Focal Adhesion Kinase (FAK) as a novel therapeutic target in HER2+ breast cancer

Glorianne Lazaro BSc (Hons)

A thesis submitted in accordance with the conditions governing candidates for the degree of:

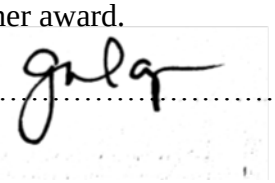
Doctor of Philosophy

Breast Cancer Molecular Pharmacology Research Group
Cardiff School of Pharmacy and Pharmaceutical Sciences
Cardiff University
Cardiff, Wales

May 2015

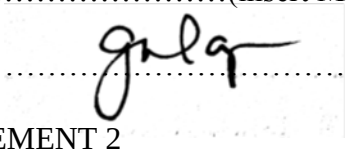
DECLARATION

This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidature for any degree or other award.

Signed  (candidate) Date ……110515……

STATEMENT 1

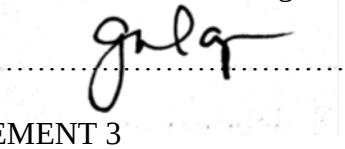
This thesis is being submitted in partial fulfillment of the requirements for the degree of ……(insert MCh, MD, MPhil, PhD etc, as appropriate)

Signed  (candidate) Date ……110515……

STATEMENT 2

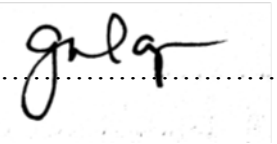
This thesis is the result of my own independent work/investigation, except where otherwise stated.

Other sources are acknowledged by explicit references. The views expressed are my own.

Signed  (candidate) Date ……110515……

STATEMENT 3

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed  (candidate) Date ……110515……

or

STATEMENT 4: PREVIOUSLY APPROVED BAR ON ACCESS

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loans after expiry of a bar on access previously approved by the Academic Standards & Quality Committee.

Signed …… (candidate) Date ……

Abstract

Focal Adhesion Kinase (FAK) is an intracellular kinase known to mediate integrin signalling following cell adhesion to the extracellular matrix. It is now emerging as a promising therapeutic target in many tumour types due to its overexpression in tumour cells and is associated with various cellular processes involved in cancer progression. Given that existing literature demonstrating that FAK plays a key role in the transduction of HER2 signalling in HER2+ cells and that the levels of FAK expression strongly correlated with HER2 overexpression in clinical samples, we explored the potential for improvement of current therapies for HER2+ breast cancer by combination treatment strategies with the small molecule FAK inhibitor, PF878.

FAK activity was assessed in a panel of cell lines reflecting HER2- (MCF7, T47D) and HER2+ (BT474, MDA-361, SKBr3) disease by Western blotting. FAK activity was relatively increased in HER2+ versus HER2- cell lines with HER2+ cells demonstrating greatest sensitivity to PF878 with respect to suppression of FAK phosphorylation at Y397. The effects of PF878 on cell proliferation as a monotherapy and in combination with Herceptin were assessed using MTT and direct coulter cell counting and by Ki67 immuno-staining. Whilst PF878 did not affect the proliferation as a monotherapy, treatment of HER2+ cells with PF878 and Herceptin combined resulted in synergistic inhibitory action on cell proliferation with an associated suppression in AKT pathway activity. This combination treatment strategy produced the greatest effects in MDA-361 cells which were intrinsically insensitive to Herceptin-monotherapy.

Inhibition of FAK activity also suppressed HER2+ cell migration in response to the (1) exogenous ligand Heregulin and (2) conditioned-media derived from fibroblasts (FCM), as assessed in Boyden Chamber migration assays. In this latter context, our data suggests that FAK may act through a STAT3-dependent mechanism to regulate fibroblast-stimulated migratory and invasive responses. Collectively, these data support a role for FAK in HER2+ breast cancer where its targeting has the potential to improve Herceptin response as well as suppress stromal-induced signalling that can contribute to disease progression and spread.

Publications

Lazaro G., Smith C., Hiscox S. 'Inhibition of FAK suppresses the pro-migratory and invasive effects of fibroblast conditioned media on HER2+ breast cancer cells, via attenuation of STAT3 phosphorylation'-Proceedings of AACR Annual meeting-San Diego, USA **April 2014**

Lazaro G., Smith C.,Goddard L., Jordan N., McClelland R., Barret-Lee P., Hiscox S.'*Targeting FAK in ER+/HER2+ breast cancer improves trastuzumab response' (Endocrine-Rel. Canc., **July 2013**)*

Lazaro G., Smith C.,Goddard L., Jordan N., McClelland R., Hiscox S. 'Inhibition of FAK activity in ER+ Her2+ breast cancer cells improves trastuzumab response' - Proceedings of BACR Tumour Microenvironment meeting- Bristol, UK **July 2013**

Lazaro G., Smith C.,Goddard L., Jordan N., McClelland R., Hiscox S. 'Inhibition of FAK activity in ER+ Her2+ breast cancer cells improves trastuzumab response' – Joint 34th EORTC-PAMM - BACR Winter Meeting, Cardiff UK **January 2013**

Acknowledgments

Foremost, I would like to express my sincere gratitude to my supervisor Dr. Stephen Hiscox firstly for giving me the opportunity to undertake this PhD, and for the continuous advice and support in all the time of the practical work, writing and completion of this thesis.

I am also grateful to Dr. Julia Gee and Dr. Kathy Taylor for insightful and constructive comments in relevance with this study. Importantly, to all the members of the Breast Cancer Molecular Pharmacology group past and present, who have taught and patiently helped me with various aspects of the practical work (and the troubleshooting issues that go along with it), but also for being such a friendly group of people who have always made my time in the lab enjoyable and well-fed with lots of cake.

A special thanks to Prof. John Eriksson and Dr. Claire Hyder for welcoming me into their lab for a placement year during my undergraduate year in Finland. My time there has given me the invaluable experience and the motivation to take on a PhD.

I would also like to thank the friends that I have made throughout the 4 years that I have spent in Cardiff and most importantly, to my parents and family who all have kept my sanity in the last few years, given me continuous encouragement and support, and remaining keenly interested in my work.

Last but not least, I would like to acknowledge Cancer Research Wales for providing the funding to carry out this work.

Contents

List of Abbreviations

AI	Aromatase Inhibitor
AIB1	Amplified in Breast 1 (Nuclear Receptor Coactivator 3)
ADAM	ADAM metallopeptidase domain
ADCC	antibody-dependent cellular cytotoxicity
AKT	(Protein Kinase B)
Ang1	Angiopoietin-1
APS	Ammonium Persulfate
AP-1	activator protein 1
ASAP1	ADP ribosylation factor [ARF]- GTPase-activating protein [GAP] containing SH3, ANK repeats, and PH domain
ATP	Adenosine Triphosphosphate
Bad	Bcl2 Associated Death Promoter
Bcl2	B cell Lymphoma 2
BRCA1	Breast Cancer Type 1 Susceptibility
BRCA2	Breast Cancer Type 2 Susceptibility
cIAP2	cellular inhibitor of apoptosis 2
Crk	Crk adaptor protein
FCM	Fibroblast Conditioned media
DCIS	Ductal Carcinoma in situ
DTT	DithiothreitolE1Oestrone
E2	Oestradiol
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
ER	Oestrogen Receptor
ERE	Oestrogen Response Element
FAK	Focal Adhesion Kinase
FAT	Focal Adhesion targeting
FERM	F for 4.1 protein, E for ezrin, R for radixin and M for moesin
FCS	Foetal Calf Serum
FRNK	FAK related non kinase
Graf	Rho-GTPase activating protei)
Grb	Growth factor receptor-bound protein
HDAC	histone deacetylases
HER2	human epidermal growth factor receptor 2
HER3	human epidermal growth factor receptor 3
HER4	human epidermal growth factor receptor 4
HRG	Heregulin
IGF	insulin growth factor

IGF1R	insulin-like growth factor 1 receptor
IHC	immunohistochemistry
IL6ST/GP130	interleukin 6 signal transducer
JAK	Janus Kinase
mAB	monoclonal Antibody
MAPK	mitogen-activated protein kinases
MDM2	Mouse double minute 2 homolog
MMP	matrix metallopeptidase
MT1-MMP	membrane-associated, type-I transmembrane MMP
MUC	Mucin
NCOR	nuclear receptor co-repressor
NES	nuclear export signals
NICE	National institute for Health and Care Excellence
NLS	nuclear localization signals
NT	non-targeting
P130 cas	p130 Crk associated substrate
PAI-1	Plasminogen activator inhibitor-1
PAK1	p21 protein (Cdc42/Rac)-activated kinase 1
PARP	poly-ADP-ribose-polymerase
PBS	phosphate buffered saline
PI3K	Phosphoinositide 3-kinase
PCNA	proliferating cell nuclear antigen
PDGFR	Platelet derived growth factor receptor
PTEN	Phosphatase and tensin homolog
Pyk2	proline-rich tyrosine kinase 2
Rac	Ras-related C3 botulinum toxic substrate
RhoA	Ras homolog gene family member A
RTK	Receptor Tyrosine Kinase
S	Serine
SHC	Shc adapter protein
siRNA	Small interfering ribonucleic acid
SOCS	suppressor of cytokine signalling
TGF-α	Transforming growth factor alpha
T	Threonine
TNBC	triple-negative breast cancer
VEC	vascular-endothelial cadherin
VEGF(R)	vascular endothelial growth factor (receptor)
WASP	Wiskott-Aldrich Syndrome protein
WT	Wildtype
XIAP	X-linked inhibitor of apoptosis protein
Y	Tyrosine

1. General Introduction

1.1 Breast cancer

1.1.1 Incidence and mortality

Breast cancer is now the most frequently diagnosed cancer among women worldwide with an estimated 1.67 million new cases diagnosed in 2012, and accounts for approximately 25% of all cancers diagnosed (GLOBOCAN 2012-latest). In the UK, women have lifetime risk of being diagnosed with breast cancer of 1 in 8. In 2011 alone, approximately 50,000 women and 390 men were diagnosed of breast cancer (Cancer Research UK 2011-latest). Over recent decades, the introduction of mammography screenings by the NHS (1988), the use of systemic adjuvant therapies and advances in treatments contributed to an observed decline in mortality especially in developed parts of the world. Despite this however, around 32 women in the UK still die from breast cancer every day (Cancer Research UK 2012-latest), highlighting the need for better therapeutic targets and strategies.

1.1.2 Risk factors

A number of risk factors have been identified that are associated with increased breast cancer risk. Of these, the majority of which correlate with increased exposure to the hormone oestrogen. As such, breast cancer is often a disease linked to older age, null parity, late-age at birth of first child, early menarche, late menopause and use of oral contraceptives (Reviewed Key et al. 2001). The use of hormonal replacement therapy (HRT) in post-menopausal women is also associated with increased risk, and further correlates with increasing total duration of use (Beral 2003). Additional risk factors also include lifestyle factors such as alcohol consumption, which has been proposed by a number of studies to increase oestrogen levels (Reviewed Gill 2000). Diet, particularly the increased intake of saturated fats are also attributed to increased risk (Boyd et al. 2003).

There is also strong evidence to suggest that familial history accounts for approximately 10% of all breast cancers (McPherson et al. 2000). Particularly in young women (under 50 y/o), it was found that there is an approximate two-fold relative risks if they have first-degree relatives (mothers, sisters, daughters) that are affected (Pharoah et al. 1997). This familial risk has been attributed through the inheritance of autosomal dominant alleles that carry genetic mutations. Two susceptibility genes, BRCA1 and BRCA2 which function as

tumour suppressors, have been identified to confer a substantial risk up to 45-90% of developing breast cancer (Cancer Research UK).

1.1.3 Breast cancer subtypes

Breast cancer is now known to be a heterogeneous disease, comprising several distinct biological (clinical) and molecular intrinsic subtypes. Identification of the biological subtype is important for prognostic information and treatment stratification whilst our increasing knowledge of molecular subtypes is helping to reveal new targets for therapeutic intervention.

1.1.3.1 Biological (clinical) subtypes

Breast tumours have been classified into at least three clinical subtypes defined by the expression of hormone receptors for oestrogen (ER), progesterone (PR), and the human epidermal growth factor receptor 2 (HER2).

Importantly, each clinical subtype is associated with a different patient outcomes as outlined in **Table 1.1 and Figure 1.1** (Onitilo et al. 2009).

Clinical subtype	Disease-free survival %
ER/PR+, HER2-	83.2
ER/PR+, HER2+	86.8
ER/PR-, HER2+	66
ER/PR-, HER2-	73.5

Table 1.1 Breast cancer clinical subtypes and their 5 year overall survival (Onitilo et al. 2009).

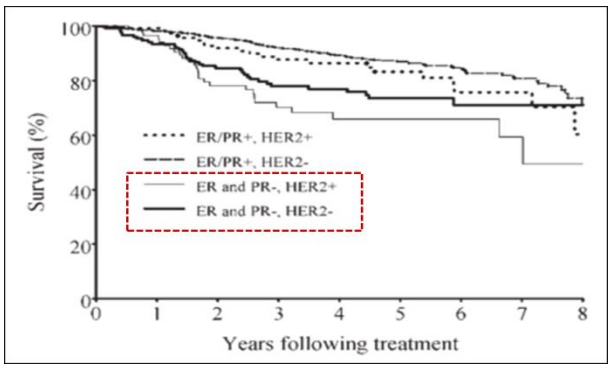


Figure 1.1 Disease free survival based on clinical subtype (Onitilo et al. 2009).

As shown above, tumours that express the hormone receptors ER and PR are associated with an overall more favourable survival .Whilst those that are HER2+ (ER/PR-) and that do not express any of these receptors (termed triple-negative breast cancers or TNBC) present with poorer outcomes (Onitilo et al. 2009).

1.1.3.2 Molecular classification of breast cancer

The availability of gene array technology has led to the further subdivision of breast cancers on the basis of their gene expression patterns, allowing for deeper understanding of their complex diversity (Perou et al. 2000; Sørlie et al. 2001). Through these, it is now apparent that there are at least 5 distinct breast cancer intrinsic subtypes: the separation of ER+ tumours into at least two distinctive groups termed (1) Luminal A and (2) Luminal B, and the emergence of the (3) normal-like, (4), HER2-enriched (HER2+) and (5) basal subtypes.

The luminal A and luminal B subtypes are both ER+ and share molecular characteristics such as the expression of keratin8/18 and GATA3 which are typical of the luminal cells that surround the mammary ducts (Creighton 2012). The luminal B subtype tumours however are distinct in that they present with higher expression of a cluster of genes associated with proliferation including Ki67 and PCNA; the expression of this cohort of genes are likely responsible for the more aggressive behaviour exhibited by these tumours which in turn underlies the association of this subtype with a poorer outcome versus the Luminal A subtype (Perou et al. 2000). Luminal B tumours are also reported to have differential frequencies of gene aberrations relative to Luminal A tumours; these genes include *TP53, PTEN, PI3KCA MAP3K1* and *MAP2K4* which again may be critically important in defining this particular subtype (Creighton 2012). The HER2+ subtype accounts for up to 30% of all breast tumours and is associated with amplification of the *ErbB2* gene locus, resulting in the overexpression of the HER2 receptor protein as wells as neighbouring genes that exist in the ErbB2 gene locus (e.g. Grb7) that may likely contribute to the behaviour HER2+ breast tumours (Kauraniemi et al. 2003). The basal subtype of tumours include the TNBC clinical subtype described above and are also associated with the expression of the basal cluster genes including keratin 5,6/17, integrin-β4 and laminin. Moreover, basal tumours possess the highest frequency of p53 gene mutations (82% of cases) when compared to all the other subtypes. Lastly, the normal breast-like group have been associated with the reduced

expression of luminal-associated genes and instead associated with the genes expressed in the adipose tissue and other non-epithelial cell types (Perou et al. 2000).

As shown below, these intrinsic classifications of breast tumours carry further prognostic significance whereby luminal subtypes are associated with a more favourable survival outcome, whilst the HER2+ and basal subtypes present with the worst (Sørlie et al. 2001; Sotiriou et al. 2003). The normal-like subtype has comparable prognosis to the luminal subtypes, although the significance of its genetic profile in relation to breast cancer biology remains very vague.

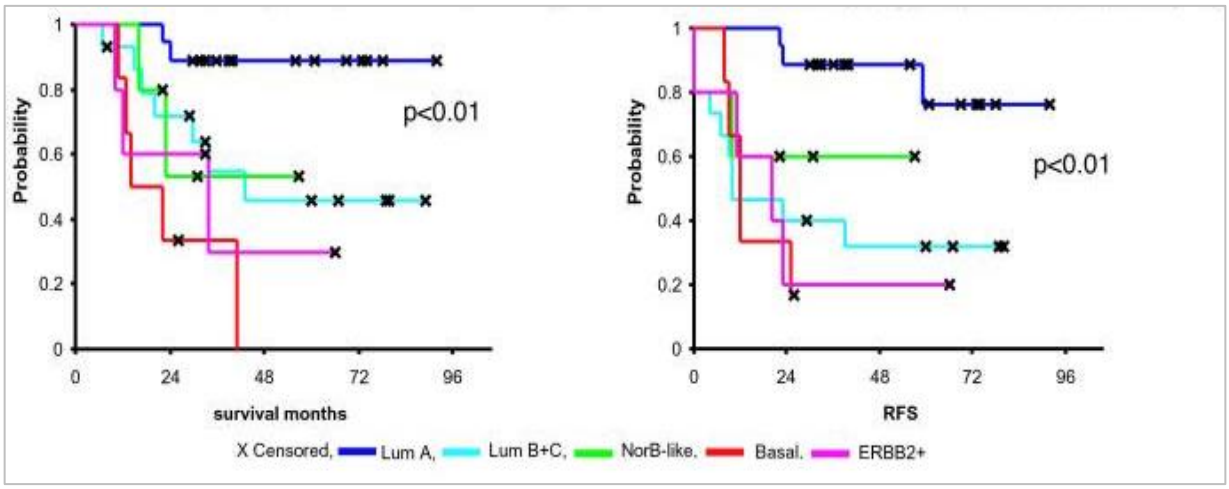


Figure 1.2 The Kaplan–Meier curves analysis of the 49 breast cancer patients, uniformly treated in a prospective study, based on different gene expression classification, showing difference in overall survival and relapse-free survival (RFS) between the subtypes, with the triple-negative and HER2+ subtypes associated with the shortest survival times (Sørlie et al. 2001).

Such classifications have also been widely important in identifying associations with distinct tumour histopathological features and the development of models to predict risk of relapse and response to treatments (Weigelt et al. 2008; Parker et al. 2009).

Although current treatment decisions are largely based on the clinical subtypes, the contribution of these gene-expression profiling approaches will likely impact practice with regards to tailoring treatment to a patient's molecular subtype. Indeed, such approaches have been developed, such as the Oncotype DX testing, which is based on identifying the 21 genes in tumour specimens and used to determine the 10 year risk of recurrence specifically in ER+/HER2- node-negative patients that will receive endocrine therapy. Moreover, the

test is also valuable in predicting response to chemotherapy (Carlson & Roth 2013), and has now been validated for clinical use by in the NHS (NICE UK). A more robust test is based on the PAM50 gene signature, aimed at identifying the expression profile of 50 genes to classify a tumour as one of the molecular breast cancer subtypes, and based on these, to similarly measure the risk of recurrence (Parker et al. 2009). This however is still currently under consideration for use in the UK by NICE.

1.1.4 Therapeutic targeting of breast tumours

As mentioned, the presence of receptor proteins (ER, PR and HER2) in the tumour is current practice that allows for the selection of appropriate therapy. To date, ER+ tumours are treated with endocrine therapy and HER2+ patients can receive therapies that target HER2 (discussed later). TNBC tumours however, due to lack of expression of these receptors are not amenable to targeted therapy, which partly contributes to their poorer prognosis. Their current mainstay treatment is adjuvant chemotherapy. Numerous therapeutic efforts are currently being explored for TNBC, including poly-ADP-ribose-polymerase (PARP)-1 inhibitors, EGFR inhibitors as wells as other tyrosine kinase inhibitors (Gluz et al. 2009).

1.1.4.1 ER functions and its therapeutic targeting

Approximately 70% of breast tumours express the oestrogen receptor (ER) and are therefore likely to be growth stimulated by the action of oestrogens. There are two known ER receptors; ERα and ERβ, the latter only relatively recently discovered. ERβ shares very similar sequence homology to ERα, particularly within its DNA and C-terminal ligand-binding domains, whilst its N-terminal is considerably shorter (Dickson & Stancel 2000). Since the role of ERβ in breast cancer is yet to be clearly established, the use of the term 'ER' in this thesis refers to the ERα receptor.

The binding of oestradiol (E2) (most potent naturally occurring oestrogen) to the ER triggers the phosphorylation of several of its serine/threonine residues, allowing for receptor dimerization (**Figure 1.3**). These dimers bind to DNA at oestrogen response elements (EREs), present in the promoter regions of oestrogen-responsive genes. Subsequent to this, co-activators (e.g. A1B1) are recruited to facilitate the transcriptional modulation of genes (Carroll & Brown 2006). The ER can also interact with other transcription factors such as c-

Fos/Jun to promote expression of AP-1 response element-regulated genes. Ultimately, these pathways regulate activity of a large number of target genes implicated in tumourigenesis which include*; c-myc, cyclin D1, IGF-IR* and *VEGF* (Carroll & Brown 2006).

In addition to ER inducing effects through transcription of genes, it can exert non-genomic functions which are attributed to a pool of ER that resides in the cytoplasm or at the plasma membrane (**Figure 1.3**). Membrane-associated ER was been shown mediate activation of growth factor receptors EGFR and IGF1-R (Kahlert et al. 2000; Razandi et al. 2003), as well as downstream signalling proteins such as Src, PI3K and MAPK resulting in pro-survival signalling (Klinge et al. 2005; Wong et al. 2002; Sun et al. 2001).

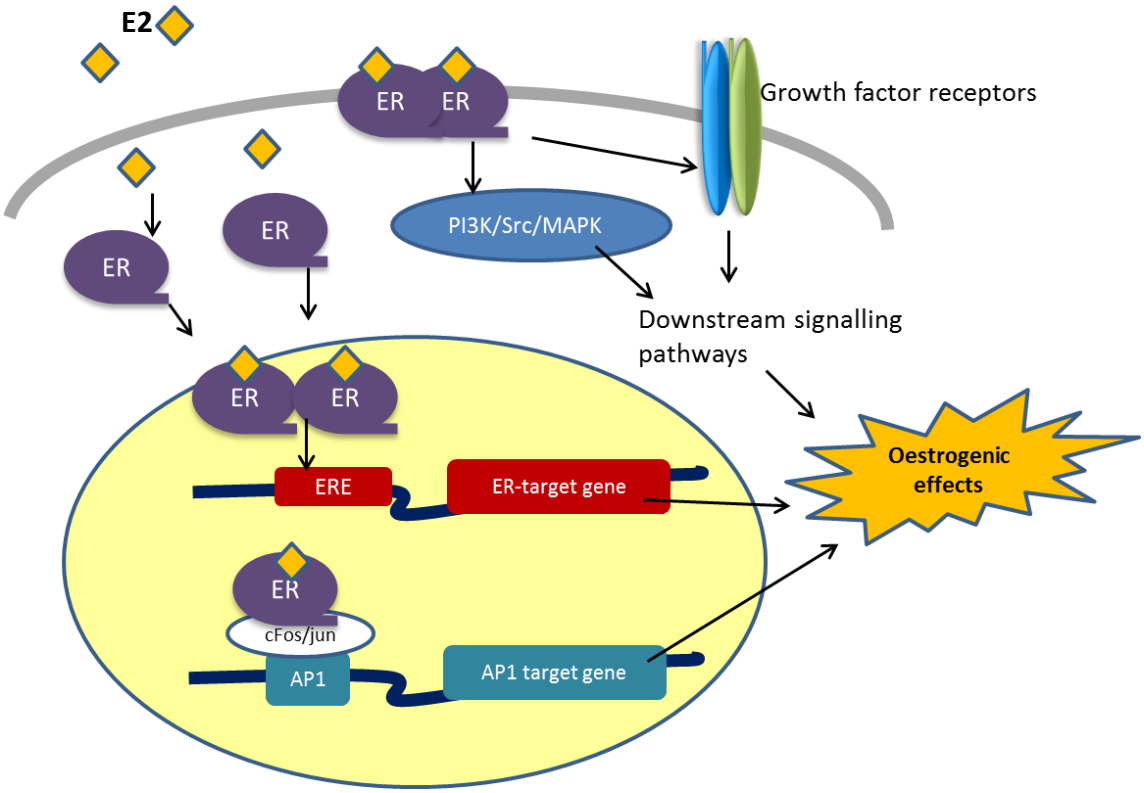


Figure 1.3 Schematic diagram mechanisms by which ERα to regulate gene expression of ERE-regulated genes and AP1-regulated genes upon binding of oestradiol (E2). ER can also directly bind and regulated activity of growth-factor receptors and downstream kinases. Collectively, these regulate processes including cell survival and proliferation.

A major strategy for the treatment of ER+ breast cancers is to prevent E2 from activating the ER. Early interventions relied on ovarian suppression, either pharmacologically using luteinizing hormone releasing factor (LHRH) agonists, surgically by surgical bilateral oophorectomy or by ovarian irradiation, altogether to prevent production of E2. These however present with the major disadvantage of being irreversible, compromising fertility in pre-menopausal women (Jones 2004). More recently, endocrine agents have been developed that either target the ER directly to prevent E2 binding, or alternatively to suppress the synthesis of E2 in the body.

