Acquired Tamoxifen Resistance and Promotion of Angiogenic Responses in Breast Cancer

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Acknowledgements

List of Abbreviations

4-OH-T	4-hydroxy-tamoxifen
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
AI	Aromatase Inhibitor
AIB1	Amplified in Breast 1 (Nuclear Receptor Coactivator 3)
Akt	(Protein Kinase B)
APS	Ammonium Persulfate
Bad	Bcl-2 Associated Death Promoter
Bcl-2	B-cell Lymphoma 2
BRCA1	Breast Cancer Type 1 Susceptibility
BRCA2	Breast Cancer Type 2 Susceptibility
BSA	Bovine Serum Albumin
CAM	Chorioallantoic Membrane
CD31	Cluster of Differentiation 31
CM	Conditioned Media
CNS	Central Nervous System
CXCR	C-X-C Chemokine Receptor
CYP2D6	Cytochrome P450 2D6
DAB	3'3'-diaminobenzidine
DCIS	Ductal Carcinoma *in situ*
DMSO	Dimethyl Sulphoxide
dNTP	Deoxyribonucleotide Triphosphate
DPX	Di-n-butylphthalate in Xylene
DTT	Dithiothreitol
E1	Oestrone
E2	Oestradiol
E3	Oestriol
ECACC	European Collection of Cell Cultures
ECIS	Electric Cell-Substrate Impedance Sensing
ECL	Enhanced Chemiluminescence
ECM	Extra-cellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
ER	Oestrogen Receptor
ERα	Oestrogen Receptor
ERβ	Oestrogen Receptor
ERE	Oestrogen Response Element
EtBr	Ethidium Bromide
FAK	Focal Adhesion Kinase
FasR	Faslodex Resistant Cell Model
FCS	Foetal Calf Serum

FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
GTP	Guanosine Triphosphate
HER1,2,3,4	Endothelial Growth Factor Receptors 1,2,3,4
HGF	Hepatocyte Growth Factor
HIF-1α	Hypoxia Inducible Factor Alpha
HR-	Hormone Receptor Negative
HR+	Hormone Receptor Positive
HRE	Hormone Response Element
HRP	Horse-radish Peroxidase
HRT	Hormone Replacement Therapy
HUVEC	Human Umbilical Vein Endothelial Cells
IDC	Invasive Ductal Carcinoma
IL-8	Interleukin-8
ILC	Invasive Lobular Carcinoma
MAPK	Mitogen-Activated Protein Kinase
MEK	Mitogen-Activated Protein Kinase Kinase (MAPKK)
MMLV	Molony-murine leukemia virus
MMP	Matrix Metalloproteinases
MTS	2-((4-Azido-2,3,5,6-tetrafluorobenzoyl)amino)ethyl Methanethiosulfonate
NO	Nitric Oxide
p53	Protein 53
PARP	Poly (ADP-ribose) Polymerase
PBS	Phosphate Buffered Saline
PFS	Progression Free Survival
PI3K	Phosphoinositide 3-Kinase
PLC	Protein Lipase C
PMSF	Phenylmethylsulfonyl fluoride
PR	Progesterone Receptor
PRMT1	Protein Arginine N-methyltransferase 1
RH	Random Hexamers
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium dodecyl sulphate
SEM	Standard Error of Mean
SERD	Selective Oestrogen Receptor Downregulator
SERM	Selective Oestrogen Receptor Modulator
SH2	Src Homology 2
SMRT	Silencing Mediator for Retinoid or Thyroid-Hormone Receptors (Nuclear Receptor Co-repressor 2)
SP1	Specificity Protein 1
STAT3	Signal Transducer and Activator of Transcription 3
TamR	Tamoxifen Resistant Cell Model
TBS	Tris-Buffered Saline
TEM-8	Tumour Endothelial Marker 8

TEMED	Tetramethylethylenediamine
TER	Transendothelial Electrical Resistance
TGF-beta1	Transforming Growth Factor Beta
VEGF	Vascular Endothelial Growth Factor
VEGFR2	Vascular Endothelial Growth Factor Receptor 2
VHL	Von Hippel–Lindau

Summary

In the treatment of pre-menopausal women with oestrogen positive (ER+) breast cancer, tamoxifen represents a first line of adjuvant treatment with demonstrable benefits. Despite this, resistance is frequently acquired to tamoxifen with an associated poor prognosis. Breast cancer cell models have revealed the importance of growth factor signalling networks in sustaining growth of endocrine-resistant cancers and, more recently, their ability to promote a highly migratory and invasive phenotype, together with the expression of genes with pro-angiogenic ontology. The potential of endocrine-resistant cells to elicit angiogenic responses, however, remains unknown. Real-time PCR was used to validate results from preliminary Affymetrix-based gene profiling of pro-angiogenic gene expression in endocrine-sensitive MCF7wt cells and their endocrine resistant counterparts. The expression of pro-angiogenic factors in conditioned media (CM) from these cells was assessed by ELISA. The proliferative and migratory effects of conditioned media on vascular endothelial cells (HUVEC and HECV cells), was determined by MTS cell proliferation assay, wound closure assays and Matrigel tubule formation assays. Changes in endothelial cell migration following co-culture with endocrine-resistant cells were examined using Boyden-chamber chemotaxis assays. Growth factor signalling and migration pathway activation in endothelial cells in response to CM was determined by Western blotting. TamR cells were found to express high levels of IL-8 and VEGF at an mRNA level compared with expression in MCF7wt cells. High levels of VEGF protein were also confirmed in the conditioned media from TamR cells versus their endocrine-sensitive

counterparts. TamR conditioned media promoted *in vivo* and *ex vivo* endothelial cell proliferation, as well as *in vitro* endothelial cell migration and the formation of tubules to a greater extent than that seen in MCF7wt CM treated cells. TamR conditioned media was found to stimulate VEGFR2 phosphorylation and downstream activation of MAPK and Akt in endothelial cells compared to MCF7wt CM. Pharmacological inhibition of VEGFR2 activity in endothelial cells suppressed TamR-induced endothelial cell proliferation and VEGFR phosphorylation. Further pharmacological manipulation of Src kinase in TamR cells revealed a Src kinase dependent mechanism of VEGF production in these cells. In addition, *in vivo* examination of TamR xenografts illustrated higher presence of endothelial cells in these tissues than in MCF7wt xenografts. These data suggest acquired tamoxifen resistance is accompanied by development of a Src kinase-dependant pro-angiogenic phenotype which, if recapitulated *in vivo*, may promote tumour progression. Therapeutic targeting of Src signalling may prove beneficial in such cases.

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Chapter 1: General Introduction

1.1 Breast Cancer

Breast cancer represents a heterogeneous group of diseases of the breast characterised by an uncontrolled proliferation of cells that line either the ducts or lobes.

Breast cancer is currently the most common malignancy in women worldwide and is the leading cause of cancer-related mortality (Benson *et al.*, 2009; Clarke, 1996; Gabriel *et al.*, 2005). Globally, more than 1.2 million cases are diagnosed every year (representing 10–12% of the female population) with around 500,000 deaths attributable to the disease. In the UK alone, over 45,000 new cases are diagnosed annually which constitutes a 50% increased incidence of breast cancer over the last 25 years (Office for National Statistics, 2008). Despite a higher prevalence of breast cancer in industrialised than in non-industrialised countries, incidence rates are also steadily increasing in less affluent societies (Porter, 2008). Increased rates of disease diagnosis have been attributed to earlier detection as a result of screening programs coupled with better and more varied treatments, and thus, decreased mortality (D. Maxwell Parkin *et al.*, 2006).

1.1.1 Risk Factors

A number of factors are described that contribute to an increased risk of developing breast cancer. These are varied and include diet, obesity, reproductive history and socioeconomic status. However, the most prominent are age and gender. Incidence rates rise rapidly every 10 years and especially

between the ages of 35 and 39 years, slowing around 50 years, corresponding to the average onset of menopause, and subsequently increase to a maximum after 80 years (Figure 1.1) (Anderson *et al.*, 2005; Benson *et al.*, 2009; Cancer Research UK, 2009a). The incidence of hormone-receptor-negative (HR-) tumours increases rapidly until age 50 years and then levels-off or decreases. In contrast, the incidence of hormone-receptor-positive tumours (HR+) is similar up to the age of 50 years, but then continues to climb at a slower pace. Thus HR- tumours tend to occur earlier in life and HR+ tumours are more common in older women. The peak ages of onset for these two tumour phenotypes are 50 and 70 years of age, respectively, and they seem to have different underlying causes and pathobiology (Benson *et al.*, 2009).
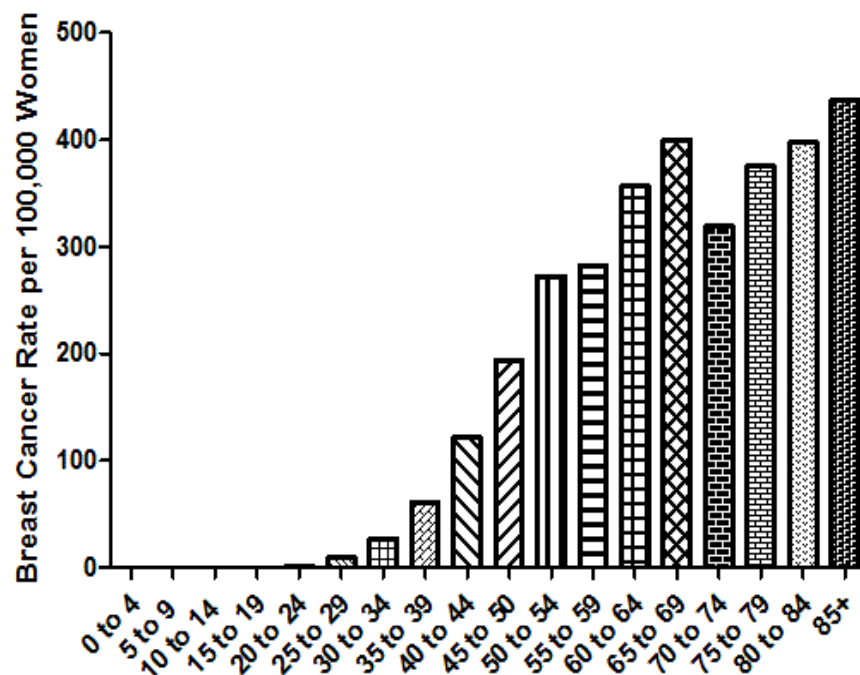
Figure 1.1 Breast Cancer Incidence Rates per 100,000 Women. Adapted from (Cancer Research UK, 2012)

Some dietary factors are implicated in the development of breast cancer including alcohol consumption which has been proposed to increase oestrogen exposure (Nagata *et al.*, 1997). Foods that are high in fat may also contribute to breast cancer by also elevating circulating oestrogen (Boyd *et al.*, 1993).

Exposure to ionising radiation is associated with a higher incidence rate of breast cancer development. In women who survived the atomic blasts in Nagasaki and Hiroshima during the Second World War the incidence rate was very high (Land, 1995). In addition, women who had received mantle radiation therapy in order to treat Hodgkin's lymphoma also had a greater incidence of developing breast cancer (Bhatia *et al.*, 1996). In both cases, the progression to breast cancer was attributed to the exposure to radiation during adolescence when breast tissue is actively developing (Martin *et al.*, 2000).

1.1.1.1 Genetic Factors

Two genes, *BRCA1* and *BRCA2*, which function as tumour suppressors have become recognised as powerful predictors for risk of developing breast cancer. *BRCA1* has been suggested to play a role in a diverse set of cell functions including transcription, cell cycle control and DNA damage repair. It has been shown to associate with RNA polymerase II and to enhance the transactivation of p53 (Anderson *et al.*, 1998). The function of BRCA2 seems less well defined although mutations in the gene are associated with widespread chromosomal rearrangements. In addition, the absence of the gene results in shortening and dysfunction of telomeres (Min *et al.*, 2011).

Women who have an abnormal *BRCA1* or *BRCA2* gene have up to a 60% risk of developing breast cancer; increased risk of developing ovarian cancer is about 55% for women with *BRCA1* mutations and about 25% for women with *BRCA2* mutations (Breastcancer.org, 2010).

1.1.1.2 Racial Differences

There are pronounced racial differences in the incidence and mortality of breast cancer (Byrne *et al.*, 1995). In younger patients breast cancer incidence rates are higher in African-American women, whereas rates are greater in their Caucasian counterparts at 50 years of age and older. Black women have a higher proportion of oestrogen receptor-negative tumours than white women do and are therefore less likely to receive endocrine treatment (Jatoi *et al.*, 2005). Within the Ashkenazi Jewish population, 2.0% – 2.5% of women carry mutant *BRCA1* and *BRCA2* genes and 12% of breast cancers can be attributed to mutations in these genes which has led to increased screening in these populations (Warner *et al.*, 1999).

1.1.1.3 Oestrogen Exposure

One of the most significant risk factors for breast cancer is the exposure to endogenous and exogenous oestrogens over one's lifetime, given the role of the ER as a promoter of breast tumour development and progression. Thus, early menarche (<12 years *vs.* 16 years) and late menopause (>55 years *vs.* <45 years) are associated with a relative risk increase of about 20% (Hankinson *et al.*, 2004). Use of hormone replacement therapy (HRT) with exogenous oestrogen in postmenstrual women has an associated increased risk of

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developing breast cancer reportedly in the region of 35% after 10 years of use, although cancers developing in these women tend to have more favourable prognosis (Colditz, 2005). For this reason it is contra-indicated in survivors of breast cancer as it can double the risk of relapse (Holmberg *et al.*, 2008).

1.1.2 Breast Anatomy

The breasts are mammary glands whose primary function in females is to provide nutrition to infants. They are predominantly made up of adipose and glandular tissue with connective tissue throughout (Ramsay *et al.*, 2005). They are located bilaterally within the subcutaneous layer of the thoracic wall. In terms of anatomy they may be divided into three main sections; the mammary gland itself, the mammary papilla and the areola. The mammary gland (Figure 1.2) consists of lobes, of which there are usually between 15 to 25 arranged in a radial pattern around the areola. The lobes themselves are divided up into lobules which are the functional units of milk production. The lactiferous duct describes the principal duct of each lobe which drains into the mammary papilla (Drake, 2005).

Cancers of the breast are most commonly found to originate in either the lobules or in the ducts. The area between the lobes is primarily made up of adipose and connective tissue (Drake, 2005). Fascicles of connective tissue, known as Cooper's ligaments, run anteroposteriorly from the clavicle, permeating the lobes and lobules and giving structure and support to the breast tissue. Breast cancer can affect these ligaments and cause them to pull on the skin causing a characteristic dimpling of the surface of the breast (Figure 1.3).
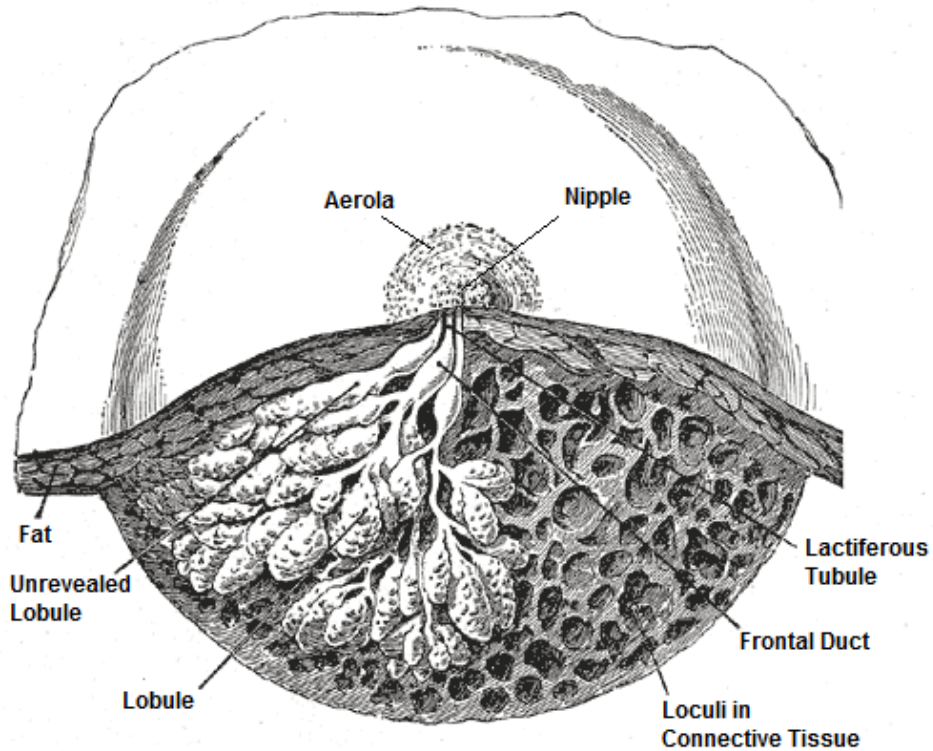
Figure 1.2 Tissue anatomy of right breast. Absence of skin and subcutaneous layer allows observation of the lobes, frontal ducts and lactiferous tubules. Image adapted from Gray's 20th Edition.
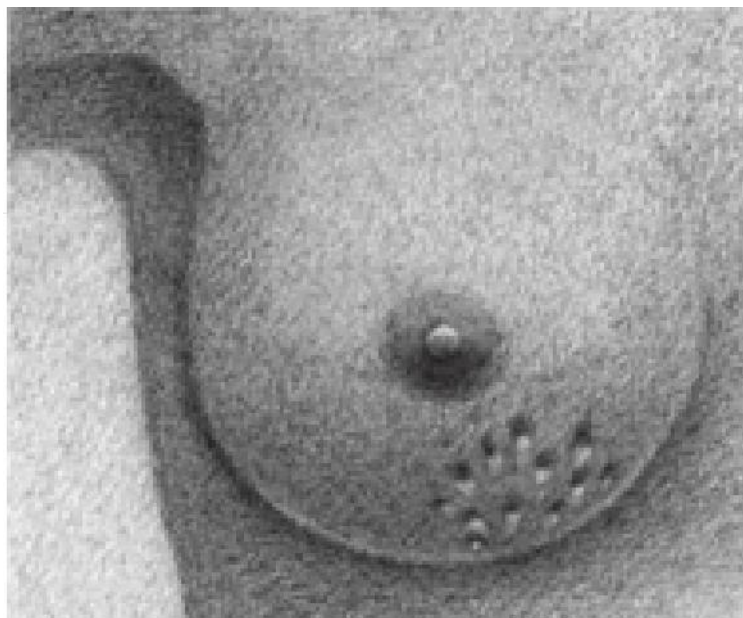
Figure 1.3 Skin Dimpling as a Result of Carcinoma Proximal to Coopers Ligaments. Image freely available under Creative Commons, no rights reserved.

1.1.3 Breast Cancer Subtypes, Classifications and Staging

As mentioned previously, breast cancer is a heterogeneous disease and as a consequence, no one treatment option is sufficient for all breast cancers. Thus the treatment must be adapted to match the clinical tumour subtype. The majority of breast cancers arise from epithelial tissue (carcinomas) or from the glandular epithelial tissue itself (adenocarcinoma). More rarely, cancers of the connective tissue of the breast may develop which are referred to as angiosarcomas (Vorburger *et al.*, 2005). Classification systems for breast cancers are commonly used to describe properties found during diagnosis and form a method of devising treatment regimens based on these properties. These methods of classification also play an important role as different breast cancer subtypes have different prognoses. Thorough classification often involves documenting the tumour histopathology, grade, stage, presence of specific receptors and mutations in known DNA markers. Classification of tumours is now based on how far the tumour cells have differentiated from their tissue of origin and organises them into the following categories.

1.1.3.1 Carcinoma *in situ*

Ductal Carcinoma *in situ* (DCIS) is the most common form of non-invasive breast cancer, representing 20% of diagnosed breast cancers (Sanaz A, 2011). Ductal refers to its origin within the breast where, although it is hyperplastic, it is localised to the duct and does not invade into the surrounding tissue, hence *in situ* (Allred, 2010). Lobular Carcinoma *in situ* behaves in a similar fashion in that the cells remain confined within the affected lobule.

1.1.3.2 Inflammatory Breast Cancer

Frequently, presentations of localised breast inflammation are subsequently diagnosed as an aggressive form of breast cancer termed inflammatory breast cancer. These cancers account for 2.5% of all diagnosed and are characterised by high incidence of metastases, both local and distant, rapid progression and low overall survival (Robertson *et al.*, 2010). Other forms of breast cancer are rarer, accounting for less than 1% of incidences and include male breast cancers and tumours of the nipple.

1.1.3.3 Invasive Ductal or Lobular Carcinoma

The majority of breast cancers at diagnosis are either of invasive ductal carcinoma (IDC) or invasive lobular carcinoma (ILC) type. These cancers are not confined to their site of origin and have invaded into the surrounding stroma. IDC is the most common type of breast cancer with an incidence rate of about 55% (Eheman *et al.*, 2009), whereas ILC only accounts for 8 – 15% (Pointon *et al.*, 1999).

1.1.3.4 Clinical Classification

There are a number of dominant clinical subtypes of invasive ductal breast cancers, characterised primarily by the expression of specific receptors. The presence of these receptors not only defines the subtype but has a significant bearing on which drug treatments are likely to prove effective. In breast cancer, as in others such as ovarian and prostate cancer, they can respond to the action of various hormones and deprivation of this signalling can reduce the growth of these tumours. In breast cancer, hormone status usually refers to the

expression of oestrogen-receptor alpha (ERα) and the progesterone receptor (PR). Approximately 60 – 70% of breast cancers present as ER+ and are associated with an overall better prognosis, therefore the presence or absence of detectable ER is an important prognostic indicator. In addition, HER2 status is a widely used marker in breast cancer classification. Outlined below (Table 1.1) are the major clinical subtypes and their associated five year overall and disease free survival percentages (Onitilo et al., 2009).

Subtype	Overall Survival	Disease-free Survival
ER/PR+, HER2+	88.7%	83.2%
ER/PR+, HER2-	90.3%	86.8%
ER/PR-, HER2+	78.8%	66.0%
ER/PR-, HER2-	79.0%	73.5%

Table 1.1 Five year overall and disease-free survival by tumour subtype. ER = oestrogen receptor, HER2 = human epidermal growth factor receptor 2, PR = progesterone receptor, + = positive, - = negative. Adapted from (Onitilo et al., 2009)

In addition to these three main breast cancer markers, the nuclear antigen, Ki-67 is being trialled as a potential fourth marker. This protein is expressed during proliferation but not while a cell is in G0. Tumours which demonstrate high Ki-67 result in higher disease reoccurrence and shorter survival times and so it has become widely reported as possessing good prognostic value (Yerushalmi et al., 2010). In terms of predicting response, some studies have shown it may have value in predicting benefit of certain chemotherapy regimens in some ER+ breast cancers (Penault-Llorca et al., 2009) as well as in ER- tumours, although

this may be as a result of increased proliferation in these tumours (Jones *et al.*, 2010).

1.1.3.5 Molecular Markers

A further level of complexity regarding classification of breast cancer is suggested from studies that have determined the genetic profile of breast cancer cell lines and tissue. Initial approaches (e.g. that described by Charles M. Perou (2000)), using array analysis of tumour gene signatures, sought to classify tumours based on the clustering pattern of gene expression with the subsequent identification of several molecular subtypes of breast cancer (Perou *et al.*, 2000). These markers include HER2, Ki-67, *c-myc*, p53 and E-cadherin. Following on from this work, five dominant molecular subtypes have been identified.

The luminal A and luminal B subtypes are both ER positive and share molecular characteristics of the luminal cells that surround the mammary ducts. However, whereas luminal A is associated with good prognosis, luminal B is associated with a much poorer prognosis. The explanation behind why luminal B cancers have a worse prognosis when both they and luminal A cancers are ER+ has been attributed to; the relative low expression of PR and the fact they are highly proliferative (Creighton, 2012). HER2 sub-type tumours, those that overexpress the HER2 receptor, occur as 25% of breast cancer tumours and are associated with a more aggressive phenotype, and a poor prognosis (Hicks *et al.*, 2008). Tumours of the basal subtype originate from the basal layer of the breast epithelium but these cells are generally ER and PR negative and do not

overexpress HER2 (these are often termed triple negative). Finally, the normal-like subtype expresses increased levels of basal-genes but reduced expression of luminal associated genes (Sørlie *et al.*, 2001; Sørlie *et al.*, 2003). The normal-like subtype accounts for 6-10% of breast cancers, they tend to be small in diameter and be associated with a good prognosis (Perou *et al.*, 2000).

A novel approach to molecular classification of tumours was published this May in which Curtis *et al.* proposed that by assessing single-nucleotide polymorphisms, in addition to copy number aberrations and copy number variants, that genetic profiles of breast cancers resolve into ten potential "integration clusters" some of which e.g. IntClust5 are associated with a poor prognosis clinically (Curtis *et al.*, 2012).

Although currently, the classification of tumours based on molecular subtypes is of greatest use in research and has not been implemented into standard medical practice, it represents an important area of study in the context of personalised medicine.

1.1.3.6 Stage and Grading for Prognostic Information

Histopathology classifies tumours based on observations made by a technician when examining biopsy material taken from the patient in addition to information from the physical exam and imaging of the breast. Methods for analysing these observations vary between institutions; however, one commonly used system is the TNM Classification of Malignant Tumours. TNM stands for 'Tumour, Node and Metastasis' where Tumour refers to the size and location of tumour, Node

describes if there has been any involvement of lymph nodes and Metastasis refers to the presence or absence of secondary tumour sites.

Tumour size is an important factor as generally the smaller the tumour is on initial examination, the better the prognosis for the patient. The score assigned to "T" can also describe spread of the tumour towards the skin or the chest wall.

Lymph node involvement, donated by the letter "N" is concerned with the presence or absence of relatively large numbers of cancer cells being detected in the lymph nodes (~200 cells). However, as technology and our understanding of tumour progression evolve, we possess the capability to detect isolated tumour cells using immunohistochemistry and PCR techniques.

Metastasis, designated "M", uses information from imaging studies to determine the presence of secondary tumour sites in the blood, bone marrow or distant organs. For each of the letters T, N and M a number is assigned which donates severity and when taken together can help direct treatment and predict prognosis (Cancer Research UK, 2009b; Güth *et al.*, 2007). Using the Nottingham Criteria each feature is assigned a score between 1 and 3 (with 1 being the best and 3 being the worst) then combining these values to give a total score (Bloom *et al.*, 1957; Elston *et al.*, 1991). Depending on the score, tumours are assigned into one of three grades; Score 3-5 Low Grade (I), Score 6-7 Intermediate Grade (II), or Score 8-9 High Grade (III). If a tumour presents as well differentiated, low grade tumour it will have a better prognosis than those that are poorly differentiated and therefore high grade (Balslev *et al.*, 1994).

1.1.4 Current Treatments in Breast Cancer

First-line treatment for many forms of breast cancer is surgical excision of the primary tumour (or more radically, mastectomy) followed by radiation therapy to remove secondary tumour sites and stray cancer cells. In women with slowly progressive ER+ metastatic breast cancer, endocrine therapy is given rather than chemotherapy to target remaining cells. This recommendation is based upon reduced toxicity of endocrine treatment, and generally longer durations of response as compared to cytotoxic chemotherapy, with no difference in overall survival (Wilcken *et al.*, 2003). Occasionally, neoadjuvant therapy is carried out to reduce the size of tumours and increase the success of surgical intervention.

1.1.4.1 HER2+ breast cancer

The human epidermal growth factor family of receptors consists of four members, HER1 (EGFR2), HER2, HER3 and HER4. They are bound by a variety of growth factors which are outlined below (Figure 1.4) with the consequence of receptor activation being an array of functional and cell survival related processes (Hynes *et al.*, 2005). The *HER2* gene is known to be amplified in around 25% of invasive breast cancers leading to significant overexpression of the HER2 receptor on these cells where it is associated with poor outcomes. (Slamon *et al.*, 1987). In light of this, a number of approaches to target HER2 in the clinic have been developed. The humanised monoclonal antibody trastuzumab (Herceptin) binds to the extracellular domain of HER2 and results in reduced downstream activation leading to cell cycle arrest. In a clinical setting, trastuzumab has anti-tumour activity as a monotherapy and in

conjunction with chemotherapeutics (Inoue *et al.*, 2010; Marty *et al.*, 2005). Despite its success, resistance to trastuzumab therapy will commonly develop after a year of treatment, with a number of mechanisms suggested to underlie resistance including cross talk between HER2 and other HER family members and increased signalling through PI3K and Src kinase dependent pathways (Berns *et al.*, 2007; Muthuswamy, 2011; Nahta *et al.*, 2005).

In addition to therapy with trastuzumab, the dual HER2 and EGFR inhibitor, lapatinib (Tykerb), has demonstrated potent anti-tumour effects in both preclinical (Rusnak *et al.*, 2001) and clinical studies (Baselga *et al.*, 2012; Konecny *et al.*, 2006) as a monotherapy and in combination therapies. It has shown significant improvement in time to progression when added to treatment regimens with chemotherapeutic agents such as capecitabine (Cameron *et al.*, 2008). Novel therapies for HER2- overexpressing breast cancers include trastuzumab-DM1 (T-DM1), which is a conjugate of the antibody trastuzumab to a cytotoxic, mertansine. In preclinical studies it has been shown to have potent anti-tumour effect on HER2 overexpressing cells as well as trastuzumab and lapatinib resistant models (Junttila *et al.*, 2011).
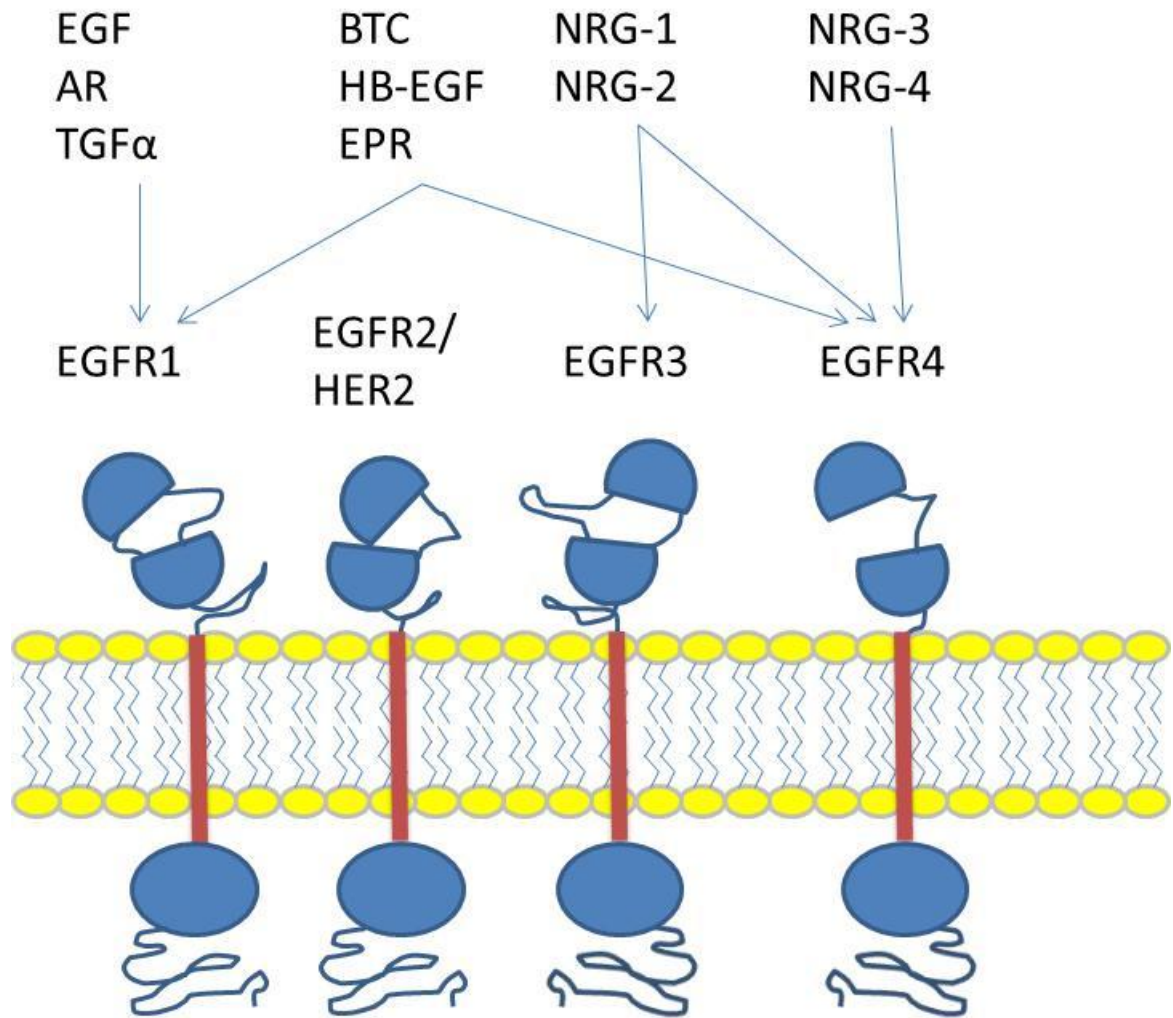
Figure 1.4 EGFR ligands and their receptors. As shown here, no ligand directly binds HER2. The other receptors form homo- and heterodimers on ligand binding and HER2 can heterodimerise with any of these. This results in autophosphorylation of tyrosine residues in the cytoplasmic domain of the receptor, leading to initiation of downstream signalling. Adapted from (Marmor *et al.*, 2004)

1.1.4.2 Triple Negative Breast Cancer

Tumours that do not express both the oestrogen and progesterone receptors and also have low or non-amplified expression of HER2 are known as triple-negative breast cancers. Due to the absence of these proteins, these cancers are resistant to most targeted therapies and are frequently associated with a very aggressive phenotype and a poor prognosis (Dent *et al.*, 2007). Due to a lack of molecular targets current treatment options include chemotherapeutics such as the DNA-targeting cytotoxic cisplatin and PARP inhibitors. More recently, EGFR inhibition is being investigated as a potential therapeutic option with some success (Naoto T. Ueno, 2011; O'Shaughnessy *et al.*, 2011; Silver *et al.*, 2010).

1.1.4.3 Chemotherapy

There are several different chemotherapy regimens that are used in the clinic at the moment. Most of which are combination therapies typically employing an anthracycline agent such as doxorubicin and a taxane such as docetaxel (von Minckwitz, 2007). Although this is widely used, there remain challenges with increased toxicity in combining agents. The addition of the nitrogen mustard cyclophosphamide was introduced after a successful phase 3 trial carried out by the Breast Cancer International Research Group in 1997 that referred to the three drug combination as TAC (Taxane Anthracycline Cyclophosphamide). The trial illustrated that the TAC improved disease free-survival time when compared to the previous chemotherapy regimen (FAC) consisting of anthracycline and cyclophosphamide with fluorouracil replacing taxane use

(Nabholtz *et al.*, 2002). Further study has confirmed that TAC does provide a better therapeutic advantage over use of FAC; however, this comes with increased toxicity. Incidence of neutropenia and congestive heart failure is higher in patients on the TAC regimen than on FAC. As a result of the lack of tissue specificity and high incidence of toxicity, the development of targeted therapies in the treatment of breast cancer has become a major research goal.

1.1.4.4 Anti-oestrogen Therapy

Oestrogens are a class of steroid hormone that play a vital role in the regulation of the female oestrous cycle in humans and are the primary female sex hormone. These hormones function in the regulation of ovulation, pregnancy and lactation. In addition, they also play a role in maintaining proper function of the immune system, CNS, bone and the cardiovascular system, both in males and females. There are three family members of oestrogens, oestrone (E1), oestradiol (E2) and oestriol (E3) with the primary functional oestrogen in pre-menopausal women being oestradiol.

Oestrogens bind the oestrogen receptor (ER) which has two isoforms alpha (ERα) and beta (ERβ) (Katzenellenbogen *et al.*, 2000). These receptors function as transcription factors and so regulate the expression of a variety of genes.

