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The role of AIB-1 and PEA-3 in a signalling pathway mediating breast cancer invasion

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MB, BCh, LRCPSI, BAO (Hons) 2004

IMRCS 2006

A thesis submitted to the Royal College of Surgeons in Ireland, and presented to the Faculty of Medicine for the degree of Doctor of Medicine
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Dedication

This thesis is dedicated to my family, and especially my parents, for their never-ending support and encouragement

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This thesis is a product that would not exist without the support and encouragement of numerous people. I am indebted to Dr. Leonie Young, whose advice and guidance has been key to this work.

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I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree of **Doctor of Medicine (M.D.)** is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. 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.

Signed _____

Date 4/11/2009

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Summary

Tumour invasion and metastasis is mediated in part through the action of Cyclooxygenase-2 (COX-2) and Matrix Metalloproteinases (MMP). COX-2 mediated production of prostaglandins drives tumour growth through cell proliferation and angiogenesis, while MMPs have a role in degrading the extracellular membrane, which in turn promotes cell motility and invasiveness. The over-expression of these genes has been shown to be a marker of cancer cells with a higher metastatic potential. Unfortunately, the exact mechanism involved in their regulation is yet to be fully elucidated. The transcriptional regulatory region of COX-2 and MMP-9 genes contains binding sites for Ets transcription factors, such as PEA-3. It has previously been reported that expression of PEA-3 is associated with the co-activator AIB-1, HER2 positivity, metastasis, and reduced disease free survival. Furthermore, an AIB-1 knockout mouse model demonstrates PEA-3 dependant MMP production, resulting in reduced metastatic potential. Translational and molecular approaches were combined to study the role of PEA-3 and AIB-1 in the transcriptional regulation of MMP-9 and COX2 in breast cancer.

The expression levels of HER-2, p-ERK, PEA-3, AIB-1, MMP-9 and COX-2 were assessed in a tissue microarray of breast cancer patients (n=560). Strong associations were present between AIB-1, PEA-3, and the target proteins COX-2 and MMP-9 ($p<0.001$, $p=0.017$ and $p=0.004$ respectively). In addition, PEA-3 was found to significantly associate with MMP-9 expression ($p=0.008$). In HER2 positive

patients PEA-3, but not AIB-1 or MMP-9, was associated with reduced disease free survival ($p<0.0044$, $p= 0.4491$, and $p= 0.0666$ respectively). A stable breast cancer cell line over-expressing PEA-3 was created. Morphological changes suggesting an invasive and motile phenotype were observed with the development of this cell line. Furthermore, the production of the target proteins COX-2 and MMP-9 was increased compared to parental MCF-7 cells. In addition, up-regulation of the growth factor pathway in these cells further augmented this increase, strengthening our hypothesis that the proteins of interest are downstream targets of this pathway. Concomitant silencing of AIB-1 was found to result in reduced levels of both COX-2 and MMP-9. The expression of HER-2 and phospho-ERK also increased in the PEA-3 transfectant cell line, supporting the role of PEA-3 in the signalling pathway.

Understanding signalling networks important in the development and progression of breast cancer enables the identification of new markers of response to treatment. This data supports a role for the transcription factor PEA-3 and its co-activator AIB-1 in the production of MMP-9 and COX2 in breast cancer. Hence, targeting the PEA-3/AIB-1 pathway is a point of potential intervention in the management of breast cancer and may in turn contribute to halting its progression.

Abbreviations

bFGF –	Basic fibroblast growth factor
AIB-1-	Amplified-in-Breast Cancer 1
AF-	Activating Function
ATCC-	American Type Culture Collection
A/G-	Agarose
COX-	Cyclooxygenase
DAB-	Diaminobenzidine
DBD-	DNA Binding Domain
dH_2O -	Distilled Water
DMSO-	Dimethyl Sulphoxide
DNA -	Deoxyribonucleic Acid
E2-	Estradiol
EGF-	Epidermal Growth Factor
EGFR-	Epidermal Growth Factor Receptor
ER-	Estrogen Receptor
ERE-	Estrogen Response Element
ERK-	Extracellular Signal-Regulated Kinase
Ets-	E26 transformation-specific
FCS-	foetal calf serum
GPCR-	G Protein Coupled Receptor
HAT-	Histone Acetyl Transferase
HER-	Human Epidermal Growth Factor Receptor

HSP-	Heat Shock Protein
IgG-	Immunoglobulin
LB broth-	Luria –Bertani broth
LBD-	Ligand Binding Domain
LXXXLL-	Leucine – amino acid – Leucine
MA-	milli amperes
MAPK-	Mitogen Activated Protein Kinase
mER-	Membrane Estrogen Receptor
MISS-	Membrane-initiated Steroid Signalling
mRNA-	Messenger Ribonucleic Acid
PAGE-	Polyacrylamide Gel Electrophoresis
NR-	Nuclear Receptor
NSAIDS-	Non-steroidal Ant-inflammatory Drugs
PEA-3-	Polyoma Virus Enhancer activator 3
p-ERK-	Phospho Extracellular Signal-regulated Kinase
PBS-	Phosphate Buffer Saline
PI3K-	Phosphatidyl Inositol 3-Kinase
PLC-	Phospholipase C
PTEN-	Phosphate and Tension Homolog
RTK-	Receptor Tyrosine Kinase
SDS -	Sodium Dodecyl Sulphate
SERM-	Selective Estrogen Receptor Modulator
SRC-	Steroid Receptor Co-activator

TGF-	Transforming Growth Factor
TIMP-	Tissue Inhibitor of Matrix Metalloproteinase
TKI-	Tyrosine Kinase Inhibitor

Chapter I

Introduction

1.1 Breast Cancer

Breast Cancer is one of the leading causes of cancer mortality in women worldwide (WHO fact sheet N297). In Ireland, around 2000 women are newly diagnosed with the disease each year (Irish Cancer Society, Action Breast Cancer Book, 2008). An initial rise in the number of breast cancers detected in Ireland accompanied the establishment of the National Breast Screening Programme service in 2000 (The National Cancer Registry, 2008). While it is difficult to clearly define the trend in the incidence of breast cancer in Ireland independent of screening effects, it is projected that the incidence will see a further rise with the extension of the breast screening services in other parts of the country. In addition, recent statistics have revealed that the mortality in Ireland was found to be 33% higher than in the USA, and was in the upper third of rates in European countries (The National Cancer Registry Ireland Report 2006). Approximately 200,000 new cases of breast cancer are diagnosed each year in Europe at a total annual treatment cost of 7 billion euro. It has also been predicted that the number of breast cancers diagnosed will increase by 4% annually (The National Cancer Registry Ireland, 2008).

The management of breast cancer has changed dramatically in the last two decades with improvements in detection and advances in both surgical techniques and adjunctive therapies. The challenge today lies in the development of diagnostic and treatment modalities that provide maximum benefit while reducing the morbidity associated with the disease and its treatment.

1.2 Aetiology

The aetiology of breast cancer is multi-factorial. It is well established that factors such as steroid hormones and their receptors, peptide growth factors, oncogenes, and tumour suppressor genes play an important role in the development of breast cancer (Keen et al, 2003; Elledge et al, 2000; Clark G, 2000). An understanding of these signalling networks will enable the development of a more targeted approach in the treatment of breast cancer.

1.2.1 Steroid Hormone Receptors

Breast cancer is mainly hormone driven, with around 50-80% of cancers expressing an estrogen receptor, with the expression varying depending on patient age (Osborne, 1998). Risk factors for breast cancer such as early menarche, late menopause and nulliparity have been correlated with an increased risk of developing the disease through prolonged exposure to circulating estrogen. Estrogen has a specific target in breast tissue, the estrogen receptor (ER), and it is mainly through this receptor that its effects are mediated. The estrogen receptor is now known to exist in two genes, ERα and ERβ. ERβ was discovered in 1996, almost forty years after the discovery of ERα (Kuiper et al, 1996; Gorski et al, 1968; Jensen and DeSombre, 1972). This discovery further added to our understanding of the role of the estrogen receptors in breast cancer. Estrogen exerts a wide variety of its effects on growth, development, and differentiation by binding primarily to this receptor protein. Ligand binding to the ER allows these receptors to function as

transcription factors leading to the transcription of target genes critical to several biological processes (Katzenellenbogen et al, 2000).

The ER proteins contain several important functional domains. The hormone-binding domain in the region E of the ER also contains an estrogen-inducible transcription activating function called AF-2. A second constitutively active transcription activation function (AF-1) is located on the proximal region of the receptor in the growth factor receptor-binding domain (NHD also known as the AF-1) (figure 1.1). The DNA binding domain and the hinge region reside between the two transcription-activating functions, AF-1 and AF-2 (Osborne et al, 2003). The following diagram, adapted from the Gustafsson review (1999), compares the amino acid sequences of the two receptors.

Figure 1.1: Comparison of the primary structures of ERα and ERβ, respectively. The figures above the receptor representations indicate the number of amino acids, with number 1 being the most N-terminal. The numbers within the ERβ receptor represent the degree of homology (%) between respective domains in the two receptors. Abbreviations: growth factor binding domain (NHD), otherwise known as AF-1, DNA-binding domain (DBD) is responsible for binding at estrogen response elements (ERE) on the chromosome. Ligand binding domain (LBD) is otherwise known as AF-2. (Gustafsson, 1999).

1.2.2 Activation of the Estrogen Receptor

Activation of the ER can occur via two independent pathways. The classical and alternative pathways are described below. Figure 1.2, illustrates the two modes of action of the ER.

1.2.2.1 The Genomic Pathway

Upon entering the cell, estradiol (E2) binds to the ER (E2-ER), which then dissociates from heat shock proteins, such as HSP90, and undergoes conformational changes. It then auto-phosphorylates and dimerises before binding to the estrogen response element (ERE), which lies upstream of the estrogen dependent genes (Ring et al, 2004). The ER complex then recruits co-activator proteins such as steroid receptor co-activators (SRCs) and drives gene transcription (Klinge et al, 2004). This is referred to as the classical pathway.

ER has been also shown to have the ability to regulate gene expression without interacting directly with DNA. This occurs through association with other transcription factors such as the Fos/Jun activating protein-1 (AP-1) complex (Schiff et al, 2003; Ring et al, 2004). This is referred to as the non-classical mode of action.

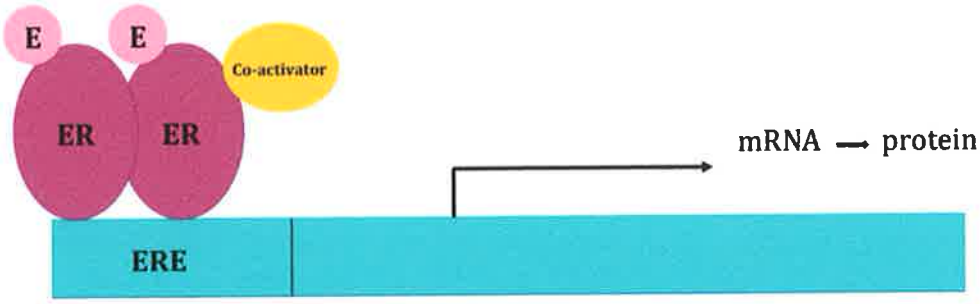
1.2.2.2 The Non-Genomic Pathway

There is evidence to suggest that ER can regulate cellular functions through non-genomic mechanisms of action, which is also referred to as membrane-initiated steroid signalling (MISS). Binding of estrogen to the ER may transactivate the epidermal growth factor (EGF) family of receptors and their downstream pathways, such as MAP kinase (MAPK) and PI3 kinase (PI3K). The exact mechanism of growth factor pathway activation is yet to be fully elucidated.

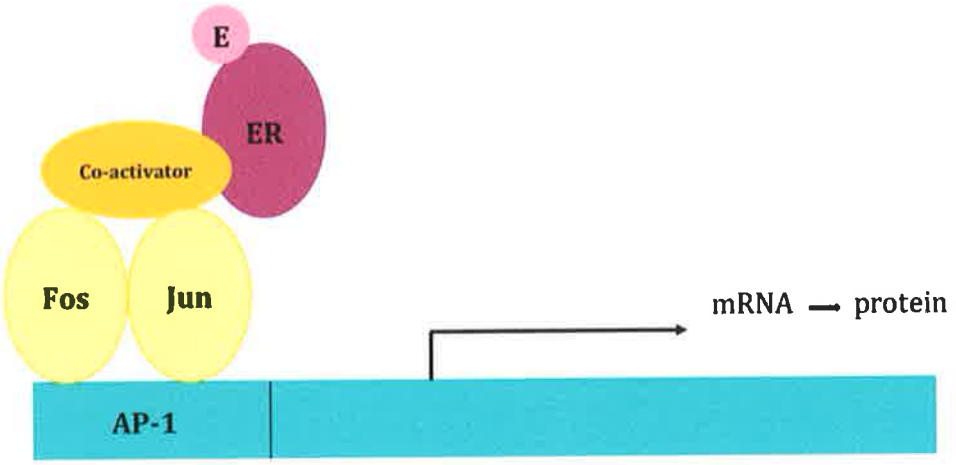
The discovery of novel membrane ERs (mER) provides a plausible mechanism by which estrogens can initiate rapid steroid actions at the cell surface and act in specific nuclear ER negative target cells. Peitras and Szego were the first to describe the binding of estrogen to membrane receptors (Pietras and Szego, 1977; Pietras and Szego 1980). Subsequent work has shown that estrogen can activate calcium flux, phospholipase C activation, and inositol triphosphate generation (Levin ER, 2001; Le Mellay et al, 1997; Lieberherr et al, 1993; Tesarik and Mendoza, 1995). Recent studies have implicated GPR30, a G-protien-coupled seven transmembrane receptor target that resides in the plasma membrane as a target for mediating this signalling pathway in breast cancer (Thomas et al, 2005). Once G protiens are activated, estrogen may then trigger signalling cascades, which mediate several cellular biological functions. The identification of a novel membrane estrogen receptor (mER) that activates growth factor pathways indicates that estrogen (and anti-estrogen) signalling in human breast cancer is therefore more complex than previously recognized.

These seemingly disparate molecular pathways of ER activation also communicate with each other and often work together to alter cellular growth. Importantly for cancer, the cross-talk between these pathways serves to amplify cell survival and proliferation signals, and growing evidence suggests that they may also contribute to resistance to various forms of endocrine therapy (Osborne and Fuqua, 1994).

Genomic pathway: Classical action of ER



Genomic pathway: Non-classical action of ER



Non-genomic pathway

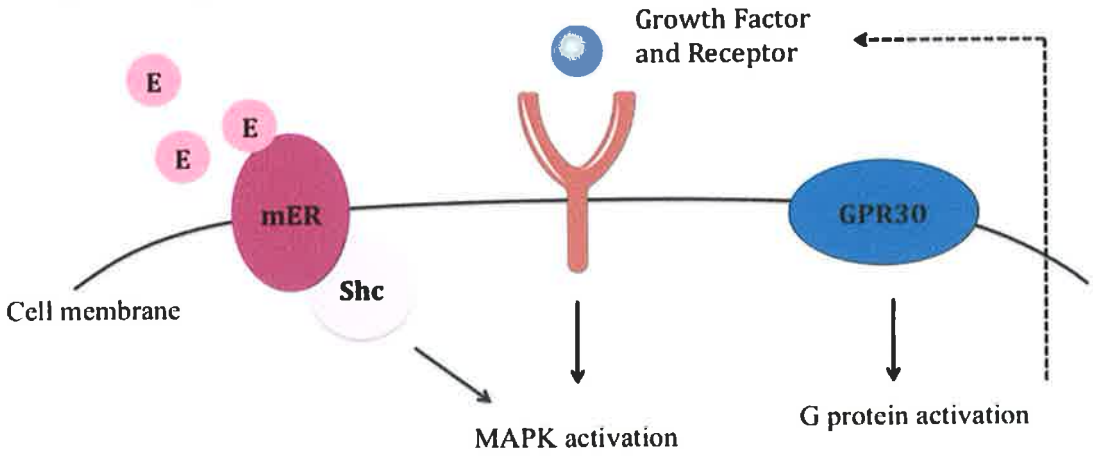


Figure 1.2: The genomic (classical and non-classical) and the non-genomic modes of action of the estrogen receptor are shown. Activation of the estrogen receptor occurs via these two pathways, as described above. Abbreviations; Estrogen receptor (ER), Estrogen (E), Estrogen response element (ERE), activating protein-1 (AP-1), mitogen-activated protein kinase (MAPK). (Ring et al, 2004; Speirs and Walker, 2007).

1.2.3 Anti-estrogens

The expression of the estrogen receptor (ERα) is one of a few prognostic factors, along with axillary lymph node status, tumour size, and histological grade and subtype (Clark G, 2000). The expression of ER has also been shown to be an important predictor of potential response to endocrine therapy. The ER has been a successful target for prevention and treatment of breast cancer and it serves as an important clinically useful biomarker. Tumours expressing ER are often stimulated by estrogen, and as a consequence, respond to therapies designed to block estrogen signalling (Osborne, 1988). At present, estrogen antagonist therapy is the most effective treatment for women with ER-positive breast cancer. Current endocrine therapies are based on targeting the estrogen receptor by antagonising ER function with a selective estrogen receptor modulator (SERM) such as Tamoxifen, reducing levels of estrogen with an aromatase inhibitor such as Arimidex and down regulating ER levels with pure anti-estrogens such as fluvestrant (Faslodex).

Various ligands modify the receptor in different ways. SERMs, such as Tamoxifen, bind to the ER and induce receptor dimerisation and binding to ER elements on target genes. However, the conformation of the receptor is different when bound by Tamoxifen than when the receptor is bound by estrogens, and Tamoxifen-bound receptors interact with a distinct set of co-regulatory molecules. Coregulators such as Steroid Receptor Co-regulators, p300/CBP, p68 RNA hilcase and L7/SPA have been shown to increase the partial agonist action of tamoxifen on ERE promoters (Jackson et al, 1997; Smith et al, 1997, Lavinsky

et al, 1997; Webb et al, 1998; Endoh et al, 1999). It is well established that Tamoxifen exerts both agonist and antagonist effects depending somewhat on the species, tissue or gene on which it is acting. Tamoxifen exerts predominantly agonistic effects in bone and in the endometrium, conversely its action is antagonistic on genes that function in cell proliferation and survival in the breast. The agonist/antagonist profile of Tamoxifen and other SERMs may be in part related to the particular expression of co-regulatory proteins within the cell (Smith et al, 1997; Jackson et al, 1997; Dutertre and Smith, 2000).

Steroidal anti-estrogens, such as fulvestrant, also bind to the ER. However, this class of agents has an entirely different mechanism of action. These drugs inhibit ER dimerisation and binding to DNA. Furthermore, they antagonize both the AF-1 and AF-2 transcription activating functions of ER, whereas the SERMs only inhibit AF-2. Finally, the steroidal anti-estrogens induce ER degradation and ER loss from the cell (Osborne et al, 2003).

1.2.3.1 Tamoxifen and Resistance

The number of women receiving hormonal therapy for breast cancer has increased by 10% from 1995 to 2000. (National Cancer Registry, Ireland, 2006). Tamoxifen is a first line hormonal therapy used in ER-positive breast cancer, and therapy results in a 40-50% reduction in the annual odds of tumour recurrence leading to prolonged disease free and overall survival (Early Breast Cancer Trialists Collaborative Group, 1998). Unfortunately, there are patients who present with primary resistance to Tamoxifen despite the presence of an

ER. Furthermore, some patients who initially respond to hormonal therapy eventually acquire Tamoxifen resistance leading to tumour progression and death. The mechanisms behind this resistance remain poorly understood (Normanno et al, 2005). It has been proposed that changes in cellular regulatory mechanisms can mediate this resistance to treatment. An up-regulated expression of epidermal growth factor receptor (EGFR) and HER-2 have been shown (Nicholson et al, 2004). Dowsett et al, showed an increase in HER-2 expression in patients with acquired resistance. In addition, p38 expression has been proposed to contribute to a tamoxifen-resistant phenotype (Dowsett et al, 2005; Nicholson et al, 2004).

1.3 Peptide Growth Factors and Their Receptors

A number of peptide growth factors and their receptors have also been implicated in normal breast development and in carcinogenesis. These include members of the epidermal growth factor, transforming growth factor- ß (TGF-ß), and insulin-like growth factor families.

1.3.1 Epidermal Growth Factor Receptor Family

The HER or erbB proteins are members of the subclass I of the receptor tyrosine kinase (RTK) superfamily. These proteins possess an extracellular ligand-binding domain, a membrane-spanning region and a cytoplasmic domain with tyrosine kinase activity. This group contains four members: epidermal growth factor receptor (EGFR/erbB1/HER-1), erbB2/neu/HER-2, erbB3/HER-3, and

erbB4/HER-4. These trans-membrane receptors have similar structure, however, 25-30% would differ in their overall homology (Keen et al, 2003). Although these receptors are structurally homologous, naturally occurring ligands have been discovered only for HER-1, HER-3 and HER-4 (Arteaga, 2003). There are at least 25 known ligands that can bind to the HER family members. This includes epidermal growth factor (EGF), TGF-a, amphiregulin, heparin-binding EGF (HbEGF), ß-cellulin, epiregulin, cipto-1, neuregulin, and heregulin (Olayioye et al, 2000). Ligand binding to these receptors leads to their homo and hetero dimerisation through which signal transduction is then elicited. To date there have been no ligands identified for HER-2, however, it appears to be the preferred hetero-dimerisation partner for other HER family members, resulting ultimately in signal transduction by hetero-dimerisation with other HER family members (Yarden et al, 2001).

1.3.2.1 HER2/neu (ErbB-2)

The HER2/neu growth factor receptor tyrosine kinase, which belongs to the EGFR/HER family, has been shown to be one of the most important oncogenes in human breast cancer. The HER-2 protein, which is also known as c-erbB-2 or neu, is a member of subclass 1 of the superfamily of receptor tyrosine kinases. After hetero-dimerisation, HER-2 complexes initiate intracellular signalling via the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3′- kinase (PI3K) and phospholipase C (PLC) pathways (Benz et al, 1997). In breast cell lines and model tumour systems, over-expression of the HER-2 gene has been associated with increased mitogenesis, malignant transformation, increased cell

motility, invasion and metastasis. (Goel et al, 2004). In human breast cancer, amplification of the HER-2 gene is found in 15-30% of primary invasive tumours (Slamon et al, 1987). In these cases, up to one hundred copies of the gene may be present per cell, which equates to a 50-fold increase in gene copy number per cell (Osborne et al, 2003). This increased gene copy number can lead to an increase in the number of receptors per cell from 20,000-50,000 up to 2 million. Gene amplification or increased production of HER-2 is generally found to correlate with adverse prognosis, particularly in node-positive breast cancer patients (Winston et al, 2004). Laboratory studies suggest that ER-positive breast cancers that over-express HER-2 may be less responsive to Tamoxifen than breast cancers with low HER-2 expression (Lipton et al, 2005). Furthermore, co-expression of HER-2 with SRC-3 has been associated with increased resistance to endocrine therapy (Osborne et al, 2003). The mechanisms for this resistance are, however, not yet clear. Furthermore, ligand-independent activation of the ER by MAPKs, which themselves are phosphorylated and thereby activated by HER-2 signalling in such tumours can occur, leading to the possible development of resistance.

1.3.3 Mitogen-Activated Protein Kinase (MAPK) Pathway

Cell proliferation and differentiation has been shown to be regulated by the activation of intracellular protein serine/threonine kinases, also named mitogen-activated protein kinases (MAPKs) and extracellular signal-regulated kinases (ERKs) (Cobb and Goldsmith, 1995; Reddy et al, 1999). A GTP binding protein, p21ras, binds to and activates the c-raf protein kinase. Raf

phosphorylation then activates MAP kinase kinase (MAPKK), also known as MEK, which in turn phosphorylates and activates MAPKs, Erk, JNK and p38. Erks are activated in response to mitogenic signals while JNKs and p38/SAPKs are stress-activated kinases (Hsu et al, 2004). MAPKs when activated translocate to the nucleus and can regulate several transcription factors associated with carcinogenesis. MAPK pathways phosphorylate many different transcription factors, modulating DNA binding affinity, nuclear stabilization and interaction with co-regulators thereby regulating gene expression (Karin et al, 1994). 15-20% of all human tumours have an activating mutation in one of the three *ras* genes (N-, K-, H-ras) (Downward et al, 2003). Enhanced activation of MAPKs has been shown to be increased in primary breast cancer compared to normal tissue as well as tissue from patients suffering from non-malignant diseases (Sivaraman et al, 1997). It is evident that MAPK activation of SRC-3 stimulates the recruitment of p300 and associated histone acetyl transferase activity suggesting that the ability of growth factors to modulate estrogen action may be mediated through MAPK activation of the nuclear receptor co-activator SRC-3 (Font de Mora et al, 2000).