Today, endocrine therapy is now regarded as the 'gold standard' for these ER+ tumours. Endocrine therapy acts to interfere with ER signalling, which is usually given as an adjuvant following surgery of the primary tumour.

There are three main categories of endocrine therapy;

1) Selective oestrogen receptor modulators (SERM) prevent oestrogen from binding to ER.

2) Selective oestrogen receptor degraders (SERD) trigger degradation of ER.

3) Aromatase inhibitors (AI) bind to the aromatase enzyme which is responsible for the production of oestrogens in peripheral tissues, resulting in a reduction of circulating oestrogen and depriving the tumour of oestrogen.

1.1.4.2 Selective oestrogen receptor modulators (SERMs)

The most widely used SERM is Tamoxifen, which competes with E2, for binding to the ER receptors, and in doing so, is able to prevent the action of oestrogen, interfering with oestrogen signalling and transcription of ER-regulated genes. Tamoxifen has been available and in use for ER+ breast cancers for over 30 years and has proven effective and preventing disease recurrence. In a meta-analysis of patient data from 20 clinical trials (n=10,645) (Early Breast Cancer Trialists' Collaborative Group (EBCTCG)), 5 years Tamoxifen adjuvant treatment significantly reduced recurrence rates and mortality throughout the first 15 years after diagnosis than those in the control group following surgery (Davies et al. 2011). Continuing the treatment for up to 10 years led to further benefits in the reduction of recurrence rates and mortality (Davies et al. 2013). However, Tamoxifen is not without side

effects and its use is associated with an increased risk of endometrial cancer, deep vein thrombosis and pulmonary embolus (Hernandez et al. 2009; Swerdlow & Jones 2005). In breast tissues, Tamoxifen binds to the ER, antagonising oestrogen activity. But at the same time in other tissues (e.g. endometrium), Tamoxifen promotes ER activation and thus acts as a partial agonist, hence it is termed as "selective oestrogen receptor modulator" (SERM) (Davies et al. 2011). This is likely to be caused by the differential expression of co-activators and co-repressors involved in the transcriptional regulation of ERE-target genes in specific tissue types (Graham et al.2000).

1.1.4.3 Selective oestrogen receptor degraders (SERDs)

The limitations of SERMs outlined above have led to the development of alternative endocrine therapies with differing modes of action and are termed selective oestrogen receptor degraders (SERDs), an example of which is Faslodex (Wakeling et al. 1991). Faslodex has a higher affinity for the ER and has been shown to inhibit ER-mediated gene transcription more effectively than Tamoxifen. Additionally and in contrast to Tamoxifen, Faslodex also abrogates the non-genomic cytoplasmic functions of ER and induces the rapid turnover and degradation of ER. Most importantly, Faslodex lacks the partial-agonist effects observed in Tamoxifen and hence it is also regarded as a 'pure anti-oestrogen'(reviewed Howell 2006).

1.1.4.4 Aromatase Inhibitors (AIs)

Aromatase inhibitors (AIs) act through inhibiting the aromatase enzyme which is responsible for the production of oestrogens in peripheral tissues, to reduce the levels of circulating oestrogen. AI's are subdivided into non-steroidal which reversibly bind to aromatase (e.g. anastrozole and letrozole) and steroidal (e.g. exemestane) agents which are irreversible. Results from the ATAC trial (anastrozole, tamoxifen, or the combination of tamoxifen and anastrozole) demonstrated a significant improvement in 5 year disease free survival (DFS) compared to Tamoxifen (Howell et al. 2005). To date, AIs (anastrozole, exemestane or letrozole) are approved for use in postmenopausal ER+ breast cancer patients in an adjuvant setting, and also as extended treatment for patients that have received the standard adjuvant Tamoxifen therapy (NICE UK).

1.1.5 HER2 functions and its therapeutic targeting

HER2 is a trans-membrane receptor tyrosine kinase (RTK) encoded for by the ErB2 proto-oncogene. Amplification of ErbB2 gene and the consequent overexpression of HER2 is found in up to 30% of breast tumours and shown to be associated with a more aggressive phenotype and poor patient outcome (Slamon et al. 1987; Liu et al. 1992). HER2 is a member of ErbB receptor family, which includes EGFR, HER3 and HER4 (**Figure 1.4**). They possess an N-terminal extracellular domain (ECD), a trans-membrane domain and an intracellular tyrosine kinase domain. Several known ligands such as the epidermal growth factor (EGF), transforming growth factor alpha (TGFα), amphiregullin, heregulins (HRG) and beta-cellulins can bind and specifically activate these family of receptors (Olayioye et al. 2000). Ligand binding induces receptor homo/heterodimerization stimulating their intrinsic tyrosine kinase activity, resulting in the trans/auto-phosphorylation of specific tyrosine residues within their intracellular regions. These phosphotyrosine residues then serve as docking sites for several downstream proteins (eg. Grb2, PI3K, Shc, Crk, Src) to stimulate signalling pathways such as the Ras/MAPK and the PI3K/AKT pathway, as well as a number of transcription factors eg. c-Fos, c-Jun and c-Myc to regulate diverse biological processes such as cell proliferation, survival/apoptosis and migration (Park et al. 2008; Schulze et al. 2005).

Accordingly, aberrant activation of these as a result of receptor overexpression as seen in breast cancers will enhance and prolong signalling and thus promote tumourigenic cellular processes (Park et al. 2008; Olayioye 2001).

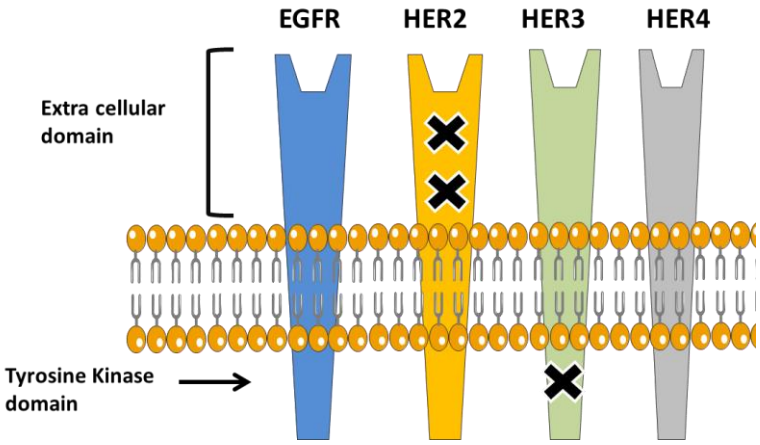


Figure 1.4 ErbB family of receptors illustrated. HER2 inactive ligand binding domain and the HER3 inactive tyrosine kinase domain depicted with X.

Interestingly, although the HER2 receptor is devoid of a functional ligand-binding domain (**Figure 1.4**), it still plays an important role in signal transduction by forming heterodimers with other ErbB receptors. In fact, HER2 is the preferred dimerization partner for all other ErbB receptors. This is attributed to the fact that HER2 exists in a (1) constitutively open ligand-bound conformation, (2) is endocytosed at a much slower rate relative to other ErbB receptors, and (3) more frequently recycled back to the cell surface thereby supporting a prolonged activation of downstream signalling pathways (Nahta 2011; Graus-Porta et al. 1997). Notably, HER3 does not exert intrinsic tyrosine kinase activity and can only be activated by heterodimerisation with another ErbB receptor. In this regard, HER2/HER3 heterodimers are considered to be the most potent oncogenic EGFR receptor family heterodimer combination (Alimandi et al. 1995; Yarden 2001).

Current therapeutic strategies employed to treat HER2+ tumours are aimed at targeting the HER2 receptor itself. The humanised murine HER2-targeted monoclonal antibody (mAb 4D5) Trastuzumab (Herceptin, Genentech)(Carter et al. 1992) was developed for this purpose and approved in the United Kingdom as an adjuvant treatment in 2006.

<u>The mechanism of action of Herceptin is thought to involve different aspects:</u>

1) Inhibition of HER2-mediated downstream signalling, particularly of the PI3K/AKT and MAPK pathways. This can result from the Herceptin-mediated uncoupling and inactivation of HER2/HER3 heterodimers (Yakes et al. 2002; Junttila et al. 2009), and also from the inhibition of Src association with HER2 and thereby preventing Src-mediated phosphorylation of the phosphatase and tensin homolog (PTEN). This consequently stabilises PTEN, increasing its membrane localization and activity; that is to prevent the activation of the PI3K/AKT pathway (Nagata et al. 2004).

2) Inhibition of HER2 cleavage and shedding of the p95HER2 fragment from the cell surface, known to retain the ability to form heterodimers with HER3 (Molina et al. 2001) and thus, high expression levels of this fragment correlated with poor patient outcomes and metastatic progression (Molina et al. 2002; Sáez et al. 2006).

3) Induction of antibody-dependent cellular cytotoxicity (ADCC) (Collins et al. 2012). This is supported by clinical data wherein Herceptin-treated patients demonstrate higher

infiltration of leukocytes and natural killer cells in the tumours (Gennari et al. 2004; Arnould et al. 2006).

4) Inhibition of angiogenesis. *In vivo* studies have demonstrated that Herceptin has reduced the expression of pro-angiogenic factors such as VEGF, TGF-α, Ang-1 and PAI-1, which correlated with significantly reduced diameter and volume of tumour blood vessels, decreased vascular permeability and importantly, decreased tumour growth (Izumi et al. 2002).

1.1.6 Therapeutic resistance

1.1.6.1 Resistance to endocrine agents

The expression of ER is indeed a marker of response to endocrine therapy however, resistance remains a problem, and this can be *de novo* (existing before treatment) or acquired (developed at some point during treatment) resulting in disease recurrence, frequently at distant sites (Osborne & Schiff 2011).

A number of mechanisms have been proposed for this such as various modifications to ER function/expression; by genetic mutations such as the Tyr537Asn missense mutation identified in the ER gene or epigenetic modifications such as CpG island hypermethylation (Zhang et al. 1997; Ottaviano et al. 1994). Over-expression of ER co-activators (e.g.AIB1) and in converse, downregulation of co-repressors (e.g.NCoR), may consequently alter the transcriptional activation by ER (Ring & Dowsett 2004). In other studies, the endocrine therapy Tamoxifen has been shown to also trigger a compensatory induction of parallel growth-signalling pathways via cross-talk with EGF receptor family members (particularly EGFR and HER2), which leads to increased activity of AKT and MAPK, enabling cells to adapt and sustain proliferation (Jordan et al. 2004; Gee et al. 2001).

It is also reported that ER+ tumours that also express the HER2 receptor is correlated with higher relapse rates and mortality (Cheang et al. 2009). Evidence from clinical trials showed that patients with a HER2+ status showed a significantly poor overall response rate to endocrine therapy (Carlomagno et al. 1996; Houston et al. 1999; Dowsett et al. 2001). *In vitro,* breast cancer cell lines transfected with HER2 displayed oestrogen-independent growth and resistance to endocrine therapy (Pietras et al. 1995). Together, these suggest a

cross-talk between the ER and HER2 signalling pathways, and the understanding of this ER and HER2 cross-talk is particularly important particularly in the context of therapeutic resistance.

It is recognised that a critical mediator in which cross-talk can occur is attributed to a pool of ER that resides in the cytoplasm or at the plasma membrane. As described in section 1.1.4.1, ER can directly activate intracellular kinases such as Src and RTKs including EGFR and IGF1-R (Razandi et al. 2003; Kahlert et al. 2000). Conversely, the downstream components of HER2-signalling, AKT and MAPK signalling for example can result in ER activation by direct phosphorylation at ser167 and ser118 residues (Yamashita et al. 2008). Activation of MAPK could also regulate activity of ER indirectly by phosphorylation of the co-activator A1B1, enhancing its interactions with ER dimer/ERE complex (Font de Mora & Brown 2000). Interestingly, it has also been shown that MCF7 cells that have developed resistance to Tamoxifen expressed elevated EGFR/HER2 signalling with an accompanying increase in AKT and MAPK activity (Knowlden et al. 2003; Jordan et al. 2004). It is therefore clear that cross-talk between ER and RTK signalling such as that mediated by HER2 is critical to the survival of cancer cells and is widely implicated in the development of resistance to endocrine therapies.

1.1.6.2 Resistance to Herceptin

Despite the fact that Herceptin has had significant success in HER2+ tumours, approximately 20% and 70% of early and metastatic HER2+ breast cancer patients respectively will display *de novo* resistance to Herceptin (Wilken & Maihle 2010). In a study, the inclusion of Herceptin with chemotherapy significantly increased the time to disease progression (TTP) to 7.4 compared to 4.6 months in patients that received chemotherapy alone (Slamon et al. 2001). Despite this benefit, this data indicates that following an initial response, acquisition of resistance and disease progression can occur within a year of Herceptin treatment initiation, thereby representing a significant hurdle. Several mechanisms underlying either the *de novo* or acquired resistance to Herceptin have been proposed. Amongst those mechanisms suggested to contribute to resistance, the following have attracted most attention.

1) Increased expression of the p95HER2 fragment. Truncated HER2 may be generated by alternative splicing or proteolytic cleavage of the full-length HER2 by the action of MMPs (Codony-Servat et al. 1999; Arribas et al. 2011). Transcriptome analysis of cells transfected with the p95HER2 fragment revealed up-regulated genes often involved in metastasis (e.g.MMP1, ANGPTL4, MET), and indeed, *in vivo* studies on p95HER2 transgenic mouse strains, have shown shorter tumour latency period and far more aggressive and metastatic tumours than those mouse strains with full-length HER2 (Pedersen et al. 2009). Clinically, elevated p95HER2 levels also correlated with significantly shorter progression-free survival in HER2+ metastatic breast cancer patients (Sperinde et al. 2010).

2) *PIK3CA* mutations or low/loss of *PTEN* expression. These lead to hyper-activation of the AKT survival pathway, and such an amplification has been linked with shorter time-to-progression and poorer patient survival (Berns et al. 2007; Razis et al. 2011).

3) Alternative compensatory growth pathways. Persistent inhibition of the HER2 signalling pathways by Herceptin could evoke alternative growth pathways, an example of this is the insulin growth factor receptor 1 (IGFR-1) pathway. This has been shown in a study by Lu et al., whereby Herceptin inhibited growth of MCF7/HER2+ cells (IGFR-1 expressing) only with concomitant treatment of IGFR-1 blocking antibodies. They also demonstrate that IGFR-1 transfection of HER2+ SKBR3 cells rendered them resistant to Herceptin (Lu et al. 2001). Recent studies also implicate alterations in intracellular components of the IGF-1 pathway particularly the balance between IGF-binding proteins IGFBP-2 and IGFBP-3 in the process of resistance (Lu et al. 2001; Dokmanovic et al. 2011).

4) HER2 epitope masking. A study on the Herceptin-resistant JIMT cell line, revealed higher levels membrane-associated glycoprotein known as mucin (MUC4) compared to sensitive cell lines. This protein has been shown to contribute to the masking of membrane proteins including HER2, which may interfere with Herceptin binding. Accordingly, knockdown of MUC4 expression by siRNA increased the binding of Herceptin (Nagy et al. 2005). MUC4 mRNA and protein expression were also elevated in a ER+/HER2+ xenograft tumours, which have become resistant to the combination of endocrine and HER2-targeted therapy (Chen A et al. 2012).

5) Other resistance mechanisms. These include the up-regulation of ErbB ligands. Herceptin-treatment of HER2+ breast cancer cells was reported to trigger a negative-feedback loop that led to the upregulation of ADAM17 and the subsequent cleavage of ErbB ligands including heregulin and betacellulin, which in turn bind to ErbB receptors and consequently transactivate HER2 (Gijsen et al. 2010). Integrin-mediated signalling and the activation of downstream mediators including FAK and Src have also been implicated in Herceptin resistance in various independent studies (Yang et al. 2010; Zhang et al. 2011; Peiró et al. 2014). Moreover, increased expression of the Met and the EphA receptor (Shattuck et al. 2008; Zhuang et al. 2010), miR-21 (Gong et al. 2011) and deregulated glycolysis (Zhao et al. 2011) are also reported to contribute to Herceptin resistance.

1.1.7 Other HER2-target therapies

Since relapse following Herceptin treatment frequently occurs, understanding resistance mechanisms are therefore crucial to aid the optimisation of existing treatments and the development of new therapeutic strategies for this disease.

Accordingly, alternative ways of inhibiting the HER2 pathway have been investigated resulting in the development of novel anti-HER2 therapeutics. Lapatinib (Tykerb®; GlaxoSmithKline), an ATP competitive inhibitor of the intracellular kinase domain of EGFR and HER2, was able to induce apoptosis in HER2+ SKBR3 derived-Herceptin-resistant cells (Nahta et al. 2007). In clinical studies, Lapatinib in combination with capecitabine improved progression-free survival (PFS) in patients with advanced HER2+ breast cancer who have progressed following treatment regimens comprising of chemotherapy and Herceptin (Geyer et al. 2006). In a Phase III study, the combination of Herceptin with Lapatinib resulted in a significantly higher pathological complete response (pCR) rate than those given Herceptin alone (Baselga, Bradbury, et al. 2012). Lapatinib in combination with chemotherapy was also shown to significantly improve overall survival by a mean of 3.2 months (Guan et al. 2013).

Another emerging HER2 targeted therapy is Pertuzumab (Omnitarg®; Genentech) which, like Herceptin, is a humanized monoclonal antibody that binds to the extracellular dimerization domain II of HER2 and inhibits hetero-dimerization of HER2 with other ErbB receptors. In a study of 808 patients, those receiving Docetaxel + Herceptin had a median PFS of 12.4

months versus those treated with Docetaxel + Herceptin + Pertuzumab (median PFS of 18.5 months) (Baselga et al. 2012).

More recently, trastuzumab-DM1 (TDM1) (Kadcyla® Roche), which is a conjugate of the antibody Trastuzumab (Herceptin) to a cytotoxic drug emantasine has been shown to be effective even in HER2+ cells as well as those that are Herceptin-resistant (Lewis Phillips et al. 2008). This therapy is now currently being investigated as a potential therapeutic option in early and metastatic HER2+ breast cancer in number of clinical trials; MARIANNE (NCT01120184) and KATHERINE (NCT01772472) .

Other strategies are also currently under investigation which include inhibitors of Heat Shock protein 90 (Hsp90) to induce HER2 degradation, as well as vaccines that elicit a response to the HER2-antigen (Meric-Bernstam & Hung 2006; Ladjemi et al. 2010).

1.2 Focal Adhesion Kinase (FAK)

Focal Adhesion Kinase (FAK) also emerged as a key effector of HER2 signalling, and the levels of FAK expression strongly correlated with HER2 overexpression in breast tumour samples (Benlimame et al. 2005; Xu et al. 2009; Schmitz et al. 2005; Behmoaram et al. 2008). FAK is involved in many different pathways involved in the regulation of cellular process including survival, growth, migration and invasion; many of these have been implicated in cancer. Targeting of FAK thus may be an appropriate therapeutic option in many cancers, including breast cancer. This thesis will focus on FAK and explore its role and therapeutic potential in breast tumours that overexpress HER2.

1.2.1 Structure and activity

FAK was originally described in 1992 as a 125kD protein that was tyrosine phosphorylated in Rous sarcoma virus-transformed chick embryos. Immuno-staining analysis showed cellular localisation of this protein at specialised regions on the cell surface termed focal adhesions (FAs). Then, the role of FAK was found to serve as a key signalling component at these sites in response to integrin clustering and adhesion to the extracellular matrix component fibronectin (Kornberg et al. 1992; Schaller et al. 1992).

FAK is now understood to be a ubiquitous non-receptor tyrosine kinase and a key component of focal adhesions, essentially providing a link between the extracellular environment and the cell cytoplasm. It is known to be activated following integrin-clustering and also following the activation of several growth factor receptors where it serves to integrate these diverse signals to regulate biological processes such as cell adhesion, proliferation, survival, apoptosis, migration and invasion. As such, FAK is described to be essential for physiological processes such as embryonic development and wound healing. Interestingly, the role of FAK in the former may well involve its ability to promote vascularisation of tissues and organs, since endothelial cell FAK deletion disrupts mice vasculature (Braren et al. 2006) and FAK-null mice have severely impaired development of the cardiovascular system (Ilić et al.1995).

1.2.2 FAK structure

The human FAK amino acid sequence is highly conserved between species with approximately 90% sequence homology to mouse and frog (Schaller 2010). It is comprised of three well-defined domains; (1) a central tyrosine kinase domain flanked by (2) N-terminal and (3) C-terminal domains (**Figure 1.5**).

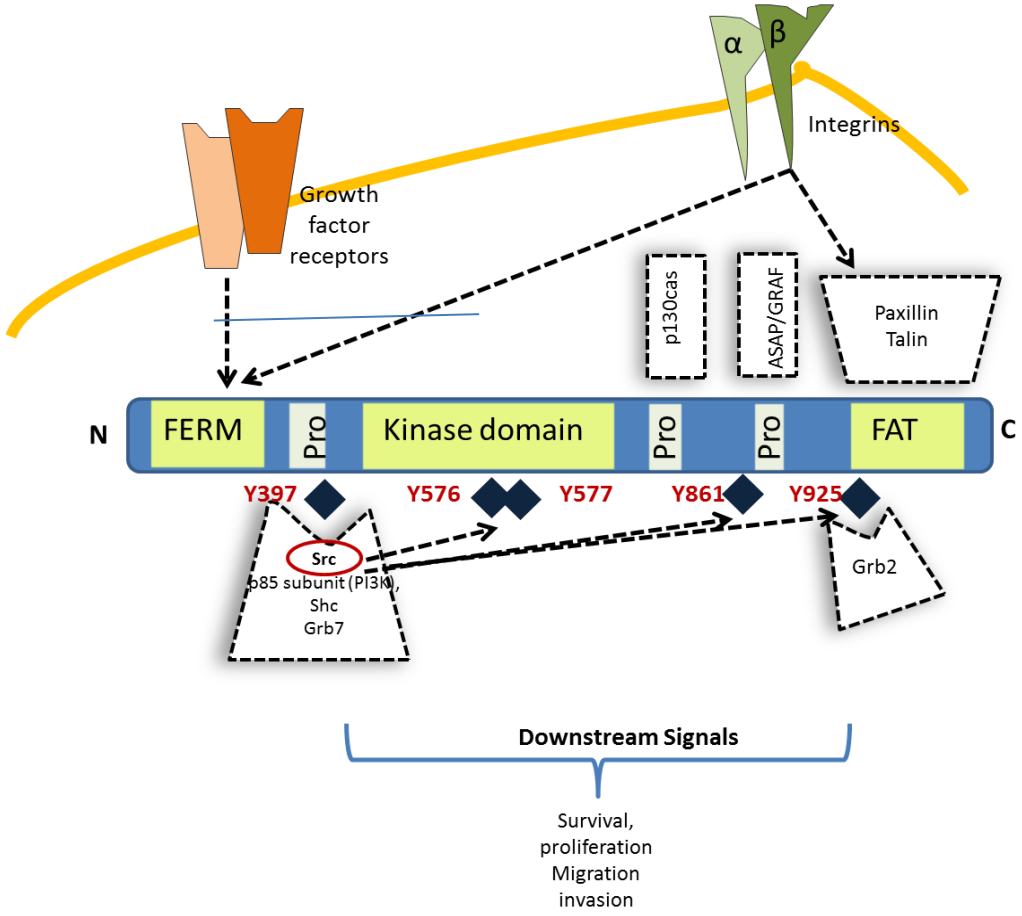


Figure 1.5 FAK structural domain and its interacting partners. Phosphorylated tyrosine residues (Y397,Y576,Y577,Y861,Y925) are represented above. These phosphotyrosine residues as well as proline-rich domains (Pro) provides binding sites for a number of targets which in turn elicit signals that regulate various cellular processes. Four-point-one, ezrin, radixin, moesin (FERM), Focal Adhesion target (FAT), ADP ribosylation factor [ARF]- GTPase-activating protein [GAP] containing SH3, ANK repeats, and PH domain (ASAP).

1.2.3 FERM domain

The FERM domain within the N-terminal region is structurally conserved in many proteins such as talin, JAKs, ERM (ezrin-radixin-moesin) and many non-receptor protein phosphotyrosine phosphatases (Girault et al. 1999). The FERM domain of FAK directly

interacts with the kinase domain of the protein to repress its activity and thus acts as negative regulator as has been experimentally supported by studies which have shown that deletion of the FERM domain promotes an increase in FAK activity (Cooper et al. 2003). Adding to this, structural studies have shown that the FERM domain directly binds the kinase domain, and sterically isolates the tyrosine residues to prevent phosphorylation (Lietha et al. 2007).

The FERM domain also mediates protein-protein interactions with the cytoplasmic tails of β1 integrins and growth factor receptors such as PDGFR and EGFR. These interactions release the auto-inhibitory conformation of the FERM domain, resulting in FAK activation, (Schaller et al. 1995; Sieg et al. 2000; Golubovskaya et al. 2002).

Further analysis of the FERM domains reveal potential nuclear export signals (NESs) and some nuclear localization signals (NLSs) which allows events at the cell periphery to be transferred co-ordinately to the nucleus (Frame et al. 2010). The relevance of nuclear FAK is highlighted by a study showing the FERM domain acting as a scaffold to facilitate p53 and Mdm2 association leading to p53 ubiquitination and promotion of cell survival, an event thought to occur in cases of cellular stress or reduced integrin signalling (Lim et al. 2008).

