Molecular Functions of the Androgen Receptor and BEX2 in Breast Cancer

By

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ABSTRACT

This commentary on my publications concerns the molecular functions of the androgen receptor (AR) and Brain Expressed X-Linked 2 (BEX2) in breast cancer. It is notable that the presented articles have made significant contributions to the fields of cancer genomics, cancer biology and experimental therapeutics with broad applications in breast cancer and other malignancies. The first chapter outlines gene expression microarray studies and explains genomic data resulting in the discovery of BEX2 in breast cancer. Chapter two elaborates on my publications regarding the molecular functions of BEX2 in promoting breast cancer cell growth and survival by modulating the mitochondrial apoptotic pathway and G1 cell cycle. In addition, this chapter explains the cross-talk between BEX2 and the NF-κB, c-Jun/JNK and ErbB2 pathways and provides a summary of my studies on the association of BEX2 expression with clinical and pathological features in breast tumors. Chapter three presents my publications on a cross-talk between AR and ErbB2-ERK signaling in estrogen receptor-negative breast cancer. Furthermore, this chapter summarizes *in vitro* and *in vivo* studies on the combined inhibition of AR and ErbB2-ERK signaling as a therapeutic strategy in molecular apocrine breast cancer. In addition, this chapter discusses the molecular functions of the Prolactin-Induced Protein, an established target gene of AR, in invasion, cell cycle and adhesion across different subtypes of breast cancer. Chapter four details my studies on the transcriptional network and novel target genes of AR in breast cancer. This chapter includes findings on AR-mediated regulation of Factor VII and the discovery of a novel protein, SRARP (C1orf64), as an AR coregulator with corepressor functions in breast cancer. In conclusion, chapter five provides a discussion on the impact of my publications in advancing different fields of cancer research and the emerging clinical applications of the presented studies in breast cancer.

CHAPTER 1: GENOMIC STUDIES IN BREAST CANCER

1.1 Methodology for gene expression microarray

I started my research on gene expression microarrays as a postdoctoral fellow at the University of Cambridge in October 2002. The aim of this project was to conduct gene expression profiling in human breast tumors. At the time, gene expression profiling was a relatively new technology and the methodologies used in this field were not adequately optimized. Therefore, I carried out studies in optimization of RNA purification and labelling techniques for expression microarray applications and published the results (1, 2).

Purification of amplified RNA (aRNA) is a key step in preparing labelled probes for expression microarrays. Generation of aRNA for expression microarrays is performed by global amplification using *in vitro* transcription, which involves multiple purification steps. A major concern during these steps is the exclusion of transcripts based on size, leading to selection bias in subsequent expression microarray analysis (1). In view of this, I investigated various purification methods to identify the best approach for each step in the generation of aRNA using indirect labelling (1). The identified purification protocol provides good yield, purity, coupling efficiency and preservation of different-sized transcripts. This process also generates labelled targets for microarray hybridization with an optimal coefficient of repeatability (1).

Another source of variability in expression microarray analysis is the quantity of fluorescent dye used per experiment. At the time of this study, there was insufficient data to determine the optimal labelled dye requirements for microarray applications. Therefore, I addressed this question and demonstrated that the quantity of Cy3 dye affects expression microarray results performed on tumor specimens (2). Notably, this

study showed that signal-to-noise ratios and coefficients of variation are significantly improved by increasing Cy3 levels in indirect aRNA labelling. Therefore, optimal dye levels reduce variability and improve reliability of expression microarray experiments.

Data presented in (1) and (2) were critical for subsequent expression microarray studies and the discovery of BEX2 (Brain Expressed X-Linked 2) as a novel gene in breast cancer. Notably, BEX2 has a small transcript size with an open reading frame (ORF) of 460 bp and a 160 aa protein product. Consequently, the application of optimized purification techniques that preserved small transcripts contributed to the identification of the BEX2 gene in breast cancer.

1.2 Gene expression profiling in primary breast tumors

Following optimization of the microarray methodology, gene expression profiling in 135 primary (early-stage) breast tumors was undertaken using a cohort of frozen tumor samples. The long follow-up and conservative use of adjuvant therapy made this cohort ideal for a prognostic study. The results of expression microarray analysis and a robust prognostic signature obtained from this study were published (3). RNA amplification, purification and labelling methods were carried out as previously described (1, 2). Oligonucleotide microarrays containing 22,575 features were used for this study (Agilent Human 1A 60-mer Oligo Microarray) and the expression data was deposited at MIAMExpress at the EBI with accession number E-UCon-1. I performed paired dye-reversal hybridizations in 127 samples and one-way hybridization in eight samples owing to limited amount of RNA, in addition to a second set of 45 biological replicate hybridizations in 24 samples (3).

The expression analysis in the above cohort identified a prognostic signature of 70 genes. This signature was further validated in two independent external data sets, which

collectively contained a total of 715 patients (3). A common prognostic module of 29 genes that was associated with survival in both our cohort and the two external data sets was identified. Interestingly, the 29 overlapping genes were consistently overexpressed in poor prognosis tumors and these signature genes were connected to cancer development functions such as oncogenesis, cell cycle progression and chromosome dynamics (3). It is notable that the gene expression data obtained from this study was applied to identify novel genes with a differential expression pattern in breast cancer, resulting in the identification of the BEX2 gene in this disease.

1.3 Discovery of BEX2 in breast cancer using expression profiling

Genes that have an important biological function in cancer generally show a differential expression pattern with a relatively higher expression in a subset of tumors. It is notable that in expression microarray analysis of breast tumors, a two-channel platform (Cy3 and Cy5 dyes) was utilized, which included a dye-reversal design with a subset of tumor samples applied as the reference pool (3). Therefore, this study provided an ideal dataset to investigate genes having a differential expression pattern in breast cancer that can act as classifiers.

To achieve this aim, further analysis of expression microarray data identified BEX2 as the gene with the highest frequency of significant log2 ratios ($p < 0.05$) across the studied cohort of breast tumors (4). This finding indicated that the expression of BEX2 varied significantly across most tumor samples. Furthermore, cancer outlier profile analysis (COPA), an established methodology to identify biologically relevant genes (Tomlins et al., 2005), showed that BEX2 and its close homologue, BEX1, ranked among the top genes with an outlier profile in the dataset (4). In addition, BEX2 appeared to have a relatively higher expression in estrogen receptor-positive (ER+) tumors.

The mutual presence of BEX2 and BEX1 in COPA analysis was striking and led to further investigation of their expression profiles. BEX2 expression relative to the common reference pool separated the tumors into two significantly different ($p < 1 \times 10^{-5}$) groups with BEX2 overexpression and underexpression detected in and 20 and 115 samples, respectively. BEX1 expression also varied significantly between the two groups ($p < 1 \times 10^{-5}$), reflecting a strong correlation between BEX2 and BEX1 expression patterns (Pearson correlation coefficient (CC) = 0.94). In addition, quantitative real time-PCR (qRT-PCR) was performed on a subset of breast tumors and demonstrated a strong correlation between the microarray and qRT-PCR data for BEX2 expression (4).

The next question asked was, if there were a group of genes that significantly correlated with BEX1 and BEX2 (BEX+ cluster). A correlation analysis and Monte-Carlo simulation identified 35 genes with significant correlations to the expression of BEX1 and BEX2 in breast tumors (4). Furthermore, to identify genes that could differentiate between tumors expressing high levels of BEX genes from the remainder of cases, the samples were divided into two groups based on the expression ratios of BEX2 (BEX+ log2 > 0; BEX- log2 < 0). Supervised analysis using a class prediction algorithm generated an optimal classifier consisting of 37 genes with a correct classification rate of 85% (4).

To validate the findings, further supervised analysis using t-test/ANOVA and significance analysis of microarray (SAM) was conducted. The results showed that approximately 2/3 of the genes overlap among all supervised methods and with the BEX+ cluster. These findings indicate a distinct expression signature differentiating BEX+ and BEX- samples, which is reproducible using different methods of analysis. To gain further insights into the potential functional significance of the BEX-signature, the Ingenuity Pathways Analysis software was employed and a significant enrichment of the BEX-signature for developmental, apoptosis and cell proliferation functions was found (4).

It is notable that BEX2 belongs to a family of genes, including BEX1, NGFRAP1 (alias BEX3), BEXL1 (alias BEX4) and NGFRAP1L1 (alias BEX5), which at the time of our study were mainly identified as developmental genes expressed in brain tissue (Alvarez et al., 2005; Brown and Kay, 1999). Therefore, our genomic studies for the first time revealed BEX2 as a novel classifier in breast cancer that is associated with a gene-signature enriched for key cellular functions. In view of the gene ontology categories associated with the BEX-gene signature, the role of BEX2 in apoptosis and cell cycle was investigated in my subsequent studies.

CHAPTER 2: MOLECULAR FUNCTIONS OF BEX2 IN BREAST CANCER

2.1 Molecular functions of BEX2 in apoptosis and cell cycle

To investigate the molecular functions of BEX2, the transcriptional regulation of BEX2 in breast cancer cells was studied (4). It is notable that BEX2 is not amplified in breast cancer and is not expressed in normal breast tissue. Therefore, BEX2 overexpression is likely to be a result of transcriptional activation of this gene in breast tumors (4). Importantly, I found that both ceramide and Nerve Growth Factor (NGF) can markedly induce BEX2 expression in breast cancer cells (4). Ceramide is a mediator of apoptosis generated in response to apoptotic stimuli and may be studied by the treatment of cells using a synthetic ceramide analogue (C2), (El Yazidi-Belkoura et al., 2003). NGF is a neurotrophic factor that interacts with its receptor p75NTR and protects breast cancer cells against C2-mediated apoptosis through the activation of NF-κB pathway (Descamps et al., 2001).

In view of these findings, it was hypothesized that BEX2 may have a function in ceramide-mediated apoptosis. This hypothesis was tested in breast cancer cell lines MCF-7 (ER+) and MDA-MB-231 (ER-) using BEX2 overexpression and siRNA-silencing experiments (4). Notably, BEX2 overexpression rescued cancer cells from C2-induced apoptosis (4). In addition, siRNA-silencing studies revealed that BEX2 expression is required for NGF-mediated anti-apoptotic response. Furthermore, BEX2 protects MCF-7 cells against apoptosis induced by the anti-estrogen drug tamoxifen (4). In this process, tamoxifen-mediated apoptosis is significantly reduced by both NGF and BEX2 overexpression. Treatment with the NF-κB inhibitor SN50 and BEX2-silencing remove the protective effects of BEX2 overexpression and NGF, respectively. These data indicate that activation of the NGF/BEX2 pathway inhibits tamoxifen-induced apoptosis

and this effect of BEX2 appears to be mediated through the NF-κB pathway. Furthermore, BEX2 expression regulates p50 NF-κB DNA binding and p65 NF-κB phosphorylation, suggesting that BEX2 functions upstream of NF-κB in modulating apoptosis (4).

I continued my studies on the molecular functions of BEX2 in my independent research laboratory as a clinician-scientist faculty at The University of Queensland from December 2007 to October 2012. Having shown that BEX2 is a part of the NGF/NF-κB pathway in breast cancer, I next characterized the therapeutic implications of this pathway (5). NGF effects on apoptosis and cell proliferation are mediated through p75NTR and p140TrkA receptors, respectively (Descamps et al., 2001). Notably, it was demonstrated that a p75NTR inhibitor Pep5, p140TrkA inhibitor K-252a and NF-κB inhibitor BAY11-7085 have pro-apoptotic and anti-proliferative activities in breast cancer cells (5). A synergy in combining NGF receptor inhibitors with the conventional breast cancer treatments tamoxifen and taxol was also demonstrated (5). These findings suggest that the inhibition of the NGF/NF-κB pathway is a potential treatment strategy in breast cancer. Therefore, as a part of the NGF/NF-κB pathway, BEX2 may present a novel therapeutic target in this disease.

