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The role of orphan nuclear receptor DAX-1 (NR0B1) in human breast cancer cells: expression, proliferation and metastasis

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The role of orphan nuclear receptor DAX-1 (NR0B1) in human
breast cancer cells: expression, proliferation and metastasis

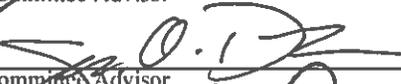
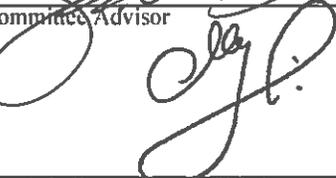
By

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*Thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science in Biology*

College of Arts and Sciences
University of San Francisco
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Abstract

The orphan nuclear hormone receptor DAX-1 (Dosage Sensitive Sex Reversal, Adrenal Hypoplasia Congenita on the X Chromosome, gene 1) plays an important role in the development of adrenal and gonadal tissues and functions as a global negative-regulator of steroidogenesis. In addition, it is known to be involved in several diseases including some cancers. Herein, we describe our examination of the role of DAX-1 in breast cancer, specifically its influence on proliferation and metastasis and its expression during progressive stages of disease. In an effort to understand how DAX-1 influences breast cancer cell proliferation and metastasis, we used MCF7 breast cancer cells and MCF10A normal breast cells and manipulated their DAX-1 expression to increase DAX-1 expression by adenovirus infection in MCF7 cells, or knockdown expression of DAX-1 through the use of RNAi in MCF10A cells. We found a trend toward increased cell proliferation when DAX-1 expression was knocked down, and decreased proliferation when DAX-1 is overexpressed. In addition, we looked at the influence of DAX-1 on breast cancer cell proliferation when the estrogen receptor α (ER α) activity is inhibited by the antagonist, Fulvestrant. To gain a better understanding of the transcriptional role of DAX-1 in breast cancer, we utilized PCR arrays to analyze changes in gene expression in the presence of DAX-1. We identified several genes with roles in breast cancer, estrogen receptor signaling and metastasis whose expression was significantly influenced by overexpression of DAX-1 in MCF7 cells. To investigate expression of DAX-1 through progressive stages of disease we utilized IHC and bioinformatics techniques. We found DAX-1 to be expressed more frequently and at higher levels at earlier stages of breast cancers and at very low levels regardless of stage in hormone receptor-positive (ER and PR) patients. Through these studies, we hypothesize that DAX-1 has the potential to be utilized clinically as biomarker for predicting disease progression and for tailoring more personalized treatment plans. There may even be a role for DAX-1 as a possible therapeutic for later stage or hormone receptor-positive patients.

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List of Abbreviations

AF-1:	Activation function 1
AF-2:	Activation function 2
AHC:	Adrenal hypoplasia congenita
AI:	Aromatase Inhibitor
AMH:	Anti-Mullerian hormone
AR:	Androgen Receptor
AV:	Adenovirus
CAR:	constitutive androstane receptor
Cas9:	CRISPR-associated gene 9
CCND1:	Cyclin D1 gene
CDK:	Cyclin dependent kinase
cDNA:	complementary DNA
ChIP:	Chromatin Immunoprecipitation
CIS:	Carcinoma <i>in situ</i>
CO ₂ :	Carbon Dioxide
CRISPR:	Clustered Regulatory Interspaced Short Palindromic Repeats
DAPI:	4,6-Diamidino-2-phenylindole
DAX-1:	Dosage sensitive sex reversal Adrenal hypoplasia congenital X chromosome, gene 1
DBD:	DNA binding domain
DHT:	5- α -dihydrotestosterone
DMD:	Duchenne muscular dystrophy
DMEM:	Dulbecco's Modified Eagle's Medium

DMSO:	Dimethyl Sulfoxide
DNA:	Deoxyribonucleic acid
DSS:	Dosage-sensitive sex
EDTA:	Ethylenediaminetetraacetic acid
EGFR:	Epidermal growth factor receptor
EdU:	5-ethynyl-2'-deoxyuridine
ER:	Estrogen Receptor
ER α :	Estrogen Receptor alpha
ER β :	Estrogen Receptor beta
ERE:	Estrogen Response Element
ESR1:	estrogen receptor 1
FBS:	Fetal Bovine Serum
FGF:	Fibroblast growth factor
FOS:	Fos proto-oncogene, AP-1 transcription factor subunit
FXR:	farnesoid X receptor
GKD:	glycerol kinase deficiency
HDR:	Homology Directed Repair
HER2:	Human epidermal growth factor receptor 2
HH:	Hypogonadotropic hypogonadism
HNF4:	Hepatocyte nuclear factor 4
HRE:	Hormone Response Element
HRT:	Hormone Replacement Therapy
HSP:	Heat Shock Protein

IGF-1:	insulin-like growth factor 1
IGF-2:	insulin-like growth factor 2
IHC:	Immunohistochemistry
ITGB3:	integrin subunit beta 3
KO:	Knock Out
LBD:	Ligand binding domain
LHR-1:	Liver receptor homolog-1
LXR:	liver X receptor X
LXXLL:	Leu-x-x-Leu-Leu
MAFF:	MAF bZIP transcription factor F
mESC:	mouse Embryonic Stem Cell
mRNA:	messenger RNA
NHR:	Nuclear Hormone Receptor
N-coR:	Nuclear receptor co-repressor
NR0B1:	Nuclear Receptor Subfamily 0 Group B Member 1
nt:	nucleotide
ONR:	Orphan nuclear receptor
PBS:	Phosphate-Buffered Saline
PCR:	Polymerase Chain Reaction
PR:	Progesterone Receptor
qPCR:	quantitative PCR
RAR:	retinoic acid receptor
rhEGF:	recombinant human Epidermal Growth Factor

RNA:	RiboNucleic Acid
RNAi:	RNA interference
RNAse:	Ribonuclease
RORB:	RAR related orphan receptor B
SF-1:	Steroidogenic Factor-1
SFN:	stratifin
siRNA:	small interfering RNA
SOX9:	SRY-box 9
SRY:	Sex-determining region Y
STAR:	Steroidogenic acute regulatory protein
TALLEN:	Transcription-Activator Like Effector Nuclease
TGF- α :	Transforming growth factor alpha
WISP2:	WNT1 inducible signaling pathway 2
WT1:	Wilms Tumor 1
ZNFs:	Zinc-Finger Nucleases

Chapter 1

General Introduction

Breast Cancer Overview

Breast cancer is the most commonly diagnosed cancer in women worldwide and the second leading cause of cancer-related deaths. The American Cancer Society estimates that around 252,710 new cases of invasive breast cancer, and 63,410 new cases of carcinoma *in situ* (CIS) will be diagnosed, and around 40,610 women will die from breast cancer this year. Since 2007, death rates from breast cancer have been falling in women older than 50 but have remained unchanged in younger women. The decrease in death rates among older women is thought to be due to increased awareness and earlier detection, as well as improved treatments^[1]. It is not clear why the same trend has not been seen in younger women but lifestyle factors such as alcohol consumption, diet and exercise, and hormonal factors such as later and fewer pregnancies, use of the combined oral contraceptive and HRT, earlier menses and later menopause, have all be suggested as possible influences in the continued incidences of cancer arising in these women.^[2]

Breast cancer encompasses a group of heterogeneous diseases that can be categorized into several subtypes based on differences at a clinical, histopathological, and molecular level. The subtypes are used to assess a patient's prognosis and determine the appropriate therapy. There have been five molecular subtypes of breast cancer identified to date (Table 1.1).

Table 1.1: Molecular subtypes of breast cancer ^[3]

	Hormone receptor (ERα and/or PR)	HER2	Ki-67	Characteristics
Luminal A	Positive	Negative	Low	Low-grade, slow growing, best prognosis
Luminal B	Positive	Positive or negative	High	Faster growing than Luminal A with a slightly worse prognosis
Triple negative/basal-like	Negative	Negative		Common in women with <i>BRCA1</i> gene mutation
HER2 enriched	Negative	Positive		Faster growing than luminal cancers but respond well to targeted therapies
Normal-like	Positive	Negative	Low	Good prognosis

Hormone receptor status is one of the major factors on which the categorization is based. The most clinically relevant molecules for breast cancer are estrogen receptor (ER) and progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), Ki-67, CK5/6, epidermal growth factor receptor (EGFR) and androgen receptor (AR). Hormone receptor positive breast cancer is the most common form and current adjuvant therapies include Aromatase Inhibitors (AIs), which suppress estrogen production in postmenopausal women, Tamoxifen, which is a selective ER modulator, and ovarian suppression, which results in the cessation of the production of estrogen by the ovaries in premenopausal women. Despite the many therapies developed and survival rates being some of the best among cancer patients^[1], receptor negative breast cancers remain difficult to treat, and drug resistance hampers long term survival rates^[4]. Therefore, a continued effort to understand the role of molecular mechanisms underlying the

pathophysiology of breast cancer initiation, growth and progression is essential for the development of tissue-specific treatments.

NHR Overview

Some of the hormone receptors mentioned above, namely the steroid receptors ER, PR and AR, are known as nuclear hormone receptors. Nuclear hormone receptors (NHRs) are eukaryotic, intracellular proteins activated by ligand binding to act as transcription factors. They directly regulate gene expression by interacting with specific DNA sequences upstream of their targets ^[5] (Figure 1.1). Lipophilic ligands, including endogenous hormones and vitamins A and D, pass through the cell membrane via diffusion and bind to their NHR. Ligand binding triggers dissociation of the NHR from heat shock proteins (HSP) and binding as either hetero-dimers or homo-dimers to another NHR. Dimers then translocate to the nucleus via active transport and, along with co-activators or co-repressors, bind to the DNA at specific sequences known as hormone response elements (HRE). The target gene downstream of the HRE is then either up or downregulated, the DNA is transcribed into mRNA which moves out of the nucleus via a nuclear pore, is transcribed into a protein by ribosomes and finally results in a cellular response.

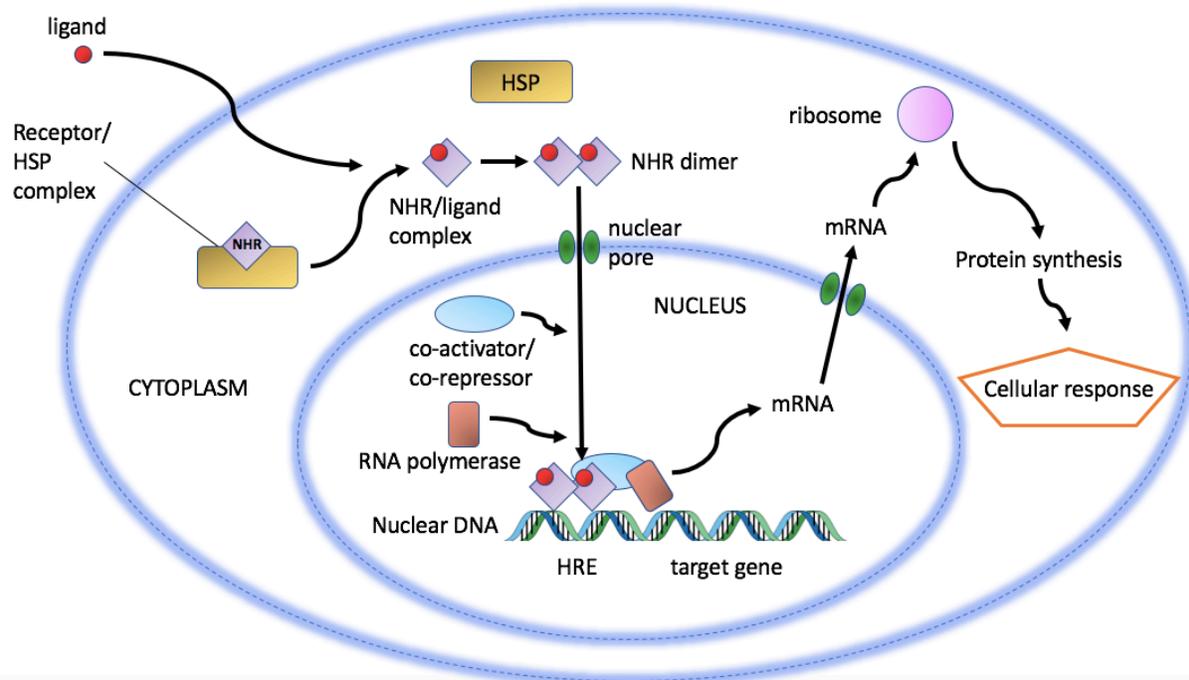


Figure 1.1: Typical nuclear hormone receptor action. Lipophilic ligands cross the cell's plasma membrane and bind directly with the NHR located in either the cytoplasm or the nucleus. Binding causes dissociation of the receptor/HSP complex and homo- or hetero-dimerization of the NHRs. NHR then moves into the nucleus and binds to the HRE, upstream of the target gene, along with co-activators or co-repressors and additional proteins such as RNA polymerase, to up or down regulate the gene. The resulting mRNA moves out of the nucleus to the cytoplasm for translation, resulting in a cellular response.

NHR Structure

The NHRs are a superfamily to which seven subfamilies belong, identified as NR0 through NR7, based on sequence similarity in the conserved DBD and LBD regions of the genes^[6]. These sub-categorizations help dictate the gene nomenclature rules.

NHRs are composed of 6 conserved regions, designated A-F, making up the typical functional structure that includes: an N-terminal domain (A/B), a DNA binding domain (DBD) (C), a hinge region (D), a ligand binding domain (LBD) (E), and a C-terminal domain (F)(Figure 1.2).



Figure 1.2: Structural organization of nuclear hormone receptors. The general structural organization of a typical nuclear hormone receptor depicting an N-terminal domain (A/B), a DNA binding domain (C), a hinge region (D), a ligand binding domain (E), and a C-terminal domain (F).

The N-terminal region is highly variable in sequence among NHRs, has a regulatory role in transcription transactivation, and includes the ligand independent activation function 1 (AF-1) which binds the NHR to coactivators ^[7, 8]. The DBD is classified as a type-II zinc finger motif. The two zinc fingers bind as a dimer to their Hormone Response Element (HRE), a short sequence of DNA located in the promoter region of the gene, anchoring the receptor in place. The DBD is highly conserved among receptors. The hinge region connects the DBD to the LBD and is thought to have a role in nuclear localization, DNA binding and co-activator recruitment ^[9]. The LBD is also highly conserved in structure among this group of receptors. This structure is known as an alpha helical sandwich, where three anti-parallel alpha helices are flanked by three alpha helices on one side and two on the other. Aside from binding with its ligand, the LBD mediates homo- and hetero-dimerization, binds to co-activator and co-repressor proteins, and contains a ligand dependent activation function 2 (AF-2). The C-terminal region, or carboxyl-terminal domain, is highly variable in sequence between receptors and in most cases, has unknown functions ^[10, 11]. Ligands that bind to NHR include, steroid hormones such as estrogen, progesterone and androgens, and various other lipid soluble signals such as thyroid hormone, retinoic acid and vitamin D ^[5, 12, 13]. Based on the ligand it binds, an NHR can be subcategorized as a steroid hormone receptor, non-steroid hormone receptor or an orphan nuclear receptor (ONR) when the ligand has not been identified ^[13].

NHR Function

NHRs function primarily as transcription factors regulating genes involved in a wide variety of biological processes including cell proliferation, development, metabolism, reproduction and disease. These actions depend on several endogenous mechanisms, including ligand binding, posttranslational modifications of amino acids, protein dimerization, nuclear transfer, protein-protein interactions with co-activators and co-repressors, and cooperative binding with other transcription factor to DNA ^[10, 14, 15]. The mechanism of action of NHRs is often represented by the inactive cytoplasmic receptor binding with its ligand and translocation to the nucleus where gene regulation takes place (Figure 1.1). While this model is valid for some steroid hormones, many NHRs are exclusively nuclear and can bind to DNA in the absence of a ligand, acting as gene repressors or promoters via other transcription factors ^[16]. Some NHRs have also been found to function in the cytoplasm regulating cellular functions; for example in the cytoplasm of endothelial cells, estrogen can act through its receptor to rapidly activate cellular pathways that control cell migration and control vascular tone ^[17].

DAX-1: A Unique NHR

A class of receptors within the NHR superfamily are the Orphan Nuclear Receptors (ONR) named so because their physiological ligands are unidentified. Whether all of these ONRs have ligands is unclear as many can act in their absence. DAX-1 is an atypical ONR and the focus of this research. It is an unusual NHR for several reasons; it lacks several conserved functional domains of the NHR superfamily including the modulatory domain harboring an AF-1 transactivation unit, a classical DBD, and a hinge region (Figure 1.3). Instead of the highly-conserved zinc finger motif of the DBD, there is a repeated Alanine/Glycine-rich peptide sequence of 65-67 amino acids. There are three full repeats and one incomplete repeat that transitions directly with the LBD ^[18]. Within each repeat is an LXXLL (Leu-x-x-Leu-Leu)-like motif, that is thought to mediate protein/protein interactions in the absence of AF-1. DAX-1

is grouped in the family of NHRs due to its highly-conserved LBD. However, another characteristic that makes it atypical is that in this region there is an unusually long insertion of 26 amino acids between helices H6 and H7. This feature is conserved across human and mouse proteins suggesting that it has a significant role in its function. The C-terminal region consists of 12 α -helices, a repressor domain and a region similar to the ligand-inducible domain typical of other NHRs.

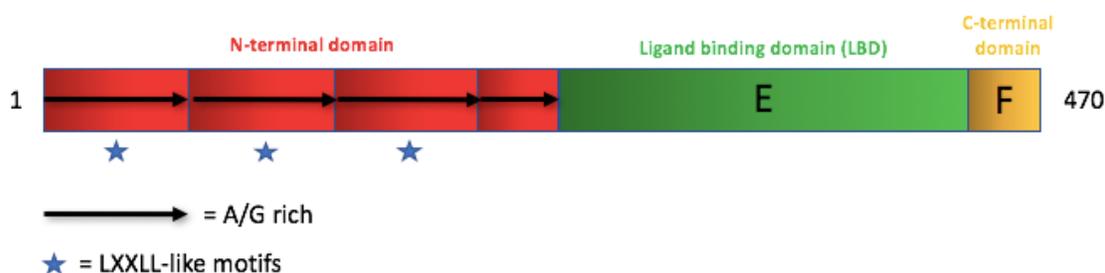


Figure 1.3: Structural organization of human DAX-1 protein. The DAX-1 protein consists of 470 amino acids with an N-terminal domain including 3 complete and one incomplete Alanine/Glycine (A/G) rich repeats and LXXLL-like motifs found within each one, a LBD and a C-terminal domain.

The genomic location of DAX-1 was identified by Muscatelli *et al.*, who noted the association of X-linked adrenal hypoplasia congenita (AHC) with glycerol kinase deficiency (GKD) and Duchenne muscular dystrophy (DMD), both of which are caused by mutations or deletions on the short arm of the X chromosome^[19]. Further studies showed DAX-1 to be encoded by the two-exon gene *NR0B1* (Nuclear Receptor family 0, Group B, member 1) located on the short arm of the X chromosome between position 21.3 and 21.2, from base pair 30,082,120 to 30,087,136^[20]. DAX-1 was initially described in relation to a disorder of sex development, known as dosage-sensitive sex (DSS) reversal, causing male to female phenotypic sex reversal in 46,XY individuals with a normal SRY gene^[20, 21]. Mutations in the same gene were then found in male patients with AHC. One of the main symptoms of AHC is adrenal insufficiency resulting in a lack of male sex hormones leading to hypogonadotropic hypogonadism (HH) after puberty. Given its involvement in two separate disorders the gene was named DAX-1, Dosage sensitive

sex-reversal (DSS), Adrenal hypoplasia congenita (AHC) locus on the X chromosome gene 1^[21].

Normal DAX-1 function

Since its discovery, there have been numerous studies into DAX-1's role in development, physiology and disease. Because DAX-1 lacks the classic DBD, its mechanism of action has been explained through interactions with other NHRs, regulating their activities. DAX-1 acts as a transcriptional corepressor in the majority of its interactions with these NHRs; examples include; Steroidogenic Factor-1 (SF-1), androgen receptor (AR), progesterone receptor (PR), estrogen receptor (ER), Nur77, hepatocyte nuclear factor 4 (HNF4), liver X receptor X (LXR), farnesoid X receptor (FXR), and constitutive androstane receptor (CAR)^[18, 22]. The common mechanism of DAX-1 transcriptional repression is through binding with the AF-1 domain of another NHR via its LXXLL motifs and recruiting other corepressor proteins, competing with coactivators for target gene binding^[23, 24].

In the adult, DAX-1 expression is restricted primarily to the adrenal glands, testes, lungs and pancreas. DAX-1 influences hormone production by acting as a coregulatory protein that inhibits the transcriptional activity of other nuclear hormone receptors through heterodimeric interactions^[25]. Notably for this research, DAX-1 expression has been shown to correlate negatively with steroidogenic gene expression and can act as a repressor of steroidogenesis *in vivo*^[26, 27]. During embryonic development, DAX-1 expression has a restricted pattern to several endocrine tissues, including adrenal glands, pituitary glands, hypothalamus, ovaries and testes^[28, 29]. Historical studies showed DAX-1 plays a key role in sex determination along with SF1, SRY, SOX9 and AMH^[20, 21, 30-32]. Specifically, DAX-1 functions as an anti-testes gene by acting antagonistically to SRY through binding to the STAR promoter. It can be detected in the genital ridge and adrenal cortex 33 days post ovulation (d.p.o.)^[30] where it is regulated by WT1 to influence gonadal differentiation. More recently DAX-1 expression has been found in mouse

embryonic stem cells (mESCs) leading to the elucidation that, in addition to inhibiting steroidogenesis, DAX-1 also plays a significant but largely undefined role in the maintenance of pluripotency of mESCs [33-38]. In humans however, DAX-1 is only expressed at low levels in ES cells, and its expression is inconsistent during differentiation.