The two oestrogen receptor subtypes have different localisations with ERα and ERβ being expressed predominantly by cells in both male and female reproductive tracts, ERα has also been detected in the female breast, endometrium and hypothalamus whereas ERβ has been detected in several of

the major organs, endothelial cells and the intestine of both males and females (Couse *et al.*, 1997). As the oestrogen receptors are steroidal hormone receptors, they are not confined to the cell membrane and can bind oestrogen in the cytosol. On activation by oestrogen (Figure 1.5) this receptor forms a dimer that can be either a homodimer (ERαα/ERββ) or a heterodimer (ERαβ). On binding to oestrogens, the receptor translocates into the nucleus where it dimerises and can then bind to specialised promoter regions on DNA, termed oestrogen response elements, the activation of which leads to the transcription of gene sequences for a plethora of proteins. In addition to binding ER in the cytosol and inducing effects through transcription of genes, oestrogen can also bind ER in the cell membrane and induce signal transduction through second messenger systems including MAPK, PI3K, AKT, cAMP and through intracellular calcium changes (Klinge *et al.*, 2005; Marino *et al.*, 1998; Morley *et al.*, 1992).
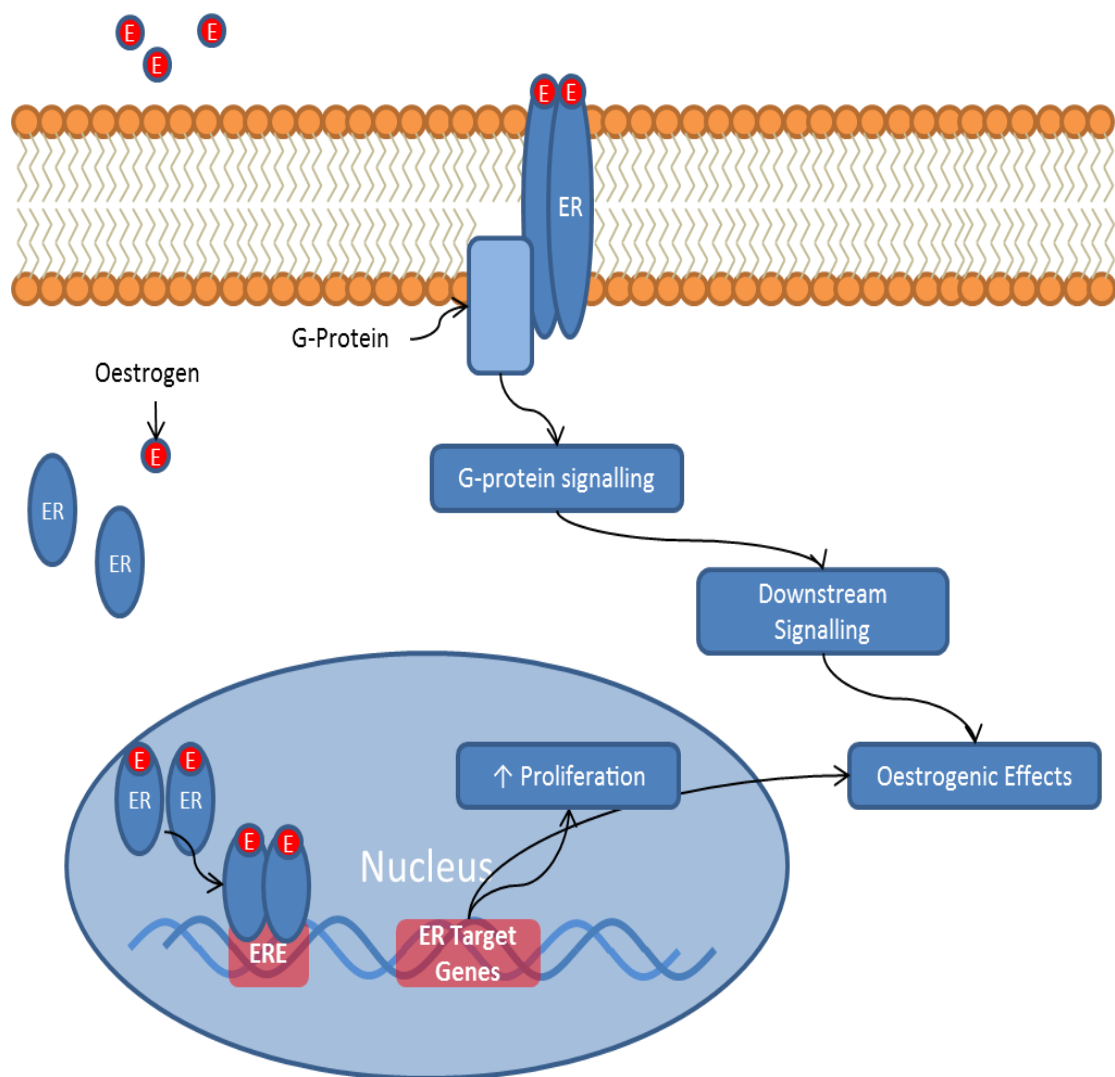
Figure 1.5 Genomic and non-genomic activity of oestrogen receptor binding.

In the absence of oestradiol, ER remains complexed to chaperone proteins such as HSP90. Binding of E2 results in receptor phosphorylation followed by dimerisation before the receptor translocates to the nucleus. Here it binds to the oestrogen response elements of oestrogen regulated genes and recruits co-repressors and co-activators of the gene which can increase or decrease gene expression.

Phosphorylation of membrane bound ER at Y357 causes complex formation of the ER with G proteins and non-receptor tyrosine kinases such as Src and PI3K. Resulting signalling through MAPK and Akt leads to oestrogenic effects such as proliferation and cell survival.

Ultimately, the ability of E2 to promote the growth of ER+ breast cancers creates the therapeutic option to antagonise ER-E2 interaction for breast tumours that express the receptor. Endocrine therapy is applied in two ways; ablative therapy to remove the source of the oestrogen and additive therapy to act upon the cancer itself to prevent the proliferative action of oestrogen, primarily through antagonising the oestrogen receptor.

Ablation of ovarian function is carried out by bilateral oophorectomy, hormone manipulation or by radiation therapy directed at the ovaries; however this treatment is only suitable for pre-menopausal women. In post-menopausal women the majority of oestrogen is synthesised by the adrenal glands. In addition, oestrogen may be synthesised by enzyme conversion of circulating hormones, such as testosterone and androstendione into oestrogen and oestrone. The enzyme aromatase is responsible for this reaction and therefore presents a further target in the manipulation of oestrogen synthesis (Macedo *et al.*, 2009). The three main classes of endocrine agents that are used in breast cancer therapy are summarised in Table 1.2.

Class	Mechanism of Action	Example
Selective Oestrogen Receptor Modulator	Binds to ER and has a mixed agonist/antagonist effect	Raloxifene, tamoxifen
Selective Oestrogen Receptor Modulator	Binds to ER with a pure antagonistic effect	Fulvestrant
Aromatase Inhibitor	Irreversibly bind aromatase enzymes (Steroidal) or reversibly compete for enzyme (Non-steroidal)	Exemestane (Steroidal), Anastrozole (Non-Steroidal)

Table 1.2 Classes of anti-oestrogen used in ER+ breast cancer therapy.

1.1.4.4.1 Selective Oestrogen Receptor Modulators

Blockade of the ER receptor in ER positive tumours elicits a dramatic response in restricting growth of primary tumours as well as those that have metastasised (Lønning, 2004; O. Abe, 2005). Tamoxifen was initially developed as a novel contraceptive candidate (Jordan, 2003) and it was not until the mid-1970s that development began in earnest of tamoxifen as an adjuvant treatment for ER+ breast cancer.

The action of tamoxifen differs depending on the tissue on which it is acting. In breast tissue, it acts as an ER antagonist resulting in inhibition of oestrogen-dependent growth. In contrast, the action of tamoxifen in other tissues, such as the endometrium, is one of an ER agonist, with resultant stimulation of cellular proliferation hence tamoxifen is dubbed a "selective oestrogen receptor modulator" (SERM). These tissue-specific properties are thought to be as a result of coregulatory proteins expressed differently by specific tissue types. These coregulators may be co-activators which increase transcription of specific

genes regulated by oestrogen response elements or co-repressors which suppress expression (Graham *et al.*, 2000). There is a small increase in risk of developing endometrial cancer with tamoxifen (Fisher *et al.*, 1994), however in many cases the benefits outweigh the risks. As the target of tamoxifen is the ER, tumours that are ER negative do not respond well to treatment, however, even those that are weakly ER positive (<1% of ER+ breast cancers) still show substantial response (Early Breast Cancer Trialists' Collaborative, 2011; Qureshi *et al.*, 2010).

Tamoxifen is metabolised in the liver by enzymes CYP2D6 and others to its active metabolites 4-hydroxytamoxifen (Figure.1.6) and N-desmethyl-4-hydroxytamoxifen (endoxifen). After binding to the oestrogen receptor, the nuclear complex still binds to oestrogen response elements on DNA. This way, tamoxifen and its metabolites not only competitively inhibit the binding of oestrogen but recruit co-repressor proteins including nuclear receptor co-repressor 1 and 2, to further inhibit typical oestrogenic action (Hoskins *et al.*, 2009; Shang *et al.*, 2000).

Treatment with tamoxifen remains a popular treatment option for women who have significant ovarian oestrogen production. Typical treatment regimens last for five years with studies showing that reduced mortality rates in this treatment group when compared to patients that have not received adjuvant tamoxifen, last well beyond 10 years from the beginning of treatment (Early Breast Cancer Trialists' Collaborative, 2011).

Tamoxifen
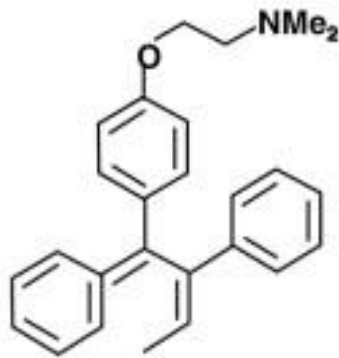
4-hydroxy-tamoxifen
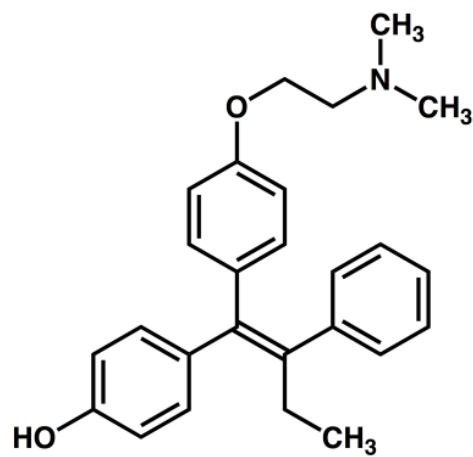
Figure 1.6 Structures of the anti-oestrogen tamoxifen in comparison to its more active metabolite 4-hydroxytamoxifen. Adapted from (Osborne *et al.*, 2004)

1.1.4.4.2 Selective Oestrogen Receptor Downregulators

The unwanted agonistic effects of tamoxifen on uterine tissues created a necessity for anti-oestrogen therapies that lacked these potentially problematic effects (Fornander *et al.*, 1993). Subsequently, the "pure" anti-oestrogen fulvestrant (Wakeling *et al.*, 1991) was developed. Fulvestrant represents a class of anti-oestrogens known as 'SERDs' (selective oestrogen receptor downregulators), so termed due to their ability to bind to, and promote the degradation of, the oestrogen receptor (Howell, 2002).

In preclinical testing, fulvestrant showed: a higher affinity for the ER than tamoxifen, no oestrogenic activity in the uterus and suppression of tumour growth in mice for a longer period than tamoxifen (Wakeling *et al.*, 1991). A further advantage of this agent was that it appeared to be effective in ER+ breast cancer cells that had acquired resistance to tamoxifen (Coopman *et al.*, 1994). In the clinic, fulvestrant resulted in reduced detectable markers of cell cycle proliferation including pS2 and Ki-67 (DeFriend *et al.*, 1994) and, in contrast to tamoxifen, no evidence was seen of ovarian hyperstimulation or elevation in endometrial thickness (Thomas *et al.*, 1994). Current trials are focusing on the use of fulvestrant as a monotherapy and in conjunction with other targeted therapies in postmenopausal women with ER positive breast cancer. Initial data support a role for fulvestrant in combination with anastrozole and gefitinib (Massarweh *et al.*, 2011; Oakman *et al.*, 2011; Pritchard *et al.*, 2010).

1.1.4.4.3 Aromatase Inhibitors

In premenopausal women, the ovaries are the main source of oestrogen production. In postmenopausal women, however, oestrogen production is more abundant in other tissues including breast and adipose tissue (Nelson *et al.*, 2001). These tissues contain the aromatase enzyme which is responsible for the conversion of androgens to oestrogens. Thus, in postmenopausal women the aromatase enzyme represents a potential therapeutic target through which the oestrogenic environment of the tumour can be perturbed which may lead to suppression in tumour growth.

Development of aromatase-inhibiting compounds commenced in the early 1980s with the discovery of aminoglutethimide. However the poor specificity for aromatase of this compound brought about the need for more specifically-targeted drugs (MacNeill *et al.*, 1992). The second generation aromatase inhibitors (AIs) included compounds such as formestane; however these compounds did not block aromatase action sufficiently to produce a therapeutic benefit. The third and currently used generation of AIs includes the compounds anastrozole and exemestane which have both high selectivity and potency in inhibiting aromatase (Howell A, 2005). These third generation compounds can be further subdivided into steroidal and non-steroidal aromatase inhibitors. Steroidal AIs such as exemestane irreversibly bind aromatase which results in its degradation. Non-steroidal AIs such as anastrozole and letrozole reversibly bind aromatase (Geisler, 2011). At the moment there is little evidence regarding which aromatase inhibitor is best for any specific breast cancer subtype (Schneider *et al.*, 2011). Results from clinical trials using these compounds as

monotherapy or as a second line treatment after failure on tamoxifen, have shown they provide clinical benefit and seem to be superior to use of tamoxifen monotherapy in post-menopausal women who have localised breast tumours that are ER+ (Baum *et al.*, 2002; Goss *et al.*, 2003).

1.1.4.4.4 Contrasting Tamoxifen and Aromatase Inhibitors in the Treatment of Breast Cancer in Postmenopausal Women

Despite both tamoxifen and anastrozole being indicated for use in hormone positive breast cancer in either post-menopausal women or those that have received ovarian ablation therapy, the results of the now famous ATAC trail cemented the preferential use of anastrozole over tamoxifen. This was as a result of the observed increase in disease free survival, time-to-reoccurrence and reduced incidence of distant metastasis development and contralateral breast cancers with the use of anastrozole as well as a documented reduction in the reporting of side-effects (Howell A, 2005). Similar data has been collected by the TEAM trial examining tamoxifen versus exemestane with increases in disease free survival when exemestane was combined with tamoxifen or when exemestane was used as a monotherapy (van de Velde *et al.*, 2011). In pre-menopausal women, and in those with functioning ovaries that produce oestrogen, tamoxifen is still indicated as a first line adjuvant.

1.2 Endocrine Resistance

Despite the clinical effectiveness of endocrine agents in the treatment of ER+ breast cancer, the success of these agents is limited by the phenomenon of resistance. About 60% of ER+ breast cancer patients who initially respond to

tamoxifen will require further therapy as a result of recurrence or metastasis. Around a third of early breast cancers and almost all advanced breast cancers, initially responsive to tamoxifen, will relapse (acquired resistance) (Horobin *et al.*, 1991; Ring *et al.*, 2004) whilst a significant minority of tumours will fail to respond to endocrine agents despite being hormone receptor positive (*de novo* resistance). A number of important mechanisms have been suggested to underlie *de novo* and acquired resistance which are discussed in the following sections.

1.2.1 *De novo* Resistance

Intrinsic resistance to endocrine treatment in ER+ breast cancer may arise due to reduced expression of ERα. Additionally, reduced expression of the cytochrome p450 family enzyme, CYP2D6, responsible for the metabolism of tamoxifen into its active metabolite, 4-OH-tamoxifen may reduce the efficacy of tamoxifen therapy (Hoskins *et al.*, 2009).

1.2.2 Acquired Resistance

In ER+ tumours that initially respond to treatment with tamoxifen, two-thirds of these patients will eventually relapse with anti-oestrogen resistant tumours. This resistance is therefore a major issue clinically. Initially, 90% of primary breast cancers and 50% of metastases are effectively treated by anti-oestrogens as demonstrated by reduced tumour volume, improved symptoms, and decreased circulating tumour markers. However, after a variable period progression occurs and is commonly accompanied by resistance to treatment (Gonzalez-Angulo AM, 2007) despite continued expression of ERα (Riggins *et al.*, 2007).

By determining the mechanisms by which cancer cells become resistant to anti-oestrogen therapy we can begin to develop methods of preventing its occurrence.

1.2.2.1 ER

Loss of expression or function of ERα during treatment can confer tamoxifen resistance, however this only occurs in a minority of cases (~15%) (Gutierrez *et al.*, 2005). Many tumours retain expression of ER (both ERα and ERβ) indicating that it may be more common for oestrogen receptors to lose function possibly due to mutations in the genes that encode the receptor (Ring *et al.*, 2004). Mutation studies have illustrated that amino acid substitution within the ER can lead to normal expression of the receptor but with a loss of functionality in terms of 4-hydroxytamoxifen binding or the ability of the ER to mediate transcription of ERE regulated genes (Mahfoudi *et al.*, 1995; Schafer *et al.*, 2000). However this is not a common occurrence and so does not account for the majority of acquired tamoxifen resistance (Karnik *et al.*, 1994). In addition to genetic changes, epigenetic modifications such as CpG island hypermethylation of ERα can cause inactivation of ER gene transcription. The problem clinically therefore, is that ERα gene expression may not relate to ERα function.

Following the discovery of a second ER receptor, ERβ, studies have shown that its gene expression is elevated in patients with acquired tamoxifen resistance compared with tamoxifen-sensitive patients (Speirs *et al.*, 1999), suggesting that ERβ represents a factor for predicting poor prognosis, data which has been corroborated by another group who reported that expression of ERβ is

associated with a more aggressive tumour phenotype (Novelli *et al.*, 2008). Additionally, ERβ predicted response in patients who were determined to be ERα- but ERβ+, illustrating that ERβ modulation of oestrogenic activity is sufficient to illicit a therapeutic response in the absence of ERα activity (Honma *et al.*, 2008).

In pre-menopausal women tamoxifen treatment results in an increase in the levels of oestrogen (Ravdin *et al.*, 1988). Although this could hypothetically limit the capability of tamoxifen to bind the ER by saturating the receptor with oestrogen, this has not been shown to be linked with acquisition of resistance (Ring *et al.*, 2004).

1.2.2.2 Co-factors

As oestrogen function can also be modulated by interactions with co-activators (such as AIB1) and co-repressors (such as SMRT) (McKenna *et al.*, 1999), the alteration of these proteins in acquired tamoxifen resistance can further alter the outcome of receptors binding to the ERE (Smith *et al.*, 2004). Elevated AIB1, for example, has been associated with a better outcome in patients who have not received adjuvant tamoxifen, but a poorer outcome in those who did (Osborne *et al.*, 2003). It has been hypothesised that these activators function to increase agonistic activities of tamoxifen and acquisition of resistance (Ring *et al.*, 2004). Furthermore, changes in post-translational modifications that regulate ERα function that affect how ER interacts with transcriptional co-regulators and other signalling molecules have been shown to contribute to acquired endocrine resistance (Musgrove *et al.*, 2009).

Finally, altered expression of classical effectors of apoptosis and cell survival can predict a poor response to anti-oestrogen treatment, these include Bcl-2, p53, c-Myc and Bad (Cannings *et al.*, 2007; Linke *et al.*, 2006; Planas-Silva *et al.*, 2007).

1.2.2.3 Growth Factor Receptor Crosstalk

One mechanism by which resistance to endocrine therapies has been suggested to develop occurs via crosstalk between growth factor signalling pathways and the ER. Studies investigating the relationship between ER and HER2 signalling have illustrated that the crosstalk between these systems plays a pivotal role in the development of resistance to ER targeted therapies (Arpino *et al.*, 2008). Tumours that are initially responsive to tamoxifen tend to have low levels of HER2 and EGFR. However, on acquisition of tamoxifen resistance such tumours are found to have elevated levels of these receptors which can lead to increased EGFR/HER2 pathway activity (Gutierrez *et al.*, 2005; Meng *et al.*, 2004). *In vitro* studies have illustrated that tamoxifen resistant cells in culture express elevated EGFR and HER2 with an accompanying increase in activation of these receptors as well as their downstream components including MAPK and AKT (Nicholson *et al.*, 2004). The correlation between this increase in second messenger signalling and the acquisition of tamoxifen resistance is not fully understood but it has been suggested that contributing factors include: decreased ERα expression, mediated by increased MAPK signalling; loss of EGFR and HER2 repression by ER activity; increased mitogenic signalling; inhibition of apoptosis and ligand-independent activation of the ER (Musgrove *et*

al., 2009). Additionally, overexpression of proteins like the Src family of tyrosine kinases in breast cancer has been associated with the acquisition of resistance. Src kinase forms a phosphorylation dependent signalling complex with Focal Adhesion Kinase (FAK) and BCAR1 that results in increased cell survival (Cowell *et al.*, 2006). When overexpressed *in vitro*, the BCAR1 protein of this complex can confer tamoxifen resistance to cells and conversely deletion of the substrate domain of the protein confers enhanced tamoxifen sensitivity (Dorssers *et al.*, 1993).

Data from our in house cell models has revealed that acquired anti-oestrogen resistance is associated with the development of an aggressive phenotype with demonstrable increases in invasion and migration. We hypothesise that these alterations in behaviour are mediated by an increased level of Src kinase activity on acquisition of tamoxifen resistance (Hiscox *et al.*, 2006b). These findings may explain why clinical observations of high Src kinase is associated with a poor prognosis (Morgan *et al.*, 2009). Further studies have demonstrated that acquired tamoxifen resistance influences tumour cell interaction with neighbouring cell types. Other studies have illustrated that development of resistance to the anti-oestrogen fulvestrant is accompanied by an elevated expression of the HGF receptor c-Met *in vitro* (Hiscox *et al.*, 2006a). Additionally, tamoxifen resistant cells have enhanced adherence to endothelial cells (Wadhawan, unpublished data) and increased Src kinase signalling in tamoxifen resistant cells can uncouple FAK activation from an adhesion dependent process. All these elements are important in metastasis and suggest that the process of acquired resistance may ultimately produce cells that have

an augmented capacity to influence their microenvironment. One aspect of this that remains to be explored is whether acquired resistance alters the angiogenic capacity of breast cancer cells.

1.3 Angiogenesis

In addition to uncontrolled growth of cells at the site of the primary tumour, many breast cancers can also be characterised by an increased invasiveness and the development of metastasis to proximal and distant sites (Suva *et al.*, 2009). In 70% of these cases where patients die from their cancer, metastases are found within bones (Coleman, 2006) and in ~15% of these there is evidence of central nervous system and brain metastases (Patil et al., 2007).

Metastasis is the single most important factor affecting mortality in cancer patients. Over 90% of cancer-related mortality is due to metastatic disease rather than problems associated with primary tumour growth (Weigelt et al., 2005). Metastasis is facilitated by an established vascular network through which tumour cells can migrate to distant sites resulting in the establishment of new tumour sites.

A vascular network is an essential component to facilitate the supply of oxygen and nutrients to tissue as well as playing an important role in homeostasis and removal of waste. Angiogenesis is the process by which new vasculature is developed from pre-existing vasculature; this differs from vasculogenesis, which is the process by which a vascular network is spontaneously developed.

The process of angiogenesis can be subdivided into two categories depending on how the vessel development occurs. If new vasculature develops from pre-existing endothelial cells and "sprouts" into the surrounding tissue it is referred to as "sprouting angiogenesis" (Adair *et al.*, 2010). This form of vascular development from pre-existing vessels was the first recognised form of angiogenesis and its process is well characterised. Initially, growth factors that function in stimulating angiogenesis (angiogenic factors) activate receptors on endothelial cells that are present on pre-existing vasculature. These endothelial cells then release enzymes (proteases), the function of which is to disrupt the basement membrane and allow migration of endothelial cells away from the parent vessel (Lamalice *et al.*, 2007) . Migrating endothelial cells proliferate in the extracellular matrix (ECM) towards the higher ECM concentration of angiogenic factors (chemotaxis) but are also drawn by haptotaxis, which causes cells to migrate towards an increasing concentration of immobilised ligand, which in the case of endothelial cell migration are the components of the ECM to which endothelial cell integrins bind (Giroux *et al.*, 1999). Finally, evidence suggest that mechanotaxis also plays a role as fluid stress on endothelial cells on the surface of the inner vessel wall can stimulate migration away from the parent vessel (Li *et al.*, 2005).

In contrast, splitting, or intussusceptive angiogenesis, occurs when the capillary wall extends into the lumen and splits a single pre-existing vessel into two (Paku *et al.*, 2011).

Angiogenesis is stimulated in conditions of oxygen deprivation which triggers induction of factors regulated by hypoxia-inducible factor-1α which in turn initiates production of pro-angiogenic factors that function in the formation of new blood vessels through a number of downstream effects, including proliferation and migration of vascular endothelial cells, survival of immature endothelial cells and induction of vascular permeability in capillaries (Yancopoulos *et al.*, 2000). Examples of pro-angiogenic factors include; vascular endothelial growth factor (VEGF) as well as, basic fibroblast growth factor and transforming growth factors alpha and beta (Jain, 2003). There are also anti-angiogenesis factors such as angiostatin, endostatin and thrombospondin-1 that have an opposite effect and it is the balance of pro- and anti-angiogenic factors that determines the rate and progression of angiogenesis (Cao, 2001; Chavakis *et al.*, 2002). Deregulation of angiogenesis due to an increase of pro-angiogenic factors and a reduction in the activity of anti-angiogenic factors leads to increased vascularisation of tumours and therefore increases the sustainable mass of the primary tumour as well as increasing the incidence of metastases, facilitated by intravasation (Loges *et al.*, 2009).

1.3.1 The VEGF-VEGFR Axis and Angiogenesis

The VEGF family of growth factors consist of six described members; VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental-derived growth factor (PDGF). VEGF-A describes 6 isoforms of the protein, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$. As the most abundant isoform,

VEGF$_{165}$ is often shortened to VEGF and is referred to as such in the majority of literature (Harper *et al.*, 2008). VEGF is produced by a variety of cell types including smooth muscle cells, cancerous cells and endothelial cells themselves. VEGF production is primarily stimulated under conditions of hypoxia wherein low oxygen concentrations inhibit the enzyme HIF prolyl-hydroxylase (which requires oxygen as a substrate). In normoxic conditions this enzyme hydroxylates proline residues on the alpha subunit of the transcription factor hypoxia-inducible factor (HIF). In these conditions the activity of the enzyme leads to ubiquitiniation and degradation of HIF. When oxygen is absent, HIF-1α (the HIF family member primarily involved in angiogenesis) is stabilised and functions in the upregulation of genes that lead to an angiogenic response, including VEGF (Adair *et al.*, 2010).

VEGF elicits a response on binding to receptors present on endothelial cells in the vasculature as well as in the lymphatic system. Receptors for VEGF have also been found in cancer cells of various origins including thyroid (Vieira *et al.*, 2005) and indeed, breast (Schmidt *et al.*, 2008). The presence of these receptors on tumour cells suggests that these cells have developed autocrine loops that may stimulate tumour cell growth and migration. Receptors for VEGF (VEGFRs) are a family of membrane tyrosine kinase receptors and are comprised of three family members; VEGFR-1(Flt-1), VEGFR2 (Flk-1/KDR) and VEGFR3 (Flt-4). VEGF mediates its effect by binding, primarily, to VEGFR1 and VEGFR2. VEGF has a higher affinity for VEGFR1 with a K_d of 10pM whereas it's affinity for VEGFR2 receptors is lower with a K_d of approximately 75 to 125pM (Shepro, 2006). This proves interesting as VEGFR2 is the receptor

through which VEGF mediates most of its proangiogenic effect, leading some to hypothesise that in normal conditions, VEGFR1 acts to sequester VEGF and prevent its binding to VEGFR2 (Kowanetz *et al.*, 2006; Olsson *et al.*, 2006).

When VEGF binds VEGFR2 (Figure 1.7), the receptor undergoes dimerisation and oligomerisation resulting in activation of the intracellular receptor tyrosine kinases that phosphorylate specific tyrosine residues on the cytoplasmic terminals of the receptor. Since most endothelial cells express both VEGFR-1 and -2, VEGF-A can induce homodimers of each receptor type, as well as VEGFR-1/VEGFR-2 heterodimers. Heterodimerisation of the soluble extracellular domains of VEGFR-1 and -2 has been demonstrated *in vitro* (Kendall *et al.*, 1996). Src homology 2 (SH2) domains, such as those present on phospholipase C (PLC), can bind the phosphorylated tyrosine residue, and in the case of PLCγ, mediate the activation of a mitogen-activated protein kinase (MAPK) cascade which in turn, leads to proliferative and survival responses in endothelial cells (Takahashi *et al.*, 2001).
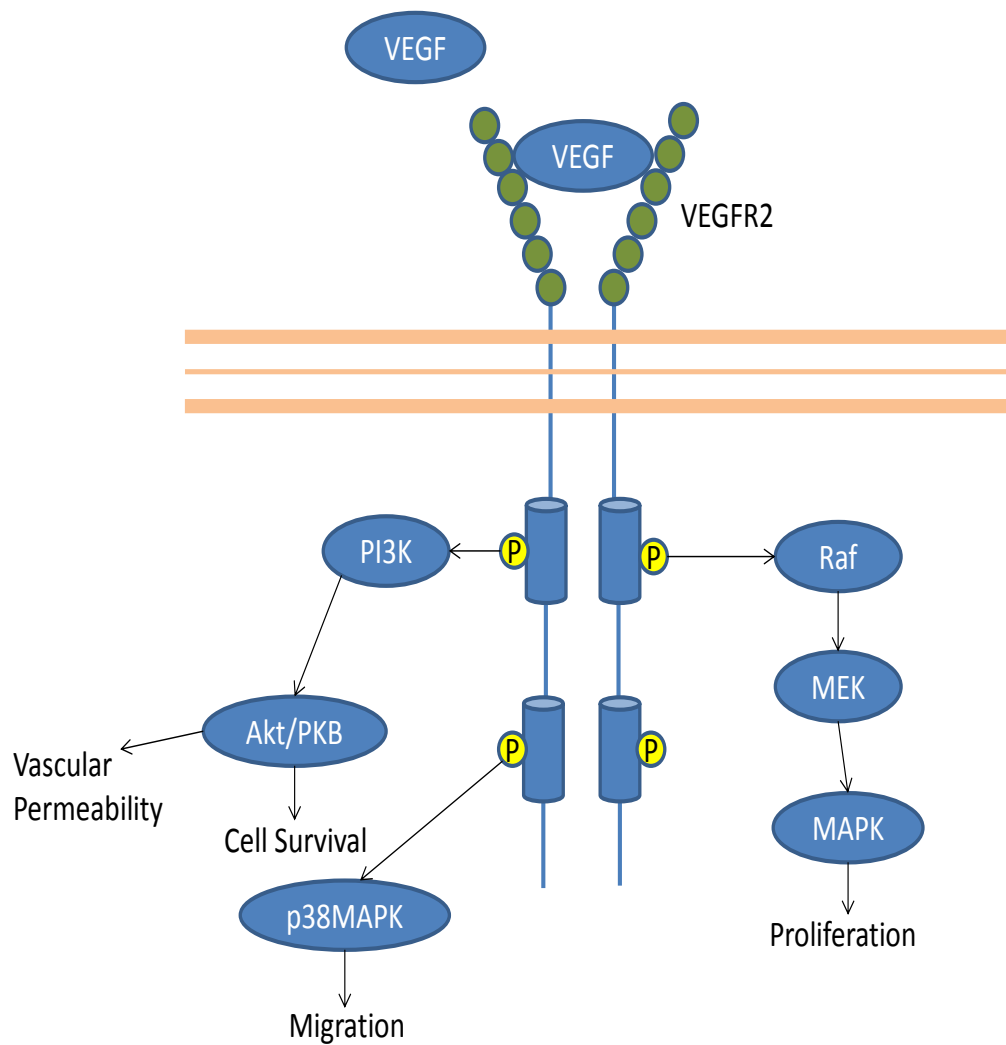
Figure 1.7 Binding of VEGF to VEGFR2. Binding of VEGF to its receptor results in receptor dimerisation and stabilisation of the dimer, allowing for autophosphorylation and following, activation of downstream signalling

In addition, VEGFR2 activation can induce the binding of SH2 adaptor protein B (SHB) to the intracellular tyrosine kinase domain at phosphorylated Tyr1175. This causes activation of phosphoinositide 3-kinase (PI3K) which results in elevated phosphatidylinositol 3,4,5-triphosphate levels and activation of downstream Akt (Dayanir *et al.*, 2001). Akt functions in inhibiting apoptosis as well as regulating production of nitric oxide (NO) through stimulation of endothelial nitric oxide synthase (eNOS) (He *et al.*, 1999). The importance of nitric oxide in regulation of angiogenesis has only been demonstrated in the activity of eNOS. Removal of eNOS in knockout mice resulted in impaired angiogenesis (Murohara *et al.*, 1999).

Src kinase is a member of the Src family kinase group non-receptor tyrosine kinases. The term "Src family kinases" encompasses a group composed of 9 family members; Src, Lyn, Yes, Fgr, Fyn, Lck, Hck, Blk and Frk. These molecules are widely reported to function in response to stimuli from growth factor receptor binding, including EGFR and VEGFR, as well as the activation of adhesion molecules that extend into the ECM (Belsches-Jablonski *et al.*, 2001; Lesslie *et al.*, 2006; Thomas *et al.*, 1997; Twamley-Stein *et al.*, 1993). Src family kinases lead to downstream activation of second messenger systems like PI3K, Focal adhesion kinase (FAK) and MAPK signalling cascades (Eliceiri *et al.*, 1999). Due to its capability to influence gene expression through Stat3, Src can induce increased transcription of genes that regulate angiogenesis such as VEGF. Certain methylation events that occur in breast cancer by PRMT1 activity (which transiently methylates ERα at R260) cause the formation of cytoplasmic complexes that contain ERα in addition to Src kinase as well as

PI3K and FAK. Although these events occurs frequently in breast cancer, their outcome in terms of tumour response remains unknown (Le Romancer *et al.*, 2008).

Activation of PI3K by VEGF-A is dependent on FAK (Abedi *et al.*, 1997), an integral tyrosine kinase in cell migration and anchorage-dependent differentiation. FAK is a protein tyrosine kinase which is recruited at an early stage to focal adhesions (found at the cell membrane where the cytoskeleton interacts with proteins of the extracellular matrix) and which mediates many of the downstream responses. The kinase activity of FAK is activated by the Src after which, FAK can interact with a number of down-stream signalling proteins, including the adaptor protein Grb2 and the p85-subunit of IP3. Studies using inhibition of Src have shown it not only reduces the activity of FAK but also suppresses VEGF expression as well as inhibiting VEGF-stimulated proliferation of endothelial cells (Ali *et al.*, 2006; Han *et al.*, 2006; Mitra *et al.*, 2006).

1.3.2 Angiogenesis in Breast Cancer

In the clinic, microvessel density has had some support as an indicator of poor prognosis (Arnes *et al.*, 2012). Women who presented with fibrocystic disease and who had the highest vascular density were found to have the highest risk of developing breast cancer (Guinebretière JM, 1994). Microvessel density, when found to be relatively high in premalignant lesions of the breast correlated with a higher risk of lesions developing into breast cancer and higher density of microvessels is found in more aggressive variants of DCIS (Guidi *et al.*, 1997).

Deregulation of angiogenesis in breast cancer is often attributed to an alteration in the balance between pro-angiogenic and anti-angiogenic signalling. This deregulation occurs in the cancer cells themselves but can affect the surrounding stroma, as a result of paracrine signalling. The fine balance between pro- and anti-angiogenic signalling is often referred to as the "angiogenic switch". In normal tissue, hypoxia sets off a cascade of events which results in the expression of pro-angiogenic factors that "flip the switch" and induce growth and migration of the endothelia. In breast cancer, factors known to be deregulated include VEGF, which has been shown to be expressed at a higher concentration in the ECM of breast cancer tumours.

The correlation between increased VEGF expression in clinical tissue and poor prognosis has been illustrated (Ragaz *et al.*, 2004) and has also been tied to poor tamoxifen response (Foekens *et al.*, 2001). Expression of the main regulator of VEGF production, HIF-1α, has also been shown to be increased in clinical tissue from breast cancer (Wang *et al.*, 1995). Other evidence indicates that deregulation of VEGF may not be entirely as a result of increased HIF-1α expression. It has been suggested that VEGF may be regulated by oestrogen (Ruohola *et al.*, 1999) but this seems to contradict further studies. Supplementation of ovariectomised mouse xenografts with E2 increased *VEGF* mRNA significantly over unsupplemented mice (Suriano *et al.*, 2008). Although an *in vitro* study that blocked oestrogen action using tamoxifen found that VEGF remained increased in their cells (Bogin *et al.*, 2002).

Other factors responsible for deregulation of angiogenesis in breast cancer discovered in preclinical models include matrix metalloproteinases (MMP). The function of these enzymes is to degrade the basement membrane surrounding cancer cells and so they can aid the migration of cancer cells into the vasculature as well as aiding migration of endothelial cells towards the tumour site (Pellikainen *et al.*, 2004). In breast cancer these enzymes are found to have an increased expression which can lead to a more invasive and metastatic tumour phenotype (Bartsch *et al.*, 2003).

Several methods have been implemented in targeting different aspects of angiogenic signalling. Inhibition can be carried out by: directly targeting the ligand (VEGF protein) or before it is translated by targeting the mRNA; by targeting the receptors to which it binds, the VEGFR family of receptors; or by inhibiting downstream signalling components of the VEGF pathway. As such there is little data for its efficacy in treatment of acquired resistance but there is pre-clinical and clinical data supporting a role for the mediators of angiogenesis, such as VEGF, in the biology of tumour development that may be ER regulated. Targeted anti-angiogenic therapy may provide increased therapeutic benefit in endocrine-resistant cancers or delay the onset of resistance by limiting the mechanisms by which resistance may occur.

1.4 Conclusions

Although many women progress well on endocrine therapy the majority receiving tamoxifen will eventually relapse with endocrine-resistant disease. This relapse is not only associated with reduced efficacy of primary anti-

hormone treatment but also presents with a more invasive and motile phenotype which presents a major clinical issue. The capacity of anti-endocrine-resistant cancers to induce angiogenesis, however, has not been explored.

1.5 Aims

We hypothesised that development of acquired endocrine resistance in breast cancer is associated with acquisition of a proangiogenic phenotype. The principle aims of this thesis were to investigate (i) how acquisition of endocrine resistance in breast cancer affected the cells' angiogenic capacity and (ii) to identify the underlying pro-angiogenic mechanism(s). In order to achieve these aims, the following objectives were set:

- To characterise the expression of pro-angiogenic factors in a panel of breast cancer cell lines.
- To characterise the pro-angiogenic capacity of factors proven to be overexpressed in anti-hormone resistant breast cancers on the growth and migration of endothelial cell models (HUVEC, HECV).
- To establish whether *ex-vivo* models of anti-hormone resistant breast cancer demonstrate differences in angiogenic responses.

Chapter 2: Materials and Methods

2.1 Materials and Suppliers

Below is a table of the materials and supplies used for the investigations detailed in this thesis, arranged alphabetically with the appropriate supplier. These suppliers are detailed at the end of the table.