My laboratory next carried out a comprehensive study to investigate a functional role for BEX2 in cell growth and survival using MCF-7 (ER+), T-47D (ER+), and MDA-MB-231 (ER-) breast cancer cell lines (6). The effect of BEX2-siRNA silencing on the induction of apoptosis was examined using an Annexin V-flow cytometry assay (6). Notably, there was a significant increase at the baseline level of apoptosis following BEX2-silencing in all three cell lines (6). This effect was most prominent in MDA-MB-231 cells, which demonstrated a 5-fold increase in the level of apoptosis. Furthermore, BEX2-silencing significantly sensitized all the cell lines to the pro-apoptotic effects of ceramide, NF-κB

inhibitor BAY11-7085, chemotherapeutic agent adriamycin and mitochondrial pro-apoptotic agent staurosporine (STS), (6). Therefore, BEX2 expression protects breast cancer cells against apoptosis (see Figure 1).

Subsequent experiments examined whether the effect of BEX2 on apoptosis is mediated through the mitochondrial intrinsic pathway (6). This was first studied by a mitochondrial permeability transition (MPT)-based apoptosis assay that detects a change in the mitochondrial transmembrane potential by fluorescent staining. MPT assays showed BEX2 overexpression has a protective effect against the induction of mitochondrial apoptosis by ceramide and STS (6). These findings were further confirmed by detecting a cytosolic release of cytochrome c from mitochondria following BEX2-siRNA silencing. To investigate an underlying mechanism for the protective effect of BEX2 against mitochondrial apoptosis, a potential role for BEX2 in the regulation of Bcl-2 protein family was also explored. Importantly, BEX2-silencing modulated the phosphorylation and expression of key Bcl-2 family members in the direction of promoting a pro-apoptotic imbalance in breast cancer cells (6). For instance, BEX2-silencing led to a significant reduction in the phosphorylation level of anti-apoptotic protein Bcl-2 and an increase in the expression of pro-apoptotic genes BAK1 and PUMA.

Moreover, the effect of BEX2 expression on cell cycle was examined using flow cytometry analysis (6). These studies revealed a significant increase in the G1 cell population after BEX2-silencing in all three breast cancer lines suggesting a G1 cell cycle arrest, which was associated with a reduction in cyclin D1 expression and an increase in p21 levels. Notably, cyclin D1 and p21 are key proteins that promote and inhibit the G1 progression, respectively (Kim et al., 2000; Wang et al., 2007). Therefore, it appears that BEX2 expression is required for the G1 phase progression in breast cancer cells (see Figure 1).

Finally, to elucidate a biochemical mechanism for the effect of BEX2 on protein phosphorylation, the possibility of BEX2 regulation of PP2A (protein phosphatase 2A) activity was investigated. This hypothesis was suggested due to ceramide being a modulator of PP2A (Ruvolo et al., 1999) and our findings demonstrating a strong interplay between BEX2 and ceramide signaling. Notably, BEX2-silencing significantly increased PP2A activity by 1.5-fold, indicating that BEX2 expression regulates PP2A phosphatase activity in breast cancer cells (6). Therefore, the regulation of PP2A provides a possible underlying mechanism for BEX2-mediated cellular effects in breast cancer (see Figure 1).

Altogether these studies suggest that BEX2 expression promotes cell growth and survival in breast cancer cells and has a pro-oncogenic function in this disease.

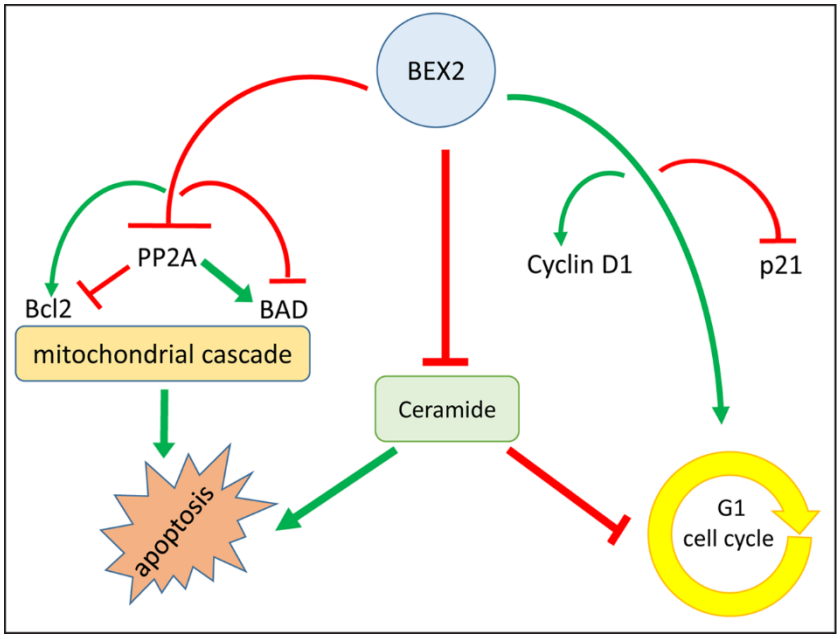

Figure 1. A schematic diagram of BEX2 interactions with the mitochondrial apoptosis pathway and G1 cell cycle. Green arrow: stimulatory effect; Red crossed-line: inhibitory effect.

2.2 BEX2 cross-talk with NF-κB, c-Jun/JNK and ErbB2 signaling

To better understand the molecular functions of BEX2, my laboratory next investigated the underlying mechanisms for BEX2 transcriptional activation and the signaling cross-talk involved in this process and published the results (7, 8). Bioinformatics analysis of the BEX2 promoter identified six AP-1/c-Jun and three NF-κB/RelA putative binding sites (7). Next, luciferase reporter assays were performed to test the effect of candidate transcription factors on the activation of BEX2 promoter and direct DNA binding was assessed using Chromatin Immunoprecipiation (ChIP) assay. These experiments demonstrated that BEX2 is a target gene for c-Jun and p65/RelA in breast cancer cells and in order to bind and activate the BEX2 promoter, c-Jun and p65 require phosphorylation at Ser63 and Ser468 sites, respectively (7).

The fact that BEX2 transcription is strongly regulated by c-Jun and p65 raises the question whether BEX2 has a role in the cellular activities mediated by these proteins. Furthermore, we have previously demonstrated that BEX2 expression is necessary for NGF-mediated activation of NF-κB (4). To investigate the effect of BEX2 on p65 activation we assessed the nuclear localization of p65 following BEX2 overexpression in breast cancer cells (7). Notably, BEX2 overexpression increased the percentage of nuclear-only p65 staining by 3-fold and this effect was completely reversed with the addition of IκBα phosphorylation inhibitor BAY11-7082. These data suggest that BEX2 overexpression increases the nuclear localization of p65 in a process that requires IκBα phosphorylation. The observed effect of BEX2 on p65 nuclear transport can be explained by the fact that BEX2 expression regulates the phosphorylation of p65 and IκBα. In this respect, BEX2-silencing results in a reduction of phospho-p65/total-p65 and phospho-IκBα/total-IκBα ratios by 0.65- and 0.6-fold, respectively and also inhibits ceramide-mediated induction of p65 DNA binding (7).

To further investigate cross-regulation between BEX2 and its transcription factors, the effect of BEX2 expression on the phosphorylation of c-Jun (Ser63) was subsequently examined in breast cancer cells (7). Notably, BEX2-silencing led to a marked reduction in c-Jun phosphorylation by 3 to 8-fold as well as a 2.4-fold decrease in the activity of c-Jun-N-terminal Kinase (JNK). These findings indicate that BEX2 expression is necessary for c-Jun phosphorylation and JNK kinase activity. Moreover, using the stably transfected c-Jun lines generated in MCF-7 cells (c-Jun+), it was shown that BEX2 expression is required for c-Jun-mediated induction of cyclin D1 and cell proliferation. In addition, BEX2-silencing increased PP2A activity in c-Jun+ stable lines, presenting further evidence for BEX2-mediated regulation of PP2A (7).

Collectively, these results suggest that BEX2 has a functional cross-talk with c-Jun and p65/RelA in breast cancer (see Figure 2). In this feedback process, BEX2 is a target gene for c-Jun and p65/RelA. BEX2, in turn, regulates the phosphorylation of c-Jun, p65 and IκBα in addition to JNK kinase activity. Furthermore, BEX2-mediated inhibition of PP2A provides a possible mechanism for these functional effects by regulating the phosphorylation and activation of proteins in the NF-κB and c-Jun/JNK pathways.

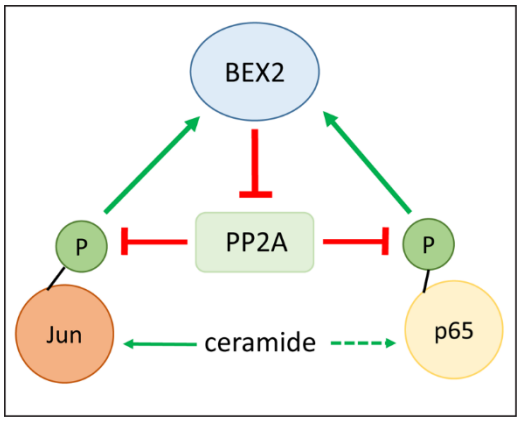

Figure 2. BEX2 interplay with c-Jun, p65 and PP2A. Green arrow: stimulatory effect; Red crossed-line: inhibitory effect. P: phosphorylated protein.

A feedback loop between BEX2 and ErbB2 is another biologically significant interplay that was revealed as a result of these studies (8). Following the generation and validation of a rabbit polyclonal BEX2 antibody, BEX2 protein expression was examined on a large cohort of breast tumors using immunohistochemistry (IHC) staining. Notably, BEX2 positive tumors identified a subset of breast cancers with the overexpression of ErbB2 (ErbB2+) and phosphorylated c-Jun (ph-Jun+) proteins. This led me to investigate an underlying mechanism for the observed association between BEX2 expression and ErbB2+/ph-Jun+ status in breast cancer (8). Functional studies revealed that ErbB2 overexpression leads to an induction of c-Jun and ph-Jun expression in breast cancer cells. Furthermore, ErbB2-overexpressing cells demonstrated a 2-fold increase in BEX2 protein expression compared to the control and this effect was abrogated by the co-transfection of TAM67 (dominant negative c-Jun) or c-Jun siRNA-silencing in ErbB2-transfected cells (8). These findings indicate that overexpression of ErbB2 induces BEX2 expression mediated through the activation of c-Jun signaling (see Figure 3).

To further examine an association between the overexpression of BEX2 with that of ErbB2 and c-Jun, we studied the possibility of ErbB2 induction by c-Jun and BEX2 in breast cancer cells. Transient and stable overexpression of c-Jun in MCF-7 line (c-Jun+) were carried out using a c-Jun plasmid and both models showed an induction of ErbB2 expression by c-Jun compared to the control cells (8). Next, the effect of BEX2 on c-Jun-mediated induction of ErbB2 was determined using a co-transfection of c-Jun and BEX2 vectors in MCF-7 cells. This showed a 1.5-fold higher ErbB2 level in BEX2+/c-Jun+ cells compared to that of control-vector/c-Jun+ line. Furthermore, the overexpression of BEX2 alone led to a 2.5-fold increase in ErbB2 expression and this effect was reversed with the co-transfection of a TAM67 construct. In addition, ChIP studies revealed that the effect of BEX2 on ErbB2 expression is mediated through an increase in c-Jun binding to

the ErbB2 promoter following BEX2 overexpression. Therefore, BEX2 overexpression induces ErbB2 transcription and enhances c-Jun-mediated induction of ErbB2 in breast cancer cells.