DAX-1 Function in Disease

In view of the action of DAX-1 as a transcriptional regulator, many studies have examined its role in different diseases, including cancers. Early studies linked DAX-1 with adrenocortical neoplasms, finding that it influenced steroid biosynthesis by tumors, hence putatively linking DAX-1 with cell proliferation and tumor formation [39]. It has since been found to play a role in many forms of cancers including; bone [40-43], lung [44, 45], prostate [46], uterine [47], ovarian [38], breast and other soft tissue tumors [48-50]. In the context of breast cancer, DAX-1 immunoreactivity has been found to directly correlate with androgen receptor (AR), estrogen receptor alpha (ER α) and estrogen receptor beta (ER β) expression and lymph nodal metastasis. Intriguingly, all of these nuclear hormone receptors are used for clinical categorization of tumors [15, 48]. Similarly, DAX-1 immunoreactivity also directly correlates with the stage of ovarian carcinomas and with ER α and ER β expression in endometrial cancer [38, 47]. However, it has an inverse relationship with prostate and endometrial carcinoma staging [46, 47]. Higher DAX-1 expression in lung cancer patients has been associated with increased risk of disease recurrence and a higher metastatic rate [44, 45].

DAX-1 Role in Breast Cancer

DAX-1 mRNA is present at low levels in normal breast tissue and is down regulated but continues to be present in many ER positive breast cancer cell lines [51]. It is a positive prognostic factor in node negative breast cancer, being correlated with smaller tumor size, earlier disease stage and increased survival [50, 51]. DAX-1 acts as a corepressor for ligand bound ER, inhibiting basal and estrogen dependent cell

proliferation^[50, 52]. Unpublished data from our own laboratory supports this by showing DAX-1 to be a suppressor of tumorigenesis in MCF7 breast cancer cells^[53].

The actions of steroid hormones, most notably estrogens and progesterone, are driving forces in the development and progression of breast cancer. In addition, androgens can also play a role due to their ability to be converted into estrogens via the enzyme p450 aromatase^[48]. Just over one third of all cancers occurring in the breast tissue are hormone dependent diseases, utilizing local or systemic estrogens for tumor cell growth and proliferation^[54]. There are two types of ERs, ER α and ER β . Estradiol (or 17 β -estradiol), the predominant form of naturally occurring estrogen, binds with the same high affinity to both receptors and to the estrogen response element (ERE) in a similar if not identical fashion. ER α and ER β are products of different genes; the major difference is in their tissue distribution^[55], with ER α being found in the endometrium, ovarian stromal cells, hypothalamus and breast cancer cells^[56], while ER β expression is seen in the ovarian granulosa cells, kidney, brain, bone, heart, lungs, intestinal mucosa, prostate and endothelial cells^[57]. While the ER α form is primarily responsible for growth and proliferation^[58], the role of ER β is currently unclear. Some studies suggest that ER β opposes the actions of ER α and clinical evidence has indicated that the loss of ER β expression is associated with poor prognosis and resistance to endocrine therapy^[59]. Data from our laboratory shows that DAX-1 binds to ER α and ER β with the same affinity. It does so via interactions with DAX-1's LXXLL motifs in the N-terminal repeat domain, specifically motif 3^[50, 52].

In breast cancer, ER α interacts with many target genes^[60], and one of the most important targets is the proliferation gene for Cyclin D1 (CCND1). Cyclin D1 is a major regulator of entry into the G1 stage of the cell cycle^[61], and it is well established that it plays a crucial role in breast cancer growth and progression^[49, 62-64]. Studies have shown DAX-1 to be a suppressor of Cyclin D1 activity^[49].

Unlike estrogens, androgens have been shown to have a play a protective role in breast cancer and have been directly correlated with DAX-1 expression ^[65,66]. Studies into the relationship between ER, AR and DAX-1 have shown that AR may repress ER through DAX-1 activity. In MCF7 cells (AR/ER α positive), expression of DAX-1 has been shown to be induced by ligand bound AR, and in association with the co-repressor N-CoR, it binds to the SF-1/LRH-1 region of the aromatase promoter suppressing its activity and hence ER activity ^[67]. When ER α positive breast cancer cells were treated with the non-aromatizable androgen 5- α -dihydrotestosterone (DHT), DAX-1 was seen to be recruited by AR and mediated a reduction in Cyclin D1 activity slowing proliferation ^[49,67].

Chapter 2

DAX-1 influence on breast cancer cell proliferation and metastasis

Introduction

Cell proliferation and metastasis play critical roles in the progression and behavior of cancer development. Altered control of cell growth is the primary characteristic feature of a malignant neoplastic cell population and increased proliferation in breast cancer tumors correlates strongly with poor prognosis [68]. The principle cause of death in cancer patients is from metastatic tumor development at secondary sites in the body. It is a complex process that is only partially understood at the biochemical and molecular level. In this chapter, we explore the influence DAX-1 has on breast cancer cell proliferation and metastasis.

The adult female breast is composed of epithelial lactiferous ducts in a fibrous tissue framework and surrounded by fat. Normal breast growth and regression is cyclical, influenced by many complex interactions of hormones and mitogenic growth factors including, estrogen, progesterone, androgens, glucocorticoids, prolactin, thyroid hormone, insulin and insulin-like growth factors (IGF-1 and IGF-2), fibroblast growth factors (FGFs), and TGF- α [69-72]. These influences are endocrine, the majority arising from the ovarian tissue in an autocrine manner and secreted by the mammary cells themselves, as well as paracrine, secreted by the surrounding stromal cells. Proliferation of ER positive breast cancer tumors is caused by estradiol, produced by the actions of the enzyme aromatase, binding to ERs and activating expression of proliferative genes including Cyclin D1 [73]. Cyclin D1, encoded by the *CCND1* gene, is a member of the cyclin family of proteins that controls a cell's progression through their cell cycle by activating cyclin dependent kinases (CDK). Cyclin D1, specifically, is required for progression through G₁ phase of the cell cycle (Figure 2.1). It dimerizes with CDK4 and CDK6 to regulate G₁/S phase transition

and entry into S phase ^[61]. Overexpression of Cyclin D1 is found in more than 50% of human breast cancers and causes mammary tumors in transgenic mice. The normal cell cycle is disrupted when Cyclin D1 gene expression or function is dysregulated. Further studies have shown that Cyclin D1 has other roles in regulating gene expression through promoting cell migration and inhibiting mitochondrial metabolism, adding to the pathology associated with its dysregulation ^[74, 75].

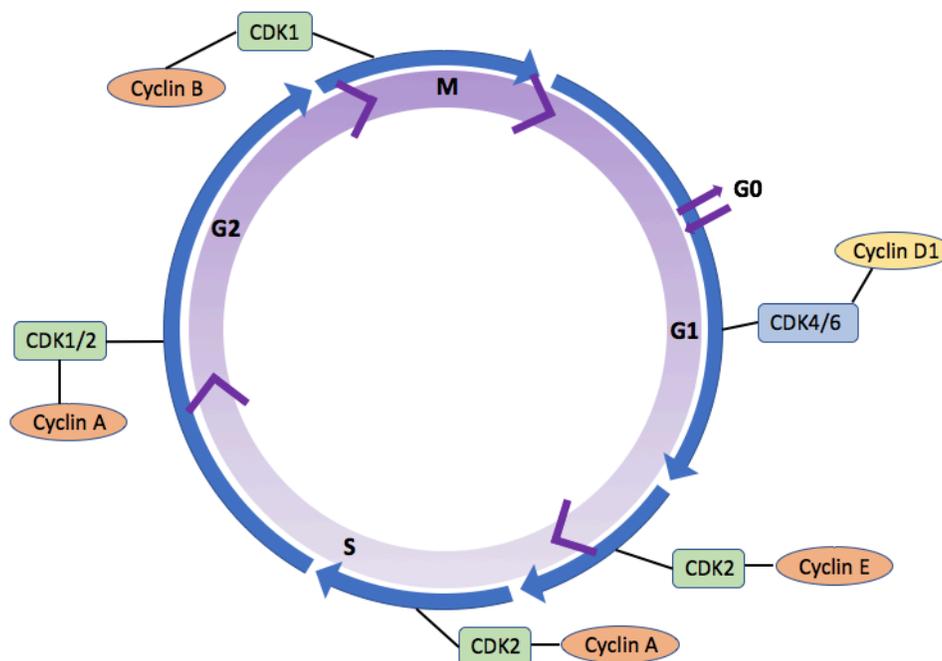


Figure 2.1: Mitotic cell cycle. G₁ and G₂ are both cell growth phases, S is a DNA synthesis phase and M is the mitotic phase where cell division occurs. G₀ is the resting phase, so not technically part of the cell cycle. Cyclin D1 interacts with CDK4 and 6 to regulate G₀ to S phase transition, Cyclin E binds with CDK2 phosphorylating the cell cycle inhibitor p27^{Kip1} allowing the cell to move from G₁ phase, past the R (restriction) point (indicated by the purple arrow) into S phase. Cyclin A associates with CDK2 during S phase and is involved in initiation and replication in the nucleus, it also associates with CDK1 late in S phase until G₂ phase where it is involved in activation and stabilization of Cyclin B/CDK1 complex, Cyclin B is required for progression of the cell into and out of M phase of the cycle.

Previous unpublished research from our laboratory has shown that in a subset of breast cancer patients, the DAX-1 gene is expressed in normal breast tissue but is down regulated or completely absent

in matched tumor tissue (Figure 2.2). This observation led to the hypothesis that DAX-1 plays a protective role in breast cancer and that its downregulation leads to a more proliferative phenotype.

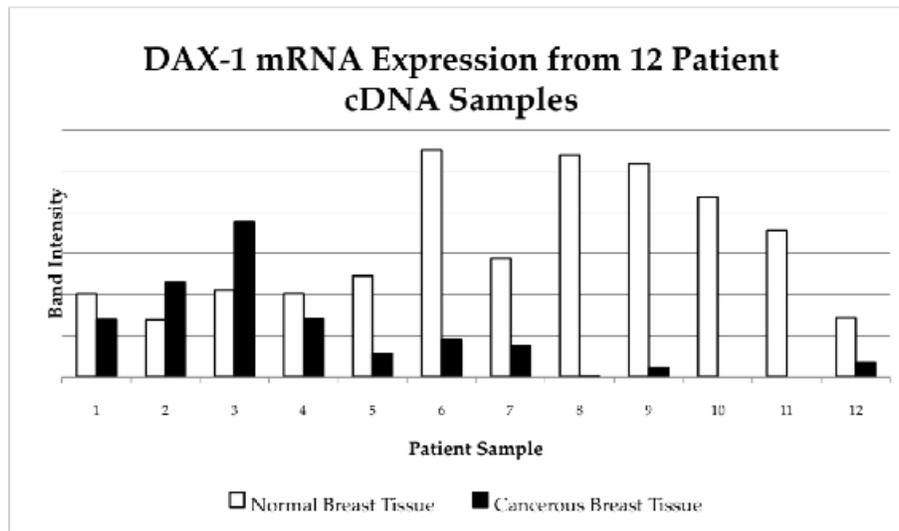


Figure 2.2: DAX-1 mRNA expression from 12 patient matched cDNA samples. Normal breast tissue samples are represented by white bars; adjacent cancerous breast tissue is represented by black bars. Expression level is measured by PCR band intensity from cDNA. 10/12 patients had reduced or no DAX-1 expression in the cancerous tissue compared with normal tissue. (Unpublished data)

Research investigating the molecular mechanisms of DAX-1 repression in the breast is limited. However, one study did find DAX-1 to be recruited by AR to form a multiprotein repressor complex, involving histone deacetylase 1, to repress the Cyclin D1 gene *CCND1* [49]. Preliminary work from our laboratory determined DAX-1 also inhibits ER induced Cyclin D1 expression. We have shown that DAX-1 binds to the promoter near the ERE sequence, thereby downregulating Cyclin D1 expression and inhibiting cell proliferation. DAX-1's influence on other proliferative genes has not yet been studied. We propose that DAX-1 acts as a negative regulator of other proliferation genes in addition to Cyclin D1 in breast cancer cells.

Based upon our current understanding of the mechanism of action of DAX-1 from the literature available and from what we have seen in our own laboratory, we hypothesize that DAX-1 suppresses cell proliferation in breast cancer cells, likely via interactions with other transcription factors and cellular proteins to act as a co-repressor of the cell cycle regulatory genes.

In the context of metastasis, DAX-1's role is poorly understood. Tumor metastasis involves a series of discrete biological processes that allow the tumor to spread from the primary site to distant locations in the body. This process involves a multi-step cascade of coordinated cell adhesion and contractility, along with proteolytic remodeling of the extracellular matrix (ECM)^[76]. It begins with additional mutations of the tumor cell's genome giving the cells the ability to invade the tissue surrounding the primary tumor. Once the metastatic cells breach the lymphatic system or vascular tissue, they are transported in the blood to distant sites. Cells are usually extravasated from the bloodstream in the first capillary bed encountered, colonizing the foreign tissue. This colonization involves a complex set of alterations to make the tissue receptive to the invading cells, involving its cellular composition, immune status, blood supply, ECM and virtually every other aspect of its properties^[77]. One important process to note is epithelial-mesenchymal transition (EMT) by which cells transit between epithelial and mesenchymal states. It is a process normally seen in embryonic development but pathological EMT processes are seen in many cancers and require molecular changes, decreased cell-cell recognition and adhesion, and increased potential for cell motility^[78]. Metastasis is driven by instability in the cells genome involving progressive loss of checks on normal chromosome stability, DNA repair and regulated gene expression. Very few metastatic cells that make it into the bloodstream actually go on to develop into secondary lesions, but those that do are often lethal. There is no cure for metastatic cancer. Current treatments are aimed at prolonging survival and maintaining a quality of life with cytostatic therapies as opposed to cytotoxic ones^[77]. Currently, therapeutics are aimed at the trunk of tumorigenesis pathways

such as receptor tyrosine kinases, and there is very little in the way of therapies aimed further down the metastatic pathways^[79]. With a deeper understanding of metastasis, in particular the later stages such as metastatic colonization, more effective druggable targeted pathways may be identified that will enhance the efficiency of current therapies^[79].

In the context of breast cancer, distant metastases are the cause of 90% of deaths^[80]. The most common site of breast cancer metastasis is the bone followed by lungs, regional lymph nodes, liver and brain^[81]. It has been shown that ER positive breast cancers have a predilection to metastasize to the bone^[82]. Established prognostic markers of breast cancer metastasis are reviewed in Table 2.1. Larger tumor size, auxiliary lymph node metastases and higher histological grade are all well-established markers and are used to base therapy decisions. In patients with node-negative tumors approximately one third will still develop distant metastases; therefore, angioinvasion is used as an additional predictor of metastasis. The urokinase-type plasminogen activator (uPA) enzyme pathway, comprised of factors such as uPA and its inhibitor PAI1, is involved in the early stages of the metastatic cascade which include degradation of the ECM. Patients with high levels of uPA activity have significantly shorter disease-free intervals than patients with low levels of activity^[83].

Steroid receptor positive tumors tend to be less aggressive than negative ones, and this is currently used clinically as a prognostic marker for disease progression. However, studies have shown ER signaling to promote metastasis in ER positive breast cancers with complex pathways involving coregulatory proteins (Figure 2.3). For example, deregulation of ER-coregulator signaling can lead to aberrant expression of Snail that leads to loss of expression of the cell adhesion molecule E-cadherin and invasive growth. Disruption of several other coregulators of ER α , including MTA1, AIB1, SRC-1 and PELP1, have been shown to lead to breast cancer metastasis^[84]. MTA1 (metastasis-associated protein 1), a commonly deregulated coregulator in breast cancer, promotes transcriptional repression of ER, leading to

metastatic progression. AIB1 (amplified in breast cancer 1) and SRC-1 (steroid receptor coactivator-1) are both nuclear receptor coactivators that interact with nuclear hormone receptors to enhance their transcriptional activator function and have been shown to promote breast cancer metastasis by interactions with the transcription factor PEA3 (polyoma enhancer activator 3). Specifically, AIB1 acts by promoting PEA3-mediated matrix metalloproteinase 2 (MMP2) and 9 (MMP9) expression leading to the breakdown of the ECM. SRC-1 acts through coactivation of PEA3-mediated Twist expression, regulating EMT transition. In addition, recent studies have found deregulation of the ER α coregulator PELP1 (proline, glutamate and leucine rich protein 1) in invasive and metastatic breast tumors ^[84]. Our own studies, described above, identified both ER α and PELP1 to be upregulated by DAX-1 overexpression in MCF7 cells, which were originally derived from a metastatic source. Although treatments targeting estrogen and ER are well established in the treatment of breast cancer, initial or acquired resistance to hormone therapies is a common feature of metastatic breast tumors and a major problem for long term treatment.

In breast cancers, an important marker for predicting metastatic potential and therefore patient prognosis is HER2. While only 15 – 20% of invasive breast cancers express abnormally high levels of HER2, approximately half of these will spread to the brain, a common metastatic site in breast cancer^[85]. As discussed in previous chapters, HER2 over expression has been well established as a promoter of breast cancer progression and metastasis. It is a transmembrane receptor with constitutive tyrosine kinase activity. Many aspects of tumor progression are favorably affected by overexpression of HER2 including increased motility of both intravasating and extravasating cells, decreased apoptosis, enhanced signaling interactions with the microenvironment and regulation of adhesion, among a multitude of other functions^[86]. The current targeted therapy for patients with metastatic HER2 breast cancer is trastuzumab, a monoclonal antibody directed against the receptor.

Table 2.1: Breast cancer metastasis prognostic markers ^[77].

<i>Marker</i>	<i>Use in clinic</i>	<i>Metastatic determinants</i>	<i>Details</i>	<i>References</i>
<i>Tumor size</i>	Established	Tumors under 2cm in diameter have low risk of metastasis; 2-5cm have high risk; over 5cm very high risk of metastasis	Independent prognosis marker	[87-90]
<i>Auxiliary lymph-node status</i>	Established	If no lymph node-metastasis, risk of distant metastasis is low; if lymph-node metastasis is present, risk of distant metastasis is high; if > 4 lymph-node metastasis, risk of distant metastasis is very high	Related to tumor size	[87, 89, 90]
<i>Histological grade</i>	Established	Grade 1 tumor = low risk of metastasis; Grade 2 tumor = intermediate risk of metastasis; Grade 3 = high risk of metastasis	Related to tumor size	[87, 89, 91]
<i>Angioinvasion</i>	Established in patients with lymph-node negative tumors	Presence of tumor emboli in over 3 blood vessels is associated with metastasis	In patients with node-negative tumors	[92, 93]
<i>uPA/UPII protein level</i>	Newly established marker	High protein levels are associated with high risk of metastasis	Independent prognosis marker	[83, 94-98]
<i>Steroid-receptor expression</i>	Established for adjuvant therapy decision	Low steroid-receptor levels are associated with metastasis	Short term predictor of metastasis risk (5 years); related to histological grade	[87]
<i>HER2 protein expression</i>	Established for adjuvant therapy decision	ERBB2 (HER2 gene) amplification and HER2 overexpression are associated with high metastatic risk	In patients with lymph-node positive tumors	[99-101]

(Table adapted from Weigelt et al. Table 2.)

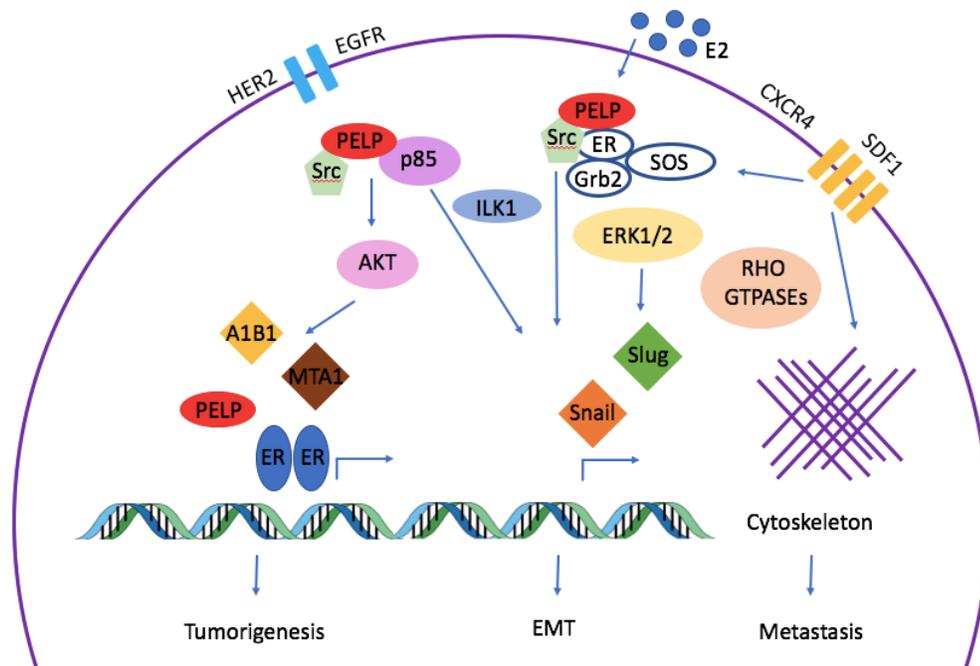


Figure 2.3: Schematic representation of hormonal regulation of metastasis^[84]. ER α mediated signaling involves nuclear as well as extranuclear actions and growth factor signaling cross talk. Estrogen signaling has the potential to activate extranuclear signaling that activates several kinase cascades, which have the potential to alter cytoskeleton, affect EMT and enhance cell migration. Deregulation or ER α -mediated signaling crosstalk will have implications in estrogen mediated tumor progression to metastasis. (Adapted from Saha Roy *et al.* figure 2)

As described in Chapter 1, DAX-1 is associated with a higher metastatic rate in lung cancers^[44, 45]. However, essential cellular components controlling the metastatic pathways in breast cancer have not been completely elucidated. Further research is needed to understand the molecular mechanisms involved in breast cancer metastasis and for the development of targeted therapeutics. Current prognostic criteria only poorly predict the risk of a breast cancer developing into metastatic disease, and as a result patients are being subjected to cytotoxic chemotherapies unnecessarily. Therefore, new prognostic markers, potentially including DAX-1, could lead to more accurate identification of patients at low and high-risk of developing metastatic disease, pinpointing those who would benefit from specific adjuvant therapies.

