EXPLORING THE ROLE OF CD44 IN TAMOXIFEN RESISTANT BREAST CANCER

A thesis presented for the degree of

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by

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SUMMARY

Resistance to endocrine therapy in breast cancer is associated with poor prognosis. Cell models of acquired tamoxifen resistance have implicated altered growth factor receptor signalling, especially the ErbB family of receptor tyrosine kinases, in development of the accompanying aggressive phenotype. Microarray analysis of an in vitro wtMCF-7 based model of acquired tamoxifen resistance ('Tam-R') identified upregulation of CD44, a transmembrane glycoprotein, known to interact with ErbB receptors and influence breast cancer progression. We investigated the hypothesis that CD44 overexpression in Tam-R cells can modulate ErbB activity and promote an adverse phenotype. CD44 gene overexpression was validated by RT-PCR and its protein expression determined by Western blotting and immunocytochemistry. CD44 contribution to intracellular signalling and phenotype of Tam-R cells (migration, invasion and growth), both endogenous and in response to hyaluronan (HA), was determined using Western blotting, immunocytochemistry and functional assays including wound healing, Boyden chamber migration, Matrigel™ invasion and growth assays in the presence or absence of siRNA-mediated CD44 knockdown. Interactions between CD44 and ErbB receptors were investigated using immunofluorescence and immunoprecipitation. CD44 was overexpressed at gene and protein level in Tam-R versus wtMCF-7 cells and whilst this did not influence the endogenous phenotype of Tam-R cells, it enhanced their sensitivity to HA as evidenced by HA-induced MAPK, EGFR and HER2 activation and increased migration. HA-induced migration was attenuated following treatment with the MAPK inhibitor PD098059, gefitinib as well as trastuzumab. CD44 was found to associate with HER2 and HER3 at the cell surface whilst HA stimulation appeared to modulate ErbB dimerisation patterns. Our data suggest that CD44 overexpression sensitises tamoxifen-resistant cells to HA thereby modulating ErbB dimerisation and enhancing migration. These observations may have importance in vivo where the tumour microenvironment can provide a rich source of HA to promote the progression of tamoxifen-resistant tumours.

LIST OF PUBLICATIONS, PRESENTATIONS AND AWARDS

Publications

- Hiscox, S., B. Baruah, et al. (2012). "Overexpression of CD44 accompanies acquired tamoxifen resistance in MCF7 cells and augments their sensitivity to the stromal factors, heregulin and hyaluronan." *BMC Cancer* 12: 458.

Presentations

- Baruah B.P., C. Smith, et al. (2008). "Overexpression of CD44 in acquired endocrine resistant breast cancer modulates ErbB activity and promotes an invasive phenotype." San Antonio Breast Cancer Symposium, December 2008 (Poster session).
- Baruah B.P., P. Barrett-Lee, et al. (2010). "CD44 overexpression in tamoxifen resistant breast cancer cells promotes a migratory phenotype.**"** Breast Cancer Initiative Day, Cardiff University, January 2010 (Poster session).
- Baruah B.P., S. Hiscox. (2010). "CD44 overexpression in tamoxifen-resistant breast cancer cells modulates ErbB activity and promotes an aggressive phenotype". British Breast Group Meeting, 2010 (Oral presentation).

Awards

- International AACR-Astra Zeneca Scholar-in-Training Award, San Antonio Breast Cancer Symposium, San Antonio, December 2008.
- 'Best Poster' Prize, Breast Cancer Initiative poster day, Cardiff University, Biosciences Building, January 2010.

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LIST OF ABBREVIATIONS

4-OH-TAM	4-hydroxy tamoxifen
ABC	ATP-binding cassette transporter proteins
AF-1, AF-2	Transcription activation function sites on ER
AI	Aromatase inhibitors
Akt	Serine/threonine-specific protein kinase
ANOVA	Analysis of variance
APS	Ammonium persulphate
BARD1	Gene which associates with BRCA1
BRCA1	A human caretaker gene
CD44s	CD44 standard
CD44-siRNA	CD44-specific siRNA
CD44v(number)	CD44 variant. For example, CD44v6
cDNA	Complementary DNA
CRE	Cyclic AMP responsive elements
CSC	Cancer stem cell
DBD	DNA binding domain (on ER)
DCIS	Ductal carcinoma in situ
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
E1	Oestrone
E2	Oestradiol

E3	Oestriol
EDTA	Ethylene-diamine-tetraacetic acid
EGFR	Epidermal growth factor receptor (ErbB1)
EM	Experimental medium
EMT	Epithelial mesenchymal transition
ER	Oestrogen receptor
ErbB	Family of structurally-related receptor tyrosine kinases
ERE	Oestrogen responsive elements
ERM	Protein family consisting of ezrin, radixin and moesin
ERα & ERβ	Oestrogen receptor alpha and beta
FAK	Focal adhesion kinase
FCS	Foetal calf serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEM	Glycolipid enriched membrane microdomain
HA	Hyaluronan
HAS	Hyaluronan synthase
HB-EGF	Heparin binding EGF like growth factor
HER2	Human epidermal growth factor receptor 2 (ErbB2)
HER3	Human epidermal growth factor receptor 3 (ErbB3)
HER4	Human epidermal growth factor receptor 4 (ErbB4)
HGF	Hepatocyte growth factor
HRG-β1	Heregulin beta 1
HRP	Horseradish peroxidase
hrs	Hours

ICC	Immunocytochemistry
IGF-1R	Insulin-like growth factor 1 receptor
IL-6	Interleukin-6
kDa	Kilodalton
Ki-67	Proliferation marker
LBD	Ligand binding domain (on ER)
MAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MEK	Kinase in the MAPK/ERK pathway
Met	Proto-oncogene for hepatocyte growth factor receptor
MMLV	Moloney Murine Leukaemia Virus
mRNA	Messenger RNA
MW	Molecular weight
NT	Non-targeting siRNA
p-(protein)	Phosphorylated form of protein; for example, p-MAPK
PBS	Phosphate buffered saline
PI3K	Phosphoinositide 3-kinase (part of PI3K-Akt pathway)
RA	Rheumatoid arthritis
RISC	RNAi-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
ROK	Rho-associated protein kinase
RT	Room temperature
RTK	Receptor tyrosine kinases

SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SERD	Selective oestrogen receptor downregulator
SERM	Selective oestrogen receptor modulator
SF-RPMI	Serum free RPMI-1640 medium
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
Src	Non- receptor tyrosine kinase protein encoded by SRC gene
T-(protein)	Total form of the protein of interest; for example, T-MAPK
T75	Cell culture flask ($75cm^2$)
Tam-R	Tamoxifen-resistant cell line derived from wtMCF-7 cells
TBST	Tris-buffered saline with Tween-20
TGF-α	Transforming growth factor-alpha
VCAM-1	Vascular cell adhesion molecule 1
VEGFR-2	Vascular endothelial growth factor receptor-2
W+5%	Phenol-red free RPMI with 5% charcoal-stripped FCS and glutamine (200mM)
WB	Western blotting
wtMCF-7	Wild type MCF-7 cells
AIB1	Amplified In Breast Cancer 1
HRT	Hormone replacement therapy
PFS	Progression-free survival
TTP	Time to progression
DFS	Disease-free survival
GnRH	Gonadotropin releasing hormone

ORR	Overall response rate
CBR	Clinical benefit rate
OS	Overall survival
MBC	Metastatic breast cancer
PDGF	Platelet-derived growth factor
HGF	Hepatocyte growth factor
RTK	Receptor tyrosine kinase
TKI	Tyrosine kinase inhibitor
CSC	Cancer stem cell
EMT	Epithelial mesenchymal transition
CTC	Circulating tumour cell
MIC	Metastasis-initiating cell

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Chapter One

Introduction

1.1 EPIDEMIOLOGY OF BREAST CANCER

Breast cancer is now a major worldwide public health problem and its incidence varies widely within regions and countries but is highest in the more developed regions of the world, in urban populations and in Caucasian women (Porter 2009). More recently, there has been a trend of increasing incidence in Central Europe, Eastern Europe and Asia where traditionally the incidence has been comparatively low (Hery, Ferlay et al. 2008). Although the reasons for this are not yet fully understood, a number of factors have been suggested to play a role. Some of the reasons cited for an increase in the incidence of postmenopausal breast cancer in the economically developed countries include reproductive risk factors (such as delay in childbearing and nulliparity), weight gain, alcohol consumption and higher use of hormone-replacement therapy (Hesketh, Lu et al. 2005; Kamangar, Dores et al. 2006).

In the United Kingdom (UK), breast cancer is now the most commonly occurring female cancer and in 2010 there were 49,961 new cases of breast cancer. Of these, 49,564 (99%) occurred in women and 397 in men (less than 1%) resulting in a female: male ratio of 125:1. females and this amounts to an Age-Standardised (AS) incidence rate of 125.9 per 100,000 women (Office for National Statistics, June 2010; Welsh Cancer Intelligence and Surveillance Unit, 2012; Information and Statistics Division National Health Service (NHS) Scotland, 2012 and Northern Ireland Cancer Registry, 2012). There has been an overall increase in breast cancer incidence rates in the UK since the mid-1970s including a sharp rise in the late-1980s due to the introduction of the national screening programme, but since the mid-2000s, the incidence has been very stable. The recent stable trend is likely to be due to the reduced use of hormone-replacement therapy (HRT) which has been shown to increase the risk in some women (Beral 2003; Farquhar, Marjoribanks et

al. 2009). Overall, breast cancer is the second most common cause of cancer related deaths in women after lung cancer accounting for 11,556 female cancer deaths in 2010. However, the mortality from breast cancer in the developed nations is on the decline, especially in the UK (Autier, Boniol et al. 2010). It is believed that the advent of national screening, specialisation of care and improved therapy are responsible for this decline (Sant, Francisci et al. 2006). There has been a significant improvement in the clinical management of breast cancer through the development of endocrine therapy.

1.2 OESTROGENS AND BREAST CANCER

1.2.1 Historical Context

The first link between breast cancer and hormones was made by Albert Schinzinger who presented his theory at the 18th Congress of German Surgeons in 1889 (Love and Philips 2002). He observed that the outcome for breast cancer was better in older women and argued that oophorectomy would make younger women prematurely old, thereby causing atrophy of the breast and of any cancer (Schinzinger 1889). The next major development happened when George Thomas Beatson performed bilateral oophorectomies on two women with breast cancer (Beatson 1896). This influenced an English surgeon, Stanley Boyd, to try surgical oophorectomy as a treatment for breast cancer. He did not believe in the effect of thyroid extract and used oophorectomy on its own and published a paper about his first five patients in 1897 (Boyd 1897). Progress in this area was greatly influenced by the discovery of oestrogen in 'follicular fluid' by Allen and Doisy in 1923 (Allen and Doisy 1983). In 1935, Professor Antoine Lacassagne postulated that if oestrogen secreted by the ovary was responsible for breast cancer then it should be possible to develop an agent to suppress this effect and he was hopeful that such an agent would be available soon (Lacassagne 1936). In 1962, Jensen proposed the oestrogen receptor (ER) as a mechanism of oestrogen action specific to target tissues (Jensen 1962).

1.2.2 Oestrogens: Structure and Function

An understanding of endocrine therapy in breast cancer needs a detailed knowledge of oestrogens, their receptors and the mechanism of action on breast tissue. Oestrogens (derived from the Greek: 'estrus' = sexual desire and 'gen' = generate) are a group of naturally occurring steroidal compounds which are primarily secreted by the ovaries in adult females and derived from cholesterol synthesis via androgen precursors (**Figure**

1.1). The three major naturally occurring oestrogens in women are oestrone (E1), oestradiol (E2), and oestriol (E3).

Oestradiol (**Figure 1.2A**) is the most common form of oestrogen in humans. Luteinising hormone (LH) and follicle stimulating hormone (FSH) influence the ovarian follicles to secrete oestrogens, primarily E2 (Owen 1975). However, oestrone (E1) is the predominant oestrogen in postmenopausal women and breast cancer incidence is highest in this age group. During the menstrual cycle and pregnancy, oestrogens stimulate growth and development of mammary glands and this observation led scientists in the earlier decades to investigate the potential role of oestrogens in the development of breast cancer (Bulbrook 1972). There is now evidence of the link between oestrogens and the development and progression of human breast cancer (MacMahon 2006; Russo and Russo 2006). Although the exact mechanisms are less well understood, binding of oestrogen to its specific nuclear receptor alpha (ERα) and subsequent stimulation of breast cell proliferation, through its action on growth factor production, is the most widely accepted mechanism. Other suggested mechanisms include a direct genotoxic effect by increasing mutation rates through a cytochrome P450-mediated metabolic activation and induction of chromosome abnormality or aneuploidy (Kulendran, Salhab et al. 2009). The exact role of exogenous oestrogen and the risk of breast cancer is an issue of ongoing research. A recent Cochrane review looked at 19 trials involving 41,904 women and concluded that in healthy women, combined continuous HRT (containing oestrogen and progesterone) significantly increased the risk of breast cancer whereas long-term oestrogen-only HRT did not significantly increase the risk of breast cancer.(Farquhar, Marjoribanks et al. 2009).

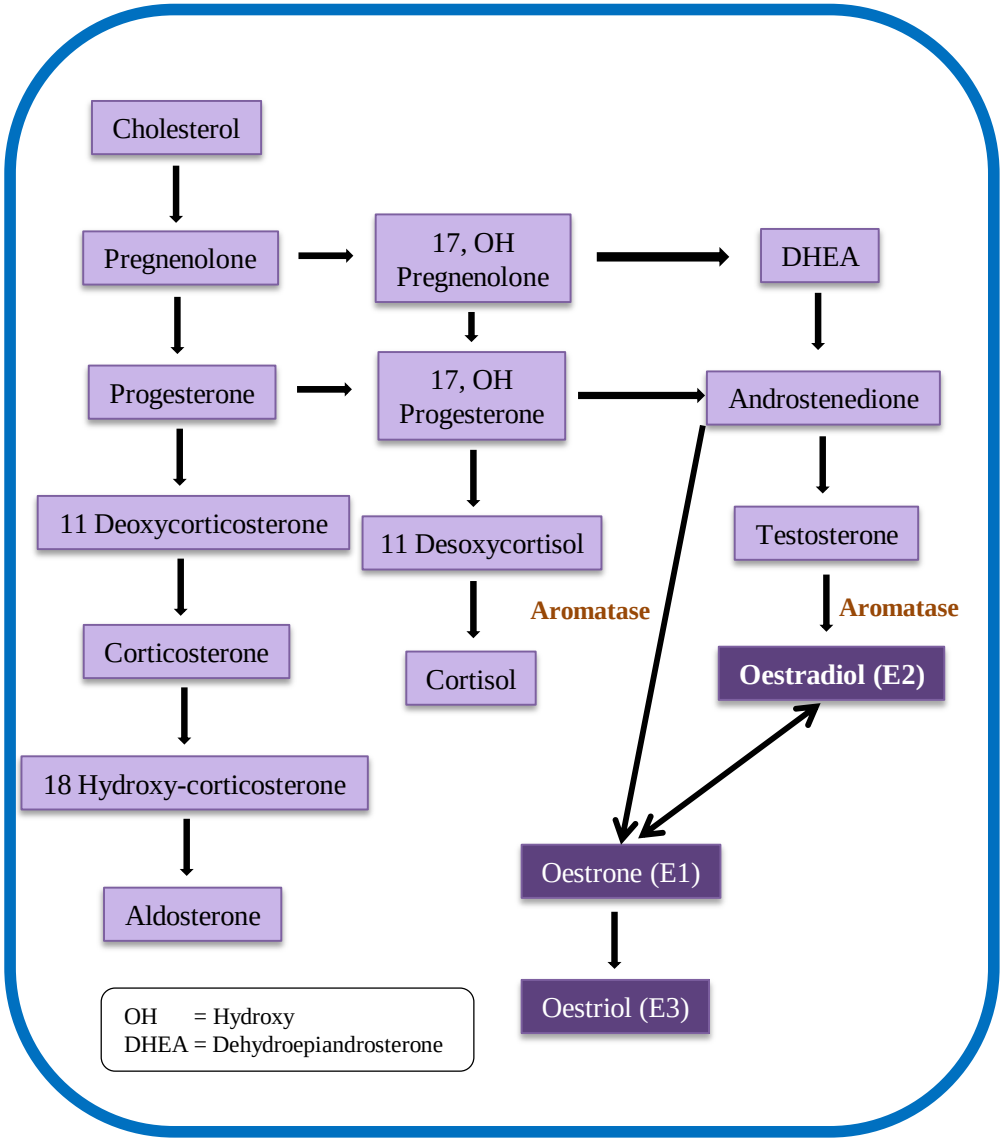


Figure 1.1 Pathway of reproductive hormone synthesis
Oestrogens (derived from the Greek: 'estrus' = sexual desire and 'gen' = generate) are a group of naturally occurring steroidal compounds which are primarily secreted by the ovaries in adult females and derived from cholesterol synthesis via androgen precursors. Oestradiol (E2) is the most commonly occurring oestrogen. Aromatase is an enzyme that is a member of the cytochrome P450 superfamily and is responsible for aromatisation of androgens into oestrogens. Adapted from Tsilchorozidou et al. 2004.

1.2.3 Oestrogen Receptor (ER)

It is now clear that oestrogen action is primarily mediated through oestrogen receptors (ER), which are members of a large superfamily of nuclear receptors that function as ligand-activated transcription factors (Evans 1988; Beato 1989). These receptor proteins share a common structural and functional organisation, with several distinct domains (Katzenellenbogen 1996). There are two main types of ER: ER alpha (ERα) and ER beta (ERβ). Whilst ERα has traditionally been considered to be the main receptor involved in breast cancer there is now evidence to indicate that ERβ is also implicated but its exact role needs to be investigated further (Hartman, Strom et al. 2009). The basic domain structure of ERα (from here onwards referred to as 'ER') is shown in **Figure 1.2B** (Kumar, Zakharov et al. 2011). The N-terminal domains A and B contain transcription activation function (AF-1) activity. Domain C is one of the two highly conserved domains seen in this family of receptors which binds to DNA, called the DNA-binding domain or DBD (Schwabe, Chapman et al. 1993). Domain D separates the DBD from the hormone binding zone or ligand-binding domain (LBD) (Tanenbaum, Wang et al. 1998). Domain E or LBD is encoded within a domain of 250 amino acids and also contains AF-2 activity, the ligand–activated transcription activation zone.

A

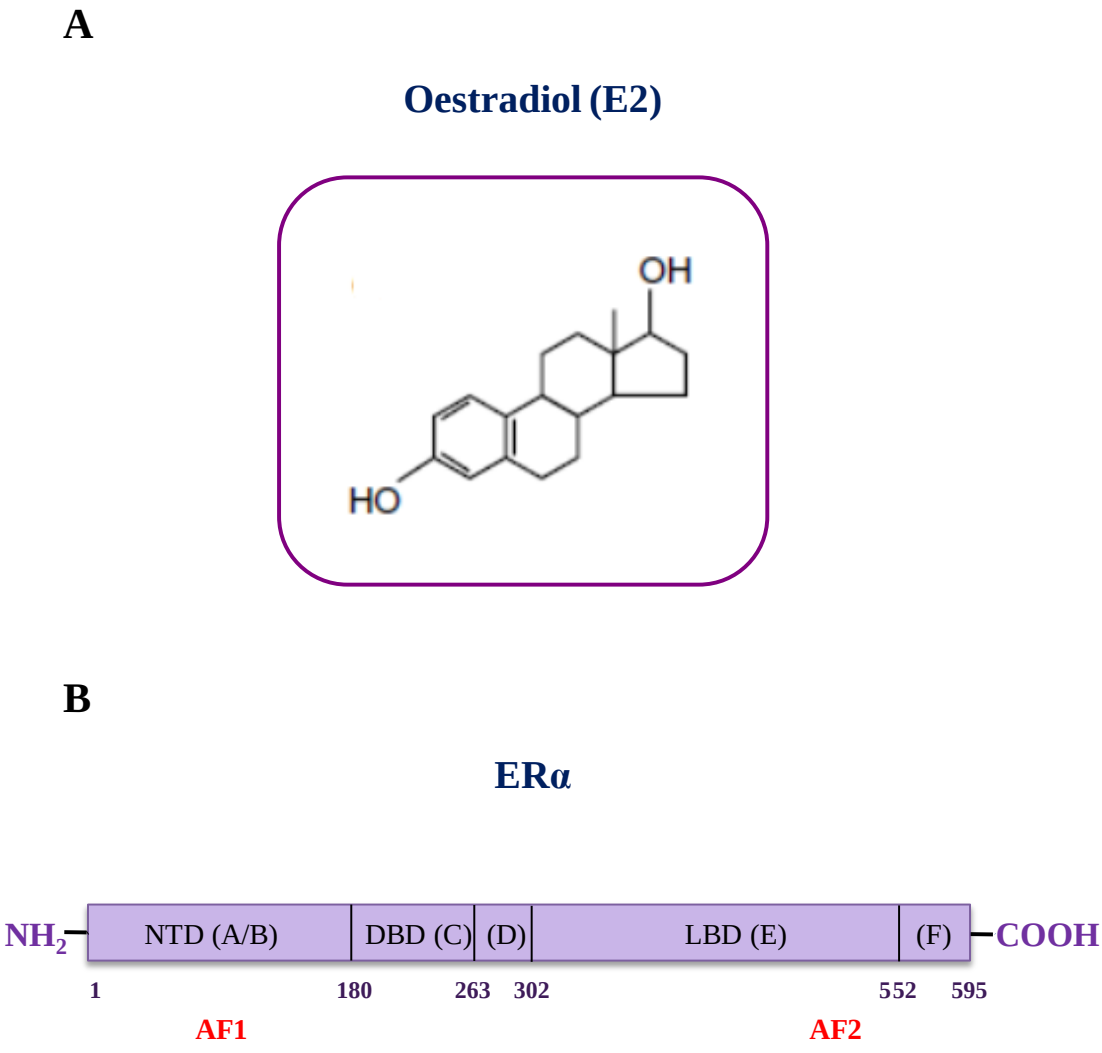


Figure 1.2 Structure of oestradiol and schematic structure of the oestrogen receptor
Oestradiol or E2 has the chemical formula $C_{18}H_{24}O_2$ (A) and its actions are mediated via the oestrogen receptor (ER) which has two main forms: ERα and ERβ. ERα is the main type (B) and is made up of 595 amino acids and contains several domains including the N-teminal domain (NTD), DNA-binding domain (DBD) and a ligand-binding domain (LBD). It has two areas with transcription activation function, namely, AF-1 and AF-2. Adapted from Kumar et al. 2011, Howell et al. 2005.

1.2.4 ER Signalling

ER signalling occurs through two general mechanisms, namely, genomic and non-genomic (**Figure 1.3**). These mechanisms are described below:

1.2.4.1 Genomic ER signalling

Genomic ER signalling proceeds via ER binding to DNA in either a direct (referred to as the 'classical' mechanism) or an indirect ('non-classical' mechanism) manner and together they are known as the 'nuclear-initiated steroid signalling' (NISS) pathway.

1. *'Classical' ER signalling:* In the absence of oestrogen, ERs are mainly found in an inactive monomeric state bound to heat shock proteins in the nucleus of the cell. When oestradiol diffuses through the cell membrane into the nucleus and, binds to the LBD on the ER, it triggers receptor conformational change, phosphorylation and finally dimerisation (Gruber, Tschugguel et al. 2002). The ligand-receptor complex binds to oestrogen responsive elements (EREs) present in the promoter regions of ER target genes to form a complex with other transcription factors (which may act as co-activators or co-repressors) leading to activation or suppression of gene expression.

2. *'Non-classical' ER signalling:* ER activation of genes lacking ERE sequence such as cyclin D1 and insulin-like growth factor 1 receptor (IGF-1R) is explained by this mechanism whereby ER interacts with other transcription factors rather than directly binding to DNA itself (Eeckhoute, Carroll et al. 2006; Maor, Mayer et al. 2006). The promoter elements include AP-1 sites, cyclic AMP response elements (CREs) and Sp-1 sites and these directly bind to the transcription factors Jun/Fos, Jun/ATF-2 and Sp-1 respectively (Sabbah, Courilleau et al. 1999; Kushner, Agard et al. 2000; Saville,

Wormke et al. 2000). ER interaction with these transcription factors facilitates their binding to DNA and, thus, indirectly promotes transcription.

Oestrogen action through either of these mechanisms results in the upregulation of genes regulating cell proliferation and survival and the downregulation of genes with anti-proliferative or pro-apoptotic activity ultimately leading to stimulation of growth and inhibition of apoptosis (Frasor, Danes et al. 2003). Two distinct transactivation domains in the ER mediate gene upregulation: AF-1, which is hormone independent, is regulated by phosphorylation and located in the amino-terminal A/B region, and AF-2, which is fully hormone dependent and located in the carboxy-terminal E region (Tora, White et al. 1989). For ER transcriptional activity to occur, AF-1 and/or AF-2 must be activated and either can be dominant although often there is a synergism between the two (Metivier, Penot et al. 2001; Osborne, Schiff et al. 2001). Oestrogen causes conformational ER changes which uncover AF-2 allowing for modulation by co-activators and co-repressors which respectively enhance or reduce transcription. Instead, AF-1 is activated by phosphorylation via kinases which are downstream of growth factor receptors such as the epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2) and IGF-1R (Zilli, Grassadonia et al. 2009). Both the ER and its associated regulatory proteins can be modulated by phosphorylation at various sites and this process involves numerous cellular kinases and introduces a further range of regulatory influences on the process of ER mediated transcription. The kinases implicated in this include Akt (a serine/threonine-specific protein kinase), mitogen activated protein kinase (MAPK) and MEK1 (kinase associated with MAPK) amongst others. For example, ER can be phosphorylated at AF-1 by MAPK (ERK1/2) which are downstream of the HER2 pathway at the serine-

118 position (directly by ERK1/2) and serine-167 by ribosomal S6 kinase activated via ERK1/2 (Kato, Endoh et al. 1995; Joel, Smith et al. 1998).

1.2.4.2 Non-genomic ER signalling

Oestrogens are known to cause rapid activation of intracellular signalling molecules (within minutes) following cellular exposure and these actions cannot be explained in terms of trasnscriptional activation but have been postulated to occur through ERs located at the plasma membrane and localised to caveolae (Kim, Lee et al. 1999). As such, this pathway has been referred to as the 'membrane-initiated steroid signalling' (MISS) pathway. Association of ERs with caveolin-1 likely provides a scaffolding platform for complexing with several other signalling molecules including G-proteins and Src (Okamoto, Schlegel et al. 1998). Oestrogen directly binds to these cell surface ERs resulting in G-protein activation which consequently stimulates various kinases including MAPK, protein kinase C and phospholipase C (Razandi, Pedram et al. 2003). Oestrogen induced activation of MAPK occurs through transactivation of EGFR (Filardo, Quinn et al. 2000). This can lead to activation of other growth factors and cytoplasmic kinases which can then phosphorylate ER and its coregulators.

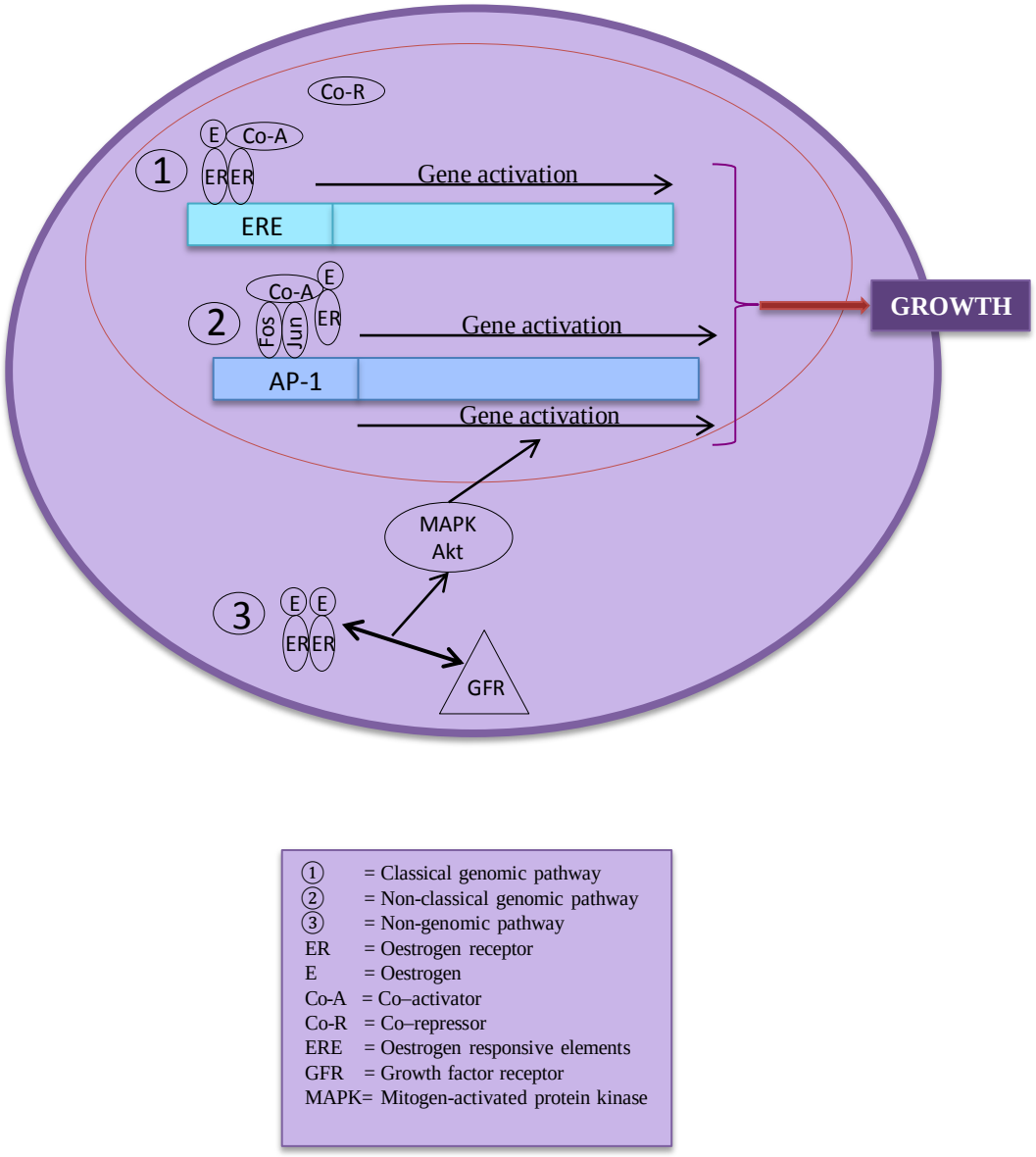


Figure 1.3 Mechanisms of oestrogen receptor (ER) signalling
ER signalling can proceed via genomic (classical and non-classical) and non-genomic pathways. The classical genomic pathway (1) involves binding of the ligand-receptor complex to EREs, whereas the non-classical genomic pathway (2) involves ER interaction with transcription factors such as Fos, Jun without directly binding to DNA. The non-genomic pathway (3) involves ER interaction with growth factor receptors which trigger activation of kinases such as MAPK and Akt leading to gene activation via phosphorylation. Adapted from Ring et al. 2004 and Nicholson et al. 2005.

1.3 ER-TARGETED THERAPY IN BREAST CANCER

ER is expressed in up to 75% of breast cancers and given its role in breast cancer proliferation it represents a potential target (Nadji, Gomez-Fernandez et al. 2005). As such, ER antagonists have been developed starting with tamoxifen. Tamoxifen was discovered by Arthur Walpole at AstraZeneca (Macclesfield, England) and was found to possess the properties of a selective oestrogen receptor modulator (SERM).

The main categories of ER-targeted drugs that have a clinical role in the management of ER-positive breast cancer are discussed below:

1. *Selective oestrogen receptor modulators (SERMs):* These agents act by inhibiting oestrogen binding to ER. They bind to the LBD of the ER and owing to their structural difference compared to ER, they induce a unique conformational change in the LBD which prevents the co-activator binding to AF-2 thereby inhibiting the transactivation function (Singh and Kumar 2005). This has no effect on the AF-1 domain which is free to promote gene transcription thereby explaining the partial agonist action noted with SERMs. Tamoxifen was the first SERM in clinical use but others have now been developed such as toremifene, raloxifene and arzoxifene. However, these agents have not proved to be more efficacious than tamoxifen and exhibit a high level of cross-resistance with tamoxifen.

   Tamoxifen is a non-steroidal triphenylethylene derivative which binds to ER leading to conformational changes which are distinctly different to oestrogen. Oestrogen binds to the hydrophobic pocket of the LBD and it is sealed inside by helix 12 resulting in AF-2 activation. In contrast, tamoxifen prevents helix 12 from sealing the

binding pocket (Shiau, Barstad et al. 1998). This prevents AF-2 activation due to an inability to bind co-activators. In genes where transcription is driven by AF-1, tamoxifen is found to have an agonistic effect (McDonnell, Clemm et al. 1995). Thus, depending on the predominance of AF-1 or AF-2 activated genes in a tissue, tamoxifen can exert either an agonistic or antagonistic role respectively. The tissue specificity of tamoxifen also depends on the expression levels of co-activators and co-repressors in a given tissue. For example, in breast tissue tamoxifen recruits a co-repressor complex and thus acts as an antagonist (Shang and Brown 2002).

The clinical efficacy of tamoxifen in the treatment of ER-positive breast cancer is now well established. Current indications for tamoxifen include primary treatment of advanced breast cancer, standard adjuvant therapy for premenopausal patients, adjuvant therapy in postmenopausal patients including patients not eligible for aromatase inhibitors (AIs) and as part of a sequence strategy prior to switch-over to AIs (Mao, Yang et al. 2012; Rao and Cobleigh 2012). It is also indicated as preventative therapy for both high-risk premenopausal and postmenopausal patients and has better efficacy than raloxifene (Cuzick, DeCensi et al. 2011). This is discussed in detail later.

2. *Selective oestrogen receptor downregulators (SERDs):* The search for agents with pure oestrogen antagonism and little or no agonistic effect has led to the development of the novel agent fulvestrant which has nearly the same affinity for ER as oestradiol (Howell and Abram 2005). Fulvestrant binds to ER monomers and inhibits dimerisation, inactivates AF-1 and AF-2 function, reduces ER translocation to nucleus and, also, accelerates ER degradation (Carlson 2005). The exact clinical role and dose

of fulvestrant is currently evolving. The phase III COmparisoN of Faslodex In Recurrent or Metastatic breast cancer (CONFIRM) study showed an improvement in progression-free survival (PFS) for fulvestrant 500 milligrams versus 250 milligrams in postmenopausal patients who had progressed on prior endocrine therapy, and, pooled evidence from other clinical trials have also demonstrated evidence of better efficacy when used at a higher dose (Howell and Sapunar 2011). 'Fulvestrant fIRst-line Study comparing endocrine Treatments' (FIRST) was a phase II, randomised trial comparing fulvestrant (500 milligrams) with anastrozole (1 milligram) as initial endocrine for postmenopausal women with ER-positive advanced breast cancer (Robertson, Lindemann et al. 2012). It was noted that fulvestrant was associated with a 34% reduction in risk of progression with the median time to progression (TTP) being 23.4 months for fulvestrant and 13.1 months for anastrozole (*p*-value = 0.01). More importantly, overall response to subsequent endocrine therapy was comparable between the treatment groups.

3. *Aromatase inhibitors (AIs):* This group of drugs inhibits the activity of the cytochrome P450 aromatase enzyme **(Figure 1.1)** responsible for production of oestrogen from androgens in peripheral tissues of postmenopausal women (Smith and Dowsett 2003). Type I steroidal drugs, such as formestane and exemestane, are androgen substrate analogues which bind competitively but irreversibly to the enzyme and are essentially 'inactivators'. In contrast, Type II non-steroidal inhibitors, such as anastrozole and letrozole, are triazoles which bind reversibly to the enzyme and fit into the substrate binding site resulting in very good potency and specificity (Miller 2003). The clinically relevant third generation AIs include the steroidal AI,

exemestane, and the non-steroidal AIs, anastrozole and letrozole (di Salle, Ornati et al. 1992; Yates, Dowsett et al. 1996; Bhatnagar 2007).

In premenopausal patients with ER-positive breast cancer, tamoxifen at a dose of 20 milligrams per day for 5 to 10 years is currently considered standard therapy as discussed in the European Society of Medical Oncology (ESMO) guidelines (Colleoni, Gelber et al. 2006; Senkus, Kyriakides et al. 2013). In the subgroup of patients who become postmenopausal following 5 years of tamoxifen, letrozole for 5 years has been noted to improve disease-free survival (DFS) significantly (Goss, Ingle et al. 2003). Patients in whom tamoxifen is contraindicated can be treated using gonadotrophin-releasing hormone (GnRH) agonist, either alone or in combination with an AI (Cuzick, Ambroisine et al. 2007).

There is currently huge debate about the optimal adjuvant therapy in postmenopausal patients with ER-positive breast cancer especially with regard to the duration and type of endocrine agent. Both AIs (steroidal and non-steroidal) and tamoxifen have been shown to be efficacious in this setting. The optimal duration of adjuvant tamoxifen therapy has been investigated as part of several clinical trials. Recent evidence from the 'Adjuvant Tamoxifen: Longer Against Shorter' (ATLAS) trial shows that adjuvant tamoxifen therapy for 10 years as opposed to 5 years leads to significant reduction in recurrence and mortality, particularly after the 10^{th} year (Davies, Pan et al. 2013). Results of the 'adjuvant Tamoxifen Treatment offer more?' (aTTom) trial show that in ER-positive disease, continuing tamoxifen for 10 years instead of 5 years produces further reductions in recurrence, from year 7 onward, and breast cancer mortality after year 10 (Gray, Rea et al. 2013). A meta-analysis of randomised trials comparing 5 years of adjuvant tamoxifen

versus no tamoxifen performed by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) which included 20 trials and 21,457 patients showed that adjuvant tamoxifen safely reduces 15 year risks of breast cancer recurrence and death (Davies, Godwin et al. 2011). Multiple randomised trials have now also proven the efficacy of AIs in the adjuvant therapy of early breast cancer including the landmark 'Anastrozole, Tamoxifen Alone or in Combination' (ATAC) trial which demonstrated improved DFS and time to recurrence in the anastrozole group over the tamoxifen group in ER-positive early breast cancer (Cuzick, Sestak et al. 2010). A meta-analysis of randomised trials of AIs compared with tamoxifen either as initial monotherapy (cohort 1) or after 2 to 3 years of tamoxifen (cohort 2) was published in 2010 (Dowsett, Cuzick et al. 2010). In cohort 1, at 5 years, AI therapy was associated with an absolute 2.9% decrease in recurrence (p-value < 0.00001) and a absolute 1.1% decrease in breast cancer mortality (p-value = 0.1). In cohort 2, at 3 years from treatment divergence, AI therapy was associated with an absolute 3.1% decrease in recurrence (p-value < 0.00001) and an absolute 0.7% decrease in breast cancer mortality (p-value = 0.02). The authors concluded that "AIs produce significantly lower recurrence rates compared with tamoxifen, either as initial monotherapy or after 2 to 3 years of tamoxifen". Another meta-analysis examined 9 randomised trials (28,632 women) and examined the role of AIs as an alternative to tamoxifen in the form of monotherapy (instead of tamoxifen), sequenced therapy (tamoxifen switched to an AI) and extended therapy (following 5 years of adjuvant tamoxifen) (Josefsson and Leinster 2010). They found that DFS was significantly improved for monotherapy (p-value = 0.002) and sequenced therapy (p-value < 0.00001). Of note, overall survival was prolonged for patients who switched from tamoxifen to AI therapy (p-value = 0.001) but no such difference in survival was noted for monotherapy (p-value = 0.39) or extended therapy (p-value = 0.67). Current evidence thus supports the use of AIs upfront (both

non-steroidal AIs and exemestane), switch over following 2 to 3 years of tamoxifen (non-steroidal AIs and exemestane) or as part of an extended adjuvant therapy regime following 5 years of tamoxifen (letrozole and anastrozole) (Senkus, Kyriakides et al. 2013).

Optimal endocrine therapy in postmenopausal women with advanced ER-positive breast cancer is also currently being debated particularly since trials have shown conflicting outcomes. A recent meta-analysis examined the evidence AIs versus tamoxifen as initial endocrine therapy in this group of patients (Xu, Liu et al. 2011). 6 trials consisting of 2560 patients were collectively analysed and a significantly improved overall response rate (ORR) and the clinical benefit rate (CBR) in favour of AIs (*p*-value = 0.002 and 0.0009 respectively) was noted. No significant difference was noted in overall survival (OS) between the two groups (*p*-value = 0.10).

1.4 RESISTANCE TO TAMOXIFEN THERAPY

Although there has been a significant improvement in outcomes in breast cancer through the use of endocrine therapy, de novo and acquired resistance to endocrine agents is a major limiting factor. In the adjuvant setting, nearly 25% of newly detected breast cancers are ER-negative and are unlikely to respond to tamoxifen therapy thus demonstrating de novo resistance (Nadji, Gomez-Fernandez et al. 2005). Among the remaining 75% of ER-positive breast cancer patients, recent meta-analysis data from the EBCTCG suggests that approximately 33% patients (approximately 25% overall) will develop recurrence by 15 years despite 5 years of adjuvant therapy with tamoxifen thus exhibiting acquired resistance (Davies, Godwin et al. 2011). Taken together, this indicates that nearly 50% patients in the adjuvant setting are affected by resistance to tamoxifen therapy. In the setting of metastatic breast cancer, recent meta-analysis evidence also shows that tamoxifen when used as first-line therapy produces initial clinical benefit in nearly 50% of patients and, even in the patients who do display an early response, eventual relapse due to acquired resistance is extremely common (Ring and Dowsett 2004; Xu, Liu et al. 2011). Even more worryingly, in both the laboratory and clinical setting, tamoxifen resistant cell lines and tumours exhibit more aggressive behaviour. We have previously demonstrated that upon acquisition of tamoxifen resistance, ER-positive MCF-7 cells display an aggressive phenotype accompanied by higher invasiveness and motility in vitro (Hiscox, Morgan et al. 2004). Therefore, defining the mechanisms of tamoxifen resistance has recently become an area of international research interest with a view to improving outcomes.

The exact mechanisms for tamoxifen resistance are, as yet, not fully understood although several key pathways and their related mechanisms have been described.

1.4.1 ER Expression

Lack of ER expression is the best negative predictor for benefit from tamoxifen. Alterations in ER expression or activity is, therefore, likely to be important in the development of resistance. In fact, lack of ER expression is the most important mechanism for de novo tamoxifen resistance and most ER-negative tumours do not respond to tamoxifen (Campbell, Blamey et al. 1981; Stewart, King et al. 1982; Ring and Dowsett 2004). Gutierrez et al. showed that ER expression is lost in only 17% of tumours with acquired tamoxifen resistance (Gutierrez, Detre et al. 2005). Also, up to 20% patients who relapse on tamoxifen respond to AIs or fulvestrant (Howell, Robertson et al. 2002). Loss of ER expression thus appears to be less important in the setting of acquired resistance. Although mutations in ERα which cause functional loss of ER activity have been noted in vitro, Roodi et al. showed that only 1% primary breast cancers have point mutations in the ER gene (Roodi, Bailey et al. 1995). This suggests that ER mutations are unlikely to be an important mechanism of resistance. There is conflicting evidence regarding the role of ERβ in tamoxifen resistance and further work is needed to elucidate this further. Post-translational or transcriptional modifications of ER via splice variants or alteration of messenger RNA (mRNA) stability have also been implicated in resistance (Adams, Furneaux et al. 2007; Shi, Dong et al. 2009).

1.4.2 Co-repressor and Co-activator Expression

Co-regulator proteins play an important role in the transcriptional machinery of ER and alteration in their expression is now considered to be an important pathway for development of resistance. In an in vitro wtMCF-7 model, Shou et al. demonstrated that tamoxifen has an agonistic action in breast cancer cells that overexpressed ‘Amplified In Breast Cancer 1’ (AIB1), an ER co-activator, and this resulted in de novo resistance

(Shou, Massarweh et al. 2004). Osborne et al. showed that high AIB1 expression, as determined by Western blotting, correlated well with poor DFS in patients treated with tamoxifen (Osborne, Bardou et al. 2003). Experimental studies have also implicated another co-activator, SRC-1, and this has been linked to AI resistance in a metastatic setting (McBryan, Theissen et al. 2012). Downregulation or decreased activity of co-repressors can also contribute to resistance. Lavinsky et al. have noted that decreased levels of the co-repressor N-CoR are linked with acquired tamoxifen resistance in a mouse model of human breast cancer (Lavinsky, Jepsen et al. 1998). Increased activity of various transcriptional factors involved in ER signalling such as AP-1 and NFkappaB has also been implicated in development of resistance (Zhou, Yau et al. 2007).

1.4.3 Cell cycle and Apoptosis Regulators

Both positive and negative cell cycle regulators appear to have an impact on tamoxifen sensitivity and resistance, in particular, positive regulators such as cyclins E1 and D1 (Butt, McNeil et al. 2005; Chu, Hengst et al. 2008). Possible mechanisms include activation of cyclin dependent kinases which are critical for the G1 phase and minimising the role of negative cell cycle regulators such as p21 and p27. Regulators of apoptosis including bcl-XL, bcl2 and caspase 9 can affect tamoxifen induced apoptosis and thus affect its efficacy (Kumar, Mandal et al. 1996).

It is worthwhile mentioning here that resistance to AIs is also an emerging problem with the now widespread use in both metastatic and adjuvant settings (Miller and Larionov 2012). Unlike resistance to tamoxifen, mechanisms underlying resistance to AIs are less clearly understood. Data from athymic mice have pointed to a role of HER2/MAPK and their interaction with ER as a probable mechanism (Brodie, Macedo et al. 2010; Sabnis

and Brodie 2010). As with tamoxifen resistance, it is probable that multiple mechanisms exist and complex interplay between these various mechanisms underlies resistance.

1.4.4 Cross-talk with Receptor Tyrosine Kinases (RTKs)

ER signalling is intimately associated with growth factors and signalling pathways in a bi-directional fashion especially receptor tyrosine kinases (RTKs) which are high affinity cell surface receptors for various growth factors, hormones and cytokines. Cross-talk between these pathways can promote resistance as can the ability of growth factor signalling to provide a growth stimulus independent of ER. For example, ERα can transactivate IGFR, HER2 and EGFR via phosphorylation and, conversely, kinases such as MAPK (ERK 1/2) which are downstream of HER2 can phosphorylate ER at AF-1 (Ring and Dowsett 2004).

The ErbB family of growth factor receptors in particular have been implicated in the development of tamoxifen resistance. The evidence supporting the role of ErbB family in tamoxifen resistance and the currently available agents targeting these receptors are discussed in detail in **Section 1.5**. The evidence implicating other RTKs in tamoxifen resistance is less compelling compared to the ErbB family and this is discussed further in this section.

There is accumulating evidence of synergistic interactions between IGF-1R, ER and EGFR/HER2. Knowlden et al. have noted IGF-1R and EGFR cross-talk in two in vitro models of acquired tamoxifen resistance (Knowlden, Hutcheson et al. 2005). Haluska et al. showed that enhanced EGFR and HER2 signalling can overcome the effects of the IGF-1R inhibitor in vitro and Lu et al. noted that in a HER2 overexpressing cell line,

increased IGF-1R signalling inhibits the action of trastuzumab (Lu, Zi et al. 2001; Haluska, Carboni et al. 2008). Using their MCF7/IGF-1R cell model which overexpresses IGF-1R, Zhang et al. showed that the IGF-1/IGF-1R may play a causal role in tamoxifen resistance via MAPK/ERK and PI3K-Akt signalling (Zhang, Moerkens et al. 2011). Hou et al. noted synergistic effect of tamoxifen and the IGF-1R inhibitor BMS-754807 in a murine xenograft model of ER-positive breast cancer (Hou, Huang et al. 2011). Interestingly, Drury et al. did not find any evidence for gain in IGF-1R levels in tumours of patients with acquired tamoxifen resistance (Drury, Detre et al. 2011). Indeed, Fagan et al. have noted the presence of IGF-1R downregulation in tamoxifen-treated xenografts and found that the IGF-1R specific antibody dalotuzumab did not enhance the effect of tamoxifen (Fagan, Uselman et al. 2012). IGF-1R directed agents have been assessed as part of clinical trials in breast cancer. A randomised phase II trial which included 156 patients with advanced hormone receptor positive breast cancer did not find any additional benefit of ganitumab, a monoclonal antibody which blocks IGF-1R, when combined with hormonal therapy (Robertson, Ferrero et al. 2013). Soria et al. noted promising response of AVE1642, a monoclonal antibody with specificity towards IGF-1R, in their subset of patients with breast cancer amongst other solid tumours (Soria, Massard et al. 2013).