Finally, the contribution of BEX2 to the level of ErbB2 overexpression was examined in an ErbB2-amplified HCC-1954 cell line. Notably, there was a 25% reduction in ErbB2 protein level in HCC-1954 cells following BEX2-siRNA silencing compared to the control-siRNA cells (8).

Taken together, these findings suggest a functional interplay involving ErbB2, c-Jun and BEX2 in breast cancer (see Figure 3). In this process, c-Jun is a transcriptional activator of both BEX2 and ErbB2. Furthermore, ErbB2 overexpression results in c-Jun–mediated induction of BEX2 expression and BEX2, in turn, enhances c-Jun–mediated transcriptional activation of ErbB2. These findings strongly suggest that BEX2 has a significant biological role in the ErbB2–c-Jun signaling pathway.

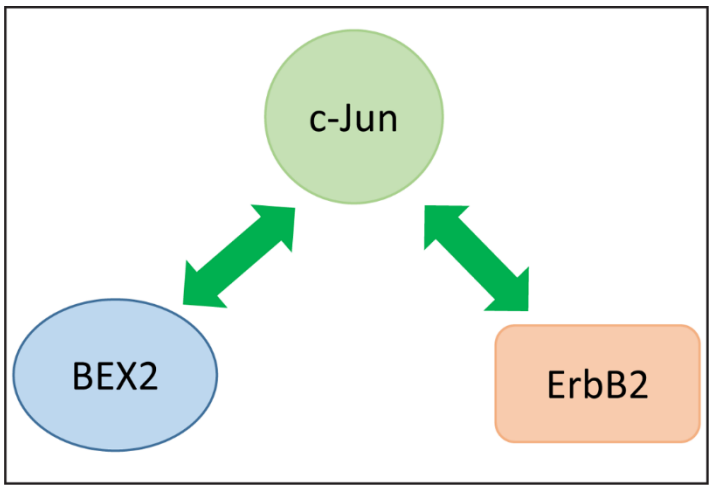

Figure 3. Functional interplay between BEX2, c-Jun and ErbB2 (Green arrow: stimulatory effect).

2.3 Association of BEX2 expression with clinical and pathological features

I have conducted several studies using breast tumor specimens to better understand the molecular functions of BEX2 and to identify clinical and pathological features associated with BEX2 expression in breast cancer. Initially, these studies were conducted by assessing BEX2 mRNA expression on frozen breast tumors (4, 6). Moreover, once my group successfully generated and validated a rabbit BEX2 antibody, BEX2 protein expression using IHC staining on breast tumors was investigated (7, 8).

First, we posed the question whether BEX2 expression influences the outcome of patients treated with tamoxifen (4). The cases were divided into BEX2 overexpression (BEX2+) or BEX2 under-expression (BEX2-) using one of two criteria: (a) log2 ratios normalized to the median value on microarrays showing at least 2-fold expression difference, and (b) qRT-PCR of samples with < 2-fold expression difference by microarray analysis showing ΔCT< -1 standard deviation (SD) or ΔCT> +1 SD of the mean. Survival analysis of these cases showed a better disease-free interval in patients with BEX2+ (n= 16; 95% confidence interval: 121–161 months) *vs.* those with BEX2- tumors (n= 14; 95% confidence interval: 63–123 months). The better prognosis in tamoxifen-treated BEX2+ patients may be due to NF-κB inactivation in these cases (4).

Another application of tumor samples was to validate our *in vitro* findings regarding cross-talk between BEX2 and the signaling pathways of mitochondrial apoptosis and NF-κB (6). To achieve this, the association of BEX2 expression with Bcl-2 and p65 NF-κB levels was studied in breast tumors using qRT-PCR. A cohort of 24 frozen breast tumors was divided into BEX2+ and BEX2- samples with at least 3-fold BEX2 expression difference between these two groups. Next, Bcl-2 mRNA expression and the percentage of tumor cells with p65 nuclear protein were measured using qRT-PCR and

immunofluorescence, respectively. Notably, Bcl-2 expression and p65-nuclear staining were significantly higher in the BEX2+ compared to BEX2- samples by 3.7- and 2-fold, respectively ($p < 0.01$). Therefore, BEX2 overexpression is associated with higher levels of Bcl-2 expression and p65 NF-κB activation, which supports signaling cross-talk demonstrated in our *in vitro* studies (6).

In addition, my laboratory examined a potential correlation between BEX2 and c-Jun protein levels in breast tumors using IHC staining (7). For this purpose, we first optimized and validated a rabbit polyclonal BEX2 antibody for IHC applications and subsequently studied the correlation between BEX2 and c-Jun protein levels on 35 primary breast tumors. Notably, there was a strong correlation between the percentage of cells that showed BEX2 and c-Jun staining in this cohort with a Pearson CC of 0.8 ($p< 0.01$). This strong positive correlation further supports a functional interplay between BEX2 and c-Jun in breast cancer (7).

Following the generation of a BEX2 antibody, we examined the expression pattern of BEX2 protein in a TMA cohort of 225 breast tissue specimens using IHC (8). Notably, BEX2 was not expressed in normal breast tissue. In contrast, approximately 50% of breast cancers and 7% of benign breast lesions had BEX2 expression. Furthermore, BEX2+ samples showed a diffuse cytoplasmic staining for this protein and there was also nuclear staining in tumors that contained a high level of BEX2 (8).

Subsequently, comparison of the expression of biomarkers between BEX2+ and BEX2- breast tumors found an approximately 2-fold higher prevalence of ErbB2 overexpression and amplification in BEX2+ compared to BEX2- breast tumors (8). These findings indicate a significant association between the overexpression of ErbB2 and BEX2 in breast tumors. Furthermore, the percentage of BEX2+ tumors was approximately 4-fold

higher in ErbB2+/ph-Jun+ tumors compared to that of ErbB2-/ph-Jun- samples (83% vs. 22%, $p < 0.01$). A receiver operating characteristic (ROC) analysis demonstrated that BEX2+ staining is a strong predictor for the ErbB2+/ph-Jun+ status in breast tumors with an area under curve of 0.81 ($p< 0.01$). Of note, BEX2+/ErbB2+/ph-Jun+ tumors accounted for about 15% of all cases and had a relatively higher p53 and a lower ER expression. These data suggest that BEX2+ staining identifies a distinct subset of breast tumors with the overexpression of ErbB2 and ph-Jun (8).

CHAPTER 3: MOLECULAR FUNCTIONS OF AR IN BREAST CANCER

3.1 Signaling cross-talk between AR and the ErbB2/ERK pathway

ER-negative (ER-) breast tumors constitute around 30% of all cases and in contrast to ER-positive (ER+) tumors, where ER signaling has a key biological role, ER- cancer is heterogeneous and there is limited knowledge about the pathogenesis of this disease. In view of this, I commenced my research on ER- breast cancer during a transition period from the University of Cambridge to my independent research laboratory at the University of Queensland. Notably, I have continued my work on this topic ever since during my faculty position at the University of Queensland (2007 to 2012) and my subsequent positions as an Associate Professor and Group Leader at the University of Iowa (2012 to 2015) and the University of Hawaii (since 2016).

My initial findings on the role of androgen receptor (AR) signaling in ER- tumors emerged from an additional analysis of expression microarray data (3). This analysis, and the related functional data, were published (9). In this study, it was found that 50% of ER- tumors were AR+ and 28% were both AR+ and ErbB2+. Notably, the subtype of ER- tumors with a “steroid-response” signature that includes AR and ErbB2 genes is termed “molecular apocrine” (Farmer et al., 2005). Next, MDA-MB-453 and Sum-190 cell lines were characterized as molecular apocrine to conduct functional experiments (9).

Using molecular apocrine cell lines, a functional cross-talk between the AR and ErbB2 pathways was demonstrated (9). In this process, the stimulation of AR and ErbB2 signaling leads to cross-regulation of steroid-response genes; AR, ErbB2, FOXA1, XBP1, TFF3 and KLK3. Moreover, AR activation by testosterone enhances the proliferation of molecular apocrine cell lines and this effect can be reversed using anti-androgen flutamide and the anti-ErbB2 AG825 (9). Conversely, the growth stimulatory

effect of ErbB2 activation by heregulin can also be inhibited with flutamide, suggesting cross-talk between the AR and ErbB2 pathways affecting cell proliferation. Importantly, there is a synergy between flutamide and AG825 on cell proliferation and apoptosis, indicating a therapeutic advantage in the combined blockage of AR and ErbB2 pathways. In addition, as opposed to the physiological transient phosphorylation of extracellular signal–regulated kinase (ERK1/2) by testosterone, the addition of ErbB2 inhibition leads to persistent phosphorylation of ERK1/2, which negatively regulates the downstream signaling and cell growth. These findings suggest cross-talk between AR and ErbB2 in molecular apocrine cells involving the ERK pathway (9), (see Figure 4).

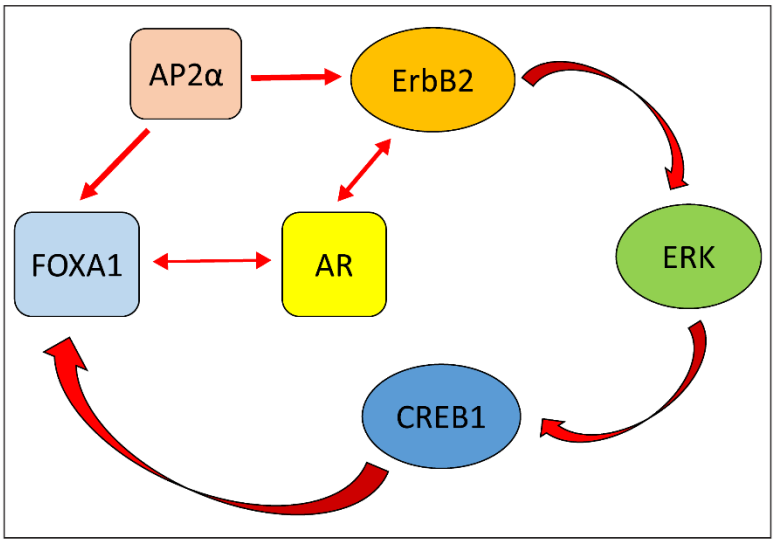

Figure 4. AR and FOXA1 interplay with the ErbB2-ERK pathway (Red arrows depict stimulatory effects).

Furthermore, my group investigated cross-talk between FOXA1 and ErbB2 in molecular apocrine breast cancer (10). FOXA1 is a notable gene in the molecular apocrine signature that has a dual transcriptional regulatory function with the ability to both facilitate and restrict key transcription factors such as AR (Sahu et al., 2011). However,

there has been limited data available regarding the function of FOXA1 in ER- breast cancer.