Materials & Methods

Reagents

Serial dilutions of Fulvestrant (Tocris Bioscience, Bristol, UK) aka ICI 182, 780 or Faslodex[®], were made in DMSO and used to treat cells at final concentrations of 1 μ M, 100nM, 10nM, 1nM, 100pM, 10pM or 1% DMSO for untreated cells.

Cell Culture

Cells were either obtained from American Type Culture Collection (AACT[®], Manassas, VA) or Sigma-Aldrich[®] (St. Louis, MO). All cell lines were routinely passaged, cultured, and maintained at 37°C in a humidified 5% CO₂ tissue culture incubator.

MCF7 human breast adenocarcinoma cells were cultured in DMEM/F12 (Thermo Fisher Scientific, Waltham, MA) with no phenol red. Phenol red bears a structural resemblance to nonsteroidal estrogens and has been shown to bind to the estrogen receptors of MCF7 cells stimulating their proliferation, therefore it was not used in their culture^[102]. Media was supplemented with 2.5mM L-Glutamine, 10% FBS (AACT[®], Manassas, VA), 1% penicillin/streptomycin, 1% amphotericin B (both from Lonza Group Ltd, Basel, Switzerland), 1% Anti-Anti (100X) Antibiotic-Antimycotic and 1% kanamycin sulfate (100X) (both from Gibco by Life Technologies, Waltham, MA)

MCF10A human mammary epithelial cells were cultured in in DMEM/F12 (Thermo Fisher Scientific, Waltham, MA) with no phenol red supplemented with 5% horse serum (AACT[®], Manassas, VA), 1% penicillin/streptomycin (Lonza Group Ltd, Basel, Switzerland), 100ng/ml cholera toxin (Sigma-Aldrich[®], St. Louis, MO), 20ng/ml rhEGF (Lonza Group Ltd, Basel, Switzerland), 500ng/ml hydrocortisone

(Sigma-Aldrich[®], St. Louis, MO), 10µg/ml insulin (Thermo Fisher Scientific, Waltham, MA), 1% amphotericin B (Lonza Group Ltd, Basel, Switzerland), 1% Anti-Anti (100X) Antibiotic-Antimycotic and 1% kanamycin sulfate (100X) (both Gibco by Life Technologies, Waltham, MA)

To passage and maintain, cells were treated with 0.25% Trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA) and incubated at 37°C until released from flask. Cells were suspended and passaged at a 1:4 ratio.

For a period of 24 to 48 hours prior to experiments, cells were seeded and maintained in their respective culture media as described above but with only 1% serum. In this way cell growth was arrested until complete media was introduced, synchronizing the cell growth cycles.

RNAi to knockdown DAX-1 expression in MCF10A cells

A set of three different target-specific 19-23 nucleotide siRNA oligo duplexes of human NR0B1 gene, designed to knockdown gene expression, were obtained from MyBioSource (San Diego, CA). The three siRNAs were pooled prior to transfection. Pooled siRNA at a concentration of 100nM was combined with the transfection reagent Lfectine RU50 (MednaBio, Hayward, CA) and incubated at room temperature for 15 minutes. The transfection mix was added to MCF10A cells 24 hours after they were seeded. Cells were incubated with the siRNA for 24 hours before further cell manipulation. As a control, cells were treated with Lfectine RU50 only. Successful gene knockdown was determined via qPCR. (Appendix AIII).

CRISPR-Cas9 knockout of DAX-1 expression in MCF10A cells

MCF10A cells were seeded in a six well plate at a concentration of 2.5×10^5 in 3ml antibiotic free growth medium per well 24 hours prior to transfection. To attain optimal transfection, wells were treated with a

DAX-1 CRISPR/Cas9 KO plasmid (Santa Cruz Biotechnology, Inc., Dallas, TX) with concentrations varying between 1-3 μ g and a transfection reagent volume varying between 5-15 μ l in Plasmid Transfection Medium. At the same time, cells were also co-transfected with an HDR plasmid containing a puromycin resistance gene. Cells were incubated for 72 hours and the media replaced after 48 hours. Successfully transfected cells were identified using puromycin selection. For these cells, optimal puromycin concentrations of 8 μ g/ml was determined prior to transfection. Growth media was removed and replaced with fresh media containing 8 μ g/ml of puromycin every two to three days for nine days. Surviving cells were then recovered in complete growth medium. Successful DAX-1 KO was determined via standard PCR to screen for the presence of absence of the DAX-1 gene (Appendix AII.1 & AII.2).

Induction of DAX-1 expression in MCF7 cells by viral transfection

Recombinant human NR0B1 adenoviral particles were obtained from Creative Biogene Biotechnology (Shirley, NY). To induce DAX-1 expression, MCF7 cells were incubated with the adenoviral particles at a concentration of 2.62×10^8 VP/ml for 24 hours before further cell manipulation (Appendix I).

ER transfection

Prior to transfection, 1 μ g pcDNA3.1-HEO (expressing the human Estrogen Receptor α gene, Figure 2.4) and 50 μ l Lafectine RU50 (MednaBio, Hayward, CA) were incubated together, at room temperature, for 15 minutes. The plasmid/Lafectine RU50 transfection mix was then added to MCF10A cells and incubated at 37°C.

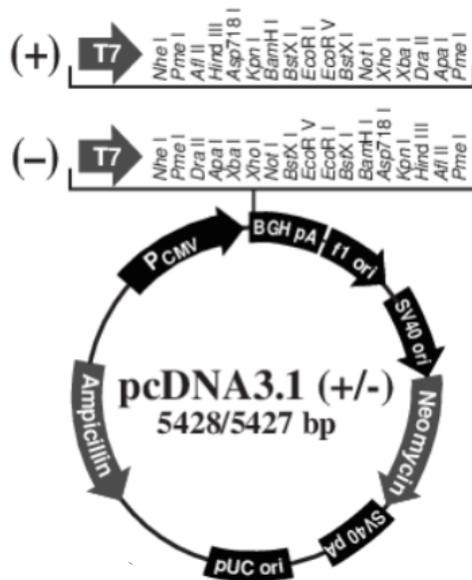


Figure 2.4: pcDNA3.1 (+/-) vector. Summary of the features of the 5.4 kb pcDNA3.1 vector utilized in transient transfection assays. Figure provided by Invitrogen Life Technologies.

RNA extraction and cDNA synthesis

Cells were harvested using 0.25% Trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA) and collected in an RNase free 1.5ml microcentrifuge tube. Total RNA was isolated using the RNeasy[®] Mini Kit (Qiagen[®], Austin, TX) as per the manufacturer's instructions. RNA was measured for concentration and purity using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). A QuantiTect[®] Reverse Transcription Kit (Qiagen[®], Austin, TX) was used to synthesize cDNA from the isolated RNA according to manufacturer's instructions.

PCR

cDNA synthesized as described above was used as a template for standard PCR. iProofTM High-Fidelity DNA Polymerase (Bio-Rad, Hercules, CA) Kit with the 5x iProofTM GC Buffer was used to make a PCR master mix, along with specific primers listed in Table 2.3. The MJ Mini Personal Thermal Cycler (Bio-Rad, Hercules, CA) was used to perform the PCR with the conditions described in Table 2.2. Primers were designed using the PrimerQuest tool available at IDTdna.com (Table 2.3).

Table 2.2: Thermocycler conditions for standard PCR

Step	Temperature	Time
Initial Denaturation	98°C	30 secs
29 Cycles:		
Denaturation	98°C	10 secs
Annealing	(see Table 2)	20 secs
Elongation	72°C	20 secs
Final Extension	72°C	5 mins
Hold	4°C	∞

Table 2.3: List of Primers used for standard PCR and two-step amplification qPCR

	Gene Name	Forward Primer 5'-3'	Reverse Primer 5'-3'	Annealing Temp
Housekeeping gene	GAPDH	CCA TCACCA TCTTCCAGGAGCG	AGAGA TGA TGACCCTTTTGGC	58°C
Targets	DAX-1	GGGTAAGAGGCGCTACCAG	GCTTGA TTTGTGCTCGTGGG	67°C
	ER α	GTCCTGTGGCA TCCACGAACT	TACTTGCCTCAGGAGGAGCAA	56°C

qPCR

cDNA synthesized as described above was used as a template for qPCR. qPCR reactions were performed in triplicate using the BioRad CFX96 Real-Time PCR system (BioRad, Hercules, CA) following the protocol below with varying initial denaturation time and annealing temperatures. qPCR reactions were prepared using 10 μ L of SYBR Green Master Mix (Life Technologies, Carlsbad, CA) or QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA), 0.5 μ L of 10 μ M forward and reverse primers (Table 2.3), 7.5 μ L of dH₂O, and 2 μ L of cDNA. GAPDH housekeeping gene was used as control and experimental genes were compared to GAPDH as a baseline. Error bars on qPCR results represent standard deviation of the mean following the $\Delta\Delta C_t$ method.

Table 2.4: Thermocycler conditions for two-step amplification qPCR

Step	Temperature	Time
Initial Denaturation	95°C	5 minutes
45 Cycles:		
Denaturation	95°C	15 seconds
Annealing + Plate read	50-65°C	30 seconds
Hold	4°C	∞

qPCR Arrays

cDNA synthesized as described above, at an RNA concentration of over 500ng/ul, was used as a template for qPCR. cDNA was added to RT² SYBR Green Master mix and the mixture aliquot across the array. Triplicate reactions were performed for each gene. Profiler™ PCR Array Human Breast Cancer (PAHS-131Z), RT² Profiler™ PCR Array Human Estrogen Receptor Signaling (PAHS-005Z) and RT² Profiler™

PCR Array Human Tumor Metastasis (PAHS-028Z) qPCR, each containing a profile of 84 related genes (gene lists available at <https://www.qiagen.com/us/shop/pcr/primer-sets/rt2-profiler-pcr-arrays/?catno=PAHS-131Z#geneglobe>, <https://www.qiagen.com/us/shop/pcr/primer-sets/rt2-profiler-pcr-arrays/?catno=PAHS-005Z#geneglobe> and <https://www.qiagen.com/us/shop/pcr/primer-sets/rt2-profiler-pcr-arrays/?catno=PAHS-028Z#orderinginformation>), were obtained from Qiagen[®] (Austin, TX). qPCR was performed using a Bio-Rad CFX96 Thermocycler and results were analyzed using the Qiagen Data Analysis Center^[103].

Cell counts

Prior to plating, a representative sample of cells in a single cell suspension were stained with Trypan Blue Solution, 0.4% (Gibco by Life Technologies, Waltham, MA) to determine cell viability and a live cell count. Cells were counted using a hemocytometer and then seeded in 24 well plates at a concentration of 0.05×10^6 in low serum medium. Culture medium was changed to complete media and Fulvestrant was added at the concentrations described above. Cells were cultured for 72 hours after addition of Fulvestrant and then trypsinized returning them to a single cell suspension, and counted using a hemocytometer. Experiments were carried out in triplicate.

Proliferation Assay

In order to quantitatively measure proliferation of the MCF10A and MCF7 cells, the Click-iT[®] EdU HCS Alexa Fluor[®] 488 (Invitrogen, Eugene, OR) kit was used. Cells were seeded in an 8-well glass chamber slide in low serum media as described above. MCF10A and MCF7 cell culture media was changed to complete media 24 hours after seeding. MCF7 + AV cells had virus added as described above and MCF10A RNAi cells were transfected with siDAX-1 as described above, and cultured for a further 24

hours prior to further manipulation. MCF10A cells were transfected with ER α receptor as described above at least two hours prior to further manipulation. Fulvestrant was added to the cells at concentrations ranging from 10pM-1 μ M with the exception of an untreated control that received 0.1% DMSO and an EdU control that received no additions. After a 72-hour incubation with Fulvestrant, 10 μ M EdU was added to each well with the exception of the control. Cells were incubated with EdU for 24 hours before staining with the Click-iT[®] EdU HCS kit. Briefly, culture media was removed and cells were fixed using 3.7% formaldehyde in PBS and permeabilized using 0.1% Triton[®] X-100 in PBS. EdU detection was achieved by adding the Click-iT[®] reaction cocktail containing a 1 X Click-iT[®] EdU reaction buffer, CuSO₄, Alexa Fluor[®] 488 azide and 1 X Click-iT[®] EdU buffer additive. Cells were incubated with the cocktail for 30 minutes at room temperature protected from the light. After removing the cocktail, the cells were washed in a Click-iT[®] reaction rinse buffer. The chamber cassettes were then removed from the slides and mounted using ProLong[™] Gold Antifade Mountant with DAPI (Molecular Probes[®] by Life Technologies[™], Eugene, OR) as a nuclear stain. Slides were imaged using a Zeiss A1 Axio Observer fluorescence microscope with Zen digital imaging software (Carl Zeiss Microscopy, LLC, Thornwood, NY) and analyzed using ImageJ software. Experiments were carried out in triplicate.

Statistical Analysis

Statistical significance was calculated using the two-tailed t-test formula built into Microsoft Excel. Data was found to be statistically significant if $p < 0.05$ (*) and highly statistically significant if $p < 0.005$ (**).

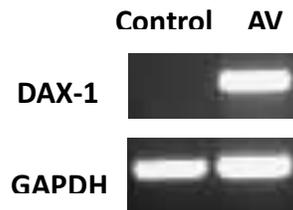
Results

Expression of DAX-1 in human adenocarcinoma cells (MCF-7) by adenoviral transduction.

As mentioned previously and shown in Figure 2.2, it is common to see a downregulation or even complete absence of DAX-1 expression in breast cancer cells. Therefore, we used MCF7 human adenocarcinoma cells, which do not express any DAX-1, as our breast cancer model cell line. To investigate the effect of the loss of DAX-1 in breast cancer cells we sought to compare wild-type MCF7 cells with MCF7 cells that were expressing DAX-1. This was achieved by viral transduction of MCF7 cells with a human recombinant NR0B1 adenovirus (Appendix I). We used this method because adenoviruses have 100% gene delivery efficiency as opposed to retroviruses or lentiviruses which have only a 30% efficiency. Also, there is no integration with the host system.

Expression levels of GAPDH (control housekeeping gene) and DAX-1 were examined by PCR and qPCR (Figure 2.5). Successful transduction was confirmed by the presence of DAX-1. Control MCF7 cells did not express DAX-1 whereas the viral transduced cells strongly expressed DAX-1. These cells will be referred to for the remainder of this thesis as MCF7 + DAX-1 cells.

A.



B.

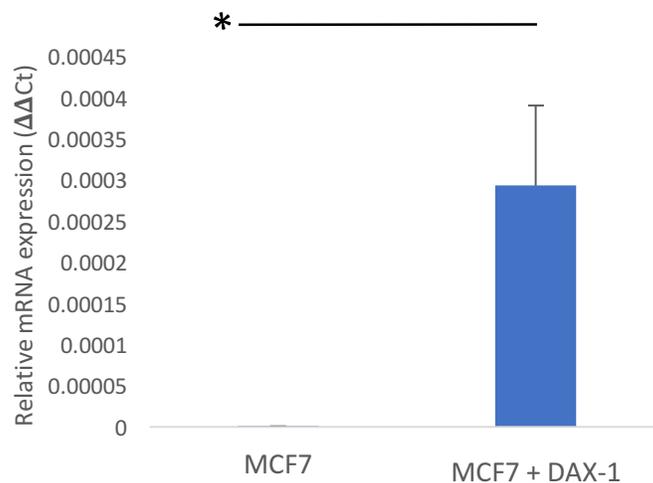


Figure 2.5: Successful DAX-1 Expression in MCF7 Cells by Adenoviral Transduction. DAX-1 was introduced to MCF7 cells using adenoviral (AV) transduction. Wild-type MCF7 cells were used as the control. A. DAX-1: Control shows no detection of DAX-1 cDNA by PCR, strong expression of DAX-1 cDNA is detectable in cells treated with NR0B1 adenovirus. GAPDH: Both the control and AV conditions show bands with strong expression of the housekeeping gene GAPDH. B. qPCR results showing very little DAX-1 expression in wild-type MCF7 cells and significant expression of DAX-1 in MCF7 + DAX-1 cells. $\Delta\Delta Ct$ was used as a measure of relative mRNA expression. Error bars represent standard deviation of the mean (* = $p < 0.05$).

Preliminary assessment of DAX-1s influence on cell proliferation in MCF7 cells

As a preliminary method to assess the effects of DAX-1 on MCF7 cell proliferation, we performed cell counts using MCF7 and MCF7 + DAX-1 cells as well as MCF10A cells, as the control. A known number of cells were plated in serum free medium to halt cell growth, synchronizing their cells cycles. MCF + DAX-1 cells were treated with DAX-1 adenovirus added 24 hours after plating. Cells were then

switched to complete medium and cultured for an additional 72 hours and cell counts were performed using a Neubauer hemocytometer (Figure 2.6).

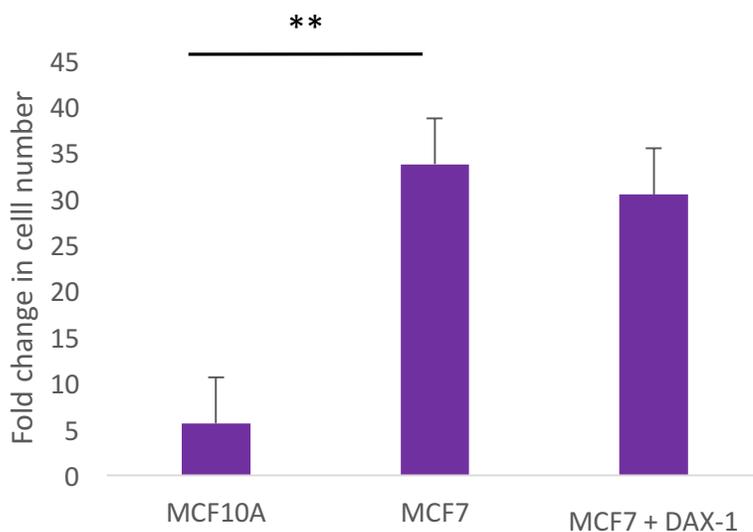


Figure 2.6: Fold change in cell number after 72 hours culture, when DAX-1 is expressed in MCF7 cells. MCF10A cell numbers increased around 5-10 fold, MCF7 cells number increased around 35-40 fold and MCF7 + DAX-1 cell numbers increased around 30-35 fold. Only the fold difference between the MCF10A cells and the MCF7 cells were statistically significant. Error bars represent standard deviation of the mean (** = $p < 0.005$).

MCF10A cells are the least proliferative at only a 5-10 fold increase after 72 hours culture; MCF7 cells were the most proliferative with a 35-40 fold increase in cell numbers after 72 hours; and MCF7 + DAX-1 cells showed a slightly reduced rate of proliferation (30-35 fold increase). These results fit with expectations. That is, normal breast cells are slowly proliferating while the breast cancer cells are growing much more aggressively. When DAX-1 is introduced the breast cancer cells, there is a detectable decrease in the proliferation rate, consistent with what we know from the literature and previous work from our own laboratory that DAX-1 is correlated with smaller tumor size^[53].

Knockout of DAX-1 in human epithelial breast cells (MCF10A) by CRISPR-Cas9

After demonstrating that DAX-1 appears to have a suppressive effect on MCF7 cells, we wanted to next examine the effect of removing DAX-1 from our MCF10A control cell line. Therefore, to knockout DAX-1 expression in MCF10A cells, which normally express DAX-1, we used the CRISPR/Cas9 system. This technique is based on an adaptive immune defense mechanism used by archaea and bacteria to degrade foreign genetic material (Appendix II). The CRISPR/Cas9 system was only recently developed as a genome-editing tool in 2012^[104], but has been quickly adopted due to its simplicity, high efficiency and versatility when compared with other genome editing tools such as, ZFNs and TALENs.

Expression levels of GAPDH (control housekeeping gene) and DAX-1 were examined by PCR (Figure 2.7). Successful knockdown was confirmed by the absence of DAX-1. Control MCF10A cells expressed high levels of DAX-1 whereas the CRISPR/Cas9 cells did not express any DAX-1.

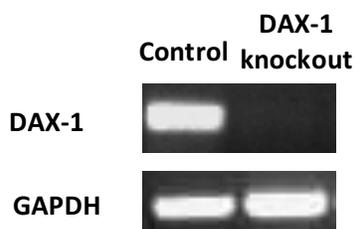


Figure 2.7: Confirmation of DAX-1 Knockout in MCF10A cells by CRISPR/Cas9 system. The control lane demonstrates strong levels of DAX-1 expression in the MCF10A cells whereas the DAX-1 K.O. cells have no detectable levels of DAX-1 expression. The housekeeping gene GAPDH was used as a control and demonstrates strong expression in both samples

Due to cell line contamination and circumstances out with our control, there were not enough stock cells to carry out any further experiments with this cell line by the time this thesis was written.

Knockdown of DAX-1 in human epithelial breast cells (MCF10A) by RNAi

As an alternative to total DAX-1 knockout with CRISPR, we used RNAi technology to knockdown DAX-1 expression in MCF10A cells. This method is less effective than CRISPR in that it only knocks down gene expression rather than completely blocking it, and the effects of siRNA are only transient as opposed to CRISPR where the knockout is permanent. However, siRNA technology is sufficient for knockdown of DAX-1 in the experiments we performed as they were short term experiments only lasting for a few days (Appendix III).