1.3.4 Herceptin (trastuzumab) and Other Growth Factor Targeted Therapies

The past two decades have seen the development of several agents that are directed against the HER (ErbB) receptors. These include monoclonal antibodies that act by binding to the extracellular domain of the receptor and small molecule tyrosine kinase inhibitors (TKIs), which directly inhibit tyrosine

kinase phosphorylation by interacting with the ATP of the enzyme substrate-binding site (Normanno et al, 2003).

Trastuzumab (Herceptin) is a humanised monoclonal antibody with high specificity for the ErbB-2 receptor. To date it has served as a first and second line single agent in the treatment of HER-2 positive breast cancer (Baselga et al, 1999; Vogel et al, 2002) and in combination with chemotherapy (Demonty et al, 2007). Studies have shown that the addition of trastuzumab to first-line chemotherapy shows significant improvement in response rate, time to disease progression and in overall survival (Slamon et al, 2001).

Lapatinib is a newer agent oral dual tyrosine-kinase inhibitor of both EGFR and ErbB-2. Pre-clinical studies have shown reduced growth in various tumours that over-express EGFR or ErbB-2 (Rusnak et al, 2001). Promising results have also been reported with the use of lapatinib as a single agent in the treatment of relapsed or refractory inflammatory breast cancer, as reported in a phase II study (Spector et al, 2006). Current trials (ALTTO and neoALTTO) are currently evaluating the efficacy of lapatinib as a single agent or in combination with traztuzumab, compared to trastuzumab as a single agent in the treatment of HER-2 positive tumours in the adjuvant and neo-adjuvant settings.

1.3.5 Resistance to Tyrosine Kinase Inhibitors

Breast cancer is a heterogenous disease and can develop as a result of several different genetic and epigenetic alterations. Activation of various oncogenic pathways has been shown in breast cancer, and these pathways interact in order to sustain tumour proliferation and growth. It is felt that around 30% of patients with advanced HER-2 positive breast cancer respond to trastuzumab as first line therapy, and the response rate drops to 15% in pre-treated patients. In addition, the majority of patients will become resistant to the drug during the course of treatment (Normanno et al, 2009). The majority of patients with metastatic breast cancer who initially respond to trastuzumab demonstrate disease progression within one year of treatment initiation (Normanno et al, 2009). Pre-clinical studies suggest that trastuzumab resistance occurs through activation of multiple receptor pathways such as the insulin-like growth factors receptor (which appears to be involved in a cross-talk with HER-2 in resistant cells), loss of function of the PTEN tumour suppressor gene which leads to increased AKT signalling decreasing the sensitivity to trastuzumab, and decreased interaction between trastuzumab and its target receptor (Nahta et al, 2006). Resistance to target based therapy is felt to be a consequence of the complex mechanisms involved in regulating breast cancer growth. As such, evidence suggests that it is unlikely that tumour inhibition will results from the blockade of a single oncogene.

1.4 Cross talk between ER and HER pathways

Growth factors and estrogen receptor pathways work together to alter cellular growth. Growing evidence suggests that this type of cross talk between the two pathways may also contribute to resistance to different types of endocrine therapy (Kent et al, 2003; Johnston et al, 2003). Growth factors such as epidermal growth factor and insulin like growth factors when bound to their receptors initiate a kinase-signalling cascade in a number of pathways including MAPK, Erk1, Erk2, PI3 and JNK (Pearson et al, 2001). On the other hand, enhanced steroid signalling can lead to transcription of growth factor related genes which can in turn result in increased signalling within growth factors pathways via a positive feedback loop, estrogen triggers rapid yet transient activation of the MAPKs, Erk-1 and Erk-2 (Filardo et al, 2002). Furthermore, estrogen can also function in a non-genomic fashion by interacting with cell surface G-protein coupled receptors (GPCR). Estrogen activation of GPCR can lead to activation of tyrosine kinase receptors and hence initiation of the MAPK cascade (Filardo et al, 2002). The MAPK pathways can interact with the ER at different levels; they phosphorylate the ER and phosphorylate ER co-activators and co-repressors that are important for ER function. For instance, phosphorylation of SRC-3 co-activator increases ER activity (Kato et al, 1995). Thus, it is possible that abnormally increased signalling within these growth factors pathways may account for loss of some estrogen dependence, resulting in anti-estrogen resistant tumours (Schiff et al 2003).

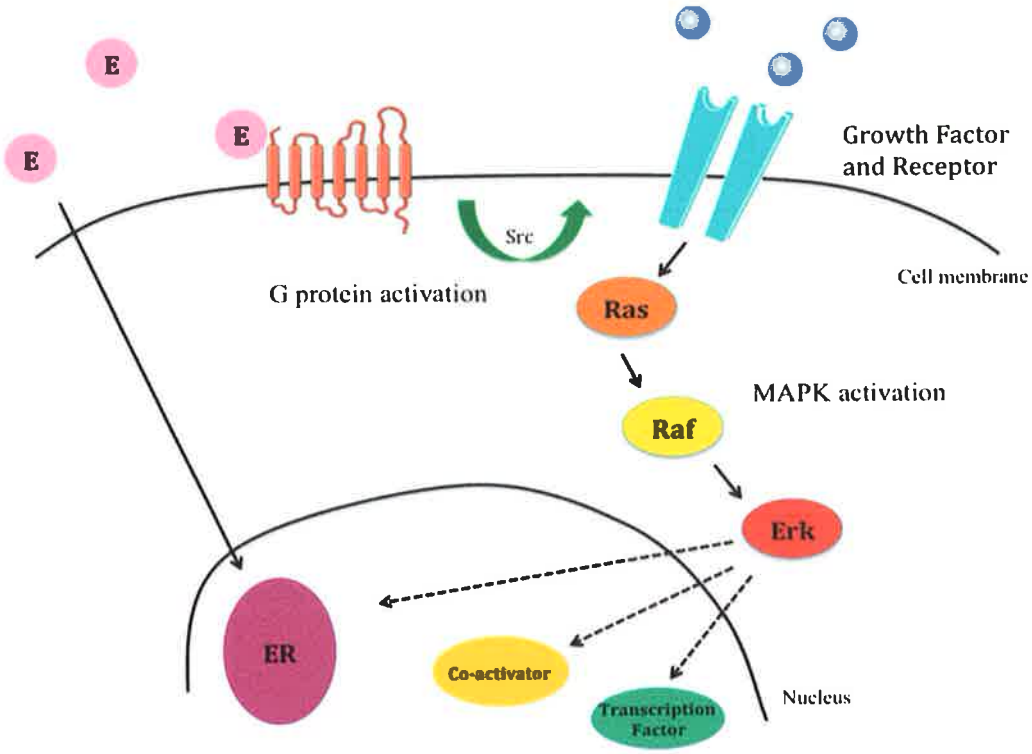


Figure 1.3: Schematic representation of crosstalk between ER and the growth factor pathway. G-protein coupled receptors (GPCR) can activate ErbB signalling though freeing membrane tethered ligands enabling them to bind to the ErbB receptors, or through activating Src which phosphorylates the intracellular domains of the ErbB receptors. HER activation can have a positive feedback effect through the Ras-MAPK pathway, which also activates the transcription of ErbB ligand genes.

1.5 Co-regulatory Proteins

Gene regulation is influenced not only by ligand binding but also by the presence of specific co-regulatory proteins present at rate–limiting levels that modulate transcription (McKenna et al, 1999). The binding of these co-regulators to ligands results in the activation or repression of the estrogen receptors transcriptional activity. Thus, the co-regulators are designated co-activators and co-repressors, respectively. A large number of these proteins

have been discovered using various screening strategies. Co-regulatory proteins interact with nuclear receptors at a conserved LXXL motif within the receptor interacting domain of the protein to drive target gene expression (Heery et al, 1997).

1.5.1 Co-activators

A co-activator is defined according to its physical interaction with nuclear receptors (NRs) in biochemical analysis and its ability to enhance nuclear receptor (NR)-dependent transcription in transient transfection assays. Most unbound co-activators that directly interact with NRs form distinct protein complexes with downstream intermediate factors for chromatin remodelling or for interaction with general transcription factors. Ligand-activated and DNA-bound NRs or other classes of transcription factors can efficiently recruit these co-activator complexes to specific promoters. The concentration and function of each individual component in these co-activator complexes can be regulated through transcriptional control and various post-translational modifications and degradation by multiple signalling pathways. The use of these co-activator complexes by NRs may provide platforms for sophisticated transcriptional regulation (Xu et al, 2003). One of the best-characterized groups of NR (nuclear receptor) co-activators is the p160 family, which includes SRC-1, SRC-2/TIF-2/GRIP-1 and SRC-3/ACTR/AIB1 (McKenna and O'Malley, 2002; Glass and Rosenfeld, 2000).

1.5.1.1 Amplified-in-Breast Cancer-1 (AIB-1)

Amplified-in-breast cancer 1 (AIB-1) (also known as SRC-3, ACTR, and NCOA3) oncogene was initially identified in an amplified chromosomal 20q region in breast cancer cells (Guan et al 1996). AIB-1 is a member of the p160 steroid receptor co-activator (SRC) family, which also contains SRC-1 and SRC-2. AIB-1 plays an important role in cell growth and survival. Its over-expression or over-activation in breast cancer cells enhances estrogen-induced cyclin D1 expression, epidermal growth factor activation, cell proliferation, and anti-estrogen resistance (Lahusen et al, 2007; List et al, 2001; Planas-Silva, 2001; Qin et al, 2008).

AIB-1 has been found to be amplified in around 5-10% of human breast cancers and its mRNA and protein are over-expressed in approximately 30% of breast tumour (List et al, 2001; Anzick et al, 1997). SRC-3/AIB-1 has been shown to be over-expressed and amplified in many cancer cells and primary tumours, including breast cancers, ovarian cancers, endometrial cancers, gastric cancers and prostate cancers (Anzick et al, 1997; List et al, 2003; Glaeser et al, 2001; Sakakura et al, 2000; Gnanapragasam et al, 2001). The over-expression of AIB-1 in transgenic mice has been shown to result in increased mammary epithelial cell proliferation, the development of mammary hyperplasia and tumorigenesis (Tilli et al, 2005; Torres-Arzayus et al, 2004). Furthermore, AIB-1 knockout mice display decreased mammary gland development during pregnancy, abnormal reproduction function, and mammary gland growth retardation (Xu et al, 2000). Observations to date using both *in vitro* and animal models indicate

that AIB-1/SRC-3 plays an important role in several growth factor induced pathways that are relevant to breast cancer survival and proliferation.

1.6 Transcription Factors

The first member of the Ets (E26 transformation-specific) family gene, *v-ets,* was originally identified as *gag-myb-ets,* a fusion oncogene of the avian transforming retrovirus E26 that induces erythroblastic and myeloblastic leukaemia in chicken (Leprince et al 1983). Ets family members can be divided into several subfamilies depending on their DNA binding domains. Most of these members have the Ets domain at the C-terminal region. The flanking DNA sequences as well as a purine-rich core appear to be important to determine the preferential binding of individual Ets family proteins (Wang et al, 1992). Thirty members of this family have been identified. A characteristic feature of this family is that they share an evolutionary conserved Ets domain of about 85 amino acid residues that mediate binding to a purine-rich DNA sequences with a central GGAA/T core consensus and additional flanking (Graves et al, 1998). Ets proteins contain the pointed domain (PNT), which is important for protein-protein interaction. Ets proteins are a family of mitogen-activated protein kinase (MAPK) dependent transcription factors, which have been implicated as downstream effectors of HER2 signalling (Galang et al, 1996). Most of Ets family proteins are phosphorylated in response to growth factors and cellular stress.

1.6.1 Ets target genes and cell proliferation

Among the multiple Ets target genes that are important for cancer progression are those that function in control of cell proliferation (cyclins and cdks), adhesion (cadherins, integrins, cell adhesion molecules), motility/migration (hepatocyte growth factor receptor c Met, vimentin), cell survival (Bcl-2), invasion (uPA &uPAR, PAI, MMPs, TIMPs, heparanase), extravasion (MMPs, integrins), micrometastasis (osteopontin, parathyroid hormone related peptide, chemokines/chemochine receptors, CD44) and establishment and maintenance of distant site metastasis and angiogenesis (integrin ß3, VEGF, Flt-1/ KDR, Tie2) (Hsu et al, 2004; Sementchenko et al, 2000; Oikawa et al, 2003).

1.6.2 Ets family proteins and other cellular proteins

Ets family proteins regulate gene expression by functional interaction with other transcription factors and co-factors on DNA-binding sites. Depending on the precise sequence context, binding of an Ets protein near other transcription factors results in a higher affinity interaction and synergistic repression or activation of specific target genes. A more recent proteomic approach has identified several Ets associated proteins (EAPs) that modulate Ets activation through different mechanisms such as blocking DNA binding, and inhibiting synergistic interaction with co-factors (Li et al, 2000). Many Ets family proteins like Elk-1, Ets-1, Ets-2 and ER81 interact with co-activators CBP/p300 to activate transcription (Jayaraman et al, 1999). Myers et al described a relationship between Ets-1 and Ets-2 with members of p-160 co-regulators

namely the co-activators SRC-1, SRC-3 and the co-repressor NCoR on DNA binding sites (Myers et al, 2005).

1.6.3 Polyoma Virus Enhancer Activator 3 (PEA-3)

The PEA-3 group is composed of three members; Erm (also called Etv5), Er81 (also called Etv1), and PEA-3 (also called E1Af or Etv4) (Launoit et al, 2006). These share a highly conserved ETS-domain and two conserved trans-activating domains, one located at the amino-terminus (AD) and the other at the carboxy-terminus (Ct) (Launoit et al, 1997). The PEA-3 group of genes is structured over more than 15kbp of genomic DNA; Erm on chromosome 3 at position 3q27-29, E1AF on chromosome 17 at position 17q21, and Er81 on chromosome 7 at position 7q21 (Launoit et al, 2006).

Figure 1.4: Schematic representation of the PEA-3 protein, consisting of 480 amino acids, acidic domain, trans-activation domain and an Ets domain.

The transcription factor PEA-3 undergoes post-translational modification to regulate its transcriptional capacity. This occurs through its phosphorylation, as it is a target of the MAPK pathway. Phosphorylation of specific serine and threonine residues has been shown to increase the trans-activation capacity of PEA-3 (Janknecht et al, 1996; O'Hagan et al, 1996; Bosc et al, 2001). PEA-3 is

also regulated by the ubiquitin-proteosome pathway following conjugation to ubiquitin (Takahashi et al, 2005).

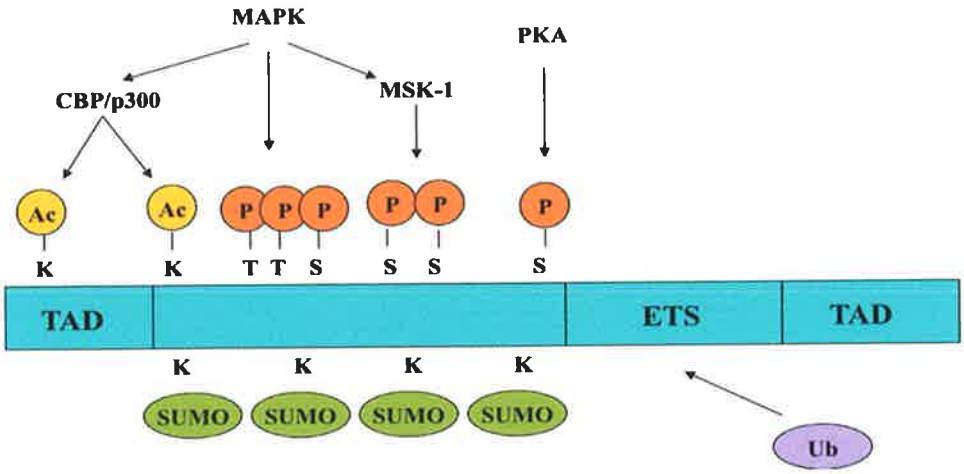


Figure 1.5: Schematic representation of the PEA-3 group of transcription factors with the different functional domains and regulating pathways. There are two transcriptional activating domains (TAD). Post-translational modifications up-regulating (above the protein) or down-regulating (below the protein) the transcriptional activating of PEA-3 group members are also shown. Abbreviations; Acetyl group (Ac), phosphate group (P), sumo peptide (SUMO), ubiquitin (Ub). (Launoit et al, 2006)

1.7 Breast cancer invasion

Alteration in the tumour microenvironment is necessary for tumour progression. Tumour invasion results from loss of physiological growth controls and normal apoptotic response, as well as changes in cell physiology and sustained angiogenesis.

1.7.1 Cyclooxygenase 2 (COX-2)

The cyclooxygenase (COX) genes encode for enzymes involved in the conversion of arachidonic acid to prostaglandins. The two main COX isoforms, which were first described in the 1990s, are COX-1 and COX-2 (Fu et al, 1990; Xie et al, 1991). COX-1 has been shown to be mainly constitutively expressed, and is responsible for major physiological functions such as gastric cyto-protection, regulation of renal blood flow, and platelet aggregation (Smith et al, 2000). COX-2, on the other hand, is an inducible form and is expressed by cells that are involved in the inflammatory processes and in a range of solid malignancies (Smith et al, 2000; Gasparini et al, 2003). COX-2 is rarely expressed in normal tissue. Its over-expression has been shown to promote angiogenesis and cell proliferation, and inhibit apoptosis (Boland et al, 2004; Kirpatrick 2001; Barnes 2005).

In breast cancer, COX-2 has been found to be over-expressed in approximately 70% of cases of in situ and 60% of invasive tumours (Boland et al, 2004; Barnes et al, 2007). Furthermore, the increased expression of COX-2 in cancer cells has been shown to be a response to activation by growth factors and oncogenes, and has been shown to be related to increased risk of tumour recurrence and decreased survival in invasive breast cancer. Studies on transgenic mice over-expressing COX-2 have demonstrated early mammary tumour formation, fewer apoptotic cells and reduced expression of pro-apoptotic proteins BAX and BClx(L) (Liu et al, 2001). Of particular interest to breast cancer, is the activation of the MAPK pathway in HER-2 positive breast cancers, which confers these

tumours to having a poorer prognosis (Slamon et al, 1987; Barnes et al, 2007). The transcriptional up-regulation of the COX-2 genes by the ErbB-2 gene expression has been found in both cultured cancer cells, translational studies, and in animal models (Dillon et al, 2008; Subbaramiah et al, 2002; Howe et al, 2001). A significant correlation in the expression of both of these genes has been reported in several human cancers such as breast cancer, colon cancer, and cholangiocarcinoma (Howe et al, 2001; Endo et al, 2002).

Non-steroidal anti-inflammatory drugs (NSAIDs) have anti-inflammatory properties that act against both COX-1 and COX-2 isoforms. Their regular use has been suggested to have chemopreventative effects against the development of breast cancer, leading to a 28% risk reduction with their use for ten or more years (Barnes et al, 2007). In addition, their regular use has been shown to significantly decrease the growth of several solid tumours (Howe et al, 2002). Despite the fact that numerous studies have proven the efficacy of COX-2 inhibitors in cancer treatment, the exact mechanism leading to COX-2 over-expression has yet to be fully elucidated.

1.7.2 Matrix Metalloproteinases (MMP)

MMPs are a family of structurally and functionally related endopeptidases, which are involved in the degredation of the extracellular membrane (ECM). These enzymes also have a role in the regulation of tissue remodelling events. Abnormal or elevated expression of these enzymes has been shown to be linked to pathological processes such as tumour growth, invasion and metastasis

(Duffy et al, 1998; Chambers et al, 1997). MMPs contain specific domains that have been found to be conserved between members. The catalytic activity of the enzyme depends on the presence of zinc ions at the catalytic active site (Duffy et al, 2000). When MMPs are synthesised, they are excreted in an inactive zymogen form and can be subsequently activated by the loss of the 10kDa amino-terminal domain (Duffy et al, 2000). This activation is then responsible for the cleavage of different components of the ECM. The four main MMP subgroups can be divided into; collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2 and MMP-9), stomeolysins (MMP-3, MMP-7, MMP-10, MMP-11), and membrane MMPs. Furthermore, tissue inhibitors of metalloproteinases (TIMPs) are involved in inhibiting the proteolytic activity of MMPs. They form high affinity stoichiometric, non-covalent complexes with the active MMPs as well as the precursor forms. In addition, they have been shown to have a role in stimulating cell proliferation *in vitro* (Duffy et al, 1998; Henriet et al, 1999).

1.7.2.1 Matrix Metalloproteinase 9 (MMP-9)

MMP-9 is one of the two gelatinases (including MMP-2) that have been implicated in tumour cell invasion and metastasis because of their unique ability to degrade the Type IV collagen, a major component of the basement membrane, and other essential extracellular matrix components (Wu et al, 2008). MMP-9 and its natural inhibitor, tissue inhibitor of metalloproteinase-1 (TIMP-1) may be involved in early cancer development and subsequent progression. Elevated serum and tissue levels of both enzymes have been found in cancer patients (Somiari et a, 2006; Talvensaari-Mattila et al, 2005; Jinga et

al, 2006; Hanemaaijer et al, 2000; Wu et al, 2008). The exact mechanism involved in up-regulating the expression of MMP-9 has yet to be fully elucidated.

1.8 Hypothesis

Ets transcription factors have been shown to have a role in promoting tumour invasion and metastasis, with several of the involved genes containing an Ets binding site for these transcription factors. Important genes involved in tumour progression include COX-2 and MMP-9. The exact mechanism involved in the regulation of these proteins is yet to be fully elucidated. We hypothesise that the Ets transcription factor, PEA-3, will require a co-regulator, such as AIB-1, to induce the transcription of the genes involved in tumour progression. Furthermore, we suspect that an active MAPK pathway may be central to this process and may induce up-regulated expression of COX-2 and MMP-9 through the activation of PEA-3 and AIB-1 (Figure 1.6).

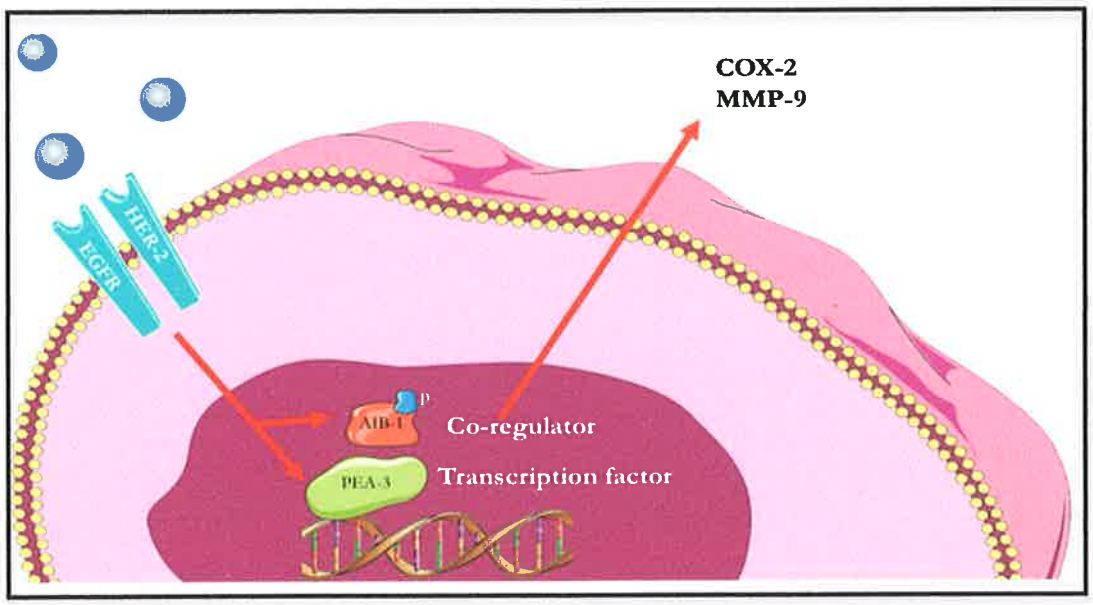


Figure 1.6: Schematic representation of the hypothesis; Activation of the growth factor pathway with growth factor ligands, such as EGF, activates the MAPK pathway which in turn leads to a cascade of reactions which may activate PEA-3, a member of the Ets transcription factor family, as well as the p160 co-regulatory protein AIB-1. These may potentially co-associate and mediate the transcription of target genes such as COX-2 and MMP-9.