Using IHC studies on a TMA cohort, it was observed that ER-/ErbB2+ tumors had approximately 2.5-fold higher frequency of FOXA1 overexpression at 72% compared to that of ER-/ErbB2- tumors at 30% (10). This led to identification of a cross-regulation network between FOXA1 and ErbB2 signaling in ER- breast cancer (10). Two possible mechanisms were revealed to explain the association between FOXA1 and ErbB2 overexpression. In one, ErbB2 signaling genes CREB1 and c-Fos regulate FOXA1 transcription, and in the other, AP2α regulates the expression of both FOXA1 and ErbB2 (see Figure 4). Moreover, FOXA1, in turn, regulates the transcription of ErbB2-signaling genes. Importantly, this cross-regulation connects FOXA1 to key signaling elements in ER- breast cancer such as the ERK-CREB1 axis, NF-κB, and AP2α (10).

Collectively, this work (9 and 10) identified functional interplay between AR-ErbB2 and FOXA1-ErbB2 signaling with therapeutic implications in ER- breast cancer (see Figure 4).

3.2 AR as a therapeutic target in ER-negative breast cancer

In parallel to studies leading to the discovery of a signaling cross-talk between AR and the ErbB2-ERK pathway, my group also investigated the therapeutic implications of this cross-talk in ER- breast cancer. First, we identified a synergy between AR and ErbB2 inhibitors in molecular apocrine cells that involved the modulation of ERK phosphorylation (9). In view of this finding, the therapeutic efficacy and signaling effects of combined treatments with AR and ERK inhibitors were investigated in ER-/AR+ breast cancer using *in vitro* and *in vivo* models and with the application of complementary approaches (11, 12). In one study, we examined the effect of AR blocker flutamide

combined with a Cdc25A phosphatase inhibitor PM-20, which persistently phosphorylates ERK leading to the inhibition of ERK signaling (11). In another study, the effect of a mitogen-activated protein kinase kinase (MEK) inhibitor CI-1040 was investigated in combination with flutamide in molecular apocrine cancer (12).

In vitro experiments were conducted with MDA-MB-453, HCC-1954, and HCC-202 cell lines. Drug synergy was assessed by a combination index (CI) method (Zhao et al., 2004). Firstly, cell viability and apoptosis for the combination therapies were measured using MTT and annexin V assays, respectively and secondly the concentrations of each monotherapy that resulted in a level of reduction in cell viability and apoptosis similar to that observed with each of the combination therapies were identified (11, 12). Subsequently, CI values for the combined treatments were calculated as follows: CI = [Ca,x/ICx,a] + [Cb,x/ICx,b]. Ca,x and Cb,x are the concentrations of drug A and drug B used in combination to achieve x% drug effect and ICx,a and ICx,b are the concentrations for single agents to achieve the same effect. A CI less than 1 indicates synergy (11, 12).

For *in vivo* studies, a xenograft mouse model was developed using MDA-MB-453 cells. Flutamide treatment was carried out with 25 mg/60-day slow-release pellets and control group received placebo pellets. PM-20 experiments were performed by daily intraperitoneal (IP) injections, MEK inhibitor treatment was by daily oral gavage of CI-1040, and control groups received only solvents by identical administration.

Based on the results of these studies, synergy between AR and ERK inhibitors was noted in molecular apocrine cancer. In this respect, CI values for flutamide combination with either PM-20 or CI-1040 were synergistic in all three cell lines at four different dose combinations (11, 12). Furthermore, *in vivo* studies demonstrated that the combination

therapies have a significantly higher therapeutic efficacy in reducing tumor growth, cellular proliferation and angiogenesis compared to the monotherapies with these agents (11, 12). In addition, our data suggested that flutamide and CI-1040 synergize in both primary and acquired resistance to ErbB2 inhibitor trastuzumab, the standard targeted treatment for ErbB2+ breast cancer. Importantly, the therapeutic effect of combination therapy in trastuzumab-resistant cells was associated with the abrogation of increased ERK phosphorylation developed in trastuzumab resistance (12).

Moreover, the effect of combination therapy of flutamide with PM-20 on the regulation of ERK signaling targets RSK and Elk-1 was investigated (11). RSK and Elk-1 are activated by ERK through phosphorylation and play a key role in ERK-mediated signal transduction (Cruzalegui et al., 1999; Doehn et al., 2009). There was a significant reduction in the phosphorylation levels of RSK and Elk-1 using different concentrations of the combination therapy and this synergistic effect was most evident at concentrations of PM-20 that monotherapy had marginal, or no effect, on the phosphorylation of these targets (11). These findings demonstrate that the combination of PM-20 and flutamide results in significant inhibition of ERK signal transduction that, in turn, can lead to a marked reduction in cell viability and growth.

In summary, our work (11, 12) demonstrates that AR-ERK signaling is a promising therapeutic target in molecular apocrine breast cancer. Furthermore, the combination therapy with AR and MEK inhibitors may overcome trastuzumab resistance. Therefore, a combination therapy strategy with AR and ERK inhibitors may provide an attractive therapeutic option for ER-/AR+ breast cancer.

3.3 Molecular functions of AR target gene, Prolactin-Induced Protein

The discovery of a cross-talk between AR and the ErbB2-ERK pathway in molecular apocrine subtype provided the opportunity to study other functionally important genes in this disease. In this respect, my group investigated the transcriptional regulation of the top twelve ranked genes in molecular apocrine signature by AR-ERK signaling (13). Modulation of AR and ERK signaling in molecular apocrine cell lines was carried out using flutamide and CI-1040, respectively. Importantly, we found that Prolactin-Induced Protein (PIP) is the most regulated gene by AR-ERK signaling and validated this finding using a mouse xenograft model. In addition, using IHC staining, AR+/ER- tumors showed a markedly higher expression of PIP (57%) compared to AR-/ER- tumors (16%). Next, the mechanism of PIP regulation by AR-ERK signaling was investigated using luciferase reporter and ChIP assays. These studies revealed that PIP is a target gene of the ERK-CREB1 pathway and is induced by AR activation (13).

I have reviewed the literature on PIP in the context of our studies (14). It is known that PIP is one of the most abundant proteins in breast cancer, which has both intracellular expression and extracellular secretion (Clark et al., 1999; Wick et al., 1989). The available data indicate that PIP is a transcriptional target of AR and prolactin-STAT5 signaling (Carsol et al., 2002). From a biochemical standpoint, PIP has aspartyl protease activity that degrades fibronectin (Caputo et al., 2000). Furthermore, the importance of PIP in cell proliferation has been demonstrated by the fact that purified PIP promotes growth of breast cancer cells (Cassoni et al., 1995). Despite this information, the molecular functions of PIP have remained largely unknown.

To study the extracellular function of PIP, we examined the effect of PIP expression on cell invasion using siRNA-silencing in MDA-MB-453 cells (13). Notably, there was a

marked reduction in cell invasion by approximately three-fold following PIP-silencing. It is known that the enzymatic degradation of fibronectin releases fragments that bind to integrin-β1 and activate the intracellular signaling (Hocking et al., 1998). In view of PIP's ability to degrade fibronectin, we hypothesized that PIP may be required for integrin-β1 activation. Therefore, the effect of PIP-silencing on integrin-β1 interactions with integrin-linked kinase 1 (ILK1) and ErbB2 was investigated using co-immunoprecipitation (co-IP) assays. Notably, PIP-silencing led to a 70% to 90% reduction in integrin-β1 binding to ILK1 and a 90% decrease in ErbB2-integrin-β1 interaction. In addition, there was nearly a complete recovery of integrin-β1 binding to ILK1 and ErbB2 following the addition of fibronectin fragments in PIP-silenced cells. Furthermore, PIP expression was necessary for the phosphorylation of integrin-β1 signaling targets ERK-CREB1 and Akt (13). These findings suggest that PIP expression is necessary for the outside-in activation of integrin-β1 mediated through the fragmentation of fibronectin (see Figure 5).

Moreover, IHC staining from a cohort of 210 breast cancers showed that PIP has a high level of expression in 85% of tumors, including luminal A (ER+), luminal B (ER+) and ER-/AR+ subtypes (15). Therefore, we next carried out a comprehensive investigation of PIP molecular functions using siRNA-silencing in a broad group of breast cancer cell lines, analysis of expression microarray data and mass spectrometry (MS). These studies demonstrated that PIP is required for the progression through G1 phase, mitosis and cytokinesis in luminal A, luminal B and molecular apocrine subtypes. In addition, defects in mitotic transition and cytokinesis, following PIP-silencing, are accompanied by an increase in aneuploidy of breast cancer cells. Furthermore, PIP expression is associated with a transcriptional signature enriched with cell cycle genes and it regulates key genes in this process including cyclin D1, cyclin B1, BUB1 and FOXM1 (15).

To identify the molecular mechanisms involved in PIP-mediated cellular functions, we performed MS to identify binding partners for endogenous PIP (15). These studies revealed 156 PIP-interacting proteins. Of note, β-tubulin was established as a top PIP-binding partner in MS and also showed a strong interaction with PIP in co-IP assays (15). To test whether PIP binding to β-tubulin has a regulatory effect on microtubule polymerization, the effect of PIP-silencing on both polymerized and soluble fractions of tubulin was measured. Importantly, the ratio of polymerized to soluble tubulin was markedly reduced following PIP-silencing (15). This finding suggests that PIP may be required for tubulin polymerization in breast cancer (15). Moreover, MS and co-IP studies revealed that PIP interacts with actin-binding proteins including Arp2/3 and is required for the inside-out activation of integrin-β1 mediated through Talin-1 (see Figure 5).

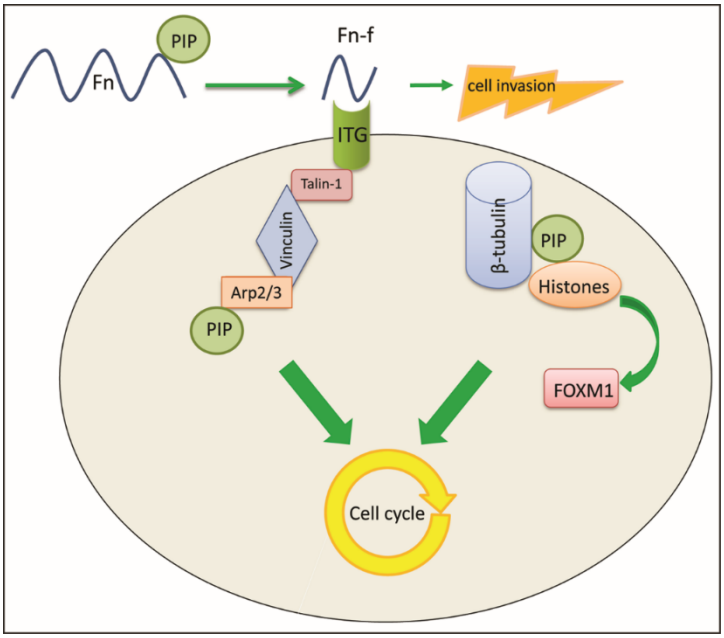

Figure 5. PIP regulation of cell cycle and invasion. The identified extracellular and intracellular interactions of PIP that can regulate cell cycle and invasion are depicted. Fn: fibronectin, Fn-f: fibronectin fragments, ITG: integrin-β1, green arrow: positive regulation. This figure is a modified adaptation (15).

Another subset of PIP-binding proteins as shown by our MS studies are involved in cell adhesion. Therefore, we subsequently examined the effect of PIP on the regulation of cell adhesion using PIP-silencing in breast cancer cells (16). These studies revealed that PIP expression is necessary for cell adhesion depending on the type of adhesion surface and cell line features. For instance, in T-47D and MFM-223 cells, a fibronectin matrix induces the baseline adhesion and reverses PIP silencing-mediated reduction of cell adhesion. However, in BT-474 cells, the baseline adhesion is not induced by fibronectin and PIP-silencing leads to a marked reduction in cell adhesion to both uncoated and fibronectin-coated plates. In an attempt to explain an underlying mechanism for PIP regulation of cell adhesion, we found that PIP expression is necessary for the formation of α-actinin/actin-rich podosomes at the adhesion-sites of breast cancer cells (16).

