approaches to mimic aromatase inhibitor-resistance and the mechanisms by which AR drives resistance in the different cell line models appeared to be heterogeneous in these models. AI-resistant T47D derivatives through continuous exposure to DHT in estrogenic-free culture conditions have demonstrated a complete loss of ER (Fujii et al., 2014). In contrast, an alternative model developed by ectopically overexpressing aromatase and AR in MCF7 cells retained the expression of ER (Rechoum et al., 2014). Other models established include the long-term treatment of aromatase-overexpressing MCF7 cells with anastrozole or letrozole, however, the levels of ER expression in these cells were not reported in the study (Ali et al., 2015).

Critically, these models represent distinct models of AI-resistance. It is important to note that the majority of recurrent breast tumours in patients retain ER expression (Aurilio et al., 2014; Kuukasjarvi et al., 1996). The T47D AI model likely represents the ~24-36% of recurrent breast tumours (Aurilio et al., 2014; Kuukasjarvi et al., 1996) which have lost their ER positivity. This heterogeneity in mechanisms may be attributed to the different cell lines and methodology adopted in developing AI-resistance.

It is plausible that AR in the ER-AR+ T47D AI-resistance model has now acquired the capacity to promote proliferation of these cells and functions in a biologically similar manner to AR in the ER-AR+ molecular apocrine breast cancers (Farmer et al., 2005; Fujii et al., 2014). This is supported by the finding that treatment of these resistant cells with bicalutamide abrogated DHT-induced growth in these cells. Whilst this has not been demonstrated, comparing the AR-chromatin-binding sites in these resistant cells to those of AR-binding sites in the MM453 cells and the ER binding sites in the treatment naïve T47D cells would determine if AR in these resistant cells now bind to "proliferative sites" bound by AR and ER in molecular apocrine breast cancer and ER+ breast cancer, respectively.

On the other hand, ER+AR+ AI-resistance model may represent a distinct mechanism of AR driving AI resistance which may be similar to the role of AR in the tamoxifen resistance as detailed above (Rechoum et al., 2014). Elevated IGF1R level, leading to enhanced downstream Akt signalling was reported to be a major mechanism of resistance in these cells (Rechoum et al., 2014). Elevated IGF1R signalling has also been previously reported to be elevated in MCF7 cells exhibiting tamoxifen resistance

(Massarweh et al., 2008). Increased IGF1R has been demonstrated to induce resistance to endocrine therapies through hyperactivation of downstream MAPK/ERK and PI3K/Akt signalling pathways (Y. Zhang et al., 2011) and both signalling nodes are capable of activating ER signalling in a ligand-independent manner (Bunone et al., 1996; Kato et al., 1995; Simoncini et al., 2000). Given that a positive feedforward loop between IGF1R and AR has been reported to contribute to disease progression in prostate cancer, it is possible that this crosstalk similarly contributes to endocrine-resistance via ligand-independent activation of ER (Fan et al., 2007; Yanase & Fan, 2009). Furthermore, the reported ability of DHT to activate ER, in a non-canonical manner, suggests that AR can contribute to ER signalling independently of estradiol in this setting (Rechoum et al., 2014).

Lastly, the model reported by Ali et al. was characterised by an overexpression PSAP, an activator of AR and activated AR signalling leading to increased migratory and invasive capacity (Ali et al., 2015). Blockade of AR with bicalutamide inhibited cell proliferation in these cells which suggests that AR plays a role in proliferation (Ali et al., 2015). Overall, these observations implicate AR signalling in the acquisition of AI-resistance and that AR can potentially contribute to this process through multiple mechanisms.

1.11. Caveats of cell line work

There are inherent caveats to the use of cell lines as preclinical models. For example, long-term passage of cells under culture conditions can impose a selection pressure on signalling pathways. Transcriptomic analysis of clinical patient samples belonging to 6 cancer types, including breast cancer, with cell lines typically used to represent these cancers, have demonstrated that the transcriptome of these cell lines clustered more closely to other cell lines of a different origin than to the cancers they were supposed to model (Gillet et al., 2011). This suggests that growth of these cells *in vitro* induced activation of similar signalling pathways required for the continued survival of these cells under culture conditions.

Long-term culture can also result in extensive clonal selection and eventual loss in heterogeneity in cell populations (Hait, 2010). This phenotypic drift has been demonstrated in the MCF7 cell line where cells derived from different laboratories have been demonstrated to differ with respect to hormone receptor expression (ER and PR)

levels, copy number alterations and chromosomal makeup (Nugoli et al., 2003; Osborne et al., 1987). Given the strong influence of cell culture on the biology of these "cancer models", it is not unexpected that *in vitro* cell line models are poor predictors of clinical response (Hait, 2010).

Furthermore, there is evidence to suggest that resistance mechanisms identified using cell line models are not clinically relevant. For example, increased upstream growth signalling pathways involving IGFR-1 or HER2, leading to hyperactivity of downstream survival signalling nodes such as MAPK or PI3K pathway, were frequently reported to be contribute to resistance against tamoxifen or long term-estrogen deprivation (Martin et al., 2003; Shou et al., 2004; Stephen et al., 2001; Y. Zhang et al., 2011). However, clinical trials assessing the efficacy of combined inhibition of ER with HER2 (Burstein et al., 2014) or IGF1-R (Robertson et al., 2013) in patients who have previously progressed on an endocrine therapy, were disappointing. Multiple independent studies have suggested that AR signalling is implicated in the growth of endocrine-resistant cells but it is important to note that the majority of these studies utilized genetically-modified cell lines and it remains to be determined how clinically-relevant these models are (Ali et al., 2015; Ciupek et al., 2015; De Amicis et al., 2010; Rechoum et al., 2014). Collectively, these observations highlight the importance of using multiple and more translationally-relevant models of breast cancer to evaluate the resistant mechanisms.

1.12. Clinical relevance of endocrine-resistant patient-derived xenografts

Patient-derived xenograft (PDX) models are surgically-derived human malignant tissues which can maintain a consistent pattern of growth when transplanted into mice. PDX models originating from different cancer types including breast cancer have been developed (reviewed in (Byrne et al., 2017)). The biology of PDX models has been comprehensively characterized in relation to the originating donor samples and it is now well-established that PDXs retain the genomic, transcriptomic and histological characteristics of the tumour of origin (Dobrolecki et al., 2016). Moreover, these PDX models also capture the intra-tumoural clonal heterogeneity that is inherent in the donor tissue (Eirew et al., 2015). Given the significant phenotypic overlap between the tumour of origin and PDX models, it is not unexpected that the response of PDX models is highly concordant with that of the tumour of origin (Marangoni et al., 2007; X. Zhang et

al., 2013). As a result PDX models are now considered the "gold-standard" and are widely used in the preclinical evaluation of novel therapeutics.

Patient samples derived from ER+ patients who have progressed on prior endocrine therapies have also been adopted in the development of PDX models. These treatment-resistant models represent a highly valuable source of "renewable" materials which allow for the evaluation of novel therapeutic strategies in the context of endocrine-resistance, given that these tumours have been exposed to and have progressed on either a single line or multiple lines of endocrine-therapies *in vivo*. Notably, these endocrine-resistant PDX models possess a range of clinically-relevant *ESR1* aberrations such as *ESR1* amplification, hotspot mutations and fusion with *YAP1* (Chandarlapaty et al., 2016; DeRose et al., 2011; Fribbens et al., 2016; Jeselsohn et al., 2015; Li et al., 2013; Niu et al., 2015; Schiavon et al., 2015; Spoerke et al., 2016). A summary of the characteristics of these PDX models pertaining to the type of prior endocrine-therapies, *ESR1* status and growth response to E2 from two studies is provided in **Table 1.2**. Of particular interest is that of *ESR1* mutations which can be detected in up to 40% of endocrine-resistant patients and which represent a major mechanism of clinical resistance against the most commonly used endocrine therapies such as aromatase inhibitors (Chandarlapaty et al., 2016; Fribbens et al., 2016; Jeselsohn et al., 2015; Niu et al., 2015; Schiavon et al., 2015; Spoerke et al., 2016). Importantly, the efficacy of AR modulators on these clinically-relevant models of endocrine-resistance has yet to be reported.

	Model	Prior ET	*ESR1* status	Growth response to estradiol
WHM	9	AI/Ful	WT	enhanced
	16	AI/Ful	AMP	reduced
	18	AI/Ful	*ESR1*-YAP1 fusion	no effect
	20	Tam/AI/Ful	*ESR1* (YS537S)	no effect
	24	Tam/AI/Ful	WT	enhanced
	26	AI/tam	WT	n/a
HCI	HCI-005	Tam/AI/Ful	*ESR1* (L536P)	enhanced
	HCI-011	AI	*ESR1* (G559A)	enhanced

Table 1.2. Examples of endocrine-resistant PDX models.

Details of endocrine-resistant PDX models in relation to their treatment history, *ESR1* status and growth response to estradiol were adapted from (Li et al., 2013) (WHM) and (DeRose et al., 2011)(HCI). AI = aromatase inhibitor, Tam = tamoxifen, Ful = fulvestrant, WT = wild-type and AMP = amplified.

1.13. Justification and aims

ER+ breast cancer constitutes 70% of all breast cancer cases and the development of resistance against standard ER targeted therapies is prevalent and represents a major clinical challenge. AR has emerged as a possible therapeutic target in the treatment of these resistant breast tumours and clinical agents, such as AR agonist enobosarm and AR antagonist enzalutamide, are available. However, the functional consequences of AR signalling are controversial in this setting and the role of AR in endocrine-resistant breast cancer is still unclear (Ali et al., 2015; Boni et al., 2014; Ciupek et al., 2015; De Amicis et al., 2010; Kono et al., 2016; Overmoyer et al., 2015; Rechoum et al., 2014). While it has been reported that increased AR activity antagonizes ER signalling in ER+ endocrine-sensitive cell line models it remains to be determined if this effect exists in the resistant context (Lanzino et al., 2005; Peters et al., 2009; Poulin et al., 1989). Contemporary preclinical studies using predominantly genetically-modified cell line models have reported that inhibition of AR was associated with growth inhibition but the clinical relevance of these observations has yet to be validated in endocrine-resistant PDX models (Ali et al., 2015; Ciupek et al., 2015; De Amicis et al., 2010; Rechoum et al., 2014). Considering that *ESR1* mutations in the ligand binding domain are present in up to 40% of endocrine-resistant breast cancer, the efficacy of AR agonists in tumours harbouring *ESR1* mutations has yet to be reported (Chandarlapaty et al., 2016; Fribbens et al., 2016; Jeselsohn et al., 2015; Niu et al., 2015; Schiavon et al., 2015; Spoerke et al., 2016).

The heavily debated role of AR in ER+ breast cancer has led to widespread confusion about how to clinically implement currently available AR target therapies. This confusion has resulted in clinical trials being conducted simultaneously with drugs that either stimulate or inhibit AR activity, largely predicated on studies that employ poor model systems that do not faithfully recapitulate clinical disease. While there is evidence from clinical studies demonstrating that activating AR in ER+ endocrine-resistant patients, regardless of the type or number of endocrine-therapy they had progressed on, was associated with suppression of tumour growth (Boni et al., 2014; Kono et al., 2016; Overmoyer et al., 2015), trials adopting the opposite approach of antagonizing AR in ER+ breast cancer have also been set up (Krop et al., 2018; O'Shaughnessy et al., 2016) (**Table 1.3**). Thus, it is still unclear what the right therapeutic strategy is to target AR in ER+ breast cancer.

Therapy	Phase	Breast Cancer Subtype	Sponsor	NCT	Enrolment
Enzalutamide	1	Advanced ER+HER- breast cancer	Pfizer	NCT01597193	Apr 2012 - Jan 2018
Enzalutamide plus Exemestane	2	Advanced ER+HER-2 breast cancer	Pfizer	NCT02007512	Dec 2013 - Mar 2019
Enzalutamide plus Fulvestrant	2	Advanced ER+HER-2 breast cancer	University of Colorado	NCT02953860	Jul 2017 - Nov 2018
Enzalutamide plus Fulvestrant	2	Locally advanced ER+ breast cancer	University of Colorado	NCT02955394	Sep 2017 - Nov 2018
Bicalutamide plus aromatase inhibitor	2	Advanced ER+HER-2 breast cancer	Xu fei	NCT02910050	Sep 2016 - Dec 2018
Abiraterone acetate plus exemestane	2	Advanced ER+HER-2 breast cancer	Janssen	NCT01381874	Aug 2011 - Aug 2018

Table 1.3. Recent clinical trials assessing efficacy of AR antagonists in ER+ breast cancer.

The expression of nuclear AR is often used as a proxy for the activation of AR in breast cancer and high AR expression has been associated with a better patient outcome in primary breast cancer (Aleskandarany et al., 2016; Gonzalez et al., 2008; Jiang et al., 2016; Vera-Badillo et al., 2014). Importantly, it is unclear which genes are directly regulated by AR in the context of endocrine-resistant breast cancer and how these genes are related to outcome in ER+ primary breast cancer. Lastly, there is some evidence to suggest that AR signalling may be implicated in other tumourigenic processes such as invasion and metastasis (Ali et al., 2015; Feng et al., 2017).

Given the above, the general aims of this thesis are

Aim 1 (Chapter 3)

To investigate the effect of antagonizing endogenous AR using *in vitro* cell line models using a combination of siRNA-mediated and pharmacological antagonism approach and to validate the efficacy of AR antagonism in an endocrine-resistant PDX model.

Aim 2 (Chapter 4)

To assess the efficacy of DHT and enobosarm on the growth of endocrine-resistant PDX models, harbouring either a wild-type or hotspot mutation in *ESR1,* and the impact of these agents on ER signalling in these models.

Aim 3 (Chapter 5)

To elucidate and understand the biology of AR-regulated genes in an endocrine-resistant PDX and determine if these genes are prognostic in ER+ primary breast cancer.

Aim 4 (Chapter 6)

To evaluate the consequences of AR activation on invasion and on spontaneous metastasis in a clinically-relevant breast cancer model *in vivo*.

CHAPTER 2

2. Materials and Methods

2.1. *In vitro* experiments

2.1.1. Cell line culture and reagents

Breast cancer cell lines were obtained from different sources. The suppliers of the base media and additives used for the culture of these cell lines are listed in **Table 2.1**. A summary of the source of the cell lines and their growth conditions is listed in **Table 2.2.** Notably, there are two batches of endocrine-resistant cell line used. The first batch of MCF7 tamoxifen resistant (TamR) and MCF7 long term estrogen deprived (LTED) cells were obtained from the Brown laboratory based at the Dana-Farber Cancer Institute (Boston). The second set of endocrine-resistant MCF7 cells were obtained from the Caldon laboratory at the Garvan Institute of Medical Research (Sydney). All cell lines have been verified to be of MCF-7 origin through short tandem repeat (STR) profiling and were routinely assessed for mycoplasma contamination at the Garvan Molecular Genetics (GMG) Facility. Cells were cultured in a humidified incubator at 37°C with 5% CO_2. For passaging, cells were first washed with phosphate-buffered saline (PBS; #14190144, ThermoFisher) before 0.05% Trypsin/EDTA (ThermoFisher) was added to fully submerge the cells. Trypsin then was fully aspirated and the cells were incubated at 37 °C for 5 min. Cells were resuspended in complete growth media and transferred to a new flask at a split ratio of 1:5-10. Post revival, cell lines were only maintained up to 10 passages before a new vial was revived. Cells were cryopreserved in 10% dimethyl sulfoxide (DMSO, Sigma) in full growth media at a density of 1 x 10^6 cells per mL in cryovials (Corning). Cryovials were then placed in Styrofoam racks in -80℃ to freeze the cells. Revival of cells was performed by first thawing the cells in a 37℃ water bath and then dispensing these cells (1 mL) into 9 ml of full media in a 15 ml tube. Tube was then centrifuged at 2,000 rpm for 2 min to pellet the cells. Supernatant was aspirated and cells were resuspended in full media and transferred to an appropriate vessel for culture.

Item	Catalogue #	Supplier
RPMI1640	#11835030	ThermoFisher
Phenol-red free RPMI1640	#11835030	
Charcoal-stripped serum (CSS)	#12676029	
Hepes	#15630106	
Tamoxifen	T176	Sigma
17β-estradiol (E2)	E2758	
Fetal bovine serum (FBS)	SH30406.02	GE Healthcare
Insulin	#60726	Novo Nordisk

Table 2.1. Suppliers of base media and additives

Cell line	Source	HR status	Base media	Supplements	Tam
Endocrine-sensitive MCF7	Michigan Cancer Foundation	ER+PR+	RPMI1640	10% FBS, 20 mM Hepes 0.28 IU/mL insulin	
MCF7 TamR	Brown laboratory	ER+PR-	RPMI1640	10% FBS, 20 mM Hepes 0.28 IU/mL insulin	5 μM
MCF7 LTED		ER+PR+	Phenol-red free RPMI1640	10% Charcoal-stripped serum (CSS) 20 mM Hepes 0.28 IU/mL insulin	
Parental MCF7	Caldon laboratory	ER+ PR+	Phenol-red free RPMI1640	5% CSS & 1 pM E2	
MCF7 TamR		ER+ PR-			1 μM
MCF7 LTED		ER+ PR+		5% CSS	
T47D	ATCC		RPMI1640	10% FBS & 20 mM Hepes	

Table 2.2. Source, hormone receptor (HR) status and growth conditions of cell lines

2.1.2. Drug treatment

Cell lines were treated with AR agonists 5α-dihydrotesterone (DHT) and enobosarm (Eno), AR antagonist enzalutamide (Enz) and PI3K inhibitor GDC-0941. The source, solvent used for resuspension, stock and final concentrations of these agents are listed in **Table 2.3**.

Name	Class	Source	Solvent	[Stock]	[Final]
DHT	AR agonist	Sigma (#A8380)	Ethanol	1 µM	1 nM
Enobosarm	AR agonist	GTx®	Ethanol	100 µM	100 nM
Enzalutamide	AR antagonist	Medivation	Dimethyl sulfoxide	10 mM	10 µM
GDC-0941	PI3K inhibitor	Selleck (#S1065)	Dimethyl sulfoxide	10 µM	5-10 nM

Table 2.3. Detail of agents used for in vitro experiments

2.1.3. siRNA transfection

AR knockdown (KD) was performed using two different small interfering (siRNA) oligos: ARsiRNA A (ARsiA) 5'-GGAACUCGAUCGUAUCAUU-3' (#4390824; ID 1539, ThermoFisher) and AR siRNA B (ARsiB) 5′-CAGUCCCACUUGUGUCAAA-3′ as previously described (Y. Wang et al., 2011) (Sigma). Control cells were transfected with nonsense siRNA (NS) (#4390843, ThermoFisher). Reverse transfection of these siRNAs was carried out using Lipofectamine^{TM} RNAiMAX (#137781509, ThermoFisher) as per manufacturer's instructions at a final siRNA concentration of 10 nM in 6 cm^2 plate. Briefly, 1.25 µL of 20 µM siRNA and 2.5 µL Lipofectamine^{TM} were each made up to 250 µL volumes with Opti-MEM^{TM} (#11058021, ThermoFisher) and incubated for 5 min at room temperature. The two solutions were mixed and incubated at room temperature for another 20 min prior to the addition to 200k cells in 2 mL of antibiotic-free full media in 6 cm^2 plates. Growth media was replaced the following day and cells were harvested at the indicated time points or trypsinized and re-seeded.

2.1.4. CrispR-based AR knockout

T47D cells were seeded into 6-well plates and were transfected when confluency reached 80%. Knock out of AR in these cells was achieved by transfecting them with AR Double Nickase Plasmid (#SCZSC-400026-NIC-2, Santa Cruz) and control cells were transfected with the Control Double Nickase Plasmid (#SCZSC-437281, Santa Cruz). Transfection of these plasmids was carried out using the Lipofectamine^{TM} 3000 Transfection Reagent (L3000015, ThermoFisher) as per manufacturer's instructions. Briefly, 3.75 µL of Lipofectamine^{TM} was mixed with 125 µL of Opti-MEM and incubated at room temperature for 5 min. After which, it was added to 125 µL of Opti-MEM containing 1 µg of plasmid (AR Double Nickase or Control) and 2 µL p3000 Solution. Then this solution was added to each well with 2 mL of growth media. After 2 days, media was replaced and 2 µg/mL puromycin (Sigma) was added to select for positively-transfected cells. Media with puromycin was replaced every 2 days and cells were selected in puromycin for a total of 6 days after which the cells were cultured in growth media in the absence of puromycin. Non-transfected T47D cells were used as a positive control for the effect of puromycin at this dose and all non-transfected cells were dead after puromycin selection for 6 days.

2.1.5. AlamarBlue cell viability assay

This assay was carried out using AlamarBlue cell viability assay (#DAL1100, ThermoFisher Scientific) according to manufacturer's instructions in 96-well format. Typically, 2-4k cells were seeded into each well of a 96-well plate in 100 µL of growth media. Media was gently aspirated the next day and replaced with media containing 1x concentration of indicated treatment regimes in 200 µL volumes. Media was replaced with fresh media and 1x treatment regime every 2-3 days. On day of harvest, media was gently replaced with 100 µL of fresh media with 10% AlamarBlue solution. Plates were incubated at 37 ^{o}C for 3h prior to the detection of fluorescence using FLUOstar OPTIMA (BMG Labtec) at excitation and emission wavelengths of 544 nm and 590 nm respectively. For each assay, 6-8 technical repeats were performed for each condition and experiments were repeated 3 times.

2.1.6. Cell count assay

50k cells were seeded into 6 cm^2 plates in 2 mL media and growth media replaced with 2 mL of fresh media containing 1x concentration of indicated treatment regime. Media was replaced every 2-3 days accordingly. Cells were washed once with PBS (ThermoFisher), trypsinized and then resuspended in 1 mL of full media. Cell suspension was gently vortex and 50 μL of cells was mixed with an equal amount of trypan blue (#15250061, ThermoFisher) and 10 μL of this mixture was loaded onto the Countess^{TM} Cell Counting Chamber Slides (#C10312, ThermoFisher) and cell number quantified using the Countess Automated Cell Counter (ThermoFisher).

2.1.7. Colony forming assay

This assay was performed in 12-well plates with cells seeded at a density of 3k cells per well. Cells were seeded in their own growth conditions and were treated the following day at a final volume of 1 mL per well in 12-well plates. Media was completely replaced every 2-3 days and cells were harvested after 9 days of treatment. At harvest, media was gently aspirated and cells were stained in 1 mL of crystal violet staining solution [0.05% w/v crystal violet (#C0775, Sigma), 1% formaldehyde (#28906, Pierce), and 1% methanol in PBS] for 20 min at room temperature following by 2 min washes in a bucket of running water. Plates were air-dried overnight. Images were scanned using the Perfection V800 Photo scanner (Epson) with a DPI of 1200 dots per inch (DPI). Area occupancy was analysed using an ImageI plugin, ColonyArea (Guzman et al., 2014), and results were expressed relative to controls.

2.1.8. Propidium-iodide based fluorescence activated cell sorting (PI-FACS)

Cells were cultured and treated in 6 cm^2 plates for this assay. At the point of harvest, supernatant was transferred to a 10 mL tube and adherent cells were washed once with ice cold PBS prior to trypsin treatment for 3 min at 37℃. Cells were resuspended in 700 μL PBS and 300 μL ice cold high-grade ethanol (100%) in a 1 mL tube and fixed overnight in -20℃. To prepare the cells for PI staining, cells were pelleted at 3,000 rpm at room temperature for 3 min, supernatant aspirated and then resuspended in 1 mL PBS. Cells were pelleted again and then resuspended in 0.5 mL PI staining solution comprising of 2 μg/mL PI (#P4864, Sigma) and 500 μg/mL RNase A (R6148, Sigma) in PBS. Cells were passed through a 35 μm mesh (#35225, Corning) to reduce

aggregation of cells prior to fluorescence analysis on the BD FACSCanto^{TM} II (Becton Dickinson). Quantification of proportion of cells in the different cell cycle phases was performed on the ModFit LT^{TM} (Verity) and processing of the individual cell cycle plots were performed on FlowJo^{TM} v10 (FlowJo LLC).

2.1.9. Subcellular fractionation

Separation of the nuclear and cytoplasmic fractions was performed on cells seeded in 10 cm^2 plates using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (#78833, ThermoFisher). The protocol used was a modified version of the manufacturer's instructions. Cells were first washed in ice-cold PBS and then resuspended in 200 µL of cytoplasmic extraction buffer I (CER I). Resuspension was vortexed at the highest setting for 15 sec and then incubated on ice for 10 min. 11 µL of CER II was added to the resuspension and the mixture was vortexed at maximum speed for 5 sec. Cell debris was pelleted by centrifuging the resuspension at maximum speed for 5 min at 4℃. 100 µL of cytoplasmic extract from the top of the supernatant was transferred to a new tube. The remaining supernatant was aspirated and the pellet was subjected to washes with 100 µL of CER I and then twice with 1 mL of PBS. The pelleted nuclei were resuspended in 100 µL of nuclear extraction buffer (NER), vortexed at maximum speed for 15 sec and then subjected to 2 cycles of 10 min incubation on ice and 15 sec vortex. Finally, the resuspension was centrifuged at maximum speed for 15 min after which 90 µL of the nuclei extract was transferred to a new tube.

2.2. *In vivo* experiments

2.2.1. Animals, surgical procedure for PDX implantation

Animal experiments were undertaken with the excellent assistance of Rhiannon Coulson and Aliza Yong. Animal procedures were performed on the approved Garvan Animal Ethics Committee Protocol 15/25. Mice of the NOD-scid IL2Rg^{null} (NSG) background from the Australian BioResources (Garvan Institute for Medical Research) were used for the propagation of PDX tumours. ER+ endocrine-resistant PDX models evaluated include Gar15-13, which was developed in-house under the Human Research Ethics Committee (HREC) approved protocol at the St Vincent's Hospital (HREC/16/SVH/29), and the previously-described HCI-005 model which was developed at the Welm laboratory (Huntsman Cancer Institute, University of Utah) (DeRose et al., 2011).

All surgical procedures were carried out on a heat pad and after mice were completely anaesthetised, with 3-4% of isoflurane, as determined by a lack of footpad pinch reflex. Systemic and local analgesia was achieved with subcutaneous injection of 5 mg/kg ketoprofen and topical application of bupivacaine respectively. External small incisions (<1 cm) proximal to the 4th inguinal fat pad were made with the tip of micro-dissecting scissors. The skin above the incision site was lifted using a pair of curved forceps and micro-dissecting scissors were used to separate the skin from the connective tissues. Once a "pocket" has been created and with the skin still lifted, the 4th mammary fat pad was located and gently pulled out such that the fat pad was partially exposed from the incision site. Once exposed, the tip of forceps with sharp edges at 45℃ (#5/45, Dumont) were used to create a "pocket" in the tip of the mammary gland and a small chunk of PDX tumour (~ 2 mm^{3}) was inserted into this "pocket", after which, the exposed gland was released and gently guided back inward. An estrogen pellet was inserted subcutaneously, when necessary, via the incision site before it was sealed with an Autoclip® wound clip (BD Primary Care Diagnostics). Post-operative local analgesia was achieved with the topical application of 100 µL of 8 mg/kg bupivacaine. Mice were removed from the heat pad and transferred to their respective housing boxes once they had recovered.

2.2.2. Surgical procedure for intraductal injection

On the day of injection, MCF7 TamR and LTED cells were trypsinized and resuspended in their respective full media, at a concentration of 1.25×10^7 cells per mL, and kept on ice. NSG mice used for these intraductal experiments were age-matched within experiments and were typically between 6-12 weeks old at the time of injection. Procedure was performed on a heat pad and mice were completely anaesthetised, with 3-4% of isoflurane, as determined by a lack of footpad pinch reflex. Systemic analgesia was achieved with subcutaneous injection of 5 mg/kg ketoprofen. The area around the 4th mammary pad was completely shaven with a shaver and cleaned with ethanol. Using a pippete, 10 µL Trypan Blue solution (T8154, Sigma) was dropped around the nipple area and the area which excluded the blue dye indicated the location of the nipple. Once the nipple had been identified, a fine tweezer was used to lift up the skin underneath the nipple such that the nipple was now protruding. A pair of spring scissors was used to trim the top of the nipple off while the nipple was still being lifted. Once the top of the nipple has been snipped off, the duct was stained with Trypan Blue solution by dropping 5 µL of Trypan Blue dye on top of it and ethanol was used to clear excess Trypan Blue solution covering the skin. A 1x cell injection solution was made up which comprised of 80% cells and 20% Trypan Blue solution. Lifting up the nipple with a fine tweezer, 10 µL of cell solution with 100k cells was injected straight into the exposed duct using a customized 15 µL Hamilton syringe with 0.5 cm blunt end needle. Successful injection of cells into the mammary ducts rendered the mammary ducts blueish upon examination. This procedure is illustrated in **Fig. 2.1**. Mice were removed from the heat pad and transferred to their respective housing boxes once they had recovered.
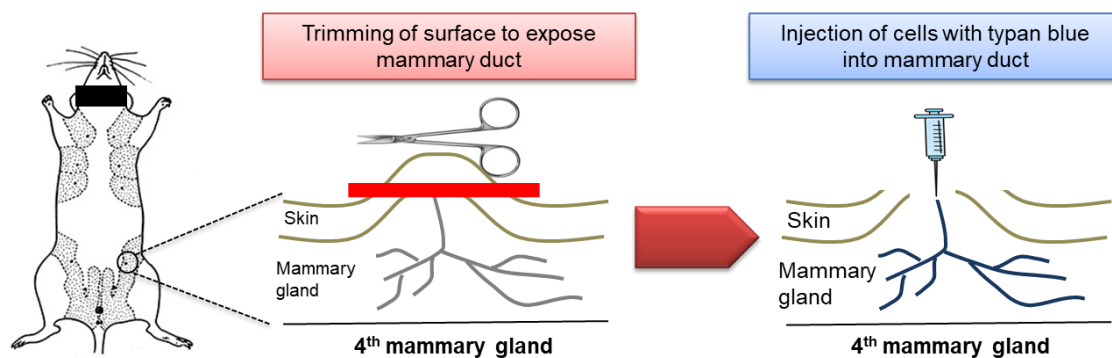
Figure 2.1. Schematic of the intraductal injection technique

2.2.3. Treatment of tumour-bearing mice

Electronic callipers were used to measure the tumours twice weekly when they became palpable and tumour volumes were calculated based on the formula Length x Width x Width x 0.5 (Marangoni et al., 2007). Mice were randomized to the different treatment arms when tumours reached 150-200 mm^3 using an online Prism tool (https://www.graphpad.com/quickcalcs/randomize1.cfm). The therapeutic agents used in the treatment of the PDX models include E2, DHT, Eno and Enz and information pertaining to the dose, mode of administration, source and solvent used for resuspension of these agents are listed in **Table 2.4**. Enobosarm was first resuspended in 100% TWEEN®-80 (Sigma) in a 10 mL tube and incubated in a 60℃ water bath for 30 min. After which it was vortexed at maximum speed for 30 sec and the appropriate amount of Baxter water was added to achieve 25% TWEEN®-80. The resuspension was re-incubated in the water bath for another 30 min and any remaining undissolved enobosarm was fully resuspended into solution by pipetting. Enzalutamide was resuspended in 25% TWEEN®80 and sonicated using Bioruptor® (Diagenode) at medium setting for 5 mins at 30 on/off intervals to achieve a homogenous suspension.

Name	Dose	Mode of administration	Source	Others
E2	0.72 mg	subcutaneous implantation	Innovative Research of America (#NE-121)	90-day slow-release
DHT	10 mg	subcutaneous implantation		60-day slow-release
Eno	10 mg/kg **daily**	oral gavage	Selleck (S1065)	25% TWEEN®-80
Enz	20 mg/kg **daily**	oral gavage	Medivation	25% TWEEN®-80
Ful	200 mg/kg **weekly**	subcutaneous injection	MedChemExpress (HY-13636)	Peanut oil

Table 2.4. Detail of agents used for *in vivo* experiments

2.2.4. Tumour harvest

Mice were euthanized by cervical dislocation when the tumours reached the ethical or predefined endpoint. After weighing the tumour, a quarter of the tumour was chopped into small chunks and snap-frozen on dry ice whereas another quarter was fixed overnight at 4℃ in 10% neutral-buffered formalin (Australian Biostain). The remaining half of the tumour was embedded in cryoprotective optimal cutting temperature (OCT) compound and snap-frozen on dry ice. All frozen samples were kept at -80℃. Formalin was replaced with 80% ethanol and tumour samples were kept in the fridge at 4℃ for ~ a week and then sent to the Histology Facility (Garvan Institute) for tissue processing and paraffin embedding.

2.3. Protein analysis

2.3.1. Protein extraction and quantification

Cell pellets were lysed in 1x radioimmunoprecipitation assay buffer (RIPA;10 mM Tris-HCl pH 8.0, 1 mM ethylenediaminetetraacetic acid, 1% Triton-X-100, 0.1% sodium-deoxycholate, 0.1% sodium dodecyl sulfate, and 140 mM sodium chloride) supplemented with 1X Halt™ Protease and Phosphatase Inhibitor Cocktail and 50 mM EDTA (#78440, ThermoFisher). Bio-Rad Protein Assay Dye Reagent (#5000006, Bio-Rad) (Bradford, 1976). Protein standards were home-made by dissolving bovine serum albumin (#7906, Sigma) in water to a final concentration of 1 mg/mL. A standard curve with 0, 5, 10, 15, 20 μg/μL of protein was constructed for protein estimation of unknown samples and 200 μL of samples and standards were loaded onto a 96-well plate and absorbance was read at wavelength of 595 nm using FLUOstar OPTIMA (BMG Labtec). Samples were typically diluted to 0.5-1.5 μg/μL in 4x Laemmli sample buffer (#1610747, Biorad) and 1x RIPA buffer.

2.3.2. Immunoblotting

20-30 μg of protein samples and 5 μL of Precision Plus Protein™ Dual Color Standards (Biorad, #1610374), were loaded into each well of a mini-protean TGX gel (Biorad #456-1085) and separated in TruPage SDS Express Running Buffer (Sigma, PCG3003) at 80V for 10 min and then at 130V for another hour using the PowerPac™ HC Power Supply (#1645052, Biorad). Separated proteins were transferred electrophorectically onto 0.45 μm polyvinlidene (PVDF) Immobilon®-FL membrane (#1PFL00010, Millipore) for an hour at 100 V in transfer buffer (10.5% glycine, 2.25% trisaminomethane and 20% methanol in distilled water). Membranes were blocked in Odyssey® blocking buffer (#927-40000, LI-COR) for 20 min at room temperature before overnight incubation with primary antibodies in Odyssey® blocking buffer at 4℃. The details of these antibodies pertaining to their supplier, species in which they were raised in and dilution factor are listed in **Table 2.5**. After overnight incubation, the membranes were washed in PBS for 9 min and PBS was replaced every 3 min. The primary antibodies were detected with IRDye® 680RW donkey anti-mouse (#926-68072, LI-COR) or IRDye® 800CW donkey anti-rabbit (#926-32213, LI-COR) antibodies. These antibodies were diluted 1:20,000 in Odyssey® blocking buffer and membranes were incubated with the secondary antibodies for 30 min at room

temperature. The secondary antibodies were detected using the Odyssey® CLx Imaging System (LI-COR). The final images were processed in Adobe Photoshop CS6.

Cell Signalling			
AR (N-terminal)	5153	rabbit	1:1000
Phospho-AKT (s473)	9271	rabbit	1:500
total Akt	2920	mouse	1:500
ER-α	8644	rabbit	1:1000
Phospho-ERα (serine 118)	2511	mouse	1:1000
FoxO3a	2497	rabbit	1:1000
GAPDH	5174	rabbit	1:1000
Histone 3	9715	rabbit	1:1000
phosho-histone 3 (serine10)	9701	rabbit	1:500
PR	3153	rabbit	1:1000
PTEN	9559	rabbit	1:1000
Abcam			
FOXA1	23738	rabbit	1:1000
AR (C-terminal)	52615	rabbit	1:1000
Lamin A	8980	mouse	1:1000
Santa Cruz			
Cyclin A	596	mouse	1:200
Sigma			
Alpha-tubulin	T9026	mouse	1:5000

Table 2.5. Details of primary antibodies used for immunoblotting

2.3.3. Densitometric analysis

ImageJ was used to measure the density of the blots and to allow for a more quantitative comparison of blots. The density of bands of interest is normalized to that of a loading control (eg. GAPDH) and data is represented as fold change from the control group. A description of the methodology can be found at the following link (https://di.uq.edu.au/community-and-alumni/sparq-ed/sparq-ed-services/using-imagej-quantify-blots).

2.3.4. Immunofluorescent staining

MCF7 TamR cells grown on poly-L-lysine-coated coverslips were treated with vehicle or 10 μM enzalutamide for 48 h in the absence or presence of 10 nM DHT. Coverslips were fixed in ice-cold methanol overnight at -20℃. Cells were immunostained with primary antibodies against AR (#5153, Cell Signaling), ER (#M7047, DAKO) and alpha-tubulin (#T6199, Sigma). Fluorescent secondary antibodies were used to detect the primary antibodies (1:250) and DNA was counterstained with 4,6-diamidino-2-phenylindole (DAPI;1:250;#62248,ThermoFisher). Coverslips were mounted onto glass slides with ProLong^{TM} Gold Antifade Mountant (#36930, ThermoFisher) and microscopy was carried out using the Automated Upright Microscope System (Leica). DAPI staining was used to define nuclear regions and fluorescent nuclear ER signals were quantified to assess changes in nuclear ER in the different conditions using ImageJ.

2.3.5. Carmine whole mount staining

Euthanized mice were placed on a dissecting board with the ventral side facing upwards and the extremities pinned onto the board. A small incision was made between the hips using a pair of fine scissors followed by the use of blunt scissors inserted into the cut to separate out the skin from the peritoneum. A mid-line cut was performed using blunt scissors and the skin was pinned onto the dissecting board to reveal the intact mammary glands. Using tweezers to hold onto the connective tissues, the mammary glands were gently lifted away from the skin and then separate from the skin using a pair of fine scissors. The mammary glands were carefully placed on a slide and the mammary glands were flattened by evenly spreading out the connective tissues. The slides with the glands were then placed and fixed overnight in 10% neutral-buffered formalin (ThermoFisher) in a slide holder. Carmine staining of these mammary glands was performed with the following steps;

Day 1: Soaked in acetone for 6 h with fresh acetone replaced after every 2 hours and then incubated in fresh acetone overnight

Day 2: Soaked in 70% ethanol for an hour followed by a quick rinse in distilled water and then soaked in Carmine alum solution [0.2% w/v Carmine (Sigma), 0.5%(w/v) aluminium potassium sulfate (Sigma), 1 thymol crystal (Sigma)] overnight

Day 3: Rinsed in distilled water following by immersion in 70% ethanol and 95% ethanol for 2 h each and then in 100% ethanol overnight

Day 4: Soaked in SlideBrite (Pangalark Laboratory Technology) for 1 h and then stored in methyl salicylate (ThermoFisher Scientific)

Imaging of the carmine staining was carried out on the Leica Application Suite Software on the Leica MZ12 microscopy system. Reversal of carmine staining was achieved with the following steps;

Day 1: Soaked in 100% ethanol

Day 2: Soaked in 95% ethanol and then 75% ethanol for 2 h each and then storing the mammary glands in 70% ethanol prior to processing them for paraffin embedment.

2.3.6.   Immunohistochemical staining

Immunohistochemical staining was performed at the Dame Roma Mitchell Cancer Research Laboratories by Marie Pickering, Geraldine Laven-Law and Zoya Kikhtyak. The details pertaining to the source, species and dilution for the primary antibodies used are listed in **Table 2.6**. Formalin-fixed and paraffin-embedded tissues were cut at 2 μm and melted onto slides. Slides were incubated at 125 °C for 5 min and then cooled to 90°C for 10 sec in a 10 mM citrate buffer for antigen retrieval. An Avidin/Biotin Blocking Kit (I#004304, ThermoFisher) and goat serum (G9023, Sigma) was used to block non-specific binding. Slides were incubated overnight at 4°C in a humid chamber. The following day, slides were incubated for 1h at room temperature with the appropriate goat anti-mouse biotinylated IgG (ER, Ki67, SEC14L2; Agilent) or goat anti-rabbit biotinylated IgG (AR; Agilent), followed by a Streptavidin-HRP (Agilent) incubation to amplify signal. 3, 3'-diaminobenzidine (Sigma) was used to visualize and detect the secondary antibody. Tissues were then counterstained with dilute Lillie-Mayer's haematoxylin (Australian Biostain), dehydrated, cleared, mounted and scanned using the Nanozoomer S360 (Hamamatsu). Images were processed using the NDP Viewing software (Hamamatsu). Quantification of intensity of IHC images was performed using ImageJ.

Primary antibodies for IHC				
Name	**Supplier**	**Catalog**	**Species**	**Dilution**
AR	Santa Cruz	SC-816	rabbit	1:1000
SEC14L2		SC-271902	mouse	1:1000
FKBP5		SC-271547	mouse	1:1000
ER-α	Agilent	M7047	mouse	1:300
Ki-67		M7240	mouse	1:400
BCL2	DAKO	#M0887	mouse	1:400
PR	Novocastra (A/B)	#NCL-L-PGR-AB	Mouse	1:1000

Table 2.6. Details of primary antibodies used for IHC

2.3.7. Quantification of Ki-67

IHC images of Ki-67 at 80 x were processed using the NDP Viewing software and 3-4 random images, representative of the overall Ki-67 staining pattern or intensity, were saved as JPEG files. These files were then processed in ImageJ and manual cell counting was performed using the ImageJ plugin Cell Counter (https://imagej.nih.gov/ij/plugins/cell-counter.html). Proliferation index was determined by calculating the percentage of Ki-67 positive cell and >1,000 cells from at least three random 80x magnification fields were counted for each tumour (Dowsett et al., 2011). Intensity of Ki-67 was performed by first converting the JPEG files to 8-bit images and then quantified using the "Measure" function.

2.4. Molecular Analysis

2.4.1. RNA extraction

RNA was extracted from snap frozen cell line pellets and tumour chunks using RNeasy Plus Mini Kit (#74134, Qiagen) as per manufacturer's instructions. Prior to RNA extraction from the frozen tumour chunks, they were first homogenized in 0.5 mL Soft tissue homogenizing CK14 tubes (KT03961-1-203.05, Bertin Instruments) using the Precellys 24 tissue homogenizer (P000669-PR240-A, Bertin Instruments) on the standard settings. Briefly, appropriate amount of Buffer RLT was added to the cell line pellets or homogenized tumour lysates and the supernatant was transferred to a gDNA Eliminator spin column in the supplied 2 mL collection tube. The tubes were centrifuged for 30 sec at maximum speed in a centrifuge at room temperature. Equivalent amount of 70% high-grade ethanol was added to the flow-through and the solution was transferred to an RNeasy spin column attached to a 2 mL collection tube. The tubes were centrifuged at maximum speed for 15 sec after which the flow-through was discarded. The columns were subsequently subjected to a series of washes with 700 µL of Buffer RW1 (twice) and 500 µL of Buffer RPE. For each wash, the tubes were centrifuged at maximum speed for 15 sec after which the flow-through was discarded. At the end of the last wash with Buffer RPE, new collection tubes were placed under the spin columns and these columns were centrifuged at maximum speed for 2 min. Lastly, 30-40 µL of RNAase-free water was added to the spin columns and centrifuged at maximum speed for 2 min to re-suspend the purified RNA.

2.4.2. Real time-quantitative PCR (RT-qPCR)

The quality and quantity of RNA were assessed using NanoDrop^{TM} (ThermoFisher). The ratio of absorbance at 260 nm to 280 nm (A260/280) for these samples was assessed as a measurement of RNA purity and only samples with A260/280 ratio of ~2 were used for downstream RT-qPCR analysis. 1-2 µg of RNA was reverse-transcribed to complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor, in 20 µL volumes, as per manufacturer's instructions (#4374967, ThermoFisher). cDNA samples were diluted to 60 µL prior to RT-qPCR reactions. Transcripts of interest were detected with either the Taqman^{TM} system (ThermoFisher) on the ABI PRISM$^{®}$ 7000 cycler (Perkin-Elmer Applied Biosystems) or

with the iQ^{TM} SYBR^{TM} Green$^{®}$ system (Biorad) on the CFX384 Touch^{TM} cycler (Bio-Rad). For both systems, assays were carried out in 10 µL volumes in 384-well plates and 2-3 technical reactions for performed for each assay. The setup of each assay using either the Taqman Gene Expression Master Mix (#4369016, ThermoFisher) or the iQ^{TM} SYBR Green supermix (#1708880, Bio-Rad) per 10 µL reaction volume and the cycling conditions are shown in **Table 2.6** and **Table 2.7** respectively. The probes and primers used in this thesis are listed in **Table 2.8.** The cycle threshold (Ct) value of genes of interest was normalized to that of housekeeping gene (*GAPDH*) for RT-qPCR performed on RNA extracted from cell lines. For RT-qPCR performed on RNA extracted from tumour samples, the Ct value of genes of interest was normalized to the mean of two housekeeping genes (*PUM1* and *IPO8*). This Ct value for each gene of interest post normalization is referred to as delta Ct (ΔCt),

ΔCt = Ct (gene of interest) – Ct (housekeeping gene)

The expression of genes in treatment groups relative to the control group was calculated using the delta delta Ct (ΔΔCt) calculation as per the formula,

ΔΔCt = ΔCt (treated group) – ΔCt (control group)

Finally, the relative fold change in linear phase was computed based on the formula 2 to power of negative ΔΔCt ($2^{-\Delta\Delta Ct}$).

Taqman^{TM}		iQ^{TM} SYBR Green®	
TaqMan^{TM} Gene Expression Master Mix	5 µL	iQ^{TM} SYBR Green Supermix	5 µL
Taqman probe	0.5 µL	Forward primer	0.5 µL
		Reverse primer	0.5 µL
cDNA	1 µL	cDNA	2 µL
Rnase-free water	3.5 µL	Rnase-free water	2 µL
Total	10 µL	Total	10 µL

Table 2.7. Set up for RT-qPCR reactions

ABI PRISM® 7000			CFX384 Touch^{TM}		
Temp	**Time**	**# cycles**	**Temp**	**Time**	**# cycles**
2 min	50°C	1	95°C	3 min	1
10 min	95°C	1	95°C	15 sec	40x
15 sec	95°C	40x	55°C	15 sec	
1 min	60°C		72°C	30 sec	

Table 2.8. Cycling conditions for RT-qPCR

Taqman™ Probes	
AR	Hs00907244_m1
CTGF	Hs00170014_m1
CXCL8	Hs00174103_m1
ESR1	Hs01046816_m1
GAPDH	Hs03929097_g1
PGR	Hs01556702_m1
PTEN	Hs02621230_s1

Primers for iQ™ SYBR Green system		
AR	Forward	CCACTTGTGTCAAAAGCGAAAT
	Reverse	CCACTTGTGTCAAAAGCGAAAT
BCL2	Forward	ACATCGCCCTGTGGATGACT
	Reverse	GGGCCGTACAGTTCCACAAA
CCND1	Forward	CAGAGGCGGAGGAGAACAAA
	Reverse	AGGGCGGATTGGAAATGAACT
ESR1	Forward	CACATGATCAACTGGGCGAA
	Reverse	CGCCAGACGAGACCAATCA
FKBP5	Forward	AAAAGGCCAAGGAGCACAAC
	Reverse	TTGAGGAGGGGCCGAGTTC
IPO8	Forward	GGCACCACTCAGCGAGGAT
	Reverse	AAGGTGAAGCCTCCCTGTTG
PR	Forward	CGCGCTCTACCCTGCACTC
	Reverse	TGAATCCGGCCTCAGGTAGTT
PUM1	Forward	AGCAAGGACAGCAGCAGGTT
	Reverse	CAGCTGCCACAAGGGGAT
SEC14L2	Forward	GCCGAATCCAGATGACTATTTTCT
	Reverse	GATGTTGTCAATGTCCTTTTGCTT

Table 2.9. Probes and primers used for RT-qPCR

2.4.3. Chromatin immunoprecipitation-PCR

The constituents of the buffers used for this procedure are listed in **Table 2.10**. Transfection of MCF7 TamR cells with NS or AR siA was performed in 15 cm^2 plate in biological triplicates. Protein-DNA complexes were crosslinked in 1% high grade formaldehyde (#28906, ThermoFisher) in RPMI media for 10 min. Following which, formaldehyde was quenched with the addition of glycine at a final concentration of 125 mM for 5 min at room temperature. Cells were washed twice with ice-cold PBS and cells in each plate were harvested in 500 μL of PBS supplemented with 1x protease inhibitor and cells were transferred to 15 mL tube. Cells were pelleted at 2,000 rpm and snap frozen overnight. In parallel, 100 μL Dynabeads beads (ThermoFisher) were washed three times in ice cold 0.05% BSA in PBS (BSA/PBS), re-suspended with 5 μg of IgG (SC-2027, Santa Cruz) or ER antibody (SC-543, Santa Cruz) in 500 μL of cold BSA/PBS and then incubated overnight at 4℃ on a rotator. The next day, the samples were sequentially lysed with LB1, LB2 and LB3. After which the samples were transferred to 1.5 TPX microtubes (M50050, Diagenode) and sonicated 30 times on a 30 sec on and off cycle on the Bioruptor® Pico (Diagenode).