1.9 Specific Aims

1. Investigate the molecular interaction between the co-activator AIB-1 and the transcription factor PEA-3 in breast cancer cell lines.
2. Examine the functional importance of AIB-1 and PEA-3 on the production of the downstream target proteins COX-2 and MMP-9.
3. Explore the role of PEA-3 on the production of the upstream target HER-2 and its role in regulating the growth factor pathway in breast cancer cells.
4. Evaluate the clinical relevance of the AIB-1/PEA-3 interaction and their target proteins in a cohort of breast cancer patients.

Chapter II

Materials and Methods

2.1 Cell Culture

2.1.1 Breast cancer cell lines

The main breast cancer cell line used in the studies described in this thesis is the MCF-7 cell line (ATCC). These epithelial cells are derived from a metastatic pleural effusion (Soule et al, 1973). The MCF-7 line retains several characteristics of differentiated mammary epithelium including the ability to process estradiol via estrogen receptors and the capability of forming domes. MCF-7 cells were maintained in minimal essential medium (MEM) (Eagle, Invitrogen) supplemented with 2mM L-glutamine (Sigma-Aldrich), 100μg/ml penicillin and 100μg/ml streptomycin (Sigma-Aldrich) and 10% (v/v) foetal calf serum (Gibco). A stable PEA-3 over-expressing cell line (MCF-7/PEA-3) was developed, as described in Section 2.4, using the parental MCF-7 cell line. These cells were maintained in the same medium, supplemented with 500μg/ml of Geneticin (Invitrogen).

Other cell lines used for comparison purposes in various investigations included SKBR-3 cells (ER negative and HER-2/neu positive. ECACC, Wiltshire, UK), MDA-MB231 (ER negative and HER-2/neu positive, ECACC, Wiltshire, UK), HCA-7 (colon cancer cells. ECACC, Wiltshire, UK). The cells were maintained in RPMI media (Gibco) supplemented with 10% foetal calf serum, 50U/ml penicillin-streptomycin, Leibovitz L-15 media (Gibco) supplemented with 10% foetal calf serum and 50U/ml penicillin streptomycin, and DMEM (Gibco) supplemented with 10% foetal calf serum, 50U/ml penicillin-streptomycin and 2mM L-Glutamine, respectively. MCF-10A cells were a gift from Dr. Ann Hopkins, Royal College of Surgeons in Ireland.

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^{0}C in a Heraeus Hera Cell incubator (Kendro Laboratory Products, Hanau Germany). Cells were visualised with a Nikon eclipse TS100 inverted microscope (Nikon, Tokyo Japan).

2.1.3 Culturing of cells from cryo-storage

Cryovials containing cells were removed from storage in liquid nitrogen and thawed to 4^{0}C. The cellular contents were transferred to a sterile universal container containing 5 ml of the required culture medium. The cell suspension was centrifuged at 1,800 rpm for 5 minutes. The supernatant was discarded and the pellet resuspended in 2 ml of fresh medium. This suspension was added to a 25cm^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^{0}C.

2.1.4 Routine culture

Cells were maintained in 75cm^2 flasks containing 10 ml of supplemented medium. Routine passaging was performed when cells reached approximately 90% confluency. Culture medium was decanted from the culture flask and adherent cells were detached from the flask by incubating the cells in 3 ml of a trypsin-EDTA solution at 37^{0}C for approximately 3 minutes. Cells were removed

and added to 10 ml of culture medium and centrifuged at 1,800 rpm for 5 minutes. The supernatant was then discarded and the cell pellet was resuspended in 2 ml of culture medium and transferred to a tissue culture flask containing an appropriate volume of growth medium (4 ml for a 25 cm^2 flask, 8 ml for a 75 cm^2 flask).

2.1.5 Cell culture treatment conditions

In the case of the MCF7 and MCF7/PEA-3 cell lines, cell culture treatments were performed when the cells were 90% confluent. The culture medium was decanted from the flask and the cells were washed in sterile PBS (Gibco). For 24 hours prior to treatment all cells were grown in serum free MEM at 37°C in 5% CO_2 atmosphere. The serum-free medium was decanted and the cells were then incubated in serum-free MEM (control), and stimulated with Epidermal Growth Factor (EGF) (Sigma- Aldrich) at a concentration of 10 ng/ml. The cells were then harvested and the cell pellets stored at -20°C.

2.1.6 Long-term storage of cells

Cells were trypsinised and pelleted as described above. The cell pellets were resuspended in 2 ml of a dimethyl sulphoxide (DMSO) (Sigma-Aldrich) (10%)/FCS (90%) mix on ice. This solution was immediately aliquoted into cryovials and incubated at –80°C overnight. The vials were then cryopreserved at -196°C.

2.2 Western Blot analysis

2.2.1 Determination of total protein using Bicinchonic acid (BCA) assay

This procedure was performed using the Pierce BCA protein assay kit (Pierce, Il USA). The method is based on the principle that in the presence of protein, copper (Cu^{+2}) is reduced to the cuprous cation (Cu^{+1}) that is chelated by bicinchonic acid to form a purple reaction product. This water-soluble complex exhibits a strong absorbance at 570 nm.

2.2.2 Protein extraction and BCA Protocol

Ten µl of pefabloc (Roche Diagnostics, Mannheim Germany) was added to 1 ml of lysis buffer (Appendix). Eighty µl of this mix were added to each cell pellet. The samples were placed on ice and vortexed at 10 minute intervals for 30 minutes. The samples were then centrifuged at 13,000 rpm for 20 minutes at 4°C. The resultant supernatant (protein lysate) was transferred into a chilled eppendorf tubes and kept on ice. Protein lysate samples (3 µl) were diluted in dH_2O. Standards (Table 2.1) and diluted samples (25 µl) were pipetted in duplicate into a 96 well plate. The reaction mix was made up with forty-nine parts of Solution 1 of the Pierce BCA protein assay kit (containing Na_2CO_3, $NaHCO_3$, BCA detection reagent and sodium tartrate in 0.5 M NaOH) with one part of Solution 2 (4% (w/v) $CuSO_4$). Reaction mix (200 µl) was added to each well. The samples were incubated at 37°C for 30 minutes. A standard curve consisting of 2000, 1500, 1000, 750, 500, 250, 125 and 25 µg/ml of bovine serum albumin (BSA) in dH_2O was created. The absorbance of the samples was

analysed at 570nm using a spectrophotometer. A graph of absorbance at 570 nm versus concentrations of standards was plotted and protein concentration determined as shown in Figure 2.1.

Table 2.1: Concentration of standards. Standards for comparison of the protein samples were made by adding increasing volumes of the stock protein albumin to decreasing volumes of distilled water.

	Stock/Albumin	Distilled water (µl)	Concentration (µg/ml)
A	0	100	0
B	10	90	200
C	20	80	400
D	30	70	600
E	40	60	800
F	50	50	1000
G	60	40	1200
H	70	30	1400

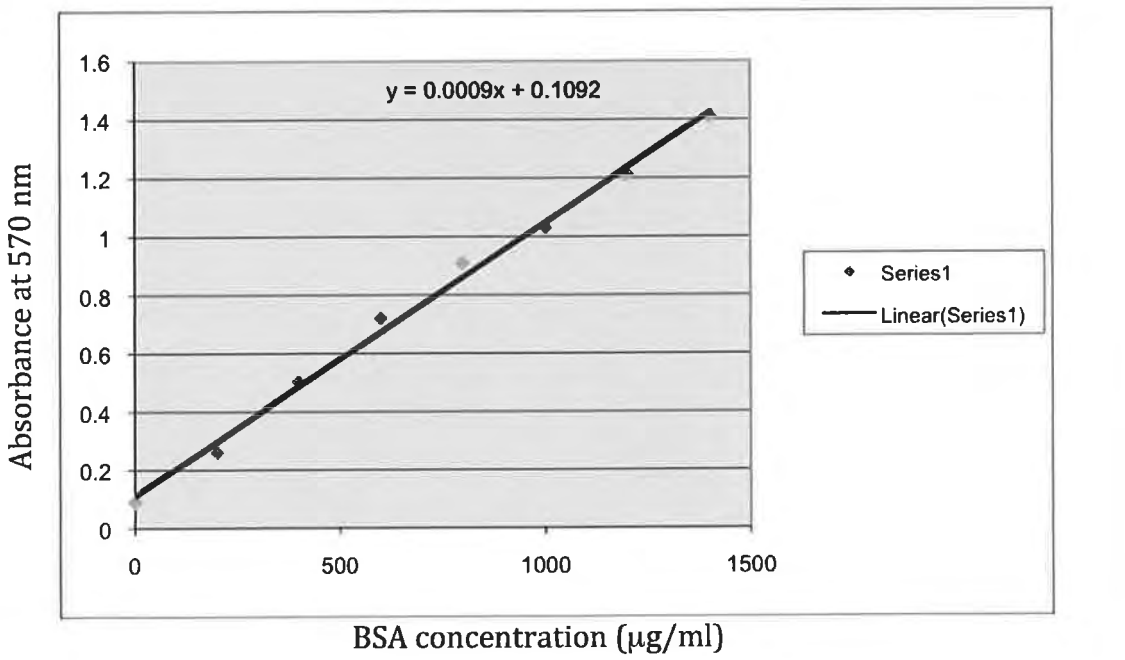


Figure 2.1: Example of a standard curve derived using the BCA protein assay. Concentration of protein calculated in µg/ml.

2.2.3 Concentration of media

Centrifugal filtration allows for concentration of medium that contains dilute secreted protein (MMP-9 is a secreted protein). In the case of the MCF-7 and MCF-7/PEA-3 cell lines, cell culture treatments were performed when the cells were 90% confluent. The culture medium was collected in a 15ml falcon tube. 4ml of sample was added to an Amicon Ultra Filter Unit (Millipore, 30k) and then spun at 400xg for 15 minutes in a centrifuge rotor (Eppendorf Centrifuge 5810R). The concentrated solute was collected into a sterile eppendorf and the protein concentration was determined as shown above.

2.2.4 Poly Acrylamide Gel Electrophoresis (PAGE)

Electrophoresis is one of the most commonly used techniques for molecular separation, which depends upon the charge distribution of the molecules being separated. A detergent SDS (Sodium Dodceyl Sulphate) is added to the protein samples which renders them negatively charged by their attachment to the SDS anions. The charged protein samples are then separated on a polyacrylamide gel in a process known as SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis). Polyacrylamide gels are formed from the polymerisation of two compounds, acrylamide and N, N-methylene-bis-acrylamide which is initiated by the addition of ammonium persulphate and N,N,N,N-tetramethylethylenediamine (TEMED). The separation of molecules within a gel is determined by the relative pore size within the gel, which depends on the amount of acyrlamide in the gel. The total amount of acrylamide in the gel is denoted as a percentage of the total volume (% running gel). As the total amount of the acrylamide decreases, the pore size of the gel increases allowing proteins of larger molecular weight to pass through the pores. Hence while a 10% running gel containing 6.7mls of acrylamide is used for PEA-3, COX-2 and MMP-9 (molecular weights of 62, 72 and 92 kDa respectively) a 6% running gel containing 4mls of acrylamide is used in the case of HER-2 and AIB-1 (molecular weights 185 and 160 kDa). A 12% gel was used for p-ERK (molecular weight 44 kDa). The proteins are immobilized on the gel and transferred onto a nitrocellulose membrane, from which individual proteins can be detected with specific antibodies.

2.2.5 Preparation of gels

Polyacrylamide gels were cast in a Bio-Rad gel system (Bio-Rad laboratories, Hercules CA USA). A 12% gel was used in the case of p-ERK, a 10% running gel was used for PEA-3, COX-2 and MMP-9, and a 6% gel for HER-2 and AIB-1 due to their molecular weights. The composition of the 12%, 10% and the 7% running gel are outlined in Table 2.2.

Table 2.2: Components of 12%, 10% and 6% gels are outlined. The reagent amount (ml) is outlined for the different concentrations.

	12%	10%	6%
Bis:Acrylamide (30%)	8.0	6.7	4.0
1.5 M Tris-HCL (pH 8.8)	5.0	5.0	5.0
Distilled dH_2O	6.6	7.9	10.6
10% SDS	0.2	0.2	0.2
10% APS	0.2	0.2	0.2
TEMED	0.008	0.008	0.16

TEMED (Sigma) and fresh Ammonium Persulphate APS (Sigma) were added immediately before use and the running gel was allowed to polymerise at room temperature for 15 minutes. A film of 70% isopropanol (Sigma) was placed on the running gel during polymerization to ensure level setting of the gel. When the running gel had set the isopropanol was removed and the gel washed with distilled water to remove any remaining reagent. The 4% stacking gel was composed as outlined in Table 2.3.

Table 2.3: Components and volumes (ml) of reagents used for the stacking gel.

	Volume
Bis:Acrylamide (30%)	5.5
1.5 M Tris-HCL (pH 8.8)	1.3
Distilled dH_2O	1.0
20% SDS	0.08
10% APS	0.08
TEMED	0.008

Following pouring of the fresh stacking gel, a comb was placed to form the wells and the gel was allowed to polymerise for 15 minutes. The stacking gel ensures that all of the protein samples loaded begin resolving on the underlying polyacrylamide at the same time when the current is applied.

2.2.6 Loading of samples

Fifty-100µg of protein for PEA-3, p-ERK, COX-2 and MMP-9 and 100-120µg of protein HER-2 and AIB-1 were made up with equal volumes of 2x reducing lamellae buffer (Sigma-Aldrich) and heated to 100^0C for six minutes. The SDS sample buffer acts as an anionic detergent to disrupt nearly all non-covalent interactions in the native proteins in the sample. The samples are heated to ensure full protein denaturation and solubilisation. Five µl of molecular weight markers (Kaleidoscope TM pre-stained standards, Bio-Rad Hercules, CA USA) were loaded directly onto the gel.

The gel was run at 80V for 20 minutes until the samples reached the running gels. Following this the gel was run at 110V for 120 minutes for PEA-3, p-ERK, COX-2 and MMP-9 and at 110V for 180-240 minutes for HER-2 and SRC-3. A running buffer (Appendix II) of 10% Tris/Glycine/SDS Buffer allowed for the even distribution of the electric current.

2.2.7 Semi-dry blotting transfer of proteins

After electrophoresis the gel was removed from the glass plates and the stacking gel was discarded. The proteins on the gel separated by SDS-PAGE were then transferred onto a nitrocellulose membrane using an ATTO semi-dry transfer system. Prior to the transfer procedure, 10-14 pieces of Whatman 3MM filter paper (Whatman International, Maidstone UK), the nitrocellulose membrane (Biorad) and the polyacrylamide gel were all soaked in Transfer Buffer (Appendix II). The semi-dry blotting system was assembled as shown in Figure 2.2. Excess buffer and air bubbles were removed before the apparatus lid was secured into place. Transfer was performed at a constant current of 250 mA for 60-100 minutes for p-ERK, PEA-3, COX-2 and MM-9 and for 90-120 minutes for HER-2 and AIB-1. In order to confirm that protein transfer was successful, the nitrocellulose membrane was incubated with Poinceau S (Sigma) solution for 60 seconds. This marks protein bands red. The Poinceau S solution was washed off with distilled H_2O.

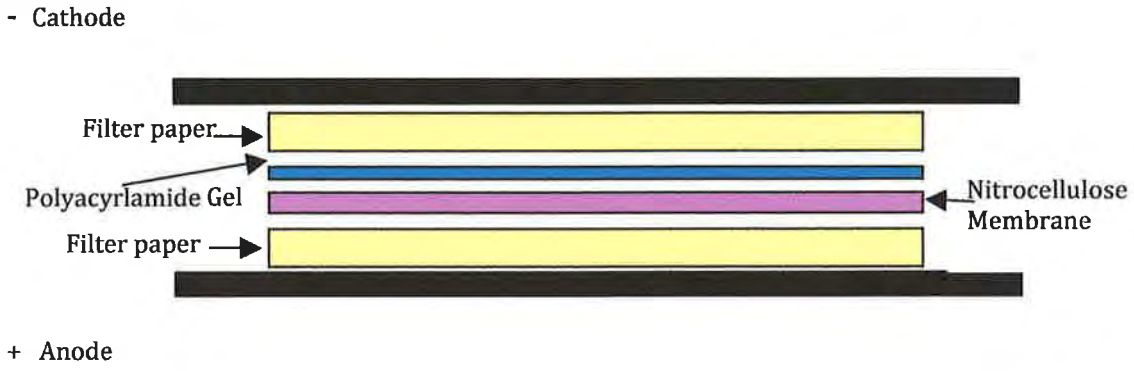


Figure 2.2: Schematic representation for the transfer of a polyacrylamide gel onto nitrocellulose membrane in western blotting.

2.2.8 Detection of Antigen

After confirmation of protein transfer, non-specific binding sites on the membrane were blocked by incubating the membrane with 5% powdered milk (Marvel) in 0.05% Triton X-100/Tris buffered saline (TBS) (Sigma) for 60 minutes with continuous rocking at room temperature. The membrane was subsequently incubated overnight with primary antibody; rabbit antihuman p-ERK (1:1000) (Cell Signaling), mouse anti-human PEA-3 (1:100) (Santa Cruz), rabbit anti-human COX-2 (1:100) (Santa Cruz), mouse anti-human MMP-9 (1:100) (Santa Cruz), rabbit anti-human AIB-1 (1:250) (Santa Cruz), and rabbit anti-human HER-2 (1:100) (Prestige Antibodies) in 5% powdered milk in 0.05% Triton X-100/TBS. The membrane was then washed 3 times for 10 minutes each with 0.05% Triton X-100/TBS prior to incubation with the corresponding HRP (Horseradish Peroxidase) conjugated secondary antibody (Santa Cruz Biotechnology) at a concentration of 1:1000-1:15,000 in 5% powdered milk in 0.05% Triton X-100/TBS.

The membrane was again washed 3 times for 10 minutes each with 0.05% triton X-100/TBS. Protein bands were detected by the addition of a chemiluminescent substrate (Luminol system, Santa Cruz Biotechnology) for the housekeeping gene β-actin, COX-2 and p-ERK or intensified luminescence (Pierce Il, USA) for HER-2, PEA-3, AIB-1, and MMP-9. Light emitted during an enzyme-catalysed decomposition reaction was captured by exposure to Fuji X-ray film for 30 seconds to 20 minutes. The size of the bands was determined using molecular weight markers.

2.3 Immunoprecipitation

Co-immunoprecipitation is a process that allows the examination of a protein-protein interaction. In brief, an antibody directed against a particular antigen is added to the cell lysate forming an antibody-antigen complex along with lysis buffer. A protein A/G agarose complex is added to the lysate, binding the antigen-antibody complex and precipitating the complex out of solution. (Protein A/G is a genetically engineered protein that combines the IgG binding domains of both protein A and Protein G). It is a gene fusion protein expressed in *E.Coli.* It binds to all human IgG subclasses making it a good choice for performing immunoprecipitation experiments). If the second protein of interest does interact with the target protein (antigen) then it will be precipitated out in the complex. After centrifugation, non-specific protein will be removed in the supernatant. The complex is then washed to further remove any non-specific protein binding. The precipitate is then resuspended in SDS sample buffer and run on a SDS-PAGE gel to probe for the second protein of interest in the

complex. Whole cell lysates were isolated from cells under control conditions as described in Section 2.2.2. Cell lysate (1 mg) was immunoprecipitated with anti-SRC-3 for 60 minutes at 4^{0}C with end-over-end rocking. The precipitates were collected for 60 minutes on a protein A/G agarose complex (Santa Cruz Biotechnology Inc). 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 RIPA buffer (Appendix I) and centrifuged at 5,000 rpm for 60 seconds (RIPA buffer is used to lyse cultured mammalian cells that allows the extraction of membrane, nuclear and cytoplasmic proteins). The precipitates were then resuspended in Laemelli SDS sample buffer (Sigma-Aldrich, Germany) and resolved on a 10% SDS-PAGE gel as previously described in Section 2.2.5. Following transfer, the membranes were probed with the corresponding antibody (PEA-3) and conjugated secondary antibody and labelled bands were detected as for standard western blotting.

2.4 Mammalian cell transfection

The ability to introduce nucleic acids into cells has enabled the advancement of our knowledge of genetic regulation and protein function within eukaryotic cells, tissues and organisms. The process of introducing nucleic acids into cells by non-viral methods, such as the DEAE-dextran and calcium phosphate techniques, is defined as "transfection". Progress in transfection technology was relatively slow until the advent of molecular biology techniques for cloning plasmid DNA. These techniques provided the means to prepare and manipulate DNA sequences and the ability to prepare virtually unlimited amounts of

relatively pure DNA for transfection experiments additional methods, such as electroporation and liposome-mediated transfer, were developed to enable more efficient transfer of the nucleic acids to a broad range of cultured mammalian cells. Specially designed cationic lipids, such as Lipofectamine™ 2000 Transfection Reagent (Invitrogen), facilitate DNA and siRNA delivery into cells. The basic structure of cationic lipids consists of a positively charged head group and one or two hydrocarbon chains. The charged head group governs the interaction between the lipid and the phosphate backbone of the nucleic acid, and facilitates DNA condensation. Often cationic lipids are formulated with a neutral co-lipid or helper lipid, followed by extrusion or microfluidisation, which results in a unilamellar liposomal structure with a positive surface charge when formulated in water. The positive surface charge of the liposomes also mediates the interaction of the nucleic acid and the cell membrane, allowing for fusion of the liposome/nucleic acid ("transfection complex") with the negatively charged cell membrane. The transfection complex is thought to enter the cell through endocytosis: liposome complex by forming a membrane bound/intracellular vesicle. Once inside the cell, the complex must escape the endosomal pathway, diffuse through the cytoplasm, and enter the nucleus for gene expression. Cationic lipids are thought to facilitate transfection during the early steps of the process by mediating DNA condensation and DNA/cellular interactions. With cationic lipid reagents, the DNA solution is not deliberately encapsulated within the liposomes, rather, the negatively charged DNA binds spontaneously to the positively charged liposomes, forming DNA-cationic lipid reagent complexes.

2.4.1 Expression vectors

PEA-3 was purchased as Human PRF IOH46096 (Invitrogen). Using Gateway Technology, PEA-3 was cloned into an Invitrogen expression vector pcDNA-DEST47 (Figure 2.3). This was performed by Dr. Aoife Quinn. Gateway Technology uses an efficient and rapid method of cloning without the use of restriction enzymes and ligases. To ensure the PEA-3 insert was intact in the new expression vector, sequencing of the entire insert was performed (AGOWA, Germany).

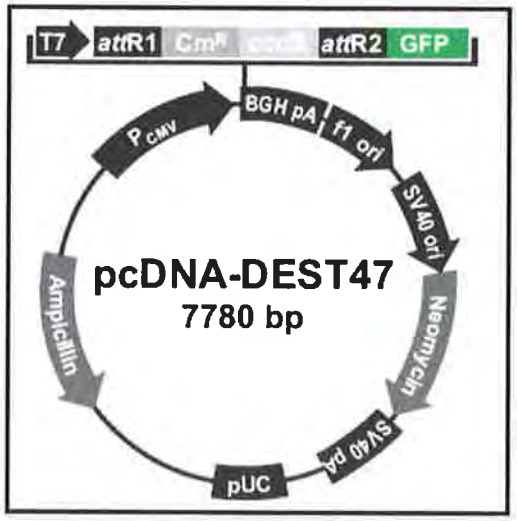


Figure 2.3: Schematic representation of the pcDNA-DEST47 vector (Invitrogen)

2.4.2 Transformation of competent *E.coli* with mammalian expression vector

Transformation is the process in which plasmid DNA is introduced into a bacterial host cell. *E.coli* Top 10 (50 µl) (one shot-Invitrogen) were mixed with the 2µl pcDNA-DEST47-PEA-3. It is important that pipette mixing is not performed at any stage. This mixture is incubated on ice for 30 minutes. Heat shocking at 42 °C for 30 seconds is then performed. The samples are placed back in ice for 1 minute. SOC medium (250 µl) is added to the mixture and then incubated and shaken (250 rpm) at 37 °C, for 1 hour. 200 µl of this reaction is then spread onto Ampicillin resistant LB Agar plates.

2.4.3 Preparation of the LB-Agar plate

LB medium was prepared according to the compositions given in Appendix I. After autoclaving the medium, it was cooled to less than 30 °C and antibiotics were added (see below). LB medium (30 ml) was poured into a standard 90 mm Petri dish and left to cool. Bacterial cells were streaked on LB agar (Appendix I) containing the appropriate concentration of antibiotics and left at 37°C overnight. The antibiotic chosen depended on the resistance of the vector. Ampicillin (50 µg/ml) was added to LB Agar and LB Broth.