MCF10A cells were plated in serum free medium 24 hours prior to transfection. 100nM siDAX-1, was introduced to the cells using lafectine RU50 as the transfection reagent. Figure 2.8 shows qPCR results confirming successful knockdown of DAX-1 in MCF10A cells. These cells will be referred to as MCF10A – DAX-1 for the remainder of this thesis.

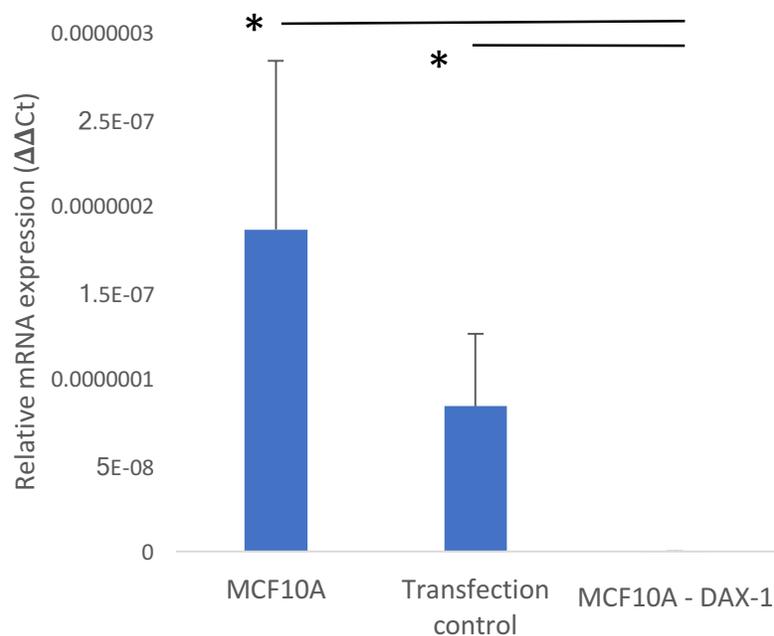


Figure 2.8: Confirmation of DAX-1 Knockdown by siRNA. qPCR results showing DAX-1 expression in wild-type MCF10A cells and the lafectine only transfection control, and no expression of DAX-1 in MCF10A - DAX-1 cells. $\Delta\Delta Ct$ was used as a measure of relative mRNA expression. Error bars represent standard deviation of the mean (* = $p < 0.05$).

DAX-1s influence on both normal (MCF10A) and cancerous (MCF7) breast cell proliferation: EdU Assay

To obtain a less arbitrary measure of cell proliferation than the cell counts described above, we used an EdU HCS assay (Appendix IV). The reason for choosing this method over BrdU, for example, is that the EdU method is simple and efficient, requiring no denaturation steps or harsh treatments, it is gentle on samples giving better preservation of cell morphology, antigen structure and DNA integrity, and it provides consistent results.

Cells manipulated to induce or knockdown DAX-1 were incubated with EdU for 24 hours. Cells were then fixed, permeabilized and the Click-iT[®] performed labelling the newly synthesized DNA with Alexa Fluor[®] 488 which is a green fluorescent dye. Slides were then visualized and analyzed under Keyence BZ-9000E microscope with the BZ viewer and analyzed using BZ analyzer software. Figure 2.9 shows the results of these experiments:

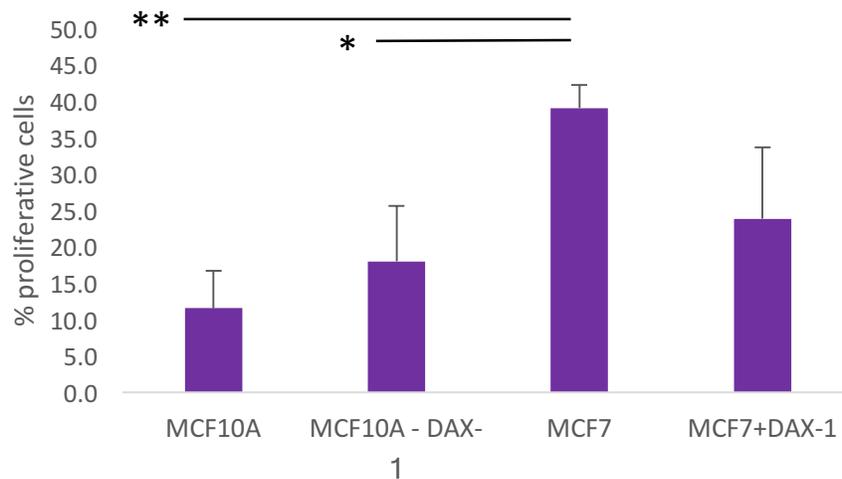


Figure 2.9: MCF10A and MCF7 cell proliferation +/- DAX-1 expression. MCF10A cells had the lowest percentage of proliferating cells, < 20%. MCF10A -DAX-1 cells have a higher percentage of proliferating cells, 20-25%. MCF7 cells had the highest percentage of proliferative cells, ~ 40%. MCF7 + DAX-1 cells had a lower percentage proliferative cells when compared with MCF7 cells, 25-35%. Error bars represent standard deviation of the mean (* $p < 0.05$) (** $p < 0.005$).

MCF10A cells were found to be the least proliferative out of all four cell types with less than 20% of cells staining positive for EdU. MCF10A – DAX-1 were more proliferative at around 20-25%. MCF7 cells were highly proliferative with around 40% of cells staining positive for EdU. Finally, MCF7 + DAX-1 cells were more proliferative than either of the MCF10A populations, but less than the MCF7 alone cells, with 25-35% proliferation. There were significant ($p < 0.05$) differences between the percentage of proliferating cells in the MCF7 group and the MCF10 – DAX-1 group, and highly significant ($p < 0.005$) differences between MCF7 and MCF10A cell proliferation. Removing DAX-1 from normal breast cells results in an increase in cell proliferation and that addition of DAX-1 to breast cancer cells not normally expressing DAX-1 results in a suppression of cell proliferation.

DAX-1 influence on breast cancer gene expression in human adenocarcinoma (MCF7) cells

In an attempt to gain a clearer understanding of the mechanism involved in the suppression of cancer cell proliferation when DAX-1 is present, we performed a qPCR array analysis. The array contained 84 breast cancer related genes along with controls. Using wild type MCF7 cDNA as our baseline control compared with MCF7 + DAX-1 cDNA as our model, we obtained information on the changes in breast cancer gene expression when DAX-1 is overexpressed. We found significant downregulation of seven genes, including the cell cycle regulators *CCND1*, *CCNA1* and *SFN*, growth factors *VEGFA*, *EGF*, and *ABCBI*. Only one gene was found to be upregulated, the apoptosis regulator *BCL2* (Figure 2.10).

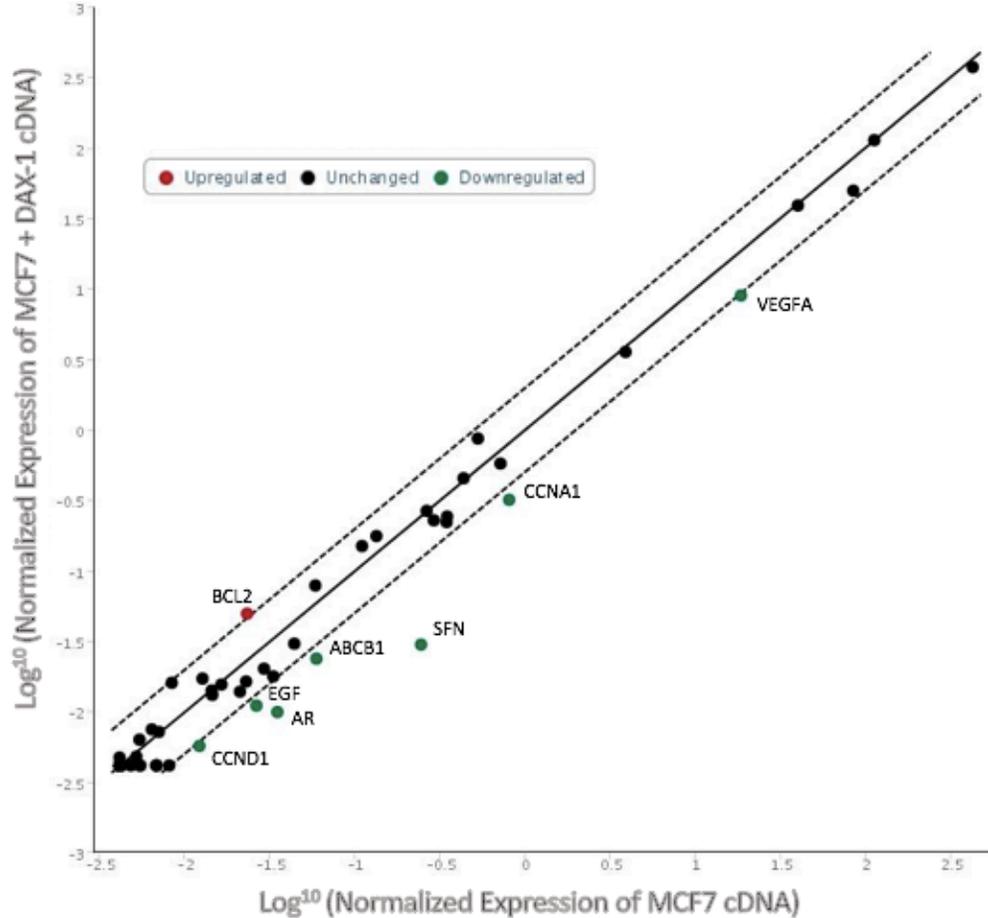


Figure 2.10: Changes in breast cancer gene expression when DAX-1 is overexpressed in MCF7 cells. Scatter plot showing Log^{10} values for normalized expression of breast cancer genes in MCF7 and MCF7 + DAX-1 cDNA. The central line indicates unchanged gene expression. The dotted line represents a twofold change in gene expression. Red dots represent gene upregulation and green dots represent gene down regulation. BCL2 was found to be upregulated and ATP binding cassette subfamily B member 1 (*ABCB1*), androgen receptor (*AR*), cyclin A1 (*CCNA1*), cyclin D1 (*CCND1*), epidermal growth factor (*EGF*), stratifin (*SFN*) and vascular endothelial growth factor (*VEGFA*) were found to be downregulated.

BCL2 expression increased 2-fold and the majority of the downregulated genes showed a two- to four-fold decrease in gene expression. *SFN* had the most dramatic downregulation decreasing eight-fold in response to DAX-1 overexpression (Figure 2.11).

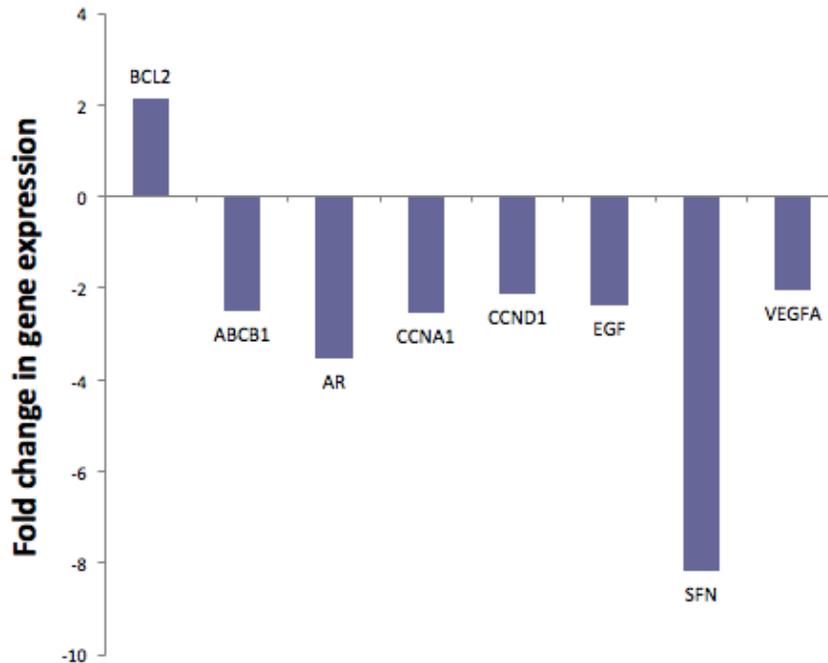


Figure 2.11: Fold change in breast cancer gene expression when DAX-1 is overexpressed. Data from qPCR array showing up- and downregulation of breast cancer genes in response to overexpression of DAX-1 in MCF7 cells.

Expression of ER α in human epithelial breast cells (MCF10A) by plasmid transfection.

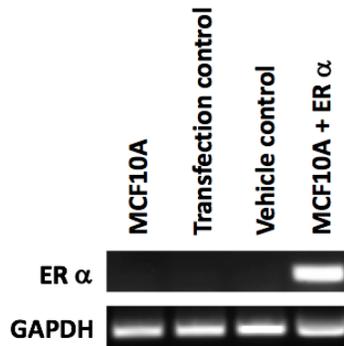
We next wanted to explore the influence of DAX-1 in breast cell proliferation when ER α is inhibited. However, in normal breast tissue, ER α is expressed at very low levels, if at all in the majority of women ^[105]. We wanted to remain consistent with our MCF10A normal breast cancer cells line as the control but they do not express ER α . Consequently, we induced ER α expression in MCF10A cells before further manipulation.

Expression of ER α in MCF10A cells was achieved using Lafectine RU50 as the lipophilic transfection reagent (Figure AV). Cells were transfected with either an empty plasmid (VC - vehicle control) or plasmid containing wild-type ER α DNA sequence. Following transfection, cells were incubated for 24 hours before further manipulation.

Expression levels of GAPDH (control housekeeping gene) and ER α were examined by PCR and qPCR (Figure 2.12). As expected, ER α was not detected in the control MCF10A cells, nor was it found

in the lafectine only control cells or the empty plasmid control. Successful transfection is shown by the strong expression of ER α in the MCF10A + ER α cells.

A.



B.

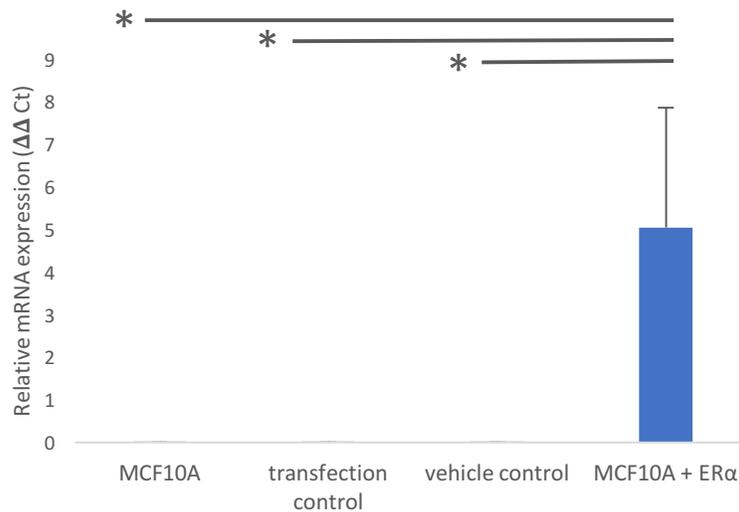


Figure 2.12: Confirmation of ER α expression in MCF10A cells after plasmid transfection. A. PCR results showing no expression of ER α in MCF10A cells or the transfection and vehicle controls. Successful expression of ER α after plasmid transfection is seen in the last two samples. GAPDH was used as a control. B. qPCR results showing no ER α expression in wild-type MCF10A cells, transfection control and vehicle control, and ER α expression in MCF10A + ER α cells. $\Delta\Delta Ct$ was used as a measure of relative mRNA expression. Error bars represent standard deviation of the mean (* = $p < 0.05$).

Suppression of ER α in human epithelial breast cells (MCF10A) and human adenocarcinoma cells (MCF7) by the total estrogen antagonist, Fulvestrant.

To suppress ER α we used Fulvestrant (aka ICI 182, 780 or Faslodex[®]), which is a selective estrogen receptor degrader (SERD) used to treat late stage ER-positive metastatic breast carcinomas. Upon binding to ER α , Fulvestrant induces a misfolding of the protein leading to increased surface hydrophobicity and subsequently degradation of the receptor by the cells own mechanisms^[106].

DAX-1 influence on breast cancer cell proliferation when ER α activity is inhibited

All four cells types discussed above were treated with Fulvestrant in dose response experiments, where increasing concentrations of the drug from 10pM to 1 μ M dilute in DMSO, untreated (UT) control cells were given DMSO only. Cells were cultured with the drug for 72 hours before being collected for staining and analysis using the EdU HCS assay. The same trends across the four cell types are seen in the untreated cells as were found in the initial EdU HCS experiments (Figure 2.13). No significant differences were seen in the proliferation rates of the MCF10A and MCF10A – DAX-1 cells when Fulvestrant was added. MCF7 cells again showed the highest rate of proliferation in the untreated cells but when the drug was added a marked decrease in proliferation was seen that continued to drop when cells were exposed to higher concentrations of Fulvestrant. MCF7 + DAX-1 showed less cell proliferation in the UT cells compared to the MCF7 only cells, and an overall decrease in the rate of proliferation when Fulvestrant is added. However, the decrease did not change in a dose-dependent manner.

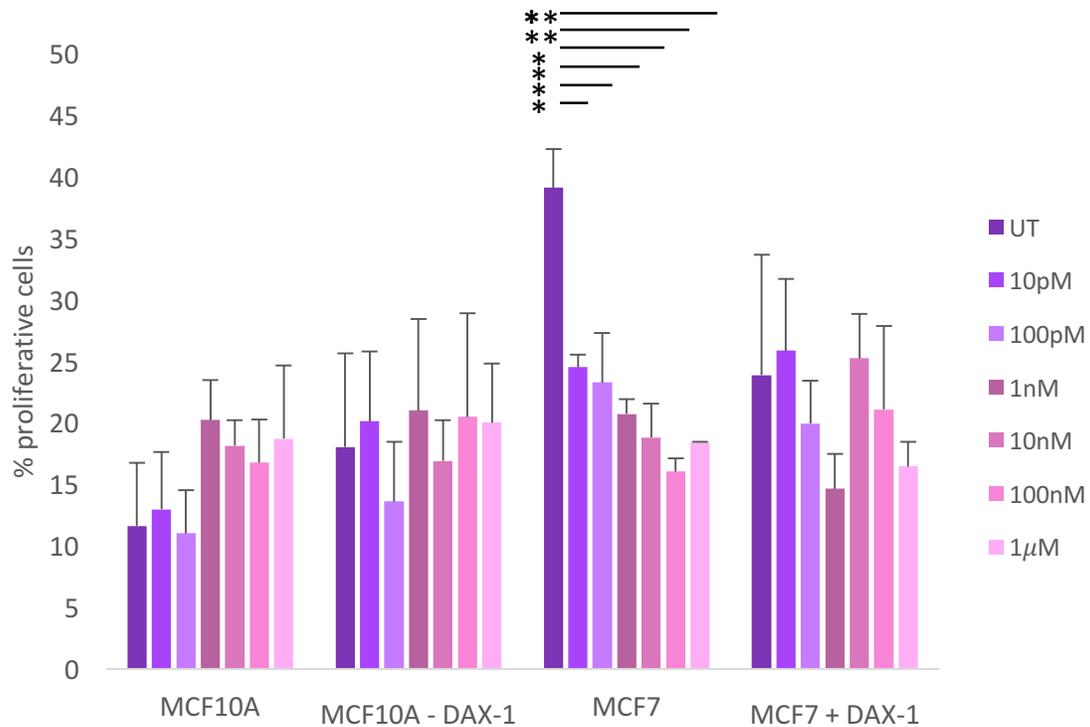


Figure 2.13: Proliferation after 72 hours in culture with increasing concentrations of Fulvestrant. Percentage of proliferating cells, determined through EdU HCS assay staining, of normal and cancerous breast cells with/without DAX-1 expression cultured for 72 hours with increasing concentrations of Fulvestrant. UT = untreated. Error bars represent standard deviation of the mean (* = $p < 0.05$, ** = $p < 0.005$).

DAX-1 influence on estrogen signaling gene expression in human adenocarcinoma cells (MCF7) cells

To further investigate estrogen receptor interactions with DAX-1 in breast cancer, we performed a qPCR array analysis for 84 estrogen signaling genes. Using wild type MCF7 cDNA as our baseline control compared with MCF7 + DAX-1 cDNA as our model, we obtained information on the changes in estrogen signaling gene expression when DAX-1 is overexpressed (Figure 2.14). We found significant downregulation of *FOS* and WNT1 inducible signaling pathway 2 (*WISP2*). Upregulation was seen in the cell cycle inhibitor amyloid beta precursor protein binding family B member 1 (*APBB1*); *BCAR1*, which also functions in cell cycle control; the tumor suppressor candidate caveolin 1 (*CAV1*); creatine kinase B (*CKB*), an enzyme involved in energy homeostasis; cathepsin D (*CTSD*); estrogen receptor binding site associated, antigen 9 (*EBAG9*); estrogen receptor 1 (*ESR1*). As well as the transcription factors MAF bZIP

transcription factor F (*MAFF*); proline, glutamate and leucine rich protein 1 (*PELPI*) and X-box binding protein (*XBPI*). Also, transforming growth factor beta 3 (*TGFB3*).