Collectively, these studies suggest that PIP has a versatile function in breast cancer, resulting from a diverse range of intracellular and extracellular binding partners (see Figure 5) and, as a consequence of these interactions, PIP can regulate key processes involved in cell cycle such as integrin-β1 activation and microtubule polymerization. Importantly, our publications on this topic provide a strong rationale for the tantalizing possibility of PIP as a therapeutic target in breast cancer.

CHAPTER 4: DISCOVERY OF NOVEL AR TARGET GENES

4.1 Investigation of AR transcriptional network in breast cancer

The next step in my research on the molecular functions of AR was aimed at the identification of an AR-transcriptional network in breast cancer. These studies were conducted and published during my faculty positions at the University of Iowa and University of Hawaii (17, 18). Traditionally, the studies of AR target genes have been limited to a few cell lines and largely concentrated on the application of ChIP-sequencing (Ni et al., 2011; Robinson et al., 2011). As a result, key target genes and coregulators of AR in breast cancer have remained unknown. To address this shortcoming, an integrative genomic analysis was undertaken to discover AR co-expressed genes in breast cancer (17, 18). The study of AR co-expressed genes in large genomic datasets presents an innovative approach to investigate AR molecular functions and novel target genes. This approach ensures that different breast cancer subtypes are appropriately represented and that identified genes are highly correlated with AR expression in a large non-biased model of this disease.

As a first step, I analyzed an expression microarray data from a cohort of 52 breast cancer cell lines by Neve *et al.* (Neve et al., 2006). Correlation of AR expression with that of every gene in the dataset was calculated (17). This analysis resulted in identification of three hundred AR co-expressed genes that had a $|CC|$ of > 0.5 with AR expression. Moreover, functional annotation of the AR co-expressed genes revealed a total of thirteen gene ontology functions that significantly associated with the AR-transcriptional network and had fold enrichments ranging from 1.5 to 33. It is notable that the majority of these functional groups were related to cell cycle or cellular metabolism (17). The dataset was further analyzed to find genes that have a highly correlated expression pattern with AR. For this purpose, a cutoff $|CC|$ of > 0.6 with AR expression

was adopted to identify this gene set; termed “AR-gene signature”. This analysis revealed 35 genes in the dataset, excluding the AR gene itself, which correlated with AR expression at this cutoff. The set of genes with the strongest positive correlation with AR included F7 (gene encoding FVII) and a group of transcriptional regulators such as NFATC4 and SPDEF (17).

Next, the genomic analysis was expanded to refine the AR-gene signature, using an additional dataset from 50 breast cancer cell lines (Kao et al., 2009). Notably, out of a total of 65 unique cell lines in the combined analysis, 37 cell lines were in common and 28 cell lines varied between the two cohorts (18). First, a list of highly co-expressed genes with AR was identified in each dataset using a cutoff of $|CC|$ of ≥ 0.6 and then a combined “AR-gene signature” was generated by compiling the subset of AR co-expressed genes in both cohorts. The combined AR-gene signature included a total of 98 genes, besides AR itself. Importantly, a novel gene, C1orf64 showed the highest positive correlation with AR expression in this signature at a CC value of 0.737 (18). Furthermore, there were only three other genes in the AR-signature that had a positive $CC > 0.7$ namely, SIDT1, F7 and PATZ1. In addition, approximately 75% of the signature genes, which had an overlap between the two datasets, significantly correlated with AR expression in both cohorts. This indicates a high level of reproducibility in signature genes despite the differences between the two platforms (18).

The combined AR-gene signature was subsequently examined for possible AR-binding sites using publically available AR ChIP-sequencing (ChIP-seq) data in breast cancer cells. To achieve this, a list of AR-signature genes with a detectable AR-binding signal in their 5 kb promoter regions were identified. Next, an average AR ChIP-seq signal was calculated for each promoter binding detected in the signature genes. A total of 31 genes in the AR-signature, including C1orf64 and F7, had detectable AR ChIP-seq signals (18).

It is notable that 30 out of 57 positively co-expressed genes in the AR-signature had ChIP-seq-detected AR binding. These data suggest a positive coexpression pattern with AR in combination with a detectable AR binding in ChIP-seq can potentially predict the transcriptional targets of AR. Importantly, these studies led me to investigate F7 and C1orf64 as novel AR target genes in breast cancer.

4.2 Coagulation Factor VII is an AR target gene

The association between FVII and AR expression was subsequently investigated using IHC in a TMA cohort of 209 breast tumors (17). These studies revealed that AR staining is higher in tumors with FVII scores of 2 to 3 compared to those with lower FVII scores (0 to 1) at 52% and 36%, respectively ($p<0.01$). In addition, a ROC analysis was applied to predict FVII expression scores based on the positive or negative status of AR staining in breast tumors. Notably, AR status could reliably predict FVII expression with an area under the curve of 0.664 (17). These findings suggest a positive association between the protein levels of FVII and AR in breast tumors.

In view of the highly correlated expression pattern between AR and FVII, the possibility of AR-mediated transcriptional regulation of F7 was examined in breast cancer cells. In this respect, AR+/ER+ cell line T-47D and AR+/ER- line MFM-223 were tested to assess the effect of AR activation by dihydrotestosterone (DHT) on F7 transcription (17). Notably, DHT treatment significantly increased F7 expression by 1.5 to 2.5-fold ($p< 0.01$), indicating that AR activation results in an induction of F7 transcription in breast cancer cells. Moreover, in agreement with the F7 transcription data, FVII was induced at the protein level following AR activation and there were several putative AR binding sites in the F7 promoter region from bioinformatics analysis (17). To investigate whether F7 is a direct AR target gene, ChIP assays were performed using an AR antibody and primer-sets in the 2 kb region of F7 promoter. Importantly, ChIP data suggested that AR binds

to the F7 promoter in a region close to the ATG start codon. This suggests AR as a direct transcriptional activator of F7 in breast cancer (17).

Furthermore, the effect of AR-mediated induction of FVII on the activity of this protein was assessed by measuring the conversion of FX to FXa (17). T-47D cell line was treated with either DHT or vehicle control for 48h followed by cell lysate extraction and measurement of Tissue Factor (TF)-FVII activity. To assess the contribution of endogenous FVII to the overall TF activity, assays were carried out for each experiment in the absence (lysate) or presence of exogenous FVII (lysate + FVII). Next, the ratio of TF-FVII activity in each cell lysate was calculated relative to that of lysate + exogenous FVII and compared between the DHT-treated and control groups. Notably, AR activation significantly increased the ratio of endogenous TF-FVII activity/total TF activity from 0.57 in the control group to 0.80 in the DHT-treated cells ($p < 0.03$). This indicates that AR activation induces FVII activity in breast cancer cells (17).

Taken together, we can propose a model for the regulation of ectopic FVII expression by AR in breast cancer cells. In this model, AR induces FVII expression and leads to an increased activity of FVIIa/TF complex that, in turn, converts FX to FXa. This activation of coagulation FVII by AR provides a novel mechanism for the transcriptional regulation of ectopic FVII expression in cancer. Importantly, this study implicates a potential role for AR signaling in the pathobiology of thromboembolic events and the regulation of FVII/TF signaling pathway in breast cancer (17).

4.3 Discovery of a novel protein, C1orf64 (SRARP), as an AR coregulator

Genomic data suggested a novel gene, C1orf64, is highly co-expressed with AR in breast cancer and there is a detectable AR binding to the C1orf64 promoter by ChIP-seq. Therefore, I investigated C1orf64 as an AR target gene in breast cancer (18). The

transcriptional studies were carried out in breast cancer cell lines T-47D (ER+/AR+) and MFM-223 (ER-/AR+). Notably, DHT-induced AR activation led to a marked reduction in C1orf64 expression by 2 to 10-fold compared to controls using qRT-PCR ($p< 0.01$). Conversely, AR-siRNA silencing increased C1orf64 expression by 3 to 4-fold in breast cancer cells ($p< 0.01$). Furthermore, DHT treatment of T-47D and MFM-223 cells markedly reduced C1orf64 protein levels by 3.3 to 6.7-fold using western blot analysis (18). These findings suggest that AR activation has a profound repressive effect on C1orf64 expression in AR+ breast cancer cells.

To examine whether C1orf64 is a direct AR target gene, ChIP assays were used to assess the promoter region of this gene in T-47D and MFM-223 cell lines (18). A total of six primer sets were employed for the 2 kb promoter region of C1orf64 to generate amplicons using qRT-PCR. ChIP assays revealed a significant enrichment by 80-fold ($p< 0.01$) for AR binding to an amplicon located at -1648 to -1527 bp region on the C1orf64 promoter. This strongly suggests that C1orf64 is a direct transcriptional target of AR in breast cancer.

To further investigate the association between C1orf64 and AR in breast cancer, C1orf64 co-expressed genes were assessed in primary and metastatic breast tumors (18). In this respect, two large cohorts were analyzed using *ONCOMINE* database (Bos et al., 2009; Grossman et al., 2016; Rhodes et al., 2004). Co-expression analysis in these datasets showed that AR is highly correlated with C1orf64 expression in both primary and metastatic breast tumors with CC values of 0.667 and 0.573, respectively (18). Furthermore, the association studies with clinical and pathological features in breast cancer demonstrated that C1orf64 expression is relatively higher in lobular histology, lower grade and ER+ tumors (18).

Moreover, functional studies identified an interplay between AR and C1orf64 in breast cancer (18). In this interplay, AR activation directly represses C1orf64 transcription and C1orf64, in turn, interacts with AR as a corepressor and negatively regulates AR-mediated induction of PIP expression (see Figure 6). This negative regulation of AR is further supported by the fact that C1orf64 has a profound repressive effect on the AR luciferase reporter activity in breast cancer cells (18). In addition, the corepressor effect of C1orf64 results in a reduction of AR binding to the PIP promoter using ChIP assays. Another aspect of this interplay involves cross-talk between the AR and ER signaling in ER+ cells. In this respect, AR activation abrogates ER-mediated induction of progesterone receptor (PGR). In contrast, C1orf64 is necessary for PGR expression; therefore, the repression of C1orf64 by AR has an inhibitory effect on the positive regulatory function of C1orf64 on ER activity (see Figure 6).

Of note, a combination of bioinformatics and biochemical studies revealed that C1orf64 is a phosphothreonine protein and an interacting partner of 14-3-3 protein in breast cancer cells (18). In addition, C1orf64 is predicted to be phosphorylated by Casein Kinase 1 at a T152 site adjacent to a 14-3-3 interacting motif (18). 14-3-3 is a chaperone, and scaffolding protein, that binds serine/threonine-phosphorylated residues and regulates key proteins involved in various cellular processes such as intracellular signaling and gene transcription (Mackintosh, 2004; Zilliacus et al., 2001). However, there was no direct interaction between the endogenous 14-3-3 and AR in breast cancer cells (18). In view of these points, a plausible mechanism for the C1orf64 coregulatory effect on AR may involve a competition with 14-3-3 binding to this receptor or acting as a scaffolding protein in a complex that includes 14-3-3 and AR (see Figure 6).