2.4.4 Plasmid purification

Plasmid DNA was purified using the Endo-free plasmid maxi kit (Qiagen). The kit yields highly purified endotoxin free DNA. A single colony was picked and inoculated in a starter culture [5 ml of LB medium (I) containing Ampicillin] incubated for 8 hours at 37°C with vigorous shaking (~ 250 rpm), the starter culture was then diluted (1/1000) in a pre-warmed sterile selective LB medium grown at 37°C for 12-16 hours with vigorous shaking (~ 250 rpm). DMSO stocks were made of all culture mixtures (365 µl of cultured broth and 35 µl of DMSO) the stocks were transferred to -80°C immediately. The bacterial cells were harvested by centrifuge at 6000x g in Beckman JS-13 rotator. Cells were re-suspended in buffer P1 (10 ml) (RNAase is added) for bacteriolysis, then of Buffer P2 (10 ml) was gently mixed but thoroughly by inverting 5-6 times and incubated at room temperature for 5 minutes. Chilled buffer P3 (10 ml) was added and mixed with the lysate immediately and gently by inverting 4-6 times. The lysate was then filtered by the QIAfilter cartridge supplied and incubated at room temperature for 10 minutes. Buffer ER (2.5 ml) was added to the lysate and incubated on ice for 30 minutes. The lysate was then allowed to enter the QIAGEN resin by gravity flow. DNA was then eluted with buffer QN after which elute was then collected in endotoxin free polycarbonate centrifuge tube. DNA was then precipitated by adding isopropanol (10 ml) at room temperature. Samples were mixed and centrifuged immediately at 9500 rpm in a Beckman JS-13 rotator for 30 minutes at 4°C. The supernatant was carefully decanted. Pellets were then washed with 5 ml of endotoxin free 70% (v/v) ethanol at room temperature and centrifuged at 2500g for 60 minutes at 4°C. The ethanol

removes the precipitated salts making the DNA easier to re-dissolve. The pellets were air-dried and re-dissolved in nuclease free water (500 µl).

2.4.5 Determination of the DNA yield

An Eppendorf Biophotometer spectrophotometer was used to assess and quantify DNA at wavelengths 260 nm and 280 nm. DNA (1 µl) was mixed with water (49 µl) prior to reading.

2.4.6 Preparing and running standard Agarose DNA gels

Agarose powder (Promega) was mixed with 1x electrophoresis buffer (TAE) at a concentration of 1.5% (0.75 mg Agarose in 50 ml TAE), and then heated in a microwave oven until completely dissolved. Ethidium bromide was added to the gel (final concentration 0.5 µg/ml) to facilitate visualization of DNA after electrophoresis. After cooling the solution to ~25^{0}C, it was poured into a casting tray containing a sample comb and allowed to solidify at room temperature. After the gel has solidified, the comb was removed. The gel, still in its plastic tray, was inserted horizontally into the electrophoresis chamber and covered with buffer. Samples were loaded in the wells. The lid and power leads were attached, and a current was applied at 100 V for 60 minutes. The gel was placed on an ultraviolet transilluminator and the image recorded.

2.4.7 Transient transfection with plasmid DNA protocol

$0.5x10^6$ cells were grown in a 6-well plate 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 eppendorfs as follows: Solution A: 400 ng of pcDNA-Dest47-PEA-3 diluted in 375 µl of reduced serum medium Opti-MEM (Gibco). 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 dropwise fashion and incubated at 37°C for 4 hours after which full medium was added. Protein levels and transcriptional activity were determined 24 hours post transfection. Transfection efficiency was assessed by examining the cells under an inverted fluorescent microscope. Transfected cells appear green (Figure 2.4).

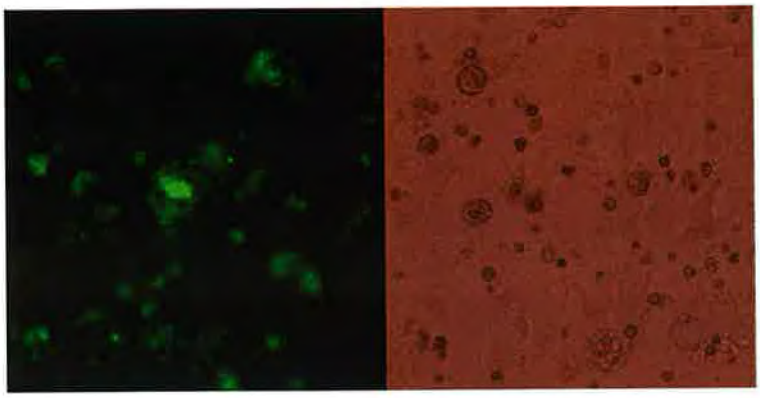


Figure 2.4: **Transfection efficiency**. MCF-7 cells transfected with pcDNA-DEST47-PEA-3. Green cells are cells that have successfully been transfected with the vector. The cells appear green (left) due to the presence of a GFP tag on the vector. The above figure demonstrates transfection efficiency of 72%. Cells were examined 24 hours after transfection. (Images visualised at 20 x magnification using an Olympus 1x51 and FITC filter).

2.4.8 Development of stable cell lines

Stable cell lines were created using the MCF-7 cell line. Cells were transiently transfected with PEA-3 in pcDNA-DEST47. Confirmation of transfection was checked at 24 hours using microscopy for the presence of the GFP tag. Cells were then grown with MEM and Geneticin® (G418 Sulfate) (Invitrogen). Cells were grown with MEM and Geneticin until stable cell growth was achieved. Cells were checked regularly to ensure that they were still over-expressing PEA-3 by western blotting for PEA-3 protein. Colon cancer cells, HCA-7 cells were used as positive controls as they inherently express PEA-3 (Result illustrated in Chapter 3).

2.5 Gene Silencing

2.5.1 Short Interfering RNA (siRNA)

Using RNAi technology, scientists can turn off the genes they want to study, this technique provides an exciting new opportunity to examine the cellular phenotype in the absence of gene expression and to assign gene function and analyze disease. RNA interference mechanisms are a recent technology, and have only been used in mammalian cells since 2001. In mammalian cells the introduction of short sequence-specific RNA duplexes that are 21-23bp long was able to initiate post-transcriptional gene knockdown and avoid triggering non-specific effect (Elbashir et al, 2001; Caplen et al, 2001). The RNAi process occurs in eukaryotes by the cleavage of long double-stranded RNA (dsRNA) into 21-23 nucleotide short (or small) interfering RNA (siRNA) duplexes, facilitated by an enzyme called Dicer. The siRNA associates with an intracellular multi-protein RNA induced silencing complex (RISC). This complex recognizes and cleaves complementary cellular mRNA. The cleaved mRNA is targeted for degradation, ultimately leading to knock down of post-transcriptional gene expression in the cell.

Target sequences for siRNA are identified by scanning the length of the desired gene for amino acid (AA) sequences. The AA and downstream 19 nucleotides are recorded and compared to an appropriate genome data base to eliminate any sequences with significant homology to other genes, those sequences that appear to be specific to the gene of interest are the potential siRNA target sites. The antisense siRNA strand is the reverse complement of the target sequence

(sense strand). The sense strand of the siRNA is the same sequence as the target mRNA sequence except that it will lack the 5' sequence.

2.5.2 AIB-1 siRNA

The AIB-1 siRNA was pre-designed and purchased from Ambion.

2.5.3 siRNA Transfection Protocol

1 x 10^5 cells were grown in antibiotic free media 10% FCS for 24 hour (serum free media was used for stimulation studies) in a 6-well plate. Cells were transfected when 50% confluent.

Oligomer-Lipofectamine 2000 complexes were prepared as follows:

Solution A: 60 pmol of siRNA SRC-3 (Ambion) was diluted in 300µl of Opti-MEM serum reduced media.

Solution B: 5µl of Lipofectamine 2000 was diluted in 300µl Opti-MEM serum reduced media. 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 then 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 either diluted with OPTI-MEM. Gene knockdown reached maximum at 24 hours and did not differ much after 48 and

72 hours transfection. Gene knockdown efficiency was tested at protein level by western blot analysis.

2.6 Breast tissue sample collection

Paraffin embedded breast cancer specimens were obtained following ethical approval and consent of patients with a positive diagnosis of breast cancer. Samples were sourced from the Departments of Histopathology at Beaumont Hospital and St Vincent's University Hospital, Dublin, Ireland. Only patients who were free of metastasis at the time of surgery were included. Dr. Tony Stafford constructed a Tissue Microarray (TMA) using 560 patients' breast cancer samples. Clinico-pathological details and 10 year follow up data was collected on all patients on the TMA with 560 samples. These microarrays were utilized to determine expression levels of HER-2, p-ERK, AIB-1, PEA-3, COX-2 and MMP-9. These results were compared with known patient and histological parameters.

2.7 Clinico-pathological parameters

Variables analysed include tumour size, tumour grade, tumour stage, estrogen receptor status, Her-2/neu receptor status (Appendix II). Histological grading was performed using the Elston-modified Scarff-Bloom-Richardson system (Elston et al, 1991). All patients underwent total or segmental mastectomy with level I, II and III axillary dissection. Tumours were localised initially by the pathologist on the haematoxylin and eosin stained tumour slides. Time to

disease progression (TDP) was defined as the time from the initiation of treatment until the disease progressed or death.

2.8 Immunohistochemistry

Immunohistochemistry is the localization of antigens in tissue sections by the use of labelled antibodies as specific reagents through antigen-antibody interactions that are visualized by a marker such as an enzyme or a fluorescent label. An unlabelled primary antibody is incubated on the tissue section, binding the antigen of interest. A biotinylated secondary antibody directed against the primary antibody is then applied. A streptavidin-biotin complex (ABC) which possesses biotin binding sites is then added and cross reacts with the biotin molecules on the secondary antibody, amplifying the signal intensity.

2.8.1 Staining protocol

Five micron thick tissue sections were cut from paraffin embedded breast microarray tissue blocks and mounted on Superfrost Plus slides (BDH, Poole, UK). Sections were de-waxed by passage through containers of xylene (BDH) for 5 minutes each and rehydrated by immersion in industrial methylated spirits (IMS) (Lennox Chemicals, Dublin, Ireland) of decreasing concentrations (100%, 90%, 70%) for three minutes in each container. The sections were then washed in PBS (Sigma-Aldrich, Steinheim Germany) for 5 minutes. A liquid-repellent pen (Dako, Denmark) was used to mark out the tissue on the slide. Endogenous peroxidase activity was blocked using 3% hydrogen peroxidase

(BDH) in PBS for 20 minutes. Antigen retrieval was performed by immersing sections in 0.01 M sodium citrate buffer (Sigma-Aldrich) pH 6.0 and microwaving on high power for 7 minutes, then on low-medium power for 5 minutes. Sections were blocked in the appropriate serum (Sigma-Aldrich) for 90 minutes. All the primary antibodies were diluted in PBS. Sections were incubated with primary antibodies; rabbit anti AIB-1 (3μg/ml) and mouse anti PEA-3 (50μg/ml) (Santa Cruz Biotechnology, CA USA), rabbit anti COX-2 (8 μg/ml) (Cayman, USA), mouse anti MMP-9 (4 μg/ml) (Chemicon, Temecula, CA) antibodies and rabbit anti phospho-p44//42 MAPK (Thr202/Tyr204) (Cell Signalling Tech, MA, USA) for 60 minutes at room temperature. Sections were subsequently incubated with the corresponding biotin-labelled secondary, anti-rabbit antibody and anti human mouse antibody in 1 in 2000 ml PBS containing 1% corresponding blocking serum (Vector Laboratories, Burlingame, CA USA) for 30 minutes, followed by peroxidase-labelled avidin biotin complex (Vector Laboratories, Burlingame, CA USA).

Sections were developed in 3, 3-diaminobenzidine tetrahydrochloride (DAB) for 2 minutes and counterstained with haematoxylin (Sigma-Aldrich) for 3 minutes. Negative controls were performed using matched IgG controls (Santa Cruz Biotechnology, CA USA) and omission of the primary antibody. Sections were then passed through increasing concentrations of IMS (70%, 90% and 100%) for 5 minutes and then xylene for 10 minutes. Cover slips (BDH) were then applied to the sections with DPX mountant (BDH).

2.8.2 Immunohistochemistry scoring system

Immunostained slides were scored for p-ERK, AIB-1, PEA-3, COX-2 and MMP-9 using the Allred scoring system (Allred DC et al, 1993; Harvey et al, 1999). While this scoring system was initially described for staining of ER and nuclear markers, it has been subsequently applied to scoring of non-nuclear proteins (Dillon et al, 2008; Qin et al, 2008; Mack et al, 1997; Redmond et al, 2009; Henriksen et al, 2007). In brief, each entire slide was evaluated using light microscopy. First, a proportion score was assigned, which represented the estimated portion of positively stained tumour cells (none=0, <1%=1, >1%<10%=2, >10%<33%=3, >33%<66%=4, >66%=5). Next 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. 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. HER2 status was evaluated using the DAKO (Glostrup, Denmark) HerceptTest immunocytochemical assay. Scoring was assessed in accordance with the manufacturer's instructions.

2.9 Immunofluorescent microscopy

Immunofluorescent microscopy was carried out using the same procedure as that used for immunohistochemistry with a secondary fluorochrome conjugated antibody instead of a biotinylated antibody. Sections were incubated in goat

serum for 60 minutes. Mouse antihuman PEA-3 (50μg/ml in 10% human serum) was placed on each slide for 90 minutes. The sections were washed in PBS and incubated with goat anti-mouse secondary fluorochrome conjugated antibody for Ets-2 rabbit anti-goat antibody (TRITC labelled fluorescent antibody which emits a red signal when positive at 570nm) (1 in 100 in PBS) (Sigma-Aldrich) for 60 minutes. The slides were washed in PBS for 5 minutes and blocked in goat serum for 90 minutes. Each slide was incubated with rabbit anti-human AIB-1 (10μg/ml in 10% human serum) for 90 minutes, followed by a PBS wash for 5 minutes. The slides were incubated in the corresponding fluorochrome conjugated rabbit anti-human secondary antibody (a FITC labelled secondary antibody which emits a green signal when positive at 525nm) (1 in 100 in PBS) (Sigma-Aldrich) for 60 minutes. Sections were rinsed in PBS and mounted using fluorescent mounting media (Dako). Negative controls were performed using matched IgG (Dako) and omission of the primary antibody.

2.10 Statistical analysis

STATA version 9 statistical program (STATA/SE 10 Educational Lab, Texas USA) was used in the statistical analysis. Univariate analysis was performed using Fisher's exact test for categorical variables and Wilcoxon's test for continuous variables. Multivariate analysis was carried out using Cox's proportional hazard model. A *p*-value of less than 0.05 was considered significant. Survival times between groups were compared using the Wilcoxon test adjusted for censored values.

2.11 Wound healing assay

Cells were seeded in a 6-well plate until reaching confluency. A wound was then created by using a 10μl pipette tip on the cell monolayers. Photographs were taken at 0 and at 24 hours.

2.12 3D cell culture

Three-dimensional culture systems have been shown to provide an important tool to interrogate how cancer genes influence glandular architecture as well as model the early changes involved in the process of carcinogenesis (Debnath et al, 2003). 3D culture of MCF-10A, MCF-7, MCF-7/PEA-3 and SKBR-3 cells was performed. Chamber slides were coated with 100% Matrigel (BD Biosciences) Cells were diluted to a final concentration of 2.5-7.5x10^4 cells/ml. 200μl of the cell suspension and 200μl of the appropriate media and 4% of Matrigel mix were added to each well. Appropriate media for each cell line (Section 2.1.1) and 2% Matrigel was prepared and the medium was changed every four days. Cells were cultured for 14-18 days and then fixed with methanol and acetone (1:1) at -20°C for 20 minutes and blocked in PBS. Figure 2.6 illustrates the method for 3D cell culture.

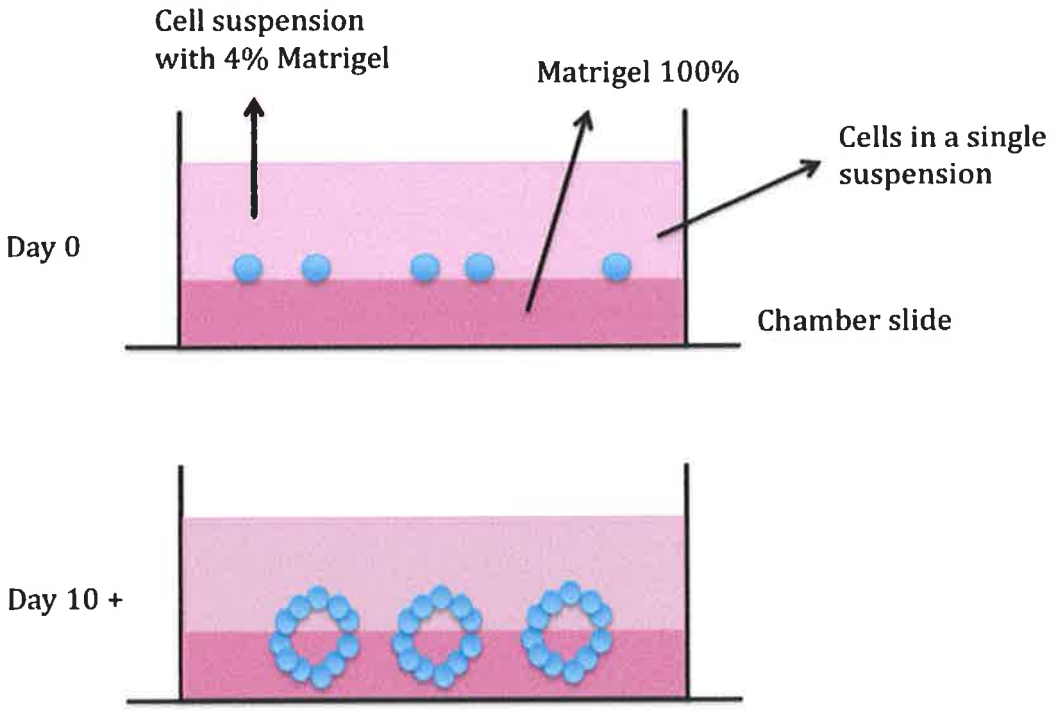


Figure 2.6: Schematic representation for the method of 3D cell culture on Matrigel. The well of the chamber slide is initially coated with 100% Matrigel and allowed to solidify. Cells are seeded onto this bed as a single suspension in appropriate medium and 4% Matrigel. The medium is replaced every 4 days. Cells proliferate, form clusters and subsequently form acini. (Debnath et al, 2003)

For immunoflorescence, cells were permeabilised with 0.5% triton in PBS for 10 minutes at 4^0C. Cells were then incubated in the primary antibodies against AIB-1 and PEA-3 overnight at 4^0C. Cells were subsequently incubated with fluorescent conjugated secondary antibodies (Molecular Probes, Invitrogen) at room temperature for 60 minutes (including Phalloidin conjugated antibody). After counterstaining with DAPI, the slides were mounted after adding anti-fade solution (Dako). Slides were examined by confocal microscopy.

Chapter III

PEA-3 promotes breast cancer cell progression through the up-regulation of HER-2, p-ERK, COX-2 and MMP-9

3.1 Introduction

Tumour invasion in the surrounding tissues and metastasis to distant organs is a major characteristic of malignant cells. Tumour cells must be able to pass through the extra-cellular matrix of stroma and vascular tissue in order to metastasise. In addition, tumour cells must escape normal cellular processes such as apoptosis and regulated proliferation.

The invasive potential of cancer cells is mediated in part through the action of Cyclooxygenase-2 (COX-2) and Matrix Metalloproteinases (MMP). COX-2 is the rate-limiting enzyme involved in the production of prostaglandins. Over-expression of COX-2 has been shown to contribute to cell proliferation, inhibition of apoptosis, and the promotion of angiogenesis (Sheng et al, 2001; Tsuji et al, 1998; Dohadwala, 2001). Furthermore, studies have demonstrated that increased production of prostaglandins stimulates the expression of the aromatase enzyme, resulting in further treatment challenges (Davies et al, 2002). A link has been established between HER-2 over-expression and the up-regulation of COX-2 in human breast cancer (Subbaramaiah et al, 2002). Inhibitors of COX-2 have been shown to reduce the risk of developing breast cancer (Singh-Ranger et al, 2002). They may act as chemo-preventative as well as chemo-therapeutic agents. Their use in combination therapy with hormonal agents, such as aromatase inhibitors, can decrease angiogenesis and aromatisation (Bundred et al, 2005). In addition, their use in conjunction with tyrosine kinase inhibitors can act synergistically to inhibit the HER-2-ras-raf pathway (Bundred et al, 2005). Unfortunately, due to documented cardio-

toxicity, the use of COX-2 inhibitors has been put to question, and some studies elucidating their efficacy and use have been halted (Singh-Ranger et al, 2002, Dang et al, 2004). However, further clinical trials on this subject will clarify the safety profile of this class of drugs.

Matrix Metalloproteinases, on the other hand, are involved in degrading the extracellular matrix as well as inducing angiogenesis, steps crucial in tumour progression. Evidence suggests that certain MMPs, such as MMP-2 and MMP-9, play a role in breast cancer initiation and growth (Press et al, 1997; Talvensaari-Mattila et al, 2001). Their expression has been associated with grade and stage of breast cancer, and their elevated serum levels have been detected in patients with metastatic disease (Zucker et al, 1993). MMP inhibition has been shown to decrease metastasis and tumour growth in mouse xenograft models (Sledge et al, 1995). Several inhibitors have been developed and entered into clinical trials. Unfortunately, their broad spectrum of use, failure to demonstrate an acceptable response and musculoskeletal toxicity, has prevented their use thus far (Miller et al, 2002). Therefore, an understanding of the signalling pathways involved in the production of both COX-2 and MMP-9 are essential in order to devise new targeted therapies.

The promoter region of genes coding for COX-2 and MMP-9 has been shown to contain an Ets binding domain, which is the binding site for Ets transcription factors (Howe et al, 2001; Gum et al, 1996). PEA-3 is a member of the Ets-related transcription factors, which consists of three members, PEA-3 (also known as E1AF or ETV4), ER81/ETV1, and ERM1/ETV5 (Xin et al, 1992; Brown et al,

1992; Monte et al, 1994). The human PEA3 gene is transcriptionally up-regulated in breast tumour cell lines and in 93% of HER-2/neu positive human breast tumours (Launoit et al, 2000). PEA-3 has been implicated in a number of biological processes including cell proliferation, remodelling, and differentiation. Our group has previously shown a positive role for PEA-3 in HER-2 mediated breast tumour progression, with its up-regulated expression correlating with tumour grade, nodal status, and disease free survival (Myers et al, 2006; Fleming et al, 2004).

In this part of the study, the Ets protein PEA-3 was stably transfected into the MCF-7 cell line. MCF-7 cells are non-invasive ER positive breast cancer cells. These cells inherently do not express PEA-3, therefore, creating a stable cell line over-expressing PEA-3 allows for investigating the functional importance of this Ets transcription factor in tumor progression and invasion. The resultant changes in cell morphology were monitored with the development of the new cell line, MCF-7/PEA-3. In addition, the production of the target proteins of interest COX-2 and MMP-9 was investigated. Furthermore, the role of PEA-3 in up-regulating the expression of HER-2 and phospho-ERK 1/2 was explored. The effect of up-regulating the growth factor pathway through treatment with epidermal growth factor (EGF) was studied in relation to the production of the target proteins.

3.2 Results

3.2.1 Forced expression of PEA-3 in MCF-7 breast cancer cells

To examine the role of the Ets transcription factor PEA-3 in tumour invasion and in the production of target genes involved in this process, the non-invasive MCF-7 breast cancer cells were transfected with a PEA-3 expression vector in order to create a cell line over-expressing PEA-3. MCF-7 are non-invasive, estrogen receptor positive breast cancer cells. These cells inherently do not express the Ets transcription factor PEA-3. Here, the expression levels in both cell lines were determined using Western Blotting analysis. As shown in Figure 3.1, the expression of PEA-3 was demonstrated in the MCF-7/PEA-3 cell line, compared to no expression in the parental MCF-7 cells. MCF-7/PEA-3 cells were used for experiments reported in Chapter 3 and Chapter 4.