Material	Supplier
Activated charcoal	S
Agarose	BL
Ammonium persulfate (APS)	S
Anti-mouse horseradish-peroxidase-linked IgG (source: sheep)	A
Anti-rabbit horseradish-peroxidase-linked IgG (source: donkey)	A
Aprotinin	S
Arcrylamide/bis-acrylamide (30% solution (v/v), 29:1 ratio)	S
Bio-Rad D_C Protein Assay (Reagents A, B and S)	BR
Bovine Serum Albumen	S
Bromophenol blue	BDH
Cell culture medium: RPMI 1640 and Phenol-red-free RPMI 1640	I
Cell Proliferation Reagent WST-1	R
Cell scrapers	GB
Chemiluminescent Supersignal® West HRP Substrate (Pico, Dura, Femto)	P
Coulter counting cups and lids	SAG
Cryotube^{TM} vials (1.8ml, starfoot, round)	N
Dimethyl sulphoxide	S
Disposable cuvettes	F
Di-thiothreitol (DTT)	S
dNTPs (dAPT, dCTP, dTTP, dGTP; 100mM)	A
Endothelial Cell Growth Medium (Basal medium + Growth supplements)	ECACC
Ethidium Bromide (EtBr)	S
Ethylene diamine tetraacetic acid	S
Ethylene glycol-bis (2-amino-ethlether)-N,N,N',N'-tetraacetic acid	S
Filter paper (Grade 3)	W
Filter paper (No.4)	W
Foetal Calf Serum (FCS)	I
General laboratory glass and plasticware	F
Glacial acetic acid	F
Glycerol	F
Glycine	S
Hydrochloric acid (HCL; 5M)	F
Hyperladder^{TM} IV	BL
Interleukin-8 (IL-8)	S

Isoton® azide-free balanced electrolyte solution	B
Kodak MXB Autoradiography film (blue sensitive; 18cm x 24 cm)	GR
Leupeptin	S
L-glutamine	I
Lower buffer for SDS-PAGE gels (Tris 1.5M, pH 8.8)	BR
Molony-murine leukemia virus (MMLV) reverse transcriptase	I
N,N,N',N'-tetramethylene-diamine (TEMED)	S
Nitrocellulose transfer membrane (Protran® BA85; 0.45µm pore size)	S&S
pH calibration buffers tablets (pH 4, 7 and 10)	F
Phenylarsine oxide	S
Phenylmethylsulfonyl fluoride (PMSF)	S
Pipette tips	GB
Polyoxoethylene-sorbitan monolaurate (Tween 20)	S
Ponceau S Solution (0.1% (w/v) in 5% acetic acid)	S
Precision Plus Protein^{TM} All Blue Standards (10-250kDa)	BR
Random hexamers (RH)	A
RNase-free H$_2$0	S
Sodium azide	S
Sodium dodecyl sulphate (SDS)	S
Sodium fluoride (NaF)	S
Sodium hydroxide (NaOH; 5M)	F
Sodium molybadate (Na$_2$MoO$_4$)	S
Sodium orthovanadate (NaVO$_4$)	S
Solvents (acetone, chloroform, ethanol, formaldehyde, isopropanol and methanol)	F
Sterile bijou tubes	BB
Sterile cell culture plasticware (i.e. flasks, dishes and plates)	N
Sterile Falcon tubes (15ml and 50ml)	SAG
Sterile phosphate buffered saline (PBS)	I
Sterile syringe filters (0.2µm)	C
Sterile universal containers (30ml)	GB
Taq DNA Polymerase (BioTaq^{TM}; 5U/µl)	BL
TRI Reagent	S
Tris HCL	S
Triton X-100	S
Trizma (Tris) base	S
Trypsin/EDTA 10x solution	I
Upper buffer for SDS-PAGE gels (Tris 0.5M, pH 6.8)	BR
Vascular Endothelial Growth Factor (VEGF)	S

Table 2.1 Materials and their suppliers. [A] Amersham, Little Chalfont, UK; **[B]** Beckman Coulter UK Ltd, High Wycombe, UK; **[BDH]** BDH Chemicals Ltd, Poole, UK; **[BB]** Bibby Sterlin Ltd, Stone, UK; **[BL]** Bioline Ltd, London, UK; **[BR]** Bio-Rad Laboratories Ltd, HERTS, UK; **[C]** Corning Inc., Corning, NY, USA; **[ECACC]** European Collection of Cell Cultures, Salisbury, UK; **[F]** Fisher Scientific UK Ltd, Loughborough, UK; **[GR]** Genetic Research Instrumentation, Rayne, UK; **[GB]** Greiner Bio-One Ltd, Gloucestershire, UK; **[I]** Invitrogen, Paisley, UK; **[N]** Nunc Int, Roskilde, Denmark; **[P]** Pierce and Warriner Ltd, Cheshire, UK; **[R]** Roche Diagnostics Ltd, Burgess Hill, UK; **[S]** Sigma-Aldrich, Poole, Dorset, UK; **[SAG]** Sarstedt AG and Co., Nümbrecht, Germany; **[W]** Whatman, Maidstone, UK; **[S&S]** Schleicher and Schuell, Dassell, Germany

2.2 Cell Culture

2.2.1 Breast Cancer Cell Lines

To model hormone sensitive breast cancer, MCF7wt (Michigan Cancer Foundation – 7) wild type (MCF7wt) cells were used as these cells represent an ER+, PR+ invasive ductal carcinoma. These cells have been are used extensively to study ER+, hormone-endocrine-responsive breast cancer. The MCF7wt cells used were a gift from AstraZeneca Pharmaceuticals but originally obtained from the American Type Culture Collection (ATCC Number HTB-22^{TM}). MCF7wt cells were routinely cultured in RPMI + 5% foetal calf serum (FCS). For experiments where MCF7wt cells were used, they were maintained in experimental medium, wRPMI + 5% stripped foetal calf serum (SFCS) + 2% L-Glutamine which is commonly referred to as w&5. wRPMI differs from RPMI in that it does not contain phenol red, as phenol red has an oestrogenic effect. In addition, stripped serum replaces whole serum and is produced by charcoal stripping whole serum. Charcoal stripping removes any lipophilic material in media such as lipid based growth factors, cytokines and importantly, steroid hormones.

The T47D (ER positive, HER2 negative) breast cancer cell line, was used to represent a second model of ER+ breast cancer. These cells were purchased from the American Type Culture Collection (Manassas, VA). T47D cells were routinely cultured in RPMI + 5 % FCS and for experiments were cultured in experimental media.

2.2.1.1 Establishment of MCF7wt models of acquired tamoxifen and fulvestrant resistance

As a model of acquired Tamoxifen resistance, 'TamR' cells were created by culturing MCF7wt cells in the presence of 4-hydroxytamoxifen (4-OH-T, 100nM) for a six month period. After an initial period of growth inhibition, cells begin to regrow (at around three to four months), suggesting they have acquired resistance to tamoxifen. These cells were maintained for a further three months to develop a stable phenotype after which the cells could then be used for routine cell culture. To maintain the selective pressure ensuring continued tamoxifen-resistance, cells were maintained in the presence of 4-hydroxytamoxifen in w&5 (w&5+ 4-OH-T) (Knowlden *et al.*, 2003).

A model of acquired Faslodex resistance was developed by a similar method as above. MCF7wt cells were cultured over a period of twelve months in the presence of 100nM Faslodex to produce MCF7wt cells with acquired faslodex resistance (FasRs). These cells were routinely cultured in wRPMI + 5%SFCS + 2% L-Glutamine and in the presence of 100nM Faslodex to maintain selective pressure, as with the TamR cell model.

2.2.1.2 Oestrogen Deprived Cells

To model a further acquired resistant state (aromatase inhibitor resistance), MCF7wt cells were grown in oestrogen deprived media (wRPMI + 5%XSFCS + 2% L-Glutamine) for a period of three to six months to produce MCF7-X cells. The XSFCS used was charcoal stripped foetal calf serum that had been additionally heated to 65°C for 30 minutes to remove growth factors present in

the serum. They were routinely cultured in wRPMI + 5% XSFCS + 2% Glutamine. Breast cancer cell lines used are summarised in Table 2.2.

Cell line	Gene Cluster	ER	PR	HER2	TP53
MCF7wt	Luminal	+	+	-	+/-^{WT}
TamR	Luminal	+	+	-	
FasR	Luminal	-	+	-	
T47D	Luminal	+	+	-	++M
MCF7wt-X	Luminal	+	+	-	

Table 2.2 Characterisation of breast cancer cell lines used. ER, Oestrogen Receptor status; HER2, overexpression of HER2; PR, progesterone receptor status; TP53, protein levels and mutational status of TP53**.** Adapted from (Neve *et al.*, 2006)

2.2.1.3 Endothelial Cell Lines

To investigate the behaviour of endothelial cells in response to various treatments we used the Human Umbilical Vein Endothelial Cell line (HUVEC) as well as a second endothelial cell line, Human Vascular Endothelial Cells (HECV). HUVEC cells were obtained from ECACC and maintained in specialised endothelial cell growth medium supplemented with a growth factor cocktail supplied with the medium both of which were also supplied by ECACC. Although the constituents of the growth factor cocktail are not defined by the company it does not contain PDGF or VEGF. HUVECs were only kept for a maximum of 9 passages as they are a primary cell line and prolonged culture results in senescence (Rhim *et al.*, 1998).

HECV cells, a gift from Professor Wen Jiang, Cardiff University School of Medicine, and originally from Dr G. Di Domenico, Biology and Cellular and Molecular Pathology Dept, Naples, Italy are a more robust cell line that allows continuous culture allowing for longer experiments than HUVECs. HECV cells were routinely maintained in DMEM + 10% FCS whilst HUVECS were cultured in an endothelial cell growth media supplied by ECACC. Both cell lines were grown in w&5 when required for experiments.

2.2.2 Cell Culture Techniques

All cell-culture was carried out under sterile conditions in a MDH Class Laminar Flow Hood. All equipment and consumables were purchased sterile for single use or were sterilised at 119°C using an autoclave. Before use, the interior surfaces of the cabinets and the equipment within were sprayed with 70% (v/v) ethanol in water to ensure a sterile working environment. 70% ethanol can precipitate intracellular proteins in bacteria better than a higher concentration and so is the standard preparation for sterilisation use.

Cells were typically maintained as monolayers in 75cm^2 flasks (T-75) and grown in a Sanyo MCO-17AIC incubator (Sanyo E&E Europe BV, Loughborough, UK) at 37°C with a humidity atmosphere containing 5% CO_2. The culture media was changed every 3 to 4 days and cells visually assessed for confluency during culture using a Nikon Eclipse TE200 phase-contrast microscope (Nikon-UK Ltd, Kingston-upon-Thames, UK) and passaged during log-phase growth (approximately 70-90% confluency.)

2.2.2.1 Passaging of Cells

To passage cells, media was first removed and cell monolayers dissociated by the addition of 5ml of a trypsin/EDTA solution (0.05%/0.02% in PBS respectively). Trypsin is a proteolytic enzyme that causes detachment of cells from the growth surface by cleaving cell-cell and cell-matrix adhesions whilst EDTA acts to chelate Ca^{2+} and Mg^{2+} ions present in FCS that otherwise act as trypsin inhibitors. After addition of the trypsin/EDTA solution, the flasks were returned to the incubator for 3 to 5 minutes until the cells had detached. Trypsin/EDTA was neutralised with an equal volume of serum-containing media and the cells were pelleted by centrifugation for 5 minutes at 1000rpm. The cell pellet was gently re-suspended in 5ml of the appropriate medium and gently mixed to ensure no cell clumps were evident. A proportion of this suspension, typically 500µl (1/10 of cell suspension volume), was diluted in the appropriate medium and used to seed and additional T-75 flask which was then cultured as normal. Prior to setting up cells for an experiment, the re-suspended cells were counted using a Coulter^{TM} Multisizer II Cell Counter (Beckman Coulter UK Ltd, High Wycombe UK) to enable seeding at an appropriate cell density.

2.2.2.2 Cell Freezing

To ensure an adequate supply of cellular material, stocks of established cell-lines were generated by making up several suspensions of $1 \times 10^{6,}$ low passage cells in 1ml of the appropriate media with a 10% concentration of FCS or SFCS and 7.5%DMSO. These cells were then placed in specialised cryotubes and frozen to -80°C for 24 hours. Following this cells were then placed in liquid

nitrogen for long-term storage. When required, cells were recovered from storage by removing the cryotubes from the liquid nitrogen and rapidly thawed to limit exposure of the cells to liquid DMSO. Once thawed, cells were removed to a sterile universal tube and diluted with ~9ml of the appropriate medium before being spun for 5min at 1000rpm to remove trace DMSO. Cells were transferred to the appropriate size flask, usually a T-25, and placed in an incubator at 37°C with 5% CO_2 overnight. Medium was changed after 24 hours and cells then cultured as normal.

2.2.3 Conditioned Media

Conditioned media was obtained from MCF7wt, TamR, FasR and MCF7X cells as follows. Cell lines were cultured in their own specific growth medium until they reached ~70% confluency after which medium was replaced with experimental medium, w&5. These cells were returned to incubate at 37.5°C for 4 days before 5ml of solution was collected, clarified by centrifugation for 5min at 1000rpm and filtered using a 0.2µm syringe filter. Media were stored at -20°C until required for further experiments.

2.3 Micro-array Analysis

An initial screen of pro- and anti-angiogenic factors expressed by breast cancer cell lines was examined at a gene level using microarray analysis. A list of factors implicated in angiogenesis was compiled from analysis of the literature and factors commonly included in commercially available angiogenesis pathway analysis kits. These genes were screened against our existing in-house microarray database based on a U133A gene array using GeneSifter software.

The TamR, FasR, T47D and MCF7-X arrays were interrogated for the mRNA expression of the genes from our list of angiogenic factors. These were all compared to the expression of the same factors by our MCF7wt array. Using the GeneSifter heat-map tool, the most significantly upregulated and downregulated genes in our cell lines compared to MCF7wt controls were determined.

2.4 RT-PCR

Gene expression in our breast cancer cell lines and in HUVEC and HECV cells after treatment with conditioned media was assessed using semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) as detailed below.

2.4.1 RNA Extraction & Isolation

Cells were seeded into 6-well plates at a density of 1.5×10^4 cells/well and cultured until they had reached log phase growth, then treated as indicated. Media was removed and cells were washed twice in PBS after which 150µl of TRI reagent was added to each well and allowed to stand for 5 minutes. The resulting lysate was passed through a pipette tip several times to ensure homogeneity of the sample, and transferred to a 1.5ml Eppendorf tube where they were allowed to stand for 5 minutes to ensure complete dissociation of the nucleoprotein complexes. To each ml of TRI Reagent, 200µl of chloroform was added followed by vortexing. Samples were left to stand for 10 minutes in order to separate the aqueous and organic phases of the lysate. The resulting mixture was centrifuged at 12,000rpm for 15 minutes at 4°C. This further separates the two mixtures into 3 phases: a lower red organic phase containing protein, a

white interphase containing DNA and a colourless upper aqueous phase containing RNA.

The aqueous phase was transferred to a clean RNase-free Eppendorf and 500µl of isopropanol was added to each, vortexed and allowed to stand for ~10 minutes at room temperature. Samples were centrifuged at 12,000rpm for 15 minutes at 4°C to precipitate the RNA. The supernatant was removed from the tube and the remaining pellet was washed in 1ml of 75% ethanol. The sample was mixed by vortex and centrifuged at 7,500rpm for 5 minutes at 4°C supernatant removed and pellets were air dried for 5-10 minutes but not allowed to dry completely as this would greatly reduce its solubility. The pellet was then dissolved in 20-50µl of RNase free distilled water and quantitated on a spectrometer by taking readings at 260nm (reads amount of nucleic acids) and 280nm, from which the purity of the RNA sample and its concentration is calculated.

2.4.2 Reverse Transcription (RT)

All RT and further PCR work was carried out in a sterile enclosure. RNA was reverse transcribed using the Molony-murine leukaemia virus (MMLV) reverse transcriptase enzyme to generate cDNA that was complementary to mRNA samples used. The RT mixture contained total RNA, dNTP mix, to provide free nucleotides, dithiothreitol (DTT) to ensure RNasin^{TM} activity in the next step, and random hexamers (RH) which act as random mRNA primers as well as RT buffer and RNase free water (sample volumes are detailed in Table 2.3). This

mixture is heated to 95°C in a PTC-100 thermocycler for 5 minutes to denature the RNA, and then quickly cooled on ice.

To this denatured sample MMLV-RT and RNasin[TM] were added and returned to the thermocycler where it is heated to 22°C for 10 minutes, 42°C for 42 minutes and finally, 95°C for 5 minutes.

Reagent	1 Sample	5 Samples	10 Samples
5x RT Buffer	6µl	30µl	60µl
0.1M DTT	3µl	15µl	30µl
Random hexamers	0.3µl	1.5µl	3µl
25nm dNTPs	0.6µl	3µl	6µl
RNase free water	3.3µl	16.5µl	33µl
RNAsin	1µl	5µl	10µl
MMLV-RT	0.8µl	4µl	8µl
Final Volume	15µl	75µl	150µl

Table 2.3 Final reagent volumes in reverse transcription reaction mix. RT-Buffer, Reverse Transcription Buffer; DTT, dithiothreitol; dNTPs, deoxynucleotide triphosphates; MMLV-RT, Molony-murine leukaemia virus reverse transcriptase.

2.4.3 Polymerase Chain Reaction (PCR)

Each PCR reaction mixture used consisted of; dNTP mix, PCR buffer, $MgCl^{2+}$, forward and reverse primers for sequences on the specific genes of interest (Table 2.5) as well as Taq polymerase enzyme in a 200µl Eppendorf. This

mixture was made up to 25μl with RNase free-dH$_2$0 and 1μg of total cDNA sample was added to each (sample volumes are detailed in Table 2.4). Each reaction was briefly mixed and centrifuges to ensure homogeneity before being placed in the thermocycler. Samples were run according to the following protocol; denaturation at 95°C for 5 minutes, then repeated cycles of denaturation, annealing at 55°C and extension at 72°C, commonly repeated for 30 cycles before a final extension and cooling to 4°C. Depending on the primer pair used, variations were made on the number of cycles used and the annealing temperature. PCR data was normalised in the presence of β-actin, a housekeeping gene. To accomplish this, a PCR reaction was carried out for each cDNA sample using primers for β-actin.

Reagent	1 Sample	5 Samples	10 Samples
25nM dNTPs	2µl	10µl	20µl
PCR Buffer	2.5µl	12.5µl	25µl
$MgCl^{2+}$	0.75µl	3.75µl	7.5µl
Forward Primer	0.63µl	3.13µl	6.25µl
Reverse Primer	0.63µl	3.13µl	6.25µl
Taq	0.2µl	1µl	2µl
RNase free water	17.8µl	89µl	178µl
Final Volume	24.51µl	122.55µl	245.1µl

Table 2.4 Final reagent volumes in polymerase chain reaction mix. dNTPs, deoxynucleotide triphosphates.

2.4.4 Agarose Gel Electrophoresis

PCR products were separated and visualised using agarose gel electrophoresis as follows. 8µl of PCR sample were mixed with 10µl of loading buffer containing bromophenol blue and sucrose and loaded into a well of a 1% agarose gel (1% agarose in Tris-Acetate-EDTA [TAE] buffer containing 10mg/ml ethidium bromide). A DNA ladder measuring 100-1000Kb was used to mark product size. Gels were run in TAE buffer at 100 volts for 30 minutes prior to visualisation and photographing on a UV illuminator.

Gene Name	Primer Sequence	Predicted Product Size (bp)
VEGF-A, Fwd VEGF-A, Rev	5'CTGAAATGAAGGAAGAGGAG 3'CACAGCAGTCAAATACATCC	567bp
VEGF-C, Fwd VEGF-C, Rev	5'CGGGAGGTGTGTATAGATGTG 3'ATTGGCTGGGGAAGAGTTTG	583
Il-8, Fwd Il-8, Rev	5'ATGACTTCCAAGCTGGCCGT 3'CCTCTTAAAAACTTCTCCACACC	285
VEGFR2, Fwd VEGFR2, Rev	5'CAATGGAGGGGAACTGAAGAC 3'TCTGGCTACTGGTGATGCTGT	536
VEGFR3, Fwd VEGFR3, Rev	5'GAGCAGCCATTCATCAACAAG 3'GGTAGTCCCAGTCAAAGGTG	400
Human IL-8 Receptor Alpha Fwd Human IL-8 Receptor Alpha Rev	5'GCTAGGGCTAAGGTGTTTTC 3'GTCCTCTTCAGTTTCAGCAA	364
Human IL-8 Receptor Beta Fwd Human IL-8 Receptor Beta Rev	5'ATCTATGCCCTGGTATTCCT 3'GGTCCTTCGGAAAAGTAAGA	451
HIF-1 Alpha Fwd HIF-1 Alpha Rev	5'TCCATGTGACCATGAGGAAA 3'CCAAGCAGGTCATAGGTGGT	486
β-Actin Fwd β-Actin Rev	5'CTTTCTGGGTATGGAATCCT 3''GGAGCAATGATCTTGATCTT	204

Table 2.5 PCR Primers used and product size predicted for RT-PCR of proangiogenic factors in breast cancer cells.

2.5 SDS-PAGE/Western Blotting

Changes in the basal expression or activation of proteins in response to treatments were assessed using SDS-PAGE and Western blotting with immunoprobing using phospho and total antibodies.

2.5.1 Cell Lysis

Cells were seeded into 6-well plates, 60mm or 100mm Petri-dishes at the required density and cultured until they reached log-phase growth, at which point they were treated indicated. Media was then aspirated from the dishes and cells were washed twice with ice-cold PBS solution before addition of Triton-X100 lysis buffer solution containing a cocktail of protease and phosphatase inhibitors as detailed below (Table 2.6).

Inhibitor	Final Concentration
Sodium Orthovanadate	2mM
Phenylmethylsulfonyl Fluoride	1mM
Sodium Fluoride	25mM
Sodium Molybadate	10mM
Phenylarsine	20µM
Leupeptin	10µg/ml
Aprotinin	8µg/ml

Table 2.6 Constituents of protease inhibitor cocktail.

Cellular material was collected using a cell-scraper and transferred to a 1.5ml Eppendorf, in which the lysates were homogenised briefly using a pipette before being clarified by centrifugation for 15 minutes at 13,000rpm, 4°C. The supernatant was then removed and stored at 20°C.

2.5.2 Protein Concentration Assay

To ensure equal protein loading for SDS-PAGE, the concentration of total soluble protein was determined using the Bio-Rad D_c Protein Assay kit (a modified version of the Lowry protein assay). A standard curve (such as in Figure 2.1) was constructed using increasing concentrations of BSA solution from 0 to 1.45mg/ml in TX100 lysis buffer to give 50µl solutions of each concentration. To this solution, 0.25ml of Reagent A (containing 5µl of Reagent S) and 2ml of Reagent B were added. The solutions were left for 10 minutes for full colour development and read on a Cecil CE2041 spectrophotometer (Cecil Instruments, Cambridge, UK) at 750nm, These readings were then compared with those of the BSA based standard curve to give the concentration of total soluble protein in the lysates.
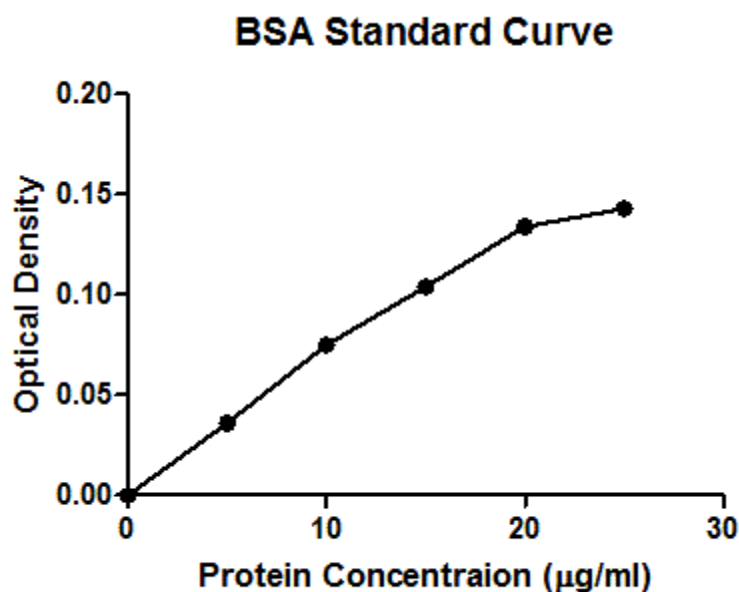
Figure 2.1 Standard curve constructed from known BSA concentrations from which unknown concentrations of protein can be interpolated.

2.5.3 SDS-PAGE Analysis

SDS-PAGE (Sodium-Dodecyl-Sulphate-Polyacrylamide Gel Electrophoresis) was performed using a Bio-Rad Mini-Protean® III apparatus powered by a Powerpac Basic™ power pack (both from Bio-Rad Laboratories Ltd. Herts, UK). The gel system used was discontinuous, consisting of a 5% (w/v) acrylamide/bis-acrylamide stacking gel at pH6.8 and a typically 8-10% (w/v) acrylamide/bis-acrylamide resolving gel at pH8.8.

A pair of glass plates, consisting of a cover and a backing plate, were cleaned with ethanol before being assembled in the gel casting apparatus, ensuring the plates were well clamped together to avoid leakage. The constituents of the resolving gel (Table 2.7) in the absence of TEMED were mixed in a universal

container before the addition of the catalyst as it would be adverse to cause polymerise and cross-linkage of the acrylamide and bis-acrylamide prematurely. After the addition of TEMED, the resolving gel was quickly and carefully mixed and dispensed between the two glass plates until the level reached approximately 1.5cm below the top of the plates (to allow room for the stacking gel). This space was topped-up with distilled water to prevent drying out of the gel and to create a level surface. Gels were left to set at room temperature for 5-10 minutes.

Once set the plates were rinsed in distilled water and any excess water was removed using sections of filter paper. Stacking gel was prepared, as described in Table 2.8, and on addition of the catalyst TEMED was carefully overlaid upon the resolving gel. Typically a 10-well comb mould was inserted into the stacking gel which was also allowed to set for 5-10 minutes at room temperature. Once set, moulding combs were removed and the gel was clamped into the electrophoresis apparatus and both inner and outer reservoirs of the tank were flooded with SDS-Page running buffer.

	10ml 8% Gel (w/v) (70-200kDa)	10ml 10% Gel (w/v) (20-100kDa)	Final Concentration in Gel
30% Acrylamide/bis-acrylamide solution*	2.7ml	3.3ml	Varies with % Gel
dH_2O	4.6ml	4.0ml	-
TRIS (1.5M, pH8.8)	2.5ml	2.5ml	375mM
SDS (10% solution in H_2O)	100µl	100µl	0.1%(w/v)
APS (10% solution in H_2O)	100µl	100µl	0.1%(w/v)
TEMED	50µl	50µl	0.05%(v/v)

Table 2.7 Constituents of the Resolving gel used for SDS-PAGE. * Ratio of

Acrylamide to bis-Acrylamide, 29:1

	10ml 5% Gel (w/v)	Final Concentration in Gel
30% Acrylamide/bis-acrylamide solution*	1.65ml	5%(v/v)
dH_2O	5.7ml	-
TRIS (1.5M, pH8.8)	2.5ml	125mM
SDS (10% solution in H_2O)	100µl	0.1%(w/v)
APS (10% solution in H_2O)	50µl	0.05%(w/v)
TEMED	25µl	0.2%(v/v)

Table 2.8 Constituents of the Stacking gel used for SDS-PAGE.

* Ratio of Acrylamide to bis-Acrylamide, 29:1

Cell lysates containing a known amount of total soluble protein (15-150µg) were

diluted as appropriate using 3 x Laemmli samples loading buffer and heated to

100°C for 5-10 minutes to denature and reduce the proteins in the sample. The

cell lysates, along with a protein molecular weight marker were carefully loaded

into the wells of the stacking gel. Electrophoresis was then performed (Figure

2.2) at 80mA (constant current) until the sample buffer dye had run the length of

the gel.
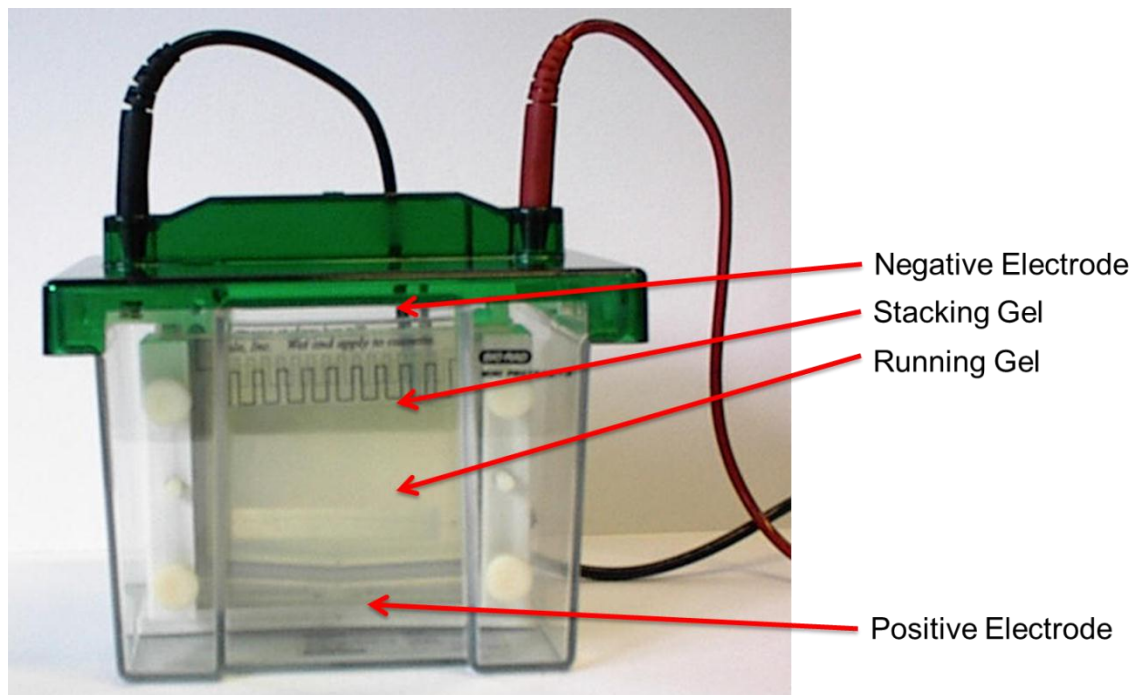
Figure 2.2 SDS-PAGE Apparatus. Proteins migrate downwards through the gel with smaller proteins moving faster and therefore, further and larger proteins moving more slowly, therefore remaining near the top of the gel.

2.5.4 Western Blotting

Proteins were transferred from SDS-PAGE gels to a nitrocellulose membrane using Bio-Rad Mini-Protean® III apparatus.

For each gel to be transferred, two pieces of grade 3 filter paper and one piece of Protran B85 nitrocellulose membrane (0.45µM pore size) were cut to the same size as the gel and pre-soaked along with two Teflon sponge pads, for at least 15 minutes in Western blot transfer buffer. Following electrophoresis the gel plates were separated, the stacking gel was carefully removed and discarded, and the resolving gel carefully transferred to a tray containing distilled water to wash off any excess SDS.

The Western blot transfer stack (Figure 2.3) was assembled following the manufacturer's instructions in the order, positive electrode, sponge, filter paper, nitrocellulose membrane, SDS-PAGE gel, filter paper, sponge, negative electrode. A 50ml Falcon tube was used after the application of each layer, excluding the gel layer, to remove trapped air by gently rolling it across the surface of each layer. The cassette was then placed into the gel transfer apparatus with the gel (black side of cassette) nearest the negative (black) electrode and the membrane (clear side of the cassette) positioned near the positive electrode (red). The transfer apparatus was placed into a tank in the presence of an ice-block to prevent over-heating of the gel during transfer. The tank was then filled with transfer buffer and run at 100V constant voltage for 1 hour.
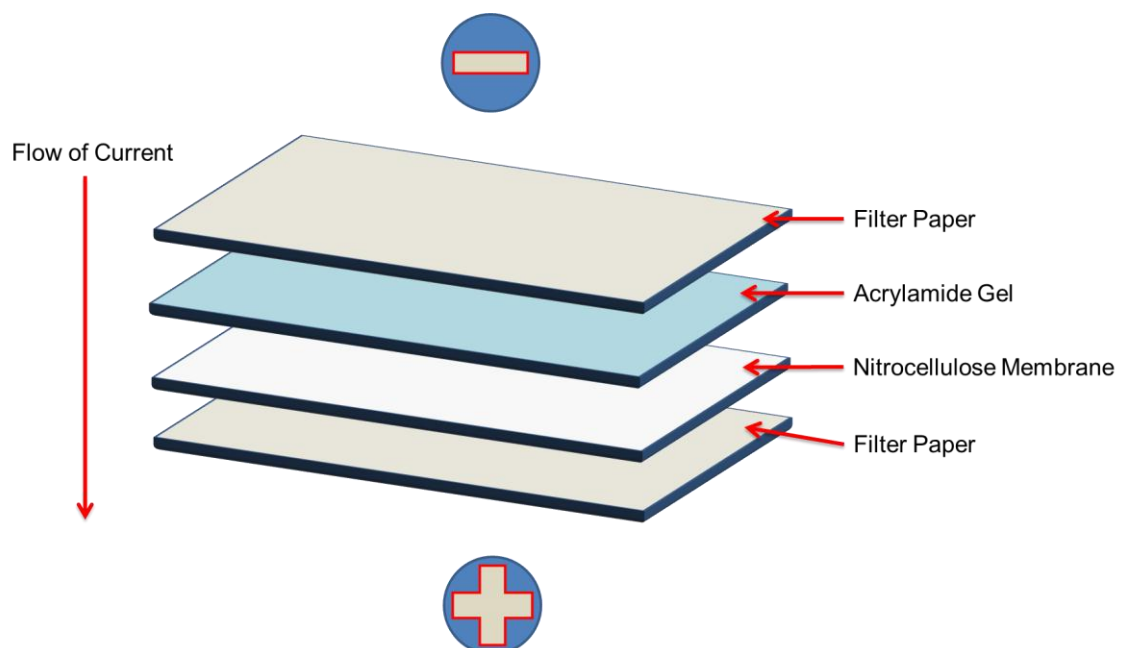
Figure 2.3 Set-up of transfer apparatus. Illustrating the flow of current through the acrylamide gel which causes migration of proteins into the nitrocellulose membrane

2.5.5 Immunoprobing of Western Blots

Nitrocellulose membranes were removed from the transfer cassette and washed twice with Tris buffered saline (TBS). To assess integrity of the membranes and efficiency at which the transfer has been carried out membranes were washed briefly in Ponceau S solution (0.1% (w/v) in 5% acetic acid) which is then easily removed following several washes with 0.05% (v/v) TBS-Tween.

After blocking in 5% (w/v) Marvel milk solution for 2 hours, the membrane was washed in TBS-Tween (2x5 minutes) and incubated overnight in a primary antibody specific for the protein of interest (Table 2.7). Primary antibodies were diluted in TBS-Tween containing 5% (w/v) Western Blocking Reagent and 0.05% (w/v) sodium azide. The primary antibody dilution and incubation conditions used for each protein varied, and so, had to be optimised for each specific antibody.

Primary Antibody	Concentration Used
VEGFR2	1:1,000
p-MAPK	1:1,000
p-Akt	1:1,000
p-Src	1:1,000
GAPDH	1:50,000

Table 2.7: Concentration of primary antibodies used for Western blotting analysis of relative protein levels

Following primary antibody incubation the membrane was washed with TBS-Tween (3x10 minutes) and incubated with the appropriate secondary antibody

diluted in TBS-Tween containing 5% Western blocking reagent. Secondary antibodies were typically used at a dilution of 1:10,000 and incubation was for 1 hour at room temperature. Further washes in TBS-Tween (4x10 minutes) and TBS (2x10 minutes) followed, before antibodies bound to the protein of interest were visualised using enhanced chemiluminescence (ECL).

Chemiluminescence was performed using a luminol/peroxide based ECL reagent (SuperSignal[TM] West Pico, SuperSignal[TM] West Dura or SuperSignal[TM] West Femto). The ECL reagent (500µl) was made following the manufacturer's instructions and carefully pipetted over the blot within a light-proof cassette. Luminol in the ECL reagent is oxidised by HRP in the presence of peroxide to produce an excited state product, which then decays to a lower energy state by releasing photons of light. This light is captured on x-ray film, with exposures ranging from seconds to hours depending on signal strength. X-ray films were developed using an X-O-graph Compact X2 X-ray developer and the bands obtained were scanned and analysed using a Bio-Rad GS-690 Imaging Densitometer.

2.6 ELISA for VEGF Concentration in Conditioned Media

ELISA plates were coated with capture antibody (0.5µg/ml) overnight before being washed (x4) in 0.05% Tween-20 in PBS and treated with block buffer (1% BSA in PBS, sterile filtered) for an hour at room temperature. A standard curve was constructed by using a range of VEGF concentrations (0 – 1ng/ml) and samples of unknown concentration were added to each well of the ELISA plate in triplicate and incubated at room temperature for 2 hours.

After incubation with plates were washed and blotted dry before addition of 0.25µg/ml detection antibody and incubation for 2 hours at room temperature. After washing and blotting of plates, avidin-HRP was made up to 0.5µl/ml in diluent and 100µl was added to each well. Plates were left to incubate for 30min at room temperature. After a final washing and blotting step, 100µl of ABTS solution (which acts as a substrate for peroxidase linked antibodies) was added to each well and the plates monitored for the development of a colour change which was read on a spectrophotometer (absorbance read at 405nm, reference at 650nm).

2.7 MTS Cell Proliferation Assay

The cell proliferation reagent MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) involves the metabolism of the tetrazolium salt to fomazan, a dark red product by cellular enzymes (Cory *et al.*, 1991). An increased number of viable cells results in an increase in mitochondrial dehydrogenase enzymes in the sample and therefore an increased amount of fomazan dye formed, which is directly correlated with the number of viable cells in the sample.