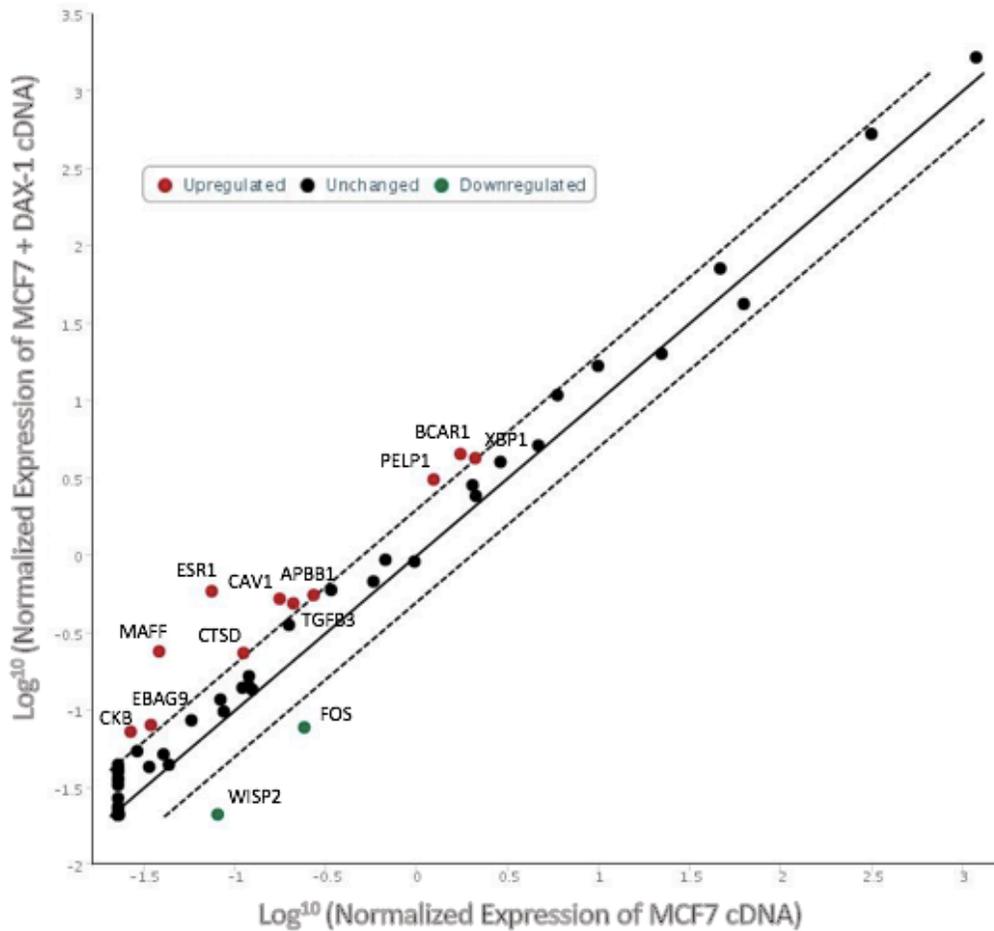


Figure 2.14: Changes in estrogen signaling gene expression when DAX-1 is overexpressed in breast cancer (MCF7) cells. Scatter plot showing Log¹⁰ values for normalized expression of breast cancer genes in MCF7 and MCF7 + DAX-1 cDNA. The central line indicates unchanged gene expression. The dotted line represents a twofold change in gene expression. Red dots represent gene upregulation and green dots represent gene down regulation. *FOS* and WNT1 inducible signaling pathway 2 (*WISP2*) were found to be downregulated whereas amyloid beta precursor protein binding family B member 1 (*APBB1*), *BCAR1*, caveolin 1 (*CAVI*), creatine kinase B (*CKB*), cathepsin D (*CTSD*), estrogen receptor binding site associated, antigen 9 (*EBAG9*), estrogen receptor 1 (*ESR1*), MAF bZIP transcription factor F (*MAFF*), proline, glutamate and leucine rich protein 1 (*PELPI*), transforming growth factor beta 3 (*TGFB3*), X-box binding protein (*XBPI*).

While most of these genes were upregulated 2- to 4-fold, *ESR1* and *MAFF* had the greatest increase at around 6- to 8-fold. *FOS* and *WISP2* were both down regulated by approximately a 4-fold decrease (Figure 2.15).

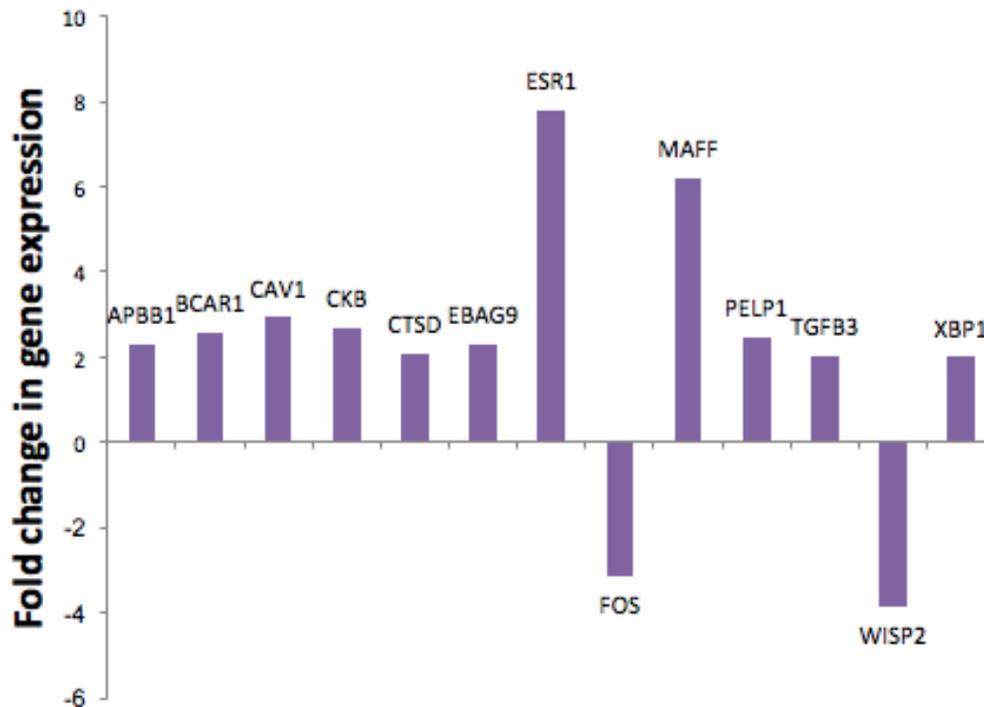


Figure 2.15: Fold change in estrogen signaling gene expression when DAX-1 is overexpressed. Data from qPCR array showing up- and downregulation of estrogen signaling genes in response to overexpression of DAX-1 in MCF7 cells.

Regulation of metastatic genes by DAX-1 in human adenocarcinoma cells (MCF7) cells

To investigate DAX-1 interactions with known metastatic genes, we performed a qPCR array analysis including 84 metastatic genes along with controls. Using wild type MCF7 cDNA as our baseline control compared with MCF7 + DAX-1 cDNA as our model, we obtained information on the changes in metastatic gene expression when DAX-1 is overexpressed. We found significant downregulation of ITGB3, VEGFA, KISS1R, RORB and PTEN gene expression (Figure 2.16 and 2.17). ITGB3 (integrin subunit beta 3) is a gene encoding for a transmembrane receptor known to participate in cell adhesion as

well as cell surface mediated signaling. Intriguingly, it was the most significantly downregulated gene at 5.53-fold. VEGFA (vascular endothelial growth factor A) is a growth factor involved in multiple metastatic processes including, cell adhesion, regulation of the cell cycle, cell growth and proliferation. It was found to be downregulated by 2.53-fold. KISS1R (KISS1 receptor) is a G-protein coupled receptor involved in cell growth and proliferation. The receptor binds metastin, a peptide encoded by the metastasis suppressor gene KISS1. KISS1R was down regulated by 2.77-fold. RORB (RAR related orphan receptor B) is an orphan nuclear receptor similar to DAX-1. It acts as a transcription factor with the ability to bind to HREs as a monomer or homodimer to enhance expression of downstream genes. It was downregulated 4.28-fold. PTEN (phosphatase and tensin homolog) is a cell cycle regulator identified as a tumor suppressor that is mutated in a large number of cancers at high frequency. It was downregulated by 2.07-fold. No upregulation of any of the genes was seen. The greatest fold change was seen in ITGB3 at 5.5-fold, then RORB at 4.5-fold. KISS1R, PTEN and VEGFA were all downregulated by 2-3-fold. (Figure 2.17)

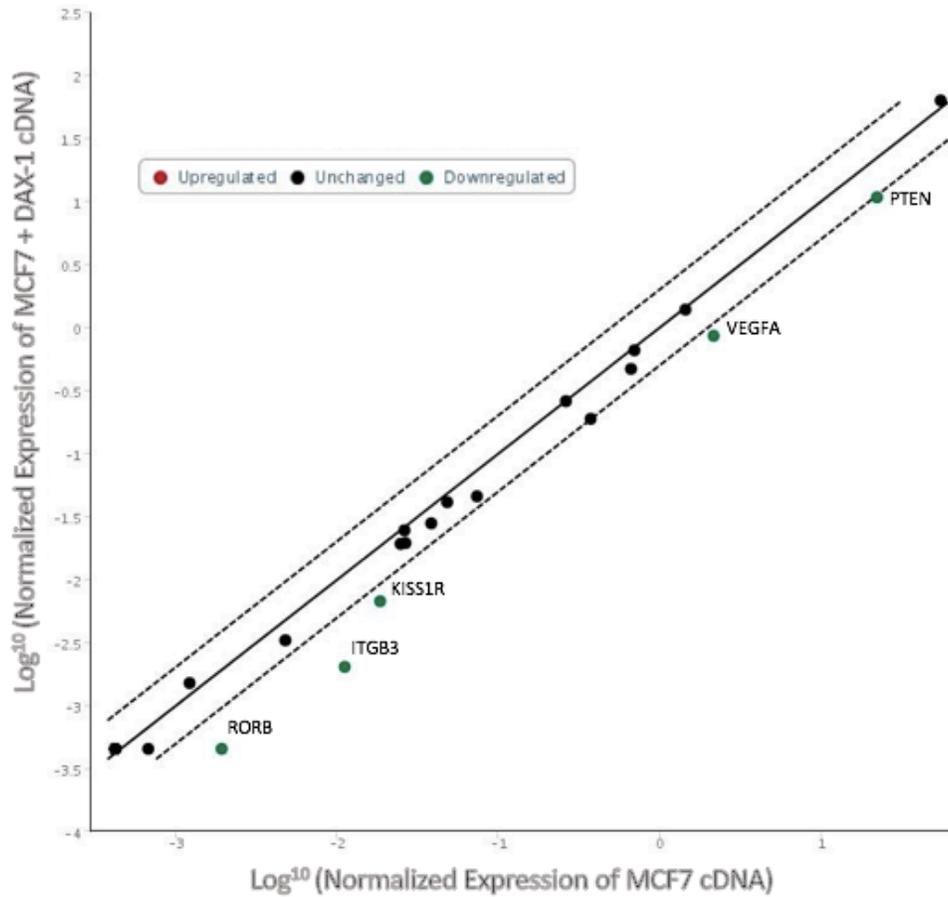


Figure 2.16: Changes in metastatic gene expression when DAX-1 is overexpressed in breast cancer (MCF7) cells. Scatter plot showing Log_{10} values for normalized expression of breast cancer genes in MCF7 and MCF7 + DAX-1 cDNA. The central line indicates unchanged gene expression. The dotted line represents a twofold change in gene expression. Red dots represent gene upregulation and green dots represent gene down regulation. Significant downregulation was seen in integrin subunit beta 3 (*ITGB3*), *KISS1* receptor (*KISS1R*), phosphatase and tensin homolog (*PTEN*), RAR related orphan receptor B (*RORB*) and vascular endothelial growth factor A (*VEGFA*).

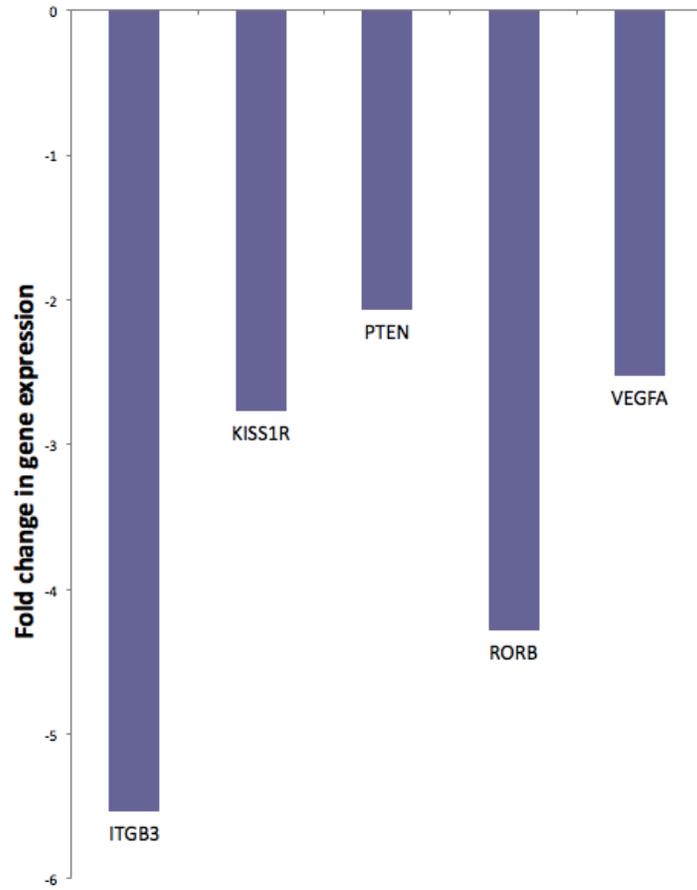


Figure 2.17: Fold change in metastatic gene expression when DAX-1 is overexpressed. Data from qPCR array showing downregulation of metastatic genes in response to overexpression of DAX-1 in MCF7 cells.

Discussion

DAX-1 acts as a repressor of breast cancer cells proliferation through downregulation of gene expression

Results from the cell count and EdU experiments show that when DAX-1 is overexpressed in breast cancer (MCF7) cells, a decrease in cell proliferation occurs. The mirror image effect is seen in normal breast (MCF10A) cells when DAX-1 expression is removed. These findings fit with the previous reports of DAX-1 being correlated with smaller tumor size and as a repressor of breast cancer cell proliferation mechanisms^[53].

Results from gene analysis obtained from the qPCR arrays provided corroborating findings of prior research specifically DAX-1 is mainly a suppressor of breast cancer promoting genes. When DAX-1 was overexpressed in the MCF7 cells, a downregulation of the cyclin D1 gene (*CCND1*) was seen. This further supports previous studies showing that DAX-1 represses cyclin D1 by several different mechanisms, including acting as a co-repressor with ER α inhibiting its promotion of *CCND1* expression and the recruitment of DAX-1 by AR to suppress cyclin D1 expression^[49].

The qPCR array also showed downregulation of two other genes encoding for cell cycle regulators; *CCNA1* and *SFN*, not previously linked with DAX-1. *CCNA1* encodes for Cyclin A1 which binds both CDK2 and CDC2 kinases, giving two distinct kinase activities, one appearing in S phase, the other in G2, and thus regulating separate functions in cell cycle. This cyclin was found to bind to important cell cycle regulators, such as Rb family proteins, transcription factor E2F-1, and the p21 family proteins. *SFN* encodes for Stratifin which is a cell cycle checkpoint protein that binds to translation and initiation factors and functions as a regulator of mitotic translation.

In addition to cell cycle regulators we also found downregulation of several growth factor encoding genes, namely *VEGFA*, *EGF*, and *ABCBI*. *VEGFA* encodes for vascular endothelial growth factor A,

which induces proliferation and migration of vascular endothelial cells, and is essential for both physiological and pathological angiogenesis. This gene is upregulated in many known tumors and its expression correlates with tumor stage and progression. *EGF* encodes epidermal growth factor, which acts as a potent mitogenic factor that plays an important role in the growth, proliferation and differentiation of numerous cell types. *ABCB1* encodes the ATP binding cassette subfamily B member 1 protein is known to be responsible for decreased drug accumulation in multidrug-resistant cells and often mediates the development of resistance to anticancer drugs.

The downregulation of AR by DAX-1 identified by our qPCR array has been previously described^[107]. Holter *et al.* demonstrated that DAX-1 inhibits ligand-dependent transcription activation as well as interactions between the N- and C- terminal regions of AR^[107]. Other studies have also shown DAX-1 to interact with and function as a negative coregulatory of AR^[108, 109]. However, Lanzino *et al.* found that in MCF7 cells, DAX-1 binds to the androgen responsive region of the cyclin D1 promoter along with AR and HDAC1 forming a repressor complex inhibiting *CCND1* transcription^[49].

The only upregulated gene from this array was the apoptosis regulator *BCL2* which has been shown to be a positive prognostic marker in ER positive and triple negative breast cancers^[110]. Studies have shown DAX-1 to be associated with BAD/BCL2 ratio and its overexpression in MCF7 cells induces apoptosis^[67]

All of the genes highlighted from our analysis support DAX-1's role as a suppressor of breast cancer proliferation and other closely related cancer mechanisms such as apoptosis. In addition, *CCNA1*, *SFN*, *VEGFA*, *EGF* and *ABCB1* have all been identified as potential novel targets of DAX-1 that warrant further investigation.

Breast cancer cell proliferation in the absence of ER α is no further suppressed by the presence of DAX-1

Although we found that proliferation decreases when DAX-1 is overexpressed in MCF7 cells, there does not appear to be any additive effect of DAX-1 when ER α activity is inhibited. Given that Fulvestrant binds antagonistically to ER α and results in its ultimate degradation by the cell ^[105], we predict that the lack of anti-proliferative activity seen in the presence DAX-1 is due to it being unable to bind to ER α and have its suppressive effects. If this is the case then the use of SERDs or any other therapeutic that alters the structure or ER α , as a treatment for breast cancer may inadvertently be cancelling out the potential beneficial effect of other cellular components, such as DAX-1. More research is needed to determine the consequences of ER α disruption by anticancer drugs and potentially the development of improved adjuvant therapies harnessing the advantageous properties of breast cancer suppressors such as DAX-1.

DAX-1 upregulates many estrogen signaling genes in breast cancer (MCF7) cells

Our results from the estrogen signaling array show DAX-1 to upregulate the majority of genes it influences. At first sight this a contradictory outcome to what we know from the literature where DAX-1 acts as a co-repressor in the majority of its interactions. However, several of these genes are involved in repression of proliferation and tumor progression (e.g. *APBB1* has a role in regulating transcription and has been observed to breast cancer progression by downregulating thymidylate synthase expression ^[111]). *CAVI* encodes a protein found in most cells types and is a tumor suppressor gene candidate. *CAVI* along with *NROB1* have both been identified as targets of EWS/FL1 in Ewing sarcoma tumors but has not been linked in breast cancer ^[112]. *BCAR1* encodes an adaptor protein that functions in multiple cellular pathways, including cell motility, apoptosis and cell cycle control, and its dysregulation has been associated with effects in a wide range of different pathways including in breast cancer. *CTSD* mutations

have been shown to be involved in the pathogenesis of breast cancer. *TGFB3* encodes for a secreted ligand of the TGF β superfamily of proteins whose binding leads to the recruitment and activation of SMAD family transcription factors and is known to have a role in cell differentiation and possibly wound healing. Therefore, the upregulation of these genes could be directly involved in the mechanisms used by DAX-1 to have its protective effects in breast cancer. In addition, the downregulated gene *FOS* forms part of the transcription factor complex AP-1 and has been implicated as a promotor of cell proliferation, differentiation and transformation. Therefore, the downregulation of *FOS* in the presence of DAX-1 also conforms with DAX-1 being a suppressor of cell proliferation. *CKB* encodes for a cytoplasmic enzyme involved in energy homeostasis and it has not been previously linked with DAX-1. However, *CKB* has also been shown to directly mediate cell invasion and lung metastasis in breast cancer. It may be that DAX-1 is involved in *CKB*'s metastatic pathway.

The MAFF protein is a transcription repressor and has been shown to play a role in cellular stress response and to promote metastasis in breast cancer. PELP1 and XBP1 are both transcription factors, PELP1 is thought to be involved in the progression of several cancers and XBP1 is an ER coactivator and has been linked with SERM resistance in multiple ER positive cells lines. *EBAG9*, is an estrogen response gene whose protein is a tumor-associated antigen that is expressed at high frequency in breast cancer. DAX-1 upregulation of these genes is difficult to explain, but highlights the complexity of the pathways involved in this disease.

WISP2 encodes a member of the WNT1 inducible signaling pathway (WISP) protein subfamily; the gene may be downstream in the WNT1 signaling pathway that is relevant to malignant transformation. The WNT1 pathway is a complex signaling pathway involving a signaling transduction cascade that is a key mediator in both embryonic development and in certain cancers^[113, 114]. There is currently little published evidence linking DAX-1 with either WISP2 or the WNT1 pathway. However, studies from our

laboratory have highlighted DAX-1 as a negative regulator of Dickkopf 1 (*Dkk1*) gene expression. *Dkk1* is known to interact with components of the WNT1 pathway in mouse embryonic stem cells and prostate cancer cells^[115, 116]. This link with DAX-1 and the WNT1 signaling pathway and the significant 4-fold down regulation seen in *WISP2* expression when DAX-1 is overexpressed in breast cancer cells, highlights *WISP2* it as a potential target for DAX-1 that warrants further investigation.

DAX-1 downregulates several metastatic genes in breast cancer (MCF7) cells

The role of DAX-1 in breast cancer metastasis has not been well studied. It has been associated with earlier disease stage which leads us to hypothesize that the loss of DAX-1 seen in later stage breast cancers may contribute to the tumors ability to metastasize. We compared metastatic gene expression levels of late stage breast cancer cells (MCF7) expressing negligible levels of DAX-1, with metastatic gene expression levels of the same MCF7 cells treated to exogenously express DAX-1.

