Investigation of the role of HMGB2 in endocrine resistant breast cancer

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I declare that this thesis, which I submit to RCSI and W.I.T for consideration of the award of M.Sc. is my own personal effort. Where any of the content presented is the result of input from any other source other than my own work this is duly acknowledged in the text. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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Abbreviations

ABC	Avidin-biotin complex
AD	Activation domain
AF-1 and AF-2	Activation function domains
AI	Aromatase inhibitor
AIB1	Amplified-in-breast cancer 1
AP-1	Activating protein
BCA	Bichinchonic Assay
bFGF	Basic fibroblast growth factor
bp	Base pairs
BCAS3	Breast Cancer Amplified Sequence 3 gene
BRCA	Breast cancer susceptibility gene
BSA	Bovine serum albumin
CARM1	Coactivator-associated arginine methyltransferase 1
CBP	CREB-binding protein
Cdk1	Cyclin-dependent kinase 1
cDNA	Complementary DNA
CDS-FCS	Charcoal dextran-stripped fetal calf serum
ChIP	Chromatin Immunoprecipitation
Co-IP	Co-Immunoprecipitation
DAB	3,3-diaminobenzidine tetrahydrochloride
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DBD	DNA binding domain
DCIS	Ductal carcinoma *in situ*
dH_2O	Distilled water
DFS	Disease-free survival
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E_2	17β-estradiol
ECL	Enhanced chemiluminescent reagent

EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
Erb	Erythroblastic Leukemia Viral Oncogene
ERE	Estrogen response elements
ERK	Extracellular signal-regulated kinase
HAT	Histone acetyltransferases
HDAC	Histone deacetylases
HER-2	Human epidermal growth factor receptor 2
HMGB2	High Mobility Group Box 2 gene
HRP	Horseradish peroxidase
IGF-1	Insulin-like growth factor 1
IMS	Industrial methylated spirits
JNK	C-Jun NH2-terminal kinase
LiCl	Lithium chloride
MAPK	Mitogen-activated protein kinase
MEM	Minimal essential medium
$MgCl_2$	Magnesium chloride
MMP	Matrix metalloproteinases
MTA1	Metastasis associated protein 1
NCoA	Nuclear co-activator
NCoR	Nuclear co-repressor
PCR	Polymerase Chain Reaction
PELP1	Proline glutamic acid leucine rich protein 1
PI3K	Phosphatidylinositol-3 kinase
PR	Progesterone receptor
qPCR	Quantitative PCR
RIPA	Radioimmunoprecipitation
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Ser	Serine
SERM	Selective estrogen receptor modulator
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptor

SNP	Single nucleotide polymorphisms
SRC	Steroid receptor coactivators
TAE	Tris acetate-EDTA
Taq	Taq polymerase (*Thermophilus aquaticus*)
TBS	Tris buffered saline
TBS-T	Tris buffered saline plus 1% Tween
TMA	Tissue microarray
TNFα	Tumour necrosis factor-α
UV	Ultraviolet
v/v	Volume/volume
w/v	Weight/volume

Investigation of the role of HMGB2 in endocrine resistant breast cancer

Abstract

The high mobility group box 2 protein (HMGB2) is an abundant chromatin remodelling protein with an affinity for unusual DNA structures. It induces architectural modifications to DNA structure, thereby allowing easier access for transcriptional machinery to promoters of interest. Immunohistochemical staining of our patient tissue microarray revealed that patients who are positive for HMGB2 in their primary tumour have a reduced risk of breast cancer recurrence. This favourable outcome could be due to the interaction between the estrogen receptor α (ERα) and HMGB2, as ERα positive tumours promote the formation of a luminal type tumour, which are less aggressive in general. HMGB2 was identified as a steroid receptor coactivator 1 (SRC1) binding partner in an endocrine resistant cell model, but not the endocrine sensitive model. In contrast to HMGB2, SRC1 has previously been associated with a reduced disease free survival. It is possible that the role of HMGB2 changes as the tumour develops resistance to an endocrine therapy. This study demonstrates that HMGB2 increases the interaction between ERα and SRC1 in an endocrine resistance breast cancer cell line. We have shown that HMGB2 binds to the estrogen regulated breast cancer amplified sequence 3 (BCAS3) promoter in an endocrine resistant cell line. Furthermore, estrogen and tamoxifen treatment increase this level of binding. HMGB2 has also been shown to regulate protein expression of BCAS3 in endocrine resistance. A knock-down study of HMGB2 resulted in a decreased expression of BCAS3. Conversely, over-expressing HMGB2 resulted in increase in BCAS3 protein expression. The findings in this study suggest that a transcriptional complex of SRC1/ERα/HMGB2 regulate the transcriptional activity of BCAS3 in endocrine resistant breast cancer.

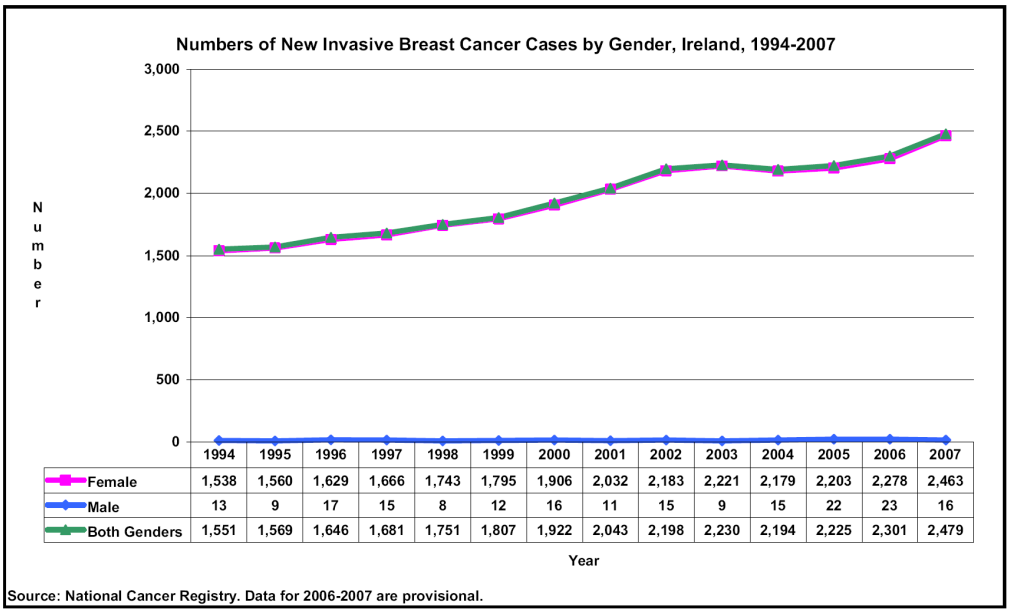
Chapter I

Introduction

1.1 Introduction to breast cancer

1.1.1 Breast Cancer Epidemiology

Cancer is a multi-factorial disease; meaning that a number of factors influence the onset and the progression of the disease. These factors include your genetic make-up, age, exposure to carcinogens, your immune system, bacterial/viral infections and physical well being (diet, weight etc.) to name but a few. "Cancer" (medically known as "malignant neoplasm") is synonymous with the uncontrolled proliferation of abnormal cells in the body. There are approximately 200 different types of cancer. More specifically, breast cancer poses a serious worldwide threat to women's health. Between the years of 1980 and 2006, a total of 16,775 women died from breast cancer in Ireland; averaging 621 women annually (HSE breast cancer statistics). Data from the Health Service Executive website (illustrated in figure 1.1) demonstrates the increasing trend in breast cancer incidence.



	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007
Female	1,538	1,560	1,629	1,666	1,743	1,795	1,906	2,032	2,183	2,221	2,179	2,203	2,278	2,463
Male	13	9	17	15	8	12	16	11	15	9	15	22	23	16
Both Genders	1,551	1,569	1,646	1,681	1,751	1,807	1,922	2,043	2,198	2,230	2,194	2,225	2,301	2,479

Figure 1.1: The annual average number of newly diagnosed breast cancer cases from 1994-2007 inclusive. With the exception of 2004, increasing numbers of female cases were observed annually. An increasing trend of 3.7% on average was recorded in female breast cancer cases between 1994 and 2007. Time trend analysis was conducted using log- linear regression with the annual percentage change measuring the average annual rate of change over the time series.
http://www.hse.ie/eng/services/Find_a_Service/National_Cancer_Control_Programme/Health_Professional_Information/Breast_Cancer_Statistics.html

1.1.2 Genetic susceptibility to breast cancer

A woman's susceptibility to developing breast cancer significantly increases if she inherits a mutated BRCA1 or BRCA2 gene (breast cancer susceptibility genes). These tumour suppressor genes transcribe protein products that are vital in controlling the well ordered cell cycle. The frequency of BRCA1 and BRCA2 mutations in the general population is 0.051% and 0.068% respectively (Antoniou *et al.,* 2002). These levels of frequency correspond to 1 in 974 individuals carrying a mutated BRCA1 gene and 1 in 734 carrying a mutated BRCA2 gene.

1.1.3 Molecular classification of breast cancer

Traditionally, prognostic predictions for breast cancer patients have been driven by a few determinants such as hormone receptor status, HER2 (human epidermal receptor 2) status, lymph node status and tumour size. However over the years, these determinants have widened, thereby providing a more detailed and accurate molecular classification. In order to create a new system of molecular classification, over 8,000 human genes were analysed from 65 breast tumour samples (Perou *et al.,* 2000). This gene expression analysis subsequently identified 5 types of breast cancer, luminal A, luminal B, basal, Erb-B2 and normal-like.

Luminal A tumours generally adhere to the following criteria, Estrogen Receptor (ER) positive, and/or Progesterone Receptor (PR) positive, Human Epidermal Receptor 2 (HER2) positive, and have a low Ki67 score. This molecular class accounts for between 42-59% of breast tumours. Ki67 is the classical marker of proliferation in cancer cells. High Ki67 is a prediction of poor prognosis (Urruticoechea *et al*., 2005). Luminal type cells are depicted in figure 1.2. Out of the 5 tumour types - luminal A tumours tend to have the best prognosis with high survival rates and a low rate of recurrence.

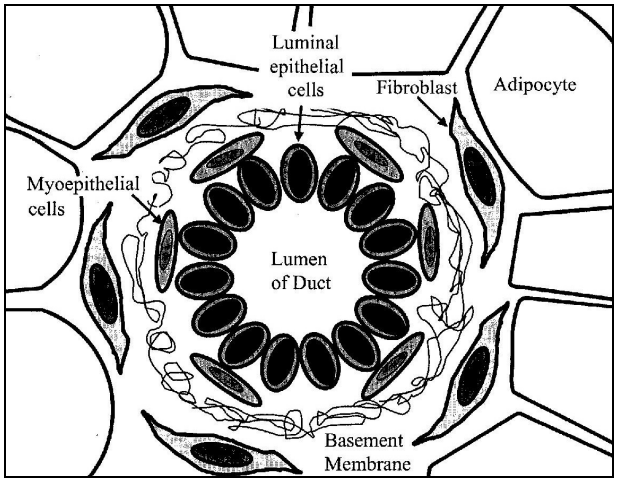


Figure 1.2: Luminal epithelial cells are located on the inner lining of the mammary duct.
http://www.grin.com/en/doc/252702/the-regulation-and-function-of-progesterone-receptor-isoforms-a-and-b-in

Luminal B tumours are generally ER positive, PR positive, HER2 positive or negative, and have a high Ki67 score. Luminal B tumours are considered to be more aggressive than their luminal A counterparts, their high Ki67 means that they proliferate at a faster rate and they are more likely to metastasise. Survival rate is high for women with luminal B tumours but not as high as luminal A (Cheang *et al.,* 2008).

Basal tumours are often referred to as "triple negative" tumours, meaning that they lack all 3 of the distinctive nuclear receptors - ER, PR and HER2. These tumours are comprised of cells that resemble the outer myoepithelial cells of the mammary duct (Honeth *et al.,* 2005). The association between this phenotype and BRCA1 hereditary tumours is particularly strong (Honeth *et al*., 2005). They are often aggressive tumours and prognosis is generally not as favourable as luminal type tumours.

Erb-B2 is a tyrosine kinase receptor, also known as HER2. It is over-expressed on 25-30% of tumours and is associated with an increase risk of recurrence (Slamon *et al.,*

1987). Other characteristics of the Erb-B2 tumour are that it is ER negative and PR negative. Seven to 12% of breast cancers possess this molecular profile.

It is questionable as to whether "normal type" breast tumours are a distinct molecular subtype. Nonetheless, this category is associated with a favourable prognosis (Carey *et al.*, 2006).

1.2 Hormone signalling in breast cancer

1.2.1 The Estrogen Receptor α

Approximately 70% of all breast cancers are estrogen receptor positive (Gaub *et al.,* 1990). There are two types of estrogen receptor, ERα and ERβ, which both belong to the nuclear hormone receptor family (Green *et al.,* 1986). They are located on the 6th and 14th chromosome respectively (6q25.1 and 14q23.2). Substantial research has been conducted on the involvement of ERα in breast cancer development. Over the years, a strong focus has been placed on estrogen driven breast cancer. Estrogen, which is a steroid hormone, is a key player in the regulation of many genes; these genes are largely involved in cell proliferation, angiogenesis (the growth of new blood vessels) and metastasis. 17β-estradiol is the main estrogen found in the body, estrone and estriol are also present at lower levels. All three forms of estrogen bind to ERα.

Structurally, ERα follows the same principles as other steroid receptors and has a DNA binding domain, a ligand binding domain, a dimerisation domain and a number of transcriptional activating domains (Carson-Jurica *et al.,* 1990). Activation function 1 (AF-1) is located in the N terminal region. This activating domain has the potential to

be activated independently of a bound ligand. Activation function 2 (AF2) however, is located in the C-terminal domain and its activation is dependent upon ligand bound stimulation. Therefore, the activity of the receptor can be determined by which activation function domain is being activated (Barkhem *et al.,* 2002).

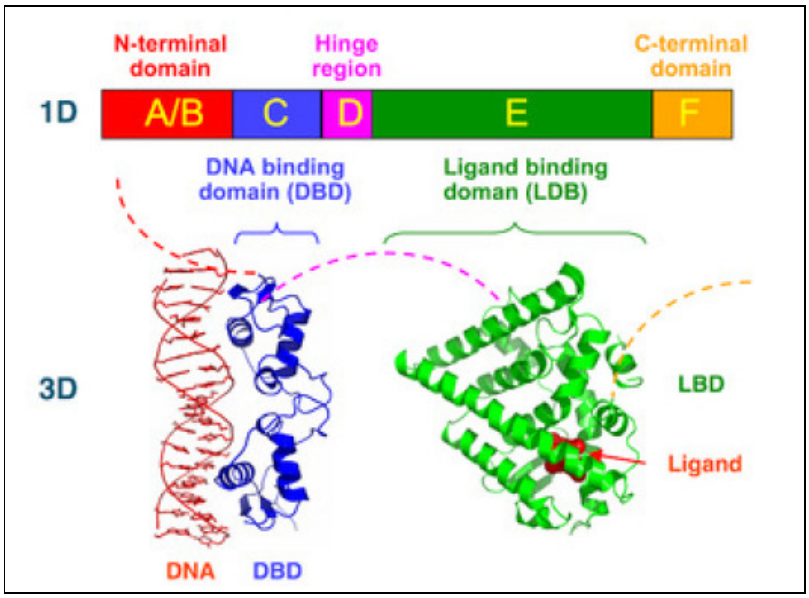


Figure 1.3: 1D structure shows the basic structure of the nuclear receptor. 3D structure shows the DNA binding domain (DBD) and the ligand binding domain (LBD). Both domains are connected via the flexible hinge region.
http://en.wikipedia.org/wiki/Nuclear_receptor

ERα acts as a ligand activated transcription factor (Shiau *et al.,* 1998). The classical mechanism of ERα activation involves estrogen binding, leading to phosphorylation of the receptor. This in turn causes the receptor to dissociate from its chaperone protein heat shock protein 70 (HSP70) in the cytosol, thereby altering the conformation of the ERα. Once a conformational change has been induced, the receptor is free to dimerise with another receptor or co-activator protein (Rosenfeld *et al.,* 2001) and this dimer is translocated to the nucleus. In the nucleus, it is available to bind to estrogen responsive elements (EREs) on the promoter of estrogen responsive genes (Nilsson *et al.,* 2001).

However, ERα does not work on its own in the regulation of estrogen responsive genes. Steroid receptor function is influenced by a family of transcriptional co-regulators which can either enhance or dampen transcriptional activity depending on whether it is a co-activator or a co-repressor respectively. Members of the p160 family of co-activators (including AIB1 and SRC1) have been shown to be elevated in breast cancer (Anzick *et al.,* 1997). The co-activator protein forms a complex with the dimerised ERα, which in turn can exert its full transcriptional response.