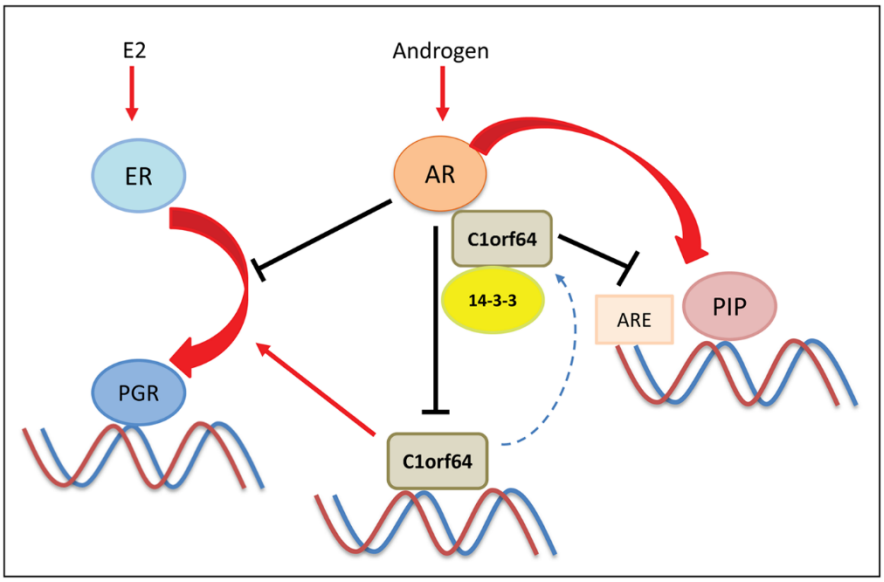

Figure 6. The negative interplay between AR and C1orf64 in breast cancer (18). Red arrows denote a stimulatory effect and black lines indicate a repressive function. ARE: androgen response element.

In summary, AR activation directly represses C1orf64 transcription in breast cancer cells and C1orf64, in turn, interacts with AR as a corepressor and negatively regulates AR-mediated transcriptional activities (see Figure 6). Importantly, C1orf64 interacts with the chaperone protein 14-3-3, presenting an underlying mechanism for the molecular functions of C1orf64 by modulation of the chaperone activity of this key protein. Therefore, there is an interplay between C1orf64 and AR with significance in the biology of breast cancer. Following the discovery of C1orf64 as a novel AR coregulator, this was communicated to HUGO Gene Nomenclature Committee (HGNC) for updating the nomenclature of this gene. As a result, my proposal for updating the nomenclature of C1orf64 to “Steroid Receptor Associated and Regulated Protein” (SRARP) was accepted by HGNC in July 2017. Moreover, my publication has been featured by the Universal Protein (UniProt) Consortium (http://www.uniprot.org) with respect to the biological function and nomenclature of SRARP.

CHAPTER 5: IMPACT AND APPLICATIONS

In this commentary, I have discussed my publications on the molecular functions of AR and BEX2 in breast cancer. It is notable that these publications have made significant contributions to the fields of cancer genomics, cancer biology and experimental therapeutics with broad applications in breast cancer and other malignancies.

Methodologies outlined in chapter 1 have documented applications for RNA purification and expression microarray analysis in various fields such as cancer genomics, nucleic acid amplification and developmental biology (Coussens et al., 2007; Lawson et al., 2010; Suslov and Steindler, 2005). Furthermore, the presented microarray dataset in breast tumors has been highly cited in genomic and cancer biology studies over the past decade (3). For example this study has been applied in meta-analysis expression profiling of breast cancer (Gyorffy and Schafer, 2009), discovery of molecular mechanism for C/EBPδ activity (Pawar et al., 2010), and identification of predictive biomarkers for distant recurrence in breast cancer (Aleskandarany et al., 2015).

Furthermore, my publications on the pro-oncogenic function of BEX2 in breast cancer and the pathways involved in this process opened up a new field of research and, in particular, impacted the study of BEX2 in brain tumors. It is notable that an initial study had demonstrated a possible tumor suppressor function for BEX2 in glioblastoma, an aggressive brain cancer (Foltz et al., 2006). However, our findings in breast cancer impacted the subsequent studies on BEX2 that suggested a pro-oncogenic function for this gene in brain malignancies. In this respect, data by Le Mercier *et al.* and Zhou *et al.* showed that BEX2 promotes cell migration and invasion in oligodendroglioma and glioblastoma brain tumor cells (Le Mercier et al., 2009; Zhou et al., 2012b). In addition, there is evidence suggesting that β-catenin is involved in BEX2-mediated induction of cell invasion and migration of glioma cells (Nie et al., 2015).

In fact, publications (4-8) have contributed to the subsequent studies into the role of BEX2 in apoptosis and proliferation of glioma cells and have revealed that many of our findings regarding BEX2 function are reproducible in glioma (Meng et al., 2014; Seznec et al., 2010; Zhou et al., 2012a). For instance, siRNA-silencing of BEX1 and BEX2 sensitizes glioma cells to apoptosis mediated by a dominant-positive variant of p53 (Seznec et al., 2010). In addition, BEX2 expression protects glioma cells against apoptosis mediated through the JNK pathway and is required for glioma cell proliferation through the NF-κB p65 (Meng et al., 2014; Zhou et al., 2012a). It is notable that, besides glioma, our findings on BEX2 function have been validated in other cancers. Most recently, it has been shown that BEX2 promotes cell proliferation through the JNK/c-Jun pathway and regulates JNK/c-Jun phosphorylation in colorectal cancer (Hu et al., 2017). In addition, another group has shown that BEX2 expression is required for cell proliferation and Hepatitis B Virus-mediated development of hepatocellular carcinoma (Huang et al., 2017). Therefore, our BEX2 work has significantly contributed to the advancement of our understanding about this gene in malignancies.

Moreover, our initial articles on AR signaling have made an original contribution to the characterization of molecular apocrine subtype and have been widely cited in studies about the pattern of AR expression in ER- breast cancer (Collins et al., 2011; Niemeier et al., 2009; Park et al., 2010). In addition to AR, our findings on the steroid-response signature have impacted the research conducted on other genes in this signature. For instance, publications on FOXA1 interplays with AR and ErbB2 signaling (9, 10), have contributed to the subsequent studies on FOXA1 function in basal subtype and ErbB2-induced breast cancers (Bernardo et al., 2013; Hodgson et al., 2013). Another example is the TFF3 gene, a member of the steroid-response signature (9), which was later shown

to be involved in breast cancer development and metastasis (Kannan et al., 2010; Pandey et al., 2014).

Importantly, the publications on AR cross-talk with ErbB2 signaling have impacted the emerging application of AR as a therapeutic target in the management of ER-/AR+ breast cancer (9, 11, and 12). In this respect, a phase II trial of bicalutamide has shown therapeutic efficacy and minimal toxicity in metastatic AR+/ER- breast cancer patients (Gucalp et al., 2013). Additional trials are ongoing that will address the clinical applications of AR inhibition in breast cancer. For example, there is currently an ongoing study to assess the efficacy and safety of AR inhibitor enzalutamide with ErbB2 inhibitor trastuzumab in patients with ErbB2+/AR+ metastatic or locally advanced breast cancer (NCT02091960). However, there is a need for further combination therapy trials to examine our preclinical findings that demonstrated a synergy between the inhibitors of AR and ERK signaling in ER-/AR+ breast cancer.

Finally, our work on AR target genes have contributed to a number of research fields. In this respect, we demonstrated that PIP regulates invasion, cell adhesion and cell cycle (13-16). Besides their impact on breast cancer research, these publications have been cited in other fields of research such as studies on PIP-mediated regulation of immune response (Li et al., 2015; Xiang et al., 2016). In addition, the identification of novel AR target genes provides a major advancement in the field of AR signaling with broader implications (17, 18). For instance, following the discovery of F7 as an AR co-expressed and target gene in breast cancer, a similar coexpression pattern between AR and F7 has been reported in prostate cancer (Shahbazi et al., 2016). Most recently, the publication of C1orf64 as a novel AR coregulator and discussions with HGNC resulted in updating the nomenclature of this gene to SRARP and opened a novel field of research in cancer biology.

APPENDIX A: List of Publications (Numbered-Style)

(*, denotes that Naderi A is the corresponding author)

1. **Naderi A**, Ahmed AA, Barbosa-Morais NL, Aparicio S, Brenton JD, Caldas C. Expression microarray reproducibility is improved by optimizing purification steps in RNA amplification and labeling, BMC Genomics, 2004; 5(1): 9. PMID: 15005798, PMCID: PMC343272.

https://www.ncbi.nlm.nih.gov/pubmed/15005798

2. ***Naderi A**, Ahmed AA, Wang Y., Brenton JD, Caldas C. Optimal amounts of fluorescent dye improve expression microarray results in tumor specimens. Molecular Biotechnology, 2005; 30(2):151-4. PMID: 15920285.

https://www.ncbi.nlm.nih.gov/pubmed/15920285

3. **Naderi A**, Teschendorff AE, Barbosa-Morais NL, Pinder SE, Green AR, Powe DG, Robertson JF, Aparicio S, Ellis IO, Brenton LD, Caldas C. A gene-signature to predict survival in breast cancer across independent data sets. Oncogene, 2007; 26(10):1507-16. PMID: 16936776

https://www.ncbi.nlm.nih.gov/pubmed/16936776

4. ***Naderi A**, Teschendorff AE, Biegel J, Cariati M, Ellis IO, Brenton JD, Caldas C. BEX2 is overexpressed in a subset of primary breast cancers and mediates nerve growth factor /nuclear factor-κB inhibition of apoptosis in breast cancer cell lines. Cancer Research, 2007; 67(14): 6725-36. PMID: 17638883.

https://www.ncbi.nlm.nih.gov/pubmed/17638883

5. ***Naderi A** and Hughes-Davies L. Nerve growth factor/nuclear factor-κB pathway as a therapeutic target in breast cancer. Journal of Cancer Research and Clinical Oncology, 2009; 135 (2):211-6. PMID: 18716795.

https://www.ncbi.nlm.nih.gov/pubmed/18716795

6. ***Naderi A**, Liu J, and Bennett IC. BEX2 regulates mitochondrial apoptosis and G1 cell cycle in breast cancer. International Journal of Cancer, 2010; 126(7):1596-1610. PMID: 19711341.

https://www.ncbi.nlm.nih.gov/pubmed/19711341

7. ***Naderi A**, Liu J, and Hughes-Davies L. BEX2 has a functional interplay with c-Jun/JNK and p65/RelA in breast cancer. Molecular Cancer, 2010; 9(1):111. PMID: 20482821; PMCID: PMC2881879.

https://www.ncbi.nlm.nih.gov/pubmed/20482821

8. ***Naderi A**, Liu J, Francis GD. A feedback loop between BEX2 and ErbB2 mediated by c-Jun signaling in breast cancer. International Journal of Cancer, 2012; 130(1): 71-82. PMID: 21384344.

https://www.ncbi.nlm.nih.gov/pubmed/21384344

9. ***Naderi A** and Hughes-Davies L. A functionally significant cross-talk between androgen receptor and ErbB2 pathways in estrogen receptor negative breast cancer. Neoplasia, 2008; 10(6):542-8. PMID: 18516291; PMCID: PMC2386539.

https://www.ncbi.nlm.nih.gov/pubmed/18516291

10. ***Naderi A**, Meyer M, Dowhan DH. Cross-regulation between FOXA1 and ErbB2 signaling in estrogen receptor-negative breast cancer. Neoplasia, 2012; 14(4): 283-96. PMID: 22577344.