Growing evidence points to a role of the vascular endothelial growth factor (VEGF) receptor in tamoxifen resistance. Tamoxifen has been noted to induce VEGF both in preclinical models and clinical specimens. For example, VEGF can be induced by tamoxifen in smooth muscle cells obtained from human saphenous vein (Bausero, Ben-Mahdi et al. 2000). Banerjee et al. noted a 38% rise in serum VEGF levels following 12 weeks of neoadjuvant tamoxifen treatment in patients with operable ER-positive breast

cancer (Banerjee, Pancholi et al. 2008). Elevated VEGF and VEGFR levels have been linked to tamoxifen insensitivity and resistance. Qu et al. showed that induction of VEGF expression in implanted xenografts to clinically relevant levels in a doxycycline regulated manner led to tamoxifen-resistant tumour growth and lung metastases (Qu, Van Ginkel et al. 2008). Using multivariate analysis, Foekens et al. noted that elevated VEGF level was an independent prognostic factor for poor response to tamoxifen in patients with advanced breast cancer (Foekens, Peters et al. 2001). Using immunohistochemical analysis of tumour specimens from 564 patients with premenopausal breast cancer randomly assigned to 2 years of tamoxifen or no treatment, Ryden et al. noted that patients with ER-positive and low VEGFR2 tumours showed improved recurrence-free survival (*p*-value = 0.001) following adjuvant tamoxifen but tamoxifen was not effective in patients with high VEGFR2 tumours (Ryden, Jirstrom et al. 2005). Linderholm et al. noted that elevated VEGFR2 levels correlated with early tamoxifen resistance whereas high VEGF levels predicted late recurrences on tamoxifen therapy (Linderholm, Hellborg et al. 2011). Sanchez et al. noted that patients with elevated VEGF levels had better outcomes when treated with 5 years of tamoxifen instead of two years (Sanchez, Sundqvist et al. 2010). Drugs targeting VEGF/VEGFR have been noted to be effective in preclinical models and in the clinical setting. Small molecule TKIs have shown promise in the setting of tamoxifen resistance in preclinical models but they have not been specifically assessed in clinical trials in this setting. Patel et al. demonstrated that brivanib alaninate, a VEGFR2 inhibitor, can reduce tumour volumes when used in tamoxifen resistant xenografts namely MCF7-Tam (Patel, Sengupta et al. 2010). Coxon et al. reported that the combination of motesanib (AMG 706), a highly selective small molecule angiokinase inhibitor with activity against VEGF receptors 1,2 and 3, and tamoxifen in an MCF-7 based athymic mice model led to significantly greater reduction in xenograft

growth than either drug alone (Coxon, Bush et al. 2009). A recent systematic review noted that phase III trials of sunitinib (SU11248, Sutent®) have not shown any significant benefit (Mackey, Kerbel et al. 2012). This review also noted that randomised phase II trials of sorafenib (Nexavar®) have shown significant benefit when used along with first and second line chemotherapy. Other TKIs with activity against VEGF receptors which are currently under development against breast cancer include motesanib (AMG706), pazopanib (Votrient®), regorafenib (BAY73-4506), cediranib (AZD 2171, Recentin®) and axitinib (Inlyta, AG013736) (Taylor, Chia et al. 2010; Martin, Roche et al. 2011; Rugo, Stopeck et al. 2011; Wilhelm, Dumas et al. 2011; Cristofanilli, Johnston et al. 2013; Hyams, Chan et al. 2013; Johnston, Gomez et al. 2013). Monoclonal antibodies against VEGF receptors have also been developed and include the VEGF-A specific antibody bevacizumab (Avastin®) and ranibizumab (Lucentis®) which is derived from an antibody fragment with higher VEGF-A specificity as compared to bevacizumab (Braghiroli, Sabbaga et al. 2012). A Cochrane review of the benefit of bevacizumab in patients with hormone receptor refractory or resistant metastatic breast cancer (MBC) concluded that the benefit of adding bevacizumab to first or second line chemotherapy is at best modest as there is no improvement in OS although there was a significant improvement in progression-free survival and ORR (Wagner, Thomssen et al. 2012). Many of the small molecule tyrosine kinase agents that are effective against VEGF receptors also show cross activity against the platelet-derived growth factor receptor (PDGF) which has also been implicated in resistance to endocrine agents (Weigel, Ghazoui et al. 2012; Weigel, Banerjee et al. 2013).

Other RTKs that have been implicated in tamoxifen resistance include the hepatocyte growth factor (HGF) receptor family (c-Met family) and the fibroblast growth factor

receptor (FGFR) family. Activation of Ron RTK, a member of the c-Met family, has been shown to confer tamoxifen resistance in murine and human breast cancer cell lines (McClaine, Marshall et al. 2010). Various agents which are active against the HGF/c-Met axis are currently being investigated in clinical trials in breast cancer (Blumenschein, Mills et al. 2012). The role of the FGFR family in tamoxifen resistance is currently not clear. Turner et al. demonstrated that FGFR1 overexpressing cell lines demonstrate tamoxifen resistance and siRNA mediated FGFR1 knockdown restores tamoxifen sensitivity (Turner, Pearson et al. 2010). Tomlinson et al. have shown that MCF-7 cells cultured with FGF-1 exhibit reduced tamoxifen sensitivity in vitro (Tomlinson, Knowles et al. 2012). They also observed that FGFR3 is overexpressed in tamoxifen-resistant tumours and FGFR3 overexpression in MCF-7 cells leads to reduced tamoxifen sensitivity.

Signalling elements such as MAPK and phosphoinositide 3-kinase -Akt pathway (PI3K-Akt) which are known to play key roles in RTK signalling are also upregulated in models of tamoxifen resistance suggesting that effects of EGFR and HER2 in the setting of tamoxifen resistance may be mediated through RTK downstream signalling pathways. Cytoplasmic proteins such as Bcar and Cas, which are linked to growth factor receptors, may also be involved in tamoxifen resistance. Van der Flier et al. measured Bcar1/p-130Cas protein levels in 937 primary breast carcinomas and concluded that levels of Bcar1/p-130Cas protein correlated well with de novo resistance to tamoxifen (van der Flier, Brinkman et al. 2000). The PI3K-Akt pathway has also been shown to be upregulated in acquired tamoxifen resistance by our group (Jordan, Gee et al. 2004). There is evidence from clinical trials indicating benefit of targeting this pathway using mTOR inhibitors in endocrine resistant breast cancer. A randomised phase II trial of

everolimus, an mTOR inhibitor, in combination with tamoxifen versus tamoxifen alone in patients with ER-positive, HER2-negative MBC with prior AI exposure showed that the combination of tamoxifen and everolimus increased the CBR, TTP and OS compared to tamoxifen alone (Bachelot, Bourgier et al. 2012). The BOLERO-2 trial was an international, randomised, double-blinded phase III study comparing combination of everolimus and exemestane versus exemestane alone in postmenopausal women with ER-positive, *HER2-negative* advanced breast cancer with disease refractory to letrozole or anastrozole (Beaver and Park 2012). The median PFS was 10.6 months for the combination versus 4.1 months for exemestane alone (p-value < 0.001) thus suggesting that addition of everolimus to exemestane is a potential treatment option in this patient subgroup. Schiff et al. have also shown that increased phosphorylated JNK and Jun levels (kinases activated by cellular stresses) and AP-1 activity is noted in breast tumours that convert to a tamoxifen-resistant phenotype (Schiff, Reddy et al. 2000).

1.5 ERBB RECEPTORS AND TAMOXIFEN RESISTANCE

In particular, the ErbB family of RTKs have been implicated in promoting de novo and acquired resistance in breast cancer. In humans, the ErbB family includes a group of four structurally related receptor tyrosine kinases namely EGFR (Epidermal growth factor receptor or ErbB1/HER1), HER2 (Human epidermal growth factor receptor 2 or ErbB2/*neu*), HER3 (Human epidermal growth factor receptor 3 or ErbB3) and HER4 (Human epidermal growth factor receptor 4 or ErbB4) (Hynes and Lane 2005). Typically they have an extracellular region which binds to ligands, a membrane linked region and a cytoplasmic domain which contains tyrosine kinase activity. ErbB receptors can be activated by a variety of ligands which typically are members of the EGF family of growth factors (Yarden and Sliwkowski 2001). ErbB receptors can exist as both monomers, homodimers or heterodimers and although HER2 has no identified ligand, it is the preferred receptor for dimerisation with other ErbB receptors and, this initiates the most potent signal transduction pathway in the entire ErbB family (Rubin and Yarden 2001). Ligand binding to these receptors leads to the formation of homo- / heterodimers and induces the formation of receptor homo- / heterodimers, tyrosine kinase domain activation and consequent phosphorylation on specific tyrosine targets within the cytoplasmic domain (Schlessinger 2004). These phosphorylated residues then serve as attachment sites for various proteins thus leading to activation of important downstream signalling pathways. Two of the most important pathways that are activated, certainly as far as breast cancer is concerned, are the MAPK pathway and the PI3K-Akt axis which also involves the 'mammalian target of rapamycin' (mTOR) receptor downstream to the PI3K-Akt pathway. Another important group of effectors are the STAT (signal transducer and activator of transcription) proteins especially STAT3 which has been implicated in breast cancer (Yu and Jove 2004). Interestingly, STAT3 has been shown to be activated

via EGF and Src kinases in breast cancer cell lines (Garcia, Yu et al. 1997; Yu and Jove 2004). Src kinase itself is also activated via the ErbB pathway and this has been demonstrated in breast cancer cell lines.

ErbB receptors have also been implicated in the pathogenesis of both de novo and acquired tamoxifen resistance in experimental studies along with promotion of an aggressive phenotype. Firstly, ErbB overexpression has been demonstrated in models of tamoxifen resistance by various authors. Long et al. demonstrated higher expression of EGFR in the acquired tamoxifen-resistant cell line (ZR-75-9al 8 microM) as compared to the endocrine-sensitive parent cell line, ZR-75-1 (Long, McKibben et al. 1992). Li et al. have also demonstrated elevated EGFR levels in their wtMCF-7 derived model of acquired tamoxifen resistance called MCF7/TAM (Li, Zhang et al. 2013). A recent study by Jin et al. has shown that EGFR upregulation in acquired tamoxifen resistance occurs via direct binding of HOXB7, an ERα responsive homeobox gene, to the EGFR promoter thus providing a mechanistic clue (Jin, Kong et al. 2011). Block et al. developed a panel of tamoxifen resistant cell lines and showed increased EGFR and HER2 expression in their T47D-TR cells whilst their MCF-7-TR cells only showed HER2 overexpression (Block, Grundker et al. 2012). Liu et al. noted that HER3 also contributes to HER2-mediated tamoxifen resistance and suggested that HER3 may be a clinically relevant therapeutic target in addition to HER2 in the setting of tamoxifen resistance (Liu, Ordonez-Ercan et al. 2007). The role of HER4 is less clear with studies suggesting that loss of HER4 expression may be associated with tamoxifen resistance (Naresh, Thor et al. 2008). Secondly, transfection studies indicate that experimentally induced ErbB overexpression can directly lead to tamoxifen resistance in previously tamoxifen sensitive cell lines. For example, Benz et al. reported that when wtMCF-7 cells were transfected with full length HER2 cDNA, the HER2 overexpressing clones were hormone dependant

but resistant to tamoxifen (Benz, Scott et al. 1992). Thirdly, ErbB overexpression in acquired tamoxifen-resistant cell lines and induced ErbB overexpression via transfection and resultant tamoxifen resistance in previously tamoxifen sensitive cell lines have been directly to the development of an aggressive phenotype. Knowlden et al. have observed that elevated levels of EGFR-HER2 heterodimers regulate an autocrine growth pathway in our tamoxifen-resistant wtMCF-7 derived 'Tam-R' cells (Knowlden, Hutcheson et al. 2003). Thrane et al. have recently showed that in a model of wtMCF-7 derived tamoxifen resistance, EGFR along with ERα sustain the higher growth rates noted in these cells (Thrane, Lykkesfeldt et al. 2013). Several transfection studies have shown that increased EGFR and HER2 expression in breast cancer cell lines exhibiting de novo hormone sensitivity has been shown to promote hormone independent proliferation (van Agthoven, van Agthoven et al. 1992; Miller, el-Ashry et al. 1994). For example, Liu et al. transfected the ER-positive wtMCF-7 cell line with a HER2 expression vector and found that transfected clones had a growth advantage in an oestrogen depleted state in vitro and the clones with higher HER2 expression could form transient tumours in ovariectomised mice as well as mice receiving tamoxifen (Liu, el-Ashry et al. 1995).

Evidence from several clinical studies also support the critical role of the ErbB family in tamoxifen resistance. Wright et al. initially noted that in their series of 221 breast cancer patients with known ER and EGFR status, expression of EGFR and HER2 protein appeared to have additive effects in lowering the possibility of response to endocrine agents. Interestingly, none of the eight patients with EGFR-positive, HER2-positive breast cancer responded to endocrine therapy but this needs to be interpreted cautiously due to the low sample size (Wright, Nicholson et al. 1992). Nicholson et al. studied the protein expression of EGFR, HER2 and Ki67 (proliferation marker) using immunocytochemistry in a series 105 breast cancers who received endocrine therapy.

They found that EGFR expression correlated with higher rates of cell proliferation (as determined by Ki67 expression) and insensitivity to hormonal agents, whereas, HER2 expression only had weak correlation with hormone insensitivity and did not influence proliferation (Nicholson, McClelland et al. 1993). De Placido et al. reported that HER2 levels were highly predictive of poor response to adjuvant tamoxifen in patients with early breast cancer (De Placido, De Laurentiis et al. 2003). Dowsett et al. reported that a proportion of patients who developed tamoxifen resistance switched from a HER2-negative status prior to endocrine therapy to a HER2-positive status at the time of relapse (Ring and Dowsett 2004). Giltnane et al. investigated the relationship between EGFR expression, measured using a new fluorescence-based method of protein expression in situ called automated quantitative analysis, and tamoxifen response using a tissue microarray constructed from a cohort of 564 patients enrolled in a randomised trial for adjuvant tamoxifen treatment in early breast cancer (Giltnane, Ryden et al. 2007). They noted a significant beneficial effect of tamoxifen in the group with low EGFR expression (p-value = 0.013) in contrast to no effect in the group with low EGFR expression (p-value = 0.7) by using a Cox model. Larsen et al. explored the role of activated ErbB receptors and their influence on outcome of patients treated with endocrine therapy and found that HER2 was associated with a worse outcome to endocrine therapy (Larsen, Bjerre et al. 2012).

Major advances have been made in therapeutic targeting of ErbB receptors especially as part of clinical trials in the last few years and this is discussed in detail below. The current status of anti-ErbB agents in relation to endocrine therapy and resistance to hormonal agents is also discussed with particular emphasis on tamoxifen resistance.

1.5.1 Therapeutic Targeting of ErbB

There are now several anti-ErbB agents that are in routine use, being used as part of clinical trials or in development as therapeutic agents for breast cancer. They can be divided into two broad categories: anti-receptor monoclonal antibodies and small molecule TKIs.

1.5.1.1 Monoclonal Antibodies

Various monoclonal antibodies with specificity against ErbB family receptors have been developed with varying degrees of clinical success. The currently available anti-ErbB monoclonal antibodies are as follows:

1. **Trastuzumab (Herceptin®):** Trastuzumab is an intravenous humanised monoclonal antibody which targets the extracellular domain of HER2 with high affinity resulting in inhibition of downstream signalling. Slamon et al. originally demonstrated in the H0684g trial that addition of trastuzumab increases the clinical benefit of first-line chemotherapy in patients with MBC which overexpresses HER2 (Slamon, Leyland-Jones et al. 2001). Vogel et al. subsequently demonstrated that trastuzumab was also effective as a single-agent in this setting (Vogel, Cobleigh et al. 2002). Trastuzumab is now widely used in the management of HER2-positive MBC either alone or in combination with chemotherapy and hormonal therapy. A recent meta-analysis by Harris et al. showed that the addition of trastuzumab improved OS, PFS and ORR in HER2-positive MBC (Harris, Ward et al. 2011). A recent Cochrane review which included 8 trials with 11991 patients concluded that trastuzumab improves DFS and OS in patients with early and locally advanced HER2-positive breast cancer (Moja, Tagliabue et al. 2012). Current meta-analysis evidence also clearly supports the

efficacy of trastuzumab when used as adjuvant therapy in HER2-positive breast cancer and supports the use of trastuzumab in the neo-adjuvant setting along with chemotherapy as it offers substantial benefit in terms of pathological complete response (Valachis, Mauri et al. 2011; Yin, Jiang et al. 2011). Whilst trastuzumab is indicated only in patients who were HER2-positive at initial presentation, Heitz et al. noted that 11.5% of tumours switch receptor status from HER2-negative to positive following development of recurrence, thus, providing a rationale for anti-HER2 therapy in selected patients who were originally HER2-negative (Heitz, Barinoff et al. 2013). There is growing evidence for the benefit of concurrent use of trastuzumab with hormone therapy. A retrospective review of 897 patients with ER-positive, HER2-positive stage I – III breast cancer revealed that addition of hormonal therapy, either tamoxifen or AI, conferred a survival benefit when added to chemotherapy and trastuzumab (Hayashi, Niikura et al. 2013). Koeberle et al. recently published early evidence suggesting that a combination of letrozole and trastuzumab is beneficial in ER-positive, HER2-positive patients who have progressed on AI and trastuzumab monotherapy alone (Koeberle, Ruhstaller et al. 2011). There is also evidence to suggest that combining trastuzumab with hormonal therapy may reverse trastuzumab resistance. For example, Chen et al. noted that addition of tamoxifen improves herceptin sensitivity in the ER-positive, Trastuzumab resistant BT/HeR cells in vitro thus providing a rationale for tamoxifen and trastuzumab combination therapy in this setting (Chen, Wang et al. 2008). Conversely, trastuzumab may be able to overcome resistance to hormonal therapy. Using xenograft studies utilising ER-positive cells stably transfected with aromatase gene (MCF7-Ca) and cells with acquired letrozole resistance (LTLT-Ca), Sabnis et al. showed that trastuzuamb can reverse letrozole resistance (Sabnis, Schayowitz et al. 2009).

2. **Pertuzumab (Perjeta®):** Pertuzumab is a novel intravenous recombinant humanised monoclonal antibody that exerts its action via inhibition of HER2 dimerisation. It is the first in a new class of therapeutic agents called HER2 dimerisation inhibitors (Zagouri, Sergentanis et al. 2013). In June 2012, pertuzumab was approved by the United States Food and Drug Administration (FDA) for use with trastuzumab and docetaxel in patients with metastatic HER2-positive breast cancer (Blumenthal, Scher et al. 2013). This was primarily based on the results of the landmark phase III randomised, double-blind, placebo-controlled CLinical Evaluation Of PertuzumAb and TRAstuzumab (CLEOPATRA) trial which included 808 patients with HER2-positive MBC (Baselga, Cortes et al. 2012). The trial reported that when pertuzumab was used as first line therapy along with trastuzumab and docetaxel in patients with MBC, the median PFS was significantly higher in the pertuzumab group compared to the control group (p-value < 0.001) with no increase in cardiac toxicity. Previous preclinical studies and earlier phase trials had shown evidence of improved activity when pertuzumab was combined with trastuzumab. For example, Scheuer et al. demonstrated enhanced anti-tumour activity with pertuzumab when used along with traztuzumab in HER2-positive xenograft models and a previous phase II trial showed that the combination of pertuzumab and trastuzumab was active and safe in patients with metastatic HER2-positive disease which progressed on trastuzumab therapy (Scheuer, Friess et al. 2009; Baselga, Gelmon et al. 2010).

3. **Cetuximab (Erbitux®):** Cetuximab is an intravenous chimeric (mouse/human) monoclonal antibody with specificity towards EGFR. Uberall et al. showed that cetuximab enhanced the anti-proliferative effect of trastuzumab in the EGFR and

HER2 overexpressing SK-BR-3 cell line (Uberall, Krizova et al. 2011). Although currently licensed for use in colorectal and head and neck cancer, cetuximab is currently being investigated in trials for breast cancer. For example, a recent phase II randomised trial showed that addition of cetuximab to cisplatin doubled the ORR and PFS in patients with triple negative MBC (Baselga, Gomez et al. 2013).

4. **Panitumumab (Vectibix®):** Panitumumab is a solely human monoclonal antibody which targets EGFR and is used as treatment of advanced colorectal cancer (Yang, Jia et al. 2001). It is currently in the developmental phase as an agent for breast cancer.

1.5.1.2 Tyrosine Kinase Inhibitors (TKIs)

Synthetic small molecule inhibitors with activity against the tyrosine kinase domain of the ErbB receptors have been developed and examined in several clinical trials as agents against breast cancer. The currently available anti-ErbB TKIs are as follows:

1. **Gefitinib (ZD1839 / Iressa®):** Gefitinib was the first agent with selective activity against the tyrosine kinase domain of EGFR and acts by binding to the ATP-binding site thereby inhibiting the anti-apoptotic Ras signalling cascade. Although currently in routine use for the treatment of non-small cell lung carcinomas, gefitinib is not yet licensed for the treatment of breast cancer and is currently only indicated for use as part of trials. There has been significant interest in the role of gefitinib in the setting of ER-positive breast cancer and in particular hormone-resistant breast cancer. In a wtMCF-7 based cell model of ER-positive breast cancer, Gee et al. demonstrated that gefitinib increased the anti-proliferative capacity of tamoxifen and prevented the development of tamoxifen resistance via inhibition of EGFR signalling (Gee, Harper

et al. 2003). In a wtMCF-7 based murine xenograft model which does not overexpress ErbB receptors, Massarweh et al. showed that gefitinib improved the anti-tumour effect of tamoxifen and delayed acquisition of acquired resistance thereby providing a rationale for combined use of tamoxifen and gefitinib (Shou, Massarweh et al. 2004). Using a model of ER/HER2-positive breast cancer exhibiting de novo tamoxifen resistance (MCF-7/HER2-18), Shou et al. showed that gefitinib is able to eliminate ER-HER2 cross-talk and restore tamoxifen sensitivity (Shou, Massarweh et al. 2004). Block et al. also showed that gefitinib is able to restore tamoxifen sensitivity using a model of acquired tamoxifen resistance, namely T47D-TR (Block, Grundker et al. 2012). Hiscox et al. demonstrated that gefitinib is able to inhibit the enhanced invasive and motile phenotype that is noted in vitro following the development of acquired tamoxifen resistance (Hiscox, Morgan et al. 2004). Several trials have investigated the role of gefitinib in breast cancer including gefitinib monotherapy. Green et al. reported that gefitinib at a dose of 500 milligram per day had a low CBR in hormone-resistant (CBR 0%) and ER-negative (CBR 7.7%) advanced breast cancer in a multicentre phase II trial (Green, Francis et al. 2009). In another phase II trial, gefitinib (500 milligram/day) was shown to be well tolerated with a CBR of 53.6% in tamoxifen-resistant breast cancer and 11.5% in ER-negative breast cancer (Gutteridge, Agrawal et al. 2010). Use of gefitinib in combination with anti hormonal agents has also been investigated. In a randomised phase II trial including 93 patients, Cristofanilli et al. reported that the combination of anastrozole and gefitinib is associated with a marked improvement in PFS (14.7 versus 8.4 months) as opposed to anastrozole combined with placebo (Cristofanilli, Valero et al. 2010). However, in a randomised phase II trial which included postmenopausal women with hormone receptor positive MBC, Carlson et al. did not note any benefit of the combination of

anastrozole and gefitinib over anastrozole or gefitinib monotherapy (Carlson, O'Neill et al. 2012). Osborne et al. reported the results of a randomised phase II trial assessing tamoxifen plus placebo or gefitinib in ER-positive MBC (Osborne, Neven et al. 2011). They noted an improved PFS in patients receiving a combination of gefitinib and tamoxifen except patients who progressed or recurred following prior AI therapy. Other trials have assessed the role of gefitinib in other settings. A recent prospective, single arm phase I/II trial of gefitinib, trastuzumab and docetaxel in patients with advanced HER2-positive MBC found that this combination was well tolerated and clinically effective as demonstrated by complete and partial response rates of 18 and 46% respectively and a stable disease rate of 29% (Somlo, Martel et al. 2012). Massarweh et al. reported the results of a small (15 patients) phase II neoadjuvant trial of gefitinib, anastrozole and fulvestrant in newly diagnosed patients with ER-positive breast cancer and noted that this combination consistently reduced proliferation as assessed via Ki-67 levels (Massarweh, Tham et al. 2011).

2. **Lapatinib (Tyverb®):** Lapatinib is an orally active TKI which targets EGFR and HER2. Konecny et al. noted significant anti-proliferative effect of lapatinib alone and in combination with trastuzumab on HER2 overexpressing breast cancer cell lines as well as xenografts in athymic mice (Konecny, Pegram et al. 2006). In an in vivo model of HER2 overexpressing breast cancer, Scaltriti et al. noted complete tumour remissions following 10 days of treatment with lapatinib and trastuzumab (Scaltriti, Verma et al. 2009). A Phase I dose escalation and pharmacokinetic study of lapatinib in combination with trastuzumab in patients with heavily pre-treated MBC showed that this combination was well tolerated and clinically active (Storniolo, Pegram et al. 2008). Following these and other preclinical and early clinical evidence of

effectiveness and safety of this drug in breast cancer models, several trials have investigated the use of lapatinib in various clinical settings. A meta-analysis of Phase III trials by Amir et al. showed that lapatinib was only clinically effective against patients with HER2-positive disease and not HER2-negative disease irrespective of EGFR status (Amir, Ocana et al. 2010). Berghoff et al. reported that although lapatinib did not improve OS in patients with HER2-positive MBC who progressed on trastuzumab, it was noted to significantly improve OS (p-value = 0.022) in the subset of patient with bone metastases (Berghoff, Bago-Horvath et al. 2013). The Phase III EGF104900 trial showed a significant 4.5 month median OS benefit of lapatinib and trastuzumab combination over lapatinib monotherapy in patients with heavily pre-treated HER2-positive MBC (Blackwell, Burstein et al. 2012). A meta-analysis of HER2 targeted agents in MBC by Harris et al. noted that addition of lapatinib to standard chemotherapy / AI therapy improved TTP, OS, PFS and ORR (Harris, Ward et al. 2011).

The role of lapatinib in the setting of ER-positive disease and endocrine resistance has also been investigated. Leary et al. investigated the effect of lapatinib in two in vitro models of acquired endocrine resistance: a model of long-term oestrogen deprivation and a model of tamoxifen resistance following long-term tamoxifen treatment (Leary, Drury et al. 2010). Both models showed modest upregulation of HER2 and lapatinib was able to restore hormone sensitivity in both models. Chu et al showed that lapatinib when combined with tamoxifen showed significant decrease in tumour volume in an acquired tamoxifen-resistant athymic mouse xenograft model (Chu, Blackwell et al. 2005). A systematic review by Fleeman et al. noted that when used as first-line therapy for hormone receptor positive MBC which overexpresses HER2,

both lapatinib plus AI and trastuzumab plus AI were clinically more effective than AI monotherapy (Fleeman, Bagust et al. 2011). A clinical trial comparing lapatinib combined with letrozole versus letrozole and placebo in patients with postmenopausal ER-positive MBC showed that the combination of lapatinib and letrozole significantly improves PFS (*p*-value = 0.019) and CBR (*p*-value = 0.003) in patients with ER-positive, HER2-positive MBC (Johnston, Pippen et al. 2009). A recent systematic review which included results of 18 studies noted that lapatinib plus letrozole was clearly superior to letrozole alone in terms of PFS, TTP and ORR (Riemsma, Forbes et al. 2012).

3. **Erlotinib (Tarceva®):** It is a reversible TKI with specific activity against EGFR and acts via reversibly binding to the ATP-binding site on the receptor (Raymond, Faivre et al. 2000). It has been shown to have anti-tumour activity in several preclinical studies. Zhang et al. demonstrated that erlotinib can inhibit tumour proliferation and metastasis in an EGFR driven xenograft model of inflammatory breast cancer (Zhang, LaFortune et al. 2009). Saxena et al. showed that erlotinib can inhibit the invasive and migratory phenotype induced by leptin and IGF1-R via EGFR transactivation in breast cancer cell lines namely, MDA-MB-468 and MDA-MB-231(Saxena, Taliaferro-Smith et al. 2008). Erlotinib is currently in trial phase for use as an agent against breast cancer. A multicentre Phase II trial of erlotinib monotherapy and another Phase II trial of erlotinib and bevacizumab combination therapy did not show any significant clinical benefit in the setting of advanced breast cancer (Dickler, Rugo et al. 2008; Dickler, Cobleigh et al. 2009). However, Twelves et al. reported encouraging ORR and an acceptable toxicity profile of the combination of erlotinib with capecitabine and docetaxel in MBC (Twelves, Trigo et al. 2008). Britten et al.

demonstrated preliminary clinical efficacy of the combination of erlotinib and trastuzumab in HER2-positive MBC in a Phase I/II trial (Britten, Finn et al. 2009). Interestingly, Montagna et al. have reported that metronomic chemotherapy along with erlotinib and bevacizumab is clinically effective against HER2-negative MBC (Montagna, Cancello et al. 2012).

Even in the setting of ER-positive disease, there is some early evidence for this drug. Erlotinib was able to inhibit the pro-migratory effect of leptin and IGFR-1 via EGFR transactivation in ER-positive wtMCF-7 cells (Saxena, Taliaferro-Smith et al. 2008). Khajah et al. showed that erlotinib was able to significantly inhibit proliferation and invasion of endocrine resistant pII cells which were established through stable transfection of wtMCF7 cells with ER-directed shRHA plasmid (Khajah, Al Saleh et al. 2012).

4. **Vandetanib (Caprelsa™):** Vandetanib is an oral multi TKI which has inhibitory effect primarily on VEGFR and amongst the ErbB family on EGFR (Carlomagno, Vitagliano et al. 2002). Sarkar et al. reported that vandetanib enhances the anti-proliferative and apoptotic effects of paclitaxel on breast carcinoma cells (Sarkar, Mazumdar et al. 2011). Mi et al. demonstrated that vandetanib could reverse multidrug resistance in a P-glycoprotein expressing breast cancer cell line (Mi and Lou 2007). In a multicentre phase II trial of vandetanib monotherapy, Miller et al. showed that the drug was well tolerated but did not show any definite clinical benefit in advanced MBC (Miller, Trigo et al. 2005). In patients with pre-treated advanced breast cancer, combination of vandetanib plus docetaxel was well tolerated but there was no additional clinical benefit when compared with docetaxel plus placebo (Boer,

Lang et al. 2012). Vandetanib is currently still in the development phase as a therapeutic agent for breast cancer.

5. **Neratinib (HKI-272):** Neratinib is an oral TKI with irreversible activity against EGFR, HER2 and HER4 (Wissner and Mansour 2008). In preclinical studies it has been shown to have potent activity against breast cancer cell lines which overexpress HER2 (Rabindran, Discafani et al. 2004). It has also been shown to be effective in the setting of de novo and acquired trastuzumab resistance in breast cancer cell lines as well as in a xenograft model (Canonici, Gijsen et al. 2013). Chow et al. reported that the combination of neratinib and paclitaxel showed a higher response rate compared to neratinib alone in a Phase I/II study (Chow, Xu et al. 2013). Martin et al. reported the results of a Phase II study comparing neratinib monotherapy versus lapatininb plus capecitabine chemotherapy in patients with advanced HER2-positive breast cancer where neratinib was shown to be a safe and effective single agent in this setting (Martin, Bonneterre et al. 2013). Neratinib is currently in the clinical trial phase as an agent against breast cancer and is currently being investigated in Phase III trials (Canonici, Gijsen et al. 2013).

6. **Canertinib (CI-1033):** Canertinib is a pan-ErbB TKI that binds to the intracellular domains of the ErbB receptors and has irreversible activity against EGFR, HER2 and HER4 (Smaill, Rewcastle et al. 2000). It is still in the early development phase as a breast cancer drug (www.selleckchem.com).

1.6 CD44

CD44 (Cluster of Differentiation 44) is a transmembrane glycoprotein that was first described as a lymphocyte homing receptor (Gallatin, Weissman et al. 1983) and later found to be a member of the cartilage link protein family (Goldstein, Zhou et al. 1989; Stamenkovic, Amiot et al. 1989). It has various isoforms and exhibits functional heterogeneity in terms of its role in physiological processes including acting as an adhesion molecule for lymphocyte homing, influencing extracellular matrix remodelling during embryogenesis and triggering release of cytokines via adhesion to macrophages. CD44, along with a select few other markers including CD24, is also a widely used marker of breast cancer stem cells (CSCs). CD44, therefore, continues to be the subject of intensive research.

1.6.1 CD44 Structure

The CD44 gene is located on the short arm of human chromosome 11 and contains up to 20 exons (Screaton, Bell et al. 1992). Exons 1 to 17 encode the extracellular domain of the CD44 protein, exon 18 encodes the transmembrane domain and exons 19 and 20 are responsible for the cytoplasmic domain of the protein (**Figure 1.4A)**. Exons 6 to 15 are variably inserted into the gene via alternative splicing of mRNA to produce a number of CD44 isoforms. The resultant CD44 molecule is a single chain glycoprotein which has a globular extracellular domain, a short transmembrane domain and a cytoplasmic domain (**Figure 1.4B**).

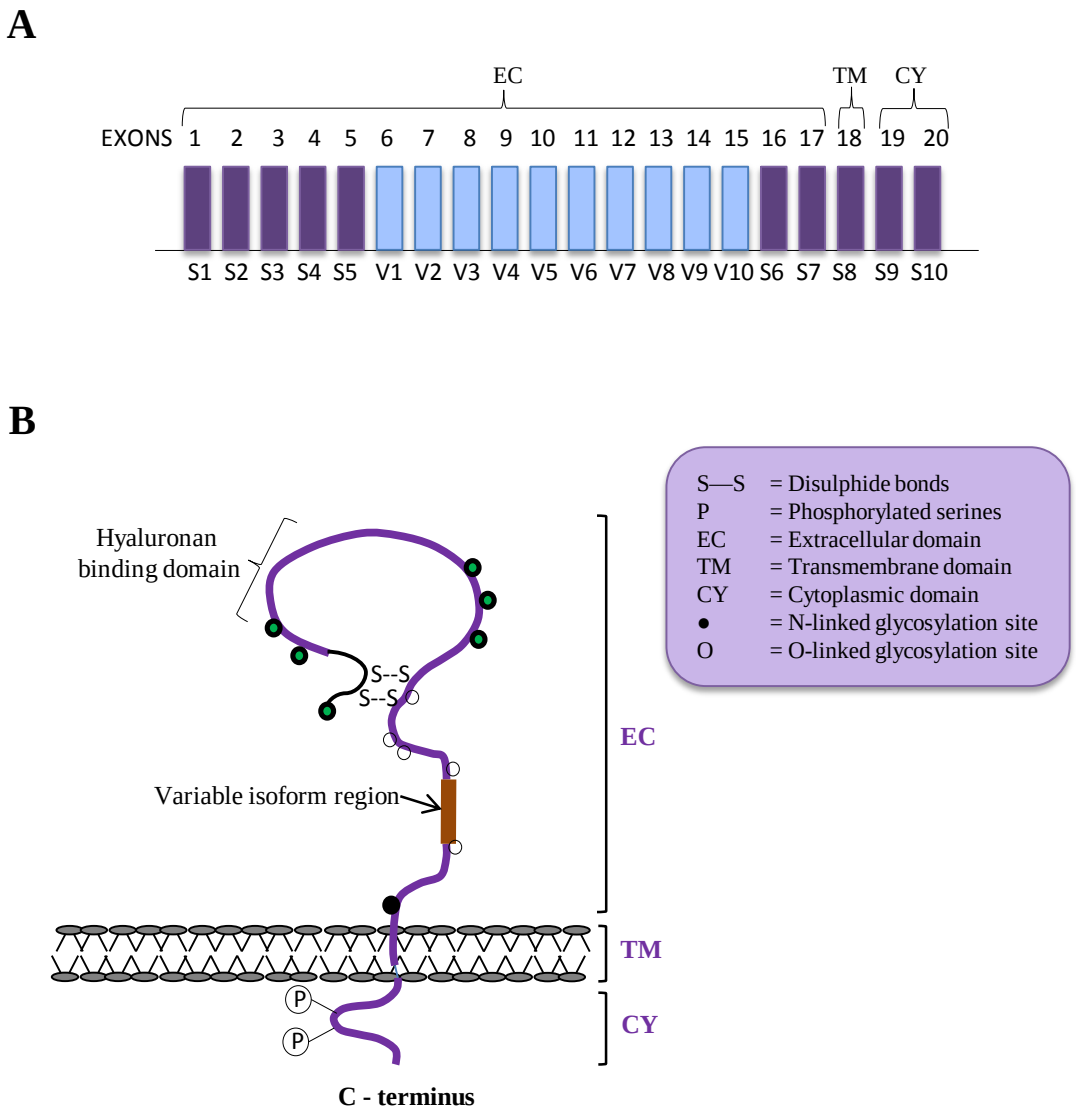


Figure 1.4 CD44 gene and protein structure
The CD44 gene is located on the short arm of human chromosome 11 and contains up to 20 exons (A). Exons 1 to 17 encode the extracellular domain of the CD44 protein, exon 18 encodes the transmembrane domain and exons 19 and 20 are responsible for the cytoplasmic domain of the protein. Exons 6 to 15 are variably inserted into the gene via alternative splicing of mRNA to produce a number of CD44 isoforms. The resultant CD44 molecule (B) is a single chain glycoprotein which has a globular extracellular domain, a short transmembrane domain and a cytoplasmic domain. Adapted from Zoller 2011.

The extracellular domain undergoes post-translational modifications via N-glycosylation and O-glycosylation and has binding sites for various matrix proteins including fibronectin, laminin and collagen (Goldstein, Zhou et al. 1989; Jalkanen and Jalkanen 1992; Ishii, Ford et al. 1993). CD44 isoforms arise through exon splicing of the CD44 gene and result in elongation of the extracellular region of the molecule through insertion of exon products within a region near the transmembrane domain. This heavily glycosylated polypeptide stalk-like variable portion of the molecule is thought to be responsible for the variation in structure and function between CD44 isoforms (Screaton, Bell et al. 1993).

The relatively well-conserved and short transmembrane domain is responsible for CD44 dimerisation and cross-linking thus allowing CD44 to be incorporated into glycolipid enriched membrane microdomains (GEM's) enabling close association with different cell signalling molecules (Foger, Marhaba et al. 2001).

The cytoplasmic domain does not vary significantly amongst the isoforms and allows CD44 to associate with cytoplasmic proteins such as ankyrin, ezrin, radixin and moesin thereby accounting for many of the effects of CD44 on signal transduction and cell migration (Mori, Kitano et al. 2008; Fehon, McClatchey et al. 2010).

The smallest CD44 isoform, termed CD44 standard (CD44s), lacks any of the variant exons and is the most widely present of all CD44 isoforms (Isacke and Yarwood 2002). Originally seen in brain cells, leucocytes and red blood cells, it is now known that most vertebrate cells express CD44s in the membrane (Telen, Rogers et al. 1987; Lucas, Green et al. 1989; Naor, Wallach-Dayan et al. 2008). The actual size of CD44s varies between different cells and tissues due to differences in the extent of glycosylation. The CD44 variants, which are named based on expression of the variant exons: v1 to v10, are less widely expressed in normal tissues, some only in malignant cell lines, and are known to have functions distinct from CD44s (Ruiz, Schwarzler et al. 1995; Borland, Ross et al. 1998). The variant isoforms have additional binding sites for other molecules near the transmembrane domain at the polypeptide stalk such as the heparin sulphate site on CD44v3 (Bennett, Jackson et al. 1995). The functional relevance of CD44 standard and the variant isoforms is discussed in **Section 1.6.3**.

1.6.2 Hyaluronan, the CD44 Ligand

The major ligand for CD44 is hyaluronan (HA), a large glycosaminoglycan which is a linear polymer containing between 2,000 to 25,000 disaccharides of glucuronic acid and N-acetylglucosamine with a molecular weight between 10^2 to 10^4 kDa. HA is synthesised at the inner aspect of the plasma membrane by three related hyaluronan synthases (HAS1, HAS2 and HAS3), extruded into the peri-cellular space and eventually degraded by one of many hylauronidases (Weigel, Hascall et al. 1997). HA is present in most mammalian tissues and plays a crucial structural role in extracellular tissue due to its hydrodynamic characteristics and by interacting with other extracellular matrix components. HA interacts closely with matrix proteoglycans such as versican and aggrecan and the negative charge on the chondroitin sulphates on these proteoglycans forces HA to form a

thick gel-like coat on the cellular surface (Evanko, Tammi et al. 2007). The interaction between HA and the chondroitin sulphates also regulates the properties of the matrix and an increase in the proportion of proteoglycans leads to a matrix that is stiffer and less permeable (Heldin, Suzuki et al. 1995). HA and other matrix proteoglycans can also play a role in proliferation and migration in various cell types and influence cell adhesion (Evanko, Tammi et al. 2007). HA also plays a role in tumour progression including breast cancer. Using a mouse mammary tumour virus (MMTV-Neu) transgenic model of spontaneous breast cancer, Koyama et al. demonstrated that stromal HA overproduction stimulates tumour growth through angiogenesis (Koyama, Hibi et al. 2007). Ghatak et al. showed that in the TA3/St breast cancer cell line, endogenous HA is necessary for the constitutively high levels of HER2 thereby demonstrating that hyaluronan can have an impact on cell signalling via its effect on RTKs (Ghatak, Misra et al. 2005).

HA is also known to bind to the "Hyaluronan Mediated Motility Receptor" (HMMR) which is more commonly referred to as RHAMM and has been given the designation CD168 (Turley 1982). RHAMM is predominantly an intracellular cytoplasmic protein that is implicated in tumour progression by promoting genomic instability through its association with 'Breast Cancer 1' or BRCA1 (human caretaker gene whose mutations lead to an increased risk of breast cancer) and the BRCA1-associated ring domain protein 1. It is transported to the extracellular space from the cytoplasm via unconventional cytoplasmic protein export mechanisms. Once in the extracellular microenvironment, RHAMM can interact with CD44 and this is considered to enhance CD44-mediated signalling through MAPK (Hamilton, Fard et al. 2007). It is thought to contribute to cancer progression by enhancing and activating the oncogenic properties of CD44 (Maxwell, McCarthy et al. 2008).

1.6.3 Role of CD44 in Cellular Signalling and Function

CD44 is known to interact extensively with HA in the stromal microenvironment. The binding site for HA lies in the extracellular domain of CD44 and growing evidence points to HA-CD44 interactions as a mechanism of eliciting intracellular signalling pathways that result in cytoskeleton modulation (Underhill and Toole 1979; Aruffo, Stamenkovic et al. 1990; Toole and Slomiany 2008). Using human melanoma cells transfected with CD44, Bartolazzi et al. showed that changes in glycosylation of CD44 have a direct effect on its interaction with hyaluronic acid thereby providing an important mechanism for regulation of CD44 function (Bartolazzi, Nocks et al. 1996). Zohar et al. demonstrated that CD44 was localised primarily at the leading edge of migrating fibroblasts and CD44-negative mouse fibroblasts displayed impaired migration (Zohar, Suzuki et al. 2000). CD44 binding to HA also triggers its metabolism and CD44 may thus mediate hyaluronan homeostasis (Kaya, Rodriguez et al. 1997). These and other experimental studies suggest a role of CD44 in regulation of adhesion of cells to the matrix as well as migration through the stroma. CD44 proteins have been found to act as platforms for growth factors and matrix metalloproteinases (MMP's) including MMP9 and MMP7 (Ponta, Sherman et al. 2003).

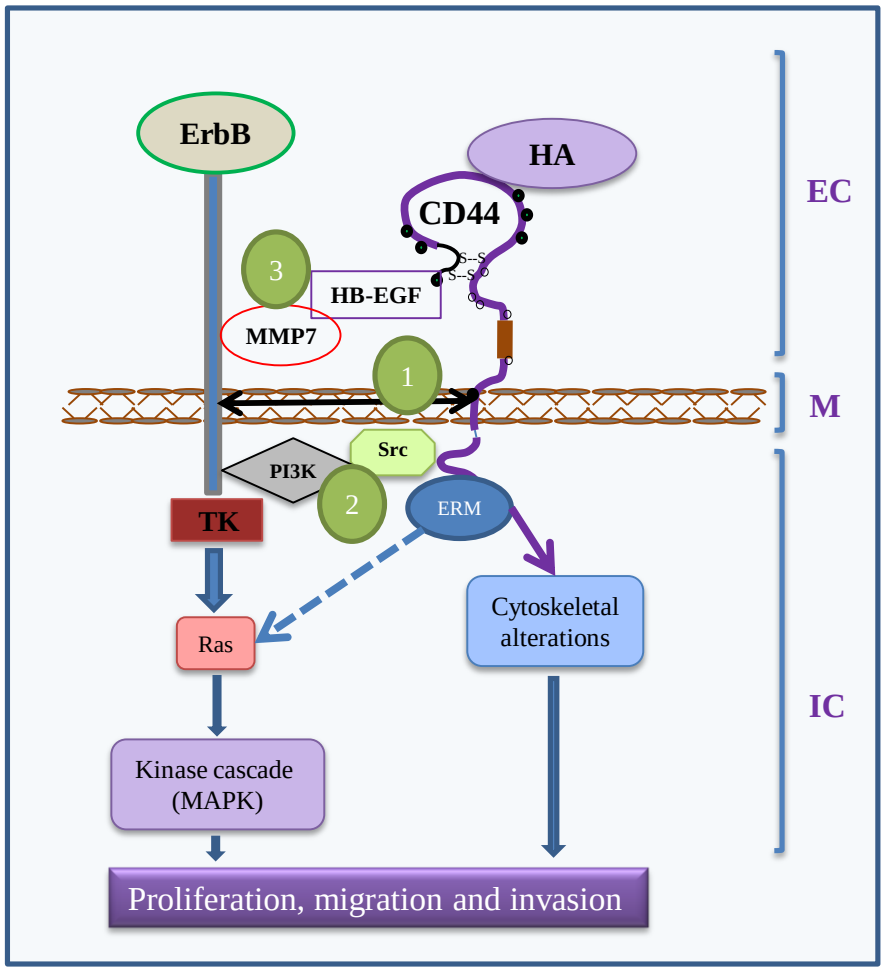
As well as exogenous matrix proteins and growth factors, CD44 has also been shown to interact with RTKs and influence their signalling behaviour. CD44 functions as a co-receptor for activation of Met, a tyrosine kinase, and it appears that the v6 splice variant is a key requirement for this activation. Orian-Rousseau et al. demonstrated that a CD44 isoform containing exon v6 sequences is necessary for Met activation by its ligand hepatocyte growth factor (HGF) in many established cell lines including primary keratinocytes (Orian-Rousseau, Chen et al. 2002). Met plays a critical role in embryonic

development and wound healing and aberrant signalling in cancer promotes tumour progression through proliferation, angiogenesis and metastasis and CD44 appears to play a part in these functions (Steffan, Coleman et al. 2011). CD44 can also function as a co-receptor for the ErbB family of RTKs which are known to be involved in signal transduction controlling normal cell growth and differentiation. CD44 is known to directly associate with members of the ErbB family. Using co-immunoprecipitation and fluorescence resonance energy transfer (FRET) analysis, Gorlewicz et al. demonstrated that CD44 co-immunoprecipitates with both HER2 and HER3 in adult rat skeletal muscle Schwann cells (Gorlewicz, Wlodarczyk et al. 2009). CD44 and ErbB association has been noted to have important implications in terms of cell signalling and function. Using patient matched oral and dermal fibroblasts, Meran et al. showed that CD44 and EGFR interaction directly activates MAPK leading to cellular proliferation in these fibroblasts (Meran, Luo et al. 2011). Yu et al. have demonstrated that HER4 can be associated with various CD44 isoforms and this complex can play a role in regulation of female reproductive organ remodelling (Yu, Woessner et al. 2002).

CD44 can interact with proteins that attach to the actin cytoskeleton and evidence points to the Band 4.1 superfamily of proteins (ERM proteins and Merlin) as being most relevant in this context. The ERM protein family consists of three proteins namely, ezrin, radixin and moesin which cross-link actin filaments to the plasma membrane and are involved in many cellular functions such as migration and regulation of cell shape (Tsukita and Yonemura 1997). Tsukita et al. showed that in cultured fibroblasts CD44 is precisely co-localised with ERM family members which act as a link between the cytoplasmic domain of CD44 and the cytoskeleton (Tsukita, Oishi et al. 1994). ERM proteins appear to be necessary for CD44 signal transduction through Met to Ras and

MAPK, a process that is closely linked to activity of Merlin ('Moesin-Ezrin-Radixin-Like-ProteiN'), another member of the same superfamily, which also attaches to CD44 but does not link with actin (Ponta, Sherman et al. 2003). Thus, the interaction between CD44, ERM proteins and Merlin can influence signal transduction as well as cell shape and motility.

A schematic representation of the interaction between CD44, HA and ErbB receptors and the consequent influence on cell signalling and function is shown in **Figure 1.5**.



HA = Hyaluronan
MMP7 = Matrix metalloproteinase 7
HB-EGF = Heparin binding EGF-like growth factor
ERM = 'Ezrin, radixin, moesin' family
TK = Tyrosine kinase
MAPK = Mitogen-activated protein kinase
PI3K = Phosphoinositide 3-kinase
EC = Extracellular
M = Membrane
IC = Intracellular

Figure 1.5 Schematic representation of CD44 interaction with HA and ErbB receptors and effect on downstream signalling and cell function

CD44 interacts with ligands such as HA and with receptor tyrosine kinases such as the ErbB family which leads to activation of several downstream signalling pathways with resultant changes in cellular phenotype. CD44 can interact with ErbB either directly via disulphide bonds or dimerisation (1), via intracellular intermediates such as Src and PI3K (2) or via extracellular intermediates such as HB-EGF and MMP7 (3). Adapted from Ponta et al. 2003, Zoller et al. 2011.

1.6.4 Physiological and Pathological Roles of CD44

Both CD44 standard and the variant isoforms have a wide range of functional implications in various physiological and non-malignant pathological processes. CD44 was initially recognised as a protein involved in lymphocyte homing and is now known to aid lymphocyte rolling, activation and homing to gut-associated lymphoid tissue (Berg, Goldstein et al. 1989). CD44 has several other roles in the immune-haematopoietic system including aiding pro-thymocyte homing from the bone marrow to the thymus (Spangrude and Scollay 1990; Wu, Antica et al. 1991). CD44 is known to play a role in embryogenesis especially in limb bud development and chondrogenesis (Knudson 2003). During limb bud development, a reduction in the matrix HA content and cross-bridging of mesodermal cells is essential, and CD44 plays a role in both reduced production of peri-cellular HA coats and HA-mediated cross-bridging of mesodermal cells (Culty, Nguyen et al. 1992). During chondrogenesis, CD44-mediated hyaluronan turnover is crucial in cartilage formation from mesenchymal cells (Nicoll, Barak et al. 2002).

There has been an increasing understanding of the role of CD44 in inflammatory and autoimmune processes following the observation that its expression is upregulated during inflammation in haematopoietic and parenchymal cells (Foster, Arkonac et al. 1998). CD44 is implicated in the pathology of rheumatoid arthritis (RA) and is known to induce interleukin-6 (IL-6) and vascular cell adhesion molecule-1 (VCAM-1) in synovial cells as well as induce apoptosis in these cells (Fujii, Tanaka et al. 1999; Fujii, Tanaka et al. 1999; Fujii, Fujii et al. 2001). Other inflammatory diseases suggested to involve CD44 include inflammatory colitis (CD44v7), atherosclerosis and asthma (Jain, He et al. 1996). CD44 has been noted to be involved in the interaction between pathogens and the host cell and there is evidence that bacteria such as *Streptococcus pyogenes* attach to cells via

interactions between their hyaluronan-rich cell wall and CD44 on the host cell surface (Cywes, Stamenkovic et al. 2000). There is evidence to suggest that CD44 can initiate signalling events that can alter host cell immunity and make them more susceptible to bacterial infections. Jung et al. demonstrated that Met activation by *Listeria monocytogenes* is dependent on CD44v6 which promotes bacterial invasion into target cells (Jung, Matzke et al. 2009). Cwyes et al. showed that Group A *Streptococci* colonisation of the oropharynx in wild-type mice which could be blocked by administration of CD44 monoclonal antibody, and no colonisation was seen in transgenic mice deficient in CD44 (Cywes, Stamenkovic et al. 2000).

1.6.5 Role of CD44 in Cancer Biology

The first clear link between CD44 and cancer emerged when Gunthert et al. found that a CD44 variant protein conferred metastatic ability to a non-metastatic rat pancreatic carcinoma cell line (Gunthert, Hofmann et al. 1991). In an attempt to identify and clone genes responsible for metastasis, they generated monoclonal antibodies recognising antigenic determinants on a cell line from a metastasising variant of a spontaneous rat pancreatic adenocarcinoma (BSp73ASML). The antibodies were then used to screen a bacterial complementary DNA (cDNA) expression library and one of the cDNA clones was found to code for a variant form of CD44 (CD44v4-7), which when transfected into the non-metastasising BSp73AS cell line conferred metastasising potential to this cell line. Similar observations were made in the highly metastatic 13762 NF mammary carcinoma cell line as well by this group (Gunthert, Hofmann et al. 1991).