A non-classical mode of ERα activation also exists, whereby the receptor can regulate gene expression without directly binding to the DNA (Gottlicher *et al.,* 1998). As was previously stated in the classical mode of the ERα activation, the ERα dimer complex binds to an ERE on the promoter of an estrogen responsive gene. However, an alternative mechanism exists in order for ERα to regulate transcription when a full ERE sequence is not present on the promoter of a target gene (Klinge *et al.,* 1997). Some estrogen responsive genes have a weak or half ERE site but yet they are still strongly regulated by ERα. This is because ERα employs the use of transcription factors (e.g. c-jun and c-fos) to act as mediators between the receptor complex and the half ERE on the promoter of the target gene (Citatiello *et al.,* 2004). In addition to weak ERE sites, other DNA sequences are targeted by this non-classical mode of regulation, AP-1 and SP-1 DNA sequences (Duan *et al.,* 1998). Co-activator proteins can also be apart of this intricate complex. It is the balance between the ligand and receptor, coactivator proteins, co-repressor proteins, c-jun, c-fos, and the regulatory DNA sequence that determines the level of transcriptional activity that is exerted.

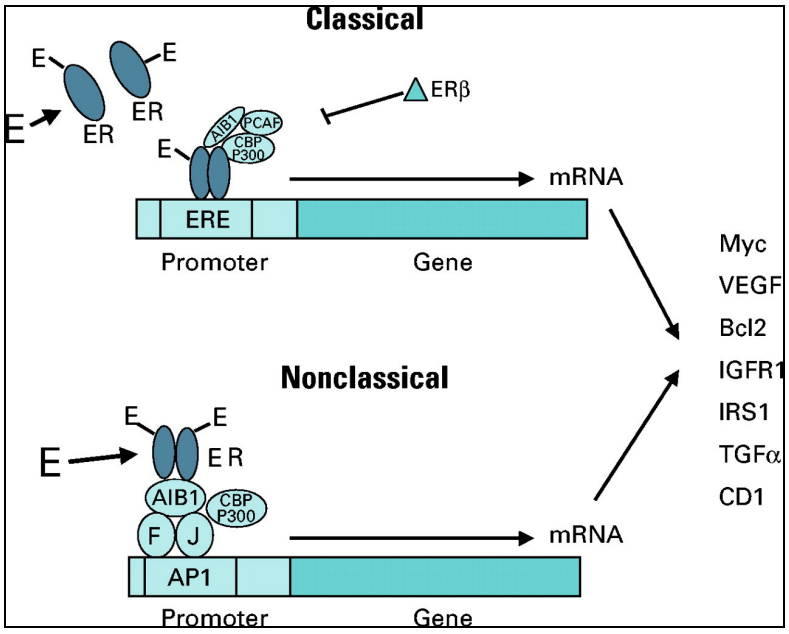


Figure 1.4: Classical and non-classical regulation of estrogen responsive genes.
The classical mode depicted at the top of the diagram shows estrogen being bound to the ER which dimerises to form a transcriptional complex with a number of coactivator proteins (AIB1, pCAF and CBP300). This complex then binds to an ERE site on its target promoter of the gene of interest. The non-classical mode underneath illustrates the c-jun and c-fos heterodimer being brought into the equation. The AP1 DNA sequence substitutes the classical ERE sequence (Osbourne and Schiff 2005).

Estrogen receptors are not solely confined to the nucleus; they are also located in the cytosol and plasma membrane (Razandi *et al.,* 2004) and exert their non-genomic affects to influence target genes. It is also sometimes referred to as "membrane initiated steroid signalling" (MISS). The ER activates growth factor signalling pathways such as cellular tyrosine kinases, mitogen activated protein kinases, phosphatidylinositol 3 kinase and Akt (Osborne *et al.,* 2001). These kinase pathways can in turn phosphorylate and activate coregulators and nuclear ERs, thereby enhancing transcriptional effects in the nucleus (Shou *et al.,* 2004). In the activation of nuclear ERα, the MAP kinase pathway phosphorylates a serine residue at position 118 in AF1, thereby stimulating ligand independent transcriptional activation (Kato *et al.,* 1995).

In combination with the recruitment of co-activator proteins in endocrine resistance, the recruitment of co-repressor proteins may be just as important. Tamoxifen bound ER may only exert its antagonistic qualities if co-repressors are recruited to enforce its competitive antagonism. Co-repressor proteins such as NCoR (nuclear co-repressor) and SMRT (silencing mediator of retinoic acid and thyroid hormone receptors) get recruited to ER in the presence of tamoxifen (Jackson *et al.,* 1997). SMRT has been shown to decrease transcriptional activation of target genes in the presence of tamoxifen. Conversely, in the presence of estrogen, SMRT was unable to override the powerful agonist properties of estrogen on its receptor (Smith *et al*., 1997). Fleming *et al.,* (2004), demonstrated that ERα preferentially binds to SRC1 in the presence of β-estradiol. Conversely, ERα will be bound by SMRT if tamoxifen is present in the cellular milieu. This shows the balance between co-repressor and co-activator proteins and how this balance will dictate how ERα functions, i.e. whether ERα will enhance an estrogen regulated gene or not.

1.2.2 Endocrine therapies for breast cancer

Endocrine therapies as adjuvant treatments for breast cancer are not a recent phenomenon. In fact, these therapies have been around, in some shape or form for the last 100 years. In the early years, endocrine therapies consisted of surgical removal of estrogen sources (ovaries, adrenal or pituitary gland). In recent years however, there has been significant advances in the development of endocrine therapies. There is an array of therapies available today, which are dependent on the patient's age, the advancement of the tumour and the molecular classification of their breast cancer.

The mainstay or most traditional endocrine therapy that has been used is tamoxifen, which was approved by the Food and Drugs Administration in 1977. Tamoxifen is a selective estrogen receptor modulator (SERM), meaning that it targets ERα and subsequently modulates its activity. Tamoxifen is metabolised by the hepatic enzyme cytochrome P450 2D6 (CYP2D6) which converts it into its active endoxifen. This drug is tissue specific as it acts as an antagonist for breast tissue; however it acts as a partial agonist in the endometrium. A number of studies have linked tamoxifen treatment with endometrial cancer (Bergman *et al.,* 2000). However, the benefits from tamoxifen on breast cancer survival outweigh the increased risk of endometrial cancer. Tamoxifen binding to ERα allows dimerisation of the receptor and binding to the DNA, however transcriptional activation is inhibited, as AF2 stimulation is prevented (Berry *et al.,* 1990).

Fulvestrant is an alternative endocrine therapy to tamoxifen and many studies have taken place to compare the two drugs (Howell *et al.,* 2004). Unlike tamoxifen, fulvestrant is a pure antagonist with no known agonist properties, thereby eliminating the heightened risk of endometrial cancer. Fulvestrant possesses a much greater affinity to bind to ERα than tamoxifen does. It binds to the ER, however dimerisation of the receptor is inhibited. Impaired dimerisation leads to degradation of the receptor, causing a disruption in receptor nuclear localisation and a failure to recruit transcriptional co-activators (Dauvois *et al.,* 1993).

Another more recent form of endocrine therapy is the use of aromatase inhibitors (AIs), which are generally given to postmenopausal women. The main source of estrogen in premenopausal women is the ovaries; however postmenopausal women generate their

only estrogen source from an enzyme called aromatase, which converts androgens to estrogen. The AI drugs target the aromatase enzyme, either in a steroidal or a non-steroidal capacity (Brueggemeier *et al*., 2005).

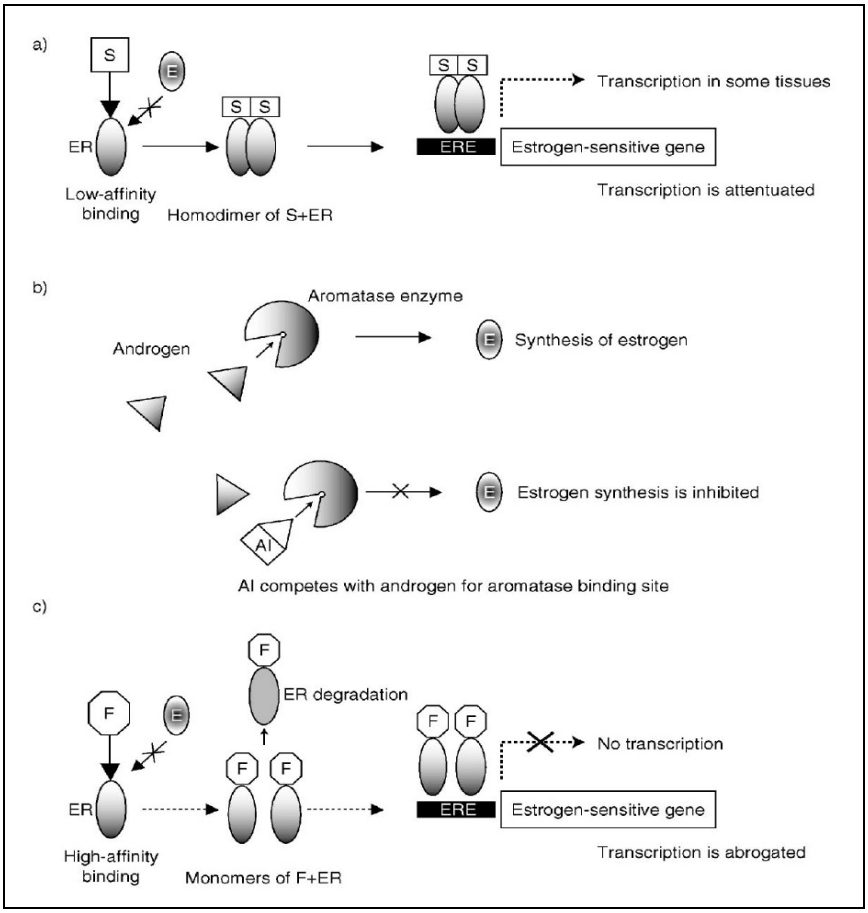


Figure 1.5: Endocrine therapies for breast cancer. (a) SERMs (labelled‘S’), such as tamoxifen competitively binds to the ER. Dimerisation of the receptor and translocation to the nucleus occurs, however transcriptional activity of the estrogen responsive gene is subdued, compared to that of estrogen. (b) The aromatase inhibitors (AIs) prevent the conversion of androgen to estrogen in postmenopausal women by blocking the aromatase enzyme binding site. (c) Fulvestrant (labelled “F”) competitively binds to the ER and promotes degradation of the receptor, thereby limiting transcription of target genes. (Mossis and Wakeling 2002)

1.2.3 Resistance to endocrine therapies

Although most patients in general respond well to endocrine treatments mentioned, a high percentage of people (up to 40%) develop resistance to the therapy, leading to tumour recurrence.

De novo or intrinsic resistance to tamoxifen is primarily due to patients lacking expression of ERα. Another mechanism for tamoxifen resistance has been established in ERα positive patients. As was previously discussed, tamoxifen is metabolised in the liver by an enzyme called P450 2D6 (CYP2D6) which breaks tamoxifen down into its active endoxifen. However, a small cohort of women (approximately 8% of Caucasian women) have been shown to possess genetically deficient alleles of CYP2D6, meaning that they are unable to correctly metabolise tamoxifen. Unsuccessful metabolism of the drug renders it ineffective in impeding tumour progression, resulting in decreased disease free survival (Wu *et al.,* 2009).

Acquired resistance is the more common type of resistance to endocrine therapies.

Over-expression and increased phosphorylation of co-activators (particularly amplified in breast cancer 1 - AIB 1) can lead to a constitutive activation of ERα, even in the absence of estradiol (Osborne *et al.,* 2003). AIB1 is over-expressed in 50% of breast tumours (Anzick *et al.,* 1997). Co-activators target ERα to activate the receptor.

There is considerable evidence showing that the cross talk between growth factor receptor pathways and ER makes a significant contribution to endocrine resistance. One such pathway is the epidermal growth factor pathway, of which HER2 is a member. Activated growth factor pathways stimulate downstream kinase cascades. The kinase

pathways can phosphorylate ER at serine 118 position which is located within the AF1 domain of the ER. Activation of the AF1 domain will stimulate the ligand independent nature of the receptor (Kato *et al.,* 1995). The interaction between growth factor signalling pathways and ER is not unidirectional. Like wise, ligand bound ER can itself simulate downstream kinases, such as the insulin-like growth factor receptor (IGFR) and the extracellular signal related kinase (ERK1/2) mitogen activated protein kinase (MAPK) pathways (Kahlert *et al.,* 2000).

1.2.4 SRC1 role in endocrine resistance

SRC1 is a 160kDa protein that interacts with both AF1 and AF2 on the ERα (Onate *et al.,* 1998). This ability to transactivate different components of ERα helps to drive transcription at an enhanced rate. SRC1 has been reported to be an independent predictor of disease recurrence regardless of endocrine treatment, with elevated levels of SRC1 associating with a worse prognosis. Furthermore, translational studies have revealed that an increased level of co-localisation of AIB1 and SRC1 with ERα occurs in endocrine treated patients who have suffered from recurrent disease (Redmond *et al.,* 2009). These co-activators facilitate the assembly of preinitiation protein complexes at target gene promoter regions. They enable specific transcription factors to be brought into the direct vicinity of their respective genes for regulation.

A condensed chromatin structure (heterochromatin) in which the DNA is tightly wound around histone proteins hinders the accessibility of the transcription factors to the promoters of which they regulate. SRC1 possesses an inherent histone acetlytransferase (HAT) activity, although on its own it has a relatively weak acetyltransferase capability.

SRC1 interacts with another HAT called p300/CBP-associated factor (PCAF) which catalyses the acetylation of lysine 14 in histone 3 (Lau *et al.,* 2000). Acetylation of a histone brings with it a negative charge; thereby neutralising the positive charge of the histone. Neutralisation of the histone results in a decreased interaction with the negatively charged DNA. This loss of interaction causes a relaxation in the chromatin structure from that of the condensed heterochromatin to a more open and accessible euchromatin. By initiating epigenetic changes within the chromatin structure SRC1 in combination with p300/CBP can promote a looser chromatin arrangement, thereby permitting the assembly of transcription machinery including RNA polymerase II and transcription factors (Spenser *et al.,* 1997).

A study by Fleming *et al.,* (2004) describes how SRC1 can be used as a predictive indicator in HER2 positive breast tumours. In a HER2 positive population on endocrine treatment, those who are also positive for SRC1 have a greater probability of disease recurrence than those who are negative for SRC1. The steroid receptor co-activator drives the aggressiveness of the HER2 phenotype. Qin *et al.,* (2009), uncovered a mechanism behind the increased metastatic potential that is associated with SRC1 positivity. SRC1 is a co-activator for a protein called Twist, which is known to be the master regulator of metastasis. Over-expression of SRC1 leads to the up-regulation of Twist, which in turn promotes cell invasion and metastasis.

1.3 High Mobility Group Box 2 protein

In conjunction with steroid receptor co-activator proteins to facilitate transcription of hormone responsive genes there are also cofactor proteins which assist further in the binding of the ER to ERE. Not all ERE are full palindromic sequences but instead half sites separated by a 3-bp sequence. Half sites can be limiting to the transcription of its respective target gene as preinitiation complexes are not as readily bound to drive transcription. One such cofactor protein is the High Mobility Group Box (HMGB) protein. It is thought that the presence of HMGB proteins can enhance binding of ER to a ERE half site by as much as 6 fold (Das *et al.,* 2004) and they can also act as mediating factors for transcription factors in the regulation of transcriptional activity. HMGB1 and HMGB2 proteins are comprised of three domains; a C terminal acidic domain and two homologous N-terminal DNA binding domains (DBDs) also called HMG boxes. Both proteins are encoded by different genes but remain identical by 82% of their amino acids (Melvin *et al.*, 1999).

HMGB proteins are abundant non-histone proteins (~1 molecules for every 10 nucleosomes) that bind non-specifically to chromatin (the minor groove in DNA in particular) to bring about structural rearrangement (Melvin *et al.,* 1999). They can promote DNA bending by creating DNA fragment circularisation thereby localising enhancer regions with their respective promoter elements (Paull *et al,* 2007). HMGB1 and HMGB2 proteins specifically interact with class 1 nuclear receptors and do not influence the transcription of class II nuclear receptors which reside in the nuclear membrane regardless of a bound ligand (Melvin *et al.,* 1999). Structural alterations in DNA induced by HMGB proteins are transient as the proteins sporadically jump

between different chromatin fibres searching for appropriate binding sites. In fact, HMGB1 has been shown to alternate between different chromatins every second (Scaffidi *et al.,* 2001). Although binding of HMGB2 to DNA sequences is said to be a random occurrence, there is evidence to suggest that this nuclear protein does prefer to bind to unusual structures of DNA, such as cruciform structures or cisplatin modified DNA (Stephanovsky and Moss, 2009).