Buffers	Constituents
LB1	50mM HEPES-KOH, pH 7.5
	140mM NaCl
	10% glycerol
	1mM EDTA
	0.5% NP-40
	0.25% Triton X-100
LB2	10mM Tris-HCl, pH 8.0
	200mM NaCl
	1mM EDTA
	0.5mM EGTA
LB3	10mM Tris-HCl, pH 8.0
	100mM NaCl
	1mM EDTA
	0.5mM EGTA
	0.1% Na-Deoxycholate
	0.5% *N*-laurylsarcosine
RIPA	50mM HEPES-KOH, pH 7.5
	500mM LiCl
	1mM EDTA
	1% NP40
	0.7% Na-Deoxycholate
Elution	50mM Tris-HCl, pH 8.0
	10mM EDTA
	1% SDS

Table 2.10. Constituents of buffers used for ChIP-PCR

Samples were diluted 1:10 with 1% Triton-X and 1x protease inhibitor in LB3 buffer and then centrifuged at maximum speed for 10 min at 4℃. An aliquot of each sample was kept aside as "input" sample. The remaining of the samples was equally divided and incubated with either IgG-bound beads or ER-bound beads overnight on a rotator at 4℃. The next day, the beads were washed 6 times with ice-cold RIPA buffer and once with TE buffer (pH 8.0) after which 200 μL of elution buffer was added to the beads and samples were incubated overnight at 65^0C. The following day, 200 μL of supernatant was transferred to a fresh Eppendorf tube and 200 μL of TE buffer added to each sample. Depletion of RNA and protein was achieved with the addition of RNase A (40 μg/mL) and Proteinase K (400 μg/mL) respectively. Contaminating proteins were removed using the green 5PRIME Phase Lock Gel (Quantabio) as per the manufacturer's instructions. DNA was purified via ethanol immunopreciptation overnight at -80, resuspended in 40 μL UltraPure Dnase/Rnase-free water

(ThermoFisher) and quantified using Qubit^{TM} dsDNA HS assay (#Q32851, ThermoFisher). Equivalent volume of DNA from each chromatin-immunoprecipitated sample was used for the subsequent RT-qPCR using the iQ^{TM} SYBR Green system. The primers were used to assess occupancy on the chemokine (C-X-C motif) ligand 8 (*CXCL-8*) gene promoter and the intergenic control regions are listed in **Table 2.11**. For each chromatin precipitated sample, ER occupancy at the *IL8* promoter was represented as fold change post normalization to the intergenic control.

Location	Type	Sequence
Distal	Forward	AAGCCCTGGACAAATATACT
	Reverse	CTATTCAATGACATCTGTGGTT
Proxmial	Forward	ATAAGTTCTCTAGTAGGGTGATGA
	Reverse	TGCTCTGCTGTCTCTGAA
Intergenic control	Forward	GGAACAGGCAACACATAA
	Reverse	CTCATACATATCTCTTAGGTCAG

Table 2.11. Primers used to assess ER binding in *CXCL8* promoters

2.5. Gene expression profiling

2.5.1. Microarray

All subsequent bioinformatics analyses were performed by Dr Heloisa Milioli.

RNA extracted from 3 biological replicates of MCF7 TamR cells, transfected with NS control or AR siRNA A, were sent to the Ramaciotti Centre for Genomics Facility (UNSW) for microarray-based gene expression profiling. Quantify control check performed on these samples indicated that the A260/280 ratio for all samples was ~1.8. Microarray hybridization was performed on the Affymetrix PrimeView™ Human Gene Expression Array. CEL files were further processed in the R software (v 3.4.3) environment using affy (Gautier et al., 2004) and limma (Ritchie et al., 2015) packages from Bioconductor (Gentleman et al., 2004). The CEL files and normalized expression matrix have been uploaded onto Gene Expression Omnibus (GEO, accession number 115270). Normalized log2 probe signals were computed using robust multi-array average (RMA) (Irizarry et al., 2003). The subsequent differential expression vectors between AR knockdown and control samples were set for each gene at a q-value threshold of 0.05. Probes were then annotated using the Affymetrix Archived NetAffx Annotation Files for PrimeView.

2.5.2. Next generation sequencing and gene expression analysis

Integrity of the RNA extracted from the short-term treated PDX tissues were assessed using the Experion™ RNA StdSens Analysis Kit (700-7111, Biorad) on the Experion™ Automated Electrophoresis System (Biorad). This analysis give an RNA Quality Indicator (RQI) score of between 1 to 10, with 1 indicative of highly degraded RNA sample and 10 indicative of intact RNA of the highest integrity. All the RNA extracted from the PDX samples had RQI ≥ 9. Quantitation of RNA was achieved with Nanodrop™ 2000 (ThermoFisher). 2 μg RNA of each sample was supplied to the Genomics Core Facility at the South Australian Health and Medical Research Institute (SAHMRI) where the samples were processed and sequenced. Conversion of the RNA into sequencing libraries was performed using the TruSeq® Stranded mRNA Library Prep kit (#20020594, Illumina) and these libraries were sequenced on the NextSeq (Illumina) with 30 million single-end reads targeted for each sample. Processing of the sequencing data generated from the high-throughput sequencing was carried in *R*. The FASTQ files were aligned to the HG38 human genome using the *Spliced Transcripts*

Alignment to a Reference (*STAR*) aligner (v2.4). Differential expression analyses between control and treated samples were performed using *edgeR* (v3.20.1) (M. D. Robinson et al., 2010) and *limma* (v3.34.1) (Ritchie et al., 2015) packages and significant differentially expressed genes were defined at a threshold of q-value of <0.05.

2.5.3. Gene set enrichment analysis (GSEA) & enrichment map analysis

GSEA was carried out by comparing differentially expressed genes against Hallmark, Curated and Gene Ontology gene sets from the Molecular Signature Database (MSigDB) (Subramanian et al., 2005). Two approaches to perform GSEA were undertaken. The first approach was performed using the "Investigate Gene Set" function embedded within the online GSEA portal (http://software.broadinstitute.org/gsea/msigdb/annotate.jsp) and it examined the overlap of a pre-defined list of genes against published gene set. The second approach required the ranking of all the differentially expressed genes identified in the RNA-seq using the signal to noise metric and processing of this "ranked" list of genes in the GSEAPreranked tool (http://www.broad.mit.edu/tools/software.html)(Paltoglou et al., 2017).

The output file from the GSEAPreranked tool with an .rpt extension was used as the source file for enrichment map analysis and this was also processed on the GSEA software which provided an output file in CytoScape (http://www.cytoscape.org).

2.5.4. Development a survival-related AR gene signature

The identification of an AR gene signature which was highly associated with survival was derived from DHT-induced genes in Gar15-13 using a previously reported approach (Tishchenko et al., 2016). This methodology involves a series of filtering steps including 1) gene matching, 2) identification of cancer-specific genes and 3) estimation of the prognostic value of each gene. The Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset, which constitutes 1230 cases of clinically-annotated ER+ luminal breast cancer patients with long-term survival information, was used to investigate the prognostication potential of the DHT-induced gene set.

CHAPTER 3

3. Inhibition of AR in endocrine-resistant breast cancer

3.1 Introduction

The AR is a steroid hormone receptor that is important in the development of male-specific phenotype. AR has a well-established function as a ligand-activated transcription factor involved in the regulation of target genes but it also has non-canonical signaling actions which are independent of its ligand-driven transcriptional activity (Zarif & Miranti, 2016). AR signaling plays a critical role in the progression of prostate cancer and inhibitors of AR are a mainstay for treating advanced stages of this disease. These anti-androgenic therapeutic agents may have broader clinical utility, as AR signaling has been implicated in the pathogenesis of other malignancies such as ovarian teratocarcinoma (Chung et al., 2014) and some sub-types of breast cancer (Chia et al., 2015; Hickey et al., 2012).

Breast cancer is a highly heterogeneous disease comprised of several major immunohistochemical subtypes. About 70% of all cases are classified as estrogen receptor positive (ER+) and the remaining 30% estrogen receptor negative (ER-) breast cancers are sub-classified into two broad categories: HER2-enriched subtype that is human epidermal growth factor receptor 2 positive (HER2+) and triple-negative breast cancer (TNBC), which lacks expression of all three biomarkers (ER, progesterone receptor (PR) and HER2). AR is expressed across all subtypes of breast cancer and approximately 90% of ER+ (Niemeier et al., 2010), 60-75% of HER2+ (Micello et al., 2010; Niemeier et al., 2010) and 10-36% of TNBC (Micello et al., 2010; Niemeier et al., 2010) are immunopositive for AR.

The consequences of AR activity are subtype-dependent. In the ER+ breast cancer, multiple *in vitro* studies have reported a growth inhibitory effect of 5 alpha-dihydrotestosterone (DHT), the most potent cognate ligand for AR, in endocrine-sensitive ER+AR+ cell line models of breast cancer (Greeve et al., 2004; Lapointe et al., 1999; Macedo et al., 2006), and this effect is reported to be mediated via antagonism of ER signaling (Lanzino et al., 2005; Lapointe et al., 1999; Macedo et al., 2006; Peters et al., 2009). These studies suggest that AR activity is tumour suppressive in ER+ breast cancer, a concept supported by clinical studies showing that AR expression is an independent prognostic marker of good outcome in ER+ breast cancers (Aleskandarany

et al., 2016; Ricciardelli et al., 2018). In contrast, AR activity is associated with increased proliferation in the ER- breast cancer (Chia et al., 2011; Lehmann et al., 2011; Ni et al., 2011; J. L. Robinson et al., 2011)

Standard-of-care endocrine therapy for ER+ breast cancer includes selective ER modulators (SERMs) such as tamoxifen, aromatase inhibitors (AIs) that inhibit peripheral estrogen biosynthesis, and selective ER degraders (SERDs) such as fulvestrant. While endocrine therapies have improved the survival of patients with early-stage ER+ disease, approximately one third of women will eventually acquire resistance to endocrine therapy, leading to disease progression and death (EBCTCG, 2005). The expression of AR is reported to be higher in endocrine-resistant breast cancer but the role of AR in these tumours is controversial (De Amicis et al., 2010; Fujii et al., 2014).

AR activity has been reported to promote proliferation, as growth suppression of endocrine-resistant cells was observed with the use of AR inhibitors such as bicalutamide and enzalutamide (Ali et al., 2015; Ciupek et al., 2015; D'Amato et al., 2016; De Amicis et al., 2010; Rechoum et al., 2014). These preclinical studies have contributed to the initiation of clinical trials with AR inhibitors including enzalutamide in endocrine-resistant breast cancer (ClinicalTrials.gov NCT Identifiers 02953860, 02007512 & 02580448). However, it is noteworthy that these studies largely utilized genetically-modified MCF7 cell line models of endocrine-resistance, including ectopic overexpression of AR (De Amicis et al., 2010) or knockdown of Rho GDP dissociation inhibitor (GDI) (Ciupek et al., 2015) in MCF7 cells to mimic tamoxifen resistance. MCF7 cells with ectopic overexpression of aromatase with or without ectopic AR overexpression were used as models of aromatase inhibitor-resistance (Ali et al., 2015; Rechoum et al., 2014). There is only one study utilizing non-genetically modified cell line models of endocrine-resistance which demonstrated that enzalutamide inhibited *in vitro* and *in vivo* growth of tamoxifen-resistant (TamR) MCF7 cells (D'Amato et al., 2016). Critically, the effect of AR antagonism on an endocrine-resistant patient-derived xenograft (PDX), now considered the most clinically relevant immunodeficient pre-clinical disease model, has yet to be reported.

Hence, the specific aims of this chapter are to

a. Understand the functional role of endogenous AR by knocking down AR using an siRNA-mediated approach in endocrine-resistant breast cancer cell line models

b. Assess the efficacy of clinical AR antagonist enzalutamide in cell line and PDX models of endocrine-resistance

3.2 Results

3.2.1 Expression levels of hormone receptors and pioneer factor FOXA1 in Myles Brown's endocrine-resistant cells

To assess the role of AR in endocrine-resistant cells, we used tamoxifen-resistant (TamR) and long-term estrogen deprived (LTED) cells obtained from Myles Brown of the Dana-Farber Cancer Research Institute. These cells have not been genetically-modified and were developed through long-term exposure to tamoxifen and estrogen deprived media, respectively.

We first evaluated the expression of steroid hormone receptors and the critical pioneer factor FOXA1 (Hurtado et al., 2011) in the endocrine-resistant cells. Western blots were performed to compare ER, AR, PR and FOXA1 levels relative to endocrine-sensitive (ES) MCF7 cells (**Fig. 3.1a**). ER expression was retained in the endocrine-resistant models and was slightly increased in the TamR cells compared to the ES and LTED cells. AR expression was increased in both endocrine-resistant models relative to ES cells, with higher levels in the LTED model (**Fig. 3.1a**). In the TamR cells, PR expression was lost and FOXA1 expression was increased relative to ES cells, consistent with previously published data (Fu et al., 2016).

Next, we validated the resistance of MCF7 TamR cells to the growth-inhibitory effect of tamoxifen. Intriguingly, while tamoxifen antagonized the viability of ES MCF7 cells by ~ 25%, and this modest growth inhibitory effect is consistent with the high serum concentration used in the culture of these cells (Butler et al., 1981), it stimulated rather than inhibited the growth of MCF7 TamR cells (Fig. 3b). The growth-agonistic effect of tamoxifen in MCF7 TamR cells has been reported previously by other groups (Ciupek et al., 2015; Jeselsohn et al., 2017).
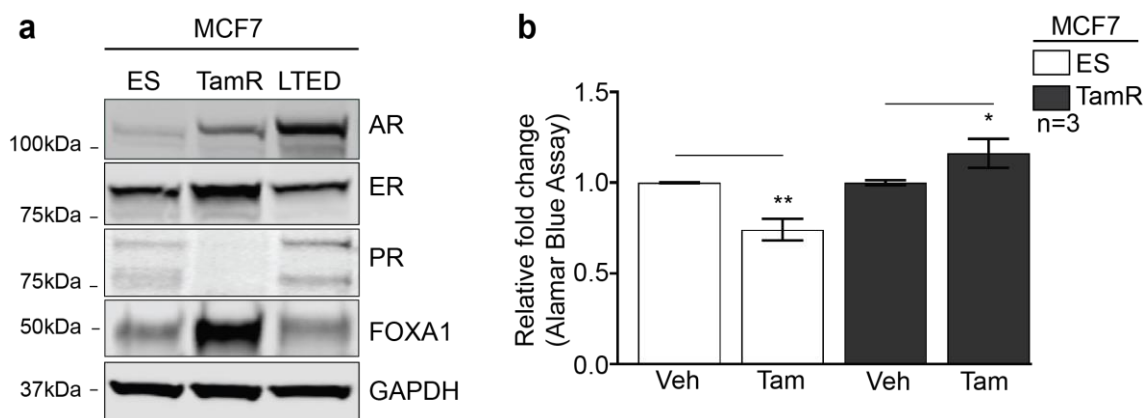
Figure 3.1 Expression levels of hormone receptors and pioneer factor FOXA1 in endocrine-resistant relative to endocrine-sensitive (ES) MCF7 cells.

a) Expression of steroid hormone receptors including AR, ER, PR and FOXA1 in ES, tamoxifen-resistant (TamR) and long-term estrogen deprived (LTED) MCF7 cells by immunoblotting. **b)** Evaluation of tamoxifen (Tam) response in ES and TamR MCF7 cells using AlamarBlue assay. Cells were treated with 5μM Tam for 4 days and viability of Tam-treated cells was compared to vehicle (Veh)-treated cells. * p <0.05 and ** p<0.01 using Student's t-test. Error bars = SEM from 3 biological replicates.

3.2.2 AR knockdown reduces proliferation of endocrine-resistant cells

To assess the effect of AR loss on proliferation of these endocrine-resistant cells, we performed transient knockdown of AR using two specific AR siRNAs (AR siA and siB). Both resulted in reduction of AR transcript and protein levels as demonstrated by real-time quantitative PCR (RT-qPCR) and immunoblotting respectively (**Fig. 3.2a, b**). AR knockdown significantly reduced proliferation of TamR and LTED cells relative to nonsense (NS) siRNA-transfected control cells, as determined by cell counting and Alamar Blue viability assays (**Fig. 3.2c, d**). These observations support a mitogenic role for AR in endocrine-resistant breast cancer.
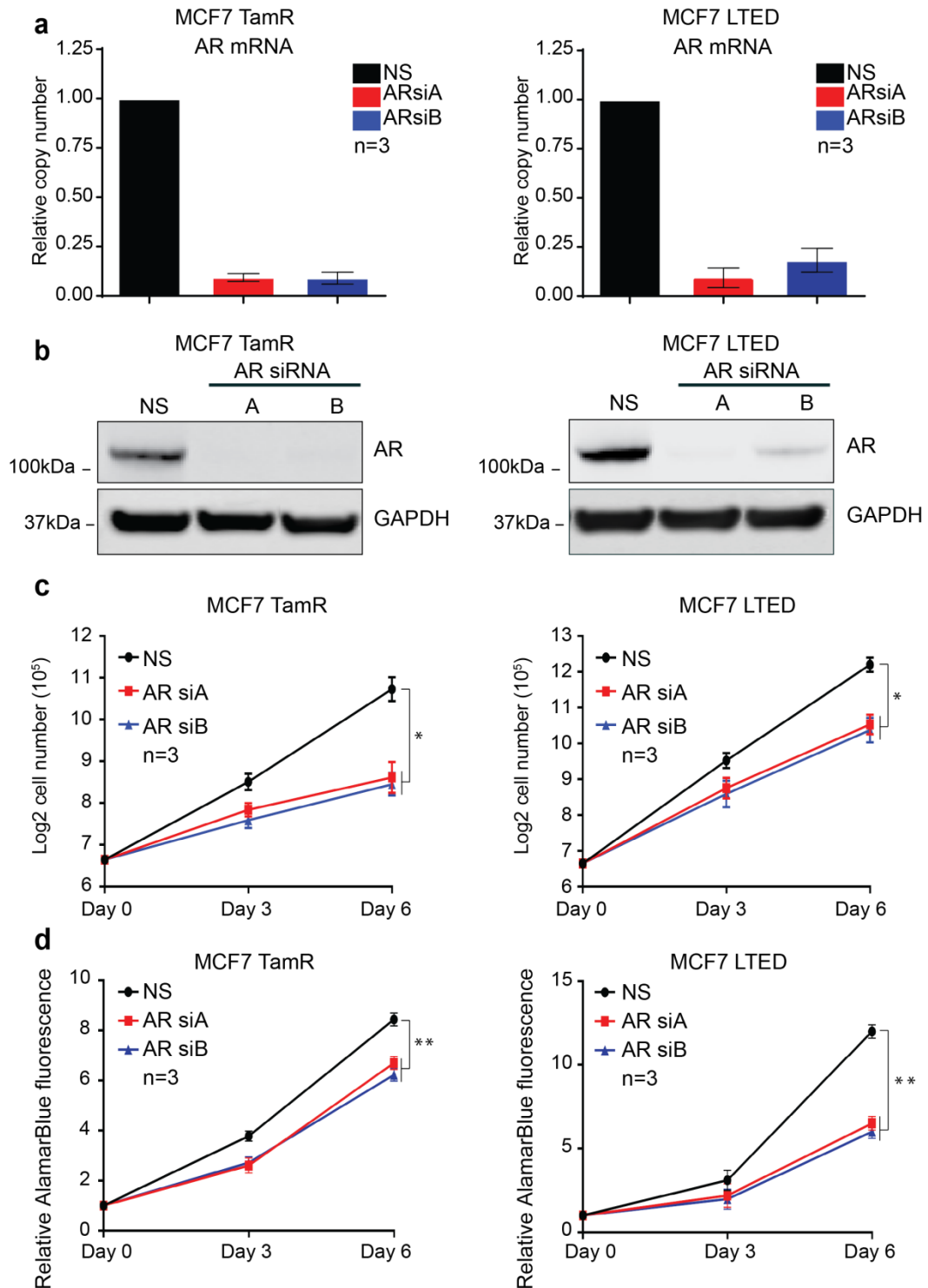
Figure 3.2. AR knockdown reduces proliferation of ER+/AR+ endocrine-resistant cells.
TamR and LTED cells were transfected with either nonsense (NS) siRNA or 2 AR-specific siRNAs (ARsiA and ARsiB) for 4 days prior to harvest. Knockdown at the message and protein levels was confirmed using **a)** real-time quantitative PCR (RT-qPCR) and **b)** immunoblotting. **c)** The effect of transient AR knockdown on the growth of TamR and LTED cells was assessed 3 and 6 days post-transfection using cell count (**c**) and AlamarBlue assay (**d**). * p <0.05, ** p<0.01 using Student's t-test. Error bars = SEM from 3 biological replicates.

3.2.3 AR knockdown restores characteristics of classical ER signalling in MCF7 TamR cells

To identify AR-regulated genes, global gene expression profiling was performed post-transient knockdown of AR in MCF7 TamR cells. Gene expression profiling of MCF7 TamR cells transfected with nonsense siRNA and AR siRNA A was performed in three biological replicates and validation of efficient AR knockdown two days post transfection with AR siRNA A was first confirmed at the transcript and protein levels prior to gene expression profiling. A schematic summarizing the experimental design is outlined in **Fig. 3.3a**. RT-qPCR and immunoblotting analysis indicated that AR was efficiently knocked down at both the mRNA and protein levels 2 days post transfection in all 3 biological replicates (**Fig. 3.3b, c**). Interestingly, ER protein levels were increased post AR knockdown (**Fig. 3.3c**). Microarray profiling was performed on these samples and principal component analysis (PCA) indicated uniformity of the samples as the samples clustered in a siRNA-specific manner (**Fig 3.3d**). Expression profiling identified 3,274 differentially expressed genes (**differential gene set**) at a false discovery rate (q-value) <0.05 with roughly equal number of up- and down-regulated genes. A heatmap of the top 50 differential genes revealed C-X-C motif ligand 8 (*CXCL-8*) and connective tissue growth factor (*CTGF*) as the top two downregulated genes (**Fig. 3.3e**). Of note, the overexpression of these genes, under the aberrant regulation by ER, has recently been reported to drive tamoxifen-resistance in breast cancer (Fu et al., 2016).
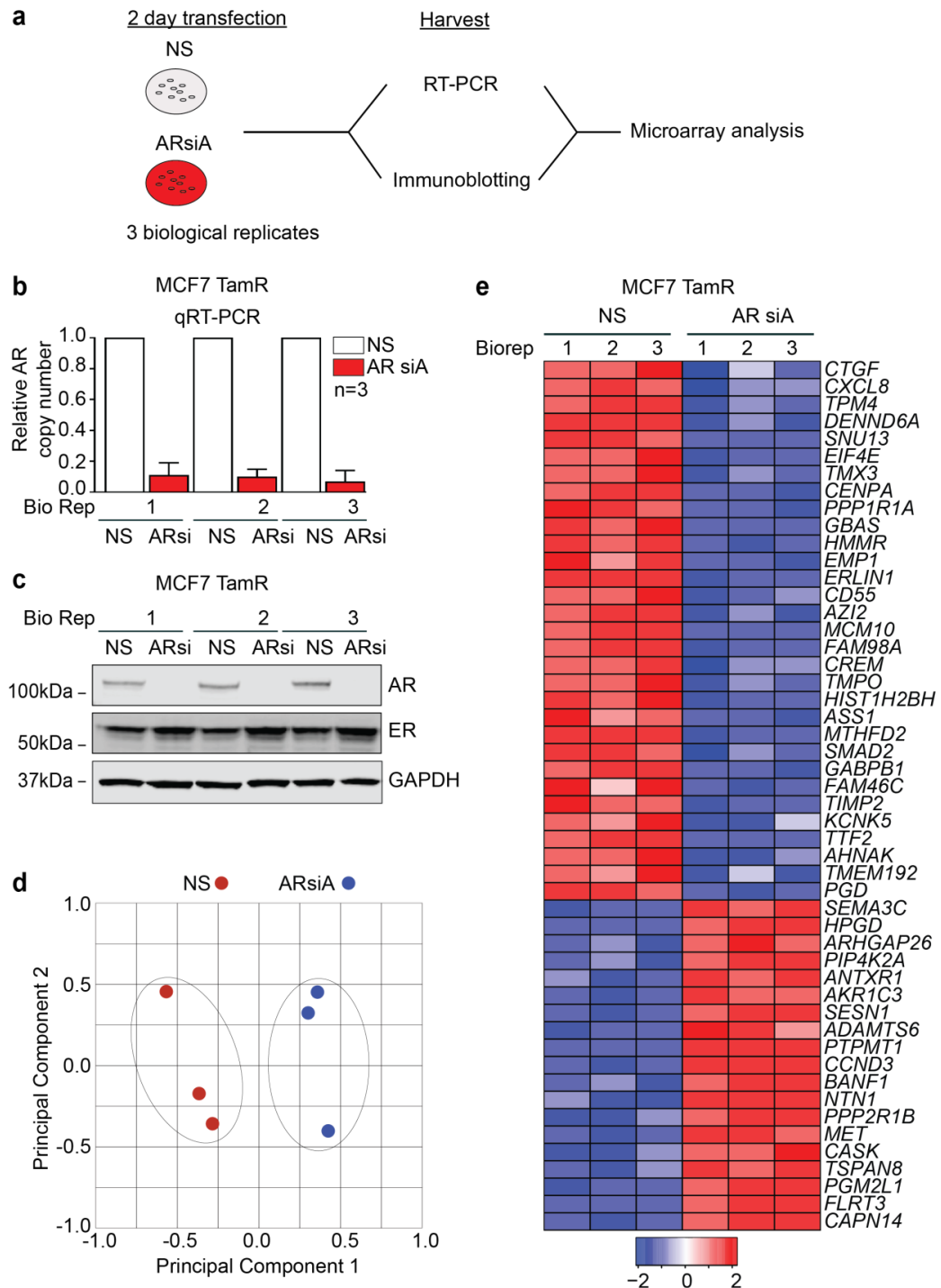
Figure 3.3. Gene expression profiling following AR loss in MCF7 TamR cells.
The effect of AR knockdown on global gene expression in MCF7 TamR cells was assessed. **a)** Schematic illustration of the design of this experiment. MCF7 TamR cells were transfected with either NS or ARsiA for 2 days prior to harvest. Efficient AR knockdown was validated at the mRNA level using **b)** RT-qPCR and **c)** at the protein level via immunoblotting. Each reaction was performed in 3 technical replicates and error bars = SD. **d)** Principal component analysis plot based on the expression profile of the biological replicates transfected with either NS or ARsiA. **e)** Heatmap of the top 50 differentially expressed genes with the most significantly up- (red) and down-(blue) regulated genes post AR knockdown in MCF7 TamR cells.

Gene Set Enrichment Analysis (GSEA) of the differential gene set against that of the hallmark gene sets from Molecular Signature Database (MSigDB) indicated that upregulated genes were enriched in pathways involved in ultra-violet (UV) response, PI3K-Akt signalling, p53 and estrogen response. On the other hand, pathways involved in E2F, G2/M checkpoint, androgen response and estrogen response were enriched in the downregulated genes (**Fig. 3.4**). Given the observed bidirectional enrichment of genes involved in estrogen response, an overlap of the different gene sets with the Hallmark Estrogen Response Early gene set (Broad Institute) was performed to identify the genes affected by AR knockdown (**Fig. 3.5a**). *AR* is part of the Hallmark Estrogen Response Early gene set and was downregulated in this analysis as expected. Notably, transcript levels of classical ER-regulated genes such *PGR*, *IGF1R* and *RARα* were increased upon the loss of AR in these MCF7 TamR cells and this trend is consistent with the increase in ER protein levels (**Fig. 3.3c**). RT-qPCR was carried out to validate the observed increases in *ESR1* and *PGR* mRNA levels as well as reductions in both *IL-8* and *CTGF* mRNA levels (**Fig. 3.5b**). Moreover, given that the binding of ER to the distal and proximal promotors of *CXCL8* was reported to contribute to the increased expression of this gene leading to endocrine-resistance, we demonstrated that knockdown of AR also significantly reduced ER binding at these sites by ~50% (**Fig. 3.5c**). Furthermore, an increase in the expression levels of *ESR1* and *PGR* were also detected in the LTED cells post AR knockdown (**Fig. 3.5d**). This effect was therefore not unique to the TamR cells. Collectively, these observations suggest that loss of AR in endocrine-resistant MCF7 cells is associated with increased classical ER signalling.
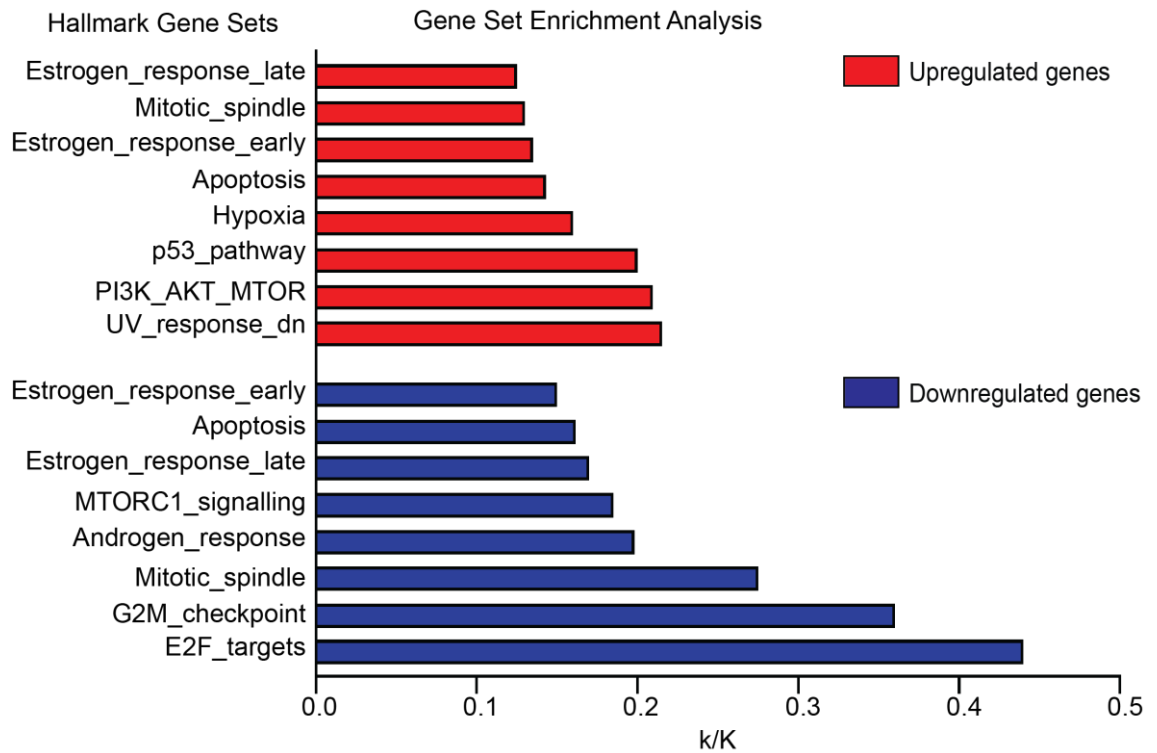
Figure 3.4. GSEA analysis of genes affected by AR loss in MCF TamR cells.
Differentially expressed genes identified from microarray profiling were subjected to MSigDB hallmark GSEA. The top 8 enrichment groups for the up- and down-regulated genes, with q values of <0.001 are presented.
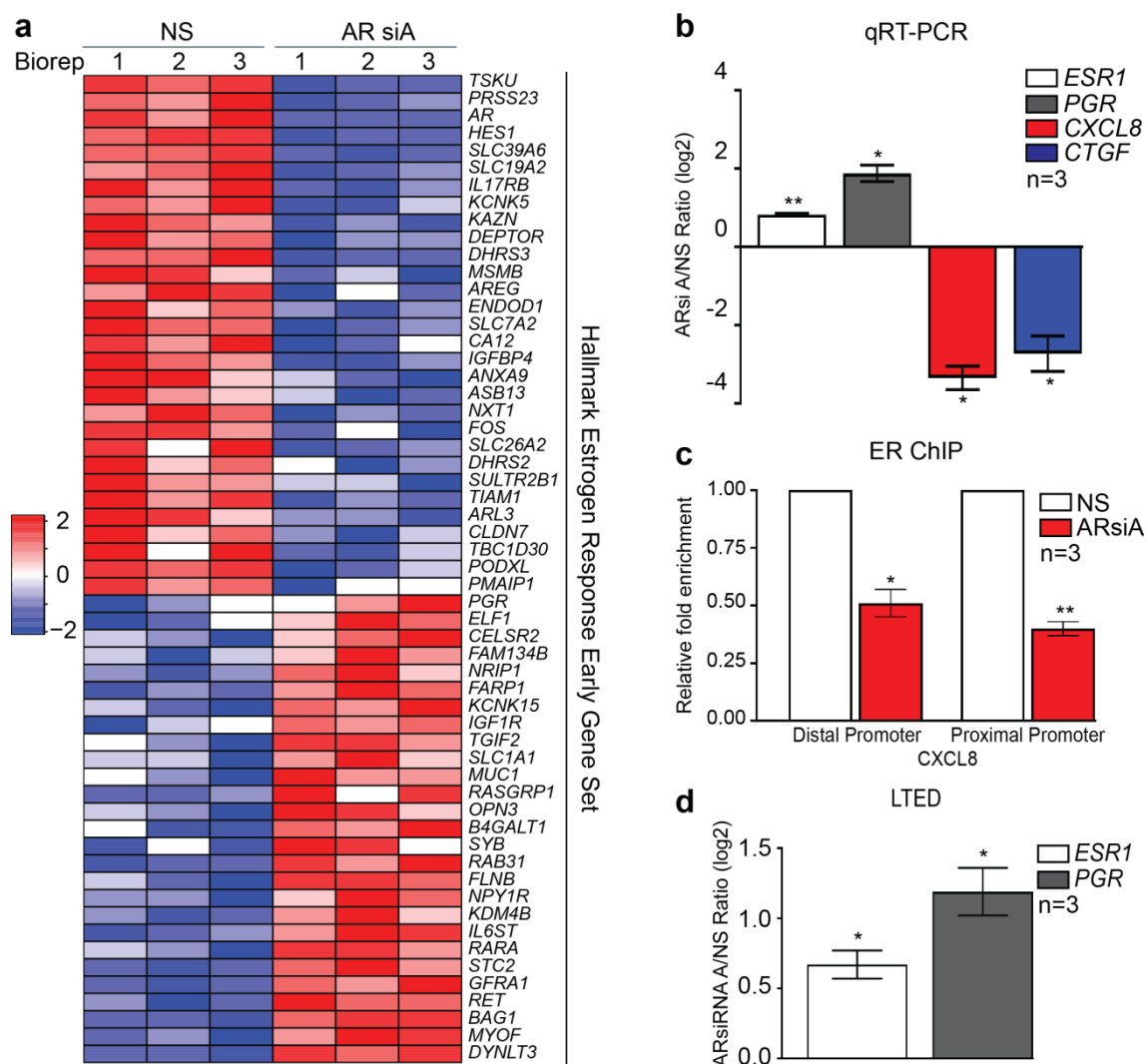
Figure 3.5. AR knockdown in MCF7 TamR cells restores characteristics of classical ER activity.
a) The differential gene set induced by the transient knockdown of AR in MCF7 TamR cells was overlapped with that of the MSigDB hallmark Estrogen Response Early signature. **b)** RT-qPCR was performed to validate changes in the selected genes *ESR1*, *PGR*, *CXCL8* and *CTGF* identified in the expression profiling. Changes in these genes post AR knockdown are presented as log2-fold change of the expression of each gene in AR siRNA-transfected cells relative to NS siRNA-transfected cells. **c)** ER binding at distal and proximal *CXCL8* promoters post AR knockdown was assessed using chromatin immunoprecipitation (ChIP). **d)** RT-qPCR was performed to assess changes in *ESR1* and *PGR* in the MCF7 LTED cells 2 days post AR knockdown. For **b** and **c** data are represented as fold change relative to NS-transfected cells. * $p<0.05$, ** $p<0.01$ using Student's t-test. Error bars = SEM from 3 biological replicates.

Next, we investigated the subcellular localization of ER post AR knockdown. Using immunofluorescence, we detected an increase in nuclear ER signal in TamR cells post AR knockdown (**Fig. 3.6a, b**). This finding was corroborated using immunoblotting for ER in cytoplasmic and nuclear fractions of TamR cells and LTED cells (**Fig. 3.6c, d**). Given that ER is a transcription factor and its nuclear localization is critical for its transcriptional activity, these results accord with our previous observations that loss of AR increased features of classical ER signalling. These results led us to hypothesize that the loss of AR may re-sensitize MCF7 TamR cells to the growth inhibitory effect of tamoxifen.
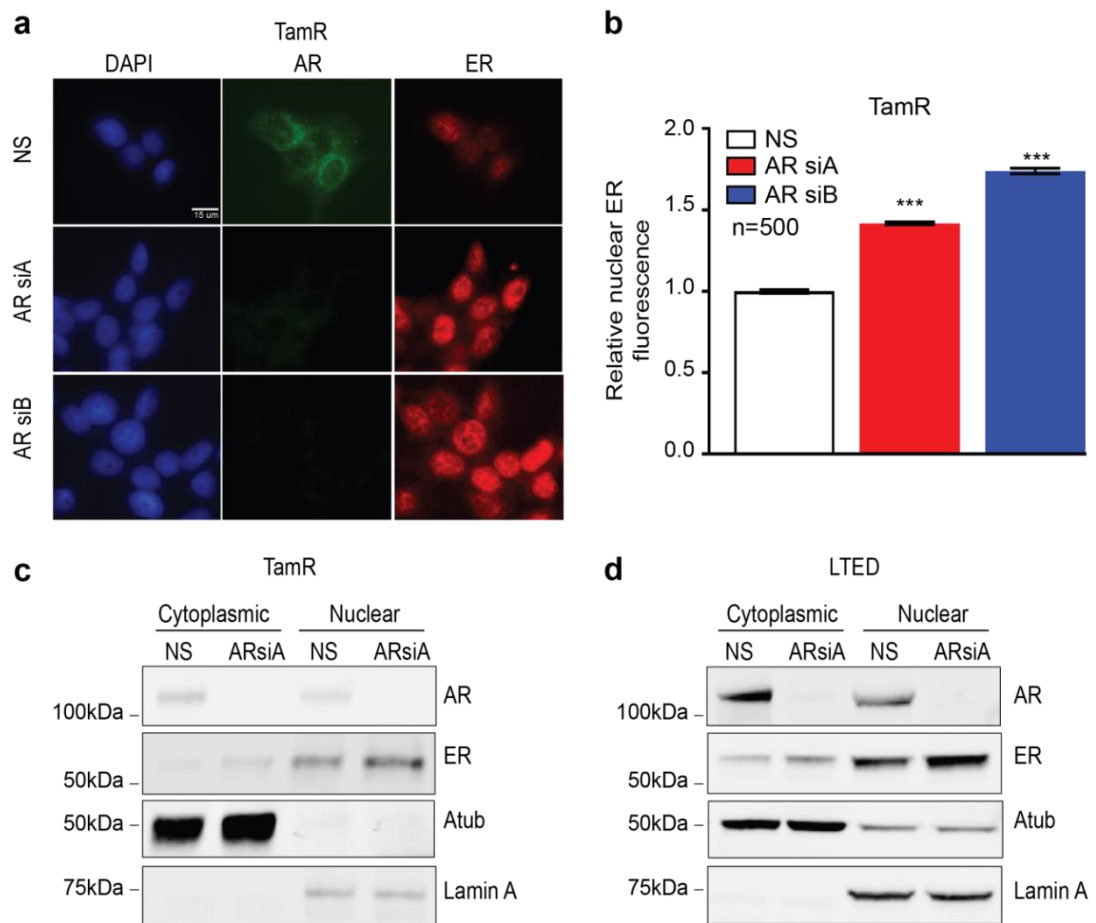
Figure 3.6. AR knockdown increases nuclear ER in endocrine resistant MCF7 cells.
a) The effect of AR knockdown on nuclear ER in MCF7 TamR cells was assessed using immunofluorescence and immunoblotting. Representative images of MCF7 TamR cells stained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI), anti-AR (green) or anti-ER (red) antibodies post 2 day transfection with NS, AR si A or AR siB. **b)** Quantification of nuclear ER intensity in 500 cells using ImageJ. Subcellular fractionation was performed on **c)** MCF7 TamR and **d)** LTED cells post AR knockdown prior to immunoblotting with AR, ER, cytoplasmic marker α-tubulin (Atub) and nuclear marker lamin A. *** $p < 0.001$ using Student's t test for NS vs AR siA or siB.

3.2.4 AR knockdown restores growth-inhibitory effect of tamoxifen in MCF7 TamR cells

Next, we evaluated the effect of tamoxifen on the proliferation of MCF7 TamR cells following AR loss. A schematic illustrating the design of the experimental setup is depicted in **Fig. 3.7a**. Tamoxifen increased the viability of NS-transfected cells relative to vehicle controls as previously observed (**Fig. 3.7b**). In contrast, tamoxifen significantly reduced viability of cells which had been transfected with AR siA or siB as compared to vehicle-treatment (**Fig. 3.7b**). Colony forming assays recapitulated these findings, whereby fewer colonies were observed in tamoxifen-treated cells following transfection with AR siA- or siB relative to vehicle (**Fig. 3.7c, d**). Tamoxifen modestly increased the colony formation of these cells following transfection with NS siRNA. Given that phosphorylation of ER at serine-118 was predictive of response to tamoxifen in early-stage breast cancer (Murphy et al., 2004), we assessed if AR knockdown altered phosphorylation of ER at this site. AR knockdown in TamR cells increased both expression of ER, as previously observed, and phosphorylation of ER at serine-118 in a manner proportional to the level of increase observed with total ER (**Fig. 3.7e**). These observations indicated that loss of AR in TamR cells was sufficient to re-sensitize these cells to tamoxifen.
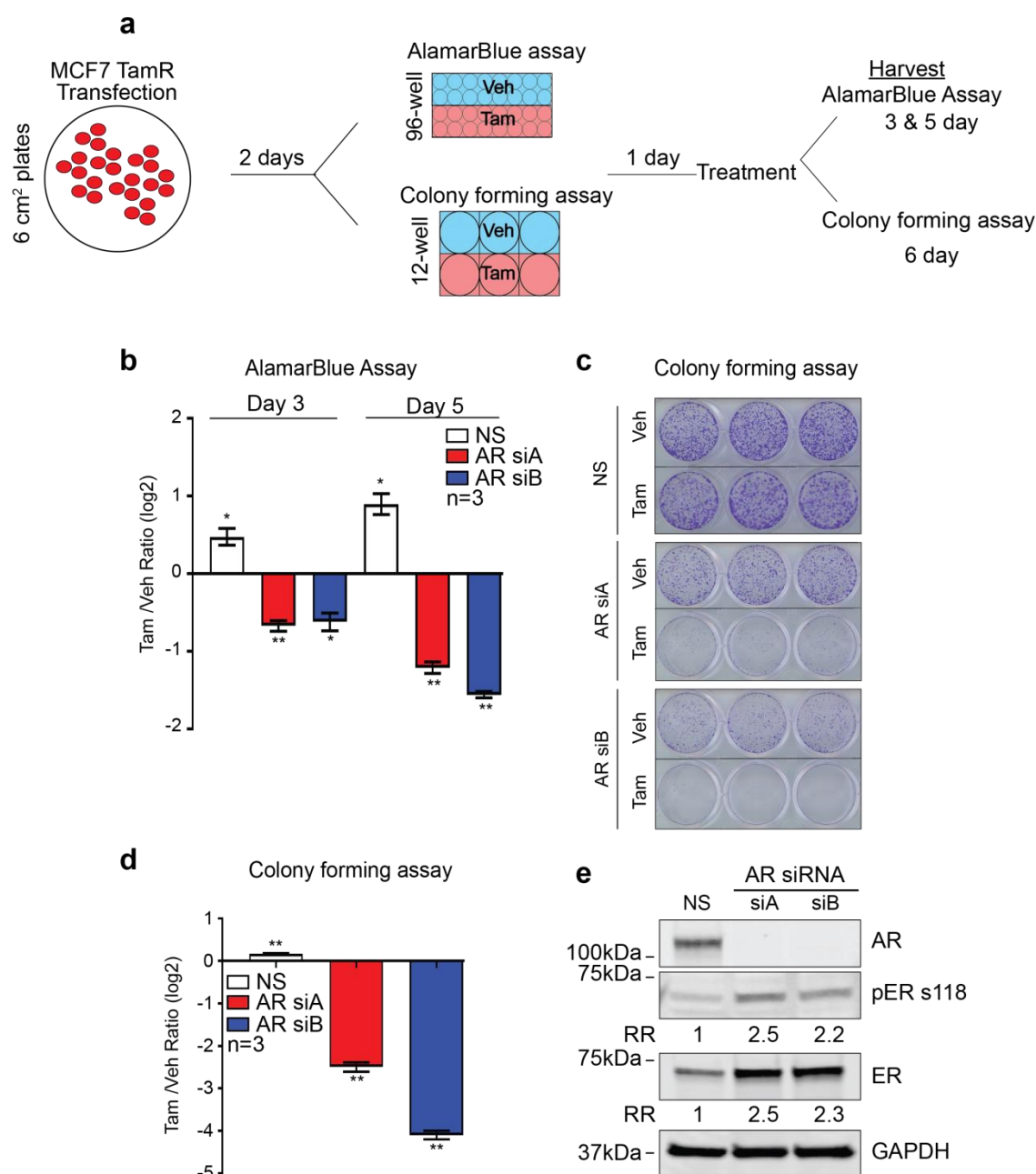
Figure 3.7. AR knockdown re-sensitizes MCF7 TamR cells to tamoxifen.

The effect of AR knockdown on the response of MCF7 TamR cells to tamoxifen was evaluated using AlamarBlue and colony formation assays. **a)** Schematic illustrating the design of the experimental setup where MCF7 TamR cells were transfected with either NS, siRNA A or B for 2 days prior to reseeding into either a 96-well plate or 12-well plate for AlamarBlue assay or colony-forming assay respectively. **b)** The viability of MCF7 TamR cells was assessed using AlamarBlue assay in response to 3 and 5 days treatment with Veh or 5 µM Tam after transfection with NS, AR siA or AR siB RNA for 2 days. Data are presented as the fold change of Tam-treated cells to Veh-treated cells in log2 ratio. **c)** 6 day colony-forming assay assessing the response of MCF7 TamR to Tam as per the treatment and transfection conditions in (**a**). **d)** Quantification of the colony area coverage using ImageJ with data presented as the fold change of Tam- to Veh-treated cells in log2 ratio. **e)** Cell lysates extracted from MCF7 TamR cells transfected with NS, AR siA or AR siB for 2 days, were immunoblotted for AR, phospho-ER at serine-118 (pER s118), ER and GAPDH (RR; relative ratio from densitometry). * p<0.05, ** p<0.01 using Student's t-test. Error bars = SEM from 3 biological replicates.

3.2.5 AR knockdown reduces PI3K-Akt signalling pathway

We postulated that inhibition of the PI3K-Akt signalling pathway contributed to the re-sensitization of MCF7 TamR cells to tamoxifen. This hypothesis was predicated on a recent report where inhibition of the AKT signalling pathway increases the reliance of ER+ breast cancer on classical ER signalling (Bosch et al., 2015) and on our previous observation that PI3K signalling was enriched in the MCF7 TamR cells with transient loss of AR (**Fig. 3.4**). To examine the effect of AR knockdown on this pathway, changes in phosphorylation of Akt at serine-473 and the expression of forkhead box O3 (FOXO3a), a transcription factor negatively regulated by the Akt signalling pathway (Guo & Sonenshein, 2004), were investigated (**Fig. 3.8**). Loss of AR was associated with a reduction in the phosphorylation of Akt and an increase in FOXO3a and these observations suggest that AR knockdown reduced the PI3K/Akt signalling activity.