HUVEC cells were set-up on 'Day 0' in 96-well plates at a confluency of 2000cells/well. Cells were allowed to settle overnight in their growth medium before being quiesced for 12 hours in serum deprived medium on 'Day 1'. On 'Day 2' cells were treated by aspirating quiescent media and replacing it with conditioned media and appropriate controls.

Cells were left in treatment media for a further 1, 3 and 5 days before media was aspirated and a solution of 10% MTS reagent in wRPMI was added. Cells were incubated at 37°C for 2 hours before being read in a microplate reader at 450nm with a reference reading of 650nm.

Optimisation of the MTS assay involved finding an appropriate incubation time within the 0.5 – 4 hour range, and it was found that incubation for 2 hours resulted in the ideal absorbance readings to show a proliferative effect without becoming saturated.

2.8 Tubule Formation Assay

To assess the ability of conditioned media from breast cancer cells to promote endothelial tubule formation (Kubota *et al.*, 1988), HUVECs were cultured in conditioned media on a Matrigel base layer. Neat Matrigel was dispensed into the wells of the 96-well plate and placed in an incubator for an hour to set. HUVECs were harvested using Trypsin/EDTA and resuspended in growth serum deprived medium before being seeded at a cell density of 15,000 cells/10µl of medium. These cells were made up to a final volume of 100µl in separate aliquots of conditioned media and appropriate controls before being dispended into the Matrigel coated 96-well plate. Cells were returned to the incubator at 37°C with 5% CO_2 for 1 hour after which, they were removed and photographed using a light microscope at x10 magnification and digital camera light-microscope attachment. Matrigel was kept at 4°C to prior to usage in order to prevent premature gelling. 96-well plates and pipette tips were cooled to -20°C to prevent increasing the temperature of Matrigel while dispensing.

2.9 Electronic Cell-Substrate Impedance Sensing (ECIS)

The effect of breast cancer cell conditioned media on the permeability of an endothelial cell monolayer can be determined using electronic cell-substrate impedance sensing (ECIS) (Giaever et al., 1993). ECIS is a dedicated system to measure the resistance across a cell monolayer. At the heart of the system are plastic arrays on which cells can be cultured and treated (Figure 2.4). These arrays possess gold electrodes on their surfaces which create a measureable electrical current that can be continually measured. Through a computer program that monitors this current, they may be used to determine the fidelity of a cell monolayer and whether or not this can be disrupted or enhanced by external factors, in this case, those present within breast cancer cell-conditioned media. In the event that a particular treatment disrupts endothelial cell-cell interactions, a detectable drop in resistance can be observed.

Figure 2.4 8W10E ECIS array; on which endothelial cells may be grown and subsequently treated to determine the effects of treatment on: cell attachment and migration, wound healing and metastatic potential
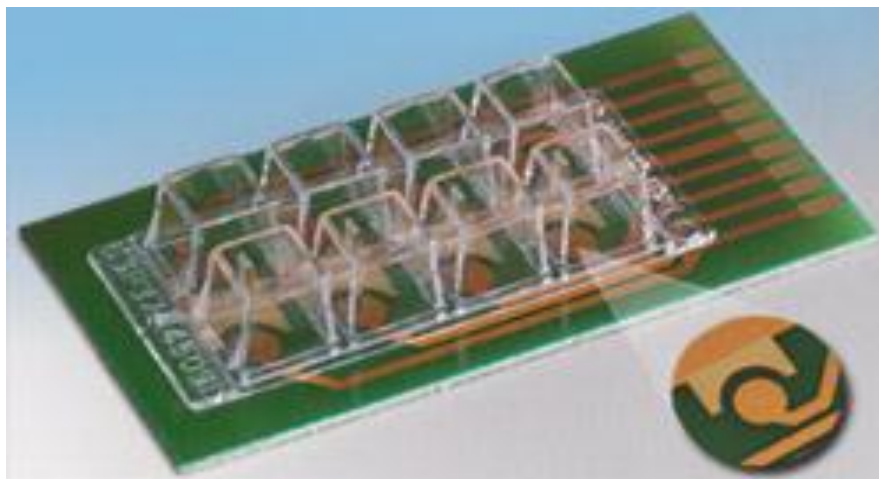
For our work, we used the ECIS Zθ (Applied Biophysics, Wold Laboratories Ltd. UK) system for measuring cell-cell adhesion of endothelial cells, (HECV cells).

This system was based in Cardiff University's School of Medicine within Professor Jiang's group. ECIS arrays were precoated with cysteine before use for 30 minutes to bind the gold surface and produce a molecular monolayer which is known to increase reproducibility Array wells were then flooded with serum-containing medium for 15 minutes to allow serum proteins to be adsorbed by the array monolayer. Following pre-treatment, HECV cells were trypsinised and counted, before seeding out at a cell concentration of 300,000 cells per array well. ECIS arrays were then immediately placed in the incubator and connected to the docking stations after which recording was begun. Using the computer associated with the ECIS equipment, impedance was assessed continually over 24 hours. When a plateau of impedance was observed, exhibiting an established monolayer of cells, arrays were removed and growth media were replaced with treatment media. After culturing for 48 hours, arrays were removed and data were analysed using the ECIS program software.

2.10 Chick Chorioallantoic Membrane Assay

Assessment of the effect of conditioned media treatment on *in vivo* angiogenesis was assessed using the chick chorioallantoic membrane (CAM) assay. Fertilised eggs were purchased chilled at 12°C to slow development of the embryo without reducing viability. These eggs may be stored at 12°C for up to one week before viability begins to deteriorate. On receipt of eggs they were transferred to a specialised egg incubator (Brinsea Mini ECO) that maintains temperature at 37.5°C and rotates eggs periodically to improve survivability by mimicking natural incubation procedures.

On day three post-fertilisation, eggs were prepared by spraying with 70% ethanol which reduced contamination risk. A small hole was bored in the wider end of the egg to depressurise the air sac. To ensure that removal of shell does not result in albumin or yolk spilling some albumin was removed prior to windowing. Eggs were laid on their side to allow removal of 2 to 3 ml of albumin using a syringe inserted into the pre-made hole. Care was taken to avoid disruption or removal of yolk as this can seriously reduce embryonic viability. The hole was then sealed using tape. Following this, eggs were pierced by inserting a sharp tweezers into the side of each egg ensuring not to apply surplus force which can result in breaking of shells. From this initial hole, an approximately $1cm^2$ window is made allowing observation of the developing embryo. This hole is then covered with tape and returned to the incubator overnight.

The following day (4 days post initial incubation), embryos were examined for viability. Embryos were determined to be viable if vessels are intact, the embryo continues to move and there is no discolouration of the yolk. Viable embryos were deemed suitable for testing of angiogenic stimuli. Heat sterilised filter paper was soaked in w&5 media or conditioned media from MCF7wt and TamR cell lines for 1 hour prior to treatment of CAM tissue. Filter papers were placed on CAM tissue proximal to embryos with one treatment (MCF7wt or TamR conditioned media) and one control on each embryo. Exposed CAM tissue was covered with tape and eggs were returned to the incubator.

Following treatment, eggs were removed from the incubator daily and photographed *in situ* to observe gross vascular changes and determine whether

these were directed towards the filter papers impregnated with conditioned media from our cell lines.

2.11 CD31 Immunohistochemistry on Breast Cancer Cell Xenografts

Paraffin embedded sections (3nM) of MCF7wt and TamR mouse xenografts (courtesy of Mr Chris Smith and Dr Julia Gee) were dewaxed by soaking twice in xylene for 7 minutes. Samples were presented as two sections of tissue per slide (one to act as a control). These samples were then rehydrated by soaking in decreasing concentrations of ethanol for 3 minutes twice (100%, 90% and 70% respectively) before being place in a trough of distilled water for 5 minutes. Samples were transferred to a coplin jar containing PBS for 5 minutes. Slides were then transferred into a humidity chamber with a single drop of 3% hydrogen peroxidase solution, which functions to eliminate endogenous peroxidases that can interfere with the specificity of an assay. Using pre-made citrate buffer (10mM Sodium citrate, pH 6.0) antigen retrieval was carried out using a pressure cooker for 2 minutes. Samples were then gradually cooled with slow running tap water before control and test samples were separated on each slide using a Dako, water repellent pen. Slides were soaked briefly in 0.5% Tween-20 in PBS. Primary antibody was then added to test samples on each slide (1/15 dilution of 1µg/ml rat anti-mouse CD31 primary antibody). Slides were transferred to a humidity chamber and placed in an incubator set to 23°C overnight. The following day, slides were washed twice with PBS for 5 minutes, followed by one wash with 0.5% Tween-20 in PBS. At this point, secondary antibody was applied to both test and control samples on each slide

(1/50 dilution of goat anti-rat HRP linked antibody). Slides were then incubated in humidity chambers placed in an incubator set to 23°C for 2 hours. Following incubation with secondary antibody, slides were washed by dipping in Tween-PBS solution.

To develop HRP linked antibody, DAB (3'3'-diaminobenzidine) substrate solution, which stains brown in the presence of HRP was prepared as per then manufacturer's instructions and 50µl of DAB solution was applied to each sample and incubated at room temperature for 10 minutes. Slides were washed in distilled water for 5 minutes twice before the addition of counterstain (10% haematoxylin in distilled water) for 10 minutes. Excess stain was removed by rinsing slides, placed in a coplin jar under a cold water source for 5 minutes to blue. Finally slides were mounted in DPX (di-n-butylphthalate in xylene) solution.

Analysis of CD31 staining was carried out by randomisation of MCF7wt and TamR slides before imaging of 5 random sections from the outer and inner parts of each sample using a light-microscope. Images were then overlaid with a counting grid that divided samples into 1mm^2 squares which allowed analysis of large and small cell groups based on the following criteria; large vessels occupied an area greater than one grid, medium vessels transected between two and three sides of one counting grid, and small vessels only transected one side of a counting grid. Total counts of large, medium and small vessels were made for inner and outer sections of each sample before they were grouped as MCF7wt and TamR.

2.12 Rat Aortic Ring Assay

Tissue was obtained from 150g Sprague-Dawley rats that were sacrificed by bleeding from the right femoral artery under anaesthetic (phenobarbital). Excision of the descending thoracic artery was performed before tissue was washed with sterile PBS to remove trace blood. Tissue sections were debrided of fibrous and adipose tissue using a scalpel before being cut into 1mm transverse sections. These sections were then embedded in a Matrigel plug in separate wells of a 12-well plate. Sections were treated with conditioned media from MCF7wt and TamR cells as well as appropriate controls (w&5 as a negative control and VEGF 10ng/ml as a positive control). These were then returned to incubators and allowed to grow over 10 days at 37°C. Media were changed on day 5 of culture. Formation of tubule structures was observed over the course of the experiment by taking photos daily and counting the structures observed in 5 random fields for each section.

2.13 Data Analysis and Statistics

GraphPad Prism version 5.01 was used to analyse data. Student's independent t-test was used for analysis of all results comparing pairs of data while one-way analysis of variance tests with Tukey's multiple comparison tests used for comparing more than two groups of data. All data were considered significant when $p \leq 0.05$. Results are, where appropriate, expressed as mean ±SEM.

Chapter 3: Characterisation of Pro-angiogenic Signalling in Endocrine-Sensitive and Endocrine-Resistant Breast Cancer Cells

3.1 Introduction and Aims

In the treatment of ER+ breast cancer the use of anti-oestrogen therapy using tamoxifen has been shown to be effective in suppressing tumour growth and preventing recurrent disease, providing benefit up to five years post treatment (Powles *et al.*, 2007). Despite this, around two-thirds of patients receiving tamoxifen will relapse with resistant disease (Dorssers *et al.*, 2001). Resistant disease frequently occurs as metastasis clinically and is associated with a poor prognosis (Ring *et al.*, 2004).

Acquired resistance to tamoxifen has been associated with elevated expression and activity of growth factor receptors such as EGFR and HER2, as well as intracellular kinases that drive survival, proliferation (Gee *et al.*, 2005) and other aggressive cell behaviours such as motility and invasion (Hiscox *et al.*, 2004). Whether acquired endocrine resistance affects the cells pro-angiogenic capacity remains unknown. Therefore, we sought to investigate whether there are changes in the regulation of angiogenesis which may prove to be functionally important in explaining phenotypical changes that are present on acquisition of endocrine resistance.

3.2 Results

3.2.1 Microarray Analysis of Pro-Angiogenic Factor Expression

To explore whether the gain in aggressive cellular characteristics extended to an angiogenic phenotype, our in-house microarray database of ER+, endocrine-sensitive (MCF7wt, T47D cells), ER+, endocrine-resistant (TamR, MCF7X cells) and ER-, faslodex-resistant MCF7wt cells was screened for the presence of genes with a pro-angiogenic ontology (Burmi *et al.*, 2006).

In studying whether the factors that regulate angiogenesis were altered on acquisition of anti-oestrogen resistance, a list of genes encompassing pro- and anti-angiogenic factors was compiled from a selection of papers reporting factors found to be associated with pro-and anti-angiogenic ontologies (Hoeben *et al.*, 2004; Robert, 2007; Roy *et al.*, 2006) as well as pro-angiogenic pathways from online sources (Cell Signalling, 2012). This list was screened using our in-house microarray database encompassing MCF7wt, TamR, FasR, X-cells and T47D cell arrays.

We investigated the expression of 322 genes that included pro-angiogenic factors including; FGF, VEGF and PDGF as well as negative regulators such as; Angiopoietin 2 and TSP-1. We used GeneSifter software to identify the most statistically significant upregulated and downregulated genes from our dataset between pairs of *in vitro* cell lines.

Array data comparing MCF7wt gene expression to that of TamR for the factors we selected (Figure 3.1) illustrated that the TamR cell line had higher expression levels of pro-angiogenic ligands such as VEGF and IL-8. In addition,

the levels of the anti-angiogenic glycoprotein Thrombospondin 1 were seen to be decreased in TamR cells in comparison to MCF7s.

Examination of array data for MCF7wt cells when compared to FasRs (Figure 3.2) demonstrated that there is an increased expression of interleukin-8 and despite also having a reduced expression of THBS1, there is also a reduction of positive regulators of angiogenesis such as VEGF-C.

Array data that compared MCF7wt expression profiles for angiogenic factors to those that came from array data examining oestrogen-deprived MCF7wt cells (MCF7X cells, a model of oestrogen deprivation) demonstrated a significant increase in the expression of VEGF-A (Figure 3.3). Figure 3.4 shows the most significantly upregulated and downregulated genes from the regulators of angiogenesis we examined in T47D cells versus MCF7wt cells. Here, we saw an increased number of negative angiogenic regulators being upregulated compared to MCF7wt.

Key:
−2   0   2
Fold Change

MCF7wt | TAMR

#	Gene
1	Interleukin 8
2	Fibroblast growth factor 13
3	nn80f11.s1 NCI_CGAP_Co9 Homo sapiens cDNA clone IMAGE:1090221 3', mRNA sequence....
4	Transforming growth factor, beta 1
5	qc74g06.x1 Soares_placenta_8to9weeks_2NbHP8to9W Homo sapiens cDNA clone IMAGE:17...
6	Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collag...
7	Midkine (neurite growth-promoting factor 2)
8	Interferon, alpha 13
9	Transforming growth factor, beta 1
10	Fibronectin 1
11	Vascular endothelial growth factor
12	Follistatin
13	Small inducible cytokine subfamily B (Cys-X-Cys), member 10
14	Angiopoietin 2
15	Forkhead box O1A (rhabdomyosarcoma)
16	Acid fibroblast growth factor-like protein
17	Colony stimulating factor 3 (granulocyte)
18	Vascular endothelial growth factor
19	SEE ALSO
20	tw46c12.x1 NCI_CGAP_Ut1 Homo sapiens cDNA clone IMAGE:2262742 3' similar to gb:X...
21	Follistatin
22	Platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) on...
23	Collagen, type XVIII, alpha 1
24	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
25	Runt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene)
26	Thrombospondin 1
27	CD59 antigen p18-20 (antigen identified by monoclonal antibodies 16.3A5, EJ16, E...
28	CD59 antigen p18-20 (antigen identified by monoclonal antibodies 16.3A5, EJ16, E...
29	601236272F1 NIH_MGC_44 Homo sapiens cDNA clone IMAGE:3608680 5', mRNA sequence.
30	Collagen, type XVIII, alpha 1
31	Stromal cell-derived factor 1
32	Fibroblast growth factor 2 (basic)
33	CD59 antigen p18-20 (antigen identified by monoclonal antibodies 16.3A5, EJ16, E...
34	Tissue inhibitor of metalloproteinase 2
35	qi08f09.x1 Soares_NhHMPu_S1 Homo sapiens cDNA clone IMAGE:1855913 3', mRNA seque...
36	Transforming growth factor, beta 2
37	7i82e10.x1 NCI_CGAP_Ov18 Homo sapiens cDNA clone IMAGE:3341226 3' similar to SW:...
38	Transforming growth factor, beta 2
39	tw46c12.x1 NCI_CGAP_Ut1 Homo sapiens cDNA clone IMAGE:2262742 3' similar to gb:X...
40	Ephrin-B2
41	B-cell translocation gene 1, anti-proliferative
42	7g91e06.x1 NCI_CGAP_Co16 Homo sapiens cDNA clone IMAGE:3313858 3', mRNA sequence...
43	601483690F1 NIH_MGC_69 Homo sapiens cDNA clone IMAGE:3886055 5', mRNA sequence.
44	AL535380 LTI_FL013_FBrn1 Homo sapiens cDNA clone CS0DF009YN13 5 prime, mRNA sequ...

Figure 3.1 Expression profile of regulators of angiogenesis in TamR vs. MCF7wt cells using the Affymetrix HG-U133A cDNA array.
MCF7wt and TamR cells were cultured under basal conditions to log-phase growth after which point they were harvested for total RNA using TriReagent (Sigma). RNA samples were DNAse1 treated to remove genomic DNA and purified using the Quiagen RNeasy mini kit. RNA samples were then quantified and tested for RNA integrity by spectrophotometry before being applied to Affymetrix HG-U133A Genechip analysis (Central Biotechnology Services, Cardiff University, Cardiff, UK). The Genechip arrays were scanned and analysed using the Microarray Suite 5.0 software (Affymetrix) and the quality of the data was verified through analysis of internal control gene expression. The gene expression profiles for the regulators of angiogenesis show above, presented as the most significantly upregulated and downregulated genes from the selection.

Figure 3.2 Expression profile of regulators of angiogenesis in FasR vs. MCF7wt cells using the Affymetrix HG-U133A cDNA array.

MCF7wt and FasR cells were cultured under basal conditions to log-phase growth after which point they were harvested for total RNA using TriReagent (Sigma). RNA samples were DNAse1 treated to remove genomic DNA and purified using the Quiagen RNeasy mini kit. RNA samples were then quantified and tested for RNA integrity by spectrophotometry before being applied to Affymetrix HG-U133A Genechip analysis (Central Biotechnology Services, Cardiff University, Cardiff, UK). The Genechip arrays were scanned and analysed using the Microarray Suite 5.0 software (Affymetrix) and the quality of the data was verified through analysis of internal control gene expression. The gene expression profiles for the regulators of angiogenesis show above, presented as the most significantly upregulated and downregulated genes from the selection.

Figure 3.3 Expression profile of regulators of angiogenesis in MCF7-X vs. MCF7wt cells using the Affymetrix HG-U133A cDNA array.

MCF7wt and MCF7-X cells were cultured under basal conditions to log-phase growth after which point they were harvested for total RNA using TriReagent (Sigma). RNA samples were DNAse1 treated to remove genomic DNA and purified using the Quiagen RNeasy mini kit. RNA samples were then quantified and tested for RNA integrity by spectrophotometry before being applied to Affymetrix HG-U133A Genechip analysis (Central Biotechnology Services, Cardiff University, Cardiff, UK). The Genechip arrays were scanned and analysed using the Microarray Suite 5.0 software (Affymetrix) and the quality of the data was verified through analysis of internal control gene expression. The gene expression profiles for the regulators of angiogenesis show above, presented as the most significantly upregulated and downregulated genes from the selection.

Key:

-2   0   2

Fold Change

	MCF7wt	T47D	
1			artemin
2			chemokine (C–X–C motif) ligand 5
3			troponin I type 3 (cardiac)
4			epidermal growth factor (beta–urogastrone)
5			interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte …
6			thrombospondin 1
7			KIT ligand
8			serine peptidase inhibitor, Kazal type 5
9			artemin
10			interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte …
11			endothelin 1
12			endoglin
13			KIT ligand
14			fibroblast growth factor 1 (acidic)
15			chemokine (C–C motif) ligand 3 /// chemokine (C–C motif) ligand 3–like 1 /// che…
16			angiopoietin 2
17			neuregulin 1
18			chemokine (C–C motif) ligand 14 /// chemokine (C–C motif) ligand 15
19			endoglin
20			angiogenin, ribonuclease, RNase A family, 5
21			granulin
22			artemin
23			thrombospondin 1
24			thrombospondin 1
25			insulin–like growth factor 1 (somatomedin C)
218			chemokine (C–X–C motif) ligand 14
219			transforming growth factor, beta 2
220			chemokine (C–X–C motif) ligand 6 (granulocyte chemotactic protein 2)
221			placental growth factor
222			TIMP metallopeptidase inhibitor 3
223			collagen, type IV, alpha 3 (Goodpasture antigen)
224			TIMP metallopeptidase inhibitor 3
225			serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type…
226			transforming growth factor, beta 2
227			serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type…
228			platelet–derived growth factor beta polypeptide (simian sarcoma viral (v–sis) on…
229			neuropilin 1
230			vasohibin 1
231			insulin–like growth factor binding protein 3
232			amphiregulin
233			insulin–like growth factor binding protein 3
234			ephrin–B2
235			transforming growth factor, beta 1
236			collagen, type XVIII, alpha 1
237			ephrin–B2
238			collagen, type XVIII, alpha 1
239			serpin peptidase inhibitor, clade B (ovalbumin), member 5
240			fibroblast growth factor 13
241			epiregulin
242			neuropilin 1
243			neuropilin 1

Figure 3.4 Expression profile of regulators of angiogenesis in T47D vs. MCF7wt cells using the Affymetrix HG-U133A cDNA array.

MCF7wt and T47D cells were cultured under basal conditions to log-phase growth after which point they were harvested for total RNA using TriReagent (Sigma). RNA samples were DNAse1 treated to remove genomic DNA and purified using the Quiagen RNeasy mini kit. RNA samples were then quantified and tested for RNA integrity by spectrophotometry before being applied to Affymetrix HG-U133A Genechip analysis (Central Biotechnology Services, Cardiff University, Cardiff, UK). The Genechip arrays were scanned and analysed using the Microarray Suite 5.0 software (Affymetrix) and the quality of the data was verified through analysis of internal control gene expression. The gene expression profiles for the regulators of angiogenesis show above, presented as the most significantly upregulated and downregulated genes from the selection.

3.2.2 Selection and Validation of Pro-Angiogenic Factor Expression in Breast Cancer Cell Lines

The information obtained from the Affymetrix dataset revealed significant deregulation of pro- and anti-angiogenic molecules in cell models of acquired anti-oestrogen resistance. This list was interrogated to select a number of factors based on whether: (i) they were positive regulators of angiogenesis and were upregulated in endocrine-resistant cell lines vs. the endocrine-sensitive MCF7wt cells; (ii) a well-established role in angiogenesis; (iii) potential targetability and finally (iv) possessed a link with existing data concerning our acquired resistant cell lines.

A major factor influencing the selection of these genes was that our cell models of resistance are known to possess elevated FAK and Src activity. These molecules are known to be deregulated after acquisition of tamoxifen resistance in MCF7wt cells (Hiscox *et al.*, 2006b; Planas-Silva *et al.*, 2006).

The two factors chosen to be taken forward were VEGF and IL-8. IL-8 was the most highly upregulated factor in TamR vs. MCF7wt and the second highest in FasR vs. MCF7wt analysis. VEGF, which is well established as a pro-angiogenic factor was also significantly upregulated in TamR vs. MCF7wt. Both of these molecules are well characterised in the literature and so, would not be difficult to obtain inhibitors, ligands or other tools for examination of their function and the role they may play in altered angiogenesis of endocrine-resistant breast cancer cells (Dai *et al.*, 2007; Li *et al.*, 2003). Both FAK and Src have been shown to influence the expression of IL-8 and VEGF respectively (Eliceiri *et al.*, 1999; Eliceiri *et al.*, 2002; Summy *et al.*, 2005; Trevino *et al.*,

2005) and therefore may represent a mechanism through which VEGF and IL-8 are overexpressed in the resistant cell models. Moreover, access to pharmacological inhibitors selective for these molecules would potentially allow us to further investigate the role of FAK/Src in the angiogenic capacity of our resistant cells.

3.2.3 Validation of VEGF and IL-8 Gene Expression in Endocrine-Sensitive and Resistant Breast Cancer Cells

Elevated expression of VEGF and IL-8 could contribute to a more angiogenic phenotype in endocrine-resistant cells and so, examination of mRNA expression of these ligands was carried out using semi-quantitative RT-PCR on RNA from our panel of breast cancer cells. These cells were grown under the same culture conditions as that in which the functional assays were performed. PCR products were resolved on 2% agarose gels with ethidium bromide and bands were analysed using densitometry.

Analysis of IL-8 expression across our *in vitro* cell lines (Figure 3.5) revealed that it was upregulated in all four cell lines examined, however, this was only significant in TamR cells when compared to MCF7wt cells. Expression of VEGF-A was greatly increased in TamR cells vs. MCF7wt as is VEGF-C.
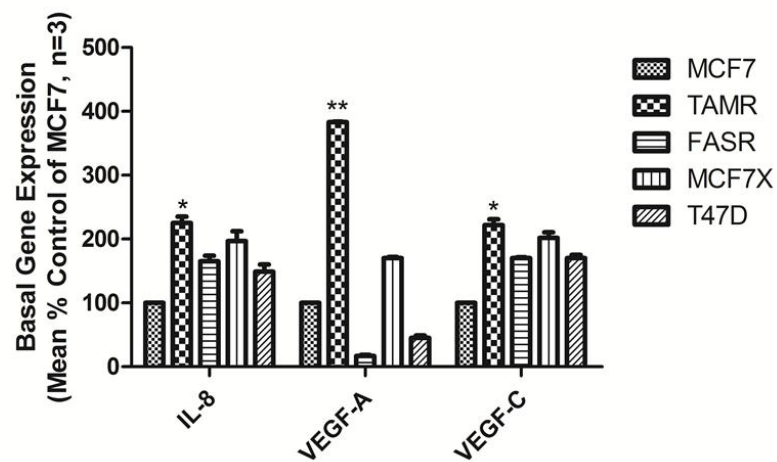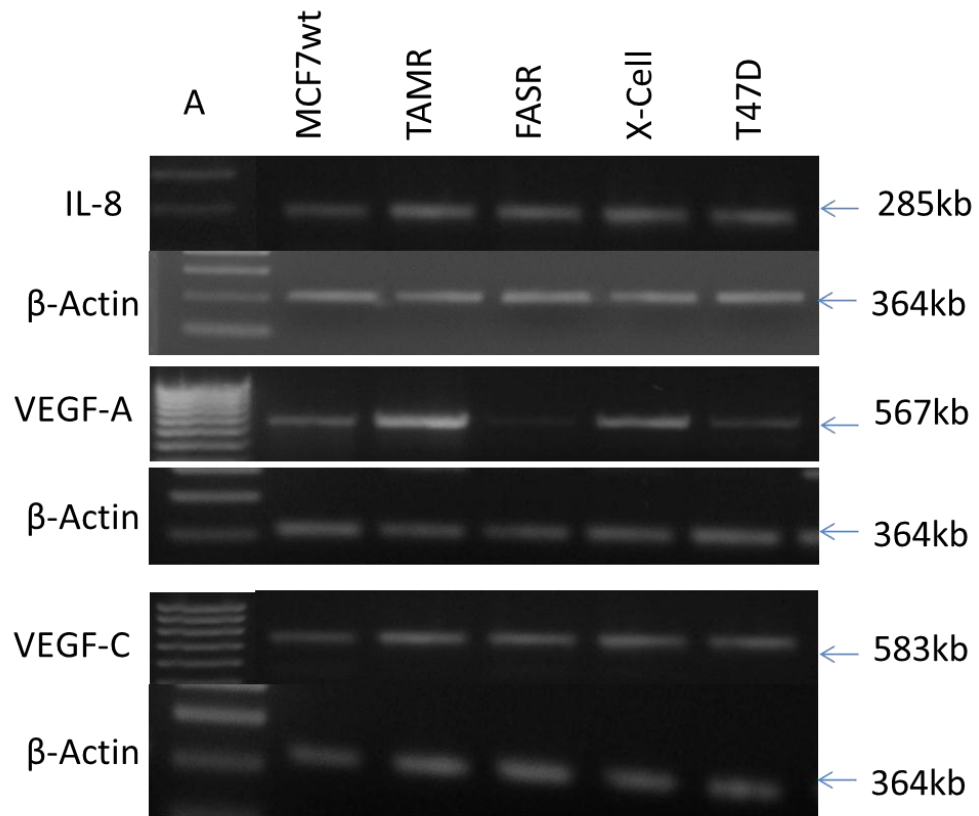
Figure 3.5 Basal IL-8 and VEGF levels in breast cancer cell lines as measured by semi-quantitative PCR

MCF7wt, TamR, FasR, X-Cell and T47D cells at 70% confluency were cultured in wRPMI + 5% foetal calf serum + 2% glutamine for 96 hours. Total RNA was then extracted from these cells using TriReagent as per the manufacturer's instructions. IL-8, VEGF-A and VEGF-C levels were determined by RT-PCR using specific primers and the PCR products were resolved on a 2% agarose gel using ethidium bromide electrophoresis. Bands were analysed by densitometry and data, corrected using Beta-actin, are presented as mean % control ± SEM, n=3; *, $p < 0.005$ vs. MCF7wt; **, $p < 0.0001$ vs. MCF7wt.

3.2.4 VEGF but not IL-8 Induces Endothelial Cell Proliferation

Having confirmed that VEGF and IL-8 gene expression were both higher in TamR cells compared to their endocrine-sensitive MCF7wt counterparts, we sought to investigate whether this was responsible for the increases seen in endothelial cell proliferation. Thus, we investigated the response of endothelial cells to purified VEGF and IL-8 using MTS assays.

Whilst treatment with VEGF resulted in a dose-dependent increase in endothelial cell growth (Figure 3.6), IL-8 did not have any significant effect on proliferation (Figure 3.7). Interestingly, at low concentration IL-8 appeared to be growth suppressive.
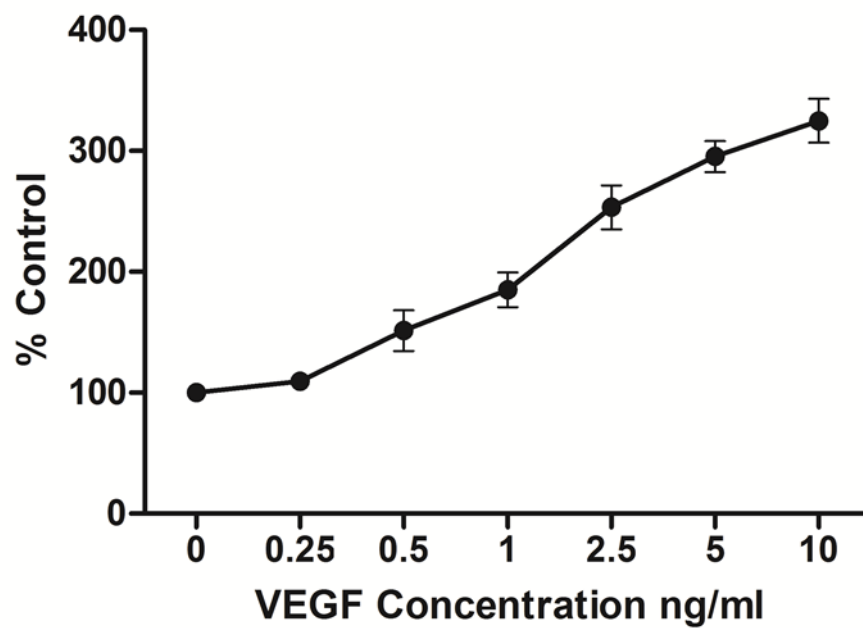
Figure 3.6 Dose response of VEGF stimulation on HUVEC cell proliferation
HUVEC cells were seeded onto 96-well plates in basal media and grown to 70% confluency before being quiesced in serum free media for 24 hours. Following this, media were removed and endothelial cells were treated with increasing concentrations of VEGF. Cell proliferation was assessed using MTS reagent. Data are presented as % Control ± SEM (n=3)
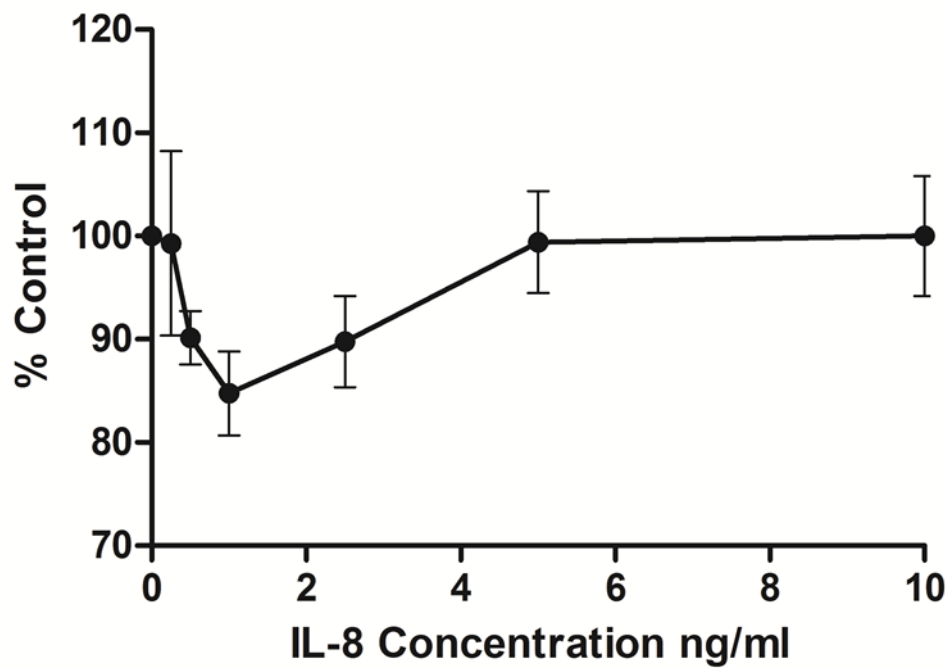
Figure 3.7 Dose response of IL-8 stimulation on HUVEC cell proliferation
HUVEC cells were seeded onto 96-well plates in basal media and grown to 70% confluency before being quiesced in serum free media for 24 hours. Following this, media were removed and endothelial cells were treated with increasing concentrations of IL-8 in w&5 or a w&5 control. Cell proliferation was assessed using MTS reagent. Data are presented as % Control ± SEM (n=3)

3.3 Analysis of VEGF Protein Expression in Breast Cancer Cell Conditioned Medium

Having identified that VEGF but not IL-8 stimulated HUVEC growth, we next sought to characterise breast cancer conditioned media for VEGF protein using ELISA. These data illustrated that VEGF was increased in media from endocrine-resistant breast cancer cell lines, TamR, FasR and T47D but only significantly in the TamR cell media (Figure 3.8).
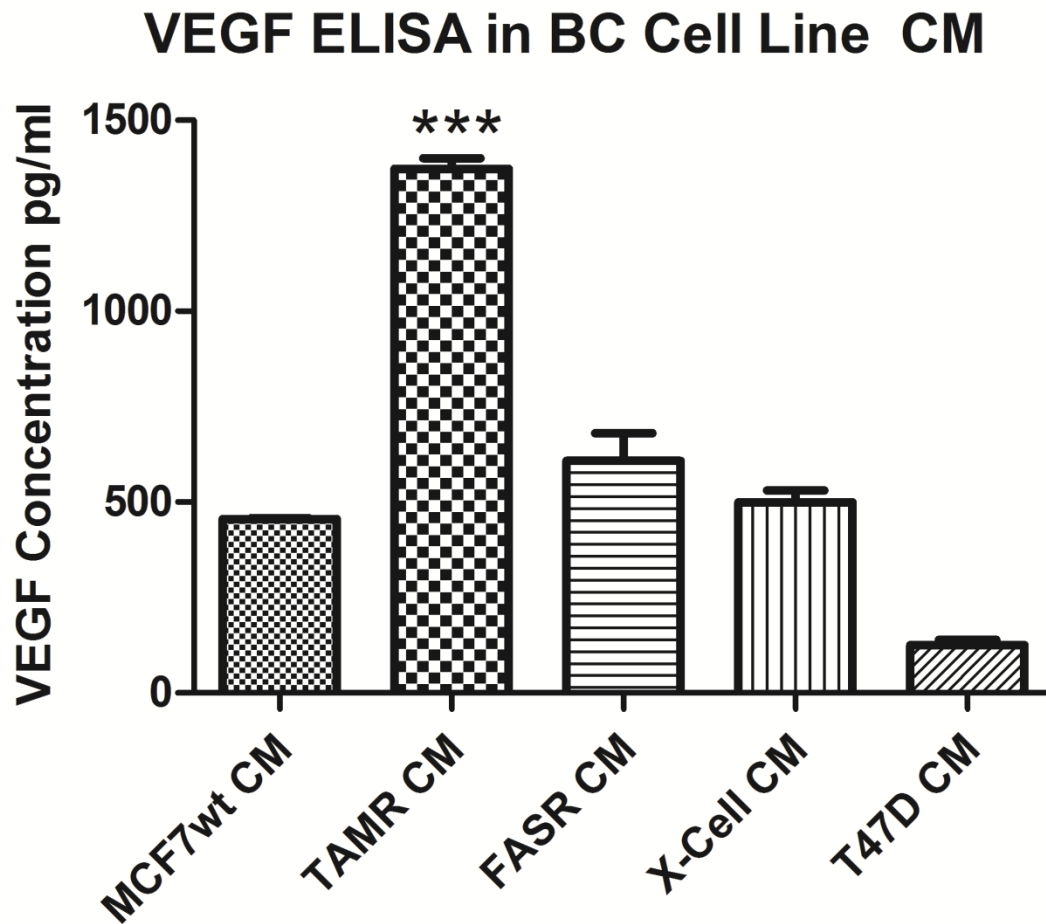
Figure 3.8 Basal protein levels of VEGF in conditioned media taken from breast cancer cell lines as measured by ELISA.
MCF7wt, TamR, FasR, X-Cell and T47D cells were grown in w&5 for a period of 4 days after which conditioned media was collected, clarified and stored at -20°C. ELISA was performed on conditioned media and protein concentrations were normalised to cell counting experiments performed after collection of media. Data are mean VEGF in pg/ml of media and are shown ± SEM. ***, $p<0.0001$ vs. Control (n=3).