The ability of CD44 to promote a metastatic phenotype has been demonstrated in human melanoma through experimental inhibition of CD44 function using in vivo administration

of GKW.A2, a monoclonal antibody specific for human CD44. Use of GKW.A2 has been shown to inhibit the growth and metastatic potential of tumour cells in the SMMU-2 human melanoma cell line (Guo, Ma et al. 1994). Hernandez et al. also noted that CD44 silencing in a human melanoma cell line (SK-mel-131) caused a reduction in cell proliferation and migration in vitro as well as decrease in cell adhesion to vitronectin, fibronectin and hyaluronan (Hernandez, Miquel-Serra et al. 2012). Kim et al. observed that HA-induced CD44 and EGFR interaction augments the motile behaviour of a human melanoma cell line (B16F10) via activation of protein kinase C signalling (Kim, Lee et al. 2008).

CD44 is overexpressed in several malignant tumours where it may contribute to further genetic aberrations. Overexpression of CD44 gene in colorectal neoplasms seems to be an early event often seen prior to gene alterations (for example, K-ras and p53) indicating that CD44 is likely to be involved in cell activation and proliferation following APC (Adenomatous polyposis coli) gene mutation or alteration of DNA methylation (Kim, Yang et al. 1994). A number of CD44 variants have been found to be overexpressed in colonic adenocarcinomas (Higashikawa, Yokozaki et al. 1996). Yamaguchi et al. noted that patients whose tumours expressed CD44v8-10 had a significantly greater relative risk of death and a higher recurrence rate compared with those whose tumours were CD44v8-10 negative and multivariate analysis confirmed CD44v8-10 expression to be an independent prognostic marker (Yamaguchi, Urano et al. 1996). Huang et al. demonstrated that CD44v6 plays a role in promoting liver metastasis in colorectal cancer through an osteopontin mediated mechanism that leads to increased adhesion with endothelial cells and weakened intercellular communication (Huang, Pan et al. 2012). In an in vitro model of CD44 overexpressing colon cancer cells (SW480), Cho et al. have

demonstrated a clear epithelial to mesenchymal transition (EMT) and CD44-mediated inhibition of membrane associated E-cadherin and β-catenin (cell-cell adhesion proteins) complex resulting in increased cell invasion and migration (Cho, Park et al. 2012). Du et al. showed that a single CD44-positive cell from a primary colorectal cancer specimen could form a sphere in vitro with stem cell properties which subsequently could generate a xenograft tumour resembling the original tumour. They also demonstrated that knockdown of CD44 prevented clonal formation and inhibited tumourigenicity in their xenograft model (Du, Wang et al. 2008). In primary gastric malignancies, CD44v9 expression correlates with tumour recurrence and increased mortality (Mayer, Jauch et al. 1993) whilst expression of CD44 variant isoforms (v3, v6, and v10 containing isoforms) in head and neck squamous cell carcinomas are associated with advanced disease (Wang and Bourguignon 2011). Altered CD44 expression functions as a marker of poor prognosis in most haematological malignancies (Hertweck, Erdfelder et al. 2011). For example, in acute lymphoblastic leukaemia, total CD44 and CD44v6 function as prognostic markers whereas in Hodgkin's lymphoma, CD44v10 displays strong prognostic relevance. CD44 is also implicated in other malignancies such as pancreatic adenocarcinoma. Zoller et al. have shown that in a rat pancreatic adenocarcinoma cell line, CD44v6 promotes motility and apoptosis resistance through activation of signalling pathways involving FAK (Focal adhesion kinase), PI3K-Akt, and MAPK activation (Jung, Gross et al. 2011).

Interestingly, and in contrast to these studies, there are reports that CD44 expression correlates with improved prognosis in some malignancies. For example, overexpression of CD44 standard is associated with reduction in metastatic potential in prostatic cancer

whilst loss of CD44, both standard and variant isoforms, appears to be a marker of poor prognosis (Gao, Lou et al. 1997; De Marzo, Bradshaw et al. 1998).

1.6.6 CD44 and Breast Cancer

As mentioned previously, the initial link between CD44 and breast carcinoma was noted by Gunthert and colleagues in the 13762NF rat mammary carcinoma cell line where expression of CD44 variants conferred metastatic potential (Gunthert, Hofmann et al. 1991). Studies using experimental breast cancer models suggest that CD44 (standard and variant isoforms) may act, both directly and indirectly, as a regulator of their aggressiveness. Such activities appear to arise through the ability of CD44 to promote degradation of the extracellular matrix, cell migration, angiogenesis and metastasis (Trochon, Mabilat et al. 1996). CD44v3-mediated activation of Rho (protein involved in termination of transcription) and PI3K-Akt pathways appear to contribute to breast cancer cell growth and invasion whilst Rho kinase (ROK) mediated binding of ankyrin to the CD44v3, CD44v8 and CD44v10 isoforms contributes to increased migration. Moreover, in the MDA-MB-231 breast cell line, CD44 interaction with ROK plays a pivotal role in promoting Akt signalling and cytokine production during breast cancer progression (Bourguignon 2001; Bourguignon, Singleton et al. 2003).

Additional evidence implicating CD44 in breast cancer arises from studies on human breast cancer tissue. CD44s, CD44v3 and CD44v6 are all known to be upregulated in in-situ and invasive ductal carcinoma of the breast and this happens prior to HA upregulation during the process of cancer progression (Auvinen, Tammi et al. 2005). There is no definitive evidence that CD44s is linked to an adverse outcome in breast cancer; rather, CD44s expression has been reported as a favourable prognostic factor in

patients with node-negative breast cancer (Diaz, Zhou et al. 2005). Moreover, CD44s is expressed at a higher level in tubular carcinoma of the breast, which rarely metastasises, as compared to micropapillary carcinoma which is more aggressive with significantly higher metastatic potential (Gong, Sun et al. 2005). In contrast, the presence of CD44 variants, in particular CD44v3 and CD44v6, correlates with adverse features in breast carcinomas. CD44v3 expression has been linked to lymph node metastases, whilst, CD44v6 expression has been associated with increased tumour grade and has been shown to be an independent prognostic factor in breast carcinomas (Kaufmann, Heider et al. 1995; Sinn, Heider et al. 1995; Rys, Kruczak et al. 2003). However, it should be noted that the role of CD44 in breast cancer is not yet fully elucidated and there are still conflicting data suggesting that it may represent a positive prognostic element. For example, a study by Jansen et al. failed to find a relationship between CD44v6 and breast cancer prognosis in a cohort of 338 patients, whilst, loss of CD44s expression was suggested to promote lung metastasis in a mouse model of mammary carcinoma (Jansen, Joosten-Achjanie et al. 1998; Lopez, Camenisch et al. 2005).

Of particular interest has been the discovery that CD44 is a reliable marker of breast cancer stem cells (CSCs). CSCs are a subgroup of tumour cells that grow on serial transplantation in xenogenic models, have self renewal ability and tumours derived from CSCs can replicate the heterogeneity seen in parental tumours thereby establishing the differentiation ability of these cells. They are also highly resistant to apoptosis. Several malignancies of haematopoietic and epithelial origin are known to express CD44 in their CSCs. The presence of CSCs in breast cancer is now considered highly likely and there have been several studies indicating their presence (Pece, Tosoni et al. 2010; Ali, Dawson et al. 2011). CD44 and CD24 were first used by Al-Hajj et al. to prospectively isolate

CSCs from patients with breast cancer using specific cell surface markers and they showed that these CD44-positive, CD24 low/negative cells ($CD44^{+}$/ $CD24^{low/-}$) were able to form tumours in NOD/SCID mice and the subsequent xenografts using these cells exhibited the phenotypic diversity representative of the original tumours (Al-Hajj, Wicha et al. 2003). Since then CD44 has been used as a reliable marker of breast CSCs in various studies in conjunction with CD24 ($CD44^{+}$/ $CD24^{low/-}$).

CD44 has also been implicated in chemotherapy resistance in breast cancer. Cain et al. used surface proteomic signatures in wtMCF-7 and the multidrug-resistant NCI/ADR-RES cell lines to show that CD44 was an independent surface biomarker of drug resistance (Cain, Hauptschein et al. 2011). CD44 activation has been shown to activate MDR1/P-glycoprotein (multidrug resistance gene) and the anti-apoptotic gene bcl-XL via beta catenin signalling in an in vitro model of breast cancer resulting in chemoresistance (Bourguignon, Xia et al. 2009). There is evidence that CD44-ErbB interplay may contribute to chemoresistance. For example, in the multidrug-resistant MCF-7/Adr human breast carcinoma cell line, Misra et al. showed that CD44-HA interaction contributes to drug resistance by formation of a complex with HER2 thereby activating PI3K which then activates Akt and anti-apoptotic events in these cells (Misra, Ghatak et al. 2005). Current evidence points to a role of CD44 in breast cancer stem cells in promoting resistance. For example, Phuc et al. showed that CD44 downregulation using siRNA increases sensitivity of breast CSCs to the chemotherapeutic agent doxorubicin (Van Phuc, Nhan et al. 2011). CSCs are known to have multidrug resistance and one of the main mechanisms appears to be the high levels of ATP-binding cassette (ABC) transporter proteins, particularly ABCG2 which confers a high ability to eliminate drugs through efflux pumping mechanisms (Hirschmann-Jax, Foster et al. 2004). This and other

mechanisms allow CSCs to have intrinsic resistance to not just chemotherapy but radiation therapy as well. For example, Philips et al. demonstrated that $CD44^{+}/CD24^{-}$ enriched cells isolated from wtMCF-7 and MDA-MB-231 cell lines exhibited more resistance to radiation treatment than corresponding non $CD44^{+}/CD24^{-}$ cells (Phillips, McBride et al. 2006).

It has been recently hypothesised that breast CSCs which express high levels of CD44 may play a role, atleast partly, in endocrine resistance in breast cancer (O'Brien, Howell et al. 2009). Evidence supporting this hypothesis includes the observations that breast CSCs are ERα low or negative and ER expression is the most consistent marker for indicating response to endocrine therapy and, therefore, breast CSCs are unlikely to be endocrine sensitive. Many of the mechanisms that are implicated in the aggressive behaviour of the breast CSCs are also pathways that are known to interact with CD44. For example, Farnie et al. showed that the EGFR pathway was activated in DCIS and treatment with gefitinib, an EGFR inhibitor, resulted in a clear reduction in mammosphere formation efficiency (Farnie, Clarke et al. 2007). Duru et al. demonstrated that a HER2-mediated prosurvival signalling network accounts for the aggressive phenotype noted in breast CSCs (Duru, Fan et al. 2012).

1.7 EXPERIMENTAL HYPOTHESES

Resistance to tamoxifen therapy in breast cancer is associated with poor prognosis clinically and development of an aggressive phenotype in vitro. Preliminary data from our group using an in vitro model of acquired tamoxifen resistance ('Tam-R' cells), have shown that these cells overexpress CD44 gene transcripts. These cells also utilise ErbB receptors known to interact with CD44. Based on these observations and the current evidence indicating a role of ErbB family receptors in tamoxifen resistance, we formulated the following hypotheses to be investigated in this MD project:

1. CD44 is overexpressed at both gene and protein level in the Tam-R model of in vitro acquired tamoxifen-resistant breast cancer
2. CD44 overexpression is responsible for promoting the aggressive phenotypic features noted in the Tam-R cells
3. CD44 overexpression sensitises the Tam-R cells to the stromal factor HA which is normally found within the tumour microenvironment
4. CD44 interacts with ErbB receptors in Tam-R cells thereby influencing Tam-R cell phenotype

1.8 THESIS AIMS AND OBJECTIVES

The goal of hypothesis testing during this project would be met through the following aims and objectives using the Tam-R model of acquired resistance:

1) Validate CD44 gene overexpression by RT-PCR and characterise CD44 expression at the protein level using Western blotting and immunocytochemistry
2) Investigate the role of CD44 as a mediator of the adverse phenotype noted in these cells using functional assays (migration, invasion and growth)
3) Determine whether increased CD44 expression augments their sensitivity to exogenous HA using Western blotting, immunocytochemistry and functional assays (migration and growth)
4) Explore the interaction between CD44 and members of the ErbB family using Western blotting, immunocytochemistry, immunofluorescence and immunoprecipitation

Chapter Two

Materials and Methods

2.1 MATERIALS

Table 2.1 is a list of all the materials and equipment used during the course of this study along with their relevant suppliers.

Table 2.1 List of materials and equipment used and their suppliers

Material / Equipment	**Supplier**
Acetic acid	Fisher Scientific UK Ltd, Loughborough, UK
Acrylamide / bis-acrylamide (30% v/v)	Sigma-Aldrich, Poole, Dorset, UK
Adobe Photoshop 6	Adobe Systems Europe Ltd, UK
Agarose	Bioline Ltd, London, UK
Alexa Fluor® 488 fluorescent dye	Invitrogen, Paisley, UK
Ammonium persulphate (APS)	Sigma-Aldrich, Poole, Dorset, UK
Amphotericin B (Fungizone®)	Invitrogen, Paisley, UK
Antibiotics (Penicillin and Streptomycin)	Invitrogen, Paisley, UK
Anti-mouse horseradish peroxidise-linked IgG (Source: sheep)	Amersham, Little Chalfont, UK
Anti-rabbit horseradish peroxidase-linked IgG (Source: donkey)	Amersham, Little Chalfont, UK
Aprotinin	Sigma-Aldrich, Poole, Dorset, UK
Bio-Rad D_c Protein Assay: Reagents A, B and S	Bio-Rad Laboratories Ltd, UK

Bio-Rad Mini-Protean® 3 apparatus	Bio-Rad Laboratories Ltd, UK
Bovine Serum Albumin (BSA)	Sigma-Aldrich, Poole, Dorset, UK
Bromophenol Blue (BPB)	BDH Chemicals Ltd, Poole, Dorset, UK
Cecil CE2041 spectrophotometer	Cecil Instruments, Cambridge, UK
Cell Culture Medium: RPMI 1640 and Phenol red-free RPMI 1640	Invitrogen, Paisley, UK
Cell Scrapers	Greiner Bio-One Ltd, Gloucestershire, UK
Chemiluminescent Supersignal® West HRP Substrate (Dura and Femto)	Pierce and Warriner Ltd, Cheshire, UK
Corning Transwell® inserts	Fisher Scientific UK Ltd, Loughborough, UK
Cotton swabs	Johnson & Johnson, Maidenhead, UK
Coulter Counter counting cups and lids	Sarstedt AG and Co, Nümbrecht, Germany
Coulter™ Multisizer II	Beckman Coulter Ltd, High Wycombe, UK
Crystal violet	Sigma-Aldrich, Poole, Dorset, UK
Denley BA852 Autoclave	Thermoquest Ltd, Basingstoke, UK
DharmaFECT® 1 lipid transfection reagent	Thermo Scientific Dharmacon Ltd, UK
Disposable cuvettes	Fisher Scientific UK Ltd, Loughborough, UK
Di-thiothreitol (DTT)	Sigma-Aldrich, Poole, Dorset, UK
dNTP's (dGTP, dCTP, dATP, dTTP)	Amersham, Little Chalfont, UK
Ethidium bromide	Sigma-Aldrich, Poole, Dorset, UK

Ethylene diamine tetraacetic acid (EDTA)	Sigma-Aldrich, Poole, Dorset, UK
EZView™ Red agarose beads	Sigma-Aldrich, Poole, Dorset, UK
Fibronectin (1μg/ml)	Sigma-Aldrich, Poole, Dorset, UK
Filter Paper	Whatman, Maidstone, UK
Foetal Calf Serum (FCS)	Invitrogen, Paisley, UK
Fotodyne 3-3002 UV transilluminator	Fotodyne Inc, USA
General Laboratory Glass and Plastic utilities	Fisher Scientific UK Ltd, Loughborough, UK
Glass coverslips	BDH Chemicals Ltd, Poole, Dorset, UK
Glass slides	Fisher Scientific UK Ltd, Loughborough, UK
Glutamine	Invitrogen, Paisley, UK
Glycine	Sigma-Aldrich, Poole, Dorset, UK
GS690 Imaging densitometer	Bio-Rad Laboratories Ltd., UK
Hamamatsu C4742-96 digital camera	Hamamatsu Photonics Ltd, Hertfordshire, UK
Hydrochloric acid (HCl)	Fisher Scientific UK Ltd, Loughborough, UK
Hyperladder™ IV (100-1000bp)	Bioline Ltd, London, UK
IEC Micromax RF Microcentrifuge	Thermo Electron Corporation, Hampshire, UK
Isoton® II azide-free balanced electrolyte solution	Beckman Coulter Ltd, High Wycombe, UK
Jouan C312 centrifuge	Thermo Fischer Scientific Inc, MA, USA
Kodak MXB Autoradiography film (blue sensitive; 18cm x 24cm)	Genetic Research Instrumentation (GRI), Rayne, UK

Labonco Purifier PCR Enclosure	GRI, Rayne, UK
Laminar flow cabinet	Bioquell UK Ltd, Andover, UK
Leica DM-IRE2 fluorescence microscope	Leica Microsystems Imaging Solutions Ltd, Cambridge, UK
Leupeptin	Sigma-Aldrich, Poole, Dorset, UK
Lower Buffer for SDS-PAGE Gels (TRIS 1.5M, pH 8.8)	Bio-Rad laboratories Ltd, Herts, UK
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich, Poole, Dorset, UK
Marvel Skimmed Milk Powder	Premier International Foods, Lincs, UK
Matrigel™ Basement Membrane	BD Biosciences, Oxford, UK
Microcentrifuge Tubes	Elkay Laboratory Products, Basingstoke, UK
Molecular Analyst™ Version 1.5	Bio-Rad laboratories Ltd, Herts, UK
Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase	Invitrogen, Paisley, UK
N,N,N',N'-tetramethylethlyene-diamine (TEMED)	Sigma-Aldrich, Poole, Dorset, UK
Nikon Eclipse TE200 phase-contrast microscope	Nikon UK Ltd, Kingston-upon-Thames, UK
Nitrocellulose transfer Membrane (Protran® 0.45µm pore size)	Schleicher and Schuell, Dassell, Germany
Olympus BH-2 microscope	Olympus Corporation, UK
ON-TARGET*plus*® siRNA Control Reagent	Thermo Scientific Dharmacon Ltd
PCR oligonucleotide primers	Invitrogen, Paisley, UK

pH calibration buffer tablets (pH4, 7 and 10)	Fisher Scientific UK Ltd, Loughborough, UK
Phenylarsine oxide	Sigma-Aldrich, Poole, Dorset, UK
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich, Poole, Dorset, UK
Pipette Tips	Greiner Bio-One Ltd, Gloucestershire, UK
Polaroid GelCam camera	GRI, Rayne, UK
Polyoxyethylene-sorbitan monolaurate (Tween-20)	Sigma-Aldrich, Poole, Dorset, UK
Ponceau S solution (0.1% w/v in 5% acetic acid)	Sigma-Aldrich, Poole, Dorset, UK
Potassium chloride (KCl)	Sigma-Aldrich, Poole, Dorset, UK
PowerMAC G5 computer	Apple Computer Inc, California, USA
Powerpac Basic power pack	Bio-Rad Laboratories Ltd, Herts, UK
Precision Plus Protein™ All Blue Standards (10-250kDa)	Bio-Rad laboratories Ltd, Herts, UK
PTC-100 thermal cycler	Bio-Rad laboratories Ltd, Herts, UK
Random Hexamers (RH)	Amersham, Little Chalfont, UK
RNase-free H_2O	Sigma-Aldrich, Poole, Dorset, UK
RNasin® ribonuclease inhibitor	Sigma-Aldrich, Poole, Dorset, UK
Sanyo MCO-17AIC incubator	Sanyo E&E Europe BV, Loughborough, UK
siRNA buffer (5x)	Thermo Scientific Dharmacon Ltd, UK
SMARTpool siRNA against CD44 (ON-Target *plus*®)	Thermo Scientific Dharmacon Ltd, UK

Sodium azide	Sigma-Aldrich, Poole, Dorset, UK
Sodium chloride (NaCl)	Sigma-Aldrich, Poole, Dorset, UK
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich, Poole, Dorset, UK
Sodium fluoride (NaF)	Sigma-Aldrich, Poole, Dorset, UK
Sodium hydroxide (NaOH)	Fisher Scientific UK Ltd, Loughborough, UK
Sodium molybdate (Na_2MoO_4)	Sigma-Aldrich, Poole, Dorset, UK
Sodium orthovanadate ($NaVO_4$)	Sigma-Aldrich, Poole, Dorset, UK
Solvents (acetone, chloroform, ethanol, formaldehyde, isopropanol and methanol)	Fisher Scientific UK Ltd, Loughborough, UK
Sterile bijou vials (5ml)	Bibby Sterilin Ltd, Stone, UK
Sterile cell culture plasticware	Nunc Int, Roskilde, Denmark
Sterile disposable serological pipettes (5ml, 10ml, 25ml)	Sarstedt AG and Co, Nümbrecht, Germany
Sterile Falcon tubes	Sarstedt AG and Co, Nümbrecht, Germany
Sterile phosphate buffered saline (PBS)	Invitrogen, Paisley, UK
Sterile syringe filters (0.2µm)	Becton Dickinson (BD) UK Ltd, Oxford, UK
Sterile syringe needles	Sherwood-Davis & Geck, Gosport, Hampshire, UK
Sterile syringes (BD Plastipak™, 10ml)	Becton Dickinson (BD) UK Ltd, Oxford, UK
Sterile Universal Containers (30ml)	Greiner Bio-One Ltd, Gloucestershire, UK
Sub-cell® Electrophoresis System	Bio-Rad laboratories Ltd, Herts, UK

Sucrose	Fisher Scientific UK Ltd, Loughborough, UK
Taq DNA polymerase (BioTaq™; 5U/µl)	Bioline Ltd, London, UK
Tri Reagent® RNA Isolation Reagent	Sigma-Aldrich, Poole, Dorset, UK
Tris HCl	Sigma-Aldrich, Poole, Dorset, UK
Triton X-100	Sigma-Aldrich, Poole, Dorset, UK
Trizma (Tris) Base	Sigma-Aldrich, Poole, Dorset, UK
Tryspin/EDTA 10x solution	Invitrogen, Paisley, UK
Upper Buffer for SDS-PAGE Gels (Tris 0.5M, pH6.8)	Bio-Rad laboratories Ltd, Herts, UK
Vectashield® hard-set mounting medium containing DAPI nuclear stain	Vector laboratories Inc, Peterborough, UK
Western Blocking Reagent	Roche Diagnostics, Mannheim, Germany
X-ray film developer and fixer solutions (X-O-dev and X-O-fix)	X-O-graph Imaging system, Tetbury, UK

2.2 CELL CULTURE

The majority of work in this study utilised the ER-positive, hormone-sensitive wild-type MCF-7 cell line ('wtMCF-7') and its tamoxifen-resistant derivative cell line ('Tam-R').

2.2.1 Routine Cell Culture: wtMCF-7

2.2.1.1 Routine cell culture

Human mammary carcinoma wtMCF-7 cells were previously donated to the breast cancer group by AstraZeneca Pharmaceuticals (Macclesfield, Cheshire), being originally obtained from the American Type Culture Collection (ATCC® Number HTB-22™). wtMCF-7 cells were retrieved from liquid nitrogen storage and routinely cultured in RPMI 1640 medium containing phenol red as an indicator of pH and supplemented with 5% foetal calf serum (FCS), antibiotics (penicillin:100IU/ml, streptomycin:100µg/ml), anti-fungal agent (2.5µg/ml amphotericin B, Fungizone®) and 200nM glutamine. Cell cultures were maintained as monolayers in $75cm^2$ flasks (T75) at 37ºC in a humidified 5% CO_2 atmosphere using a Sanyo MCO-17AIC incubator (Sanyo E&E Europe BV, Loughborough, UK). The culture medium was changed at regular intervals (3-4 days). Culture flasks were monitored via phase-contrast microscopy (Nikon Eclipse TE200; Nikon UK Ltd, Kingston-upon-Thames, UK). Cells were routinely assessed during culture by light microscopy and passaged once they reached 90% confluency.

2.2.1.2 Cell passaging

Cell culture medium was aspirated and the adhered cells separated using 10ml mixture of trypsin (0.05%) and EDTA (0.02%) in PBS. Following 3-5 minutes in the incubator to allow cells to detach, trypsin was neutralised by the addition of 10mls of routine culture medium. The cell suspension was centrifuged at 1000rpm for 5 minutes (Jouan C312,

Thermo Fischer Scientific Inc, MA, USA), the supernatant discarded and the cell pellet was resuspended uniformly in 10mls of the appropriate medium. $1/10^{th}$ of this uniform cell suspension was subsequently added to 15ml fresh medium and seeded into a T75 flask and cultured under routine conditions described above. Cell lines were generally used for up to a maximum of 25 passages to avoid adverse phenotypic changes that are seen in higher passages. Once cells were discarded, having reached a high passage number, cryopreserved cells of a low passage number were revived for routine culture. All cell culture experiments were performed in laminar flow cabinets (Bioquell UK Ltd, Andover, UK) with full sterile precautions. Routine culture equipment was sterilised via autoclaving at 119ºC in a Denley BA852 autoclave (Thermoquest Ltd, Basingstoke, UK).

2.2.2 Routine Cell Culture: Tam-R

To explore the role of CD44 in acquired tamoxifen resistance in vitro, we used the Tam-R cell line. These are a tamoxifen-resistant derivative of wtMCF-7 cells used for studies into endocrine resistance generated by continuous exposure (6 months) of wtMCF-7 cells to 4-hydroxytamoxifen (4-OH-TAM, 10^{-7}M in ethanol) (Knowlden, Hutcheson et al. 2003). After an initial drop in the wtMCF-7 growth rates due to the suppressive effects of tamoxifen, a gradual increase in the growth rate of the remaining viable cells was observed indicating outgrowth of cells resistant to 4-OH-TAM. The new cell line was cultured in medium containing 4-OH-TAM for a further 4 months prior to original characterisation studies. Following retrieval from liquid nitrogen storage, Tam-R cells were routinely cultured in phenol-red free RPMI with 5% charcoal-stripped FCS, glutamine (200mM), antibiotics (penicillin:100IU/ml, streptomycin:100µg/ml) and anti-fungal agent (2.5µg/ml amphotericin B, Fungizone®). This medium is referred to as

'W+5%'. Tam-R cell cultures in W+5% were maintained, monitored and passaged using the technique described for wtMCF-7 cells in **Section 2.2.1.1 and 2.2.1.2**.

2.2.3 Preparation for Use of Cells in Experiments: wtMCF-7 and Tam-R

Due to the potential oestrogenic stimulus arising from the phenol red indicator and steroid hormones within the serum, the culture conditions for wtMCF-7 cells were altered 24 hours prior to any experiments. After washing cells 3 times with Dulbecco's phosphate-buffered saline (PBS), the medium was changed to the experimental medium (EM) containing phenol-red free RPMI with 5% charcoal-stripped FCS and glutamine (200mM) but lacking antibiotics or antifungals. Tam-R cells were switched over to EM containing 4-OH-TAM 24 hours prior to use in experiments.

To ensure experimental accuracy, cells were seeded onto experimental dishes after determination of cell concentration and number using a Coulter Counter. Once harvested using trypsin/EDTA as described above, the cells were passed through a sterile 25G syringe needle to obtain a single cell suspension. A 100µl aliquot of this suspension was mixed carefully with 10mls Isoton® II and cell number determined using a Coulter™ Multisizer II (Beckman Coulter Ltd, High Wycombe, UK). Cell number counts were performed in triplicate and then averaged and multiplied by a factor of 200 to obtain a count of the total number of cells in the flask. Cells were then seeded at the required density as dictated by the experimental design in the appropriate medium.

2.2.4 Treatments

The experiments in this study required use of different treatments in cultured cells these are listed below along with their final concentrations (**Table 2.2**). Full details of treatment concentration and duration are listed in the relevant sections of the results chapter.

Table 2.2 List of treatments used in this study

Treatment	Function	Concentration	Manufacturer
Gefitinib (Iressa®)	Tyrosine kinase inhibitor	1µM	AstraZeneca, UK
Heregulin (HRG-β1)	ErbB ligand	10ng/ml	Sigma Ltd, Dorset, UK
Hyaluronan (low molecular weight)	CD44 ligand	200µg/ml	R&D Systems, Oxford, UK
Hyaluronan (medium molecular weight)	CD44 ligand	200µg/ml	R&D Systems, Oxford, UK
Hyaluronan (high molecular weight)	CD44 ligand	200µg/ml	R&D Systems, Oxford, UK
PD098059	MAPK inhibitor	50µM	Alexis, Bingham, UK
Transforming Growth Factor Alpha (TGFα)	Growth factor	10ng/ml	Sigma Ltd, Dorset, UK
Trastuzumab (Herceptin®)	Monoclonal Antibody against HER2	100nM	Roche, UK

2.3 GENE EXPRESSION ANALYSIS

Gene expression in wtMCF-7 and Tam-R cells was analysed using the semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR) technique (Saiki, Scharf et al. 1985; Mullis, Faloona et al. 1986; Mullis and Faloona 1987). All experiments were conducted in a Labonco Purifier PCR Enclosure (GRI, Rayne, UK) which was rubbed down with 70% ethanol prior to use to minimise contamination.

2.3.1 Total RNA Isolation, Quantitation and Gel electrophoresis

Cells were seeded into 60mm dishes ($5x10^5$ cells per dish), cultured until log phase growth was reached and total RNA extracted using TRI Reagent ® RNA Isolation Reagent (1ml per $10cm^2$ at room temperature). Lysates were collected using a cell scraper and pipetted into 1.5ml microcentrifuge tubes. Chloroform (0.2ml per 1ml TRI Reagent®) was added to the lysates to achieve phase separation. Samples were then vortexed, incubated at room temperature (15 minutes) and centrifuged at 11,300 rpm (15 minutes at 4°C) using a microcentrifuge (IEC Micromax RF Microcentrifuge; Thermo Electron Corporation, Hampshire, UK). Isopropanol was then added to the aliquoted upper aqueous phase to precipitate RNA and the process repeated. RNA pellets were subsequently washed twice with ethanol (75% in H2O, 1ml per pellet), vortexed and centrifuged for 5 minutes (9000rpm at 4°C). Each pellet was then resuspended in 20µl RNAase-free sterile H_2O.

This was followed by determination of RNA concentration. 1µl RNA was dissolved in 499µl RNAase-free H_2O and the absorbance was measured at a wavelength of 260nm (A_{260}) using a spectrophotometer (Cecil CE2041; Cecil Instruments, Cambridge, UK). RNA concentration was calculated using the following formula: RNA concentration

(µg/ml) = A_{260} x 40 x Dilution Factor. RNA quantification was then performed by measuring the absorbance at a wavelength of 280nm (A_{280}). The A_{260}/ A_{280} ratio was calculated to determine protein contamination (acceptable range = 1.8 – 2.0).

Agarose gel electrophoresis was performed by resolving RNA samples on a 1% (w/v) agarose gel in Tris-Acetate-EDTA buffer (TAE, pH 8.3; **Appendix VI**) containing ethidium bromide (1µl of a 10g/ml solution per 100ml agarose gel). Electrophoresis of samples (500ng RNA dissolved in 5µl loading buffer; **Appendix VI**) was performed using a Sub-cell® Agarose Electrophoresis System linked to a power pack (Bio-Rad laboratories Ltd., Herts, UK) in a tank with TAE buffer at constant voltage (80V for 1 hour). A Fotodyne 3-3002 UV transilluminator was used to visualise gels under UV light and photographs taken with a Polaroid GelCam camera (GRI, Rayne, UK). Good-quality RNA was indicated by a 28S band appearing twice as strong as the 18S band.

2.3.2 Reverse Transcription

Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase enzyme was used to prepare complementary DNA (cDNA) using a standard protocol. 6µl of total RNA-RNAase-free H_2O solution (containing 1µg RNA) was mixed with 6µl of master mix (2µl of 100µM random hexamer oligonucleotides and 4µl dNTP mix comprising 0.625mM of dGTP, dCTP, dATP and dTTP). Samples were denatured at 65°C for 5 minutes and subsequently cooled at 4°C prior to addition of 8µl of RT master mix (4µl First Strand Buffer at 5x concentration, 2µl of 0.1M DTT, 1µl of RNasin™ RNase inhibitor and 1µl MMLV reverse transcriptase). Following incubation at 37°C for 50 minutes and inactivation, aliquotted samples were stored at -20°C until further use.

Amplification was performed using primers for CD44s (Forward: 5' GAC ACA TAT TGT TTC AAT GCT TCA GC 3'; Reverse: 5' GAT GCC AAG ATG ATC AGC CAT TCT GGA AT 3') and β-actin (5' GGA GCA ATG ATC TTG ATC TT 3'; Reverse: 5' CCT TCC TGG GCA TGG AGT CCT 3').

2.3.3 Polymerase Chain Reaction (PCR)

The master mix solution used for PCR contained 2µl of 100mM dNTP mix, 2.5µl of 10x NH_4 reaction buffer, 0.625µl of 20µM forward primer, 0.625µl of 20µM reverse primer, 0.2µl Taq polymerase enzyme, 0.75µl of 50mM $MgCl_2$ and 17.8µl sterile RNAase-free H_2O making up a volume of 24.5µl per reaction. This master mix solution was added to 0.5µl cDNA and denatured at 100°C in a PTC100-thermal cycler (Bio-Rad laboratories Ltd, Herts, UK) with a suitable negative control for each reaction. All PCR reactions included primers specific for β-actin as an internal control and were performed in a semi-quantitative manner using 27 cycles so that products were in the linear range of amplification (Denature 94°C, elongate 72°C and anneal 62°C).

Electrophoresis of PCR cDNA products (15µl PCR product and 5µl loading buffer – **Appendix VI**) was performed as described before along with a molecular weight marker (Hyperladder™ IV 100-1000bp; 5µl). Visualisation of cDNA products using UV transillumination was carried out followed by imaging and subsequent scanning with a GS690 Imaging densitometer connected to a computer running Molecular Analyst version 1.5 (both Bio-Rad Laboratories Ltd, Herts, UK).

2.4 ANALYSIS OF PROTEIN EXPRESSION

Identification of cellular proteins and semi-quantitative assessment of protein expression was performed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Towbin, Staehelin et al. 1979) and Western blotting (Laemmli 1970). Protein specific antibodies were used for analysis of protein expression in wtMCF-7 and Tam-R in basal conditions and following various treatments and modulation of experimental conditions.

2.4.1 Cell Lysis

Cells were seeded into 35mm, 60mm or 100mm petri dishes at an appropriate cell density and cultured until log-phase growth was reached. Cell confluency and viability was assessed using a Nikon Eclipse TE200 phase-contrast microscope (Nikon UK Ltd, Kingston-upon-Thames, UK). Appropriate treatments were then added according to experimental design and cells were continued to be cultured till experimental end point was reached. After this time cells were washed with ice cold PBS twice and lysed using lysis buffer containing protease and phosphatise inhibitors (**Appendix VI**). Cell material was collected using a cell scraper and the lysates transferred to a 1.5ml centrifuge tube and left on ice for 20 minutes. Lysates were then clarified by centrifugation (13,000rpm for 15 minutes at 4°C) and the supernatants stored at -20°C until required.

2.4.2 Protein Concentration Assay

Total soluble protein concentration in cell lysates was carried out using the Bio-Rad D_c Protein Assay Kit (Bio-Rad Laboratories Ltd., UK) using a modification of the original Lowry method (Lowry, Rosebrough et al. 1951). Initially, a BSA standard curve was generated by using BSA protein concentration standards ranging between 0 to 1.45 mg/ml

(**Appendix VI**) analysed at 750nm using a spectrophotometer (Cecil CE2041). Subsequently, lysates were diluted 1:5 in lysis buffer (final volume of 50µl in cuvette) in preparation for absorbance measurement. To each sample was added 250µl Reagent A (containing 20µl Reagent S per1ml Reagent A) and 2ml Reagent B and the solutions were incubated at room temperature for 5 minutes and absorbance read as previously described. Total soluble protein concentration in the samples was calculated using the BSA standard curve and the absorbance of the lysate samples.

2.4.3 SDS-PAGE Analysis

SDS-PAGE was used to separate proteins according to size from the original whole cell lysates using the method originally described by Laemmli as mentioned earlier. This included a stacking gel (4-5% w/v) and a resolving gel (8% w/v) as described in **Table 2.3 & Table 2.4**, and was performed using the Bio-Rad Mini-Protean® III apparatus powered by a Powerpac Basic powerpack (Bio-Rad Laboratories, UK). The Bio-Rad gel casting system was cleaned with ethanol and the resolving gel mixture was poured (leaving sufficient space for the stacking gel) into the gel cassette and overlaid with distilled water. The gel was then allowed to set at room temperature for at least 30 minutes before being overlaid with the stacking gel.

Table 2.3 Recipe for stacking gel

Composition	**4% gel (w/v) (10ml)**	**5% gel (w/v) (10ml)**	**Final Concentration**
Acrylamide / bis-acrylamide (30% solution)	1.3ml	1.67ml	4-5% w/v
dH_20	6.1ml	5.83ml	-
Tris (0.5M, pH6.6)	2.5ml	2.5ml	125mM
SDS (10% solution in dH_20)	100µl	100µl	0.1% (w/v)
APS (10% solution in dH_20)	50µl	50µl	0.05% (w/v)
TEMED	10µl	10µl	0.1% (v/v)

Table 2.4 Recipe for resolving gel

Composition	**8% gel (w/v) for 10ml**	**Final Concentration**
Acrylamide / bis-acrylamide (30% solution)	2.7ml	8%
H_20	4.6ml	-
Tris (1.5M, pH8.8)	2.5ml	375mM
SDS (10% solution in H_20)	100µl	0.1% (w/v)
APS (10% solution in H_20)	100µl	0.1% (w/v)
TEMED	20µl	0.2% (v/v)

The water overlying the gel was discarded once the gel was set and the stacking gel solution poured in. A 10 or 15-well comb (1.5mm width) was then inserted into the stacking gel solution and gel allowed to set at room temperature. The gels were positioned in the electrophoresis apparatus, appropriate amount of SDS-PAGE running buffer (**Appendix VI**) was added to the inner and outer reservoirs, and the combs were gently removed.

Cell lysates were prepared for loading onto gels by mixing volumes containing 20-60µg protein with 3x sample loading buffer containing DTT (**Appendix VI)** and denatured by heating for 5 minutes at 100°C in a dry bath. 10µl of a molecular weight marker (Precision Plus All Blue Standards 10-250kDa, 10µl) was loaded alongside the experimental samples into the wells of the stacking gel (20-60µg/lane) to aid assessment of the molecular weight of the detected proteins. Electrophoresis of the gels was performed at a constant voltage (160V) until the samples had migrated the required distance down the gel. After electrophoresis, the gels were prepared for protein transfer by immersing the cassette in transfer buffer (**Appendix VI**).

2.4.4 Western Blotting

Western Blotting is the method of transferring size-fractionated proteins onto a nitrocellulose membrane using electrophoresis. Proteins from the SDS-PAGE gels were transferred to a nitrocellulose membrane using the Bio-Rad mini-Protean system described earlier. A ‘gel sandwich’ was created using two pieces of filter paper, two Teflon sponge pads and one piece Protran® nitrocellulose membrane (0.45µm pore size, cut to match gel size) pre-soaked in transfer buffer (30 minutes) per SDS-PAGE gel for assembling the transfer cassette (**Figure 2.1**). Following completion of SDS-PAGE

electrophoresis, the resolving gel was carefully removed from the cassette and placed in a tray containing distilled water. A 5ml pipette tip was rolled over the 'gel-sandwich' to ensure removal of any air bubbles. The cassette was then placed into the transfer apparatus ensuring that the membrane was adjacent to the anode. This is to ensure that the negatively charged or reduced proteins migrate in the appropriate direction, namely gel to membrane, thus reducing the risk of loss of proteins during transfer. The transfer cassette was placed into the transfer tank with an ice-pack and then filled with cold transfer buffer (4°C). The transfer process involved electrophoresis at 100V constant voltage for 60-120 minutes (adjusted depending on the molecular size of the protein of interest).

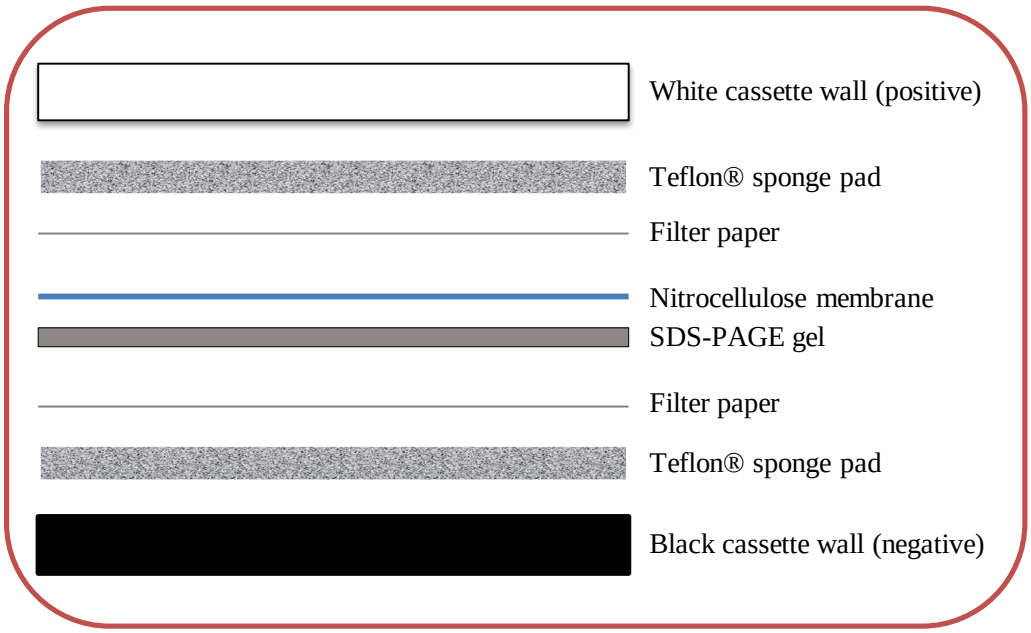


Figure 2.1 Schematic representation of the Western blotting transfer cassette ('gel sandwich')

A schematic representation of the Western blotting transfer cassette used during the transfer process as described in **Section 2.4.4** is depicted above. A Teflon® sponge was put onto the black plastic wall of the transfer cassette, followed by a piece of grade 3 filter paper. The SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) gel was then carefully placed on the filter paper taking care to keep the correct orientation and avoid formation of air bubbles. The gel was kept wet using transfer buffer and the nitrocellulose membrane was laid on top. The cassette was completed by putting a piece of filter paper and then another Teflon® sponge. This 'gel sandwich' was then rolled with a large pipette tip to remove any air bubbles and the cassette was closed. All components were carefully pre-soaked in transfer buffer prior to use.

2.4.5 Immunoprobing of Western Blots

Following completion of transfer, the nitrocellulose membrane was washed with distilled water (3 x 5 minutes) and then stained with Ponceau S solution (0.1% w/v in 5% acetic acid) to demonstrate adequate transfer of proteins. The membrane was destained with TBS-Tween (TBST: 0.5% v/v Tween-20 in 1x TBS, **Appendix VI**) and then incubated in a blocking solution (Milk, 5% w/v in TBS contain 0.1% v/v Tween-20) for at least 60 minutes at room temperature using a roller. Subsequently, membranes were washed with TBST (3 x 5 minutes) and incubated under gentle agitation with the primary antibody according to the incubation conditions considered optimal for the antibody in question. The list of primary antibodies used in the experiments are listed in **Table 2.5** and antibodies were prepared for incubation by diluting in TBST containing 5% v/v Western Blocking Reagent and 0.05% sodium azide (preservative).

After incubating with the primary antibody, membranes were washed with TBST (3 x 10 minutes) at room temperature and then incubated with the appropriate horseradish-peroxidase (HRP) conjugated anti-rabbit or anti-mouse secondary antibody at room temperature for 60 minutes. Excess secondary antibody was carefully washed using TBST (3 x 10 minutes) and the protein of interest visualised with luminol/peroxide-based Enhanced ChemiLuminescence/ECL reagents (Supersignal™ West Dura or Supersignal™ West Femto) applied according to manufacturer's instructions (500µl per membrane) in an auto-radiography cassette (Kricka, Schmerfeld-Pruss et al. 1991; Mattson and Bellehumeur 1996). Luminol is oxidised by horseradish-peroxidase (in the secondary antibody) in the presence of peroxide, a process which releases photons that can be detected on X-ray film. Exposures range from a few seconds to several hours depending on the signal strength. X-ray films were developed using an X-O-graph

compact X2 X-ray developer (X-O-Graph Imaging system, Tetbury, UK). The images obtained were scanned and analysed using a Bio-Rad Imaging GS-690 densitometer. Variation in loading was normalised using β-actin or GAPDH which are used as internal loading controls (Liao, Xu et al. 2000). Blots shown later in the results section are representative of a minimum of three separate experiments and densitometry values were used for semi-quantitative statistical analysis.

Table 2.5 List of antibodies used during immunoprobing of Western blots

Antibody	Species	Dilution	Incubation conditions	Supplier
Primary Antibodies				
CD44	Mouse	1:2000	O/N 4°C + 2 hours RT	Stratech Scientific, UK
p-MAPK (42/44)	Rabbit	1:1000	O/N 4°C	Cell Signaling Technology Inc, USA
T-MAPK	Rabbit	1:2000	O/N 4°C	Cell Signaling Technology Inc, USA
p-EGFR (1068)	Rabbit	1:2000	2 hours RT	Cell Signaling Technology Inc, USA
T-EGFR	Rabbit	1:2000	2 hours RT	Cell Signaling Technology Inc, USA
p-HER2 (1248)	Rabbit	1:2000	2 hours RT	Invitrogen, Paisley, UK
T-HER2	Rabbit	1:2000	2 hours RT	Cell Signaling Technology Inc, USA
Secondary Antibodies				
GAPDH	Mouse	1:15000	1 hour RT	Abcam, Cambridge, UK
β-actin	Mouse	1:20000	1 hour RT	Sigma-Aldrich, Poole, Dorset, UK

(Conditions: RT = Room Temperature, O/N = Overnight)

2.5 IMMUNOCYTOCHEMISTRY

Immunocytochemistry (ICC) uses antibodies that target specific peptides or protein antigens in the cell via specific epitopes. Unlike Western blotting, ICC provides information about the in-situ localisation of the protein in the cell..

2.5.1 Culture on Coverslips and Cell Fixation

Cells were passaged and counted as described previously before being seeded onto autoclaved, sterile 3-aminopropyltriethoxysilane-coated (TESPA) coverslips at a density of 1 x 10^4 cells/cm^2 within 35mm dishes. Following a 24-hour period to allow cells to adhere to the coverslip, the growth medium was changed to the experimental medium (EM) containing appropriate treatments. At completion of the experiment, dishes containing the coverslips were placed on ice and transferred to a laminar flow cabinet, where cell fixation was performed according to the immunocytochemical assay being performed (**Table 2.6**). The details of the individual cell fixation methods used are listed below:

1) *Formal Saline*: After aspirating medium from the 35mm dishes containing the coverslips, a 3.75% Formal Saline solution (containing 450ml tap water, 50ml 37% formaldehyde solution and 4.5g sodium chloride) was added to each dish (1ml per dish) for 10 minutes. This was followed by a 100% ethanol wash for 5 minutes and subsequently a phosphate-buffered saline (PBS) wash for an additional 5 minutes.

2) *Phenol Formal Saline:* After aspirating medium from the 35mm dishes containing the coverslips, a 2.5% (w/v) Phenol Formal Saline solution (prepared by dissolving phenol in a formal saline solution) was added to each dish (1ml per dish) for 5

minutes. This was followed by a 100% ethanol wash for 5 minutes and subsequently a PBS wash for an additional 5 minutes.

Once fixed, the coverslips were stored in the -20°C in sucrose storage medium (**Appendix VI**) prior to the immunocytochemical assay.

2.5.2 Immunocytochemical assays

On the day of the planned assay, stored coverslips (placed in 35mm dishes) were removed from the freezer and washed in PBS (2 x 5 minutes) followed by a wash with 0.02% Tween-PBS (5 minutes) to remove the storage medium and allow the primary antibody applied to be distributed evenly across the coverslip aided by the detergent effect of Tween. The appropriate primary antibody was then applied to the coverslips (humidified by suspending over a a water bath in plastic towers) and incubated (between 60 minutes to overnight) at room temperature or 23°C (if left overnight) depending on the specific assay being performed. Following incubation of the primary antibody, coverslips were washed again in PBS/Tween-PBS depending on the assay in question. One drop of the secondary antibody (Dako peroxidise-labelled EnVision^{TM}, Dako Ltd., Cambridgeshire, UK) from the original plastic container was applied to the coverslips and incubated at room temperature for 60 minutes. Coverslips were washed again with PBS/Tween-PBS as appropriate to the assay and immunolabelled proteins were visualised with a chromagen (diaminobenzidine tetrahydrochloride and hydrogen peroxide substrate) for 10 minutes followed by counterstaining with either methyl green or 5% hematoxylin. The counterstain was then washed off and coverslips air dried in preparation for the immunostaining analysis.

2.5.3 Assessment of immunocytochemistry using H-score

Final assessment of immunostaining was carried out using a microscope (Olympus BH-2) fitted with a dual viewing attachment to allow assessment by two independent observers simultaneously. Nuclear, membrane and cytoplasmic staining was assessed for CD44, MAPK and Ki-67 as appropriate. The intensity of immunostaining and percentage of cells exhibiting positive staining was estimated using a minimum evaluation of 2000 cells per coverslip. The data collected from these assessments is used to calculate a score that reflects the degree of expression of the protein of interest called the H-score (Gee, Robertson et al. 1994). Depending on the intensity of staining, the cells are classified into four groups: no staining (score = 0), low (score = 1), moderate (score = 2) and high (score = 3) intensity of staining respectively and a corresponding percentage value is allocated to each of the four groups (no staining = a, low = b, moderate = c, high =d; where, a + b + c + d = 100%). The H-score is the mathematical score derived from the following equation: H-score = (a * 0) + (b * 1) + (c * 2) + (d * 3) [Range = 0 - 300]. The H-score is used for statistical analysis of the level of protein expression between experimental triplicates.

Specific steps were followed while creating scale bars for the immunocytochemistry images in an attempt to maintain uniformity and accuracy. A standardised scale specifically designed for use with microscopes was used for calibration of images. The scale was photographed at various magnifications and these images were used to superimpose a scale bar on images obtained at the corresponding magnifications using Powerpoint software. The scale bar and the original image were merged together (using the ‘group’ function on the Powerpoint software) before any further modifications to the images was performed to preserve the fidelity of the scale bar.