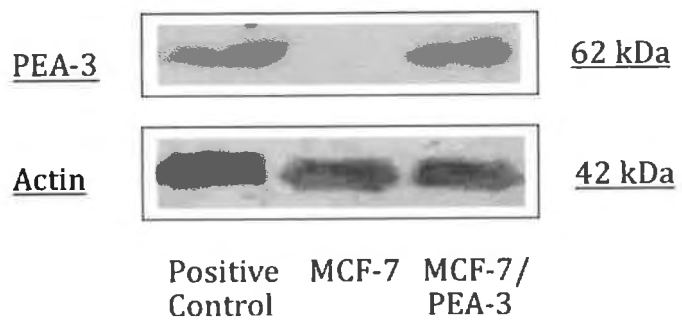


Figure 3.1: Western blot analysis of PEA-3 expression in protein lysates from positive controls (HCA-7 colon cancer cells), parental MCF-7 cells, and MCF-7/PEA-3 transfectants. As demonstrated, PEA-3 was observed in the PEA-3 transfectants and the positive control, compared to no expression of PEA-3 in MCF-7 breast cancer cells.

3.2.2 EGF upregulates PEA-3 expression in MCF-7/PEA-3 cells

PEA-3 expression is mainly regulated by Ras-dependant mitogen activated protein kinase (MAPK) pathways (O'Hagan et al 1996). Activation of the MAPK cascades could occur by upstream receptors such as the tyrosine kinase ErbB2 receptor and growth factor ligands. To assess the effects of an up-regulated MAPK pathway on the expression of PEA-3, cells from the MCF-7 and MCF-7/PEA-3 cell lines were treated with epidermal growth factor (EGF). An elevated expression of PEA-3 was noted in treated compared to untreated cells (Figure 3.2). This compared to no production of PEA-3 in the parental cell line, as shown previously. In addition, an active MAPK pathway did not induce an increased expression of the Ets transcription factor in the MCF-7 cells treated with EGF.

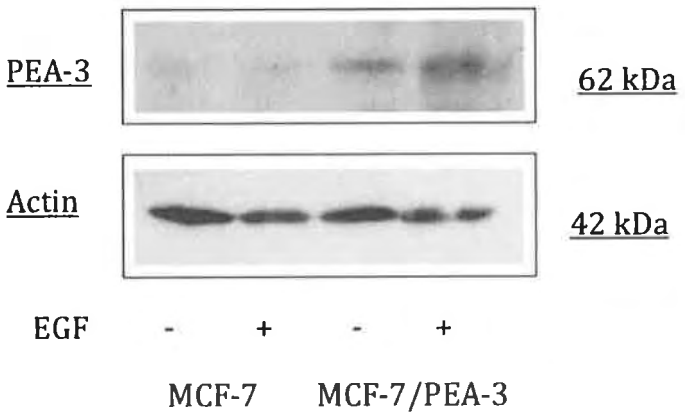


Figure 3.2: Western blotting analysis of PEA-3 expression in MCF-7 and MCF-7/PEA-3 cells. MCF-7 parental cells and MCF-7/PEA-3 cells were treated with vehicle control (-) and 10ng of EGF (+). Cells were harvested at 24 hours post treatment. Treatment of MCF-7 cells with EGF showed no increase in PEA-3 expression, however, treatment in MCF-7/PEA3 cells resulted in elevated expression of PEA-3 compared to untreated cells.

3.2.3 Morphology of PEA-3 transfected cells (MCF-7/PEA-3)

Tumour progression is associated with the ability of cells to grow independently of growth signals, lose apoptotic sensitivity, increase proliferation, develop angiogenesis, and finally an ability to invade and metastasize (Hanahan et al, 2000). These events necessitate a loss of cell-to-cell contact and motility in order to invade the surrounding tissues.

The expression of PEA-3 has been associated with poor outcome in breast cancer patients. Here we demonstrate morphological changes associated with transfection of PEA-3 into MCF-7 cells. MCF-7 cells are morphologically round and found to be growing in clusters. These cells demonstrate close cell-to-cell contact. MCF-7/PEA-3 cells, on the other hand, appeared to develop pseudopodial-like protrusions with loose cell-to-cell contact (Figure 3.3). This feature would suggest enhanced cell motility, and therefore, enhanced invasion capacity.

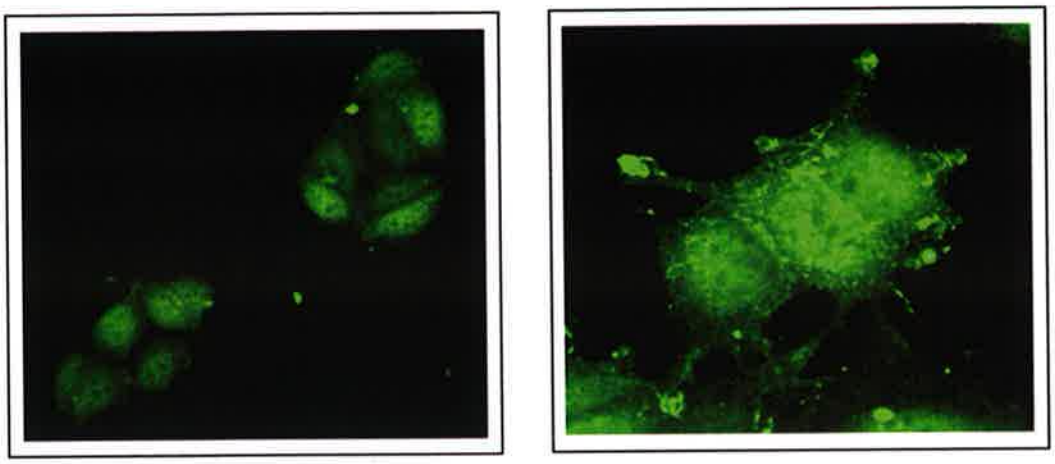


Figure 3.3: Immunofluorescence staining of MCF-7 parental cells and MCF-7/PEA-3 cells with an actin primary antibody. Morphologically, MCF-7 cells (left) were noted to be round, growing in organised clusters, while PEA-3 transfectants (right) appeared to develop pseudopodia, which suggests increased cell motility.

The 3D cell culture of MCF-7, MCF-10A, MCF-7/PEA-3 and SKBR-3 cells was also performed as described in Section 2.14. Cells were grown over a 14 day period. Over the two week period MCF-10A and MCF-7 cells appeared to develop mammary epithelial spheres with acini. MCF-10A cells are typically used as a normal control in the study of breast cancer. These cells are similar to normal human breast epithelial cells, and would form spheres when grown in 3D culture conditions. MCF-7/PEA-3 cells, on the other hand, proliferated into undifferentiated structures (Figure 3.4). SKBR-3 cells are also shown in Figure 3.4. These cells are highly invasive and express HER-2. In this part of the study, these invasive cells were used to studyand demonstrate the contrast in cellular stucture of phenotypically invasive cells. These results indicate that MCF-7/PEA-3 cell proliferate into less differentiated and organized structures, and would therefore support the hypothesis that PEA-3 is required for mammary tumour cells to progress into malignant growths.

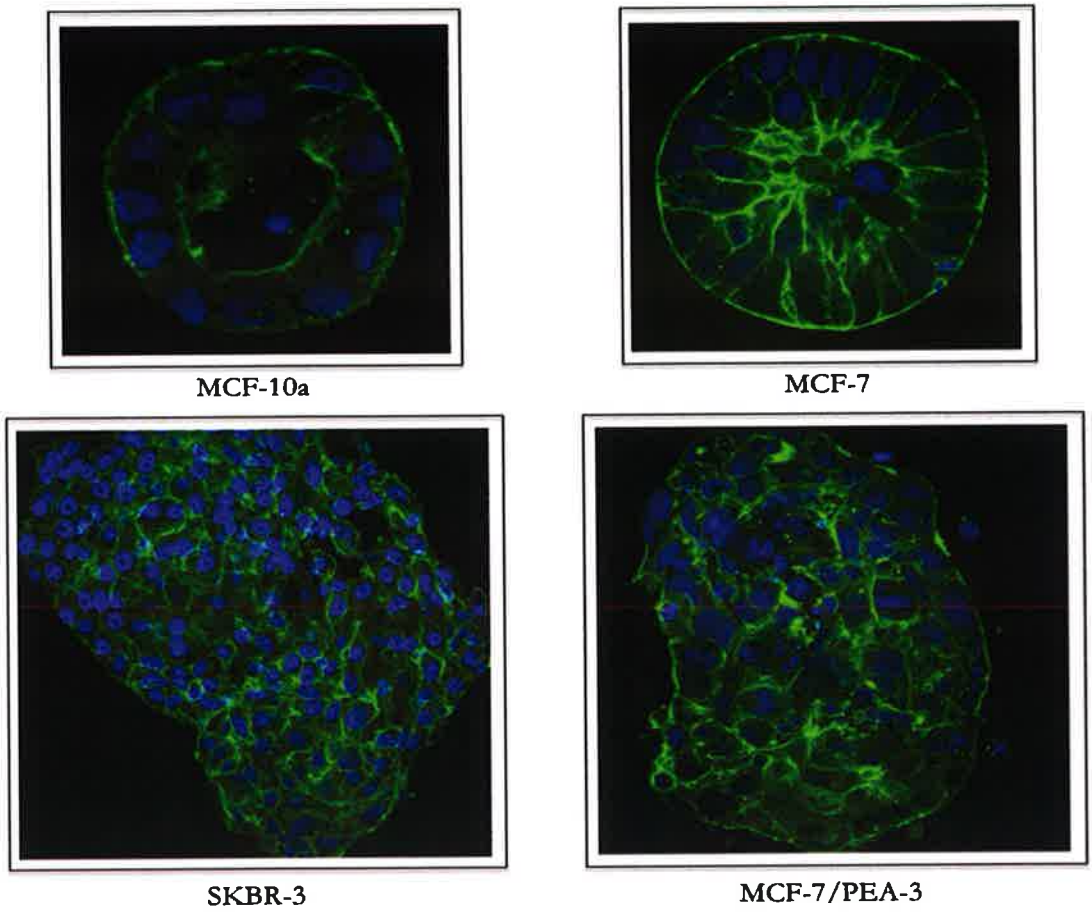


Figure 3.4: Morphological appearance of MCF-7, MCF-10A, and MCF-7/PEA-3 and SKBR3 cells in 3D cultures. Cells were cultured for 14 days and immunofluorescent staining was performed using an antibody against F-Actin. Cell nuclei were visualised by DAPI staining. Fluorescent-labelled 3D cell cultures were examined and imaged under a confocal microscope. MCF-10A and MCF-7 cells appeared to develop into well differentiated and organised structures, while MCF-7/PEA-3 and SKBR-3 cells demonstrated a loss of this cellular organisation, which is attributed to their invasive nature.

3.2.4 PEA-3 is required for cell migration

The role of PEA-3 in enhancing cell motility and promoting migration was explored. A wound healing assay showed that, 24 hours after a wound was made on the monolayer of cells, the PEA-3 transfectants, MCF-7/PEA-3,

demonstrated cell migration into the denuded area in several areas of the wound, in comparison to parental MCF-7 cells which displayed no cell migration (Figure 3.5). MDA-MB231 cells were used as a positive control, as they show a higher degree of invasion and motility.

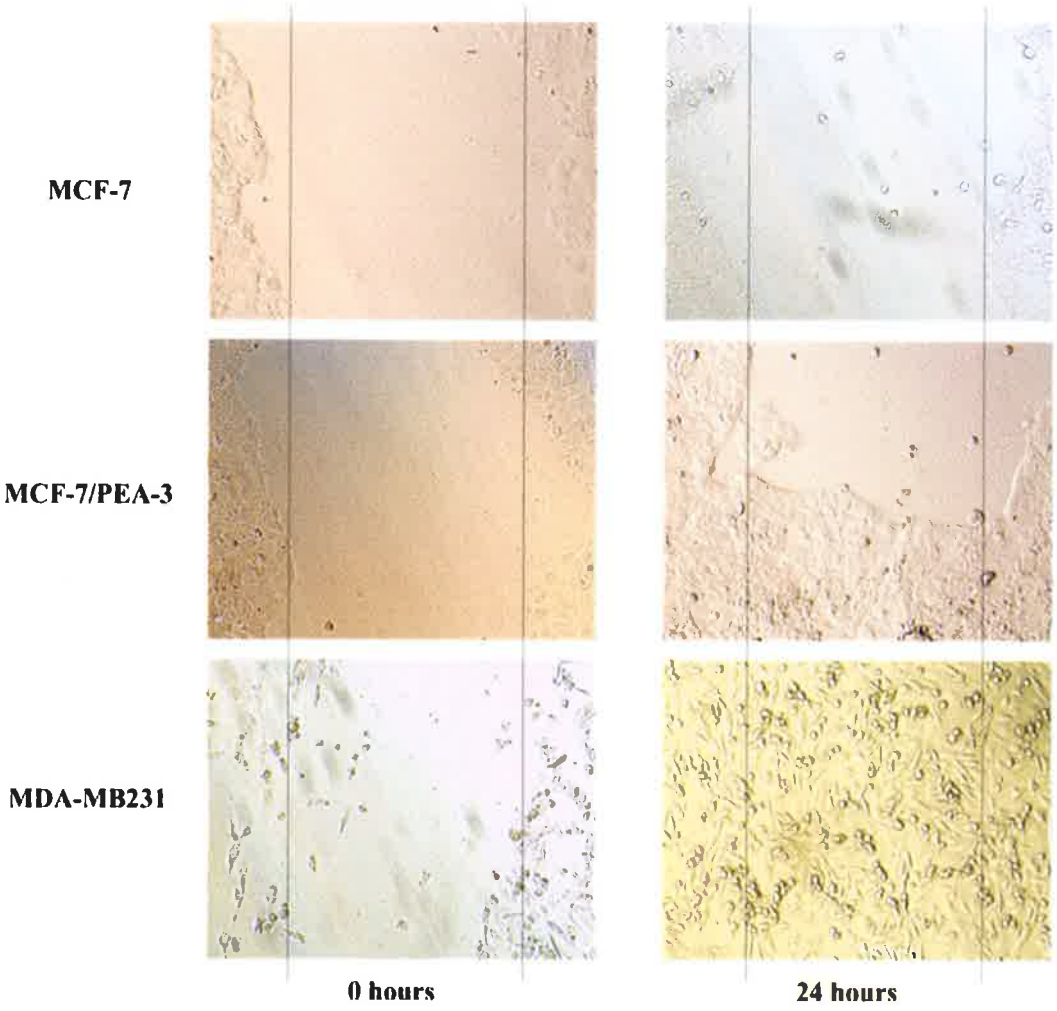


Figure 3.5: Wound healing assay with MCF-7 controls, MCF-7/PEA-3 cells and MDA-MB231 cells. Cells were examined 24 hours after a wound was created in the cell monolayer. MDA MB231 cells were used as a positive control. MCF-7/PEA-3 cells showed migration into the wound assay after 24 hours, compared to no migration demonstrated in the MCF-7 cell line. Vertical lines represent the approximate site of the wound edges for each of the cell lines.

3.2.5 PEA-3 regulates HER-2 and phospho-ERK expression in MCF-7 breast cancer cells

Activated MAP kinases have been shown to directly phosphorylate and elevate the activity of a subset of nuclear transcription factors including members of the Jun, Fos, and Ets families (Treisman et al, 1996; O'Hagan et al, 1997). PEA-3 has been reported to be expressed in around 93% of HER-2 positive breast tumours (Benz et al, 1997). Data suggests that the PEA-3 protein can itself regulate the transcription of the PEA-3 gene by binding to sites on the PEA-3 promoter (Benz et al, 1997). In addition, PEA-3 has been found to transcriptionally up-regulate the expression of HER-2 (Sheppard et al, 2001, Myers et al, 2006). Therefore, we investigated the functional importance of PEA-3 in the production of HER-2. Western blotting analysis and immunoflorescent studies were used to assess the expression HER-2 in MCF-7/PEA-3 cells. PEA-3 was found to up-regulate the expression of HER-2 (Figures 3.6 and 3.7).

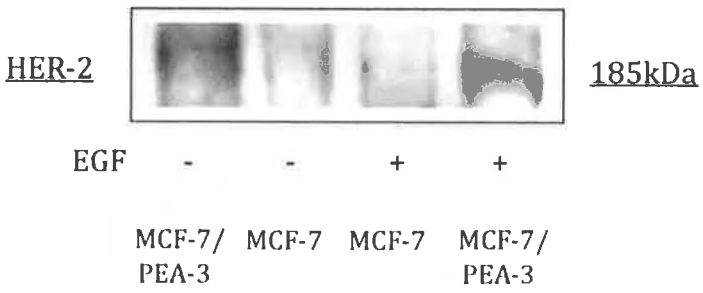


Figure 3.6: Western blot analysis of HER-2 expression in protein lysates from parental MCF-7 cells and MCF-7/PEA-3 transfectants treated with vehicle control (-) and 10ng of EGF (+). As demonstrated, HER-2 was expressed in the PEA-3 transfectants treated with EGF, compared to no expression of HER-2 in parental MCF-7 breast cancer cells.

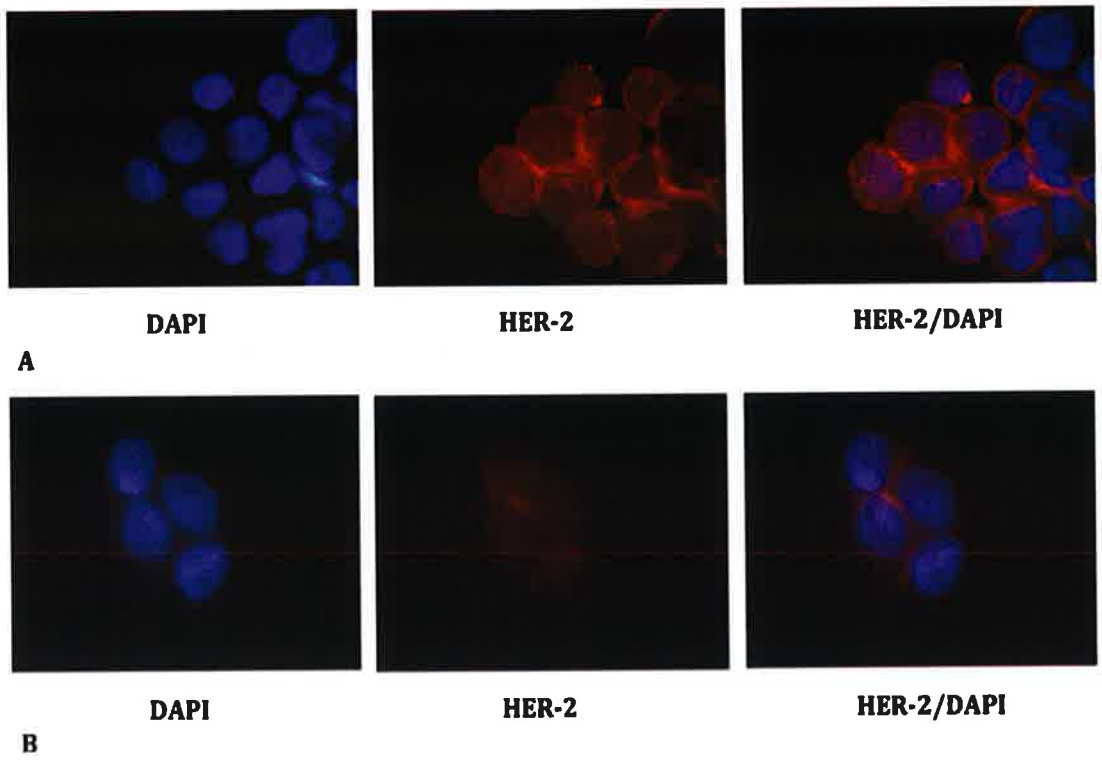


Figure 3.7: Immunofluorescent staining of HER-2. Staining was performed using an antibody against HER-2. Cell nuclei were visualised by DAPI staining. As demonstrated, HER-2 staining was more abundant in MCF-7/PEA-3 cells (A) compared to MCF-7 cells (B).

Cell proliferation and differentiation has been shown to be regulated by the activation of intracellular protein serine/threonine kinases, also named mitogen-activated protein kinases (MAPKs) and extracellular signal-regulated kinases (ERKs) (Cobb and Goldsmith, 1995; Reddy et al, 1999). In order to assess the activity of the downstream effectors of the MAPK pathway in our study cell lines, the expression of phospho-ERK was assessed. As shown above, results demonstrated an up-regulation in the expression of HER-2 in the PEA-3 positive cell line. Therefore, it is predicted that this would facilitate enhanced

signalling through this pathway. Figure 3.8 illustrates the expression of p-ERK in the MCF-7/PEA-3 cell line, suggesting increased activity through the growth factor signalling pathway.

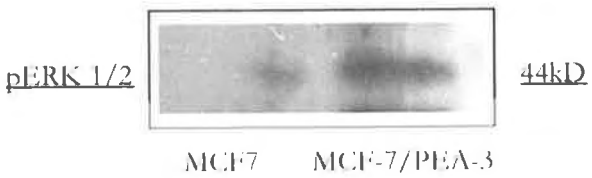


Figure 3.8: Western blot analysis of p-ERK expression in protein lysates from parental MCF-7 cells and MCF-7/PEA-3 transfectants. As demonstrated, p-ERK was expressed in the PEA-3 transfectants, compared to no expression of p-ERK in parental MCF-7 breast cancer cells.

3.2.6 Induced expression of COX-2 and MMP-9 in PEA-3 transfectants

The expression of the two target genes COX-2 and MMP-9 was examined in MCF-7 and MCF-7/PEA-3 breast cancer cells to investigate the functional role of the transcription factor, PEA-3, in this signalling pathway leading to their production. Western blotting analysis revealed an elevated expression of both target proteins in the transfected cells only, supporting a role for PEA-3 in promoting a more invasive cellular phenotype (Figure 3.9).

Furthermore, an activated MAPK pathway, which was found to up-regulate the expression of PEA-3 as demonstrated above, is also expected to result in increased production of COX-2 and MMP-9. The two cell lines, MCF-7 and MCF-7/PEA-3 were treated with EGF, and the expression of the target proteins was

probed for using Western Blotting. As shown in figure 3.9, EGF resulted in increased COX-2 and MMP-9 in treated compared to untreated cells. These results support a role for an active MAPK pathway in up-regulating the expression of PEA-3, which mediates further production of target proteins involved in degrading the ECM and in promoting angiogenesis.

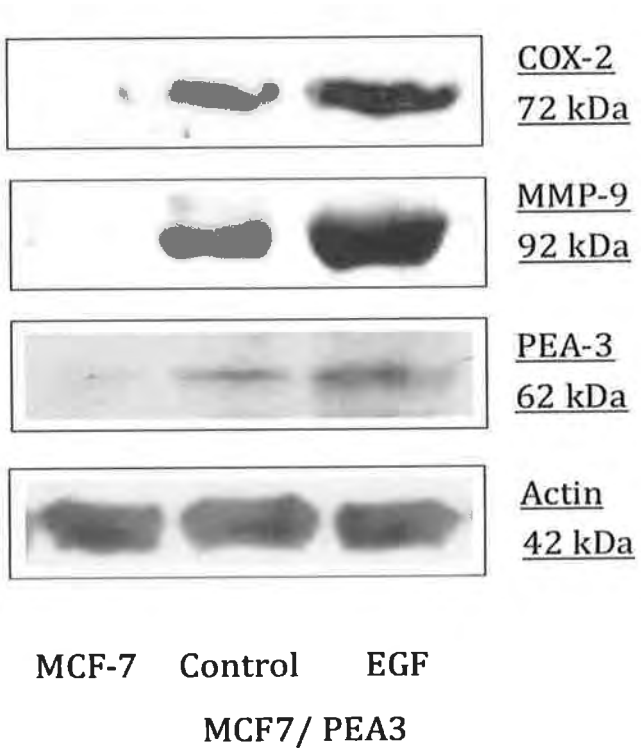


Figure 3.9: The expression of COX-2 and MMP-9 in MCF-7 and MCF-7/PEA-3 cells using Western Blotting analysis. Cell lysates were used to probe for COX-2 and concentrated conditioned media was used to probe for MMP-9. Expression of both COX-2 and MMP-9 was upregulated in the PEA-3 transfectants. Cell lines were then treated with vehicle control and 10ng of EGF, showing an increased expression of COX-2 and MMP-9 in treated cells.

3.3 Discussion

Tumour metastasis is a result of numerous changes in gene expression and cellular behaviour. An understanding of these pathways leading to tumour progression will aid in the development of novel treatments and in the prevention of tumour spread.