3.3.1 Exploration of VEGF Production by TamR cells: HIF-1α

Typically, regulation of angiogenesis is under the control of HIF 1-alpha (Pugh *et al.*, 2003). Under hypoxic conditions the breakdown of HIF-1α is inhibited which frees the transcription factor to bind DNA and upregulate the expression of several genes implicated in angiogenesis. To assess whether altered HIF-1α expression was contributing to the elevated VEGF seen in conditioned media taken from TamR cells we first examined the mRNA expression of the gene in breast cancer cells. RT-PCR analysis (Figure 3.9) of HIF 1-alpha expression did not show any alteration in expression of the gene at an mRNA level between the cell lines we examined.

Examination of HIF1-α protein expression (Figure 3.10) revealed no significant difference between MCF7wt and TamR cell expression of the transcription factor although the expression level in FasR, MCF7X and T47D cell did demonstrate a lower level of the protein when compared to MCF7wt. Interestingly, alterations in the expression of HIF 1-alpha did not seem to correlate with differences seen in expression of VEGF and IL-8 (Figure 3.5).
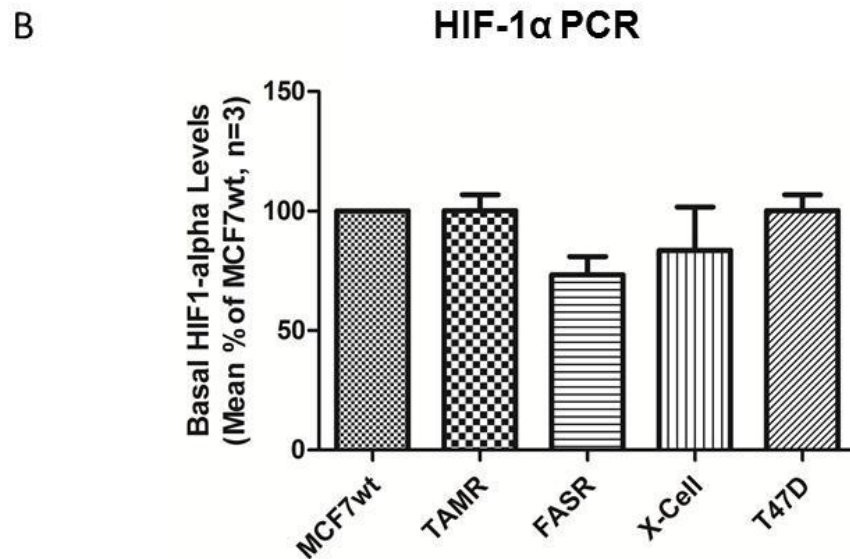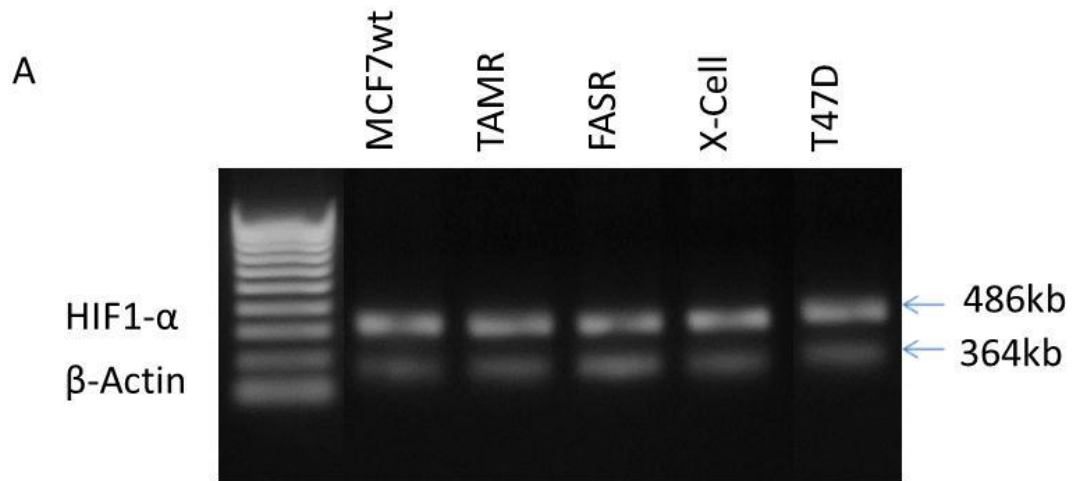
Figure 3.9 Basal HIF1-alpha levels in breast cancer cell lines as measured by semi-quantitative PCR

MCF7wt, TamR, FasR, X-Cell and T47D cells at 70% confluency were cultured in wRPMI + 5% foetal calf serum + 2% glutamine for 96 hours. Total RNA was then extracted from these cells using TriReagent as per the manufacturer's instructions. HIF1-alpha levels were determined by RT-PCR using primers specific for HIF1-alpha and the PCR products were resolved on a 2% agarose gel using ethidium bromide electrophoresis. Bands were analysed by densitometry and data, corrected using Beta-actin, are presented as mean % control ± SEM, n=3.
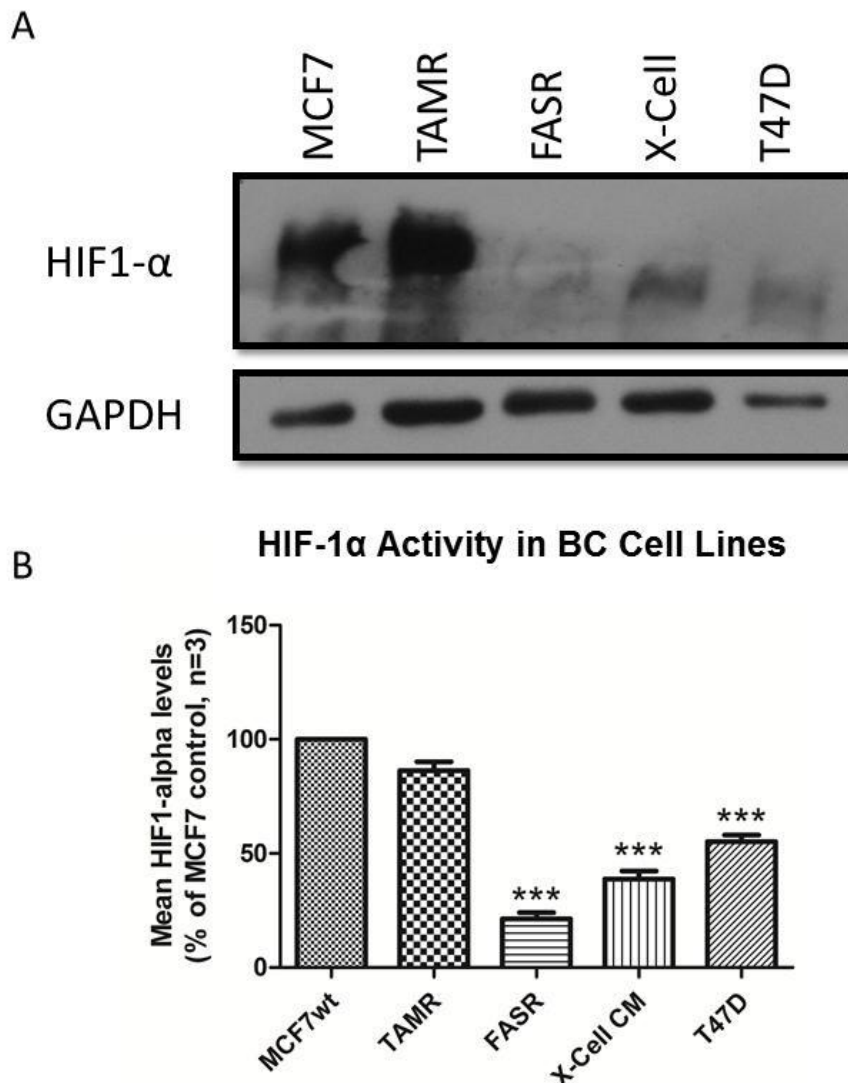
Figure 3.10 Basal levels of HIF1-alpha in breast cancer cell lines as determined by Western blotting

MCF7wt, TamR, FasR, MCF7X and T47D cells were cultured from 70% in wRPMI + 5% stripped foetal calf serum + 2% glutamine for 96-hours before being lysed for proteins as described previously. Total soluble protein (20μg/lane) was subjected to SDS-PAGE and Western blot analysis before membranes were probed with antibodies specific for HIF 1-alpha (A). Densitometry was conducted on the bands obtained and the data, which were corrected for loading with GAPDH are presented as mean % control (MCF7wt) ± SEM, n=3.

3.3.2 Exploration of VEGF Production by TamR Cells: ER

Several groups have published data linking regulation of VEGF to oestrogen receptor signalling (Buteau-Lozano *et al.*, 2002; Ruohola *et al.*, 1999), although not all reports demonstrate this (Bogin *et al.*, 2002; Hyder *et al.*, 1998). Nevertheless, considering endocrine resistance occurs as a consequence of altered ER signalling, we sought to examine whether this would affect the production of VEGF by our tamoxifen resistant cells. In addition, we sought to determine whether ER stimulation or inhibition in our endocrine-sensitive MCF7wt cells would have any effect on their VEGF production.

Examination of VEGF concentration in conditioned media (Figure 3.11) from MCF7wt and TamR cells treated with tamoxifen, faslodex, oestradiol or control media illustrated that there was no significant difference between any treatment group and the control for that group.
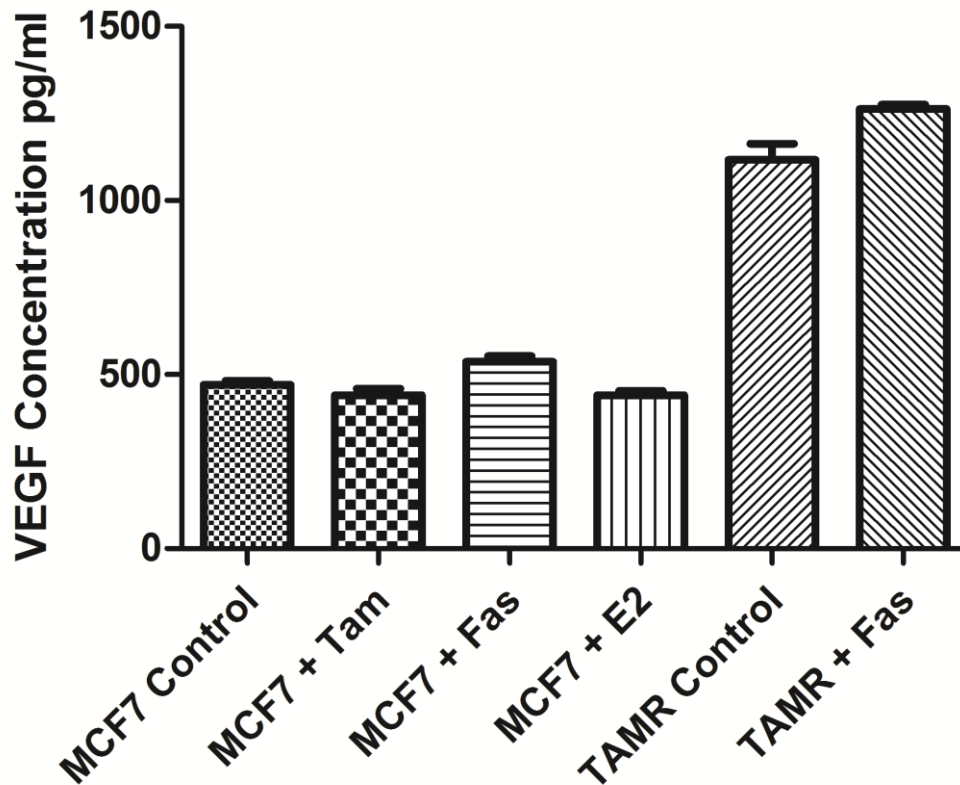
Figure 3.11 VEGF Concentration in conditioned media taken from breast cancer cell lines that were treated for 4 days with endocrine agents
MCF7wt and TamR cells were set up in experimental media and allowed to settle overnight before media were replaced with w&5 containing Tamoxifen (4×10^{-7}M), Faslodex (1×10^{-7}M) or E2 (1×10^{-9}M). Media were collected 96 hours later and an ELISA was performed to determine VEGF concentration in each sample. Media were normalised to total cell counts, determined at 96 hours post treatment. All results were ns compared to MCF7wt control.

3.3.3 Exploration of VEGF Production by TamR Cells: Src kinase

TamR cells possess elevated signalling through several growth factor pathways

which results in increased phosphorylation of downstream second messengers

such as MAPK and Akt (Figure 3.12). Previous work (S. Hiscox, unpublished data) has already suggested a role for Src kinase, a factor known to be upregulated in acquired anti-oestrogen resistance models, as a key regulator of pro-angiogenic signalling. In addition we were able to inhibit the main Src-family member using a small molecule inhibitor, Saracatinib (AZD0530). Examination of ELISA data from experiments carried out on conditioned media taken from TamR cells that had been pre-treated with the drug (Figure 3.13) illustrated that removal of Src kinase signalling leads to a significant reduction in the production of VEGF by the cell. Inhibition of other intracellular kinases (Figure 3.13) known to be upregulated in TamR cells in comparison to MCF7wts illustrated that the removal of MAPK and PI3K/Akt signalling at doses known to reduce their activity, does not have a significant effect on the reduction of VEGF production by TamR cells.
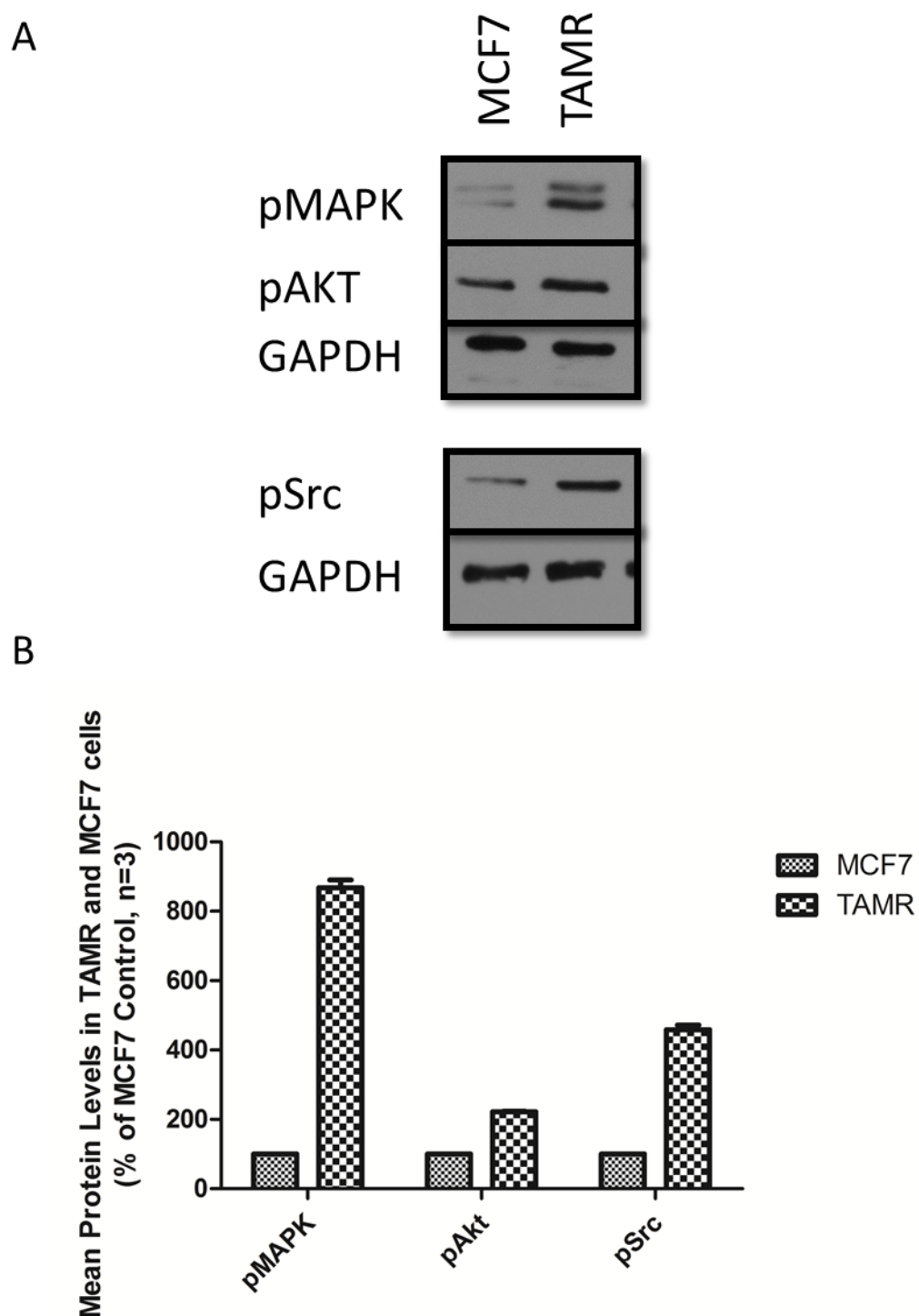
Figure 3.12 Basal levels of intracellular signalling molecules in MCF7wt and TamR cells as determined by Western blotting

A. MCF7wt and TamR cells were cultured from 70% in wRPMI + 5% stripped foetal calf serum + 2% glutamine for 96-hours before being lysed for proteins as described previously. Total soluble protein (20µg/lane) was subjected to SDS-PAGE and Western blot analysis before membranes were probed with antibodies specific for phospho-MAPK, phospho-Akt and phospho-Src (A).

B. Densitometry was conducted on the bands obtained and the data, which were corrected for loading with GAPDH are presented as mean % control (MCF7wt) ± SEM, n=3.
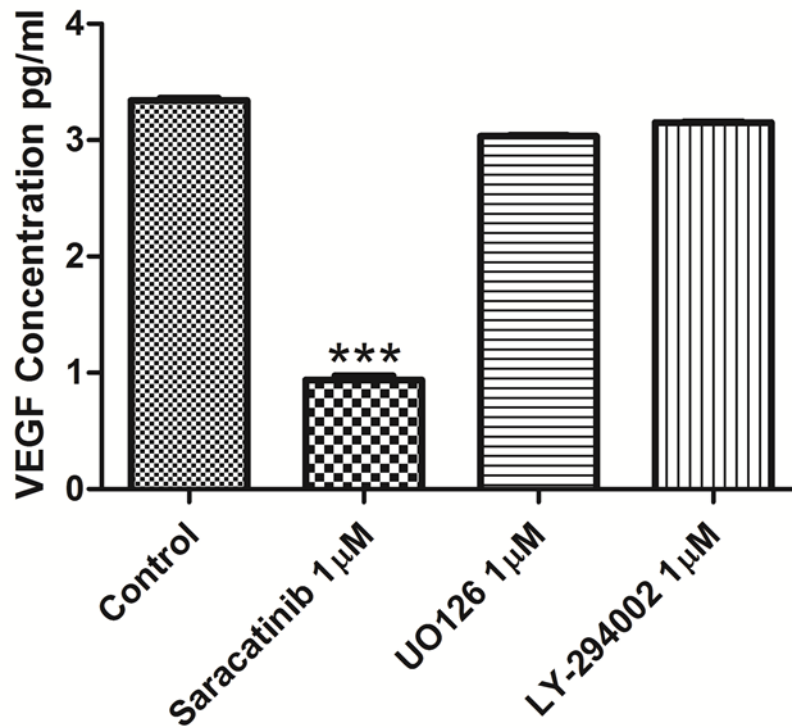
Figure 3.13 Basal protein levels of VEGF in conditioned media taken from breast cancer cell lines as measured by ELISA ± Src inhibition.
TamR cells were grown in wRPMI + 5% stripped foetal calf serum + 2% glutamine ± 1μM saracatinib OR 1μM UO126 OR 1μM LY-2940002 for a period of 4 days after which conditioned media was collected, clarified and stored at -20°C. ELISA was performed on conditioned media and protein concentrations were normalised to cell counting experiments performed on collection of media. Data are mean VEGF in pg/ml of media and are shown ± SEM. ***, p<0.00001 (n=3).

3.4 Discussion

The aim of this chapter was to determine the extent of the deregulation of angiogenic factors in endocrine-resistant breast cancer cells and to conduct a preliminary exploration of a mechanism by which this occurs.

3.4.1 Basal Angiogenic Characteristics of Breast Cancer Cells

Resistance to treatment with anti-oestrogens is a major clinical problem and the aggressive phenotype associated with acquisition of resistance is of serious concern. The presence of a more invasive and motile phenotype has already been established in *in vitro* models but what is less well understood is how deregulation of angiogenesis may play a role in resistant disease.

Deregulation of growth factor receptor expression and activation in acquired anti-oestrogen resistance has been clearly demonstrated (Knowlden *et al.*, 2003; Massarweh *et al.*, 2008) and provides growth signalling in the presence of endocrine agents. Here, we took a panel of breast cancer cell lines that represent different forms of anti-oestrogen resistance and examined whether angiogenic factors were also altered. Importantly, we were looking for factors that fit specific criteria. This would ensure that we took forward the best possible candidates for further investigations.

Our criteria demanded that the "ideal" candidate factors would be;

> 1. Upregulated, pro-angiogenic factors. Deregulation of angiogenesis that leads to increased endothelial cell growth and increased vascularisation could manifest as a result of increased pro-angiogenic

signalling or reduced anti-angiogenic signalling. We ultimately sought to reverse any changes that would bring about a pro-angiogenic phenotype and this would be much more straightforward through inhibition of pro-angiogenic factors than it would be to increase the expression or activity of anti-angiogenic factors. In addition, this would prove more beneficial in identifying a druggable target with clinical relevance.

2. Well established in the literature. Having determined factors that play a role in pro-angiogenic signalling we sought to explore the mechanism by which this deregulation came about. Selecting factors that are well-documented and whose mechanism is well understood would facilitate this process.

3. Druggable Targets. As mentioned, we wanted to explore reversal of pro-angiogenic signalling and so, choosing factors that are easily druggable or have commercially available inhibitors would enable this.

4. Possess an established link with Src and FAK. Having established the importance of pro-invasive kinases Src and FAK in our cell models of endocrine resistance, and the documented link between these factors and angiogenesis (Eliceiri *et al.*, 2002; Lin *et al.*, 2003) we hypothesised that these factors may play a role in altered pro-angiogenic signalling. We therefore favoured pro-angiogenic factors that are regulated by these kinases.

All three models of resistance demonstrated deregulation of pro- and anti-angiogenic factors. TamR vs. MCF7wt cells and FasR vs. MCF7wt cells both

showed significant upregulation of commonly reported pro-angiogenic factors including the pro-angiogenic chemokine, IL-8 and the potent angiogenic growth factor, VEGF, both of which fit our criteria for factor selection. MCF7-X cells also demonstrated significant upregulation of VEGF; however, this was accompanied by an increased expression of several anti-angiogenic agents including Thrombospondin 1.

IL-8 is a pleiotropic chemokine expressed in a variety of tissues including colon, lung and prostate which is also expressed by endothelial cells where it functions in promotion of metastasis and angiogenesis (Keping, 2001). IL-8 has previously been shown to bind to CXCR1 and CXCR2 receptors and promote proliferation and survival signalling in endothelial cells (Li *et al.*, 2003). The expression of this chemokine has also been shown to be elevated in breast cancer tumours that do not express ER (Freund *et al.*, 2002) which may contribute to an invasive phenotype in cancer cells (Green *et al.*, 1997).

VEGF-A is the most important VEGF family member in promotion of angiogenesis. VEGF-C predominantly functions in lymphangiogenesis, or development of new lymphatic vessels (Stacker *et al.*, 2002). For our purposes, therefore, VEGF-A is of greater importance and in this work is commonly referred to as VEGF. VEGF exerts its effect primarily by binding to VEGFR2 on the cell surface of endothelial cells resulting in proliferation and vascularisation of the surrounding tissue (Adams *et al.*, 2000). VEGF mRNA and protein expression has already been shown to be elevated in *in vivo* MCF7wt cells after treatment with tamoxifen (Bogin *et al.*, 2002) and additionally, to be regulated by the action of oestrogen, through binding of ER-α and c-Myc to VEGF promoter

sites. Our data, however, suggests than in our MCF7wt cell model short-term treatment with tamoxifen did not increase the protein expression of VEGF as measured by ELISA of conditioned media from these cells. Differences in our experimental procedure may explain the disparity in our results. Our studies were carried out using a 4 day treatment in comparison to the previous study in which cells were treated for 14 days. Additionally, Bogin *et al.* used MCF7wt cells growing as subcutaneous xenografts while we used *in vitro* MCF7wt cells.

PCR analysis of IL-8 and VEGF expression in our panel of breast cancer cell lines (Figure 3.5) confirmed that the mRNA levels for these ligands in our acquired tamoxifen resistant breast cancer cell model is indeed elevated compared to the MCF7wt cells. Although, IL-8 can enhance the survival and proliferation of both tumour cells and endothelial cells, IL-8 failed to induce HUVEC cell growth. Moreover, we found that we could not detect the expression of CXCR receptors in our two endothelial cell models (data not shown). This is supported by similar work which suggests a role for IL-8 as an indirect mediator of angiogenesis that may work with a cohort of other factors to produce an angiogenic response (Petzelbauer *et al.*, 1995). For this reason, we eliminated IL-8 from our study and continued with VEGF as a candidate mediator of a proangiogenic phenotype in acquired tamoxifen resistant breast cancer.

Data from mRNA analysis was supported by protein analysis of VEGF expression by ELISA (Figure 3.8) which demonstrated that conditioned media taken from TamR cells had significantly higher concentrations of VEGF than MCF7wt media. However, elevated VEGF concentrations were not observed in

conditioned media taken from FasR, X-Cell or T47D cells and in addition, these cells lines did not significantly induce endothelial cell growth (Chapter 4) as a result of this we eliminated these cell lines from further investigations.

3.4.2 Mechanism of VEGF Production in Breast Cancer Cells

Having identified VEGF as a potential pro-angiogenic candidate associated with acquired tamoxifen resistance, we sought to explore the mechanism by which VEGF ligand was increased in TamR cells.

3.4.2.1 Classical Regulation of VEGF Production: HIF-1α

Angiogenesis is stimulated in response to low concentrations of oxygen through the action of HIF-1α. In normoxic conditions, the alpha subunits of this protein are hydroxylated by proline-hydroxylases, the function of which is limited by oxygen concentration. The effect of HIF-1α hydroxylation is subsequent ubiquitination of the protein by VHL E3 ubiquitin ligase enzymes followed by degradation by the proteasome. In a hypoxic environment the action of the proline-hydroxylase enzyme is inhibited and so, HIF is free to upregulate the expression of target genes (Huang *et al.*, 1998). HIF-1 alpha binds to response elements upstream of genes not only implicated in angiogenesis, such as VEGF, but in tumour progression by mediating cell proliferation and survival (iNOS, IGF2) (Laughner *et al.*, 2001).

Here, however, we present data that suggests that although HIF-1 alpha is detectable at both mRNA and protein levels in both the endocrine-sensitive MCF7wt and the anti-oestrogen resistant TamR cells, there is no significant

difference seen between them in contrast to VEGF, which is greatly upregulated in TamR cells. Lack of correlation between HIF-1α expression/activity and VEGF protein expression suggests a HIF-1α independent mechanism. Previous work supports a model in which angiogenic signalling through increased VEGF production can continue in the absence of altered HIF-1α signalling.

It has been proposed that although loss of HIF-1α in tumour cells does indeed limit tumour growth, it does not eliminate the tumour and growth may eventually resume in an HIF-1α independent manner (Sutphin *et al.*, 2004). In colon cancer cells, induction of mutant variants of HIF-1α by RNA interference did not completely eliminate VEGF production (Mizukami *et al.*, 2004). The same group also found that after the knock-down of HIF-1 alpha, the expression of the chemokine IL-8 was increased under hypoxic conditions and that inhibition of IL-8 reduced the hypoxic stimulation of VEGF production (Mizukami *et al.*, 2005). A study in which siRNA was used to knock-down the expression of HIF-1α in a hepatocellular carcinoma cell line found detectable levels of VEGF production that could be further reduced using inhibitors that targeted Akt/PI3K and the transcription factor SP1 (Sae Byeol Choi, 2011). In exploring HIF-1α independent mechanisms relevant to our own cell models we then sought to explore VEGF regulation in endocrine-resistant cells based on signalling through the well-documented receptor implicated in endocrine resistance, the ER itself.

3.4.2.2 HIF-1α Independent Regulation of VEGF Production: ER

Previous evidence linking ER to the regulation of VEGF supports the hypothesis that alterations in ER signalling as a result of endocrine resistance could alter VEGF production by tumour cells. Pre-clinical evidence suggested that VEGF transcription may be regulated by oestrogen activity through ERα via SP-proteins (Stoner *et al.*, 2004), and that VEGF regulation may be controlled by a complex ERα-ERβ mechanism which alters the effect of E2 binding (Buteau-Lozano *et al.*, 2002).

Clinically, increased circulating VEGF levels have been reported in tamoxifen treated patients with metastatic disease compared to those not receiving tamoxifen, which may illustrate a deregulation of VEGF production as a result of treatment with tamoxifen (Adams *et al.*, 2000). Having established a rationale for examining ER regulation of VEGF, we analysed the production of VEGF by MCF7wt and TamR cells that had been treated for 96-hours with oestradiol stimulation, tamoxifen inhibition or fulvestrant inhibition. However, as Figure 3.11 illustrates, no treatment group showed a significant difference in VEGF levels when compared to control. Although we are not the first to suggest that ER may not play a role in regulation of VEGF in these cell lines (Bogin *et al.*, 2002; Guo *et al.*, 2003; Hyder *et al.*, 1998), it must be taken into consideration that the use of short-term stimulation or inhibition may not be sufficient to produce alterations in VEGF production and that longer term treatment may be required. Having found no correlation between ER activity and the production of increased VEGF by endocrine-resistant cells, we explored the involvement of Src kinases. These pro-invasive enzymes are known to be deregulated in

tamoxifen resistance *in vitro* and have been previously implicated in the regulation of VEGF in the literature (Eliceiri *et al.*, 1999; Hiscox *et al.*, 2006b).

3.4.2.3 HIF-1α Independent Regulation of VEGF Production: Src Kinase

On examination of Src kinase inhibition as well as In Figure 3.12, data illustrates that TamR cells exhibit a higher activity of the intracellular signalling molecules MAPK and Akt as well as increased Src kinase activity. As discussed previously, alterations in signalling through these second messenger hubs can result in altered transcription and expression of pro-angiogenic signalling molecules such as VEGF. When we examined what effect inhibition of these proteins had on the concentration of VEGF in conditioned media taken from TamR cells we found that only inhibition of Src kinase resulted in reduced VEGF (Figure 3.13)

Signalling through MAPK has been shown to directly upregulate expression of VEGF by directly phosphorylating the alpha subunit of HIF-1 and increasing transcriptional activity of the protein. Akt signalling on the other hand also increases VEGF expression by binding HIF-1 alpha, however, the mechanism is slightly different in that Akt stabilises the protein binding of HIF-1 alpha to the HRE (Sodhi *et al.*, 2001). Thus both MAPK and Akt can independently regulate the activity of HIF-1 alpha. However, in our study we have demonstrated that inhibition of these kinases did not reduce VEGF production by TamR cells which may support the hypothesis that the increases seen in VEGF concentration in TamR conditioned media versus MCF7wt conditioned media are not as a result of elevated MAPK or Akt signalling.

Inhibition of Src kinase, however, resulted in a significant reduction in the VEGF concentration in TamR conditioned media. This would suggest that the increases seen in TamR CM versus MCF7wt CM are the result of alterations in signalling through Src kinase and it is this change that mediate increases in VEGF expression. Regulation of angiogenesis by the Src family kinases has not been a huge subject of interest; however, the evidence that already exists demonstrates that Src plays a vital role in the regulation of endothelial cell function and angiogenesis. Src null mice exhibit normal vascular system development; however, mice deficient in Src, Fyn and Yes do not survive past approximately 10 days, which is the same time period that VEGFR null mice undergo embryonic lethality (Donald *et al.*, 2005). The role of Src in VEGF regulation is usually attributed to its ability to increase VEGF mRNA production by binding to HIF-1 alpha and STAT3 at the VEGF promoter region, therefore increasing its expression (Gray *et al.*, 2005).

3.5 Chapter Summary

In this chapter, we have shown that;

- MCF7wt models of acquired tamoxifen resistance and fulvestrant resistance overexpress genes for a number of key positive regulators of angiogenesis

- VEGF protein expression is significantly increased in TamR cells which reflects changes seen in gene expression

- Alterations seen in VEGF expression do not appear to be due to deregulation of HIF 1-alpha expression or signalling through the ER but rather arise as a consequence of increased Src activity in these cells

Chapter 4: *In vitro* Assessment of Conditioned Media Effects on Endothelial Cell Function

4.1 Introduction

Having established that key pro-angiogenic factors are deregulated in endocrine-resistant breast cancer cell models, the next element of this project was to study whether this translated into an increased capacity to promote angiogenic events in endothelial cells. Two cells lines were used to model endothelial cell function: HUVEC and HECV.

Human umbilical vein endothelial cells (HUVECs) are a primary cell line that are isolated from cultures of mixed umbilical cord vein cells before fibroblasts and other non-endothelial cell are removed by centrifugation and enzyme action. They are typically used between passages 8 and 10 to maintain primary cell characteristics (Rhim *et al.*, 1998).

Although a good model of endothelial cells, the HUVEC cell line can be challenging to use *in vitro* due to their passage limitations. To address this, and also to limit cell line specific effects, we sought to use an alternative model of endothelial cells, namely, the HECV cell line. These are primary endothelial cells that are more suitable for continuous culture and retain primary cell characteristics for longer, allowing them to be maintained in cell culture for ~15 passages.

4.2 Results

4.2.1 Effect of Breast Cancer Cell Line Conditioned Media on Endothelial Cell Proliferation

The ability of breast cancer conditioned media to promote the proliferation of endothelial cells was determined by MTS assay. Endothelial cells (HUVEC and HECV) were grown to 70% confluency being treated for 72 hours with conditioned media after which an MTS assay was performed to assess proliferation.

All breast cancer cell types induced HUVEC proliferation (Figure 4.1 A) but a significant increase was only observed in endothelial cells treated with TamR conditioned media. These growth effects were also shown in HECV cells (Figure 4.1 B) again illustrating that TamR conditioned media increases endothelial cell proliferation to a greater extent versus other cell lines.
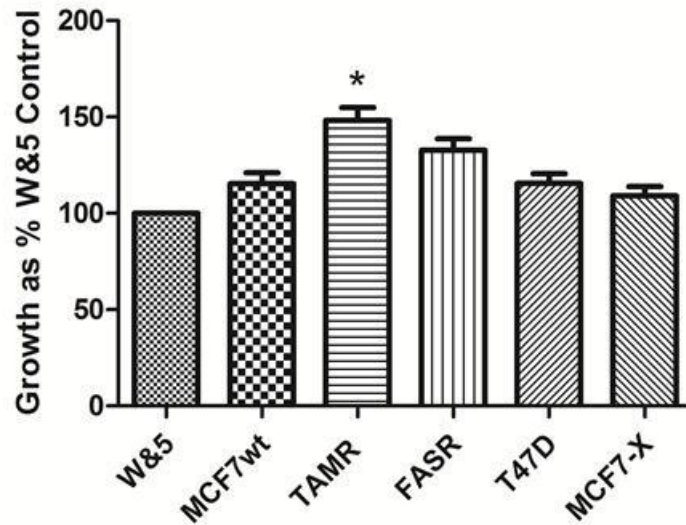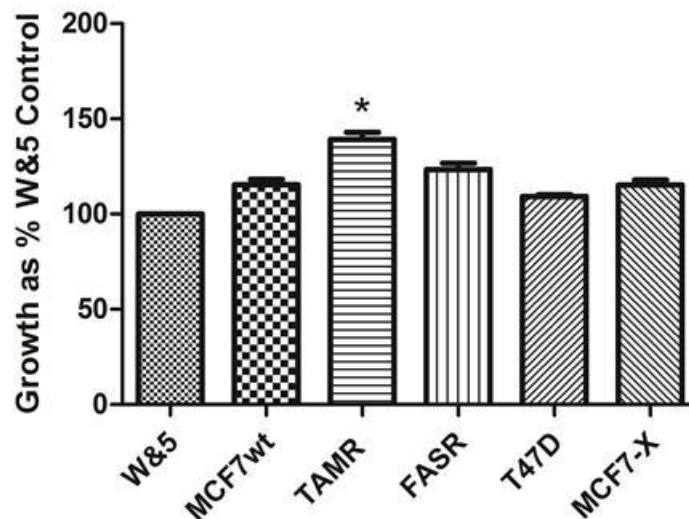
Figure 4.1 Effect of conditioned media from several breast cancer cell lines on the proliferation of HUVEC and HECV endothelial cells as determined by MTS assay.