1.2.4 Central kinase domain

The central tyrosine kinase domain is necessary for its catalytic activity and mediating downstream signalling activity. Located within this is the major autophosphorylation site for FAK, tyrosine 397 (Y397) which modulates FAK-mediated signalling by creating high-affinity binding sites for SH2-containing proteins such as Src, PI3K, phospholipase Cγ (PLCγ), and growth-factor-receptor-bound protein-7 (GRB7) (Schaller et al. 1994; Zhang et al. 1999; Han & Guan 1999; Chen 1994). Binding of Src is involved in the activation of Src which, in turn, allows Src to phosphorylate additional tyrosine residues including two within the central kinase domain, Y576 and Y577, which are necessary for maximal FAK kinase activity (Calalb et al. 1995). Phosphorylation at Y397 and Y576/Y577 residues were found to be crucial for efficient disassembly of focal adhesions and consequently for cell migration (Hamadi et al. 2005). More recently, additional factors have been identified that can activate FAK through binding to the FERM domain such as has been shown with the RET receptor that can directly phosphorylate the Y576/577 residues (Plaza-Menacho et al. 2011).

1.2.5 C-terminal domain

The C-terminal domain harbours the FAT sequence and two proline-rich domains. The FAT domain is required for localization of FAK to focal adhesions and is thought to occur by the direct interaction of the FAT domain with other proteins such as paxillin and talin, that can in turn interact with the cytoplasmic tails of β1 integrins clustered at focal adhesions (Schlaepfer et al. 2004; Hildebrand et al. 1993)

The phosphorylation sites Y861 and Y925 are also situated within the FAT domain. The Src-dependent phosphorylation of the Y861 residue was shown to regulate interaction of p130Cas adaptor protein to the FAK C-terminal proline-rich regions, given its proximity to these sites (**See Figure 1.5**). This was found to mediate the Ras-induced transformation of fibroblasts (Lim et al. 2004). pFAK Y861 is important in promoting the association of FAK with an αvβ5 integrins following VEGF stimulation of endothelial cells (Eliceiri et al. 2002). As such, pFAK Y861 may have an essential role in VEGF-mediated vascular responses. pFAK Y925 on the other hand, similarly phosphorylated by Src, provides a binding site for the Grb2 adaptor protein, which subsequently recruits SOS, to activate Ras/MAPK survival signalling (Mitra et al. 2005).

The two C-terminal proline-rich domains mediate interactions SH3 domain-containing proteins including p130Cas as described above. One of the downstream events following p130Cas activation is the binding of the Crk adaptor protein and subsequent activation of Rac, to promote membrane ruffling, lamellipodia formation and actin reorganization (Sharma & Mayer 2008). MAPK activation has also been suggested to occur following p130Cas activation, an alternative pathway in addition to that mediated by pFAK Y925/Grb2 interactions (Schlaepfer et al. 1997). p130cas also regulates survival signalling, by activating the Ras/Rac1/Pak1/MAPK kinase 4 (MKK4) pathway to activate c-Jun NH2-terminal kinase (JNK), a pathway observed to be activated in the absence of growth factors (Almeida 2000).

Other SH3-domain containing proteins that bind to the proline-rich domains include Graf and ASAP1, which have been implicated in the regulation of focal adhesion assembly and cytoskeletal remodelling respectively (Schlaepfer et al. 2004).

1.2.6 FAK splice variants

The human FAK gene consists of at least 44 exons and at least eight un-translated regions (Corsi et al. 2006) and multiple FAK transcripts have been reported as a result of alternative splicing or the use of alternative promoters. Whilst most alternative FAK isoforms are reported in rat and mouse, some have been identified in the adult human brain (Corsi et al. 2006). The most well-characterised variant is FRNK (FAK related non kinase), which consists of just the C-terminal domain of FAK. FRNK competes with FAK for localisation at focal adhesions (via the FAT domain), and because it lacks the N-terminal and kinase domain it is able to attenuate normal FAK signalling functions (Schaller et al. 1993). FRNK expression in mammalian cells is often restricted, although it has been detected in rat aortic smooth muscle cells and is up-regulated two weeks after induced vascular injury (Taylor et al. 2001). FRNK has been considered to act as a naturally-occurring dominant negative FAK and its expression has been shown to inhibit cell proliferation, cell adhesion, cell invasion and promote apoptosis (Schlaepfer et al. 2004).

More recently, an alternatively spliced FAK transcript lacking exon 33 was identified in breast and thyroid tumour specimens. Interestingly this FAKdel33 mutant exhibited higher FAK phosphorylation at Y397, Y576/7 and Y925 residues than the WT-FAK, and was found to be associated with enhanced cell migratory behaviour (Fang et al. 2013).

1.2.7 Regulation of FAK kinase activity

It has been has previously demonstrated that point mutations (Lys to Glu) within the kinase domain residues FAK-K578 and FAK-K581 resulted in a "super-FAK" which displayed adhesion-independent elevated catalytic activity compared with wild-type FAK (Gabarra-Niecko et al. 2002). The occurrence of these spontaneous mutations in FAK however is not reported, although the characterisation of this active FAK mutant provides a useful experimental tool to elucidate the catalytic activities of FAK.

There are also identified proteins that serve to inhibit FAK activity, one which is the FAK-interacting protein 200 (FIP200) which directly binds to the kinase domain of FAK (Abbi et al. 2002). Protein tyrosine phosphatases (PTPs), of which PTPα has been shown to positively regulate FAK phosphorylation (Zeng et al. 2003), and conversely SHP2 acts to

dephosphorylate FAK in response to various stimuli (Mañes et al. 1999; Vadlamudi et al. 2002). There is also evidence that PTEN is able to interact with FAK and induce its de-phosphorylation, resulting in the inhibition of migration and invasion of the glioblastoma cell line U87MG (Tamura et al. 1998; Tamura et al. 1999).

1.2.8 FAK family members

The only other member of FAK family is proline-rich tyrosine kinase 2 (Pyk2), also called cell adhesion kinase β (CAKβ). It shares a similar structural organization as FAK, with approximately 45% amino acid sequence similarity. It is highly expressed in the central nervous system, and whereas FAK is often activated by integrin engagement, Pyk2 is activated by various stimuli that lead to the increase in intracellular calcium levels (Yu et al. 1996). Pyk2 null mice are viable, without significant developmental defects, however, it has been shown to crucial for the migratory behaviour of macrophages (Okigaki et al. 2003). Pyk2, unlike FAK, are often located in the peri-nuclear regions than in focal adhesions suggesting that they may interact with different proteins (Klingbeil et al. 2001). Although, it has been shown that Pyk2 can partially rescue the cell motility defects in FAK−/− mouse fibroblasts, which is attributed to its ability to interact with the cytoplasmic tails of β1 integrin at focal adhesions (Klingbeil et al. 2001).

Since many functions of FAK are shared by its isoforms, particularly Pyk2, there is considerable interest in understanding its distinct functions in cancer progression, although it still remains unclear.

1.2.9 Role of FAK in cancer

Given that FAK is the central hub of multiple signalling pathways that regulate various cellular processes, it is not surprising that FAK is also implicated in cancer development and spread. For example, evidence shows that FAK activity promotes the anchorage-independent growth of kidney epithelial cells and renders them resistant to an apoptotic response known as anoikis (Frisch et al. 1996). This was one of the early suggestions of the important role of FAK in cancer.

Over the recent years, over-expression and/or increased activity of FAK has been reported in a wide variety of human cancers including the larynx, colon, acute myeloid leukaemia, melanoma and breast (van Nimwegen & van de Water 2007; Gabarra-Niecko et al. 2003; Kahana et al. 2002; Recher et al. 2004; Watermann et al. 2005). Contradicting work regarding the clinical relevance of FAK expression have also been reported; whereby FAK overexpression was not correlated with prognosis of pancreatic tumours (Furuyama et al. 2006), whilst reduced FAK expression correlated with poorer outcome in cervical tumours (Gabriel et al. 2006). Moreover, a recent study also demonstrated that pFAK Y397 dephosphorylation resulting from Ras activation was correlated with increase migratory and invasive behaviour of 3Y1 rat fibroblasts (Zheng & Lu 2014).

In a study of several breast cancer cell lines, amplified copy numbers of the FAK gene were present a number of breast cancer cell lines (Agochiya et al. 1999). High FAK gene copy number in invasive breast tumour specimens as detected by fluorescence in situ hybridization (FISH) was significantly associated with shorter overall survival and RFS (Yom et al. 2011). FAK protein overexpression has been linked to progression of ductal carcinoma in situ (DCIS) and infiltrating ductal carcinoma (IDC) lesions, as well as in invasive and metastatic lesions (Owens et al. 1995; Lightfoot et al. 2004; Lark et al. 2005). Furthermore, FAK overexpression has been associated with poor tumour prognostic markers including high mitotic index, nuclear grade 3, architectural grade 3, ER- PR- status (Lark et al. 2005). A more recent study has also demonstrated that the overall patient survival was significantly worse in those with high FAK expression in metastatic tumour (logrank $p = 0.003$) and correlated with a younger age of patients, lymphovascular invasion and with the TNBC subtype (Golubovskaya et al. 2014).

Mouse studies have also demonstrated the role of FAK in breast cancer. Mammary epithelial-specific FAK knockout mice (FAK $^{flox/flox}$) exhibit a delay in tumour formation, and once present, tumours are slow-growing, smaller and with significantly impaired ability to metastasise to the lungs compared to the FAK$^{+/+}$ and FAK $^{+/flox}$ mice. Additionally, tumour cells from FAK $^{flox/flox}$ mice display proliferation and cell-cycle progression gene expression changes, as well as suppressed activity of Src, MAPK and p130cas (Provenzano et al. 2008).

Interestingly, FAK is emerging as a major player in stem cells. Luo et al. (2009) also demonstrated that these FAK $^{flox/flox}$ mice have depleted mammary cancer stem cell (MaCSCs) content in their primary tumours. Notably, the isolated MaCSCs had impaired self-renewal and migratory capabilities *in vitro*, and subsequent transplantation onto a NOD-SCID mice revealed compromised tumourigenicity and impaired maintenance of self-renewal ability, altogether suggesting an important role of FAK in the regulation of MaCSCs, which are now thought to be critical to tumour persistence and drivers of drug resistance and disease recurrence (Guan 2010).

Unequivocally, FAK is regarded as a key mediator of signals from both integrins and growth factor receptors and signalling downstream of FAK regulates a variety of cellular processes. The evidence associating FAK with these processes will be described below, with relevance to those implicated in malignancy and tumour progression.

1.2.9.1 Cell survival and proliferation

Early findings of Frisch et al. (1996) implicated FAK as a mediator of anchorage-independent survival. Subsequently, experimental evidence confirmed this showing for example, that overexpression of FAK in glioblastoma cells rendered them resistant to ionising radiation-induced apoptosis (Kasahara et al. 2002). In this case, FAK appeared to act through a mechanism involving FAK-induced PI3K/AKT survival pathway activation and induction of inhibitor-of-apoptosis proteins cIAP-2 and XIAP which are endogenous inhibitors of caspases. Further studies by this group and others subsequently revealed that FAK-overexpressing cells had increased expression of cell cycle regulators such as cyclins D3 (Yamamoto et al. 2003) and D1 (Zhao et al. 2001; Zhao et al. 2003) likely to promote cell cycle progression. Importantly, inhibition of FAK has been shown to enhance cytotoxicity of chemotherapeutic drugs (Halder et al. 2005; van Nimwegen et al. 2006; Chen et al. 2010) potentially by removing their FAK-induced survival advantage rendering them more sensitive to chemotherapeutic agents.

1.2.9.2 Cell migration and invasion

FAK has long been regarded as a regulator of cell migration and invasion and therefore a potential supportive element in the process of metastasis. Early studies have demonstrated

that fibroblasts from FAK-deficient mice had increased number of focal adhesions and an augmented migratory capacity, implicating a role for FAK in the turnover of focal adhesions (Ilić et al. 1995). Overexpression of FAK in chinese-hamster ovarian (CHO) cells stimulated cell migration relative to that of cells expressing only endogenous levels of FAK (Cary et al. 1996). This study and others (Owen et al. 1999; D. J. Sieg et al. 1999; Hamadi et al. 2005) have also illustrated the requirement for FAK kinase activity for this migratory response. Additional studies have also identified that the ability of FAK to control cell migration arises through engagement with multiple effectors which in turn regulate focal adhesion dynamics and cytoskeletal arrangement (**see table 1.2**)

FAK target	Resulting migratory-associated effects	Reference
paxillin	Focal adhesion disassembly	(Webb et al. 2004)
talin	Focal adhesion disassembly	(Chen et al. 1995)
MAPK/ERK	-Phosphorylates and activates MLCK to promote actomyosin contractility. -calpain activation resulting in focal adhesion disassembly	(Webb et al. 2004; Cuevas et al. 2003)
p 130cas	- Binds with the adapter protein Crk, leading to Rac activation to enhance membrane ruffling and lamellipodia formation. -Focal adhesion disassembly	(Webb et al. 2004; Cho & Klemke 2002; Sharma & Mayer 2008)
Grb7	-recruited at focal adhesions to modulate paxillin, MAPK and AKT activity	(Han et al. 2000) (Chu et al. 2009)
PI3K/AKT	-cooperates with p130cas to promote migration -phosphorylates Girdin, an actin-binding protein to facilitate lamellipodia formation	(Reiske et al. 1999; Enomoto et al. 2005)
α-actinin	-Release of actin stress fibres from focal contacts	(Izaguirre et al. 2001)
p190RhoGAP	-decrease RhoA activity to promote focal adhesion disassembly -Establishment of cell polarity	(Ren et al. 2000; Tomar et al. 2009)
P190 RhoGEF	-increase RhoA activity and promote focal adhesion assembly -Stabilisation of microtubules at the leading edge of the migrating cell	(Zhai et al. 2003; Y. Lim et al. 2008; Palazzo et al. 2004)
Cdc42	-subsequent activation of N-WASP to stimulate the Arp2/3 complex to initiate actin fibre assembly	(Sanchez et al. 2013)

Table 1.2 Key FAK effectors involved in FAK-mediated migratory processes

FAK has also been proposed to function in cell invasion, a process distinct from but involving migration in that it requires the active proteolytic degradation of the surrounding matrix (McCawley & Matrisian 2000). FAK overexpression in *v-src* transformed fibroblasts was associated with the co-localization of FAK/Src complex together with β1 integrins at invadopodia protrusions and correlated with increased cell invasion through Matrigel (Hauck et al. 2002). Accordingly, expression of a dominant-negative FRNK in these fibroblasts inhibited invasion through Matrigel, as well as suppressed experimental lung metastases in nude mice, without effects on cell migration. These were attributed with the decrease in the expression and secretion of MMP2 (matrix-metalloproteinases-2) (Hauck 2002). Further studies also support the role for FAK signalling in the expression of other proteases, such as MMP9 and MT1-MMP (Wu et al. 2005; Hsia et al. 2003), altogether supporting the role for FAK in cell invasion, particularly by regulating MMP-mediated matrix degradation.

1.2.9.3 Angiogenesis

In vivo studies using targeted deletion of FAK in endothelial cells showed multiple phenotypes attributed to impaired embryonic angiogenesis which contributed to embryonic lethality (Shen et al. 2005). Conversely, overexpression of FAK in vascular endothelial cells potentiated angiogenesis in response to induction of skin-wound and hind-limb ischaemia in a transgenic mice (Peng et al. 2004). These along with the earlier work by Ilic (1995), suggested the role for FAK in angiogenesis; the process by which new blood vessels are formed from pre-existing ones. With regards to the tumour, angiogenesis is essential to facilitate the supply of oxygen and nutrients and an avenue in which tumour cells can migrate to distant sites (Liekens et al. 2001). The role of FAK in tumour angiogenesis was attributed to its phosphorylation at Y925, whereby it activates the MAPK signalling pathway, associated in part in regulating VEGF expression (Mitra et al. 2006). pFAK Y861 was also shown in a separate study to mediate VEGF-regulated endothelial cell migration and survival (Abu-Ghazaleh et al. 2001). *In vivo,* mice that have FAK specifically deleted in their ECs and were subcutaneously injected with melanoma cells displayed impaired tumour angiogenesis which resulted in decreased tumour growth when compared to control. *In vitro* isolation of these ECs with FAK deletion demonstrated impaired migratory response to VEGF, decreased proliferation and enhanced apoptosis (Tavora et al. 2010). In a separate study,

endothelial FAK deletion also resulted in decreased growth of brain tumours and is associated with tumour vasculature that are longer, thinner, less branched and have reduced permeability (Lee et al. 2010). In accordance, it has been recently demonstrated that endothelial FAK mediates the phosphorylation of vascular endothelial cadherin (VEC), in response to VEGF stimulation, subsequently leading to VEC internalisation and disturbance of endothelial cell-to-cell junctions, enabling tumour cell transmigration. They have further shown *in vivo* that FAK inhibition prevented VEGF-induced tumour cell extravasation into lung micro-vessels, and significantly inhibited the formation of lung metastasis (Jean et al. 2014).

1.3 FAK as a potential therapeutic target

Taken together, due to the substantial evidence linking elevated FAK protein levels with a range of human tumour types including the breast, together with the role for FAK in various aspects of tumour progression, FAK is emerging to be a novel therapeutic target in cancer. Indeed, these studies have prompted the development of various FAK inhibitors, with the focus on targeting the autophosphorylation site of FAK, Y397. Some of these inhibitors and their progress in the clinical setting are summarized in **Table 1.3** below.

Inhibitor	Target	*In vitro/In vivo* anti-tumourigenic effects, clinical trials	References
TAE-226	FAK,Pyk2,IGF1-R	Breast, glioma, oesophageal cancers	(Wang et al. 2008; Shi et al. 2007; Golubovskaya, Virnig, et al. 2008)
PF-562271 (PF271)	FAK, Pyk2	Pancreatic, ovarian, breast, lung, colon, glioblastoma cancers. ➢ Phase I clinical trial (Pfizer)	(Stokes et al. 2011; Ward et al. 2013; Roberts et al. 2008; Infante et al. 2012)
PF045558878 (PF878/VS-6063/defactanib)	FAK, Pyk2	Breast*, ovarian*, thyroid cancer ➢ Ongoing Phase I,II trials in patients with non-haematological malignancies (Verastem Inc.). ➢ Ongoing Phase I/Ib trial in ovarian cancer (Verastem Inc.)	(Xu et al. 2014; Pachter et al. 2014; Kolev et al. 2014; O'Brien et al. 2014)
PND-1186 (VS4718)	FAK, Pyk2	Breast*, lung (mesothelioma), leukaemia, ovarian cancer* ➢ Ongoing Phase 1 trial in	(Walsh et al. 2010; Kolev et al. 2014; Trombino et.al. 2014)

		patients with metastatic non-haematological malignancies (Verastem Inc.)	
Y15 (Compound 14)	FAK (allosteric inhibitor of FAK, also shown to inhibit scaffolding functions)	Thyroid, colon, pancreatic, breast, lung, melanoma, glioblastoma cancer	(O'Brien et al. 2014; Heffler et al. 2013; Golubovskaya, Nyberg, et al. 2008; Golubovskaya et al. 2013)
GSK2256098	FAK	Pancreatic ductal adenocarcinoma, glioblastoma, ➢ Ongoing Phase 1 trial in patients with lung (mesothelioma) or advance solid tumours (GlaxoSmithKline)	(Chen et al. 2012; Zhang et al. 2014)

Table 1.3 Compounds targeting FAK that are currently in preclinical and clinical trials. All compounds are ATP-competitive kinase inhibitors with the exception of Y15, which is an allosteric inhibitor.
*Particularly potent in the cancer stem cell population

1.4 FAK and HER2 in breast cancer

As has already been described, FAK mediates multiple signalling pathways crucial to tumour progression and their overexpression correlates with poorer patient prognosis in breast cancer. A number of regulators of FAK have been identified including integrins and growth factor receptors, of which HER2 represents an important component. For example, Heregulin (ErbB receptor ligand) stimulated HER2 activation results in the Src-dependent phosphorylation of FAK at Y861 which is accompanied by changes in focal adhesion distribution and the formation of membrane ruffles in HER2-overexpressing MCF7 cells (Vadlamudi et al. 2003). Moreover, re-expression of wild-type FAK in $FAK^{-/-}$ HER2/3 overexpressing cells rescued their anchorage-independent growth-defective phenotype. In addition, these cells induced a more aggressive tumour growth and increased incidence of lung metastases compared with $FAK^{-/-}$HER2/3 cells when implanted into the mammary-fat pad of a severely immunosuppressed SCID mice (Benlimame et al. 2005). Moreover, FAK is key to the transduction of HER2 signalling in HER2-overexpressing MCF10A breast cancer cells, and in MUC4-overexpressing ovarian cells which also, have elevated HER2 activity (Ponnusamy et al. 2008; S. E. Wang et al. 2005). In breast tumours, a correlation between HER2 and FAK expression has been reported. In particular, the levels of total FAK protein and pFAK Y861 also appear to be higher in HER2 overexpressing breast tumours, which

further correlated with increased AKT activity and poorer tumour differentiation (Schmitz et al. 2005). More recently, it was shown that Herceptin induced alterations in the appearance, localisation and dynamics of focal adhesions as well as suppressed invasion in $FAK^{+/+}$ HER2+ cells, much less so in $FAK^{-/-}$cells, though this was rescued upon re-expression of FAK, further supporting the link between HER2 and FAK signalling (Xu et al. 2009).

1.5 Aims and Objectives

In light of the evidence linking FAK and HER2 and the fact that HER2 cancers are a poor prognostic subtype that frequently exhibit resistance to targeted therapy, we hypothesised that FAK might represent an important target in HER2+ cancers.

To investigate this hypothesis we set the following objectives:

- Explore the functional relevance of FAK in a panel of cell models reflective of different HER2+ breast cancer subtypes paying particular interest to the role of FAK in cell proliferative responses and the underlying signalling mechanisms.

- Investigate the role of FAK in HER2-mediated migratory responses implicated in metastatic processes.

- Explore whether the use of Herceptin in combination with the novel FAK inhibitor (PF878) (targeting the Y397 autophosphorylation site) might be a useful strategy to enhance/sensitize to the growth suppressive effects of Herceptin.

2. Methodology

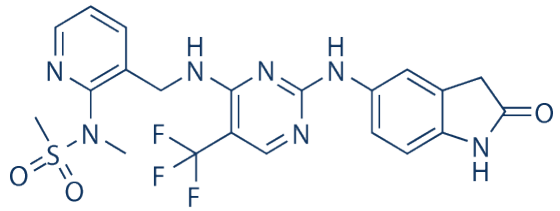
2.1 Materials and Reagents

2.1.1. Key drugs or reagents used

- The FAK inhibitors used in this project were PF-04554878 ('PF878', a gift from Pfizer) and PF-562271 ('PF271', purchased from Tocris Bioscience (Roberts et al. 2008)). Both compounds work as potent competitive ATP inhibitors of FAK, suppressing its kinase activity. Stock solutions were prepared in DMSO at a concentration of 5mM.

  The structures of PF271 (N-Methyl-N-(3-{[2-(2-oxo-2,3-dihydro-1H-indol-5-ylamino)trifluoromethyl-pyrimidin-4-ylamino]-methyl}-pyridin-2-yl)-methanesulfonamide) and PF878 (N-methyl-4-(4-((3-(N-methylmethan-3-ylsulfonamido)pyrazin-2-yl)methylamino)-5-(trifluoromethyl)pyrimidin-2-ylamino)benzamide are illustrated below. PF878, is a derivative of PF271 but with a side group modification to improve oral bioavailability.

A



B

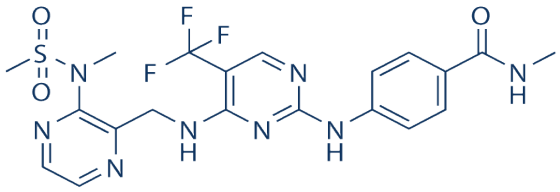


Figure 2.1 Chemical structures of the FAK inhibitors (A) PF-562271 and (B) PF-04554878

- Trastuzumab (Herceptin, Roche Ltd) is a recombinant humanized monoclonal antibody produced in Chinese Hamster ovary (CHO) cells that selectively binds to the extracellular domain of HER2 receptor. Herceptin, provided in the form of

white to pale yellow lyophilized powder, was reconstituted in RNAase-free sterile water to give a stock concentration of 100 µM.

- Heregulin β1 (HRG, Sigma Aldrich UK), was provided in the form of lyophilized powder containing 2.5 mg Bovine serum albumin (BSA) and was reconstituted in RNAase-free sterile water to give a stock concentration of 100µg/ml.

- 'Stattic' is an irreversible STAT3 inhibitor (Sigma Aldrich) that directly interferes with the SH2 binding site of STAT3. The chemical name is 6-Nitrobenzo[b]thiophene-1,1-dioxide and the structure illustrated below. A 110mM stock solution was made by reconstituting 25mg/100µl of DMSO and stored in aliquots at -20°C where it is stable for up to one month.

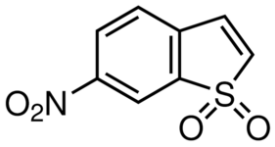


Figure 2.2 Chemical structure of Stattic $C_8H_5NO_4S$

- All drugs/reagents were diluted to the final concentration in fresh cell culture medium on the day of experiments.

2.1.2 Other materials

All other materials used throughout this study along with the companies from which they were sourced are listed in table 2.1 below.