MMP-9 degrades type IV collagen, which is a major component of the basement membrane. The enzymatic activity has been shown to be elevated in metastatic tumour cells (Sato et al 1992, Bernhard et al 1994). In addition to the capability of escaping from the tight molecular structure of normal cellular growth, tumour cells undergo transformation processes of the surrounding microenvironment. Over-expression of COX-2 is known to correlate with aggressive and invasive potential of tumour cells (Basu et al, 2006; Ristimaki et al, 2002). One of the mechanisms by which COX-2 modulates this process is angiogenesis. This occurs through the production of pro-angiogenic factors (Barnes et al, 2004).

Ets family members have been shown to be expressed at mRNA level both in primary human breast cancer and in breast cancer cell lines, and their expression has been associated with disease progression and metastasis (Span et al, 2002; and Myers et al, 2005). Activation of these transcription factors by MAPK dependant pathways, in breast cancer cell lines, has been shown to lead to a persistent expression of tumour related genes (Benz et al, 1997). Putative

Ets target genes have been identified based largely on the finding of Ets binding domains in their upstream transcriptional regulatory regions.

Our investigations here assess the importance of the Ets transcription factor, PEA-3 in promoting alterations is cell morphology, growth, and in the production of target proteins which enhance tumour cell progression. We found that PEA-3 mediated the development of alterations in cell morphology. Breast cancer cells over-expressing PEA-3 were also found to have an increased expression of the target proteins COX-2 and MMP-9. PEA-3 expression was also noted to result in increased expression of HER-2 and p-ERK, thereby, suggesting activity through the growth factor pathway. As PEA-3 is a target in the MAPK pathway, it was found that inducing the activation of this pathway through EGF treatment results in up-regulated expression of not only PEA-3 itself, by creating a positive feedback loop, but also in its target proteins COX-2 and MMP-9. These results support a role for PEA-3 in promoting tumour cell progression.

In conclusion, PEA-3 is an important mediator in the activation of COX-2 and MMP-9 promoters. It has also been suggested to be a marker of tumour cells with a higher metastatic potential. In this part of the study we demonstrated a functional role for PEA-3 in the production of HER-2, p-ERK, COX-2 and MMP-9 in breast cancer cell lines. We also observed a significant increase in the production of PEA-3 and its target genes with the up-regulation of the MAPK pathway. An alteration in tumour cell morphology was also noted in association with PEA-3 expression.

Chapter IV

The role of AIB-1 in PEA-3 mediated production of COX-2 and MMP-9

4.1 Introduction

Amplified in breast cancer (AIB-1) is a member of the p160 family of steroid receptor co-regulators (SRC), which also includes SRC-1 and SRC-2 (TIF2, GRIP1) (Anzick et al, 1997; Onate et al, 1995; Tilli et al, 2005; Xu et al, 2003). AIB-1, also known as SRC-3/ACTR/NCOA-3, is an oncogene initially identified in an amplified chromosomal sequence in the 20q11-12 region in breast cancer cells. Not only has AIB-1 been identified in breast cancer, but it has also been localized in prostate cancer, ovarian cancer, pancreatic and gastric cancers (Anzick et al, 1997; Sakakura et al, 2000; Ghadimi et al, 1999). Around 5-10% of human breast cancers are found to have an amplified AIB-1 gene (Anzick et al, 1997; Bautista et al, 1998; List et al, 2001). Furthermore, its mRNA and protein are over-expressed in approximately 30% of tumours. In addition, AIB-1 over-expression has been associated with HER-2 expression, as well as poor prognosis in those patients treated with the selective estrogen receptor modulator, tamoxifen (Fleming et al, 2004; Osborne et al, 2003). Elevated expression and activation of AIB-1 has been shown to enhance estrogen-induced expression of cyclin D1, epidermal growth factor receptor activation, cell proliferation, and anti-estrogen resistance (Lahusen et al, 2007; List et al, 2001; Planas-Silva, 2001; Yuan et al, 2007).

AIB-1 has been shown to be involved in cell migration and proliferation. Mutations in the Taiman gene, a Drosophila protein related to AIB-1, for example, results in defects in migration of the border cells in the Drosophila ovary. Mutants cells also exhibited abnormal accumulation of E-cadherin, B-

catenin and focal adhesion kinase (Liao et al, 2003). In addition, loss of AIB-1 in *in vitro* and *in vivo* models results in reduced estrogen mediated inhibition of apoptosis and cell growth and reduced estrogen dependent colony formation in soft agar and tumour growth in nude mice (Liao et al, 2003). These findings suggest that co-activators can regulate the invasive potential of tumour cells, as well as having a role in hormone-regulated cell proliferation.

Proteins encoded for by the SRC family are around 160kD in size, and would have an overall sequence similarity of around 40% (Liao et al, 2003). The conserved region of these genes contains multiple LXXLL motifs (L represents leucine and X any amino acid). These motifs are responsible for the interaction of the SRC family with nuclear receptors, transcription factors, and co-integrators (such as CBP, p300, p/CAF, CARM1 and PRMT1) (Torchia et al, 1997; Heery et al, 1997; Onate et al, 1998; Li et al, 1998). Our group has previously described a positive association between nuclear receptor co-regulatory proteins (SRC-1 and AIB-1) and non-steroid receptor transcription factors (Ets-1 and Ets-2) in mediating endocrine-independent growth (Myers et al, 2005).

The Ets proteins are a family of mitogen-activated protein kinase (MAPK)-dependent transcription factors. These proteins have been implicated as downstream targets of HER-2 and have been shown to be associated with poor patient prognosis (Galang et al, 1996). Ets proteins contain a conserved winged helix-turn-helix DNA-binding domain, which regulates gene transcription through the binding of Ets transcription factors to promoter regions of their

target genes. In studies using reporter assays, the PEA-3 group members have been found to enhance the transcription of several MMP family gene promoters, vimentin, the intracellular adhesion molecule ICAM-1, osteopontin, uPA, and cyclooxygenase genes (Lanouit et al, 2006). Specifically related to our hypothesis, the promoter region of genes coding for COX-2 and MMP-9 have been shown to contain an Ets binding domain, which is the binding site for Ets transcription factors (Howe et al, 2001; Gum et al, 1996).

In this part of the study, we investigated the functional role of AIB-1 in the production of the target proteins COX-2 and MMP-9. MCF-7 cells and PEA-3 transfectants (MCF-7/PEA-3), described in Chapter 3, were used to investigate the relationship between AIB-1 and PEA-3 using co-immunoprecipitation and immunofluorescence studies. Knock-down studies were then used to characterize the functional importance of AIB-1 in the production of COX-2 and MMP-9. Data reported here supports a role for AIB-1 in PEA-3 mediated production of COX-2 and MMP-9. Enhanced understanding of the role of co-regulators in promoting tumour progression provides us with an attractive target in the treatment of breast cancer. Targeting AIB-1 or its interaction with the transcription factor PEA-3 is a promising avenue, which could only be utilized with furthering our understanding of the molecular mechanisms involved in tumour progression.

4.2 Results

4.2.1 Expression of AIB-1 in MCF-7 and MCF-7/PEA-3 breast cancer cells

To investigate the role of AIB-1 in the pathway leading to the production of COX-2 and MMP-9, the expression of this protein was assessed in our experimental cell lines. A stable cell line over-expressing PEA-3 was generated, as described in Section 3.2.1. This cell line was used for the investigations presented in this chapter. The expression of AIB-1 in the PEA-3 transfectants and in the parental cell line, MCF-7 is shown in Figure 4.1 using Western Blotting.

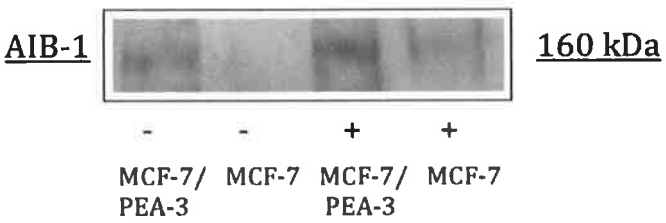


Figure 4.1: Western blotting analysis showing the expression of AIB-1 in MCF-7 and MCF-7/PEA-3 cells. Cells were treated with vehicle control (-) and 10ng of EGF (+). As demonstrated, AIB-1 was expressed in the MCF-7/PEA-3 cell line. EGF up-regulated the expression of AIB-1.

4.2.2 Co-association between AIB-1 and PEA-3

4.2.2.1 Co-immunoprecipitation

Co-immunoprecipitation allows for the identification of protein-protein interaction. Cell lysates from MCF-7 and MCF-7/PEA-3 cells were used to immunoprecipitate AIB-1 protein using an AIB-1 antibody. Following this, an antibody against PEA-3 was used to probe for the expression of the transcription factor. As shown in Figure 4.2, a co-association is demonstrated between AIB-1 and PEA-3.

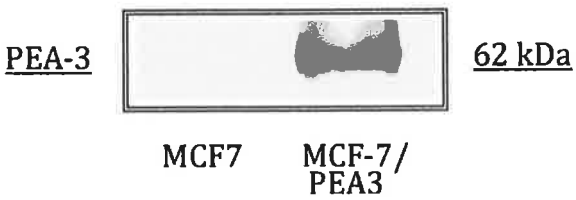


Figure 4.2: PEA-3 and AIB-1 co-associated when PEA-3 was transfected in MCF-7 cells. An antibody against AIB-1 was used for co-immunoprecipitation in MCF-7 and MCF-7/PEA-3 cells. Immunoblotting for PEA-3 revealed a co-association with AIB-1 in the MCF-7/PEA-3 cells.

4.2.2.2 Immunofluorescence

Immunofluorescence staining was used to localize AIB-1 and PEA-3 in breast cancer cell lines MCF-7 and MCF-7/PEA-3. This was performed in order to demonstrate this interaction as well as assess the cellular location of this. Using immunofluorescence, AIB-1 and PEA-3 were successfully co-localised in the nucleus of MCF-7/PEA-3 cells. This is illustrated in Figure 4.3. These results are supported by immunohistochemistry findings to be discussed in chapter 5.

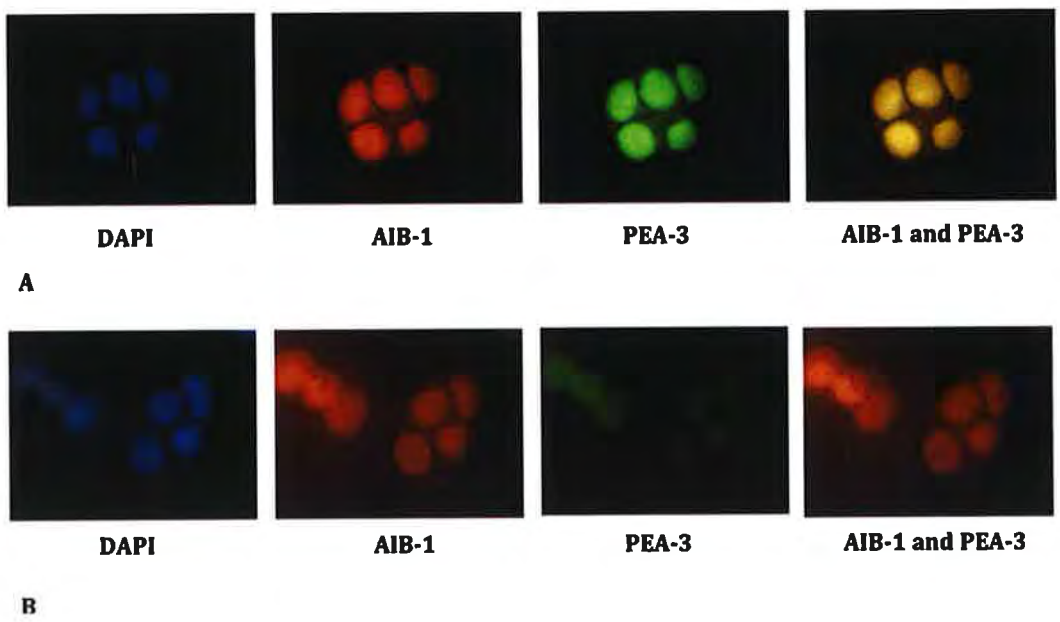


Figure 4.3: Immunofluorescent staining of AIB-1 and PEA-3. Staining was performed using an antibody against AIB-1 and PEA-3. Cell nuclei were visualised by DAPI staining. As demonstrated, co-location of AIB-1 and PEA-3 in the nucleus of MCF-7/PEA-3 cells (A) is shown. MCF-7 cells are shown in section B.

4.2.3 AIB-1 knockdown results in a decrease in the expression of the target proteins COX-2 and MMP-9

The role of AIB-1 in promoting breast cancer cell invasion and metastasis through the production of the target proteins COX-2 and MMP-9 was examined. Silencing of AIB-1 was induced by transfection of the MCF-7/PEA-3 cell line with an siRNA against AIB-1. Studies have shown that AIB-1 is constitutively expressed in MCF-7 cells. The PEA-3 transfectant cells (MCF-7/PEA-3) are therefore ideal to study this relationship and to demonstrate the functional importance of AIB-1 in this process. Using Western Blotting, the expression of AIB-1 was assessed in MCF-7/PEA-3 cells and those where AIB-1 was silenced. This is demonstrated in Figure 4.4.

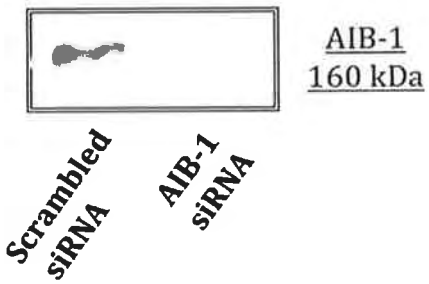


Figure 4.4: AIB-1 expression in MCF-7/PEA-3 cells. Cells were transfected with scrambled siRNA and siRNA against AIB-1. Cells were harvested at 24 hours post transfection. Expression of AIB-1 was found to be reduced following transfection of MCF-7/PEA-3 cells with an siRNA against AIB-1.

The expression of the target proteins COX-2 and MMP-9 was also assessed in the cell lines (MCF-7/PEA-3 and MCF-7/PEA-3/siAIB-1). Interestingly, expression of both proteins appeared to be reduced (Figure 4.5). In addition, and as predicted, there was no change in the expression of PEA-3, which illustrates that knockdown of AIB-1 has no effect of the expression of PEA-3. These results in combination would support a role for the co-regulator AIB-1 in PEA-3 mediated production of COX-2 and MMP-9.

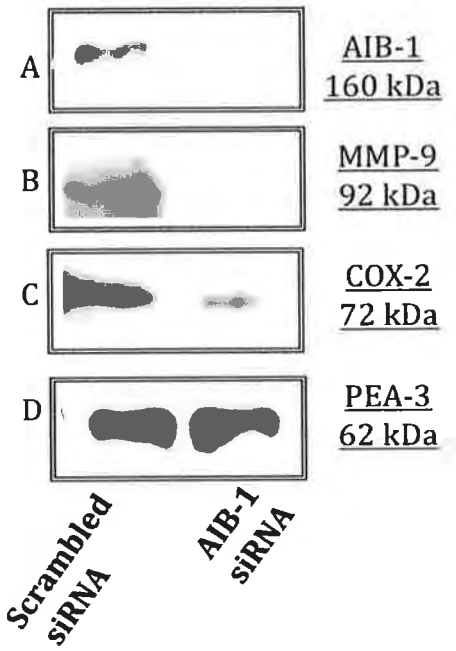


Figure 4.5: Cells were transfected with scrambled siRNA and siRNA against AIB-1. Cells were harvested at 24 hours post transfection. Knockdown of AIB-1 resulted in decreased expression of both COX-2 and MMP-9. There was no change in the expression levels of PEA-3 detected.

4.3 Discussion

The enzymes COX-2 and MMP-9 have been shown to be a marker of tumour cells with a higher invasive and metastatic potential. COX-2, as described previously, is the rate-limiting enzyme involved in the production of prostaglandins. It promotes cell proliferation and induces angiogenesis. MMPs are similarly involved in promoting tumour progression. MMPs are involved in degrading the extracellular membrane, which includes laminin, collagen, fibronectin, and proteoglycans (Weinberg, 2007). MMP-2 and MMP-9 are type IV collagenases that degrade one of the major components of the extra-cellular membrane in breast cancer cells, collagen. Due to their nature of action, both COX-2 and MMP-9 have been found to be over-expressed in tumour cells compared to normal breast tissue.

In this part of the study, we addressed the role of AIB-1 in the production of the target proteins COX-2 and MMP-9. Firstly, we demonstrated a co-association between the AIB-1 and PEA-3. These results are consistent with other studies using cultured cells, which have demonstrated that AIB-1 is a co-activator for PEA-3, as well as AP-1 and NF-kB (Goel et al 2004; Warbajh et al, 2007; Yan et al, 2006). To examine the functional importance of AIB-1 in the pathway leading to the production of proteins involved in tumour progression, knock-down of AIB-1 was performed and cells were assessed for the expression of the target proteins of interest. This part of the study demonstrated that AIB-1 is essential in the pathway mediating PEA-3 induced production of COX-2 and MMP-9. This, in turn, is supported by recent animal studies which have demonstrated that

genetic ablation of AIB-1 reduces lung metastasis in mice and recipient mice bearing transplanted tumours which lack AIB-1 (Qin et al, 2008). In these models, tumorigenesis and lung metastasis was induced through the activation of Shc-Ras-mitogen-activated protein kinase (MAPK) and c-Src-phosphatidylinositol-3 kinase- Akt pathways that are normally stimulated by receptor tyrosine kinases (Qin et al, 2008). Results from several studies on a number of tumour types, which have been shown to express AIB-1, have demonstrated that AIB-1 can induce the expression of different MMPs in a cell context-specific manner (Yan et al, 2008, Li et al 2008). This expression was shown to promote cancer cell invasion and metastasis.

It is known that the promoter region of these genes (COX-2 and MMP-9) contains an Ets binding domain, the binding site for Ets transcription factors. Co-immunopricipitation and immunoflorescence studies reported here demonstrate that AIB-1 and PEA-3 were found to co-associate when PEA-3 was transfected into the MCF-7 cell line. This supports the role of AIB-1 as a co-regulator for PEA-3, and in turn would have a role in mediating breast cancer cell progression. Potential therapeutic targets are highlighted here. Firstly, targeting PEA-3 and AIB-1 individually. This, however, would be challenging given the role of these factors in normal cell growth and differentiation. The second potential target is the PEA-3/AIB-1 interaction. Interrupting this protein-protein interaction could potentially reduce tumour cell growth, the ability to invade, promote angiogenesis and escape from normal cell controls.

Chapter V

Associations between HER-2, p-ERK, AIB-1, PEA-3, COX-2 and MMP-9 and clinicopathological parameters in breast cancer

5.1 Introduction

Breast cancer is the leading cause of cancer related deaths in women worldwide. In Ireland, around 2000 women are newly diagnosed with the disease each year. The disease incidence is also found to be in the upper third of rates recorded amongst European countries (The National Cancer Registry Ireland Report, 2006).

During normal growth and differentiation, cell proliferation is typically rigidly controlled. In cancer states, cancer cells escape normal growth controls and continue to proliferate in an uncontrolled manner. It is felt that one of the ways by which this transformed state occurs is through an over-expression of normally regulated growth factors and their receptors. These in turn would signal to downstream targets and mediate the production of factors, which promote the sustainment of this new tumour profile.

While systemic therapies typically included cytotoxic chemotherapy or hormonal therapy, advances in knowledge regarding other forms of targeted therapies lead to the development new modalities for the treatment of breast cancer. Recently, we have seen the emergence of biological therapies, which work through interfering with specific targeted molecules needed for carcinogenesis and tumour growth, rather than by simply interfering with rapidly dividing cells. Therefore, an understanding of the molecular mechanisms involved in breast cancer progression and the enhanced ability to

identify potential biomarkers of aggressive disease allows for further exploration of this therapeutic path.

In the previous chapters, interactions between AIB-1 and PEA-3 were shown to regulate the production of COX-2 and MMP-9. The role of an up-regulated growth factor pathway in relation to the production of these target proteins was investigated. To further examine the role of these biomarkers in breast cancer and its progression, the expression of these proteins was examined in a series of archived paraffin embedded tissue microarrays from a cohort of patients with primary breast cancer. Their expression was then compared to known clinico-pathological parameters.

5.2 Clinicopathological variables

A tissue microarray (TMA) was constructed using breast cancer tissue from 560 patients with primary breast cancer (TMA was constructed by Dr. Tony Stafford). Ten-year follow-up on these patients is available. Patients were treated with hormonal therapy, chemotherapy and surgery. Clinico-pathological variables included tumour size, tumour grade, nodal status, ER and HER status and time to disease progression.

Immunohistochemistry was performed on these TMAs looking at the expression of HER-2, p-ERK, AIB-1, PEA-3, COX-2 and MMP-9. Dr. Aisling Redmond, Dr. Tony Stafford, and Dr. Mary Dillon performed staining of these slides as part of their own studies and work in the lab. In order to ensure the specificity of the

primary antibodies in detecting the expression of the proteins of interest, the TMA was also stained with the corresponding species specific IgG as a negative control. Each TMA was called by two independent sources that were blinded to the patient data. In collaboration with the above mentioned investigators, their results were analysed in relation to the hypothesis presented in this thesis.

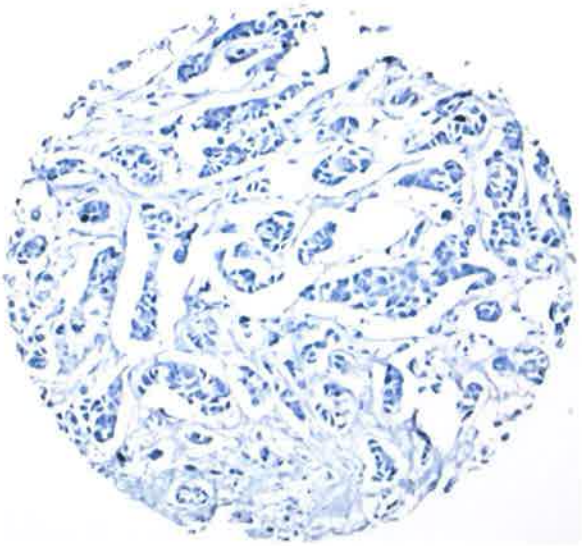
5.3 Results

Expression levels of HER-2, p-ERK, PEA-3, AIB-1, COX-2 and MMP-9 were determined using immunohistochemical analysis. Associations between the transcription factor, co-activator, and their target proteins were analysed and correlation with survival was determined. Statistical analysis was performed by Dr. Aisling Redmond using the Stata statistical package.

5.3.1 HER-2

Over-expression of the EGFR family member HER-2 is associated with an adverse prognosis in human breast cancer (Slamon et al, 1997). These tumours tend to be highly proliferative and poorly differentiated. The Her-2 (erbB2) gene encodes a 185-kDa transmembrane receptor with tyrosine kinase activity and belongs to the family of epidermal growth factor receptors. Over-expression of HER-2 occurs in 20-30% of breast cancers (Ross et al, 1998). In the study population (n=560), Her-2 expression was found in 20% of patients. Immunohistochemical expression was found in the membrane of the tumour cells (Figure 5.1).

A



B

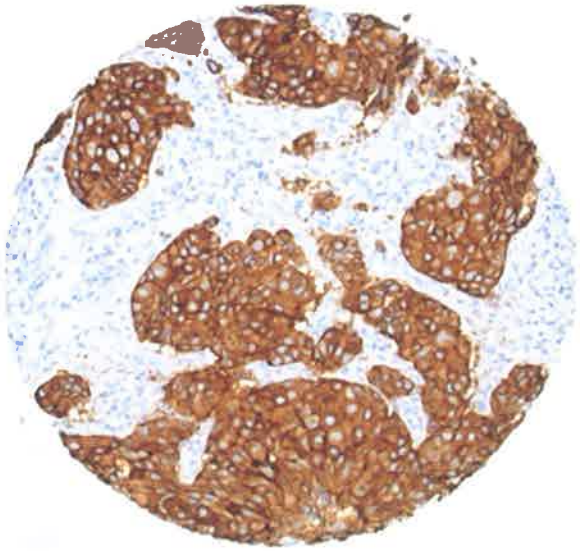
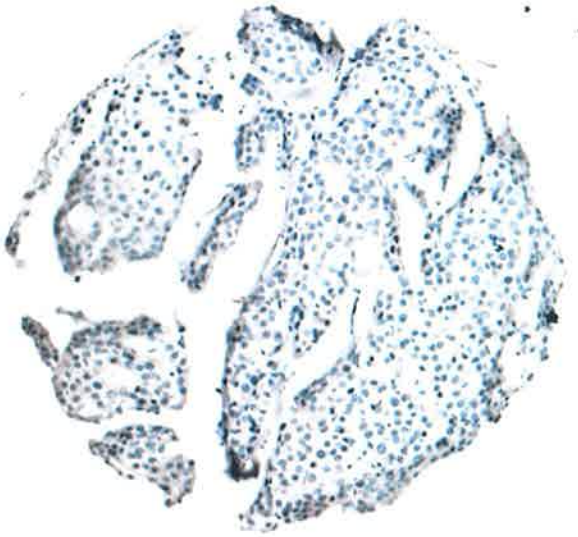


Figure 5.1: Immunohistochemistry illustrates membranous staining of Her-2 (B). Figure 5.1(A) represents the core IgG negative control.