HUVEC and HECV cells were seeded onto 96-well plates in basal media and grown to 70% confluency before being quiesced in serum free media for 24 hours. Media were then replaced with neat conditioned media from breast cancer cell lines (MCF7wt, TamR, FasR, T47D, and MCF7-X) or an untreated media as control. Cell proliferation was assessed using MTS reagent. Data are presented as % Control ± SEM (* $p < 0.05$ vs. Control, n=3)

4.2.2 Blockage of Endothelial Cell VEGFR2 Attenuates their Response to TamR Conditioned Media

Having determined that only TamR conditioned media significantly increased cell proliferation versus MCF7wt conditioned media; it was taken forward for further investigations. Endothelial cells respond to stimulation with VEGF which has previously been shown to be upregulated in TamR cells versus endocrine-sensitive cells. Therefore we wished to further investigate whether VEGF receptors represent a major factor for TamR conditioned media induced endothelial cell proliferation.

To examine this, HUVEC cells were pre-treated with the small molecule inhibitor ZM323881 to block signalling through the VEGFR2 receptor, as this receptor mediates most of the pro-angiogenic effects of VEGF. As illustrated in Figure 4.2, this pre-treatment blocked the capacity of TamR conditioned media to induce cell proliferation of both HUEVC and HECV cell lines.
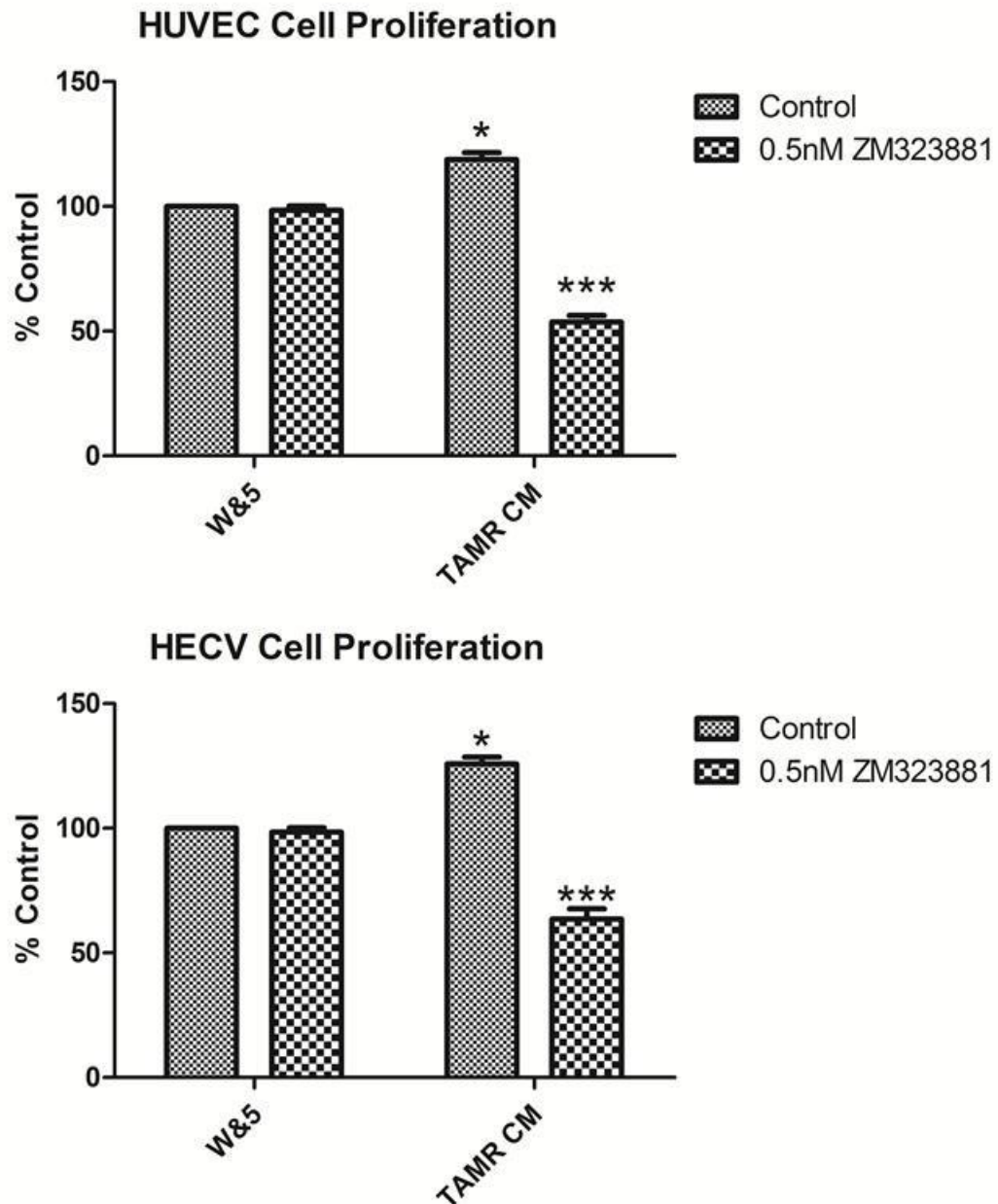
Figure 4.2 Cell proliferation of endothelial cells after treatment with conditioned media from TamR cell lines, ± the VEGFR2 inhibitor ZM323881
HUVEC and HECV cells were seeded onto 96-well plates in basal media and grown to 70% confluency before being quiesced in serum free media for 24 hours. Following this, media were removed and endothelial cells were treated with w&5 containing 1μM ZM323881 or a w&5 control. After 1 hour of treatment with the inhibitor, media were once again removed and replaced with conditioned media from TamR cells. Cell proliferation was assessed using MTS reagent. Data are presented as % Control ± SEM (* p<0.05 vs. w&5 Control, *** p<0.0001 vs. TamR CM control, n=3)

4.2.3 Reductions in VEGF Concentration by Src kinase Inhibition Result in Reduction of Cell Proliferation by TamR Conditioned Media

Previous data in this project has suggested that VEGF production is regulated by Src kinase. Therefore, Src may represent a target with which to reduce the angiogenic capacity of TamR conditioned media. To investigate this, TamR cells were treated with the Src-inhibitor, AZD0530, prior to collection of conditioned media for endothelial cell growth assays. Conditioned media from Src-inhibited TamR cells failed to induce growth of endothelial cells to the same extent as untreated TamR conditioned media (Figure 4.3).
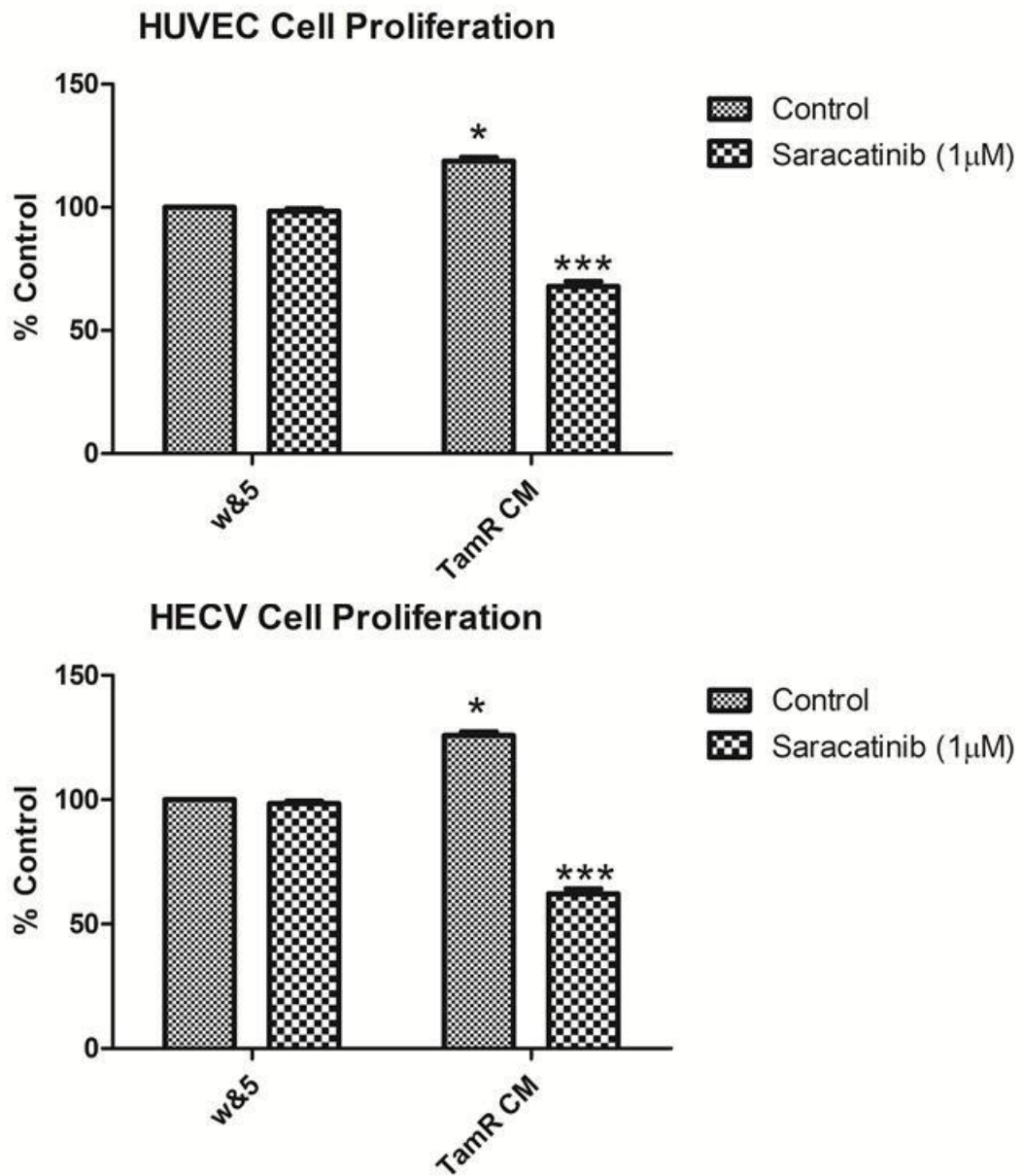
Figure 4.3 Cell proliferation of endothelial cells after treatment with conditioned media from TamR cells, ± the Src kinase inhibitor, Saracatinib

HUVEC and HECV cells were seeded onto 96-well plates in basal media and grown to 70% confluency before being quiesced in serum free media for 24 hours. Following this, media were removed and endothelial cells were treated with TamR conditioned media pre-treated with 1μM Saracatinib or TamR conditioned media control. After 72 hours cell proliferation was assessed using MTS reagent. Data are presented as % Control ± SEM (* p<0.05 vs. w&5 Control, *** p<0.0001 vs. TamR CM control, n=3)

4.2.4 TamR Conditioned Media Promotes VEGF Signalling in Endothelial Cells

Pro-angiogenic signalling through VEGF relies on the ligand binding its receptor, primarily VEGFR2, on endothelial cells. This activates signalling through Src/PI3K, leading to cell survival, and MAPK, signalling resulting in cell proliferation. Through Western blot analysis, endothelial cell signalling was examined in the presence of conditioned media with or without pre-treatment with the Src kinase inhibitor saracatinib.

Analysis of phosphorylation of HUVEC and HECV VEGFR2 (Figures 4.4 and 4.7) after stimulation with MCF7wt and TamR conditioned media illustrated that TamR conditioned media increases the phosphorylation of VEGFR2 to a greater extent than MCF7wt CM. Conditioned media from TamR cells that had been pre-treated with the Src inhibitor, however, had a reduced capacity to activate VEGFR2. TamR conditioned media also increased the activity of VEGFR2 related downstream signalling molecules in HUVECs and HECVs (Figures 4.5 and 4.8). Akt, Src and MAPK were all found to be phosphorylated to a greater extent in cells treated with TamR conditioned media than in cells treated with MCF7wt media. Finally, Src inhibition in TamR cells resulted in reduced phosphorylation of downstream signalling molecules in HUVECs and HECVs when stimulated with conditioned media from drugged cells (Figures 4.6 and 4.9).

HUVEC
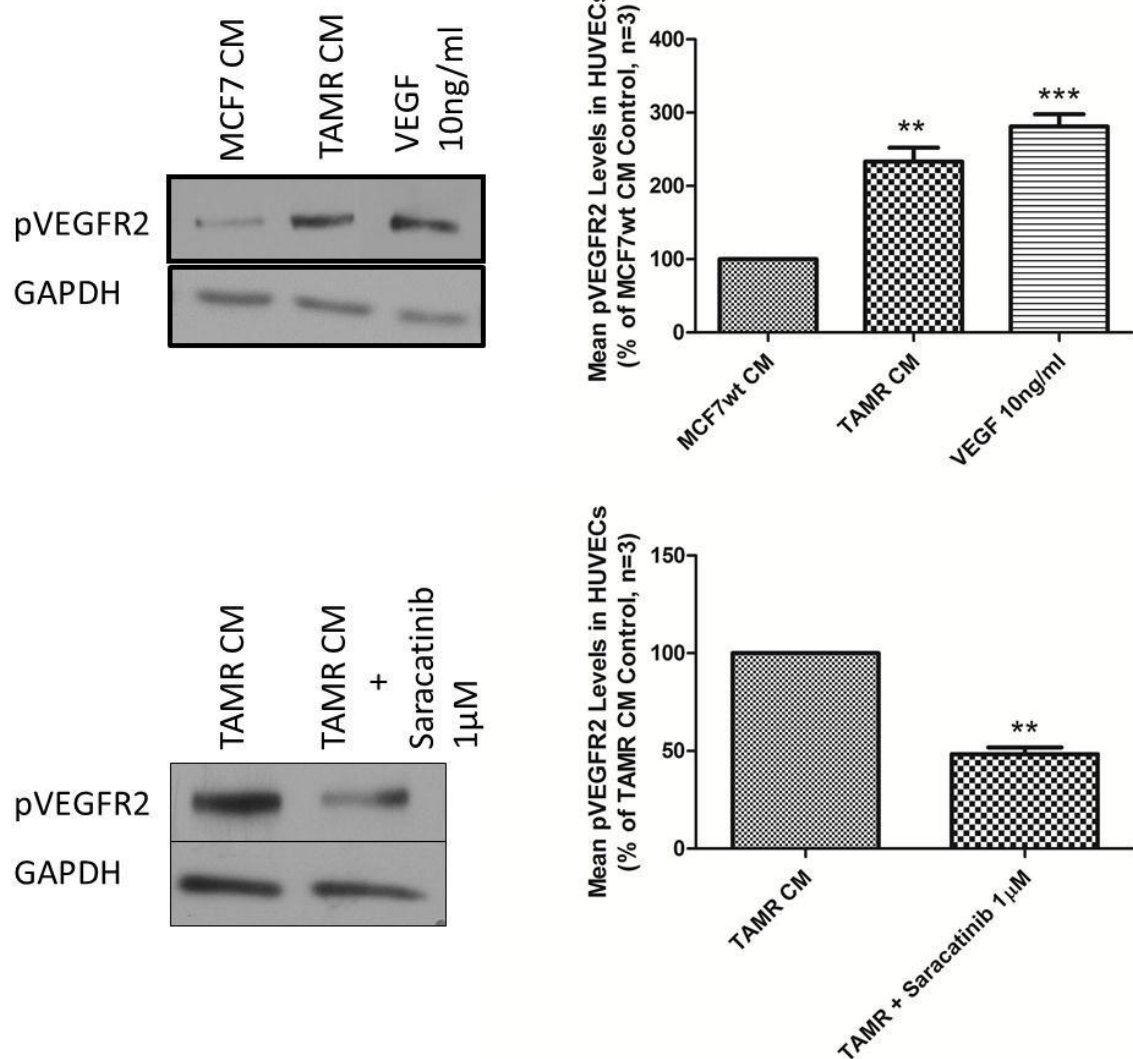
Figure 4.4 Effect of MCF7wt and TamR conditioned media treatment on activation of VEGFR2 in HUVEC cells and the effect of TamR Src kinase inhibition on TamR conditioned media activation of pVEGFR2

HUVEC cells were seeded onto 12-well plates in basal media and grown to 70% confluency before being quiesced in serum free media for 24 hours. Following this, media were removed and endothelial cells were treated with conditioned media or conditioned media from cells that had been pre-treated with Saracatinib. Cells were lysed as described in materials and methods (Section 2.5). Total soluble protein (20µg) was subjected to SDS-PAGE/Western blot analysis and the membranes were probed with antibodies specific to VEFGR2. Densitometry was carried on bands obtained and results were corrected for loading using GAPDH, presented as % Control ± SEM (** $p<0.01$ vs. Control, *** $p<0.001$, n=3)

Figure 4.5 Effect of MCF7wt and TamR conditioned media treatment on signalling through Akt, Src and MAPK in HUVECs

HUVEC cells were seeded onto 12-well plates in basal media and grown to 70% confluency before being quiesced in serum free media for 24 hours. Following this, media were removed and endothelial cells were treated with conditioned media. Cells were lysed as described in materials and methods (Section 2.5). Total soluble protein (20µg) was subjected to SDS-PAGE/Western blot analysis and the membranes were probed with antibodies specific to Akt, Src and MAPK. Densitometry was carried on bands obtained and results were corrected for loading using GAPDH, presented as % Control ± SEM (* p<0.05 vs. Control, ** p<0.01, n=3)
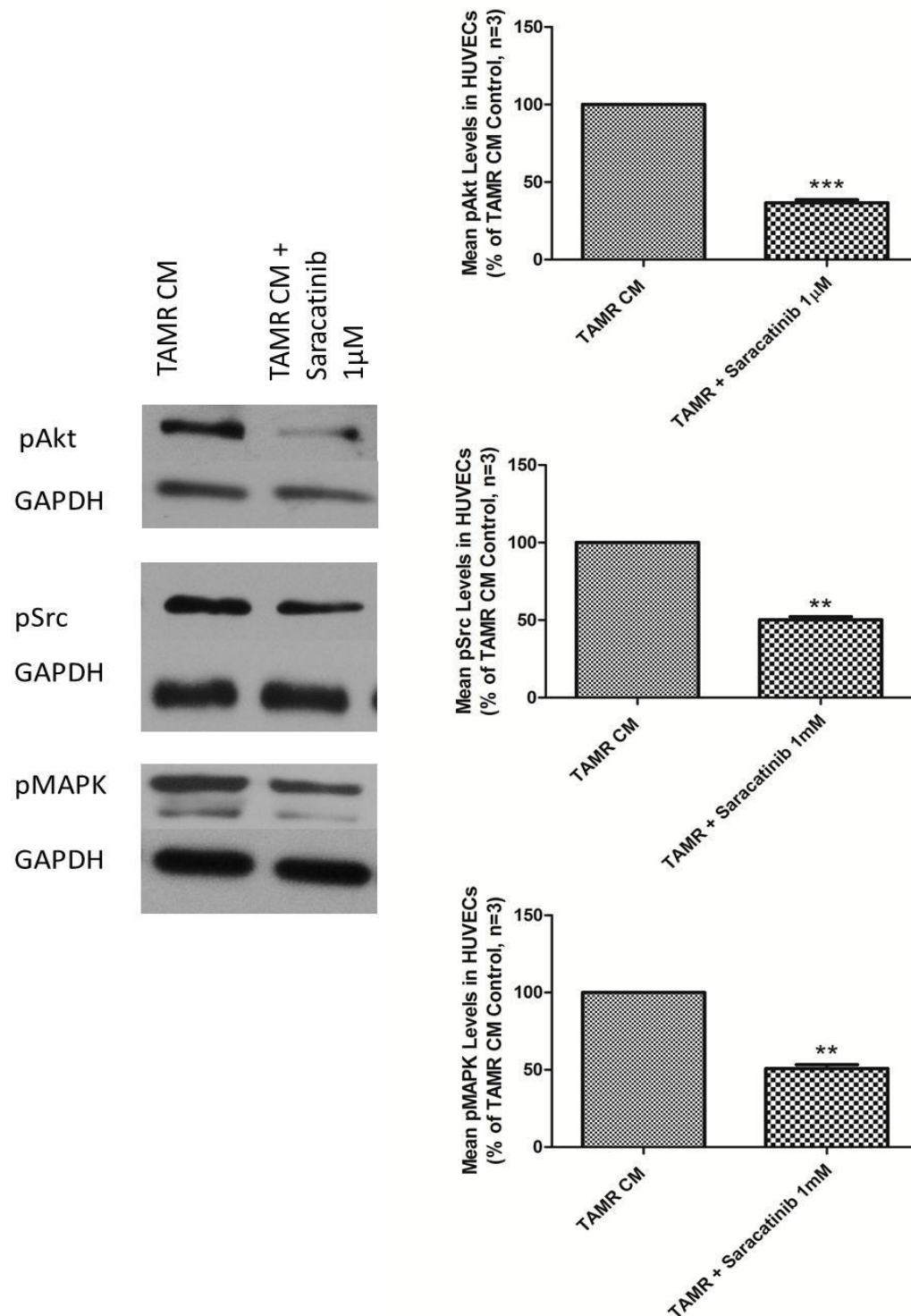
Figure 4.6 Effect of TamR Src kinase inhibition on TamR conditioned media activation of HUVEC pAkt, pSrc and pMAPK

HUVEC cells were seeded onto 12-well plates in basal media and grown to 70% confluency before being quiesced in serum free media for 24 hours. Following this, media were removed and endothelial cells were treated with conditioned media or conditioned media from cells that had been pre-treated with Saracatinib. Cells were lysed as described in materials and methods (Section 2.5). Total soluble protein (20µg) was subjected to SDS-PAGE/Western blot analysis and the membranes were probed with antibodies specific to Akt, Src and MAPK. Densitometry was carried on bands obtained and results were corrected for loading using GAPDH, presented as % Control ± SEM (* $p<0.05$ vs. Control, ** $p<0.01$ vs. Control, *** $p<0.001$ vs. Control n=3)

HECV

Figure 4.7 Effect of MCF7wt and TamR conditioned media treatment on activation of VEGFR2 in HECV cells and the effect of TamR Src kinase inhibition on TamR conditioned media activation of pVEGFR2

HECV cells were seeded onto 12-well plates in basal media and grown to 70% confluency before being quiesced in serum free media for 24 hours. Following this, media were removed and endothelial cells were treated with conditioned media or conditioned media from cells that had been pre-treated with Saracatinib. Cells were lysed as described in materials and methods (Section 2.5). Total soluble protein (20µg) was subjected to SDS-PAGE/Western blot analysis and the membranes were probed with antibodies specific to VEFGR2. Densitometry was carried on bands obtained and results were corrected for loading using GAPDH, presented as % Control ± SEM (** p<0.01 vs. Control, *** p<0.001, n=3)

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Figure 4.8 Effect of MCF7wt and TamR conditioned media treatment on signalling through Akt, Src and MAPK in HECVs

HECV cells were seeded onto 12-well plates in basal media and grown to 70% confluency before being quiesced in serum free media for 24 hours. Following this, media were removed and endothelial cells were treated with conditioned media. Cells were lysed as described in materials and methods (Section 2.5). Total soluble protein (20µg) was subjected to SDS-PAGE/Western blot analysis and the membranes were probed with antibodies specific to Akt, Src and MAPK. Densitometry was carried on bands obtained and results were corrected for loading using GAPDH, presented as % Control ± SEM (* $p<0.05$ vs. Control, ** $p<0.01$, n=3)

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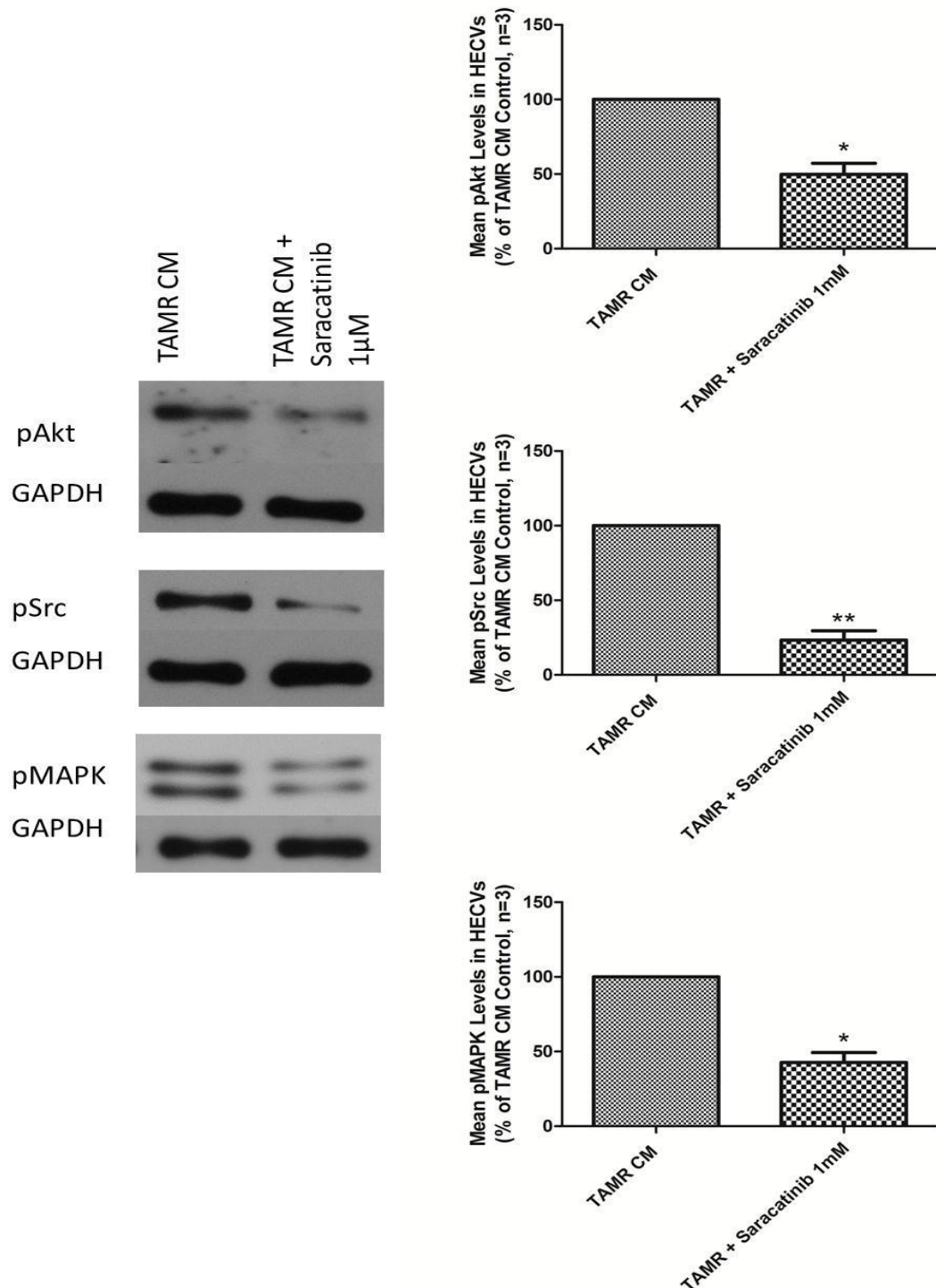
Figure 4.9 Effect of TamR Src kinase inhibition on TamR conditioned media activation of HECV pAkt, pSrc and pMAPK
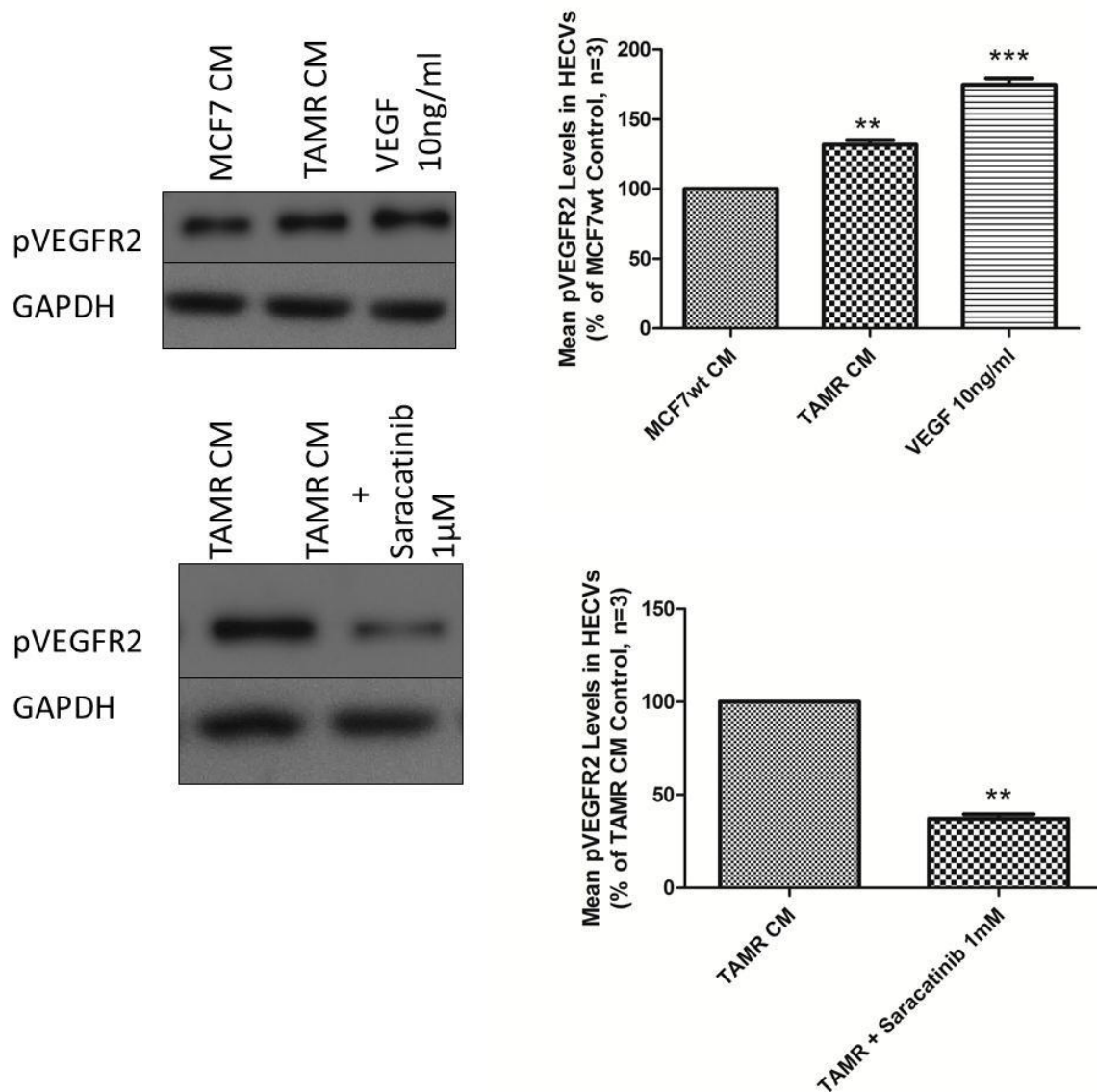
HECV cells were seeded onto 12-well plates in basal media and grown to 70% confluency before being quiesced in serum free media for 24 hours. Following this, media were removed and endothelial cells were treated with conditioned media or conditioned media from cells that had been pre-treated with Saracatinib. Cells were lysed as described in materials and methods (Section 2.5). Total soluble protein (20µg) was subjected to SDS-PAGE/Western blot analysis and the membranes were probed with antibodies specific to Akt, Src and MAPK. Densitometry was carried on bands obtained and results were corrected for loading using GAPDH, presented as % Control ± SEM (* p<0.05 vs. Control, ** p<0.01 vs. Control, *** p<0.001 vs. Control n=3)

4.2.5 TamR Conditioned Media Increases the Migratory Capacity of Endothelial Cells to a Greater Extent than that of MCF7wt Media

Migration of endothelial cells has been shown to play an essential role in the process of angiogenesis. To examine the effect of paracrine signalling from breast cancer cells on the migratory capacity of endothelial cells, we carried out two tests of migration using our *in vivo* endothelial cell lines. Wound healing assays carried out illustrated that the capacity of endothelial cells to close over an area created on a super-confluent monolayer of cells is increased in the presence of conditioned media from TamR cells. Data in Figure 4.10 and 4.11 illustrate similar responses in both HUVEC and HECV cell lines.