Of the 86 metastatic genes analyzed we found that overexpression of DAX-1 resulted in significant downregulation of 5 genes; *ITGB3*, *KISS1R*, *PTEN*, *RORB* and *VEGFA*. No genes were found to be upregulated, consistent with the previously identified role of DAX-1 as primarily a repressor of gene transcription.

ITGB3 encodes for integrin subunit β 3 (also identified as β 3 integrin) that is known to participate in cell adhesion as well as cell-surface mediated signaling. It has been found to be essential for breast cancer epithelial-mesenchymal transition (EMT) and metastasis^[117-119]. Through *in vivo* studies, it has been found that the functional disruption of β 3 integrin through silencing with siRNA, alleviated triple negative breast cancer primary tumor burden and significantly inhibited metastasis^[120]. DAX-1 and *ITGB3* have not previously been linked, but our study showing downregulation of *ITGB3* in response to DAX-1 over-expression in breast cancer cells highlights this gene as a potential target for further investigation.

KISS1R encodes for the KISS1 receptor, which is a galanin-like G protein-coupled receptor that binds metastin, a peptide encoded by the metastasis suppressor gene KISS1. Interestingly, the tissue distribution of the expressed gene suggests that it is involved in the regulation of endocrine function, and this is supported by the finding that this gene appears to play a role in the onset of puberty^[121]. Mutations in this gene have been associated with hypogonadotropic hypogonadism, one of the disorders where DAX-1 was first identified^[19, 122].

PTEN encodes for phosphatase and tensin homolog that functions as a tumor suppressor. It acts by negatively regulating the AKT/PKB signaling pathway, which promotes cell survival and growth in response to extracellular signals, and is mutated in a large number of cancers at high frequency including breast cancers^[123]. Other studies have shown that methylation of its promoter region is also a major mechanism leading to its decreased expression in breast cancer cells^[124]. *KISS1R* and *PTEN* downregulation by DAX-1 over-expression is difficult to explain with our current understanding of their mechanisms of action. Further investigation into their association within the context of breast cancer is needed to understand their interactions more fully.

RORB encodes for RAR (retinoic acid receptor) related orphan receptor B, a member of the NR1 subfamily of nuclear hormone receptors (NHR). It is a DNA-binding protein that can bind as a monomer or as a homodimer to hormone response elements upstream of several genes to enhance their expression. It is known to regulate genes involved in the circadian rhythm, disruption of which has been linked to breast cancer^[125]. It has also been identified as a suppressor of metastasis in ovarian carcinomas^[126]. Beside these studies, there has been little research of the role of RORB plays in breast cancer metastasis or its interactions with DAX-1. We do know, however, that DAX-1 binds to other NHRs including ER α and SF1 through its LXXLL motifs in the N-terminal region. As RORB is a NHR, it is feasible that it could play a role as a transcription factor interacting with DAX-1 to regulate gene expression.

VEGFA, also already identified as a gene that is downregulated by DAX-1 in Chapter 2, encodes for vascular endothelial growth factor A. This gene is a member of the PDGF/VEGF growth factor family. It induces proliferation and migration of vascular endothelial cells and is essential for both physiological and pathological angiogenesis. Upregulation of the gene is seen in many known tumors and its expression is correlated with tumor stage and progression^[127, 128]. DAX-1 and *VEGFA* have not been linked previously in the context of breast cancer.

Our study has found conflicting results with DAX-1 expression in breast cancer cells showing a downregulation of genes involved in both metastasis promotion and suppression. The process of metastasis is a complex one involving multiple different cell functions and molecular pathways and as can be seen from our results, DAX-1 appears to be involved in more than one of these processes. In the first instance, it appears to be involved in promotion of metastasis by suppressing *KISS1R* which binds with metastin, a tumor suppressor, however the *KISS1R*/metastins mechanism of action is not well understood. In addition, *PTEN*, known to act as tumor suppressor, is also downregulated by DAX-1. However, *PTEN* is part of a complex signaling pathway that is highly regulated by multiple mechanisms. DAX-1 could be interacting with any number of factors involved in these processes, possibly having beneficial effects that override the effects of downregulating *KISS1R* and *PTEN* expression. Downregulation of *ITGB3* and *VEGFR* fits more easily with what we know about DAX-1 acting as a suppressor of metastasis in other cancers. *RORB* is an interesting NHR not previously linked with DAX-1, that may be involved in a possible feedback loop with DAX-1. However, for all of these genes, it is not known if DAX-1 is acting as a transcription factor to directly suppress their expression or if it is influencing some other pathway or factor that then subsequently is resulting in the genes' downregulation. Our results have identified several genes that are downregulated by DAX-1 in the MCF7 cells. Further

investigation of these genes is necessary in order to untangle their role breast cancer metastasis in the context of DAX-1.

Chapter 3

DAX-1 and other receptor status during progressive stages of invasive ductal carcinoma

Introduction

Invasive ductal carcinoma (IDC) constitutes approximately 80% of all breast cancers ^[3]. As the name suggests, this type of breast cancer begins in the ducts and then breaks through the basement membrane to invade the surrounding breast tissue. Over time, IDC will then metastasize to the lymph nodes and other areas of the body. More than 250,000 women will be diagnosed with invasive breast cancer each year and most will be IDC. Women over 55 years old are most commonly diagnosed IDC; however, it also affects around 1 in 1,000 men ^[3].

The breast cancer stage describes the extent of the disease and is one of the most important factors in determining the prognosis and treatment options. Table 3.1 give details of each stage based on the American Joint Committee on Cancer (AJCC) TNM scoring system. The TNM system takes into account: the size of the tumor (**T**) and if it has spread to nearby areas; whether the cancer has reached nearby lymph nodes (**N**); and whether the cancer has metastasized (**M**).

Table 3.1: Breast Cancer Stages. (adapted from the American Cancer Society, Stages of Breast Cancer Table)¹¹

<i>Stage</i>	<i>TNM score</i>	<i>Description</i>
<i>0</i>	Tis, N0, M0	Cancer has not spread to lymph node or distant sites.
		Includes, <i>ductal carcinoma in situ</i> (DCIS), <i>lobular carcinoma in situ</i> (LCIS), and Paget disease of the nipple
<i>IA</i>	T1, N0, M0	The tumor is 2 cm or less across (T1) and has not spread to the lymph nodes (N0) or metastasized (M0)
<i>IB</i>	T0 or T1, N1mi, M0	The tumor is 2 cm or less across (T0 or T1) with micrometastases in 1 to 3 axillary lymph nodes (N1mi). The cancer has not spread to distant sites (M0)
<i>IIA</i>	T0 or T1, N1 (but not N1mi), M0	The tumor is 2 cm or less across (or is not found) (T1 or T0) and either: Spread to 1-3 axillary lymph nodes and larger than 2 mm across (N1a) OR Tiny amount of cancer found in internal mammary lymph nodes (N1b) OR Both of the above (N1c) The cancer has not spread to distant sites (M0)
		OR
<i>IIB</i>	T2, N0, M0	The tumor is between 2 -5 cm across (T2) but has not spread to the lymph nodes (N0) or metastasized to distant sites (0)
	T2, N1, M0	The tumor is between 2 -5 cm across (T2) and spread to 1-3 axillary lymph nodes and/or tiny amount of cancer found in internal mammary lymph nodes (N1). The cancer has not spread to distant sites (M0)
	OR	
	T3, N0, M0	The tumor is larger than 5 cm across but does not grow into the chest walls or skin (T3). The cancer has not spread to the lymph nodes (N0) or to distant sites (M0)

IIIA

T0 to T2, N2, M0

Tumor is not more than 5 cm across (T0 to T2). It has spread to 4 to 9 axillary lymph nodes, or has enlarged the internal mammary lymph nodes (N2). The cancer has not spread to distant sites (M0)

OR

T3, N1 or N2, M0

The tumor is larger than 5 cm across but does not grow into the chest walls or skin (T3). It has spread to 1 to 9 axillary lymph nodes or to internal mammary lymph nodes (N1 or N2). The cancer has not spread to distant sites (M0)

IIIB

T4, N0 to N2, M0

The tumor has grown into the chest wall or skin (T4), and one of the following applies:

- It has not spread to the lymph nodes (N0).
- It has spread to 1 to 3 axillary lymph nodes and/or tiny amounts of cancer are found in internal mammary lymph nodes on sentinel lymph node biopsy (N1).
- It has spread to 4 to 9 axillary lymph nodes, or it has enlarged the internal mammary lymph nodes (N2).

The cancer hasn't spread to distant sites (M0).

IIIC

any T, N3, M0

The tumor is any size (or can't be found), and one of the following applies:

- Cancer has spread to 10 or more axillary lymph nodes (N3).
- Cancer has spread to the infraclavicular lymph nodes (N3).
- Cancer has spread to above the infraclavicular lymph nodes (N3).
- Cancer involves axillary lymph nodes and has enlarged the internal mammary lymph nodes (N3).
- Cancer has spread to 4 or more axillary lymph nodes, and tiny amounts of cancer are found in internal mammary lymph nodes (N3).

The cancer hasn't spread to distant sites (M0).

IV

any T, any N, M1

The tumor can be any size (any T) and may or may not have spread to nearby lymph nodes (any N). It has spread to distant organs or to lymph nodes far from the breast (M1). The most common sites are the bones, liver, brain or lungs.

It is known that the steroid hormone receptors ER, PR and AR play an important role in cancer progression in IDC patients. It has been shown that expression of ER, PR and HER2 by tumors is associated with disease prognosis. Approximately two thirds of all breast cancer tumors express ER and PR and are known as hormone receptor positive breast cancers. They tend to grow more slowly than those that do not express these receptors. This characteristic and the fact that there are more targeted treatment options available for hormone receptor positive patients, means that in the short term, their prognosis tends to be better than receptor negative patients. HER2 is a growth promoting protein and its overexpression is seen in 1 in 5 breast cancer tumors. HER2 positive cancers tend to grow and spread faster than HER2 negative cancers, but again targeted therapies are available to treat positive tumors ^[1].

Immunohistochemistry staining of breast cancer tissue reveals 73-75% of all breast cancers are ER positive^[1]. ER expression generally coincides with PR expression and is seen in half of all HER2 positive breast cancers. ER and PR interactions with their hormones are known to promote tumor cell proliferation and metastasis. The mechanism of action of ER in breast cancer is complex and as yet, not fully understood. One of the most well documented mechanisms is through circulating estrogens that bind to ERs, leading to receptor dimerization and binding to EREs upstream of specific target genes. As described previously, one well-known target of ER is the cyclin D1 gene, which leads to the activation of cell proliferation pathways^[62, 63]. Other ER interactions are also known to promote growth of breast cancer cells, including targeting expression of signaling components of the insulin-like growth factor system.

Ligand bound ER α has been shown to influence gene expression by binding with other transcription factors and not directly to DNA. In addition, in the absence of estrogen, extracellular signaling can also induce ER-mediated transcription ^[129]. PR acts in a similar way to ligand bound ER, although through different signaling pathways, to exert its proliferative influence in breast cancer cells. Specifically, circulating progesterone binds to PR, activating the receptor to recruit a series of coactivator and corepressors such as SRC-1, SRC-2 and SRC-3, CBP/p300 and others ^[130]. These PR complexes bind to progesterone responsive elements (PRE) in the DNA and initiate transcription of target genes. In one study, high PR expression was correlated with DAX-1 in breast cancer patients ^[51], but no subsequent studies have explored this association any further. ER and PR are screened for in biopsy samples using IHC, where $\geq 1\%$ positive staining is identified as positive ^[3]. When one or both of these receptors are found to be present, the patient is diagnosed as having hormone receptor-positive breast cancer. Hormone receptor-positive breast cancers tend to be less aggressive and have a better short-term outlook than hormone receptor-negative breast cancers, largely because of drugs that have been developed to target the receptors or the hormones themselves.

HER2 is overexpressed in 20-30% of breast cancers. It is associated with more aggressive disease, higher recurrence rate and increased mortality ^[99, 131, 132]. HER2 is member of the human epidermal growth factor receptor family, encoded by the ERBB2 gene. This family of receptors are membrane-bound receptor tyrosine kinases; they all contain extracellular ligand-binding domains, a transmembrane domain, and an intracellular domain capable of interacting with a multitude of signaling molecules. All members of the family exhibit both ligand-bound and ligand-independent activity. HER2 has no known ligand ^[133] and has the ability to heterodimerize with the other members of the epidermal growth factor receptor family. Dimerization results in autophosphorylation of tyrosine kinase residues within the cytoplasmic domain and initiation of several signaling pathways involved in cell proliferation and apoptosis

suppression, including, MAPK, PI3K/Akt, PKC and STAT, among others. Overexpression of HER2 has become one of the hallmark clinical markers screened for in breast cancer patients. Biopsy samples are often tested for HER2 using IHC giving results of either 0, 1+, 2+ or 3+. An assignment of 0-1+ means the cancer is HER2 negative, 2+ is an inconclusive result and further testing with FISH is often required, and 3+ is classed as HER2 positive. There are targeted therapies available for HER2 positive patients, such as trastuzumab (Herceptin[®]).

There are several problems with the current handling and treatment of breast cancers. One being that often patients are screened only once to identify the phenotype of their breast cancer, but studies have shown that ER, PR and HER2 status can change throughout tumor progression ^[134]. Another issue is that although there have been many adjuvant therapies developed attempting to target the breast cancer more specifically based on hormone receptor status and HER2 status, they still tend to be too general and result in unfavorable side effects. For example, selective estrogen receptor modulators (SERMs), including Tamoxifen and Toremifene, block estrogen receptors in breast tissue suppressing estrogen/receptor activity. SERMs, however, upregulate the actions of estrogen in other tissues such as bone, liver and uterine cells and have been shown to lead to uterine cancer, blood clots and stroke. Selective estrogen receptor degraders (SERDs), such as Fulvestrant discussed in Chapter 2, are another hormone receptor targeted therapy. SERDs target all ERs in the body, blocking and damaging the receptor. They have only been approved by the FDA for use in post-menopausal women with late stage metastatic cancer and have been associated with osteoporosis. Aromatase inhibitors (AIs) block the production of estrogen by targeting the aromatase enzyme. Ovarian suppression can be achieved through oophorectomy, LHRH analogs and chemotherapy drugs. Both AIs and ovarian suppression target estrogen production giving rise to symptoms of menopause. More specifically targeted therapies for hormone receptor positive patients include CDK4/6 inhibitors, which block cell division, and Everolimus, which blocks mTOR, a protein

involved in cell proliferation. Both of these targeted therapies are only used to treat post-menopausal women with advanced breast cancer and have severe side effects including low blood cell counts and increased risk of serious infection. HER2 targeted therapies are also available in the form of monoclonal antibodies targeting the receptor and result in the block of ligand binding and tyrosine kinase inhibitors, which prevent phosphorylation and subsequent activation of the signal transduction pathways, leading to apoptosis and decreased proliferation. Overall, these drugs can lead to heart damage and congestive heart failure ^[1].

Another common proliferation marker screened for in many cancers, including breast, is the nuclear protein Ki-67. It is present during all active phases of the cells cycle (G1, S, G2, M) but absent during quiescence (G0). Ki-67 levels are low in G1 and S phases but peak early in mitosis leading to its strong association with cell proliferation and growth. Studies have identified Ki-67 involvement in the early stages of polymerase I-dependent ribosomal RNA synthesis, but beyond this its exact role is obscure and there is little published work on its overall function ^[135, 136]. The Ki-67 labelling index is used to classify tumors as low, intermediate or highly proliferating according to nuclear staining of Ki-67 of $\leq 15\%$, 16-30%, and $>30\%$. Ki-67 status is used to plan patient treatment and as a predictor of response to therapy.

As described in Chapter 1, androgens have been shown to play a protective role in breast cancer and have been directly correlated with DAX-1 expression ^[65, 66]. However, the research available examining androgens and AR actions in breast cancer is limited. From our own findings (Chapter 2) and in a study by Holter *et al.*, AR is downregulated when DAX-1 is overexpressed. Other studies have shown that AR may repress ER through DAX-1 activity. In MCF7 cells (AR/ER α positive), expression of DAX-1 has been shown to be induced by ligand bound AR, and in association with the co-repressor N-CoR, it binds to the SF-1/LRH-1 region of the aromatase promoter suppressing its activity and hence

ER activity^[67]. When ER α positive breast cancer cells were treated with non-aromatizable androgen 5- α -dihydrotestosterone (DHT), DAX-1 was seen to be recruited by AR and mediated a reduction in Cyclin D1 activity slowing proliferation^[49, 67]. From these studies, it is evident that AR and DAX-1 are working together to negatively regulate breast cancer progression; however, the mechanisms by which this occurs remain unclear.

In this chapter, we investigate DAX-1 expression in invasive ductal carcinoma subjects expressing major breast cancer markers. Correlations between DAX-1 expression and the clinical stage were then analyzed, as well as ER, PR, AR, Ki67 and HER2 antigen expression.

Materials and Methods

Immunohistochemistry (IHC)

A breast cancer tissue microarray was obtained from US Biomax inc. (BR1504a) (Figure 3.1). Table 3.2 provides a breakdown of the patient demographics. The 4 μ m sections were deparaffinized and subjected to heat induced epitope retrieval. Immunohistochemical staining was then performed on the array using the following primary antibodies: anti-NR0B1/DAX1 raised in rabbit (ab60144 Abcam, SF, CA, USA) and anti-Androgen Receptor (AR V7 specific) raised in mouse (AR 441 ThermoFisher Scientific, Waltham, MA, USA). After incubation overnight, secondary antibodies, Goat anti-rabbit IgG conjugated with FITC (31635 ThermoFisher Scientific, Waltham, MA, USA) and Goat anti-mouse IgG conjugated with APC-Alexa Fluor 750 conjugate (21006 ThermoFisher Scientific, Waltham, MA, USA), were added to the array. The array was mounted using ProLong™ Gold Antifade Mountant with DAPI (Molecular Probes® by Life Technologies™, Eugene, OR) as a nuclear stain. Images were taken using a Zeiss A1 Axio Observer fluorescence microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) at x20 magnification with Zen digital imaging software (Carl Zeiss Microscopy, LLC, Thornwood, NY) and analyzed using ImageJ software.

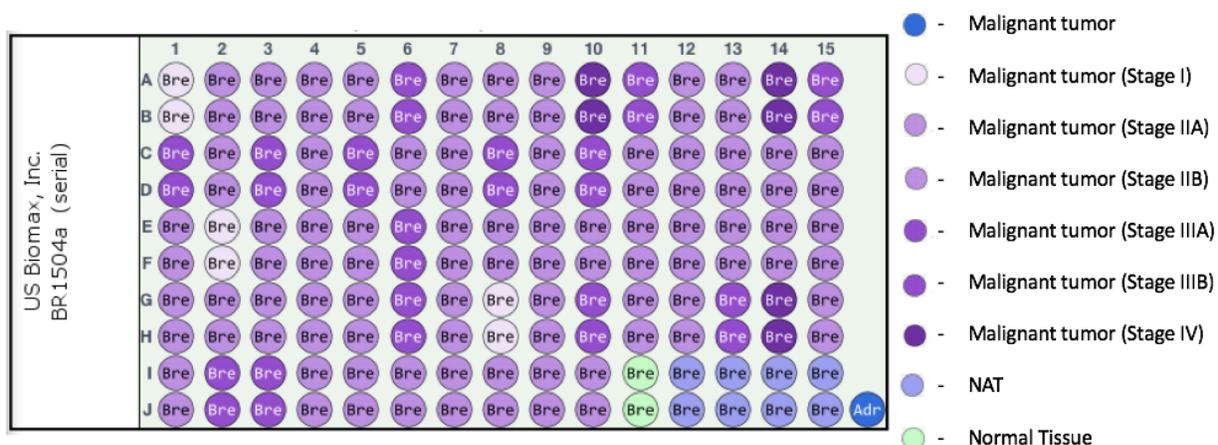


Figure 3.1: Breast Cancer Tissue Microarray Panel Display. Breast cancer tissue microarray with cancer adjacent breast tissue, containing 70 cases of invasive ductal carcinoma, 4 cases of cancer adjacent breast tissue and 1 case of normal breast tissue, duplicate cores per case. Bre = Breast tissue, Adr = Adrenal gland tissue^[137].

Table 3.2: Demographic data of the study participants.

DISEASE STAGE	AGE (YEARS)	NUMBER
<i>I</i>	45-62	3
<i>IIA</i>	31-70	32
<i>IIB</i>	27-75	18
<i>IIIA</i>	47-59	4
<i>IIIB</i>	34-69	10
<i>IV</i>	50-58	3

Bioinformatics

The results shown here are in whole or part based upon data generated by the Breast Cancer portion of The Cancer Genome Atlas (TCGA) Research Network: <http://cancergenome.nih.gov/>. Analysis was

conducted in ‘R’, a free language and environment for statistical computing, using the TCGAbiolinks package to download and manipulate data from the TCGA database^[138, 139]. An overview of the data is available online: <https://wiki.nci.nih.gov/display/tcga/rnaseq+version+2>.

Results

DAX-1 expression during progressive stages of invasive ductal carcinoma compared with other breast cancer markers

A tissue array containing breast tumor samples from 70 patients at progressive stages of invasive ductal carcinoma, in duplicate, was stained for the presence of DAX-1 and AR. Tissue was counterstained with DAPI to visualize the cells nuclear content. Patient age, pathology diagnosis, tumor grade, stage, TNM score, ER, PR, Ki67 and Her2 status were all known. The percentage of positive staining for both DAX-1 and AR on sections of the same disease stage varied widely. Samples were classed as receptor positive if there was $> 0.5\%$ staining. DAX-1 positive samples were identified at all stages of disease. AR positive samples were seen at all disease stages with the exception of stage IIIA samples. DAX-1 expression was detected wherever AR was and occasionally seen in other areas. Staining of both receptors was found to be exclusively nuclear (Figure 3.2).