Using a potent inhibitor of the PI3K signalling pathway, GDC-0941, which inhibits the catalytic PI3K p100α and p110δ subunits, we demonstrated that inhibition of PI3K-Akt signalling pathway recapitulates features of AR loss such as increases in ER and FOXO3a (**Fig. 3.9a**) and re-sensitization of MCF7 TamR cells to tamoxifen (**Fig. 3.9b**). These observations demonstrate that inhibition of the PI3K-Akt signalling pathway may contribute to the re-sensitization of MCF7 TamR cells to the growth inhibitory effect of tamoxifen.
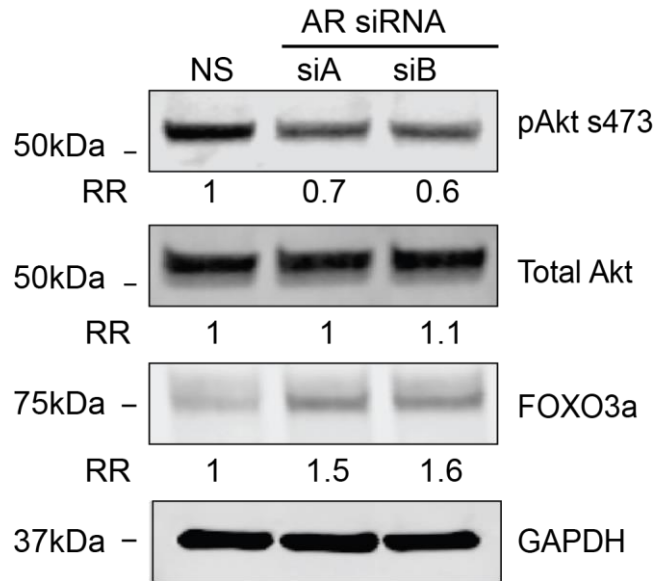
Figure 3.8. AR knockdown in MCF7 TamR cells reduces the PI3K-Akt signalling pathway.
Protein lysates derived from cells transfected with either NS, AR siA or AR siB were used for immunoblotting with phospho-Akt at serine 473 (pAkt s473), total Akt, FOXO3A and GAPDH. Densitometry was performed using imageJ (RR= relative ratio).
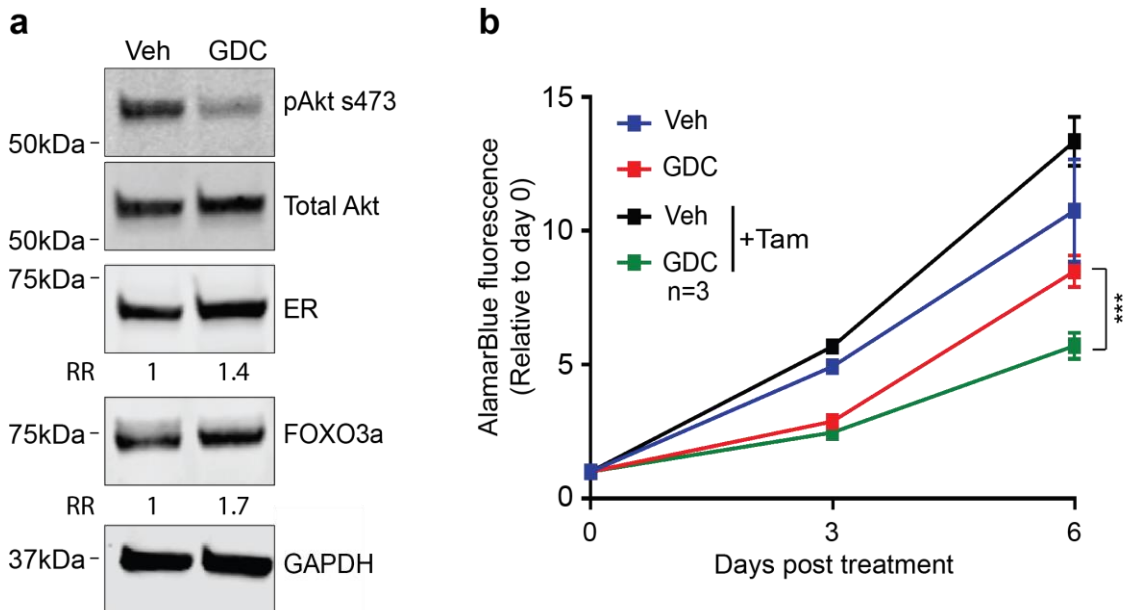
Figure 3.9. Inhibition of Akt re-sensitizes MCF7 TamR cells to tamoxifen.
a) Cell lysates extracted from MCF7 TamR cells treated with vehicle or 125 nM GDC-0941 (GDC) for 2 days were subjected to immunoblotting for pAkt s473, total Akt, ER FOXO3a and GAPDH (RR; relative ratio). **b)** AlamarBlue assay evaluating the effect of 50 nM GDC on the viability of MCF7 TamR cells in the presence and absence of 5 µM tamoxifen at the indicated time points. *** $p < 0.001$ using Student's T test. Error bars = SEM from 3 biological replicates.

3.2.6 Enzalutamide does not recapitulate effect of AR knockdown

Next, we determined if enzalutamide, which works in part by blocking nuclear localization of ligand-bound AR (Tran et al., 2009), could reproduce the effect achieved with AR knockdown in MCF7 TamR cells. We first validated the efficacy of enzalutamide at a dose of 10 μM enzalutamide as this dose has been used antagonize AR signalling in both prostate (Hoefer et al., 2016) and breast cancers *in vitro* (Cochrane et al., 2014). Using immunoblotting, we demonstrated that enzalutamide slightly reduced total AR by ~10% in their basal growth media without DHT supplementation. In the presence of DHT supplementation, where AR protein is elevated presumably as a result of increased AR nuclear localization and stabilization, enzalutamide treatment completely abolished this effect (**Fig. 3.10a**). Using immunofluorescence, we confirmed that DHT-induced nuclear localization of AR and that this process was abrogated by enzalutamide (**Fig. 3.10b**). Hence these results indicate that the dose of enzalutamide used *in vitro* was efficacious and corresponded to its known mechanism of action.

In contrast to siRNA-mediated loss of AR, functional antagonism with enzalutamide had no effect on the colony formation of MCF7 TamR cells relative to vehicle-treated cells (**Fig. 3.11a, b**). Similarly, treatment with enzalutamide had no effect on the viability of TamR cells, and it did not re-sensitize TamR cells to the inhibitory effects of tamoxifen (**Fig. 3.11c**). In contrast to the effect of transient AR knockdown (**Fig. 3.5b**), enzalutamide increased *CTGF* (1 log2-fold change, $p < 0.05$) and modestly decreased *ER* (-0.28 log2-fold change, $p < 0.05$) and *CXCL8* levels (-0.34 log2-fold change, $p < 0.05$) (**Fig. 3.11d**). The expression of *PGR* remained unchanged with enzalutamide. PI3K-Akt signaling pathway was also unperturbed with enzalutamide treatment in these cells (**Fig. 3.11e**). Furthermore, treatment with enzalutamide had no effect on the viability of LTED cells, suggesting its lack of efficacy was not limited to one form of endocrine-resistance (**Fig. 3.11f**). These results demonstrate that pharmacological inhibition of AR does not recapitulate the effect of AR knockdown in endocrine-resistant breast cancer cells *in vitro*.
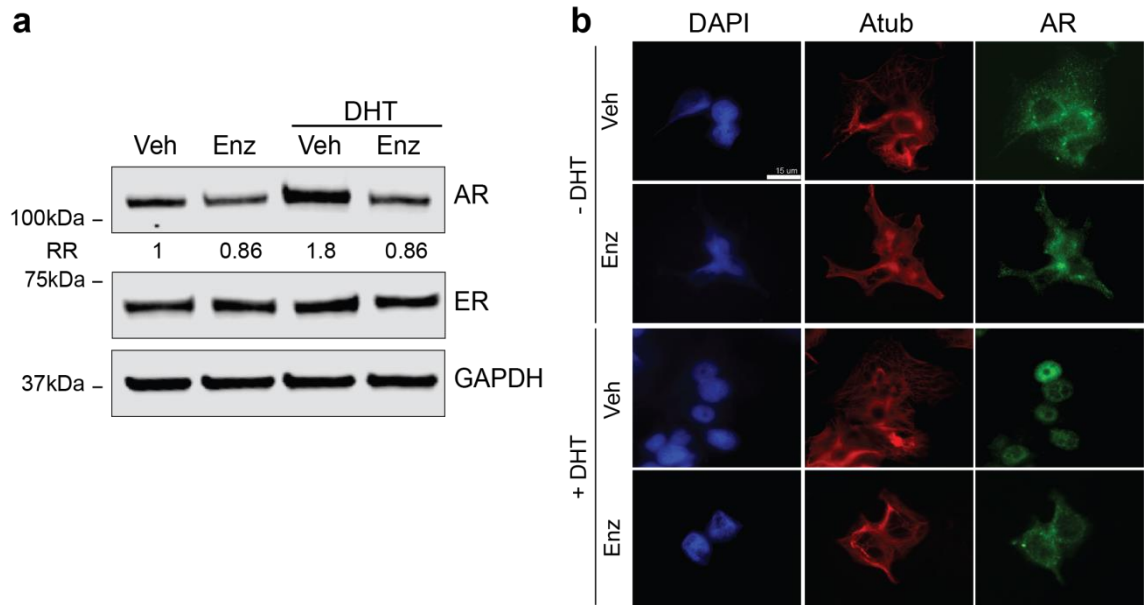
Figure 3.10. Enzalutamide (Enz) prevents DHT-induced AR nuclear localization in MCF7 TamR cells.

The effect of 10 µM Enz on AR expression and subcellular localization was evaluated using immunoblotting and immunofluorescence. Cells were treated with enzalutamide for 48 h in the presence or absence of 10 nM DHT prior to harvest for **a)** immunoblotting for AR, ER and GAPDH and **b)** immunofluorescence with DAPI (blue) and antibodies against cytoplasmic marker A-tubulin (Atub; red) and AR (green). Densitometric analysis was performed using ImageJ and data are represented relative to vehicle (Veh)-treated cells in the absence of DHT (RR; relative ratio).
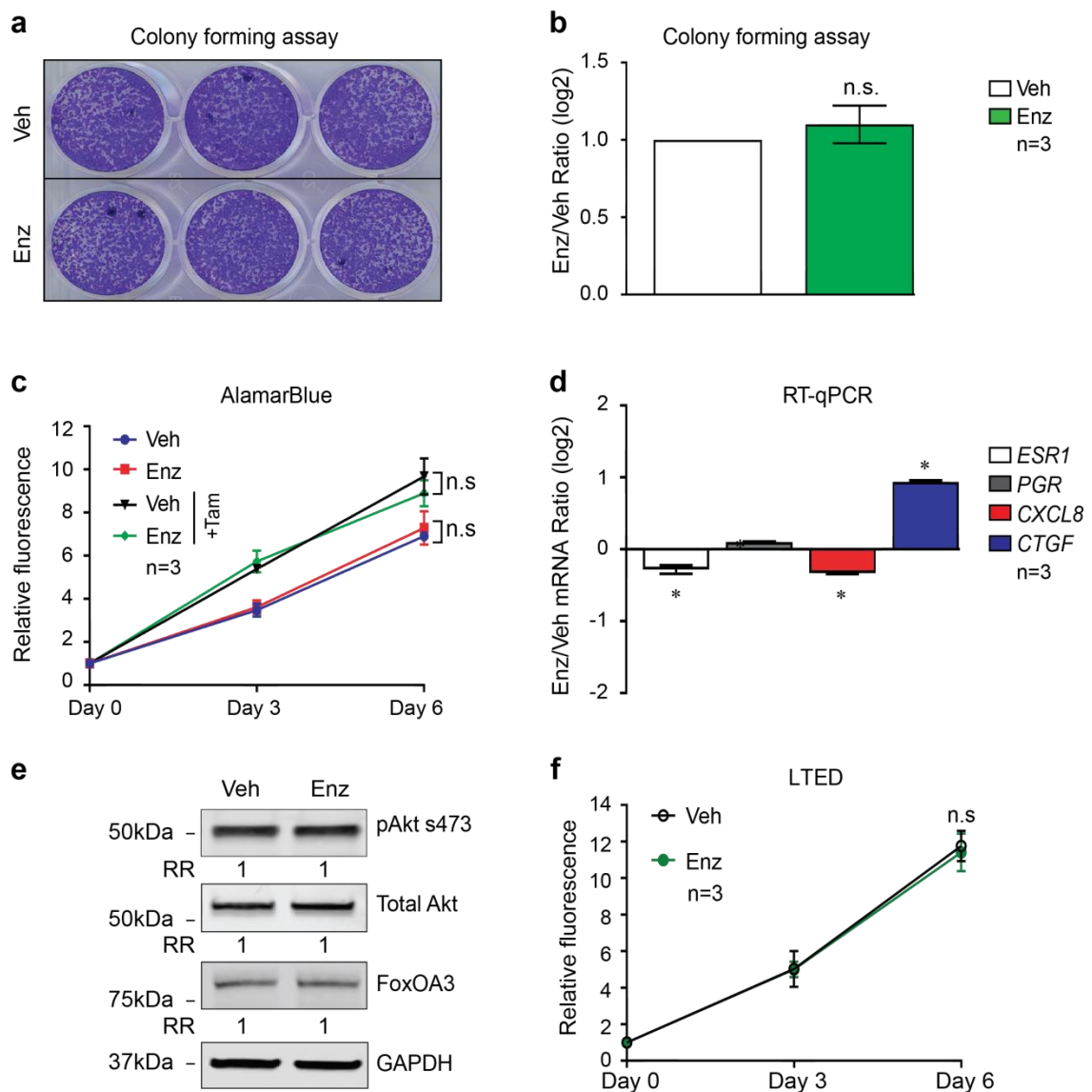
Figure 3.11. Enzalutamide does not recapitulate the effect of AR knockdown in endocrine-resistant cells.

a) Colony forming assays evaluating the effect of Veh or Enz treatment on MCF7 TamR cells for 9 days. b) Quantification of the colony area coverage using ImageJ and data are presented as the fold change of Enz to Veh-treatment. c) AlamarBlue assessing the effect of 10 μM Enz on the viability of MCF7 TamR cells at 3 and 6 days post-treatment in the absence or presence of 5 μM Tam. Data are presented as relative to day 0. d) RT-qPCR was performed to determine the effect of 48 h Enz treatment on the mRNA levels of *ESR1*, *PGR*, *CXCL8* and *CTGF* in MCF7 TamR cells. e) Cell lysates extracted from MCF7 TamR cells treated with either Veh or 10 μM enzalutamide (Enz) for 2 days were immunoblotted for phospho-Akt at serine 473 (pAkt s473), total Akt, FOXO3A and GAPDH (RR; relative ratio from densitometry). f) AlamarBlue assessing the effect of 10 μM Enz on the viability of MCF7 LTED cells at 3 and 6 days post-treatment. Data are presented as relative to day 0. Data are presented as log2 ratio relative to Veh treatment. * $p < 0.05$, n.s = not significant, using Student's t-test. Error bars = SEM from 3 biological replicates.

3.2.7 Characteristics of endocrine-resistant Gar15-13 PDX model

Next we examined the consequences of enzalutamide treatment *in vivo*. We first report on the development of a unique ER+ PDX model (Gar15-13) which was established from a metastatic liver biopsy. This metastatic tissue was obtained from a postmenopausal patient who had tumour recurrence one year into adjuvant treatment with an aromatase inhibitor. The patient had a primary tumour which was ER+PR-HER2- and the metastatic liver tumour was similarly ER+PR-HER2-. IHC staining performed in our laboratory confirmed the expression of AR in the liver met sample (results not shown) and the core characteristics of donor tissue are summarised in **Fig. 3.12a**. Interestingly, Gar15-13 tumours grew *in vivo* independent of estradiol (E2) supplementation (**Fig. 3.12b**). The resulting PDX tumour was subjected to whole genome sequencing (WGS) and immunostaining for hormone receptors ER, PR and AR, proliferation marker Ki-67 and luminal marker cytokeratin 8/18 (CK8/18) (**Fig. 3.12d**). WGS sequencing indicated that 15-13D has a wild-type *ESR1* and mis-sense mutations in *TP53* and *AR* (**Fig. 3.12c**). IHC staining indicated that Gar15-13 was immuno-positive for ER and AR and immuno-negative for PR, similar to the expression profile of its donor tissue (**Fig. 3.12d**). IHC staining also indicated that Gar15-13 was immuno-positive for the luminal marker cytokeratin 8/18 (CK8/18) and proliferation marker Ki-67.

a Patient Characteristics

Age	ER	PR	HER2	AR	Last endo therapy	Site of sample
69	Pos	Neg	Neg	Pos	Aromatase inhibitor	Liver

b

(Tumour volume graph: Tumour volume (mm³) vs Days post implantation, -E2 n=6)
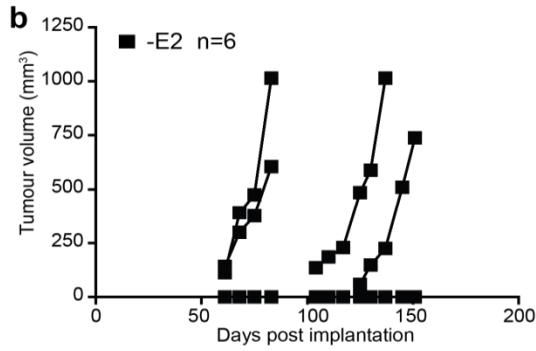
c Mutational profile

Gene	Types	AA change
TP53	missense	L536P
AR	missense	P392S

d IHC

(IHC staining panels: ER, PR, AR, Ki-67, CK8/18)
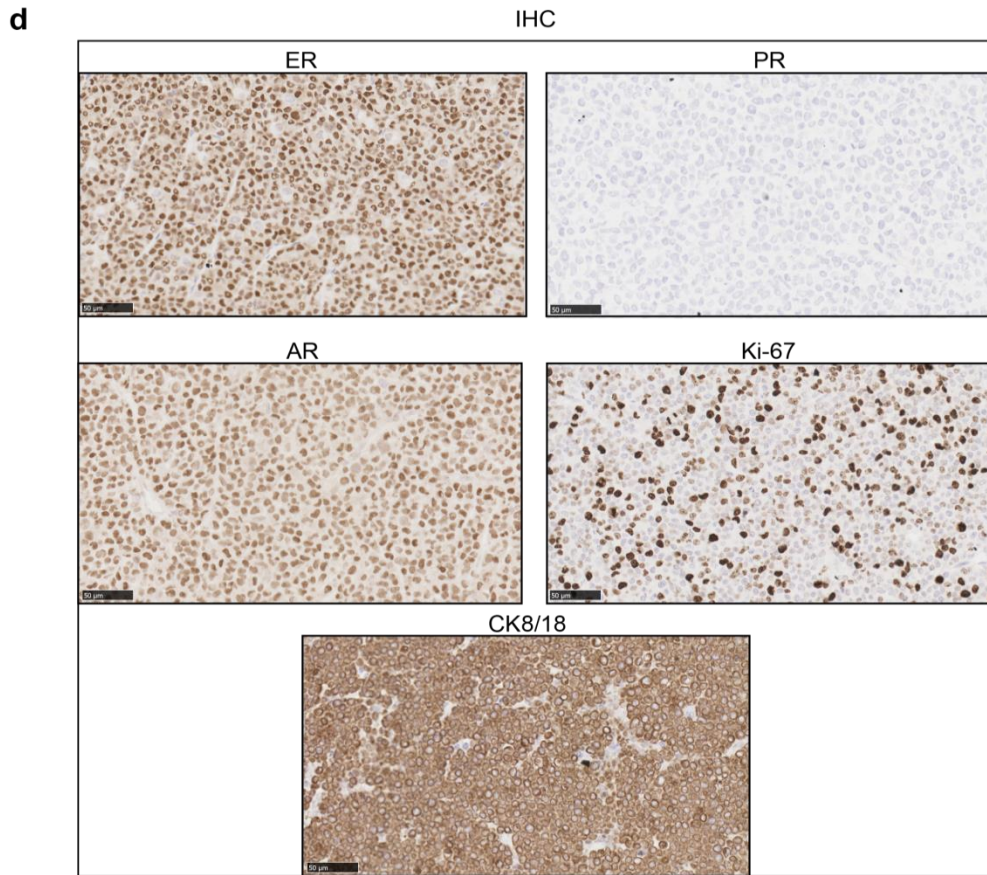
Figure 3.12. Establishment of the ER+AR+ endocrine-resistant Gar15-13 PDX tumour.
a) Characteristics of metastatic breast cancer tumour sample derived from a liver biopsy of a patient who progressed after 12 months of non-steroidal aromatase inhibitor treatment. Clinical report confirms the positivity of ER expression along with the lack of PR expression and HER2 amplification. **b)** Growth kinetics of PDX tumours when tumour chunks were implanted into the fourth mammary fat pads of NSG mice in the absence of estradiol (E2) supplementation. **c)** Whole genome sequencing of the PDX revealed missense mutations resulting in amino acid (AA) changes from leucine (L) to proline (P) and proline to serine (S) in TP53 and AR respectively. **d)** IHC staining for ER, AR, PR, Ki-67 and cytokeratin-8/18 (CK8/18) of the PDX tumour. Scale bars represent 50 μm.

Given that ER+ PDX models are typically established with E2 supplementation (Matthews & Sartorius, 2017), we investigated the effect of E2 on the growth of Gar15-13. Notably, implantation of an E2 pellet suppressed the growth of Gar15-13 in NSG mice with intact ovaries (**Fig. 3.13a**). To validate the lack of dependency of Gar15-13 on E2 for growth, Gar15-13 was implanted into a cohort of ovariectomized (OVX) NSG mice and these tumours were still able to grow in an E2-independent manner (**Fig. 3.13b**). Furthermore, implantation of an E2 pellet in tumour-bearing mice when tumours were approximately 200 mm^3 resulted in growth suppression (**Fig. 3.13c**). The tumour weights from mice which received an E2 pellet were significantly lower compared to mice which did not receive one (**Fig. 3.13d**). IHC staining revealed that long-term treatment with E2 was associated with reduction in Ki-67 but it did not increase expression of the apoptotic marker cleaved caspase-3 (CC3), suggesting that the effect of E2 on this model was primarily cytostatic (**Fig. 3.13e**). In addition, E2 treatment reduced the expression levels of ER and AR, and increased the expression of PR (**Fig. 3.13f**). The reduction in ER may reflect an increase in classical ER signaling as observed with increased PR. While E2 is widely accepted to be a mitogen in primary ER+ breast tumours, the effect of E2 in endocrine-resistant breast cancer is controversial, and E2-induced tumour-suppression in a subset of these breast cancers has previously been reported (Sweeney et al., 2014).
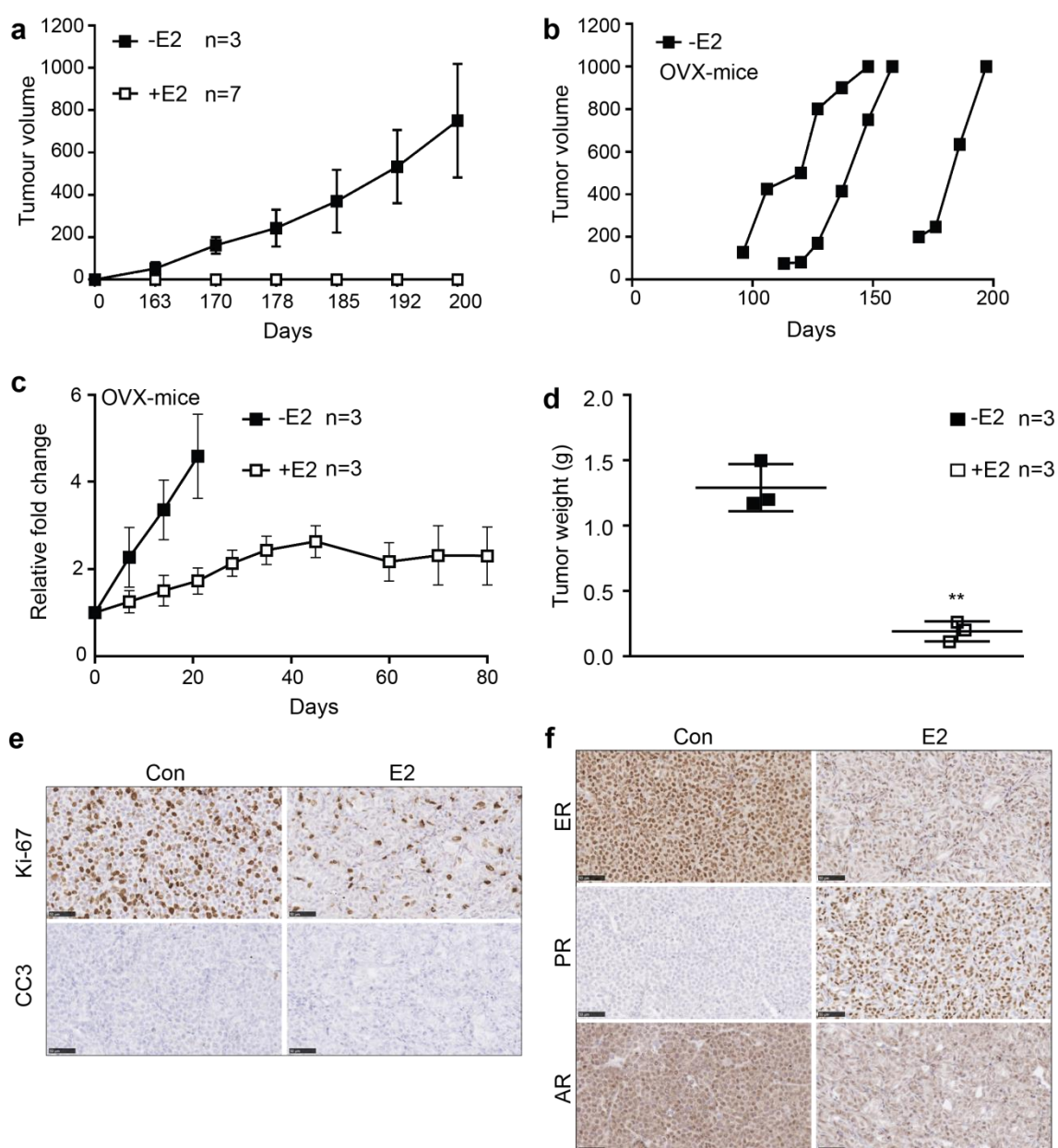
Figure 3.13. E2 suppresses tumour growth of Gar15-13 PDX tumours.
a) The effect of E2 on the growth of Gar15-13 with (n=7) or without (n=3) an E2 pellet. **b)** Donor tumour pieces were implanted into ovariectomized (OVX)-mice. **c)** The growth-suppressive effect of E2 was validated in tumour-bearing OVX-NSG mice and mice were randomized to control group (-E2, n=3) or to estradiol group (+E2, n=3) when tumour volumes reached ~200 mm^3. Data is represented as fold change from tumour volume at the start of treatment (relative tumour volume) and mice were culled when tumour volumes reached 1000 mm^3 or at day 80. **d)** Tumour weights from mice without E2 implantation versus that from mice who received an E2 pellet. Statistical analysis was based on Student's t-test, *** p <0.001. IHC staining was performed to assess changes in the expression of e) Ki-67, cleaved caspase-3 (CC-3) and **f)** ER, PR and AR between control and DHT treated tumours. Scale bars represent 50 µm.

3.2.8   Enzalutamide does not inhibit the growth of Gar15-13 PDX tumours

Lastly, we evaluated the efficacy of enzalutamide on the Gar15-13 PDX *in vivo*. Enzalutamide did not affect the basal growth of these tumours relative to vehicle (**Fig. 3.14a**), concordant with our *in vitro* findings with TamR and LTED cells. The tumour weights and proliferation indices, as measured by the proportion of Ki-67 positive cells, were similar in both treatment groups (**Fig. 3.14b-d**). Importantly, the reduced levels of nuclear AR and protein expression of an AR canonical activity-regulated gene, SEC14-Like Lipid Binding 2 (SEC14L2) provided evidence of antagonism of classic AR signaling pathways in the tumours treated with enzalutamide (**Fig, 3.14e, f**). Furthermore, the expression levels of AR-regulated genes *SEC14L2* and *FK506 binding protein* (*FKBP5*) were reduced in accordance with the inhibitory effect of enzalutamide on the transcriptional activity of AR (**Fig. 3.14g**). ER was unchanged and its expression remained predominantly nuclear with enzalutamide and the expression of an ER-target gene BCL2 was also unaffected by enzalutamide (**Fig. 3.14h**). Collectively, these results demonstrate that antagonizing AR had no effect on ER signaling and may not be the optimal treatment option for endocrine-resistant breast cancer.
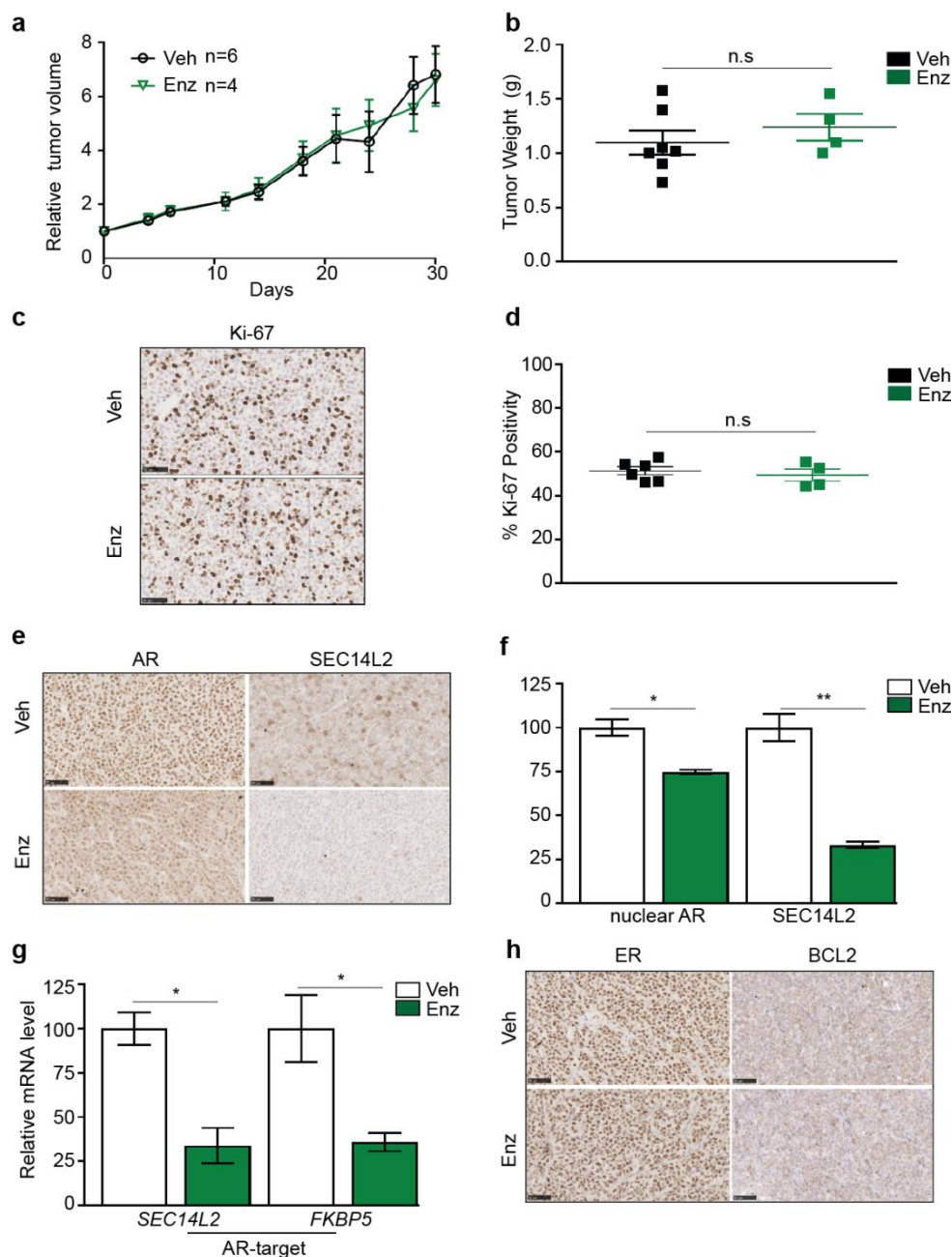
Figure 3.14. Enzalutamide does not inhibit the growth of Gar15-13 PDX tumours.

The effect of antagonizing AR using enzalutamide (Enz) on an ER+AR+ endocrine-resistant PDX was assessed. **a)** PDX-bearing mice were treated with either vehicle (Veh) or 20 mg/kg Enz when tumours reached ~150-200mm^3. Tumours were harvested when they reached the ethical endpoint of 1,000 mm^3 and data are presented as fold change of tumour volumes at harvest from baseline tumour volumes. **b)** Comparison of tumour weights derived from Veh- or Enz-treated tumours. **c)** Ki-67 IHC staining of tumours treated with Veh or Enz. **d)** Proliferation indices of tumours treated with Veh or Enz quantified as the proportion of cells positive for Ki-67 in >1,000 cells from at least 3 random high-magnification fields. IHC staining of endpoint tumours with **e)** AR and AR-target SEC14L2 and **f)** ER and ER-target BCL2.(**f**) Quantification of nuclear AR and total SECL14L2 was performed using ImageJ from 4 representative images at high magnification. (**g**) RNA extracted from tumours treated with vehicle or enzalutamide (n=3 were used to assess changes in expression levels of *SEC14L2* and *FKBP5*.* $p<0.05$, **$p<0.01$ and n.s. indicates not significant when comparing Veh vs Enz using Student's t test. Error bars = SEM. Scale bars = 50 μm.

3.2.9 Enzalutamide inhibits growth of AR-null T47D cell line

While enzalutamide is a well-established inhibitor of AR it has been reported to suppress growth of breast cancer cell lines in an AR-independent manner when used at high micro-molar concentrations (eg. >10 µM)(Thakkar et al., 2016). We assessed the potency of this "off-target" effect of enzalutamide on ER+AR-null derivative of T47D cells that were developed using the double nickase CrispR technology (Ran et al., 2013). The original CrispR system relies on the activity of Cas9 nucleases for inducing double-stranded DNA breaks at sites targeted by specific guide RNA (gRNA). The modified double nickase system on the other hand utilizes a mutant D10A Cas9 protein (Cas9n) which can only nick the strand of DNA complementary to the sgRNA. In order to induce a double-stranded break using this system, two complementary gRNA targeting regions, which are approximately 20 base pairs apart, are required to mimic a "double-stranded break" which subsequently activates the non-homologous end joining (NHEJ) DNA repair mechanism resulting in microdeletion in the target region (**Fig. 3.15a**). This approach of using a paired set of sgRNAs and Cas9n protein has been demonstrated to be more specific than the conventional Cas9-based CrispR system by 50- 1000 fold in cell line models (Ran et al., 2013).

Following transfection with Control or AR CrispR plasmids and selection for transfected cells using puromycin, we validated the loss of AR expression in the AR-null T47D cells at passages 3 and 5 using antibodies that recognize the amino-terminus (N-term) and carboxyl-terminus (C-term) of AR (**Fig. 3.15b**). Importantly, no variants of AR were detected in these AR-null T47D cells post treatment with DHT using both N-term and C-term AR antibodies (**Fig. 3.15c, d**). Enzalutamide resulted in significant growth-suppression at 20 and 40 µM concentrations in both control and AR-null T47D cells in AlamarBlue assays (**Fig. 3.16**). These results support the hypothesis that enzalutamide can elicit significant off-target effects when used at high concentrations *in vitro* (Thakkar et al., 2016).
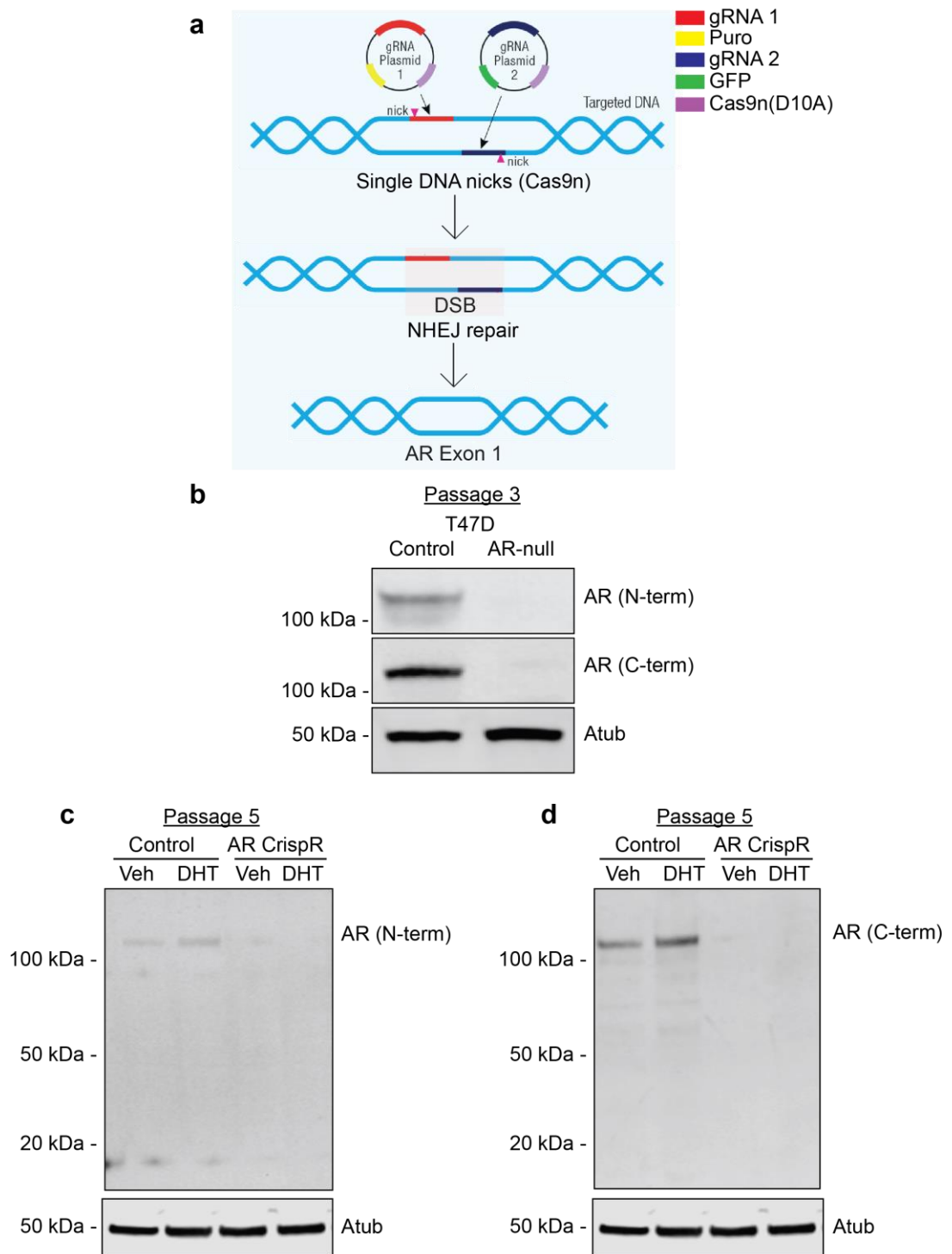
Figure 3.15. Establishment of ER+AR-null T47D cells.

a) Schematic illustrating double nickase CrispR system which comprises two plasmids. Both plasmids encode for the Cas9n protein and both have a selection marker which is either puromycin (puro) or GFP. The pair of guide RNAs (gRNA1 and 2) are encoded on different plasmids and they target DNA regions which are in close proximity. Single-stranded breaks at these sites are induced by the Cas9n and the single-stranded breaks on complementary strands of the DNA are recognized as a double-stranded by the NHEJ mechanism leading to a microdeletion of the region. T47D cells were transfected with either control or AR CrispR plasmid and positively-transfected cells were selected by exposing these cells to 2 μg/ml puromycin for a week. Validation of the loss of AR protein was done via immunoblotting of cells at **b)** passage 3 and 5 with antibodies recognizing the **c)** N-term and **d)** C-term of the AR protein.
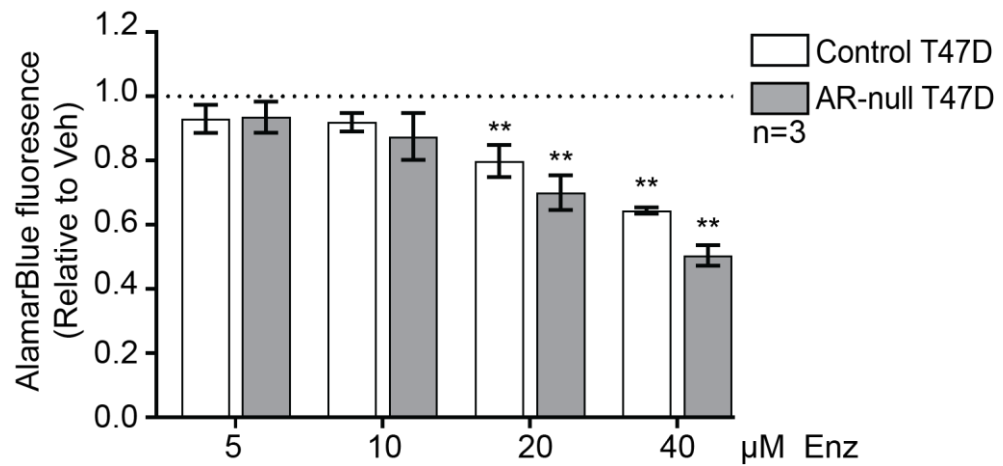
Figure 3.16. Enzalutamide reduces viability of AR-null T47D cells at high doses.
AlamarBlue assays evaluating the dose response of control and AR-knockout T47D cells to 4 days of enzalutamide (Enz) treatment. Data are represented as relative to Veh-treatment for each cell line and ** $p < 0.01$ using Student's t test comparing Enz- and Veh-treatment. Error bars = SEM from 3 biological replicates.

3.3 Discussion

AR has been implicated in the development of endocrine-resistant breast cancer but the mechanistic basis remains unclear. In this chapter, we investigated the consequences of transient knockdown and pharmacological antagonism of endogenous AR using a combination of endocrine-resistant ER+AR+ breast cancer cell lines and PDX models.

Results from our *in vitro* AR siRNA experiments to knock down AR in cell lines support the hypothesis that AR plays a role in endocrine-resistance. We found that AR was predominantly located in the cytoplasm of endocrine-resistant cells under normal growth conditions, which implicates non-canonical signaling rather than canonical nuclear AR activity. Treatment with an AR antagonist, enzalutamide, did not phenocopy the effect of AR knockdown despite demonstrable inhibitory effects on classic AR transactivation events. Likewise, enzalutamide failed to inhibit the *in vivo* growth of an endocrine-resistant PDX model, although it inhibited AR nuclear localization and transcriptional activity. Non-canonical activity of AR and other steroid hormone receptors including ER have been described previously (Bjornstrom & Sjoberg, 2005; Leung & Sadar, 2017; Zarif & Miranti, 2016), but the clinical significance of this type of AR signaling in breast cancer is not well described. These findings suggest that the role of AR in endocrine-resistant breast cancer is complex and may be modulated by conditions that impinge upon the ability of AR to signal in a non-canonical manner.

The loss of proliferative capacity observed with knockdown of endogenous AR in the endocrine-resistant cell line models supports previous findings that AR plays a role in the proliferation of breast cancer cells (Ciupek et al., 2015; De Amicis et al., 2010; Rechoum et al., 2014). Our data provide further mechanistic insight by revealing that this effect is likely due to the non-canonical signaling activity rather than canonical nuclear activity of AR, given that pharmacological inhibition of AR had no effect on the growth of these cells. AR knockdown also reduced the proliferation of endocrine-sensitive MCF7 cells (D'Amato et al., 2016; Yeh et al., 2003) which suggests that AR has a universal mitogenic role in breast cancer regardless of its state of endocrine-therapy sensitivity. Importantly, these studies also provide evidence of functional non-canonical AR activity in breast cancer cells. Yeh et al. (2003) observed that AR-null MCF7 cells had defective MAPK activity that was restored by overexpression of a

truncated form of AR lacking the ligand binding domain. D'Amato et al. (2016) revealed that estrogen induced AR nuclear translocation and binding to non-canonical DNA response elements, whereas androgen recruited AR to canonical DNA response elements. While we observed AR to be predominantly in the cytoplasm of cells, some nuclear protein was evident. Treatment with enzalutamide reduced nuclear AR but had no impact on cell proliferation, suggesting that the limited nuclear AR present in our models was not playing a significant mitogenic role. Hence, our data and that of others support the hypothesis that non-canonical activity of the AR has mitogenic effects on endocrine-sensitive and -resistant forms of MCF7 cells.

In addition to inhibiting the growth of MCF7 TamR cells, AR knockdown also restored features of classical ER signaling and sensitivity to the anti-proliferative effect of tamoxifen. While classical ER-regulated genes such as *PGR*, *IGF1R* and *RARα* were increased, downregulation of other estrogen response genes such as *FOS* and *amphiregulin* (*AREG*) was also observed. Given that AR can also regulate the expression of estrogen response genes such *FOS* (Chia et al., 2011) and *AREG* (Barton et al., 2015), it is likely that the downregulation of these estrogen response genes was a direct consequence of loss in AR activity. However, the overall consequences of AR loss appear to be an increase in classical ER signalling in the TamR cells. This effect has not been reported previously and appears to be distinct from the effect of AR loss in endocrine-sensitive MCF7 cells which reduced classical activation of ER under estradiol-stimulated conditions (Yeh et al., 2003). This dichotomous effect of AR on ER signaling in an endocrine-sensitive versus -resistant state could be ascribed to the different expression levels of AR or key nuclear co-factors that influence AR and ER function. In endocrine-sensitive MCF7 cells, low endogenous levels of AR could facilitate ER activity (D'Amato et al., 2016; Yeh et al., 2003), but the relatively higher levels of AR in MCF7 TamR cells inhibited classical ER activity in our study. Lanzino et al. (2005) demonstrated that overexpression of AR in MCF7 cells suppressed classical ER signaling via the sequestration of an AR-associated protein of 70 kDa (ARA70), a co-factor of ER and AR. Hence, it is plausible the higher AR expression in the MCF7 TamR cells results in sequestered co-regulatory factors (such as ARA70) from ER, which leads to inhibition of classical ER signaling. The knockdown of AR may increase the accessibility of ER to these factors and promote classical ER transcriptional activity. Another confounding factor is the increased expression of

FOXA1 in the MCF7 TamR cells. FOXA1 is an important coregulator for ER and AR signaling in breast cancer cells (Augello et al., 2011) and regulates ER activity in both endocrine-sensitive and endocrine-resistant breast cancer (Ross-Innes et al., 2012). However, it remains to be determined if and how loss of AR changes FOXA1 activity in both endocrine-sensitive and -resistant contexts.

Through network analysis of AR-regulated genes in the MCF7 TamR cells, we identified reduction in PI3K-Akt signaling pathway as a potential pathway underlying the re-sensitization of these cells to tamoxifen. This reduction in PI3K-Akt signaling pathway was associated with an increase in PTEN, a suppressor of the PI3K-Akt signaling. A regulatory relationship between AR and PTEN has been reported in ER+ breast cancer where activation of AR can induce upregulation of PTEN leading to inhibition of growth (Y. Wang et al., 2011). Furthermore, AR regulation of PTEN in this setting is via direct transcriptional regulation as androgen responsive elements (AREs) in the promoter of PTEN have been identified. Given that inhibiting the transcriptional activity of AR has no bearing on PTEN expression, the observed increase in PTEN level is likely an indirect consequence of AR loss.

Treatment with enzalutamide also did not affect the growth of an estrogen-independent PDX model derived from a patient who progressed on an aromatase inhibitor. Importantly, a clear distinction between the PDX model and the cell line models is the nuclear localization of AR in the PDX model, in contrast to the predominantly cytoplasmic localization in the cell line models. The lack of therapeutic efficacy in the PDX model cannot be attributed to insufficient dosing because enzalutamide effectively antagonized canonical AR signaling as evidenced by reduced AR nuclear expression and transcriptional activity. Our results do not concur with a previous study (D'Amato et al., 2016) in which enzalutamide re-sensitized MCF7 TamR cells to tamoxifen *in vitro* and significantly suppressed growth of TamR xenografts *in vivo* when given as a monotherapy. Differences in the type of xenograft and doses of enzalutamide used could account for these divergent results. Enzalutamide has been reported to suppress growth of AR-negative breast cancer cell lines *in vitro* when used at >10 μM concentrations (Thakkar et al., 2016), implicating off-target effects of enzalutamide in breast cancer cell lines. In support of this, we demonstrated that enzalutamide significantly reduced the viability of an ER+ AR-null T47D derivative cell line at doses

>10 µM. Notably, D'Amato et al. (2016) reported that enzalutamide suppressed colony formation of MCF7 TamR cells at a minimal dose of 20 µM. Likewise, the growth suppressive effect of enzalutamide on MCF7 TamR xenografts *in vivo* was achieved with 50 mg/kg, which 2 fold greater than the 10-30 mg/kg doses commonly used in prostate cancer models (Evans et al., 2011; Lin et al., 2013; Moilanen et al., 2015). We used a dose of 20 mg/kg in our *in vivo* experiments to minimize the off-target effects of enzalutamide. At this dose, there was demonstrable anti-androgenic effect in tumour cells within our PDX. Collectively, these data strongly support the concept that canonical AR signaling does not promote growth of endocrine-resistant breast cancer.

Observations from previous AR-targeted clinical studies also support the notion that canonical AR signaling does not promote growth of endocrine-resistant breast cancer and that the use of AR antagonists is ineffective in the endocrine-resistant setting. Two clinical trials have assessed the efficacy of AR antagonists in breast cancer, and both have reported no clinical benefit from AR inhibition. The first study trialed the first generation AR antagonist flutamide in non-selected patients with metastatic breast cancer (Perrault et al., 1988). The second and more recent study evaluated the efficacy of androgen biosynthesis inhibitor abiraterone acetate in patients with ER+ breast cancer who had progressed on a non-steroidal aromatase inhibitor (O'Shaughnessy et al., 2016), a similar clinical context to the patient from which our PDX model derived. In contrast, multiple clinical studies have reported that the use of AR agonists to activate canonical AR activity in endocrine-resistant breast cancer is associated with tumour suppression and a clinical benefit rate of 38-50% (Birrell, Roder, et al., 1995; Boni et al., 2014; Kono et al., 2016; Overmoyer et al., 2015). The agents used to activate AR in these studies include testosterone (Boni et al., 2014), testosterone analogue fluoxymesterone (Kono et al., 2016, medroxyprogesterone acetate, which is a synthetic progestin with androgenic activity {Birrell, 1995 #1794), and a selective AR modulator (SARM) that induces a selected spectrum of AR activity (Overmoyer et al., 2015).