Migration was also assessed using a Boyden chamber cell culture system in which conditioned media was held in the 24-well plate under the chamber and endothelial cells were seeded into the chamber itself. Cell counts of cells that had migrated through the filter towards the conditioned media were found to be higher when the conditioned media was taken from TamR cells (Figure 4.12 and 4.13).
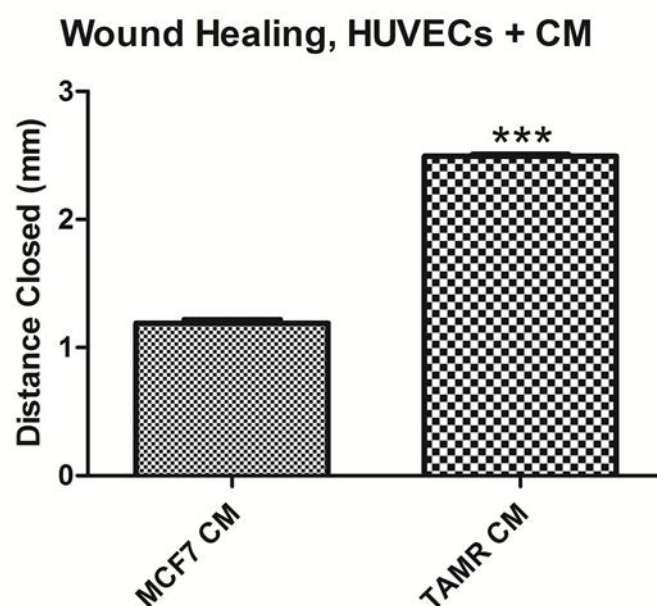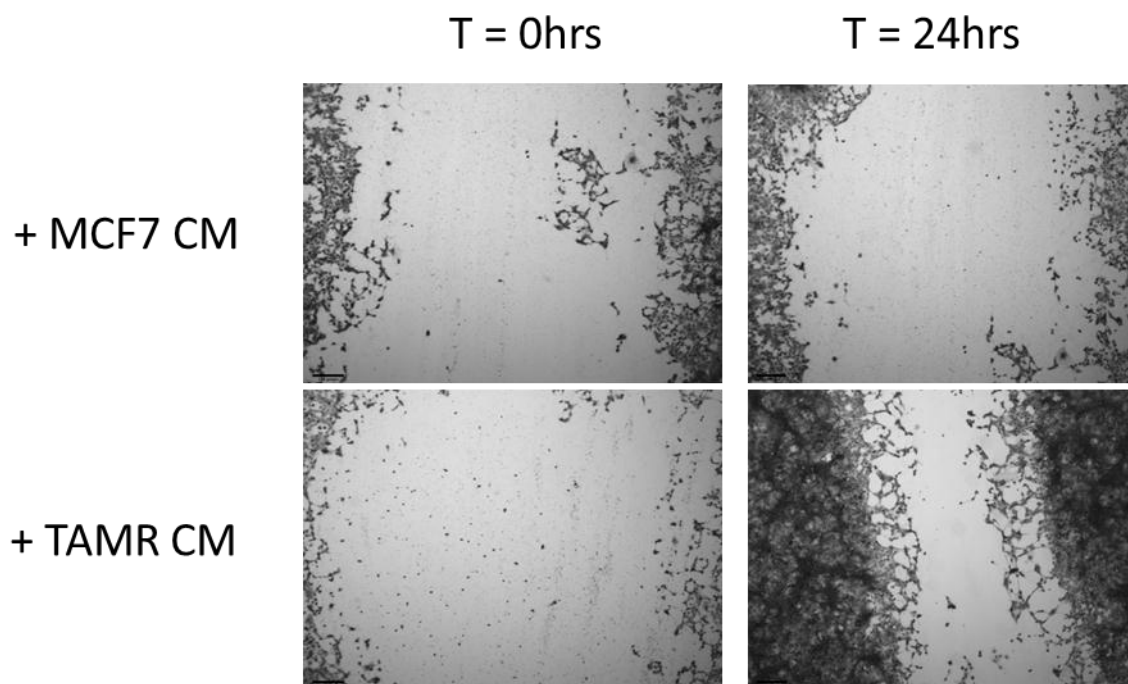
Figure 4.10 Migratory capacities of HUVECs after stimulation with conditioned media taken from MCF7wt and TamR cell lines as measured by wounding assay HUVEC cells were seeded onto 12-well plates in basal media and grown to 100% confluency before a 1cm line of cells is removed using a pipette tip and aspirator. Following this, media were removed and endothelial cells were treated with MCF7wt or TamR conditioned media. After 24 hours cell migration was assessed by light microscope photography and image analysis using ImageJ software. Data are presented as % Control distance closed ± SEM (*** p<0.001 vs. Control, n=3)
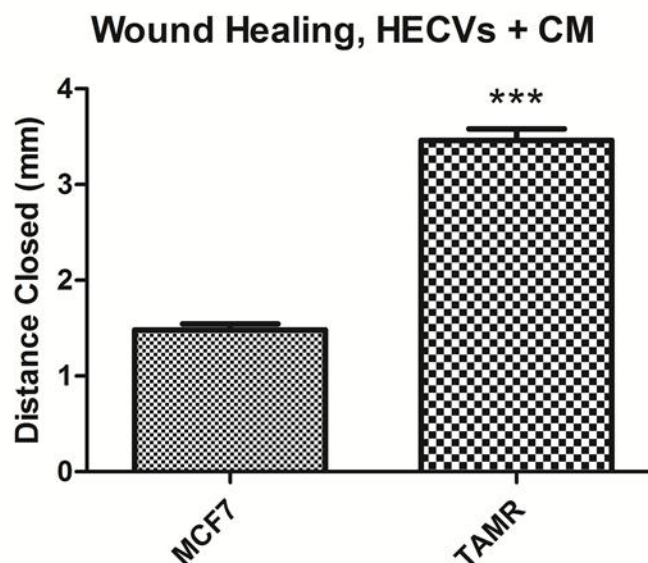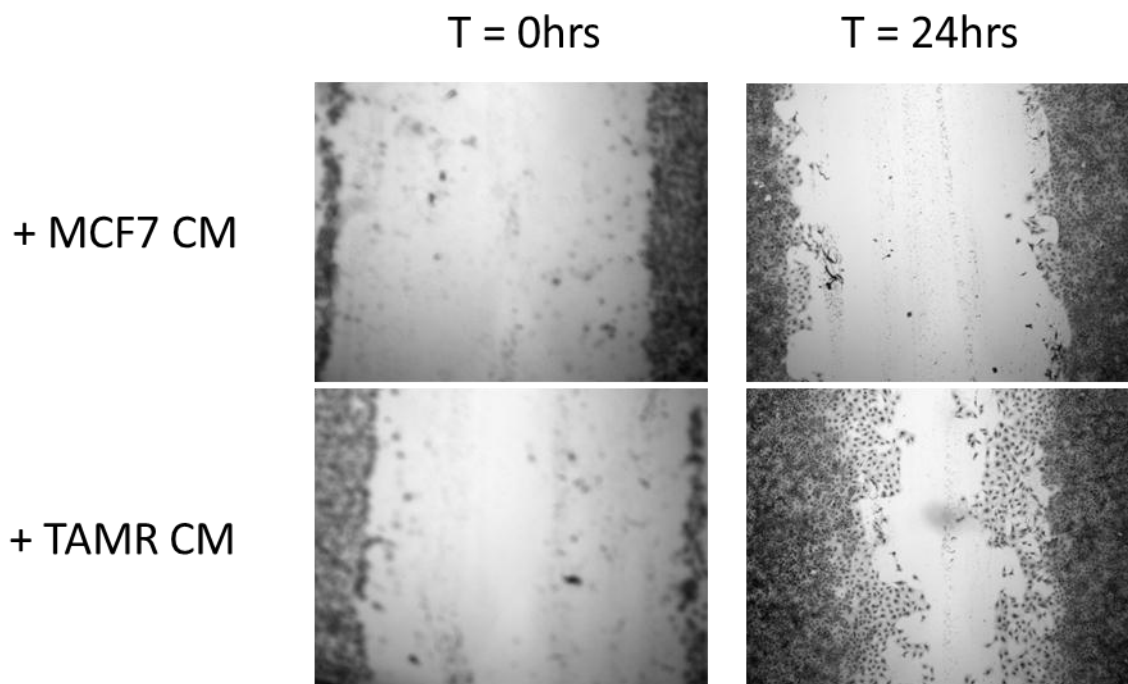
Figure 4.11 Migratory capacities of HECVs after stimulation with conditioned media taken from MCF7wt and TamR cell lines as measured by wounding assay
HECV cells were seeded onto 12-well plates in basal media and grown to 100% confluency before a 1cm line of cells is removed using a pipette tip and aspirator. Following this, media were removed and endothelial cells were treated with MCF7wt or TamR conditioned media. After 24 hours cell migration was assessed by light microscope photography and image analysis using ImageJ software. Data are presented as % Control distance closed ± SEM (*** p<0.001 vs. Control, n=3)

+ MCF7 CM

+ TAMR CM

+ TAMR + Saracatinib CM
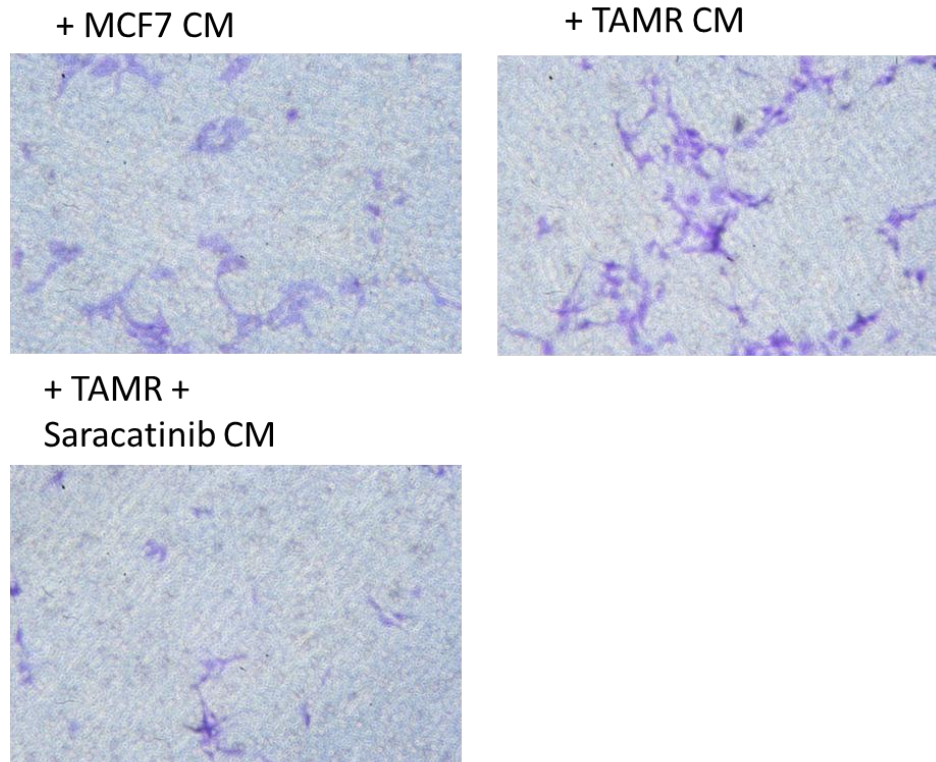
Cell Migration, Boyden Chamber; HUVEC + CM +/- Src Inhibitor
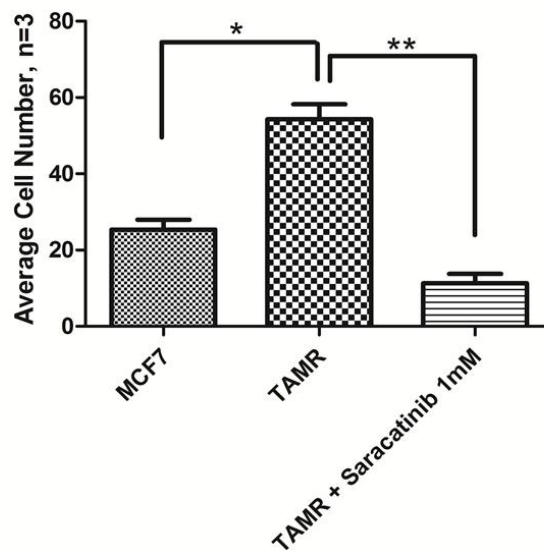
Figure 4.12 Migratory capacities of HUVECs after stimulation with conditioned media taken from MCF7wt and TamR cell lines as measured by Boyden chamber assay

HUVEC cells were seeded into Boyden chambers in w&5. Wells below Boyden chambers had 100μl of conditioned media pipetted into each. After 24 hours cell migration was assessed by light microscope photography on formalin fixed and crystal violet stained filter membranes from each Boyden chamber. Image analysis was carried out using ImageJ software. Data are presented as % Control cells counted ± SEM (*p<0.05, ** p<0.01 vs. Control, n=3)

+ MCF7 CM

+ TAMR CM

+ TAMR + Saracatinib CM
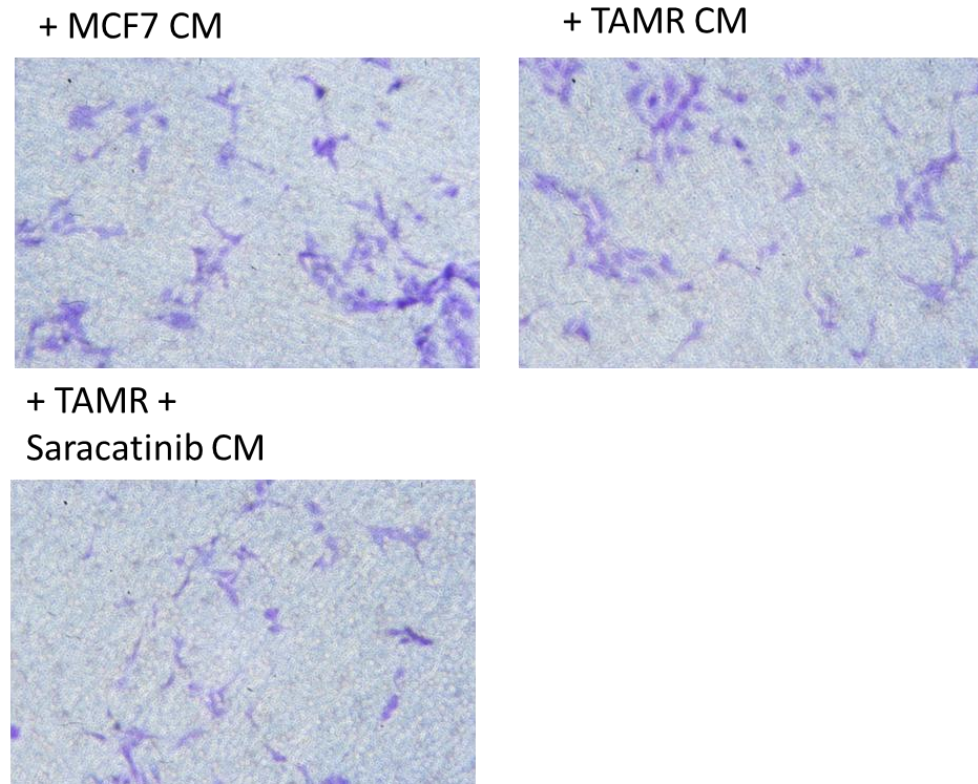
Cell Migration, Boyden Chamber; HECV + CM +/- Src Inhibitor
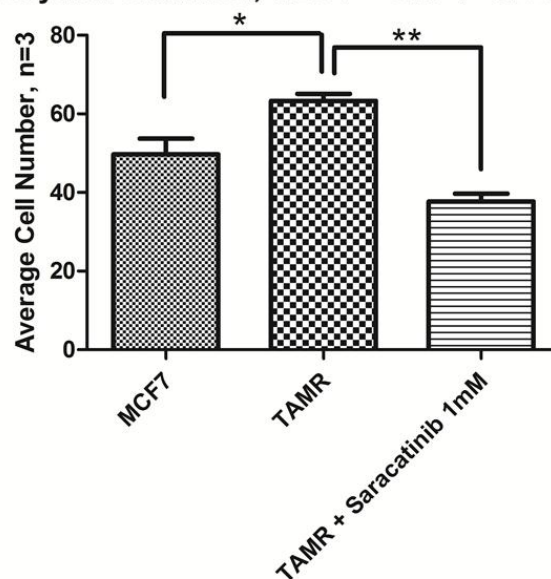
Figure 4.13 Migratory capacities of HECVs after stimulation with conditioned media taken from MCF7wt and TamR cell lines as measured by Boyden chamber assay

HECV cells were seeded into Boyden chambers in w&5. Wells below Boyden chambers had 100μl of conditioned media pipetted into each. After 24 hours cell migration was assessed by light microscope photography on formalin fixed and crystal violet stained filter membranes from each Boyden chamber. Image analysis was carried out using ImageJ software. Data are presented as % Control cells counted ± SEM (*p<0.05, ** p<0.01 vs. Control, n=3)

4.2.6 TamR Conditioned Media Increases the Tubule Formation of HUVECs

Tubule formation accompanying proliferation of endothelial cells is indicative of a more complete angiogenic process. Endothelial cells that solely proliferate would not be capable of producing novel vasculature. For our work, this presents another method of screening pro- and anti-angiogenic factors. In our experiments, we grew endothelial cells on a Matrigel basement membrane in the presence of conditioned media to assess whether it would promote or inhibit the formation of endothelial cell tubules. It was found that only HUVEC cells demonstrated a capacity to form tubules when cultured on basement membranes. HECV cells, under control conditions, did not demonstrate quantifiable tubule formation and so, were excluded from this experiment. Data in Figure 4.14 illustrate that HUVECs, when stimulated with conditioned media from TamR cells, possess a greater capacity to form these two-dimensional tube like structures.
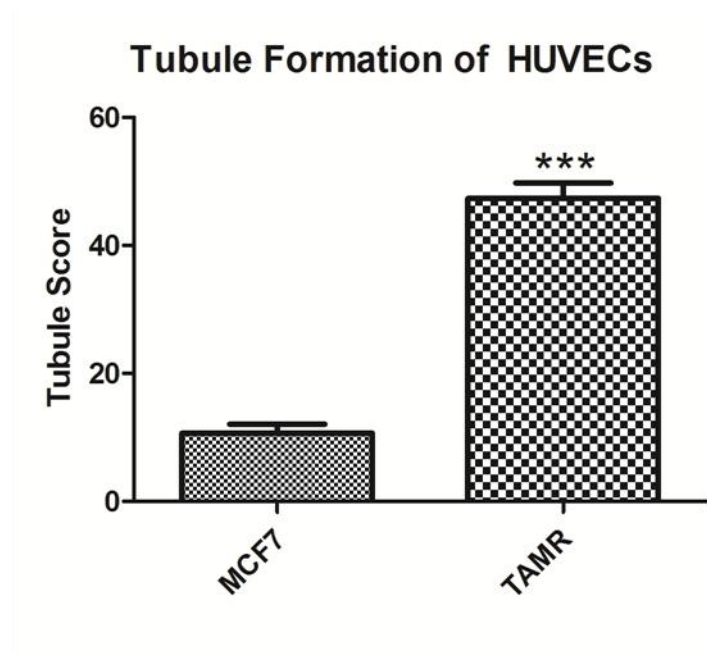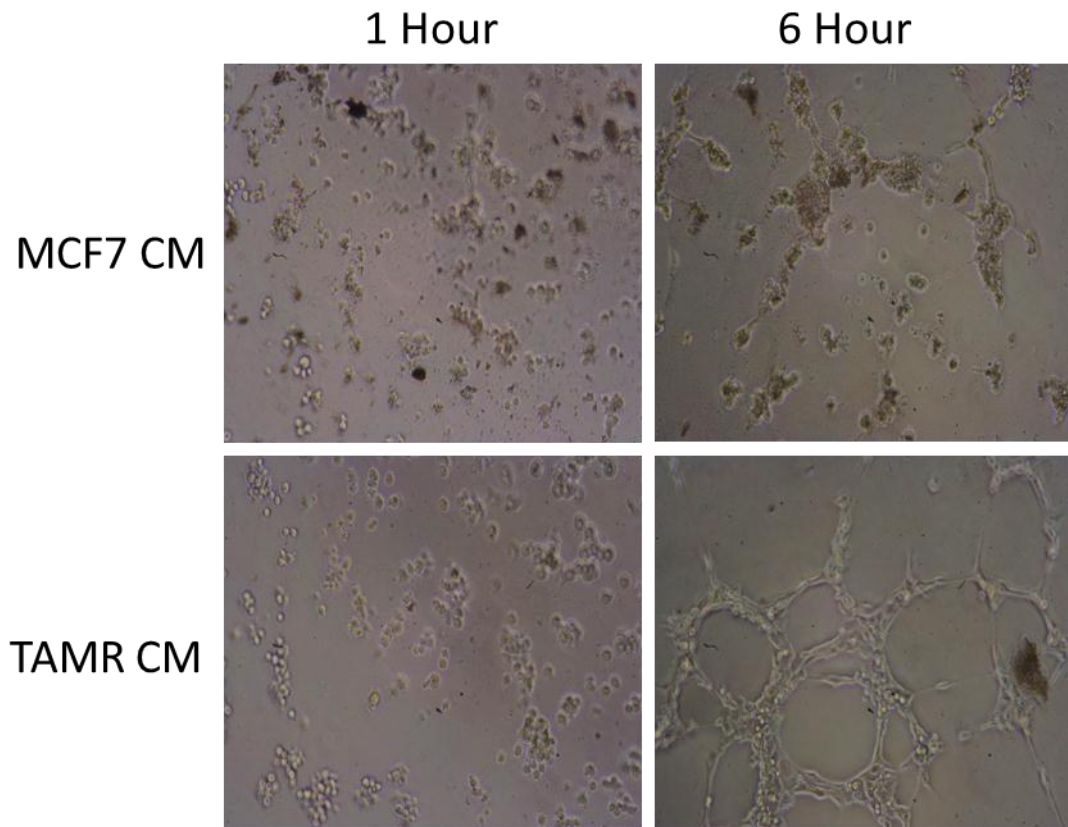
Figure 4.14 Tubule formation of HUVEC cells on Matrigel after stimulation with conditioned media taken from MCF7wt and TamR cell lines

HUVEC cells were seeded onto 24-well plates, coated with Matrigel, in conditioned media. After 6 hours tubule formation was assessed by light microscope photography on formalin fixed tubules. Image analysis was carried out using ImageJ software. Data are presented as % Control cells counted ± SEM (*p<0.05, ** p<0.01 vs. Control, n=3)

4.2.7 HECV Cell Monolayers Lose Tight Junction Integrity after Treatment with TamR Conditioned Media

The impedance of current flow across a monolayer of endothelial cells can be used to examine the integrity of tight junctions between the cells. In the presence of lowered integrity at tight junctions, a drop in the impedance of current can be measured and so, we can use various treatments to assess how different factors can illicit this effect. Initial attempts at using HUVEC cells for these experiments demonstrated that they did not form sufficient monolayers to continue using in ECIS investigations. In our experiments, a monolayer of HECV cells was treated with conditioned media from MCF7wt and TamR cell lines to observe whether this would have any effect on transendothelial electrical resistance (TER). Although this experiment was carried out in triplicate to establish significance, there was a large amount of variability in the data and so no significance could be drawn from the assay. Figure 4.15 shows a representative graph of TER over time after the addition of conditioned media from MFC7wt and TamR cell lines. Treatment with both conditioned media resulted in a drop in TER over time. Interestingly, TamR conditioned media produced a greater drop in TER.
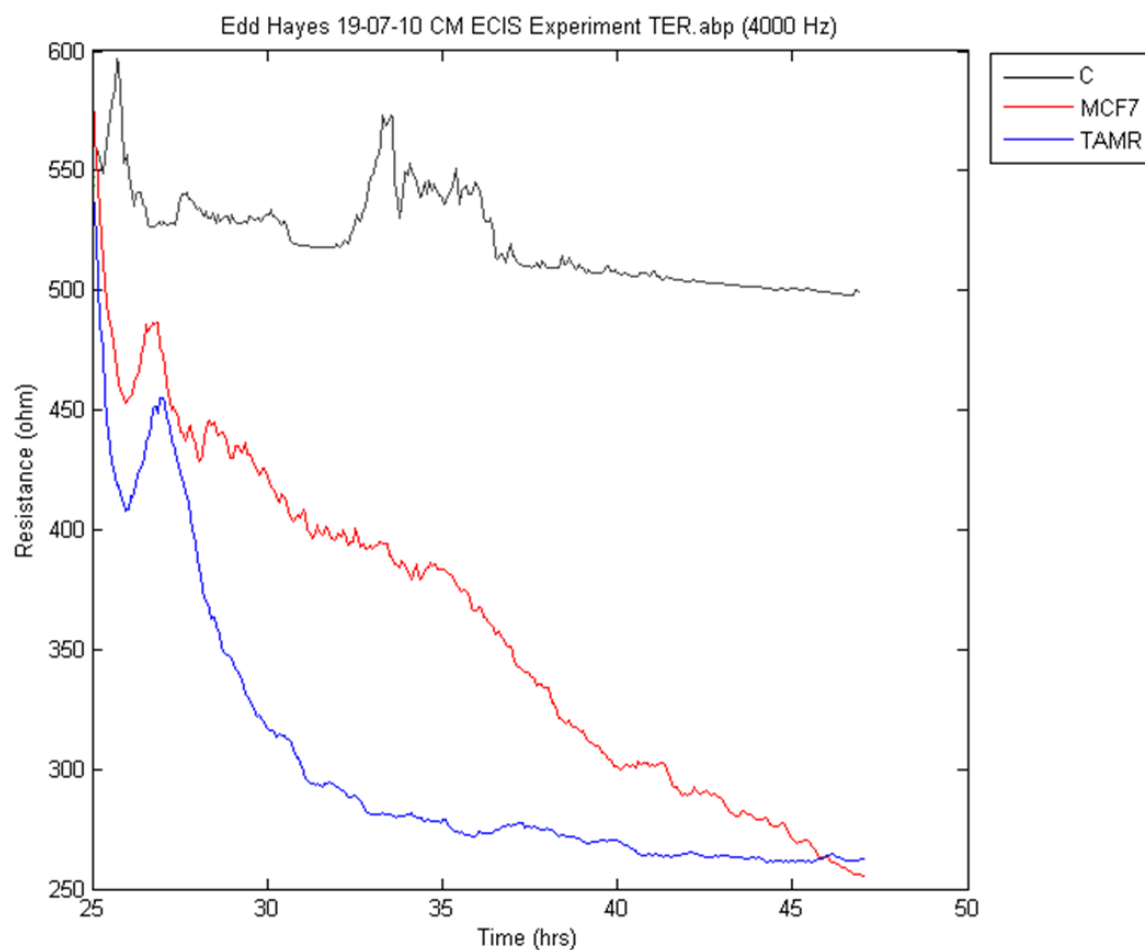
Figure 4.15 Electrical impedance across a monolayer of HECV cells after treatment with conditioned media from MCF7wt and TamR cell lines

HECV cells were seeded into 8W1E ECIS array plates in w&5 at sufficient number to produce a cell monolayer. ECIS analysis was begun and at 24 hours conditioned media were added to array wells. Data were analysed using proprietary ECIS software from which a graphical representation of TER over time could be drawn.

4.3 Discussion

4.3.1 Conditioned Media from TamR is Capable of Stimulation Endothelial Cell Proliferation through a VEGF/VEGFR2 Dependant Mechanism

The use of conditioned media to investigate paracrine stimulation between heterogeneous cell types is widely reported (Freshney, 2010). Previous observations from the Breast Cancer Molecular Pharmacology group have shown that fibroblast conditioned media is able to stimulate the migratory capacity of endocrine-resistant breast cancer cells, but it is not known whether these breast cancer cells are able to exact stimulatory effects on other cell types (Hiscox *et al.*, 2006a). In this project we have sought to determine their effects on endothelial cell function and thus explore their pro-angiogenic capacity.

The ability of TamR conditioned media to induce endothelial cell proliferation could arise as a result of the presence of several growth factors in the media secreted by these cells. Breast cancer cells have been shown to be capable of production of factors like TGF-α, VEGF, bFGF and PDGF (Ciardiello *et al.*, 2001; Rozengurt *et al.*, 1985), however, our work using microarray and PCR techniques had shown that our TamR conditioned media possessed high levels of VEGF, a key mediator of endothelial cell proliferation.

To determine whether VEGF was a central mediator of the proliferative response seen in HUVEC cells on treatment with TamR conditioned medium as opposed to other growth factors, we performed stimulation experiments on HUVEC cells with TamR conditioned media in the presence of absence of the

small molecule VEGFR2 receptor inhibitor (ZM323881). Within the VEGFR family of receptors, VEGFR2 has been shown to possess most of the proangiogenic function with VEGFR1 being required for haematopoietic stem cell recruitment and VEGFR3 regulating lymphatic endothelial cell function (Holmes *et al.*, 2007). The necessity of VEGFR2 for mediating the proliferative effects of VEGF has been demonstrated in knockout studies where suppression of VEGFR2 expression results in reduced VEGF sensitivity in contrast to knockout of VEGFR1, where cells retain their response to VEGF (Koolwijk *et al.*, 2001). Stimulation of VEGFR2 results in activation of the PI3K/AKT signalling pathway leading to a reduction in apoptotic signalling in endothelial cells (Fujio *et al.*, 1999) as well as pro-angiogenic responses mediated through nitric oxide signalling, which results in endothelial cell proliferation and migration (Kawasaki *et al.*, 2003). VEGFR2 activation also results in signal transduction through the MAPK pathway (Cross *et al.*, 2003) where activated MAPK can promote proliferation in a c-Jun/c-Fos-dependant manner (Takahashi *et al.*, 1999). Indeed, recent data point to the MAPK pathway as a key regulator of endothelial cell response to angiogenic ligands with deletion of *Erk1* or *Erk2* resulting in reduced cell proliferation and migration (Srinivasan *et al.*, 2009). Our data suggest that endothelial cells treated with TamR conditioned media demonstrate increased activity through these second messengers. Data from VEGF stimulation of endothelial cells also demonstrated this elevated signalling likely as a consequence of VEGF binding VEGFR2.

The selective VEGFR2 inhibitor ZM323881 has been demonstrated to reduce VEGF-stimulated endothelial cell proliferation *in vitro* and has an IC_{50} of 8nM

(Whittles *et al.*, 2002). In our experiments, we found that pre-treatment of endothelial cells for 1 hour with 0.5nM of the inhibitor before stimulating with conditioned media from TamR cells reduced the capability of TamR conditioned media to induce endothelial cell proliferation. Dose-response studies of endothelial cells to the inhibitor alone revealed that cell proliferation of both HUVEC and HECV cells was not significantly reduced up to 10µM of ZM323881.

These inhibitor studies suggest that VEGF present in TamR conditioned media is responsible for induction of endothelial cell proliferation via activation of VEGFR2. Having established the importance of the VEGF/VEGFR2 axis in mediating increased endothelial cell growth and suggesting that this increased VEGF concentration is produced by a Src dependant manner, we then sought to determine the effect of Src kinase inhibition on VEGF production by TamR cells.

4.3.2. Inhibition of Src Kinase in TamR Cells Attenuated their Ability to Promote Endothelial Cell Growth

Increased phosphorylation of VEGFR2 by TamR conditioned media as a result of elevated VEGF is indicative of increased pro-angiogenic signalling in endothelial cells. Inhibition of Src kinase signalling in TamR cells using the small molecule inhibitor saracatinib illustrated that, while TamR conditioned media can increase the phosphorylation of VEGFR2, the inhibition of Src kinase in TamR cells prior to collection of conditioned media is capable of reducing phosphorylation of the receptor, likely as a result of decreased VEGF ligand concentration in the media. We have demonstrated this effect in both primary

endothelial cell models with similar patterns of phosphorylation exhibited by both HUVEC and HECV cell lines. Additionally, stimulation of endothelial cells with conditioned media from Src inhibitor treated TamR cells results in decreased Akt, Src and MAPK phosphorylation than when endothelial cells are stimulated with untreated TamR media.

4.3.3 TamR Conditioned Media Increases the Migratory Capacity of Endothelial Cells

The migration of endothelial cells is a well-documented step in the angiogenic process. Chemoattractant-mediated, pro-motile signalling from neighbouring cells promotes the migration of endothelial cells towards hypoxic areas and allows reestablishment of oxygenation to those tissues. VEGF stimulation of endothelial cells results in the formation of actin structures involved in the migration of cells (Lamalice *et al.*, 2007). Filopodia orientate towards the higher concentration of chemoattractant while lamellipodia and stress fibres function in forward movement, adherence and contraction of the cell body (Small *et al.*, 2005). Increased signalling through heregulin (HRG) has been shown to increase the expression of VEGF by binding to HRG response elements upstream of VEGF promoter regions. In murine lung models, endothelial cell migration has been shown to increase on stimulation by HRG, as a result of subsequent VEGF expression. One group has generated data illustrating the increased production of heregulin by MCF7wt cells after stimulation with oestradiol (Keshamouni *et al.*, 2002) however, it remains to be elucidated what effect tamoxifen stimulation and indeed development of tamoxifen resistance has on this process. In our experiments we show that TamR conditioned media

has a greater propensity to stimulate endothelial cell migration, as illustrated in the wound healing assay and as a chemoattractant as demonstrated in the Boyden chamber assay.

A selection of Rho family small GTPases are thought to be involved in the response of endothelial cell migration to the activation of VEGFR2 by VEGF (Lamalice *et al.*, 2007). In particular, Cdc42 is known to be a key regulator of cell polarity and directionality of membrane protrusions (Cau *et al.*, 2005). In endothelial cells, Cdc42 on leading edge protrusions sense VEGF gradients as a result of VEGFR2 binding and subsequent activation of Cdc42 and stress activated protein kinases (SAPK/p38) which results in stress fibre remodelling (Lamalice *et al.*, 2003). The high concentration of VEGF in conditioned media form TamR cells therefore, should be sufficient to activate VEGFR2 mediated migratory mechanisms and results in the observed increase in migratory capacity seen in the Boyden chamber and wound healing assay. Although it should be noted that in the wound healing assay, there is no discernible concentration gradient and so we must hypothesise that although we are seeing migration not proliferation (as evidenced by the closure of space in a short time period) it is not directional migration. Rather, the cells migrate into the available space in response to increased motile signalling on the leading (wound) edge and not the reverse, due to contact inhibition.

4.3.4 TamR Conditioned Media Increases the Capacity of HUVEC Cells to Form Tube Like Structures

The formation of tubule structures when grown on a basement membrane, such as fibronectin or Matrigel, is a component of endothelial cell behaviour. As such,

it is a relatively easy *in vitro* method for examining functional angiogenesis as opposed to solely measuring proliferation, which, although obviously tied to angiogenesis, the presence of uncontrolled growth alone without formation of tubule structures would not lead to increased vascularisation of tissue. Matrigel represents an artificial basement membrane construct and is produced in abundance by a murine tumour cell line, Engelbreth-Holm-Swarm (EHS) (Kleinman *et al.*, 2005). Our HUVEC cell line exhibited visible tubule formation six hours after seeding onto Matrigel in its home media as well as in w&5 control media and so these were used as positive controls in each assay. When treated with conditioned media, TamR cell media exhibited an increased capacity to form tubules than control media. MCF7wt media, however, did not greatly affect the tubule forming capacity of endothelial cells.

The use of HUVEC cells is well established for use in tubule assays as well as several other endothelial cell models including SVEC4-10 (murine) and HMEC-1 (human) (Arnaoutova *et al.*, 2010). However, our HEVC cells, did not display successful tubule formation under control conditions in the assay. This is surprising given reports by Rmali et al. 2005 who successfully made use of tubule formation assay. Although, the same study put forward an hypothesis that loss of certain cell markers such as tumour endothelial marker 8 (TEM-8) results in an inability to form tubules *in vitro* (Rmali *et al.*, 2005). Given the nature of continuous culture cells lines, it is not unlikely that some loss of expression of certain proteins may lead to losses in functionality. It would be worthwhile to examine HECV cells of different passage to determine whether

tubule formation could be observed at an earlier time point in their culture to elucidate whether a loss in functionality has occurred.

4.3.5 Effects of TamR Conditioned Media May Reduce Cell-cell Tight Junction Integrity in Endothelial Cells

Electric cell-substrate impedance sensing (ECIS) is a method of measuring the impedance of an electrical current across a monolayer of cells, in our case endothelial cells, which depends upon the insulator capacity of cells upon gold-film electrodes. The movement of current around the cells can be used to determine changes in cell shape, which can be used to measure cellular growth and migration and also be used to assess tight junction formation and thus the integrity of intracellular contacts, an indirect indicator of monolayer permeability. This latter element is important as monolayer permeability has been implicated in tumour cell intravasation and thus haematogenous metastatic spread (Fidler *et al.*, 2002) and has been shown to be driven by a VEGF and Src mediated mechanism (Criscuoli *et al.*, 2005). Our protocol involved allowing endothelial layer monolayers to stabilise over 24 hours before challenging cells with conditioned media from MCF7wt and TamR cell lines. We observed a visible decrease in transendothelial resistance after treatment with both media when compared to w&5 control media. Despite these encouraging results, however, we found high variability within these results using the same protocol and conditions. As the use of ECIS equipment was not routine within our group it is possible that the inexperience of using this novel technique was a cause of the poor reproducibility. Further practice of this technique would clearly be invaluable as some of the results obtained would help explain whether TamR

cells are capable of modulating vascular permeability. However, despite this, the data did suggest that TamR conditioned media was able to reduce transendothelial permeability to a greater extent than endocrine-sensitive MCF7wt cells. The implications of this are interesting in that the ability of breast cancer cells to modulate intracellular communication and endothelial permeability may enhance their capacity to spread.

4.4 Chapter Summary

- Conditioned media from TamR cells has a greater capacity to induce endothelial cell proliferation than media taken from MCF7wt cells

- TamR conditioned media increases signalling through VEGFR2 and second messenger systems in endothelial cells, however, this effect is reduced when the TamR cells had been previously treated with the Src kinase inhibitor Saracatinib

 - Functional assessment of endothelial cells after stimulation with TamR conditioned media revealed that this treatment also increases the capacity of these cells to migrate and form tubules and may also increase vascular permeability by reducing the integrity of endothelial cell monolayers

Chapter 5 – *Ex vivo* and *In vivo* Analysis of Pro-angiogenic Capacity of TamR cells

5.1 Introduction

Data in the previous chapters, demonstrates that VEGF secretion is elevated in TamR cells and that this process involves Src kinase activity. Furthermore, conditioned media from TamR cells is able to promote angiogenic responses in two endothelial cell lines. However, despite this encouraging *in vitro* data, it still remains to be seen whether these effects translate into pro-angiogenic responses in a more clinically relevant context i.e. *in vivo*. Thus, the aims of this chapter were to assay the effects of TamR conditioned media on vascular development using the chick chorioallantoic membrane assay, to determine the effects of TamR conditioned media on tubule formation in a rat aortic ring assay and finally to determine whether there is altered vascularisation in TamR cell mouse xenografts when compared to MCF7wt xenografts.

5.2 Results

5.2.1 Chick Chorioallantoic Membrane (CAM) Assay

The use of the chick chorioallantoic membrane assay in the study of angiogenesis has been a commonly utilised *in vivo* tool for nearly a hundred years with evidence of the assay being carried out as far back as 1913 (Ribatti, 2004). The chorioallantois is the name given to the fusion product of the allantois, an extra-embryonic membrane in which blood vessels begin to form after three days incubation, and the chorion, which is the outermost membrane surrounding the embryo. Therefore, vessels within the chorioallantois function to

mediate gas exchange between the embryo and the environment through the porous shell.

In our experiments we wanted to determine whether proangiogenic and chemoattractant properties displayed by endothelial cells *in vitro* following stimulation with conditioned media could also be demonstrated in an *in vivo* system. Sterilised filter papers soaked in conditioned media from either MCF7wt or TamR cells or untreated media as a control were used to approximate the effect of a body of tumour cells placed in close proximity to a vascular network (modelled by the developing vasculature of the CAM tissue). We hypothesised that the increased angiogenic factors known to be present in the conditioned media from TamR cells would promote vascular development and that this would be directed towards the filter paper containing TamR conditioned media. Disappointingly, however, this methodology proved technically challenging and we encountered difficulties with maintaining sufficient numbers of viable embryos in each experiment in order to correctly compare between treatment and controls. Figure 5.1 illustrates one successful experiment where TamR conditioned media appeared to promote development of vasculature within the CAM (arrowed).
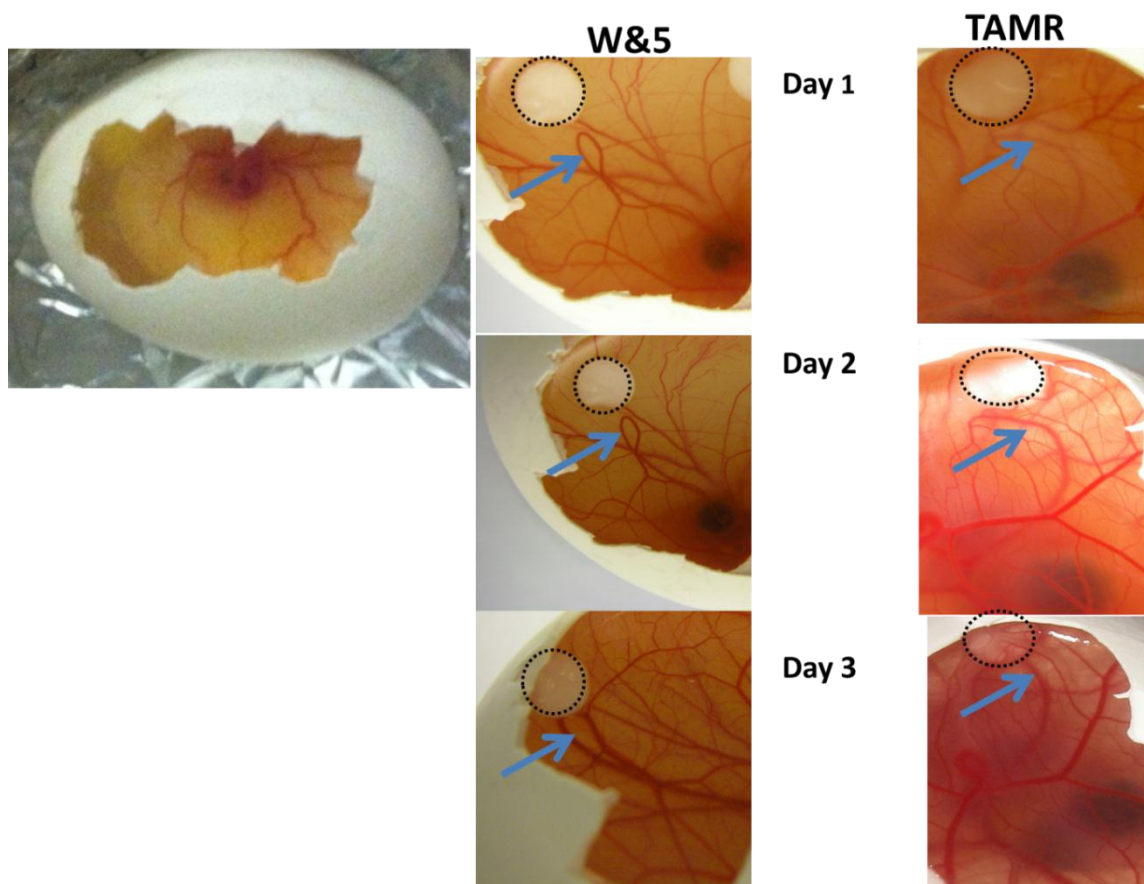
Figure 5.1 Effect of TAMR conditioned media on the vessel development in chick embryos.
Top, fertilised egg after some of the shell is removed during windowing and before treatment with filter papers. Bottom, development of vasculature at days 1, 2 and 3 post treatment. Fertilised chick eggs at day 1 post fertilisation were incubated at 37.5°C. At day 2 post fertilisation shells were opened to allow display of chorioallantoic membrane. Filter papers incubated in conditioned media from TamR cells overnight were placed on the CAM distal to the embryo. Photographs were taken at 24 hour intervals.