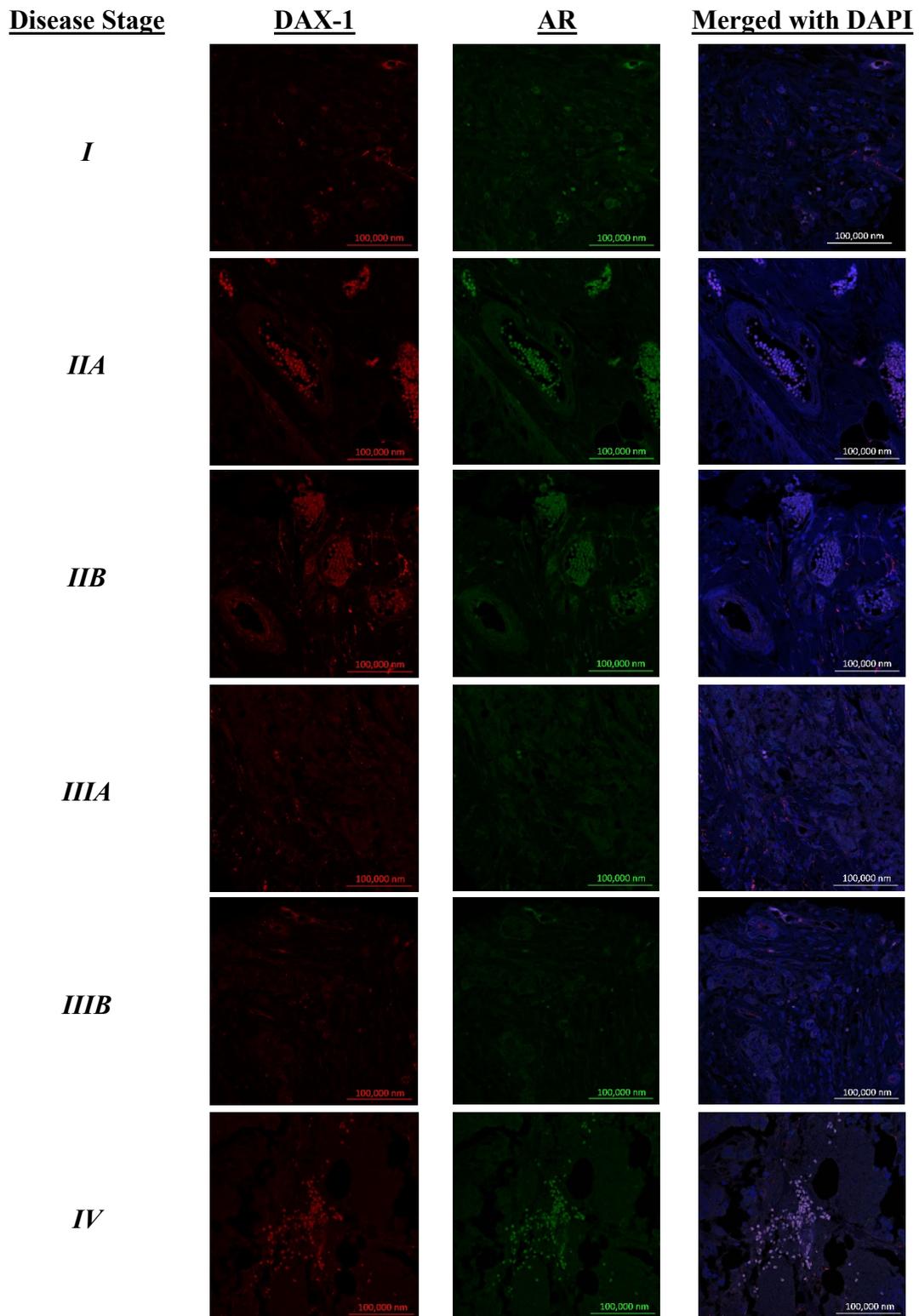


Figure 3.2: Representative IHC protein staining during progressive stages of invasive ductal carcinoma. Left column: DAX-1 staining. Middle column: AR staining. Right column: Merged DAX-1, AR ad DAPI staining. Positive staining for DAX-1 was found at every stage of disease, AR was not seen at stage IIIA. DAX-1 expression was detected wherever AR was and occasionally seen in other areas.

Around 70% of patients with earlier stage disease (I-IIB) were DAX-1 positive whereas only 20 to 30% of patients expressed DAX-1 at later stages (IIIA-IV) (Figure 3.3A). AR expression follows a similar pattern to DAX-1 with more patients being positive at earlier stages than later (Figure 3.3B). No AR expression was seen at stage IIIA. ER expression remains relatively constant throughout stage at around 50 – 70% of patients (Figure 3.3C). PR expression falls as disease stage increases with the exception of stage IIIA where around 50% of patients are PR positive (Figure 3.3D). It appears that Ki67 is expressed more frequently at higher than normal levels during early disease stage and drops later in the development of the disease (Figure 3.3E). High HER2 expression (3+ only) was observed at different stages of disease compared with DAX-1 expression (Fig. 3.3F). With the exception of stage I where there is no Her2 expression, around 20 – 30% of patients express HER2 at all other stages. Due to low sample size within some of the stages from the microarray described above, we employed bioinformatics techniques to investigate the same parameters on a far greater scale.

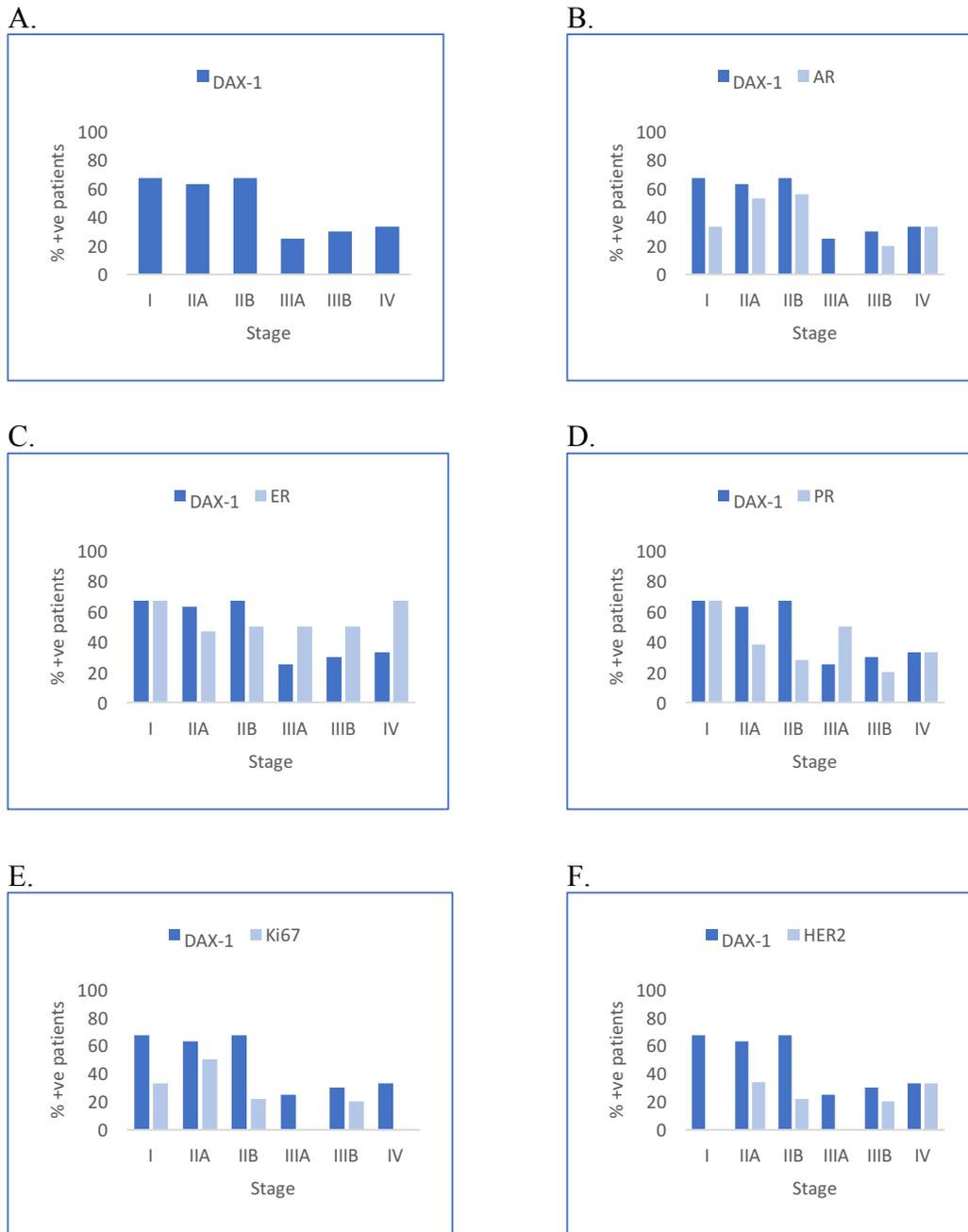


Figure 3.3: DAX-1 expression compared with other breast cancer markers during progressive stages of invasive ductal carcinoma. Data summarized from the invasive ductal carcinoma array (US Biomax inc. BR1504a) and categorized into disease stage. A: Percentage of patients showing positive IHC staining for DAX-1. B: Percentage of patients showing positive IHC staining for AR. C: Percentage of patients showing positive IHC staining for ER. D: Percentage of patients showing positive IHC staining for PR. E: Percentage of patients showing positive IHC staining for Ki67 (only intermediate and high levels on the Ki-67 labelling index). F: Percentage of patients showing positive IHC staining for HER2 (3+ only).

DAX-1 expression in receptor positive and negative breast cancer patients during progressive stages of disease

Patient data was pooled from The Cancer Genome Atlas (TCGA) Research Network and analysis was conducted in ‘R’, a free language and environment for statistical computing. A total of 1,098 patients were found to have been screened for the DAX-1 gene, NR0B1, and many other markers. DAX-1 expression was measured in RNASeq reads, which is a measure of the quantity of mRNA in a sample at the time of RNA extraction. To study correlations between DAX-1 expression and the other common breast cancer biomarker genes, *ESR1* ($ER\alpha$), *PGR* (PR) and *ERBB2* (HER2), the data was analyzed by first identifying DAX-1 expression levels in the biomarker positive and negative populations then subcategorizing these into progressive breast cancer stage, from I to IV. Comparatively low levels of DAX-1 expression (less than 50 RNASeq reads) were seen in both ESR1 and PGR positive patients at all oncological stages. ESR1 and PGR negative patients initially showed comparatively high DAX-1 expression levels during earlier oncological stages, which then decreased with progressive stage (Figures 3.4 & 3.5).

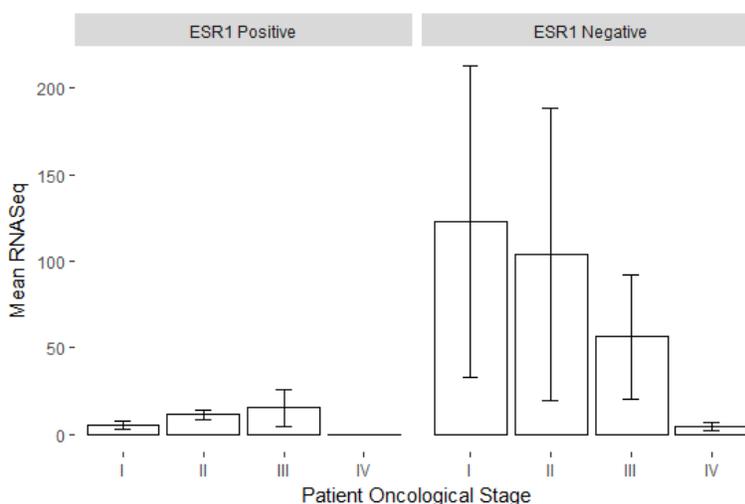


Figure 3.4: DAX-1 expression in ESR1 ($ER\alpha$) positive and negative patients categorized by breast cancer stage: Mean RNASeq reads for DAX-1 expression are shown for breast cancer patients divided by ESR1 status and oncology stage. Higher expression of DAX-1 is seen in ESR1 negative patients regardless of oncology stage. DAX-1 expression drops with progressive oncology stage in ESR1 negative patients. Error bars represent standard deviation of the mean.

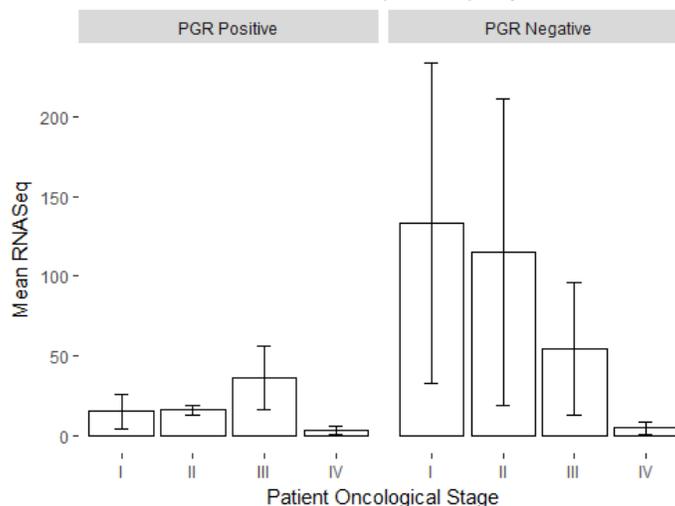


Figure 3.5: DAX-1 expression in PGR (PR) positive and negative patients categorized by breast cancer stage: Mean RNASeq reads for DAX-1 expression are shown for breast cancer patients divided by PGR status and oncology stage. Higher expression of DAX-1 is seen in PGR negative patients regardless of oncology stage. DAX-1 expression drops with progressive oncology stage in PGR negative patients. Error bars represent standard deviation of the mean.

Levels of DAX-1 expression were low, less than 60 RNASeq reads, in *ERBB2* positive and negative patients. In *ERBB2* positive patients DAX-1 expression was the highest at stage I (with a mean of 40 RNASeq reads) and dropped by approximately half in stage II and III patients and decreased again in stage IV patients. *ERBB2* negative patients show very little DAX-1 expression in stage I patients, increasing to nearly 40 RNASeq reads in stage II patients before falling to very low levels again in later stage patients (Figure 3.6).

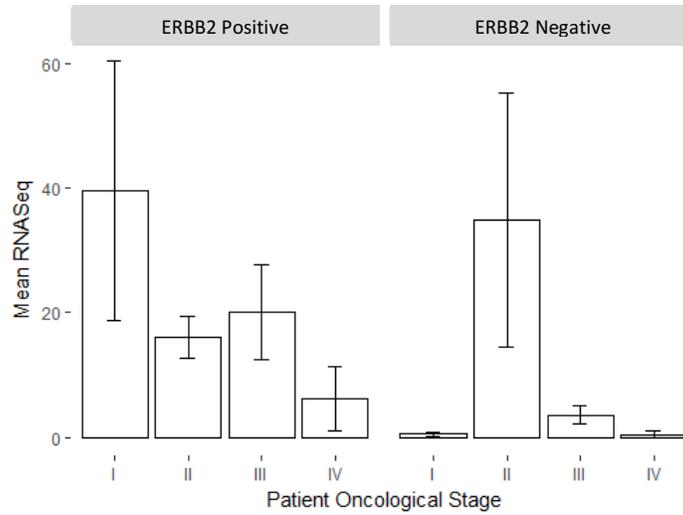


Figure 3.6: DAX-1 expression in HER2 positive and negative patients categorized by breast cancer stage: Mean RNASeq reads for DAX-1 expression are shown for breast cancer patients divided by HER2 status and oncology stage. Higher expression of DAX-1 is seen in HER2 positive patients at all stages of oncology with the exception stage II where HER2 negative patients show higher DAX-1 expression. DAX-1 expression drops with progressive oncology stage in HER2 positive patients. Error bars represent standard deviation of the mean.

Discussion

A small number of studies^[50, 51] and unpublished data from our own laboratory^[53] have shown DAX-1 to be correlated with smaller tumor size, earlier disease stage and increased survival rates in several cancers including breast. We have also shown that DAX-1 is a suppressor of tumorigenesis in MCF7 breast cancer cells. To explore this relationship further, we looked at the expression of DAX-1 in tumor tissue samples from patients with progressive stages of invasive ductal carcinoma and tumor samples from the TCGA database also at progressive stages of breast cancer. From the tissue samples, we found that the proportion of patients with DAX-1 positive tumors was high (approximately 70%) in stage I and II, and decreased to 30 - 40% in stage III and IV tumors. The tumor samples from the TCGA database showed that in certain subpopulations, i.e. ER and PR negative and HER2 positive patients, DAX-1 expression levels were higher at earlier stages of disease. These findings agree with previous findings mentioned above.

AR is emerging as an important steroid hormone receptor in the progression of breast cancer and has been shown to interact with DAX-1 to suppress cyclin D1 expression^[49, 67]. Therefore, we examined the correlation between DAX-1 and AR in the IDC tissue array. DAX-1 and AR expression were found together in the majority of patients, with the exception of a small number expressing DAX-1 only. The expression was seen in the same locations on the tissue samples and were exclusively nuclear, providing evidence that both proteins co-localize. Unfortunately, no data were available for AR expression in the TCGA database so we were unable to look at this correlation in a wider patient cohort.

We next wanted to explore the correlation between other common breast cancer biomarkers including ER, PR, HER2, Ki-67. Our tissue array showed ER expression to be consistently high, at around 50 - 70%, across the oncological stages. This is as we would expect, knowing that around two thirds of breast cancer tumors are ER positive. Similarly, PR was expressed consistently throughout oncological

stage but in a lower proportion of patients than ER. Again, this fit with what is already known, that is a lower percentage of patients are PR positive (around 65%). HER2 and Ki-67 are both expressed under normal circumstances in the breast but at elevated levels in some breast cancers. Therefore, when screening patients for these markers, scoring systems are applied as described above. In line with clinical standards, we only took patients with HER2 grade 3+ and Ki-67 levels greater than 16% as positive for these markers. Only 1 in 5 breast tumors express high levels of HER2 and our staining showed a similar pattern to this with some stages having 20 - 40% of patients expressing the marker and other stages with no patients being positive. Ki-67 showed similarly sporadic expression across the oncology stages.

Besides the DAX-1/AR correlation, no other strong correlations were seen between the proportion of patients expressing the other breast cancer markers and DAX-1. A drawback to this study was the limited sample size (Table 4.2); e.g. at some stages there were only three representative patients. This restricted the analysis that could be performed with the data. Therefore, we employed bioinformatics techniques to investigate further. Expression levels for DAX-1, ER, PR and HER2 in 1,098 breast cancer patients across the range of oncological stages, were available and we found DAX-1 expression levels to be significantly higher in ER and PR negative patients compared with positive patients. The highest levels of DAX-1 expression were seen in stage I patients. DAX-1 expression progressively decreased through the stages to its lowest levels at stage IV. DAX-1 expression in HER2 positive patients was at a similar level to those found in the receptor positive patients but did show the progressive decrease in DAX-1 expression as oncological stage increased. These data show that DAX-1 has the potential to be utilized as an additional clinical biomarker when assessing patients for prognosis and planning treatments. Specifically, if a patient is DAX-1 negative in early stage breast cancer this could indicate a more aggressive suppression of cell proliferation may be beneficial. Conversely if the patient is DAX-1 positive at early stages then a less aggressive form of treatment might be the best option, limiting the many side

effects of therapeutics. DAX-1 may even have the potential for use as a therapeutic itself. For example, if expression of DAX-1 could be re-activated in patients that have lost its expression, such as later stage patients and those that are hormone receptor positive, then cancer progression could potentially be suppressed. Further investigation into the mechanism of DAX-1s action and analysis of a larger sample size of breast cancer patients DAX-1 expression levels are needed to fully explore its potential as a biomarker and/or a therapeutic.

Thesis Summary

The research conducted throughout this thesis highlight some of the factors contributing to breast cancer biology. Biological processes are usually never simple, and breast cancer is no exception. Here we touched on breast cancer cell growth, proliferation and metastasis but there are many other processes at play. The intricacies of the cross-talk between proteins within the cell at any one time are complex and the cells response to intrinsic and extrinsic factors mediate its outcome. Development of more effective ways to fight this disease is based on our ability to untangle and understand these pathways and mechanisms of action.

The role of DAX-1 in breast cancer cells is poorly understood but previous research has shown that it does play a role in potentially more than one way. That, with the added complexity of the different characteristics this disease can take on, makes for a hugely convoluted area of study. The focus of this thesis has been on the role of DAX-1 in breast cancer cell proliferation and metastasis because these are two of the most important mechanisms by which the disease progresses. We found that when DAX-1 is overexpressed in breast cancer cells, proliferation rate drops and conversely when DAX-1 expression is knocked down in normal breast cells, proliferation rates increase. When the estrogen receptor antagonist Fulvestrant was introduced to these cells, no further potentiation of DAX-1s effects were seen suggesting that DAX-1s suppressive influence on proliferation is lost when ER is blocked. We also identified many novel key genes involved in cell growth, proliferation and metastasis, whose transcription is modulated by DAX-1. The most significant changes in gene expression were downregulation of *SFN*, *ITGB3* and *RORB* and upregulation of *ERS1* and *MAFF*. The upregulation of gene expression is of particular interest as traditionally DAX-1 has been known to act as a suppressor of gene transcription. These findings suggest a potential direct effect of DAX-1 in the growth of breast cancer cells.

Through our IHC and bioinformatics data, we found DAX-1 to be a marker commonly expressed in breast cancer cells. Expression was more frequently seen during earlier stages of disease and in ER and PR negative and HER2 positive patients. In addition, we found that DAX-1 and AR expression almost always occurred together and co-localized in the nucleus. With the addition of more AR and DAX-1 expression data in breast cancers, find more definitive associations between the two.