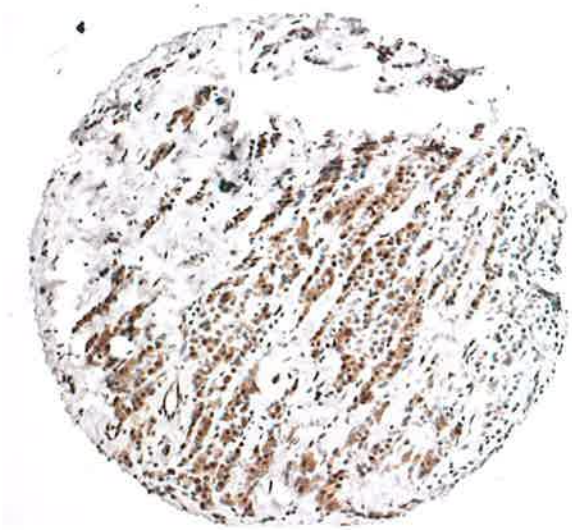
5.3.2 p-ERK

The MAPK pathway plays a pivotal role in breast cancer progression. An important pathway in this signalling cascade is the ras-raf-ERK cascade whose activation results in the phosphorylation of extracellular signal-regulated protein kinase 1/2 (ERK). Activated ERK 1/2 proteins in turn phosphorylate and activate a variety of substrates including transcription factors and protein kinases. Elevated ERK 1/2 activity has been noted in breast cancer compared to benign breast disease (Adeynika et al, 2002).

Analysis of immunohistochemical staining revealed an expression of p-ERK in 30% of patients. As expected, the expression of p-ERK was localised to the nucleus (Figure 5.2).



A



B

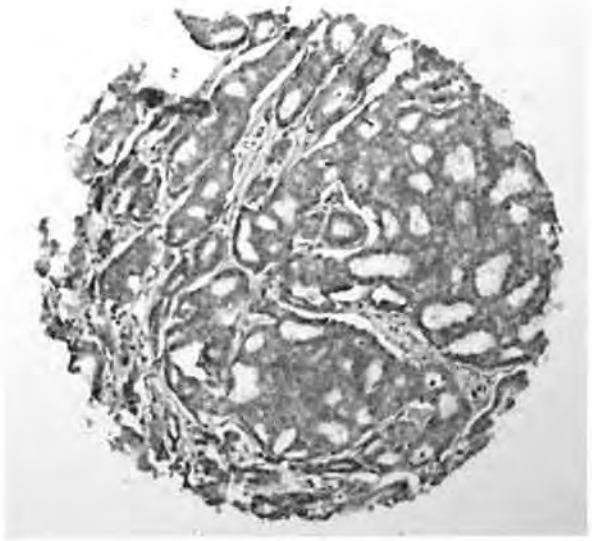
Figure 5.2: Immunohistochemical staining of p-ERK revealed localization to the cytosol of the breast cancer cells (B). Figure 5.2 (A) represents the core IgG negative control.

5.3.3 AIB-1

The steroid receptor co-activators, also known as p160 proteins, were among the first factors identified that interact with nuclear receptors and enhance their transactivation in a ligand-dependant manner (Cavailles et al, 1994). AIB-1 (SRC-3), functions mainly through interaction with transcription factors and recruitment of HATs (such as CBP, p300 and p/CAF) and histone methyltransferases (such as CARM1 and PRMT1) to the promoter for chromatin remodelling and DNA transcription (Liao et al, 2003).

Analysis of immunohistochemical staining revealed an expression of AIB-1 in 74% of patients. As expected, the expression of AIB-1 was localised to the nucleus (Figure 5.3).

A



B

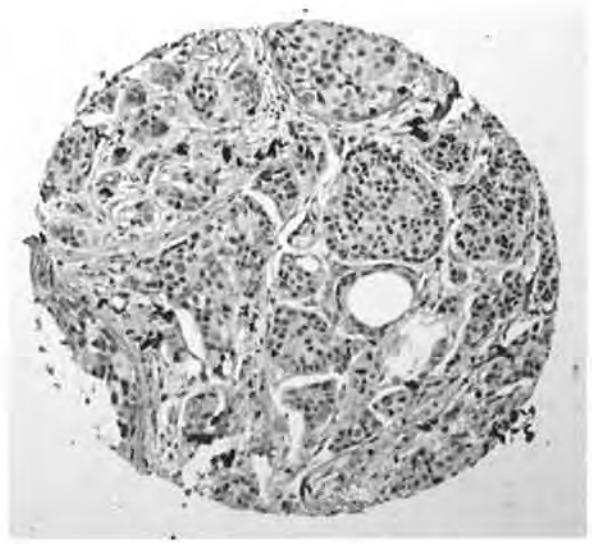
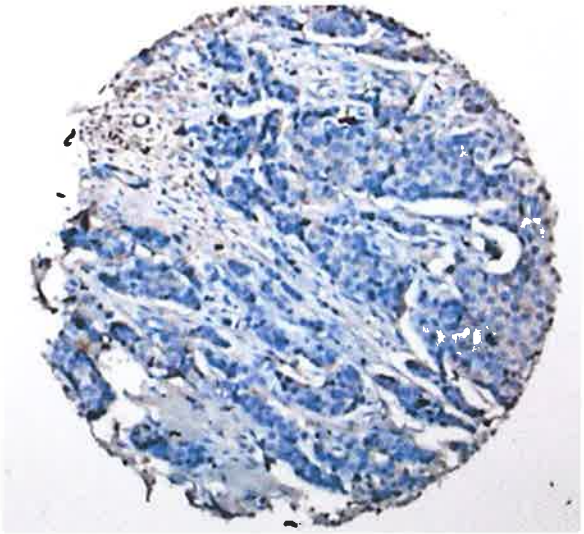


Figure 5.3: Immunohistochemical staining of AIB-1 revealed localization to the nucleas of the breast cancer cells (B). Figure 5.3 (A) represents the core IgG negative control.

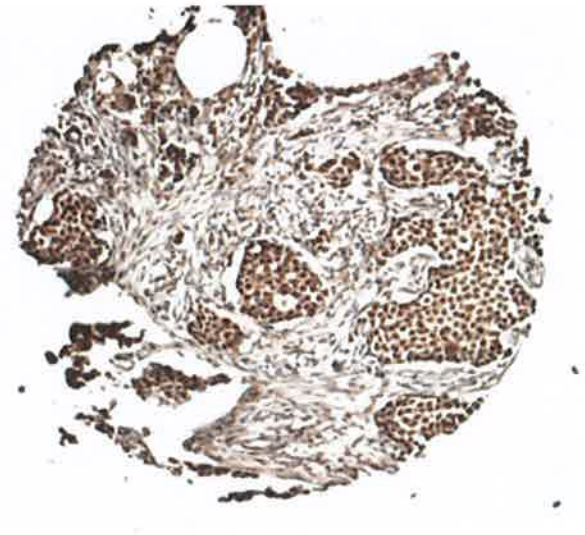
5.3.4 PEA-3

PEA-3 is over-expressed in both human and murine tumours, suggesting a role for this transcription factor in malignancy (Baert et al, 1997; Trimble et al, 1993). Several lines of evidence have emerged which support a role for PEA-3 in breast cancer progression. The Ets transcription factor family of proteins have been found to regulate the transcription of multiple genes, and their trans-activating potential is affected by post-translational modifications.

Analysis of the immunohistochemical staining of the TMA of 560 breast cancer patients revealed 53% expression of PEA-3. Expression of PEA-3 was localised to the nucleus (Figure 5.4), supporting observations reported previously from molecular studies presented in Chapter 3 and 4. Co-association was confirmed between AIB-1 and PEA-3 as previously shown, and these results are supported by the nuclear expression of both proteins on immunohistochemistry.



A



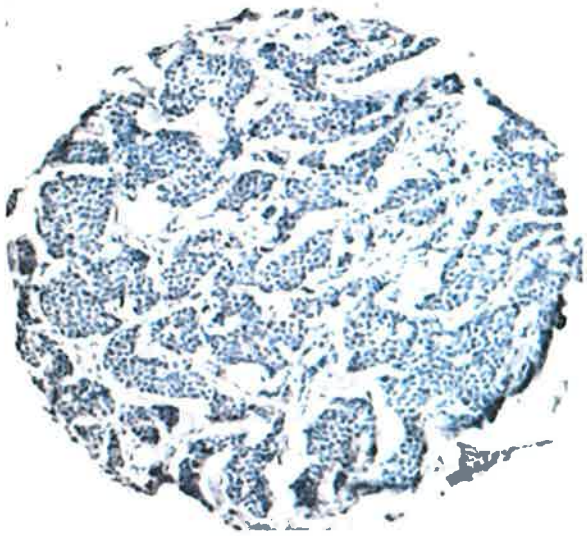
B

Figure 5.4: Immunohistochemical staining of PEA-3 revealed localization to the nucleus of the breast cancer cells (B). Figure 5.4 (A) represents the core IgG negative control.

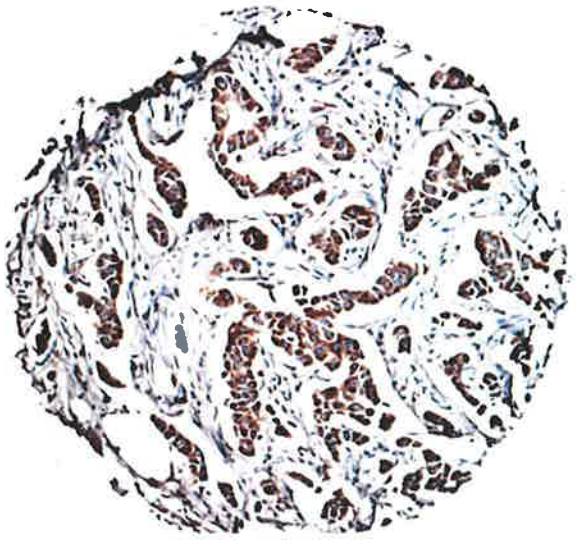
5.3.5 COX-2

Studies of breast cancer have indicated a link between the pathogenesis of the disease and the expression of COX-2. COX-2 has a role in the production of prostaglandins, which are derived from the metabolism of arachidonic acid. These mediate tumour formation and progression by inhibiting normal cellular growth regulation and apoptosis, inducing cell proliferation and the stimulation of angiogenesis. Molecular studies have revealed a varying degree of expression of COX-2 in breast tumour specimens.

The expression of COX-2 was in 48% of specimens on the TMA. The expression of this protein correlated with findings from other studies, in that it was localized to the cytoplasm, as expected from the nature of its action (Figure 5.5).



A



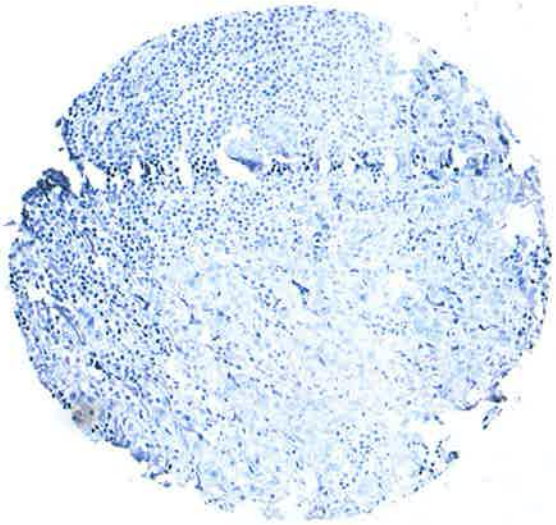
B

Figure 5.5: Immunohistochemical staining of COX-2 revealed localization to the cytoplasm of the breast cancer cells (B). Figure 5.5 (A) represents the core negative control.

5.3.6 MMP-9

The matrix metalloproteinase family of enzymes have been shown to have an integral role in the process of tumour progression. An alteration in the tumour microenvironment, in addition to angiogenesis and cell migration, contributes to tumour invasion and metastasis. As part of this process, MMPs are secreted proteins, which upon activation, contribute to tumour progression. Cellular models, which are commonly used in molecular studies, provide a limited source of tissue samples. They may, therefore, give limited information in comparison to tumour tissue. Immunohistochemical anaylsis of the patient cohort presented here supports results presented in earlier chapters.

Elevated levels of MMP-9 was found in the study population (89%). MMP-9 was predominantly expressed in the cytoplasm and extracellular matrix of the breast cancer specimens (Figure 5.6). While this expression may be regarded as high, it is important to note that MMPs are involved in all stages of tumour progression, from initial changes from in-situ to invasive context and up to distant metastasis.



A

B

Figure 5.6: Immunohistochemical staining of MMP-9 revealed localization to the cytoplasm and extracellular matrix of the breast cancer cells (B). Figure 5.5 (A) represents the core IgG negative control.

5.4 Associations

Analysis of the proteins of interest revealed statistically significant associations. These associations are shown below in Table 5.1. The association between HER-2 and AIB-1, and PEA-3 and COX-2 were not significant. This may be due to the interplay of other factors in the signalling pathway leading to the production of COX-2 and MMP-9. However, these combined results would suggest that the expression profiles of HER-2, AIB-1, PEA-3, COX-2 and MMP-9 are positively correlated. This would, therefore, substantiate results discussed in Chapter 3 and 4, which support a role for AIB-1 in PEA-3 mediated production of COX-2 and MMP-9.

Table 5.1: Associations between the expression of HER-2, p-ERK, AIB-1, PEA-3, COX-2 and MMP-9 are present here. Statistical analysis was performed using Fisher's exact test, and a *P* value of <0.05 is considered to be significant.

Associations	p-value
HER-2 and PEA-3	0.004
HER-2 and AIB-1	0.870
PEA-3 and p-ERK	0.597
PEA-3 and AIB-1	<0.001
PEA-3 and COX-2	0.31
PEA-3 and MMP-9	0.008
AIB-1 and p-ERK	0.039
AIB-1 and COX-2	0.017
AIB-1 and MMP-9	0.004

5.5 Prediction of disease free survival (DFS)

Clinicopathological parameters were further analysed in relation to the expression of HER-2, p-ERK, AIB-1, PEA-3, COX-2 and MMP-9. None of these variables were found to predict disease free survival when analysed independently. By analyzing these in the HER-2 positive cohort it was found that PEA-3 predicts poor DFS (p=0.0136). This is shown in Figure 5.7.

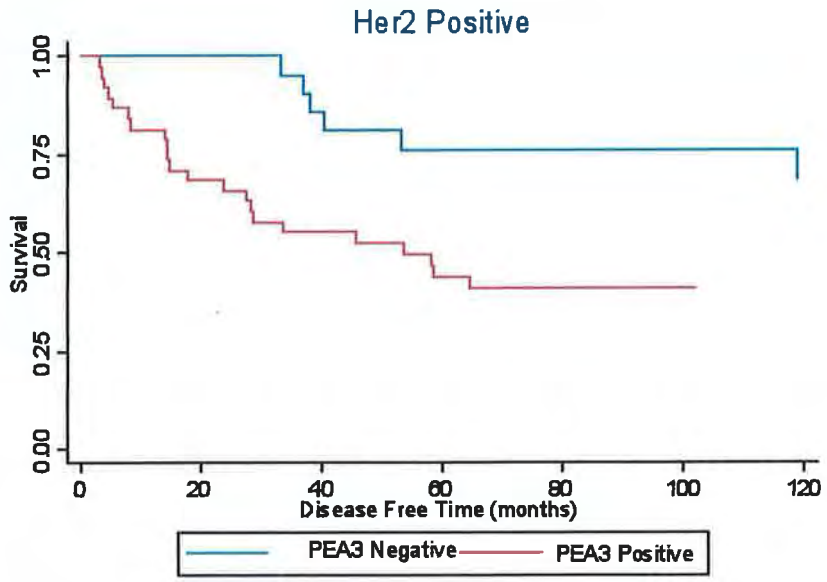


Figure 5.7: Kaplan-Meier estimate of DFS of breast cancer patients according to PEA-3 expression in a HER-2 positive population (PEA-3 positive n=21, PEA-3 negative n=43). The P value was calculated using the Wilcoxon test (p=0.0136).

5.6 Discussion

In this part of the study the protein expression of HER-2, p-ERK, AIB-1, PEA-3, COX-2 and MMP-9 was assessed in a large cohort of breast cancer specimens paraffin embedded in a TMA (n=560). It has been shown that the study of biomarker expression of both nuclear and non-nuclear proteins is feasible on TMAs compared to IHC of whole sections (Henriksen et al, 2007; Dillon et al, 2008; Qin et al, 2008). In addition, while most TMA studies use two cores from tumour specimens, the TMA used here was constructed to contain four representative core samples, providing a potentially more accurate representation of the expression of the biomarkers of interest. This unique patient population allows us to understand signalling pathways underlying the process of breast tumour progression. Identification of these markers in tumour tissue provides us with possible tissue biomarkers important in planning breast cancer patient treatment by the ability to predict those most like to respond to certain treatment modalities.

Several published studies examined the expression of HER-2, PEA-3, AIB-1, COX-2 and MMP-9 in breast cancers. Each of these proteins has been found to be up-regulated in human breast cancers compared to normal breast tissue. PEA-3, for example, was found to positively correlate with HER-2 expression, tumour grade, reduced DFS and axillary lymph node metastasis (Myers et al, 2005, Myers et al, 2006). It has also been shown that AIB-1 expression correlates positively with the over-expression of HER-2 (Myers et, 2005; Osborne et al, 2003).

While previous studies have confirmed COX-2 and MMP-9 as target proteins of the Ets transcription factor, PEA-3, the exact mechanism involved in their production is still unclear. Both COX-2 and MMP-9 play an important role in tumour progression through enhanced cell proliferation, degradation of the extracellular member and the promotion of angiogenesis. All of these changes are central in the process of invasion and metastasis. This thesis sought to study the pathway leading to their production. It was hypothesized that AIB-1 and PEA-3 are important regulators in the process of tumour progression and their activity may be enhanced by the presence of an activated MAPK pathway. The results presented here, support a role for AIB-1 as a co-regulator for PEA-3 in mediating the production of COX-2 and MMP-9. The results also illustrated the strong association between PEA-3 and HER-2, which is consistent with published studies. In combination, PEA-3 and HER-2 were found to associate with poor disease free survival. Therefore, the biomarkers studied here present potential predictors of breast cancer patient outcome, suggesting the presence or the potential to develop more aggressive disease.

Chapter VI

Discussion

6.1 Discussion

Breast cancer is the leading cause of cancer related deaths worldwide (WHO fact sheet N297). It has proven to be an increasing global public burden with more than a million new cases anticipated worldwide (World Cancer Report, 2003). Breast cancer develops in around 12% of women who live to the age of 90 years (Pasche, 2008). A positive family history is reported in 15-20% of women who develop the disease (Pasche, 2008). Early stage disease accounts for the majority of these new diagnoses, and this is largely as a result of improvements in education, screening, technology, diagnosis and treatment (Berry et al, 2005; Elkin et al, 2006). Unfortunately, despite these advances, a significant proportion of women may eventually experience a distant relapse or recurrence-related complications.

Over the last few decades, investigators have sought to understand the biology of the disease in depth in order to optimize disease specific patient outcomes. This, in turn, has led to improved patient survival through the development of new therapeutic modalities and refinements in older treatment strategies. Adjuvant therapies have become an integral part in the treatment of breast cancer, both at the early and late stages. This has accounted for significant improvements in breast cancer specific mortality (Berry et al, 2005).

Breast cancer is mainly hormone driven, with around 70% of cancers expressing an estrogen receptor (Keen et al, 2003). In addition, a number of other peptide growth factors and their receptors, namely members of the receptor tyrosine

kinase family, have been implicated in normal breast development and carcinogenesis (Keen et al, 2003). HER-2 is expressed in around 20-30% of breast cancers and is associated with poorly differentiated and highly proliferative tumours (Sorlie et al, 2001). Its over-expression has been correlated with enhanced tumorigenicity, increased metastatic potential, and resistance to chemotherapy (Wang et al, 2001). Therefore, an enhanced understanding of the pathways leading to breast cancer development and progression is crucial in managing the disease.

Current therapies include those that manipulate the estrogen receptor (ER) and those that target growth factor receptors. ER modulators such as tamoxifen or faslodex, target the ER directly, while aromatase inhibitors (AI), such as anastrozole, reduce the levels of circulating estrogens (Ring et al, 2004). While hormonal manipulation has revolutionized breast cancer treatment to date, one of the most significant limitations is the development of resistance, which effects up to 50% of patients (Schiff et al, 2004). A proposed mechanism is a shift in tumour phenotype from steroid dependence to steroid independence/growth factor dependence (Arpino et al, 2008). Targeting growth factor receptors is also a subject of great interest and several classes of receptor inhibitors have been developed. The monoclonal antibody, trastuzumab, is directed against the HER-2 receptor, while the dual tyrosine kinase inhibitor (TKI) lapatinib works on both EGFR and HER-2.

Similar to endocrine treatments, a proportion of patients who initially respond to treatment, will eventually relapse (approximately 40%)(Lu et al, 2001).

Hence, our understanding of pathways central to the process of tumour invasion and metastasis is important in order to develop improved treatment approaches. The mechanisms governing breast tumour invasion and metastasis remain a dilemma. Local and distant metastasis occurs by spread to various organs of the body (bone, lung, liver, kidney, brain) through the bloodstream and lymphatics. It has been shown that approximately 40% of patients with breast cancer have micro-metastatic disease the time of presentation, making the process of detection at the time of diagnosis and treatment difficult. Both local and distant disease recurrence in addition to mortality from the disease are inevitable consequences (Fisher et al, 1991; Veronesi U et al, 1995). To date, markers such as tumour size, differentiation, grade, lymph node positivity, estrogen receptor status, expression of HER-2/neu, other oncogene markers and genetic mutations are regarded to be useful in estimating risk and prognosis. Our ability to predict those patients with aggressive disease, which is more likely to recur, is dependant on our understanding of the molecular mechanisms underlying. There has been a continued interest in identifying prognostic biomarkers in breast cancer, which translates into an ability to identify cohorts of patients who are more likely to benefit from evolving systemic therapies.

In this thesis, we focused on the target genes COX-2 and MMP-9. The enzyme COX-2 is the rate-limiting enzyme in the metabolism of arachidonic acid into prostaglandins. An expression of COX-2 in breast tumours is associated with increased tumorigenic transformation, higher tumour grade, and decreased overall and progression-free survival (Howe et al, 2005; Liu et al, 2005; Ristimaki et al, 2002; Surowiak et al, 2005). The expression of COX-2 has been

shown to relate to angiogenesis, increased cell proliferation, high expression levels of p53 and HER-2, and the presence of axillary lymph node metastasis (Davies et al, 2003, Ristimaki et al, 2002. Surowiak et al, 2005).

MMP-9, on the other hand, is a member of the matrix metalloproteinase family of zinc and calcium-dependant extracellular enzymes. MMP-9 is one of the two gelatinases that has been implicated in tumour cell invasion and metastasis because of its unique ability to degrade type IV collagen, which is a major component of the basement membrane and other essential extracellular matrix components (Zheng-Sheng et al, 2008; Duffy et al, 2000). MMP-9 levels have been found to be significantly elevated in breast cancer patients compared to patients with benign breast disease and healthy controls. Elevated levels of MMP-9 were found to be associated with the presence of lymph node metastasis, higher disease stage, and lower overall survival and relapse-free survival. This suggests that MMP-9 may be a useful biomarker for predicting disease progression (Zheng-Sheng et al, 2008). Here, we sought to understand a common signalling pathway leading to the production of these two proteins.