Conventionally, the main antagonist of epigenetic changes has been the acetylation of histone proteins along with other post-translational modifications to make DNA sequences accessible for transcriptional complexes. More recently however, HMGB proteins are being recognised for their manipulation of nucleoprotein complexes and the effects that this brings about for transcriptional activity (Agresti *et al.,* 2003).

1.4 Breast Cancer Amplified Sequence 3 gene

The breast cancer amplified sequence 3 (BCAS3) gene is relatively ambiguous regarding its contribution to the development of endocrine resistance. It is known to be amplified in many breast tumours. The chromosomal location of BCAS3, 17q23, is a region that is rife for inverted repeat sequences of DNA (palindromic sequences). These inverted repeat sequences are highly susceptible to inter and intra-molecular recombination, resulting in the formation of unusual structures such as hairpins and cruciforms (Tanaka *et al,* 2002). As was previously mentioned, HMGB2 is prone to bind to such structures. The 17q23 is amplified in 20% of breast tumours and is associated with a poor disease free survival (Bärlund *et al.,* 2002).

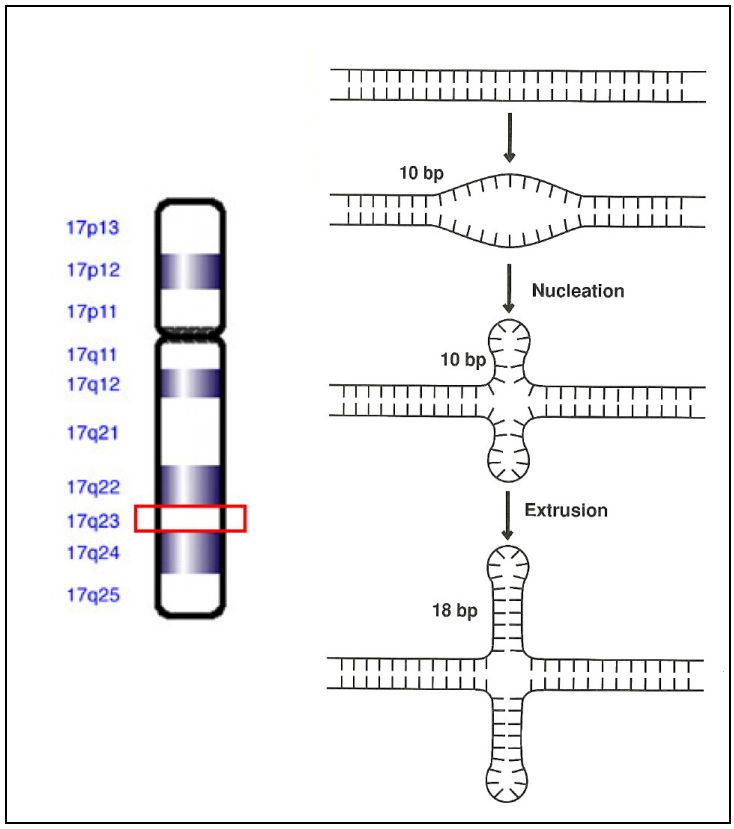


Figure 1.6: Chromosomal region 17q23 (where BCAS3 is located) is abundant in inverted repeat sequences of DNA which leave it highly susceptible to the formation of cruciform structures. *http://fmp 8.cit.nih.gov/hembase/detail.php?chrb=17q23* and *http://proj1.sinica.edu.tw/~tigpcbmb/course%20material/cb9903/cb9903.htm*

A study by Gururaj *et al.,* (2006), links BCAS3 to tamoxifen resistance in premenopausal women. A positive correlation was observed between BCAS3 and tumour grade/proliferation. Metastasis associated protein 1 (MTA1) has been shown to act as a co-activator of BCAS3, depending on post-translational modifications on the protein. If MTA1 is acetylated on lysine residue 626, it up-regulates the transcriptional activity of BCAS3 by recruiting RNA polymerase II onto an enhancer region of the BCAS3 gene (Gururaj *et al.,* 2006). MTA1 binds to the ERE that is located 12kb downstream from the transcriptional start site of BCAS3. Following stimulation of

BCAS3, a positive feedback loop between BCAS3 and ERα is initiated. However, BCAS3 requires another co-activator called PELP1 (Proline-,glutamic acid-,leucine-rich protein 1) in order to regulate ERα (Gururaj *et al.,* 2007). This feedback loop has been observed in an endocrine sensitive cell line. The same study also investigated the potential of SRC1 in activating BCAS3 in an endocrine sensitive model. SRC1 did not regulate the transcriptional activity of BCAS3. However, it is important to mention that this has not been investigated in an endocrine resistant model. A recent study by Jain *et al.,* (2012) observes that BCAS3 deficiency severely impairs cellular organization and polarity in endothelial cells by disrupting actin localization.

1.5 Hypothesis

Interactions between ERα and SRC1 have been shown to play a part in the development of endocrine resistance in breast cancer. There are many studies that have identified SRC1 to be a strong predictor of disease recurrence and poor disease free survival. Our research group employed the use of mass spectrometry in order to gain an insight into other proteins that were bound to SRC1. One such protein that was identified was HMGB2. HMGB2, a highly abundant non-histone nuclear protein, is known for its ability to induce chromatin rearrangement. This rearrangement can facilitate the recruitment of transcriptional complexes onto target promoters, thereby enhancing gene transcription. We hypothesised that HMGB2 contributes to the ability of SRC1 in promoting endocrine resistance in breast cancer.

Subsequently, our research group sought to determine which target genes this proposed HMGB2/SRC1/ERα complex may be regulating. Previous work that was carried out in

our lab discovered a gene called BCAS3 was a target for SRC1. This was revealed through the use of chromatin immunoprecipitation sequencing (ChIP-seq), combined with parallel high-throughput sequencing. In brief, this technique involves immunoprecipitating a known protein from a complex, such as SRC1. As the protein has previously been crosslinked, the immunoprecipitated protein is bound to its respective DNA fragment. This DNA fragment is sequenced on a genome sequencer. The sequenced fragments are aligned to a reference genome and mapped for enriched regions called peaks, which are identified through peak calling software packages. Our lab identified that BCAS3 was a target gene for SRC1. We hypothesised that BCAS3 is the target gene for the transcriptional complex of ERα/SRC1/HMGB2.

1.6 Aims

The aims and specific objectives of the study were defined as follows:

To investigate the role of HMGB2 in endocrine resistant breast cancer:

- In the *in vitro* setting, using breast cancer cell lines to determine the relationship with SRC1, ERα and BCAS3.
- In the breast cancer patient population using immunohistochemistry of patient tissue on a tissue microarray.

Chapter II

Materials and Methods

2.1 Cell Culture

2.1.1 Breast cancer cell lines

MCF-7 breast cancer cell line

MCF-7 cells, a well established breast cancer cell line which over-express ER and PR but not HER2 were obtained from the American Type Culture Collection (ATCC, Virginia, USA). They were cultured in minimum essential medium (MEM) (Sigma Aldrich, Stenheim, Germany) supplemented with 10% foetal calf serum (FCS) (Sigma Aldrich), 10,000 units of penicillin (Sigma Aldrich), 10mg of streptomycin (Sigma Aldrich) and 1ml of 200mM L-glutamine (Sigma Aldrich) per 100mls of medium.

LY2 breast cancer cell line

LY2 cells were kindly donated by Dr. Robert Clarke, Department of Oncology, Georgetown University, DC, USA. These cells were developed as a stable variant of MCF7 cells that are resistant to LY117018, a potent antiestrogen and are cross resistant to tamoxifen (Bronzert *et al.* 1985). The cells are sub-cultured in phenol red free MEM containing 10% charcoal dextran stripped fetal calf serum (Sigma Aldrich) to ensure low levels of certain steroid hormones with constant exposure to 10^{-8}M 4-hydroxytamoxifen (4-OHT) (Sigma Aldrich). Charcoal: dextran stripping reduces the serum concentration of many hormones and certain growth factors, such as estradiol, cortisol, corticosterone, T3, T4 and prostaglandins. L-glutamine (Sigma Aldrich) was also added to the medium; 1ml of 200mM per 100mls of medium, in addition to antibiotics; 10,000 units of penicillin (Sigma Aldrich), 10mg of streptomycin (Sigma Aldrich).

2.1.2 Cell culture environment

All cell culture techniques were performed in a sterile environment using a laminar airflow cabinet. All cells were maintained in a humid 5% (v/v) CO_2 atmosphere at 37 °C.

2.1.3 Passaging of cells

Culture medium was removed from the cell culture flask and the adherent cells washed twice with sterile PBS (Gibco) in order to remove remaining media. Cells were detached from the flask base by incubating the cells in trypsin solution at 37°C and by tapping gently on the side of the flask at regular intervals. Detached cells were centrifuged at 1,200 rpm for 4 minutes at room temperature. The resultant cell pellet was resuspended in appropriate culture medium and placed into 75cm^3 flask. In general, one confluent 75cm^3 flask of MCF7 and LY2 cells was passaged into four to five 75cm3 flasks.

2.1.4 Culturing of cells from cryo-storage

Cryovials containing cells were removed from storage at -80°C and thawed on ice. The cells were transferred to a sterile universal container containing 5 ml of the required culture medium. The cell suspension was centrifuged at 1,250 rpm for 4 minutes. The supernatant was discarded and the pellet resuspended in 2 ml of fresh medium. This suspension was added to a 75 cm^2 tissue culture flask, to which a further 8 ml of culture medium was added. The flasks containing the cells were then incubated at 37°C.

2.1.5 Cell Treatments

Prior to any experiment involving endocrine treatments, cells were sub-cultured for 72 hours in phenol red free MEM containing 10% charcoal dextran stripped fetal calf serum to avoid potential bias from steroid hormones present in FBS or phenol red.

Cells were treated with 17B-estradiol (10^{-8}M), 4-hydroxytamoxifen (4-OHT) (10^{-8}M) or 0.01% ethanol as a vehicle control.

2.1.6 Cell Counting

A haemocytometer was used to count cells. A cell pellet was re-suspended in 5mls of media and vortex mixed. A 50µl sample of this suspension was added to 50µl of tryphan blue (Sigma Aldrich) and mixed. 10µl of this mixture was then added to the chamber on the haemocytometer. The numbers of cells present in the central 1mm^2 grid were counted equating to a volume of 0.1µl. This process was repeated three times and the mean value used to estimate the number of cells/µl and from this the required volume of cell suspension was determined and seeded in the appropriate culture vessel.

2.2 Protein biochemistry

2.2.1 Protein extraction

Pefabloc protease inhibitor (10 µl) (Roche Diagnostics, Mannheim Germany) was added to lysis buffer (1 ml) (Appendix) to make the protein lysis solution. Lysis solution (100 µl) was added to each cell pellet. The samples were placed on ice for 30 minutes and vortexed at 10 minute intervals for 10 seconds each time. The samples were then centrifuged at 13,000 rpm for 20 minutes at 4^{o}C. The resultant supernatant (protein lysate) was transferred into chilled Eppendorf tubes and stored at -20^{o}C.

2.2.2 Protein quantification

This procedure was performed using the Pierce Bicinchoninic acid (BCA) protein assay kit (Pierce, Il USA). A standard curve was obtained by serially diluting 5 mg/ml bovine serum albumin (BSA) in dH_2O. A blank solution containing only dH_2O was also included. Protein lysate samples (5 µl) were diluted 1:10 in dH2O. Standards and diluted samples (25 µl) were pipetted in duplicate into a 96 well plate. The reaction mix was made up with 49 parts of Solution 1 of the Pierce BCA protein assay kit (containing Na2CO3, NaHCO3, BCA detection reagent and sodium tartrate in 0.5 M NaOH) with one part of Solution 2 (4% (w/v) CuSO4). Standards and samples were incubated with 200 µl of reaction mix at 37°C for 30 minutes. The absorbance of the samples was analysed at 450 nm using a spectrophotometer. Linear regression analysis of the BCA standard curve (y axis absorbance, x axis protein concentrations) was used to calculate the unknown protein concentrations of each sample.

2.2.3 Coimmunoprecipitation

Coimmunoprecipitation is a process that allows examination of a protein- protein interaction. Equal concentrations of total protein (1 mg) in a total volume of 1ml were pre-cleared with the addition of 6ug of rabbit IgG. Samples were then rotated with 50 µl of Protein A/G agarose beads (Santa Cruz) (previously blocked in bovine serum albumin 3% overnight at 4°C) for 4 hours at 4°C. Protein agarose was removed from the protein lysate by 5 minutes centrifugation at 1000 rpm at 4°C. Protein was immunoprecipitated with anti- ERα (6 µg) overnight at 4°C. The precipitates were collected for 4 hours on a Protein A/G agarose complex. The samples were centrifuged at 5,000 rpm for 60 seconds and the supernatant was discarded. The remaining cell precipitates were washed 3 times in radioimmunoprecipitation (RIPA) buffer (Appendix

1) and centrifuged at 5,000 rpm for 60 seconds. The precipitates were then resuspended in 2X Laemelli SDS sample buffer (Sigma-Aldrich), boiled at 95°C for 10 minutes and analyzed by SDS-PAGE and Western blotting.

2.2.4 SDS-PAGE and Western blotting

Western blotting is used to detect proteins in tissue or cell lysate. Polyacrylamide gel electrophoresis (PAGE) is used to separate proteins based on size. The gel is then transferred to a membrane using electrical current allowing for detection with a specific antibody to the protein of interest.

To enable access of the antibody to the epitope of interest it is necessary to denature the protein using an anionic denaturing loading buffer. For this purpose, the desired quantity of protein lysate, standard 60µg per sample for a western blot, was mixed with an equal volume of 2x Laemelli buffer, briefly vortexed and boiled at 95^{o}C for 5 minutes. The laemmli buffer contains sodium dodecyl sulfate (SDS) which causes proteins to become negatively charged by their attachment to SDS anions. SDS denatures proteins by "wrapping around" the polypeptide backbone. In so doing, SDS confers a negative charge to the polypeptide in proportion to its length. In denaturing SDS-PAGE separations, therefore, migration is determined by molecular weight.

Polyacrylamide gels are formed from the polymerization of two compounds, acrylamide (Sigma Aldrich) and N,N-methylenebisacrylamide which is a cross-linking agent for the gels. The polymerization is initiated by the addition of ammonium persulfate (Sigma Aldrich) along with N,N,N,N-tetramethylethylenediamine (TEMED) (Sigma Aldrich). The separation of molecules within a gel is determined by the relative size of the pores with pore size determined by the total amount of acrylamide present and the amount of

cross-linker. As the total amount of acrylamide increases, the pore size decreases. Gels were prepared on the BioRad Mini-PROTEAN Tetra Electrophoresis System (BioRad, Hercules, CA, USA) and allowed to polymerise for 40 minutes at room temperature. A 5% stacking cell was then poured to the top 12mm of the gel and a 1.5mm, 10-well comb inserted and again allowed to polymerise for 40 minutes at room temperature. The composition of the various percentage gels used is outlined in table 2.1.

Table 2.1: Gel preparation for SDS-PAGE

	6% Resolving	8% Resolving	10% Resolving	15% Resolving	5% Stacking
H_20	5.3 ml	4.6 ml	4.0 ml	3.4 ml	1.4 ml
30% acrylamide mix	2.0 ml	2.7 ml	3.3 ml	7.5 ml	0.33 ml
1.5M Tris (pH 8.80)	2.5 ml	2.5 ml	2.5 ml	3.8 ml	0.25 ml
10% SDS	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.150 ml
10% ammonium persulphate	0.1 ml	0.1 ml	0.1 ml	0.15 ml	0.1 ml
TEMED	0.008	0.006 ml	0.004 ml	0.006 ml	0.002 ml

Solutions for preparing gels for SDS-PAGE electrophoresis. Volumes (ml) provided are for preparation of 10mls of resolving gel and 2mls of stacking gel, sufficient for preparation of one standard gel on a BioRad 1.5mm plate.

Gels were placed in the BioRad electrophoresis module and the tank filled with 900mls of 1x running buffer (Appendix A). Prepared samples were carefully loaded into the relevant well. A molecular weight marker, 6µl of Precision plus protein standard (BioRad) was run alongside samples for estimation of molecular weight. Gels were run at a constant voltage of 80V for the initial 20 minutes followed by a further 90 minutes at 110V.