Our findings have identified DAX-1 as playing an important role in breast cancer and as such shows potential for use clinically. Based on our data demonstrating DAX-1's role in controlling cell proliferation, it could be that DAX-1 would be a useful biomarker when planning patient treatments. For example, if DAX-1 expression was still present in later stage patients then clinicians may consider alternatives to ER inhibitors to allow DAX-1 to continue its suppression of cell proliferation by acting through the ER. DAX-1 also holds promise as a targeted therapeutic. If normal levels of DAX-1 expression could be reintroduced to cells that had lost it, tumor progression may be slowed through both suppression of cell growth and proliferation and limit cell migration and metastasis. Whatever its potential, DAX-1's role in breast cancer warrants further investigation, and this work has laid a foundation upon which to expand our understanding of its mechanisms of action in this context.

Appendix I

Expression of DAX-1 in human adenocarcinoma cells (MCF-7) by adenoviral transduction.

Adenoviruses are replication deficient due to the deletion of their E1 gene, involved in replication of the virus, and E3 gene, involved in modulation the host immune response^[140]. Adenoviruses retain the ability to infect cells but, are unable to produce new viral particles (virions). As shown in Figure 2.3, attachment of the viral particles to the cell is mediated by high affinity binding to the Coxsackie-Adenovirus Receptor (CAR). Internalization occurs through endocytosis upon interaction with α V-integrins. Via transport mechanisms provided by microtubules, the adenovirus reaches the host nucleus and injects its DNA. After entering the nucleus, the viral DNA remains epichromosomal and, therefore does not integrate into the host cell and does not activate or inactivate the host genes^[140]. The viral DNA is then transcribed by the cells own mechanisms and the DAX-1 protein is expressed in the cells where it would not otherwise have been. As this is not a stable line and DAX-1 is only expressed transiently, cells were treated with the adenovirus at the beginning of each experiment.

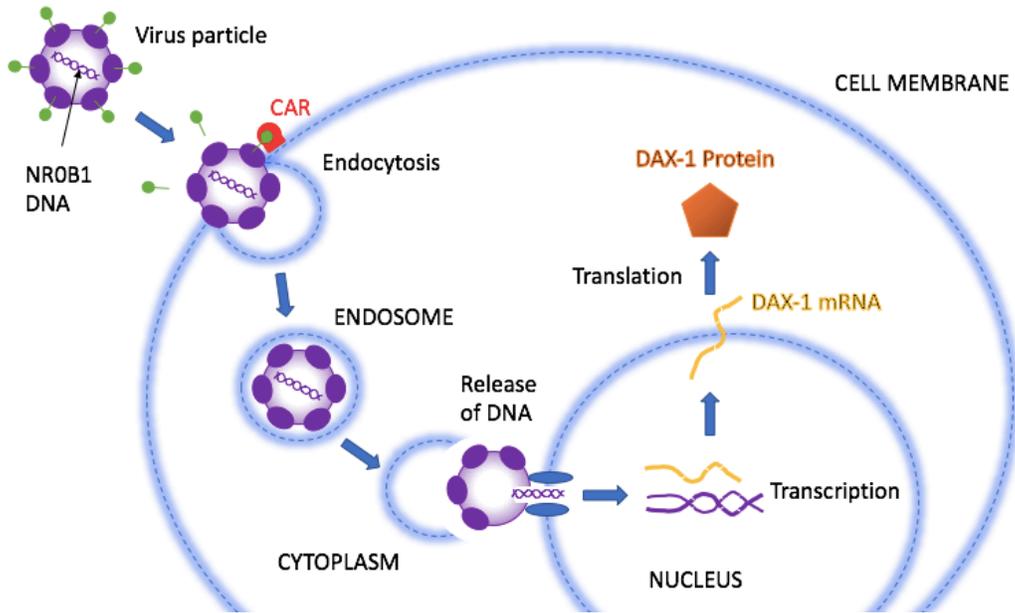


Figure AI: Simplified Illustration of the Adenovirus Transduction Method. Virus particles lacking the ability to replicate and containing the NROB1 gene, are introduced to the cell culture. The virus particle binds to the cell via the CAR and is internalized by endocytosis. The endosome moves through the cytoplasm to the nucleus where the viral DNA is released and transcription can occur. DAX-1 mRNA moves out of the nucleus to be translated into a protein by the ribosome.

Appendix II

Knockout of DAX-1 in human epithelial breast cells (MCF10A) by CRISPR-Cas9

CRISPR/Cas9 knockout plasmids were designed containing a pool of three plasmids, each encoding the Cas9 nuclease and a DAX-1 specific 20 nucleotide guide RNA (gRNA) (Figure 2.6). Cells, in this case MCF10A cells, were transfected with the plasmids. Expression of the plasmid by the cell gives rise to short CRISPR RNAs (crRNA) that guide the Cas9 protein to its complementary DNA sequence. The Cas9/crRNA complex binds to a proto-spacer adjacent motif (PAM) site and unwinds the DNA. The crRNA binds to the target DAX-1 locus in the genomic DNA adjacent to the PAM site. Cas9 cleaves the 5' exon of the gene targeted by the three gRNA plasmids at three specific sites and the DAX-1 gene is disrupted. These breaks in the DNA can be repaired via the homogenous directed repair (HDR) pathway. HDR plasmids (Figure 2.7) are co-transfected with the CRISPR/Cas9 KO plasmids and contain a puromycin resistance gene that is used for selection of cells where successful Cas9-induced DNA cleavage has occurred.

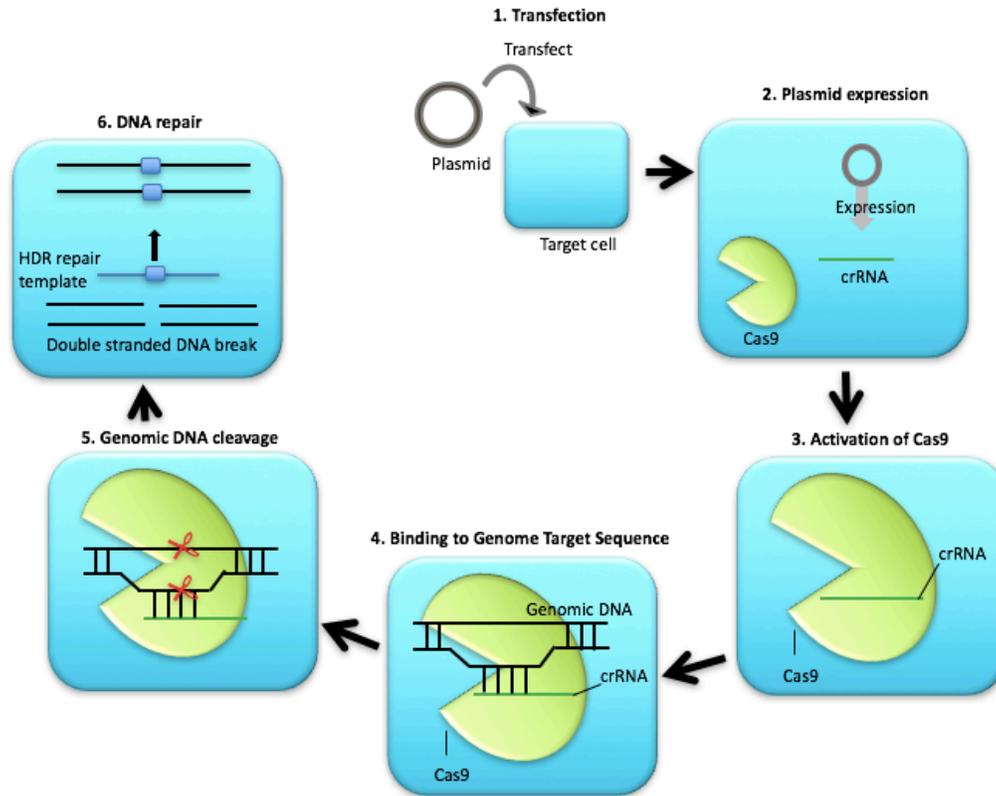
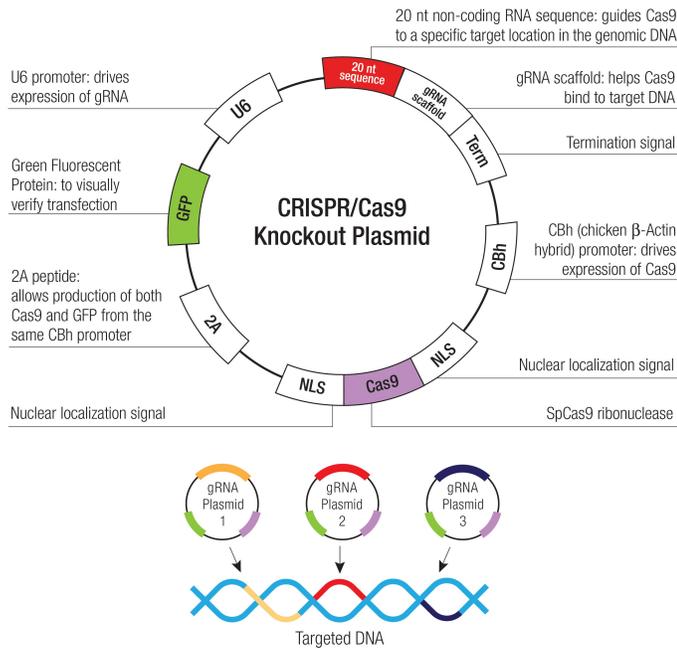


Figure AII.1: CRISPR-Cas9 knockout mechanism. 1. CRISPR/Cas9 plasmid is transfected into target cells. 2. Expression of the plasmid produces the crRNA guide strand. 3. crRNA binds to the activated Cas9. 4. The crRNA identifies the target sequence on the genomic DNA, Cas9 unwinds the DNA and crRNA binds. 5. Double stranded cleavage of the target DNA occurs. 6. The DNA is repaired by HDR.

1.



2.

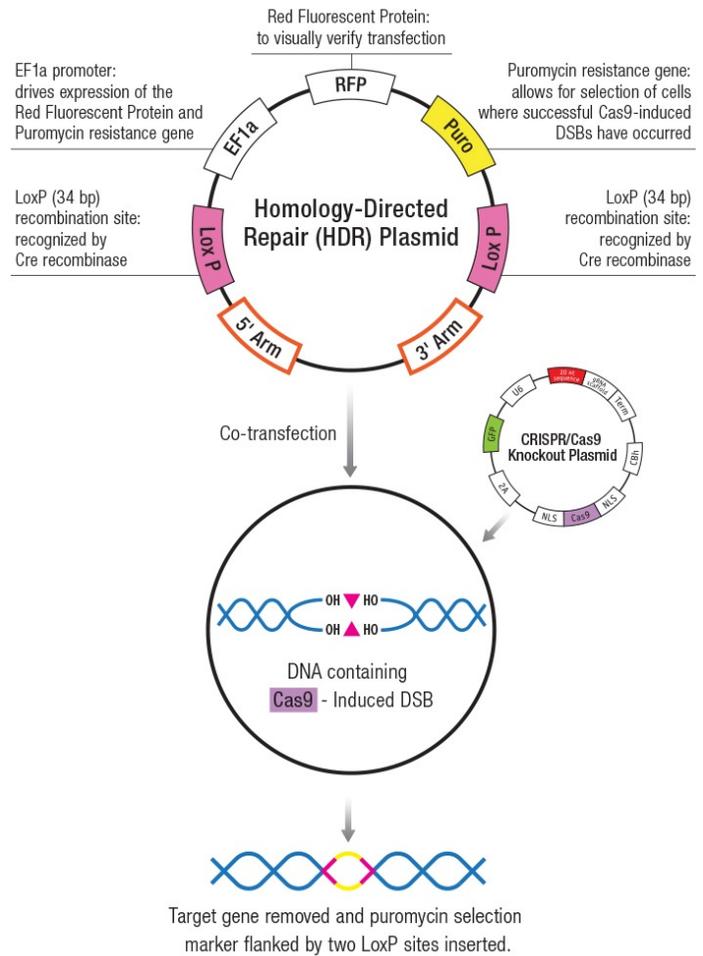


Figure AII.2: CRISPR/Cas9 knockout plasmids & HDR plasmid. 1. CRISPR/Cas9 Knockout plasmids. A pool of 3 plasmids containing sequences encoding for the Cas9 nuclease and target-specific 20 nt guide RNA. 2. Homology Directed Repair (HDR) Plasmid containing a puromycin resistance gene to allow for selection of cells successfully transfected with the DAX-1 CRISPR/Cas9 KO plasmid ^[141].

MCF10A cells were treated with a DAX-1 CRISPR/Cas9 KO plasmid in plasmid transfection medium and co-transfected with an HDR plasmid containing a puromycin resistance gene. Cells were incubated for 72 hours and those that were successfully transfected were identified using puromycin selection.

Appendix III

Knockdown of DAX-1 in human epithelial breast cells (MCF10A) by RNAi

The mechanism of action of siRNA in this case is as follows (Figure AIII): a long double stranded RNA (dsRNA) coding for the DAX-1 gene is introduced to the cell by means of lipophilic transfection. Once in the cell dsRNA is cleaved by Dicer, an endo-ribonuclease, into short interfering (siRNA) duplexes, approximately 21 nucleotides long. The siRNA then binds with an Argonaut (Ago) protein and is unwound to form single stranded siRNA known as the guide strand. The Ago/siRNA complex bind with other proteins to form the RNA induced silencing complex (RISC). The guide siRNA directs the RISC complex to the complimentary DAX-1 mRNA where it binds and Ago cleaves the mRNA which will then be degraded by the cells own mechanisms and the gene will be silenced.

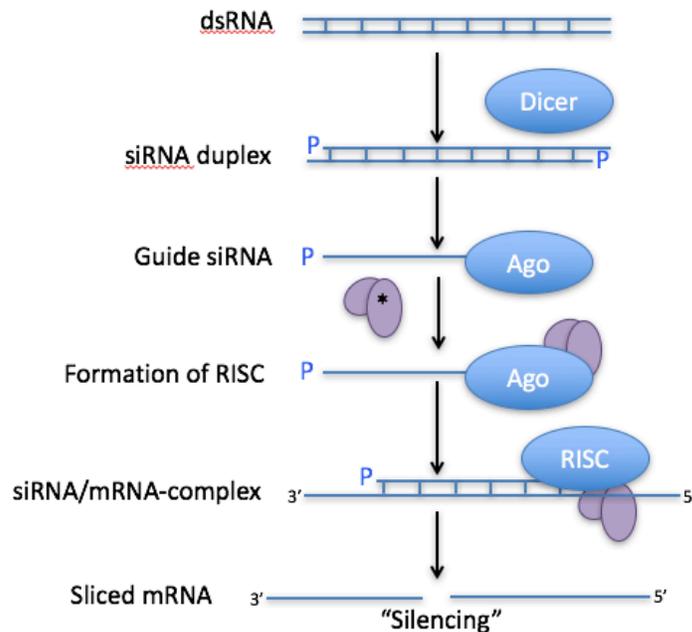


Figure AIII: siRNA Mechanism. dsRNA is cleaved by Dicer to produce siRNA duplexes which then bind to Ago which unwinds the siRNA releasing one copy to be degraded and retaining the other copy as the guide siRNA. (*) other proteins within the cells then bind to form the RISC complex which is guided to the complementary mRNA by the guide siRNA to form the siRNA/mRNA complex. The mRNA is cleaved by Ago and the gene is silenced^[142].

Appendix IV

EdU Assay

EdU (5-ethynyl-2'deoxyuridine) is a modified nucleoside thymidine analogue that, when cultured with cells, becomes incorporated in newly synthesized DNA. Once cells have been cultured with EdU for 24 hours they are fixed and detergent permeabilization allows the small molecule-based Click-iT® EdU detection reagent to gain access to the DNA. The click reaction is a copper catalyzed covalent reaction between an azide and an alkyne which attaches a fluorescent dye, specifically Alex Fluor 488 in our case, to the EdU molecule (Figure AIV.1).

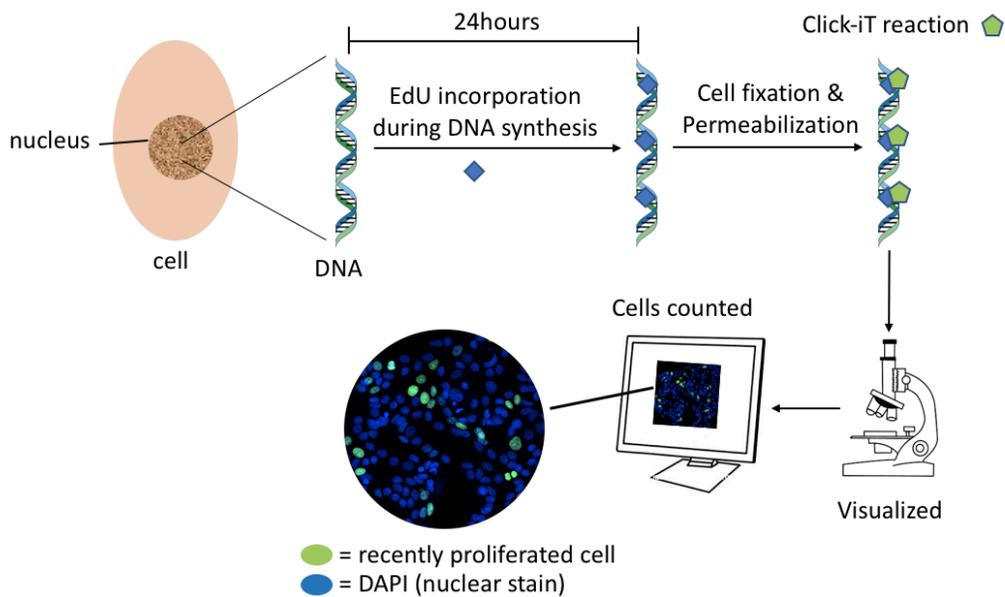


Figure AIV.1: EdU HCS mechanism of action. EdU is incorporated into newly synthesized DNA. Once cells have been cultured with EdU for 24 hours they are fixed and permeabilized. Using a highly specific click-iT reaction the EdU molecules become labelled with a fluorescent dye, specifically Alex Fluor 488 in our case. Cells are then counter stained with the nuclear dye, DAPI, visualized under a Keyence BZ-9000E microscope with the BZ viewer and analyzed using BZ analyzer software.

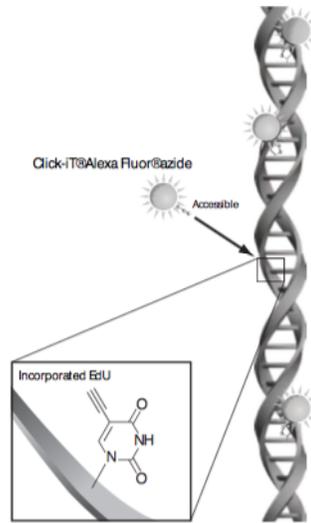


Figure AIV.2: Detection of the incorporated EdU with the Alexa Fluor®. Alexa Fluor azide binds with DNA incorporated EdU molecules via the Click-iT® reaction allowing for visualization of newly synthesized DNA ^[143].

Appendix V

Lipophilic transfection

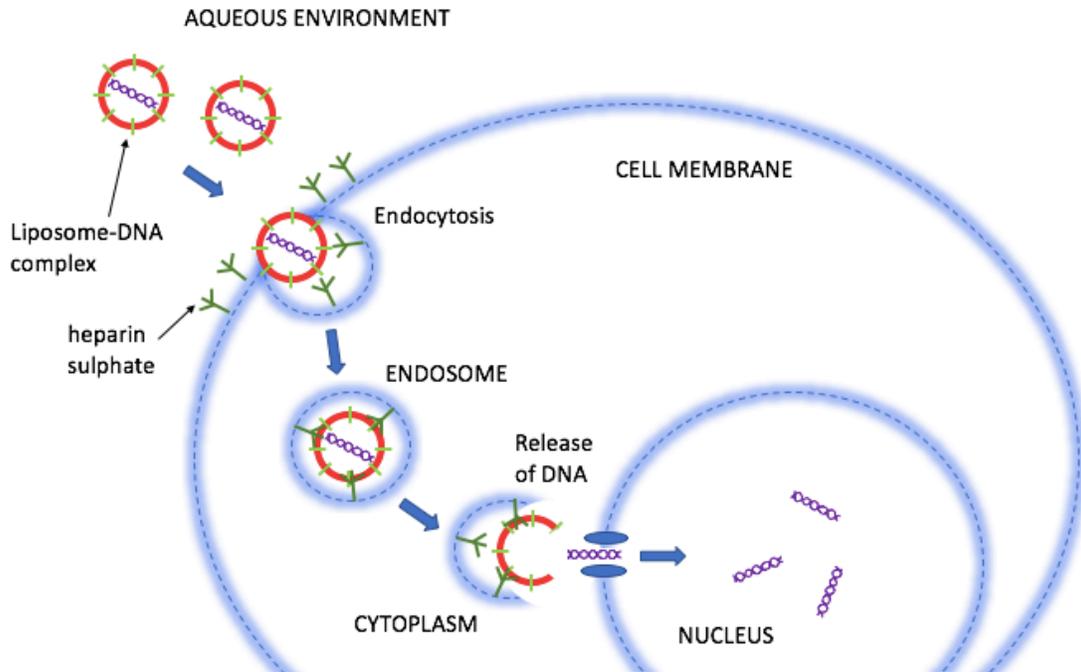


Figure AV: Lipophilic transfection. Liposomes are synthetic analogues of the phospholipid bilayer, that form spherical structures under aqueous conditions and encapsulate nucleic acids in the presence of free DNA. Access to the cell is gained by endocytosis and the liposome-DNA complex is moved through the cytoplasm to the nucleus in an endosome. The DNA is then released into the nucleus and transiently transcribed by the cells own mechanisms.

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