Table 2.6: Immunocytochemical assays and their corresponding fixation methods

Immunocytochemical assay	**Fixation method**
Primary Antibodies	
CD44	Phenol Formal Saline
p-MAPK (44/42)	Formal Saline
T-MAPK	Formal Saline
Ki-67	Formal saline
Secondary Antibodies	
Dako EnVision™ (Goat anti-mouse) or Dako EnVision™ (Goat anti-Rabbit)	Not diluted

2.6 SIRNA-MEDIATED CD44 KNOCKDOWN

To further explore the role of CD44 in Tam-R cells an siRNA based approach was taken (Hannon 2002). RNA interference (RNAi) is a term to describe gene silencing by double-stranded RNA (dsRNA) which after entering the cell is cleaved by Dicer (an RNase III–like enzyme) into double stranded small interfering RNAs (siRNAs) usually between 21-23 nucleotides in length (Bernstein, Caudy et al. 2001). The siRNAs then form part of a protein complex called the RNAi-induced silencing complex (RISC), which guides the siRNAs to the target RNA sequence (Nykanen, Haley et al. 2001). The siRNA duplex then unwinds, and the antisense strand, while still bound to RISC, directs degradation of the complementary mRNA sequence by a combination of endo- and exo-nucleases (Martinez, Patkaniowska et al. 2002). This phenomenon can be replicated, under in vitro conditions, in mammalian cells using synthetic, short double-stranded RNAs that mimic the siRNAs produced by the enzyme Dicer (Elbashir, Harborth et al. 2001). Delivery of siRNA into cells can be done via microinjection, electroporation or using a lipid-based transfection reagent. Careful early optimisation studies should be conducted in any model to ensure adequate transfection and knockdown as well as reduce non-specific effects from the various reagents used in the process. Maximum down regulation is observed in 24-48 hours but this effect can last up to 10 days.

Non-targeting (NT) and CD44-specific siRNA (CD44-siRNA), both purchased as a 5nmol lyophilised powder, were reconstituted in 250µl of 1x siRNA buffer giving a final stock concentration of 20µM which was then aliquoted in 10µl volumes and stored at -20°C. The reagents used for siRNA inhibition in this study are listed in **Table 2.7**.

Table 2.7 List of reagents used for siRNA experiments

Reagent	Function	Company
SMARTpool siRNA against CD44 (ON-Target *plus*®)	Knockdown of CD44 mRNA transcripts. Suppresses CD44 protein expression	Thermo Scientific Dharmacon Ltd, UK
ON-TARGET *plus*® Control Reagent	Negative Control	Thermo Scientific Dharmacon Ltd, UK
DharmaFECT® 1	Lipid Transfection Reagent / Vehicle	Thermo Scientific Dharmacon Ltd, UK
siRNA buffer (5x)	siRNA buffer used at 1x (in sterile RNAase free water) to resuspend siRNA lyophilate.	Thermo Scientific Dharmacon Ltd, UK

To suppress CD44 expression in TamR cells, a previously optimised in-house protocol was used for siRNA transfection. Briefly, Tam-R cells were seeded into 35mm dishes in standard medium at a cell density of 3 x 10^5 cells per well and cultured until a confluency of around 50% was reached at which point the cells were considered ready for transfection. For all siRNA experiments, a set of controls were used to ensure accurate interpretation and ensure that non-specific or off-target effects of siRNA were excluded. This included an untreated control (C), a lipid transfection control (L) and a non-targeting siRNA (NT) control along with the CD44-specific siRNA (CD44-siRNA) treated sample making a set of 4 x 35mm dishes for each experimental arm.

An appropriate amount of 5x siRNA buffer was diluted to 1x siRNA buffer using RNAase-free H_2O in a sterile eppendorf. A pre-calculated amount of 2µM siRNA solution was prepared using 1x siRNA buffer and 20µM siRNA stock in a sterile eppendorf. In Tube A, 100µl of 2µM siRNA solution was added along with 100µl of

serum-free RPMI (SF-RPMI: wRPMI + 2% glutamine but no serum) to make a total volume of 200µl and left at room temperature for 5 minutes. In Tube B, 3.2µl of the DharmaFECT® 1 lipid transfection reagent was added along with 196.8µl of SF-RPMI making a total volume of 200µl and left for 5 minutes at room temperature. For the 'NT' dish, the NT-siRNA was used instead of the CD44-siRNA at exactly similar volumes and concentration. In the 'L' dish, no siRNA was used and the volume made up with SF-RPMI. In the untreated control dish 'C', no lipid transfection reagent or siRNA was used. After 5 minutes, contents of tubes A & B were mixed gently (total volume: 400µl) and set aside for 20 minutes at room temperature to allow for lipid-micelle formation. This is then diluted 1:5 with 1600µl of EM (wRPMI + SFCS + 2% glutamine) to give a final siRNA concentration of 100nM in a volume of 2mls. The volumes of reagents were adjusted accordingly if higher or lower volumes were required during experiments for different sizes of culture vessels according to a pre-calculated table (**Appendix VI**). The medium from the 35mm dishes was then aspirated and replaced with the siRNA containing solution and left to incubate for 72 hours to allow adequate siRNA-induced CD44 inhibition. After 72 hours, the cells were considered ready for use either for lysis or for setting up migration, invasion and other assays. Experimental treatments were only added after satisfactory transfection was achieved following 72 hours of siRNA treatment which was the optimum duration noted in the previous in-house optimisation studies which was subsequently validated for this project.

2.7 CELL MIGRATION ASSAYS

Two well-established types of migration assays were used to assess cell migration during the course of this study: Wound healing assay and Boyden Chamber assay.

2.7.1 Wound Healing Assay

Wound healing assay involves creation of a defined, cell-free area in a cell monolayer across which cells migrate and this process can be monitored in real time by microscopy (Rodriguez LG 2005). It has the advantage of being a relatively simple assay which provides real-time imaging of the migration process in two dimensions

Cells were seeded onto 35mm plates and cultured until they reached at least 90% confluency. Three parallel scratches were made in the monolayer using a fine sterile pipette tip (0.5-10µl white tip roducing a standard basal wound of reproducible size. To avoid re-seeding of detached cells, plates were rinsed with culture medium (x3) to remove detached cells. A permanent mark was made in the base of the plates using a green 21G needle to provide a reference point for wound width measurements and basal images were taken (**Figure 2.2**). Cells were cultured for 36 hours at which point the assay was stopped. The migration of cells into the scratched area was imaged and photographed at 10x magnification at 0 and 36 hours using using an Olympus BH-2 phase contrast microscope attached to an Olympus DP12 digital camera (Olympus Corp, UK). The linear reference mark at the base of the dish ensured that the same areas were photographed at 0 and 36 hours for accurate comparison. The micrographs were analysed with Adobe Photoshop 6 and quantification was performed by recording measurement of the distance (in arbitrary units) between the wound edges at 10x magnification. All experiments were performed in triplicate and data expressed as mean wound closure ± SD.

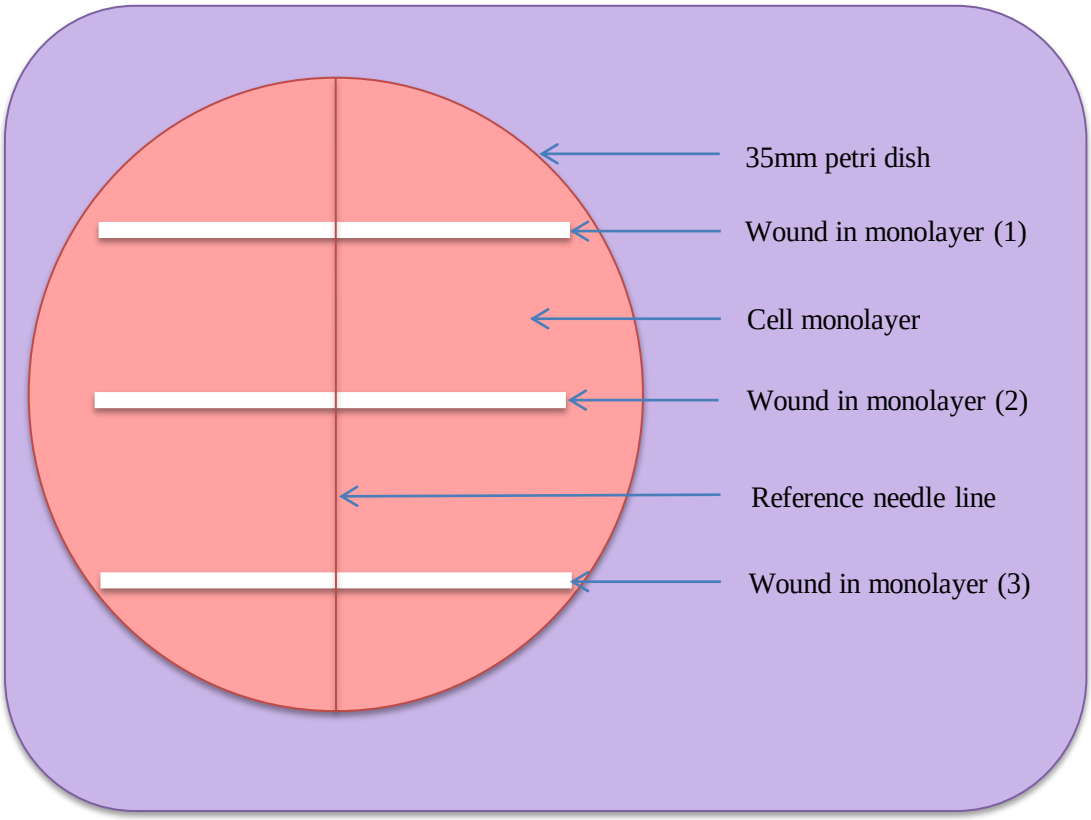


Figure 2.2 Schematic representation of a wound healing assay
Wound healing assays were used in this study to assess migratory behaviour of cells as described in **Section 2.7.1**. Cells were seeded onto 35mm plates and cultured until they reached at least 90% confluency. Three parallel scratches were made in the monolayer using a fine sterile pipette tip (0.5–10µl white) producing a standard basal wound of reproducible size. To avoid re-seeding of detached cells, plates were rinsed with culture medium (x3) to remove detached cells prior to addition of treatments. A permanent mark was made in the base of the plates using a green 21G needle to provide a reference point for wound width measurements at 0 and 36 hours.

2.7.2 Boyden Chamber Assay

The Boyden Chamber assay was used to determine the role of CD44 in basal migratory behaviour of Tam-R cells in this study. The assay was performed in modified Boyden chambers (6.5mm diameter) placed in a 24-well plate. The chemo-attractant Fibronectin (10µg/ml in PBS; Sigma, Poole, UK) was used to coat the porous polycarbonate membranes (0.8µm) in the Costar Transwell© inserts which were placed in 24-well plates containing 250µl of fibronectin solution such that the lower end of the insert membrane was submerged in the solution and incubated at 37°C for 2 hours. Inserts were then removed from the wells and placed inverted in a tissue culture hood to air dry before further use. The wells were then filled with 650µl medium with or without treatments and inserts replaced into the wells (**Figure 2.3**). The 24-well plate containing the inserts was then replaced in the incubator to equilibrate.

Cells were harvested and 200µl cell suspension (50,000 cells per membrane), with or without treatments, was pipetted into the insert and cultured for 48 hours at 37°C. The supernatant was then discarded and excess non-migratory cells removed from the insert gently using a cotton bud. The insert was then placed into a solution of formaldehyde (3.7% in PBS, 10 minutes) to fix the cells and subsequently washed with PBS (1 x 2 minutes) and stained with crystal violet (0.5% in dH_2O, 20 minutes). Membranes were washed by immersion in PBS solution (x3) to remove excess stain and left to air dry. Analysis and imaging of the membranes was carried out using an Olympus BH-2 phase contrast microscope attached to an Olympus DP-12 digital camera. Quantification was performed by viewing 5 separate fields per membrane (20x magnification) and counting the number of cells in each field. Data were then plotted at mean cells per field ± SD for a minimum of 3 separate experiments, each performed in triplicate.

2.8 MATRIGEL™ INVASION ASSAY

Several in vitro assays have been developed to understand and elucidate the mechanisms by which malignant cells acquire an invasive phenotype under in vivo conditions (Shaw 2005). Invasion assays were performed in this study using a modified version of the Boyden chamber assay (Albini, Iwamoto et al. 1987) to compare the invasive potential of wtMCF-7 and Tam-R cells basally and following CD44-siRNA knockdown.

The invasion assay was carried out using special invasion chambers (6.5mm diameter inserts Corning Transwell® inserts possessing 8µm porous membranes) placed in a 12-well plate. 50µL of the synthetic basement membrane Matrigel™ (stock solution of 11mg/ml diluted 1 in 3 with ice cold wRPMI) was used to coat the upper surface of the porous membrane of each of the inserts. The Matrigel™ was allowed to set for a minimum of 2 hours at 37°C and appropriate media, with or without treatments, were added to the well housing the insert and the plate then incubated at 37°C. The cell line of interest was harvested and resuspended in appropriate medium, with or without treatments, and 200µL of this cell suspension was added to the insert at a cell density of 50,000 per well (**Figure 2.3**). Cells were then incubated for 48 hours. For CD44-siRNA experiments, the transfection protocol described in **Section 2.6** was used prior to seeding siRNA-transfected cells into the Matrigel™ coated chambers .

Following this, the inserts were removed from the wells, the supernatant removed and Matrigel™ gently wiped off from the upper surface of the membrane using a cotton bud. The insert was then placed into a solution of formaldehyde (3.7% in PBS, 10 minutes) to fix the cells and subsequently washed with PBS (1 x 2 minutes) and allowed to air dry. The insert membrane was then carefully excised using a scalpel blade and prepared for

staining by mounting it onto a glass slide, applying a fixative containing the nuclear stain DAPI (Vectashield®) and overlaid with a glass cover slip. The membrane mounted on the slide is then allowed to set overnight at 4°C.

A Leica DM-IRE2 fluorescence microscope (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK) connected to a Hamamatsu C4742-96 digital camera (Hamamatsu Photonics UK Ltd, Hertfordshire, UK) and a PowerMAC G5 computer (Apple Computer Inc, California, USA) was used to assess the membranes and count the number of cells that had invaded the membrane. Quantification was performed by viewing 5 separate fields per membrane (20x magnification) and counting the number of cells in each field. Data were then plotted at mean cells per field ± SD for a minimum of 3 separate experiments, each performed in triplicate.

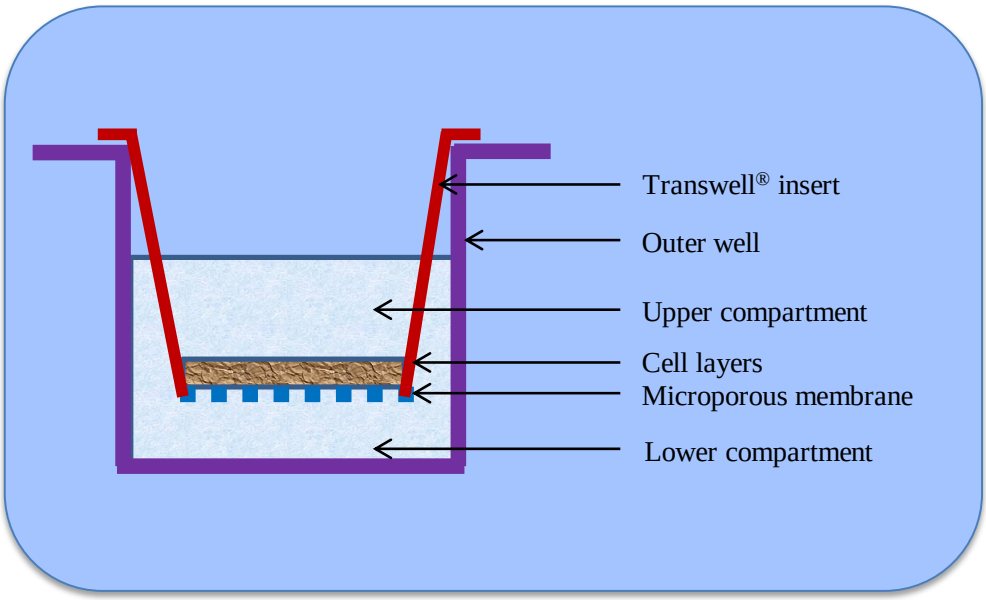


Figure 2.3 Schematic representation of a Transwell® insert used for Boyden chamber migration and Matrigel™ invasion assays
A schematic diagram of a Transwell® insert used during the migration and invasion assays in this study as described in **Section 2.7.2** and **Section 2.8** is depicted above. The microporous membrane was coated with either Fibronectin (migration assay) or Matrigel™ (invasion assay) prior to adding the cell suspension and medium with or without treatments. Adapted from catalogue schematic in www.corning.com.

2.9 CELL GROWTH ASSAY

Several assays have been designed to replicate cell growth in vitro in order to understand the mechanisms underlying this process. The growth assay used in this study is the cell counting assay standardised in our laboratory previously.

Cell growth measurements were performed by cell counting experiments using a Coulter Counter. Tam-R cells were harvested using trypsinisation, as described previously, and seeded into a 24-well plate at a density of 30,000 cells per well containing 1ml volume. Each treatment was set up in triplicate in three separate wells. Appropriate treatments were added after 24 hours, allowing the cells to adhere to the plate during this period. For siRNA experiments at least 72 hours was allowed for adequate siRNA transfection. Following treatment, the plate was incubated at 37°C in a humidified 5% CO_2 atmosphere for 5 days. Medium was then aspirated from a well and 1ml of Trypsin/EDTA was added to each well using a Finnpipette. Cells were then incubated for a further 3 to 5 minutes until cells were in suspension. Using a 5ml syringe and an orange 25G needle the suspension was passed through the syringe twice to obtain a single cell suspension and finally aspirated back into the syringe. 1ml of Isoton® II was added to the well and aspirated into the syringe. This was repeated three times making up a total syringe volume of 4mls. The contents of the syringe were then transferred into a counting pot containing a further 6mls of Isoton® II making a final volume of 10mls. The number of cells in each well was then determined using a Coulter counter. The count was repeated twice and an average count calculated for each well. This average value was multiplied by 20 to give the average cell number per well. The mean cell number ± SD of the counts from the 3 wells was subsequently calculated for each experiment performed in triplicate.

2.10 IMMUNOFLUORESCENCE STAINING

Immnuoflourescence is a technique based on the pioneering work by Coons and Kaplan which involves the use of fluorescently-labelled antibodies to tag proteins of interest in live or fixed cells. This allows for subsequent antigen detection using fluorescence microscopy (Coons 1941). The main benefit of this procedure is that it allows for dual staining, where two or more separate antigens can be labelled with different fluorophores. Image analysis of stained samples can be used to reveal whether these proteins are co-localised within the same cell or region of the cell.

Tam-R cells were cultured in 35mm dishes on glass cover slips with W+5% until cells reached log phase. The medium was then aspirated and the coverslips transferred to fresh 35mm dishes. Fixation was carried out with 3.7% formaldehyde in PBS for 15 minutes followed by permeabilisation with 0.2% Triton X-100 in PBS for 7 minutes. Coverslips were then treated with 10% normal goat serum for 30 minutes as a blocking agent followed by wash with PBS (x2) and treatment with antibodies diluted in PBS containing 1% BSA as detailed in **Table 2.8**. Coverslips were then washed after use of Alexa Flour® conjugates and mounted onto microscope slides using a hard set mounting medium (Vectashield®, Vector Laboratories Inc, Peterborough, UK) containing the nuclear stain DAPI (4',6-diamidino-2-phenylindole). Cells were viewed at 63x magnification using a Leica DM-IRE2 inverted fluorescent microscope and images taken for further analysis.

Table 2.8 List of antibodies used in immunofluorescence staining

Antibody	**Source**	**Dilution**	**Incubation conditions**	**Supplier**
Primary Antibodies				
CD44	Mouse	1:1500	2 hours RT	Stratech Scientific Ltd, Suffolk, UK
T-HER2	Rabbit	1:100	2 hours RT	Dako Ltd, Cambridgeshire, UK
T-HER3	Rabbit	1:100	2 hours RT	Santa Cruz Inc, CA, USA

2.11 IMMUNOPRECIPITATION

Immunoprecipitation involves precipitating a protein antigen out of solution using an antibody that specifically binds to that particular protein. NT and CR44-siRNA transfected Tam-R cells were treated with HA for 5 minutes and cell lysates were obtained as described in **Section 2.4** for SDS-PAGE. Lysates were centrifuged at 12,000rpm for 15 minutes, the supernatant pipetted into a fresh eppendorf and protein concentration of the supernatant determined using a Bio-Rad D_c Protein Assay Kit (Bio-Rad Laboratories Ltd, UK) using a modification of the original Lowry method (**Section 2.4.2**). The protein lysate was diluted such that 500µL of the solution contained 500µg protein and this was then divided into two parts in two different eppendorfs. One eppendorf was prepared for the specific immunoprecipitating anibody and the other for a matching isotype control antibody. Into each eppendorf, 5µL of the appropriate antiobody was added (from the antibody stock tube), lids sealed with Parafilm and tubes were left in a rotator overnight at 4°C to allow antigen-antibody complexes to form.

The following day, 20µL dye-conjugated agarose beads (EZView^{TM} Red, Sigma) were placed in a series of eppendorf tubes (corresponding to the number of immunoprecipitation samples) and washed by adding 750µL lysis buffer to each tube, vortexed and centrifuged at 8200rpm for 30 seconds at 4°C. Tubes were then returned to ice, supernatant discarded and wash repeated two more times. Cell lysates containing the antibody-antigen complexes were then removed from the rotator, briefly centrifuged at 8200rpm and contents transferred to the eppendorfs containing the equilibrated EZView^{TM} Red beads. Eppendorf lids were sealed with Parafilm and placed again in rotator for 2 hours at 4°C for the antigen-antibody complexes to attach to the agarose beads. Following this, tubes were returned to an ice filled container, briefly centrifuged at

8200rpm and the supernatant discarded. Pellets were washed with 750µL lysis buffer (containing protease inhibitors), vortexed gently and left on ice for 5 minutes. Samples were then centrifuged at 8200rpm for 5 minutes at 4°C and supernatant discarded. This wash was repeated three times and on the final wash, attempt was made to remove as much supernatant as possible without discarding any beads. The next step involvesd eluting the antigen-antibody complexes from the beads and this was carried out by adding 20µL lysis buffer and 20µL of 2x loading buffer (containing DTT) to each tube and boiling samples at 100°C for 5 minutes. Samples were then briefly vortexed and centrifuged again at 8200rpm for 5 minutes. At this point, samples were considered ready for SDS-PAGE analysis and Western blotting as described in **Section 2.4.4**. The Ponceau S stain step was avoided as only some IgG bands were usually noted and hence, was not considered essential. Blots were immunoprobed with the antibody of interest and detected using chemiluminescent reagents as described in **Section 2.4.5**.

Table 2.9 List of antibodies used in immunoprecipitation

Antibody	Source	Dilution	Incubation conditions	Supplier
CD44	Mouse	1:1500	2 hours RT	Stratech Scientific Ltd, Suffolk, UK
T-EGFR	Rabbit	1:100	2 hours RT	Cell Signaling Technology Inc, USA
T-HER2	Rabbit	1:100	2 hours RT	Dako Ltd, Cambridgeshire, UK
T-HER3	Rabbit	1:100	2 hours RT	Santa Cruz Inc, CA, USA

2.12 STATISTICAL ANALYSIS

Direct comparisons between wtMCF-7 and Tam-R cells or between control and treatment effects were assessed using a Student's *t* test with the Bonferroni adjustment factor. Student's *t* test was also used to compare differences between control and treatment groups where there were more than two comparison groups by testing each group separately. Spearman rank correlation test was used to assess whether HA effect on p-MAPK expression was dose-dependant. A *p*-value of ≤ 0.05 was considered to be statistically significant for all experiments in this study.

Chapter Three

Results

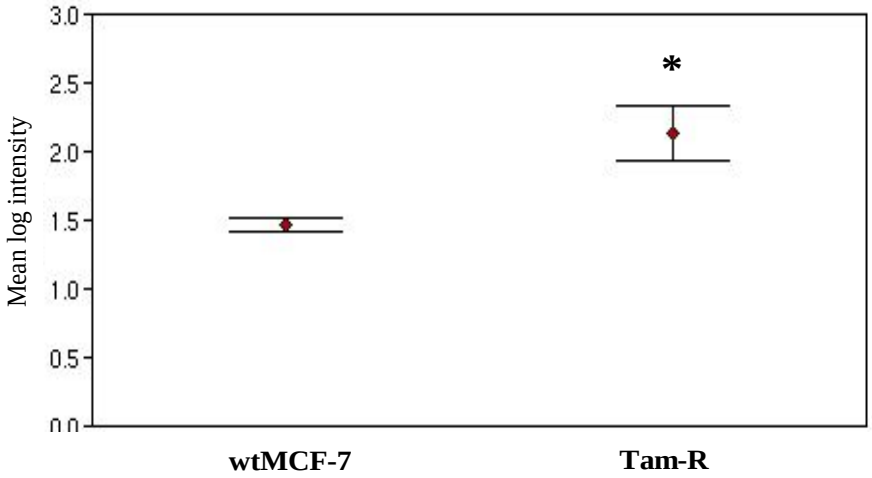
3.1 CHARACTERISATION OF CD44 PROTEIN EXPRESSION IN TAMOXIFEN-SENSITIVE AND ACQUIRED TAMOXIFEN-RESISTANT WTMCF-7 CELLS

Our previous observations arising from interrogation of our microarray database identified that CD44 was overexpressed in Tam-R compared to wtMCF-7 cells. Affymetrix® microarray analysis showed 1.4 fold higher expression of CD44 in Tam-R cells compared to wtMCF-7 cells (p-value < 0.05 on t-test, data provided courtesy of J.Gee, R. McClleland and L Farrow; **Figure 3.1A**). Our initial aim was therefore to further characterise CD44 expression in our acquired tamoxifen–resistant ('Tam-R') MCF-7 cells and compare it with the expression in their wild-type, endocrine-sensitive wtMCF-7 counterparts. To do this we compared CD44 gene and protein expression between wtMCF-7 and Tam-R cells using RT-PCR, Western blotting and immunocytochemistry.

3.1.1 CD44 mRNA Expression in Tam-R Cells and wtMCF-7 Cells

Preliminary characterisation of CD44 expression in Tam-R cells using RT-PCR revealed that CD44 mRNA levels were higher in Tam-R cells as compared to the wMCF-7 cells (**Figure 3.1B**; data provided courtesy of C. Smith and N. Jordan). This validated our microarray analysis data which showed overexpression of CD44 in Tam-R cells compared to wtMCF-7 cells. We therefore proceeded to study the protein level expression of CD44 in our Tam-R and wtMCF-7 cell lines using Western blotting and immunocytochemistry.

A



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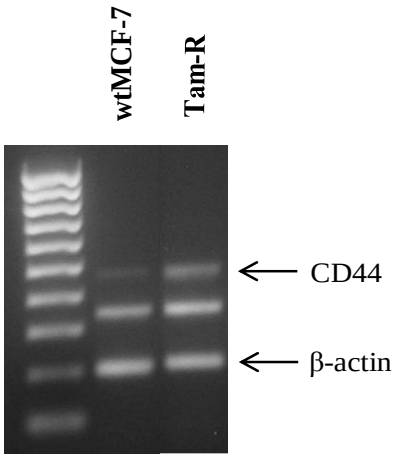


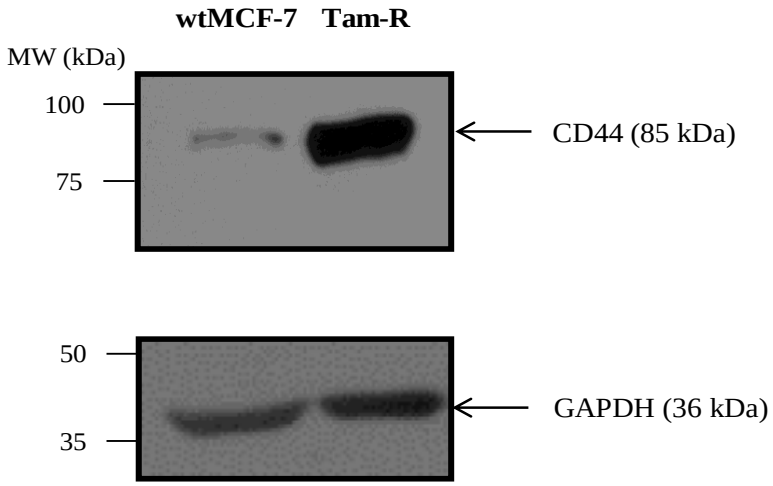
Figure 3.1 CD44 gene expression levels in wtMCF-7 and Tam-R cells
Affymetrix® microarray analysis was performed to evaluate CD44 expression in wtMCF-7 and Tam-R cells. Data is presented as comparative mean log intensity ± SD (A). This showed 1.4 fold upregulation of CD44 in Tam-R cells compared to wtMCF-7 cells (*p*-value ≤ 0.05 on *t*-test). CD44 mRNA expression was subsequently compared between wtMCF-7 and Tam-R cells using RT-PCR as described in **Section 2.3**. A higher mRNA expression of CD44 was noted in Tam-R compared to wtMCF-7 cells (B). The representative gel in (B) has been truncated to exclude other cell lines included in the original PCR which are not relevant to this project. Data provided courtesy of J. Gee, R. McClleland, L. Farrow and C. Smith.

3.1.2 Basal CD44 Protein Expression in Tam-R cells and wtMCF-7 Cells

For these experiments, wtMCF-7 and Tam-R cells were cultured in experimental medium (EM) until log-phase growth and lysed on ice using the technique described in **Section 2.4**. On Western blotting, an 85kDa protein band corresponding to the expected size of CD44 was noted in both wtMCF-7 and Tam-R cells but the band density was significantly higher in the Tam-R cells (**Figure 3.2A**). Semi-quantitative analysis using densitometry after normalising for loading using GAPDH revealed that the expression in Tam-R cells was 4.7 fold higher as compared to the wtMCF-7 cells suggesting that CD44 is overexpressed in Tam-R cells at the protein level (p-value = 0.028 on t-test; **Figure 3.2B).** It is important to mention at this point that the CD44 antibody used in this study was not specific to a particular CD44 isoform and likely recognises the standard and other variant isoforms of the CD44 protein.

This finding was further corroborated using immunocytochemistry utilising the technique described in **Section 2.5**. Basal wtMCF-7 and Tam-R cells were cultured on glass cover slips until log-phase growth was reached and then fixed using 2.5% phenol (w/v) in Formal saline for 5 minutes followed by 100% ethanol for 5 minutes. Immunocytochemical analysis showed minimal CD44 expression in wtMCF-7 cells and significantly higher expression of CD44 in the Tam-R cells although it must be mentioned that there was difference in confluency between the two cell lines which can affect protein expression levels (**Figure 3.3**). The expression of CD44 in Tam-R cells appeared heterogeneous with some cells showing quite marked overexpression compared to others. There was minimal or no expression noted in the cytoplasm and nucleus under basal conditions and CD44 was noted to be mainly localised to the cell membrane in Tam-R cells.

A



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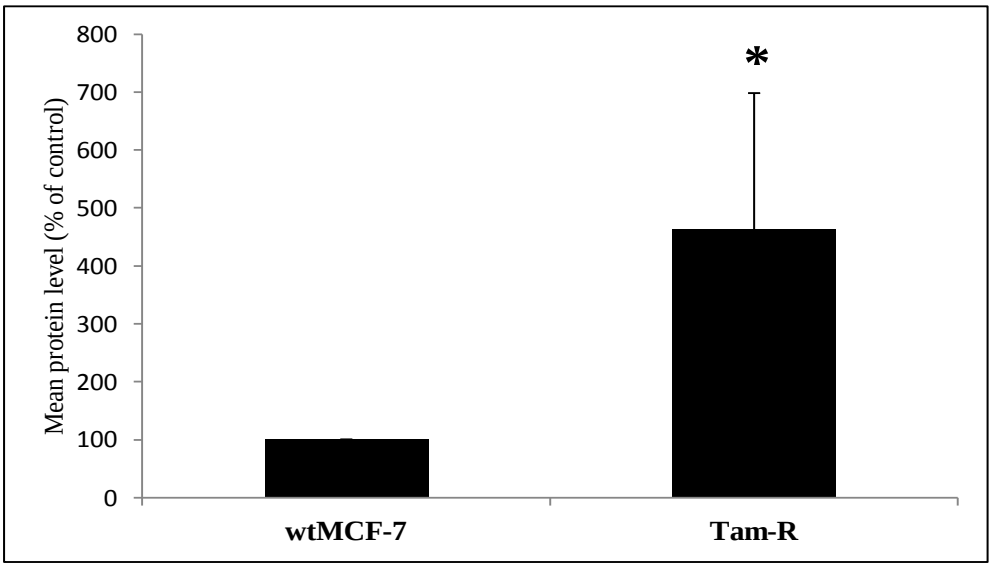


Figure 3.2 CD44 protein expression in wtMCF-7 and Tam-R cells on Western blotting
Basal wtMCF-7 and Tam-R cells were cultured in EM ± 4-OH-TAM (10^{-7}M) until log-phase growth was reached and then lysed on ice. CD44 protein expression (expected molecular weight of 85kDa) was evaluated through Western blotting using the procedure outlined in **Section 2.4**. A representative blot is shown above (A). Data was normalised using GAPDH and presented as mean protein level ± SD. CD44 expression was 4.6 fold higher in Tam-R compared to wtMCF-7 cells (Mean protein level – wtMCF-7 100% versus Tam-R 465 ± 235%, n=3, *p*-value = 0.028 on *t*-test; B).

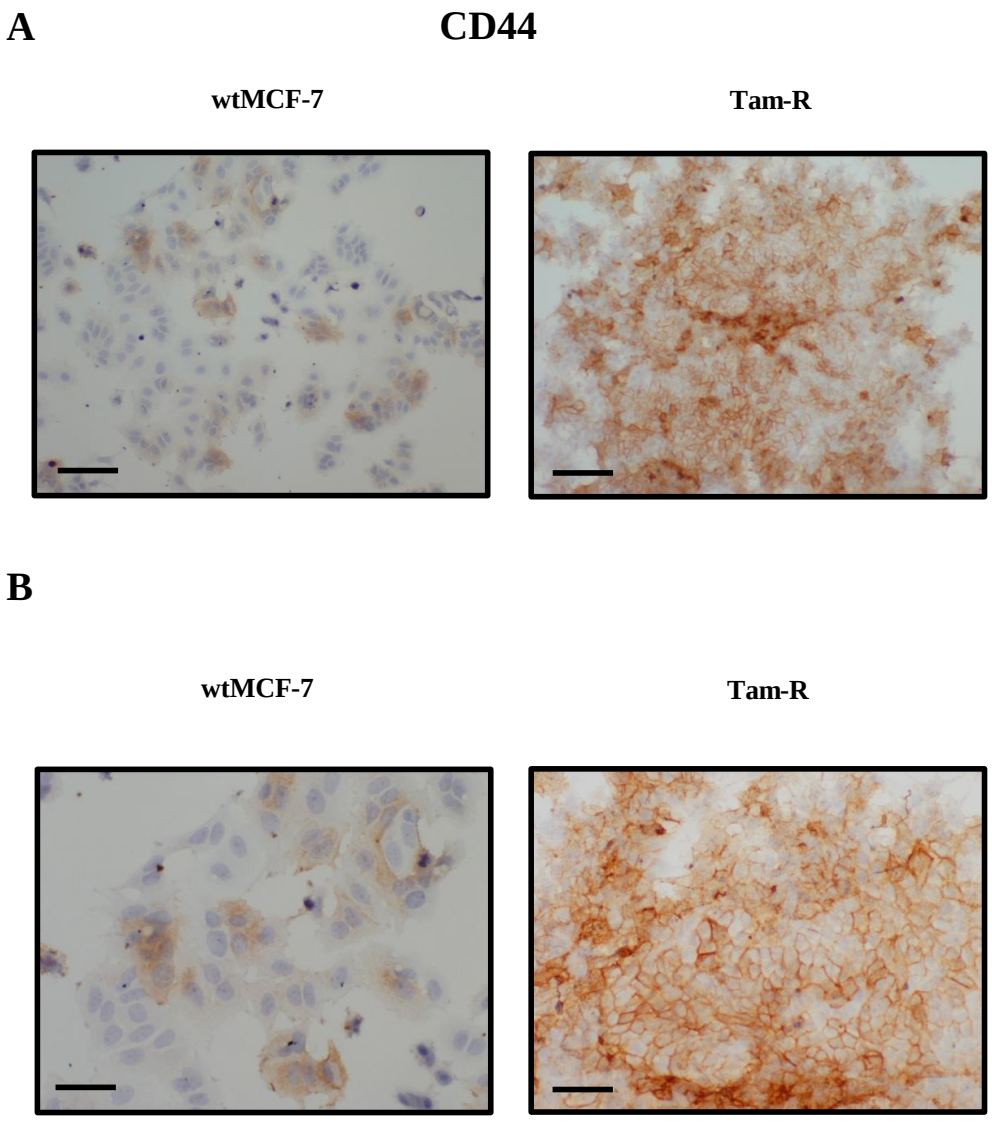


Figure 3.3 CD44 protein expression in wtMCF-7 and Tam-R cells on immuno-cytochemistry
Basal wtMCF-7 and Tam-R cells were cultured on glass cover slips until log-phase growth was reached and then fixed using 2.5% phenol (w/v) in Formal saline for 5 minutes followed by 100% ethanol for 5 minutes. CD44 expression was assessed through immunocytochemistry using the procedure described in **Section 2.5**. Representative images of CD44 expression in wtMCF-7 and Tam-R cells are shown above at 10x magnification (A) and 20x magnification (B). CD44 was overexpressed in Tam-R compared to wtMCF-7 cells and CD44 was noted to be primarily localised to the membrane (Membrane H-score – wtMCF-7 30 versus Tam-R 125, n=1). Scale bar = 50µm at 20x magnification and 100µm at 10x magnification.

3.1.3 Discussion

Significant upregulation of the CD44 gene in Tam-R cells compared to wtMCF-7 cells on Affymetrix® analysis was an interesting finding. The CD44 probes used during this analysis were not variant specific and likely detected expression of the standard form (CD44s). Our interest in further exploring the role of CD44 in the setting of tamoxifen resistance arose from published links between CD44 and breast cancer. CD44 has been shown to interact with the ErbB family of receptors which play an important role in breast cancer progression and have been implicated in poor response to endocrine therapy and endocrine resistance (Newby, Johnston et al. 1997; Wobus, Rangwala et al. 2002; Folgiero, Avetrani et al. 2008; Palyi-Krekk, Barok et al. 2008). CD44 has also been shown to enhance cell migration, angiogenesis and metastasis in breast cancer models (Trochon, Mabilat et al. 1996; Bourguignon, Singleton et al. 2003). Moreover, cell models of acquired resistance show altered expression of various growth factor receptors that may augment the cell's sensitivity to numerous factors present within the tumour microenvironment and CD44 overexpression may sensitise these cells to various stromal ligands (Hiscox, Jordan et al. 2006). *Finally, the main stromal ligand of CD44 is hyaluronan which itself has been implicated in breast cancer pathogenesis.* In light of our microarray data and, based on the above evidence that implicates CD44 in breast cancer progression, we hypothesised that CD44 may be implicated in influencing the aggressive phenotype noted in our tamoxifen-resistant Tam-R cells either directly, or by influencing the function of ErbB receptors.

We first validated the preliminary microarray data using RT-PCR to assess CD44 expression at the gene level. Despite the obvious advantages of microarray analysis, this validation was undertaken to avoid potential pitfalls due to deficiencies inherent in this technique. For example, there is potential for misidentification with other variants and members of a closely-

related gene family and possibility of statistical error due to the substantial volume of data generated (Murphy 2002). Subsequent validation experiments of CD44 expression at the gene and protein level showed significantly higher levels of CD44 in the Tam-R cells compared to their endocrine-sensitive wtMCF-7 counterparts. This was a novel finding in the setting of tamoxifen resistance although there is evidence to suggest higher CD44 expression in drug-resistant and more invasive ER-negative cell lines compared to wtMCF-7 cells. In a mouse xenograft model of human breast cancer, Fitchner et al. showed that wtMCF-7 cells express CD44 standard at much lower levels compared to the multidrug-resistant MCF7/ADR cell line derived from wtMCF-7 cells (Fichtner, Dehmel et al. 1997). They also noted that CD44s expression in wtMCF-7 cells was much lower than more aggressive breast cancer cell lines such as MDA-MB435. The underlying mechanism behind CD44 upregulation in our Tam-R model is a potential area of further investigation. However, it is unlikely that this is an ER-regulated phenomenon as wtMCF-7 and Tam-R cells have similar levels of ERα mRNA and protein (Hutcheson, Knowlden et al. 2003). It appears to be an early change in the course of development of tamoxifen resistance as CD44 upregulation has been detected as early as 3 days following 4-OH-TAM treatment in wtMCF-7 cells (S. Hiscox and R. Bellerby, unpublished observations).

On immunocytochemistry, we found that CD44 expression in the Tam-R cells was primarily localised to the cell membrane in a heterogenous fashion with minimal cytoplasmic and nuclear staining noted under basal conditions. This corresponds with the primary membrane localisation of CD44 noted in various studies of CD44 structure and function (Lucas, Green et al. 1989; Ponta, Sherman et al. 2003). Membrane associated CD44 undergoes proteolytic cleavage by metalloproteinases and gamma secretase causing release of the intracellular domain which translocates to the nucleus and influences gene transcription (Okamoto, Tsuiki

et al. 2002; De Falco, Tamburrino et al. 2012). It is possible that the small amount of CD44 detected in the cytoplasm and nucleus may represent this intracellular portion of CD44. The other possibility is that variant forms of CD44 are also being detected during these experiments. Although a full discussion and delineation of the role of the variants is beyond the scope of this thesis, following on from this project, additional isoforms are currently being investigated by our group.

3.1.4 Conclusion

Based on this data, we concluded that CD44 is overexpressed at both the gene and protein level in acquired tamoxifen-resistant Tam-R cells compared to their endocrine-sensitive wtMCF-7 counterparts. We subsequently investigated the hypothesis that this overexpression contributes to the endogenous aggressive phenotype noted in Tam-R cells.

3.2 EXPLORING THE ROLE OF CD44 AS A DETERMINANT OF THE TAMOXIFEN-RESISTANT CELL PHENOTYPE

CD44 upregulation has previously been associated with an aggressive phenotype in breast cancer cell models. For example, Li et al. have shown an increase in migratory behaviour of wtMCF-7 cells following transient transfection with CD44s (Li, Zha et al. 2012). Acquisition of tamoxifen resistance in Tam-R cells is accompanied by a dramatic and significant increase in invasiveness and motility when compared to wtMCF-7 cells (Hiscox, Morgan et al. 2004). We were keen to investigate whether CD44 overexpression played a role in this aggressive cellular behaviour. Thus, our main aim here was to investigate the role of CD44 in basal migration, invasion and growth of Tam-R cells. To do this, we used an siRNA approach to suppress CD44 expression.

3.2.1 Optimisation of CD44-siRNA Technique

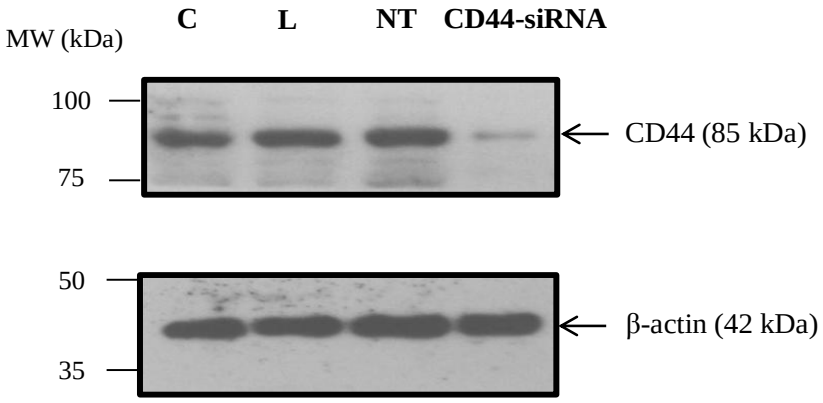
The first step was to validate an existing siRNA protocol available in the laboratory for use in our study. For the initial siRNA optimisation experiments, once Tam-R cells achieved 50% confluence they were treated with either experimental culture medium (C), medium containing lipid transfection reagent (L), non-targeting siRNA (NT) or CD44-specific siRNA (CD44-siRNA) for 72 hours prior to lysis on ice followed by analysis of protein expression. Following Western blotting, a protein band corresponding to the expected CD44 molecular weight of 85kDa was noted in all the samples but the density was markedly reduced in the cells treated with CD44-siRNA (**Figure 3.4A**). This indicated effectiveness of the siRNA protocol.

Validation of the CD44-siRNA technique was further confirmed using immunocytochemistry. Tam-R cells were cultured until they achieved 50% confluence and

treated with NT or CD44-siRNA for 72 hours prior to lysis on ice. Imunocytochemical analysis revealed clear reduction in CD44 expression levels in CD44-siRNA transfected cells compared to NT cells (**Figure 3.4B**). The siRNA method was thus noted to be effective in significantly reducing CD44 expression on both Western blotting and immunocytochemistry. Effective CD44 knockdown was confirmed in each experiment involving siRNA-mediated CD44 knockdown to ensure accurate interpretation of results.

Having established the efficacy of the CD44-siRNA knockdown technique in Tam-R cells, we proceeded to elucidate the role of CD44 in basal function of Tam-R cells with respect to its involvement in cell migration, invasion and growth.

A



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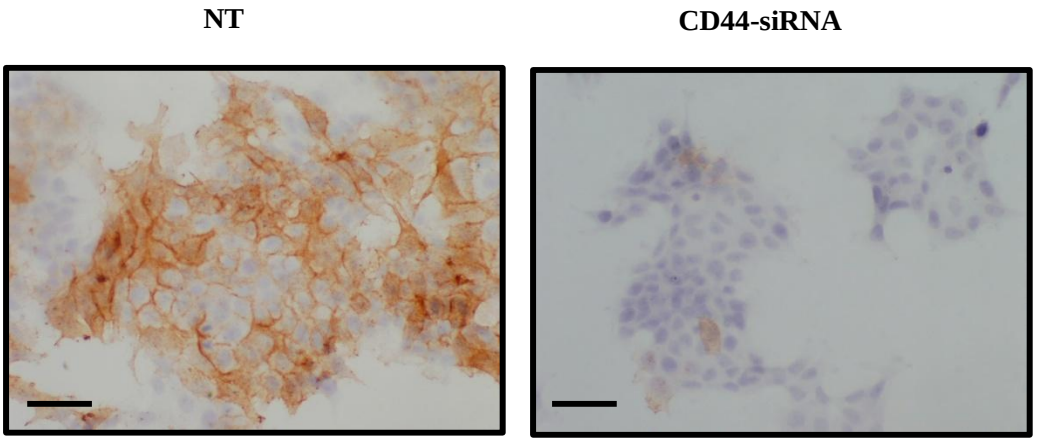


Figure 3.4 Validation of CD44-siRNA protocol in Tam-R cells
Tam-R cells were cultured in 35mm dishes until 50% confluence was reached and treated using only culture medium (C), medium containing lipid transfection reagent (L), or medium containing either non-targeting siRNA (NT) or CD44-specific siRNA (CD44-siRNA) for 72 hours prior to lysis on ice. CD44 protein expression was evaluated through Western blotting as described in **Section 2.4**. A representative blot is shown above (A). Tam-R cells were subsequently cultured on glass cover slips until 50% confluence was reached, treated as above and fixed using 2.5% phenol (w/v) in Formal saline for 5 minutes followed by 100% ethanol for 5 minutes. CD44 expression was assessed through immunocytochemistry as described in **Section 2.5**. Representative images are shown above (B). Use of CD44-siRNA significantly reduced CD44 expression in Tam-R cells on Western blotting (A) and immunocytochemistry (B) confirming efficacy of the CD44-siRNA transfection protocol. Scale bar = 50µm (20x magnification).

3.2.2 Effect of CD44 Knockdown on Migration in Tam-R Cells

The effect of CD44 knockdown on the migratory capacity of Tam-R cells was investigated using wound healing assays as described in **Section 2.7.1**. Tam-R cells were cultured until 50% confluence was reached and medium replaced with either NT or CD44-siRNA for 72 hours. Wounds were then created in the cell monolayer and cells allowed to migrate for 36 hours. Although Tam-R cells transfected with CD44-siRNA showed a trend towards reduced migration as evidenced by slower wound closure compared to NT cells, this difference was not statistically significant (*p*-value = 0.07 on *t*-test, **Figure 3.5A & B**).

To further investigate changes in Tam-R cell migration in response to CD44 manipulation, Boyden chamber migration assays were performed in the presence of CD44-siRNA as described in **Section 2.7.2**. After transfecting cells with either NT or CD44-siRNA for 72 hours, cells were harvested and seeded into the Transwell® inserts and allowed to migrate for a period of 48 hours. Following quantitation of the experiment, there was no statistically significant difference noted in the basal migratory phenotype of Tam-R cells transfected with CD44-siRNA compared to NT cells (*p*-value > 0.05/NS on *t*-test; **Figure 3.5C**).