After SDS-PAGE electrophoresis proteins are transferred using electrical current from the gel onto a membrane to allow for visualization. This transfer can be performed in wet or semi-dry conditions. Both the semi-dry and wet methods were adopted for

different proteins in this study. The gel plates were disassembled and the gels removed and placed in a cooled transfer buffer (Appendix A) and allowed to equilibrate with the buffer for 15 minutes. A sandwich of 5 pieces of Whatman filter paper / gel / nitrocellulose membrane / 5 pieces Whatman paper wetted in transfer buffer was made placed directly between the cathode and anode respectively of the semi dry transfer rig (Atto, Tokyo, Japan). A constant current of 250mA was used for transfer with transfer times varying according to molecular weight of the protein of interest and the transfer method used. The relevant times are recorded in table 2.2.

Table 2.2: Conditions used for western blotting for proteins studied

Protein	Molecular Weight	Gel %	Transfer		Primary Antibody		Secondary Antibody	
			Method	Time	Antibody	Conc	Antibody	Conc
β-Actin	42kDa	-	Semi dry	-	Mouse monoclonal	1:2000	Anti-mouse	1:5000
BCAS3	117kDa	8%	Wet	3 hours	Rabbit polyclonal	1:1000	Anti-rabbit	1:2000
ERα	68kDa	10%	Wet	3 hours	Rabbit polyclonal	1:200	Anti-rabbit	1:2000
HMGB2	26kDa	15%	Semi dry	45 mins	Mouse monoclonal	1:200	Anti-mouse	1:2000
c-MYC	67kDa	8%	Wet	3 hours	Rabbit polyclonal	1:200	Anti-rabbit	1:2000
SRC-1	160kDa	6%	Wet	3 hours	Rabbit polyclonal	1:200	Anti-rabbit	1:2000

The nitrocellulose membrane was removed from the transfer apparatus after the appropriate time and placed in 10ml of 5% non-fat dry milk (Chivers, Dublin, Ireland) in tris-buffered saline (TBS) for 1 hour at 4^{o}C with gentle rocking. The primary antibody was diluted, in a volume suited to membrane size, in 2.0% non-fat dry milk in TBST to the required concentration as documented in table 2.2. Membranes were incubated in diluted primary antibody overnight with gentle rocking at 4^{o}C. Following primary antibody incubation, the membrane was washed three times, 10 minutes each

time in TBST. The relevant secondary HRP conjugated antibody; anti-Rabbit IgG-HRP (Sigma Aldrich) or anti-mouse IgG-HRP (Sigma Aldrich) was reconstituted to the concentration specified in table 2.2 in 5% non-fat dry milk in TBST. Incubation in secondary antibody was for 60 minutes at 4^{o}C. The membrane was washed a further 3 times, 10 minutes each time in TBST and then developed with substrate solution. SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) was used as substrate solution with luminol/enhancer solution and stable peroxide solution mixed in a 1:1 ratio to form a working solution of 0.1 ml per cm^2 of membrane. The blot was incubated in the working solution for 5 minutes at room temperature and then placed in a labelled plastic membrane protector and using a dark room, exposed to X-ray film (FujiFilm, Tokyo, Japan). The film was developed by immersion in developing solution followed by fixer solution (Kodak, USA) and left to air dry.

2.2.5 Mass spectrometry

SDS–PAGE gel lanes were cut into bands and digested in-gel with trypsin according to the method of Shevchenko *et al*. (1996). The resulting peptide mixtures were re-suspended in 1% formic acid and analysed by nano-electrospray liquid chromatography mass spectrometry (Nano-LC MS/MS). A HPLC instrument (Dionex, LC Packings, UK) was interfaced with an LTQ ion trap mass spectrometer (ThermoFinnigan, CA). Protein digests were injected using an autosampler into a C18 PepMap 100 (Dionex) column was used with a length of 150mm, internal diameter (ID) of 75□M, a particle size of 3□□M and a pore size of 100 Angstrom. Chromatography buffer solutions (Buffer A, 5% acetonitrile and 0.1% formic acid; Buffer B, 80% acetonitrile and 0.1 % formic acid) were used to deliver a 60 min gradient (35 min to 45% Buffer B, 10 min to 90%, hold 10 min, 3 min to 5 %, hold for 15 min). A nanoflow rate of 2 µl/min was

used at the electrospray source. Full MS scans were recorded on the eluting peptides over the 300-2000 m/z range. Tandem MS (MS/MS) spectra were acquired in a data-dependent manner, sequentially on the first to tenth-most intense ion selected from the full MS scan. Spectra were searched using the X!Tandem algorithm (*www.thegpm.org/tandem/)* against the International Protein Index (IPI) database (http://www.ebi.ac.uk/IPI/). The list of proteins was refined manually to remove one-peptide hits, keratin and IgG contamination, and to exclude non-specific interactions (defined as any protein that had one or more distinct/unshared peptide found in the IgG control samples) according to the method of Goodall *et al.* (2010).

2.2.6 Chromatin immunoprecipitation

The purpose of a chromatin immunoprecipitaion (ChIP) assay is to determine whether proteins bind to a particular region on the endogenous chromatin of living cells or tissues. LY2 cells were cultured in 75 cm^2 tissue culture flasks to approximately 80-90% confluence. They were steroid depleted for 72 hours prior to a 45 min treatment of 17B-estradiol (10^{-8}M), 4-hydroxytamoxifen (4-OHT) (10^{-8}M) or 0.01% ethanol as a vehicle control. Proteins were crosslinked to the DNA by treating the cells with 1% formaldehyde in Eagles MEM medium lacking FBS for 10 minutes before quenching with glycine (125 mM, 5 minutes at room temperature). Cells were washed in ice-cold PBS before being scraped in a solution of PBS and protease inhibitors (10µl/ml). Sonication conditions were previously tested in the lab to yield DNA fragments averaging 200 – 500 bp as assessed by agarose gel electrophoresis and were as follows: 8 sonications (10 seconds), with 2 minutes between each; output control 4-5; duty cycle 60% (using a Branson Sonifier 250, Danbury, CT, USA). Lysates were quantified using the NanoDrop Spectrophotometer (Thermo Scientific, DE, USA). Lysates were

normalised to the sample with the lowest concentration so that all samples contained the same amount of sonicated DNA. Sonicated DNA was diluted to 2mls in ChIP dilution buffer including 1% protease inhibitor. 20µl of each diluted sample was removed to a fresh Eppendorf and incubated at -80°C, to be used as input DNA at a later stage. 75µl salmon sperm (Millipore, Protein A agarose Salmon sperm DNA) was added to the normalised DNA samples (outputs) for 30 minutes at 4°C. To achieve immunoprecipitation, 6 µg of HMGB2 (Abcam rabbit polyclonal) antibody was added to each sample. After incubation with antibody over night at 4°C, 60µl of salmon sperm was added for an additional 90 minutes of incubation. Beads were then washed consecutively for 5 minutes on a rotating platform with 1ml of each solution: (a) low salt wash buffer, (b) high salt wash buffer, (c) Lithium Chloride wash buffer and (d) 1X TE buffer twice (Appendix 1). Output and input DNA samples were re-suspended in 100µl of 10% Chelex-100 resin solution (Bio-Rad, Hercules, CA, USA). Samples were vortexed for 10 sec and heated to 95°C for 10 minutes. 2µl of proteinase K solution (Sigma-Aldrich) was added to the samples, which were vortexed and incubated at 55°C on a heating block for 30 minutes with agitation at 1,000 rpm. Samples were heated again at 95°C for 10 minutes to inactivate proteinase K, and vortexed. Samples were centrifuged at 10,500rpm for 1 minute at 4°C to elute ChIP DNA. Supernatants (80µl) were transferred to fresh tubes. DNA yield and purity was measured with the NanoDrop Spectrophotometer. DNA was submitted to PCR to amplify the promoter regions of interest (i.e. BCAS3 promoter).

2.3 Nucleic Acid Biochemistry

2.3.1 PCR

Polymerase chain reaction (PCR) is a technique used to amplify segments of DNA. The method employs a heat stable DNA polymerase to enzymatically assemble a new DNA strand from template DNA through a number of cycles using specific DNA primers for initiation of DNA synthesis.

All PCR reactions were made up in a 0.2ml domed PCR reaction tube (StarLab, Ahrensburg, Germany) to a volume of 50µl. The compositions of the PCR reactions used are recorded in table 2.3. Likewise the starting amount of template DNA will differ according to the application and varied from 1.0µl for cDNA to 30.0µl of ChIP output DNA.

Table 2.3: PCR reaction reagents and composition

Component	Volume (µl)	Final Concentration
10X PCR buffer	5.0	1x
50mM MgCl2	0.5 – 2.0	0.5 – 2.0mM
10mM dNTP Mix	1.0	0.2 mM each
Forward Primer	1.25	0.5 µM
Reverse Primer	1.25	0.5 µM
Taq DNA polymerase	0.20	1.0 unit
Template DNA	1.0 – 30.0	-
Autoclaved distilled water	39.85 – 10.85	-

PCR reaction reagents and composition for a standard 50µl PCR reaction.

Reactions were performed in a thermal cycler (DNA Engine Tetrad 2, Peltier Thermal Cycler, BioRad, Hercules, CA). There are a number of stages during each reaction:

- Initial DNA denaturation: Incubation in a thermal cycler at 94^{o}C for 3 minutes ensures denaturation of template DNA and activation of the Taq DNA

polymerase.

- DNA denaturation: heating at 94°C for 45 seconds ensures DNA denaturation at the beginning of each cycle.
- Primer annealing: following denaturation, temperature is reduced to allow primer annealing. Temperature and duration of this annealing stage is dependent on the specific primers and the conditions for all primers used are detailed in table 2.5.
- Extension: DNA polymerase synthesises a new complementary DNA strand by adding dNTPs in the 5' to 3' direction. This stage is performed at 72°C for 90 seconds.
- The denaturation-annealing-extension cycle is repeated up to 37 times depending on the product under amplification. Details of the cycle number for the various PCR reactions performed are included in table 3.
- Final elongation: 10 minute incubation at 72°C after the last cycle is used to ensure that any remaining single-stranded DNA is fully extended.

Table 2.4: Primers used

PCR	Sequence (5'-3')	Tm°	Product Size
BCAS3 Promoter For	GGGAGATGGAAACTGAAGCA	64.1	197bp
BCAS3 Promoter Rev	CTTTTAGCTGGCCACTCACC	63.7	

Primer sequences used in PCR reactions with melting temperatures (Tm°) for each primer and expected product size.

PCR products were analysed using agarose gel electrophoresis for size separation with ethidium bromide dye used as an intercalating agent. Tris/Acetate/EDTA (TAE) or Tris/Borate/EDTA (TBE) were used as buffers and the percentage of agarose gel varied

between 1.0% and 2.0% according to predicted product size. Gels were visualised under UV light and images recorded using the LAS3000 Image software (Fuji, Japan).

Table 2.5 Cycling conditions for PCR reactions used

PCR Product	MgCl Conc	Denature		Anneal		Extend		No. of Cycles
		Temp (°C)	Time (s)	Temp (°C)	Time (s)	Temp (°C)	Time (s)	
BCAS3 Promoter	1.5mM	94	45	63.0	30	72	90	30

2.3.2 Real time PCR

Real time PCR, or quantitative real time PCR, enables both detection and quantification of specific DNA sequences in a sample. The fundamental difference when compared to traditional PCR is that in real time PCR the amount of accumulated DNA template is measured throughout the reaction rather than assessing the amount of template at the end. By focusing on quantification during the exponential phase of the reaction, real time PCR allows for accurate quantification of the samples. The PCR products are detected through use of fluorescent dyes that bind to double stranded DNA.

The fluorescent dye used was SYBR green (Qiagen). Experiments were performed on the LightCycler 2.0® system (Roche Diagnostics, Forrenstrasse, Switzerland) with data analysis on the corresponding software. 2µl of ChIP output samples were used in each qPCR reaction. Reactions were prepared in standard 20µl LightCycler capillaries with the following composition:

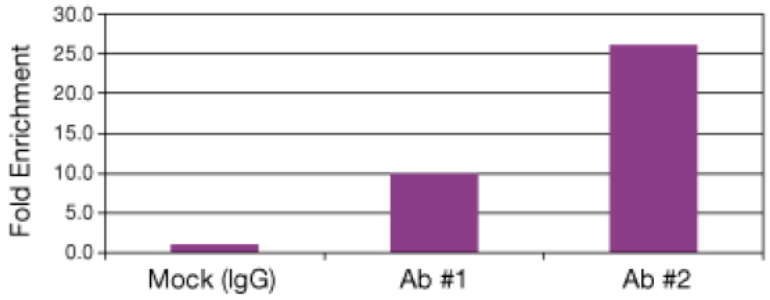
- SyberGreen Master Mix (Qiagen): 10.0µl
- Primer Mix: 2.0µl of 12.5µM primer stock
- ChIP output DNA: 2µl

- dH_20: to a total reaction volume of 20µl (i.e. 6 µl)

In order to normalise the ChIP samples on the LightCycler system, the 'fold enrichment method' was employed. This produces 'signal over background' or 'IgG' in this case. With this method, the ChIP signals are divided by the no-antibody signals, representing the ChIP signal as the fold increase in signal relative to the background signal. The assumption of this method is that the level of background signal is reproducible between different primer sets, samples, and replicate experiments. C_t which stands for the "threshold cycle," is the intersection between the DNA amplification curve and the threshold line. It provides a relative measure of PCR reaction product, i.e. target DNA.

Table 2.6: To calculate fold enrichment of ChIP samples on qPCR

Antibody	**Raw C_t**	**Non-specific adjustment (C_t IP) - (C_t mock)**	**Fold enrichment ($2\text{-}DDC_t$)**
Mock IgG	34.6	0	1
Antibody # 1	31.3	-3.3	9.8
Antibody # 2	29.9	-4.7	26.0



ChIP analysis method was replicated from Invitrogen instructions as outlined on http://www.invitrogen.com. Capillaries were then loaded onto the LightCycler instrument and the run conditions used are outlined in table 2.7.

Table 2.7: Run conditions used for qPCR reactions.

qPCR Product	Denature		Anneal		Extend		No. of
	Temp (°C)	Time (s)	Temp (°C)	Time (s)	Temp (°C)	Time (s)	Cycles
BCAS3 promoter	94	15	60	20	72	20	50

2.4 Transfections

2.4.1 Gene Silencing

Gene silencing is when the level of a particular protein of interest is reduced. In this study, silencing was carried out using RNA interference (RNAi) technology as described by Elbashir *et al*., 2001. Predesigned and validated siRNA directed against HMGB2 (Dharmacon), SRC-1 (Ambion), BCAS3 (BCAS3_7 from Qiagen Flexitube) and ERα (Ambion) were used in the knockdown studies. 2 x 10^5 cells were grown in antibiotic and serum free media for 24 hours in 6- well plates. Cells were transfected when 70% confluent. Oligomer- Lipofectamine 2000 complexes were prepared as follows:

• Solution A:

-60 pmol of siRNA SRC-1 was diluted in 250 µl of Opti-MEM serum reduced media. -50 pmol of siRNA HMGB2 was diluted in 250 µl of Opti-MEM serum reduced media.

-100 pmol of ERα siRNA was diluted in 250µl of Opti-MEM serum reduced media.

- 50pmol of BCAS3 siRNA was diluted in 250µl of Opti-MEM serum reduced media.

• Solution B:

5 µl of Lipofectamine 2000 was diluted in 250 µl Opti-MEM serum reduced medium. Solutions were incubated at room temperature for 5 minutes. The diluted oligomer was then mixed with the diluted Lipofectamine 2000 solution and incubated at room temperature for 20 minutes. The oligomer- Lipofectamine complex was added to the

cell monolayer and mixed gently by rocking the plate back and forth. Cells were incubated at 37°C for 6 hours after which the transfection media was replaced with standard MEM. Protein was harvested at 48hours from time of transfection for HMGB2 and ERα siRNA samples. Protein was harvested at 72 hours from time of transfection for SRC1 and BCAS3 siRNA samples.

2.4.2 Protein over-expression

Over-expression studies of SRC1 and HMGB2 were performed using the Lipofectamine 2000 transfection reagent (Invitrogen, catalogue number: 11668-027). This system is a non-viral method of facilitating DNA transport into the cell nucleus. The over-expression studies were transient in nature and performed using vectors for the genes of interest that had already been designed and validated within the laboratory. The vector used for HMGB2 over-expression was pSPORT6 (Invitrogen) and that for SRC-1 was pcDNA3.1 (Invitrogen). Prior to transfection, LY2 cells were steroid depleted as standard for 72 hours. Transfections were performed in media without antibiotics. $5x10^6$ cells were grown in 6- well plates in antibiotic free medium and incubated in a CO_2 incubator at 37°C until 80-90% confluent. Solutions for the transfection experiment were prepared in 1.5 ml Eppendorf tubes as follows:

• Solution A: 2 µg of HMGB2 in pSPORT6 was diluted in 375 µl of reduced serum medium Opti-MEM (Gibco® Invitrogen). 2µg of SRC1 in pcDNA3.1 was diluted in 375µl of reduced serum medium Opti-MEM (Gibco® Invitrogen).