Material	Source
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma Aldrich
3D Culture 96 well BME Cell invasion assay kit	Cultrex
5X siRNA buffer solution	Thermo Scientific
30% Acrylamide solution	Sigma Aldrich
Alexafluor Fluorophores- 488, 594	Invitrogen
TRITC-labelled actin phalloidin	Sigma Aldrich
Ammonium Persulphate (APS)	Sigma Aldrich
EZ View Red Protein G/A Affinity Gel beads	Sigma Aldrich
Fungizone	Invitrogen, Paisley UK
Penicillin/Streptomycin	Invitrogen, Paisley UK
Aprotinin	Sigma Aldrich
BioRad Protein Assay Reagents A, B, S	BioRad Laboratories Ltd
Blue sensitive X-ray film	Photon Imaging Systems
Bovine Serum Albumin (BSA)	Sigma Aldrich
Bromophenol Blue	BDH Chemicals
Cell Culture Medium (RPMI 1640 and Phenol Red-free RPMI 1640)	Invitrogen, Paisley UK
Chemiluminescence reagents (ECL,Pico, Dura, Femto,)	Fisher Scientific UK
Corning Standard Transwell Inserts (6.5mm diameter,8um pore size)	Fisher Scientific UK
Crystal Violet	Sigma Aldrich
Dimethyl sulphoxide (DMSO)	Sigma Aldrich
Di-thiothreitol (DTT)	Sigma Aldrich
Dharmafect Transfection Reagent	Thermo Scientific
DCCM-1 culture media	Biological Industries
Fibronectin (from Human plasma): 1mg/ml	Sigma Aldrich
Foetal Calf Serum	Gibco UK
Glycerol	Fisher Scientific
Glycine	Fisher Scientific UK
Leupeptin	Sigma Aldrich

L-glutamine	Sigma Aldrich
Methanol	Fisher Scientific UK
NP40	Sigma Aldrich
Ponceau S solution (0.1% w/v in 5% acetic acid)	Sigma Aldrich
Phenylarsine oxide	Sigma Aldrich
Phenylmethylsulfonyl fluoride (PMSF)	Sigma Aldrich
Precision Plus Protein Blue marker	BioRad
RhoA/Rac1/Cdc42 Activation Assay Combo Kit	Cytoskeleton Inc. USA
siRNA buffer (1X) diluted in H_20	Sigma Alrich
Sodium Azide	Sigma Aldrich
Sodium dodecyl sulphate (SDS)	Sigma Aldrich
Sodium Fluoride	Sigma Aldrich
Sodium Molybdate	Sigma Aldrich
Sodium Orthovanadate	Sigma Aldrich
Stripping buffer	Fisher Scientific UK
Tetramethylethylenediamine (TEMED)	Fisher Scientific UK
Triton-X 100	Sigma Aldrich
Tween-20	Sigma Aldrich
Trizma Base (Tris)	Fisher Scientific
Vectashield mounting medium (hardset) containing DAPI	Vectorlabs

Table 2.1 List of materials used throughout the project

All primary and secondary antibodies used for immuno-based detection assays are listed in **Table 2.2** below.

Target protein	Species	Distributor	Catalogue number
pAKT (ser473)	Rabbit	Cell signalling	4051
AKT (total)	Rabbit	Cell signalling	9272
B-actin	mouse	Sigma Aldrich	A5316
E-Cadherin	Mouse	R&D systems	BTAI
pEGFR (y1068)	Rabbit	Cell Signalling	2234
pFAK(Y397)	Rabbit	Cell signalling	3283
pFAK(y861)	Rabbit	Invitrogen	44-626-G
pFAK (y576/577)	Rabbit	Cell Signalling	3281
FAK (total)	Rabbit	Biosource	AH00502
pHER2(y1248)	Rabbit	Cell signalling	2247
HER2 (total)	Rabbit	Cell signalling	2242
pHER3(y1289)	Rabbit	Cell signalling	4791L
HER3 (total)	Rabbit	SantaCruz	SC-285
pHer4(y1284)	Rabbit	Cell Signalling	4757
pMAPK	Rabbit	Cell signalling	9101S
MAPK (total)	Rabbit	Cell signalling	9102
pSrc(Y416)	Rabbit	Cell signalling	21015
pSTAT3 (Y705)	Rabbit	Santa Cruz	SC-7993
Total Stat3	Rabbit	Santa Cruz	C-20
Src (total)	Rabbit	Biosource	44-656-G
PARP	goat	R&D systems	AF-600-NA
GAPDH	mouse	SantaCruz	SC-32233
Anti-Rabbit IgG	Goat	Cell signalling	7074
Anti-mouse IgG	Sheep	GE Healthcare	NXA931
Normal Rabbit IgG	Rabbit	Santa Cruz	SC-2027

Table 2.2 List of antibodies used throughout the project. For Western Blotting the antibodies were used at a dilution of 1:1000 with the exception of the loading controls β-actin and GAPDH which were used at a dilution of 1:15,000.

2.2 Cell Culture

The human breast cancer cell lines used in this study obtained from ATCC are shown **Table 2.3**. The tissue/organ of origin from which they were derived from and the routine culture conditions are also detailed.

Cell line	ER,PR and HER2 status	Source	Tumour type	Culture conditions
MCF7	HER2- ER+ PR+	PE	Invasive ductal carcinoma	RPMI+ 5%FCS+ 2% glutamine
T47D	HER2- ER+ PR+	PE	Invasive ductal carcinoma	RPMI+ 5%FCS+ 2% glutamine
BT474	HER2+ ER+ PR+	P.Br	Invasive ductal carcinoma	RPMI+ 10%FCS+ 2% glutamine
MDA361	HER2+ ER+ PR-	P.Br	Breast adenocarcinoma from a metastatic site in the brain	RPMI+ 10%FCS+ 2% glutamine
SKBr3	HER2+ ER- PR-	PE	Pleural effusion from Breast adenocarcinoma	RPMI+ 10%FCS+ 2% glutamine

Table 2.3. Characterisation of breast cancer cell lines used. Expression of ER, PR and HER2 detailed and adapted from (Neve et al. 2006). **PE:** Pleural Effusion **P.Br:** Primary Breast. **RPMI**: (Roswell Park Memorial Institute) medium (containing phenol red). **FCS:** foetal calf serum. All culture media were also supplemented with 10IU/ml penicillin, 10ug/ml streptomycin and 2.5ug/ml fungizone.

In other studies, we also used a lung fibroblast cell line, MRC-5. These cells were obtained from ATCC and routinely maintained in RPMI supplemented with 5%FCS, 2% glutamine, 10IU/ml penicillin, 10µg/ml streptomycin and 2.5ug/ml fungizone.

In experiments that required collection of conditioned media (Chapter 6), this was done using DCCM-1 medium, a high-protein serum-free media supplemented with 200mM glutamine 10µg/ml streptomycin, 2.5ug/ml fungizone. This is in order to avoid confounding effects caused by serum proteins.

All cells were routinely sub-cultured when they reached approximately 80-90% confluence, usually every 7-10 days. Cells were detached from the plate by the addition of trypsin/EDTA solution (0.05%/0.02% in PBS respectively) and incubated for 3minutes at 37°C in a 5% CO_2 humidified incubator. Trypsin activity was neutralised by addition of serum-containing cell culture medium and centrifuged at 1000rpm for 5 minutes to

pellet cells. The supernatant was discarded whilst the pellet was re-suspended in fresh culture medium and sub-cultured into flasks usually in a 1:6 – 1:10 ratio of the original culture.

In all experiments, the culture medium was replaced every four days unless otherwise stated. All cell culture procedures were carried out in a laminar flow safety cabinet to ensure sterility of cultures.

2.3 Cell proliferation assays

2.3.1 MTT assay

This assay involved the conversion of the water-soluble yellow compound, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), to insoluble purple formazan crystals by mitochondrial dehydrogenase enzymes in actively respiring and viable cells. The formazan is solubilized and quantified by spectrophotometric means (Mosmann 1983). The colour produced is directly proportional to the number of viable cells and is therefore used to assess the anti-proliferative effects of various drug treatments.

Cells were harvested using trypsin/EDTA as detailed in section 2.2 and seeded into 96-well plates at a density of $6x10^3$ cells/well (Day 0), and were allowed to settle for 24hrs before treatments were added as required (Day1). Plates were incubated for 5 days at 37°C in a humidified incubator, with a medium change at Day 4. On day 6, medium was removed from each well and cells gently washed with PBS. 140ul MTT solution (0.5mg/ml in phenol-red free RPMI filtered through a 0.2µm syringe filter) was added to cells and left to incubate at 37°C for 4hrs. The MTT solution was then aspirated and the cells lysed with 100 µl 10% (v/v) Triton-X100 (Sigma Aldrich) in PBS overnight at 4°C. The following day, the resulting solution was read in a spectrophotometer at 540nm. Mean absorbance values were calculated and normalised to % of its corresponding control (100%).

2.3.2 Cell growth measurement using a Coulter counting assay

This approach represents the most direct way of counting cells and useful in detecting more discrete changes in cell number than other methods such as MTT-based assays. Coulter

counter is a form of electronic counting wherein cells in single suspension pass through a small opening between the electrodes of the counter. As they pass individually through a sensor, a pulse is detected and is noted as one particle count. This assay was used for when longer growth experiments are required (7 days or more), as the MTT assay, owing to the small surface area of a 96-well plate, is limited to experiments of just 5 days.

Cells were harvested using trypsin/EDTA and seeded into 24-well plates at a density of 1x 10^5 cells/well (Day 0), and were allowed to settle for 24hrs before treatments were added as required (Day1). Plates were incubated for 6 days at 37°C in a humidified incubator. Typically, this growth assay was run for 7 to 10 days with medium change every 4 days. Following the required incubation period, medium was removed from each well and the cell monolayer was dispersed by addition of trypsin/EDTA solution (1ml/well). Thereafter, cells were drawn up into a 5ml syringe through a 25G needle three times to ensure a single-cell suspension. Cells were then washed three times with Isoton solution (1ml), also taken up into the syringe each time. Cells in were syringed into a counting cup containing 6mls of Isoton to give a total volume of 10mls and counted using a Coulter Multisizer II. A minimum of two counts were taken from each well in triplicate. Mean averaged cell count was normalized to % of its corresponding control (100%).

2.4 Antibody-based detection methods

2.4.1 SDS-PAGE and Western Blotting

2.4.1.1 Cell lysis for extraction of whole cellular contents

Cells growing in 35mm diameter petri dishes ± treatments as indicated were placed on ice and washed with PBS three times. To each dish was added an appropriate volume of lysis buffer containing fresh protease inhibitors (2mM sodium orthovanadate, 50mM sodium fluoride, 1mM PMSF, 10mM sodium molybdate, 20µM phenylarsine, 10µg/ml leupeptin and 8µg/ml aprotinin). This inhibitor cocktail prevents protein degradation and dephosphorylation by endogenous proteases and phosphatases present in the whole cell extracts. In doing so, a better yield is achieved but importantly the phosphorylated residue of interest remains intact. The cells were scraped and collected into 1.5ml Eppendorf tubes and placed on ice for a further 5 minutes. Sample were then centrifuged for 15minutes at

12,000rpm in 4°C and the resulting supernatant containing cellular proteins was retained and transferred onto fresh Eppendorf tubes for storage at -20°C or prepared for protein quantification (**see section 2.4.1.3**).

2.4.1.2. Cell lysis for isolation of cell nuclear and cytoplasmic fractions

Cells growing in 60mm diameter petri dishes ± treatments as indicated were placed on ice and washed with PBS three times, scraped, collected in 1.5ml Eppendorf tubes and centrifuged at 1000rpm for 5 minutes at 4°C. The pellet was re-suspended in 5 pellet volumes of cytoplasmic extract (CE) buffer (containing NP40, **Table 2.4**) and left on ice for 5minutes. Lysates were centrifuged as above and the supernatant (containing the CE) transferred into fresh Eppendorf tubes and temporarily kept on ice. The pellet, now containing cell nuclei, was washed with CE buffer (without NP40) by gentle pipetting and centrifuged as above. The supernatant was discarded and the pellet was re-suspended in 2 pellet volumes of the NE buffer (**Table 2.5**) and incubated on ice for 10 minutes with periodic vortex mixing to loosen the pellet. All fractions were centrifuged at maximum speed of 14,000rpm for 10minutes at 4°C for further clarification. Supernatants were transferred into fresh Eppendorf tubes and stored at -20°C until required for SDS-PAGE processing.

10X cytoplasmic buffer (CE)	**Amount in 50mls**	**Concentration (10x)**
HEPES	1.19g	100mM
KCl	2.23g	600mM
EDTA	0.18g	10mM
NP40	375µl	0.75%
On the day of experiment -Diluted to 1X with distilled H_20. -100ul of PMSF (100mM stock) and 1mM DTT added to 10mls of 1X buffer solution.		

Table 2.4 Cytoplasmic Extract Buffer

10X nuclear extract (NE) buffer	Amount in 50mls	Concentration (10x)
Tris-HCl	1.576g	200mM
NaCl	12g	4200mM
$MgCl_2$	0.15g	15mM
EDTA	0.03g	2mM
On the day of experiment Diluted to 1X with distilled H20. 100ul of PMSF (100mM stock) and 1mM DTT added to 10mls of 1X buffer solution. Add glycerol (25% v/v)		

Table 2.5. Nuclear Extract Buffer

2.4.1.3 Protein Quantitation

To determine the amount of total protein present in each sample, a Bio-Rad DC colourimetric protein assay was performed. This is based on the reaction of copper tartrate solution with the protein sample and a subsequent reduction of Folin reagent to produce a characteristic blue colour.

A series of BSA protein standards of known concentration (0, 0.25, 0.5, 0.75, 1.0 and 1.45 mg/ml) were diluted in lysis buffer in cuvettes in order to obtain a standard curve. In parallel, 10 µl of each protein lysate sample was aliquoted into separate cuvettes. To both the protein standards and lysate samples 250ul of Bio-Rad 'reagent A' (containing 20µl/ml of 'Reagent S') and 2mls of BioRad 'Reagent B' were added and gently mixed. After 5 minutes the absorbance at 750nm of each protein standard was read using a spectrophotometer and values plotted to create a standard curve. From this, the absorbance of the sample proteins can be deduced, which would be directly proportional to protein concentration. This allowed loading of approximately equal amounts of protein from different cellular samples on SDS-PAGE gels.

2.4.1.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

To be able to separate proteins according to their size by SDS-PAGE, samples were prepared by mixing the samples with appropriate volumes of 3x Laemmli sample loading (with 24mg/ml DTT (freshly added on the day). The negatively charged detergent, SDS, denatures

and coats proteins giving them a negative charge to allow their separation by size and charge when an electrical field is applied to a porous polyacrylamide gel. The glycerol increases the density of the sample solution ensuring that the sample remains in the bottom of the polyacrylamide wells, whilst the TRIS acts as a buffer to maintain pH during electrophoresis. The DTT causes the disruption of tertiary protein structures by breaking disulphide bridges to maximise the binding of SDS, whilst the bromophenol blue is a dye that allows the visualisation of the protein migration front. Prepared samples were boiled for 5 minutes at 100°C in sample buffer to ensure protein denaturation.

SDS-polyacrylamide gels were prepared as detailed in **Table 2.6**, with the use of a gel stacking apparatus (BioRad Mini Protean 3). Resolving gels (lower) of 8-12% polyacrylamide solutions were poured between 1.5 mm glass plates positioned on a casting frame. Water was added on top of each gel to ensure a flat edge, and prevent contact with air which may interfere with the polymerisation process. Gels were allowed to polymerise for 15 minutes, thereafter, the water overlay was poured out.

Final Gel % (Resolving)	**8%**	**10%**	**12%**
30% Acrylamide	5.4ml	6.6ml	8ml
dH_20	9.2ml	8ml	6.6ml
Tris pH 8.8	5ml	5ml	5ml
10% SDS	200ul	200ul	200ul
10% APS	200ul	200ul	200ul
TEMED	50ul	50ul	50ul

Table 2.6 Resolving Gel

Final Gel % (Stacking)	**5%**
30% Acrylamide	1.67ml
dH_20	5.83ml
Tris pH 6.8	2.5ml
10% SDS	100ul
10% APS	50ul
TEMED	25ul

Table 2.7 Stacking gel

Stacking gels of 5% polyacrylamide solution (**Table 2.7**) were prepared and poured on top of the resolving gel immediately followed by insertion of 10/15-well plastic combs. The stacking gel was left to polymerise for a further 20 minutes. Once set, individual gels were transferred to a gel running tank and the reservoirs were flooded with running buffer (192mM glycine, 25mM Tris, 0.1% w/v SDS) and wells were loaded with the prepared protein samples. Precision Plus Protein All Blue marker (BioRad) (**Figure 2.3**) was also loaded alongside, to allow for estimation of the protein molecular weights. Electrophoresis was performed at a constant voltage of 120V for approximately 1hr 30minutes or until desired separation is achieved.

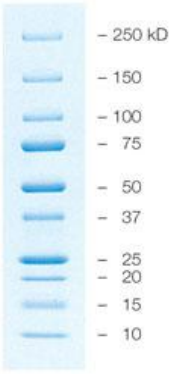


Figure 2.3 Precision Plus Protein Blue Standard.
Molecular weights in kD indicated on the right hand side.

Separated proteins were then transferred from the gel onto a nitrocellulose membrane (GE Healthcare) by construction of a Western blotting 'sandwich' system illustrated in **Figure 2.4.** Assembled cassettes were placed in a tank filled with transfer buffer (0.25M TRIS base, 1.92M Glycine, 20% methanol) and an ice block. Proteins were transferred over 60 minutes at 100-120V, allowing for the negatively charged proteins to migrate from the gel to the membrane. After the transfer, the membranes were immersed in Ponceau-S then washed briefly in distilled water to assess the efficiency of the transfer.

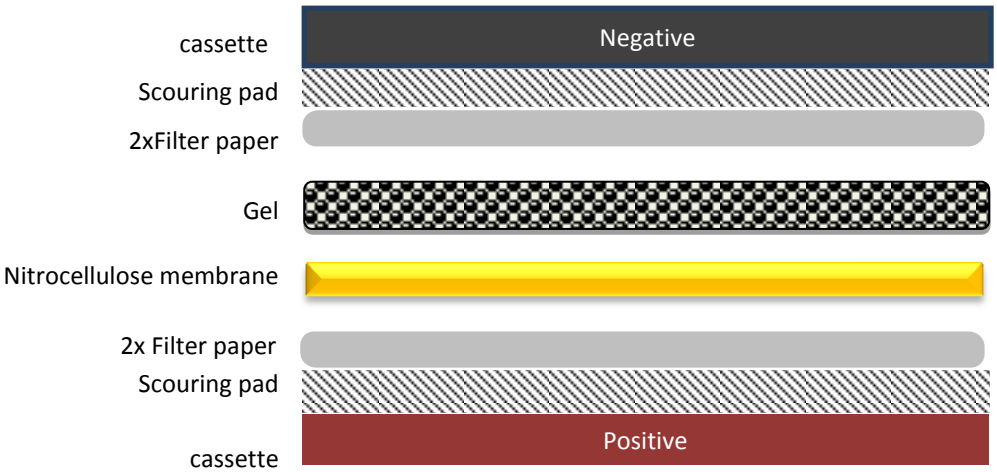


Figure 2.4. Wet transfer "Sandwich" set-up. The membrane and gel are placed in the direction of the current flow which is indicated in the apparatus diagram. All components were pre-soaked in transfer buffer prior to set-up.

Membranes were removed from the sandwich stack and blocked with 5% non-fat milk (Marvel) in TBS Tween-20 (0.05%) (TBST) and subsequently probed with primary antibodies specific for the protein(s) of interest. Membranes were incubated overnight at 4°C in falcon tubes on a roller bed then washed three times (10minutes per wash) with TBST to remove any excess unbound primary antibody and probed with Horseradish peroxidise (HRP)-conjugated secondary antibodies diluted in 1% non-fat milk in TBST for 1hr at room temperature. Membranes were subsequently washed with TBST three times before the chemiluminescence detection as detailed below.

2.4.1.5 Chemiluminescence detection

The enzymatic activity of the bound HRP-conjugated secondary antibodies was activated by incubation with freshly prepared developing solution containing a luminol-based enhanced chemiluminescence substrate (**Figure 2.5**). In the dark room, the membranes are exposed to blue-sensitive X-ray films and processed in an automated developer to visualise reactive protein bands.

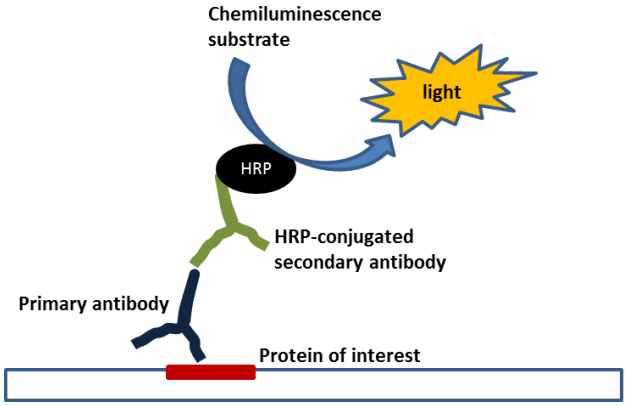


Figure 2.5. Chemiluminescence detection. The HRP-conjugated secondary antibody binds to the primary antibody specific for the protein of interest on the nitrocellulose membrane.

2.4.1.6 Stripping and re-probing membranes

In some cases, membranes were re-probed with a second primary antibody to detect additional proteins in the same lysates. Where the first and second target proteins were of similar size, membranes were first stripped in order to avoid signal carry over form the first probing. The stripping process involved washing membranes in Restore Plus Western Blot Stripping Buffer (Fisher) for approximately 15minutes at room temperature. After rinsing with TBST, membranes were exposed blue X-ray films for a minimum of 15minutes to check that the stripping had worked before incubating with antibodies. The membranes were then blocked with 5% milk/TBST and incubated with appropriate primary antibodies.

2.4.1.7 Densitometry

Densitometry analysis of Western blots was performed using AlphaDigiDoc program. Integrated density values from the bands of protein of interest are typically normalised to the loading protein control, GAPDH or β-actin. Normalised values are then converted to % values corresponding to the assigned control band (100%).

2.4.2 Immuno-fluorescence staining

Cells were grown on 0.13-0.17mm thick coverslips pre-coated with fibronectin (1:100 in sterile PBS) in 35mm culture dishes. When they reached approximately 60-70% confluence, treatments were added at doses and for the times indicated. Cells were then washed three times with PBS, fixed with paraformaldehyde ('PFA', 3.7% in dH20) for 15minutes and permeabilised using Triton-X100 (0.1% in PBS) for 8 minutes. Cells were blocked in 10% normal goat serum (in 1% BSA in PBS) for 40 minutes to block non-specific antibody binding. This was followed by 40 minutes incubation with the primary antibodies (1:100 dilution in PBS containing 1% BSA). For double labelling, all primary antibodies were mixed together prior to incubation. Cells were thoroughly washed with PBS three times and incubated with the appropriate fluorophore-conjugated secondary antibodies for 40 minutes (Alexa-fluor488, Alexa-fluor 592, Alexa-fluor-conjugated phalloidin at 1:1000 dilution). Coverslips were again washed and mounted onto glass slides using Vectashield mounting media which contained DAPI (4', 6-diamidino-2-phenylindole) as a nuclear counterstain. Cells were viewed under oil-immersion at 63X

magnification using a Leica DMIRE2 microscope. Where necessary, images captured were imported into Image J for 8-bit conversion to generate a grayscale image. This was subsequently used to generate an auto-threshold image to segment features of interest.

2.4.3 Immunoprecipitation

All procedures performed on ice unless otherwise stated.

500 µg of protein lysates were diluted in RIPA buffer to a volume of 1ml. These were incubated with 1µg of primary antibody or an equivalent amount of normal IgG as a negative control for 2hrs at 4°C on a rotator allowing for antigen/antibody complexes to form. Meanwhile, the EZview Red protein Affinity Gel Beads (Sigma Aldrich UK) were equilibrated in preparation for immunoprecipitation as follows: 30µl of the bead slurry was aliquoted into Eppendorf tubes and mixed with 700µl RIPA buffer until uniformly re-suspended and centrifuged for 30seconds at 9400rpm; this washing and equilibration step was repeated twice and after the final wash the supernatant was carefully removed and the bead pellet kept on ice.

The antigen/antibody mix were added to the pre-washed beads and incubated for 2-3hrs at 4°C with rotation, allowing for the antigen/antibody complexes to bind to the beads. The mix was then centrifuged at 9400rpm for 30seconds at 4°C and the supernatant carefully removed. The bead pellet was washed thrice by adding 750µl of RIPA buffer, gently mixed, followed by centrifugation as above. After the last centrifugation step, the supernatant was removed and the bound antigen/antibody complexes eluted by adding 25µl of RIPA buffer and 25µl of Laemmli loading buffer (2X) followed by vigorous mix by vortex; which would initiate the dissociation of the antigen/antibody complexes from the beads. Samples were then denatured by boiling at 100°C for 5 minutes. Finally, the mix was centrifuged at 9400rpm for 1min to pellet the beads, whilst the supernatant containing the immuno-precipitated proteins was collected and ran on SDS-PAGE.

2.4.4 Immunocytochemistry (ICC) analysis of Ki67

Cells were grown on coverslips in 35mm culture dishes. When they reach approximately 60-70% confluence, treatments or stimulants were added, for the necessary times. Cells

were fixed with 2.5% Phenol formal saline and washed with PBS three times followed incubation with the primary antibody (Ki-67) for 2 hours in a humidified chamber at room temperature. Coverslips were washed with PBS and incubated with the appropriate Dako Envision secondary antibody for 2hours. After washing, sections were then visualized with 3,3'-diaminobenzidine (DAB) as a chromogen for 5 minutes and counterstained with haematoxylin. Coverslips were washed with tap water, air dried, mounted onto glass slides and visualised under a light microscope. Ki67-positive cells were counted in at least 5 different fields of view and expressed as % of the mean positive cell count of all cells per field against the control (100%).