A

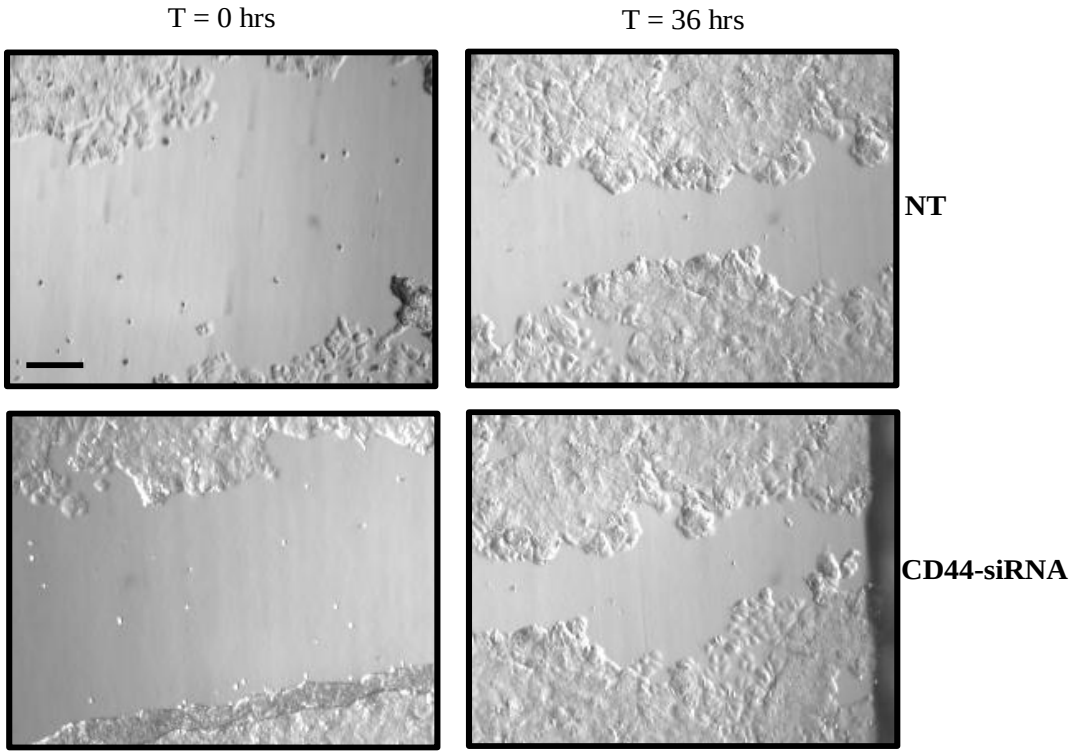
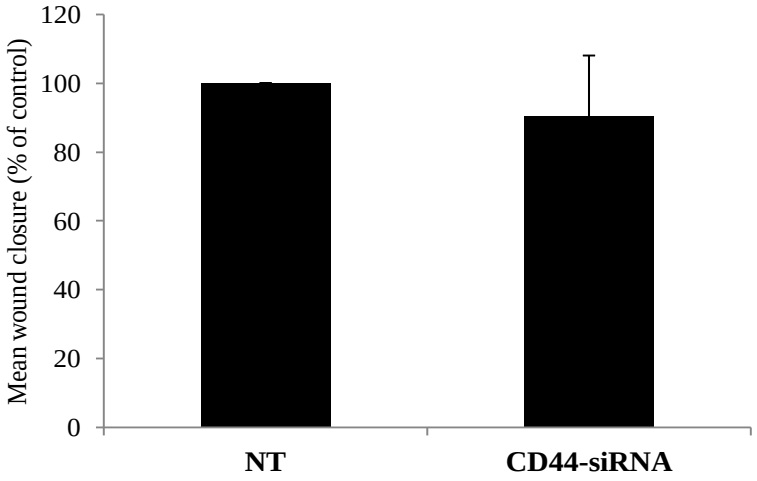


Figure 3.5A Effect of CD44 knockdown on migration in Tam-R cells
Tam-R cells were cultured until 50% confluence was reached and medium replaced with either non-targeting siRNA (NT) or CD44-specific siRNA (CD44-siRNA) for 72 hours. Wound healing assay was then performed as described in **Section 2.7.1**. Representative images are shown above (A). Results were collated after 36 hours and data presented as mean wound closure (% of control) ± SD (Mean wound closure – Control 100% versus CD44-siRNA 90.5 ± 17.6%, n=3, *p*-value = 0.07 on *t*-test; B). Subsequently, Tam-R cells were set up as above and Boyden chamber migration assay was performed as described in **Section 2.7.2** by transferring cells into Transwell® inserts containing porous polycarbonate membranes coated with fibronectin (10µg/ml). Results were collated after 48 hours and data presented as mean cell migration (% of control) ± SD (Mean cell migration – Control 100% versus CD44-siRNA 78.2 ± 33.7%, n=3, *p*-value > 0.05/NS on *t*-test; C). Use of CD44-siRNA does not have a significant inhibitory effect on endogenous migration in Tam-R cells. Scale bar = 100µm (10x magnification).

B



C

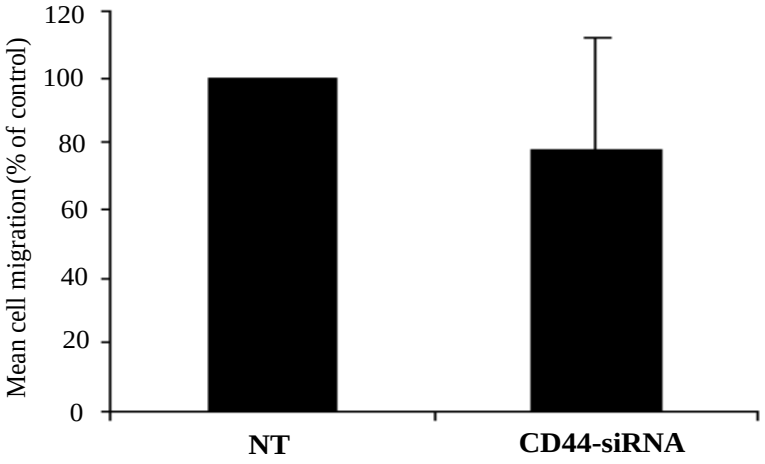


Figure 3.5B&C Effect of CD44 knockdown on migration in Tam-R cells
Tam-R cells were cultured until 50% confluence was reached and medium replaced with either non-targeting siRNA (NT) or CD44-specific siRNA (CD44-siRNA) for 72 hours. Wound healing assay was then performed as described in **Section 2.7.1**. Representative images are shown above (A). Results were collated after 36 hours and data presented as mean wound closure (% of control) ± SD (Mean wound closure – NT 100% versus CD44-siRNA 90.5 ± 17.6%, n=3, *p*-value = 0.07 on *t*-test; B). Subsequently, Tam-R cells were set up as above and Boyden chamber migration assay was performed as described in **Section 2.7.2** by transferring cells into Transwell® inserts containing porous polycarbonate membranes coated with fibronectin (10µg/ml). Results were collated after 48 hours and data presented as mean cell migration (% of control) ± SD (Mean cell migration – NT 100% versus CD44-siRNA 78.2 ± 33.7%, n=3, *p*-value > 0.05/NS on *t*-test; C). Use of CD44-siRNA does not have a significant inhibitory effect on endogenous migration in Tam-R cells. Scale bar = 100µm (10x magnification).

3.2.3 Effect of CD44 Knockdown on Invasion in Tam-R Cells

The effect of CD44 knockdown on the invasive capacity of Tam-R cells was investigated using Matrigel™ invasion assays as described in **Section 2.8**. After transfecting cells with either NT or CD44-siRNA for 72 hours, cells were harvested and seeded into the modified Transwell® inserts and allowed to invade for a period of 48 hours. After quantitation of the experiment, there was no statistically significant change noted in the endogenous invasive phenotype of Tam-R cells transfected with CD44-siRNA compared to NT cells (*p*-value > 0.05/NS on *t*-test; **Figure 3.6A**). We then proceeded to assess the effect of CD44 knockdown on growth in Tam-R cells.

3.2.4 Effect of CD44 Knockdown on Growth in Tam-R Cells

The effect of CD44 knockdown on the basal growth capacity of Tam-R cells was investigated using cell growth assays as described in **Section 2.9.** Growth assays were set up after transfecting cells with either NT or CD44-siRNA for 72 hours and the results were collated after 5 days. After quantitation of the experiment, there was no statistically significant change noted in the basal growth pattern of Tam-R cells transfected with CD44-siRNA compared to NT cells (*p*-value >0.05/NS on *t*-test; **Figure 3.6B**).

(NB: The exact duration and degree of persistence of the effect of CD44-siRNA on CD44 protein expression has not been specifically tested during this study.)

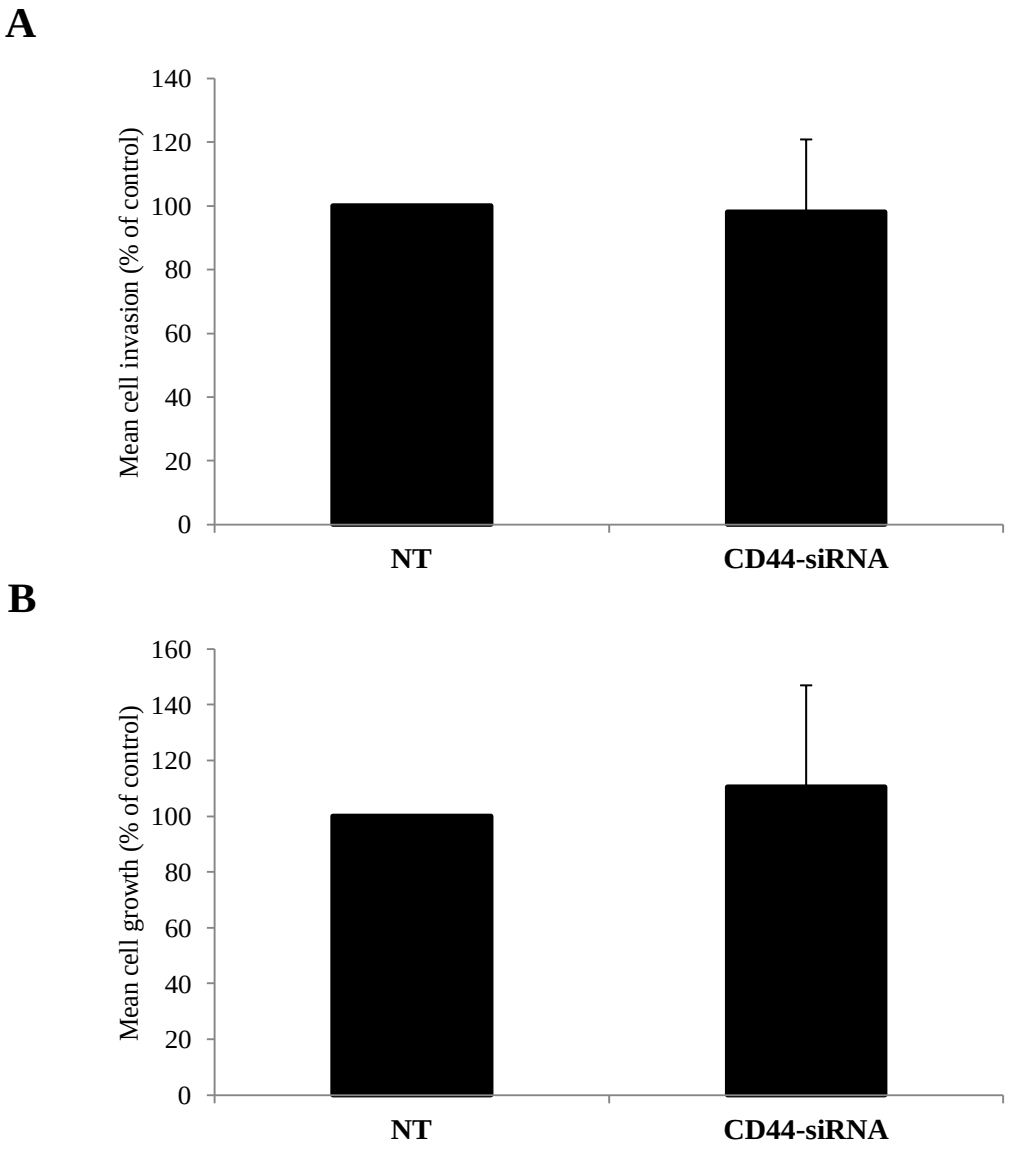


Figure 3.6 Effect of CD44 knockdown on invasion and growth in Tam-R cells
Tam-R cells were cultured in 35mm dishes and once 50% confluence was reached, the medium was replaced with either non-targeting siRNA (NT) or CD44-specific siRNA (CD44-siRNA) and cultured for a further 72 hours. Matrigel™ invasion assay was performed as described in **Section 2.8** by transferring cells into Transwell® inserts containing porous polycarbonate membranes coated with Matrigel™. Results were collated after 48 hours and data presented as mean cell invasion (% of control) ± SD (Mean cell invasion – NT 100% versus CD44-siRNA 98.2 ± 22.7%, n=3, *p*-value >0.05/NS on *t*-test; A). Cell growth assay was also performed on cells set up in a similar manner using the protocol described in **Section 2.9.** Results were collated after 5 days and data presented as mean cell growth (% of control) ± SD (Mean cell growth – NT 100% versus CD44-siRNA 110.5 ± 36.4%, n=3, *p*-value > 0.05/NS on *t*-test; B). There was no significant inhibitory effect following CD44-siRNA transfection on endogenous invasion and growth in Tam-R cells. Data provided courtesy of S. Hiscox.

3.2.5 Discussion

Adverse phenotypic features are commonly noted in the more aggressive breast cancers and include a more marked migratory phenotype, greater invasiveness and a higher growth capacity amongst other features. Similar observations have been made in our model of tamoxifen resistance where Tam-R cells show a more aggressive phenotype as compared to the endocrine-sensitive wtMCF-7 cells. Tam-R cells exhibit up to 200% higher growth capacity as compared to wtMCF-7 cells and elevated levels of MAPK has been implicated in driving this (Knowlden, Hutcheson et al. 2003). Furthermore, Tam-R cells in vitro grow as loosely packed colonies with loss of cell-cell junctions and they also exhibit altered morphological characteristics which resemble cells undergoing epithelial mesenchymal transition (EMT). Elevated Src activity has been suggested to play a role in the promotion of invasiveness noted in Tam-R cells (Hiscox, Morgan et al. 2006). Other researchers have also investigated the mechanisms underlying aggressive behaviour in resistant cell lines and have demonstrated suppression of the ER and ER-regulated genes and activated survival pathway signalling (Zhou, Capello et al. 2011; Li, Chen et al. 2013).

To examine the role of CD44 overexpression on the endogenous phenotype of Tam-R cells, we have used siRNA for post-transcriptional knockdown of CD44 during in this study. The protocol was optimised for this study with assistance from Christopher Smith who had previously used it in a model of hormonal resistance. Our original experiments to confirm siRNA-mediated knockdown of CD44 included four arms: control (C), lipid (L), non-targeting (NT) and CD44-specific siRNA (CD44-siRNA). The ‘L’ arm contained the lipid transfection reagent only whereas the ‘NT’ arm contained a non-targeting siRNA that was not specific to CD44. The main purpose of using the ‘L’ and ‘NT’ was to rule out non-specific effects that could affect the interpretation of results. We have demonstrated via Western

blotting and immunocytochemistry that transfection with NT does not have any effect on CD44 expression and the 'NT' arm has been used as the sole control arm for all further experiments involving CD44 siRNA. We preferred siRNA amongst other possible techniques for suppression of CD44 such as neutralising antibodies and short-hairpin RNAs (shRNA). The main advantage of using siRNA was the ease and efficiency of transfection, the stable quality of available reagents, minimal off-target effects and reliable suppressive effects. However, using siRNA has the disadvantage of having less future therapeutic potential in terms of drug delivery as compared to a neutralising monoclonal antibody should future experiments indicate potential for use as a therapeutic agent. .

Our data suggests that CD44 does not play a role in the endogenous aggressive phenotype noted in Tam-R cells. The role of CD44 in determining the basal aggressive phenotype of tamoxifen-resistant wtMCF-7 cells has not been investigated by any other group previously. However, the role of CD44 on migration, invasion and growth has been explored in other models of breast cancer. For example, Ouhtit et al. transfected CD44s into wtMCF-7 cells and found that the resulting cell line (MCF7F-B5) had significantly higher migratory, invasive and growth capacity compared to the parent cell line (Ouhtit, Abd Elmageed et al. 2007). In their doxycycline-regulated breast cancer xenograft model, CD44s induction did not affect the growth or local invasion of the primary tumour but promoted distant metastatic spread to the liver providing in vivo evidence of the role of CD44s in promoting invasion and metastasis.

3.2.6 Conclusion

Based on our data, we concluded that despite being overexpressed in Tam-R cells, CD44 does not appear to influence their endogenous migration, invasion or growth in vitro. We hypothesised that CD44 overexpression may sensitise Tam-R cells to stromal ligands such as HA present in the microenvironment and proceeded to investigate this further.

3.3 DETERMINING WHETHER CD44 OVEREXPRESSION IN TAM-R CELLS SENSITISES THEM TO STROMAL FACTORS

The microenvironment is known to play an important role in breast cancer progression. We proceeded to investigate potential interplay between CD44 and the stroma via its interaction with hyaluronic acid (HA), its main ligand, and the consequent effect on signalling and function of Tam-R cells as a result of this interaction. Our aim was to test the hypothesis that Tam-R cells, which overexpress CD44, would exhibit greater sensitivity towards HA compared with endocrine-sensitive wtMCF-7 cells.

3.3.1 Effect of HA Stimulation on MAPK Activity in Tam-R Cells

To investigate whether CD44 overexpressing Tam-R cells were sensitised to HA, basal Tam-R cells were cultured in experimental medium (EM) until log-phase was reached and then treated with five increasing concentrations of HA (0, 10, 50, 200 and 1000µg/ml) for 5 minutes prior to lysis on ice. The initial time point of 5 minutes was chosen as it was more likely to demonstrate early changes in phosphorylation in downstream signalling pathways. TGF-α was used as a positive control as it is known to stimulate multiple signalling pathways in our Tam-R cell model. Analysis of activity of proteins known to be involved in CD44 signalling (MAPK, Src and Akt) was then performed using Western blotting.

A dual protein band at the expected phosphorylated MAPK (p-MAPK 42/44) molecular weight of 42/44 kDa was noted at all HA concentrations (**Figure 3.7A**). The intensity of the bands was seen to increase compared to control at HA concentrations of 50µg/ml and above and this increase was noted to be dose-dependant. Although a dual protein band was also noted at the expected molecular weight of 42/44 kDa for total MAPK (T-MAPK), there was no increase in intensity following HA treatment at the indicated doses. Semi-quantitative

analysis with densitometry after correcting for loading using β-actin and T-MAPK levels showed a statistically significant 2.7 fold upregulation of p-MAPK in Tam-R cells treated with a HA dose of 200μg/ml as compared to untreated Tam-R cells (*p*-value = 0.03 on *t*-test; **Figure 3.7B**). There was a positive correlation between the dose of HA and the resulting p-MAPK expression indicating that this is a dose-dependant effect thereby suggesting a direct HA effect instead of a non-specific effect (Spearman rank correlation: Rho = +0.88). In contrast, no upregulation of p-MAPK signalling following HA treatment was seen in wtMCF-7 cells (**Figure 3.8**).

We also investigated the effect of HA on other downstream signalling pathways known to be functionally relevant in CD44 signalling including Src and Akt, but did not find any consistent response to HA treatment (**Figure 3.9**). As such, in this study we have used p-MAPK as a surrogate marker of HA stimulation in Tam-R cells.

In our study we found that p-MAPK upregulation following HA treatment was noticeable at HA concentrations at or above 50μg/ml. However, the lowest dose that produced a significant, consistent and reproducible response in our cell model was 200μg/ml. This dose of HA has been used by other researchers in their in vitro cell models including breast cancer models. For example, Bourguignon et al. used a HA dose of 200μg/ml in their SP1 cell line based migration assays and demonstrated a HA-mediated increase in migratory phenotype (Bourguignon, Zhu et al. 2000). Using the lowest dose which shows consistent measurable results has the advantage of limiting non-specific effects of the drug.

For the initial experiments above, medium molecular weight HA (132 kDa) was used. Various molecular weight forms of HA are commercially available and there is evidence in

the literature to suggest variable actions of HA depending on the molecular weight. We compared three different commercially available forms of HA in our tamoxifen-resistant model and found that medium molecular weight HA (average molecular weight of 132kDa) achieved the best p-MAPK upregulation in Tam-R cells (**Figure 3.10**). Low molecular weight HA has been reported to have an inhibitory effect on CD44 function which was an additional reason for favouring the 132kDa form of HA. In the BT-549 human breast cancer cell line and the immortalised HK-2 human renal tubular epithelial cell line, Yang et al. noted an inhibitory effect of low molecular weight HA on CD44 clustering (Yang, Cao et al. 2012). A growth inhibitory mechanism is also well described for high molecular weight hyaluronan (500kDa and above) which can trigger inactivation of ERM proteins (Ezrin, radixin and moesin) and activation of Merlin, thereby, leading to inhibition of growth and invasiveness (Ponta, Sherman et al. 2003). HA of an average molecular weight of 132 kDa at a dose of 200µg/ml has, therefore, been used for all further experiments in this study unless otherwise specified.

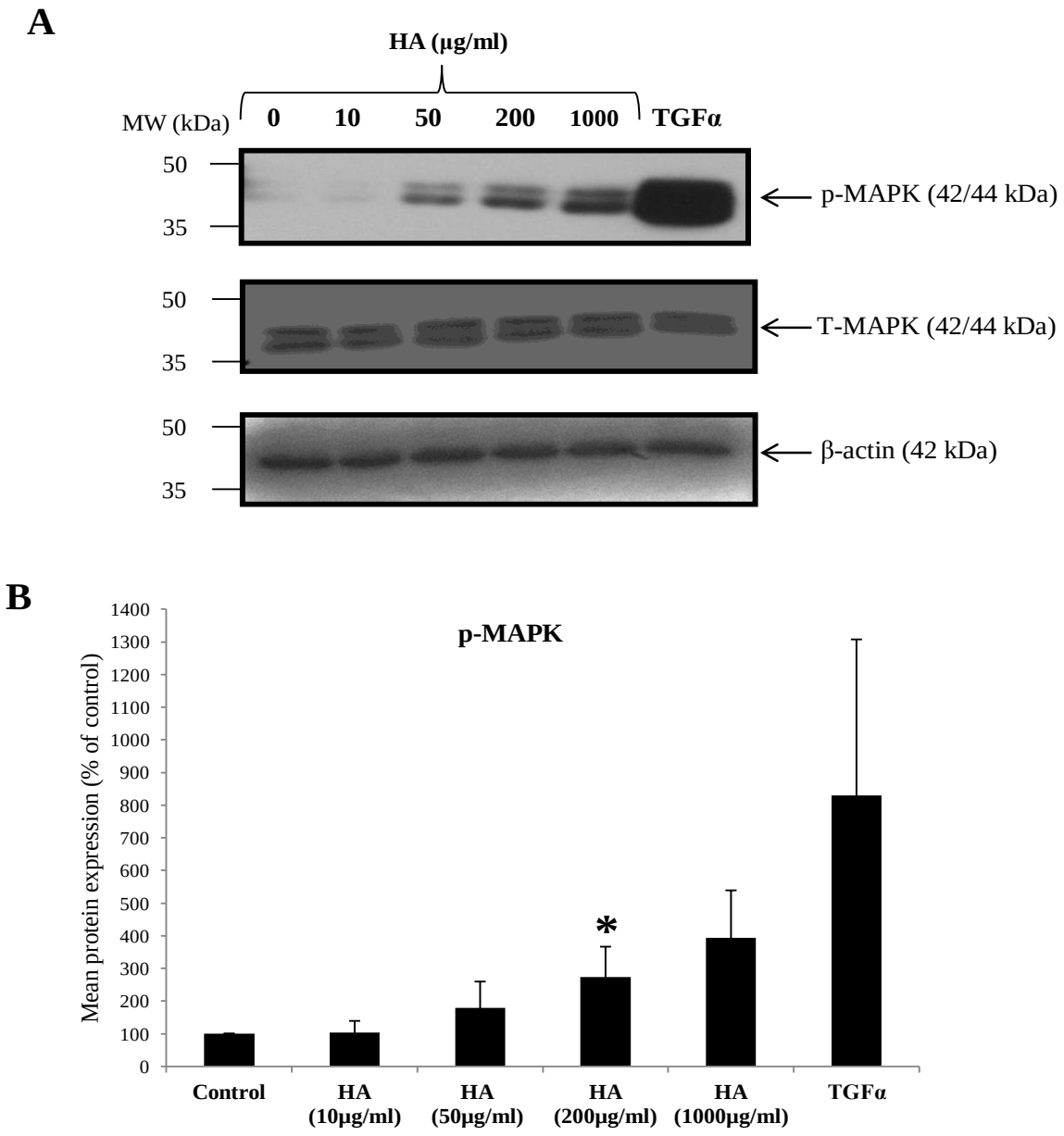


Figure 3.7 Effect of HA on MAPK signalling in Tam-R cells
Tam-R cells were cultured in experimental medium (EM + 4-OH-TAM) until log-phase growth was reached and treated with five different concentrations of HA (0, 10, 50, 200 and 1000µg/ml) for 5 minutes and then lysed on ice. Phosphorylated MAPK (p-MAPK) and total MAPK (T-MAPK) expression was evaluated through Western blotting as described in **Section 2.4**. A representative blot is shown above (A). Data was normalised using beta-actin (β-actin) and presented as mean protein expression (% of control) ± SD (Mean protein expression – Control 100% versus HA '200µg/ml' 273.2 ± 93.5%, n=3, *p*-value = 0.03 on *t*-test; B). Treatment of Tam-R cells with HA led to dose-dependent p-MAPK upregulation in Tam-R cells (Spearman rank correlation: Rho = +0.88). TGFα was used as a positive control.

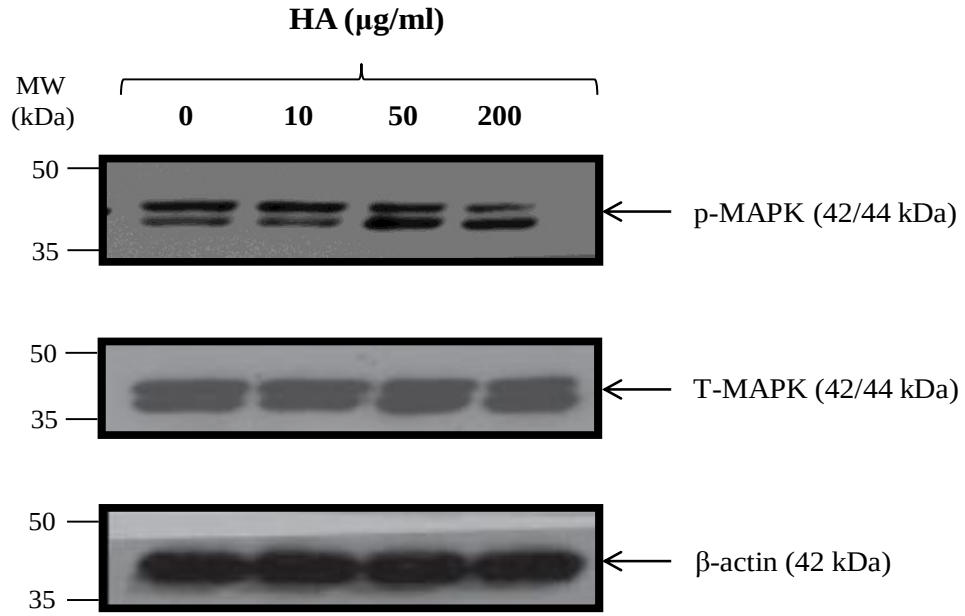


Figure 3.8 Effect of HA on MAPK signalling in wtMCF-7 cells
wtMCF-7 cells were cultured in EM until log-phase growth was reached and treated with four different concentrations of HA (0, 10, 50 and 200µg/ml) for 5 minutes and then lysed on ice. p-MAPK and T-MAPK expression was evaluated through Western blotting as described in **Section 2.4**. A representative blot is shown above. Treatment of wtMCF-7 cells with HA did not show any upregulation of p-MAPK at the indicated doses.

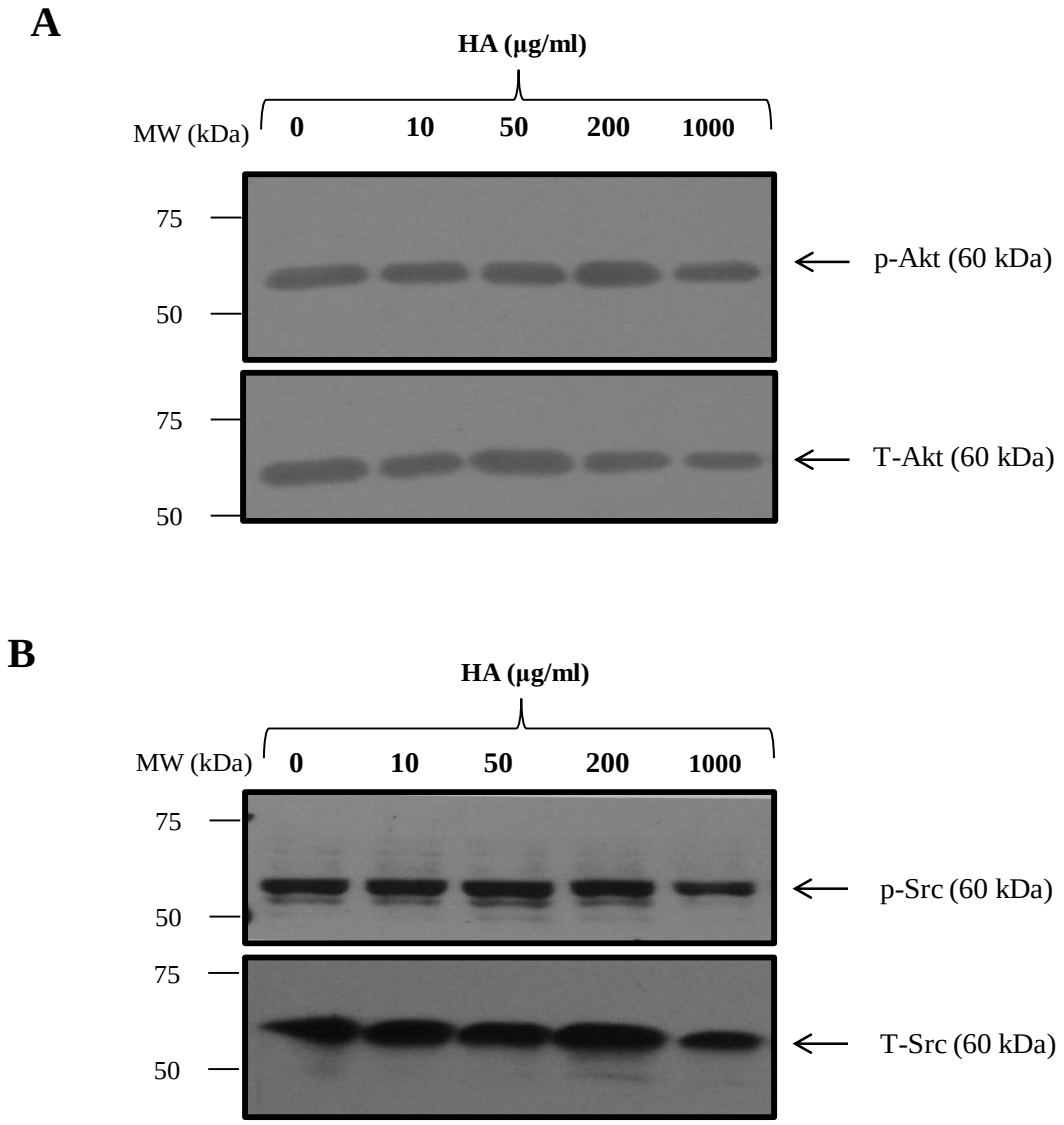


Figure 3.9 Effect of HA on Akt and Src signalling in Tam-R cells
Tam-R cells were cultured in experimental medium (EM + 4-OH-TAM) until log-phase growth was reached and treated with five different concentrations of HA (0, 10, 50, 200 and 1000µg/ml) for 5 minutes and then lysed on ice. Akt (p-Akt and T-Akt) and Src (p-Src and T-Src) expression was evaluated through Western blotting as described in **Section 2.4**. Representative blots are shown above (A, B). Treatment of Tam-R cells with HA did not lead to any detectable upregulation of Akt or Src at the indicated doses.

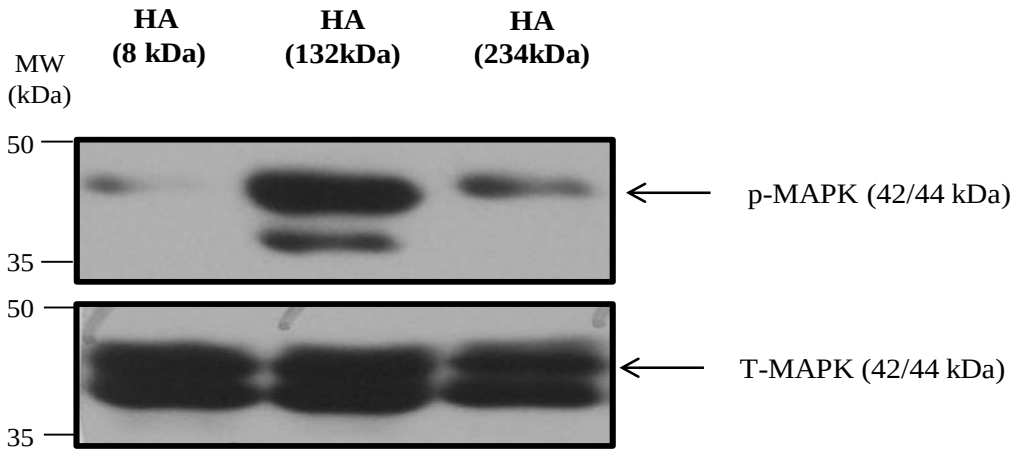


Figure 3.10 Effect of three different types of HA on MAPK signalling in Tam-R cells
Tam-R cells were cultured in experimental medium (EM + 4-OH-TAM) until log-phase growth was reached and then treated with three different commercially available molecular weights of HA namely 8kDa, 132kDa and 234kDa (average molecular weight) at a concentration of 200µg/ml for 5 minutes and then lysed on ice. p-MAPK and T-MAPK expression was evaluated through Western blotting as described in **Section 2.4**. A representative blot is shown above. The most effective p-MAPK upregulation was noted following use of medium molecular weight HA (132kDa).

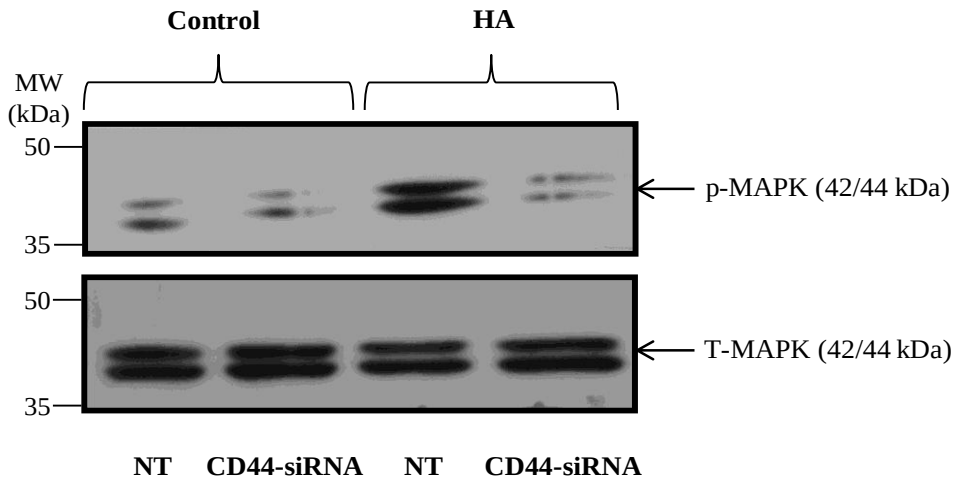
3.3.2 Effect of CD44 Knockdown on MAPK Upregulation following HA Treatment

We proceeded to assess whether the p-MAPK response to HA was clearly CD44-mediated or whether other potential mechanisms and receptors were involved in this response. Tam-R cells were transfected with CD44-siRNA following the standardised protocol described in **Section 2.6** and compared with NT cells. Once log-hase growth was reached, cells were treated with HA for 5 minutes. No significant decrease in band density at the expected molecular weight for p-MAPK was noted in CD44-siRNA treated cells compared to NT cells (**Figure 3.11A**). NT cells treated with HA demonstrated a clear increase in band density at the expected molecular weight for p-MAPK compared to untreated cells, but this increase was not observed in Tam-R cells transfected with CD44-siRNA (**Figure 3.11A**). Semi-quantitative analysis of densitometry values revealed that this differential response to HA between NT and CD44-siRNA transfected Tam-R cells was a statistically significant finding (*p*-value = 0.006 on *t*-test: NT – Control versus HA; **Figure 3.11B**).

This experimental design was repeated using immunocytochemistry as well. CD44-siRNA transfection did not have any significant effect on the basal activity of p-MAPK in Tam-R cells on immunocytochemistry. NT cells treated with HA demonstrated significant upregulation of p-MAPK but this upregulation was not evident in CD44-siRNA transfected cells (**Figure 3.12A**). Semi-quantitative analysis using H-score showed that this differential response to HA between NT and CD44-siRNA transfected cells was statistically significant (*p*-value = 0.001 on *t*-test: NT – Control versus HA; **Figure 3.12B**). On immunocytochemistry, p-MAPK was noted to be primarily localised to the nucleus and cytoplasm as expected.

These data suggested that whilst basal MAPK signalling in Tam-R cells was not significantly influenced by CD44, HA treatment and thus potentially CD44 activation, resulted in upregulation of MAPK activity.

A



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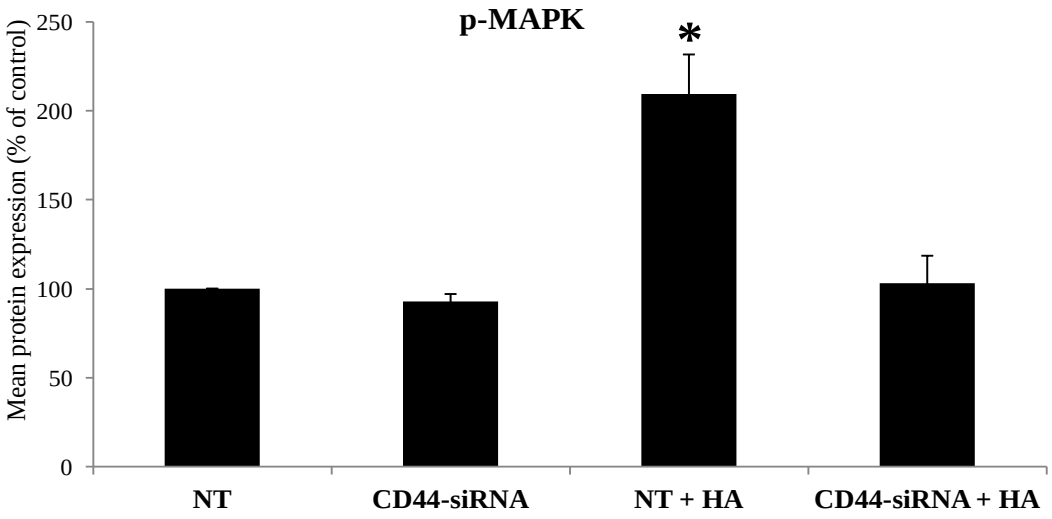

Figure 3.11 Effect of CD44 knockdown on MAPK upregulation following HA treatment in Tam-R cells on Western blotting

Tam-R cells were cultured in 35mm dishes until 50% confluence was reached and treated using either non-targeting siRNA (NT) or CD44-specific siRNA (CD44-siRNA) for 72 hours prior to lysis. Thereafter, medium was replaced with EM ± HA (200µg/ml) for 5 minutes and p-MAPK expression evaluated through Western blotting. Representative blots are shown above (A). Data was normalised and results presented as mean protein expression (% of control) ± SD (Mean protein expression – NT: Control 100% versus HA 209.3 ± 22.4% [*p*-value = 0.006 on *t*-test], CD44-siRNA: Control 92.8 ± 4.2% versus HA 103.1 ± 15.4% [*p*-value > 0.05/NS on *t*-test], n=3; B). p-MAPK upregulation in response to HA was significantly attenuated following CD44 knockdown.

A

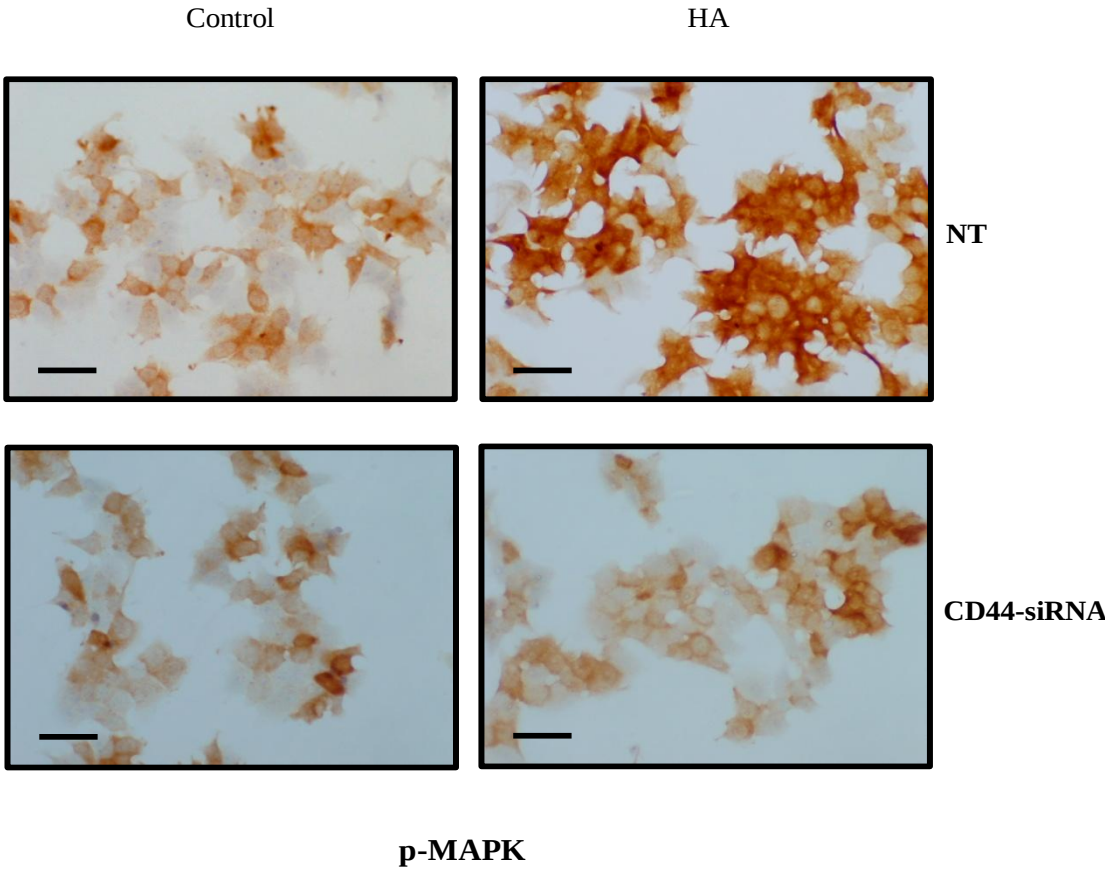

Figure 3.12A Effect of CD44 knockdown on MAPK upregulation following HA treatment in Tam-R cells on immunocytochemistry

Tam-R cells were cultured on glass cover slips until 50% confluence was reached and transfected with non-targeting siRNA (NT) or CD44-specific siRNA (CD44-siRNA) for 72 hours. Cells were then treated with EM ± HA (200µg/ml) for 5 minutes, fixed with Formal saline and p-MAPK expression was assessed through immunocytochemistry. Representative images are shown above (A). H-score was calculated and data presented as mean H-score (% of control) ± SD (Mean H-score – NT: Control 100% versus HA 200.9 ± 19.7% [*p*-value = 0.001 on *t*-test], CD44-siRNA: Control 110 ± 42.2% versus HA 128.6 ± 47.6% [*p*-value > 0.05/NS on *t*-test], n=3; B). Use of CD44-siRNA significantly attenuated p-MAPK upregulation following HA treatment. Scale bar = 50µm (20x magnification).

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B

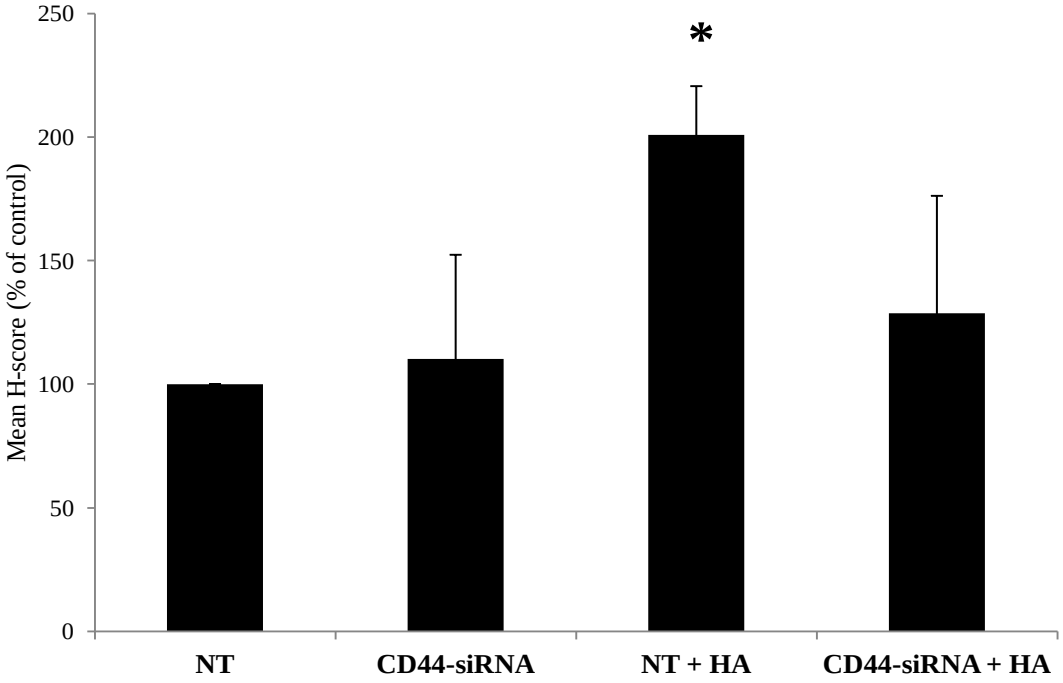

Figure 3.12B Effect of CD44 knockdown on MAPK upregulation following HA treatment in Tam-R cells on immunocytochemistry

Tam-R cells were cultured on glass cover slips until 50% confluence was reached and transfected with non-targeting siRNA (NT) or CD44-specific siRNA (CD44-siRNA) for 72 hours. Cells were then treated with EM ± HA (200µg/ml) for 5 minutes, fixed with Formal saline and p-MAPK expression was assessed through immunocytochemistry. Representative images are shown above (A). H-score was calculated and data presented as mean H-score (% of control) ± SD (Mean H-score – NT: Control 100% versus HA 200.9 ± 19.7% [*p*-value = 0.001 on *t*-test], CD44-siRNA: Control 110 ± 42.2% versus HA 128.6 ± 47.6% [*p*-value > 0.05/NS on *t*-test], n=3; B). Use of CD44-siRNA significantly attenuated p-MAPK upregulation following HA treatment. Scale bar = 50µm (20x magnification).

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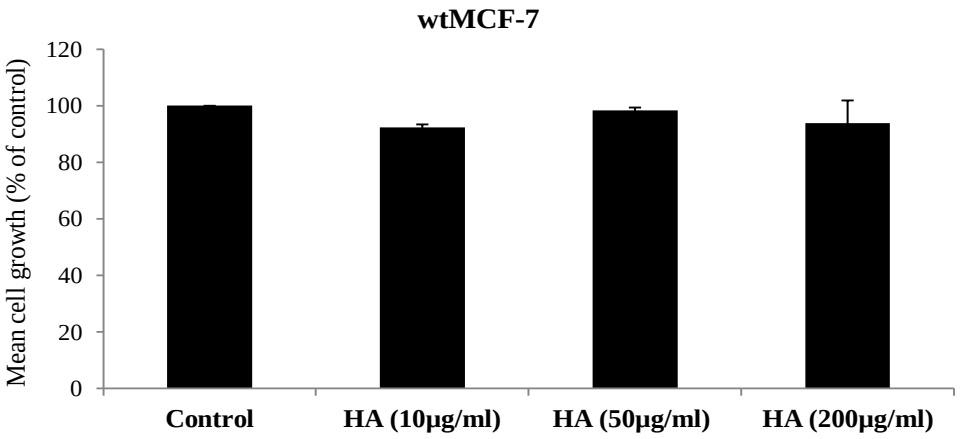
3.3.3 Effect of HA Stimulation on Growth of wtMCF-7 and Tam-R Cells

Next, we proceeded to assess the effect of HA stimulation on the growth Tam-R cells both with and without CD44 knockdown. Initial experiments explored the role of CD44 in influencing cell growth and for this experiment, wtMCF-7 and Tam-R cells were set up in 24-well plates as described in **Section 2.9** and cell counting was performed at the end of the assay to determine growth rates in response to HA. There was no effect of HA treatment on growth of wtMCF-7 cells (*p*-value > 0.05/NS on *t*-test; **Figure 3.13A**). HA produced no growth effect on Tam-R cells (p-value > 0.05/NS; **Figure 3.13B**). No further experiments were conducted to investigate the role of CD44 on growth as there was no significant effect noted following treatment with HA in Tam-R cells.

A



B

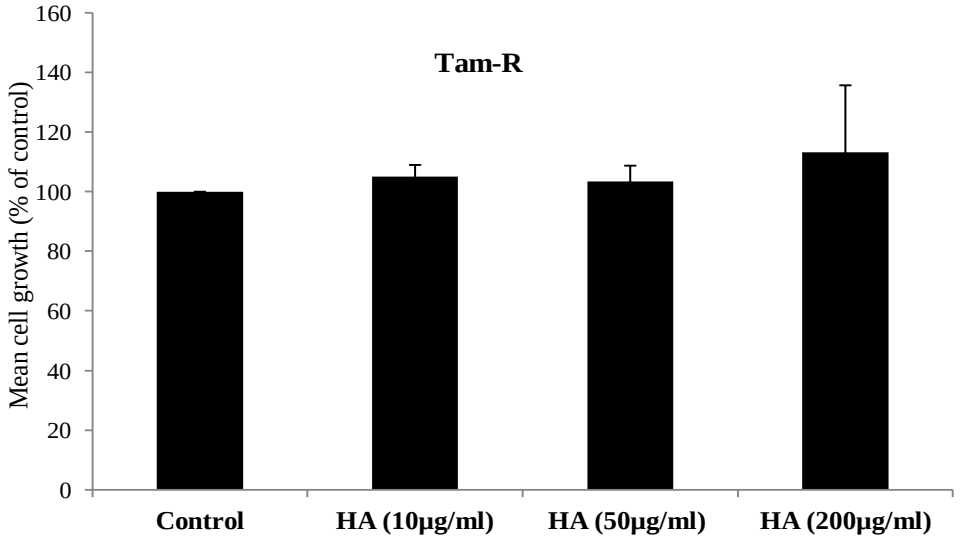


Figure 3.13 Effect of HA treatment on growth in wtMCF-7 and Tam-R cells
wtMCF-7 and Tam-R cells were cultured in experimental medium (EM ± 4-OH-TAM) and then treated with four different concentrations of HA (0, 10, 50 and 200µg/ml). Cell growth assay was performed using the protocol described in **Section 2.9.** Results were collated after 5 days and data presented as mean cell growth (% of control) ± SD. Treatment of wtMCF-7 cells with HA did not result in any effect on growth (A). Treatment of Tam-R cells with HA did not result in any growth effect (Mean cell growth – Control 100% versus HA'200µg/ml' 113.1 ± 24.4%, n=3, *p*-value > 0.05/NS on *t*-test; B). Overall, there was no effect of HA on growth of wtMCF-7 or Tam-R cells at the indicated doses.