Limitations of our *in vivo* work include the use of a single endocrine-resistant PDX model. As mentioned earlier, this PDX has only been exposed to a single line of endocrine-therapy (aromatase inhibitor) and harbors a wild-type *ESR1*. It remains to be determined if pharmacological antagonism of AR is similarly ineffective in endocrine-

resistant tumours exposed to multiple lines of endocrine-therapies and in tumours with *ESR1* mutations which confer constitutive ER activity. Furthermore, changes in the key genes (*CTGF*, *CXCL8*, *ESR1* and *PGR*) post transfection of MCF7 TamR cells with AR siRNA A should have been validated using AR siRNA B to ensure that these effects are not specific to AR siRNA A.

In summary, our study provides evidence that non-canonical AR activity facilitates an endocrine-resistant phenotype in breast cancer, but this activity cannot be inhibited pharmacologically with AR antagonist enzalutamide. The lack of efficacy associated with the use of enzalutamide in our study has implications for current and future clinical trials that aim to specifically target AR in endocrine-resistant breast cancer.

CHAPTER 4

4. Activation of AR in endocrine-resistant breast cancer

4.1 Introduction

In this chapter, we investigated the efficacy of AR agonists in ER+ endocrine-resistant breast cancer. Observations from the previous chapter have demonstrated that AR has a functional role in facilitating endocrine-resistance, but this role was confined to a non-canonical activity of AR which was not effectively targeted by the AR antagonist enzalutamide. Previous studies have established that activation of AR is associated with an anti-proliferative effect in endocrine-sensitive breast cancer cell line models {Birrell, 1995 #1669;Cops, 2008 #1670;Lapointe, 1999 #1671}. This growth-inhibitory effect has been reported to be mediated through the antagonism of ER signalling, as demonstrated by DHT-induced downregulation of ER (Lanzino et al., 2005; Poulin et al., 1989) and ER-target genes such as PR (Peters et al., 2009).

In the context of endocrine-resistant breast cancer, several clinical studies have provided evidence of a tumour suppressive effect associated with the activation of AR. These studies demonstrated that treatment with AR agonists such as testerosterone, medroxyprogesterone acetate and fluoxymesterone was associated with significant clinical benefit (Birrell, Roder, et al., 1995; Boni et al., 2014; Kono et al., 2016). However, there has been a dearth of preclinical evidence on the consequences of activating endogenous AR in endocrine-resistant models, and it remains to be determined if the observed growth-suppressive effect in the clinical studies was similarly mediated through the inhibition of ER signaling.

Selective AR modulators (SARMs) are an emerging class of agents which possess clinical utility in their ability to selectively induce AR activity. Cachexia and sarcopenia are conditions in which increased AR activity is desired and SARMs which are being investigated in this area include enobosarm (GTx®) (J. Kim et al., 2005), BMS-564929 (Bristol-Myers Squibb) (Ostrowski et al., 2007) and LGD-0433 (Viking Therapeutics). Enobosarm is the most clinically developed SARM and it has completed 2 phase 3 clinical trials for the treatment of cachexia in lung cancer (NCT01355497; 01355484).

Apart from anabolic-related conditions, the efficacy of SARMs in ER+ breast cancer is now also being investigated. Enobosarm has successfully completed a phase 2 breast

cancer study and has achieved a clinical benefit rate of 35% in patients with ER+ metastatic breast cancer (Overmoyer et al., 2015). RAD140, a SARM which was developed for the treatment of breast cancer, has recently been demonstrated to effectively antagonize ER signalling and growth in endocrine-sensitive PDX models *in vivo* (Z. Yu et al., 2017). A phase 1 trial of RAD140 in patients with ER+ metastatic breast has been initiated (NCT03088527).

SARMs may also have a better side-effect profile as compared to the traditional steroidal-based ligands used in the treatment of breast cancer. SARMs are non-steroidal in structure and will not be converted into estrogen through aromatization which may potentially promote the growth of estrogen-sensitve ER+ breast cancer cells (Lueprasitsakul & Longcope, 1990). Furthermore, SARMs also preferentially activate AR in tissues such as the muscles and the breast and its lack of activity in the androgenic tissues reduces the risk of virilization observed with traditional AR ligands (Braunstein, 2007). This tissue specificity of SARMs is reportedly due to its ability to induce recruitment of coregulators distinct from those induced by endogenous androgens in the different tissues (Chang & McDonnell, 2002; Narayanan et al., 2018). Collectively, these observations suggest that SARMs are a promising class of novel endocrine-therapy for the treatment of endocrine-resistant breast cancer.

The specific aims of this chapter are

1) Evaluate the response of i*n vitro* and *in vivo* preclinical models of endocrine-resistance to DHT and enobosarm
2) Assess the consequences of DHT and enobosarm on ER signaling in these models

4.2 Results

4.2.1 Characterization of endocrine-resistant MCF7 cells (Caldon Laboratory)

For this project, we have used a matched set of parental, TamR and LTED cells of MCF7 origin developed by the Caldon Laboratory at the Garvan Institute. These endocrine-resistant cells were derived from the same parental MCF7 cells which were were exposed to either tamoxifen (1 μM) or estrogen-deprived conditions for more than 6 months. Unlike the cells used in the previous Chapter, this set of matched endocrine-resistant cells would allow us to compare the response of AR activation in the endocrine-resistant cells relative to its parental MCF7 cells. We first determined the expression of hormonal receptors and pioneer factor FOXA1 by immunoblotting (**Fig. 4.1**). These endocrine-resistant cells exhibited a higher expression of AR relative to the parental MCF7 cells. MCF7 TamR cells demonstrated FOXA1 overexpression and PR loss relative to parental cells. ER levels were higher in both the MCF7 TamR and LTED cells relative to parental MCF7 cells. These findings were similar to the endocrine-resistant MCF7 cells obtained from the Brown Laboratory. Herein, MCF7 parental, TamR and LTED cells will refer to cells derived from the Caldon Laboratory unless otherwise stated.
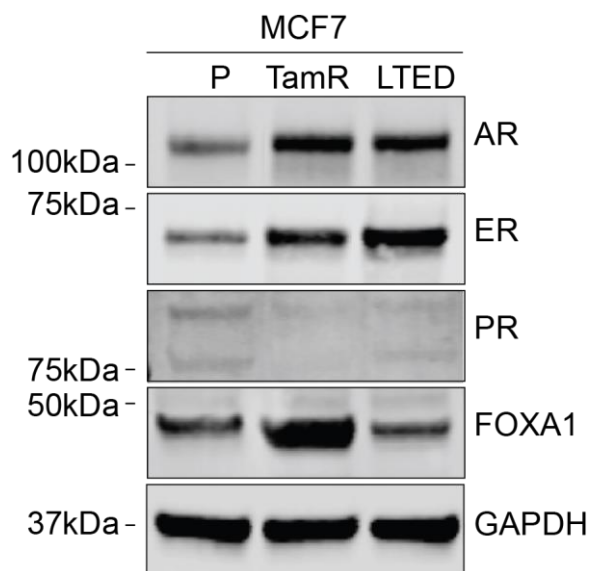
Figure 4.1. Expression levels of hormone receptors and pioneer factor FOXA1 in endocrine-resistant MCF7 cells from the Caldon Laboratory.
Protein lysates extracted from parental (P), TamR and LTED MCF7 cells grown in their respective growth media were immunoblotted for AR, ER, PR and FOXA1. GAPDH was immunoblotted as a loading control.

4.2.2 AR agonists reduce colony formation of MCF7 cells

We first validated the efficacy of AR agonists DHT and enobosarm in matched parental and endocrine-resistant MCF7 cells. The binding of AR ligands to AR induces nuclear translocation and increases the stability of AR. We observed that treatment with DHT or enobosarm for 24 hours resulted in higher AR levels (**Fig. 4.2a**), confirming the effect of ligand binding to AR. Notably, the expression of ER was unchanged in response to DHT and enobosarm at this time point.

To examine the consequences of AR agonists on growth, the effect of 9-day treatment with either DHT or enobosarm on the colony formation of these cells was assessed. Both DHT and enobosarm reduced the colony formation of parental MCF7 cells, consistent with the previously reported growth-inhibitory effect of AR activation in endocrine-sensitive MCF7 cells (Lanzino et al., 2005; Peters et al., 2009; Poulin et al., 1989) (**Fig. 4.2b-c**). A similar effect was observed in MCF7 TamR and LTED cells, with both DHT and enobosarm suppressing colony formation (**Fig. 4.2d-g**). Notable observations here were the stronger growth-inhibitory effect of AR agonists in the endocrine-resistant cells relative to that in the parental MCF7 cells, consistent with the higher level of AR in these resistant cells. The effect of DHT was also more pronounced than enobosarm in all three cell lines.

In contrast, antagonism of AR activity using enzalutamide had no effect on the colony formation of MCF7 TamR cells (**Fig. 4.3a-b**), in spite of antagonizing DHT-induced stabilization of AR (**Fig. 4.3c**), consistent with our observations in the previous Chapter. In these experiments, the growth-inhibitory effect of enobosarm was included as a positive control. Collectively, these *in vitro* observations support the notion that the activation, rather than the antagonism of AR, is the more effective therapeutic strategy in endocrine-resistant cells.
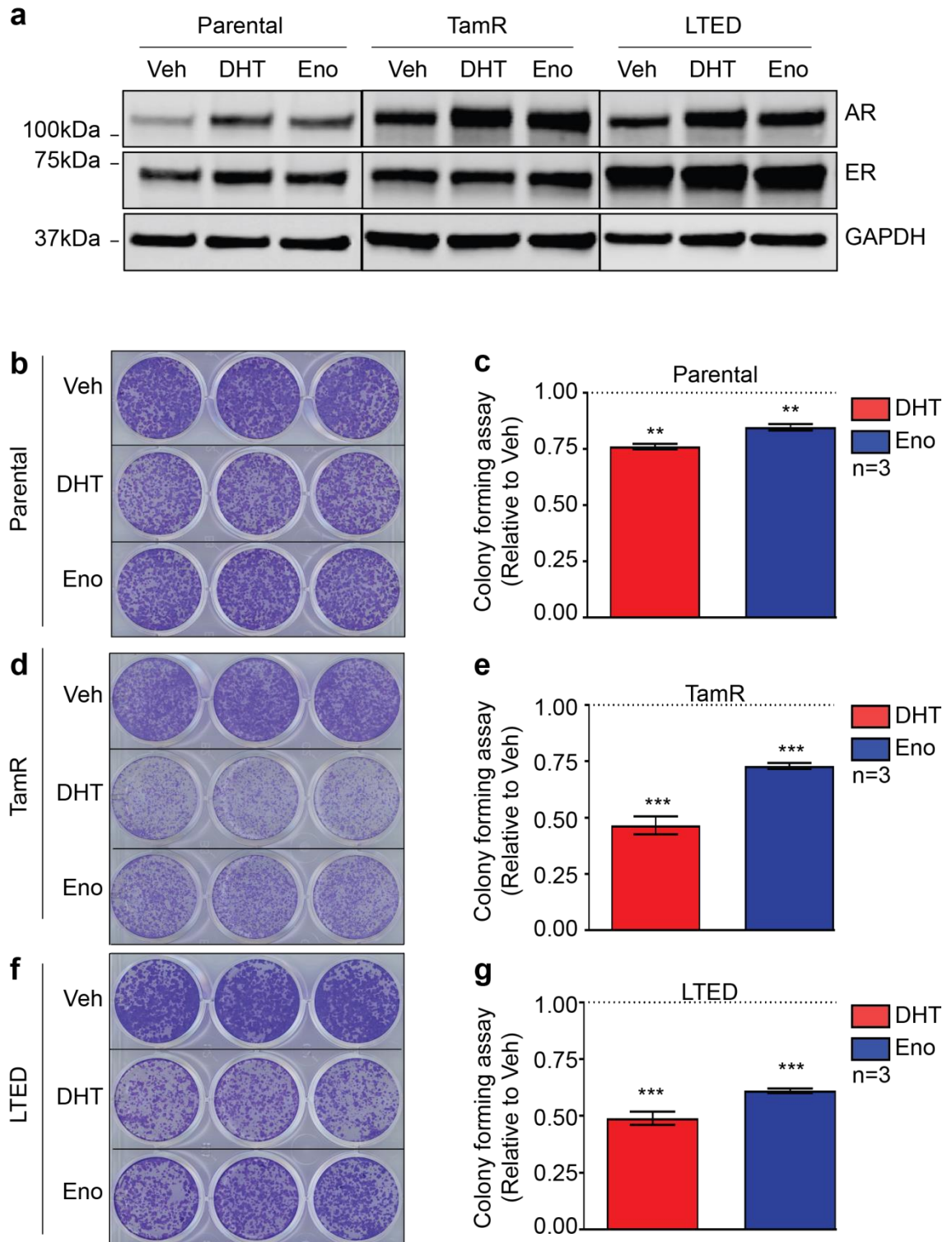
Figure 4.2. AR agonists reduce colony formation of parental and endocrine-resistant MCF7 cells.
a) Cell lines were treated with vehicle (Veh), 1 nM DHT or 100 nM Enobosarm (Eno) for 24 hrs prior to protein extraction. Levels of AR and ER were compared between Veh and AR agonist-treated samples using immunoblotting. **b, d, f**) Cells were treated with Veh, 1 nM DHT or 100 nM Eno for 9 days prior to staining with crystal violet solution. **c, e, g**) Quantification of area coverage using ImageJ plugin ColonyArea from 3 biological replicates and data are presented as relative to Veh-treated cells. Statistical analyses were based on Student's t-test, ** p-value <0.01, *** p <0.001. Error bars = SEM from 3 biological replicates.
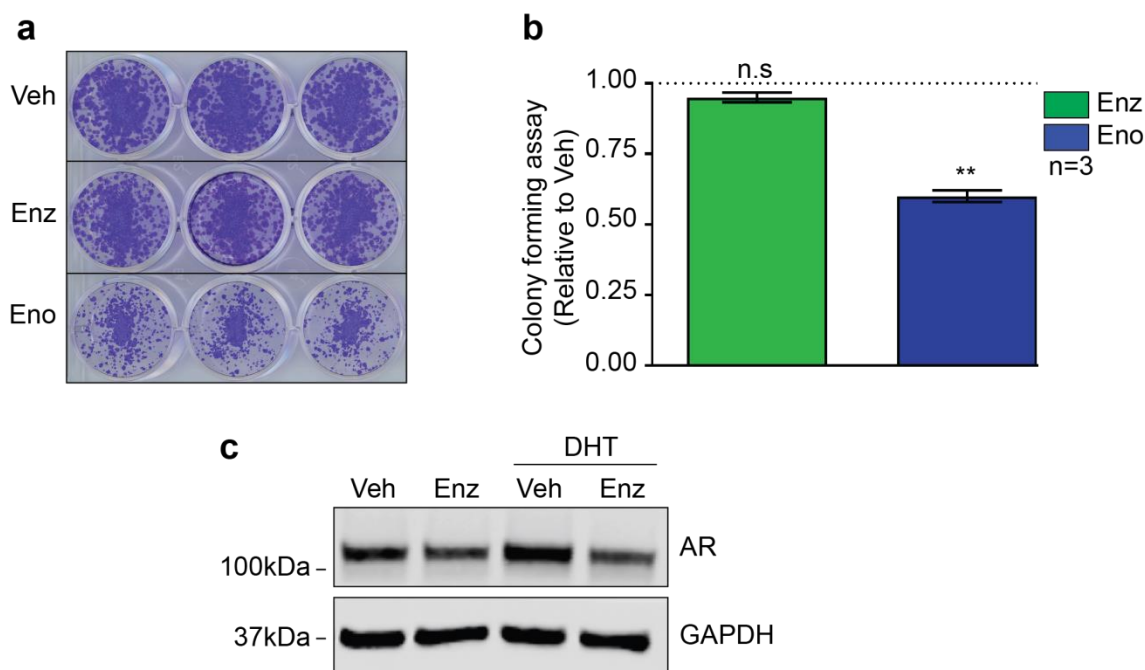
Figure 4.3. Enzalutamide does not inhibit colony formation of MCF7 TamR cells.
a) Cells were treated with either vehicle (Veh), 10 μM enzalutamide (Enz) or 100 nM Enobosarm (Eno) for 9 days prior to staining with crystal violet solution. **b)** Quantification of area coverage using ImageJ plugin ColonyArea from 3 biological replicates. **c)** Cells were treated with either Veh or 10 μM Enz, in the absence or presence of 1 nM DHT, for 24 h hours prior to protein extraction, and AR levels were compared between the different treatment groups using immunoblotting. GAPDH was immunoblotted as a loading control. Statistical analyses were based on Student's t-test. ** $p < 0.01$.and n.s. indicates not significant. Error bars = SEM from 3 biological replicates.

4.2.3   AR agonists induce G1 cell cycle arrest in MCF7 TamR and LTED cells

We further characterized the cell cycle response of MCF7 TamR and LTED cells to DHT and enobosarm using propidium iodide-based fluorescence-activated cell sorting (FACS) and immunoblotting. FACS analysis indicated a G1 phase cell cycle arrest in TamR and LTED cells treated with AR agonists as evidenced by the increased accumulation of cells in G1 phase following treatment (**Fig. 4.4a-d**). In support of this observation, immunoblotting analysis revealed reductions in the S-phase marker cyclin A and the mitotic marker phospho-histone 3 serine (pH3s10) in the cells treated with DHT and enobosarm relative to controls (**Fig. 4.4e-f**).

Furthermore, the impact of AR activation on poly(ADP-ribose) polymerase (PARP) was assessed. The cleavage of full length (FL) PARP to cleaved PARPis an early event in the initiation of apoptosis (Kaufmann et al., 1993). Treatment with AR agonists increased the cleavage of PARP in the MCF7 TamR cell line but had little effect on the MCF7 LTED cells which had a high baseline of PARP cleavage in the control cells

(**Fig. 4.4e-f**). Notably, while increased PARP cleavage post treatment with AR agonists was observed in the MCF7 TamR cells, this was not associated with increased subdiploid populations which are often used as a proxy for cell death (**Fig. 4a**). This suggests that the effect of AR agonists was still predominantly cytostatic at the time point examined. Overall, these observations indicated that AR activation induced a cytostatic effect in the resistant-derivatives of MCF7 cells regardless of the type of endocrine-therapy in which they were developed.
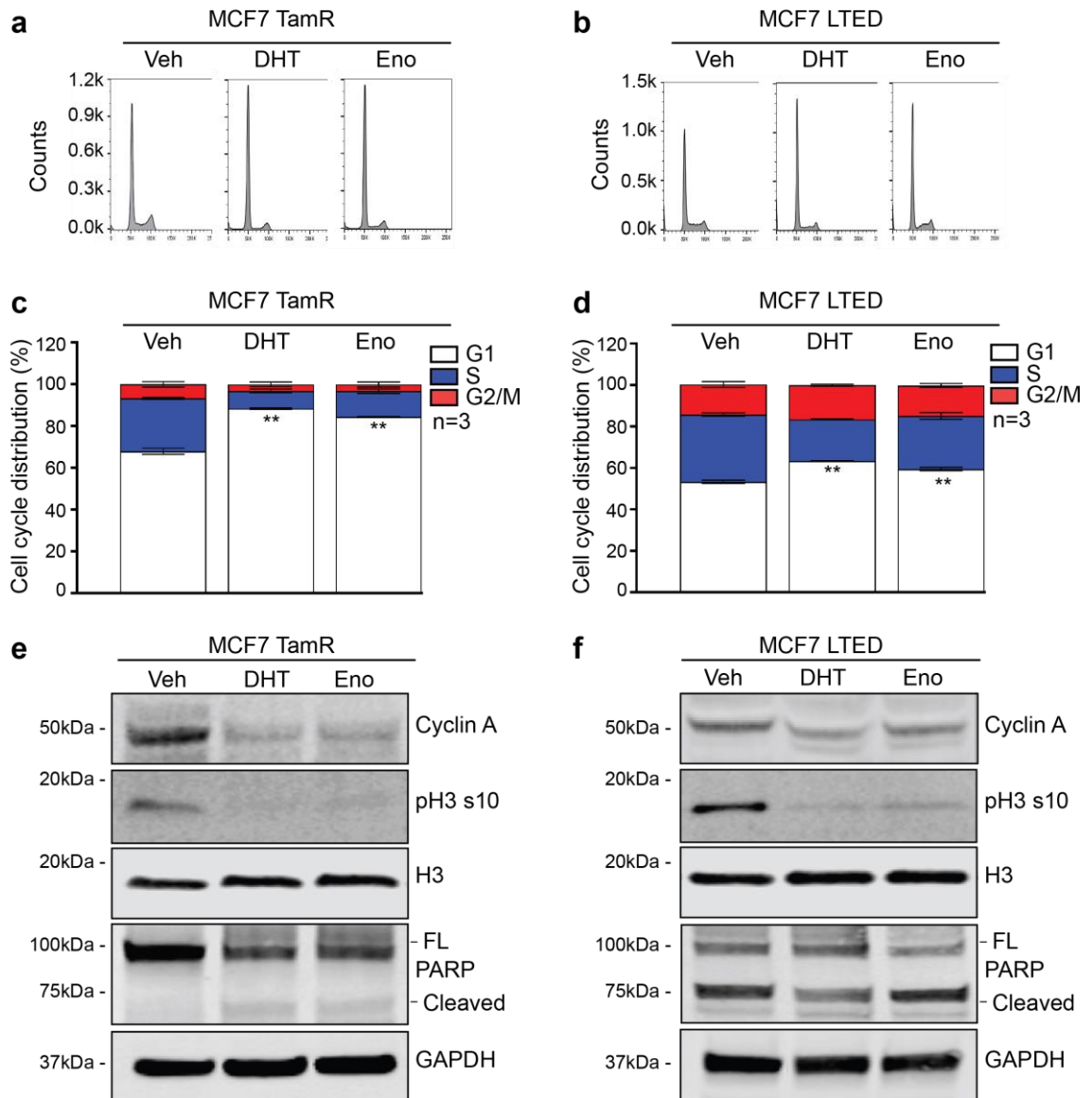
Figure 4.4. AR agonists induce G1 cell cycle arrest in endocrine-resistant MCF7 cells.

MCF7 TamR and LTED cells were treated with vehicle (Veh), 1 nM DHT or 100 nM enobosoarm (Eno) for 6 days prior to harvest for **a, b)** PI-FACS cell cycle analysis, **c, d)** quantification of cell cycle distribution, and **e, f)** immunoblotting from 3 biological replicates of MCF7 TamR and LTED cells. **e, f)** Cyclin A, phospho-histone serine 10 (pH3s10), histone 3 (H3), Poly ADP-ribose polymerase (PARP) and GAPDH (loading control) were immunoblotted using cell lysates derived from MCF7 TamR and LTED cells. Statistical analyses were based on Student's t-test. ** p-value <0.01 comparing the proportion of cells in G1 phase in the indicated treatment group vs vehicle treatment group. Error bars = SEM from 3 biological replicates.

4.2.4   AR agonists antagonize ER signalling in endocrine-resistant MCF7 cells

Immunoblotting analysis was performed to examine the impact of AR agonists on ER signalling. The expression levels of ER and PR were assessed in the parental and endocrine-resistant cells treated with DHT or enobosarm for 6 days (**Fig. 4.5**). Treatment with DHT or enobosarm reduced the expression level of ER only in the MCF7 TamR cells. In contrast, the level of ER was unaffected but the expression of PR was reduced with treatment with DHT and enobosarm in the MCF7 parental and LTED cells, providing support for the antagonism of ER transcriptional activity in these cells. These observations demonstrated that ER signaling can be inhibited by AR agonists in a cell-line specific manner. The pioneer factor FOXA1, which plays a critical role in the transcriptional activity of ER, was unchanged in all three cell lines treated with AR agonists.
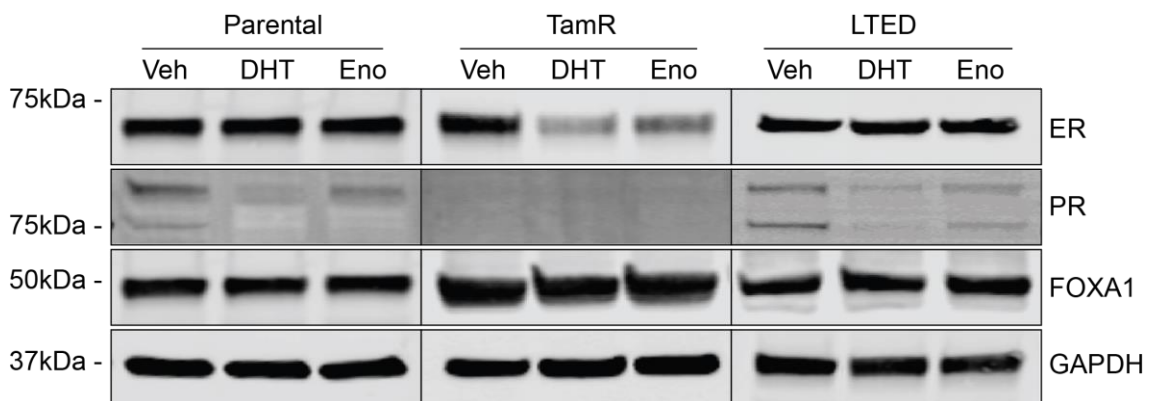
Figure 4.5. AR agonists reduce ER expression in endocrine-resistant cells.
MCF7 parental, TamR and LTED cells were treated with vehicle (Veh), DHT or enobosarm (Eno) for 6 days prior to harvest for immunoblotting. Protein extracted from the cell lysates was immunoblotted for ER, PR, FOXA1 and GAPDH (loading control).

4.2.5 *In vivo* evaluation of AR-directed therapy in PDX models

Next, we evaluated the efficacy of DHT and enobosarm on endocrine-resistant ER+ PDX models *in vivo*. These experiments involved a short-term and a long-term treatment cohort. Recipient female NSG mice were around 4-6 weeks old at the time of tumour implantation. Tumour-bearing mice were randomized and treated when tumours reached 150-200 mm^3. The short-term treatment cohort was treated for five days prior to harvest, and these samples were used for molecular analysis such as transcriptomic or ER chromatin binding analyses.

In the long-term treatment cohort, tumour-bearing mice were culled when tumours reached 1000 mm^3 (ethical endpoint) or after 60 days of treatment. A schematic illustration of a typical PDX experiment is shown in **Fig. 4.6**. For this Chapter, we focussed on the long term modulation of AR in the PDX models.
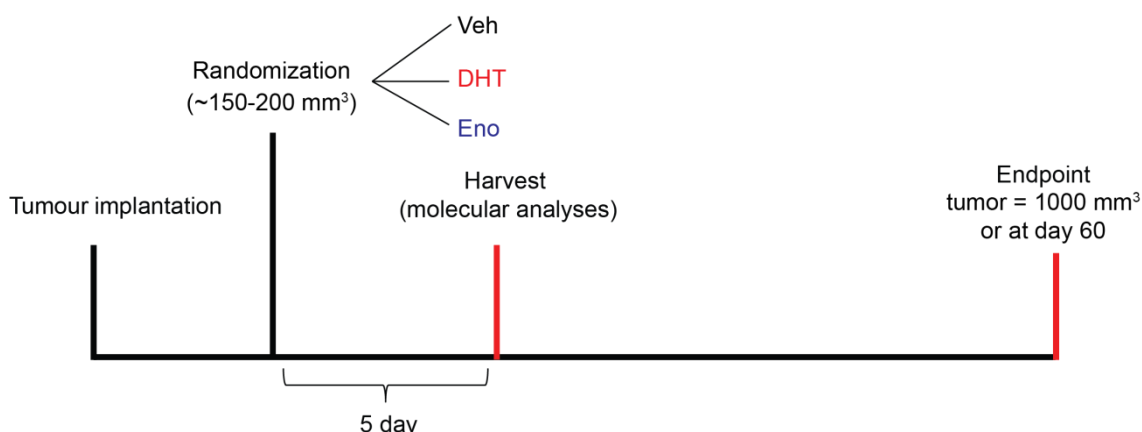
Figure 4.6. Schematic representation of a PDX experiment.
Donor tumour pieces implanted into NSG mice are randomized to vehicle (Veh), DHT or enobosarm (Eno) treatment arm when tumour volumes reached ~150-200 mm^3. One cohort of mice will be harvested five days post treatment and analyzed for molecular changes (short-term treatment). The long-term effect of treatment on tumour growthwas evaluated and mice in this cohort were culled at endpoint which was defined by tumour volumes of 1000 mm^3 or at day 60.

4.2.6 Activation of AR suppresses growth of Gar15-13 tumours

We first assessed the effect of AR agonists in the Gar15-13 PDX model. DHT had a stronger anti-proliferative effect compared to enobosarm (**Fig. 4.7a**). Consistent with the growth kinetics, the tumour weights in DHT and enobosarm treatment arms were significantly reduced relative to the controls (**Fig. 4.7b**). IHC analysis indicated that Ki-67 expression was significantly reduced with DHT and enobosarm (**Fig. 4.7c**). The proliferation index of control tumours was 50%, and this was reduced to 18% and 30% with DHT and enobosarm respectively (**Fig. 4.7d**). The strong tumour-suppressive effect of DHT was further validated by the reduction in cyclin A levels, a marker of proliferative cells in S phase, relative to controls (**Fig. 4.7e**). Importantly, administration of a high dose of fulvestrant (5 mg) (Heidari et al., 2015) on a weekly basis had no effect on the growth of Gar15-13 PDXs despite reducing the expression of ER consistent with its mechanism of action (**Fig. 4.8a-b**)(Osborne et al., 2004). However, treatment of an endocrine-sensitive ER+ PDX model with the same dosing regimen did inhibit growth of this model (results not shown) which suggests that the fulvestrant was efficacious and that the Gar15-13 PDX model was innately resistant to fulvestrant.
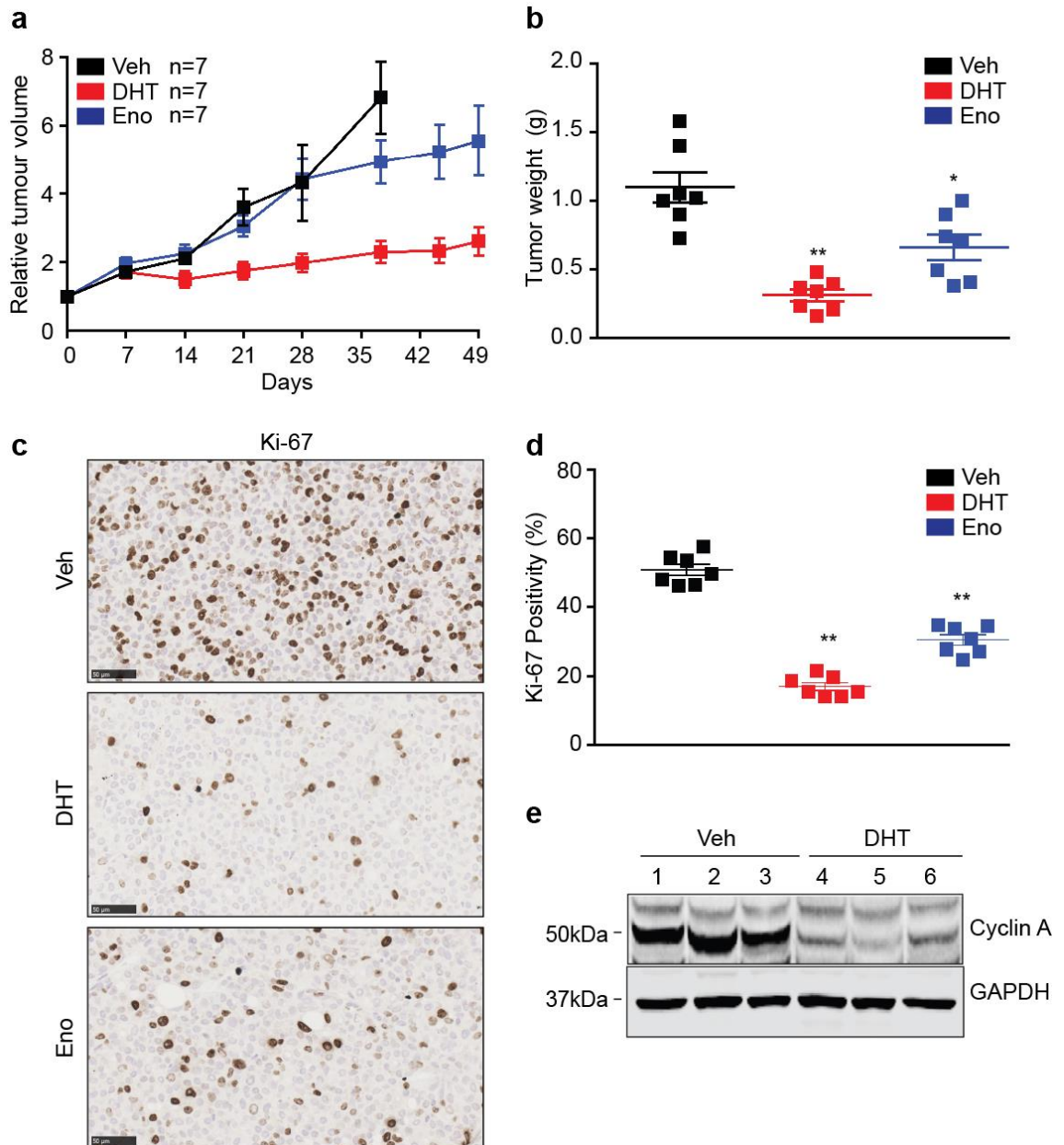
Figure 4.7. AR agonists suppress the growth of Gar15-13 PDX.
a) Tumour bearing mice were randomized to be treated with vehicle (Veh), DHT and enobosarm (Eno). Data are presented up to the point where all the animals were alive in each arm in order to the cross treatment comparisons valid and are represented as relative change from baseline tumour volume at the start of treatment (relative tumour volume). **b)** Tumour weights from long-term treated tumours harvested at endpoint were compared across the treatment groups. **c)** Endpoint harvested tumours were stained for Ki-67. Representative images at 40x magnifications are shown here and scale bar = 50 μm. **d)** Proliferation indices of tumours from different treatment groups were determined. * p<0.05 and ** p<0.01 using Student's t test **e)** Protein lysates were extracted from three Veh and three DHT-treated tumours and immunoblotted for cyclin A and GAPDH (loading control).
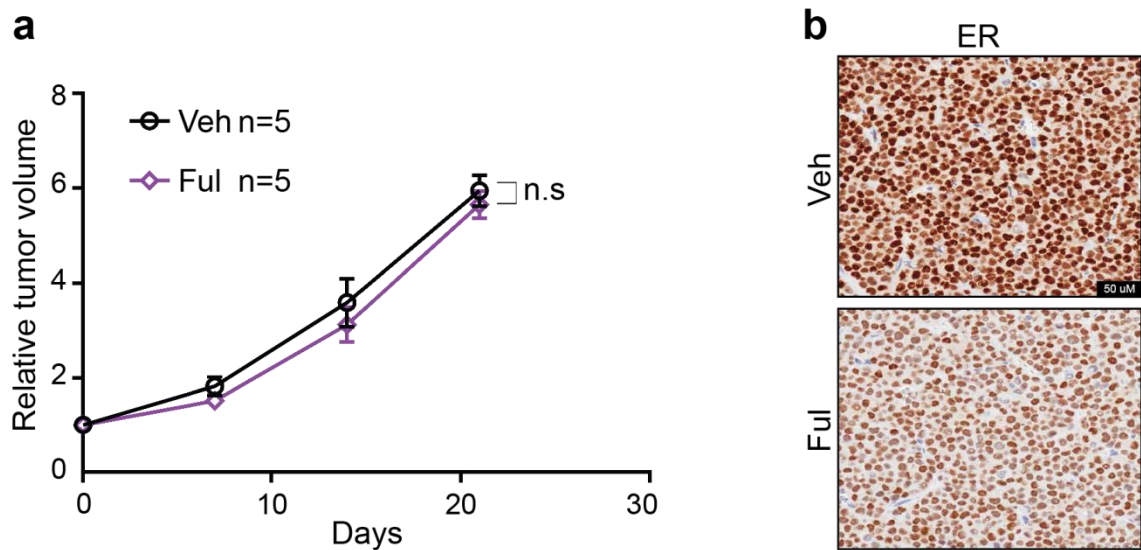
Figure 4.8. Fulvestrant does not affect growth of Gar15-13 PDX.
a) Growth kinetics of Gar15-13 treated with Veh or fulvestrant (Ful). Data are represented as relative change in tumour volume from baseline at the start of treatment (relative tumour volume). **b)** Representative images of Veh- or Ful-treated tumours stained for ER using IHC. n.s. indicates not significant when comparing the relative tumour volumes of tumours treated with Veh or Ful, at the indicated time point, using Student's t test. Error bars = SEM from 5 biological replicates.

4.2.7 Activation of AR antagonizes ER signaling in Gar15-13 PDX tumours

Further IHC analyses were performed to examine changes in AR and ER signalling as indicated by the levels of AR-regulated proteins SEC14L2 and FK506 binding protein 5 (FKBP5) and ER-regulated proteins PR and BCL2. The basal levels of SEC14L2 and FKBP5 were different with some expression of SEC14L2 evident in the control tumours while FKBP5 was completely absent in the vehicle treated samples (**Fig. 4.9a**). Treatment with DHT and enobosarm increased the protein levels of SECL14L2 and FKBP5, and these changes were concordant with enhanced nuclear localization of AR relative to control tumours (**Fig. 4.9a**). The magnitude of increase in SEC14L2 and FKBP5 was higher with DHT compared to enobosarm treatment, and supports the activation of canonical AR signalling. With regard to the impact of ER signaling, neither DHT nor enobosarm increased the expression of PR, suggesting that these ligands did not activate transcriptional activity of ER (**Fig. 4.9b**). Importantly, the expression of BCL2 was completely absent with DHT] and was strongly reduced with enobosarm treatment (**Fig. 4.9b**). The nuclear localization of ER remained largely unchanged in response to AR activation with either DHT or enobosarm. These observations demonstrated that the activation of AR was associated with downregulation of ER signaling in this endocrine-resistant PDX model, and the magnitude of growth suppression induced by AR agonists appeared to correlate with the degree of AR activation.
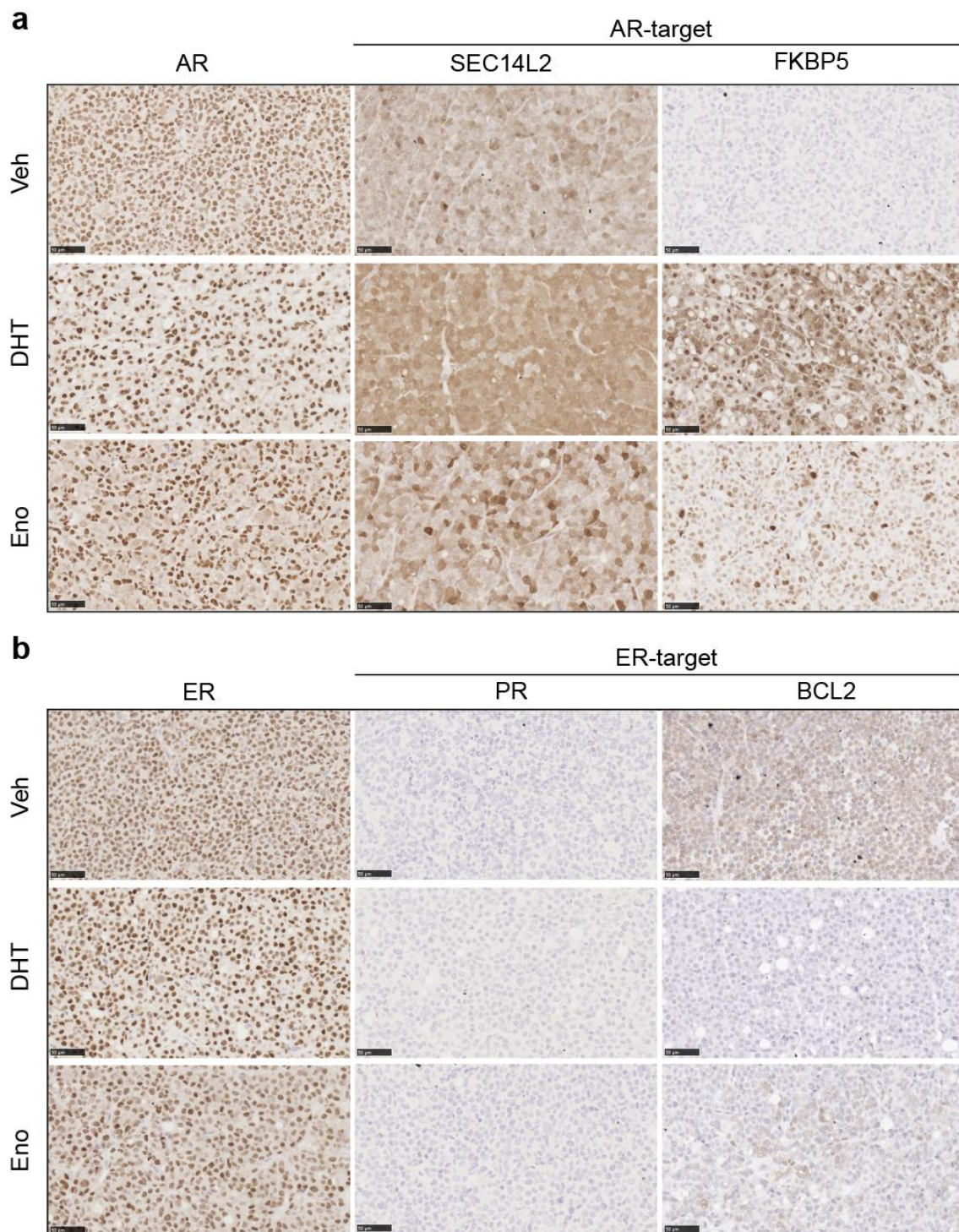
Figure 4.9. AR agonists reduce levelsof AR and ER-regulated proteins in Gar15-13 PDX tumours.

Endpoint tumours were subjected to IHC staining for AR, AR-regulated proteins SEC14L2, FKBP5 (**a**), ER, and ER-regulated proteins PR and BCL2 (**b**) following long term treatment with vehicle (Veh), DHT or enobosarm (Eno). Representative images at 40x magnifications are shown here. Scale bars = 50 μm.

4.2.8 Enobosarm reduces growth of an independent cohort of Gar15-13 PDX

Another independent Gar15-13 experiment was performed to validate the response of this model to enobosarm (**Fig. 4.10a**). In this cohort, enobosarm and DHT treatment similarly resulted in tumour growth suppression relative to controls (**Fig. 4.10a**). IHC analyses demonstrated an increase in SEC14L2 and a significant reduction in Ki-67 positivity with enobosarm, relative to vehicle treatment, as previously demonstrated (**Fig. 4.10b-c**).
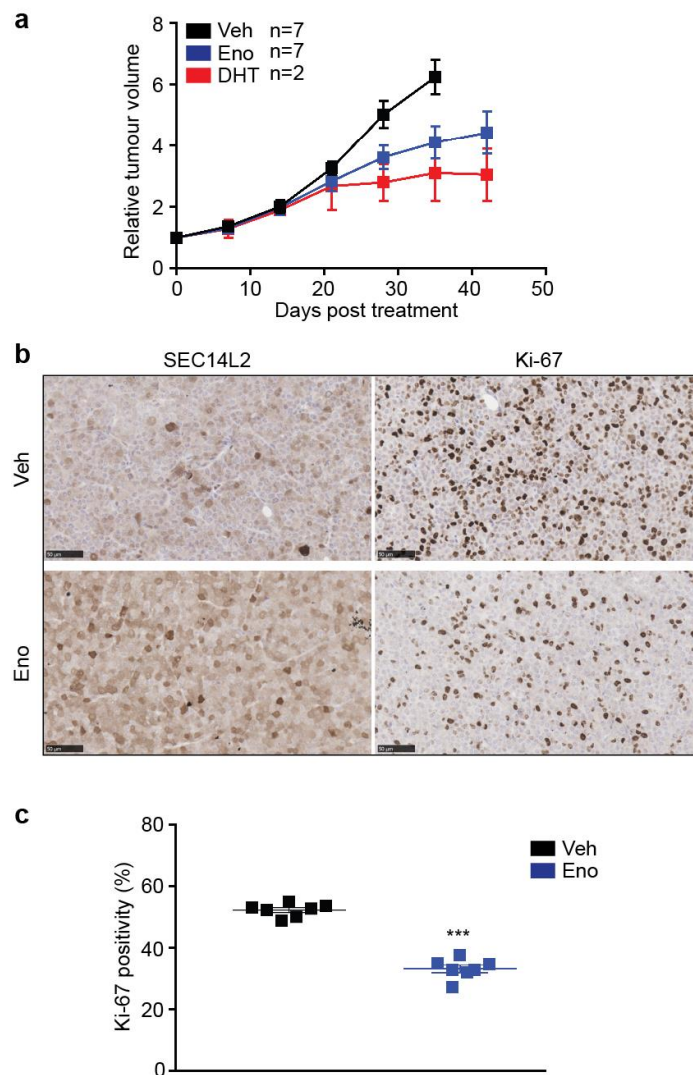
Figure 4.10. Validation of tumour-suppressive effect of enobosarm on Gar15-13 PDX tumours.
a) Growth kinetics of Gar15-13 treated with vehicle (Veh), Enobosarm (Eno) or DHT. Data are represented as relative change in tumour volume from baseline tumour volume at the start of treatment (relative tumour volume). **b)** Representative images of Veh- or Eno-treated tumours stained for SEC14L2 or Ki-67 using IHC. **c)** Proliferation indices of tumours treated with Veh or Eno. *** $p<0.001$ using Student's t test. Error bars = SEM from 7 biological replicates.

4.2.9 Characterization of HCI-005 PDX model

Moving on from the Gar15-13 model, we investigated the effect of *in vivo* AR modulation on another endocrine-resistant ER+ PDX model (HCI-005). The establishment of this PDX has been previously reported (DeRose et al., 2011). Unlike Gar15-13 PDX, this model was derived from the pleural effusion of a patient who had ER+PR+HER2- breast cancer and who had progressed on mutiple lines of endocrine therapy, including tamoxifen, an aromatase inhibitor and fulvestrant. This tumour was not HER2-amplified, similar to the Gar15-13.