2.5 *In vitro* migration assay

To monitor cellular migration, a Boyden chamber based approach was used. These assays were conducted using 24-well plate inserts (Corning) having a 6.5mm diameter microporous membrane (8 micron pore size). Inserts were first coated with fibronectin (1µl:100µl in PBS). Cells at a density of 0.5 x 10^5 cells/ml were then seeded in serum-free RPMI supplemented with 0.1% BSA (±treatments). In the lower chamber chemo-attractants were added to the experimental medium and cells were allowed to migrate to the underside of the membrane for 20hrs. Non-migratory cells were wiped from the top of the chamber suing a cotton bud whilst migratory cells were fixed with 3.7% PFA, stained with 0.5% crystal violet and allowed to dry. Stained cells were visualised by light microscopy and counted. Cell migration was quantified as mean cell number counts per field of view. Data (mean cell migration) was obtained from 5 random fields of view per insert. Each treatment was performed in duplicates for each experiment, which was independently performed three times.

2.6 *In vitro* 3D invasion assay

Cells suspended in extra cellular matrix (ECM) (Cultrex) were seeded onto a round-bottom 96 well plate and left to incubate for 72hrs to promote aggregation or spheroid assembly. "Invasion matrix" (Cultrex) was added to the spheroids and centrifuged at 200xg for 3 minutes to eliminate bubbles. After 1hr, culture medium containing chemo-attractants and/or treatments were added to the top and left for 3 days to allow cells to invade into the surrounding matrix. The spheroids were photographed at various time-points.

2.7 Detection of MMP2 and MMP9 using gelatin zymography

The principle of the assay is similar to that of SDS-PAGE with the additional incorporation of gelatin in the resolving gel at a final concentration of 0.1%. Samples under test were mixed with appropriate volumes of 2X laemmli loading buffer (1:1 ratio). Gels were ran on ice at ~70V for 4 hours and washed in 2.5% Triton X100 for 30 minutes at least three times with gentle agitation. This was followed by incubation in the 'activation' buffer containing 50mM TRIS, 10mM $CaCl_2$ (pH 7.6) at room temperature with agitation overnight. The next day, gels were stained for 1-2hrs with Coomassie Blue (0.1% Coomassie solution in 20% acetic acid at a 1:1 ratio) and de-stained with de-staining solution for approximately 1 hr, after which it was viewed under white light and photographed.

2.8 *In vitro* RNAi transfections

To transiently suppress FAK expression in our cell models, a siRNA based approach was taken using the ON-TARGETplus Human PTK2 (5747) pool of four highly potent siRNAs that target FAK (L-0003164-00-0005, Thermoscientific).

Listed are the mRNA sequences targeted by the FAKsiRNA;

1.	**GCGAUUAUAUGUUAGAGAU**
2.	**GGGCAUCAUUCAGAAGAUA**
3.	**UAGUACAGCUCUUGCAUAU**
4.	**GGACAUUAUUGGCCACUGU**

The siRNA pool above, along with a scrambled, non-targeting siRNA control, were re-suspended in 1X siRNA buffer (diluted from 5X siRNA buffer in RNAse-free sterile water) to achieve a stock concentration of 20µM.

For all experiments using siRNA, parallel controls were included which consisted of:

- *Culture medium control*- used as baseline for growth **('C')**
- *Lipid control*- cells receiving only the DHARMAfect transfection agent to confirm that the lipid and transfection procedure is minimally disruptive to the cell **('LO')**
- *ON-TARGETplus non-targeting siRNA control* – cells transfected with an siRNA designed to have no known mRNA targets, used to check for non-specific effects associated with siRNA delivery **('NTsiRNA')**

Cell transfection was carried out as follows: Cells were seeded and grown to approximately 60% confluence prior to transfection. On the day of the transfection, 20 µM stocks of the siRNA were diluted to 2µM in 1XsiRNA buffer. In two separate tubes, the appropriate volume of 2µM siRNA (to achieve a final concentration of 100nM) and DharmFECT1 Transfection agent were diluted in serum-free phenol-red-free RPMI supplemented with 200mM glutamine (SF-RPMI). After 5 minutes, the two mixtures were combined and left to incubate for 20 minutes at room temperature, allowing the formation of siRNA-containing micelles. Subsequently, these were mixed with an appropriate volume of RPMI+5%FCS and were added to the cells and left to incubate for 72hrs before functional assays were carried out. For each cell line, optimal protein knockdown time-points were assessed by Western Blotting.

2.9 Isobologram analysis

Isobologram analysis was conducted as described by Tallarida (2001), in order to determine whether two drugs act synergistically, additively or are antagonistic. To do this, firstly the $IC_{30/50}$ values for each drug are plotted on the X and Y axis of a graph and connected with a straight line, referred to as the 'line of additivity' (**Figure 2.6**) The concentrations of the two drugs that, when combined, achieved a similar effect ($IC_{30/50}$) were then plotted on the graph. Points which fall on the straight line suggest that the combination drug effect was additive, whilst those that fall above are considered to be antagonistic and those below are synergistic. Isobologram graphs indicate the mechanistic interactions between the two drugs when used in combination.

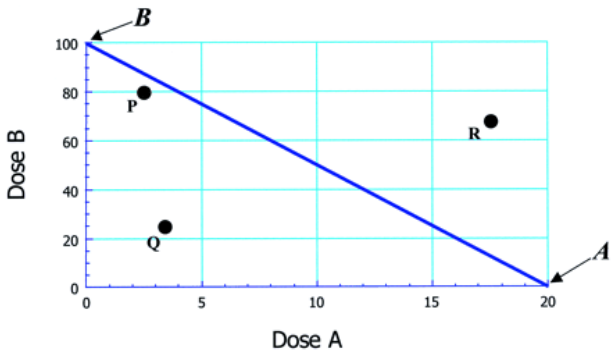


Figure 2.6. Isobologram (illustration) for some particular effect. IC_{50} dose of drug A alone is A = 20 and for drug B alone is B = 100. The straight line connecting these intercept points (additivity line). An actual dose pair such as point Q attains this effect with lesser quantities and is synergistic while the dose pair denoted by point R means higher doses are required and is therefore antagonistic. A point such as P that appears near the line are simply additive (Tallarida 2001).

2.10 Statistical analysis of data

Data was analysed using GraphPad Prism 5 software. A student's independent t-test was used to compare pairs of data while one way analysis of variance tests (ANOVA) with Tukey's multiple comparison tests (post-hoc) was used for comparing more than two groups of data. All data were considered significant when $p \leq 0.05$ *. Error bars, where appropriate, were expressed as mean ±SEM.

3. Results (I)

Targeting FAK in HER2+/ ER+ breast cancer

3.1 Introduction

Defining the ER/PR expression and HER2 amplification status in breast tumours is valuable for determining patient prognosis and guiding treatment strategy. Analysis of the molecular gene expression patterns of breast cancers further reveals a number of intrinsic subtypes, thereby deepening our understanding of the complex diversity of breast tumours and defining novel therapeutic strategies (Perou et al. 2000; Goldhirsch et al. 2011). It is now apparent that there are at least 5 distinct breast cancer subtypes: Luminal A, luminal B, normal-like, HER2+ and basal (Sørlie et al. 2001). Whilst both luminal subtypes are ER+, Luminal B are reported to be generally more aggressive than the Luminal A subtype and with significantly worse outcome, having higher relapse rates and reduced patient survival (Sørlie et al. 2001; Millar et al. 2009; Haque et al. 2012). Studies suggest that up to 30% of Luminal B breast tumours may overexpress the HER2 receptor and are associated with increased relapse risk and mortality (Cheang et al. 2009)

Elevated FAK expression has been observed in breast tumours. In one study, FAK has been shown to be elevated in pre-invasive ductal carcinoma in situ (DCIS) and infiltrating ductal carcinoma (IDC) and thus suggests that FAK may play a critical role particularly in tumours with the propensity to invade (Lightfoot et al. 2004). This is in further agreement with other studies that have identified elevated levels of FAK in invasive breast tumours (Owens et al. 1995; Lark et al. 2005). That FAK indeed has a role in tumour progression and represents attractive therapeutic target is further emphasized by numerous efforts that have been made by several groups to inhibit FAK signalling in cancer cells using small molecule inhibitors, several of which are currently in clinical trials (See Table 1.3).

Given that FAK plays a key role in the transduction of HER2 signalling in HER2+ cells and that levels of FAK expression strongly correlated with HER2 overexpression in clinical samples (Benlimame et al. 2005; Schmitz et al. 2005), and considering that FAK inhibitors are now in clinical trials, there is potential for a combination treatment approach with FAK inhibitors and Herceptin in Luminal B HER2+/ER+ breast cancer. Thus the aims of this chapter were to initially explore the role and therapeutic potential of FAK in this subtype.

3.2 Elevated FAK expression in breast cancer is associated with poorer patient survival

Previous studies of breast cancer patient samples revealed that FAK overexpression was correlated with tumours with an aggressive phenotype, high mitotic index and high nuclear grade (Owens et al. 1995; Lark et al. 2005). Extending this, we explored whether a relationship existed between FAK expression and breast cancer outcome by investigating FAK mRNA levels in a series of breast cancer tissues analysed by microarray for at least 22,227 genes which are publicly available (Kaplan-Meier analysis: www.kmplot.com) (Győrffy et al. 2010). As shown in **Figure. 3.1A**, comparison of 4142 breast cancer patient samples with no restrictions applied on tumour grade, lymph node status or endocrine/chemotherapy treatments, revealed an association between high FAK mRNA levels and a significantly worse patient RFS (logrank P = 0.00061) over a follow-up period of up to twenty-five years. When patients were split according to the luminal subtypes, high FAK mRNA levels were associated with worse RFS in tumours with luminal B features (n=1173, HER2 +/-) (logrank P=0.059), whilst no association was observed in luminal A tumours (n=553) **(Figure 3.1 B,C).** Taken together, these support several lines of evidence indicating that FAK overexpression (*PTK2* gene) is a prognostic factor for poorer survival in breast cancer patients, particularly those of the luminal B subtype.

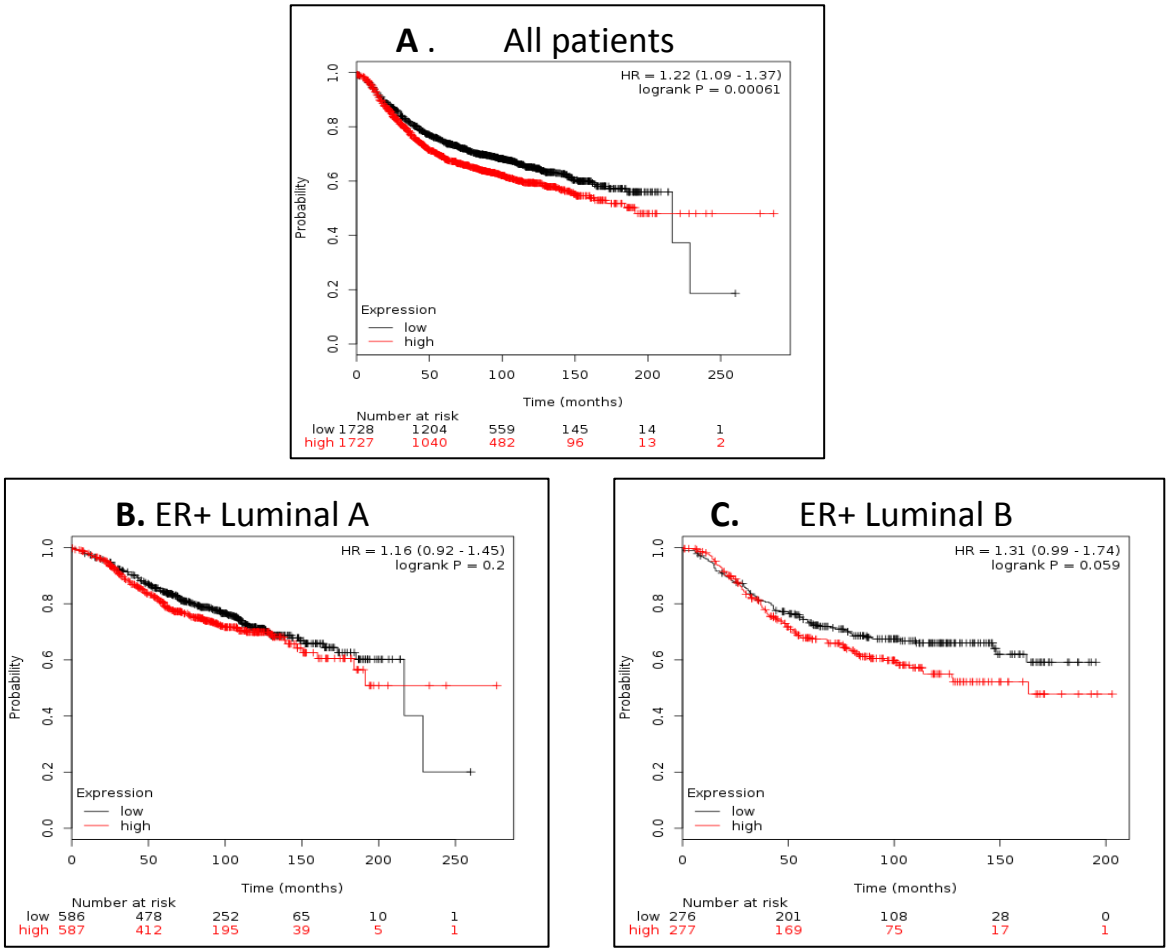


Figure 3.1. (A) The Kaplan-Meier Plotter (http://www.kmplot.com) was queried to evaluate Affymetrix microarray expression of FAK mRNA (208820_at) levels in breast cancer patient tumour samples. Selections were: RFS, grade (all), lymph node status (all) and chemotherapy treatments /endocrine treatments (all). The Hazard ratio (with 95% confidence intervals) is shown. Queries were acquired from (A) all patients (n=4142), (B) ER+ Luminal A subtype (n=1173) and (C) ER+ Luminal B subtype (n=553).

3.3 FAK activity is elevated in HER2+/ER+ versus HER2-/ER+ breast cancer cell lines

Following on from the clinical analysis, we next compared the relative expression and activity pattern of FAK in four luminal breast cancer cell lines using Western Blotting to determine whether the activity of FAK may be linked to HER2 status. These cell lines were:

- **MCF7** and **T47D** (HER2-, ER+)
- **BT474 and MDA-MB 361 (MDA361)** (HER2+, ER+)

As shown in **Figure 3.2**, we verified that MCF7 and T47D cells did not express HER2 in contrast to BT474 and MDA361 cell models, with BT474 cells exhibiting the highest HER2 expression and basal level of phosphorylation. The total protein levels of FAK were similar between these cell lines**,** however, the HER2+/ER+ cells displayed a non-significant trend for increased FAK activity (phosphorylation at Y861 but not at Y397). The extent of FAK phosphorylation at Y861 did not appear to correlate with the degree of intrinsic HER2 activity within the HER2+/ER+ cell lines, as the highest levels of FAK Y861 activity were observed in MDA361 cells, which had the lowest HER2 activity.

FAK and HER2 signalling pathways have been suggested as being linked through common downstream elements including AKT and MAPK (van Nimwegen & van de Water 2007; Schmitz et al. 2005; Park et al. 2008). Investigation of AKT and MAPK activity in these cells lines revealed a degree of variation between cell lines but no obvious relation to HER2 nor FAK expression levels.

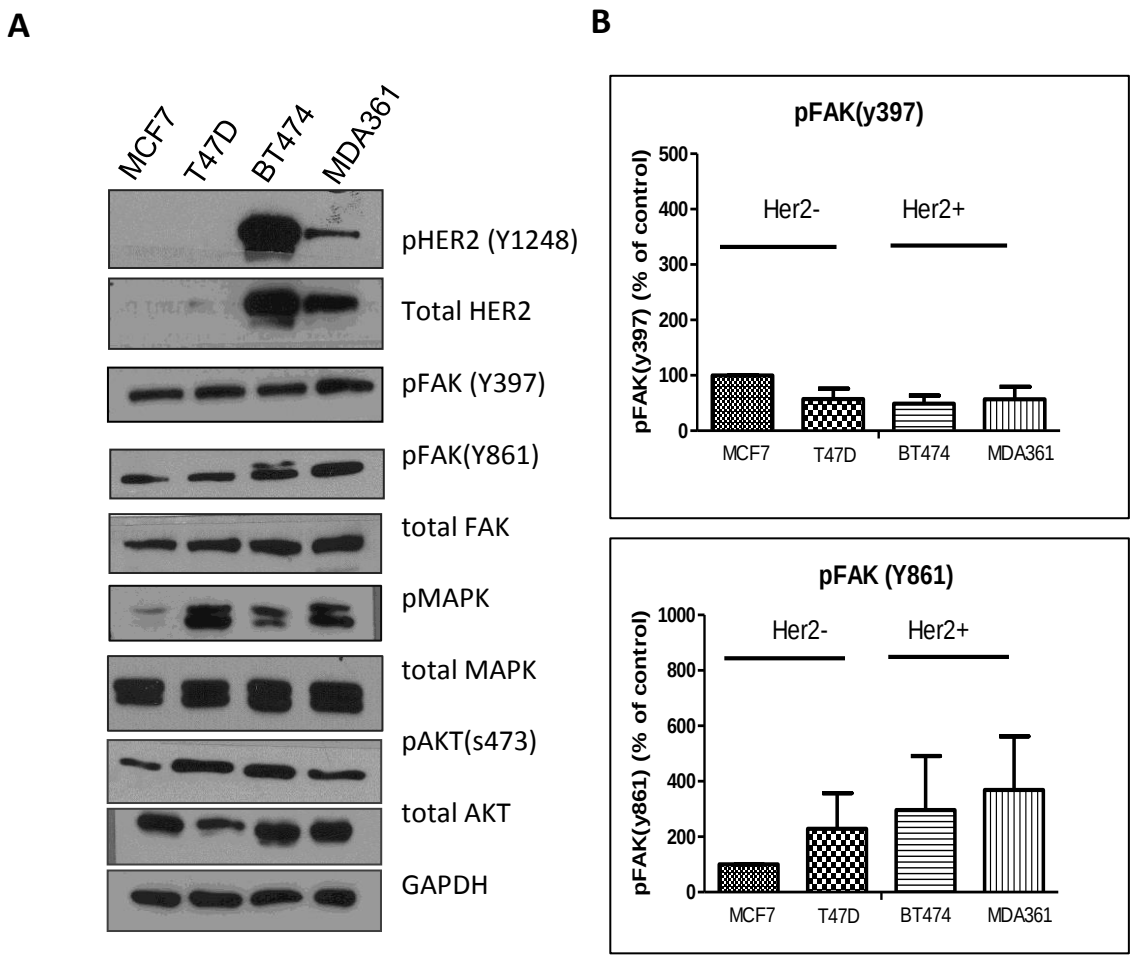


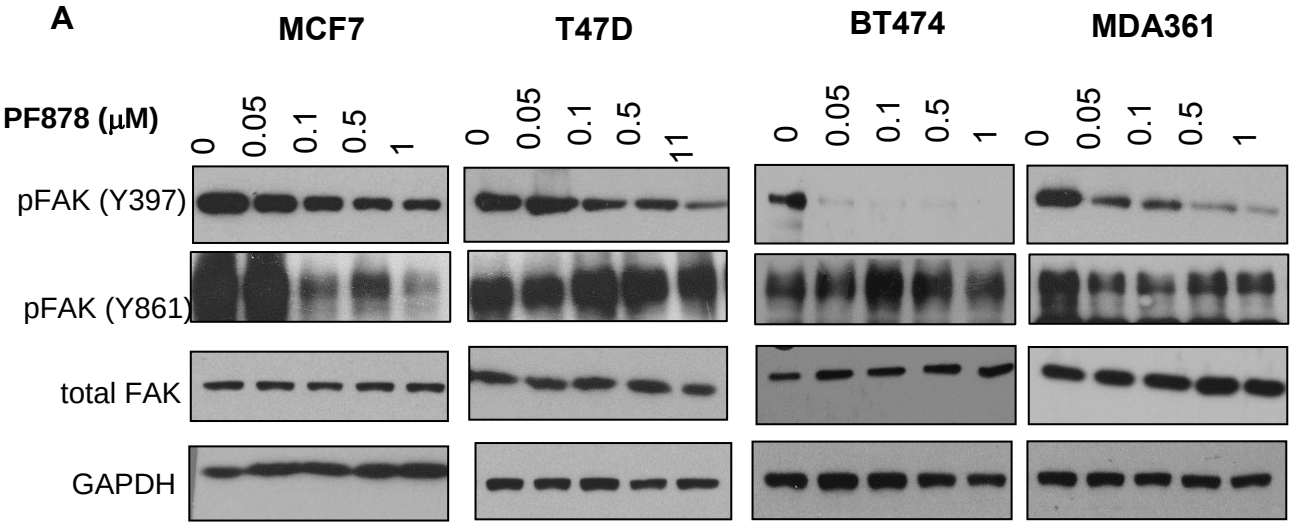
Figure 3.2. Expression of FAK in ER+/HER2- and ER+/HER2+ breast cancer cells. (A) Cell lysates from ER+/HER2- (MCF7, T47D) and ER+/HER2+ (BT474, MDA361) breast cancer cells, grown under routine cell culture conditions, lysed, process for Western Blotting and probed for FAK, HER2 and signalling elements common to these pathways. (B) Densitometric analysis showing the relative protein levels across the cells lines expressed as a ratio of active (Y397 and Y861) to total FAK and normalised to GAPDH. Representative protein levels represent the mean ±SEM from three independent experiments.

3.4 HER2+/ER+ cell lines are more sensitive to PF878 than HER2-/ER+ cell lines

As a result of the finding that FAK was partly elevated in HER2+/ER+ vs HER2-/ER+ cell lines, we then sought to determine the sensitivity of these cell lines to the small molecule FAK inhibitor, PF878.

Western blotting revealed that PF878 was able to suppress FAK phosphorylation at Y397 in both in HER2+/ER+ and HER2-/ER+ cells at sub-micromolar doses **(Figure 3.3A)**. Whilst PF878 did not affect FAK phosphorylation at Y861 in T47D or BT474 cells, treatment did result in a partial decrease of FAK Y861 activity in MCF7 and MDA361 cells. Comparison of IC_{50} values between the cell lines suggested that HER2+/ER+ cells were more sensitive to PF878 than HER2-/ER+ cells, potentially pointing to an increased reliance of these cells on FAK **(Figure 3.3B).** Within the HER2+/ER+ cell line group, BT474 cells appeared to be more sensitive to PF878 with respect to the inhibition of FAK Y397 phosphorylation versus MDA361 cells.

A



B

Cell Line	Breast Cancer Molecular Subtype	IC_{50} (μM) pFAKY397 inhibition (±SEM)
MCF7	HER2- (ER+)	0.25 ± 0.22
T47D	HER2-(ER+)	0.26 ± 0.39
BT474	HER2+(ER+)	0.05 ± 0.03
MDA361	HER2+(ER+)	0.17 ± 0.12

*

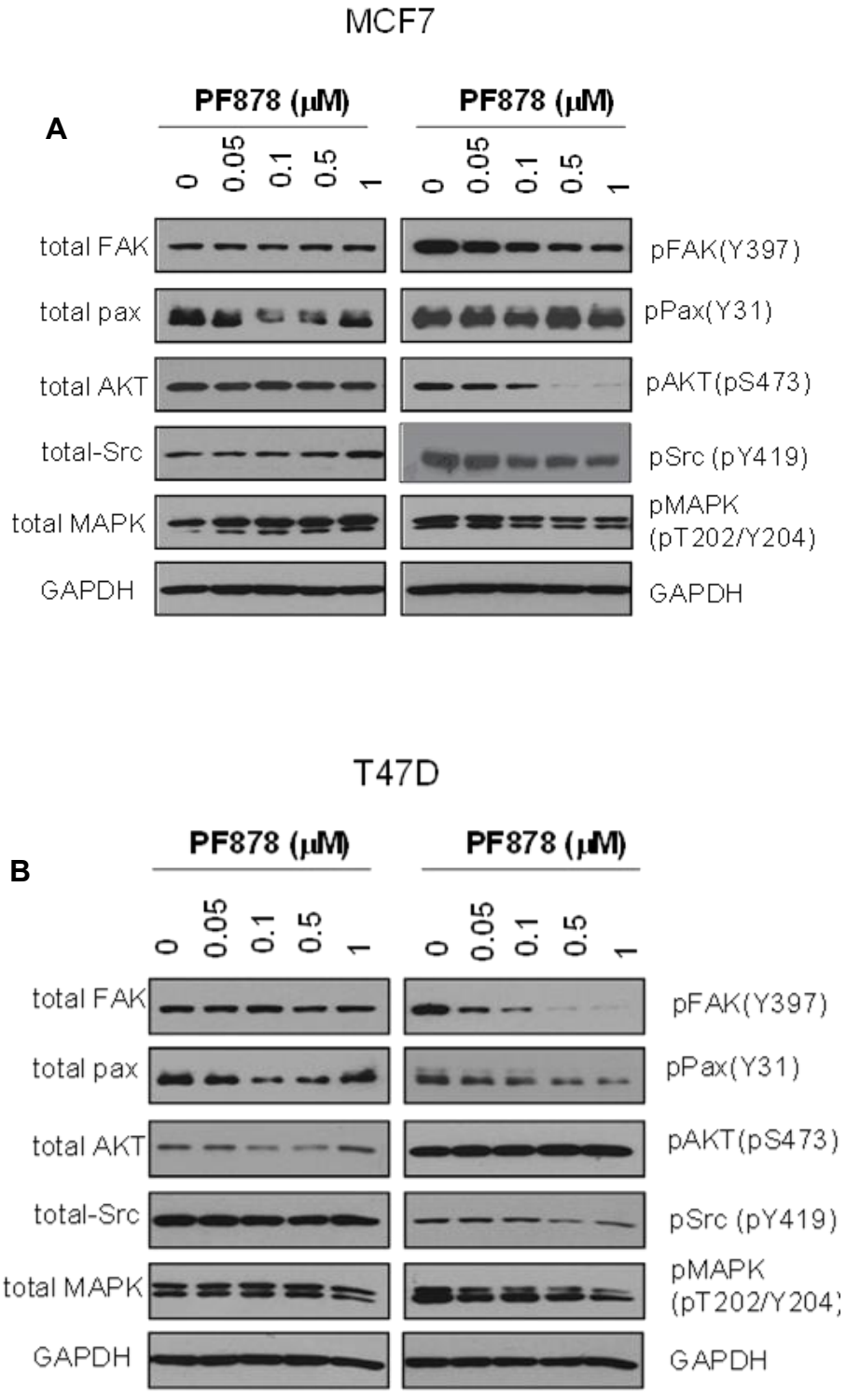
*

Figure 3.3. ER+/HER2+ cell lines are more sensitive to PF878 vs ER+/HER2- cells. (A) MCF7, T47D, BT474, MDA361 cells were treated with the PF878 (0-1μM) for 60mins. Cells were lysed, processed for western blotting and probed for FAK (total, Y397, Y861). GAPDH was used as loading control. (B) Densitometric analysis of immunoblots was carried out to calculate the IC_{50} values ± S.D for PF878. Representative blots and data are the mean of at least three independent experiments. *p=<0.05

3.5 Effect of FAK inhibition on downstream FAK- related signalling pathways

Subsequently, we investigated whether the suppression of FAK activity by PF878 resulted in the inhibition of intracellular signalling intermediates implicated in FAK and/or HER2-mediated pathways **(Figure 3.4).** In HER2-/ER+ cells, our data showed a reduction in the levels of AKT activity in MCF7 cells and of Src in T47D cells in response to PF878.