3.3.4 Effect of HA Stimulation on Migration in Tam-R and wtMCF-7 Cells

To investigate the influence of CD44 in promoting a migratory phenotype in Tam-R cells, wound healing assays were set up using Tam-R cells in the presence and absence of HA. Tam-R cells showed a clear increase in migratory response following HA treatment compared to untreated cells (**Figure 3.14A**). Wound size was measured at 0 and 36 hours and results collated and expressed as mean wound closure (% of control) compared to untreated control. The wound closure rate of Tam-R cells treated with HA was 1.8 fold higher than untreated cells (*p*-value = 0.01 on *t*-test; **Figure 3.14B).**

To assess whether this increase in the rate of wound closure was a pure migratory response and to examine the role of cell proliferation, Ki67 based immunocytochemical assay was performed in parallel on cover slips along with the standard wound healing assay. Ki67, a nuclear non-histone protein, is now well established as a marker for proliferation in breast cancer and it has been suggested that it might have a prognostic role as well (Weigel and Dowsett 2010). This assay did not show any clear difference in Ki67 expression in Tam-R cells treated with HA compared to untreated controls although it must be mentioned that a formal quantification was not performed (**Figure 3.14C)**. This suggests that the difference in the wound closure rate following HA treatment is likely to be due to a differential migratory response rather than a variable rate of proliferation. Pre-treatment with an anti-proliferative agent such as mitomycin C would help to elucidate this further by inhibiting proliferation altogether.

Subsequently, CD44-siRNA transfection studies were used to elucidate whether the increased migratory response seen in Tam-R cells was a CD44-dependant phenomenon. Wound healing assays were set up with NT and CD44-siRNA transfected cells in the presence and absence of

HA. The migratory response of Tam-R cells noted after treatment with HA was eliminated in CD44-siRNA transfected cells was still noted in the NT cells (**Figure 3.15A).** Semi-quantitative analysis of the wound healing assay confirmed that this was a statistically significant finding (*p*-value = 0.01 on *t*-test: NT – Control versus HA; **Figure 3.15B**). This suggested that the HA-induced migratory response seen in Tam-R cells is mediated through CD44.

To investigate the influence of HA on the migratory phenotype of wtMCF-7 cells, wound healing assays were set up using wtMCF-7 cells in the presence and absence of HA. Wound size was measured at 0 and 36 hours and results collated and expressed as mean wound closure compared to untreated control. We noted that wtMCF-7 cells did not show any significant difference in the rate of wound closure following treatment with HA compared to untreated controls (*p*-value = 0.13 on *t*-test; **Figure 3.16A&B**).

A

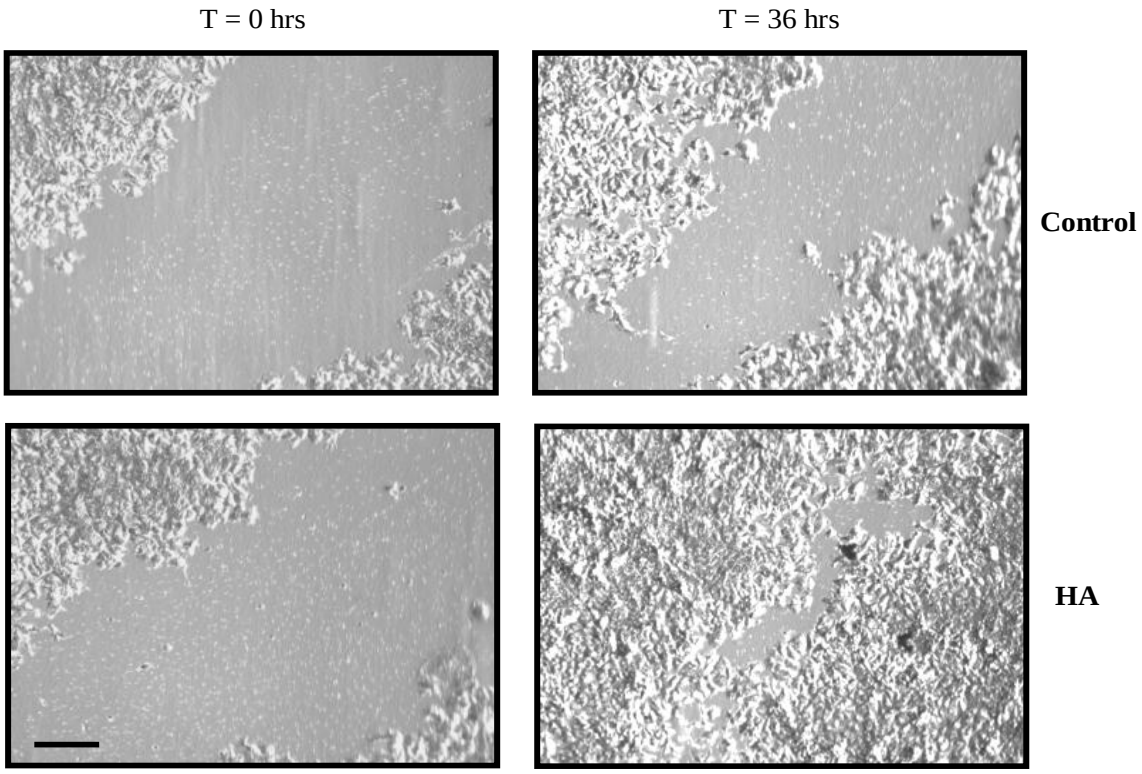
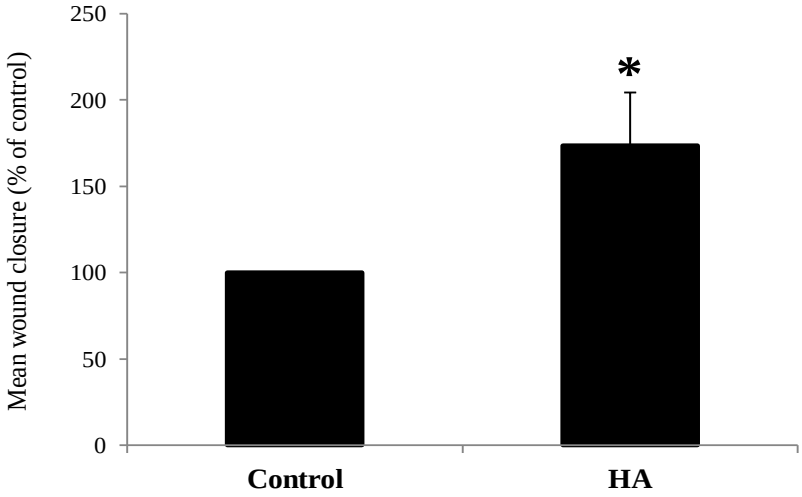


Figure 3.14A Effect of HA treatment on migration in Tam-R cells
Tam-R cells were cultured in 35mm dishes until 90% confluence was reached and wound healing assay performed as described in **Section 2.7.1** following treatment with EM ± HA (200µg/ml). Representative images are shown above (A). Results were collated after 36 hours and data presented as mean wound closure (% of control) ± SD (Mean wound closure – Control 100% versus HA 173.5 ± 30.9%, n=3, *p*-value = 0.01 on *t*-test; B). Cells were also simultaneously cultured on glass coverslips, treated as above, fixed with formal saline and immunocytochemical staining of Ki-67 performed. Representative images are shown (C). Similar levels of Ki67 staining between the control and HA treated cells suggested that the HA-induced response in Tam-R cells is more likely to be a differential migratory response rather than due to a change in rate of proliferation. Scale bar = 100µm (10x magnification).

B



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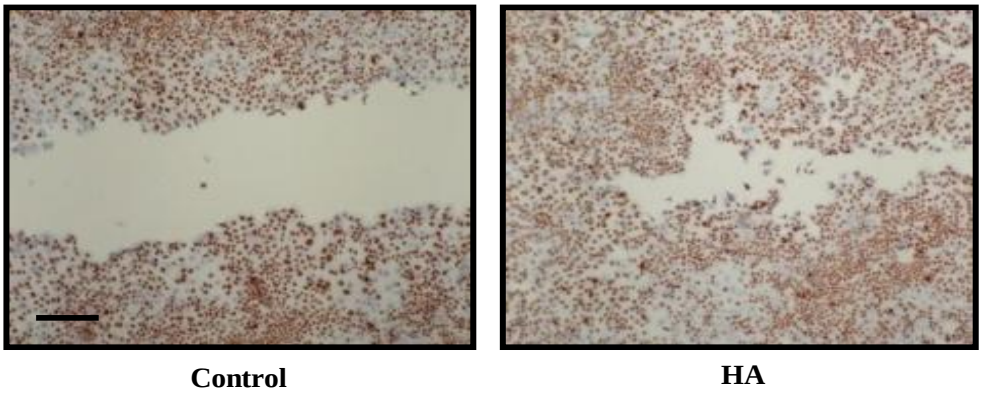


Figure 3.14B&C Effect of HA treatment on migration in Tam-R cells
Tam-R cells were cultured in 35mm dishes until 90% confluence was reached and wound healing assay performed as described in **Section 2.7.1** following treatment with EM ± HA (200µg/ml). Representative images are shown above (A). Results were collated after 36 hours and data presented as mean wound closure (% of control) ± SD (Mean wound closure – Control 100% versus HA 173.5 ± 30.9%, n=3, *p*-value = 0.01 on *t*-test; B). Cells were also simultaneously cultured on glass coverslips, treated as above, fixed with formal saline and immunocytochemical staining for Ki-67 performed. Representative images are shown (C). Similar levels of Ki67 staining between the control and HA treated cells suggested that the HA-induced response in Tam-R cells is more likely to be a differential migratory response rather than due to a change in rate of proliferation. Scale bar = 100µm (10x magnification).

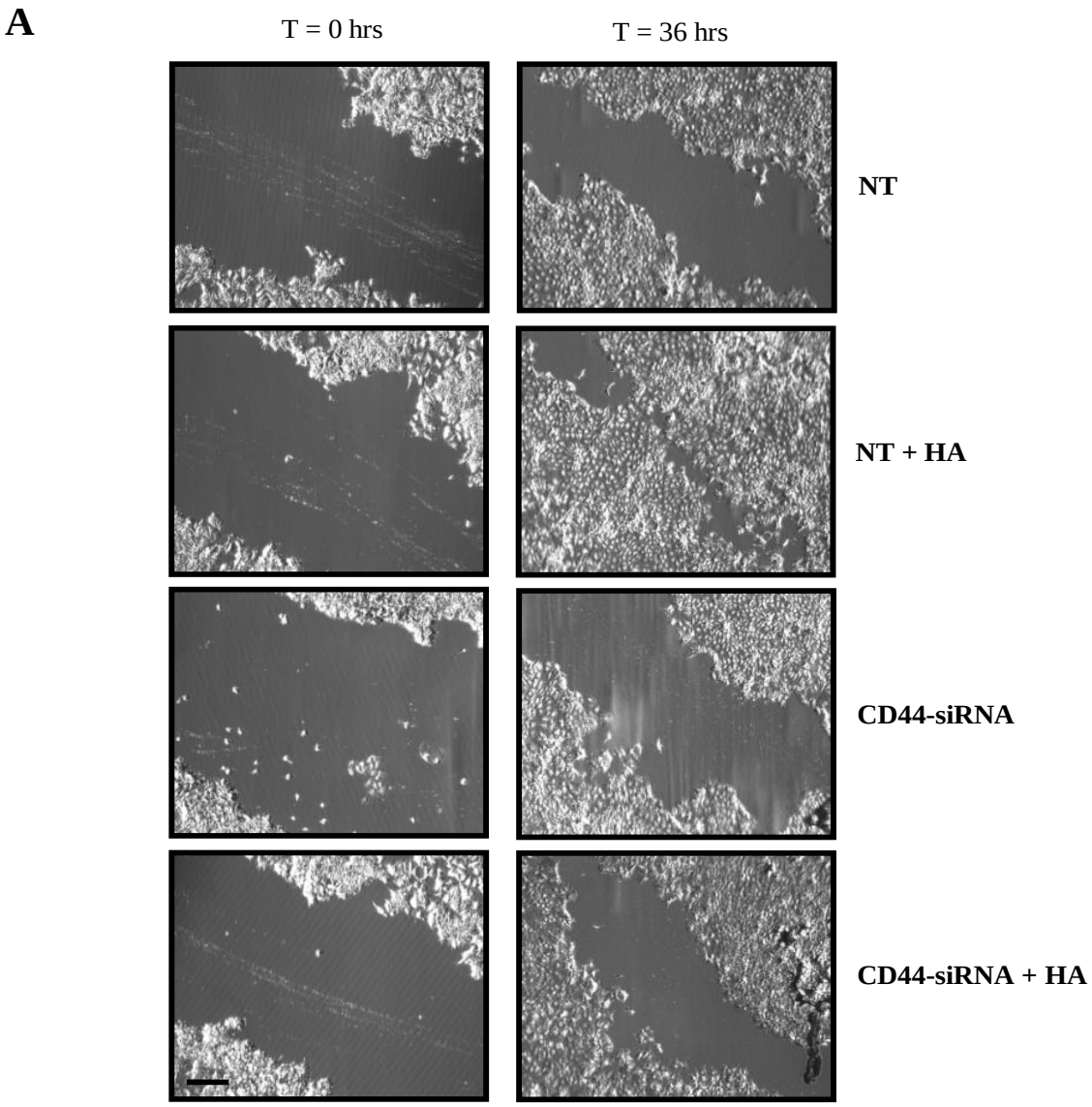
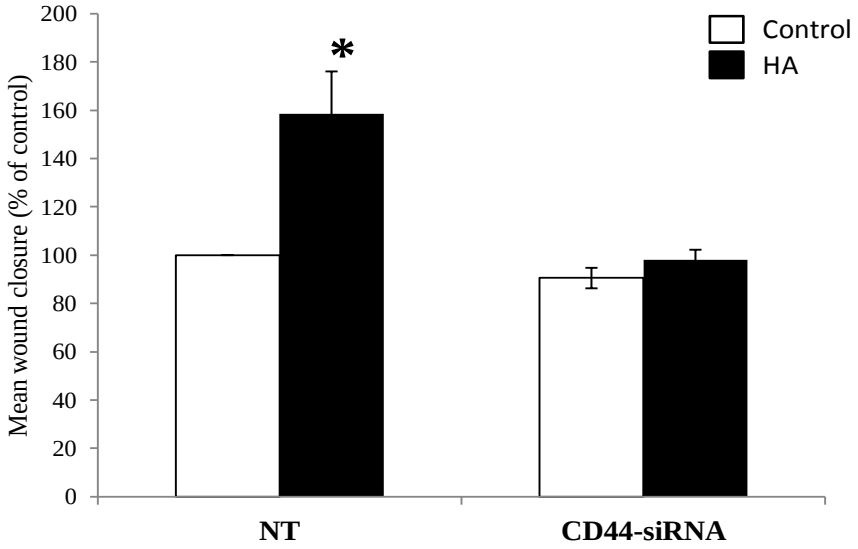


Figure 3.15A Effect of HA treatment on migration in Tam-R cells following CD44 knockdown

Tam-R cells were cultured in 35mm dishes until 50% confluence was reached. Cells were then transfected with NT-siRNA (NT) or CD44-specific siRNA (CD44-siRNA) for 72 hrs and wounds created in the monolayer as described in **Section 2.7.1** followed by treatment with EM ± HA (200µg/ml). Representative images are shown above (A). Results were collated after 36 hours and data presented as mean wound closure (% of control) ± SD (Mean wound closure – NT: Control 100% versus HA 158.5 ± 4.2% [*p*-value = 0.01 on *t*-test], CD44-siRNA: Control 90.5 ± 17.6% versus HA 97.9 ± 4.3% [*p*-value > 0.05/NS on *t*-test], n=3; B). HA-induced migratory effect in Tam-R cells is reversed following CD44-siRNA transfection. Cells were also simultaneously cultured on glass coverslips for CD44 immunocytochemical analysis which confirmed effective CD44 knockdown (C). Scale bar = 100µm (10x magnification).

B



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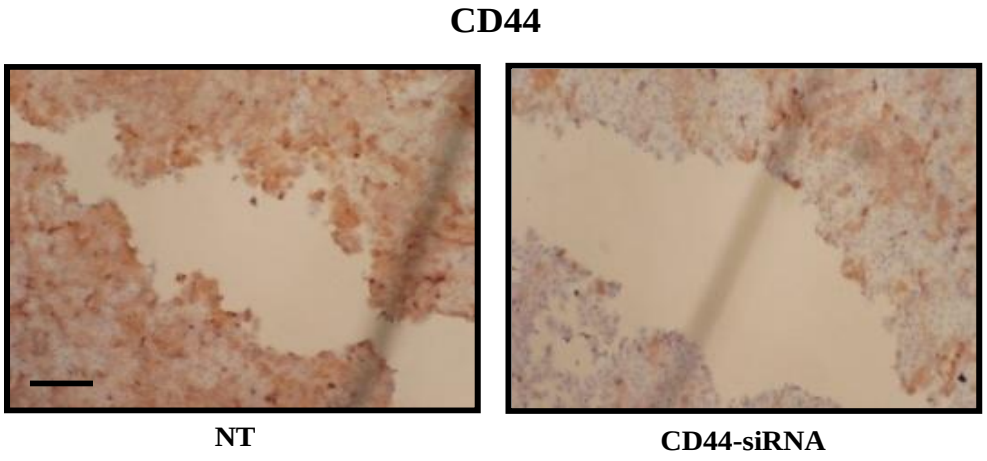


Figure 3.15B&C Effect of HA treatment on migration in Tam-R cells following CD44 knockdown

Tam-R cells were cultured in 35mm dishes until 50% confluence was reached. Cells were then transfected with NT-siRNA (NT) or CD44-specific siRNA (CD44-siRNA) for 72 hrs and wounds created in the monolayer as described in **Section 2.7.1** followed by treatment with EM ± HA (200µg/ml). Representative images are shown above (A). Results were collated after 36 hours and data presented as mean wound closure (% of control) ± SD (Mean wound closure – NT: Control 100% versus HA 158.5 ± 4.2% [*p*-value = 0.01 on *t*-test], CD44-siRNA: Control 90.5 ± 17.6% versus HA 97.9 ± 4.3% [*p*-value > 0.05/NS on *t*-test], n=3; B). HA-induced migratory effect in Tam-R cells is reversed following CD44-siRNA transfection. Cells were also simultaneously cultured on glass coverslips for CD44 immunocytochemical analysis which confirmed effective CD44 knockdown (C). Scale bar = 100µm (10x magnification).

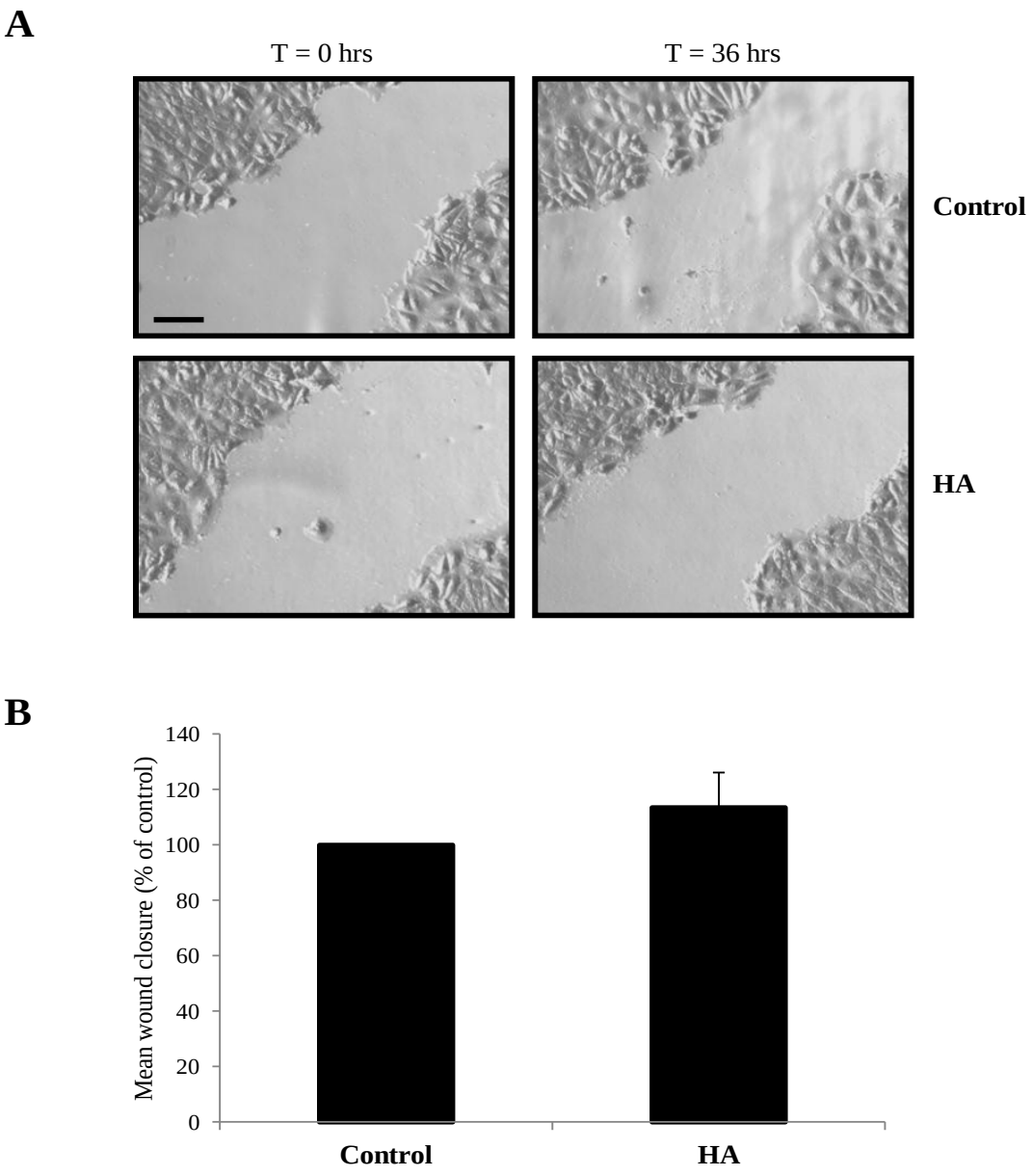


Figure 3.16 Effect of HA treatment on migration in wtMCF-7 cells
wtMCF-7 were cultured in 35mm dishes until 90% confluence was reached and wounds created in the monolayer followed by treatment with EM ± HA (200µg/ml). Representative images are shown above (A). Results were collated after 36 hours and data presented as mean wound closure (% of control) ± SD (Mean wound closure – Control 100% versus HA 113.5 ± 12.6%, n=3, *p*-value = 0.13 on *t*-test; B). Treatment of wtMCF-7 with HA did not result in a clear migratory effect. Scale bar = 100µm (10x magnification).

3.3.5 Discussion

The interplay between cancer cells and stromal elements in the surrounding microenvironment is critical in influencing tumour development and progression. In this study, we have investigated whether CD44 overexpression in Tam-R cells sensitises them to HA which is widely present in the stroma and we have used exogenous HA for experimental purposes. HA plays a crucial structural role in extracellular tissue and there is growing evidence of its role in influencing cell signalling pathways which are reported to mediate growth, survival, and migration of cancer cells via its interaction with its main cell-surface receptor, CD44 (Toole 2004; Toole and Slomiany 2008). Exogenous HA has been used by other groups to understand the role of CD44 in various malignancies (Bourguignon, Zhu et al. 2000). Binding of HA to CD44 has been previously shown to alter the actin cytoskeleton system, through ankyrin and GTPases such as Rho, with resultant induction of cell migration (Bourguignon 2008). HA has also been suggested to mediate clustering of CD44 on the cell surface with a resultant increase in cell invasion through sequestering of MMP9 (Peng, Su et al. 2007). Furthermore, various studies have shown that inhibition of HA production results in suppression of breast tumour development and metastasis providing further credence to the link between HA, CD44 and aggressive cell behaviour (Udabage, Brownlee et al. 2005; Hosono, Nishida et al. 2007; Urakawa, Nishida et al. 2011). These in vitro and in vivo data may explain why HA expression in clinical breast cancer is an independent negative prognostic factor for predicting survival (Auvinen, Tammi et al. 2000; Toole 2009).

In our study, we noted a differential effect on Tam-R cell signalling between the different molecular weights of HA, an effect that has also been noted by other researchers. For example, in the BT549 human breast cancer cell line, Yang et al. demonstrated a strong

relationship between HA size and CD44 clustering *in vivo* and also noted that HA regulates cell adhesion in a manner specifically dependent on its size (Yang, Cao et al. 2012). To our knowledge this differential effect has not been observed in a model of tamoxifen resistance before.

Although the dose-dependant p-MAPK upregulation following HA treatment suggested a direct effect, we proceeded to perform studies using CD44-siRNA to exclude the possibility that the HA action was being mediated via non-CD44 pathways. The main reasoning behind this approach was that HA is known to have another receptor which has also been shown to be associated with cell signalling processes. Known as CD168 or RHAMM ('Receptor for Hyaluronan Acid Mediated Motility') it was first isolated from cardiac embryonic cells and like CD44 also undergoes alternative splicing (Turley, Noble et al. 2002). A recent study showed that a HA-induced CD44, RHAMM and MAPK (ERK1/2) complex sustains a highly motile phenotype in the ER-negative MDA-MB-231 and Ras-MCF10A breast cancer cell lines (Hamilton, Fard et al. 2007). Moreover, Affymetrix® analysis showed that RHAMM levels are higher in Tam-R compared to wtMCF-7 cells although this was not shown to be statistically significant (**Appendix I**). Our study findings suggest that CD44, rather than RHAMM, is a mediator of MAPK upregulation following HA treatment. However, due to the possibility of co-dependance between CD44 and RHAMM, further studies using RHAMM-specific siRNA are required to draw a definitive conclusion.

Our data suggests that HA induces a CD44-mediated upregulation of p-MAPK in Tam-R cells and this was a novel and exciting finding in the setting of tamoxifen resistance as MAPK is part of the Ras-Raf-MEK-ERK/MAPK pathway which is directly downstream to EGFR. MAPK signalling via EGFR-HER2 heterodimers has previously been implicated in

our Tam-R cells (Knowlden, Hutcheson et al. 2003). The fact that this MAPK upregulation is CD44 driven suggested a link between CD44 and EGFR in our Tam-R cells.

Our data showed a significant CD44-mediated migratory effect of HA in Tam-R cells. Similar observations have been made in other breast cancer cell lines. Zohar et al. showed that a HA-CD44-ERM complex promotes migration of embryonic fibroblasts, activated macrophages, and metastatic breast cancer cells (Zohar, Suzuki et al. 2000). Bourguignon et al. demonstrated that HA-induced breast tumour migration in the SP1 cell line is mediated via CD44 where linkage between CD44v3 and Tiam1 (a guanine nucleotide exchange factor) leads to a cytoskeletal mediated enhancement of cell migration (Bourguignon, Zhu et al. 2000). Kung et al. have demonstrated a pro-migratory effect of HA in breast cancer cells via enhanced membrane-type 1 matrix metalloproteinase expression and CD44 cleaving (Kung, Chen et al. 2012). Jothy et al. noted that pan-CD44 and CD44v6-specific antibodies have a blocking effect on cell migration in the highly metastatic Hs578T breast cancer cell line (Herrera-Gayol and Jothy 1999).

HA-induced MAPK upregulation in our Tam-R cells raised the possibility that the enhanced migration may be mediated through the MAPK pathway. HA-induced phenotypic changes through involvement of MAPK signalling pathway in breast cancer cells has been noted before. For example, Fang et al. showed that when the expression vector pcDNA3.1-CD44st was cloned and stably transfected into wtMCF-7 cells, HA could interact with this transfected CD44 standard form and increase the invasion capability of wtMCF-7 cells through the MAPK pathway (Fang, Jiang et al. 2011).

3.3.6 Conclusion

Based on our data, we concluded that HA induces a CD44-mediated upregulation of MAPK signalling and enhances the migratory behaviour of Tam-R cells. Following this observation, we proceeded to investigate the interplay between CD44 and ErbB family in our Tam-R cells.

3.4 INTERACTION BETWEEN CD44 AND ErbB RECEPTORS

Several published studies indicate that CD44 can interact with and potentially transactivate receptor tyrosine kinases (RTKs), including those of the ErbB family. Our aim was to investigate whether CD44 interacted with ErbB receptors in Tam-R cells. To do this, we studied the co-localisation patterns between CD44 and ErbB receptors using immunoflourescence. We assessed the role of CD44 and HA on ErbB dimerisation and signalling in Tam-R cells using siRNA knockdown, Western blotting and immunoprecipitation. We also investigated the mechanism behind the HA-induced pro-migratory effect seen in Tam-R cells especially with regard to the potential role of ErbB receptors.

3.4.1 Co-localisation of CD44 and ErbB Receptors

Dual immunofluorescence staining was performed to investigate whether there was co-localisation of CD44 and ErbB receptors in Tam-R cells. Immunoflourescent staining of Tam-R cells grown under basal conditions revealed similar staining patterns for both CD44 and HER2 which, when the images were merged, suggested an apparent co-localisation of these receptors in the membrane (**Figure 3.17;** data provided courtesy of C. Smith and N. Jordan). Of note, HER3 was found to be expressed in the nucleus as well as the membrane but only the membrane HER3 was found to co-localise with CD44. ErbB receptors are known to be present in very low levels in wtMCF-7 cells and are barely detectable using standard techniques as reported previously by our group (Knowlden, Hutcheson et al. 2003).

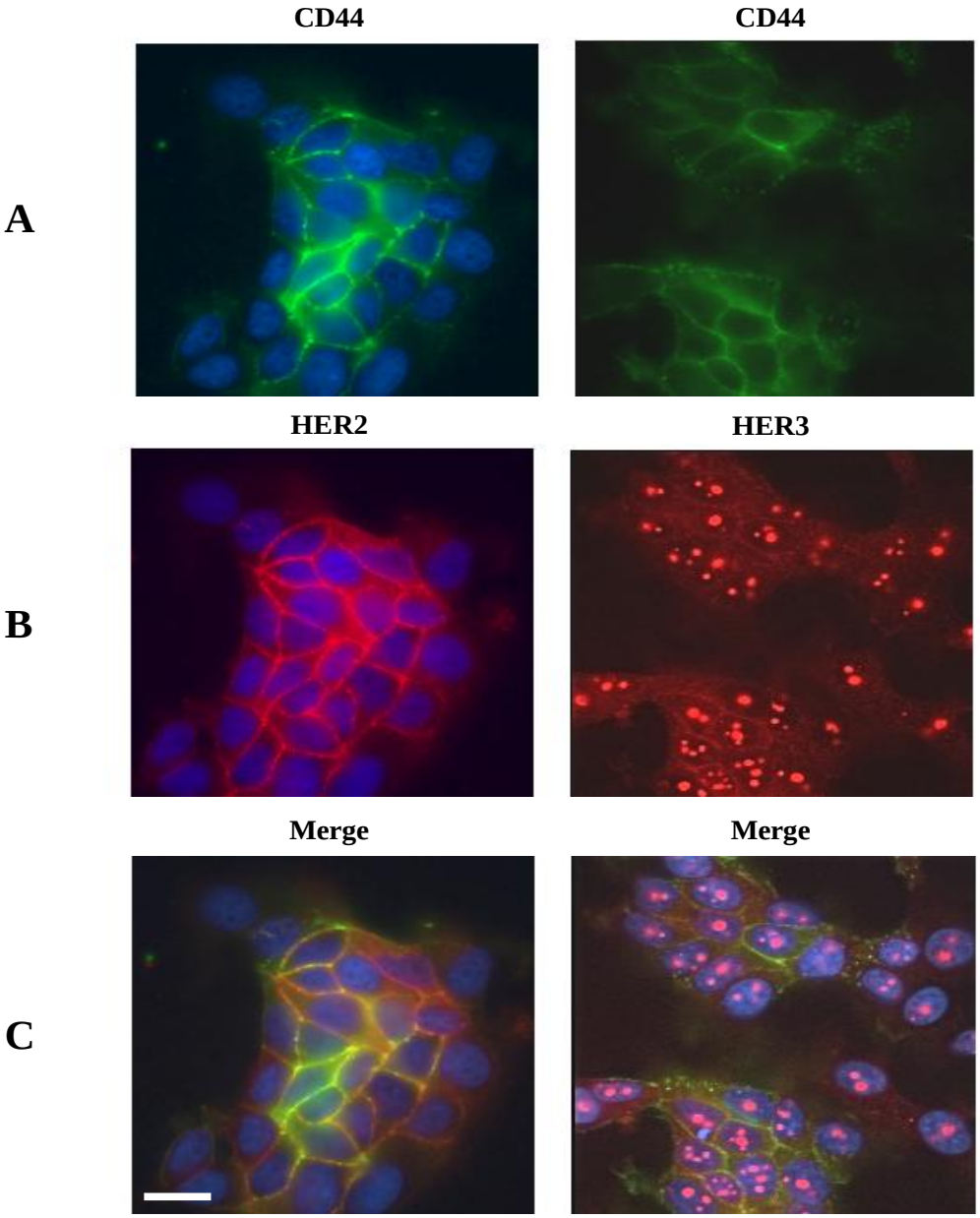


Figure 3.17 Co-localisation of CD44 with HER2 and HER3 in basal Tam-R cells
Tam-R cells were cultured in 35mm dishes with EM + 4-OH-TAM until log-phase growth was reached and cells were fixed. Immunofluorescence analysis was carried out using the method described in **Section 2.10** and slides examined under an inverted fluorescence microscope. Representative images are shown above. Immunofluorescence microscopy revealed a largely cell-surface location of CD44 (green, A). Subsequent staining for HER2 and HER3 (red) and image overlay revealed apparent co-localisation (yellow/orange) of these receptors in Tam-R cells (B,C). Of note, HER3 was expressed in the nucleus and the membrane but only the membrane HER3 co-localised with CD44. Scale bar = 25µm (63x magnification). Data provided courtesy of C. Smith and N. Jordan.

3.4.2 Role of CD44 in Basal ErbB Signalling in Tam-R Cells

Having noted that CD44 co-localises with HER2 and HER3 in Tam-R cells and given that CD44 has been linked to ErbB receptor activation in other breast cancer cell lines (Palyi-Krekk, Barok et al. 2008), we investigated whether CD44 knockdown affects the endogenous activity of ErbB members

Basal EGFR signalling was compared between Tam-R cells transfected with NT or CD44-siRNA. On Western blotting, a 175kDa protein band corresponding to the expected size of phosphorylated EGFR (p-EGFR: Y1068) was noted in both NT and CD44-siRNA transfected Tam-R cells, but the band density was significantly lower in the CD44-siRNA cells (**Figure 3.18A).** No similar decrease in band density was noted in total EGFR (T-EGFR) levels between the two groups (**Figure 3.18A).**

Subsequently, basal HER2 signalling was compared between Tam-R cells transfected with NT and CD44-siRNA. On Western blotting, a 185kDa protein band corresponding to the expected size of p-HER2 (Y1248) was noted in both NT and CD44-siRNA transfected Tam-R cells, but the band density was significantly lower in the CD44-siRNA cells (**Figure 3.18B).** There was a small decrease in band density noted in T-HER2 levels as well between the two groups (**Figure 3.18B)**. This indicates that there may be a reduction in absolute levels of p-HER2 in Tam-R cells following CD44 siRNA knockdown. Since HER2 phosphorylation is known to have important effects on downstream signalling, decrease in absolute levels of p-HER2 is likely to have an impact on signalling in Tam-R cells. It has previously been noted that CD44 knockdown using siRNA does not lead to any decrease in basal migration, invasion and growth (**Section 3.2**). Further investigation is required to understand the role played by CD44 in basal ErbB signalling and subsequent impact on function in Tam-R cells.

Having established that CD44 co-localises with HER2 and appears to play a role in endogenous ErbB activation, we proceeded to investigate the impact of CD44 activation through HA on ErbB signalling in Tam-R cells.

3.4.3 Effect of HA Stimulation on ErbB Signalling in Tam-R Cells

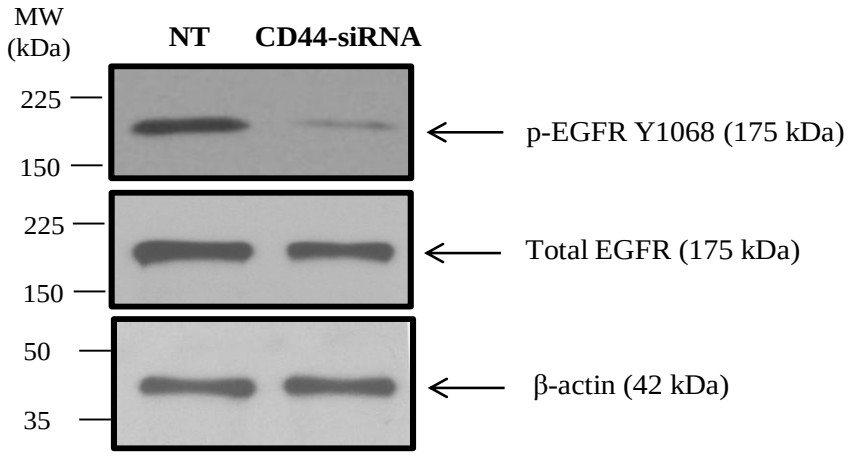
We hypothesised that HA treatment of Tam-R cells would further augment ErbB signalling. To investigate the effect of HA treatment and determine the role of CD44 activation on ErbB signalling, EGFR and HER2 expression was compared between NT and CD44-siRNA transfected Tam-R cells in the presence or absence of HA.

On Western blotting, a 175kDa protein band corresponding to the expected size of p-EGFR was noted in both NT and CD44-siRNA transfected Tam-R cells, but the band density was significantly lower in the CD44-siRNA treated cells. Moreover, following HA treatment, there was a clear increase in p-EGFR band density in NT cells but no corresponding change was noted in the CD44-siRNA transfected cells. Semi-quantitative analysis using densitometry after correcting for level of T-EGFR and loading showed a statistically significant 1.6 fold increase in p-EGFR activity following HA treatment in the NT cells which was not noted in the CD44-siRNA transfected cells (*p*-value = 0.02 on *t*-test; NT – Control versus HA **Figure 3.19A&B**).

On Western blotting, a 185kDa protein band corresponding to the expected size of p-HER2 was noted in both NT and CD44-siRNA transfected cells, but the band density was lower in the CD44-siRNA treated cells. Following HA treatment, there was a clear increase in p-

HER2 band density in the NT cells but no corresponding change was noted in the CD44-siRNA cells. Semi-quantitative analysis using densitometry after correcting for level of T-HER2 and loading showed a 1.7 fold increase in p-HER2 activity following HA treatment in the NT cells whereas CD44-siRNA transfected cells showed attenuated response to HA with only a 1.4 fold increase (*p*-value = 0.04 on ANOVA; **Figure 3.20A&B**).

A



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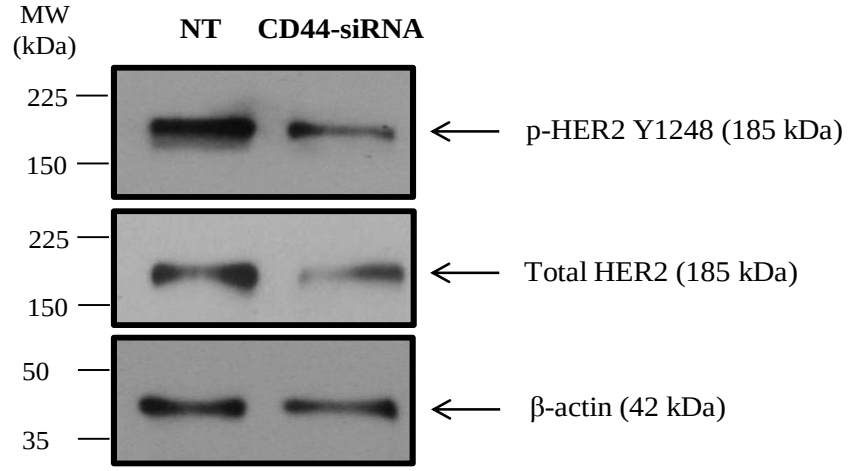


Figure 3.18 Effect of CD44 knockdown on basal EGFR and HER2 signalling in Tam-R cells

Tam-R cells were cultured in EM + 4-OH-TAM until 50% confluence was reached and treated using either non-targeting siRNA (NT) or CD44-specific siRNA (CD44-siRNA) respectively for 72 hours prior to lysis on ice. EGFR and HER2 protein expression was evaluated through Western blotting as described in **Section 2.4**. Representative blots are shown above. Use of CD44-siRNA reduced basal p-EGFR expression but did not affect T-EGFR levels (A). CD44-siRNA transfection resulted in decrease in p-HER2 and T-HER2 expression in Tam-R cells (B).

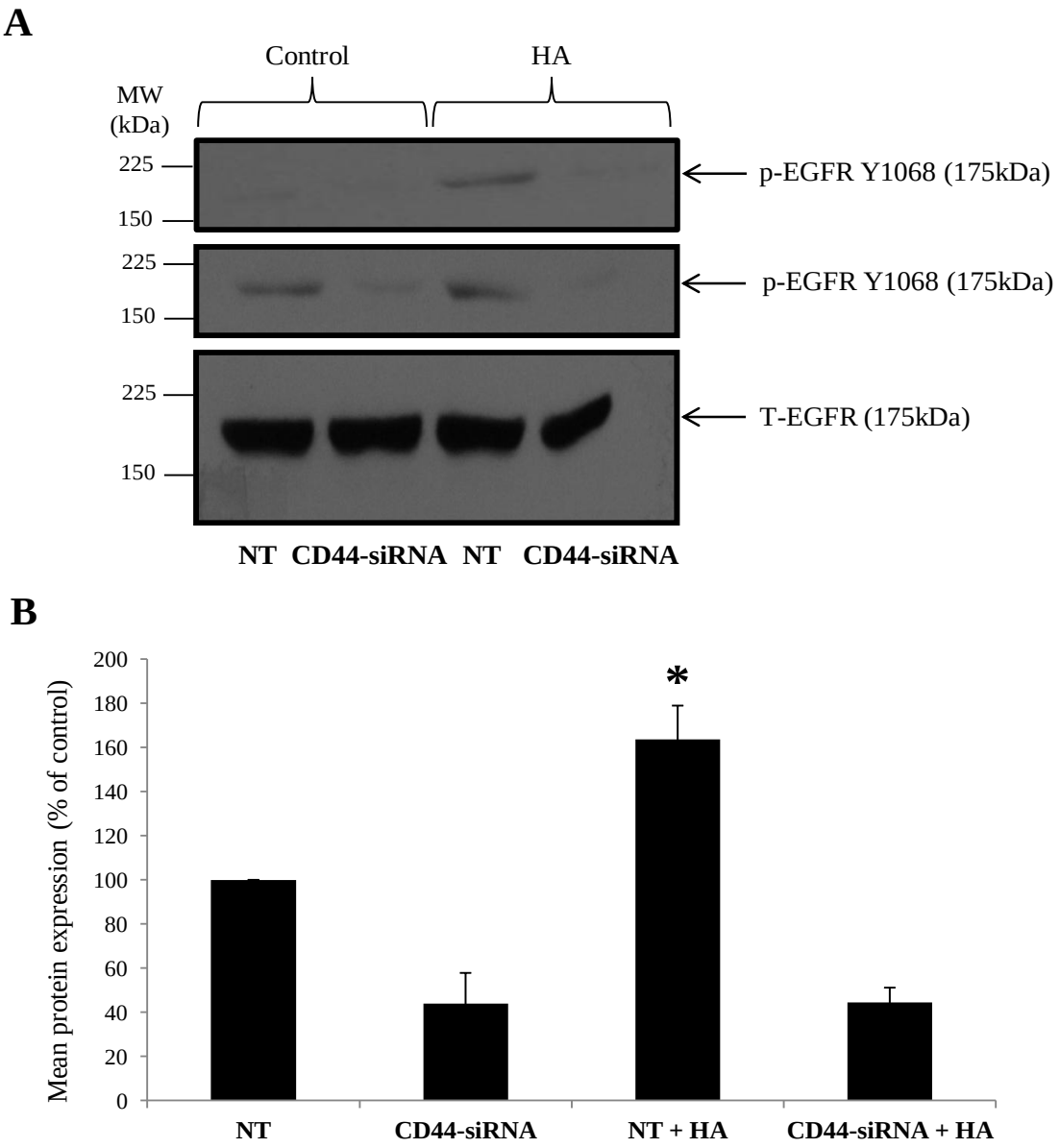


Figure 3.19 Effect of HA and CD44 knockdown on EGFR signalling in Tam-R cells
Tam-R cells were cultured in EM + 4-OH-TAM until 50% confluence was reached and treated using either non-targeting siRNA (NT) or CD44-specific siRNA (CD44-siRNA) for 72 hours. Thereafter, medium was replaced with EM ± HA (200µg/ml) for 5 minutes and EGFR expression evaluated through Western blotting. Representative blots are shown above (A). Data was normalised and results presented as mean protein expression ± SD (B). CD44-siRNA knockdown resulted in significant inhibition of basal p-EGFR activity (Mean protein expression – NT 100% versus CD44-siRNA 44.1 ± 13.8%, n=3, *p*-value = 0.02 on *t*-test; B). There was clear upregulation of p-EGFR expression following HA treatment but this response was markedly attenuated in CD44-siRNA transfected cells (Mean protein expression – NT: Control 100% versus HA 163.6 ± 15.3% [*p*-value = 0.02 on *t*-test], CD44-siRNA: Control 44.1 ± 13.8% versus HA 44.6 ± 6.6% [*p*-value > 0.05/NS on *t*-test], n=3; B).

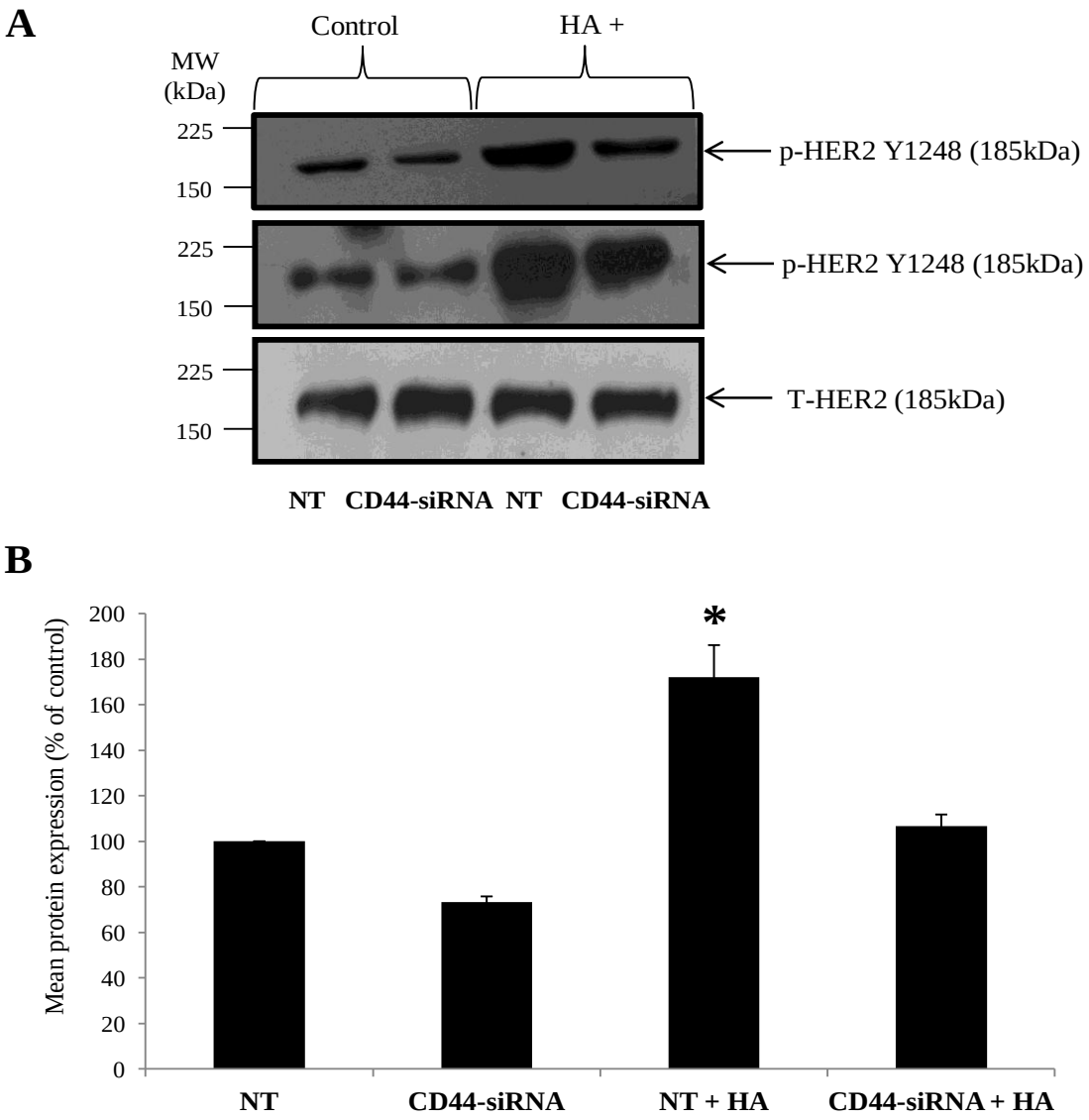


Figure 3.20 Effect of HA and CD44 knockdown on HER2 signalling in Tam-R cells
Tam-R cells were cultured until they reached 50% confluence and treated using either NT or CD44-siRNA for 72 hours. Thereafter, medium was replaced with EM ± HA(200µg/ml) for 5 minutes and HER2 expression evaluated through Western blotting. Representative blots are shown above (A). Data was normalised and results presented as mean protein expression ± SD (B). CD44 knockdown resulted in inhibition of basal p-HER2 activity but this was not statistically significant (Mean protein expression – NT 100% versus CD44-siRNA 73.4 ± 2.5%, n=3, *p*-value > 0.05/NS on *t*-test; B). There was clear upregulation of p-HER2 expression following HA treatment but this response was attenuated in CD44-siRNA transfected cells (Mean protein expression – NT: Control 100% versus HA 172.0 ± 14.1% [*p*-value = 0.001 on *t*-test], CD44-siRNA: Control 73.4 ± 2.5% versus HA 106.6 ± 5.2% [*p*-value = 0.004 on *t*-test], Overall *p*-value = 0.04 on ANOVA n=3; B).