Firstly, we confirmed that this model was able to grow in the absence of E2 supplementation (**Fig 4.11a**). Whole exome sequencing of the PDX identified a clinically important missense mutation in the hotspot region of *ESR1* (L536P) which has been previously reported to confer constitutive activity in a ligand-independent manner (Toy et al., 2017) (**Fig. 4.11b**). In addition, deletion of nucleotides in *BRCA2* and missense mutation in *mutS homolog* (MSH6; V50I) were also identified (**Fig. 4.11b**). Furthermore, the growth of this model can be enhanced with estrogen supplementation as previously reported (DeRose et al., 2011) (**Fig. 4.11a**). IHC staining indicated that this PDX tumour retained the expression of ER and PR, and it also expressed AR and the luminal marker CK8/18 (**Fig. 4.11c**).

a) Growth kinetics (Tumor volume vs Days post implantation)
- +E2 n=6
- - E2 n=3

b) Mutational profile

Gene	Types	AA change
ESR1	missense	L536P
BRCA2	INDEL	------
MSH6	missense	V50I

c) IHC: ER, PR, AR, Ki-67, CK8/18
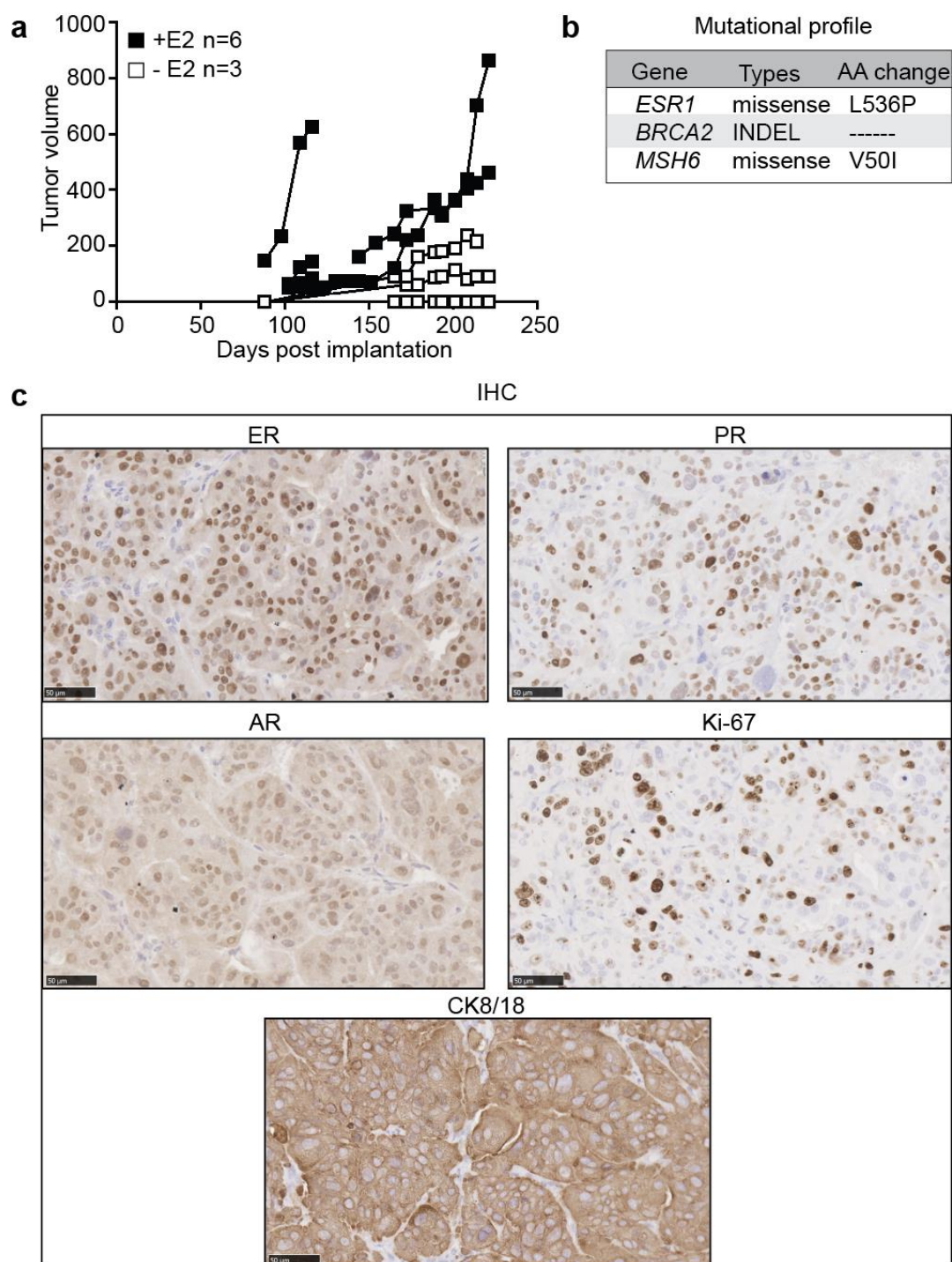
Figure 4.11. Characterization of the endocrine-resistant ER+ HCI-005 PDX.
a) Growth kinetics of HCI-005 PDX tumours implanted in NSG mice in the presence (n=6) or absence (n=3) of a 0.3 mg estradiol silastic pellet. **b)** Whole genome sequencing of HCI-005 revealed missense mutations resulting in amino acid (AA) changes from leucine (L) to proline (P) and valine (V) to isoleucine (I) in *ESR1* and *MSH6* respectively and an indel was identified within *BRCA2*. **c)** IHC staining for ER, AR, PR, Ki-67 and CK8/18 in HCI-005tumours which grew in the presence of estradiol supplementation. Scale bars = 50µm.

4.2.10 Activation of AR suppresses growth of HCI-005 PDX tumours

The effect of AR activation with DHT and enobosarm on the HCI-005 PDX was investigated. This experiment was performed in the presence of 0.3 mg estradiol silastic pellets (Dall et al., 2015) considering the slow growth rate of the tumours in the absence of estradiol in spite of harboring an activating *ESR1* mutation. Activation of AR signalling with DHT or enobosarm inhibited the growth of HCI-005 (**Fig. 4.12a**). The growth suppresive effect of enobosarm was much more effective on the HCI-005 model compared to the Gar15-13 PDX model. The weights of tumours treated with DHT or enobosarm at the end of the experiment were similar (**Fig. 4.12b**) and Ki-67 positivity was significantly reduced from 40% in the control arm to 5% and 10% with DHT or enobosarm treatment respectively (**Fig. 4.12c,d**). Furthermore, immunoblotting analysis demonstrated a reduction in the expression level of cyclin A which confirmed the cell cycle inhibitory effects of DHT in this model (**Fig. 4.12e**).
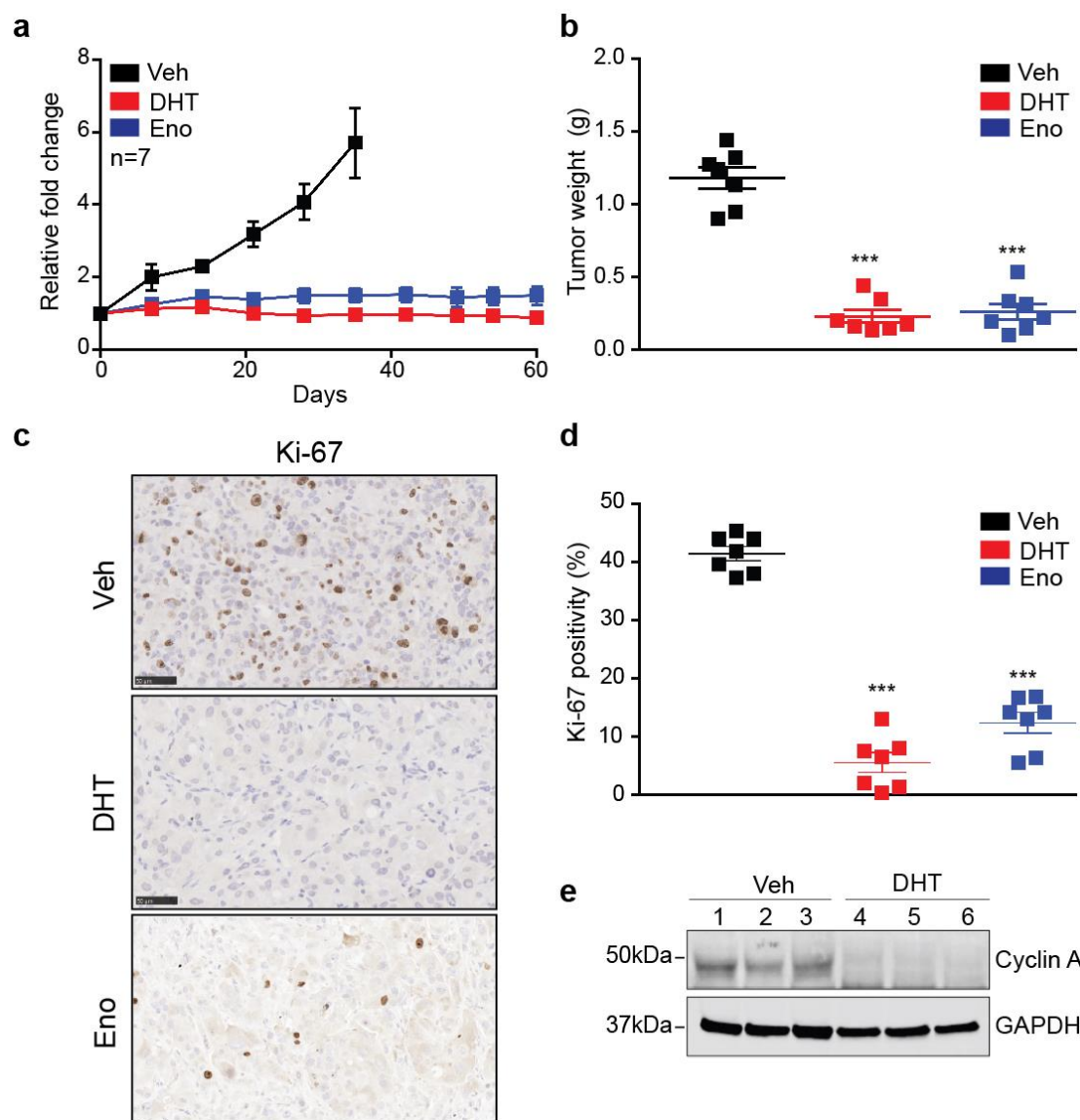
Figure 4.12. AR agonists suppress growth of the endocrine-resistant HCI-005 PDX model.
a) Tumour bearing mice were randomized to be treated with vehicle (Veh), DHT or enobosarm (Eno). Data are represented as relative change from baseline tumour volume at the start of treatment (relative tumour volume). **b)** Tumour weights from long-term treated tumours harvested at endpoint were compared across the treatment groups. **c)** Endpoint tumours were stained for Ki-67. Representative images at 40x magnifications are shown. Scale bar = 50 μm. **d)** Proliferation indices of tumours from different treatment groups were determined. *** p<0.001 using Student's t test. **e)** Protein lysates were extracted from three Veh and three DHT-treated tumours and immunoblotted for cyclin A and GAPDH (loading control).

4.2.11   AR agonists reduce ER signaling in HCI-005 PDX tumour

IHC was performed to examine the impact of AR activation on ER signaling in HCI-005. Treatment of HCI-005 with DHT or enobosarm increased AR transcriptional activity as evidenced by an increased nuclear localization of AR and expression of AR-regulated SEC14L2 and FKBP5, in keeping with its ligand-induced activity (**Fig. 4.13a**). Importantly, suppression of ER signalling, as indicated by reduced ER, and ER-regulated PR and BCL2, was associated with the activation of AR (**Fig. 4.13b**). Collectively, these phenotypic observations in endocrine-resistant breast cancer, along with published studies in endocrine-sensitive breast cancer, demonstrate that inhibition of ER signaling is a common feature of AR activation in ER+ breast cancer (Lanzino et al., 2005; Peters et al., 2009; Poulin et al., 1989). A summary of the key characteristics of the two endocrine-resistant PDX models and the consequences of DHT and enobosarm on growth and ER signaling are illustrated in **Fig. 4.14**
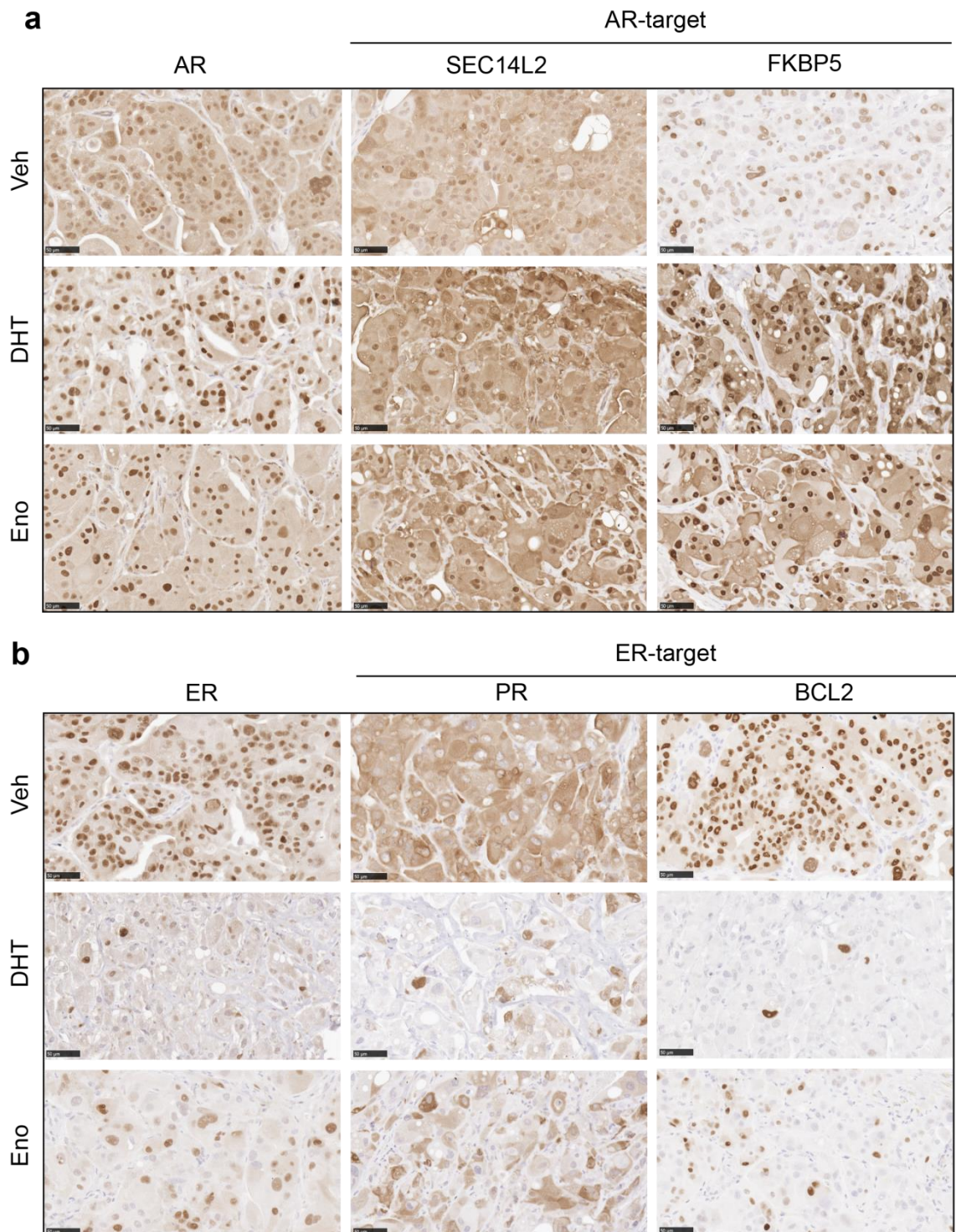
Figure 4.13. AR agonists antagonize the transcriptional activity of ER in HCI-005 PDX tumours.
Endpoint tumours following treatment with vehicle (Veh), DHT or enobosarm (Eno) were subjected to IHC staining for **a)** AR and AR-regulated proteins SEC14L2, FKBP5 and **b)** ER and ER-regulated proteins PR and BCL2. Representative images at 40x magnifications are shown here. Scale bars = 50 μm.

Figure 4.14. Summary of the phenotypes of endocrine-resistant PDX models and their response to AR agonists.

4.3 Discussion

Multiple clinical studies have reported that AR agonists are associated with tumour suppression with a clinical benefit rate of 38-50% (Birrell et al., 1995; Boni et al., 2014; Kono et al., 2016; Overmoyer et al., 2015). The agents used to activate AR in these studies include testosterone (Boni et al., 2014), testosterone analogue fluoxymesterone (Kono et al., 2016), medroxyprogesterone acetate which is a synthetic progestin with androgenic activity (Birrell, Roder, et al., 1995) and most recently with enobosarm (Overmoyer et al., 2015). However, the mechanisms by which AR activation induces suppression of these resistant-tumours remain undefined. Here we evaluated the efficacy of the AR natural ligand DHT and enobosarm on preclinical breast cancer cell lines and PDX models of endocrine-resistance. We demonstrated that AR activation with either DHT or enobosarm suppressed the proliferation of these preclinical models, with DHT typically exerting a greater growth-inhibitory effect..

Importantly, this effect was associated with a reduction in ER and ER signaling, and is similar to the effect achieved in endocrine-sensitive MCF7 cells (Lanzino et al., 2005; Peters et al., 2009; Poulin et al., 1989). However, the mechanisms by which AR agonists can inhibit ER signaling in our endocrine-resistant cells appear to be diverse, with reduced ER transcriptional activity, without alteration in the expression of ER, in the parental and LTED cells, and downregulation of ER expression in the TamR cells. The decrease in ER protein has previously been reported in the ER+ ZR-751 cell line and was observed when these cells were treated with DHT for 9 days (Poulin et al., 1989). Hence, given that we have only examined the effect of treatment for 6 days, it is possible that prolonged treatment of parental and LTED cells may similarly lead to downregulation of ER. The quick onset of ER downregulation in AR-agonist treated MCF7 TamR cells may be due to the acquired changes, such as in the overexpression of FOXA1, required to overcome tamoxifen resistance. As FOXA1 is a cofactor which interacts with ER and AR in breast cancer (Hurtado et al., 2011; J. L. Robinson et al., 2011), it can be hypothesized that high expression of FOXA1 in the TamR cells may sensitize these cells to ER loss following AR activation. Whilst this was not investigated in this thesis, a comprehensive comparison of ER and FOXA1 chromatin binding profile changes in the FOXA1-overexpressing TamR with that of parental and LTED cells post acute and chronic treatment with an AR agonist may shed light on the mechanisms underlying this differential response.

The observed *in vivo* tumour suppressive effect and downregulation of ER transcriptional activity in two endocrine-resistant PDX models following treatment with DHT and enobosarm is consistent with our *in vitro* data. Importantly, activation of AR with DHT elicited a pronounced and consistent growth-inhibitory effect on the PDX models, and this effect is independent of prior endocrine-therapy history and mutational status of *ESR1*. Our data with DHT, which is the most potent cognate ligand of AR, provide the clearest indication that canonical AR activity is tumour-suppressive and contradicts previous studies which have concluded that canonical AR activity contributes to endocrine-resistance (Ali et al., 2015; Ciupek et al., 2015; D'Amato et al., 2016; De Amicis et al., 2010; Rechoum et al., 2014). As described in Chapter 3, the contribution of AR to endocrine-resistance may be attributed to its non-canonical activity which is not effectively targeted by AR antagonist abirateterone acetate in the clinical setting (O'Shaughnessy et al., 2016).

A recent study reported that high expression of AR in primary breast cancer was associated with a good outcome (Ricciardelli et al., 2018). This, and our findings, suggest that AR signaling is anti-proliferative in breast cancer regardless of the state of endocrine-therapy sensitivity. To further test this notion, an AR gene-signature derived from DHT treatment of our endocrine-resistant PDX models may be used to stratify ER+ breast cancer patients according to outcomes in the context of primary disease and metastatic disease, and will be described in detail in the following Chapter.

While DHT inhibited tumour growth in our preclinical models, the response of enobosarm was more variable. Enobosarm resulted in a tumour growth inhibition in the HCI-005 PDX model to a similar degree as DHT, but had a weaker growth inhibitory effect in the Gar15-13 PDX model. However, it is important to note that Gar15-13D was resistant to fulvestrant which is the most potent clinical antagonist of ER and yet it was still modestly growth-inhibited by enobosarm. This suggests that AR-targeted therapies may be effective in aromatase inhibitor-resistant patients who do not adequately respond to subsequent ER-targeted therapies such as fulvestrant.

The difference in response to enobosarm could be attributed to the inherent differences between these two PDX models such as the mutational status of *AR* and growth response to E2. Gar15-13 has a missense mutation in *AR* leading to amino acid change to serine in place of proline at residue 392 (P392S) which may reduce the response of

this tumour to a "weaker" agonist like enobosarm. The consequences of AR mutations in breast cancer are currently unknown. AR mutations in primary breast cancer are rare and interrogation of the METABRIC cohort revealed a complete absence of AR mutations in 2509 non-selected breast cancers (Pereira et al., 2016) (**Appendix A**). However, a similar analysis in a metastatic breast cancer cohort comprising 213 samples (~65% ER+) (Lefebvre et al., 2016) identified genetic aberrations in *AR* in 4 (~2%) samples (**Appendix A**). Of the four mutations, three occurred in ER+ breast tumours and one was found in an ER-HER2+ breast tumour. Interestingly, the *AR* Leu57Gln mutation identfied in the ER-HER2+ breast tumour has been reported to contribute to development of androgen-independent prostate cancer (Tilley et al., 1996), which suggests that mutation at this site may allow for AR to be constitutively active and lose its dependency on androgens for activation. The consequence of this mutation in an ER-HER2+ breast cancer may be of clinical significance as AR activation has been described to contribute to the growth of ER-HER2+ breast cancer (Chia et al., 2011; Ni et al., 2011; J. L. Robinson et al., 2011). Mutiple preclinical studies have now demonstrated that inhibition of AR in this breast cancer subtype is an effective therapeutic strategy (Chia et al., 2011; Ni et al., 2011; J. L. Robinson et al., 2011). The other three *AR* mutations in the ER+ breast tumours identified in the metastatic cohort have not been described previously.

The Gar15-13 PDX model harbours a missense mutation in *AR* leading to amino acid change to serine in place of proline at residue 392 (P392S). This mutation is frequently reported to be associated with androgen insensitivity syndrome where AR fails to respond to physiological levels of androgens, resulting in impairment of masculinization of male-specifc organs (Audi et al., 2010; Ferlin et al., 2006; Hiort et al., 2000) (**Appendix A**). Hence, it is plausible that as a result of this mutation, a greater degree of AR activation is required to achieve complete growth inhibition, as demonstrated with DHT, and a weaker AR agonist such as enobosarm, can only induce partial inhibition of growth.

It is not known if this AR mutation was present in the primary breast cancer or if it was an acquired somatic mutation in response to aromatase inhibitor treatment. Aromatase catalyzes the conversion of testosterone into estradiol and inhibition of this enzyme has been demonstrated to result in an elevation of androgen levels (Takagi et al., 2010).

Furthermore, this increased level of androgens have been proposed to contribute to the growth-inhibitory effect of aromatase inhibitors (Chanplakorn et al., 2011; Macedo et al., 2006). Hence, it could be posited that this mutation may provide a survival advantage by blunting the antiproliferative effect of aromatase inhibitors. While the significance of this mutation is not addressed in this thesis, the functional significance of this mutation could be tested experimentally. This can be achieved by mutating *AR* using CrispR technology in an aromatase-overexpressing ER+ cell line (e.g MCF7) and assessing the *in vivo* growth response of this *AR*-mutant cell line to testosterone and aromatase inhibitor treatment relative to its wild-type *AR* counterpart.

Interestingly, defective canonical transcriptional activity of AR has also been implicated in breast cancer. A splice variant of AR, with a deletion in exon 3 and resulting in an approximately 105kDa truncated AR protein, has been identified in breast cancer (Zhu et al., 1997). This AR variant has also been associated with androgen insensitivity syndrome (Quigley et al., 1992). This AR splice variant has a deletion in exon 3, which encodes for the zinc finger domain critical for receptor binding to DNA (Berg, 1989), and is associated with reduced DNA-binding affinity and reduced transcriptional regulation of AR-target genes (Quigley et al., 1992). It was postulated that this splice variant of AR reduces the growth inhibitory effect of androgens on the proliferation of ER+ tumours althought this has yet to be demonstrated (Zhu et al., 1997). Collectively, there is evidence suggesting that reduced canonical transcriptional activity of AR, either via alternative splicing or point mutation, may lead to progression of ER+ breast cancer and the clinical implications of *AR* aberrations warrant further investigation.

Another key point of distinction between the Gar15-13 and HCI-005 PDX models which may contribute to the differential response to enobosarm is the growth response to estradiol. The experiments with Gar15-13 were performed in the absence of E2 as Gar15-13 not only grows in an E2-independent manner but it is also growth-suppressed by E2. The growth-inhibitory effect induced by estradiol in the Gar15-13 PDX model is associated with increased PR levels and this suggests that ER signaling is not engaged in a classical manner in the absence of estradiol. On the other hand, the experiment with HCI-005 was performed in the presence of estradiol as the growth of this model is enhanced by estradiol. Furthermore, the expression of PR in the HCI-005 model in the presence of E2 indicates ER is operating "classically" in this model. It is hence

plausible that weak activation of AR with enobosarm is sufficient to antagonize *in vivo* growth driven by "classical ER signaling" as in the HCI-005 model.

The implications of these results is that the expression of PR, a proxy for classical ER signaling, could potentially be used as a biomaker of response to enobosarm as a monotherapy. A case study has reported that a patient with a ER+PR+AR+ breast cancer, who had progressed on mutiple lines of endocrine therapies including palbociclib, obtained a durable partial response to enobosarm with progression-free survival of ~ 11 months (Vontela et al., 2017). In the recently concluded phase 2 enobosarm trial which enrolled patients with metastatic ER+ breast cancer (NCT01616758), it would be interesting to determine if the expression of PR can stratify responders from non-responders.

In this chapter, we have demonstrated that activating AR is an effective therapeutic strategy in suppressing the growth of endocrine-resistant breast cancer *in vitro* and *in vivo*. Moreover, there is some evidence demonstrating that this effect is associated with the downregulation of ER signalling. A comprehensive characterization of global gene expression changes in response to AR activation in these models will provide definitive molecular mechanisms underlying this growth-suppressive effect.

CHAPTER 5

5. Molecular characterization of AR-regulated genes in endocrine-resistant breast cancer

5.1 Introduction

AR is expressed in up to 90% of primary ER+ breast cancer and multiple studies have correlated the expression of AR with clinically-favourable prognostic factors such as low grade, small tumour size and negative lymph node status (Aleskandarany et al., 2016; Collins et al., 2011; Gonzalez et al., 2008; Jiang et al., 2016; Niemeier et al., 2010; Qi et al., 2012; Vera-Badillo et al., 2014). A recent meta-analysis, based on twenty-two studies and more than five thousand AR+ breast cancer patients, further affirmed the prognostic value of AR in early-stage ER+ disease, reporting that high expression of AR was significantly associated with improved disease-free survival and overall survival in univariate and multivariate analyses which factored the expression levels of *ESR1* and *ERRB2*, patient age, tumour grade, size and lymph node involvement (Bozovic-Spasojevic et al., 2017). The definition of AR positivity in these studies ranged from 1% to 75% (Castellano et al., 2010; Park et al., 2011; Tokunaga et al., 2013).

A recent study has proposed an optimal level of AR expression cut-off in the tumour of 78% or greater using the receiver operating characteristic method on their retrospective patient cohort to accurately be an independent prognostic marker of good outcome (Ricciardelli et al., 2018). As nuclear AR was evaluated in these studies, and nuclear localization of AR is largely driven by androgen binding, the high percentage of AR positivity required to be prognostic likely reflects the requirement for a high degree of AR activity in these tumours. This is consistent with our data in endocrine-sensitive MCF7 cells (Chapter 4), and other preclinical studies demonstrating that ligand activation of AR suppressed growth of ER+ endocrine-sensitive cell lines *in vitro* (Birrell, Bentel, et al., 1995; Cops et al., 2008; Lapointe et al., 1999). While nuclear AR is often used as a proxy for the activation of AR, it remains to be determined which downstream genes and signalling processes are regulated by ligand-bound AR in breast

cancer. Moreover, an AR gene signature predictive of survival in ER+ breast cancer has yet to be identified.

Our results showing the growth suppression of endocrine-resistant breast cancer cell lines and breast cancer PDX models by DHT and enobosarm (Chapter 4) indicate that active AR signalling is also tumour-suppressive in the endocrine-resistant context. Here we sought to utilize these models to characterize the AR-regulated genes in breast cancer and determine the underlying mechanistic basis of the anti-tumour effect of AR agonists.

The specific aims of this Chapter were to

1) Provide further molecular evidence of antagonism of ER signalling by AR activation
2) Identify the processes regulated by DHT-induced AR activation
3) Derive an AR-gene signature from DHT-treatment of preclinical endocrine-resistant breast cancer models, and assess the prognostic potential of this gene signature.

5.2 Results

5.2.1 Schematic of downstream analyses for short-term treated PDX models

We performed molecular analyses on tumours harvested following short-term (5-day) treatment. This approach was undertaken to standardise the number of doses the tumours have received, as endpoint tumours were likely to have been exposed to different doses, and to investigate the short-term transcriptomic changes associated with the activation of AR in order to minimise the effect of compensatory signalling consequences with longer term treatment. IHC analyses and confirmatory RT-qPCR analyses for selected AR- and ER-regulated genes were investigated to ascertain changes at the transcript level prior to global gene expression profiling using RNA-sequencing. Finally, a prognostic AR-gene signature was derived from DHT-treated Gar15-13 PDX using the clinically annotated ER+ patient cohort in METABRIC. A schematic of the analyses performed is outlined in **Fig. 5.1**.
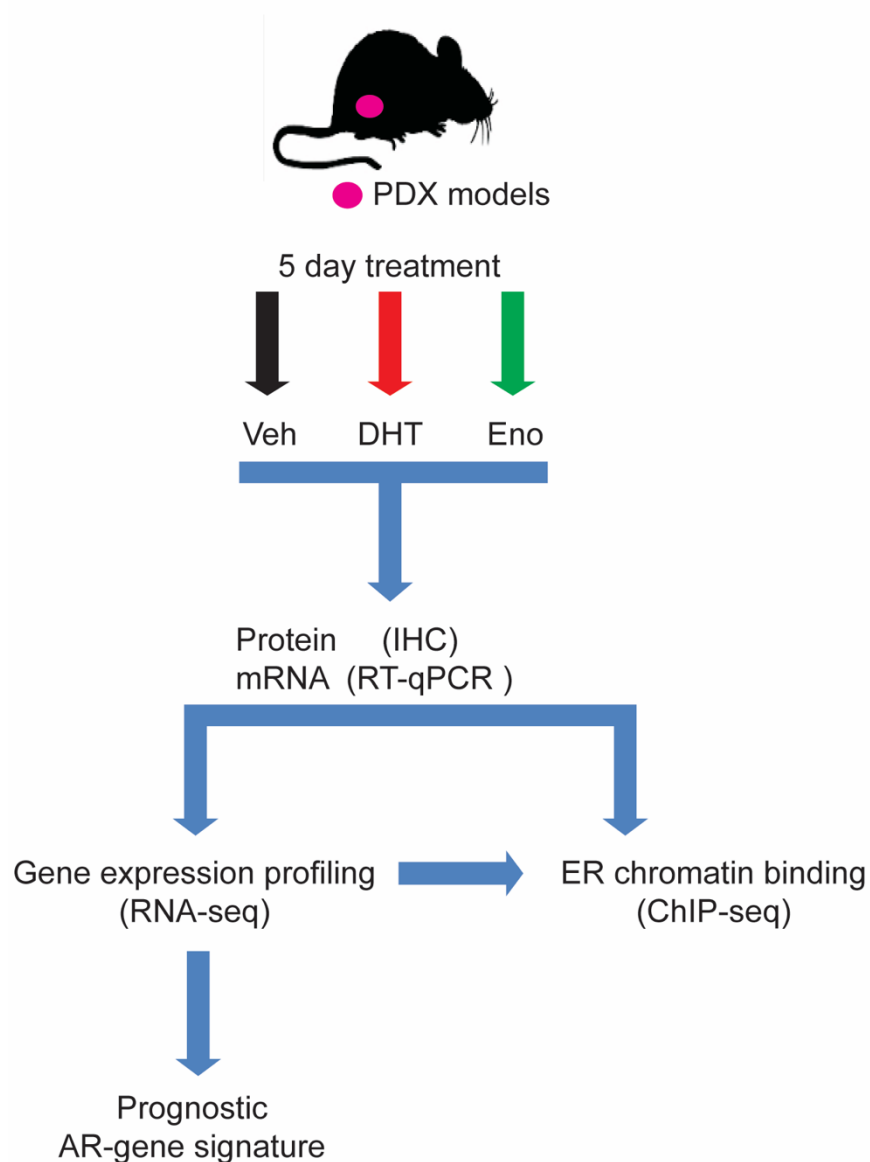
Figure 5.1. Flow of experiments and analyses in Chapter 5.
ER+ PDX models were subjected to treatment with Veh, DHT or Eno for 5 days prior to harvest. IHC and RT-qPCR were performed to determine the effects on AR and ER signalling pathways. Global gene expression profiling and ER chromatin immunoprecipitation followed by sequencing (ChIP-seq) were performed to identify differentially expressed genes and to interrogate changes in ER chromatin binding respectively. An AR gene signature associated with survival was derived from DHT-induced differentially expressed genes in Gar15-13 PDX.

5.2.2 IHC analysis of short-term treatment in HCI-005

Short-term treatment of HCI-005 PDX with DHT or enobosarm stimulated AR signalling as indicated by an increase in nuclear localization of AR and increased expression of AR target genes, FKBP5 and SEC14L2 (**Fig. 5.2a**). There was a reduction in ER with DHT only, and diminished transcriptional activity of ER (reduced levels of ER-regulated genes BCL2 and PGR) observed with both DHT and enobosarm (**Fig. 5.2a**). Treatment with DHT or enobosarm for 5 days significantly reduced proliferative capacity, as indicated by lower Ki-67 positivity, relative to control tumours (**Fig. 5.2c-d**). DHT exerted a more pronounced effect on Ki-67 than enobosarm as previously observed (**Fig. 4.11d**). Overall, short-term treatment with AR agonists elicited a similar suppressive effect on ER signalling and Ki-67 positivity as long-term treatment (**Fig 4.11d, 4.12**).
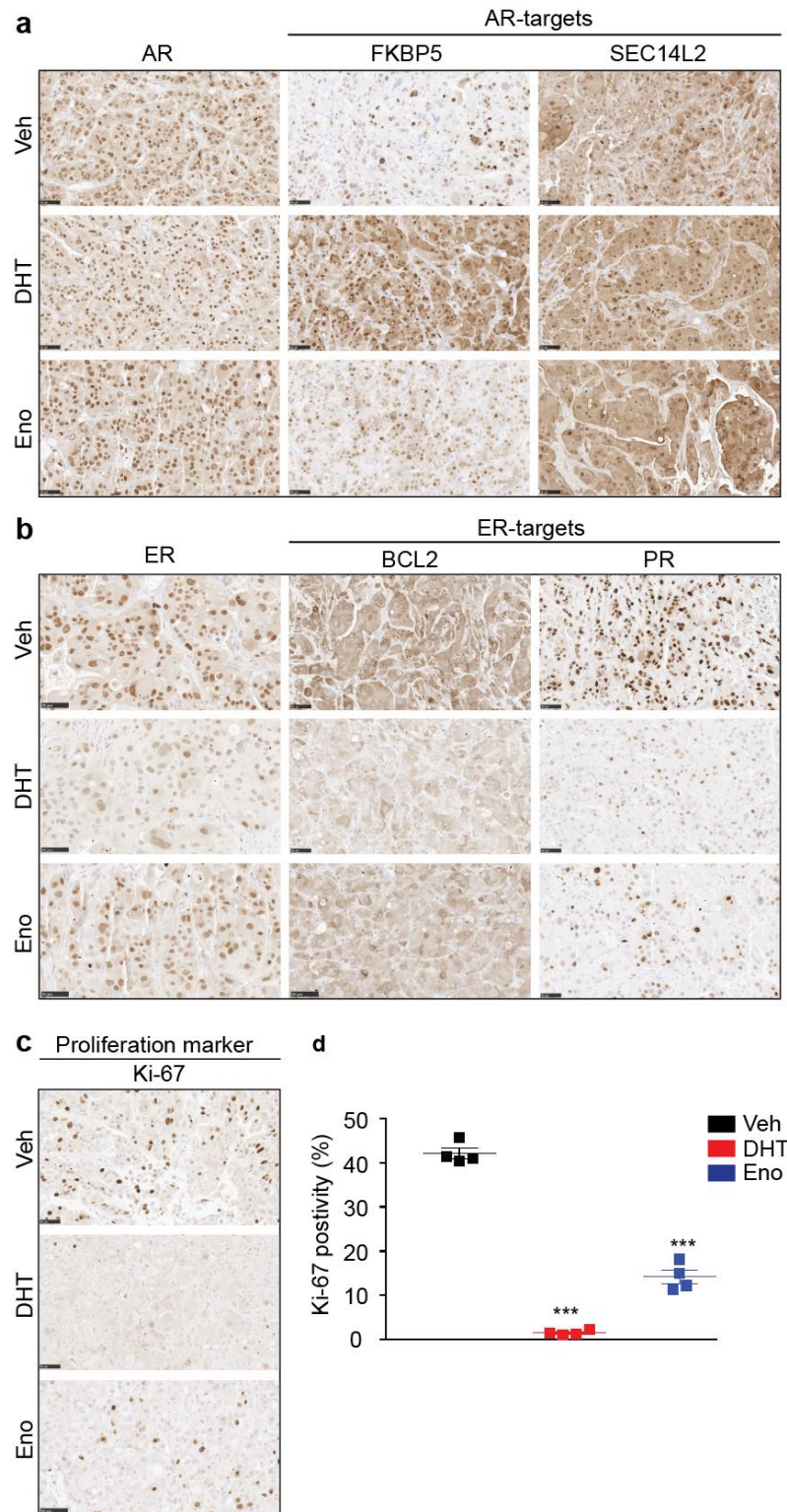
Figure 5.2. IHC analysis of tumours following 5 day AR agonist treatment in HCI-005 PDX.
Tumour-bearing mice were treated with Vehicle (Veh), DHT or enobosarm (Eno) for 5 days prior to harvest. IHC was performed for AR, AR-regulated proteins SEC14L2 and FKBP5 (**a**), ER, ER-regulated proteins BCL2 and PR (**b**), and the proliferation marker Ki-67 (**c**). Proliferation indices of tumours from the different treatment groups were determined (**d**). *** $p<0.001$ using Student's t test. Error bars = SEM from 4 biological replicates. Representative images at 40x magnification are shown. Scale bars = 50 μm.

5.2.3   RT-qPCR analysis of selected ER and AR target genes in HCI-005 PDX.

RT-qPCR was performed to ascertain changes in AR- and ER-regulated genes at the transcript level in HCI-005 PDX tumours. We observed that short-term treatment with DHT or enobosarm increased the expression levels of AR-regulated genes *SECL14L2* and *FKBP5,* while *AR* was unaffected (**Fig. 5.3a**), DHT elevated the mRNA transcript of *SECL14L2* by ~3.5-fold and *FKBP5* by ~25-fold (**Fig. 5.3b**). In contrast, DHT but not enobosarm reduced the expression of *ESR1* (**Fig. 5.3c**), consistent with the IHC results, and the expression of ER-target genes *BCL2* and *PGR* was significantly downregulated by both DHT and enobsarm **(Fig. 5.3d)**.
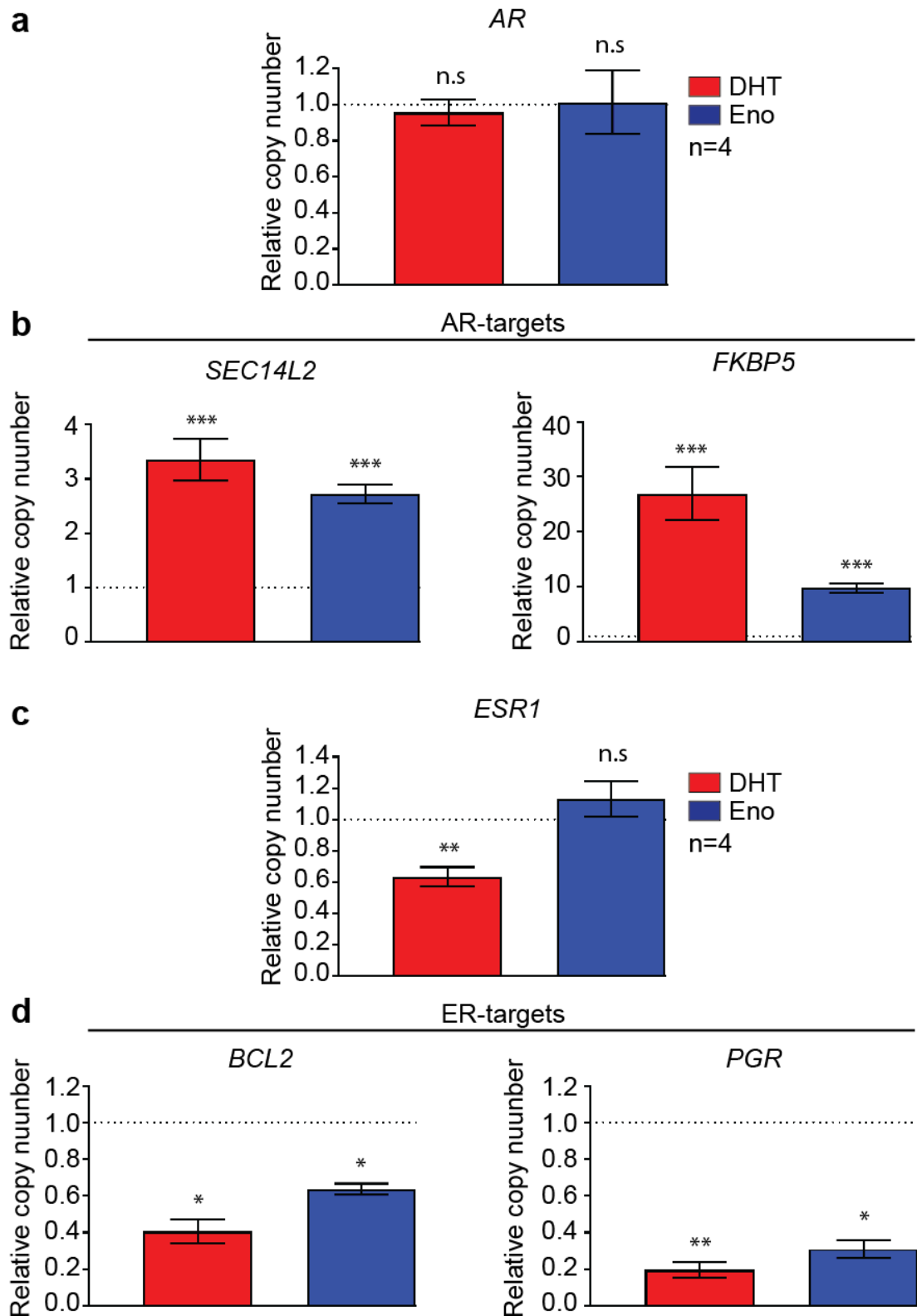
Figure 5.3. RT-qPCR analysis of tumours following 5 day AR agonist treatment in HCI-005 PDX.
Probes against *AR* (**a**), AR-target genes *SEC14L2* and *FKBP5* (**b**), *ESR1* (**c**), and ER-target genes *BCL2* and *PGR* (**d**). Copy number of target genes of interest was normalized to *PUM1* and *IPO8* and the data are represented as relative to Veh. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and n.s. = not significant using Student's t test. Error bars represent SEM from 4 biological replicates.

5.2.4 IHC analysis of tumours following 5 day AR agonist treatment in Gar15-13 PDX.

IHC analyses in this second endocrine-resistant PDX model similarly demonstrated that DHT and enobosarm induced activation of canonical AR activity as indicated by increased nuclear AR localization and expression of FKBP5 and SEC14L2 (**Fig. 5.4a**). Downregulation of ER was only observed with DHT, as with the HCI-005 PDX model, but downregulation of BCL2 was observed with both DHT and enobosarm (**Fig 5.4b**). PR remained negative with DHT and enobosarm indicating that neither drug acted as an ER agonist. Short-term treatment of Gar15-13 PDX (**Fig. 5.4c,d**) also reduced the Ki-67 positivity within the tumours (**Fig. 5.4c,d**) to levels similar to that of the endpoint treatment (**Fig. 4.7d**)
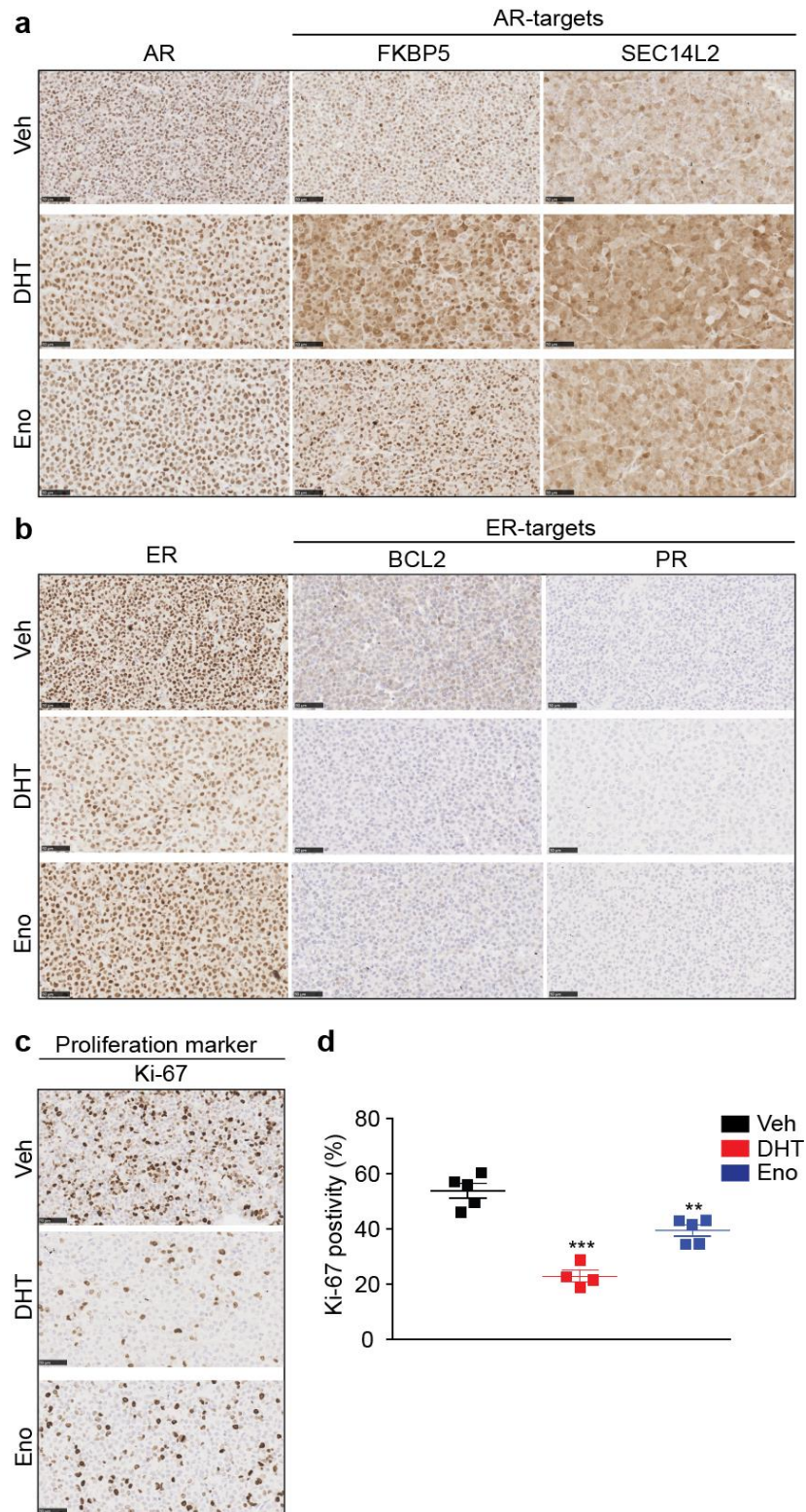
Figure 5.4. IHC analysis of tumours following 5 day AR agonist treatment in Gar15-13 PDX.
Tumour-bearing mice were treated with Vehicle (Veh), DHT or Enobosarm (Eno) for 5 days prior to harvest. Tumours were stained for AR, AR-regulated proteins SEC14L2 and FKBP5 (**a**), ER, ER-regulated proteins BCL2 and PR (**b**), and proliferation marker Ki-67 (**c**). Proliferation indices of tumours from the different treatment groups were determined (**d**). ** $p<0.01$ and *** $p<0.001$ using Student's t test. Error bars = SEM from 4-5 biological replicates. Representative images at 40x magnification are shown. Scale bars = 50 μm.