In the HER2+/ER+ cells, data showed that in BT474 cells, FAK inhibition did not affect downstream FAK-related signalling, except for paxillin, a FAK binding partner (Schaller 2001). HER2 phosphorylation at Y1248 was decreased in a dose-dependent manner in MDA361 cells with an accompanying slight reduction in the activity of Src, AKT and MAPK. In contrast, these events were not apparent in BT474 cells.



Continued on next page

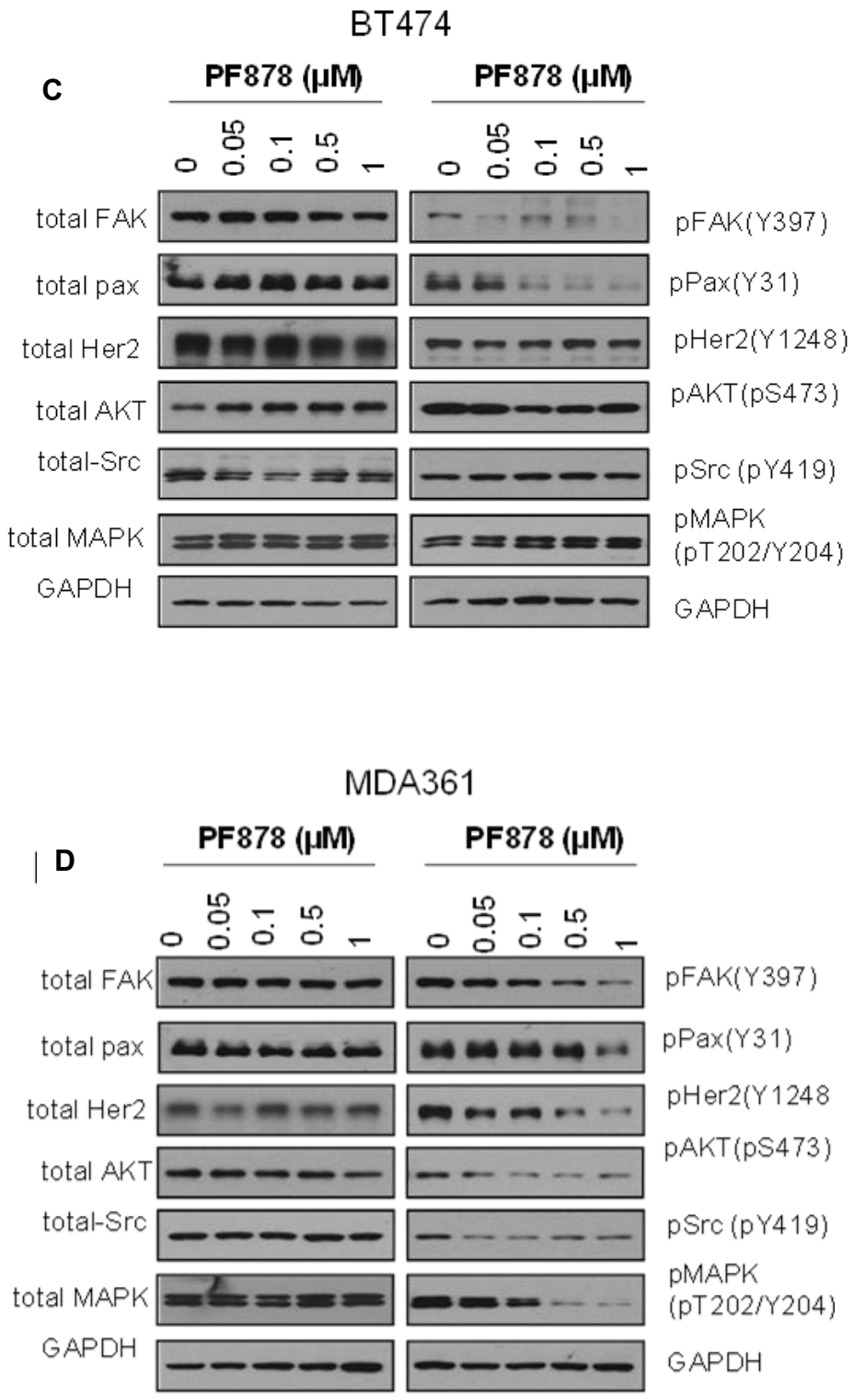


Figure 3.4. Inhibition of FAK activity suppresses endogenous FAK/HER2-related signalling in MDA361 cells. MCF7,T47D, BT474 and MDA361 cells were treated with increasing concentrations of PF878 (0–1 μM) for 60 min, following which the cells were lysed , process for Western Blotting and probed for the proteins indicated. GAPDH was used as loading control. Representative blots and data are the mean of at least three independent experiments

3.6 Inhibition of FAK suppresses HER2+/ER+ breast cancer cell migration but not proliferation

FAK is thought to function as a downstream effector of both integrin receptors, following ECM engagement, and also growth factor receptors to promote a range of cellular responses including proliferation and migration. We thus investigated the effect of FAK inhibition on these cellular characteristics. The effects of PF878 cell proliferation over a range of doses (0-1μM) that spanned the IC_{50} for FAK, despite demonstrating successful inhibition of pFAK Y397, only resulted in a modest inhibitory effect on cell proliferation across all cell lines with the greatest anti-proliferative effects observed in MDA361 cells, although this was only achieved at a dose of 1μM, much higher than its calculated IC_{50} value **(Figure 3.5A).**

Since FAK is known to be downstream of integrin receptors that bind to extracellular matrix ligands, we further investigated the hypothesis that matrix proteins might contribute to drug sensitivity (Nista et al. 1997; Kasahara et al. 2002; Pontiggia et al. 2012) and that FAK inhibition might result in greater effects on proliferation when cells are cultured in the presence of matrix components. This is based on the rationale that matrix components are known to activate integrin-mediated signalling, and may be integral in regulating cell proliferation and survival signals (Stupack & Cheresh 2002; Kim et al. 2011). Given that FAK is often coupled with integrin signalling, FAK plays a more dominant role in the presence of matrix components (Cary & Guan 1999).

As our data shows in **Figure 3.5B**, PF878 produced a modest yet more dose-dependent reduction in cellular proliferation in the presence of matrix components (fibronectin) compared with uncoated surfaces. This was particularly better observed in MDA361 cells at the higher concentration of 1μM PF878.

FAK is also important in the processes of cell migration in response to growth factor or integrin activation, a critical step in metastasis and tumour progression. FAK was shown to be involved in the formation and turnover of FAs at the leading and trailing edge of the cell, allowing for forward movement and tail retraction, to promote migration (Carragher & Frame 2004) and it has been well documented that inhibition of FAK suppresses cell migration (D. Sieg et al. 1999; Hauck et al. 2001; Hiscox et al. 2011). We thus sought to

determine the potential for PF878 to impede migration of HER2+/ER+ cells. Previous data from our group indicated that neither of these cell lines exhibited high intrinsic migratory potential in Boyden Chamber assays, suggesting that any observed differences in endogenous FAK between these cells did not affect their intrinsic migratory nature.

Thus in order to explore the role of FAK in the migratory response of BT474 and MDA361 cells, we employed Heregulin β1 (HRG), a ligand for both HER3 and HER4 receptors that can trans-activate HER2 via hetero-dimeric interactions and is known to promote cellular migration and metastasis (Plowman et al. 1993; Carraway et al. 1994; Atlas et al. 2003a). Assessment of cell morphology by bright-field microscopy revealed that in the presence of HRG (24hrs), although MDA361 cell colonies remained largely intact, cell-to-cell contacts within these colonies seemed to loosen **(Figure 3.5C),** which may indicate breakdown of cell-to-cell interactions and acquisition of an early migratory phenotype. These did not occur in BT474 cells, though a noticeable increase in protrusions amongst the cells in the periphery of the colonies were observed **(Figure 3.5C).**

In the Boyden Chamber assays, HRG stimulation greatly augmented the migratory capacity of MDA361 cells (approximately 20-fold increase), an effect that was antagonised by 1μM PF878 **(Figure 3.5D).** To further validate these data, an siRNA approach was taken to suppress both FAK expression and activity in MDA361 cells. Initial optimisation experiments were carried out to determine the concentration of FAKsiRNA to be used for functional assays; these data showed that FAK levels were suppressed using siRNA at concentrations ranging from 25 to 150nM (all for 72hrs) (**Figure 3.5E**). Although 25nM FAKsiRNA was almost equally as effective at inhibiting FAK at the higher concentrations (100 and 150nM) after short-time points, a concentration of 100nM was selected as this provided effective and sustained knockdown over a prolonged period (up to 6 days) which was required for some of the subsequent experimental work (i.e. longer term growth assays).

FAK siRNA (100nM) reduced the total protein level of FAK after 48h by approximately 40% (**Figure 3.5E**). This effect was time-dependent and was further reduced post-transfection, achieving approximately 80% inhibition at Day 6. This was also accompanied by a decrease in FAK phosphorylation at Y397 and Y861. Interestingly, the decrease in the kinase and scaffolding functions of FAK likely to occur as a consequence of the loss of the total FAK

protein, led to an increase in the expression and activity of HER2 and HER3 receptors. A similar observation was also noted for AKT. No significant changes in cell viability were observed between NTsiRNA and FAKsiRNA-transfected SKBr3 cells 72hrs post-transfection and are noteworthy when results were interpreted (**Figure 3.5F**).

Accordingly, together with the observations with PF878, FAKsiRNA transfection significantly suppressed MDA361 cell migration in response to HRG, although to a lesser extent than the pharmacological inhibitor (**Figure 3.5G**).

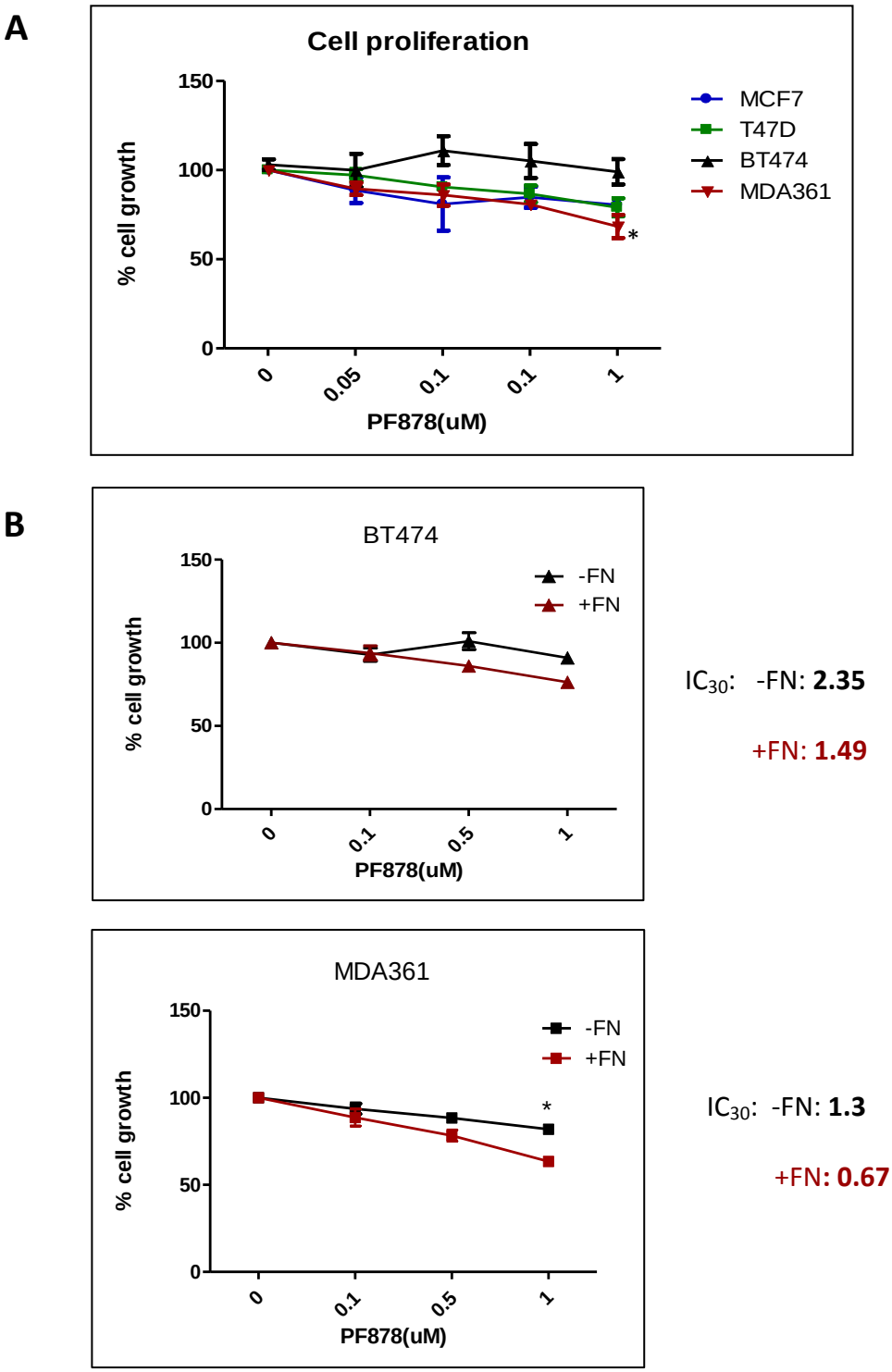


Figure 3.5. The effect of FAK inhibition on cellular proliferation. (A) The role of FAK in HER2-/ER+ and HER2+/ER+ cell growth in the absence (A) or presence (B) of fibronectin substrate was assessed using MTT assay ±PF878 (0–1 µM) over a period of 5 days. Data are mean % cell growth ±SEM (n=3). Continued.

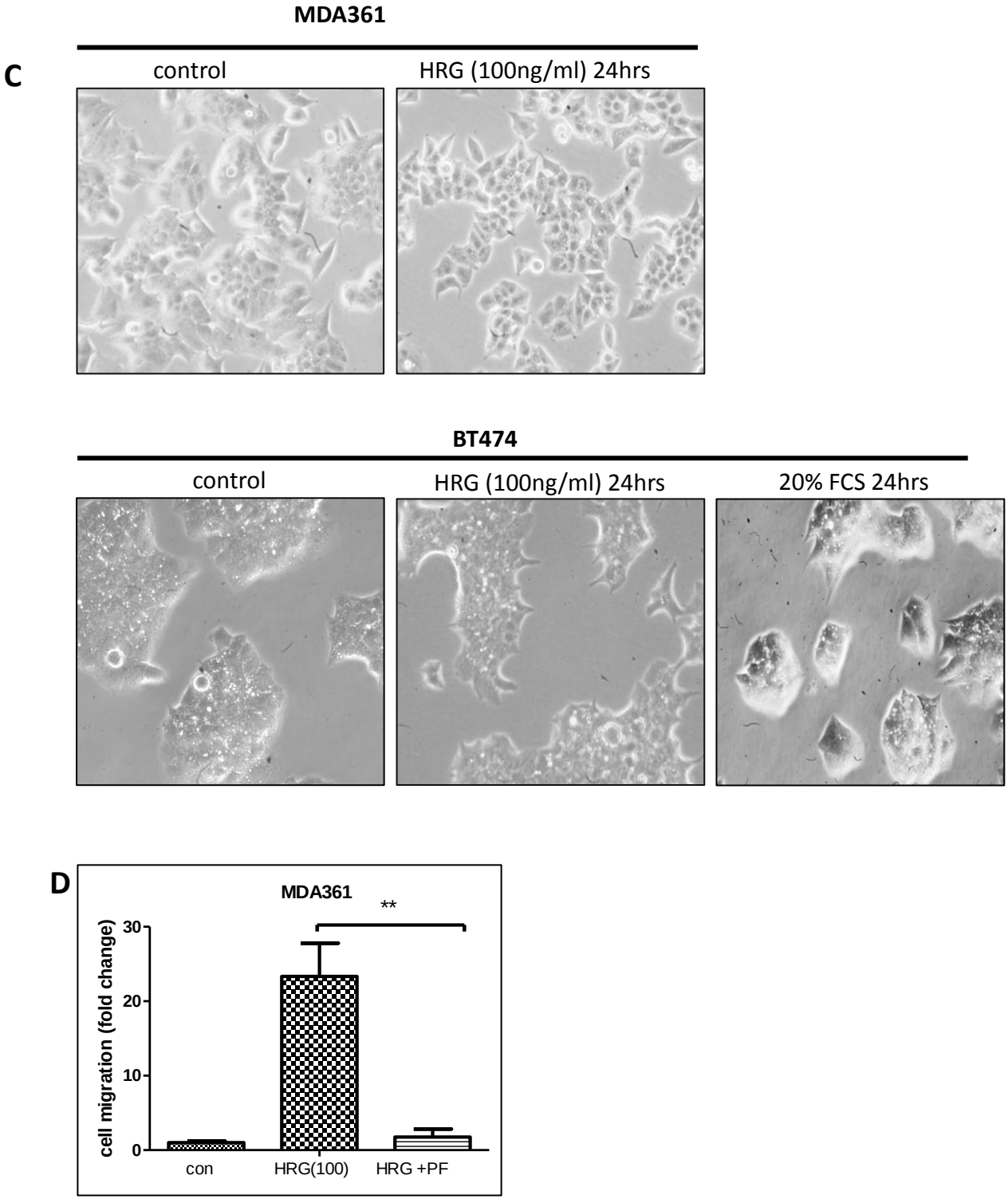
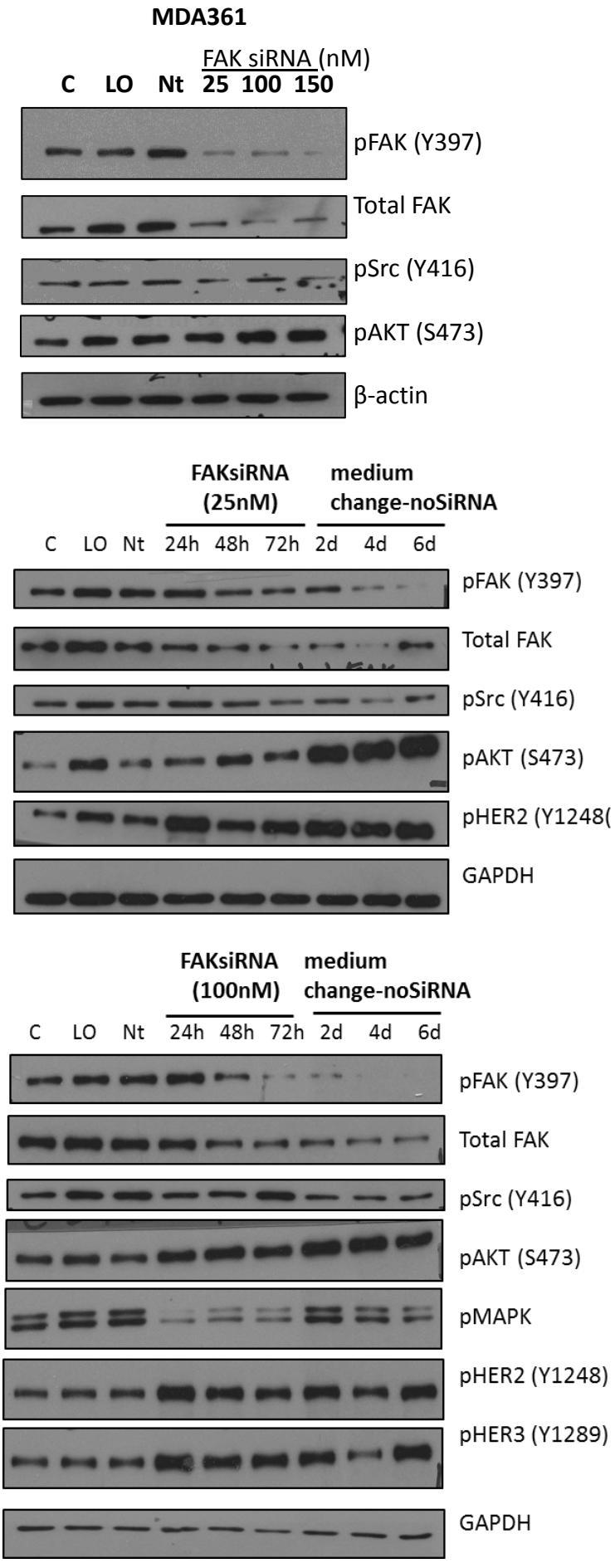


Figure 3.5. (Continued) Effect of FAK inhibition on cellular migration. (C) The effect of 24hr HRG (100ng/ml) or serum stimulation (20%FCS) in cell morphology was observed by capturing bright-field photos. (D) Heregulin-stimulated MDA361 cell migration ±PF878 (1µM) were analysed using fibronectin-coated Boyden Chambers. Continued.

E



Continued on next page

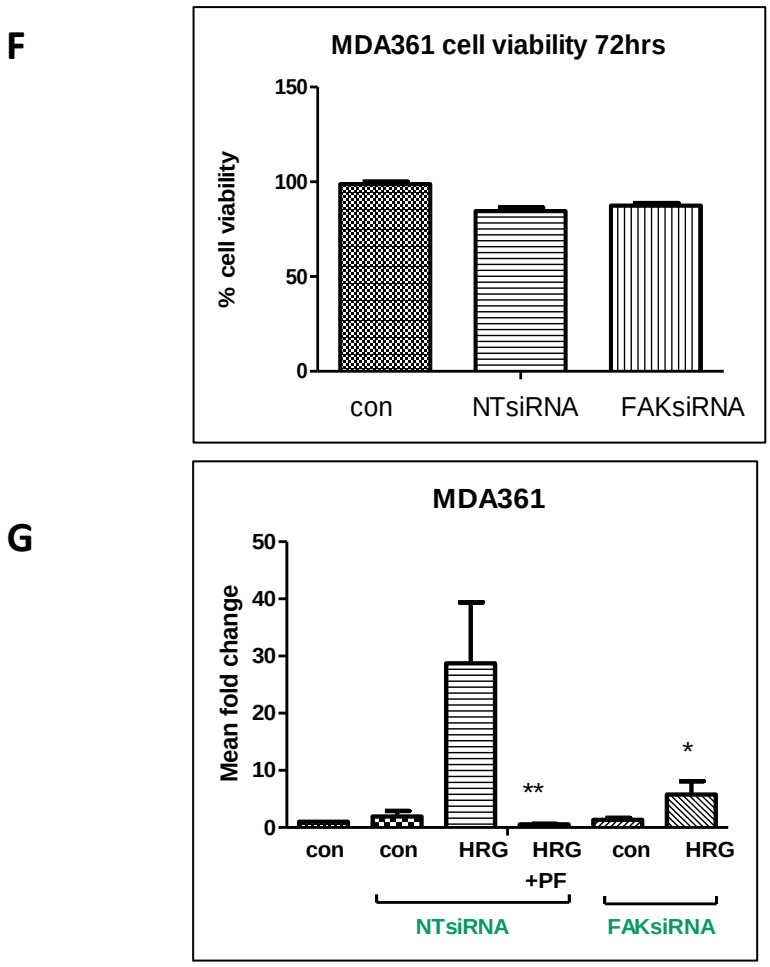


Figure 3.5. (Continued) (E) For validation, MDA361 cells were transfected with FAKsiRNA. To optimise the siRNA transfection, MDA361 cells were grown to 50% confluence and treated with FAKsiRNA (25-100nM) at for 72hrs. Cells lysates were processed for Western Blotting and probed for the proteins indicated. Further optimisation using 25nM and 100nM FAKsiRNA was performed to determine whether FAK knockdown is sustained for a longer time, 6 days. (F) Toxicity of the transfection process was evaluated by treating cells with the DharmaFECT transfecting cells with the non-targeting siRNA (Nt-siRNA) and FAKsiRNA for 72hrs and effects on cell growth were assessed by Coulter counting. (G) Following optimisation, NT/FAKsiRNA MDA361 cells were subject to cell migration assays under similar conditions as in (D). Data represent the mean numbers of migratory cells/membrane run in duplicates from three independent experiments.