5.2.2 Rat Aortic Ring Assay

The use of the aortic ring assay is considered to be a better representative of the *in vivo* environment versus 2D cultures of single cell types as the aorta consists of a number of cell types, representing both endothelial and non-endothelial cells alike. Under the appropriate conditions, endothelial cells within the aorta will begin to proliferate and migrate in order to form tubules (Go *et al.*, 2003) which begin to stretch out into the surrounding matrix into which they have been implanted.

Freshly removed rat aorta were sectioned into 1mm ringlets and inserted into Matrigel where they were then exposed to plain media or conditioned medium from either MCF7wt cells or their TAM resistant counterparts.

Under control conditions (i.e. exposure to plain media), aortic endothelial cell growth was seen after 24 hours of culture, with tubule structures becoming more pronounced until day 5, after which it appeared that the cells did not proliferate any further.

Aortic sections exposed to MCF7wt conditioned media displayed little, if any, outgrowth of endothelial cells. Rather, these treatments resulted in significant amounts of fibroblast infiltration of the Matrigel (Figure 5.2A). In contrast to this, TamR conditioned media appeared to promote the growth of large numbers of tubule-like structures within the Matrigel (Figure 5.2B).

Imaging was performed of the resultant structures to enable the quantitation of tubules; these data further demonstrated the contrasting effects of MCF7wt and TamR cell medium to promote aortic ring, endothelial cell tubule formation (Figure 5.2).
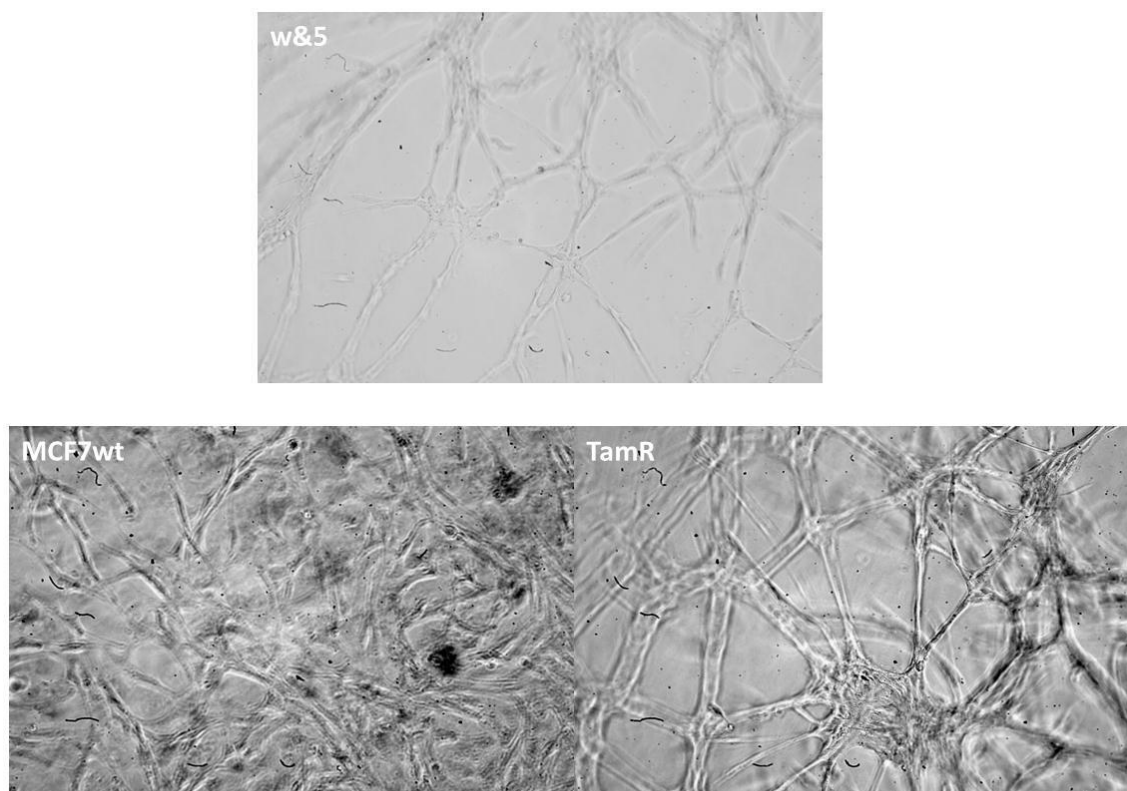
Rat Aortic Ring Assay, Tubule Scores W&5 vs. MCF7 vs. TAMR
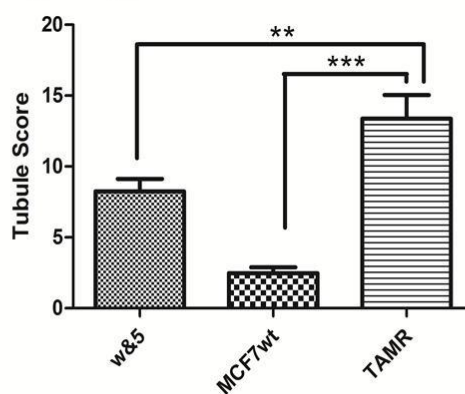
Figure 5.2 Effect of MCF7wt and TAMR conditioned media on the tubule formation of endothelial cells from rat aortic tissue grown on a Matrigel membrane.

The descending thoracic aorta was dissected from three week old Sprague-Dawley rats. Tissues were debrided of adipose and fibrous tissues, washed in warm heparin containing wRPMI before being sectioned into 2mm ringlets. Following this, sections were placed onto Matrigel membranes in MCF7wt or TamR conditioned media. After 24 hours sections were photographed using light microscopy. Tubule formations were counted with data here presented as % Control tubule formation counts ± SEM (** $p<0.001$ vs. w&5 Control, *** $p<0.0001$ vs. MCF7wt, n=3)

5.2.3 MCF7 and TAMR Xenograft Studies

Having observed that TamR conditioned media leads to an increased proliferation rates of endothelial cells *in vivo*, we hypothesised that TamR cells grown as xenografts would display a greater level of vascularisation than would their MCF7wt counterparts. We sought to determine whether this was the case by using tissue sections made available to us from mice which had subcutaneous xenografts from either MCF7wt or TamR cells. Once the xenografts were removed, fixed and sectioned, an antibody specific to the endothelial cell antigen CD31 was used to specifically stain the tumour vasculature. CD31 is appropriate for this purpose as it is known to be constitutively expressed on the surface of endothelial cells to a much higher degree versus other cell types likely to be present within the tumour e.g. immune cells, fibroblasts and the tumour cells themselves (Parums *et al.*, 1990; Righi *et al.*, 2003). Stained sections were visualised and groups of "small" or "large" CD31 positive structures were counted, where groups of cells that occupied more than $1mm^2$ were counted as "large vessels" and those that occupied less than $0.1mm^2$ were reported as "small vessels".

Examination of CD31-stained tissue sections revealed that TamR xenografts possessed higher numbers of both large and small vessels versus MCF7wt xenograft tissues (Figure 5.3).
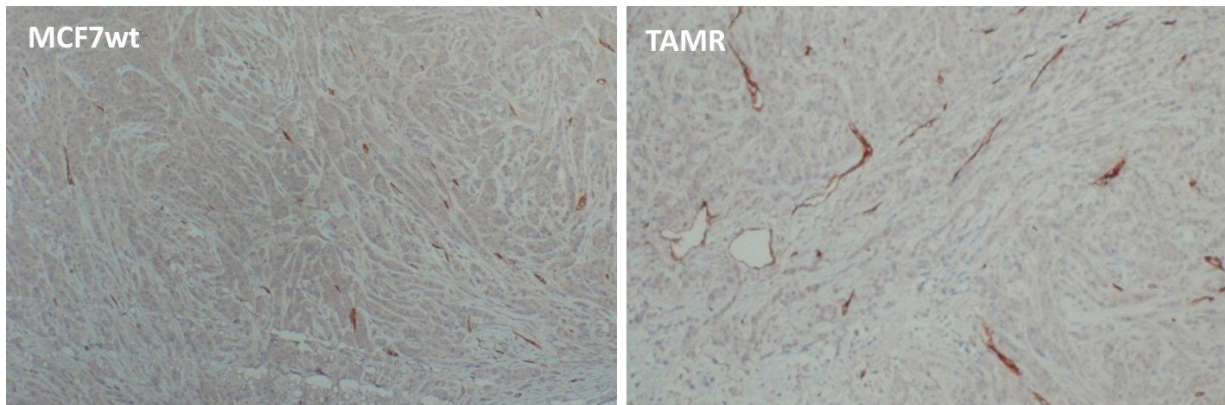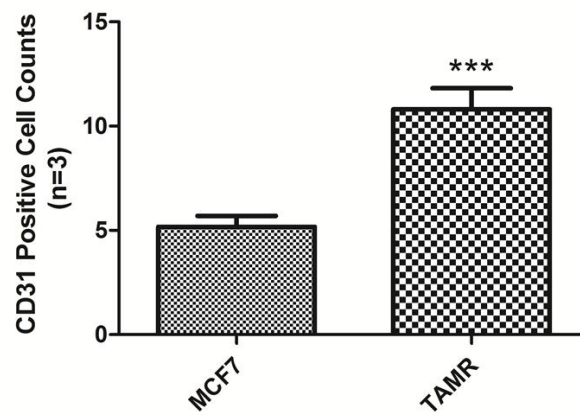
Figure 5.3 Differences in CD31 endothelial cell staining of xenograft tissue from mice grafted with MCF7wt and TAMR cells

Formalin fixed paraffin embedded xenograft tissue was prepared as discussed in Chapter 2. Antigen recovery was carried out using citrate buffer at pH 6 and heat via a pressure cooker for 2 minutes. 1/15 dilutions of rat anti-mouse CD31 antibody were applied overnight and following washes with PBS, goat anti-rat secondary was applied for 2 hours. Finally, DAB/H_2O_2 was applied for 10 minutes with subsequent haematoxylin counterstaining. CD31 staining was assessed using ImageJ software. Data are presented as % Control ± SEM (* $p<0.05$ vs. Control, n=3)

5.3 Discussion

5.3.1 Chick Chorioallantoic Membrane Assay

To further investigate the pro-angiogenic properties of breast cancer cell conditioned media in an *in vivo* context, we attempted to use the Chick Chorioallantoic Membrane (CAM) assay. This assay is well established and has been routinely used to explore regulation of angiogenesis in a more complex system compared to *in vitro* cell lines (Cavill *et al.*, 2010; Kusaka *et al.*, 1991; Ribatti *et al.*, 1999).

Unfortunately, there were significant problems with this assay, mainly arising from a very poor embryo viability (<20% survival, on average) resulting in very few usable eggs which frustratingly resulted in being unable to achieve a complete set of data with matching controls.

Studies report that embryonic viability for CAM assays ranges widely between 30% and 70% (Auerbach *et al.*, 1974; Dohle *et al.*, 2009). Many factors can influence this including time taken to transport eggs between farm and laboratory. Our supplier was located in Lincolnshire and, although they has been used routinely by other departments in Cardiff University (with reported success), transport of materials for these experiments over such long distances may lead to a reduction in viability. Factors that affect transport while in route can also reduce viability including extended periods outside of chilled or incubator environments and the conditions of transport i.e. agitation due to poor road conditions (Ponce *et al.*, 2003). We also found that contamination remained a significant factor for reduced viability. Despite stringent

decontamination of equipment and sterile working conditions we still found evidence of contamination on eggs with exposed CAM tissue, particularly after day 5 in culture. The use of filter-paper disks to apply treatment to the CAM has been previously reported in several studies in the testing of pro- and anti-angiogenic agents (Murugesan *et al.*, 2007; Sahni *et al.*, 2006). The major disadvantage of using filter paper is that the paper itself can act as an inflammatory stimulus and effect a change in the assessment of angiogenesis. As a result of poor embryo viability we were unable to obtain significant results from assays as most CAM experiments use a minimum of 12 embryos for each group including positive and negative controls. Since concluding the experimental stage of this thesis we have discovered alternative culture methodologies that may increase embryonic viability which could be utilised in future studies. However, despite these challenges, we were able to collate some data that pointed to an increased ability of TamR cell conditioned medium to promote vessel growth compared to w&5 control media.

5.3.2 TAMR, but not MCF7 Conditioned Media Promotes Proliferation and Tubule Formation in the Rat Aortic Ring Assay

The rat aortic ring assay was used to determine whether alterations in angiogenesis seen *in vitro* in response to conditioned media from tamoxifen resistant breast cancer cell models could be reproduced in a more complex biological system. These assays involve culture of dissected sections of the descending thoracic aorta from juvenile rats (Burbridge *et al.*, 2001) in a Matrigel bed and monitoring tubule outgrowth in response to specific stimuli. One benefit of using *ex vivo* tissue as opposed to primary endothelial cells *in*

vitro is that it will allow observation of the effect the surrounding cells have on the process of angiogenesis. However, this can also complicate experiments as a plethora of factors produced by other cells in the tissue, for example fibroblasts, may produce factors that influence the growth of endothelial cells through signalling that may not involve VEGF. However, the use of experimental medium (w&5) and MCF7wt conditioned media was able to control for this to a large extent.

Endothelial cells in this model represent established vasculature better in that they are not in a high proliferative state as cells in culture for several passages would be. However, the initiation of the angiogenic response may well be influenced in response to the injury arising as part of the dissection procedure (Nicosia *et al.*, 2011) which is less representative of angiogenesis in cancer. Nevertheless it remains a popular, and well validated, model for assessing angiogenesis and factors that affect angiogenesis (Feng *et al.*, 2012; Kayashima *et al.*, 2012).

Our data (Figure 5.2) illustrated a significant increase in endothelial cell outgrowth from aortic tissue and the subsequent formation of endothelial tubules following exposure to conditioned media from TamR cells versus MCF7wt cells. Previous studies have shown that stimulation of rat aortic tissue with growth factors such as VEGF results in an increased proliferative response, even after endothelial cells have begun to senesce and become reabsorbed (Nicosia *et al.*, 1997) . Interestingly, MCF7wt conditioned media treatment of aortic ring sections resulted in both a reduced proliferation of endothelial cells and also an increased proliferation of non-endothelial cells,

determined to be fibroblasts based on their morphology and incapability to form tubules.

Given that conditioned media from TamR cell lines possess elevated VEGF concentrations we hypothesise that this contributed to elevated endothelial cell proliferation. One aspect of this assay that was not investigated due to time limitations, but should be done in the future, is to perform the assay in the presence of the specific VEGFR2 inhibitor, ZM323881, in order to validate VEGF as the stimulating ligand in this system. Furthermore, to illustrate that the VEGF effecting the change is from the conditioned media and not surrounding stromal cells, cells from which conditioned media is collected could be pre-treated with inhibitors that reduce VEGF production (e.g. Src kinase inhibitor), before the media is collected for use on tissue in this system.

Although tubule formation is expected to be specific to the endothelial cells, this could additionally be confirmed in these types of assays by subsequently staining for the endothelial specific marker, CD31, or using FITC labelled GSL-1, another endothelial cells specific cell marker.

5.3.3 TAMR Xenograft Tissue is More Highly Vascularised than MCF7wt Xenograft Tissue

Murine models are routinely used in the study of cancer development and treatment. Severely immunocompromised mice will readily accept xenografts from human cells without rejection (Morton *et al.*, 2007). These models can then be used to examine the development of tumours, their metastatic and invasive properties as well as the response to treatment. Positive attributes of xenograft

studies include the ease of model development and comparatively quick turnaround from grafting to therapeutic response as well as the fact that the tumours that are developed are human in origin and so are better representatives of human tumours in situ than those from studies using alternative species' tumours i.e. genetically engineered mouse models.

CD31 was initially identified on endothelial cells and platelets (van Mourik *et al.*, 1985) where it was shown to act as a cell adhesion molecule and also as a promoter of intracellular signalling cascades that may function in the regulation of angiogenesis. (Liu *et al.*, 2012; Newman, 1999). Endothelial cell lines such as HUVEC have been shown to express CD31 at a high density both *in vitro* and *in vivo* at a level of approximately 1×10^6 per endothelial cell (Newman, 1999). When grown as monolayers, endothelial cells express CD31 along the borders of the cell at cell-cell junctions (McCarthy *et al.*, 1991). CD31 is also routinely used in the identification of endothelial cells in clinical tissue (Arnold *et al.*, 2012; Croix *et al.*, 2000; Hull *et al.*, 1996) and thus represented an appropriate marker to identify vascularisation in these xenografts. Importantly, the antibody used recognised both human and mouse antigens and so could detect murine vascularisation of the human xenograft tissue.

Our data illustrate that CD31 staining was highest in TamR xenografts, revealing a greater number of both small and large vessel structures. Previously, studies that examined the effect of VEGF inhibition in xenograft models of breast cancer cells have demonstrated that reduction of VEGF signalling leads to a reduced tumour microvessel density (Roland *et al.*, 2009). Therefore, conversely, we propose that elevated VEGF produced by TamR

cells leads to an increase in vascularisation of tissue when compared to MCF7wt xenografts. Other growth factors and signalling molecules could be mediating this change in xenografts so verification of this proposition could be carried out by inhibition of VEGF in TamR cell xenografts.

5.4 Chapter Summary

In this chapter, we have explored whether the *in vitro* pro-angiogenic properties of conditioned media from endocrine-resistant breast cancer cells observed translated into pro-angiogenic effects *in vivo*. Using a number of techniques, we have shown that:

- Despite the poor viability associated with the chick chorioallantoic assay there was a suggestion that TamR conditioned media promoted vasculature development versus MCF7wt media.

- TamR cell conditioned media increased the formation of endothelial cell tubules surrounding rat aortic ring sections grown on Matrigel versus MCF7wt cells

- Whilst both TamR and MCF7wt xenograft tissue stained positive for the endothelial marker, CD31, the extent of staining was significantly greater in TamR sections when compared to MCF7wt tissues.

Chapter 6: General Discussion

6.1 Summary of Data

Our results demonstrate that Src-dependant upregulation of VEGF in TamR cell promotes *in vitro* and *ex vivo* angiogenic responses in endothelial cells. Furthermore xenografts from TamR cells appear highly vascularised compared to MCF7wt cell xenograft tissue.

6.2 Discussion

Incidence rates of breast cancer continue to rise with time (Office for National Statistics, 2008). These data are as a result of increased screening programs coupled with improvements in treatment regimens (Glass *et al.*, 2007). However, although mortality rates are decreasing it remains the most common cause of death in women after lung cancer.

Breast cancer is a heterogeneous disease that comprises as a number of clinical and histological subtypes. Selection of therapy following tumour excision is usually determined based on the presence of ER and PR receptors and the expression level of the HER2 receptor. For ER positive cancers, treatment strategies are employed that target the binding or production of oestrogen. These treatments include selective oestrogen receptor modulators (such as tamoxifen) which bind ER and antagonise its function leading to reduction in cell proliferation (Riggs *et al.*, 2003). The advent of aromatase inhibitors has largely replaced the use of tamoxifen in postmenopausal women; however, treatment with ER receptor antagonists is still the primary choice in receptor positive pre-menopausal women.

Despite the proven benefits of tamoxifen and other ER antagonists, their clinical use is limited by resistance which occurs in two-thirds of ER+ tumours initially respond to therapy (Dorssers *et al.*, 2001). Acquired tamoxifen resistance is associated with tumour spread clinically and a poor prognosis (Ring *et al.*, 2004). *In vitro* data has also shown that breast cancer cells that become endocrine-resistant also display a more aggressive and invasive phenotype (Hiscox *et al.*, 2004), (mediated in part through the increased expression and/or activation of intracellular pro-invasive kinases including Src-kinase and focal adhesion kinase) (Hiscox *et al.*, 2007; Hiscox *et al.*, 2006b). Acquired resistance may also augment the capacity of breast cancer cells to interact with other elements (cells and proteins) in the tumour microenvironment: for example, overexpression of c-Met accompanies fulvestrant resistance in MCF7wt cells which subsequently renders these cells more sensitive to stromally produced HGF (Hiscox *et al.*, 2006a). Despite these data suggesting interplay between breast cancer cells and other cell types in the tumour microenvironment in the context of endocrine resistance, there is little data regarding whether acquisition resistance has any bearing on the angiogenic capacity of breast cancer cells that would further favour both tumour progression and spread *in vivo*. Thus, this formed the aim of this PhD thesis.

The development of blood vessels surrounding tumours can be influenced by paracrine signalling from tumour cells and the surrounding stroma (Kerbel, 2008). On acquisition of tamoxifen resistance, key signalling pathways governing tumour cell growth and survival are deregulated, which alters the development of the tumour (Ring *et al.*, 2004; Shou *et al.*, 2004). However,

some of these pathways also regulate angiogenic signalling (Koduri *et al.*, 2006; Tabernero, 2007). We hypothesised that such deregulation may also play a role in the development of a proangiogenic phenotype in anti-oestrogen resistant breast cancer cells. The clinical implications of this are that increased angiogenic signalling could sustain resistant tumour development and spread as new vasculature promotes growth and an increased risk of further metastasis.

Initial results demonstrated that the expression of genes with pro-angiogenic ontology is increased in our *in vitro* cell lines modelling tamoxifen (TamR), fulvestrant (FasR) and aromatase inhibitor (MCF7-X) resistance when compared to endocrine responsive MCF7wt cells. Gross gene deregulation was observed in the microarray profiles produced from the resistant models vs. MCF7wt, however, the gene signature of this deregulation was not consistent between the three resistant cell lines. This may be as a result of the different mechanisms by which these three agents (tamoxifen, fulvestrant and oestrogen withdrawal) act and the mechanisms by which resistance develops. One consideration in this context is that of the ER, the expression of which is retained in TamR and MCF7-X cells but is lost in FasR cells. To select out which genes represented potential candidates for further exploration, we looked for genes that fit a certain set of criteria. Our criteria demanded that these genes were; (i) positively upregulated and pro-angiogenic, to allow easy manipulation of their function by downregulation using inhibitor studies; (ii) be well established in the literature, to provide information on their mechanism and regulation; (iii) targetable, through the presence of commercially available inhibitors, to allow easy manipulation of signalling; (iv) given our previous knowledge of Src and FAK deregulation in endocrine resistant

cell models, implicated as downstream targets of these kinases. Genes that were significantly upregulated in resistant cells that fit these criteria were the growth factor, VEGF, and the cytokine IL-8.

To confirm these factors played a role in endothelial cell proliferation, we treated HUVEC and HECV cells with VEGF and IL-8 ligand. IL-8, however, failed to produce a proliferative response in either of our endothelial cell models despite having detected both CXCR1 and CXCR2 receptors in these cells. We hypothesise that IL-8 may function in cooperation with co-factor molecules including other chemokines, such as IL-6, that bind CXC receptors (Acosta *et al.*, 2008). In support of this, our experiments in which we supplemented serum-containing media with IL-8 and observed a small but significant increase in endothelial cell proliferation pointing to the presence of other factors in serum which might co-operate with IL-8 to produce endothelial cell growth. Other studies have shown that IL-8 can directly stimulate the proliferation of HUVECs *in vitro* (Li *et al.*, 2003) although these studies used whole serum and supplemented media in addition to IL-8 ligand which may explain the differences with our data.

VEGF, in contrast to IL-8, promoted endothelial cell proliferation and was taken forward for further investigation. VEGF is a key potentiator of angiogenesis and elevated expression is directly tied to increased proliferative and migratory response in endothelial cells (Hoeben *et al.*, 2004). VEGF was produced by all our resistant cell models but was highest in TamR conditioned media which also induced a significant response in promoting endothelial cell growth. In contrast, conditioned media from FasR and MCF7-X cells did not have a significant proliferative effect on

endothelial cells. This is despite findings that indicated their VEGF profile at a protein level was significantly increased over control media. However, array analysis of these two cell lines illustrated that they also possessed elevated mRNA expression of a number of anti-angiogenic agents such as thrombospondin-1, which could potentially function to reduce the pro-angiogenic effects of VEGF (Lawler, 2002). As cell proliferation is required for the development of new vasculature, only TamR cells were used in further investigations.

Validation of increased TamR VEGF expression by mRNA and protein analysis confirmed data from the only previous study to examine VEGF concentration in a model of tamoxifen resistance (Kim *et al.*, 2009). Although this group derived their tamoxifen resistant MCF7wt cell line using methodology published by our group (Knowlden *et al.*, 2003) they have not published a characterisation of these cells. We cannot therefore determine whether their TamR cell line has the same characteristics as ours which are known to overexpress EGFR, Src and FAK and so the mechanisms underlying VEGF production in this model may be different. We examined the effect of removing VEGF signalling by inhibition of the VEGFR2 receptor. This is the main effector of VEGF signalling and the majority of its actions result in angiogenic responses (Ferrara *et al.*, 2003). Inhibition of signalling through this receptor using the small molecule inhibitor ZM323881 reduced downstream signalling through MAPK and Akt as well as reducing TamR induced proliferation of endothelial cells.

To examine the mechanism by which VEGF expression is increased in TamR cells we initially explored the role of HIF1-α, which is known to be a key regulator of pro-angiogenic signalling (Ke *et al.*, 2006). VEGF is known to be

upregulated in some ovarian cancers and its expression correlates with the expression of HIF1α (Wong *et al.*, 2003). In breast cancer, HIF1α has been shown to be increased in poorly differentiated lesions and in more advanced stages of breast cancer. This also correlated with increased VEGF expression (Bos *et al.*, 2001). Clinically, increased HIF1α has been suggested as a predictive factor for endocrine resistance in ER+ cancers (Generali *et al.*, 2006). In our study, however, no difference was seen in HIF1α expression between our MCF7wt and TamR groups. Additional work using cobalt chloride to mimic the effects of hypoxia and thus to increase HIF1α, and HIF1α regulated proteins, found that although $CoCl_2$ increased HIF1α activation this did not result in increased VEGF expression nor did conditioned media from $CoCl_2$ treated TamR cells result in increased proliferative responses in endothelial cells (data not shown).

Since no significant differences in HIF1α expression or activity between our endocrine-sensitive and tamoxifen resistant cell lines were found, we next explored the role of ER given that previous work has illustrated that VEGF expression in clinical tissue from breast cancer patients is correlated with ER status (Adams *et al.*, 2000).

Despite this clinical data suggesting a link between ER and VEGF, the effect of ER modulation on VEGF expression *in vitro* are not clear. Some studies have shown that MCF7wt cells, treated with tamoxifen, demonstrated a reduced expression of VEGF mRNA whilst stimulation of the ER with oestradiol resulted in increased VEGF expression (Lee *et al.*, 2004). However, other studies in contrast suggest that tamoxifen treatment of endocrine-sensitive cell lines *in*

vitro increases the expression of VEGF (Bogin *et al.*, 2002; Ruohola *et al.*, 1999). Our own analysis revealed no significant change in VEGF expression when MCF7wt cells were treated with oestradiol, tamoxifen or fulvestrant. Furthermore, VEGF production in resistant cell models did not correlate with ER status.

Having found that neither HIF1α nor ER, function in the deregulation of VEGF, we next examined the role of Src kinase, known to be upregulated in acquired tamoxifen resistance (Hiscox *et al.*, 2006b) and to regulate expression of VEGF (Chou *et al.*, 2010; Summy *et al.*, 2005), although this mechanism is not fully understood. Our data confirmed that TamR cells have increased Src activity correlating with an increased VEGF expression. Subsequently we demonstrated that inhibition of Src reduced VEGF expression in TamR cells and also attenuated the ability of TamR conditioned media to induce endothelial cell proliferation.

The capacity of TamR cells to induce angiogenic behaviour in endothelial cell models was demonstrated by increases in proliferation, migration and formation of tubules in response to TamR conditioned media. Vascular development *in vivo* requires all three processes to produce new functional vessels (Chrzanowska-Wodnicka *et al.*, 2008). Additionally the integrity of tight junctions between endothelial cells can influence the process of angiogenesis. The degradation of these junctions allows endothelial cells to move outwards from the parent vessel towards a pro-angiogenic signal such as that from tumour cells. We made use of electric cell-substrate impedance sensing (ECIS) assays to assess the effect of conditioned media on the transendothelial resistance

(TER) of the endothelial cell line, HECVs. Despite high variation seen between experiments, we found encouraging data indicating that TamR conditioned media decreased TER to a greater extent than MCF7wt conditioned media. Reductions in TER can be suggestive of increased vascular permeability or as a reduction in tight junction integrity and therefore further experimentation will be required to determine which of these processes is responsible for the results seen (Kröll *et al.*, 2009).

To move our *in vitro* work towards an *in vivo* system, we performed rat aortic ring assays. These data illustrated that conditioned media from TamR cells induced the proliferation of *ex vivo* endothelial cells to a greater extent than MCF7wt conditioned media. We subsequently attempted to perform further *in vivo* work using the chick chorioallantoic membrane assay. Although these were disappointingly without success (due to a lack of viable embryos) one experiment did appear to show vessel growth in response to TamR conditioned media but not to w&5 control media.

Xenografts of cell lines transplanted from *in vitro* culture environments into nude-mice provide valuable insight into how tumours similar to these cell lines will behave in a more complex system (Clarke, 1996). For our purposes, we sought to examine how these cell lines would influence the surrounding tissue in terms of development of vasculature. Our data demonstrated increased presence of endothelial cell staining in TamR cell xenografts when compared to MCF7wt xenografts. This endothelial cell presence is representative of increased vascularisation of these xenografts. It has already been established that VEGF concentration is directly correlated with microvessel density in breast

cancer tumours in the clinic (Thielemann *et al.*, 2008) but whether increased microvessel density represents a useful prognostic factor remains to be elucidated (Duarte *et al.*, 2007; Nakamura *et al.*, 2003).It is important to note the limitations of xenograft studies. In our experiments, the MCF7wt xenograft group were implanted with cells that had been supplemented with oestradiol as previous experimentation has illustrated that exogenous supplementation with this ligand is necessary for the xenograft to survive in the host animal. Removal of the oestradiol supplementation from MCF7wt xenografts results in tumour regression illustrating their dependence on oestrogenic signalling (VanWeelden *et al.*, 1998). Oestradiol supplementation of TamR cells has no effect on their growth and is routinely excluded from xenograft procedures.

As an aside to our main project goals, we initially hypothesised that the presence of elevated growth factor signalling through angiogenic pathways may also lead to endocrine insensitivity in breast cancer cells through co-opting of receptor pathways such as VEGFR2. In addition, breast cancer cells that are positive for the VEGFR2 receptor have previously been shown to be directly stimulated by VEGF, resulting in increased proliferation and migration responses (Liang *et al.*, 2006; Mercurio *et al.*, 2005). Our data, however, suggest that our MCF7wt and TamRs are negative for these receptors and direct stimulation with VEGF ligand did not produce a proliferative response.

Angiogenesis is an important process in breast cancer but may be of greater importance in breast cancers that have acquired tamoxifen resistance as they may show further deregulation of proangiogenic factors including VEGF. Such

data suggest that targeted therapies that primarily inhibit VEGF may be useful in this context.

Bevacizumab is a recombinant humanised monoclonal antibody that directly binds VEGF and prevents its subsequent binding to VEGF receptors (Ferrara *et al.*, 2004). In the RiBBOn-1 (Regimens in Bevicizumab for Breast Oncology-1) and RiBBOn-2 phase III trials patients received chemotherapy and bevacizumab (or placebo) with progression-free survival (PFS) being assessed as the primary end-point (Brufsky *et al.*, 2009; Robert *et al.*, 2011). Both studies demonstrated a significant increase in PFS when patients were receiving both chemotherapy and bevacizumab. RiBBOn-2 revealed a trend towards increased overall response rate when bevacizumab was combined with chemotherapy, however, neither trial found significant differences in overall survival with the addition of bevacizumab.

Bevacizumab therapy is also associated with a number of adverse effects including severe hypertension, heart failure and a perforations of soft tissues in the nose, stomach and bowel (Tanne, 2011). As a result its indication for use in breast cancer was revoked in November 2011 by the Federal Drug Administration (Brufsky *et al.*, 2011; Pivot *et al.*, 2011; Smith *et al.*, 2011).

Small molecule inhibitors of receptor tyrosine kinases that inhibit receptors such as VEGFR2 have had some pre-clinical success. Sunitinib (Sutent) binds to VEGFR2 as well as PDDFR-β, FLT3 and c-Kit. It has demonstrated anti-tumour activity in preclinical models of breast cancer as a single agent and in combination therapy with chemotherapeutics such as docetaxel (Abrams *et al.*,

2003; Murray *et al.*, 2003). However, it did not demonstrate significant gains in progression free survival in the phase III clinical trials for use of the drug in the treatment of advanced HER-2 negative breast cancer or as a combination therapy in conjunction with capecitabine (Barrios *et al.*, 2010). Sorafenib (Nexavar) has had more success in the clinic with one phase IIb trial improves progression free survival in metastatic breast cancer when combined with capecitabine or paclitaxel (Gradishar, 2010).

The current view of these therapies is that they can give increased progression free survival and response rate when used in conjunction with a chemotherapy regimen but do not produce an increase in overall survival when compared to chemotherapy alone. Difficulties in treatment of breast cancer using anti-angiogenic monotherapy appear to include the identification of patient subgroups that will benefit and being able to administer a high enough dose to effect a positive change in tumour growth without the inset of severe side effects due to the relatively poor therapeutic index of current therapies (Mackey *et al.*, 2012). We suggest then, that in lieu of targeting VEGF directly, inhibition of upstream regulators of VEGF such as Src kinase may be beneficial in treating tumours which possess increased vascularisation and VEGF concentrations. In addition, targeting Src may also reduce further adverse aspects of resistance such as migration and invasion (Hiscox *et al.*, 2006b). Despite the numerous studies investigating anti-angiogenic therapy in breast cancer, it has not been evaluated for use in tamoxifen-resistant cancers, however, data here present a rationale for this based on the proangiogenic prolife of tamoxifen resistant cancer cells.

One phase I/II trial in lung cancer profiling Src and EGFR inhibition illustrated significant reductions in plasma VEGF which correlated with disease control (Haura *et al.*, 2010). In breast, two studies that inhibited either VEGF, using bevacizumab, or Src, using the small molecule inhibitor dasatinib, found that both methods resulted in increased VEGFR2 expression which may result as a compensatory mechanism in the event of reductions in VEGF ligand (Denduluri *et al.*, 2008; Strauss *et al.*, 2009). Data suggests that Src inhibition alters VEGF expression in clinical breast cancer, however, data from clinical trials using Src-inhibition as a monotherapy or in conjunction with anti-endocrine agents is necessary to provide a clearer picture on its role as a novel therapy. In addition to its use as a first line therapy, we hypothesise that combination therapy using Src kinase inhibitors and endocrine agents may offset the development of a pro-angiogenic phenotype on acquisition of endocrine resistance or even prevent resistance from developing (Hiscox *et al.*, 2009).

Our data demonstrate that acquired tamoxifen resistance in breast cancer confers a proangiogenic phenotype to cells. Underlying this appears to be a Src-dependant increase in VEGF which promotes VEGFR2 mediated endothelial cell growth. Although anti-angiogenic therapies have had low success in the clinical treatment of breast cancers, these have not yet been primarily directed to the treatment of endocrine-resistant tumours in which the tumours may rely more heavily on signalling through angiogenic pathways. This is excluding a handful of phase I and II trials which reported no significant improvements with treatment with bevacizumab. That said, bevacizumab, although previously popular is not the only anti-angiogenic agent. There

remains the possibility that treatment with other small molecule inhibitors targeting angiogenic ligands may prove more beneficial.

6.2 Future Perspectives

In this work, we have examined the role played by VEGF in the altered angiogenic signalling observed in tamoxifen resistant breast cancer models. From the microarray analysis carried out at the beginning of this project, several other candidate factors were found to be overexpressed in TamR cells compared to MCF7wt cells. These factors included FGF and TGFβ1 which have previously been implicated in pro-angiogenic signalling (Cross *et al.*, 2001; Pepper, 1997). The relevance of such factors could be tested using the same methodology as that used for VEGF analysis and may illustrate how altered angiogenic signalling is a multifactorial process and not wholly under the control of the VEGF/VEGFR2 axis in tamoxifen resistant cells.

Further expansion of breast cell lines used to include those that model different breast cancer subtypes would be useful in assessing how angiogenic signalling differs between them and may identify particular subtypes that would be most sensitive to anti-angiogenic therapy. Currently, our group is developing tamoxifen resistant T47D cells which would be useful in corroborating data from tamoxifen resistant MCF7wt cells as they both represent ER+ breast cancers. We also have access to TamR cells that have been cultured in the presence of tamoxifen for longer periods of time (2 years+) which may be more representative of the clinical treatment time.

Although we obtained some encouraging data from our *ex vivo* assays the use of *in vivo* functional assays to support our *in vitro* data in more complex systems would be of great benefit. *In vivo* assays could include xenograft implantation of TamR cells in nude mice ± antiangiogenic therapy or Src inhibitors. We also intend to extend our xenograft study to encompass FasR xenograft models and hypothesise that as a result of reduced VEGF expression, we may see reduced microvessel density in these samples.

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