5.2.5 RT-qPCR analysis of selected ER and AR target genes in Gar15-13

RT-qPCR was performed to ascertain transcriptomic changes in selected AR- and ER-regulated genes. 5 day treatment with DHT or enobosarm significantly increased the expression levels of AR-regulated genes *SECL14L2* and *FKBP5*, while DHT but not enobosarm reduced the expression of *AR* (**Fig. 5.5a,b**). Notably, DHT increased the expression of *SEC14L2* and *FKBP5* by ~15-20-fold. This is in contrast with the lower magnitude of *SECL14L2* (-3.5-fold) induced by DHT in the HCI-005 model (**Fig. 5.3b**). There was also evidence of reduced ER signalling at the transcriptomic level in DHT- and enobosarm-treated tumours. As observed with the HCI-005 model, only DHT reduced the transcript level of *ESR1*, while both DHT and enobosarm downregulated ER-regulated *BCL2* and *CCND1* (**Fig. 5.5c,d**)

Collectively, these results demonstrated that short-term treatment was sufficient to induce changes in AR and ER signalling pathways in two endocrine-resistant PDX models. Furthermore, the observed change at the protein level was also reflected at the transcript level and this justified the use of short-term treatment to interrogate AR-induced global gene expression changes. Importantly, these results also provided strong molecular evidence of ER activity inhibition by AR agonists in these PDX models.
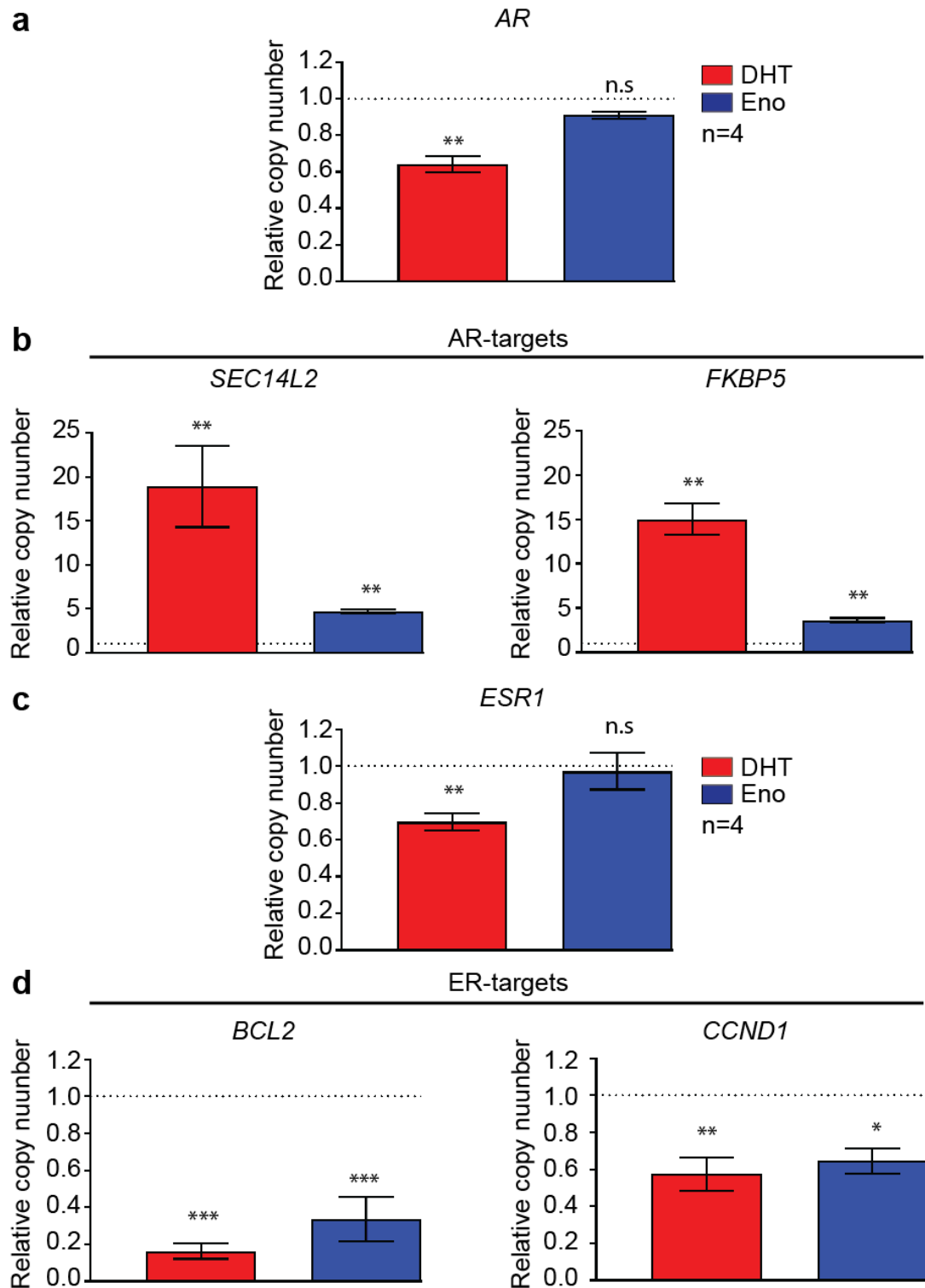
Figure 5.5. RT-qPCR analysis of tumours following 5 day AR agonist treatment in Gar15-13 PDX.
RT-qPCR was performed using probes against *AR* (**a**), AR-targets *SEC14L2* and *FKBP5* (**b**), *ESR1* (**c**), and ER-targets *BCL2* and *CCND1* (**d**) were used for RT-qPCR. Copy number of target of interest was normalized to *PUM1* and *IPO8* and data are represented as relative to Vehicle (Veh). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and n.s. indicates not significant using Student's t test. Error bars represent SEM from 4 biological replicates.

5.2.6 Global gene expression profiling of short-term treated Gar15-13 PDX tumours.

The short-term treated Gar15-13 PDX samples were subjected to next generation RNA sequencing. At a threshold of q-value<0.05, 2,726 genes and 378 genes were differentially expressed with DHT and enobosarm treatment relative to vehicle treatment, respectively (**Fig. 5.6a**). An overlap of these differential genes indicated that a significant number of genes (n=359) induced by enobosarm were also differentially regulated by DHT (**Fig. 5.6b**). However, DHT also differentially regulated a large number of unique genes (n=2,367). Amongst the differentially expressed genes, roughly equal number of genes was up- and down-regulated by DHT whereas slightly more than half of enobosarm-regulated genes were upregulated (**Fig. 5.6c**). Using a more stringent cut off at q<0.05 and absolute fold-change of >2,665 and 225 genes were defined to be differentially expressed in DHT and enobosarm treated samples respectively (**Fig. 5.6d**). At this cut-off, all of the genes regulated by enobosarm (n=225) were also regulated by DHT. Furthermore, slightly more than half of these genes regulated by DHT and enobosarm were upregulated. Based on these analyses, it can be concluded that 1) enobosarm behaved like a weak AR agonist and that 2) genes which were altered by two or more fold-change were more likely to be upregulated.
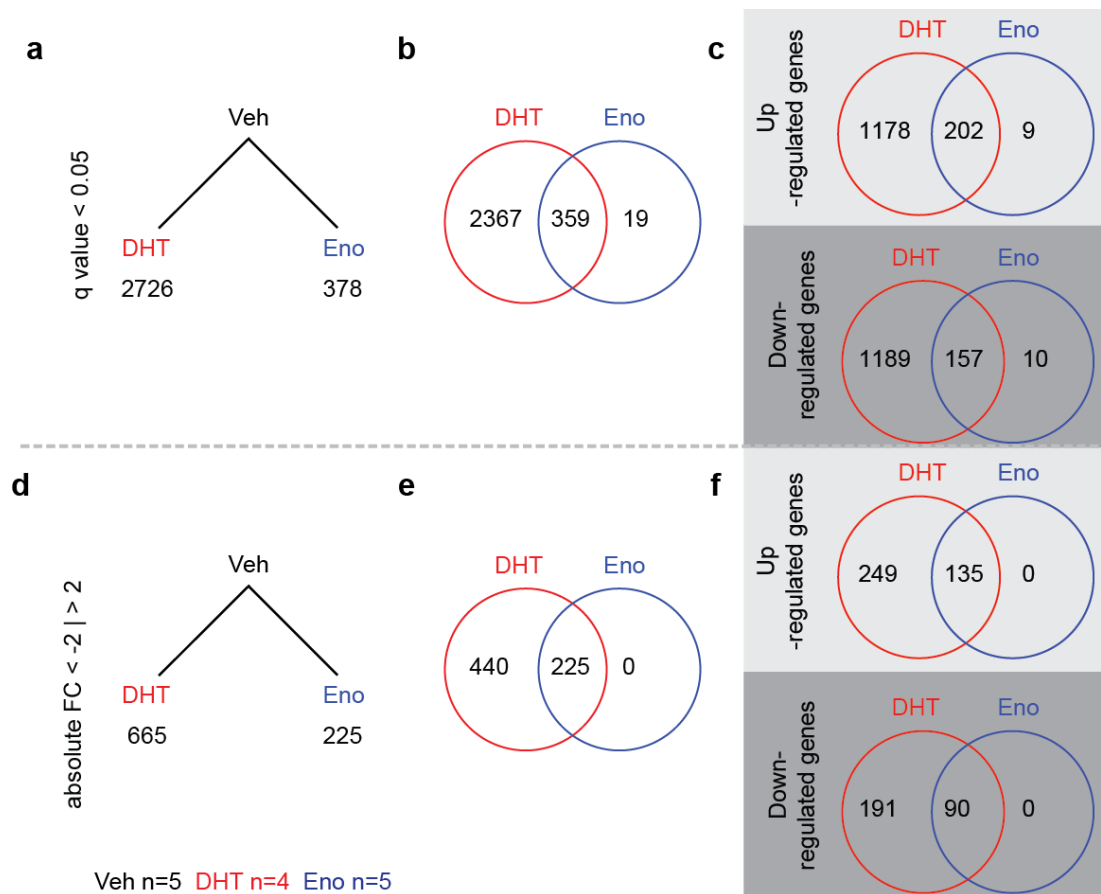
Figure 5.6. Summary of differentially expressed genes induced by AR agonists in Gar15-13 PDX tumours.

The number of differentially-expressed genes induced by treatment with DHT or Enobosarm (Eno) in Gar15-13 relative to vehicle (Veh) treatment identified from RNA-sequencing was demonstrated at different statistical cut-offs. At a statistical cut-off of q<0.05 (**a-c**), the number of differentially expressed genes identified in DHT-treated or Eno-treated tumours, relative to Veh, are shown in (**a**). The overlap between DHT- and Eno-induced genes was represented in a Venn diagram (**b**) and this overlap was applied to specifically include the up- and down-regulated genes (**c**). At a statistical cut-off of q<0.05 and absolute fold-change (FC) of >2 (**d-f**), the number of differentially expressed genes identified in DHT-treated or Eno-treated tumours, relative to Veh, are shown in (**d**). The overlap between DHT- and Eno-induced genes was represented in a Venn diagram (**e**) and this overlap was applied to include the up- and down-regulated genes (**f**).

5.2.7 Heat map of top DHT-regulated genes

A heat map based on the top 100 DHT-induced differentially expressed genes was constructed, 71 genes which were upregulated and 29 genes downregulated (**Fig. 5.7**). Established AR-regulated genes such as *SEC14L2* and *FKBP5* were amongst the most highly DHT-upregulated genes and ER-regulated *BCL2* was amongst the most significantly downregulated genes and these observations correlated with our RT-qPCR data (**Fig. 5.5b,d**). Relative to DHT, the expression of these genes was moderately affected by enobosarm consistent with our earlier observation that enobosarm acted like a weak agonist.
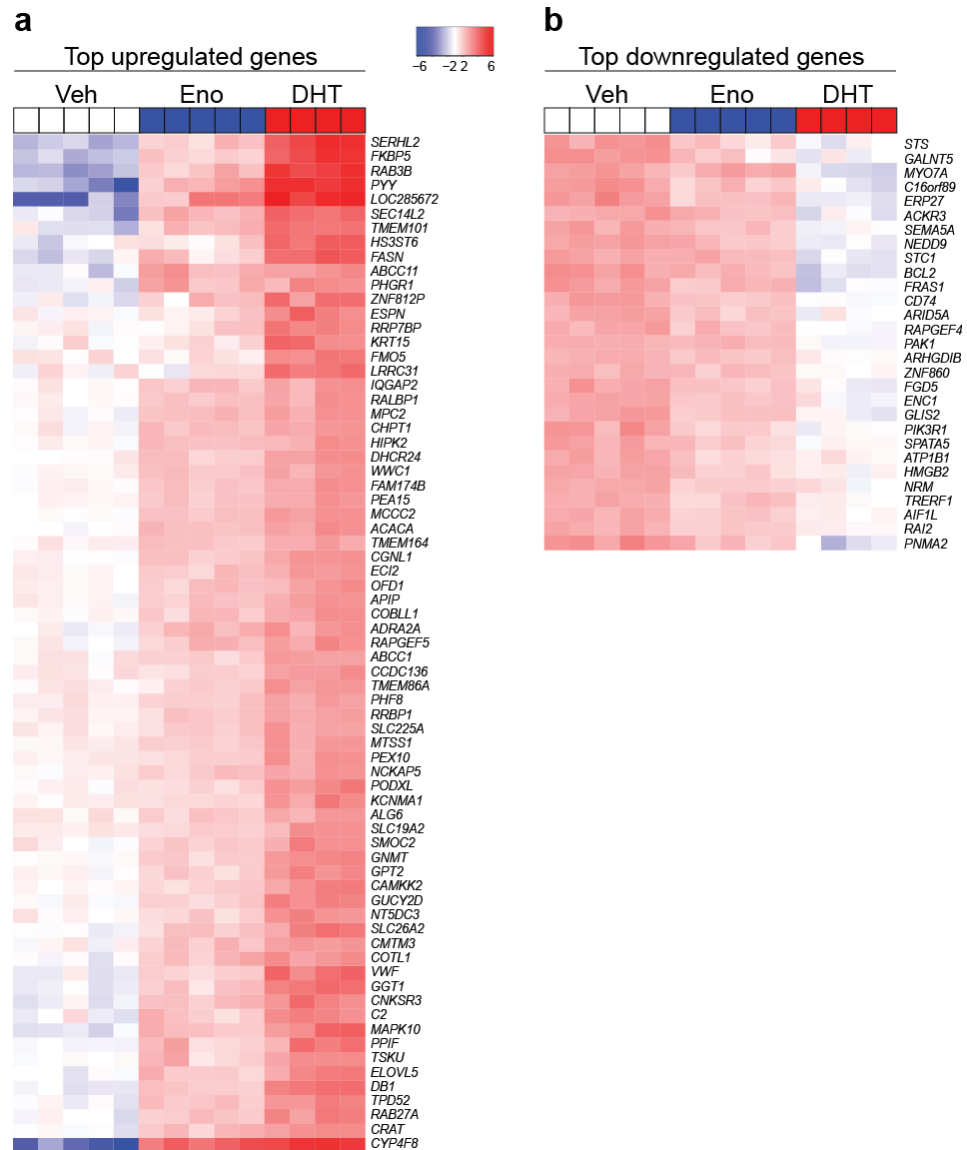
Figure 5.7. Heat map of the top 100 DHT-regulated genes in Gar15-13 PDX tumours.
The top 100 differentially expressed genes in response to DHT treatment relative to Vehicle (Veh) treatment in Gar15-13 PDX tumours was divided into upregulated (**a**) and downregulated genes (**b**). Enobosarm (Eno) induced modest changes in these genes.

5.2.8 GSEA analysis using publicly available gene signatures

To understand the biological significance of the global gene expression changes, gene set enrichment analysis (GSEA) of the differentially-expressed genes induced by DHT and enobosarm was performed and these gene sets were compared against publicly available gene signatures (Subramanian et al., 2005). The top eight positively or negatively enriched signatures in DHT- and enobosarm-treated samples are shown in **Fig. 5.8a** and **5.8b** respectively. Selected enrichment plots for DHT and enobosarm treatment are shown in **Fig. 5.8c** and **5.8d** respectively. Notably, upregulated genes in both gene sets were positively enriched in signatures pertaining to active AR signalling in ER- breast cancer and in prostate cancer. This includes the "Doane response to androgen up" which is a gene signature derived from the treatment of MDA-MB-453 breast cancer cell line, a representative ER-AR+ breast cancer cell line, with a synthetic androgen, which was the most highly enriched signature in both DHT and enobosarm treatment (Doane et al., 2006) (**Fig. 5.8a, b**). Other AR-related gene signatures observed in the upregulated genes relevant to breast cancer include the "Doane breast cancer classes up" and "Farmer breast cancer apocrine vs basal" which were both developed from comparing the transcriptomes of AR+ tumours versus AR- tumours in ER- breast cancer (Doane et al., 2006; Farmer et al., 2005). The AR gene signature "Nelson response to androgen up", which constitutes the response of the prostate cancer cell line LNCaP treated with a synthetic androgen (Nelson et al., 2002), was also highly enriched in the upregulated genes.

Intriguingly, enrichment of genes associated with endocrine-resistance was also observed with both DHT (Creighton endocrine therapy resistance 3; results not shown) and enobosarm (Creighton endocrine therapy resistance 2). The DHT-regulated genes which contributed to the enrichment of this endocrine-resistance associated gene signature were enriched in functions pertaining to androgen response and pathways related to metabolism such as xenobiotic metabolism, glycolysis and fatty acid metabolism (**Fig. 5.9**).
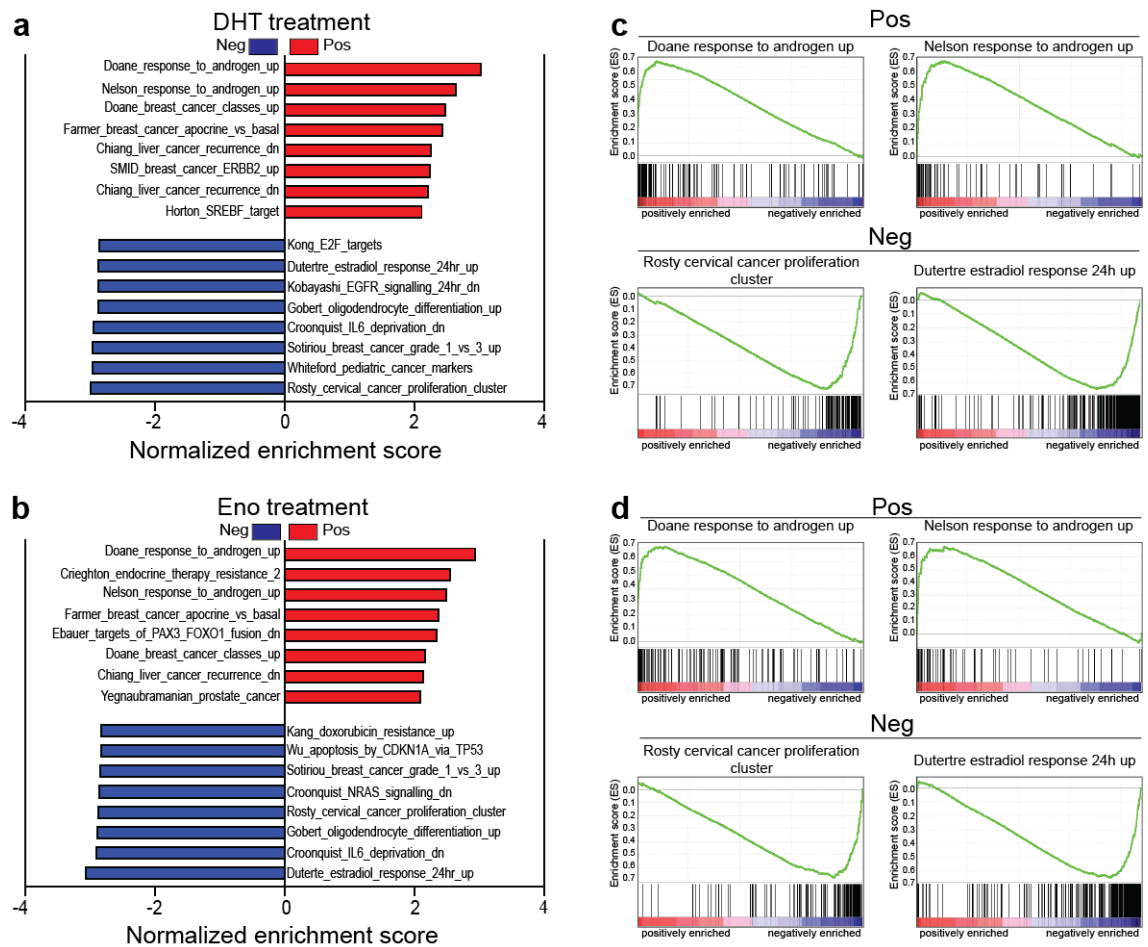
Figure 5.8. GSEA analysis using publicly-available gene signatures.

Differentially-expressed genes induced by DHT and Enobosarm (Eno) in Gar15-13 PDX tumours were assessed for the enrichment of chemical and genetic perturbations signatures (C2) from MSigDB. The top eight positively (Pos) or negatively (Neg) enriched signatures, as indicated by the normalized enrichment score, for DHT (**a**) and Eno (**b**) are shown. Individual enrichment plots for selected enriched signatures for DHT and Eno treatment are shown in (**c**) and (**d**) respectively.

a

Creighton endocrine therapy resistance 3

positively enriched   negatively enriched

b

Gene Set Name	No. of genes	q-value
HALLMARK_ANDROGEN_RESPONSE	13	5.06E-15
HALLMARK_MTORC1_SIGNALING	10	7.95E-08
HALLMARK_XENOBIOTIC_METABOLISM	10	7.95E-08
HALLMARK_GLYCOLYSIS	9	8.85E-07
HALLMARK_FATTY_ACID_METABOLISM	8	1.56E-06
HALLMARK_OXIDATIVE_PHOSPHORYLATION	8	7.81E-06
HALLMARK_PEROXISOME	6	1.95E-05
HALLMARK_BILE_ACID_METABOLISM	5	4.09E-04
HALLMARK_CHOLESTEROL_HOMEOSTASIS	4	9.66E-04
HALLMARK_ADIPOGENESIS	5	4.76E-03

Figure 5.9. Genes upregulated by DHT in Gar15-13 PDX tumours are implicated in endocrine-resistance.

a) Enrichment plot demonstrating the enrichment of Creighton endocrine therapy resistance 3 signature in DHT-induced upregulated genes in Gar15-13. **b**) The core enrichment genes regulated by DHT which contributed to this endocrine-resistant signature were compared against the Hallmark gene signatures using GSEA.

On the other hand, the signatures of significance enriched in the downregulated genes induced by DHT and enobosarm include the "Rosty cervical cancer proliferation cluster" and "Dutertre estradiol response 24h up" (Rosty et al., 2005) (Dutertre et al., 2010). The former corresponds to genes involved in proliferation or cell division and are controlled by the E2F signalling pathway whereas the latter corresponds to genes upregulated in MCF7 cells in response to estradiol treatment and is thereby a gene signature representative of canonical ER activity. Overall, the analysis of the differentially expressed genes induced by DHT and enobosarm on a global scale indicated an upregulation of AR activity and concurrent downregulation of genes implicated in proliferation and ER signalling.

5.2.9 GSEA analysis using Gene Ontology datasets

The differentially expressed genes induced by DHT and enobosarm were subsequently compared with the Gene Ontology (GO) datasets to infer biological functions (Ashburner et al., 2000). Analysis of the DHT-induced differentially expressed genes indicated that upregulated genes were engaged in metabolic pathways such as "organic acid catabolic process", "carboxylic acid catabolic process" and "alpha amino acid catabolic process" whereas the downregulated gene set was enriched in pathways pertaining to "nuclear transcribed mRNA catabolic process nonsense mediated decay", "protein localization to endoplasmic reticulum" and "multi-organism metabolic process" (**Fig. 5.10a, b**). To overcome gene redundancy in the different sets and to gain a more "global" insight into the biological functions of these genes, an enrichment map was created (**Fig. 5.10c**). Enrichment maps allow for a simplistic visualization as it organizes GO sets, known as nodes, into clusters with the overlap in the genes between the different nodes represented by interconnecting edges(Merico et al., 2010). This approach allowed for the identification of biologically significant clusters and simplified the interpretation of our analysis. Using this approach, we have identified that the upregulated genes formed a major cluster with broad functions in metabolism. On the other hand, the downregulated genes formed three major clusters with functions pertaining to G1-S progression, S phase and mitosis. Representative GO gene set enriched in each cluster is highlighted (**Fig. 5.10c**).
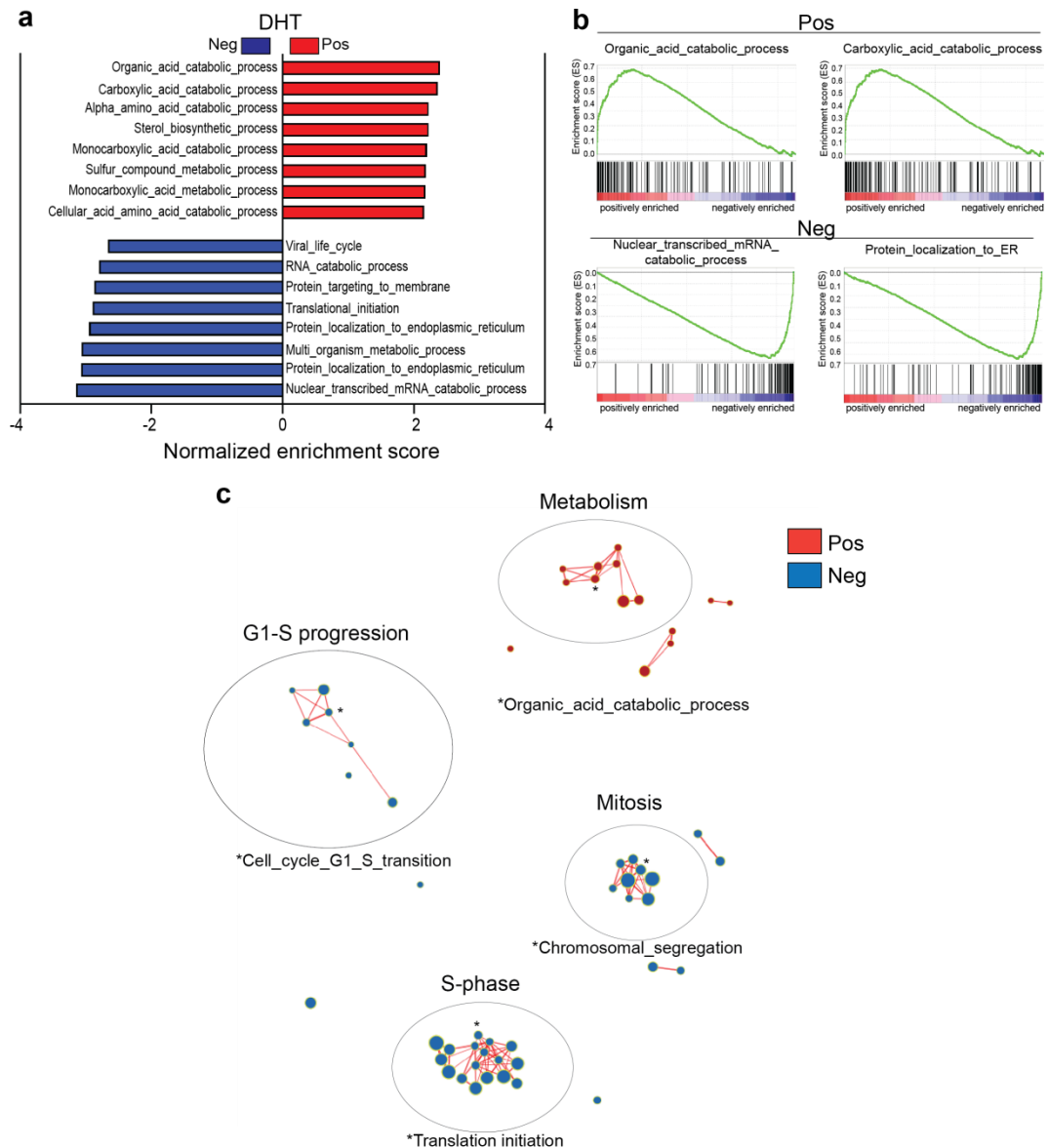
Figure 5.10. GSEA analysis of DHT-induced genes against Gene Ontology (GO) processes.

a) The top eight positively (Pos) or negatively (Neg) enriched processes, as indicated by the normalized enrichment score, for DHT. **b)** Individual enrichment plots for selected enriched processes in DHT-induced gene signature. **c)** Enrichment map of the processes identified in the GSEA analysis at a cut-off of q<0.001. Edges between the different nodes indicate an overlap of genes by 50%. A representative GO process in each cluster is indicated by (*).

GSEA of the enobosarm-induced gene set against the GO gene sets revealed a similar pattern (**Fig. 5.11**). This analysis indicated that the upregulated gene set was implicated in metabolic pathways such as "water soluble vitamin metabolic process", "organic acid catabolic process" and "glyoxylate metabolic process" whereas the downregulated gene set was enriched in pathways pertaining to "multi organism metabolic process", "nuclear transcribed mRNA catabolic process nonsense mediated decay" and "establishment of protein localization to endoplasmic reticulum" (**Fig. 5.11a, b**). Visualization of the enriched groups using the enrichment map also identified several distinct clusters (**Fig. 5.11c**). Similar to DHT treatment, the upregulated genes were enriched in "metabolism" whereas the downregulated genes were implicated in cell cycle progression and resulted in clusters with broad functions in "G1-S progression", "S phase" and "mitosis". Interestingly, a unique cluster, constituted by enrichment of genes involved in DNA recombination, was observed with enobosarm treatment. This cluster was labelled as a DNA repair cluster as genes with established roles in DNA repair such as *FEN1*(H. Sun et al., 2017), *RAD51*(King et al., 2017) and *RPA2* (Liaw et al., 2011) were part of the core enrichment genes which contributed to the enrichment of this DNA replication gene set (**Fig. 5.11d**).

Overall, these GSEA analyses with the GO gene sets demonstrate that most of the DHT- or enobosarm-induced upregulated genes were involved in metabolism. In contrast, the downregulated genes had broad roles across the different stages of cell cycle progression. Notably, our attempt to map changes in ER chromatin binding in response to treatment with AR agonists using ChIP-sequencing was unsuccessful.
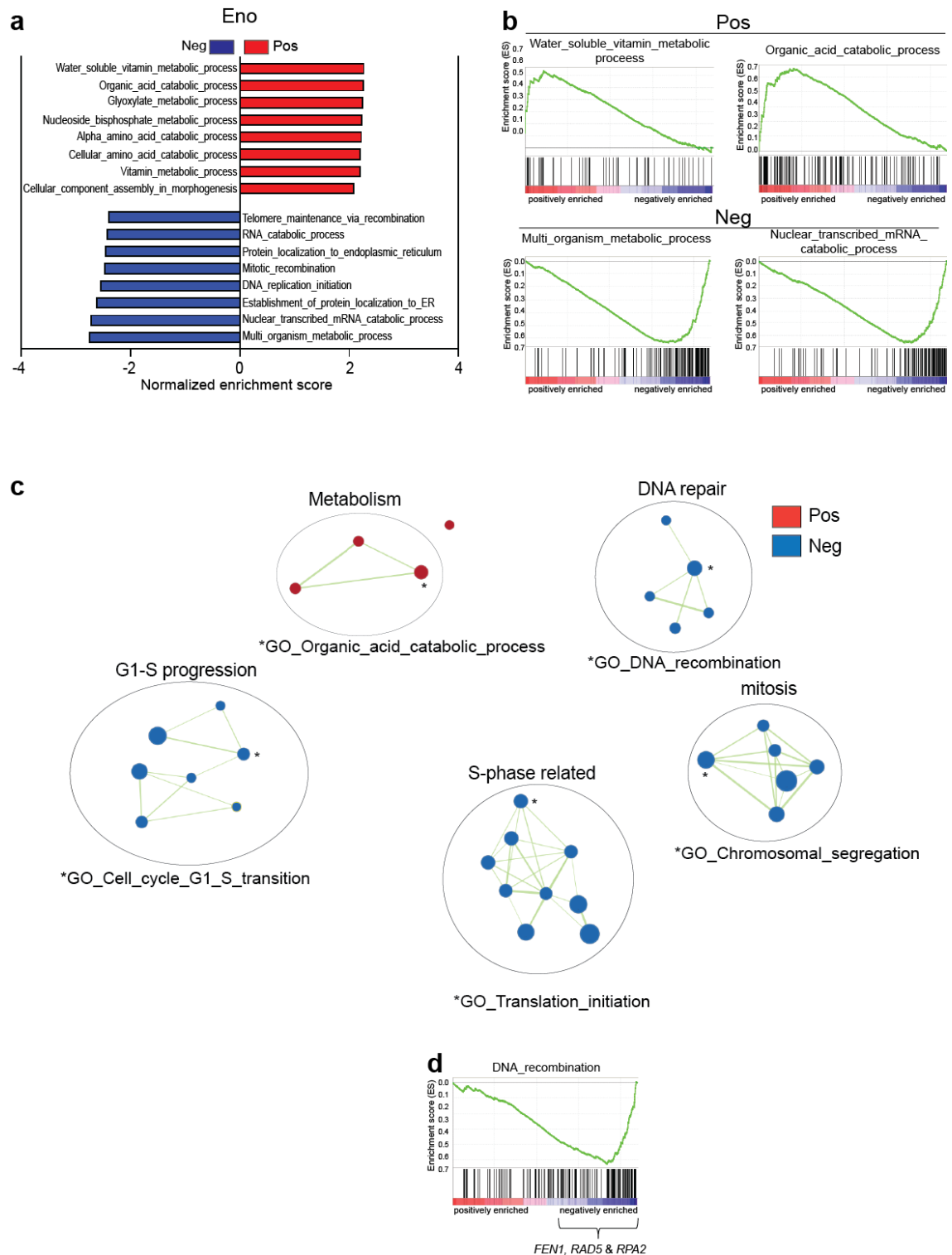
Figure 5.11. GSEA analysis of Eno-induced genes against Gene Ontology (GO) processes.

a) The top eight positively (Pos) or negatively (Neg) enriched processes, as indicated by the normalized enrichment score, for enobosarm (Eno). **b**) Individual enrichment plots for selected enriched processes in Eno-induced gene signature. **c**) Enrichment map of the processes identified in the GSEA analysis at a cut-off of q<0.001. Edges between the different nodes indicate an overlap of genes by 50%. A representative GO process in each cluster is indicated by *. **d**) Enrichment plot for GO_DNA_recombination and selected core enrichment genes.

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5.2.10 Discovery of a prognostic AR gene signature using METABRIC

Next we determined if the genes associated with DHT-induced AR activation were prognostic by applying filtering steps, based on a previously reported approach (Tishchenko et al., 2016). We started with the 660 differentially expressed genes induced by DHT based on the statistical criteria of q-value<0.05 and absolute fold-change>2. Of the 660 genes, 616 genes had matching annotated genes within METABRIC where gene expression was determined on an Illumina microarray-based platform. Next, the list was further narrowed down to "cancer specific" genes which exhibited a significant difference in gene expression in the ER+ breast tumours relative to "normal" controls within the METABRIC dataset. After this, the "cancer-specific" gene list was further refined to select for genes associated with survival. In this analysis, each gene was subjected to the Kaplan-Meier estimator to determine if their expression level was capable of stratifying two groups of patients with distinct survival outcomes, based on the top tertile (patients with high expression of this gene) and the bottom tertile (patients with low expression of the same gene). After going through the filtering steps outlined above, we established an AR gene signature which comprised of 187 genes, of which 86 and 101 genes were up- and down-regulated with DHT treatment, respectively. A summary of the above analysis including the full list of the genes in the gene signature is summarised in **Fig. 5.12**. As a confirmatory analysis, this signature was applied to ER+ cases in the METABRIC dataset using the cox regression model and it was demonstrated to be significantly prognostic (p = 5.88e-15) (**Fig. 5.13**).

a) Schematic illustrating the flow of filtering steps for the different expressed genes (DEGs), induced by DHT treatment in Gar15-13 PDX tumours and defined by a cut-off at q<0.05 and absolute fold change of 2. The final AR gene signature comprises 187 genes, 86 upregulated genes (**b**) and 101 downregulated genes (**c**).
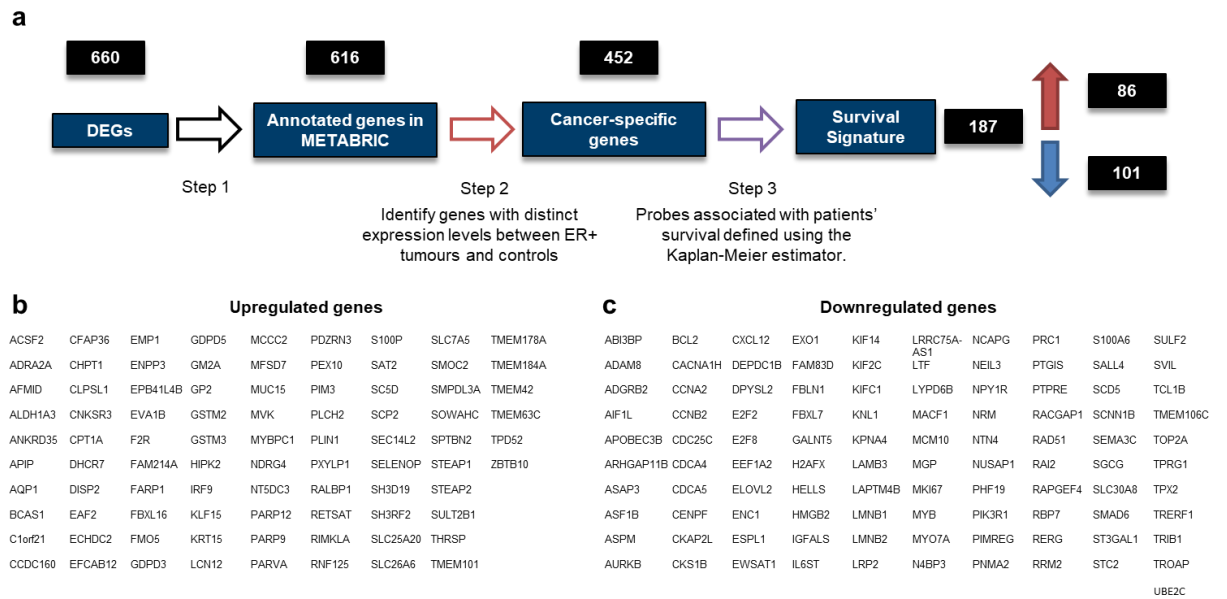
Figure 5.12. Discovery of a prognostic AR gene signature.
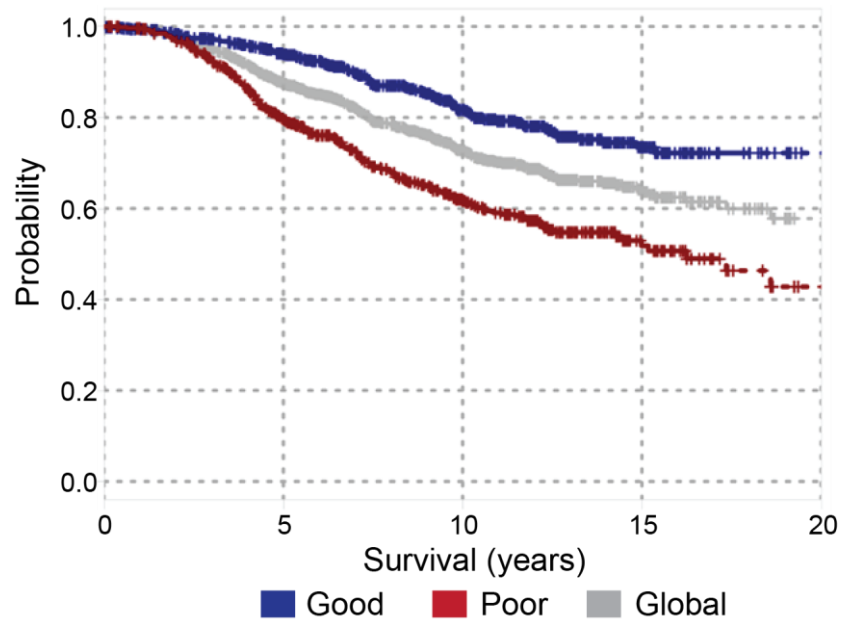
Figure 5.13. AR gene signature stratifies patients to "Good" and "Poor" prognosis.

The AR gene signature was applied across the ER+ tumours (n=1,230) in the METABRIC dataset using Cox regression which included patient age, tumour grade and positive lymph nodes as co-variates. This gene signature segregated patients into "Good outcome" (n=689, blue) and "Poor outcome" (n=541, red) with p< 5.88e15.

5.2.11   Biological significance of the AR gene signature

In order to gain a better functional insight into the AR gene signature, we performed GSEA and compared the up- and the down-regulated genes from the AR gene signature against the gene sets from the Hallmark gene signature (Subramanian et al., 2005). The downregulated genes were enriched for those genes implicated in G2-M, E2F signalling, mitosis and estrogen response whereas the upregulated genes were enriched in pathways pertaining to estrogen response, cholesterol homeostasis, apoptosis and androgen response (**Fig. 5.14a**). Notably, the downregulated genes which contributed to the enrichment of the G2M Checkpoint Hallmark signature include established proliferative genes such as *cyclin A2 (CCNA2)*, *cyclin E2 (CCNE2)*, *aurora kinase B (AURKB)* and *DNA topoisomerase 2-α (TOP2A)*. The genes contributing to the enrichment of the Estrogen Response Hallmark signature include the canonical ER targets such as *BCL2*, *CXCL12* and *MYB* (**Fig. 5.14b, c**). Signatures enriched in the upregulated genes were related to estrogen response, cholesterol homeostasis and apoptosis (**Fig. 5.15a**). On the contrary, none of the genes which were part of the Estrogen Response Early signature observed with the upregulated gene set were classical ER targets and these genes were not associated with proliferation (**Fig. 5.15b**). Through these analyses it can be concluded that the upregulated genes in this AR gene signature were likely to be acting as tumour suppressors whereas the downregulated genes were likely to be oncogenes.

a

Gene Set Name	No. of genes	FDR q-value
HALLMARK_G2M_CHECKPOINT	19	9.60E-25
HALLMARK_E2F_TARGETS	15	4.54E-18
HALLMARK_MITOTIC_SPINDLE	10	2.37E-10
HALLMARK_ESTROGEN_RESPONSE_EARLY	8	1.23E-07
HALLMARK_ESTROGEN_RESPONSE_LATE	7	2.13E-06
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	5	4.47E-04
HALLMARK_MYOGENESIS	5	4.47E-04
HALLMARK_TNFA_SIGNALING_VIA_NFKB	4	5.08E-03
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	3	9.44E-03

b

HALLMARK_G2M_CHECKPOINT

AURKB	E2F2	LMNB1	TOP2A
CCNA2	ESPL1	MKI67	TPX2
CCNB2	EXO1	NUSAP1	TROAP
CENPF	H2AFX	PRC1	UBE2C
CKS1B	KIF2C	RACGAP1	

c HALLMARK_ESTROGEN_RESPONSE_EARLY

BCL2	NPY1R
CXCL12	STC2
ELOVL2	SVIL
IL6ST	
MYB	

Figure 5.14. Gene Set Enrichment Analysis of the down-regulated genes in the AR gene signature.

a) The upregulated genes in the AR gene signature were subjected to gene enrichment analysis and compared against the Hallmark gene signatures. List of genes contributing to the enrichment of G2M Checkpoint (**b**) and Estrogen Response Early (**c**) gene signatures.

a

Gene Set Name	No. of genes	FDR q-value
HALLMARK_ESTROGEN_RESPONSE_EARLY	7	2.47E-06
HALLMARK_CHOLESTEROL_HOMEOSTASIS	4	1.92E-04
HALLMARK_ESTROGEN_RESPONSE_LATE	5	3.79E-04
HALLMARK_APOPTOSIS	4	2.01E-03
HALLMARK_ADIPOGENESIS	4	3.67E-03
HALLMARK_INTERFERON_ALPHA_RESPONSE	3	4.56E-03
HALLMARK_ANDROGEN_RESPONSE	3	4.56E-03
HALLMARK_PEROXISOME	3	4.56E-03
HALLMARK_BILE_ACID_METABOLISM	3	5.02E-03
HALLMARK_IL2_STAT5_SIGNALING	3	1.67E-02

b

HALLMARK_ESTROGEN_RESPONSE_EARLY

CHPT1	SLC7A5
DHCR7	SULT2B1
FARP1	
KRT15	
SEC14L2	

Figure 5.15. Gene Set Enrichment Analysis of the up-regulated genes in the AR gene signature.

a) The downregulated genes in the AR gene signature were subjected to gene enrichment analysis and compared against the Hallmark gene signatures. **b**) List of genes contributing to the enrichment of Estrogen Response Early gene signature.

5.2.12 Relationship between the AR gene signature and luminal subtypes

ER+ tumours can be broadly divided into luminal A and luminal B molecular subtypes with distinct gene expression profiles and survival outcomes (Sorlie et al., 2001; Sorlie et al., 2003). We investigated the relationship between the expression profiles of "Good outcome" and "Poor outcome" patient populations, previously stratified based on the AR gene signature (**Fig 5.13**), and the expression profiles specific to patients with either luminal A or B tumours. Furthermore, gene expression data from "normal breast tissues" embedded within METABRIC were included in this analysis to identify potential changes in AR signalling between normal and ER+ tumours. A heat map based on the AR gene signature clearly stratified the luminal A from luminal B tumours and the luminal A overlapped with "Good outcome" tumours. In contrast the luminal B tumours overlapped with the "Poor outcome" tumours (**Fig. 5.16a**). Interestingly, the expression of upregulated genes in the AR gene signature was highest in the normal breast tissues (control) and lowest in the luminal B tumours (**Fig. 5.16b**). On the contrary, the expression of downregulated genes in the AR gene signature was lowest in the control tumours and highest in the luminal B tumours. Overall, we demonstrated that the AR gene signature could clearly demarcate luminal A from luminal B tumours.
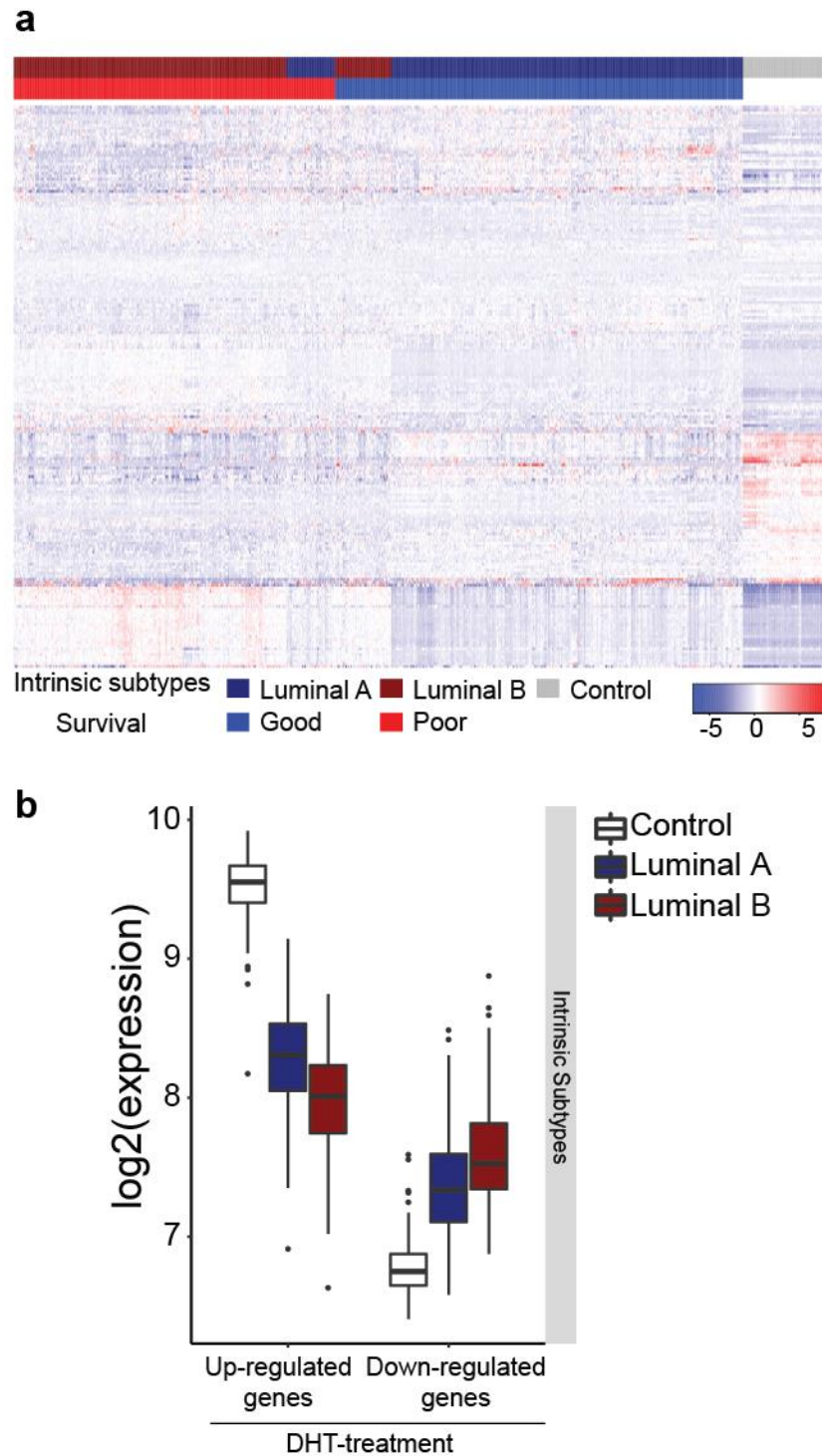
Figure 5.16. AR signature is enriched in luminal A subtype.

a) A heat map of the expression profiles of luminal A, luminal B and control breast samples from the METABRIC dataset was constructed based on expression of the AR gene signature. **b**) The expression level of the up- and down-regulated genes in the AR gene signature was compared across the luminal A, luminal B and control samples.

5.2.13 AR gene signature outperforms MammaPrint and other cancer signatures

Next, we compared the strength of our AR gene signature against the clinically used MammaPrint, cancer-related and random gene signatures. The MammaPrint signature, developed by Van't Veer and colleagues (van 't Veer et al., 2002), is a 70-gene signature that is used clinically to segregate patients with early stage breast cancer into high or low-risk of recurrence. Interestingly, there are less than 5 genes which overlap between our AR gene signature and the MammaPrint signature (results not shown). We compared the prognostic power of the AR and the MammaPrint gene signatures on clinically annotated ER+ breast cancers embedded within the METABRIC (Pereira et al., 2016) and Loi (Loi et al., 2008) datasets. Notably, the Loi dataset comprises patients who had tamoxifen as their sole adjuvant therapy. Intriguingly, the prognostic power of our AR gene signature outperformed the MammaPrint and other known cancer-related gene signatures in these datasets (**Fig. 5.17a**).