• Solution B: 4 µl of Lipofectamine 2000 reagent (Invitrogen) was diluted in 375 µl of Opti-MEM. Solutions were allowed to stand for 5 minutes at room temperature. Both solutions were mixed gently and incubated at room temperature for 20 minutes. Cells were then washed with sterile PBS. The Lipofectamine-DNA mixture was then added to

the cell monolayer in a drop wise fashion and incubated at 37°C for 4 hours after which full medium was added. Protein quantification was determined 24 hours post transfection.

2.5 Immunohistochemistry

2.5.1 Biological samples

Ethical approval for all studies on human samples was obtained from the Medical Ethics Committee, Beaumont Hospital, Dublin 9 and also from St. Vincent's, University Hospital, Elm park, Dublin 4. Biological samples were only used from patients who provided informed consent for the use of their tissue in any subsequent research. Tissue from a total of 937 patients is present on tissue microarrays, with standard clinicopathological (hormonal status, node and grade) and follow-up data recorded.

2.5.2 Staining protocol

Sections were taken at a thickness of 5µm using a microtome and mounted on Superfrost Plus slides (VWR International, Leuven, Germany). Sections were deparaffinised by passage through xylene twice for a time of 3 minutes each time. This was followed by rehydration by sequential passage for six minutes each through decreasing concentrations of industrial methylated spirits (100% → 90% → 70%). The slides were then washed twice in PBS for 5 minutes. Endogenous peroxidase activity was quenched by incubating the slides in 3% hydrogen peroxide (H_2O_2, Sigma Aldrich) for 20 minutes followed by a wash in PBS. Heat mediated antigen retrieval was performed by placing the slides in a 10mM sodium citrate buffer (pH 6.0) and heating in a closed plastic container in a domestic microwave for 7 minutes at high power and

then 5 minutes at medium power followed by 10 minutes cooling at room temperature. Binding of secondary antibody to non specific endogenous immunoglobulins was minimised by pre-incubating slides at room temperature for 90 minutes with a 3% solution of serum from the species in which the relevant secondary antibody was raised. Primary antibody concentrations were determined according to the manufacturer's instructions and subsequent optimisation. Primary antibodies were diluted in PBS to the required concentration. Details of the relevant primary antibody concentration and incubation conditions are described in table 2.8.

Table 2.8: Conditions used for immunohistochemistry

Target Protein	Blocking Solution	Primary antibody	Primary antibody concentration	Primary antibody incubation time	Secondary Antibody
HMGB2	Vectastain horse serum	Mouse monoclonal anti HMGB2 - Abnova H00003148-M05)	5.0µg/ml	Overnight at 4^0C	Anti mouse IgG

Sections were then washed in PBS containing 0.1% Tween (Sigma Aldrich) for 15 minutes. The slides were then incubated in the relevant Vectastain Elite (Burlingame, CA) biotinylated secondary antibody (PK-1600 series) diluted by a factor of 1 in 200 in a solution of PBS containing 3% of the relevant blocking serum and maintained at room temperature for 30 minutes. The signal was amplified by incubating with the Avidin-biotin complex from the Vectastain Elite kit for a further 30 minutes. Product was developed using 3,3'-Diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich) for 3 minutes followed by 3 minutes incubation with haemotoxylin (Sigma-Aldrich) for counterstaining after which slides were washed in flowing tap water for 5 minutes. Samples were then dehydrated again by passing through increasing concentrations of

IMS (70% → 90% → 100%) and then xylene for a further 6 minutes. Samples were mounted with DPX mounting solution (Sigma-Aldrich).

2.5.3 Immunohistochemistry scoring system

An intensity score was assigned that represented the average intensity of the positive tumour cells (none=0, weak=1, intermediate=2, strong=3). The proportion and intensity scores were then added to obtain a total score, which ranged from 0 to 8.

A total score of greater than three was deemed positive. Two individuals, who were blinded to the patient clinico- pathological data, scored the TMAs separately and results were entered into a database. These results were then analysed using statistical software.

2.6 3D Mammosphere Assay

Three-dimensional culture systems have been shown to provide important insights into the polarisation of mammary epithelial cells. In-vivo, these epithelial cells undergo a distinct morphological sequence of events that result in the formation of acini-like spheroids (Debnath *et al.,* 2003). Pathogenesis is associated with the disruption of this well organised, luminal structure. 3D culture of MCF-7 and LY2 cells were performed. Chamber slides were coated with 100% Matrigel (BD Biosciences). Cells were diluted to a final concentration of 2.5 x 10^4cells/ml. 200µl of this cell suspension (i.e. 5,000 cells) and 200ul of the appropriate media and 4% of Matrigel mix was added to each well, medium was changed every four days. Cells were cultured for 14 days and then fixed 4% PFA at room temperature for 20 minutes. Figure 2.1 illustrates the method for 3D cell culture.

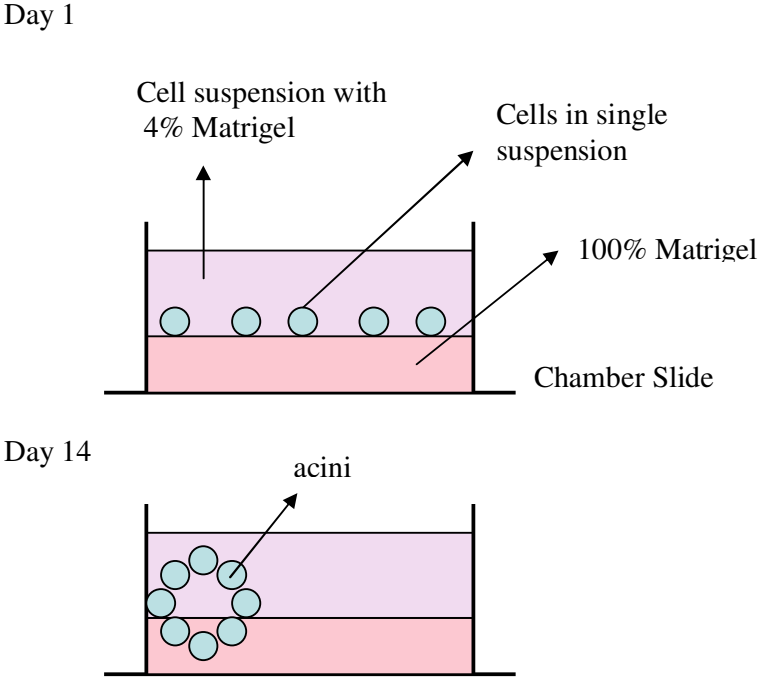


Figure 2.1: Schematic representation of the method of 3D cell culture on Matrigel. The well of the chamber slide is initially coated with 100% Matrigel and allowed to solidify. Cells are seeded onto this bed as a single suspension in appropriate medium and 4% Matrigel. The medium is replaced every 4 days. Cells proliferate, form clusters and subsequently form acini (Debnath *et al.,* 2003).

For immunoflorescence, cells were fixed with 4% PFA at room temperature for 20 minutes. The cells were subsequently permeabilised with 0.5% triton in PBS for 10 minutes at 4^{o}C. Cells were incubated with Phalloidin conjugated antibody at room temperature for 60 minutes. After counter-staining with DAPI, the slides were mounted following the application of anti-fade solution (Dako). Slides were examined by confocal microscopy.

Chapter III

Investigation of HMGB2 in the ER/SRC1 complex in endocrine resistant breast cancer

3.1 Introduction

The process of differentiation leading to an organised cellular structure is paramount in normal breast development. The normal breast gland is composed of a highly organised ductal-lobular system. This system is controlled by various polarity, prolific, apoptotic and differentiating signals. Appropriate signal control and communication between these signalling pathways determines the organisation of developing mammary glands. The lumen formation inside the epithelial tubes; caused by apoptosis is essential for milk secretion in the breast (Debnath *et al.,* 2002). Disruption of this elaborate architecture is the beginning of a breast carcinoma. *In vitro* models have been successful in recapitulating the sequence of differentiation in the glandular epithelial structure. These models essentially use a matrigel component which mimics the basal membrane in the breast. It contains the necessary proteins that are required for cell polarity such as collagen, laminin and ectactin (Kleinman *et al.,* 1982). In particular, laminin has been shown to be a key player in the maintenance of polarity (Gudjonsson *et al.,* 2002).

It is known that ERα positive tumours are histologically well differentiated and display a less aggressive phenotype, thereby resulting in a more favourable disease free survival outcome (McGuire *et al.*, 1991). Little is known about the co-activators that contribute to ERα in promoting a well differentiated tumour in breast cancer. El-Dhaheri *et al.,* (2011) demonstrate that CARM1 (co-activator associated arginine methyltransferase 1) is a co-activator for ERα; regulating ERα responsive genes. The expression of CARM1 results in an inhibition of proliferation and a stimulation of differentiation, resulting in a luminal type tumour with the well organised structure that was previously described in

section 1.1.3. The co-expression of CARM1 and ERα are indicators of a lower grade breast cancer.

SRC1 has been shown to be a primary contributor to endocrine resistance and breast cancer recurrence, resulting in a reduced disease free survival (Redmond *et al.,* 2009). Over-expression of SRC1 has been shown to mediate constitutive ERα expression in the presence of tamoxifen (Shang and Brown, 2002). More recently, it has been confirmed that SRC1 is a key regulator of the disintegrin ADAM22, which influences cellular migration and differentiation (McCartan *et al.,* 2012).

3.2 Results

3.2.1 ERα knockdown in MCF7 and LY2 cells prior to 3D culture assay

Prior to the 3D culture assay being carried out, confirmation of ERα was required. MCF7 cells and LY2 cells were transiently transfected with siRNA targeting ERα. The siRNA knockdown was confirmed by western blot analysis to measure ERα protein levels (figure 3.1). At 48hours post-transfection cells were harvested and lysates were subject to western blot analysis with an anti-ERα antibody. Considerably lower levels of ERα were observed in the cells that had been transfected with the ERα siRNA, in comparison to cells transfected with the control scrambled siRNA(siScr).

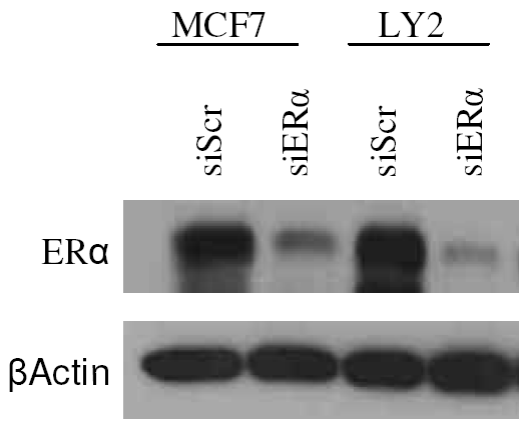


Figure 3.1: Western blot analysis demonstrates the successful transfection of ERα siRNA into MCF7 and LY2 cells prior to 3D culture assays being undertaken (n=2).

3.2.2 3D Culture Assay in MCF7 and LY2 cells with siRNA targeting ERα

In order to replicate mammary gland morphogenesis in an *in vitro* setting, the 3D culture assay was employed. This assay illustrates the differentiation potential of cells by observing their ability to develop spherical acini-like structures when cultured in Matrigel ™. Loss of ERα function by siRNA knockdown lead to a decreased ability to form an organised spherical structure with a lumen in both cell lines. The LY2 cell line appears to be less organised than the MCF7 cell line following ERα knockdown. The general assumption is that gene silencing resulting from siRNA can last between 5-7 days (depending on cell type and concentration of siRNA). This delayed ERα expression is responsible for the decrease in luminal structure.

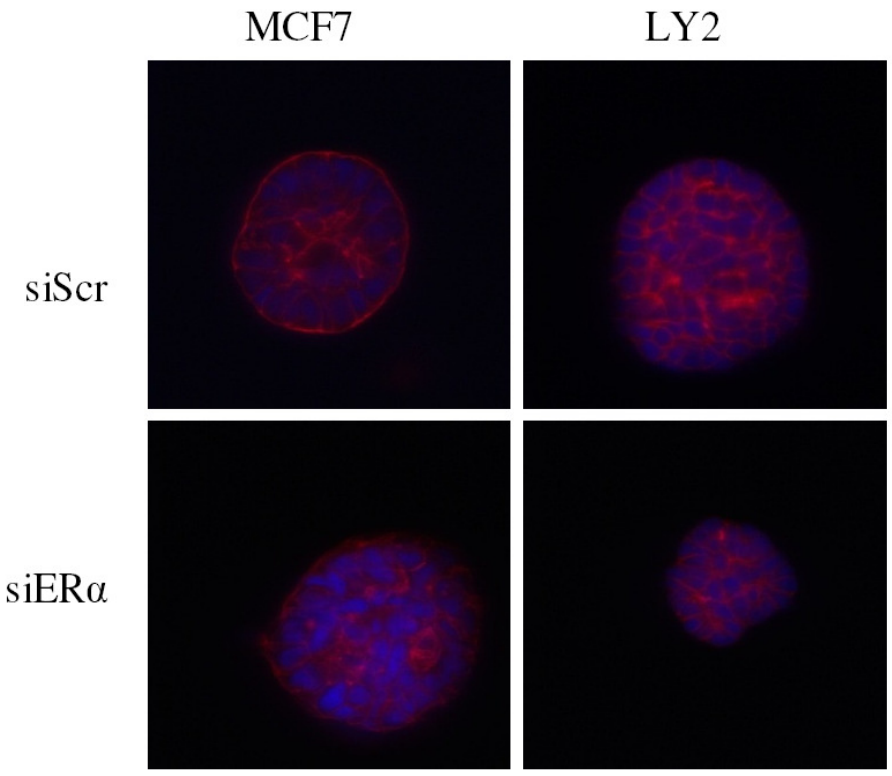


Figure 3.2: Morphological appearance of MCF7 and LY2 cells in 3D cultures following ERα siRNA transfection. Cells were cultured for 14 days and immunofluorescent staining was performed. Cell nuclei were visualised by DAPI staining. For actin staining, a fluorescently tagged phalloidin was added. Fluorescently labelled 3D cultures were examined and imaged under a confocal microscope. MCF7 cells and LY2 cells appeared to develop into well differentiated and organised structures. Conversely, when ERα siRNA was transfected into the cells, both cell lines demonstrated a loss of this cellular organisation (n=1).

3.2.3 Mass Spectrometry

As ER facilitates differentiation of breast cancer cells, interacting partners of the ER coactivator SRC-1 were investigated to understand the mechanism of action of ER in differentiation. SRC-1 can interact with a variety of transcription factors, and so novel SRC-1 interacting proteins were investigated in endocrine sensitive and endocrine resistant cell lines. The approach adopted for this was a mass spectrometry based screen. Briefly, endocrine sensitive MCF-7 and endocrine resistant LY2 breast cancer cells were pre-treated with 4-hydroxytamoxifen prior to cell lysis. An equal quantity of protein lysate was subject to co-immunoprecipitation with an antibody directed against SRC-1. Eluted proteins were separated by 1D gel electrophoresis and protein bands were excised from Coomassie blue stained gels. The resulting peptide mixtures were analysed by nano-electrospray liquid chromatography mass spectrometry. A number of SRC-1 interacting proteins were identified (126 in total), including HMGB2.

Figure 3.3: SDS Page gel from which proteins were selected for mass spectrometry. 1D MCF-7 and LY2 cells were treated with 4-OHT and were immunoprecipitated with anti-SRC-1 antibody. SRC-1 interacting proteins were separated on a one-dimensional gel, and resultant lanes were analyzed using nano-electrospray liquid chromatography mass spectrometry. A number of SRC-1 interacting proteins were identified (126 in total), including HMGB2 (n=1).

3.2.4 HMGB2 expression in the MCF7 and LY2 cell lines

Protein expression of HMGB2 was examined in MCF7 cells and LY2 cells and varying levels were observed in response to estrogen and tamoxifen treatment (figure 3.4). HMGB2 expression was increased after estrogen treatment in both MCF7 cells and LY2 cells. Tamoxifen treatment decreased the protein expression level of HMGB2 in both cell lines compared to estrogen treatment. This decrease in response to tamoxifen was more pronounced in the LY2 cell line compared to the MCF7 cell line.