https://www.ncbi.nlm.nih.gov/pubmed/22577344

11.***Naderi A** and Liu J. Inhibition of androgen receptor and Cdc25A phosphatase as a combination targeted therapy in molecular apocrine breast cancer. Cancer Letters, 2010; 298 (1): 74-87. PMID: 20605569.

https://www.ncbi.nlm.nih.gov/pubmed/20605569

12. ***Naderi A**, Chia K, Liu J. Synergy between inhibitors of androgen receptor and MEK has therapeutic implications in estrogen receptor-negative breast cancer. Breast Cancer Research, 2011; 13(2): R36. PMID: 21457548; PMCID: PMC3219199.

https://www.ncbi.nlm.nih.gov/pubmed/21457548

13. ***Naderi A** and Meyer M. Prolactin-Induced Protein mediates cell invasion and regulates Integrin signaling in estrogen receptor-negative breast cancer. Breast Cancer Research, 2012; 14(4): R111. PMID: 22817771; PMCID: PMC3680918.

https://www.ncbi.nlm.nih.gov/pubmed/22817771

14. ***Naderi A**. Prolactin-Induced Protein in Breast Cancer. Advances in Experimental Medicine and Biology, 2015; 846: 189-200. PMID: 25472539.

https://www.ncbi.nlm.nih.gov/pubmed/25472539

15. ***Naderi A** and Vanneste M. Prolactin-Induced Protein is required for Cell Cycle Progression in Breast Cancer. Neoplasia, 2014; 16(4): 329-42.e14. PMID: 24862759.

https://www.ncbi.nlm.nih.gov/pubmed/24862759

16. Vanneste M. and ***Naderi A**. Prolactin-Induced Protein regulates cell adhesion in breast cancer. Biochemical and Biophysical Research Communications, 2015; 468(4): 850-6. PMID: 26585492.

https://www.ncbi.nlm.nih.gov/pubmed/26585492

17. ***Naderi A**. Coagulation factor VII is regulated by androgen receptor in breast cancer. Experimental Cell Research, 2015; 331(1): 239-50. PMID: 25447311; PMCID: PMC4302010.

https://www.ncbi.nlm.nih.gov/pubmed/25447311

18. ***Naderi A.** C1orf64 is a novel androgen receptor target gene and coregulator that interacts with 14-3-3 protein in breast cancer. Oncotarget, 2017; 8(34): 57907-57933. PMID: 28915724, PMCID: PMC5593696.

https://www.ncbi.nlm.nih.gov/pubmed/28915724

APPENDIX B: Bibliography from other sources (Author-Date Style)

Aleskandarany, M.A., Soria, D., Green, A.R., Nolan, C., Diez-Rodriguez, M., Ellis, I.O., and Rakha, E.A. (2015). Markers of progression in early-stage invasive breast cancer: a predictive immunohistochemical panel algorithm for distant recurrence risk stratification. Breast Cancer Res Treat *151*, 325-333.

Alvarez, E., Zhou, W., Witta, S.E., and Freed, C.R. (2005). Characterization of the Bex gene family in humans, mice, and rats. Gene *357*, 18-28.

Bernardo, G.M., Bebek, G., Ginther, C.L., Sizemore, S.T., Lozada, K.L., Miedler, J.D., Anderson, L.A., Godwin, A.K., Abdul-Karim, F.W., Slamon, D.J.*, et al.* (2013). FOXA1 represses the molecular phenotype of basal breast cancer cells. Oncogene *32*, 554-563.

Bos, P.D., Zhang, X.H., Nadal, C., Shu, W., Gomis, R.R., Nguyen, D.X., Minn, A.J., van de Vijver, M.J., Gerald, W.L., Foekens, J.A.*, et al.* (2009). Genes that mediate breast cancer metastasis to the brain. Nature *459*, 1005-1009.

Brown, A.L., and Kay, G.F. (1999). Bex1, a gene with increased expression in parthenogenetic embryos, is a member of a novel gene family on the mouse X chromosome. . Hum Mol Genet *8*, 611-619.

Caputo, E., Manco, G., Mandrich, L., and Guardiola, J. (2000). A novel aspartyl proteinase from apocrine epithelia and breast tumors. The Journal of Biological Chemistry *275*, 7935-7941.

Carsol, J.L., Gingras, S., and Simard, J. (2002). Synergistic action of prolactin (PRL) and androgen on PRL-inducible protein gene expression in human breast cancer cells: a unique model for functional cooperation between signal transducer and activator of transcription-5 and androgen receptor. Molecular Endocrinology *16*, 1696-1710.

Cassoni, P., Sapino, A., Haagensen, D.E., Naldoni, C., and Bussolati, G. (1995). Mitogenic effect of the 15-kDa gross cystic disease fluid protein (GCDFP-15) on breast-cancer cell lines and on immortal mammary cells. Int J Cancer *60*, 216-220.

Clark, J.W., Snell, L., Shiu, R.P., Orr, F.W., Maitre, N., Vary, C.P., Cole, D.J., and Watson, P.H. (1999). The potential role for prolactin-inducible protein (PIP) as a marker of human breast cancer micrometastasis. Br J Cancer *81*, 1002-1008.

Collins, L.C., Cole, K.S., Marotti, J.D., Hu, R., Schnitt, S.J., and Tamimi, R.M. (2011). Androgen receptor expression in breast cancer in relation to molecular phenotype: results from the Nurses' Health Study. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc *24*, 924-931.

Coussens, A.K., Wilkinson, C.R., Hughes, I.P., Morris, C.P., van Daal, A., Anderson, P.J., and Powell, B.C. (2007). Unravelling the molecular control of calvarial suture fusion in children with craniosynostosis. BMC Genomics *8*, 458.

Cruzalegui, F.H., Cano, E., and Treisman, R. (1999). ERK activation induces phosphorylation of Elk-1 at multiple S/T-P motifs to high stoichiometry. Oncogene *18*, 7948-7957.

Descamps, S., Toillon, R.A., Adriaenssens, E., Pawlowski, V., Cool, S.M., Nurcombe, V., Le Bourhis, X., Boilly, B., Peyrat, J.P., and Hondermarck, H. (2001). Nerve growth factor stimulates proliferation and survival of human breast cancer cells through two distinct signaling pathways. J Biol Chem *276*, 17864-17870.

Doehn, U., Hauge, C., Frank, S.R., Jensen, C.J., Duda, K., Neilsen, J.V., Cohen, M.S., Johansen, J.V., Winther, B.R., Lund, L.R.*, et al.* (2009). RSK is a principal effector of the RAS-ERK pathway for eliciting a coordinate promotile/invasive gene program and phenotype in epithelial cells. Mol Cell *35*, 511-522.

El Yazidi-Belkoura, I., Adriaenssens, E., Dolle, L., Descamps, S., and Hondermarck, H. (2003). Tumor necrosis factor receptor-associated death domain protein is involved in

the neurotrophin receptor-mediated antiapoptotic activity of nerve growth factor in breast cancer cells. J Biol Chem *278*, 16952-16956.

Farmer, P., Bonnefoi, H., Becette, V., Tubiana-Hulin, M., Fumoleau, P., Larsimont, D., Macgrogan, G., Bergh, J., Cameron, D., Goldstein, D.*, et al.* (2005). Identification of molecular apocrine breast tumours by microarray analysis. Oncogene *24*, 4660-4671.

Foltz, G., Ryu, G.Y., Yoon, J.G., Nelson, T., Fashey, J., Frakes, A., Lee, H., Field, L., Zander, K., Sibenaller, Z.*, et al.* (2006). Genome-wide analysis of epigenetic silencing identifies BEX1 and BEX2 as candidate tumor suppressor genes in malignant glioma. Cancer Res. *66*, 6665-6674.

Grossman, R.L., Heath, A.P., Ferretti, V., Varmus, H.E., Lowy, D.R., Kibbe, W.A., and Staudt, L.M. (2016). Toward a Shared Vision for Cancer Genomic Data. N Engl J Med *375*, 1109-1112.

Gucalp, A., Tolaney, S., Isakoff, S.J., Ingle, J.N., Liu, M.C., Carey, L.A., Blackwell, K., Rugo, H., Nabell, L., Forero, A.*, et al.* (2013). Phase II trial of bicalutamide in patients with androgen receptor-positive, estrogen receptor-negative metastatic Breast Cancer. Clin Cancer Res *19*, 5505-5512.

Gyorffy, B., and Schafer, R. (2009). Meta-analysis of gene expression profiles related to relapse-free survival in 1,079 breast cancer patients. Breast Cancer Res Treat *118*, 433-441.

Hocking, D.C., Sottile, J., and McKeown-Longo, P.J. (1998). Activation of distinct alpha5beta1-mediated signaling pathways by fibronectin's cell adhesion and matrix assembly domains. The Journal of Cell Biology *141*, 241-253.

Hodgson, M.C., Vanostran, G., Alghamdi, S., Poppiti, R.J., Agoulnik, A.I., and Agoulnik, I.U. (2013). Reduced androgen receptor expression accelerates the onset of ERBB2 induced breast tumors in female mice. PLoS One *8*, e60455.

Hu, Y., Xiao, Q., Chen, H., He, J., Tan, Y., Liu, Y., Wang, Z., Yang, Q., Shen, X., Huang, Y.*, et al.* (2017). BEX2 promotes tumor proliferation in colorectal cancer. Int J Biol Sci *13*, 286-294.

Huang, F., Cai, P., Wang, Y., Zhou, X., Chen, H., Liao, W., Mao, Y., Zha, X., Zhang, H., and Hu, Z. (2017). Up-regulation of brain-expressed X-linked 2 is critical for hepatitis B virus X protein-induced hepatocellular carcinoma development. Oncotarget *8*, 65789-65799.

Kannan, N., Kang, J., Kong, X., Tang, J., Perry, J.K., Mohankumar, K.M., Miller, L.D., Liu, E.T., Mertani, H.C., Zhu, T.*, et al.* (2010). Trefoil factor 3 is oncogenic and mediates anti-estrogen resistance in human mammary carcinoma. Neoplasia *12*, 1041-1053.

Kao, J., Salari, K., Bocanegra, M., Choi, Y.L., Girard, L., Gandhi, J., Kwei, K.A., Hernandez-Boussard, T., Wang, P., Gazdar, A.F.*, et al.* (2009). Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. PLoS One *4*, e6146.

Kim, W.H., Kang, K.H., Kim, M.Y., and Choi, K.H. (2000). Induction of p53-independent p21 during ceramide-induced G1 arrest in human hepatocarcinoma cells. Biochem. Cell Biol. *78*, 127-135.

Lawson, M.H., Rassl, D.M., Cummings, N.M., Russell, R., Morjaria, J.B., Brenton, J.D., Murphy, G., and Rintoul, R.C. (2010). Tissue banking of diagnostic lung cancer biopsies for extraction of high quality RNA. J Thorac Oncol *5*, 956-963.

Le Mercier, M., Fortin, S., Mathieu, V., Roland, I., Spiegl-Kreinecker, S., Haibe-Kains, B., Bontempi, G., Decaestecker, C., Berger, W., Lefranc, F.*, et al.* (2009). Galectin 1 proangiogenic and promigratory effects in the Hs683 oligodendroglioma model are partly mediated through the control of BEX2 expression. Neoplasia *11*, 485-496.

Li, J., Liu, D., Mou, Z., Ihedioha, O.C., Blanchard, A., Jia, P., Myal, Y., and Uzonna, J.E. (2015). Deficiency of prolactin-inducible protein leads to impaired Th1 immune response and susceptibility to Leishmania major in mice. Eur J Immunol *45*, 1082-1091.