Furthermore, it has been reported that most random multi-gene signatures are highly likely to be linked to outcome in breast cancer and most publicly available gene signatures are not more significant than randomly-generated signatures (Venet et al., 2011). Hence we tested the strength of our AR signature against randomly defined signatures of the same size as the AR gene signature and we found that the AR signature was significantly more prognostic than the random gene signatures across the datasets tested (**Fig. 5.17b**).
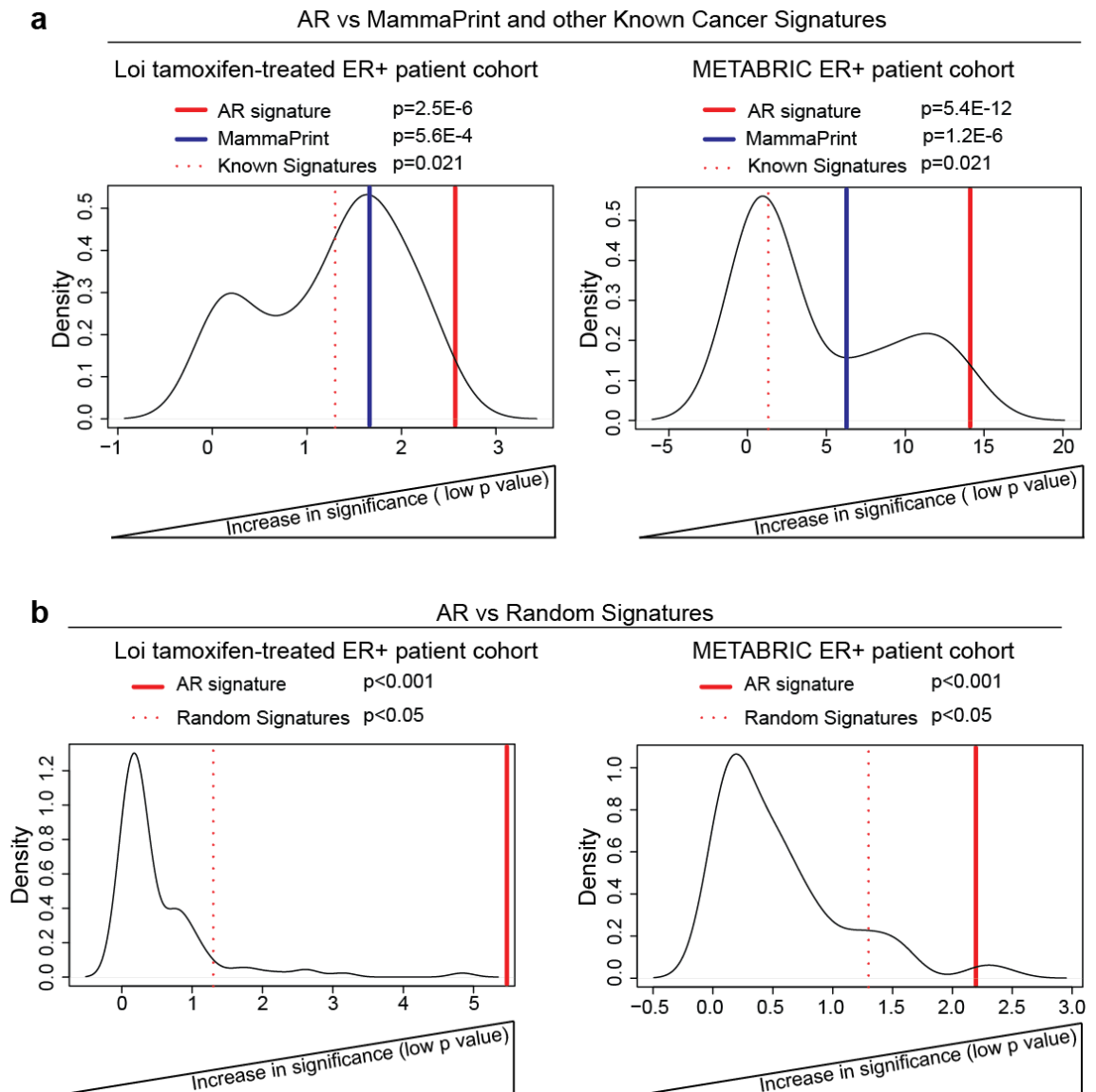
Figure 5.17. AR gene signature outperforms all other prognostic gene signatures

a) The prognostic power of the AR gene signature was compared against that of MammaPrint and other known cancer-related gene signatures in the Loi tamoxifen-treated and METABRIC ER+ patient cohorts. **b**) The prognostic power of the AR gene signature was compared against that of random signatures in the same datasets in (**a**).

5.3 Discussion

In this chapter, we have molecularly characterised the changes induced by the activation of AR in our endocrine-resistant PDX models. We have demonstrated suppression of classical ER-targets at the expression level with acute treatment of AR agonists in both PDX models. Moreover, through global profiling of gene expression changes we discovered that the genes which were upregulated in response to AR agonists were enriched for signatures pertaining to AR activity in the molecular apocrine breast cancer and prostate cancer whereas the downregulated genes were enriched for signatures associated with cell cycle and estrogen response amongst others. This inhibitory effect of AR activation on ER signalling at a global gene expression level, further supports the notion that AR activity antagonises ER signalling. Lastly, we established an AR gene signature which is prognostic in patients with primary ER+ breast cancer and in patients treated with tamoxifen.

The pivotal result in this Chapter is the discovery of a prognostic AR gene signature. Some upregulated genes in the signature are reported to possess tumour suppressive functions. For example, *SEC14L2* encodes for a vitamin E binding protein (Zimmer et al., 2000) and its expression is lower in invasive carcinomas relative to normal breast tissues. Importantly high expression of this gene is also associated with a better 5-year survival in patients with ER+ breast cancer (X. Wang et al., 2009; Xi Wang et al., 2015). *Six transmembrane epithelial antigen of the prostate 1* (*STEAP1*) encodes for a cell surface antigen and the expression of this protein is reported to be associated with a better survival in breast cancer (Xie et al., 2018). Experimentally, the expression of STEAP1 inhibited the invasion and migration of breast cancer cells through the inhibition of genes such as MMP9, MMP13 and vimentin.

Intriguingly, a number of DHT-upregulated genes in the signature, such as *choline phosphotransferase 1* (*CHPT1*) and *7-dehydrocholesterol reductase* (*DHCR7*), have been reported to contribute to disease progression (Jia et al., 2016; Voisin et al., 2017), and high expression of *DHCR7* is associated with high grade tumours and poor overall survival in ER+ breast cancer (Voisin et al., 2017). Interestingly, both of these genes are implicated in metabolism, with *CHPT1*, a direct ER-target, reported to mediate ER-induced metabolic reprogramming by increasing de novo phosphatidylcholine synthesis whereas *DHCR7* is an enzyme critical for the biosynthesis of an oncometabolite. Given

that AR is a part of the estrogen response signature, these results suggest that some of the metabolic reprogramming induced by ER signalling that is required for the evolution and progression of ER+ tumours, may be mediated by AR and further investigations are required to ascertain this.

The downregulated genes in the AR gene signature are highly enriched in proliferation including *TOP2A*, *CCNA2* and *ENP-F*. These are genes have important roles in the G2/M progression and importantly the expression of these genes is highly associated with a poor prognosis in breast cancer (Romero et al., 2011) (T. Gao et al., 2014; O'Brien et al., 2007). The downregulation of these genes by DHT may underlie the strong AR-mediated anti-proliferative effect.

Critically, this prognostic AR gene signature outperforms the clinically-used signature MammaPrint and other random or publicly available signatures in ER+ breast cancer. Not only does this signature support the studies which demonstrated that the expression of AR in primary breast cancer is an independent factor associated with good outcome (Aleskandarany et al., 2016; Gonzalez et al., 2008; Jiang et al., 2016; Vera-Badillo et al., 2014), the prognostic strength of this AR gene signature, which is a functional readout of canonical AR activity, reflects the importance of AR biology in breast cancer which is not captured by other signatures. While this AR gene signature is highly prognostic in ER+ breast tumours, the impact of this gene signature in HER2+ breast cancer should be investigated considering the reported oncogenic interaction with AR and HER2 (Chia et al., 2011; Ni et al., 2011).In regard to the role of AR in endocrine-resistant breast cancer, our observation challenges the current dogma where AR, and by inference canonical AR activity, is reported to contribute to disease progression of breast cancer (Q. Yu et al., 2011) (Cimino-Mathews et al., 2012; Ciupek et al., 2015; Cochrane et al., 2014; De Amicis et al., 2010; Fujii et al., 2014; Grogg et al., 2015; Rechoum et al., 2014). The strong prognostic power of this signature, derived from an endocrine-resistant PDX model, in the various datasets including a cohort of tamoxifen-treated patients, provides the clearest indication that active AR signalling is predominantly tumour-suppressive in ER+ breast cancer regardless of the state of endocrine-therapy sensitivity.

A recent study that has purported that AR contributes to ER+ disease progression reported that patients with nuclear AR/ER ratio of >2 were 4-times more likely to fail

tamoxifen (Cochrane et al., 2014). Other studies which contributed to the current dogma have reported that the positivity of AR was significantly higher in ductal carcinomas *in situ* (DCIS) proximal to invasive carcinomas than tumours with just DCIS (Q. Yu et al., 2011), and noted that the expression of AR was more likely to be retained and expressed in metastatic tumours relative to other hormonal receptors such ER and PR (Grogg et al., 2015). More recently, a study reported that tumours with an AR/ER ratio ≥ 2 were associated with increased frequency of metastatic lymph nodes and tumours with high histological grade (Rangel et al., 2018). It is important to note that conclusions drawn from these studies often assumed that nuclear AR equates canonical activity of AR. This assumption does not always hold true given that AR can engage in a non-canonical signalling mechanism as previously demonstrated. Furthermore, the association of expression of AR with disease progression is not an unanimous as high expression of AR expression in metastatic breast cancer has been reported to be associated with a significantly better overall survival (higher AR versus low AR; median overall survival 53.1 versus 27.2 months; p=0.001) (J. Y. Kim et al., 2017).

Lastly, it is intriguing that DHT-induced genes in Gar15-13 PDX tumours are enriched in a signature that is associated with endocrine-resistance. This Creighton endocrine therapy resistant group 3 signature is derived from a MCF7 xenograft model overexpressing HER2 (Creighton et al., 2008). While we have observed stong growth suppression in all our ER+ breast cancer models, these models are non HER2-amplified. Amplification of HER2, which is present in ~10% of ER+ breast cancer patients, is associated with a poor clnical response to endocrine-therapy (Dowsett et al., 2001; Ellis et al., 2006). Given the established interactions between AR and HER2 in the molecular apocrine breast cancer (Chia et al., 2011; Ni et al., 2011; J. L. Robinson et al., 2011), it is possible that AR activity can contribute to circumvention of ER-targeted treatment in a small proportion of ER+ breast cancer with high activity of HER2. Inhibition of AR may be more appriopriate for these tumours. It is also important to note that the primary endpoint analyzed in our PDX experiments is growth, and it remains to be determined how the activation of AR impacts on invasion and metastatic dissemination.

The major drawback of this chapter is the unsuccessful attempt at mapping out the ER chromatin binding sites in the PDX models following treatment with AR agonists in order to evaluate changes in ER binding and to identify potential direct ER target genes.

Completion of this challenging *in vivo* ChIP-sequencing technique will require further optimization in the laboratory.

In summary, we have molecularly characterized the response of two ER+ endocrine-resistant PDX models and have provided strong evidence of ER signalling inhibition with the activation of AR by DHT or enobosarm. Through global transcriptomic profiling of Gar15-13 PDX tumours, we also demonstrated an increase in genes involved in metabolism and a decrease in genes implicated in cell cycle progression following treatment with these AR agonists. An AR-gene signature conceived from Gar15-13 PDX tumours treated with DHT was strongly prognostic in the METABRIC cohort, which indicated that active canonical AR signalling has a general tumour suppressive role in ER+ breast cancer independent of endocrine-therapy sensitivity.

CHAPTER 6

6. AR signalling reduces invasion of MCF7 TamR cells *in vivo*

6.1 Introduction

In the previous chapters, we have comprehensively demonstrated that activation of AR is associated with growth inhibition of endocrine-resistant breast cancer models. However, it remains to be determined how AR activity affects other aspects of disease progression such as invasion, metastatic dissemination and colonization of distant organs, key drivers of breast cancer-related mortality. The activity of AR has been implicated in invasion (Ali et al., 2015) and metastatic dissemination of ER+ breast cancer cell lines (Aceto et al., 2018; Feng et al., 2017).

In a study by Ali et al (Ali et al., 2015), increased invasion of endocrine-resistant ER+ cell lines was observed *in vitro* with the addition of prosaposin, a multi-functional protein involved in intracellular metabolism of sphingolipid and an activator of AR in castration-resistant prostate cancer (Koochekpour et al., 2005; O'Brien et al., 1994; Sandhoff & Kolter, 2003). The stimulation of AR by DHT has also been shown to promote spontaneous metastasis to the lungs by increasing epithelial-mesenchymal transition using the fatpad implantation method (Feng et al., 2017). This effect was reported to occur via DHT-induced concurrent suppression and elevation of E-cadherin and vimentin, respectively. However, the key experiment demonstrating changes in EMT was performed using an ER- T47D cells which is well-established to be ER+ and this disparity raises questions about the accuracy of the data produced in this study. Furthermore, we have not observed changes in E-caderin and vimentin following DHT treatment in Gar15-13 PDX tumours (results not shown). Aceto and colleagues, identified that malignant cells derived from bone mets of ER+ patients were enriched in AR signalling, relative to cells from lung mets, and they demonstrated that inhibition of AR using enzalutamide was effective in inhibiting the spread of cancer cells to the bone (Aceto et al., 2018). However, the functional *in vivo* assay was performed using an ER-AR+ MDA-MB-231 cell line which is a basal-like cell line (Subik et al., 2010) and therefore their conclusion on the role of AR signalling in promoting bone metastasis in ER+ disease is not vindicated. To date, the effect of AR activation on invasion and metastatic dissemination in ER+ breast cancer has not been explored in clinically-

relevant models and hence the exact role of AR signalling on invasion and metastasis of ER+ breast cancer cells remains unclear.

Spontaneous metastasis of ER+ cells from the primary tumours developed through xenografting of ER+ cell lines is rare, a limitation of such preclincal models. Experimental metastasis of these cells can be induced using other *in vivo* techniques such as tail vein injection (Elkin & Vlodavsky, 2001; Imanishi et al., 2011) and cardiac injection (Ogba et al., 2014). However, metastasis is a multi-step process involving invasion through the basement membrane, intravasation and extravasation of the vasulature, and subsequent organ colonization. Considering that these metastases were not developed spontaneously from the systemic dissemination of the cells derived from the primary tumours as is seen clinically, the clinical relevance of the metastases developed using these experimental techniques is limited.

Metastasis of cell line xenograft tumours developed through mammary intraductal (MIND) injection has emerged a novel preclincal tool to study metastases in breast cancer (Sflomos et al., 2016). This approach can induce the establishment of ER+ tumours that recapitulates clinical features of breast cancer such as disease progression from carcinoma *in situ* to invasion and metastatic seeding. Unlike fat pad injection of cells, the MIND technique allows the injected cells to grow in the context of being surrounded by intraductal epithelium, which more accurately models breast cancer development.Hence, this *in vivo* model presents a relevant method for the evaluation of AR activity on disease progression and metastasis of ER+ breast cancer. Notably, endocrine-sensitive MCF7 cells were used in the aforementioned study (Sflomos et al., 2016) and the behaviour of endocrine-resistant cells injected intraductally has not been previously described to our knowledge.

Hence, the specific aims of this chapter are to

1) Determine the invasive and metastatic capacity of MCF7 TamR and LTED cells *in vivo* using the MIND method
2) Determine if DHT-induced AR activation affects invasion and metastatic dissemination of endocrine-resistant breast cancer cell lines.

6.2 Results

6.2.1 Establishment of MCF7 TamR and LTED MIND tumours

The ability of MCF7 TamR and LTED to form MIND tumours was first assessed. These cells were intraductally injected into a cohort of mice (MCF7 TamR, n=9; MCF7 LTED, n=8). Mice were culled sequentially over a period of 7 months as these intraductal ER+ tumours were expected to be much less proliferative relative to those established in the fat pad (Sflomos et al., 2016) (**Fig. 6.1a**). Whole mount staining of the harvested mammary glands demonstrated distention of these glands (**Fig. 6.1b, c**) compared to contralateral control mammary glands (Non-injected; **Fig. 6.1d**) 3 months post injection. At this stage, tumours were also palpable. Tumour growth rates were not reported as the cells were not luciferase-tagged nor were they measurable because of the way in which it grows intraductally in a branch-like fashion, unlike spherical tumours in the fatpad. Intriguingly, mammary glands injected with TamR cells appeared to lose their normal mammary ductal structure at 3 months, and evidence of compete invasion of the mammary gland at 5 months (**Fig. 6.1b, c**). In contrast, mammary glands injected with MCF7 LTED cells appeared to be similarly distended at 3 and 5 months following ductal injection (**Fig. 6.1b, c**), with less evidence of invasion. These observations indicated that endocrine-resistant cells were able to grow within the ducts of the mammary glands and there were differences in the capacity of TamR and LTED cells to grow in this *in vivo* ductal environment.

a

day 0 — n=8-9 — Intraductal Injection

3 months — n=2

5 months — n=4

7 months — n=2-3

Harvest

b — 3 months

TamR

LTED

c — 5 months

TamR

LTED

d — TamR

Left — Non-injected

Right — injected
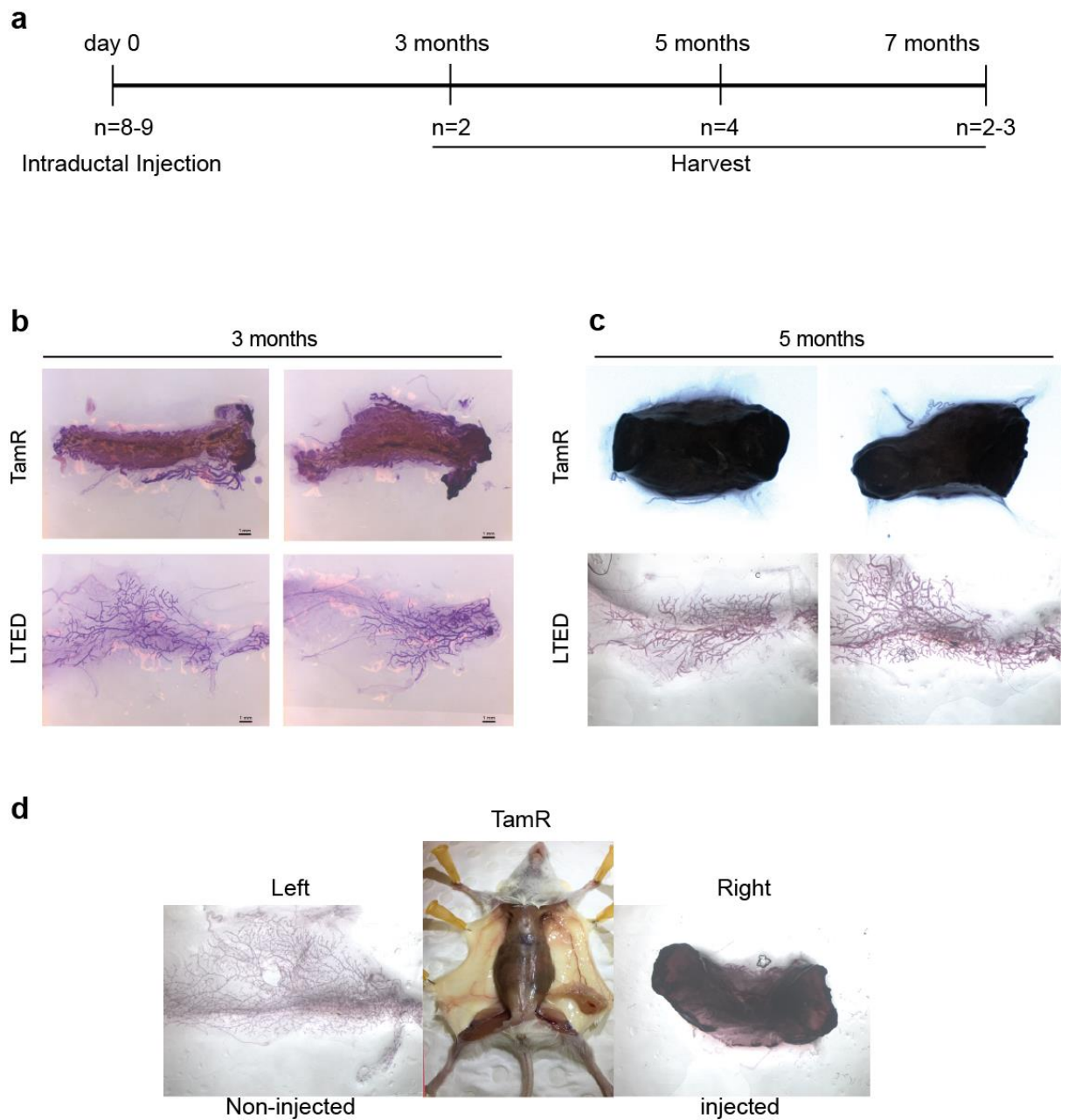
Figure 6.1. Establishment of MCF7 TamR and LTED MIND tumours

The competency of MCF7 TamR and LTED cells to establish mammary intraductal (MIND) tumour were assessed in this pilot study. **a**) NSG mice injected with these cells were harvested across different time points at 3, 5 and 7 months post injection. Carmine whole mount staining was performed to examine the histology of the mammary glands harvested 3 months (**b**) and 5 months (**c**) post injection. **d**) Carmine staining of a non-injected mammary gland and an injected mammary gland from the same mouse was performed. Representative images at low magnification are shown and scale bar = 1 mm.

6.2.2 Detection of MCF7 cells in MIND tumours

IHC was performed on tumours, 5 months following injection, to determine if the distention of the mammary gland was due to the growth of injected MCF7 cells which were engineered to express nuclear GFP. As expected, GFP+ cells were detected in MCF7 TamR and LTED MIND tumours but not in the non-injected glands (**Fig. 6.2**). These tumours were also immuno-positive for Ki-67, AR and ER. Notably, the TamR MIND tumours appeared to be more highly Ki-67 positive compared to the LTED MIND tumours. Some ER and AR expression was detected in the epithelium of non-injected normal mammary gland but they were immuno-negative for GFP, human specific CK8/18 and Ki67, consistent with endogenous mouse mammary epithelial cells,
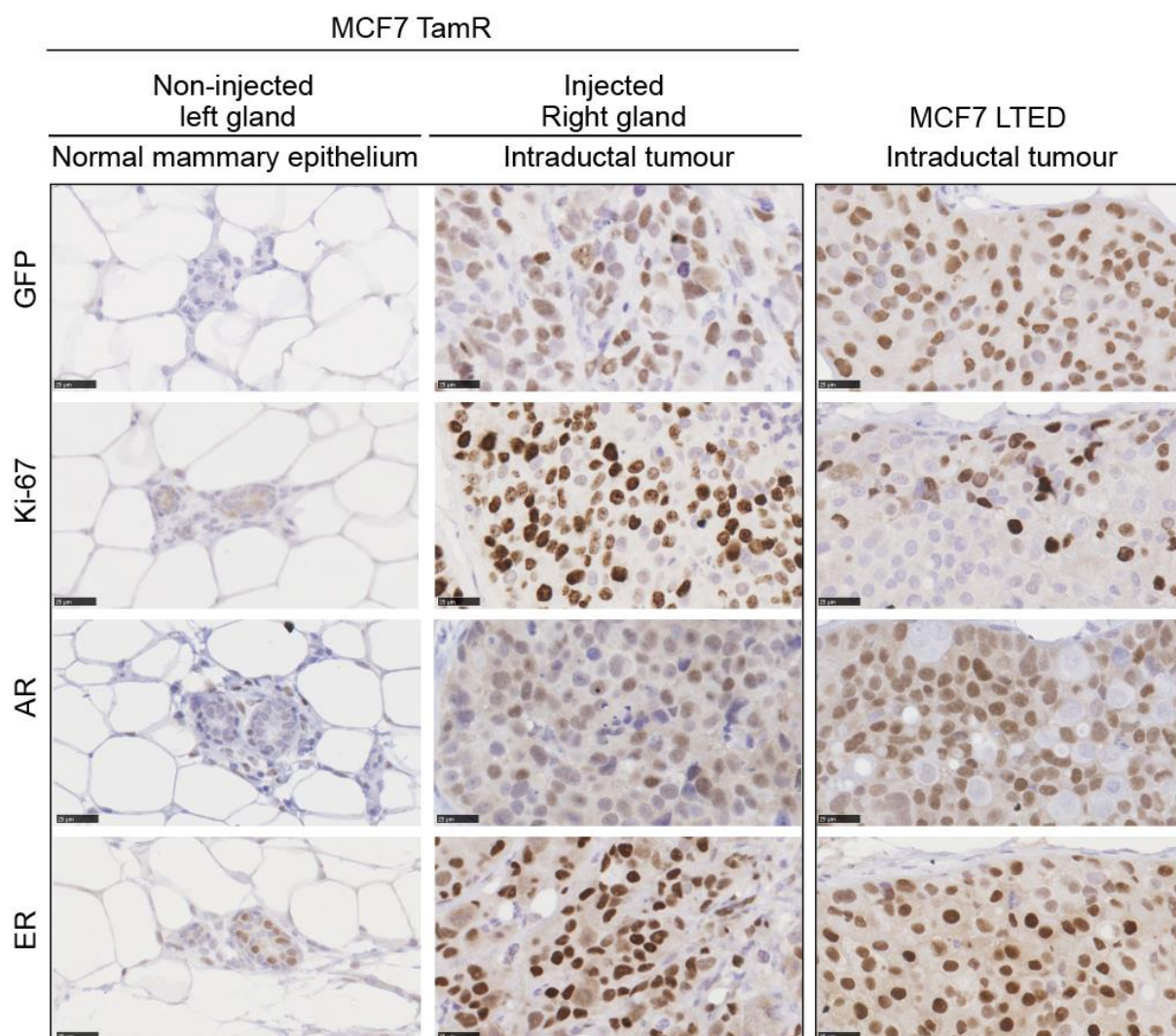
Figure 6.2. IHC analysis of MCF7 cells in MIND tumours

A control mammary gland and mammary glands, injected with TamR cells or LTED cells, were subjected to IHC staining for GFP, Ki-67, AR, and ER after 5 months. Representative images are shown and scale bars = 25µm.

6.2.3 Histological features of MCF7 TamR and LTED MIND tumours

Further histological examination of these MIND tumours indicated the presence of cells confined to the lumen similar in appearance to ductal carcinoma in situ (DCIS) and cells which have invaded similar in appearance to invasive ductal carcinoma (IDC) in the TamR MIND tumours. While there was some variation in staining intensity with respect to AR and GFP, the IDC (**Fig. 6.3a**) and DCIS like structures (**Fig. 6.3b**) were generally positive for AR, ER and GFP, and consistently stained for Ki-67 (**Fig. 6.3a,b**). In contrast, the LTED MIND cells were mostly located within DCIS like structures, and were immuno-positive for AR, ER, GFP and Ki-67 (**Fig. 6.4**). These results suggest that MCF7 TamR cells possess a higher invasive capacity than the LTED cells.
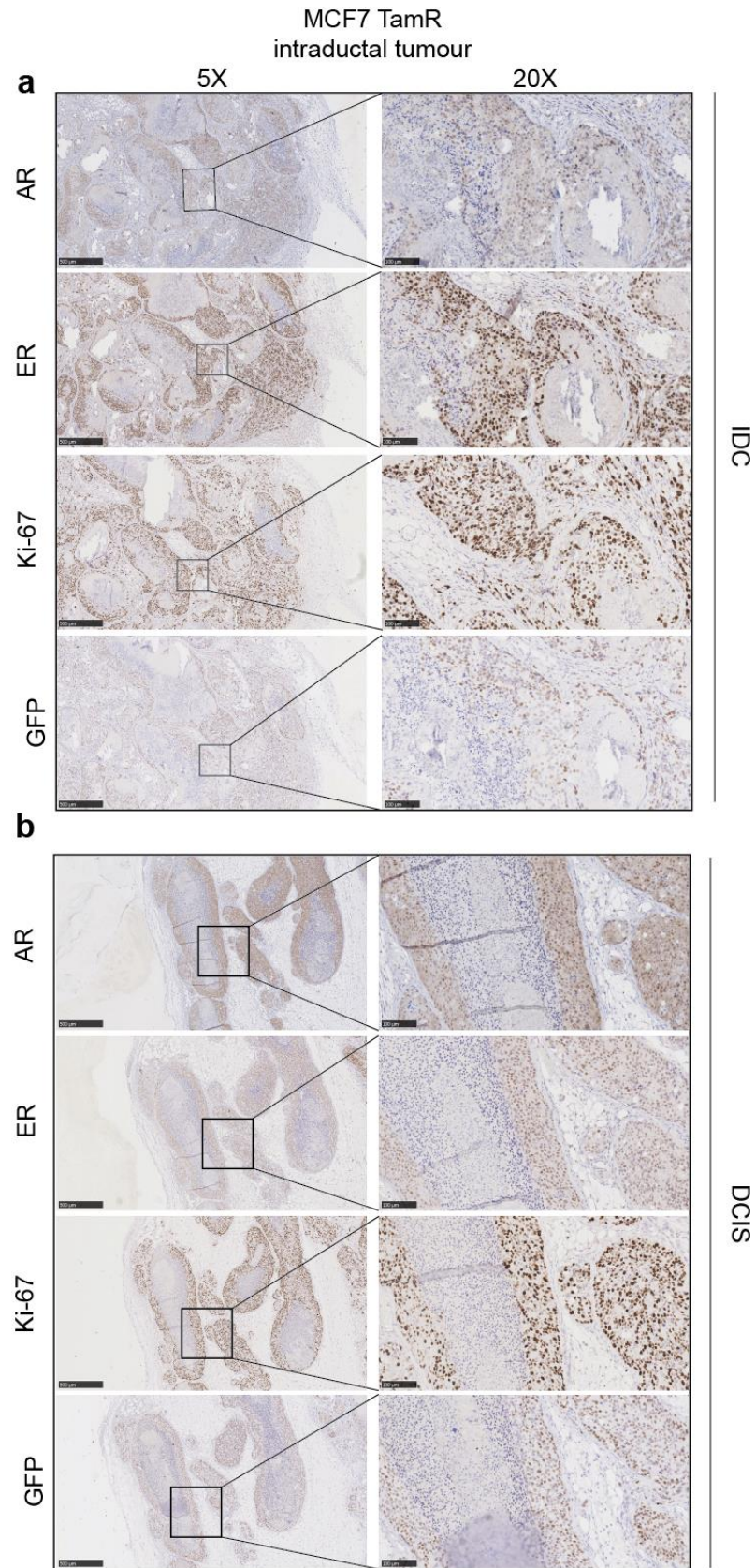
Figure 6.3. Histological features of MCF7 TamR MIND tumours

Sections of a representative TamR tumour exhibiting features of ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) like structures were subjected to IHC staining for AR, ER, Ki-67 and GFP. Scale bars = 500 μm.
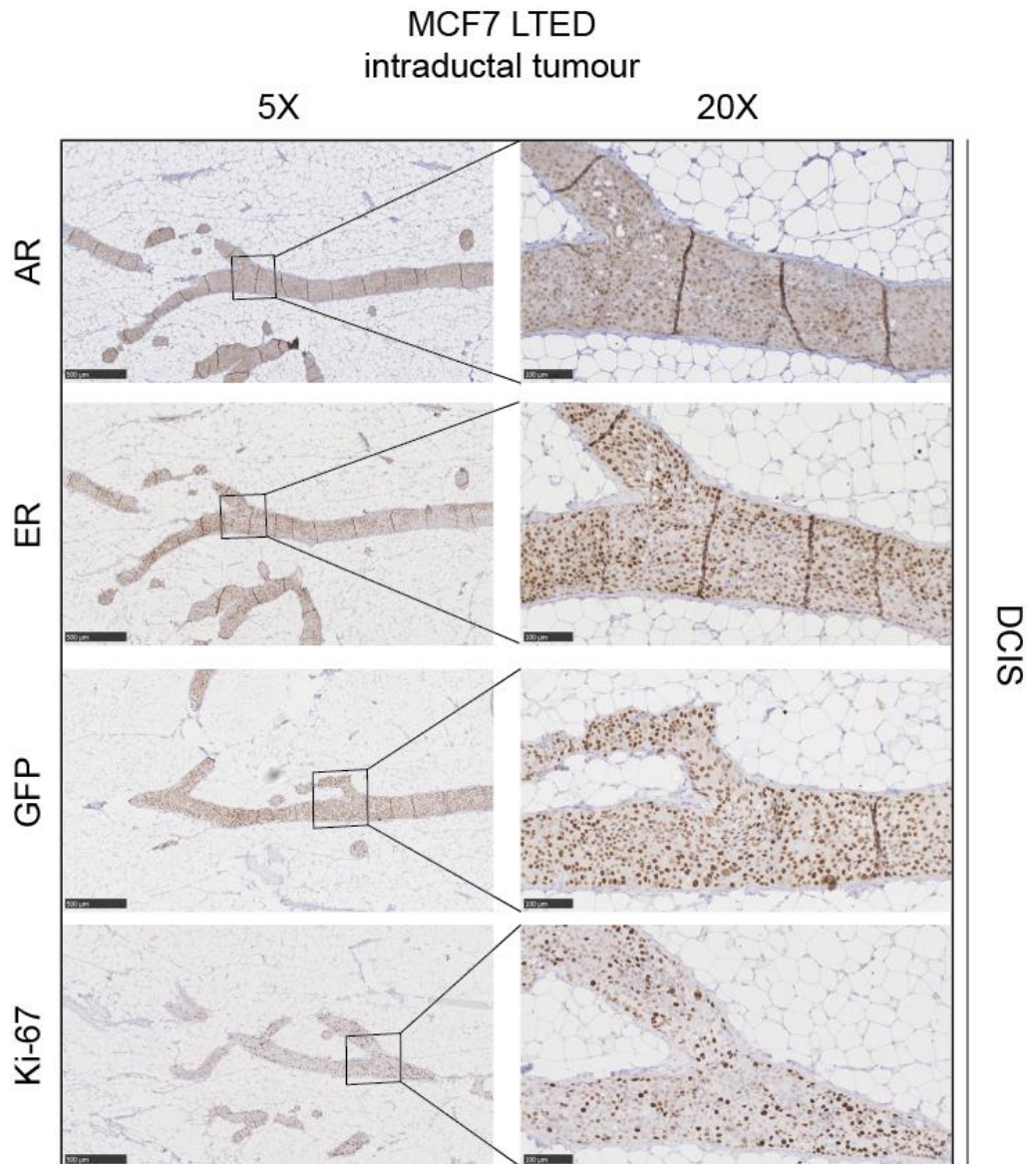
Figure 6.4. Histological features of MCF7 LTED MIND tumours.

Sections of a representative LTED MIND tumour exhibiting features of ductal carcinoma in situ (DCIS) like structure were subjected to IHC staining for AR, ER, Ki-67 and GFP.

6.2.4 Detection of metastatic cells in the lungs of injected mice

Next, we evaluated the metastatic potential of MCF7 TamR and LTED cells. Metastatic cells in the lungs of mice injected with these endocrine-resistant cells for 7 months were detected with IHC staining for GFP, AR and ER. Pockets of cells, potentially representing micro-metastases, were detected in the lungs of mice injected with TamR cells but not in the lungs of mice injected with LTED cells (**Fig. 6.5a**). These TamR metastatic cells were immuno-positive for GFP, AR and ER. The average number of GFP-positive cells in the lungs per mouse was calculated from the total number of cells identified from 4 sections of the lungs, with each section 20 µm apart. Approximately 36 cells were detected in the lungs of each mouse injected with TamR cells, in contrast to <3 cells with LTED cells (**Fig. 6.5b**). These observations suggest that the TamR cells had a higher metastatic potential than the LTED cells using this approach.

a Lung mets

	TamR	LTED	Normal non-injected
GFP			
AR			
ER			

b
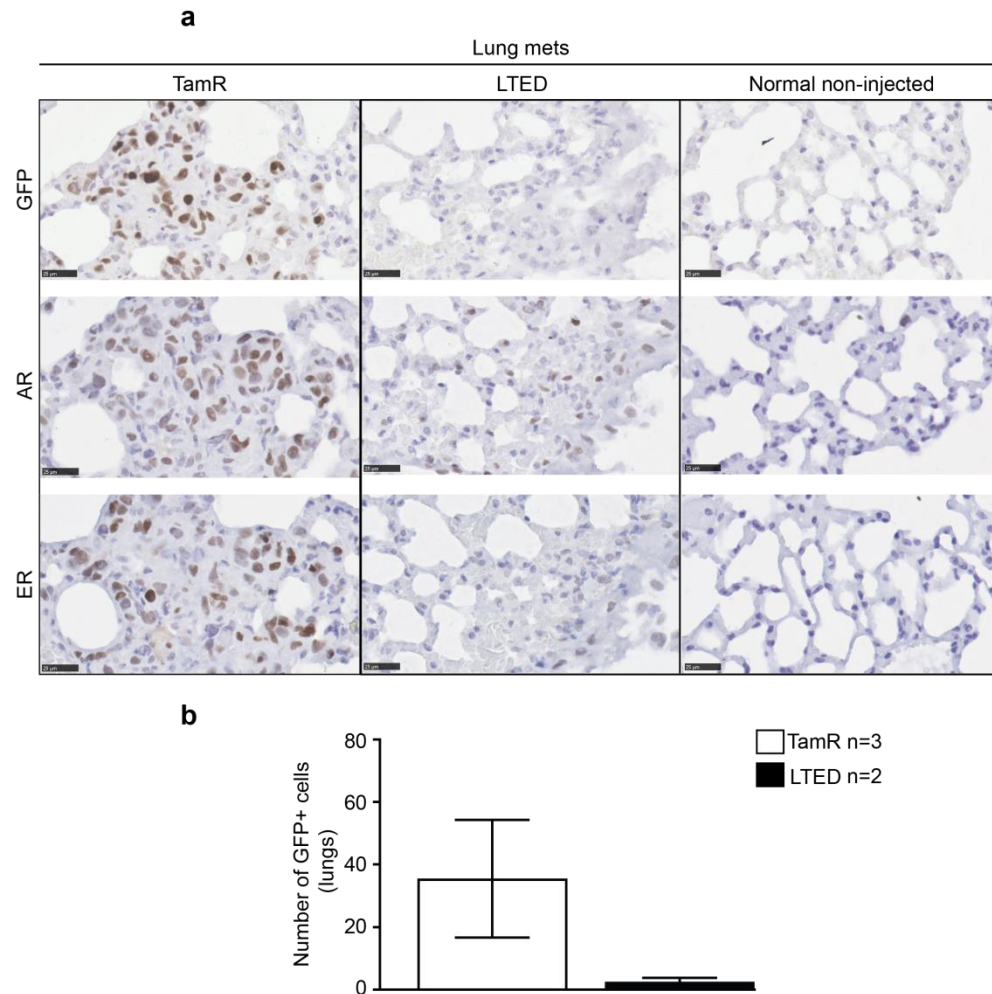
Figure 6.5. Incidence of MCF7 lung metastasis in lungs of mice 7 months post injection.

The metastatic dissemination of MCF7 TamR and LTED cells was investigated. The lungs of mice were harvested 7 months post injection and IHC staining with GFP AR and ER was performed on these cells (**a**). Lungs from non-injected mice were included as negative controls. The number of GFP+ cells was derived from the average of the number of cells identified in 4 sections of the lungs (**b**). Scale bars = 25 μm. Error bars = S.E.M.

6.2.5   Effect of DHT on invasion and metastasis of MCF7 TamR cells

Given their higher invasive and metastatic potential, the MCF7 TamR cells were chosen to evaluate the effect the DHT-induced AR activation on invasion and metastasis *in vivo*. In this experiment, the injected cells were allowed to establish for a month (incubation period) prior to allocation to either 5 day (short), 2 month (medium) or 7 month (long term) treatment group. Mice allocated to these groups were then further randomized to either the DHT group or to the control group. Changes in AR and ER signalling were evaluated in tumours treated with DHT for 5 days and the effect of AR activation on invasion was assessed in mice treated with DHT for 2 months. The effect of AR activation on metastatic dissemination of MCF7 TamR cells to the lungs was investigated in mice treated with DHT for 7 months. A summary of the experimental design is illustrated in **Fig. 6.6**.
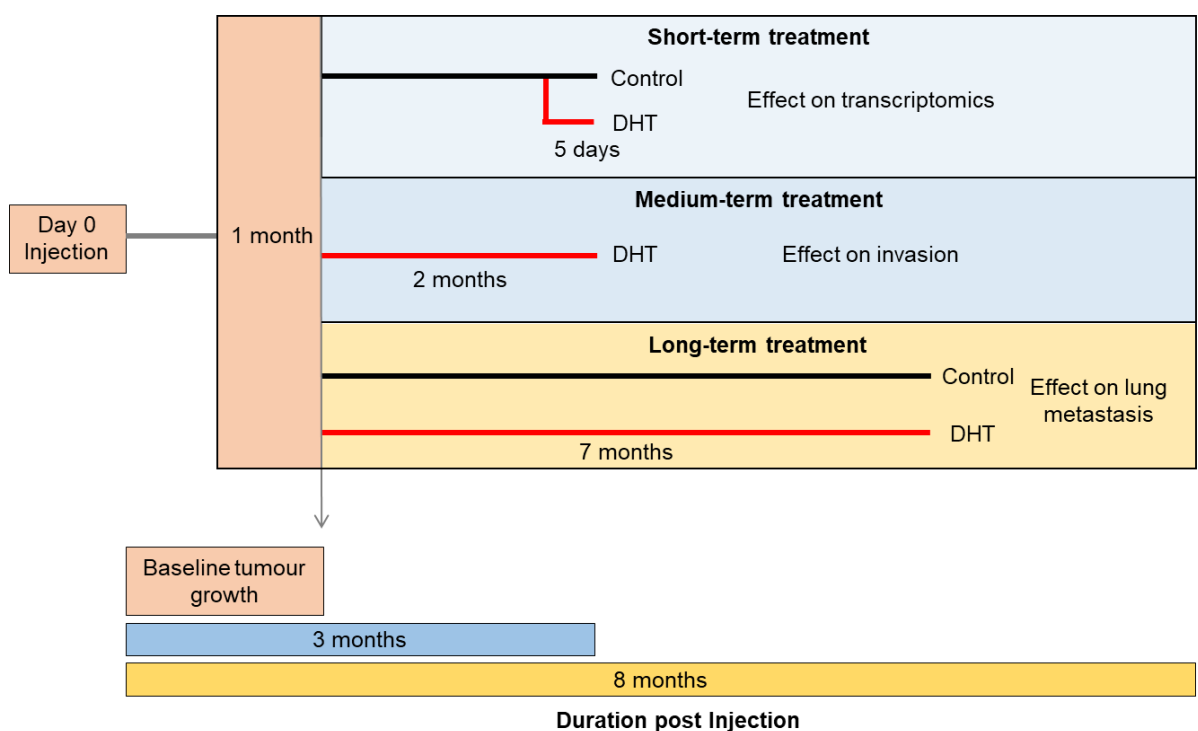
Figure 6.6. Experimental layout for evaluating the effect of DHT on invasion and metastasis in MCF7 TamR MIND tumours.

The effect of DHT on invasion and metastatic dissemination of TamR MIND tumours was assessed. Mice were injected with TamR cells were left to grow tumours for a month prior to allocation to short/medium-term treatment or long-term treatment. One mouse was culled at this point to confirm tumour establishment at baseline. The primary readout of 5 day treatment arm was the effect of DHT on transcription within the tumour. In the 2 month treatment arm, the effect of DHT on invasion was assessed. The incidence of metastasis was assessed in the 7 month treatment arm.

To ascertain the presence of TamR cells in the mammary glands at the point of treatment, mammary glands harvested from a mouse injected with TamR cells for 30 days (baseline) were stained with Ki-67 to confirm the presence of highly proliferative TamR cells (**Fig. 6.7a**). Mammary glands from a non-injected mouse stained negative for Ki-67 (**Fig. 6.7a**). Positive validation of changes in AR and ER signalling with DHT treatment was performed on the 5 day treated MIND tumours. RT-qPCR demonstrated an increase of *SECL14L2* by 20-fold and a reduction of *ESR1* by 25% in response to the DHT treatment for 5 days (**Fig. 6.7b**). Importantly, these changes were associated with reductions in the intensity of Ki-67 staining, an indirect measurement of Ki-67 positivity, in both DCIS and IDC like structures in the treated tumours (**Fig. 6.7c,d**).