The Ets transcription factor family have been linked to several types of human tumours. It has been suggested that their expression levels correlate with tumour invasion and metastasis, making it a useful predictor of tumour progression in breast cancer patients. The promoter region of both COX-2 and MMP-9 has been found to contain an Ets domain, which is the binding site for Ets transcription factors. The Ets transcription factor PEA-3 has been previously documented to have a role in promoting carcinogenesis. Here, we investigated

the role of PEA-3 as a potential regulator of COX-2 and MMP-9. Transfection studies suggested that induced expression of PEA-3 in the non-invasive MCF-7 cell line mediated the expression of COX-2 and MMP-9. Furthermore, morphological changes including the development of pseudopodial extensions and migration in a wound assay were noted in the transfected cell line, which suggest that PEA-3 supports the development of a more motile phenotype. In addition, PEA-3 transfection appeared to promote changes in cellular architecture with loss of differentiation and the ability to form acini in 3D cell culture.

Steroid co-regulatory proteins such as the p160 family, SRC-1 and AIB-1, were until recently thought to associate almost exclusively with nuclear receptors. Recent studies, however, have described p160 interactions with steroid independent transcription factors, including AP-1, NF-kappaB, and p53 (Lee et al, 1998; Na et al, 1998; Lee et al, 1999). A p160 binding region, recognised as an LXXLL domain, has been described in loop one of the Ets domain of Ets proteins (Wasylyk et al, 1993). Previous work from our lab has demonstrated the co-expression of SRC-1 and AIB-1 and the transcription factors Ets-1, Ets-2 and PEA-3 within breast tumour epithelial cells (Myers et al, 2005). In addition, recruitment of AIB-1 to the Ets protein DNA complex was observed. Results presented here support a co-association between the Ets protein PEA-3 and the co-regulator AIB-1 as demonstrated by co-immunoprecipitation and immunoflorescence studies.

In relation to the production of the target proteins COX-2 and MMP-9, the results presented here illustrate the functional importance of AIB-1 in this process. Silencing AIB-1 using an siRNA resulted in reduced expression of both COX-2 and MMP-9. There was no reduction in the expression of PEA-3, suggesting the importance of AIB-1 in the production of the downstream target proteins of interest in this thesis.

Ets proteins require activation for their functional activity. MAPK mediated phosphorylation has been shown to represent a mode of activation of Ets proteins and the promotion of Ets-DNA binding (Sharrocks et al, 1997). The MAPK pathway can be activated by growth factors or by cellular stress. External stimuli are transmitted to the nucleus upon activation, inducing the activation of numerous transcription factors. This ultimately results in changes in gene expression, which contribute to breast tumour progression. *In vitro* studies have provided evidence that PEA-3 can be activated when phosphorylated by MAPK, both through extracellular signal-related kinase (ERK) and the c-Jun N-terminal kinase (JNK)/ stress activated protein kinase (SAPK) (O'Hagan et al, 1996). The results presented in this thesis demonstrate that stimulation of the PEA-3 transfectants with EGF resulted in an up-regulated expression of COX-2 and MMP-9, compared to parental MCF-7 cells, which do not express COX-2 or MMP-9. This supports a role for an active MAPK pathway in activating PEA-3, which in the presence of AIB-1 would lead to the production of target proteins involved in tumour progression.

The HER-2 gene is frequently over-expressed not only as a consequence of gene amplification but also as a result of transcriptional up-regulation in many human breast tumour cell lines (Bosher et al, 1995). The HER-2 upstream regulatory region contains a conserved Ets binding site and mutation of this sequence has been found to reduce the transcription of related reporter genes (Scott et al, 1994). MCF-7 cells are non-invasive, estrogen receptor positive breast cancer cells. This cell line has been shown in studies to express very low levels of HER-2. This suggests the presence of a growth factor pathway which may be stimulated by enhanced signalling through growth factor receptors. Transfection of MCF-7 breast cancer cells with PEA-3 resulted in an increased expression of HER-2 as demonstrated by immunoflorescence and western blotting studies. This supports previous studies suggesting HER-2 as a target of PEA-3. In addition, enhanced growth factor signalling was demonstrated with elevated expression of p-ERK 1/2 in MCF-7/PEA-3 cells compared to parental MCF-7 cells. Taken together with the results discussed previously, these results suggest that PEA-3 regulates the transcription of HER-2, COX-2 and MMP-9. Increased HER-2 expression further enhances signalling through the growth factor pathway promoting the production of these target proteins, which are involved in breast tumour invasion and metastasis.

The translational aspect of the study supports the molecular studies by showing strong associations between the co-regulator AIB-1 and the transcription factor PEA-3. In addition, statistically significant associations between these factors and their target proteins COX-2 and MMP-9 were demonstrated. An active growth factor pathway, through an active HER-2 receptor indicates the

importance of this signalling pathway in promoting tumour invasiveness. While these proteins did not independently predict poor outcome in terms of recurrence and overall survival, it was observed that in the HER-2 positive population, PEA-3 expression was a predictor of reduced DFS. This indicates the strong relationship between these proteins and breast cancer progression in the clinical setting. Therefore, an ability to select out those patients who express the biomarkers of aggressive disease may enable us to tailor treatment accordingly. It has been consistently shown that multimodal therapy offers patients survival benefit, compared to single agent treatment.

Several areas in the pathway highlighted in this thesis would represent potential therapeutic targets. To date, there is a wealth of data available that supports the ability of COX inhibitors to reduce the formation, growth and metastasis of tumours in experimental animals. Ongoing trials are evaluating the clinical activity of COX-2 inhibitors in breast cancer. COX-2 inhibitors are showing great promise for their potential therapeutic benefit in a range of cancers. However, there has recently been a concern over their potential cardiotoxicity of COX 2 inhibitors. This was highlighted with the identification of increased cardiovascular events following treatment with Rofecoxib, a COX-2 inhibitor, in the APPROVe trial (Bombardier et al, 2000; Bresalier et al, 2005). In addition, a review published in the New England Journal of Medicine in 2005 raised concerns over long-term use of Celecoxib, another COX-2 inhibitor (Solomon et al, 2005). A subsequent review of the data stated that patients who had been taking Rofecoxib had up to three times greater risk of developing a myocardial infarction compared to patients taking Celecoxib (Kimmel et al,

2005; Barnes et al, 2007). While there is still a need to prove the safety profile of this class of drugs, there is no doubt that it will have a significant role in the future as a chemopreventative as well as a chematherapeutic agent in the treatment of breast cancer. In addition, several studies have shown a strong correlation between COX-2 and aromatase expression (Gunnarsson et al, 2006; Muller-Decker et al, 2005; Ragaz et al, 2003). The use of COX-2 inhibitors in combination with aromatase inhibitors is advocated and their use will be supported when the results of ongoing trials are analysed.

With regards to inhibition of MMP, this has been shown to decrease metastasis and local tumour growth in mouse models (Sledge et al, 1995). Several broad-spectrum MMP inhibitors have been entered into clinical trials for the treatment of several tumour types, resulting in failed phase III trails which showed little clinical improvement in outcome (Bramhallm et al, 2001; Hidalgo et al, 2001; Shepherd et al, 2001). In breast cancer, a phase III randomized clinical trail of Marimastat vs placebo, showed no improvement in progression-free survival (Sparano et al, 2002). In addition, due to the lack of specific inhibitors of individual MMPs and associated the possible musculoskeletal toxicity associated with current inhibitors, their use is currently limited (Coussens et al, 2002; Miller et al, 2002).

Herceptin (trastuzumab) is the anti-HER-2 monoclonal antibody that has revolutionized the treatment of HER-2 positive breast cancer. Newer generation tyrosine kinase inhibitors have also emerged with promising results for the future. These signal transduction inhibitors are ideal upstream targets for the

signalling pathways highlighted in this study. However, our knowledge of the complexity of these pathways emphasises the importance of developing specific targeted therapies which would act as adjuncts to current strategies. Our ability to select those patients who are more likely to benefit from such treatments is essential.

6.2 Future Work

The work presented here has highlighted several potential areas of further research. This section describes the proposed investigations that will allow us to further interrogate the role of AIB-1 and PEA-3 in mediating breast tumour progression.

Tyrosine kinase receptor inhibitors (TKI), including trastuzumab (Herceptin) have revolutionised the treatment of growth factor receptor positive breast cancer patients. Trastuzumab is a humanized mouse recombinant anti Her-2/neu monoclonal antibody that targets with high affinity and specificity the extracellular membrane portion of the HER-2/neu growth factor receptor, inhibiting signal transduction and cell proliferation (Beselga et al, 1996). In a retrospective analysis of specimens from breast cancer patients who participated in phase II and phase III trials of trastuzumab and chemotherapy treatment, those with HER-2 positive tumours had significantly improved clinical response and overall survival with the addition of trastuzumab to chemotherapy (Mass RD, 2005). In contrast, HER-2 negative patients on FISH analysis did not show benefit with the addition of trastuzumab. Furthermore, Herceptin as a single agent has been shown to be active and well tolerated as a first line treatment in females with metastatic breast cancer (Vogel et al, 2002). Lapatinib, an EGFR and HER-2 receptor inhibitor, is a newer agent that has been shown to inhibit tumour cell growth in *in vitro* and xenograft models.

While many patients initially respond to treatment with tyrosine kinase inhibitors, approximately 40% have been shown to eventually relapse. Preliminary molecular and clinical studies from our group and others support the hypothesis that inappropriate treatment of HER2 positive breast cancer patients with Herceptin can lead to tumour recurrence and the development of distant metastasis. In addition, it has been previously reported that the expression of PEA3 is associated with, HER2 positivity, metastasis and reduced disease-free survival (Myers et al, 2005).

Molecular and translational work discussed in this thesis support a role for AIB-1 as a co-regulator in PEA-3 mediated production of COX-2 and MMP-9. In addition, a role for PEA-3 in up-regulating the expression of HER-2/neu has been shown, contributing to enhanced breast cancer cell invasiveness. There are several potential therapeutic areas to target in the signalling pathway highlighted in this thesis. While several of these, such as the AIB-1/PEA-3 interaction, is a subject of current study and development, existing therapies targeting the growth factor receptors have been proven to have a role in improving clinical outcome. Capitalising unique clinical populations will promote advances made in terms of understanding how TKI therapy works and the mechanisms of acquired resistance. Patient profiling in this manner will inform the optimum drug treatment for each cancer.

Results described here have formed a basis for further work aiming to investigate the molecular mechanisms of TKI resistance. Focusing on the ER co-activator AIB1 and the transcription factor PEA-3, the role of treatment with

TKIs will be studied in relation the regulation of target gene production. The strength of PEA-3 and AIB1 and their downstream target genes, in terms of their capacity to predict response to TKI treatment will be tested in a large cohort of breast cancer patients. Furthermore, the functional relevance of the transcription factor, co-activator and their target gene expression will be examined in a clinical setting in patients undergoing neo-adjuvant TKI treatment.

Molecular characterization of the PEA-3/AIB-1 interaction in response to TKIs and associated changes in the transcription of their target genes in TKI resistant cells

A stable cell line over-expressing PEA-3 was created for the studies described in this Chapter 3 and 4. We propose that these cells (MCF-7/PEA-3), which have been found to express HER-2, are Herceptin sensitive. By creating a Herceptin resistant cell line (MCF-7/PEA-3 HerR), we aim to compare the role of Herceptin and Lapatinib on the interaction between PEA-3 and AIB-1 in the two cell lines. Expression of PEA-3 and AIB-1, as well as their interactions will be determined using standard immunoprecipitation assays. Recruitment of PEA-3 and AIB-1 to the promoter region of the target genes COX-2 and MMP-9 will be assessed by chromatin immunoprecipitation assays (ChIP). Furthermore, the transcriptional activation of COX-2 and MMP-9 will be assessed by luciferase assays. Luciferase constructs were synthesized for this thesis and preliminary work has been ongoing at the time of writing this thesis to investigate the ability of PEA-3 to

active the COX-2 and MMP-9 promoters in cells treated and untreated with EGF. Future work will involve assessing this in relation to treatment with TKIs.

Evaluate the clinical relevance of PEA-3/AIB-1 target genes in breast cancer patients treated and untreated with TKI.

It has also been proposed to assess the expression of HER-2, AIB-1, PEA-3, and their target proteins in a cohort of patients on Herceptin and Lapatinib treatment using immunohistochemistry. The expression of these markers and clinico-pathological parameters, tumour progression, and disease-free survival will be evaluated. For this study, a cohort of HER-2 positive breast cancer patients who have been treated with Herceptin/Lapatinib and a cohort that has not been treated with a TKI were identified. A tissue microarray (TMA) of these patients is currently under construction. The time to disease recurrence and death is recorded in addition to classic clinico-pathological details. At the time of writing this thesis a TMA of 62 patients was constructed. Staining for AIB-1, PEA-3, COX-2 and MMP-9 was completed. Table 7.1 shows the clinico-pathological parameters recorded on the patient cohort. This number is currently small and detailed analysis will be completed upon completed construction and staining of the TMA.

Table 7.1: Clinico-pathological details of patients included in a tissue microarray of patients treated and untreated with Herceptin/Lapatinib (n=62)

Total number of patients	62
Age range	28-86
Procedures Mastectomy Lumpectomy	 27 35
Positive sentinel node	20
Axillary lymph node dissection Positive	41 31
Histology Invasive ductal carcinoma Invasive lobular carcinoma Others	 53 5 4
Size < 2.5 cm > 2.5 cm	 26 36
Grade < III III	 21 28
ER positive	34
PR positive	21
HER2 positive	49
Hormonal treatment Tamoxifen Arimidex	 13 13
Radiotherapy	43
Chemotherapy	43
Herceptin	22
Recurrence Total HER2 positive HER2 negative	12 12 0

Objective III: Effects of TKI treatment of PEA-3, AIB-1, and their targets in breast cancer patients

Ex vivo models provide important information as to the relative expression and localisation of key markers of breast cancer development, progression, and resistance to treatment. However, as they are taken at a snapshot in time, they give little appreciation of the functionality of the proteins in terms of response to TKI or in the development of resistance. As such, an *in vivo* functional model of AIB-1/PEA-3 interactions in response to TKI would bring together data from both in vitro molecular studies and TMA studies, bringing proof of concept gained from earlier objectives. Tumour samples will be collected from patients on neo-adjuvant TKI pre and post treatment, and prior to surgery. Patients are to be randomized to treatment with one of the TKIs (Herceptin or Lapatinib). Core biopsy specimens will be formalin fixed and paraffin embedded. Immunohistochemical staining of Ki67 will assess alterations in cell proliferation as well as cell cycle complete response (CCCR). Growth factor receptors Erb1 and Erb2, members of the MAP kinase cascade, AIB-1, PEA-3 and target proteins will be examined on the sections.

Understanding signalling networks important in the development and progression of breast cancer will allow for the identification of markers of response to treatment. Data presented thus far supports a role for the transcription factor PEA-3 and its co-activator AIB-1 in the production of MMP-9 and COX2 in breast cancer. The proposed future work aims to interrogate this pathway further in order to provide further evidence for potential therapeutics targets.

Chapter VII

Conclusion

The results reported in this thesis suggest that COX-2 and MMP-9 are target genes of PEA-3/AIB-1 mediated transcription. It has also been demonstrated that PEA-3 has a role in up-regulating the expression of HER-2, leading to enhanced signalling through the growth factor pathway. This was illustrated through the associated increase in the expression of p-ERK. In addition, AIB-1 has a role in up-regulating the production of COX-2 and MMP-9, and therefore promoting tumour invasion and metastasis. Knock-down of AIB-1 resulted in reduced production of both COX-2 and MMP-9. Immunohistochemical analysis and clinico-pathological correlation from our large cohort of breast cancer patients supports the molecular studies presented in Chapter 4 and 5.

This thesis highlights the potential therapeutic target points of a pathway leading to tumour progression. Interrupting the AIB-1/PEA-3 link represents a new therapeutic avenue, which may lead to reduced production of the putative target genes COX-2 and MMP-9.

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Appendix I- Molecular Reagents

Western Blotting

Tris Buffered saline (TBS) (20X):
121.1 g Tris
175.5 g NaCl
Made up to 1 litre with dH_2O
Use at 1X final concentration

Wash buffer:
1X TBS
0.05% Triton® X-100

Blocking buffer:
1X TBS
0.05% Triton® X-100
5% Marvel

1 M Tris.HCl, pH 6.8
157.6 g Tris- HCl
Made up to 1 litre with dH_2O, pH 6.8

1.5 M Tris.HCl, pH 8.8:
236.4 g Tris- HCl
Made up to 1 litre with dH_2O, pH 8.8

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

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

Running buffer (10X)
288 g Glycine
60.6 g Tris Base
20 g 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
20 g SDS was dissolved in 100 ml of dH_2O

10% Ammonium Persulphate
100 mg/ml was dissolved in dH_2O. A fresh solution was prepared for each gel

Cell Culture

Foetal calf Serum (FCS) (GiBcoBRL®)

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

Complete Roswell Park Memorial Institute (RPMI) 1640 Medium 500 ml volume (GiBcoBRL®)
10 ml of 1 M HEPES (20 mM final concentration)
5 ml of Pen/Strep solution (50 U/ml Penicillin and 50 U/ml Streptomycin)
50 ml of FCS
5 ml L-glutamine (200mM ; 2mM final concentration)- Renewed every two weeks.

Leibovitz L-15 media 500ml volume (GiBcoBRL®)
5mls of Pen/Strep solution (50 U/ml Penicillin and 50 U/ml Streptomycin)
50ml of FCS

Dulbecco's Modified Eagle Medium (DMEM) 500 ml volume (GiBcoBRL®)
5mls of Pen/Strep solution (50 U/ml Penicillin and 50 U/ml Streptomycin)
50ml of FCS
5 ml L-glutamine (200mM ; 2mM final concentration)- Renewed every two weeks.

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. Use 1 ml of cold RIPA Buffer for every 5 × 10^6 cells (~20 µl of packed cells, which is equivalent to~40 mg of cells).

GENETICIN® (G418 Sulfate) Invitrogen
Dissolve Geneticin Selective Antibiotic in fully supplemented growth medium without antibiotics at a concentration of 5 mg/ml and filter using a 0.22 micron filter. Treat cells with 400ug/ml of Geneticin for optimum results.

Immunohistochemistry

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

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

Sodium citrate buffer
Dissolve 1.4mg of sodium citrate in 500 ml of dH_2O
Final concentration of 0.01 M sodium citrate, pH 6.0

Transient transfection assay

LB
10 g Tryptone (Bacto Tryptone- Becton, Dickson and company USA)
5 g Yeast extract (Bacto Yeast Extract- Becton, Dickson and company USA)
10 g NaCl
Autoclave

LB agar
10 g Tryptone (Bacto Tryptone- Becton, Dickson and company USA)
5 g Yeast extract (Bacto Yeast Extract- Becton, Dickson and company USA)
10 g NaCl
Agar 1.5% (Bacto™ Agar- Becton, Dickson and company USA)
Autoclave

SOC medium
20 g Tryptone (Bacto Tryptone- Becton, Dickson and company USA)
5 g Yeast extract (Bacto Yeast Extract- Becton, Dickson and company USA)
0.5 g NaCl
Dissolve and then add
10 ml 250mM KCl
5 ml 2M $MgCl_2$
Autoclaved, cooled and then
20 ml 1 M sterile glucose added

Buffer P1 (resuspension buffer)
50 mM Tris. Cl, pH 8.0
10 mM EDTA
100 μg/ml RNase A
Storage at 2-8°C

Buffer P2 (Lysis buffer)
220Mm Na OH
1% SDS
Storage at 15-25°C

Buffer P3 (neutralization buffer)
3 M Potassuim acetate pH 5.5
Storage at 2-8°C

Buffer FWB2 (QIA filter wash buffer)
1M Potassuim acetate, pH 5.0
Storage at 15-25°C

Buffer QBT (equilibration buffer)
750 mM NaCl
50mM MOPS, pH 7.0
15% isopropranol (v/v)
0.15% Tritron® X-100(v/v)
Storage at 15-25°C

Buffer QC (wash buffer)
1.0M NaCl
50 mM MOPS, PH 7.0
15% isopropanol (v/v)
Storage at 15-25°C

Buffer QF (elusion buffer)
1.25 M NaCl
50 mM Tris.Cl, PH 8.5
15% isopropanol (v/v)
Storage at 15-25°C
Buffer QN (elusion buffer)
1.6 M NaCl

50 mM MOPS, PH 7.0
15% isopropanol
Storage at 15-25°C

Buffer TE
10 mM Tris.Cl, PH 8.0
1mM EDTA
Storage at 15-25°C

Buffer STE
100mM NaCl
10 mM Tris. Cl, PH 8.0
1mM EDTA
Storage at 15-25°C

LiCl Immune Complex Wash Buffer
0.25M LiCl
1% IGEPAL-CA630
1% deoxycholic acid (sodium salt)
1mM EDTA, 10mM Tris, pH 8.1

TE Buffer
10mM Tris-HCl
1mM EDTA, pH 8.0

0.5M EDTA
0.5M EDTA, pH 8.0

5M NaCl
5M NaCl

1M Tris-HCl, pH 6.5
1M Tris-HCl, pH 6.5

SDS Lysis Buffer
1% SDS
10mM EDTA
50mM Tris, pH 8.1

3D Cell Culture

10x PBS/Glycine
1.3 M NaCl
0.13 M Na_2HPO_4
0.03 M NaH_2PO_4
1 M Glycine

10x IF Wash
1.3 M NaCl
0.13 M $NaHPO_4$
0.03 M NaH_2PO_4
0.07 M NaN_3
5.0g BSA
10.0ml Triton-X 100
2.05ml Tween-20

Appendix II- Clinicopathological details of patients included in the tissue microarray (n-560)

Patient Demographics (n=560)

Age range (years)	26.3-90.6
Average age (years)	56.3

Tumour grade	**(number and percentage)**	
Grade I	44	7.8%
Grade II	211	37.6%
Grade III	222	39.6%
Unspecified	83	14.8%

Tumour size		
< 1 cm	16	2.8%
1-2 cm	136	24.2%
>2 cm-3 cm	185	33%
>3 cm-4 cm	143	25.5%
>4 cm	80	14.3%

Nodal status		
Node negative	283	51.6%
Node positive	277	49.4%

ER status		
ER negative	170	30.3%
ER positive	359	64.1%
Unspecified	31	5.5%

HER2		
HER2 negative	317	56.6%
HER2 positive	88	15.7%
Unspecified	155	27.6%

Appendix III

Prizes

- The role of AIB-1 in PEA-3 mediated production of COX-2 and MMP-9

Sheppard Prize, Beaumont Hospital, Dublin, April 2009

- AIB-1 and PEA-3; key players in breast cancer invasion

William O'Keefe Prize, Waterford October Surgical Meeting, October 2008

Oral Presentations

- A pathway to breast cancer invasion

William O'Keeffe Presentation (Invited Speaker) at the Waterford October Surgical Meeting, October 2009

- COX-2 and MMP-9; Products of a signalling pathway regulated by AIB-1 and PEA-3

Sir Peter Freyer Surgical Meeting, Galway, September 2009

- The role of AIB-1 in PEA-3 mediated production of COX-2 and MMP-9

Sheppard Prize, RCSI/Beaumont Hospital, Dublin, April 2009

- A signalling network in invasive breast cancer; a role for PEA-3 mediated production of MMP-9 and COX-2

Sylvester O'Halloran Surgical Meeting, Limerick, March 2009

- AIB-1 and PEA-3; key players in breast cancer invasion

President's Prize, Charter Day, Royal College of Surgeons in Ireland, Feb 2009

- A pathway to breast cancer invasion

Society for Academic Research Annual Meeting (Plenary), January 2009

- AIB-1 and PEA-3; key players in breast cancer invasion

William O'Keefe Prize, Waterford October Surgical Meeting, October 2008

- A transcriptional regulatory mechanism in invasive breast cancer

Sir Peter Freyer Surgical Meeting, Galway, September 2008

- The breast cancer metastatic pathway; the role of AIB-1, PEA-3 and MMP-9

Sylvester O'Halloran Surgical Meeting, Limerick, March 2008

Poster Presentations

- A transcriptional regulatory mechanism in invasive breast cancer; role of AIB-1 and PEA-3

Research Day, Royal College of Surgeons, April 2009

- A transcriptional regulatory mechanism in invasive breast cancer; role of AIB-1 and PEA-3

31st San Antonio Breast Cancer Symposium, San Antonio, Texas, December 2008

- The transcriptional regulation of genes in invasive breast cancer a role for AIB-1 and PEA-3

British Association of Surgical Oncology Meeting, London, November 2008

- The role of AIB-1, PEA-3 and MMP-9 in modulating breast cancer progression

Research Day, Royal College of Surgeons, April 2009