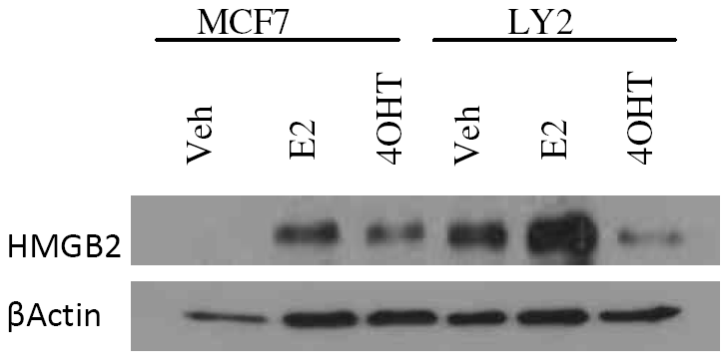


Figure 3.4: Western blot analysis of HMGB2 expression in protein lysates from MCF7 cells and LY2 cells. Cells were treated with vehicle, estrogen or tamoxifen (Veh, E2, 4OHT respectively). HMGB2 was more strongly expressed in LY2 vehicle compared to that of MCF7 vehicle. In both cell lines, treatment with estrogen resulted in elevated expression of HMGB2 compared to the vehicle samples. Tamoxifen treatment in the MCF7 cells down-regulated the expression of HMGB2 compared to the estrogen treatment, however it was still higher than the vehicle control. In the LY2 cells, tamoxifen treatment down-regulated the expression of HMGB2 compared to the estrogen treatment (n=3).

3.2.5 Tamoxifen driven HMGB2 regulates the expression of SRC1 in LY2 cells

To determine whether HMGB2 played a role in SRC1 expression, LY2 cells were transiently transfected with a siRNA targeting HMGB2 and treated with tamoxifen. The knockdown of HMGB2 was confirmed by western blot analysis with an anti- HMGB2 antibody (figure 3.5). At 72 hours post-transfection cells were harvested and lysates were subject to western blot analysis with anti- SRC1 and anti-βActin antibodies. Significantly lower levels of SRC1 protein were observed when HMGB2 was silenced, followed by tamoxifen treatment. (figure 3.5).

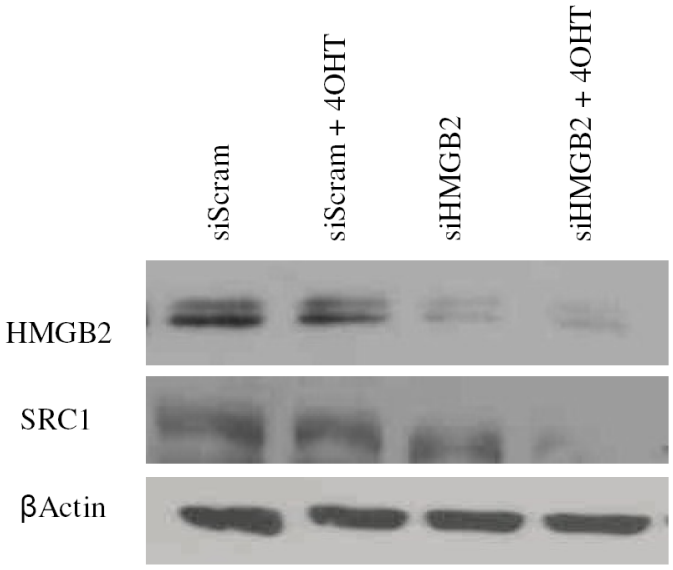


Figure 3.5: LY2 cells were transfected with HMGB2 siRNA or the scrambled siRNA. Prior to protein extraction, cells were treated with tamoxifen or vehicle for 45 minutes. Western blot analysis demonstrates that the tamoxifen driven HMGB2 regulates the expression of SRC1. In the absence of tamoxifen treatment, HMGB2 does not have the same capacity to regulate the expression of SRC1 (n=2).

3.2.6 ERα/ SRC1 interactions following HMGB2 knock down in LY2 cells

Leading on from the previous experiment, showing that tamoxifen driven HMGB2 regulates the expression of SRC1 in the LY2 cell line, we sought to determine whether HMGB2 could affect the level of interaction between ERα and SRC1. Also, we were interested in ascertaining whether tamoxifen treatment could further affect this regulation of interaction. LY2 cells were transiently transfected with a siRNA targeting HMGB2 and treated with tamoxifen. Subsequently, a co-immunoprecipitation (Co-IP) experiment was performed to determine the level of interaction between ERα and SRC1, pulling out ERα using an ERα antibody and immunoblotting for ERα and SRC1. Loss of HMGB2 function by siRNA knockdown results in a decreased interaction between ERα and SRC1. Furthermore, tamoxifen treatment in conjunction with HMGB2 knockdown resulted in a significantly lower level of interaction between ERα and SRC1 in the LY2 cell line. As seen in figure 3.5, this decreased interaction between ERα and SRC1 could be due to less SRC1 being present.

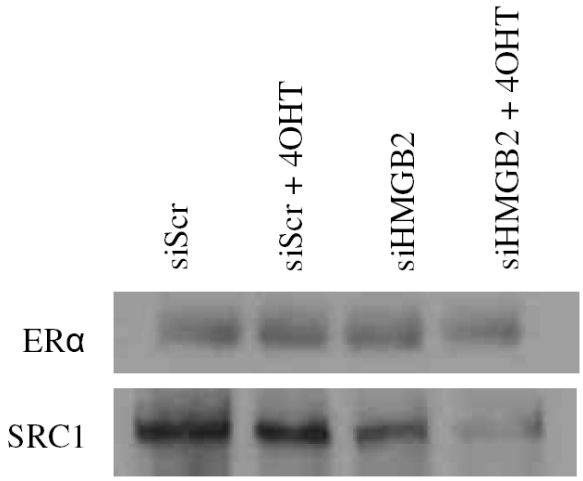
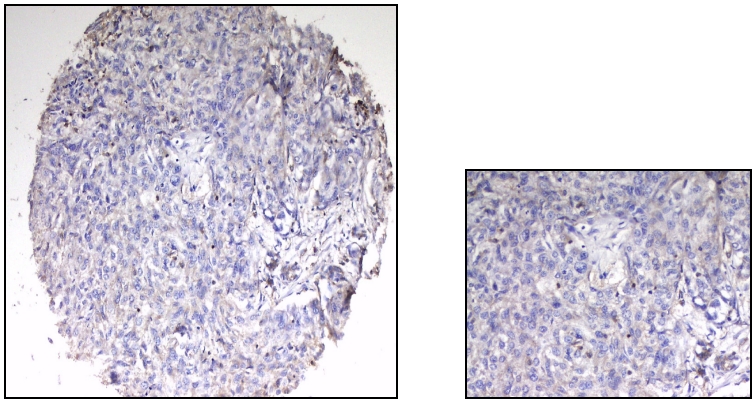


Figure 3.6: CoIP of ERα, followed by Western blot analysis of ERα and SRC1 demonstrates that the loss of HMGB2 results in less interaction between ERα and SRC1 in LY2 cells. In addition to the loss of HMGB2, tamoxifen treatment for 45 minutes further diminishes the interaction between ERα and SRC1 (n=2).

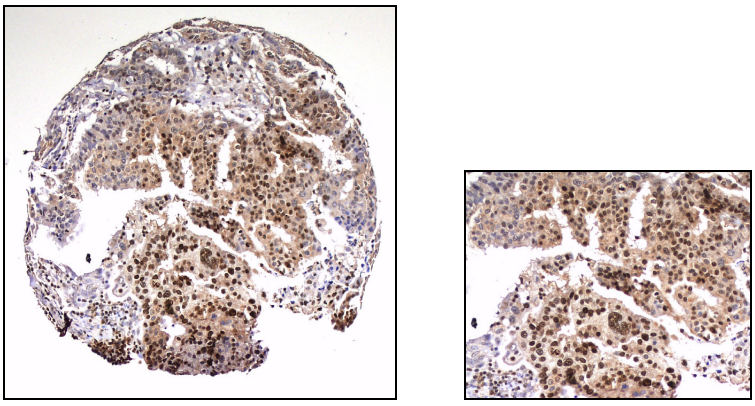
3.2.7 HMGB2 Immunohistochemistry

In order to correlate our molecular studies of HMGB2 to the clinical setting, immunohistochemistry for HMGB2 was carried out on a tissue microarray consisting of 937 patient tumour samples. HMGB2 was identified in 36% of patient tumour samples, which is indicated by brown staining in figure 3.7 (b), and is predominantly localised to the nucleus with some cytoplasmic staining. A Kaplan Meier curve was used to demonstrate survival analysis in a tamoxifen treated cohort of patients (937 patients). Patients who were negative for HMGB2 expression had reduced disease free survival (c). Both SRC1 and ER correlated with HMGB2 expression (p=0.017 and p=0.0001 respectively).

(a) HMGB2 negative



(b) HMGB2 positive



(c) Kaplan-Meier survival curve

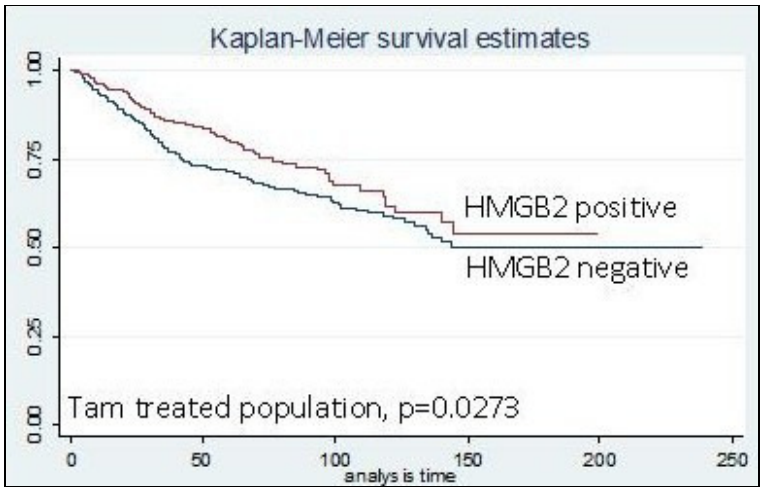


Figure 3.7: A number of tissue micro-arrays containing cores from 937 breast tumours were stained for HMGB2 using immunohistochemistry. The images above are representative of (a) tumour core negative for HMGB2 expression; (b) tumour core positive for HMGB2 expression. HMGB2 expression was identified in 36% of patient tumour samples (n=1). (c) In a tamoxifen treated cohort of 948 patients, those that did not express HMGB2 had reduced disease free survival.

3.3 Discussion

SRC1 directly interacts with the AF1 and AF2 (ligand independent and ligand dependant domains of the steroid receptor) of ERα (Onate *et al.,* 2006). It has been established that ERα promotes cellular differentiation in breast carcinomas. This differentiation results in a lower grade tumour, bringing with it a more favourable prognosis. Through gene silencing of ERα, we demonstrated the loss of differentiation in both MCF7 cells and LY2 cells (section 3.2.2). Knocking out ERα did not prevent the cells from forming into acini; however their structure was not as well organised as the control. An inverse correlation exists between luminal phenotype and tumour invasiveness. SRC1 positivity in breast cancer has been consistently associated with a strong propensity for metastasis and invasiveness. Our research group have recently published findings on how SRC1 can effect cellular differentiation independently of ER by targeting a disintegrin protein ADAM22 (McCartan *et al.,* 2012).

Our research group sought to determine other proteins which are partners of SRC1 in endocrine sensitive and endocrine resistance cell lines models (MCF7 and LY2 cells respectively) through the use of mass spectrometry. A total of 126 proteins were identified to interact with SRC1 in the LY2 cells but not in the MCF7 cells. HMGB2 was among this list of SRC1 interacting proteins (section 3.2.3). Interestingly, HMGB2 had previously been shown to increase the binding affinity of ER to a ERE half site by as much as 6 fold (Das *et al.,* 2004). Subsequently, protein expression levels of HMGB2 were analysed in MCF7 cells and LY2 cells (section 3.2.4). In both cell lines, estrogen treatment resulted in an up-regulation of HMGB2 protein expression compared to vehicle treatment.

In this project, we have shown that HMGB2 regulates the protein expression of SRC1 in LY2 cells and subsequently, this regulation is influenced by tamoxifen treatment (section 3.2.5). Knock-down of HMGB2 alone did not affect the levels of SRC1. However, in the presence of tamoxifen this knock-down decreased SRC1 protein levels. These results are indicative that HMGB2 regulates the expression of SRC1 in an endocrine resistant model. Knowing that SRC1 and ERα are well established binding partners, we sought to determine whether HMGB2 could influence this partnership. Indeed, as figure 3.6 illustrates, HMGB2 was proven to increase the level of binding between SRC1 and ERα in LY2 cells. This effect was evident without tamoxifen treatment, but even more so in the presence of tamoxifen.

Initial molecular findings lead our research group to examine HMGB2 in the clinical setting. Tumour samples from 937 breast cancer patients were stained for HMGB2 resulting in 344 staining positive, equivalent to 36% of the patients being positive for HMGB2 (section 3.2.7). Kaplan-Meier (figure 3.7) estimates of survival depict that in a tamoxifen treated population, those patients who were HMGB2 positive had a lesser chance of disease recurrence than those who were HMGB2 negative. Therefore, HMGB positivity is associated with a more favourable prognosis than HMGB2 negativity.

Chapter IV

HMGB2 regulates the expression of estrogen responsive genes in endocrine resistant breast cancer

4.1 Introduction

c-Myc is a proto-oncogene which has been found to be a key player in tumour development. This 64kDa transcription factor regulates transcription of a number of different proteins involved in cell proliferation (Kang *et al.,* 1996). Myc is critical in embryonic development and is over-expressed in 70,000 cancer deaths per annum in the U.S (http://myccancergene.org).

The regulation of *c-myc* expression is important in the estrogen driven proliferation of human breast cancer cells (Dubik *et al*., 1987). Myc over-expression is associated with a more aggressive phenotype of tumour and metastasis (Deming *et al.,* 2000). When myc is over-expressed in a hormone sensitive cell line, the cells sensitivity to anti-estrogenic treatment becomes significantly reduced (McNeil *et al.,* 2006), implying that myc exerts a large influence on the development of endocrine resistance.

More recently, BCAS3 has emerged as an estrogen responsive gene. An estrogen dependent interaction between BCAS3 and histone 3 (H3) exists in MCF7 cells (Gururaj*, et al.,* 2006). In the absence of estrogen this interaction is lost. BCAS3 has a distinct bromodomain, which recognizes the acetylated lysine residue on H3. Protein-histone interactions generally function to promote chromatin remodelling; a process that HMGB2 has been well established in (Lnenicek-Allen *et al.,* 1996). An interesting observation by Gururaj *et al.,* (2006) demonstrate that an ERE half site is located on an intronic region that is 12kb upstream from the transcriptional start site of BCAS3. The presence of this ERE half site is essential for the maximum transcriptional activation of BCAS3. It is an unusual occurrence that an intronic region would possess such

influence over transcriptional activity. Current literature is limited regarding the function of BCAS3 in breast cancer. What is known about BCAS3 is that its chromosomal location of 17q23, is amplified in about 20% of breast cancers and this amplification is associated with a higher grade tumour and therefore, a poor prognosis (Monni *et al.,* 2001).

As was previously described in section 1.3, HMGB2 has a strong affinity for unusual DNA structures (e.g. cruciform structure); incidentally these structures are a prevalent occurrence in the BCAS3 genomic sequence. As HMGB2 is a highly abundant chromatin binding protein, approximately one molecule for every 10 nucleosomes, (Melvin *et al.,* 1999). Results from section 3.2 show HMGB2 to correlate with a favourable clinical prognosis (survival curve; figure 3.7). However, it is also associated with SRC1 in the LY2 cell line (section 3.2.3), a protein that is detrimental to disease free survival (Redmond *et al.,* 2009). It is this association that required a deeper investigation into the role of HMGB2 in the endocrine resistant breast cancer cell line.

4.2 Results

4.2.1 HMGB2 regulates the expression of cMyc in LY2 cell line

Myc is a well established estrogen responsive gene and for this reason the influence of HMGB2 on Myc expression was initially examined. HMGB2 was knocked-down in the LY2 cell line and 24 hours post transfection, cells were harvested and lysates were subject to Western blot analysis with anti-HMGB2, anti-c-Myc and anti-βActin antibodies. Successful silencing of HMGB2 resulted in a decrease in c-Myc protein expression, confirming regulation of this classic ER target gene by HMGB2 (figure 4.1).

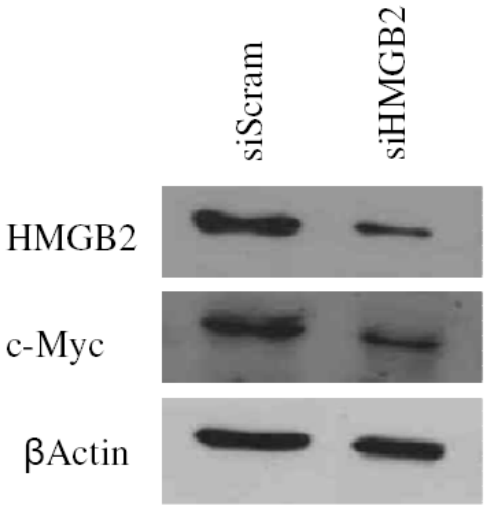


Figure 4.1: LY2 cells were transfected with siRNA targeting HMGB2 or scrambled siRNA. Forty eight hours post transfection, cells were harvested and lysates were subject to Western blot analysis with anti-HMGB2, anti-cMyc and anti-βActin antibodies. As demonstrated, HMGB2 siRNA results in a down-regulation of c-Myc expression (n=3).