A similar effect on Ki-67 was observed when tumours were treated with DHT for 2 months and the overall Ki-67 intensity was reduced in both DCIS and IDC like structures relative to the corresponding areas in the control arm (**Fig. 6.8a,b**). Activation of AR by DHT was also associated with a significant reduction in tumour weights relative to the control tumours (**Fig 6.8c**). Given that DCIS progresses to IDC, the invasive capacity of MCF7 TamR cells was determined based on the number of DCIS relative to the IDC like structures in the MIND tumours. Given the disparity in size of the DCIS like structures, only DCIS which were less than 500 μm in diameter with an intact basement membrane were included in the analysis (**Fig. 6.9a**). The number of DCIS like structures in MIND tumours exposed to DHT was significantly more than that found in control tumours (**Fig. 6.9b**). These observations suggest that DHT-induced AR activation may be able to hinder the invasion and progression of DCIS to IDC in the MCF7 TamR MIND tumours.
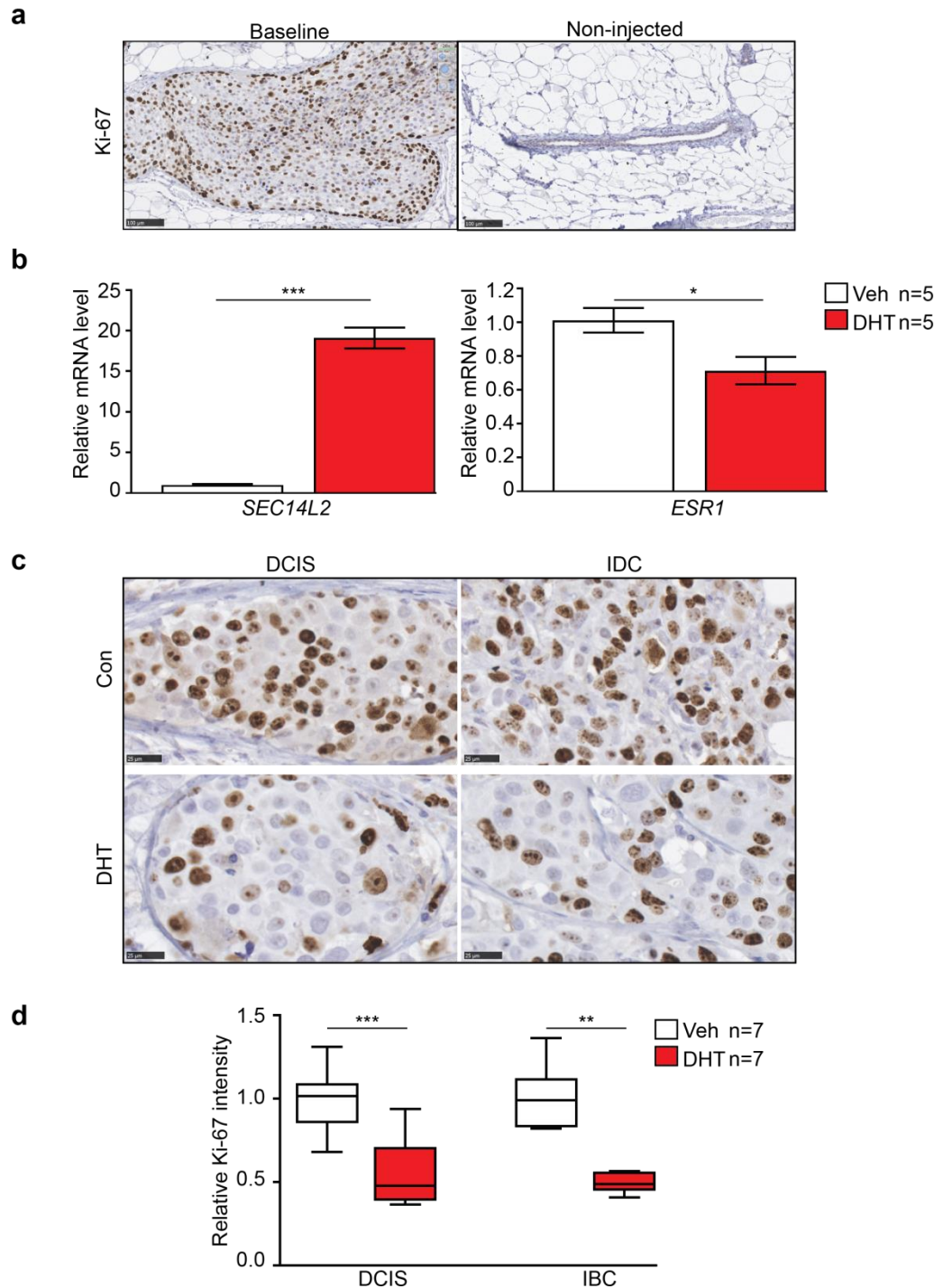
Figure 6.7. 5 day DHT treatment reduces proliferation of MCF7 TamR MIND tumours
(**a**) TamR MIND tumours established 30 days post injection and control non-injected mammary glands were subjected to IHC staining for Ki-67. RNA extracted from control (Con) or DHT-treated TamR MIND tumours were analysed for changes in the expression levels of *SECL14L2* and *ESR1* using RT-qPCR (**b**). The effect of DHT on proliferation was determined by changes in Ki-67 positivity in both DCIS and IDC like structures (**c**). Quantification of the intensity of Ki-67 staining was determined by ImageJ from 4 representative high-magnification areas. Data are represented as relative to controls. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ using Student's t-test. Error bars = S.E.M. from biological replicates. Scale bars = 100 μm (**a-b**) or 25 μm (**c**).
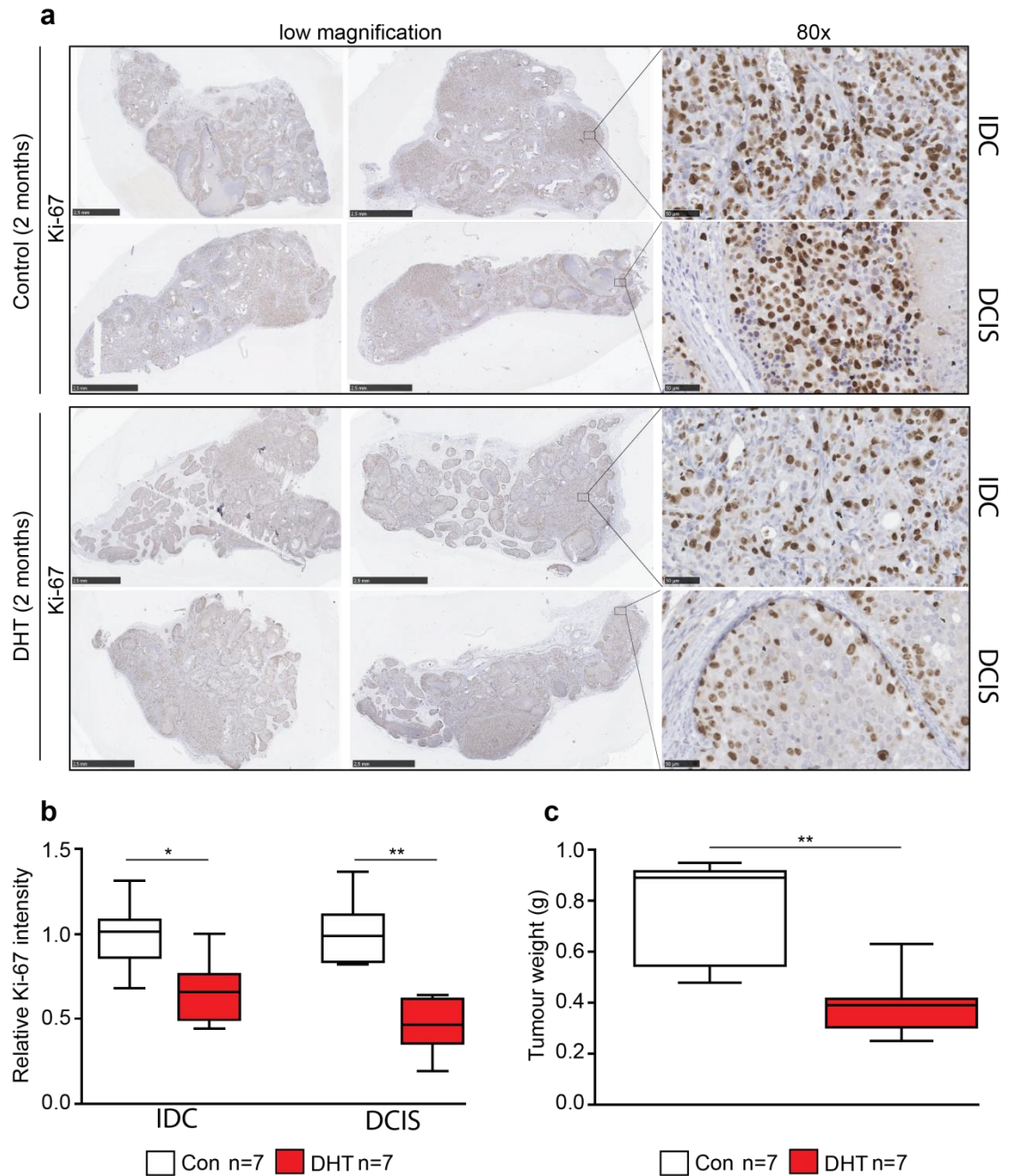
Figure 6.8. 2 month DHT treatment reduces proliferation of MCF7 TamR MIND tumours

MCF7 TamR MIND tumours treated with DHT for 2 months and harvested. Ki-67 staining of the DHT-treated and control tumours was demonstrated in four biological replicates at low magnification (**a**). Quantification of the intensity of Ki-67 staining was determined by ImageJ from 4 representative high-magnification areas (**b**). Tumour weight of control tumours versus that of DHT-treated tumours (**c**). Data in (**b**) are represented as relative to controls. * $p<0.05$ and ** $p<0.01$ using Student's t-test. Error bars = S.E.M. from biological replicates. Scale bars = 2.5 mm (low magnification) and 25 µm (80x).

a

DCIS counted

DCIS not counted

* Only DCIS ≤ 500 μm were counted

b

Veh n=7

DHT n=7
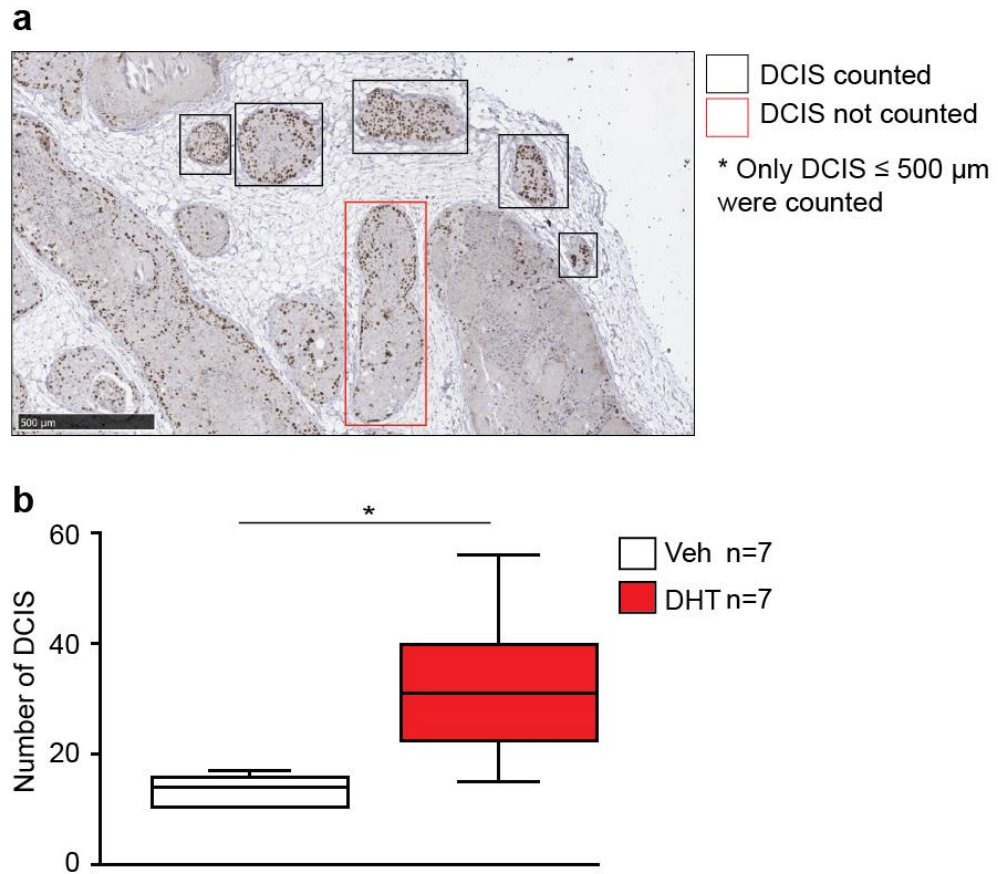
Figure 6.9. 2 months of DHT treatment reduced invasion in MCF7 TamR MIND tumours.

The effect of DHT on the progression of DCIS to IDC like structures was assessed. The effect of DHT on invasion was determined by quantifying the number of DCIS lesions <500 μm based on Ki-67 staining (**a**). The number of DCIS like structures in DHT-treated tumours was compared to that in the control tumours (b). * $p<0.05$ using Student's t-test. Error bars = S.E.M. from 7 biological replicates. Scale bars = 500 μm.

Finally, the effect of DHT-induced AR activation on the metastatic dissemination of these cells was determined. The lungs of the mice injected with these cells for 7 months were subjected to IHC staining with GFP (**Fig. 6.10a**). The number of GFP+ cells in the lungs of each mouse was quantified from 4 sections of the lungs. Treatment of the mice had no effect on the metastatic dissemination of MCF7 TamR as there was no difference in the number of GFP+ cells in the lungs of control and DHT-treated mice (**Fig. 6.10b**).
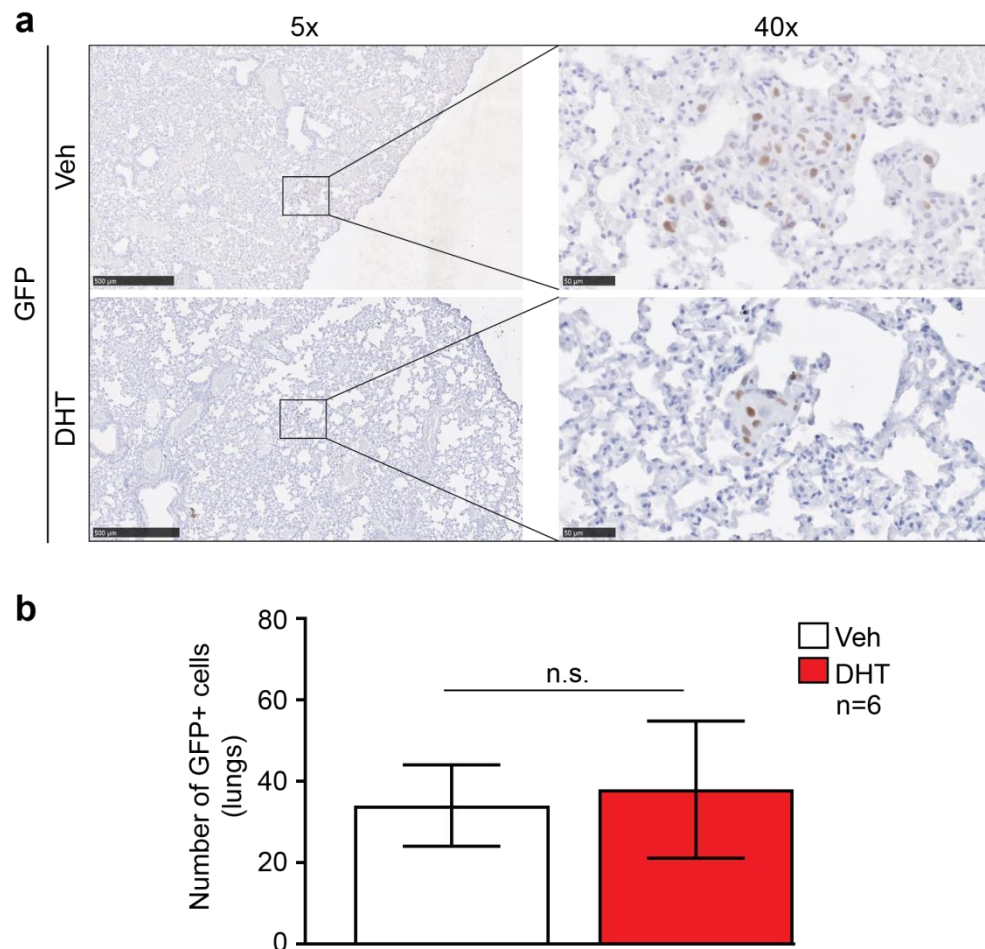
Figure 6.10. DHT does not affect metastatic dissemination of MCF7 TamR MIND tumours to the lungs

The effect of long-term DHT treatment on the metastatic colonization of lungs by MCF7 TamR MIND tumours was determined. Lungs of control mice or DHT-treated mice harvested 7 months post commencement of DHT treatment were subjected to IHC staining for GFP (**a**). The average number of GFP+ cells in the lungs per mouse was determined from 4 sections of the lungs (**b**). n.s. = not significant using Student's t-test. Error bars = S.E.M. from 6 biological replicates. Scale bars = 500 µm (5x) and 50 µm (40x).

6.3 Discussion

In this chapter, we have used an *in vivo* modelling technique which models the clinical phenotypes of ER+ breast cancer to evaluate the consequences of AR activity on invasion and metastatic dissemination of endocrine-resistant cells (Sflomos et al., 2016). We demonstrated that MCF7 TamR and LTED cells were capable of growing within the ductal microenvironment of the lungs, consistent with previously published endocrine-sensitive ER+ cells such as MCF7, T47D and ZR751 (Sflomos et al., 2016). We compared the behaviour of MCF7 TamR and LTED cells using this technique and demonstrated that MCF7 TamR cells exhibited higher proliferative, invasive and metastatic capacity *in vivo* compared to LTED cells. A comparison of the growth of these endocrine-resistant cells to the parental MCF7 cells would have determined the impact of the acquisition of endocrine-resistance on the behaviour of these cells intraductally, but this was beyond the scope of our current work.

Importantly, activation of AR by DHT significantly dampens the proliferation and invasion of the MIND cells, but did not seem to affect the dissemination and colonization of MCF7 TamR cells in the lungs. These phenotypic differences are consistent with the higher expression of FOXA1 (**Fig. 4.1**) in the TamR cells as compared to the LTED cells. High FOXA1 expression, a uniquely acquired feature of ER+ cells exposed to tamoxifen, was reported to induce increased growth factor signalling and invasion of MCF7 TamR cells *in vitro* (Fu et al., 2016). Observations from other studies which reported that tamoxifen but not estrogen-deprivation induced increased invasion, and that MCF7 TamR cells were 9-fold more invasive than the parental MCF7 cells, support our observations *in vivo* (Borley et al., 2008; Hiscox et al., 2006; LeBeau et al., 2014). Considering that patients with ER+ tumours receiving aromatase inhibitor treatment have elevated levels of androstenedione(Elliott et al., 2014) , a weak androgenic hormone and a precursor for the biosynthesis of testosterone and estrogen, it remains to be determined whether LTED cells, developed in complete hormone-deprived conditions, actually recapitulate clinical features of ER+ tumours exposed to these therapies.

The factors underlying the invasiveness of the TamR cells *in vivo* are beyond the scope of this study. It is well-established that invasion of cells from the primary tumour into the surrounding stroma requires the proteolysis of the basement membrane which acts

as a physical barrier separating the organs from the surrounding stroma. Type-4 collagen is the predominant type of collagen in the basement membrane and it can be broken down by matrix metalloproteinases-2 (MMP-2) and MMP-9 (Zeng et al., 1999). Interestingly, inhibition of MMP-9 reduced the invasion of TamR cells *in vitro* and this suggests that MMP-9 may mediate the invasion of TamR cells *in vivo* in our study (Farabegoli et al., 2011).

In regard to the response of TamR MIND tumours to DHT, we have demonstrated induction of AR-regulated *SECL142* and reduction in *ESR1* mRNA levels, and these observations parallel the response of these cells to DHT *in vitro*. Functionally, activation of AR was associated with inhibition of proliferation of cells in the MIND tumours, regardless of whether it was within DCIS or part of the IDC like structure. This observation accords with the effect of DHT on the MCF7 TamR cells *in vitro* as well as other breast cancer models examined in this thesis (Chapters 4 & 5). While DHT treatment also hindered the invasion of MCF7 TamR cells, it remains to be determined if this effect of DHT was due to inhibition of proteins specific to this process, such as MMP-9, or if it was a consequence of inhibition of proliferation. Interestingly, there appears to be an accumulation of highly Ki-67 positive TamR cells in the invading front (results not shown). This observation, if validated, would contradict the evidence suggesting that cells had to choose between proliferation and invasion where highly invasive cells had reduced proliferative capacity and vice versa (reviewed in (Kohrman & Matus, 2017)).

Intriguingly, the metastatic dissemination of MCF7 TamR cells was unperturbed by treatment with DHT-induced AR activation. This suggests that the process of metastasis is not affected by DHT-imposed growth inhibition in the primary tumours. However, there are several shortcomings in the quantification of metastasis which can limit the conclusions drawn from our data. Firstly, we have used an IHC based approach to identify GFP+ cells in a limited number of sections of the lungs (four). Secondly, we have not comprehensively evaluated dissemination to other organs. An alternative approach is the use of a luminescent-based imaging approach whereby metastatic cells in the different organs can be identified, tracked and quantified using a live-imager, and may be particularly informative considering that the activity of AR is reportedly associated with bone metastasis in ER+ breast cancer (Aceto et al., 2018). However, this

technique is not ideal for microscopic metastases. An alternative approach would be to quantify the amount of genomic GFP by RT-qPCR present in the lungs and other organs.

A question which remains unanswered from this work is if the activation of AR affects the proliferation of the cells which have already colonized secondary sites such as the lungs. In the case of the lung metastases it would be challenging to assess the effect of DHT on the proliferation of these lung metastatic cells since they only establish micro-metastases rather than macro-metastases which suggest which that the proliferative capacity of these cells is relatively low. The low proliferative capacity of these metastatic cells in the lungs could be explained by the observation that the presence of the primary tumours activates a systemic inflammatory response involving interleukin-1β which sustains metastatic cells in the lungs in a mesenchymal form and limits the proliferation of these cells (Castano et al., 2018). Given the inhibitory effect of the primary tumours, removal of the primary tumours or pharmacologic inhibition of interleukin-1β induces subsequent differentiation and proliferation of these metastatic cells. In light of these observations, an alternative approach would be to evaluate the effect of AR activation following surgical removal of the primary tumours to evaluate the proliferation of these metastatic cells in the lungs.

In summary, we have demonstrated that endocrine-resistant MCF7 cells can develop MIND tumours when injected intraductally but there are fundamental differences in the proliferation, invasion and metastasis of these cells in an endocrine-resistance specific manner with TamR exhibiting a higher tumourigenic potential than the LTED cells. Importantly, the activation of AR by DHT reduced the proliferation and invasion of the TamR MIND tumours although it did not appear to affect the metastatic dissemination of these cells to the lungs.

CHAPTER 7

7. Final Discussion

ER+ breast cancer constitutes 70% of all breast cancers and ER-targeted therapies, such as tamoxifen and aromatase inhibitors, are the mainstay in the management of this disease. However, development of resistance against these ER-targeted agents is common and novel therapies for the treatment of resistant tumours are an urgent clinical need. AR is expressed in the majority of endocrine- sensitive and -resistant ER+ breast cancer (Collins et al., 2011; Hu et al., 2011; Niemeier et al., 2010; Qi et al., 2012) (Cimino-Mathews et al., 2012; De Amicis et al., 2010; Grogg et al., 2015). The expression of AR is a well-established independent prognostic factor of good outcome in ER+ tumours (Bozovic-Spasojevic et al., 2017; Ricciardelli et al., 2018). Together, these observations are consistent with the growth-inhibitory effect of DHT on ER+ cell line models (Lanzino et al., 2005; Peters et al., 2009; Poulin et al., 1989). However, up to now, the targeting of AR in endocrine-resistant breast tumours remains controversial with both agonistic and antagonistic strategies reported to be effective, and have been evaluated in clinical trials (Chapter 1, **Table 1.3**). In this thesis, we comprehensively evaluated the biological roles of AR in endocrine-resistant breast cancer functionally and transcriptomically, utilizing *in vitro* and *in vivo* preclinical models, with siRNA and pharmacological approaches. Through this, we have provided clarity to the optimal therapeutic strategy in targeting AR in endocrine-resistant ER+HER2- breast cancer, and established a prognostic AR gene signature which outperforms other established prognostic gene signatures in ER+ breast cancer.

Contemporary preclinical studies suggest that inhibition of AR using pharmacological agents, such as enzalutamide, were effective in antagonizing the growth of cell line models of endocrine-resistance. However, these studies were largely carried out using genetically-modified cell lines, including the overexpression of AR (Ali et al., 2015; Ciupek et al., 2015; De Amicis et al., 2010; Rechoum et al., 2014). Using cell lines with endogenous levels of AR expression, we dissected the consequences of inhibiting AR using siRNA-mediated knockdown and pharmacological inhibition. Using the siRNA knockdown approach, we identified a functional role for AR in facilitating endocrine-resistance. This function of AR was associated with inhibition of classical ER signalling and sustaining an ER signalling program that promotes endocrine-resistance. However,

the lack of any efficacy with the use of AR antagonist enzalutamide on our endocrine-resistant preclinical models including an *in vivo* PDX model that grows independently of E2 supplementation, suggests that it is the non-canonical activity of AR and not its canonical transcriptional activity that contributes to this phenotype.

Non-canonical activity of AR has been reported in cell line models of breast cancer, ovarian teratocarcinoma and prostate cancer (Chung et al., 2014; Yeh et al., 2003; Zarif & Miranti, 2016). AR appears to partially regulate the activity of PI3K/Akt pathway through its non-genomic activity in our breast cancer model and this accords with a previous observation that AR physically interacts with EGFR, which lies upstream of PI3K/Akt, in ER+ breast cancer cells (Ciupek et al., 2015). Importantly, it has been reported that mainstream AR-targeted therapies, which were designed to inhibit the ligand-induced activation of AR, do not block this non-canonical activity of AR, consistent with our observations using enzalutamide (Peterziel et al., 1999).

The lack of efficacy associated with enzalutamide in our study concurs with observations from two clinical trials which similarly reported a similar phenomenon. The first study evaluated the efficacy of flutamide in non-selected patients with metastatic breast cancer (Perrault et al., 1988) whereas the second study assessed the efficacy of exemestane (an aromatase inhibitor) with or without the androgen biosynthesis inhibitor abiraterone acetate in endocrine-resistant ER+ breast cancer. The latter study supports our hypothesis that inhibition of classical AR activity is ineffective in endocrine-resistant breast tumours (O'Shaughnessy et al., 2016). However, results from a recent phase two clinical trial reported that a prolonged progression-free survival (HR= 0.44, p<0.0335) was associated with the combination of enzalutamide and exemestane relative to those whose who received only exemestane. However, this response was observed in only patients who had not received an endocrine-therapy for metastatic breast cancer and were also positive for an AR-gene signature based biomarker (Krop et al., 2018). Notably, patients with the AR signature-positive tumours constituted 35% of all patients recruited for this trial and this included the cohort of patients who had received a line of endocrine-therapy for metastatic breast cancer and who did not benefit from enzalutamide. Hence, considering the relatively low response rate and demonstrated off-target effect associated with enzalutamide treatment, further confirmatory analyses are required to demonstrate that inhibition of AR signalling is

associated with the observed extended progression free survival in the responders in this trial.

On the contrary, activation of AR using either DHT or enobosarm was associated with growth suppression in our endocrine-resistant preclinical models. These observations are consistent with the clinical studies which report a similar response when patients who had progressed on one or more lines of endocrine therapy were exposed to AR agonists such as testosterone, fluoxymesterone, medroxy progesterone acetate and enobosarm (Birrell, Roder, et al., 1995; Boni et al., 2014; Kono et al., 2016; Overmoyer et al., 2015). This growth inhibition with AR agonists in the resistant tumours demonstrates that canonical AR transcriptional activity is anti-proliferative in ER+ breast tumours independent of their sensitivity to the different types of endocrine therapies.

Mechanistically, the growth-suppression induced by AR agonists is associated with downregulation of ER signalling and this has important clinical implications. Recent studies have identified that key resistance mechanisms associated with clinical development of endocrine-resistant breast cancer are largely centred upon the persistent activation of ER (Chandarlapaty et al., 2016; Fanning et al., 2016; Li et al., 2013; Magnani et al., 2017; Schiavon et al., 2015; Toy et al., 2017). These include the emergence of *ESR1* mutations, *ESR1* amplification, *ESR1*-fusion and amplification of *cytochrome P450 Family 19 Subfamily A Member 1 (CYP19A1)*. Currently, the most prevalent resistant mechanism is the occurrence of mutations in the hotpot region of *ESR1*, which occurs in up to 40% of patients with endocrine-resistant breast cancer, and which leads to constitutive activation of ER and reduced binding affinity to ER antagonists (Chandarlapaty et al., 2016; Fanning et al., 2016; Fribbens et al., 2016; Jeselsohn et al., 2015; Niu et al., 2015; Schiavon et al., 2015; Spoerke et al., 2016; Toy et al., 2017). Amplification of *CYP19A1,* which encodes for aromatase, was observed in ~20% of patients who progressed on an aromatase inhibitor (Magnani et al., 2017). This increased expression of aromatase is reported to increase local autocrine ER signalling in these patients through increased estrogen biosynthesis from endogenous or circulating testosterone in the microenvironment. Lastly, hyper-activation of ER can also be achieved through amplification of *ESR1* (Kota et al., 2017; Li et al., 2013) and through fusion of *ESR1* with other genes at the ligand-binding domains, which renders

it constitutively active (Hartmaier et al., 2018). The discovery of these ER-activating mechanisms in endocrine-resistant breast cancer suggests that alternative methods of inhibiting ER activity, such as with the use of AR agonists, are feasible therapeutic strategies in the management of this disease.

Interestingly, amplification of *ESR1* in an endocrine-resistant PDX model (Li et al., 2013) and in an endocrine-resistant patient (Kota et al., 2017) has been associated with estradiol-induced tumour growth suppression. In the case study reported by Kota et al, the patient had relapsed on tamoxifen, aromatase inhibitors and fulvestrant, but had a partial response when given estradiol as a late-line therapy. It is possible that Gar15-13 PDX tumours similarly possesses amplification of *ESR1* given the strong growth-inhibitory effect of estradiol on Gar15-13 and the high expression level of ER (**Appendix B**) although this requires further verification.

Notably, while DHT strongly inhibited the growth of both endocrine-resistant PDX models, the growth-inhibitory effect of enobosarm was relatively more pronounced in the ER+PR+AR+ HCI-005 PDX model, relative to the ER+PR-AR+ Gar15-13 PDX model. The expression of PR in the HCI-005 is likely a reflection of ER engaging in a more classical manner in the HCI-005 model and this suggests that enobosarm may be more effective at inhibiting ER signalling when it is operating classically. A case study of an ER+AR+PR+ endocrine-resistant patient, who experienced an 11-month progression-free survival on a last line enobosarm therapy, supports the hypothesis that the expression of PR could potentially be a biomarker of response to enobosarm (Vontela et al., 2017). However, the relationship between expression of PR and response to enobosarm requires further validation, and it remains to be determined if the expression of PR could be used as a predictive biomarker for AR agonist therapy in the recently concluded phase two trial of patients with metastatic breast cancer which has yet to be reported (NCT01616758).

We have also established an AR gene signature, using the clinically annotated METABRIC ER+ patient cohort, which is strongly associated with prognosis in ER+ patients. Notably, the prognostication power of this AR gene signature outperforms random signatures and the clinically-used Mammaprint 70-gene signature and this highlights the importance of AR biology in ER+ tumours. Furthermore, expression of genes regulated by AR is enriched in luminal A tumours and these patients have a better

outcome than patients with luminal B tumours (Sorlie et al., 2001; Sorlie et al., 2003). Hence, our observation is in line with the studies which have established AR as an independent prognostic factor associated with favourable patient outcome (Ricciardelli et al., 2018). The significance of this signature is that it represents a functional readout of AR signalling when most correlative studies have used nuclear staining of AR to determine its prognostic value. Our signature demonstrates that active AR signalling is generally tumour-suppressive in ER+ breast cancer. Furthermore, this AR gene signature was derived from an endocrine-resistant PDX, which suggests that the tumour-inhibitory effect of AR signalling is independent of endocrine-therapy sensitivity.

Whilst we have demonstrated that downregulation of ER signalling is associated with activation of AR, the underlying mechanism contributing to the perturbation of ER transcriptional activity has not been elucidated. Given the reported crosstalk between ER and other hormone receptors such as AR (Hua et al., 2009; Karmakar et al., 2013; Kittler et al., 2013; Lanzino et al., 2005; Mohammed et al., 2015), we hypothesized that activation of AR interferes with the transcriptional activation of ER (**Fig. 1.3**). Recent technological advancement in the form of rapid immunoprecipitation mass spectrometry of endogenous proteins (RIME) has allowed the identification of binding partners which are bound to ER and AR in the context of breast cancer and prostate cancer, respectively (Mohammed et al., 2013; Paltoglou et al., 2017; Papachristou et al., 2018). An analysis of these binding partners identified a group of common cofactors which are bound by ER and AR (**Fig. 7.1**) (Paltoglou et al., 2017; Papachristou et al., 2018). These overlapping cofactors include the pioneer factor FOXA1, steroid receptor coactivator 2 (NCOA2) and proteins which constitute the basal active transcriptional machinery such as p300 and histone acetyltransferase 1 (HAT1). While yet to be demonstrated it is plausible that activation of AR sequesters some of these critical cofactors leading to the inhibition of ER signalling.
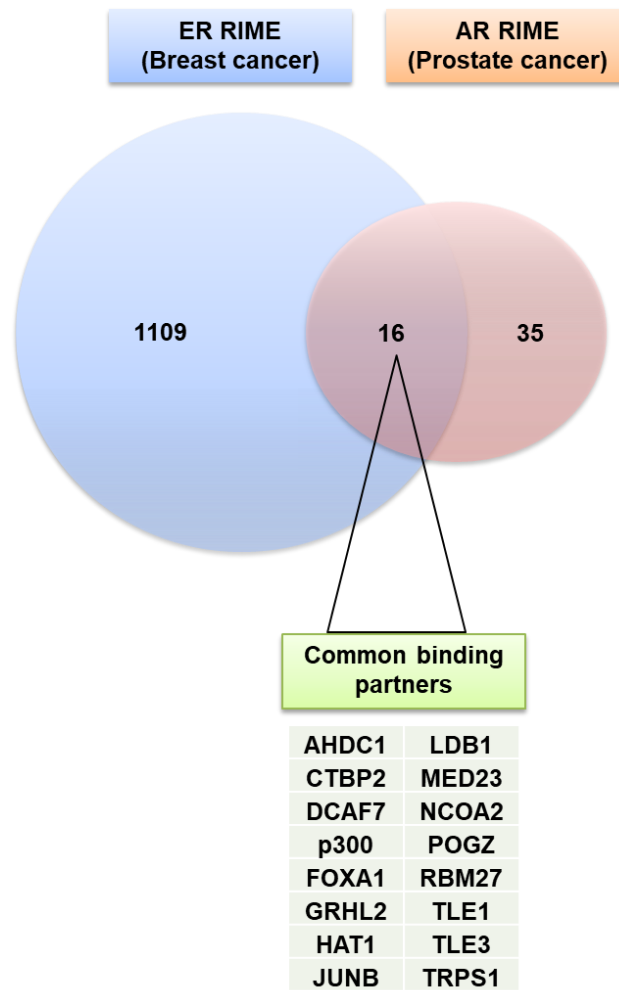
Figure 7.1. Overlap of ER and AR binding partners identified by RIME.

There are several limitations in this thesis. The first pertains to the limited number of endocrine-resistant PDX models evaluated in this thesis. Clinical mechanisms underlying endocrine-resistance are diverse and whilst *ESR1* mutations have emerged as a major mode of resistant mechanisms, the site of *ESR1* mutations are heterogeneous with different phenotypic response to ER-targeted therapies (Toy et al., 2017). The most commonly occurring *ESR1* mutations are D538G and Y537S, which are present in almost 50% and 30% of patients with *ESR1* mutations, respectively, whereas the L536P mutation in the HCI-005 PDX tumours is relatively rare and is found in only 10% of patients with *ESR1* mutations (Spoerke et al., 2016). Given that the *ESR1* mutations are reported to have variable responses to ER-targeted antagonists (Toy et al., 2017), it remains to be determined if tumours harbouring the more frequently occurring mutations respond to AR agonists in a similar manner to HCI-005 PDX tumours.

The second limitation pertains to the evaluation of enzalutamide as a monotherapy *in vivo*. Clinically the use of enzalutamide is being trialled in a combination with an ER-directed therapy such as exemestane (Krop et al., 2018). We did not evaluate the combination of ER-directed therapies and AR antagonists in our models. Given that Gar15-13 PDX tumours are resistant to fulvestrant it would be interesting to determine if enzalutamide could sensitize these tumours to the growth-inhibitory effect of fulvestrant. Lastly, we were unsuccessful in our attempt to interrogate changes in global ER chromatin binding in Gar15-13 following activation of AR with DHT or enobosarm. Successful completion of this ER ChIP-sequencing experiment, in tandem with our RNA-sequencing data, would have allowed for the identification of direct ER-regulated genes which were differentially regulated by the activation of AR.

Overall, this thesis has provided strong evidence demonstrating that AR is implicated in the sustenance of an endocrine-resistant phenotype via a non-standard signalling pathway and which is not effectively inhibited by enzalutamide as a monotherapy. On the contrary, activation of AR signalling using an endogenous ligand of AR or a clinical AR modulator effectively reduces proliferation of endocrine-resistant cells through the downregulation of ER signalling. Further supporting a tumour suppressive role of AR is the discovery of an AR gene signature, established from an endocrine-resistant PDX, which is strongly associated with prognosis in ER+ breast cancer. The translational

impact of this work is that activating AR should be the optimal therapeutic approach in targeting AR in ER+ endocrine-resistant breast cancer.

Future directions

The management of endocrine-resistant breast cancer has evolved since the commencement of this thesis and the addition of CDK4/6 inhibitors to an endocrine-therapy has proven to be particularly effective such that it is now the standard-of-care for the treatment of patients with metastatic ER+ breast cancer, (Finn et al., 2016; Hortobagyi et al., 2016; Hortobagyi et al., 2018). The mechanisms of endocrine resistance and CDK4/6 resistance have been comprehensively reviewed in (Portman et al., 2018). The clinical relevance of resistance to endocrine therapy as a single agent will decrease over time as the treatment algorithms have evolved to incorporate combinations of targeted therapies with an endocrine therapy backbone.

As a result of this development, the utility of AR agonists may have the most clinical relevance and potential for clinical development in patients who have acquired combination resistance to CDK4/6 inhibitors and ER-directed therapies, where there is no currently defined standard of care. A case study has reported of a clinical benefit associated with the use of enobosarm in a patient who has acquired resistance to multiple lines of ER-targeted therapies and to palbociclib ((Vontela et al., 2017). Larger scale studies are required to affirm the efficacy of AR agonists and to identify biomarkers of response in these patients.

On the other hand, evaluation of the efficacy of AR agonists as a replacement for ER-targeted therapy in combination with a CDK4/6 inhibitor in the endocrine-resistant setting is also warranted. In the PALOMA-3 study, response to CDK4/6 inhibitor is strongly associated with sensitivity to prior endocrine therapies and patients who were previously sensitive to endocrine therapies were more likely to benefit from CDK4/6 inhibition and achieve better overall survival (N. C. Turner et al., 2018). In this study, patients were defined as "endocrine-sensitive" if they had received an endocrine-therapy at the adjuvant setting for at least 2 years or at the metastatic setting for at least 6 months. In our hands, AR agonists suppressed the growth of 2 different endocrine-resistant PDX models *in vivo*, and a logical next step would be to evaluate its combinatory effects with CDK4/6 inhibitors. In support of this hypothesis, we have

preliminary data demonstrating that the combination of enobosarm and palbociclib is more effective than either drug alone in suppressing the growth of MCF7 TamR cells *in vitro* and Gar15-13 PDX tumours *in vivo* (**Appendix C**). However, the pertinent question which remains to be answered is how this combination compares to the standard-of-care combination comprising of fulvestrant and a CDK4/6 inhibitor.

We have recently established MFC7 combination resistant models to ER-directed therapies and CDK4/6 inhibitors through long term culture. In parallel, we have also done the same in PDX models that were derived from endocrine therapy naïve and endocrine-resistant tumour tissue from patients. More recently, we have established PDXs from two patients who have relapsed on combination endocrine therapy and CDK4/6 inhibitor therapy. These preclinical models will provide the basis for the subsequent evaluation of AR directed therapies in this important area of clinical need.

Overall, we have demonstrated that activation of AR represents a feasible therapeutic strategy in the treatment of endocrine-resistant breast cancer.

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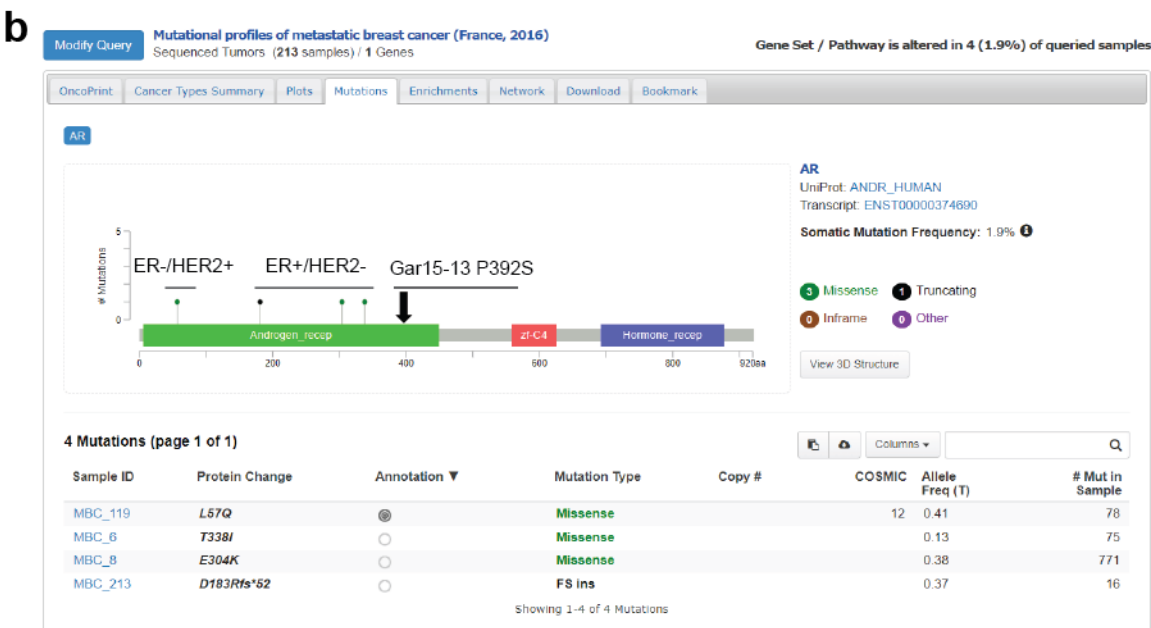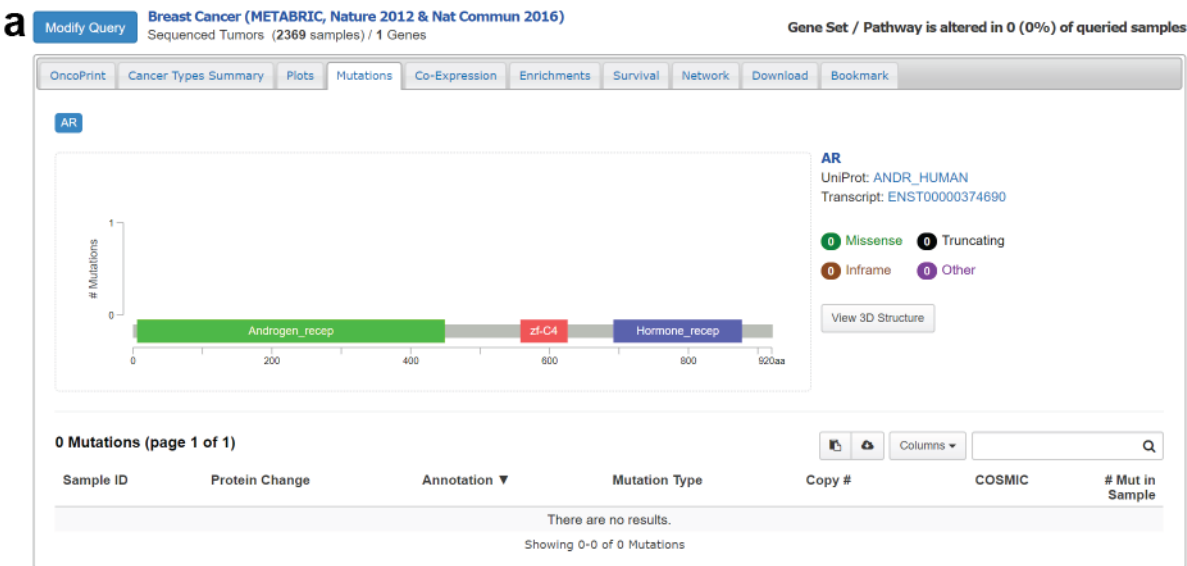
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Appendices

Appendix A

a

Breast Cancer (METABRIC, Nature 2012 & Nat Commun 2016)
Sequenced Tumors (2369 samples) / 1 Genes

Gene Set / Pathway is altered in 0 (0%) of queried samples

b

Mutational profiles of metastatic breast cancer (France, 2016)
Sequenced Tumors (213 samples) / 1 Genes

Gene Set / Pathway is altered in 4 (1.9%) of queried samples

4 Mutations (page 1 of 1)

Sample ID	Protein Change	Annotation ▼	Mutation Type	Copy #	COSMIC	Allele Freq (T)	# Mut in Sample
MBC_119	L57Q	⦿	Missense		12	0.41	78
MBC_6	T338I	○	Missense			0.13	75
MBC_8	E304K	○	Missense			0.38	771
MBC_213	D183Rfs*52	○	FS ins			0.37	16

Showing 1-4 of 4 Mutations

c

Accession #	Phenotype	Mutation type	Exon	Position	From amino acid	Reference
338	MAIS	Substitut.	1 (Nterm)	392	p.(Pro392Ser)	Hiort et al. J Clin Endocrinol Metab 85: 2810-2815, 2000
504	MAIS	Substitut.				Hiort et al. J Clin Endocrinol Metab 85: 2810-2815, 2000
767	MAIS	Substitut.				Ferlin et al. Clin Endocrinol 65: 606-610, 2006
908	CAIS	Substitut.				Appari et al. J Molecular Med: 87: 623-632, 2009
950	PAIS	Substitut.				Bhangoo al. Asian J Androl 12: 561-566, 2010
1004	PAIS	Substitut.				Audi et al. J Clin Endocrinol Metab 95: 1876-1888, 2010
1070	PAIS	Substitut.				Hughes et al. Semin Reprod Med 30: 432-442, 2012
817	Testicular cancer	Substitut.				Garolla et al. Endocrine Related Cancer 12: 645-655, 2005

Frequency of AR mutations in breast cancer and occurrence of P392S mutation in androgen insensitivity syndrome.

The frequency of AR mutations was determined in primary breast cancer from the METABRIC cohort (**a**) and in metastatic breast cancer (**b**). P392A mutation in AR is frequently observed in patients with either mild or complete androgen insensitivity syndrome (MAIS, CAIS).
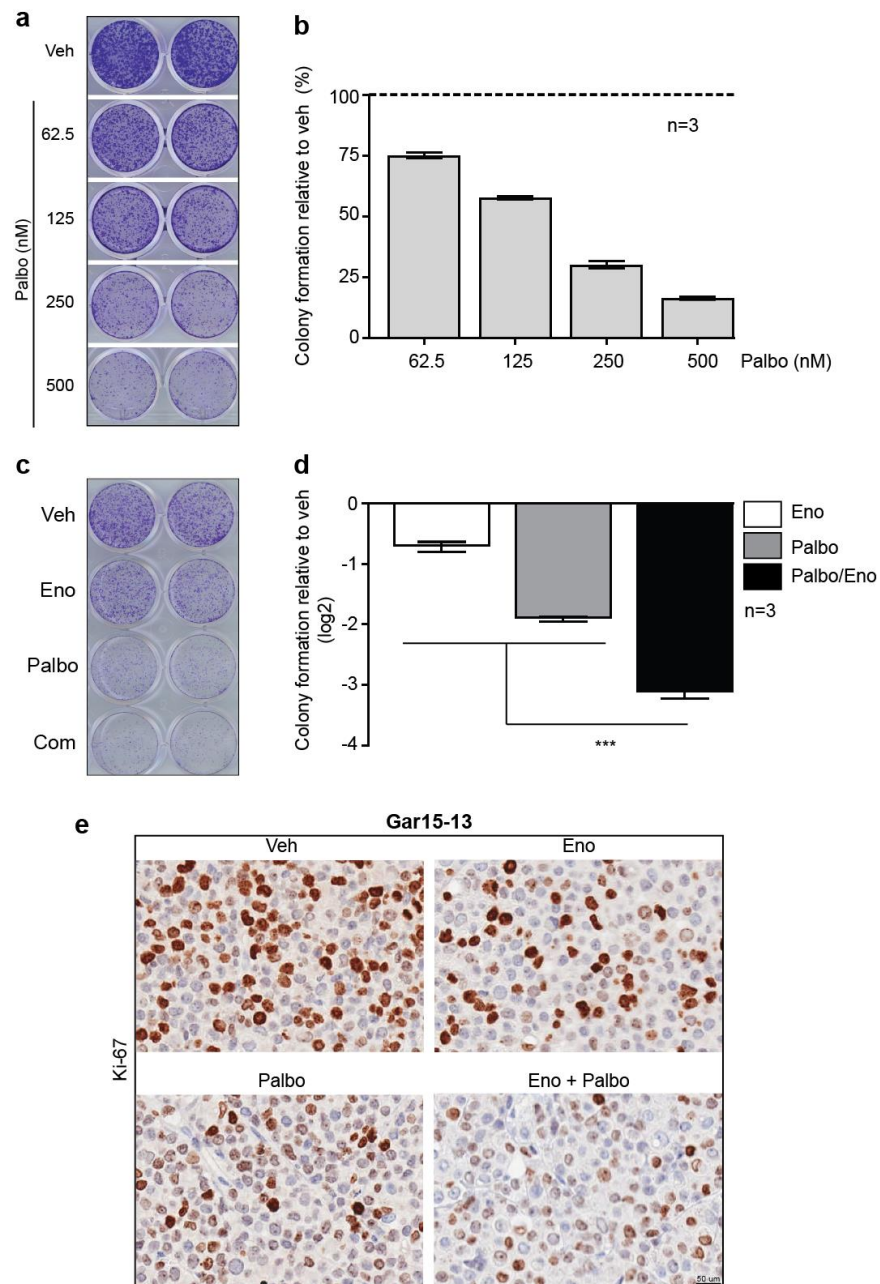
Appendix B
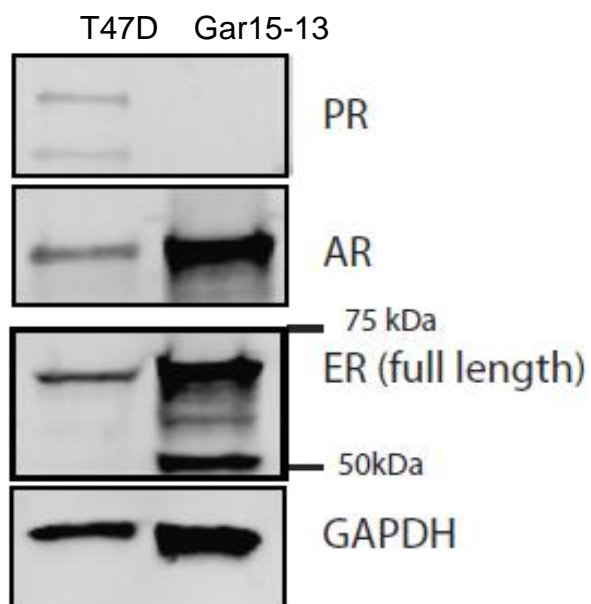
Expression level of hormone receptors in Gar15-13 PDX model
Protein lysates derived from T47D and Gar15-13 PDX model were immunoblotted for PR, AR, ER and
GAPDH.

Appendix C

Combination of enobosarm and palbociclib effectively inhibits growth of endocrine-resistant breast cancer models

a) Dose response of MCF7 TamR cells to palbociclib treatment for 6 days was assessed using colony forming assay. **b**) Quantification of colony formation in (a) was performed using ImageJ and data are presented as relative to controls. **c**) The efficacy of combined enobosarm (eno, 100 nM) and palbocicilib (Palbo, 125 nM) treatment (Com) for 6 days on the colony formation of MCF7 TamR cells relative to monotherapy with these agents was assessed. **d**) Quantification of colony formation in (**b**) was performed using ImageJ and data is presented as relative to controls in log2 scale. **e**) The efficacy of combined eno (10mg/kg/day) and palbocicilib (100 mg/kg/day) treatment for 3 weeks on the proliferation of Gar15-13 tumours relative to monotherapy with these agents was evaluated. Harvested tumours were stained with Ki-67 as readout of proliferation.