* p=<0.05,**P=<0.001.

Interestingly, HRG failed to stimulate migration of BT474 cells (**Figure 3.5H**). This relative insensitivity of BT474 cells to HRG suggests that HER2 signalling may not predominantly contribute to cell migration in these cells. Despite this lack of response to HRG, it has been previously shown that BT474 cells can migrate in response to 20% serum (Worzfeld et al. 2012). In accord, bright-field microscopy images of 20% serum-stimulated BT474 displayed more prominent cellular protrusions that differ from those formed following HRG-stimulation (**Figure 3.5C**). Thus, the assay was repeated with inclusion of serum stimulation BT474 cells resulting in enhanced migration which was subsequently attenuated by PF878 (**Figure 3.5H**).

H

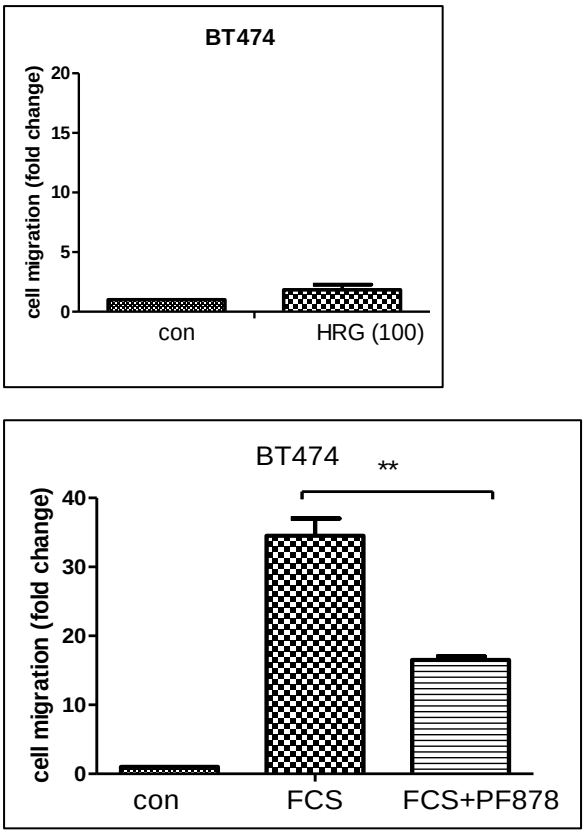


Figure 3.5.(continued) (H) HRG-stimulated and migration and serum-induced (20%) migration of BT474 cells ±PF878 (1uM). Data represent the mean numbers of migratory cells/membrane run in duplicates from three independent experiments.
* p=<0.05,**P=<0.001.

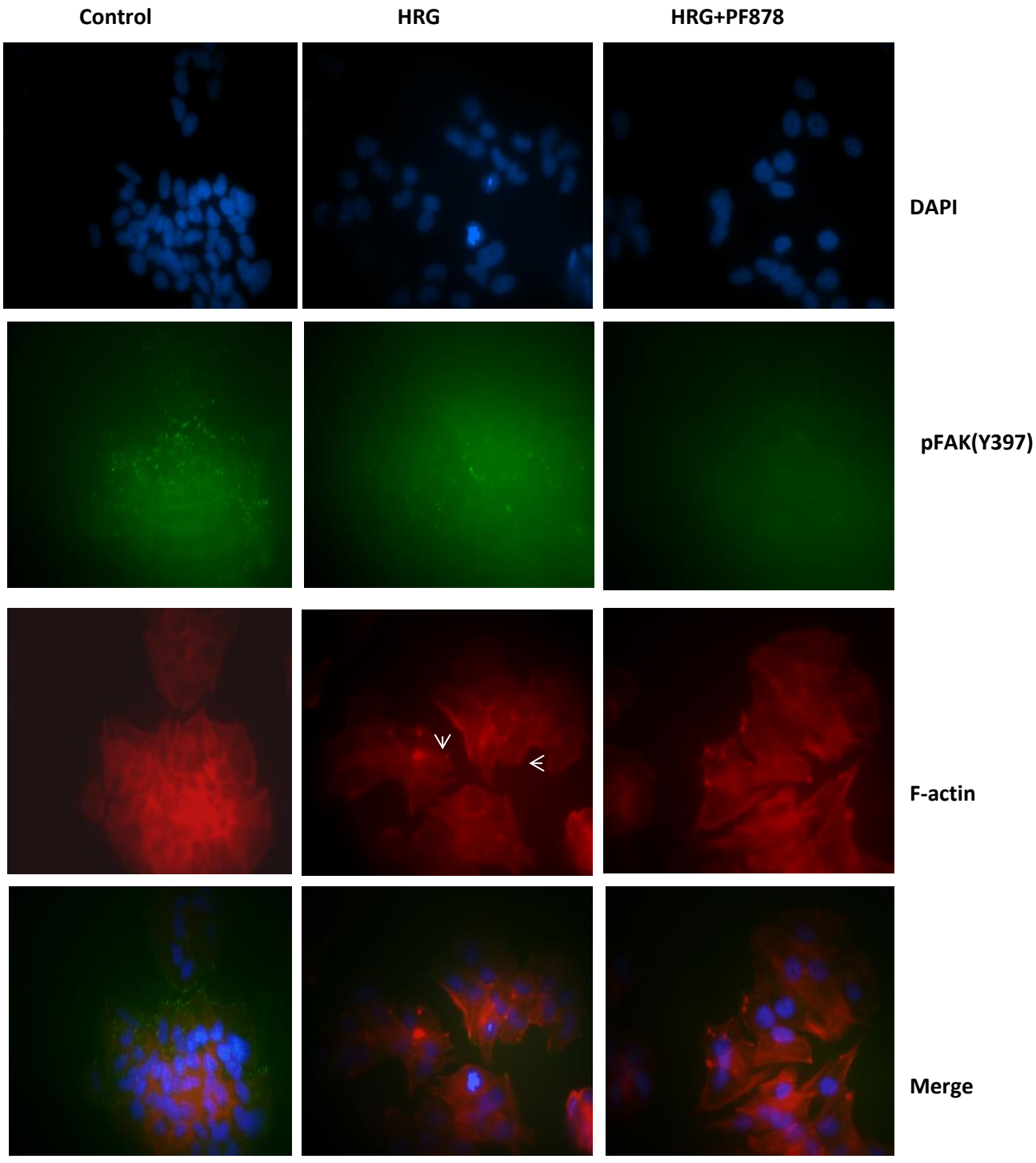
3.7 FAK plays a central role in Heregulin-induced cytoskeletal regulation and E-Cadherin loss

Efficient migration requires the dynamic regulation of focal adhesions (FAs) together with remodelling of the actin cytoskeleton. Both processes are reliant upon the functional FAK and are also closely associated with the re-localisation of cell-to-cell junction proteins such as E-Cadherin (Mitra et al. 2005, Serrels et al. 2011). Therefore, following the finding that PF878 can significantly suppress HRG-induced cell migration, we examined the localisation of FAK, filamentous-actin (F-actin) and E-cadherin in MDA361 cells following HRG stimulation.

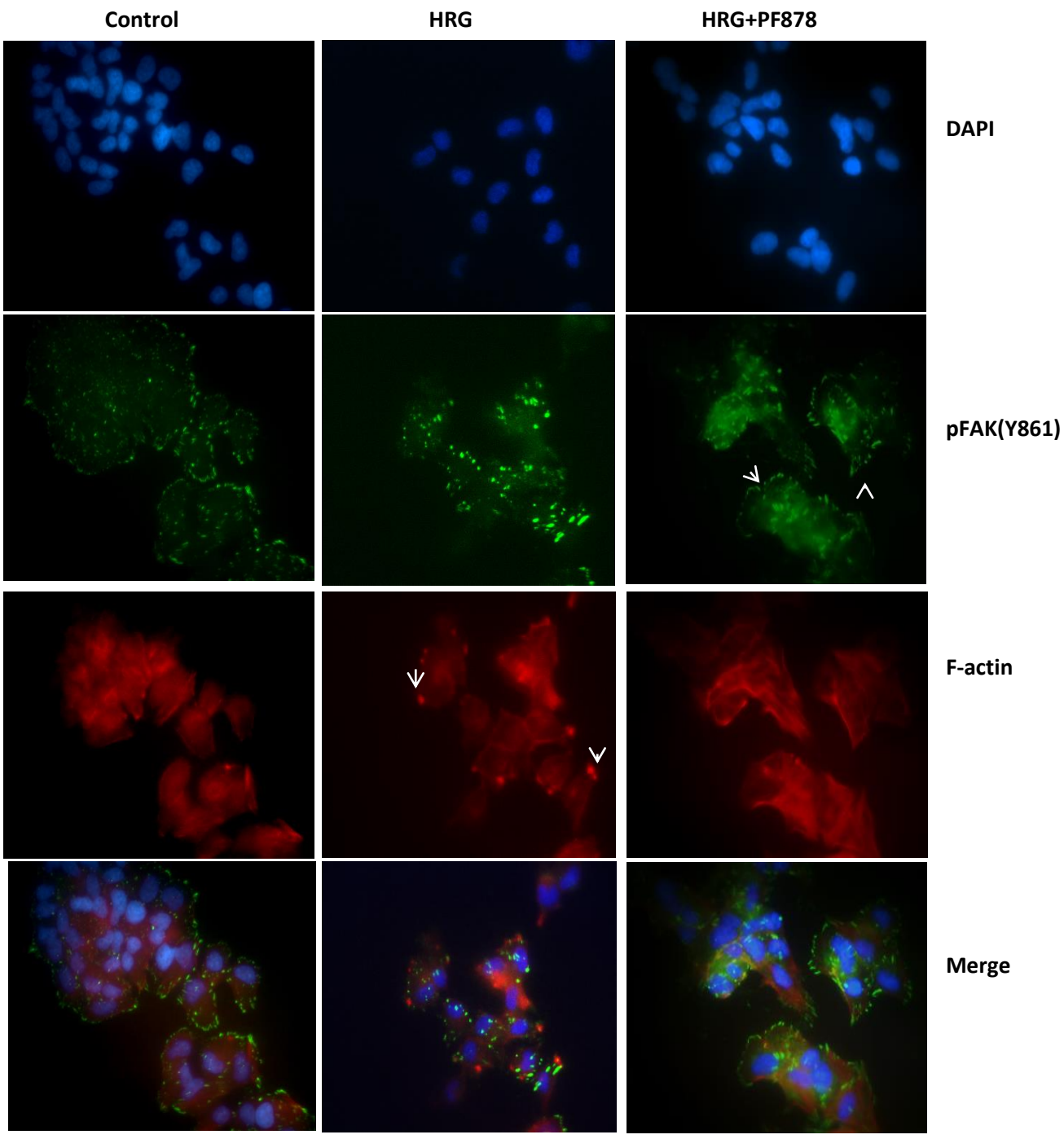
Moderately weak pFAK Y397 and strong pFAK Y861 punctate staining were observed around the cell periphery and at the ends of the actin-stress fibres, reminiscent of FAs (**Figure 3.6A**). HRG stimulation for 60mins increased the presence of F-actin-containing aggregates in some cell colonies (indicated by the arrowheads **Figure 3.6A**)**,** suggestive of an active remodelling of the actin cytoskeleton (Stricker et al. 2010). Furthermore, its co-localisation with pFAK Y861 was disrupted**.** As we were unable to detect strong pFAK Y397 staining, the exposure time to laser light was extended although this resulted in a very high background, thus making the images difficult to interpret. Further investigations revealed that the dissolution of cell-cell contacts as indicated by loss of cell-to-cell E-cadherin staining was also apparent**.**

When treated with PF878, an overall increase in pFAK Y861 staining was observed, both at the cell periphery and across the cytoplasm, whilst a near complete loss of pFAK Y397 staining was observed. pFAK Y861-containing FAs were noticeably larger, elongated and are more prominent at the periphery whilst intercellular E-Cadherin staining remained intact.

A



B



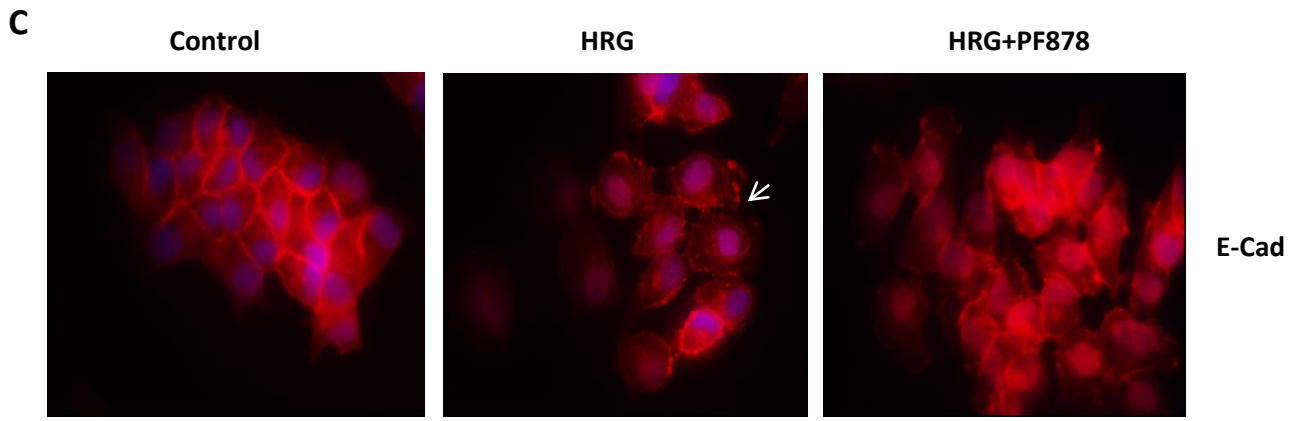


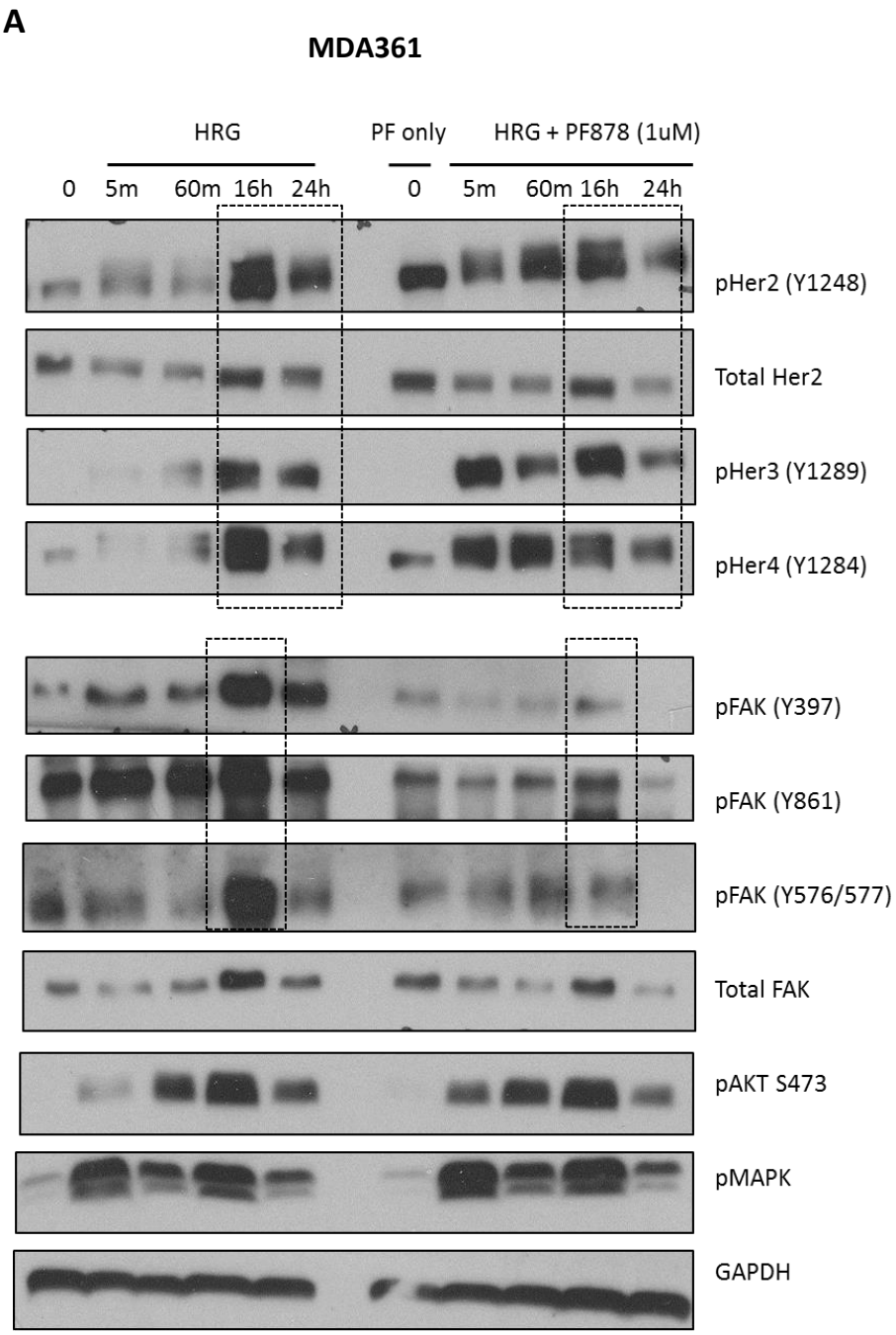
Figure 3.6. Immunofluorescence images of MDA361 cells seeded on FN-coated plates following Heregulin-stimulation migration (100ng/ml) ±PF878 (1μM) for 60mins. Cells were stained for primary antibodies: (A) pFAK Y397 (green) + Rhodamine –phalloidin (actin) (red), (B) pFAK Y861 (green) + Rhodamine-phalloidin(actin) (red), (C) E-Cadherin (red). All cells were stained with appropriate Alexa-fluor 488/594-conjugated secondary antibodies and counterstained with DAPI, a nuclear dye (blue). Magnification: x63, oil-immersion objective.

3.8 Inhibition of FAK attenuates HRG-mediated migratory signalling in HER2+/ER+ breast cancer cells

Because of the inhibitory effects exerted by PF878 on HRG-stimulated MDA361 cell migration, we wished to explore the potential signalling pathways in which FAK might play a role during this process. Cells were seeded onto fibronectin-coated plates, since migration was performed on fibronectin-coated membranes, and stimulated with HRG (100ng/ml) in the presence or absence of PF878 (1μM) for a range of times (0-24hrs) reflecting the period in which the migrations assays were performed. Cells were lysed and processed for Western Blotting for analysis of expression and activation of ErbB receptor family members and related signalling components (**Figure 3.7A**).

Our data demonstrated that HRG induced the phosphorylation of HER2, HER3 and HER4 with peak activation observed at 16hrs. This was accompanied by phosphorylation of FAK (Y397, Y861 and Y576/577) as well as the downstream signalling elements, AKT and MAPK, all of which demonstrated a similar peak at 16hrs. In the presence of PF878, HRG-induced FAK phosphorylation (Y397, Y861 and Y576/577) was much less evident. Interestingly, we observed that treatment with PF878 alone led to an increase in the activity of HER2, HER3 and HER4 although it did not alter AKT or MAPK phosphorylation status. FAK inhibition did also appear to attenuate the ability of HRG to promote maximal ErbB receptor phosphorylation (i.e. at 16 and 24hrs). Given that HRG promoted AKT and MAPK activation and that both AKT and MAPK are well described to be involved pro-survival and growth signalling pathways, we investigated whether HRG was able to induce growth effects in these cells. Moreover, we wished to determine whether FAK inhibition might suppress any such effects, although this may not occur via AKT/MAPK signalling as PF878 did not alter HRG-induced AKT or MAPK activity. However, our data revealed that despite an increase in growth-related signalling, HRG did not affect the proliferative ability of either cell line tested (**Figure 3.7B**). There was some modest suppression of endogenous growth in MDA361 cells using PF878 alone as previously demonstrated (**Figure 3.5A**)

A


MDA361
HRG
PF only
HRG + PF878 (1uM)
0
5m
60m
16h
24h
0
5m
60m
16h
24h
pHer2 (Y1248)
Total Her2
pHer3 (Y1289)
pHer4 (Y1284)
pFAK (Y397)
pFAK (Y861)
pFAK (Y576/577)
Total FAK
pAKT S473
pMAPK
GAPDH

B

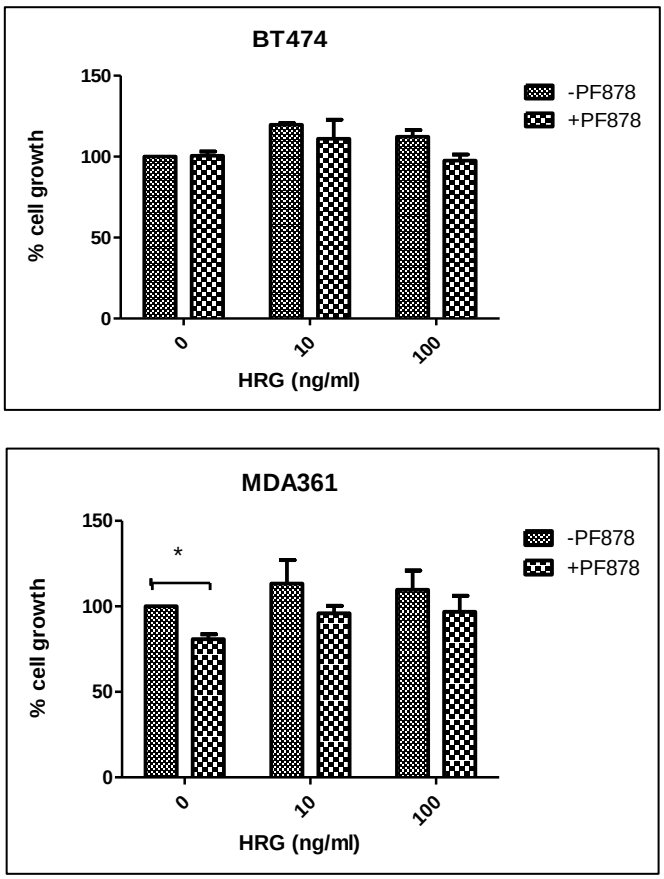


Figure 3.7. (A) HRG stimulates downstream signalling in MDA361 cells. Cells were seeded on fibronectin-coated plates and cultured to 70% confluence and treated with HRG (100ng/ml) at the indicated time-points ± pre-treatment with PF878 (1μM) for 60mins. Cells were lysed, processed for Western Blotting and probed for proteins as indicated. Representative blots shown from three independent experiments. (B) Cells were grown in 24-well dishes in the presence of HRGβ1 (10 or 100ng/ml) ±PF878 (1μM) for 7 days. Cell growth was then assessed by Coulter counting (n=3) *=$p<0.05$

3.9 Discussion

Breast cancer represents a heterogeneous group of diseases reflecting a spectrum of clinical, pathological and molecular characteristics. The ER+ breast cancer subtype is the most common, accounting for 70% of all cases diagnosed (Sørlie et al. 2001). This chapter focused on the luminal B subtype of ER+ tumours, of which up to 30% may overexpress the HER2 receptor, and have been associated with increased relapse risk and mortality (Cheang et al. 2009)

Herceptin has revolutionised treatment of HER2+ tumours, however, it has been shown that response rates are low, varying between 11-26% depending on prior therapies (Baselga et al. 1996; Cobleigh et al. 1999; Vogel et al. 2001) and thus better treatments are warranted. Moreover, inclusion of Herceptin in treatment regimens benefits HER2+/ER- tumours more than HER2+/ER+ tumours, suggesting that the presence of ER might be a limiting factor to Herceptin response (Bhargava et al. 2011; Montemurro et al. 2012). Conversely, the presence of HER2 in ER+ tumours is shown to attenuate the response of these tumours to endocrine therapy (Houston et al. 1999). Key to the development of more effective approaches for the management of HER2+ disease is the identification of novel therapeutic targets that may have a potential to improve response to existing treatments.

It has been well established that FAK is an important mediator of cell growth, survival and migration, all of which are frequently deregulated during malignancy. As already described, several studies have supported the role of FAK in various stages of tumour progression and its overexpression is often correlated with an aggressive cancer phenotype. FAK overexpression has also been reported to correlate with HER2 overexpression in breast cancers (Schmitz et al. 2005). Since both HER2 and FAK are elevated in breast cancer and given its close interplay with HER2-mediated signalling, one hypothesis is that FAK may represent a novel therapeutic target in HER2+ breast cancer; however, studies exploring this concept are limited to only a few (Benlimame et al. 2005; Xu et al. 2009; Yang et al. 2010). Thus, in this chapter, we investigated the therapeutic potential of FAK in HER2+ /ER+ breast cancer.

Our initial observations in HER2+/ER+ cell lines revealed that there was no apparent correlation between HER2 status, total FAK expression and pFAK Y397. We however

observed that the activity of pFAK Y861 was higher in HER2+/ER+ cells, an observation also supported by a previous study demonstrating a correlation between pFAK Y861 and HER2 overexpression (Schmitz et al. 2005). In addition, Vadlamudi et.al. (2003) also reported that FAK Y861 phosphorylation specifically occurs following HER2-initiated signalling. Taken together, our findings and those in the literature led us to hypothesize that HER2+/ER+ cell lines may be more reliant on FAK and thus be more sensitive to FAK inhibition. A number of pharmacological FAK inhibitors are currently at various stages of pre-clinical and clinical development for cancer, highlighting the critical role of FAK in tumour progression (Table 1.3). In this study, we used the FAK inhibitor PF878 (now VS-6063) obtained from Pfizer. PF878, though its precise mechanism are undisclosed, it works similarly to another Pfizer compound, PF271, by competitively binding to the ATP binding site of FAK at Y397 (Roberts et al. 2008). It has also been reported to inhibit Pyk2, another FAK-related kinase (Pachter et al. 2014).