3.4.4 Role of ErbB in HA-induced Migratory Response in Tam-R Cells

These data suggested that HA treatment played a clear role in activation of ErbB signalling in Tam-R cells and this appeared to be mediated by CD44. We had previously noted in **Section 3.3.4** that HA treatment elicited a clear migratory response in Tam-R cells which was reversed following CD44 knockdown. In the light of our new observation indicating upregulation of ErbB activity following HA treatment, we hypothesised that the migratory response could also be mediated by ErbB members possibly via the MAPK pathway, and we proceeded to test this hypothesis.

We performed wound healing assays to determine the role of MAPK, EGFR and HER2 signalling in this migratory response. The effect of three different drugs, namely PD098059 (50µM in dimethylsulfoxide), gefitinib (1µM in ethanol) and trastuzumab (100nM in dH_2O) on migration in Tam-R cells was assessed. PD098059 is a well established MAPK inhibitor thought to exert its effect by binding to the inactive form of MAPK1 (Alessi, Cuenda et al. 1995). Gefitinib (Iressa®) is a selective, reversible inhibitor of the tyrosine kinase domain of EGFR (Solomon, Hagekyriakou et al. 2003). Trastuzumab (Herceptin®) is a monoclonal antibody effective against the HER2 or *neu* receptor and acts by binding to the extracellular juxta-membrane domain of HER2 (Hudis 2007). These drugs have been used in our Tam-R model at the above concentrations in previous studies with proven physiological effect thus supporting their use at the stated concentrations in this study (Knowlden, Hutcheson et al. 2003; Knowlden, Hutcheson et al. 2005). Use of Akt and Src inhibitors would have further clarified the role of Akt and Src pathways in this response and this should be explored in future experiments.

Tam-R cells were cultured in 35mm dishes until they reached 90% confluence at which point wound healing assays were performed along with treatment with PD098059, gefitinib and trastuzumab in the presence or absence of HA. Results were collated after 36 hrs and presented as mean wound closure (% of control) ± SD. Representative images are shown in **Figure 3.21A**.

The effect of the three drugs on basal migration of Tam-R cells was first assessed. Although there was a trend towards reduction in migratory behaviour following treatment with all three drugs, in particular gefitinib, these changes were not found to be statistically significant (*p*-value > 0.05/NS for all three drugs compared to untreated control; **Figure 3.21A&B**).

Treatment of Tam-R cells with HA resulted in a statistically significant 1.7 fold increase in wound closure as compared to untreated cells (*p*-value = 0.02 on *t*-test**; Figure 3.21C)**. Use of the MAPK inhibitor (PD098059) resulted in attenuation of the migratory response of Tam-R cells to HA treatment (*p*-value > 0.05/NS on *t*-test; **Figure 3.21C**). Use of the EGFR inhibitor (Gefitinib) also resulted in attenuation of the migratory response (*p*-value > 0.05/NS on *t*-test; **Figure 3.21C**) as did treatment with Trastuzumab, the anti-HER2 antibody (*p*-value > 0.05/NS on *t*-test; **Figure 3.21C**). The experimental outcome suggested that the pro-migratory response following HA treatment in Tam-R cells is mediated by CD44 via MAPK and the attenuation noted following treatment with drugs that target EGFR and HER2 also suggest involvement of the ErbB receptors in this migratory response. This however needs to be examined in more detail before a definitive conclusion can be reached.

A

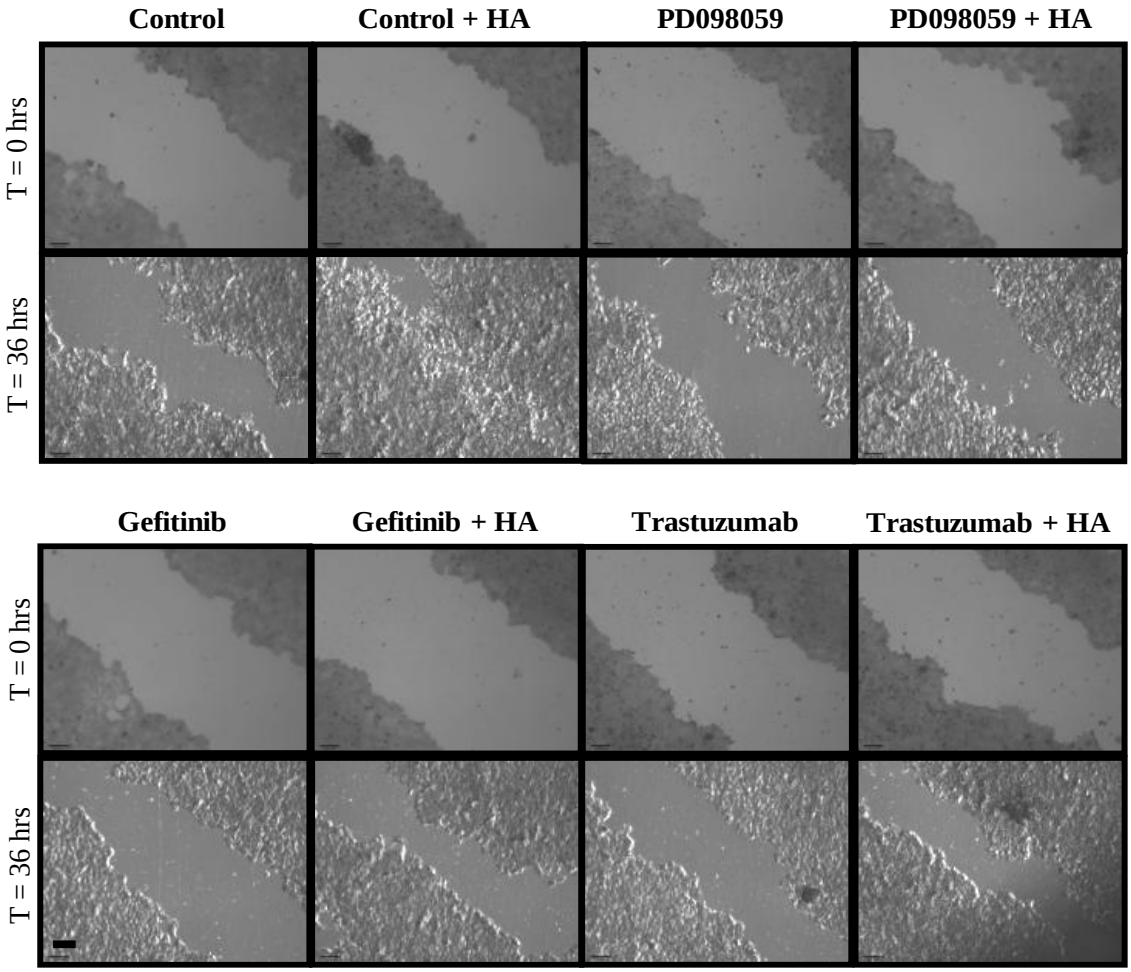
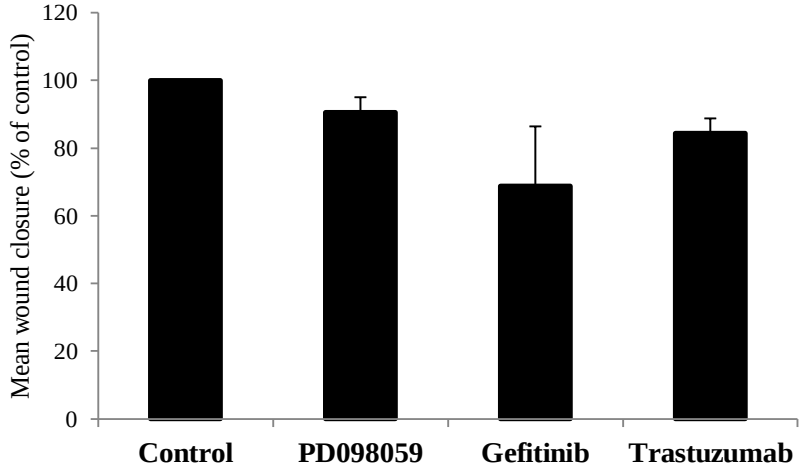


Figure 3.21A Effect of PD098059, gefitinib and trastuzumab on HA-induced migratory response in Tam-R cells

Tam-R cells were cultured in 35mm dishes until at least 90% confluence was reached and wound healing assay performed as described in **Section 2.7.1** alongside treatment with PD098059 (50µM in dimethylsulfoxide), Gefitinib (1µM in ethanol) and Trastuzumab (100nM in dH_2O) in the presence or absence of HA (200µg/ml). Representative images are shown above (A). Results were collated after 36 hours and data presented as mean wound closure (% of control) ± SD. There was no statistically significant decrease in basal migration following treatment with PD098059, Gefitinib or Trastuzumab although there was a trend towards decreased migration with Gefitinib (*p*-value > 0.05/NS on *t*-test for all three drugs compared to control; B). Treatment of Tam-R cells with PD098059, Gefitinib and Trastuzumab shows attenuation of HA-induced migratory response and is most marked after treatment with PD098059 (*p*-values on *t*-test: Untreated versus HA – Control: 0.02, PD098059: > 0.05/NS, Gefitinib: > 0.05/NS, Trastuzumab: > 0.05/NS). Scale bar = 100µm (10x magnification).

B



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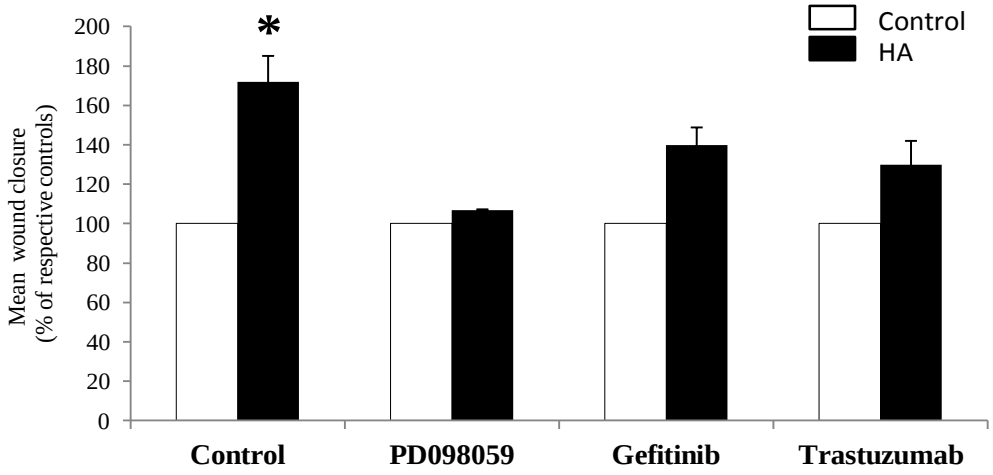


Figure 3.21B&C Effect of PD098059, gefitinib and trastuzumab on HA-induced migratory response in Tam-R cells

Tam-R cells were cultured in 35mm dishes until at least 90% confluence was reached and wound healing assay performed as described in **Section 2.7.1** alongside treatment with PD098059 (50µM in dimethylsulfoxide), Gefitinib (1µM in ethanol) and Trastuzumab (100nM in dH_2O) in the presence or absence of HA (200µg/ml). Representative images are shown above (A). Results were collated after 36 hours and data presented as mean wound closure (% of control) ± SD. There was no statistically significant decrease in basal migration following treatment with PD098059, Gefitinib or Trastuzumab although there was a trend towards decreased migration with Gefitinib (*p*-value > 0.05/NS on *t*-test for all three drugs compared to control; B). Treatment of Tam-R cells with PD098059, Gefitinib and Trastuzumab shows attenuation of HA-induced migratory response and is most marked after treatment with PD098059 (*p*-values on *t*-test: Untreated versus HA – Control: 0.02, PD098059: > 0.05/NS, Gefitinib: > 0.05/NS, Trastuzumab: > 0.05/NS). Scale bar = 100µm (10x magnification).

3.4.5 Association of CD44 and ErbB Receptors in Tam-R Cells

We noted earlier that exogenous HA can promote ErbB activation in the absence of exogenous ErbB ligands such as heregulin and this raised the possibility that CD44 may influence ErbB dimerisation either directly or indirectly. It is possible that this finding is somewhat confounded by the potential presence of low levels of ligands in the culture medium or autocrine production by the cells themselves. To investigate this, we performed immunoprecipitation using ErbB antibodies in the presence and absence of HA and also following CD44-siRNA transfection.

Under basal conditions, EGFR could not be co-immunoprecipitated with CD44 but treatment with HA resulted in EGFR-CD44 dimerisation which was reversed by CD44-siRNA (**Figure 3.22A: IP1**). Conversely, under basal conditions, CD44 and HER2 could be co-immunoprecipitated and HA treatment did not alter this interaction which reversed following CD44-siRNA transfection (**Figure 3.22B: IP4**). EGFR-HER2 dimerisation was promoted by HA and this was found to be CD44-mediated as CD44-siRNA transfection reversed this effect (**Figure 3.22B: IP6**). Under basal conditions, HER3 was preferentially dimerised with EGFR as compared to HER2. Interestingly, HA stimulation appeared to cause HER3 to dimerise preferentially with HER2 instead of EGFR and this effect was reversed by CD44-siRNA confirming that this is CD44-mediated (**Figure 3.22A&B: IP2&5**). These interactions are summarised in tabular form for clarity in **Tables 3.1 and 3.2.**

A

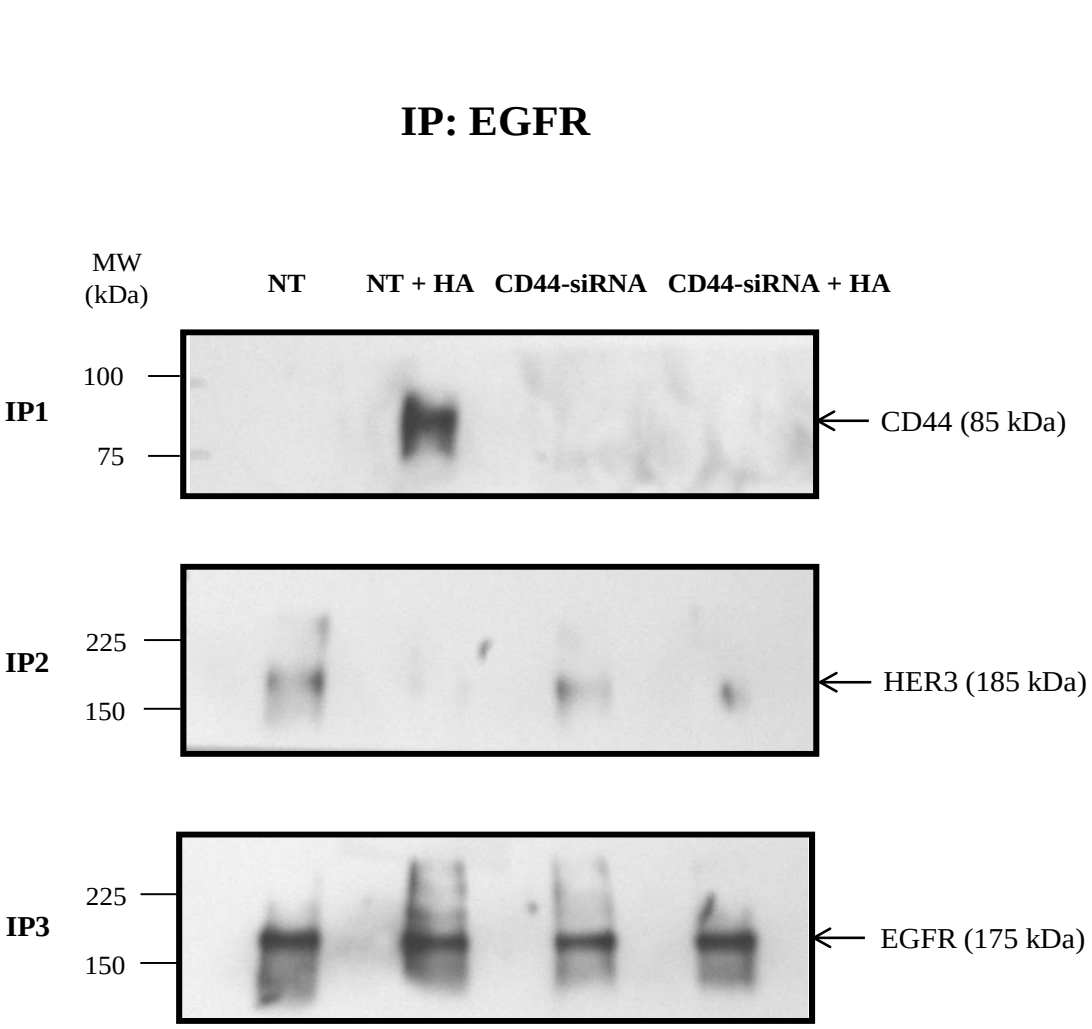


Figure 3.22A Effect of HA and CD44 knockdown on association between CD44 and ErbB receptors

Tam-R cells were cultured in EM + 4-OH-TAM until 50% confluence was reached and transfected with NT or CD44-siRNA for 72 hours. Thereafter, medium was replaced with EM ± HA (200µg/ml) for 5 minutes and CD44-ErbB interactions evaluated through immunoprecipitation as described in **Section 2.11**. Representative blots are shown above (A). Under basal conditions CD44-HER2 and EGFR-HER3 dimerisation was predominant. Following HA treatment, there was alteration in ErbB dimerisation patterns with preferential formation of CD44-EGFR and HER2-HER3 dimers which could be reversed following CD44-siRNA transfection indicating that they are CD44-mediated. Thus, HA-CD44 interaction can modulate ErbB dimerisation patterns. These interactions are presented in tabular form for clarity in **Table 3.1 and 3.2.**

B

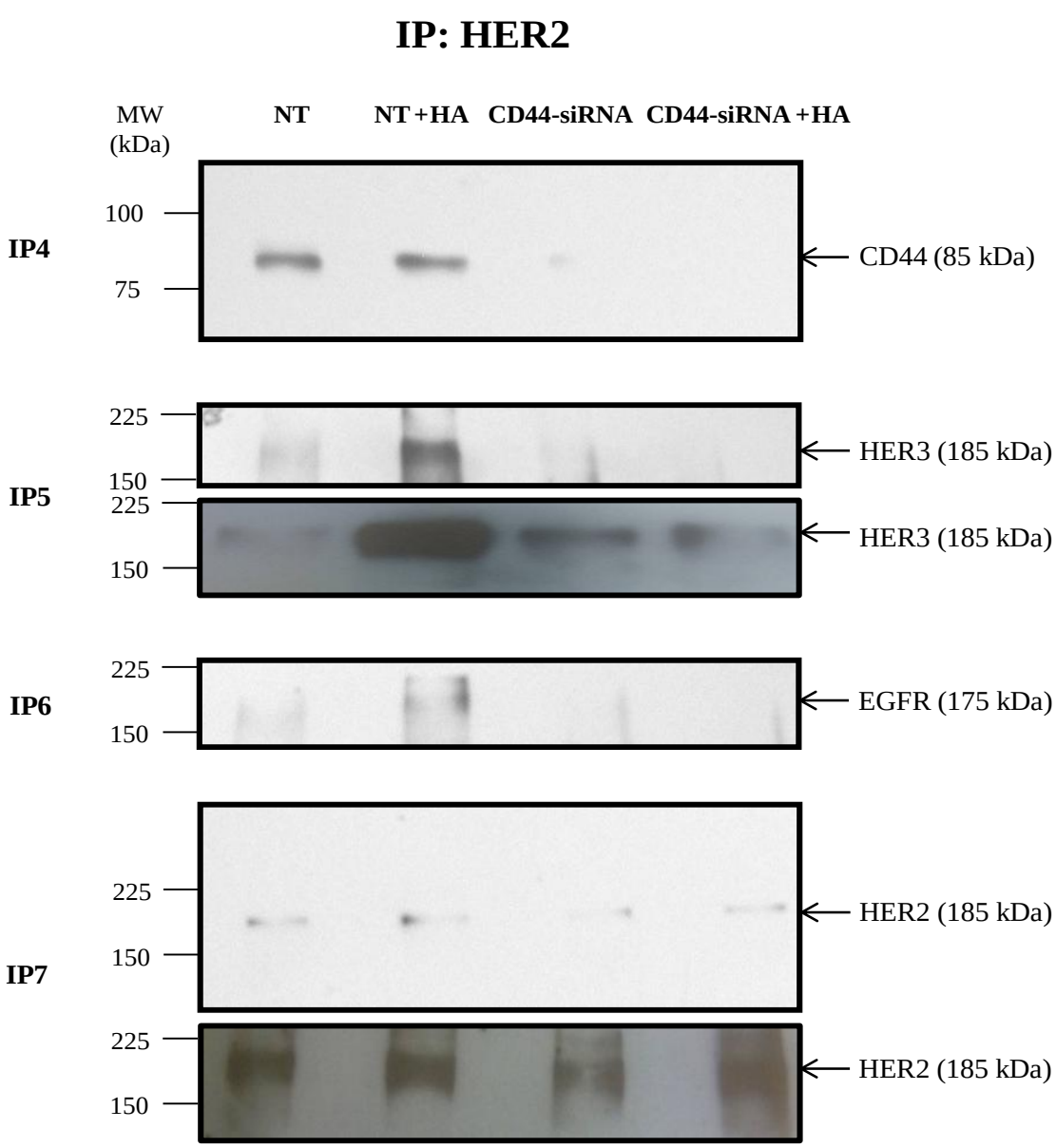


Figure 3.22B Effect of HA and CD44 knockdown on association between CD44 and ErbB receptors

Tam-R cells were cultured in EM + 4-OH-TAM until 50% confluence was reached and transfected with NT or CD44-siRNA for 72 hours. Thereafter, medium was replaced with EM ± HA (200µg/ml) for 5 minutes and CD44-ErbB interactions evaluated through immunoprecipitation as described in **Section 2.11**. Representative blots are shown above (A). Under basal conditions CD44-HER2 and EGFR-HER3 dimerisation was predominant. Following HA treatment, there was alteration in ErbB dimerisation patterns with preferential formation of CD44-EGFR and HER2-HER3 dimers which could be reversed following CD44-siRNA transfection indicating that they are CD44-mediated. Thus, HA-CD44 interaction can modulate ErbB dimerisation patterns. These interactions are presented in tabular form for clarity in **Table 3.1 and 3.2.**

Table 3.1 Immunoprecipitation patterns between CD44 and ErbB receptors in 'NT' transfected Tam-R cells before and after treatment with HA

	EGFR	HER2	HER3	CD44
EGFR	++ → ++	+ → ++	++ → +	- → ++
HER2	+ → ++	+ → +	+ → ++	+ → +

Key for Table 3.1 and 3.2

x → y = Expression level pre → post HA treatment
'-' = Minimal or absent
'+' = Present but low
'++' = High
'+++' = Very high

Table 3.2 Immunoprecipitation patterns between CD44 and ErbB receptors in 'CD44-siRNA' transfected Tam-R cells before and after HA treatment

	EGFR	HER2	HER3	CD44
EGFR	++ → ++	+ → +	++ →++	- →-
HER2	+ → +	+ → +	+ → +	- → -

3.4.6 Discussion

The ErbB family represents a group of four structurally related RTKs (EGFR, HER2, HER3 and HER4) which have all been implicated in signal transduction controlling normal cell growth and differentiation. Several growth factors serve as ErbB ligands and ErbB receptors can exist as both monomers, homodimers or heterodimers (Rubin and Yarden 2001). Although HER2 has no identified ligand, it is the preferred receptor for dimerisation with other ErbB receptors and, this initiates the most potent signal transduction pathway in the entire ErbB family (Rubin and Yarden 2001). The link between ErbB receptors and breast cancer is now well established and indeed EGFR and HER2 overexpression are associated with reduced sensitivity to endocrine agents and poorer prognosis (Wright, Nicholson et al. 1992; Nicholson, McClelland et al. 1993; Nicholson, McClelland et al. 1994). Elevated levels of EGFR-HER2 heterodimers have been suggested to provide an autocrine growth stimulus in our Tam-R cells (Knowlden, Hutcheson et al. 2003). There is clear evidence of association between CD44 and members of the ErbB family. We were, therefore, keen to understand the interaction between CD44 and ErbB receptors in our model of tamoxifen resistance and test our hypothesis that CD44 overexpression in Tam-R cells modulates ErbB activity and influences phenotype.

Knowlden et al. have previously noted that phosphorylated EGFR-HER2 and EGFR-HER3 but no HER2-HER3 heterodimerisation is evident in Tam-R cells (Knowlden, Hutcheson et al. 2003). Previous studies have also shown that EGFR and HER2 expression are higher in Tam-R compared with wtMCF-7 cells but both cell lines have similar expression levels of HER3. We have seen on immunoflourescence that CD44 and HER2 co-localise in the membrane. This is further substantiated by immunoprecipitation studies where there is evidence of CD44-HER2 as well as EGFR-HER3 but not CD44-EGFR heterodimerisation

under basal conditions. Furthermore, CD44 influences expression of both phosphorylated EGFR and HER2 but appears to influence EGFR in a more significant fashion. Our study thus adds to the growing understanding of ErbB associations and signalling mechanisms in our Tam-R cells. Interestingly, Ghayad et al. have also noted high levels of basal EGFR-HER3 dimerisation in their tamoxifen resistant wtMCF-7 derived cell line CL6.8 (Ghayad, Vendrell et al. 2009).

Other researchers have also noted the importance of the ErbB receptors in their models of tamoxifen resistance. Block et al. reported that their wtMCF-7 derived 4-OH-TAM model of resistance (MCF-7-TR) displays HER2 overexpression and EGFR was overexpressed in their T47D-derived model of tamoxifen resistance (T47D-TR) (Block, Grundker et al. 2012). Ghayad et al. showed that tamoxifen resistance in two of their wtMCF-7 derived cell lines (CL6.7 and CL6.8) was accompanied by activation of MAPK signalling and overexpression of EGFR and HER2 (Ghayad, Vendrell et al. 2009). Moreover, treatment with the MAPK inhibitor PD098059 restored endocrine response in both cell lines.

Our study findings suggest that CD44 may act as a modulator of ErbB dimerisation and consequently modify signalling and function in Tam-R cells. This is supported by data from immunoprecipitation experiments which show that HA-induced CD44 activation leads to leads to preferential formation of CD44-EGFR and HER2-HER3 instead of EGFR-HER3 heterodimers. This is particularly important in view of the evidence that HER2-HER3 heterodimerisation has been linked to heregulin-induced gefitinib resistance (Hutcheson, Knowlden et al. 2007).

We also noted during immunoflourescence that although membrane HER3 co-localises with CD44, HER3 is also detectable in the nucleus. Adilakshmi et al. have described a nuclear HER3 variant (nuc-ErbB3) that results from alternative transcription in rat primary Schwann cells and contains a functional nuclear homing signal sequence which can bind to chromatin (Adilakshmi, Ness-Myers et al. 2011). Koumakpayi et al. have reported HER3 nuclear localisation in prostate cancer cell lines and specimens (Koumakpayi, Diallo et al. 2006). Nuclear localisation of HER3 has been reported in immortalised human breast cells and breast cancer cell lines as well (Offterdinger, Schofer et al. 2002). The exact function of nuclear HER3 in our model is not clear but there is a possibility that it may be involved in altering cellular behaviour via a genetic mechanism and this needs to be examined further.

We have previously shown that HA stimulation of CD44 overexpressing Tam-R cells resulted in MAPK activation and increased migration. The data here suggest that the migratory response is mediated via the MAPK pathway. Interestingly, Lee et al. noted that bacteriophage hyaluronidase, HyIP, decreased breast cancer cell migration by inhibiting activation of MAPK suggesting a direct role of the MAPK pathway in stimulating migration (Lee, Moore et al. 2010). Our data also raises the possibility that these events may occur as a consequence of HA/CD44-induced ErbB transactivation, possibly involving both EGFR and HER2. However, this needs to be investigated in more detail.

The interplay between CD44, HA and ErbB receptors and the effect on migration as noted in this study are depicted in a schematic form in **Figure 3.22**.

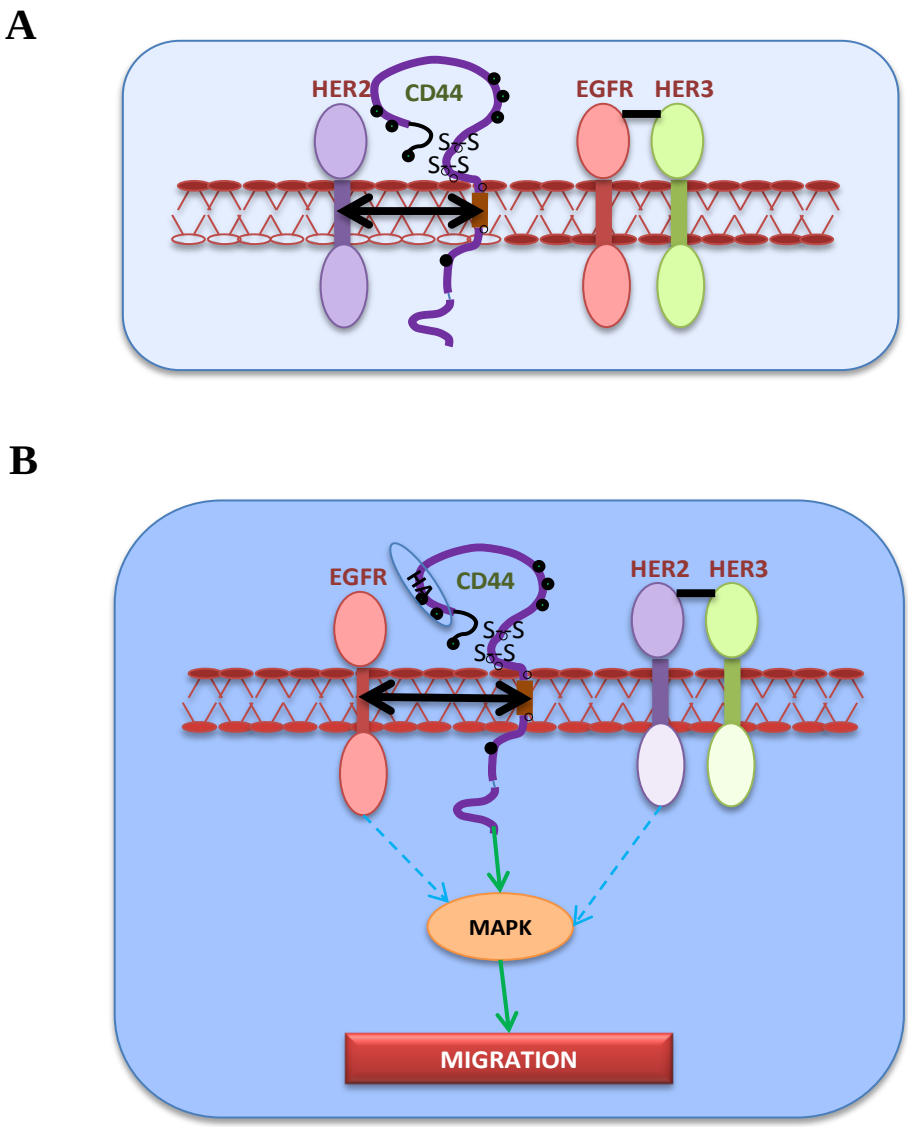


Figure 3.23 Schematic diagram of interplay between CD44, HA and ErbB receptors and the effect on migration in Tam-R cells as noted in this study
The interplay between CD44, HA and ErbB receptors in Tam-R cells as identified in this study is schematically depicted above. Immunoprecipitation studies have shown that CD44-HER2 and HER3-EGFR dimerisation is predominant in Tam-R cells under basal conditions as discussed in **Section 3.4.5** (A). HA-CD44 interaction leads to changes in ErbB dimerisation and signalling with a resultant effect on cell phenotype (B). CD44-EGFR and HER2-HER3 dimerisation is augmented following HA treatment as discussed in **Section 3.4.5**. Significant increase in the migratory phenotype of Tam-R cells is noted following HA treatment which appears to be mediated primarily via MAPK with a partial role of HER2 and EGFR as discussed in **Section 3.4.4.** Adapted from Linggi et al. 2006, Zoller 2011. (NB: CD44 is known to associate with ErbB members via its transmembrane domain and the extracellular domain in ErbB receptors is responsible for ErbB-ErbB dimerisation.)

3.4.7 Conclusion

CD44 is co-localised with HER2 and HER3 in Tam-R cells, influences basal ErbB signalling and HA-mediated CD44 activations results in alteration in ErbB dimerisation patterns.

Chapter Four

Discussion

4.1 GENERAL DISCUSSION

4.1.1 Resistance to Endocrine Therapy

Use of endocrine therapy has led to definite improvement in the prognosis of breast cancer worldwide. However, de novo and acquired resistance to currently available endocrine agents continues to be a significant limiting factor in further improving outcomes. Unfortunately, development of resistance often presents as metastatic disease and is accompanied by several features that indicate a more aggressive disease pattern compared to the primary malignancy. The prognosis associated with disease relapse following development of acquired resistance to anti-hormonal therapy is very poor. As such, there has been increasing scientific interest in understanding and elucidating the mechanisms of acquired resistance in an effort to identify novel targets and therapeutic approaches that might overcome resistance.

4.1.2 Use of In Vitro Models of Resistance

Various scientific strategies have been employed in an effort to understand the pathways involved in resistance. Of these, in vitro cell models continue to be one of the most frequently used tools in the laboratory for elucidating the molecular basis for resistance. Although simplified compared to in vivo models, they allow a cost-effective strategy for initial elucidation of the mechanisms underlying therapeutic resistance. They are especially valuable as they lend themselves well to various manipulations at the gene and protein level, and allow rapid testing of novel drugs, especially with regard to their modes of action and anti-cancer activity. Cell models of acquired tamoxifen resistance have been developed primarily through use of three main ER-positive cell lines, namely, wtMCF-7, T47D and ZR75-1 which respond well to oestrogen stimulation as well as anti-hormonal suppression (Wong and Chen 2012). The predominant strategy has been long-term culture of these cells in the presence of

anti-hormonal drugs until sustained growth is achieved despite the presence of the drug, thus, signalling the development of acquired resistance. Our group has developed several cell models based on wtMCF-7 and later T47D cells which `have been used to study signalling and function in the setting of endocrine resistance and these have provided some interesting insights into the mechanisms underlying endocrine resistance.

4.1.3 Current Evidence from Cell Models

Data from several studies modelling acquired endocrine resistance with in vitro breast cancer cell lines have suggested that prolonged exposure to active endocrine agents result in induction of growth factor mediated signalling pathways which eventually promote an endocrine-resistant state. Increased EGFR expression has been noted in wtMCF-7 based cell models of tamoxifen resistance (Ignatov, Ignatov et al. 2009). Studies from our group have also shown that Tam-R cells exhibit EGFR and HER2 overexpression at both mRNA and protein level compared with endocrine-sensitive wtMCF-7 cells (Knowlden, Hutcheson et al. 2003). This growth factor receptor induction can be noted even in the drug responsive phase of the development of resistance indicating that this is an early phenomenon in the development of resistance. Further evidence supporting the crucial role of growth factor signalling in resistance comes from transfection studies whereby overexpression of growth factor receptors in hormone-sensitive cells have rendered them endocrine-resistant. For example, transfection of wtMCF7 cells with HER2 was accompanied by a pattern of tamoxifen-resistant tumourigenic growth (Benz, Scott et al. 1992). Another observation that supports this concept is that signalling pathways that are downstream to these receptors are also significantly upregulated following development of endocrine resistance. Elevated levels of phosphorylated MAPK and upregulated Src-kinase activity have been noted in models of

tamoxifen resistance (Hiscox, Jiang et al. 2006; Knowlden, Jones et al. 2008; Morgan, Gee et al. 2009). Downregulation of negative regulators of these downstream pathways has also been noted. For example, expression of Caveolin-1, a negative regulator of the Ras-MAPK pathway which is encoded by a tumour suppressor gene, is markedly decreased as a stable tamoxifen-resistant phenotype develops in wtMCF-7 cells treated with 4-OH-TAM (Thomas, Hutcheson et al. 2009). Thus, there is reliable evidence that supports the critical role of growth factor receptors, especially receptor tyrosine kinases (RTKs) such as members of the ErbB family, in mediating signalling events that underpin the development of acquired resistance.

Apart from inducing signalling changes, induction of growth factor receptors also contribute to the development of an aggressive cell phenotype accompanied by enhanced migratory potential and invasiveness. An eight-fold increase in basal motility has been observed by Zhou et al. in their model of tamoxifen-resistant wtMCF-7 cells (Zhou, Zhong et al. 2012). Our tamoxifen-resistant Tam-R cells also display a dramatic and significant increase in invasiveness and motility compared to the wild type MCF-7 cells (Hiscox, Morgan et al. 2004). Tam-R cells also grow as loosely packed colonies with loss of cell-cell junctions and exhibit altered morphological characteristics which resemble cells undergoing epithelial-to-mesenchymal transition (EMT). Similar changes in phenotype with clear features of EMT have also been noted by Kim et al. in their TAM-R-MCF-7 model of tamoxifen resistance (Kim, Choi et al. 2009). Mediators such as Src kinase have been implicated in promoting this aggressive phenotype noted in endocrine resistance (Hiscox, Morgan et al. 2006).

Knowledge of these underlying signalling and phenotypic changes has allowed identification of potential therapeutic targets through which endocrine resistance might be overcome along with suppression of the resulting aggressive cellular characteristics. Such approaches have met with partial success in the laboratory setting. However, although results in cell models have been quite encouraging, use of such strategies in the clinical setting have yielded mixed results. This is evident, for example, when the preclinical and clinical experience with gefitinib is considered. As discussed earlier, gefitinib was the first agent with selective activity against the tyrosine kinase domain of EGFR. There was encouraging preclinical evidence that supported the possibility that gefitinib would be a potent drug in the setting of endocrine resistance. Gefitinib was shown to improve the anti-proliferative ability of tamoxifen and prevent development of tamoxifen resistance in both cell and animal models of ER-positive breast cancer (Gee, Harper et al. 2003; Shou, Massarweh et al. 2004). Gefitinib was also shown to eliminate ER-HER2 cross-talk and restore tamoxifen sensitivity in a model of ER/HER2-positive breast cancer exhibiting de novo tamoxifen resistance (MCF-7/HER2-18) as well as restore tamoxifen sensitivity in a model of acquired tamoxifen resistance, namely T47D-TR (Shou, Massarweh et al. 2004; Block, Grundker et al. 2012). Furthermore, gefitinib was shown to inhibit the enhanced invasive and motile phenotype noted in vitro in models of acquired tamoxifen resistance (Hiscox, Morgan et al. 2004). These and other studies led to the initiation of several trials which sought to investigate the benefit of gefitinib in breast cancer including trials of gefitinib monotherapy. Interestingly, the results from trials evaluating gefitinib monotherapy have shown contrasting results. For example, whilst Gutteridge et al. found that gefitinib at a dose of 500 milligram per day was well tolerated with a CBR of 53.6% in tamoxifen resistant breast cancer in their phase II trials, Green et al. reported that gefitinib at the same dose had no clinical benefit in hormone-

resistant advanced breast cancer (CBR 0%) in a multicentre phase II trial (Green, Francis et al. 2009; Gutteridge, Agrawal et al. 2010). Trials evaluating the benefit of combinational therapy of gefitinib with anastrozole have also reported conflicting results. For example, whilst Cristofanilli et al. reported that the combination of anastrozole and gefitinib is associated with an improvement in PFS (14.7 versus 8.4 months) over the combination of anastrozole and placebo in a randomised phase II trial, Carlson et al. did not find any additional benefit of the combination of anastrozole and gefitinib over anastrozole monotherapy (Cristofanilli, Valero et al. 2010; Carlson, O'Neill et al. 2012). Osborne et al. have noted an improved PFS in patients with ER-positive MBC receiving a combination of gefitinib and tamoxifen except patients who progressed or recurred following prior AI therapy (Osborne, Neven et al. 2011). Overall, despite encouraging preclinical data, the results of trials investigating gefitinib have yielded less promising results than expected. This illustrates the urgent need to identify further pathways that influence hormonal resistance and develop novel therapeutic approaches.

4.1.4 CD44 – RTK Interplay

Towards this end, we used Affymetrix® microarray analysis to screen various breast cancer cell models of resistance. This showed upregulation of CD44 in Tam-R cells compared to wtMCF-7 cells and this observation was later validated by RT-PCR studies. There is emerging evidence of the role of CD44-RTK interplay in influencing signalling and function in several malignancies including breast cancer and in the setting of endocrine resistance. CD44 can interact with and modulate the activity of various RTKs including Met, VEGFR-2, HER2 and EGFR (Singleton, Salgia et al. 2007; Tremmel, Matzke et al. 2009; Gordin, Tesio et al. 2010; Hatano, Shigeishi et al. 2010). We were particularly encouraged by published

links between CD44 and ErbB family receptors which have been shown to play an important role in various aspects of breast cancer progression. Overexpression of these receptors has also been linked with poor response to endocrine therapy (Newby, Johnston et al. 1997; Horiguchi, Koibuchi et al. 2005; Folgiero, Avetrani et al. 2008; Spears, Taylor et al. 2011). In fact, even in our Tam-R model of tamoxifen resistance, overexpression of ErbB receptors have been linked with an autocrine growth pathway (Knowlden, Hutcheson et al. 2003).

Various mechanisms via which CD44 can influence signalling and activation of ErbB members have been described in breast and other tissues. One of the mechanisms implicated in this process involves direct interaction between CD44 and ErbB family receptors. CD44 co-localises with ErbB receptors and has been co-immunoprecipitated with HER2 and EGFR in four different breast cancer cell lines namely wtMCF-7, MDA-MB-231, MDA-MB-435, and MDA-MB-436 (Wobus, Rangwala et al. 2002). CD44-HER2-EGFR complex formation has been demonstrated in cytology specimens from patients with metastatic breast cancer (Wobus, Rangwala et al. 2002). Another mechanism whereby CD44 transactivates ErbB receptors involves intracellular and extracellular intermediates. CD44-HA interaction can activate intracellular Src and phosphoinositide-3-kinase (PI3K) with consequent induction of HER2 and HER3 phosphorylation. This has been demonstrated in the TA3/St breast cancer cell line, where CD44-HA interaction sustains high levels of phosphorylated HER2, and blocking this interaction causes disruption of a complex containing HER2, CD44 and PI3K (Ghatak, Misra et al. 2005). CD44 can also activate ErbB receptors through extracellular or stromal intermediates such as the pro-form of heparin binding epidermal growth factor (HB-EGF) which is further cleaved by matrix associated metalloproteinase 7 (MMP7) allowing HB-EGF to bind and activate HER4 (Zoller 2011).

A further mechanism of ErbB transactivation involves HA-CD44 mediated alterations in ErbB dimerisation patterns and our study data supports the role of this mechanism in the setting of endocrine resistance. It has been shown in our Tam-R model that predominantly EGFR-HER2 and EGFR-HER3 heterodimers are seen basal conditions, but HER2-HER3 heterodimers are not readily detectable (Knowlden, Hutcheson et al. 2003). Data from our study shows that HA-CD44 interaction induces HER2-HER3 heterodimer formation. This pattern of dimerisation has previously been implicated in heregulin-mediated gefitinib resistance in Tam-R cells (Hutcheson, Knowlden et al. 2007). Data from our study also shows that HA-CD44 interaction leads to preferential formation of CD44-HER2 rather than CD44-EGFR heterodimers noted in the basal state, a novel finding in the setting of tamoxifen resistance. This is particularly important as the predominant mechanism of ErbB activation involves dimerisation changes when activated by eleven known ligands which include epidermal growth factor (EGF), heparin binding EGF-like growth factor (HB-EGF), transforming growth factor alpha (TGF-α), Neuregulins 1-4 amongst others (Linggi and Carpenter 2006). The exact mechanism of alterations in ErbB dimerisation patterns noted in our study following HA treatment and/or CD44 knockdown in the absence of known ErbB ligands is not fully understood. The implications of these alterations in ErbB dimerisation patterns on ErbB signalling and cellular phenotype is not yet known and merits further investigation.

CD44 has been further implicated in influencing other RTK pathways in the setting of endocrine resistance. Involvement of the insulin growth factor receptor, IGF-IR, in two models of tamoxifen resistance has been demonstrated by our group. A unidirectional IGF-

IR/EGFR cross-talk mechanism has been found to be operational in Tam-R cells which directly influences EGFR signalling and cell growth. A similar mechanism has also been detected in T47D-R cells which is a tamoxifen-resistant cell line derived from the endocrine-sensitive T47D cells (Knowlden, Hutcheson et al. 2005). CD44 interaction with Met has been noted in our wtMCF-7 derived model of fulvestrant resistance (Steve Hiscox, unpublished observations). Other ligands that interact with non-ErbB RTKs such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) have also been associated with CD44 in breast cancer models. For example, significant alterations in gene and protein expression of apoptosis mediators such as VEGF and PDGF has been noted following stimulation by osteopontin, a CD44 ligand, in a murine model of breast cancer (Mi, Guo et al. 2009). Further investigation into the role of CD44-HA interaction on the signalling and function of RTKs would provide important clues to details of associated mechanisms.

Data from our study shows significant overexpression of CD44 at both gene and protein level in the endocrine-resistant cells compared to the wild-type endocrine-sensitive cells. However, this does not contribute to the intrinsic adverse phenotype noted in these cells despite a suppressive effect of CD44 knockdown on EGFR, and to a lesser extent, HER2. The observations from our study have been further validated by in-house CD44s transfection experiments in wtMCF-7 cells showing minor induction of endogenous EGFR and HER2 activity but no resultant change in downstream signalling or eventual cellular function (**Appendix III**; data provided courtesy of R. Bellerby). This is an interesting finding suggesting that mild to moderate suppression of ErbB activity does not affect basal function. This is somewhat surprising given that EGFR-mediated growth is considered to be the major mechanism accounting for the higher proliferative activity noted in Tam-R cells. One

possible explanation for this could be that the resistant cells manage to 'switch' to another signalling pathway that partially sustains growth despite some ErbB suppression (Sierra, Cepero et al. 2010). This 'switching' is noted long-term in most resistant cells where altered growth factor signalling sustains an adverse phenotype compared to the endocrine-sensitive cells. Another possibility is that ErbB suppression simply uncovers another signalling pathway that the cells routinely rely on to a lesser extent as compared to the primary ErbB pathway, but, is nevertheless able to sustain the adverse phenotype when partial ErbB suppression is seen. In fact, this highlights one of the major problems in overcoming resistance, namely, the ability of tumour cells to use alternative mechanisms to overcome anti-hormonal therapy. Breast cancer cells can overcome the 'action' of the drug via 'reactive' mechanisms that can potentially override the growth suppressive effect of the drug. Some of these reactive mechanisms include oestrogen hypersensitivity via increased transcriptional activity of ERα, oestrogen independence, induction of growth factor signalling amongst others (Normanno, Di Maio et al. 2005).

4.1.5 CD44 Overexpression in the Context of the Microenvironment

The tumour microenvironment is increasingly being recognised as one of the vital elements of cancer development and progression. The breast microenvironment typically consists of the extracellular matrix (ECM) and various cell types present in the stroma such as fibroblasts, endothelial cells, adipocytes and immune cells along with their secretory products. It is now generally accepted that breast cancers sequentially progress through defined stages with initial epithelial hyperproliferation and progressing to *in-situ*, invasive and metastatic carcinomas and the tumour microenvironment has been shown to play an important role in each of these steps (Place, Jin Huh et al. 2011). There are various

mechanisms whereby the microenvironment influences various aspects of tumour progession. For example, malignant tissues contain abnormal fibroblasts which influence cancer cell growth and angiogenesis via secretion of factors such as stromal derived factor-1 and hepatocyte growth factor (Orimo, Gupta et al. 2005). One of the important mechanisms whereby the microenvironment impacts on cancer progression is by supplying cancer cells with ligands which can induce receptor activation and influence signalling and behaviour. In the context of our study, an important element of the breast cancer microenvironment is the presence of hyaluronic acid (HA). Data from our study shows that whilst CD44 overexpression does not contribute to the endogenous aggressive phenotype seen in Tam-R cells, it significantly augments the cellular response to HA. This leads onto the hypothesis that the true implication of CD44 overexpression in tamoxifen-resistant cells is only revealed when the tumour microenvironment and the presence of exogenous ligands in the microenvironment with potential to activate CD44, such as HA, are considered.

Indeed, even in the context of HA there is evidence, apart from our own data, to support the view that HA-CD44 interactions can have significant effects on cellular behaviour. Firstly, there is evidence to support that HA-CD44 interactions can promote aggressive phenotypic features in malignant cells. HA-CD44 interaction can alter membrane structure and promote a migratory phenotype by altering the actin cytoskeleton system (Bourguignon 2008). HA-induced CD44 clustering has been shown to influence cellular invasion via accumulation of MMP9 at the cell surface (Peng, Su et al. 2007). Secondly, it has been shown that inhibition of HA activity can result in suppression of tumour progression and metastasis. For example, use of the HA inhibitor 4-methylumbelliferone showed clear inhibition of cell growth and motility in an in vivo model of breast cancer (Urakawa, Nishida et al. 2011). Thirdly, there is

evidence from clinical studies that show that HA expression is a strong and independent predictor for poor survival in breast cancer. For example, analysis of HA levels using a biotinylated HA-specific probe in 143 paraffin-embedded tumour samples of human breast carcinoma revealed that both the concentration of HA in peri-tumoural stroma and HA-positivity in the tumour cells were strong negative predictors for overall survival (Auvinen, Tammi et al. 2000). Thus, it is very likely that CD44 overexpression may be of significant importance in an in vivo setting where HA is available in abundance. In our study, we have used exogenous hyaluronan to stimulate CD44 activation in Tam-R cells but validation of our findings in an animal model of endocrine resistance would give further valuable insights into the significance of CD44 overexpression in the presence of stromal ligands, and in particular, the presence of HA.

It fact, it is possible that the degree of CD44 overexpression itself may be a poor indicator of drug response or prognosis until other factors in the microenvironment such as the concentration of stromal HA or the presence of other ligands is considered. One potential impact of the concentration of stromal HA could be in determining the exact phenotypic feature induced in the cancer cell. In other words, at certain concentrations of HA a proliferative phenotype may be induced whereas at other concentrations, a migratory phenotype may predominate. Such a differential response to ligands is seen in wound healing for example, where the concentration of PDGF regulates whether the mesenchymal cells develop a proliferative or migratory phenotype with a higher concentration favouring mitogenesis and proliferation (De Donatis, Comito et al. 2008). This might explain an interesting observation in our study which showed a profound impact of HA on migratory behaviour of Tam-R cells but there was little or no effect on proliferation. This could be

investigated further by performing growth assays in the presence of significantly higher concentrations of HA compared to our study. In this context, it is interesting to note that the primary sensors of chemoattractant ligand levels are RTKs and in particular EGFR. These act as signal transducers and as a relay mechanism that drives cellular decision about the final migration/proliferation switch (De Donatis, Ranaldi et al. 2010). Ricono et al. have demonstrated an EGFR-based mechanism whereby cell migration and invasion proceeds without any effect on primary tumour growth in an in vivo model of pancreatic adenocarcinoma (Ricono, Huang et al. 2009). Jeanes et al. have shown that that integrins exert differential control on normal human mammary epithelial cell proliferation and migration depending on the type of β-subunit present (Jeanes, Wang et al. 2012). It is, therefore, an interesting possibility that varying concentrations of HA might have a differential effect on migration or proliferation and RTKs may be mediators of this process. This can be investigated further, for example, by determining HA concentration in tumour tissue and replicating those concentrations in experimental conditions in vitro. Another approach would be to analyse HA expression in tumour tissue, classify tumours into high- and low-HA groups and further assess ErbB expression levels and associated prognostic indices.