4.2.2 SRC1 ChIP-Sequencing

ChIPseq is a method which combines chromatin immunprecipitation (ChIP) with DNA sequencing in order to analyse proteins which are bound to DNA and their respective binding sites. Dr. Damian McCartan performed SRC1 ChIP-seq experiments. In this experiment, LY2 cells were either treated with tamoxifen or vehicle. The cells were subsequently subjected to ChIP, using an SRC1 antibody. DNA libraries were generated from the ChIP output DNA. Sequencing was carried out using the Illumina/Solexa Genome Analyser system. The sequence tags (35 nucleotides in length) were mapped to the human genome using the Eland software. The Model-based analysis for ChIPseq (Macs) software was used to analyse ChIP peaks. The peaks correspond to immuno-enriched areas - regions where SRC1 binds to the genome. One of these peaks was at the BCAS3 promoter, with an increase in peak height in tamoxifen treated LY2 cells compared to the vehicle control.

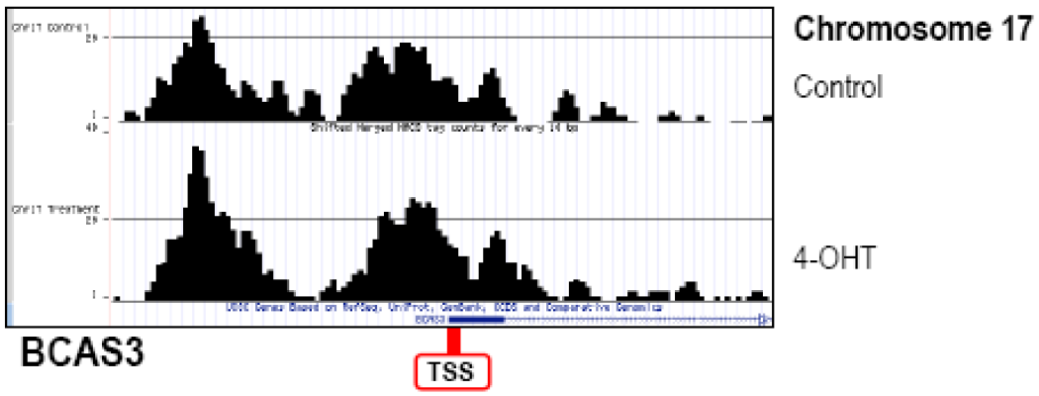


Figure 4.2: ChIPseq revealed SRC-1 occupancy at the BCAS3 promoter. Comparisons are made between peaks under control conditions and following treatment with 4-OHT. Peaks are more pronounced in tamoxifen treated LY2 cells compared to the vehicle control, indicating that tamoxifen treatment enhances SRC1 binding onto the BCAS3 promoter (n=1).

4.2.3 SRC1 regulates the expression of BCAS3 in LY2 cell line

ChIPseq has confirmed binding of SRC1 at the promoter region of BCAS3. To investigate whether SRC1 binding regulated BCAS3 expression, knock-down and over-expression studies were conducted. LY2 cells were transiently transfected with a siRNA targeting SRC1 or the cells were transiently transfected with an over-expression vector targeting SRC1. At 72 hours post-transfection, cells were harvested and lysates were subject to Western blot analysis using anti-SRC1, anti-BCAS3 and anti-βActin antibodies. Silencing SRC1 resulted in a significant decrease in BCAS3 expression. Likewise, over-expression of SRC1 resulted in an increase in BCAS3 expression (figure 4.3). Dr. Damian McCartan carried out the over-expression study in figure 4.3.

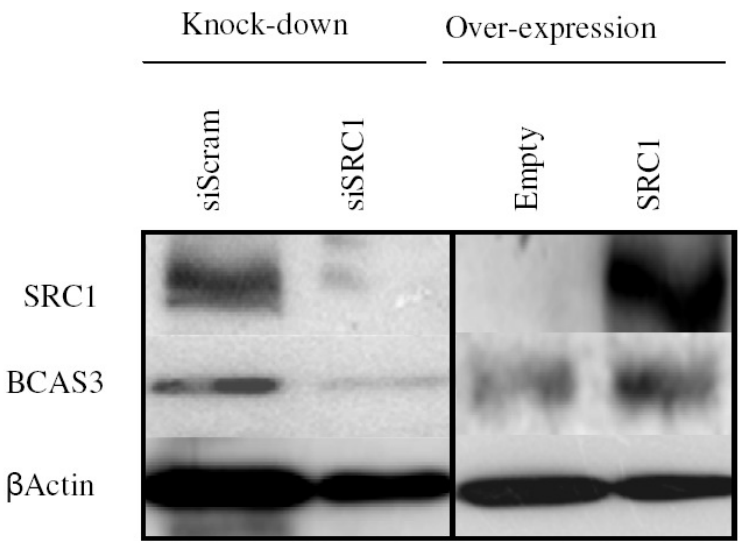


Figure 4.3: LY2 cells were transfected with siRNA SRC1 or they were transfected with an over-expression vector of SRC1. Western blot analysis demonstrates that SRC1 regulates the expression of BCAS3 in LY2 cells (n=3)

4.2.4 HMGB2 regulates the expression of BCAS3 in LY2 cell line

As HMGB2 was identified by mass spectrometry as an SRC1 interacting protein, it was hypothesised that HMGB2 may regulate protein expression of BCAS3 in the LY2 cell line. To test this hypothesis, LY2 cells were transiently transfected with a siRNA targeting HMGB2 or they were transiently transfected with an over-expression vector targeting HMGB2. At 24 hours post transfection, cells were harvested and lysates were subject to Western blot analysis with anti-HMGB2 and anti-BCAS3 antibodies. A decrease of HMGB2 expression resulted in a down-regulation of BCAS3 protein level (figure 4.4). Similarly, when HMGB2 was over-expressed, there was a concomitant up-regulation of BCAS3.

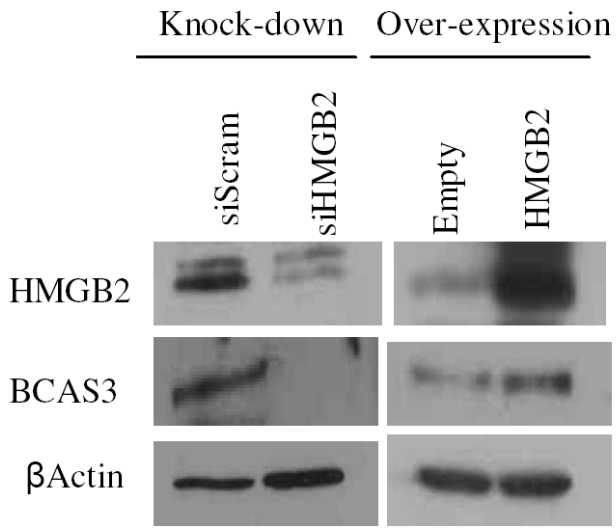


Figure 4.4: LY2 cells were transfected with siRNA targeting HMGB2 or control siRNA. Forty-eight hours post transfection, cells were harvested. Cells were also transfected with an over-expression vector targeting HMGB2 or a control empty vector and harvested at 24 hours post transfection. Lysates were subject to Western blot analysis with anti-HMGB2, anti-BCAS3 and anti-βActin antibodies. As demonstrated, HMGB2 siRNA results in a down-regulation of BCAS3 expression. Conversely, the over-expression of HMGB2 resulted in the up-regulation of BCAS3 (n=3).

4.2.5 ERα regulates the expression of BCAS3 in LY2 cell line

To investigate if ERα is also involved in this regulation of BCAS3, LY2 cells were transiently transfected with a siRNA targeting ERα. The siRNA knockdown of ERα was confirmed by Western blot analysis. At 48 hours post- transfection, cells were harvested and lysates were subject to anti-BCAS3 and βActin antibodies. Considerably lower levels of BCAS3 protein were observed following ERα silencing in LY2 cells.

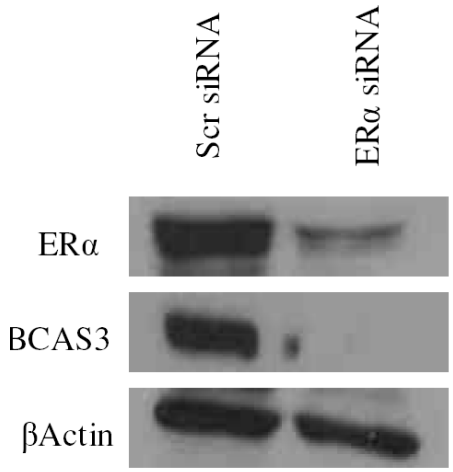


Figure 4.5: LY2 cells were transfected with siRNA ERα. Western blot analysis subsequently identified that ERα was regulating the expression of BCAS3 (n=2).

4.2.6 HMGB2 recruitment to the BCAS3 promoter via PCR; LY2 cell line

To determine whether HMGB2 is bound to the BCAS3 promoter, LY2 cells were analysed by chromatin immunoprecipitation (ChIP) assay with anti- HMGB2 antibody. The ChIP assay demonstrates whether proteins, such as chromatin remodelling proteins like HMGB2, bind to a particular region on the chromatin of living cells. The BCAS3 promoter region was amplified from crosslinked chromatin from LY2 cells that was immunoprecipitated with anti-HMGB2 antibody, a H4 antibody positive control, or an IgG antibody negative control. HMGB2 binding at the promoter region was visible under all three treatment conditions – vehicle, estrogen and tamoxifen. Whether treatment with the ligands estrogen and tamoxifen regulated the level of binding was not evident by this PCR method.

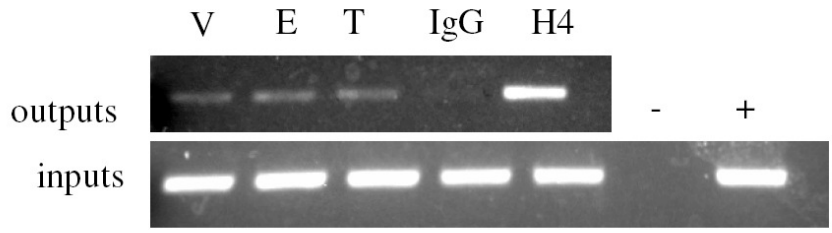


Figure 4.6: Chromatin immunoprecipitation demonstrates recruitment of HMGB2 to the BCAS3 promoter under vehicle, estrogen and tamoxifen treatment in the LY2 cell line (n=3).

4.2.7 HMGB2 recruitment to the BCAS3 promoter via Real-Time PCR in LY2 cell line

Quantitative PCR (qPCR) was utilised to determine more subtle differences in the recruitment of HMGB2 onto the BCAS3 promoter between vehicle treated, estrogen treated and tamoxifen treated LY2 cells. A validation experiment was performed by real-time PCR to validate the normal PCR results. Quantitative real-time PCR analysis is more sensitive than normal PCR, thereby facilitating the observation of more subtle changes in the recruitment of HMGB2 onto the BCAS3 promoter. This method revealed increased recruitment of HMGB2 with the estrogen and tamoxifen treatments.

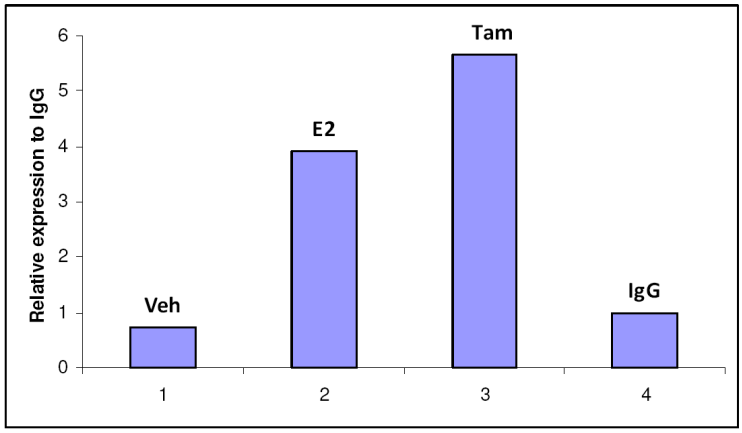


Figure 4.7: Q-PCR quantification of HMGB2 ChIP demonstrates more subtle differences in the recruitment of HMGB2 to the BCAS3 promoter. HMGB2 recruitment is enhanced following estrogen treatment and further more with tamoxifen treatment (n=1).

4.2.8 BCAS3 regulates the expression of ERα in LY2 cell line

In an endocrine sensitive model, BCAS3 has been reported to be involved in a positive feedback loop to regulate expression of ER (Gururaj *et al.,* 2007). To test whether this feedback loop also exists in the endocrine resistant setting, LY2 cells were transiently transfected with a siRNA targeting BCAS3. At 72 hours post- transfection, cells were harvested and lysates were subject to Western blot analysis using anti-BCAS3, anti-ERα and anti-βActin antibodies. Lower levels of ERα were observed when BCAS3 was silenced (figure 4.8). In accordance with our hypothesis, BCAS3 does regulate the protein levels of ERα in the LY2 cell line.

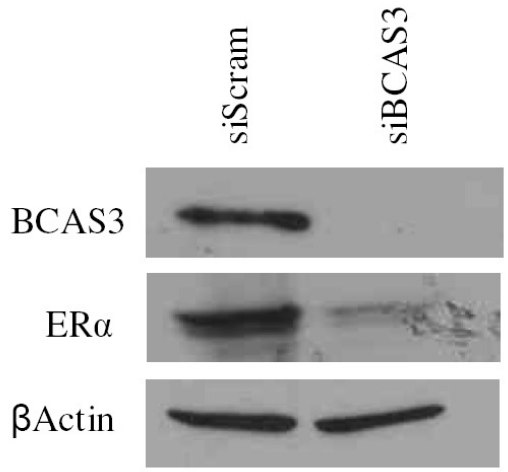


Figure 4.8: LY2 cells were transfected with siRNA BCAS3. Western blot analysis subsequently identified that BCAS3 was regulating the expression of ERα (n=3).

4.3 Discussion

It has been known since the 1980s that *c-myc* is regulated by estradiol in MCF7 cells (Dubik and Shiu 1988). In fact the steroidal treatment increases c-myc mRNA 10-fold within 20minutes. To establish that the chromatin remodelling protein, HMGB2 does function in regulating estrogen responsive genes, *c-myc* was chosen as the reference gene. Indeed, HMGB2 was observed to regulate the expression of myc in the LY2 cell line (section 4.2.1).

As we discovered that HMGB2 is a binding partner of SRC1 in LY2 cells (section 3.2.3), we hypothesised that a target gene for SRC1 could simultaneously be a target gene for HMGB2. SRC1 ChIP-sequencing analysis revealed that SRC1 binds to the promoter region of the estrogen regulated BCAS3 (section 4.2.2). It was subsequently demonstrated that HMGB2 regulates the protein expression of BCAS3 in the LY2 cell line (section 4.2.3).

There has been an abundance of literature illustrating the effect that coregulatory proteins possess in either enhancing or subduing the transcriptional activity of steroid receptors, particularly the SRC family of steroid receptor coactivators (York and O' Mally 2010). This long history of SRC investigations lead Gururaj *et al.,* (2007) to determine if co-regulatory proteins within the SRC family regulated protein expression of BCAS3 in MCF7 cells. Interestingly, SRC1 and SRC3 did not affect the protein expression of BCAS3. In this study (figure 4.3) we demonstrated that BCAS3 is regulated by SRC1 in LY2 cells; a finding that has not been previously reported.

In light of results obtained in section 4.2.5 showing that ERα regulates the expression of BCAS3 in the LY2 cell line, our research group sought to ascertain whether the ability of SRC1 to regulate BCAS3 in the LY2 cell line could be facilitated by another protein; perhaps the chromatin remodelling protein HMGB2. As is already understood of the SRC1 protein, it is tethered to the DNA (not directly bound to the DNA), thereby enhancing the transcriptional activity of a target steroid receptor. There are two activation domains within the SRC1 protein, AD1 and AD2 (Onate *et al.*, 1996). Simultaneous activation of both domains is necessary for SRC1 to exert its maximum influence steroid receptors transcriptional activity (Onate *et al.*, 1996). Figure 4.6 demonstrates the recruitment of HMGB2 onto the BCAS3 promoter in the LY2 cell line, which is visualised by traditional PCR. Real time PCR has the ability to visualise more subtle differences in chromatin binding. It is 5 times more sensitive than its normal PCR counterpart. As was observed in figure 4.7 with real time PCR results, estrogen and tamoxifen drives further recruitment of HMGB2 onto the BCAS3 promoter. These results imply that HMGB2 is a regulatory protein that binds to the BCAS3 promoter in the endocrine resistant model of LY2 cells.