Mackintosh, C. (2004). Dynamic interactions between 14-3-3 proteins and phosphoproteins regulate diverse cellular processes. Biochem J *381*, 329-342.

Meng, Q., Zhi, T., Chao, Y., Nie, E., Xu, X., Shi, Q., Hua, L., Wang, L., Zhan, W., Wang, Y.*, et al.* (2014). Bex2 controls proliferation of human glioblastoma cells through NF-kappaB signaling pathway. J Mol Neurosci *53*, 262-270.

Neve, R.M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F.L., Fevr, T., Clark, L., Bayani, N., Coppe, J.P., Tong, F.*, et al.* (2006). A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer cell *10*, 515-527.

Ni, M., Chen, Y., Lim, E., Wimberly, H., Bailey, S.T., Imai, Y., Rimm, D.L., Liu, X.S., and Brown, M. (2011). Targeting androgen receptor in estrogen receptor-negative breast cancer. Cancer Cell *20*, 119-131.

Nie, E., Zhang, X., Xie, S., Shi, Q., Hu, J., Meng, Q., Zhou, X., and Yu, R. (2015). Beta-catenin is involved in Bex2 down-regulation induced glioma cell invasion/migration inhibition. Biochem Biophys Res Commun *456*, 494-499.

Niemeier, L.A., Dabbas, D.J., Beriwal, S., Striebel, J.M., and Bhargava, R. (2009). Androgen receptor in breast cancer: expression in estrogen receptor-positive tumors and in estrogen receptor-negative tumors with apocrine differentiation. Mod Pathol. *23*, 205-212.

Pandey, V., Wu, Z.S., Zhang, M., Li, R., Zhang, J., Zhu, T., and Lobie, P.E. (2014). Trefoil factor 3 promotes metastatic seeding and predicts poor survival outcome of patients with mammary carcinoma. Breast Cancer Res *16*, 429.

Park, S., Koo, J., Park, H.S., Kim, J.H., Choi, S.Y., Lee, J.H., Park, B.W., and Lee, K.S. (2010). Expression of androgen receptors in primary breast cancer. Ann Oncol *21*, 488-492.

Pawar, S.A., Sarkar, T.R., Balamurugan, K., Sharan, S., Wang, J., Zhang, Y., Dowdy, S.F., Huang, A.M., and Sterneck, E. (2010). C/EBP{delta} targets cyclin D1 for proteasome-mediated degradation via induction of CDC27/APC3 expression. Proc Natl Acad Sci U S A *107*, 9210-9215.

Rhodes, D.R., Yu, J., Shanker, K., Deshpande, N., Varambally, R., Ghosh, D., Barrette, T., Pandey, A., and Chinnaiyan, A.M. (2004). ONCOMINE: a cancer microarray database and integrated data-mining platform. Neoplasia *6*, 1-6.

Robinson, J.L., Macarthur, S., Ross-Innes, C.S., Tilley, W.D., Neal, D.E., Mills, I.G., and Carroll, J.S. (2011). Androgen receptor driven transcription in molecular apocrine breast cancer is mediated by FoxA1. The EMBO journal *30*, 3019-3027.

Ruvolo, P.P., Deng, X., Ito, T., Carr, B.K., and May, W.S. (1999). Ceramide induces Bcl2 dephosphorylation via a mechanism involving mitochondrial PP2A. J Biol Chem *274*, 20296-20300.

Sahu, B., Laakso, M., Ovaska, K., Mirtti, T., Lundin, J., Rannikko, A., Sankila, A., Turunen, J.P., Lundin, M., Konsti, J.*, et al.* (2011). Dual role of FoxA1 in androgen receptor binding to chromatin, androgen signalling and prostate cancer. The EMBO journal *30*, 3962-3976.

Seznec, J., Weit, S., and Naumann, U. (2010). Gene expression profile in a glioma cell line resistant to cell death induced by the chimeric tumor suppressor-1 (CTS-1), a dominant-positive variant of p53--the role of NFkappaB. Carcinogenesis *31*, 411-418.

Shahbazi, S., Khorasani, M., and Mahdian, R. (2016). Gene expression profile of FVII and AR in primary prostate cancer. Cancer Biomark *17*, 353-358.

Suslov, O., and Steindler, D.A. (2005). PCR inhibition by reverse transcriptase leads to an overestimation of amplification efficiency. Nucleic Acids Res *33*, e181.

Tomlins, S.A., Rhodes, D.R., Perner, S., Dhanasekaran, S.M., Mehra, R., Sun, X.W., Varambally, S., Cao, X., Tchinda, J., Kuefer, R.*, et al.* (2005). Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science *310*, 644-648.

Wang, J., Lv, X.W., Shi, J.P., and Hu, X.S. (2007). Mechanisms involved in cermaide-induced cell cycle arresit in human hepatocarcinoma cells. Gastroenterol. *13*.

Wick, M.R., Lillemoe, T.J., Copland, G.T., Swanson, P.E., Manivel, J.C., and Kiang, D.T. (1989). Gross cystic disease fluid protein-15 as a marker for breast cancer: immunohistochemical analysis of 690 human neoplasms and comparison with alpha-lactalbumin. Hum Pathol *20*, 281-287.

Xiang, R., Oddy, V.H., Archibald, A.L., Vercoe, P.E., and Dalrymple, B.P. (2016). Epithelial, metabolic and innate immunity transcriptomic signatures differentiating the rumen from other sheep and mammalian gastrointestinal tract tissues. PeerJ *4*, e1762.

Zhao, L., Wientjes, M.G., and Au, J.L.S. (2004). Evaluation of Combination Chemotherapy: Integration of Nonlinear Regression, Curve Shift, Isobologram, and Combination Index Analyses. Clin Cancer Res. *10*, 7994-8004.

Zhou, X., Meng, Q., Xu, X., Zhi, T., Shi, Q., Wang, Y., and Yu, R. (2012a). Bex2 regulates cell proliferation and apoptosis in malignant glioma cells via the c-Jun NH2-terminal kinase pathway. Biochem Biophys Res Commun *427*, 574-580.

Zhou, X., Xu, X., Meng, Q., Hu, J., Zhi, T., Shi, Q., and Yu, R. (2012b). Bex2 is Critical for Migration and Invasion in Malignant Glioma Cells. J Mol Neurosci.

Zilliacus, J., Holter, E., Wakui, H., Tazawa, H., Treuter, E., and Gustafsson, J.A. (2001). Regulation of glucocorticoid receptor activity by 14--3-3-dependent intracellular relocalization of the corepressor RIP140. Mol Endocrinol *15*, 501-511.

APPENDIX C: Abbreviations (In alphabetical order)

aa: amino acids

ANOVA: analysis of variance

AR: androgen receptor

ARE: androgen response element

aRNA: amplified RNA

BEX1: Brain Expressed X-Linked 1

BEX2: Brain Expressed X-Linked 2

bp: base pair

CC: correlation coefficient

$|CC|$: absolute correlation coefficient

ChIP: Chromatin Immunoprecipiation

ChIP-seq: ChIP-sequencing

CI: combination index

Co-IP: co-immunoprecipitation

COPA: cancer outlier profile analysis

ΔCT: Δ Cycle Threshold value for qRT-PCR

DHT: Dihydrotestosterone

E2: estradiol

ER: estrogen receptor

ERK: extracellular-signal-regulated kinase

HGNC: HUGO Gene Nomenclature Committee

IHC: immunohistochemistry

MEK: mitogen-activated protein kinase kinase

MS: mass spectrometry

NF-κB: Nuclear Factor-κB

NGF: Nerve Growth Factor

ORF: open reading frame

PGR: progesterone receptor gene

PIP: Prolactin-Induced Protein

PP2A: protein phosphatase 2A

qRT-PCR: quantitative real time-polymerase chain reaction

ROC: receiver operating characteristic

SD: standard deviation

SRARP: Steroid Receptor Associated and Regulated Protein

STS: staurosporine

TF: Tissue Factor

TMA: tissue microarray

APPENDIX D: Declarations

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

Please note that I am the first and/or the senior/corresponding author in all of my submitted publications for PhD by Publication. For publications (5 to 18), all the studies were carried out by my independent research activities. In this respect, I am both the first and senior/corresponding author in the following manuscripts: (5 to 15), (17) and (18). In these publications, I have conceived the studies, obtained funding, carried out the vast majority of experiments (over 90%), analyzed the data, drafted the manuscripts and carried out all the required revisions. Notably, I am the sole author for publications (14), (17) and (18). For publication (16), I am the senior/corresponding author and the first author was my former post-doctoral fellow. In this publication, I conceived the study, obtained funding, personally carried out some of the experiments including the required revisions, supervised my staff, analyzed the data and drafted the manuscripts.

For publication (4), I am the first and co-corresponding author. I designed the experiments, carried out the vast majority of experiments (over 90%), performed a significant part of the data analysis, drafted the manuscript and carried out the revisions. For publications (1), (2), and (3), I am the first author and also the corresponding author in manuscript (2). In these publications, I carried out all of the experiments, contributed to the data analysis, drafted the manuscripts and carried out the revisions.

Word count and a list of figures:

Word count for the commentary: 9,243 words

APPENDIX E

PORTFOLIO OF PUBLICATIONS

FORM UPR16

Research Ethics Review Checklist

Please include this completed form as an appendix to your thesis (see the Research Degrees Operational Handbook for more information

UNIVERSITY OF PORTSMOUTH

Postgraduate Research Student (PGRS) Information				**Student ID:**	869611
PGRS Name:	Ali Naderi				
Department:	Pharmacy and Biomedical Sciences	**First Supervisor:**	Dr. Jeremy Mills		
Start Date: (or progression date for Prof Doc students)	**02/10/2017**				
Study Mode and Route:	Part-time ☒	MPhil ☐	MD ☐		
	Full-time ☐	PhD ☒	Professional Doctorate ☐		

Title of Thesis:	Molecular Functions of the Androgen Receptor and BEX2 in Breast Cancer
Thesis Word Count: (excluding ancillary data)	9,243

If you are unsure about any of the following, please contact the local representative on your Faculty Ethics Committee for advice. Please note that it is your responsibility to follow the University's Ethics Policy and any relevant University, academic or professional guidelines in the conduct of your study

Although the Ethics Committee may have given your study a favourable opinion, the final responsibility for the ethical conduct of this work lies with the researcher(s).

UKRIO Finished Research Checklist:
(If you would like to know more about the checklist, please see your Faculty or Departmental Ethics Committee rep or see the online version of the full checklist at: http://www.ukrio.org/what-we-do/code-of-practice-for-research/)

a) Have all of your research and findings been reported accurately, honestly and within a reasonable time frame?	YES ☒ NO ☐
b) Have all contributions to knowledge been acknowledged?	YES ☒ NO ☐
c) Have you complied with all agreements relating to intellectual property, publication and authorship?	YES ☒ NO ☐
d) Has your research data been retained in a secure and accessible form and will it remain so for the required duration?	YES ☒ NO ☐
e) Does your research comply with all legal, ethical, and contractual requirements?	YES ☒ NO ☐

Candidate Statement:

I have considered the ethical dimensions of the above named research project, and have successfully obtained the necessary ethical approval(s)

Ethical review number(s) from Faculty Ethics Committee (or from NRES/SCREC):	

If you have *not* submitted your work for ethical review, and/or you have answered 'No' to one or more of questions a) to e), please explain below why this is so:

This is a PhD by Publication and is based on previously published work completed following ethics guidelines

Signed *(PGRS)*:	A. Naderi	**Date:** 09/07/2018