Additional data from our group provides further evidence that lends credence to the idea that CD44 overexpression in tamoxifen resistance may be more relevant in the context of exogenous ligands present in the microenvironment. Our group has shown that CD44 knockdown using siRNA significantly attenuates the activation of signalling and augmentation of cell growth, invasion and proliferation that is normally seen in Tam-R cells following treatment with the ErbB ligand, heregulin beta-1 (**Appendix II**; data provided

courtesy of L. Goddard and N. Jordan). Heregulins are known to be present in the tumour microenvironment and have been shown to influence tumour progression and spread. For example, breast cancer associated fibroblasts overexpress heregulin in contrast with fibroblasts found in benign breast tissue (Sadlonova, Bowe et al. 2009). Interestingly, a study of clinical breast cancer specimens which looked at both stromal and epithelial heregulin expression found that only stromal heregulin levels correlated with outcome thus providing further evidence in support of our hypothesis (Visscher, Sarkar et al. 1997). Overall, it appears likely that CD44 overexpression may be most relevant in an in vivo setting where the presence of ligands in the microenvironment may serve to augment cell signalling and behaviour via CD44-mediated pathways.

4.1.6 Role of CD44 Isoforms

Although the data here points to a role for CD44 in tamoxifen resistance, the fact that CD44 may exist in several variant forms makes it challenging to understand the relative contribution of the different CD44 variants to the adverse phenotypic features seen in our model of tamoxifen resistance. Our siRNA approach used to suppress CD44 expression in Tam-R cells has been subsequently shown to result in knockdown of multiple CD44 isoforms including CD44v3 and CD44v6 (C. Smith and S.Hiscox, unpublished observations). Thus, there is a distinct possibility that several CD44 variants may collectively contribute to the HA-mediated signalling and functional changes noted in Tam-R cells, and, further work is ongoing in our laboratory to elucidate this in more detail. The role of variants is supported by studies that show variable influence of CD44s and the different variants on different aspects of tumour progression. For example, immunohistochemical staining of paraffin sections from

specimens of node-negative invasive breast carcinoma showed that CD44s appears to be a favourable prognostic indicator but there was no correlation between tumour progression and CD44v6 levels (Diaz, Zhou et al. 2005). Ma et al. also performed immunohistochemical analysis on primary breast cancer specimens and demonstrated that CD44v6 expression correlates with tumour size, nodal involvement and 5-year survival (Ma, Deng et al. 2005). Moreover, although the HA-binding ability is seen in all CD44 isoforms, the contribution of individual isoforms towards ErbB receptor dimerisation and/or activation is currently unknown. It is, therefore, possible that different CD44 isoforms or variants may play antagonistic roles. Certain variants may contribute towards development of an aggressive phenotype whilst other variants may have a neutral or even an inhibitory role. There is published evidence that would support such a hypothesis and this includes studies which indicate a more significant role of CD44s over variant CD44 isoforms with regard to promotion of tumour proliferation and metastasis. HA stimulation of CD44s has been noted to mediate breast cancer cell adhesion, motility and invasion whereas HA stimulation of CD44 variants only regulates cell motility (Afify, Purnell et al. 2009). Induction of CD44s expression in wtMCF-7 cells using a tetracycline inducible system has been shown to result in breast tumour invasion and liver metastasis in vivo (Ouhtit, Abd Elmageed et al. 2007). Unfortunately, there is currently a lack of knowledge concerning the exact roles of the standard and CD44 isoforms with regard to their role in determining response to available endocrine agents in a clinical setting. This is an area where further research needs to be undertaken to achieve a more comprehensive understanding of the role of CD44s and variant isoforms. .

Further evidence of the potential relevance of CD44 variants clinically comes from a preliminary immunohistochemical study performed by our group in a series of patients with histologically proven breast cancer (Hiscox, Baruha et al. 2012). Tumour tissue available from 69 patients with breast carcinoma who received anti-hormonal therapy with tamoxifen at City Hospital, Nottingham was investigated using immunohistochemistry. This was performed on the paraffin-embedded tissue samples after staining for CD44v3 and a H-score between 1-300 assigned to each specimen as described in **Section 2.5**. Other CD44 isoforms were not tested. It was noted following statistical analysis that the time to disease progression (p-value = 0.017) and the time to death (p-value = 0.028) were both significantly lower in the group expressing higher CD44v3 levels (**Appendix IV;** data provided courtesy of N. Jordan, J. Gee and L. Farrow)**.**

4.1.7 CD44, CSCs and Endocrine Resistance

As discussed in **Section 1.6.6**, there is a current debate on the role played by breast CSCs, which express high levels of CD44, in promoting endocrine resistance in breast cancer (O'Brien, Howell et al. 2009). Breast CSCs are known to be ERα low or negative and ER expression is known to be the most consistent marker for response to endocrine therapy. It is therefore likely that breast CSCs exhibit de novo endocrine resistance. There is recent evidence implicating CSCs in the development of EMT and it is now widely accepted that development of endocrine resistance is accompanied by a transition to a mesenchymal phenotype with associated aggressive features (Hiscox, Jiang et al. 2006; Morel, Lievre et al. 2008; Biddle and Mackenzie 2012). Interestingly, several mechanisms that are considered to be important in promoting the aggressive phenotype of breast CSCs, such as EGFR and HER2, are strongly implicated in endocrine resistance and are also pathways that are known to interact with CD44 (Farnie, Clarke et al. 2007; Duru, Fan et al. 2012). The recent evidence demonstrating presence of CD44-high circulating tumour cells (CTCs) with stem cell characteristics in patients with metastatic breast cancer makes the issue of the potential role of CSCs in resistance even more important in the clinical setting (Theodoropoulos, Polioudaki et al. 2010). Baccelli et al. noted the presence of metastasis initiating cells (MICs) amongst a population of CTC and furthermore demonstrated that these MICs expressed CD44 (Baccelli, Schneeweiss et al. 2013). Using EMT and stem cell markers including CD44, Barriere et al. showed that upto 39% of patients with primary breast cancer had CTCs with mesenchymal and stem cell properties (Barriere, Riouallon et al. 2013). Using flow cytometry and magnetic-activated cell separation, Wang et al. noted that nearly 19% of CTCs had features of CSC (Wang, Cao et al. 2012). It has also been shown that the proportion of CTCs with CSC features has a positive correlation with the stage of disease presentation

(Wang, Shi et al. 2012). There is current debate as to the extent of the role of CSCs in promoting the development of resistance to endocrine agents.

In fact, there is experimental evidence from cell models of tamoxifen resistance to support the hypothesis that tamoxifen-resistant cell lines have an enriched CSC population. In a model of tamoxifen resistance based on wtMCF-7 cells, tamoxifen-resistant cells showed enhanced mammosphere forming capacity compared to the endocrine sensitive cells, suggesting an increased CSC fraction in endocrine resistant cells (O'Brien, Farnie et al. 2008). Liu et al. developed a model of acquired tamoxifen resistance from wtMCF-7 cells (TAMR-MCF7) and specifically investigated whether the resistant cells have stem cell properties (Liu, Zhang et al. 2013). They noted that their tamoxifen resistant cell line had enhanced mammosphere forming ability and tumourigenicity in nude mice. Moreover, their qPCR analysis revealed increased mRNA expression of several stem cell markers including SOX-2, OCT-4 and CD133 in the tamoxifen-resistant cells compared to the wild-type cells. They also noted significantly higher mRNA levels of Snail, vimentin and N-cadherin and reduced E-cadherin in the resistant cell line indicating features of EMT. Yun et al. have noted a link between Notch-4, which is known to play a key role in CSCs, and tamoxifen resistance in their model of acquired tamoxifen resistance (Yun, Pannuti et al. 2013). Whilst it appears quite plausible that tamoxifen-resistant cell lines have a higher proportion of cells with CSC characteristics, it is unclear whether the heterogeneity seen in these cell lines represents a population of cells derived solely from CSCs.

The results from our study which is based on a cell model of acquired tamoxifen resistance provides certain interesting insights into this. Our study results show that CD44 is overexpressed in acquired tamoxifen-resistant Tam-R cells compared to the tamoxifen-sensitive wtMCF-7 cells. One possible explanation for this overexpression is that tamoxifen treatment of wtMCF-7 cells results in selective sparing of CSCs present in the wtMCF-7 cell population. This is entirely plausible as CSCs are known to have absent or low expression of ER and are known to be relatively resistant to chemotherapy and radiotherapy. Thus, whilst other ER-positive cells in the wtMCF-7 population are inhibited by tamoxifen, the CSCs may be inherently resistant to tamoxifen. This subselected population of CSCs may subsequently proliferate and lead to a heterogenous population of cells. In this context it is interesting to mention that there is evidence supporting the fact that CSCs can replicate the cellular hierarchy seen in parental tumours. Indeed, immunocytochemical analysis of CD44 expression in Tam-R cells shows that the CD44 expression is heterogenous with some cells showing markedly elevated expression and others with little or no expression with a high proportion of cells staining positive. It is likely that most of the cells which are CD44 positive are not actually CSCs especially in view of the fact that upto 90% of Tam-R cells express ER positivity and there are similar levels of ER mRNA and protein between wtMCF-7 and Tam-R cells (Hutcheson, Knowlden et al. 2003). To what extent these ER-positive Tam-R cells represent clonal populations derived from de novo tamoxifen-resistant CSCs is unclear at this stage. It is worthwhile discussing experimental strategies that can potentially be employed to explore this issue in more detail. One option would be to analyse the expression of other CSC markers such as CD24 and ALDH in Tam-R cells alongside CD44 using techniques such as immunofluorescence or immunoprecipitation. This would give an indication of the likely proportion of cells with CSC features amongst the heterogenous Tam-R cells. Another option

is to look at cells at the earliest stages of development of tamoxifen resistance such as after 4-6 months of tamoxifen treatment of wtMCF-7 cells and compare the proportion of cells with CSC characteristics compared to basal wtMCF-7 cells. Cells with CSC characteristics can be selected from this population of cells exhibiting early tamoxifen resistance, using flow cytometry for example, and allowed to grow in similar conditions in parallel and features of cells derived can be compared with 'normal' Tam-R cells. One potential problem with this approach could be cell yield as cell numbers tend to be relatively low during this stage of culture of early Tam-R cells.

4.1.8 Therapeutic Strategies against CD44 and HA

Our data provides a rationale for therapeutically targeting CD44 in endocrine-resistant breast cancer. This is based on two main observations. Firstly, CD44 overexpression induces a significant migratory response in Tam-R cells in the presence of HA which is ubiquitous in the microenvironment in vivo. Secondly, CD44 can modulate the response of ErbB receptors which appear to play an important role in inducing and sustaining endocrine resistance. In particular, CD44 knockdown can attenuate HA-induced activation of ErbB signalling as well as reverse HA-induced HER2-HER3 dimerisation. Thus, CD44 knockdown in vivo may serve to not only reduce the responsiveness of endocrine-resistant cells to pro-migratory stimuli from the microenvironment, it may also attenuate ErbB-mediated augmentation of aggressive phenotypic features in these cells. Therapeutically targeting CD44 in this setting may thus serve to provide additional benefit along with current modalities of therapy. Although the use of CD44 as a target in breast cancer is probably not a novel idea, as far as we are aware our study is the first to directly implicate CD44 as a potential target in tamoxifen-resistant breast cancer. It is worth mentioning at this point that even if future

studies fail to show a direct functional relevance of CD44 overexpression in an in vivo or clinical setting, the finding of CD44 overexpression could still have two potential alternative applications, namely, as a diagnostic tool and also as a target for delivery of other known drugs directly to the CD44 overexpressing malignant cells. Indeed, HA has already been used as a carrier agent to deliver drugs to cells expressing high levels of CD44. For example, Cohen et al. tried a novel nanocarrier delivery system with HA for cisplatin therapy in breast cancer and reported improved tumour control and lower toxicity in vivo in a mouse model (Cohen, Cai et al. 2009).

Interestingly, certain features about CD44 make it a good candidate as a feasible drug target. A 'druggable' target has been defined as a protein, peptide or nucleic acid with activity that can be modulated by a drug. This can consist of a small molecular weight chemical compound or a macromolecule, such as an antibody or a recombinant protein (Gashaw, Ellinghaus et al. 2012). An important feature of CD44 which lends itself well to targeting is the cell surface location. It is well known that a major feature that distinguishes target proteins which have successful drugs developed against them is their location in the cell. Bakheet et al. analysed the features of 148 human drug target proteins and 3573 non-drug targets to identify differences in their properties and noted that if a potential protein has a membrane location it makes them nearly twice as likely to be a successful drug target (Bakheet and Doig 2009). In fact, 60% of current drug targets are located at the cell surface (Overington, Al-Lazikani et al. 2006). CD44 also has a cytoplasmic domain which interacts with various intracellular signalling pathways and this allows targeting via smaller molecules which can cross the cell membrane. Another feature that favours CD44 is a fairly detailed

understanding of the three dimensional structure of the CD44 protein including details of the HA binding domain in humans (Teriete, Banerji et al. 2004). Moreover, reliable cell and tissue based assays are available against CD44 which allows monitoring of drug efficacy. CD44 has also been listed in the International Therapeutic Target Database (http://bidd.nus.edu.sg/group/ttd/ttd.asp) as a potential target.

Various potential therapeutic approaches to CD44 have been examined in pre-clinical models and in a clinical setting. One approach that has been tried in the clinical setting is the use of a monoclonal antibody to target CD44. Rupp et al. reported the safety, pharmacokinetics and preliminary efficacy of bivatuzumab mertansine in patients with CD44v6-positive metastatic breast cancer in 2007 (Rupp, Schoendorf-Holland et al. 2007). Bivatuzumab mertansine is a tumour-activated prodrug conjugate which contains maytansinoid mertansine (DM1) linked to the humanised monoclonal antibody bivatuzumab, which recognises the variant domain (v6) of CD44v6 through disulphide bonds. DM1 is an analogue of maytansine and is a potent inhibitor of tubulin polymerisation causing microtubule disruption and resulting in a potent cytotoxic effect. Twenty-four patients were treated at eight different dose levels and 50% patients were noted to have disease stabilisation in the setting of heavily pre-treated metastatic breast cancer. However, nearly 75% patients experienced transient and mild skin disorders which are possibly explained by the high levels of CD44 expression present in normal skin. Unfortunately, due to one fatal case of toxic epidermal necrolysis (TEN) that occurred in another bivatuzumab study running in parallel on patients with head and neck and oesophageal squamous cell carcinoma, the clinical development of bivatuzumab mertansine was discontinued (Tijink, Buter et al. 2006). Another strategy to target CD44 is to competitively inhibit CD44 using proteins or peptides. This has been applied in a murine

model by Yu et al. who demonstrated induction of apoptosis in pulmonary metastases of parental TA3/St breast cancer cells with the use of soluble CD44 isoforms (Yu, Toole et al. 1997). Inhibition of CD44 transcription or translation is an additional strategy. Salinomycin, an agent with potent anti-CD44 transcriptional activity, has been shown to demonstrate a definite anti-tumour effect in a murine model of breast cancer (Gupta, Onder et al. 2009). A further strategy that can be employed is to prevent CD44 activation by inhibiting HA binding to CD44. Small HA oligomers (6 to 20 monosaccharides) have been previously used to competitively inhibit HA-CD44 interaction in an in vitro model of breast cancer and this resulted in disassembly of CD44-HER2 complexes thus demonstrating the efficacy of this approach (Ghatak, Misra et al. 2005). Since the discontinuation of clinical trials on bivatuzumab mertansine, no other strategy has thus far been applied in a clinical setting. Although the fatal case of TEN was an unfortunate incident, it should not discourage from developing the concept of an anti-CD44 agent further. The main limitation appears to be the presence of CD44 in various human tissues including high levels in the skin. However, with further research it may be possible to clearly identify which CD44 isoforms predominate in various tissues. This coupled with a further understanding into the exact roles of the variants into various aspects of breast cancer progression may allow more specific targeting in the future.

Another potential strategy that emerges from our data is the benefit of targeting HA directly in tamoxifen-resistant breast cancer. The main reasoning behind this is the evidence that HA can augment the migratory phenotype of Tam-R cells and also mediate CD44-ErbB interaction. The two primary approaches that have been tried in a pre-clinical setting include

enzymatic degradation of HA and reduction of HA production by inhibiting hyaluronan synthase (HAS). Kerbel et al. have shown that HA degradation using hyaluronidase can reverse the intrinsic chemoresistance of intact murine EMT-6 mammary carcinoma spheroids (Kerbel, St Croix et al. 1996). Shuster et al. injected intravenous hyaluronidase into SCID (severe combined immunodeficiency) mice bearing xenografts of human breast carcinoma and noted that this was accompanied by decreased hyaluronan in the tumour specimens and a reduction in tumour volume of nearly 50% (Shuster, Frost et al. 2002). Inhibition of HAS through the use of siRNA-mediated silencing of the HAS2 gene has been shown to suppress the malignant phenotype of the invasive breast cancer cell line Hs578T (Li, Li et al. 2007). Thus, there have been some encouraging results from pre-clinical studies for the use of these strategies against HA but further work is needed before these can be translated into the clinical setting.

4.2 STUDY LIMITATIONS

Although novel and potentially exciting, the data here need to be interpreted cautiously in view of the study limitations. Our study uses an in vitro model of tamoxifen resistance and is limited by the drawbacks inherent in the use of such a model. The main limitation is that this study involves use of only one in vitro model of acquired tamoxifen resistance and validation in further models of resistance would help to further substantiate these findings and also potentially identify further anti-hormone specific CD44-mediated mechanisms and those generic to multiple forms of drug resistance. It is worth mentioning here that the importance of CD44 in other models of resistance is being explored through ongoing work in our laboratory. Indeed, overexpression of CD44 has also been noted in our Fas-R model of

fulvestrant (Faslodex®) resistance where it appears to stimulate migration (Rebecca Bellerby, unpublished observations).

The use of in vivo models of acquired tamoxifen resistance would provide additional insight into the role of CD44 in endocrine resistance. For example, a potential strategy would be to establish xenografts using Tam-R cells and observe the effect of a CD44-neutralising antibody on the growth characteristics of the primary tumour and development of metastases. In vivo studies are especially important in terms of translational benefit especially if drug testing is being considered at a later date as these models help in determining dosage, pharmacodynamics and toxicity profiles.

A further limitation includes the potential cross-inhibition of the siRNA technology used between the standard and variant forms of CD44 making it difficult to eliminate the role of variants in interpreting the data. Transfection studies using individual CD44 variants and standard form can help to identify and study the specific role of each in an in vitro setting. Moreover, due to resource constraints, further detailed elucidation of some of the interesting findings was not possible in this study but the additional experiments of interest and areas of potential research have been identified during the discussion in relevant sections.

4.3 CONCLUSIONS

A careful analysis of the results of this study allows the following conclusions to be reached:

- CD44 is overexpressed at both the gene and protein level in acquired tamoxifen-resistant Tam-R cells compared to their endocrine-sensitive wtMCF-7 counterparts

- CD44 overexpression in Tam-R cells does not appear to influence their endogenous migration, invasion or growth
- Overexpression of CD44 in Tam-R cells augments their sensitivity to hyaluronan with the resultant transactivation of ErbB members and development of a migratory phenotype
- CD44 is co-localised with ErbB receptors in Tam-R cells and it can modulate ErbB dimerisation

Discussion of the relevance of the results of this study in the overall context of published literature on endocrine resistance leads to further conclusions. Data from models of acquired endocrine resistance with in vitro breast cancer cell lines have established that prolonged exposure to active endocrine agents result in induction of growth factor mediated signalling pathways, especially RTKs, which contribute to the development of an aggressive cell phenotype accompanied by enhanced migratory potential and invasiveness which eventually promote an endocrine-resistant state. Knowledge of these pathways underlying signalling and phenotypic changes has allowed identification of potential therapeutic targets through which endocrine resistance might be overcome along with suppression of the resulting aggressive cellular characteristics. However, despite encouraging preclinical data, the results of trials thus far have yielded less promising results than expected. This illustrates the urgent need to identify further novel pathways that influence hormonal resistance and develop therapeutic approaches based on this understanding. Our study reveals the CD44 is overexpressed in the setting of tamoxifen resistance, but, the true implication of CD44 overexpression in tamoxifen-resistant cells is likely to be revealed only when the tumour microenvironment and the presence of exogenous ligands in the microenvironment with potential to activate CD44, such as HA, are considered. Our study also highlights the importance of CD44-ErbB

interplay but the implications of the alterations in ErbB dimerisation patterns on ErbB signalling and cellular phenotype is not yet known and merits further investigation. Although the data here points to a role for CD44 in tamoxifen resistance, the fact that CD44 may exist in several variant forms makes it challenging to understand the relative contribution of the different CD44 variants to the adverse phenotypic features seen in our model of tamoxifen resistance. Our study adds some insight into the debate on the role played by breast CSCs with high CD44 levels in promoting endocrine resistance in breast cancer but further investigation is needed prior to any definite conclusions. Our study results provide a rationale for targetting CD44 in tamoxifen resistance. However, this is currently of limited clinical relevance because of previous failure of bivatuzumab, but, identification of the predominant CD44 isoforms in different tissues and a development of a more detailed understanding of the exact roles of the variants in various aspects of breast cancer progression may allow more specific targetting in the future. Finally, although novel and potentially exciting, the data in our study needs to be interpreted cautiously in view of the study limitations.

Overall, our study results support an emerging role for CD44 as a key element determining breast cancer progression in the setting of endocrine resistance where it may represent a potential therapeutic target.

4.4 Future work

To continue to explore the role of CD44 in endocrine resistance, further work needs to be undertaken and a consideration of the results of this study helps to identify promising future areas of work. The mechanisms underlying CD44 overexpression in Tam-R cells compared to wtMCF-7 cells can be elucidated further especially with regard to the individual

contributions of the different CD44 variants. In particular, the question of whether this overexpression is a result of subselection of CSCs needs to be explored in detail. The clinical implications of the CD44 overexpression noted in our preclinical model should also be subsequently explored in clinical specimens with particular emphasis on the prognostic significance of this overexpression preferably including analysis of the role of the different variants. Studies that compare CD44 expression before and after development of resistance would be particularly interesting. It would also be interesting to further explore the mechanisms via which HA promotes a migratory phenotype. Further investigation of this effect in an animal model, perhaps with the use of monoclonal antibodies, may provide additional supporting evidence of the merit of CD44/HA targeting in the setting of endocrine resistance. Another interesting area for future work is potential ability of CD44 to alter ErbB dimerisation patterns. This certainly merits a detailed look particularly with regard to the effect of these alterations on the cellular phenotype. Use of anti-ErbB agents including dimerisation inhibitors to explore this further may have significant translational benefit.

Overall, the findings from this study need to be expanded by performing investigations in other models of endocrine resistance including animal models with particular emphasis on the role of the different variants. Translational clinical studies would also be helpful in further elucidating the role of CD44 in endocrine-resistant breast cancer.

Appendix

5.1 APPENDIX I: Affymetrix Analysis of RHAMM Expression in Tam-R Cells

Affymetrix® microarray analysis was carried out to compare RHAMM gene expression levels between wtMCF-7 cells and Tam-R cells. Gene expression of RHAMM was noted to be higher in Tam-R cells compared to wtMCF-7 cells but statistical analysis using *t*-test revealed that trend was not statistically significant (*p*-value > 0.05/NS on *t*-test; **Figure 5.1A**).

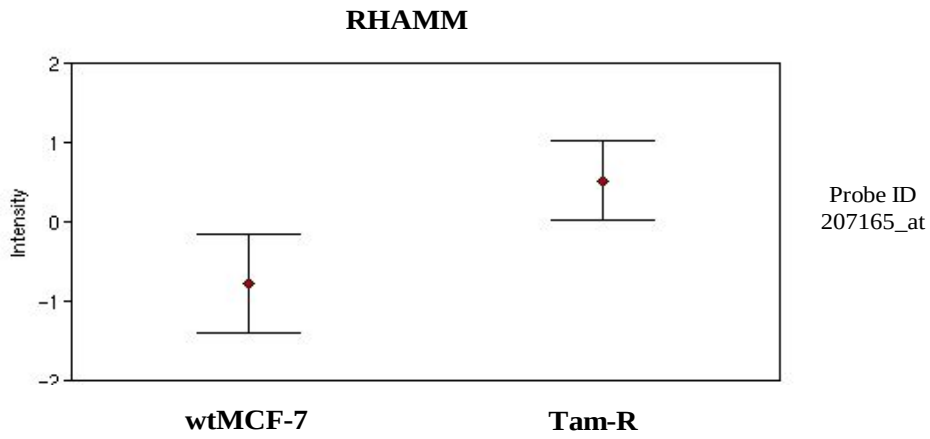


Figure 5.1 Affymetrix® microarray analysis of RHAMM expression in wtMCF-7 and Tam-R cells

Affymetrix microarray analysis was performed using basal wtMCF-7 and Tam-R cells and RHAMM expression evaluated in both these cell lines. Representative probeset results with data presented as comparative mean log intensity ± SD are shown above (*p*-value > 0.05/NS on *t*-test; A). Data provided courtesy of J. Gee and L. Farrow.

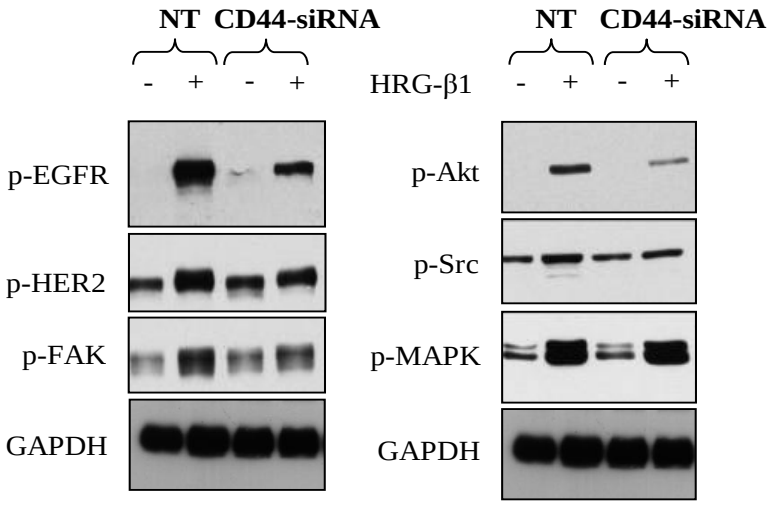
5.2 APPENDIX II: CD44 Suppression Attenuates Heregulin-induced Signalling and Invasive Phenotype in Tam-R Cells.

The role of stromal ligands and their effect in the setting of CD44 overexpression in Tam-R cells was further investigated using the ErbB ligand heregulin beta-1 (HRG-β1). Expression of p-EGFR and p-HER2 and associated downstream signalling components, namely p-MAPK, p-Akt, p-Src and p-FAK, was compared between Tam-R cells transfected with CD44-siRNA and compared with NT-siRNA transfected Tam-R cells. Treatment of NT cells with HRG-β1 resulted in activation of p-EGFR and p-HER2. There was also associated activation of p-MAPK, p-Akt, p-Src and p-FAK (**Figure 5.2**A). However, in CD44-siRNA transfected Tam-R cells there was significant attenuation of the signalling response to HRG-β1 (**Figure 5.2**A). There was a particularly marked attenuation of p-EGFR signalling.

The effect of CD44 knockdown on HRG-β1 response in Tam-R cells was also investigated from a functional standpoint. Invasion assays were set up using Tam-R cells treated with NT-siRNA and CD44-siRNA in the presence and absence of HRG-β1. Knockdown of CD44 expression significantly attenuated heregulin-induced invasion in Tam-R cells (**Figure 5.2 B**).

Overall, CD44 suppression through CD44-siRNA significantly attenuates heregulin-induced signalling and invasive phenotype in Tam-R cells.

A



B

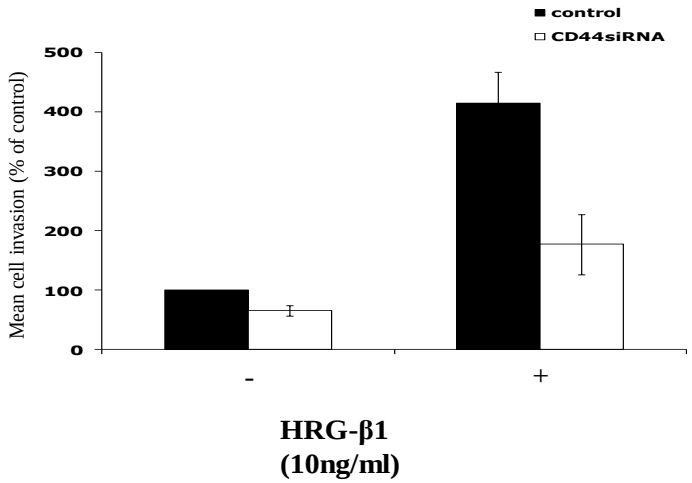


Figure 5.2 CD44 suppression attenuates heregulin-induced signalling and invasive phenotype in Tam-R cells.
Tam-R cells were treated with NT-siRNA (NT) or CD44-specific siRNA (CD44-siRNA) prior to treatment with the ErbB ligand, heregulin-beta1 (HRG- β1). On Western blotting, HRG- β1 promoted phosphorylation of EGFR and HER2 and associated downstream signalling pathways in NT cells but this response was significantly attenuated in CD44-siRNA transfected cells (A). On invasion assays, there was a marked increase in cell invasion following HRG- β1 treatment in NT cells which was significantly attenuated in cells treated with CD44-siRNA (p-value < 0.05 on *t*-test; B). Data provided courtesy of S. Hiscox, N. Jordan and L. Goddard.

5.3 APPENDIX III: Transfection of CD44s in wtMCF-7 Cells promotes HA-induced ErbB Activation and a Migratory Phenotype

To further investigate the role of CD44s in breast cancer cells, CD44s was transfected into wt MCF-7 cells which have minimal endogenous levels of CD44. This resulted in clear CD44s overexpression in the transfected cells compared to basal controls (**Figure 5.3A**).

CD44s overexpression in wtMCF-7 cells did not lead to any increase in the basal activity of p-EGFR and p-HER2. However, the wtMCF-7 cells which overexpressed CD44s showed a marked increase in p-EGFR and p-HER2 activity following treatment with HA (**Figure 5.3B**). This response was not observed in basal wtMCF-7 cells treated with HA. There was associated downstream activation of p-MAPK following HA treatment in CD44s transfected wtMCF-7 cells an effect which was not noted in normal wtMCF-7 cells.

The effect of HA on the migratory capacity of wtMCF7 cells overexpressing CD44 was subsequently investigated using wounding assays. There was no migratory response noted in basal wtMCF-7 cells following HA treatment and this was in clear contrast to the marked migratory response noted in wtMCF-7 cells overexpressing CD44 when treated with HA (**Figure 5.3C**).

Overall, CD44s overexpression in wtMCF-7 cells was accompanied by a marked activation of ErbB signalling and migratory phenotype following treatment with the CD44 ligand HA.

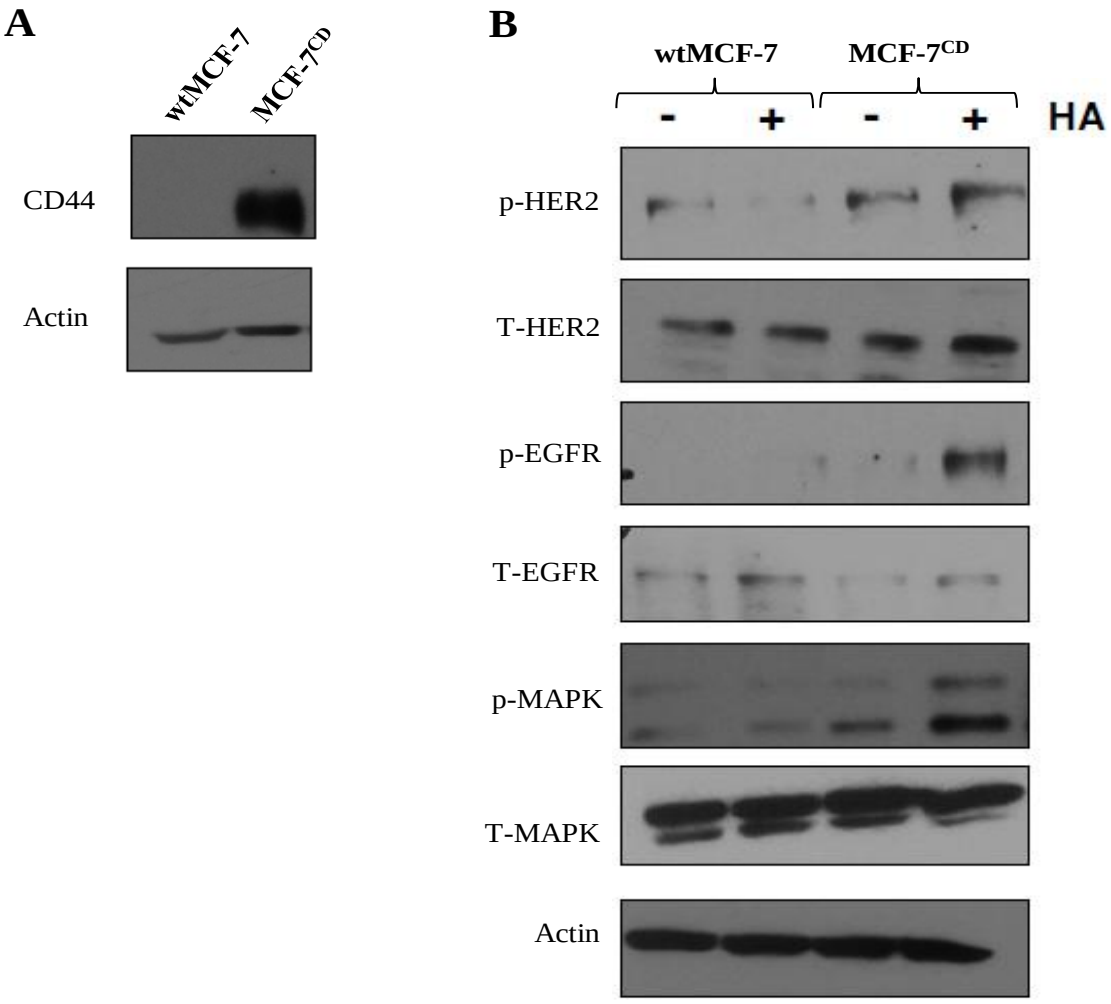


Figure 5.3A&B Overexpression of CD44 in wtMCF-7 cells augments their response to HA

The standard isoform of CD44 was transiently expressed in wtMCF-7 cells, designated as MCF-7^{CD} (A), and their response to HA subsequently examined using Western blotting and wounding assays. HA induced EGFR, HER2 and MAPK activity to a much greater degree in CD44-expressing MCF-7^{CD} cells compared to wtMCF-7 cells (B). HA treatment of CD44-expressing MCF-7^{CD} cells promoted a significant enhancement of their migratory capacity (C).

C

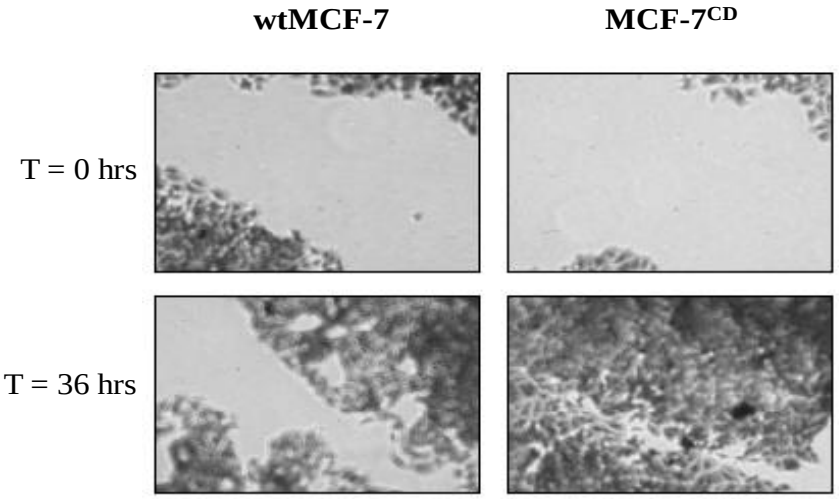


Figure 5.3C Overexpression of CD44 in wtMCF-7 cells augments their response to HA
The standard isoform of CD44 was transiently expressed in wtMCF-7 cells, designated as MCF-7^{CD} (A), and their response to HA subsequently examined using Western blotting and wounding assays. HA induced EGFR, HER2 and MAPK activity to a much greater degree in CD44-expressing MCF-7^{CD} cells compared to wtMCF-7 cells (B). HA treatment of CD44-expressing MCF-7^{CD} cells promoted a significant enhancement of their migratory capacity (C).

5.4 APPENDIX IV: High CD44v3 Expression is Associated with Poor Outcome in Breast Cancer Patients following Tamoxifen Monotherapy

To further examine the prognostic significance of additional CD44 family members, our group performed preliminary analysis of CD44v3 expression in a series of ER-positive clinical breast cancers. Other CD44 variants were not tested during this study. Tumour tissue available from 69 patients with breast carcinoma who received anti-hormonal monotherapy with tamoxifen at City Hospital, Nottingham was investigated using immunohistochemistry (**Figure 5.4A**). This was performed on the paraffin-embedded tissue samples after staining for CD44v3 and a H-score between 1-300 assigned to each specimen as described in **Section 2.4**. Patients were divided into two groups for statistical analysis based on the median H-score (55): High CD44 group (H-score >55) and Low CD44 group (H-score <55). Statistical analysis showed that the time to disease progression (*p*-value = 0.017) and the time to death (*p*-value = 0.028) were both significantly lower in the group expressing higher CD44v3 levels (**Figure 5.4B, C & D**)**.** CD44v3 was not found to have independent prognostic significance on multivariate analysis. This preliminary study shows the potential importance of CD44v3 expression in determining response to endocrine therapy in patients with primary breast cancer.

A

Patient characteristics (n=69)	Values
Mean age in years (range)	54 (25-77)
Menopausal status	
Premenopausal	26
Postmenopausal	43
Tumour grade	
I	5
II	25
III	39
Progression	
Complete response	6
Partial response	10
Static disease	14
Progression	39
Survival	
Alive	5
Cancer related death	64

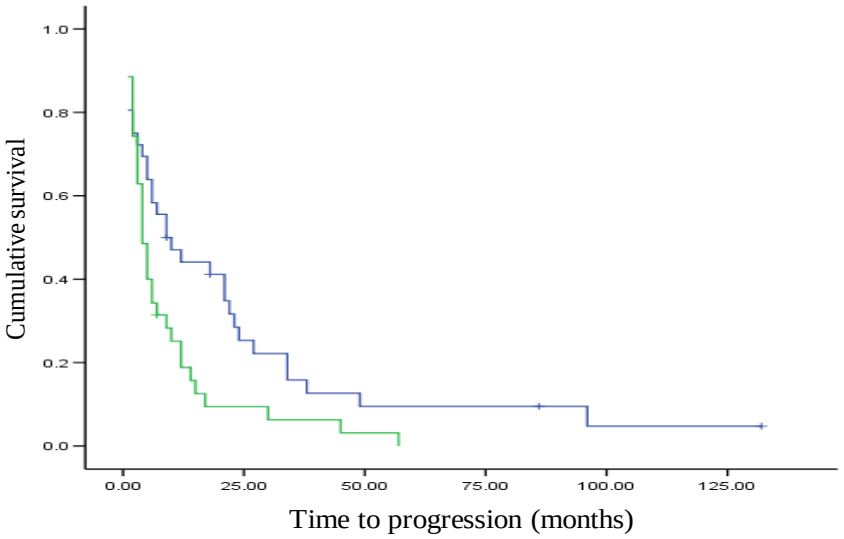
B

	Low CD44 group (H score < 55)	High CD44 group (H score >55)	p-value
Median time to death in months (95% CI)	49 (15.5 – 82.5)	23 (8.8 – 37.1)	0.028
Median time to progression in months (95% CI)	9 (1.86 – 16.1)	4 (2.5 – 5.4)	0.017

Figure 5.4A&B Higher CD44v3 expression correlates with poorer response to endocrine therapy

Tumour tissue available from 69 patients with breast carcinoma who received anti-hormonal therapy with tamoxifen at City Hospital, Nottingham was investigated using immunohistochemistry (A). Patients with low CD44 expression, as defined by a H-score <55, had longer survival (*p*-value = 0.028) and slower progression of disease (*p*-value = 0.017) (B). Comparative Kaplan-Meier curves detailing time to disease progression and time to death from initiation of therapy between the high and low CD44v3 expression groups are also shown (C,D).

C



D

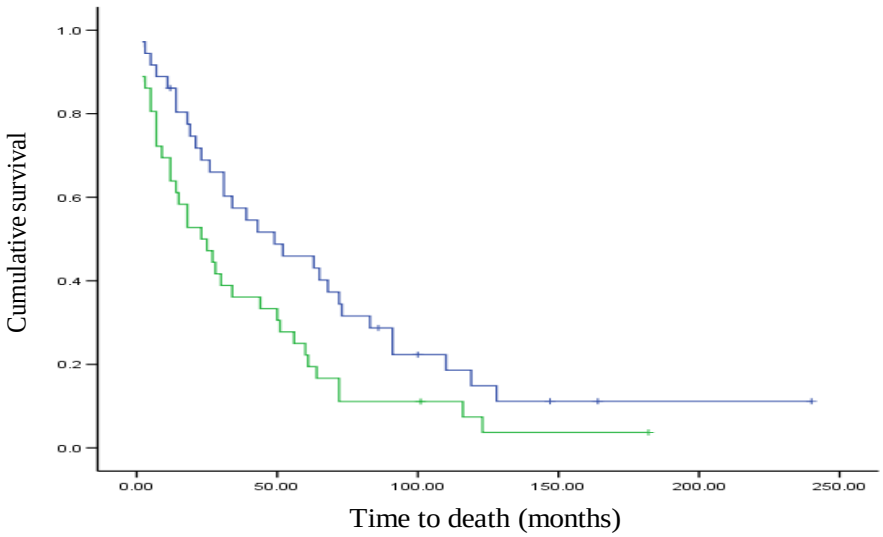


Figure 5.4C&D Higher CD44v3 expression correlates with poorer response to endocrine therapy

Tumour tissue available from 69 patients with breast carcinoma who received anti-hormonal therapy with tamoxifen at City Hospital, Nottingham was investigated using immunohistochemistry (A). Patients with low CD44 expression, as defined by a H-score < 55, had longer survival (*p*-value = 0.028) and slower progression of disease (*p*-value = 0.017) (B). Comparative Kaplan-Meier curves detailing time to disease progression and time to death from initiation of therapy between the high and low CD44v3 expression groups are also shown (C,D).

5.5 Appendix V: Summary of Therapeutic Strategies against CD44 and HA

The potential therapeutic strategies against CD44 and HA as discussed in **Section 4.1.7** are summarised in the table below (adapted from Zoller 2011):

Table 5.1 Examples of therapeutic strategies against CD44 and HA

Strategy	**Examples demonstrating benefit from use of strategy**
Anti-CD44 strategies	
Anti-CD44 antibody	Bivatuzumab induced disease stabilisation in heavily pre-treated metastatic breast cancer (Rupp, Schoendorf-Holland et al. 2007)
Competitive protein or peptide	Transfection with soluble CD44 isoforms resulted in induction of apoptosis in a murine model (Yu, Toole et al. 1997)
CD44 transcription inhibition	Loss of CD44 gene function after salinomycin use in a mouse model (Gupta, Onder et al. 2009)
Anti-HA strategies	
Inhibition of binding of HA to ligand	Small HA oligomers inhibit interaction with HER2, ezrin and hsp90 in breast cancer in vitro (Ghatak, Misra et al. 2005)
Enzymatic degradation of HA	Use of hyaluronidase reverses chemoresistance of murine EMT-6 mammary carcinoma spheroids (Kerbel, St Croix et al. 1996)
Inhibition of HA production	Inhibition of hyaluronan synthase 2 (HAS2) suppresses the malignant phenotype of breast cancer cells (Li, Li et al. 2007)
Inhibition of receptor dimerisation	Pertuzumab is currently being investigated in clinical trials of HER2 positive metastatic breast cancer

5.6 Appendix VI: Composition of Buffers and Solutions Used During this Study

The following buffers and solutions were used during this study:

1. RT-PCR: The buffers and solutions used for RT-PCR are listed in **Table 5.1.**
2. SDS-PAGE and Western blotting: The buffers and solutions used in SDS-PAGE and Western blotting are listed in **Table 5.2**. The composition of protease/phosphatase inhibitors is listed in **Table 5.3** & the BSA concentration guide used for the protein assay is listed in **Table 5.4**.
3. ICC: The solutions and buffers used during Immunocytochemistry are listed in **Table 5.5**.

Table 5.2: Composition of buffers and solutions used in RT-PCR

Buffer	**Volume**	**Composition (Final concentration)**
PCR Buffer (10x)	20ml	TRIS-HCl base: 4ml of 0.5M stock (100mM) KCl: 10ml of 1M stock (500mM) $MgCl_2$: 0.3ml of 1M stock (15mM) Gelatine: 0.1 ml 0f 2% stock (0.01% w/v) H_2O: 5.6ml
Tris-Acetate-EDTA Buffer (50x stock)	1000ml	TRIS base: 242g (2M) Glacial Acetic acid: 57.1ml (1M) EDTA (0.5M stock): 100ml (0.05M) dH_2O: Sufficient colume to make up to 1000ml pH = 8.3
Sample Loading Buffer	10ml	Sucrose: 6g (60% w/v) BPB: 0.025g (0.25% w/v) dH_2O

Table 5.3: Composition of buffers and solutions used in SDS-PAGE and Western blotting

Buffer	**Volume**	**Composition (Final concentration)**
Lysis Buffer	100ml	TRIS base: 0.61g (50mM) EDTA: 0.19g (5mM) NaCl: 0.87g (150mM) Triton X-100: 1ml (1% v/v) Distilled water (dH_2O): 99ml Add protease/phosphatase inhibitors before use
Running Buffer (10x stock)	1000ml	TRIS base: 30.2g (0.25M) Glycine: 144g (1.92M) SDS: 1g (0.1% w/v) dH_2O: 1000ml pH = 8.3 Dilute 10x prior to use
Laemmli Sample Loading Buffer (3x stock)	10ml	SDS: 0.6g (6% w/v) Glycerol: 3ml (30% v/v) TRIS Base: 3.6ml of 0.5M (180mM) BPB: 2mg (0.02%) DTT: 24 mg/ml dH_2O
Transfer Buffer	1000ml	0.25M TRIS base: 3.03g 1.92M glycine: 14.4g 20% methanol: 200ml dH_2O: 800ml
Tris-buffered saline (TBS)	1000ml	TRIS Base: 1.21g (10mM) NaCl: 5.8g (100mM) dH_2O: 1000ml
TBS-Tween		Add 0.05% Tween-20 to TBS

Table 5.4: Protease and phosphatase inhibitors used in SDS-PAGE and Western blotting

Name of inhibitor	Solvent	Stock concentration	Volume in 10ml lysis buffer (Concentration)
Sodium Orthovanadate	dH_2O	100mM	200µl (2mM)
PMSF	Isopropanolol	100mM	100µl(1mM)
Sodium molybdate	dH_2O	1M	100µl (10mM)
Sodium fluoride	dH_2O	2.5M	100µl(25mM)
Phenylarsine	Chloroform	20mM	10µl (20µM)
Aprotinin	dH_2O	2mg/ml	40µl (8µg/ml)
Leupeptin	dH_2O	5mg/ml	20µl (10µg/ml)

Table 5.5: Standard curve volumes used for Bio-Rad D_c Protein Assay

BSA concentration (mg/ml)	BSA (µl)(1.45mg/ml stock)	Cell lysis buffer (µl)
0.00	0.0	50.0
0.25	8.5	41.5
0.50	17.5	32.5
0.75	26.0	24.0
1.00	34.5	15.5
1.45	50.0	0.0

Table 5.6: Composition of buffers and solutions used in Immunocytochemistry

Buffer	**Volume**	**Composition**
Phosphate-buffered saline (PBS)	5 litres	NaCl: 42.5g Anhydrous di-potassium hydrogen orthophosphate: 7.15g Potassium hydrogen orthophosphate: 1.25g Distilled water (dH_2O): To make up 5L volume
Sucrose storage medium	250ml	Sucrose: 42.8g Magnesium chloride: 0.33g PBS: 250ml (see above) Glycerol: 250ml Store in -20°C prior to use
PBS-Tween	500ml	Tween-20: 100µl (0.02% v/v) PBS: 500ml

5.7 APPENDIX VII: Calculation Table Used for siRNA Experiments

The following calculation table was used to calculate the appropriate volumes of each of the reagents used for siRNA transfection studies:

Table 5.7 Chart used for preparing different volumes of siRNA transfection reagents

Desired volume (ml)	20µM stock (µl)	A siRNA 2µM	A SF-RPMI (µl)	B SF-RPMI (µl)	B Lipid (µl)	Total volume A+B (µl)	To dilute 1:5 add SFCS-medium	Final volume (µl)
1	5	50	50	98.4	1.6	200	800	1000
2	10	100	100	196.8	3.2	400	1600	2000
3	15	150	150	295.2	4.8	600	2400	3000
4	20	200	200	393.6	6.4	800	3200	4000
5	25	250	250	492	8	1000	4000	5000
6	30	300	300	590.4	9.6	1200	4800	6000
7	35	350	350	688.8	11.2	1400	5600	7000
8	40	400	400	787.2	12.8	1600	6400	8000
9	45	450	450	885.6	14.4	1800	7200	9000
10	50	500	500	984	16	2000	8000	10000
11	55	550	550	1082.4	17.6	2200	8800	11000
12	60	600	600	1180.8	19.2	2400	9600	12000
13	65	650	650	1279.2	20.8	2600	10400	13000
14	70	700	700	1377.6	22.4	2800	11200	14000
15	75	750	750	1476	24	3000	12000	15000
16	80	800	800	1574.4	25.6	3200	12800	16000

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