As was described in section 1.4, a positive feedback loop exists between ERα and BCAS3 in the MCF7 cell line. ERα has been shown to be recruited to a regulatory region of BCAS3 via a half ERE site (Gururaj *et al.,* 2006). This recruitment is dependant on the presence of the ERα co-regulator PELP1 (Gururaj *et al.,* 2007). Transcriptional activation of BCAS3 results in this protein acting as an ERα co-activator itself, thereby promoting a positive feedback loop effect. These authors also demonstrate that over-expression of BCAS3 stimulates pS2 expression. pS2 is a long established estrogen driven oncogene (Brown *et al.,* 1984). As BCAS3 has already been

linked to tamoxifen resistance (Gururaj *et al.,* 2006), these results together imply that over-expression of BCAS3 could be a contributor in the development of estrogen independent cell growth. The mutual co-regulatory functions between ERα and BCAS3 that have been described above have been unique to the endocrine sensitive cell model of MCF7 cells. Results illustrated in figures 4.5 and 4.8 depict that this positive feedback effect also exists in the endocrine resistant LY2 cell line.

Chapter V

General Discussion

Breast cancer is the most frequently diagnosed cancer in females worldwide. It accounts for 23% of new cancer cases which equates to 1.38 million diagnoses (Jemal *et al.*, 2011). The incidence of breast cancer increased in western countries during the 1980s and 1990s. This increase was largely due to hormone replacement therapies being prescribed to post-menopausal women (Althuis *et al.,* 2005). In contrast to the increased incidence however, death rates have been declining due to early detection and improved treatments (Autier *et al.,* 2010).

As estrogen is a well established stimulus of breast cancer, therapies have targeted either estrogen synthesis (aromatase inhibitors) or ER signalling (tamoxifen). Although tamoxifen treatment has undoubtedly been a prominent player in reducing the rate of mortality, breast cancer recurrence develops within 15years in 33% of patients (Early Breast Cancer Triallists' Collaborative Group 2005). This acquired resistance is due to the presence of co-regulatory proteins, such as SRC1, that stimulate ERα (Redmond *et al.,* 2009). Also, a bidirectional crosstalk between ER and receptor tyrosine kinase signalling can elicit a resistance to endocrine therapies (Nicholson *et al.,* 2001). Understanding the complexity of the molecular pathways that activate ER has been integral in developing endocrine targeted therapies.

5.1 HMGB2 acts as a facilitator in ERα/SRC1 interactions

SRC1 has been shown to be a prominent ERα co-activator. The co-activator interacts with both activation domains of ERα (AF1 and AF2), enabling maximal transcriptional activity (Onate *et al.,* 1998). Over-expression of SRC1 is sufficient to alter the antagonistic effects of tamoxifen on ERα (Romano *et al.,* 2010). Endocrine treated patients who have suffered from recurrence of breast cancer have increased co-localisation of SRC1 with ERα (Redmond *et al.,* 2009).

More than 70% of breast tumours are characterised as luminal subtype which is associated with ERα positivity and a more favourable prognosis (Dunnwald *et al*., 2007). The first aim of this study was to observe the formation of a luminal type tumour and visualise the effect that ERα possesses on this well organised, luminal structure. Specifically, ERα was silenced in two cell lines, MCF7 and LY2. ERα silencing was confirmed at the protein level. A decrease in luminal properties were observed in both cell lines when ERα was silenced.

As SRC1 is a well documented co-activator of ERα, mass spectrometry was employed to investigate what other proteins may be interacting with SRC1 and a cross comparison was made between MCF7 cells and LY2 cells. This experiment first identified HMGB2 as an SRC1 interacting protein, unique to the LY2 cell line. HMGB2 is a non-histone chromatin binding protein which induces structural modifications on DNA conformation (Melvin *et al.,* 1999). It sporadically binds to chromatin; however it possesses a stronger affinity for unusual DNA structures such as cruciform structures (Stephanovsky and Moss, 2009). Western blot analysis was used to confirm protein

expression of HMGB2 in the MCF7 and LY2 cell lines. An increased expression of HMGB2 was observed in LY2 cells compared to MCF7 cells. Estrogen treatment increased the protein expression of HMGB2 in both cell lines, whilst tamoxifen treatment decreased protein expression of HMGB2 compared to estrogen stimulated levels. This protein analysis of HMGB2 in the MCF7 and LY2 cell lines suggests that this chromatin binding protein may have a role to play in the development of endocrine resistant breast cancer.

To understand the molecular role of HMGB2 in tamoxifen resistance, this protein was silenced in the LY2 cell line and the effect on SRC1 protein expression was analysed. Indeed, there was regulation of SRC1 protein expression by HMGB2 and this regulation was driven by tamoxifen treatment. Without tamoxifen treatment there was no visible regulation of SRC1 protein expression. This finding implicates HMGB2 in regulating SRC1 protein expression under the influence of an endocrine therapy. The next step was to determine if HMGB2 influences the interaction between ERα and SRC1 in the LY2 cell line. HMGB2 was silenced in the LY2 cell line and a co-immunoprecipitation (CoIP) experiment was performed to determine ERα/SRC1 interactions. It was found that the presence of HMGB2 does increase ERα/SRC1 interactions in the LY2 cell line. Conversely, when the cells are treated with tamoxifen and HMGB2 is silenced, there is a dramatic decrease in the level of interaction between ERα and SRC1. However, it is possible that the decrease in ERα/SRC1 interactions are due to overall less SRC1 being present in the cellular milieu caused by the HMGB2 knockdown. Further investigations would be required to determine whether HMGB2 directly causes an increased interaction between ERα/SRC1 or if it is an indirect affect. As has already been described, expression of SRC1 has been confirmed to be an independent predictor of

disease recurrence through the increased co-localisation with ERα (Redmond *et al.,* 2009).

Immunohistochemistry was performed on a large cohort of breast tumours in order to determine the percentage of patients who are positive for HMGB2 protein expression. It was found that 36% of patients are positive for HMGB2 protein expression and that this expression is confined to the nucleus. A Kaplan Meier survival curve was employed to cross compare disease recurrence between patients who were HMGB2 positive and patients who were HMGB2 negative (all patients were on an endocrine therapy). It was found that patients who were positive for HMGB2 were less likely to suffer from disease recurrence than those patients who were negative for HMGB2. Despite this fact, identification of HMGB2 as an SRC-1 interacting protein in the LY2 cell line caused us to pursue the role of this protein in the resistant cell line, as the protein level in the primary tumours of the patients, which are taken pre-endocrine treatment, may not be reflective of the molecular environment in a resistant tumour.

5.2 HMGB2 regulates protein expression of BCAS3

The second part of this research sought to identify a novel target gene that is regulated by HMGB2 in the endocrine resistant LY2 cell line. Firstly, as HMGB2 is a known cofactor of ERα, we decided to examine the regulation of a classic ER target gene, Myc. The over-expression of the classic ER target gene Myc has been connected to a decrease in sensitivity to endocrine therapies (McNeil *et al.,* 2006). In order to confirm that HMGB2 regulates Myc in endocrine resistance, HMGB2 was silenced in the LY2 cell

line, resulting in a decrease of Myc protein expression. This is indicative that HMGB2 is implicated in the regulation of ER target genes that are associated with the development of endocrine resistance in breast cancer.

To identify a target gene that could be regulated by HMGB2, it was deemed likely that a target gene of HMGB2 could simultaneously be a target gene of SRC1 as the HMGB2/SRC1 partnership has been confirmed. Using SRC1 ChIPseq data from the lab, BCAS3 was identified as a potential target, with tamoxifen treatment increasing SRC1 binding to the BCAS3 promoter compared to no treatment. BCAS3 has previously been associated with endocrine resistance (Gururaj *et al.,* 2006). Its chromosomal location (17q23) makes it highly susceptible to recombination events producing unusual DNA structures (Barbouti *et al.,* 2004). As HMGB2 has previously been reported to bind to such structures, BCAS3 was a prime candidate for HMGB2 regulation in endocrine resistance.

The next step in affirming the regulation of BCAS3 by HMGB2 involved gene silencing and over-expression studies in the LY2 cell line. Both approaches illustrated how manipulation of HMGB2 levels resulted in alterations of BCAS3 production, thereby implicating HMGB2 in the regulation of BCAS3 protein expression. Previous studies on BCAS3 regulation have confirmed that a positive feed-back loop exists between ERα and BCAS3 in an endocrine sensitive model (Gururaj *et al.,* 2007). This study led to the hypothesis that the same feed-back loop interaction exists in the endocrine resistant LY2 cell model. To investigate this theory, ERα was silenced in the LY2 cell line, which resulted in a decreased protein expression of BCAS3. Like wise, silencing BCAS3 resulted in a decreased protein expression of ERα.

To date, there has been no confirmation that SRC1 regulates the expression of BCAS3 in an endocrine resistant breast cancer cell line. A study by Gururaj *et al.,* (2006) confirms that there is no regulation of BCAS3 by SRC1 or SRC3 in MCF7 cells. We sought to determine that SRC1 does regulate the expression of BCAS3 in an endocrine resistant setting. To test this hypothesis, SRC1 was silenced and over-expressed in LY2 cells, resulting in a decrease and an increase in BCAS3 expression respectively. These findings verify that SRC1 does regulate BCAS3 in endocrine resistance.

As our evidence suggests that the HMGB2/ERα/SRC1 transcriptional complex is an influential regulator of BCAS3 in endocrine resistance, a direct interaction between the protein complex and the BCAS3 promoter was examined. It was chosen to investigate HMGB2 as a potential DNA binding partner at the BCAS3 promoter. A ChIP experiment was employed to establish whether HMGB2 directly binds to a promoter region of BCAS3 in the LY2 cell line. Direct binding of HMGB2 onto the BCAS3 promoter was verified. Initial studies did not show any differences in the recruitment of HMGB2 between vehicle, estrogen or tamoxifen treated LY2 cells. Subsequently, a more sensitive detection method (real-time PCR) was employed to analyse HMGB2 recruitment onto the promoter of interest. This resulted in an increased recruitment of HMGB2 onto the BCAS3 promoter with estrogen treatment and a further increase with tamoxifen treatment. These findings demonstrate that HMGB2 does regulate the expression of BCAS3 and that this regulation is influenced by estrogen and an endocrine therapy such as tamoxifen.

5.3 Conclusion

Throughout this research, an insight into the functional role of the chromatin binding protein HMGB2 has been examined in an endocrine resistant breast cancer cell line model. In our tissue microarray from nearly one thousand breast cancer patients, those who were positive for HMGB2 in their primary tumour had a reduced risk of disease recurrence compared to patients who were negative for HMGB2. The clinical benefits of HMGB2 could be attributed to its partnership with ERα, as ERα is known to promote a luminal type tumour with a favourable prognosis. We identified HMGB2 as interacting with SRC-1 in the endocrine resistant cells, which seem contradictory to the clinical findings as SRC1 has previously been associated with a reduced disease free survival. It is possible that the role of HMGB2 changes as the tumour develops resistance to endocrine therapy. This work has demonstrated step by step that HMGB2 acts as a facilitator protein to enhance the interaction between ERα and SRC1 in an endocrine resistant breast cancer cell line. HMGB2 has the capacity to induce architectural modifications to DNA structure, thereby creating easier access for transcriptional machinery to bind to promoters of interest (Melvin *et al.,* 1999). HMGB2 has also been shown to increase the affinity of ER to ERE half sites by as much as 6 fold (Das *et al.,* 2004). As HMGB2 has a higher binding affinity for unusual DNA structures, the chromosomal location of BCAS3, 17q23 (a region abundant in recombination events) suggests a prime target for HMGB2. The findings in this research project suggest that a transcriptional complex encompassing ERα, SRC1 and HMGB2 regulates the transcription of BCAS3 in endocrine resistance.

Chapter VI

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Chapter VII

Appendix

7.1 Immunohistochemistry

Phosphate buffered saline

Dissolve one PBS tablet per 200ml dH_2O
Each PBS tablet contains:
0.1M phosphate buffer
0.0027M potassium chloride
0.137M sodium chloride
Autoclave and filter prior to use.

Hydrogen peroxide solution

10 ml dH_2O
1ml 30% H_2O_2
3,3'-Diaminobenzidinee
Dissolve set of tablet in 1 ml of dH_2O
Each set of tablets contains:
Di-amino-benzidine 0.7mg/ml
Urea hydrogen peroxide 1.6mg/ml
Tris buffer 0.06M

Sodium citrate buffer

Dissolve 1.4mg of sodium citrate in 500ml of dH_2O.
Final concentration of 0.01 M sodium citrate, pH 6.

7.2 Cell Culture

Fetal calf Serum (FCS) (GiBcoBRL®):

Minimal essential medium MEM Medium 500ml volume (GiBcoBRL®)
5ml of Pen/Strep solution (50 U/ml Penicillin and 50 U/ml Streptomycin)
5ml L-glutamine (200mM; 2mM final concentration). Renewed every 2 weeks.
50ml of FCS

Trypsin-EDTA

Trypsin 10 X liquid (25g/l GiBcoBRL®)
HBSS (GiBcoBRL®)

0.02% EDTA
20MM HEPES
1ml of trypsin and 1 ml of 0.02% EDTA was made up to 10ml HBSS filter prior to use through Acrodic® 32 filters (0.2µm pore size).
RIPA buffer (Pierce)

Used to lyse cultured mammalian cells.
1 ml of cold RIPA Buffer for every ~20µl of packed cells, equates to ~40mg of cells.

7.3 Western Blotting

Tris Buffered saline (TBS) (20X):

121.1g Tris
175.5g NaCl
Made up to 1 litre with dH_2O
Use at 1X final concentration, pH 8.3

Wash buffer:

1X TBS
0.05% Triton® X-100

Blocking buffer:

1X TBS
0.05% Triton® X-100
5% Molecular grade skimmed milk
1M Tris.HCl, pH 6.8
157.6g Tris- HCl
Made up to 1 litre with dH_2O, pH 6.8

1.5 M Tris.HCl, pH 8.8:

236.4g Tris- HCl
Made up to 1 litre with dH_2O, pH 8.8

Transfer buffer:

2.93g Glycine
5.8g Tris Base
0.375 g SDS
200ml Methanol
Made up to 1 litre with dH_2O

Sample buffer (5X)

2g SDS

5ml 1M Tris.HCl (6.8)
3.0ml dH2O
8ml Glycerol
2 ml 0.1% Bromophenol Blue
Make up to 20ml with dH_2O
5% ß-mercaptomethanol

Running buffer (10X)

288g Glycine
60.6g Tris Base
20g SDS
Made up to 2 litres with dH_2O

Acrylamide/Bis-acrylamide 30%

Liquid easigel 30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide was used directly.

20% SDS

20g SDS was dissolved in 100 ml of dH_2O

10% Ammonium Persulphate

100mg/ml was dissolved in dH_2O.

7.4 Chromatin Immunoprecipitation

ChIP Dilution Buffer

50µl 10% SDS
0.55 ml Triton X 100
22.334 mg EDTA (= 1.2 mM EDTA in 50mls)
131.97 mg NaCl (= 167 mM in 50mls)
49.4 mls dH_2O
pH 8.1

LiCl Immune Complex Wash Buffer

529.88mg LiCl (= 0.25M in 50mls)
0.5ml IPEGAL NP40
0.5 g deoxycholic acid
18.61 mg EDTA (= 1M EDTA)
78.8 mg Tris HCl (= 10mM in 50 mls)
49.5 ml dH_2O
pH 8.1

SDS Lysis Buffer

5ml 10% SDS
186.12mg EDTA (=10mM EDTA)
394mg Tris HCl (= 50mM in 50mls)
45ml dH_2O
pH 8.1

Low Salt Immune Complex Wash Buffer

500µl 10% SDS
0.5 ml Triton-X 100
37.22mg EDTA (= 2 mM in 50mls)
157.6mg Tris HCl (= 20mM in 50mls)
438.3mg NaCl (= 150mM in 50mls)
49 ml dH_2O
pH 8.1

High Salt Immune Complex Wash Buffer

37.22 mg EDTA (= 2mM in 50mls)
500µl 10% SDS
0.5 ml Triton-X 100
157.6mg Tris HCl (= 20mM in 50mls)
1461mg NaCl (= 500mM in 50mls)
49ml dH_2O
pH 8.1

TE Buffer

18.6mg EDTA (=1mM in 50mls)
78.8mg Tris HCl (=10 mM in 50mls)
50 mls dH_2O
pH 8.0