Both HER2+/ER+ cell lines (BT474 and MDA361) show increased sensitivity to PF878 with respect to its ability to suppress phosphorylation of Y397 compared to HER2-/ER+ cells; BT474 cells, which had the highest level of HER2 phosphorylation, demonstrated the greatest sensitivity to the compound. However, despite effective inhibition of Y397, this did not translate out into a suppression of cell growth for either HER2-/ER+ or HER2+/ER+ models with the exception of MDA361 cells, where a modest (maximally 30%) yet statistically significant suppression of growth was observed with the highest dose tested (1μM); interestingly however, this was above the calculated IC_{50} value of 0.17μM for inhibition of Y397 activity again suggesting a disconnect between FAK Y397 and growth signalling pathways. While concentrations of >5μM did exert growth suppressive effects in all cell lines (data not shown), this may likely be result of off-target (FAK-independent) effects, as has previously been suggested to occur in other cell lines (Slack-Davis et al. 2007).

Several key signalling events are known to be initiated following FAK activation. Classically, FAK activation is characterised by auto-phosphorylation at Y397, allowing for binding of Src and PI3K (Schaller et al. 1994). Src subsequently phosphorylates other FAK tyrosine residues including Y576/577, Y861 and Y925 as well as other FAK-associated proteins (Cary & Guan 1999). The phosphorylated tyrosine residues provide binding sites for SH2-domain-containing proteins, of which PI3K and Grb2 are well described, consequently leading to the

activation of the AKT and MAPK signalling pathways respectively (Cary & Guan 1999). Given this information, we investigated the hypothesis that inhibition of FAK auto-phosphorylation at Y397 might result in suppression of these elements also. FAK inhibition only affected downstream signalling in MCF7 and MDA361 cells and only in terms of a reduction in AKT and MAPK activity. Loss of AKT/MAPK in this context might arise as a result of suppressed activity of its upstream regulator, Src, as illustrated by the decrease in Src activity in response to PF878 in these two cell lines (see section 3.5).

Having investigated the effect of PF878 on elements of FAK-mediated signalling pathway components, we evaluated the functional consequences that may arise in these cells as a result of this. Both AKT and MAPK have been associated with cellular growth pathways (Yamamoto et al. 2003; Liang & Slingerland 2003; Behmoaram et al. 2008) but only MDA361 cells were growth inhibited by PF878, despite PF878 suppressing AKT and MAPK in MCF7 cells also. These conflicting data between cell lines may simply reflect the differences in the role of FAK in different tumour subtypes. Alternatively, FAK inhibition in MCF7 cells may trigger an as-yet identified alternative growth pathway, a hypothesis supported by studies showing that use of PI3K/AKT pathway inhibitors activate compensatory growth signalling in tumours, for instance via HER3 and IGFR-1 (Rodrik-Outmezguine et al. 2011; Chandarlapaty et al. 2011). A further point for consideration is that these growth experiments were carried out in RPMI media supplemented with 5% FCS. Serum is rich in various components that can include growth factor signalling and which may in turn stimulate parallel growth pathways independent of FAK. This however was unavoidable as the cells do not proliferate well in the absence of serum, which would then limit the time-frame of our growth assays (usually 7 days).

Together, the differential downstream signalling effects by PF878 on the cell lines tested might represent a context where this could be investigated further to determine the subtypes that reliant on FAK and to identify relevant predictive markers for FAK inhibitor response.

FAK is known to be critical intracellular mediator of ECM signals via its interactions with integrin receptors and cell-matrix contact to support growth factor signalling (Stupack & Cheresh 2002; Cary & Guan 1999; Kim et al. 2011). Thus we sought to investigate the

hypothesis that FAK inhibition might suppress growth in the context of matrix interactions. Previous reports have shown that pFAK Y397 inhibition, whilst not affecting the growth of cells in 2D culture, increases cellular apoptosis in 3D culture (Tanjoni et al. 2010). This in turn points to the importance of the microenvironment on FAK activity, where for example integrin-mediated FAK activation may potentiate pro-survival signalling. However, we did not observe any differences in the ability of PF878 to suppress the proliferation of HER2+/ER+ cells growing on either matrix (fibronectin)-coated surfaces or on uncoated surfaces, with the exception of MDA361 cells at 1μM PF878. This could imply that these cells may differentially express the appropriate integrin receptors for this matrix-mediated downstream signalling. Interestingly, it is now well acknowledged that differentially activated signalling pathways exist between monolayer and 3D culture settings (Baker & Chen 2012, Ganghadara et.al, *submitted*). This suggests perhaps that FAK-mediated growth signalling of HER2+/ER+ cells are significantly influenced by the distinct cell-matrix interactions present in the 3D cell culture settings than in monolayer cultures.

Whilst neither of the two HER+/ER+ cell lines displayed a high intrinsic migratory behaviour, our data do support a role for FAK in the HRG or serum-stimulated migration of MDA361 and BT474 cells respectively. In these studies, 1μM of PF878 significantly suppressed cell migration, similar to that used in the growth assays. In this regard, targeting FAK is likely to be more relevant with regards to metastatic dissemination rather than tumour growth. This finding is not unexpected since FAK is well documented to be involved in the migratory behaviour of cells; inhibition of FAK suppresses breast cancer migration and invasion, whilst FAK overexpression in $FAK^{-/-}$ cells rescue cell migration defects (Sieg et al. 1999; Walsh et al. 2010).

Interestingly, HRG only stimulated the migration of MDA361 cells, which was subsequently suppressed by PF878. The same results were obtained by knockdown of FAK expression with FAKsiRNA (100nM), which suppressed both FAK activity and expression. We became interested in this approach given the accumulating evidence that the kinase-independent, scaffolding functions of FAK mediate several important intercellular events (Bolós et al. 2010; Fan et al. 2013). If indeed the physical scaffold of FAK is required for MDA361 cell migration, then the siRNA treatment was hypothesised to lead to a much greater suppression than that of the pharmacological inhibitor. Interestingly, however, was not the

case and might therefore suggest a potential for compensatory signalling events such as that mediated by HER2/HER3 or AKT, observed upon FAK knockdown versus treatment with PF878. Alternatively, the absence of FAK might also lead to increased expression in the FAK-related Pyk2, which may compensate for loss of FAK as previously reported to occur in mouse embryonic fibroblasts, endothelial cells and cancer stem cells (Sieg et al. 1998; Weis et al. 2008; Fan & Guan 2011).

HRG does not directly bind nor physically activate HER2, but instead activates HER3/4, which subsequently recruits and activates HER2 via hetero-dimeric interactions (Carraway et al. 1994; Plowman et al. 1993). HRG activates Her3/4 receptors resulting in the activation of FAK (Vadlamudi et al. 2002; Vadlamudi et al. 2003;). These FAK-dependent mechanisms most likely involve signalling and scaffolding functions through multiple interactions with focal adhesion (FA) components. FAK, for example, binds to paxillin and p130Cas at nascent FAs, which in turn facilitate activation of ERK and MLCK reported to be necessary to FAs disassembly, a process required for the continuous forward cell protrusion (Webb et al. 2004). FAK has also been reported to regulate pathways involved in modulating membrane protrusions and cytoskeletal arrangements by interactions with Arp2/3 and RhoGTPases for example (Wu et al. 2004; Zhai et al. 2003; Serrels et al. 2007). These and other studies (Hamadi et al. 2005; Schaller 2010) are consistent with our data demonstrating that FAK activity modulates focal adhesion dynamics and actin remodelling to regulate HRG-stimulated MDA361 cell migration. Although we cannot rule out the role of pFAK Y397 in these effects due to the high background of the IF images which made interpretation of the data difficult, our data does imply that pFAK Y861 plays a role in the context of HRG-induced migration in this HER2+ cells as others have shown (Vadlamudi et al. 2002; Vadlamudi et al. 2003). Accompanying these changes was also a HRG-induced loss of intercellular E-Cadherin, a key component of adherens junctions as previously reported to occur in other breast and colon carcinoma cell lines (Okoshi et al. 2011; Kim et al. 2013). The net effect of this would be to facilitate the dissemination of individual cells from the tightly packed colonies. Accordingly, de-localisation and/or suppressed expression of E-Cadherin is widely reported in the acquisition of an invasive tumour phenotype and is correlated with metastatic tumours including the breast (Moll et al. 1993; Canel et al. 2013). Importantly, our findings implicating the role of FAK in this process is supported by the work of Canel et

al. 2010, who showed that inhibition of the Src/FAK pathway using pharmacological Src or FAK inhibition disrupts E-cadherin endocytosis and strengthens cell-cell adhesions.

In the case of BT474 cells, these were not migratory in response to HRG, which is contradictory to previous studies (Weingaertner et al. 2013; Pradip et al. 2013). The differences from ours and others' reports lie in experimental design. In these studies, HRG is present in the seeding media during the setup of the assay whereas we utilised HRG as a chemo-attractant, suggesting the possibility of HRG to influence the initial cell-adhesion processes prior to migration of BT474 cells. Alternatively, it might also be explained by the fact that these cells already have endogenously high basal auto-phosphorylation of HER2, thus HRG stimulation at 100ng/ml may prove to be a saturating concentration wherein it can no longer elicit further HER2 phosphorylation at Y1248 (**See Appendix 8.1**). Studies on the MDAMB-468 breast cancer cell line with point-mutated HER2 receptor that renders the Y1248 inactive reveal the indispensable role of this residue in cell migration (Dittmar et al. 2002). For this reason, we focused our studies on this residue. We should however also incorporate detection of other auto-phosphorylation sites in HER2 such as Y877, whose activity can also be promoted by growth factors (Bose et al. 2006; Xu et al. 2007).

Of interest, serum stimulation elicited BT474 migratory responses in agreement with a previous study (Worzfeld et al. 2012) and occurred in a FAK-dependent manner. Given the plethora of ligands within the serum, BT474 cell migration can be stimulated by as many or as few of these ligands that can trigger downstream signalling via FAK, for instance EGF and PDGF (Sieg et al. 2000). Though exploring the putative serum-induced pathways represents an interesting avenue to pursue, we chose to focus in elucidating downstream signalling mediated by HRG in MDA361 cells. To do so, we stimulated MDA361 cells with HRG over a period of 0-24 hours reflecting the time in which our Boyden chamber assays were carried out. Treatment with HRG stimulated the rapid phosphorylation of the ErbB receptors HER2, HER3 and HER4, which might arise through hetero-dimerization-mediated transactivation of HER2, with peak activation observed at 16hrs.This was accompanied with increased activity of FAK at Y397, Y576/Y577 and Y861, all of which followed a similar activation pattern, which may consequently activate MAPK and AKT. Although the activation of the ErbB receptors in response to HRG, mirrored AKT activation, more so than MAPK, which is consistent with the fact that HER3 is a potent activator of the PI3K/AKT pathway due to the

presence of several Thr-X-X-Met motifs, the binding site for the P85 subunit of PI3K (Fedi et al. 1994). These effects were sustained up to the latest time-point of 24hrs. Interestingly, this was not the case for BT474 cells (**See Appendix 8.1**), wherein levels of HER2, HER3, pFAK Y397 and Y861 activity fell below basal levels at 24hrs, lending a potential explanation to the differential sensitivity of these HER2+/ER+ cell lines to HRG.

Inhibition of FAK activity however led to increased HRG-induced phosphorylation of HER2, HER3 and HER4 receptors. Such a response was unexpected, but nevertheless indicate the possibility that the homo/heterodimeric interaction HER2 or HER4 or their auto-phosphorylation can be markedly influenced by FAK. Immuno-precipitation studies are required to confirm whether this results from the direct interaction of FAK with these ErbB receptors, as have previously been described to occur in Schwann cells and in MCF7 cells stimulated with HRG (Vartanian et al. 2000; Vadlamudi et al. 2002). What seems apparent however, was that when FAK activity was inhibited, the phosphorylation of ErbB receptors appeared to saturate, as shown by limited sensitivity to the persistent HRG stimuli. Dissecting the dynamics of each of these heterodimers in the presence of the FAK inhibitor is worthy of a separate study in its own right**.**

Expectedly, PF878 also inhibited HRG-stimulated FAK activity at Y397 and Y861, confirming the role of FAK in HRG-induced MDA361 cell migration as we have established earlier. To our surprise, PF878 failed to inhibit HRG-induced AKT and MAPK activity. Unlike FAK, the roles of AKT and MAPK in cell migration are not very well explored although a few are described. Enomoto et al. (2005) showed that AKT phosphorylates ser1416 in Girdin, an actin-binding protein to mediate formation of the lamellipodia. Although a contradictory study suggests that AKT activation inhibits cell invasion through inhibition of the NFAT transcription factor (Yoeli-Lerner et al. 2005). Increased MAPK activity on the other hand was shown to be a required for stimulating cell migration in response to fibroblast growth factors (FGFs),platelet-derived growth factor (PDGF-BB), HRG and fibronectin (Shono et al. 2001; Graf et al. 1997; Ruan et al. 2012; Klingbeil et al. 2001). Reports have also demonstrated the role for MAPK in phosphorylating the myosin-light chain kinase (MLCK), a regulator myosin and polymerization of actin stress fibres in a migrating cells (Klemke 1997). More interestingly, MAPK and MLCK were found to be key effectors of FAK–Src signalling along with paxillin and p130cas to regulate FAs disassembly (Webb et al. 2004). This

mechanism, however relevant in our context appeared to be FAK-independent, since PF878 failed to inhibit HRG-induced AKT and MAPK phosphorylation in MDA361 cells.

An obvious explanation to this lack of AKT and MAPK inhibition is the sustained and/or increased activity of the HER2/HER3 and HER2/HER4 dimers in the presence of PF878. Alternatively perhaps, HRG-stimulated AKT and MAPK activity have a more dominant role in cell proliferation and survival rather than migration as you would expect, as these are critical roles well reported in the literature. However, there was no correlation between HRG-induced AKT and MAPK and proliferation of MDA361 and BT474 cells observed, which was equally surprising. These observations may be explained by the presence of serum (5%) in the media the growth assay was performed in, in accordance with the findings of Lewis et al. (1996). In that study, HRG-stimulated growth responses were apparent at low serum concentrations (0.1-1%), whereas in higher serum concentrations (10%), HRG exerted no effects or were even growth-inhibitory in some cell lines.

Taken together the data shown that MDA361 and BT474 cells respond to HRG and serum respectively with increased migration which might suggest that in an *in vivo* context, such tumour types may display an increased aggressive behaviour since they would be exposed to numerous growth factors. The importance of the tumour microenvironment in promoting tumour progression and spread is well established; given our data on the role of FAK in this process, one may propose that FAK inhibitors might be a useful therapeutic strategy through which to suppress tumour spread.

4. Results (II)

Targeting FAK in HER2+/ ER- breast cancer

4.1 Introduction

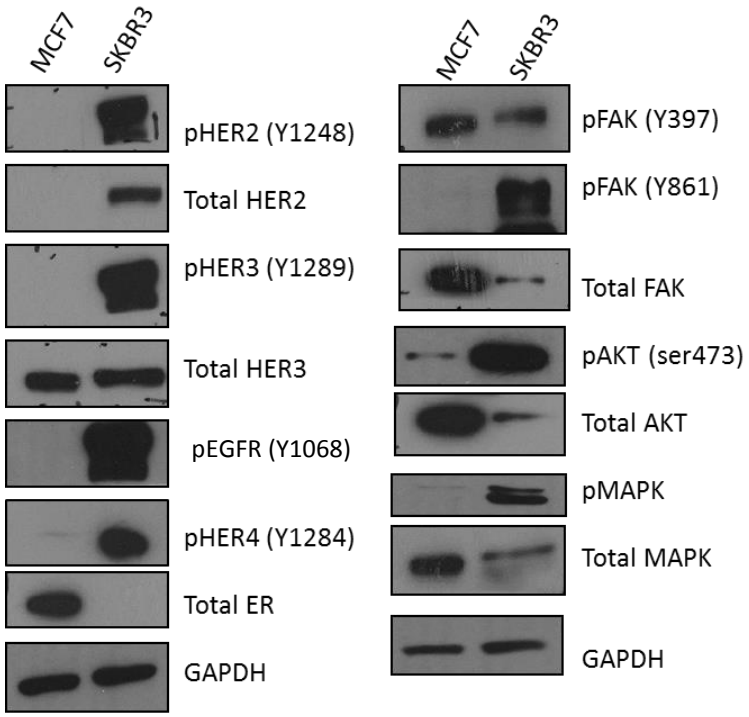
In the previous chapter we demonstrated a role for FAK in the luminal B (HER2+/ER+) subtype of breast cancer, where FAK inhibition resulted in modest growth inhibition and a significant suppression of ErbB-induced migratory responses.

In light of these data, we next wished to extend these studies to explore whether FAK also presented a potential target in HER2+/ER- disease models. HER2+/ER- tumours generally have low expression of the luminal gene cluster (see Section 1.3) and a high proliferative index (Ki67 expression)(Prat & Perou 2011) and this subtype of breast cancer is well recognised to be more aggressive, with HER2+ patients having shorter survival times versus patients with the luminal (A or B) subtype (Sørlie et al. 2001; Prat & Perou 2011). Given the link between FAK and HER2 and the fact that FAK has previously been shown to be highly expressed in HER2+/ER- breast tumours (Schmitz et al. 2005) where it is linked to an aggressive behaviour, an established role in HER2 signalling (Benlimame et al. 2005; Xu et al. 2009), and our previous chapters' data, our hypothesis was that FAK might also play a role in the proliferative and/or migratory responses of HER2+ tumours.

4.2 FAK activity is elevated in HER2+/ER- cells

To study FAK in the context of HER2+/ER- disease, we used SKBr3 cell line which represents an appropriate cell model of this type of tumour (Neve et al. 2006). Initially, we explored the expression of FAK, HER2 and other related signalling elements in these cells versus the HER2-/ER+ MCF7 cells by Western Blotting. Our data in **Figure 4.1A** verified that SKBr3 cells have elevated levels of active HER2 and were also negative for the ER. SKBr3 also displayed elevated basal activity of the other ErbB receptor family members, EGFR, Her3 and HER4 versus MCF7 cells. Whilst we observed that the levels of FAK were lower in SKBr3 cells versus MCF7, FAK phosphorylation at Y861 was greater in the HER2+ cell line as were the activity of AKT and MAPK. Moreover, the analysis also appeared to suggest that pFAK Y397 is higher when normalised to total FAK levels in SKBr3 than in MCF7 cells (**Figure 4.1B**).

A



B

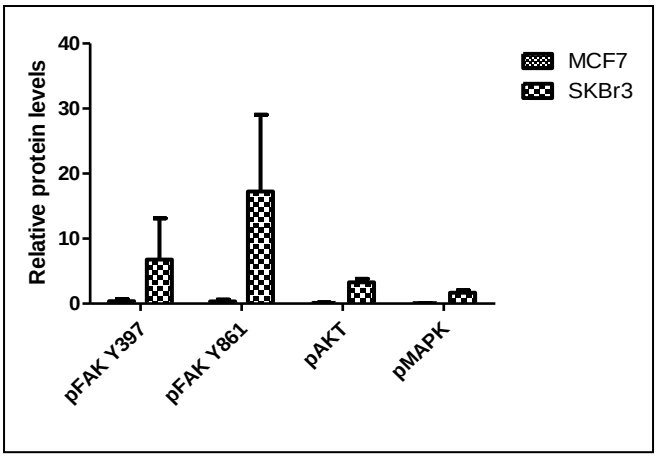


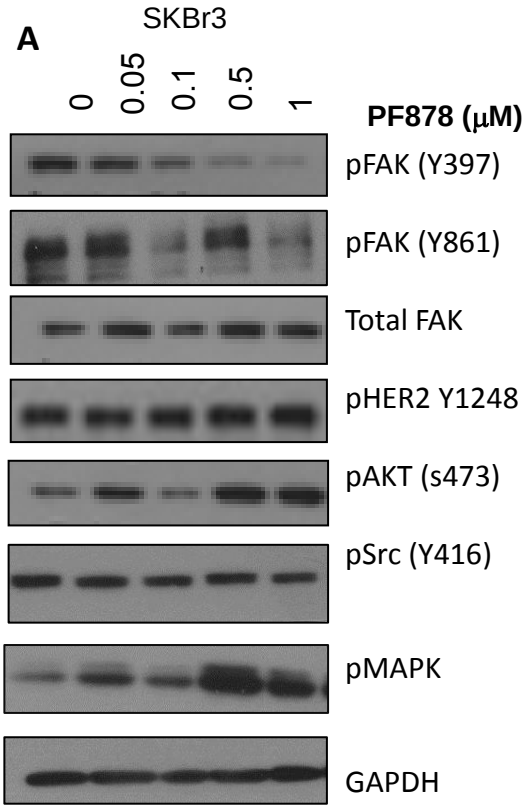
Figure 4.1 (A) Expression of FAK in ER+/HER2- and ER-/HER2+ breast cancer cells. (A) Cell lysates from HER2-/ER+ (MCF7) and HER2+/ER- (SKBr3) breast cancer cells grown under routine cell culture conditions were probed for proteins indicated. (B) Densitometric analysis values showing the levels of phosphorylated proteins normalised to their respective total protein expression. This revealed that for all signalling elements investigated, their activity as higher in HER2+ cell lines; pFAK Y397 (6-fold higher vs. MCF7), pFAK Y861 (15-fold) , pAKT (3-fold) and pMAPK (1.6-fold). Representative blot shown from three independent experiments (±SEM).

4.3 Sensitivity of HER2+/ER- cells to FAK inhibition relative to HER2-/ER+ cells

Having shown that FAK activity is elevated in SKBr3 cells, we sought to examine whether these cells are also sensitive to FAK inhibition using the small molecule inhibitor, PF878. Treatment of SKBr3 cells with increasing doses of PF878 effectively suppressed FAK phosphorylation at Y397 (**Figure 4.2A**) with an IC_{50} value of 0.166µM (SEM ±0.124) (**Figure 4.2B**). In comparison, the IC_{50} for this compound for inhibition of FAK Y397 phosphorylation in MCF7 cells was 0.25µM (SEM± 0.22). Although SKBr3 cells were more sensitive, it was not statistically significantly different to MCF7 cells (p=0.77). PF878 treatment also resulted in a modest decrease of FAK phosphorylation at Y861 in SKBr3 cells.

We subsequently examined whether FAK inhibition resulted in a loss of activity in the levels of ErbB receptors and FAK-related signalling would be inhibited in response to increasing doses of PF878. As shown, suppression of FAK activity had no effect on the activity of HER2 and Src, but led to a paradoxical increase in MAPK and AKT activity at concentrations of PF878 >/= 0.05 µM.

A



B

Cell Line	Breast Cancer Molecular Subtype	IC_{50} (µM) pFAKY397 inhibition (±SEM)
MCF7	ER+, HER2-	0.25 ± 0.22
SKBr3	ER- HER2+	0.166± 0.124

Figure 4.2. (A) SKBr3 lysates treated with the PF878 (0-1uM) for 60mins were probed for FAK (total, Y397,Y861) as well as HER2 and downstream FAK/HER2-related signalling proteins. GAPDH was used as loading control. (B) Densitometric analysis of immunoblots was carried out to calculate the IC_{50} values ± SEM for PF878. MCF7 values obtained from Chapter 3. Representative blots shown are from three independent experiments.

4.4 Inhibition of FAK suppresses HER2+/ER- breast cancer cell migration but not proliferation

We similarly investigated the effect of PF878 on cell proliferation over a range of doses (0-1μM) that spanned the IC_{50} for FAK phosphorylation at Y397. PF878 modestly suppressed the growth (approximately by 20%) for both cell lines but only at the highest concentration tested (1uM) (**Figure 4.3 A,B**). Subsequent experiments were performed in the presence of fibronectin as a substrate to which the cells could attach to test the hypothesis that matrix proteins might contribute to drug sensitivity similarly explored in section 3.6. However, our data suggested that there was no statistical difference between the effects of PF878 on MCF7 and SKBr3 cells seeded on fibronectin-coated or uncoated plates, with the exception at a higher concentration of SKBr3 cells treated with 1μM PF878 (**Figure 4.3A,B**).

Next we examined the effect of PF878 on SKBR3 cell motility using a Boyden chamber migration assay. In the absence of stimulation, the migratory capacity of SKBr3 cells was low. However, in the presence of HRG (10ng/ml), SKBr3 cell migration was significantly elevated, an effect that was antagonised when FAK was inhibited by PF878 (**Figure 4.3C**).

To validate these data obtained with the pharmacological FAK inhibitor and to further explore the role of FAK in HER2+/ER- cell function, we used a siRNA approach. Initial optimisation experiments revealed that treatment of SKBr3 cells with 100nM FAKsiRNA suppressed FAK expression, an effect that lasted for at least 6 days post-transfection (**Figure 4.3D**). In contrast to the sustained suppression of FAK, the activities of other signalling molecules known to be involved in FAK-related pathways (Src, AKT, MAPK, HER2 and HER3) returned to near basal levels within 48hrs post-transfection. The effects of FAK siRNA on growth were subsequently determined by direct Coulter counting which showed no significant changes in cell viability between control (NTsiRNA) and FAKsiRNA-transfected SKBr3 cells 72hrs post-transfection (**Figure 4.3E**). In contrast, SKBr3 cells transfected with FAKsiRNA had a significantly impaired migratory response to HRG when compared to controls (**Figure 4.3F**).

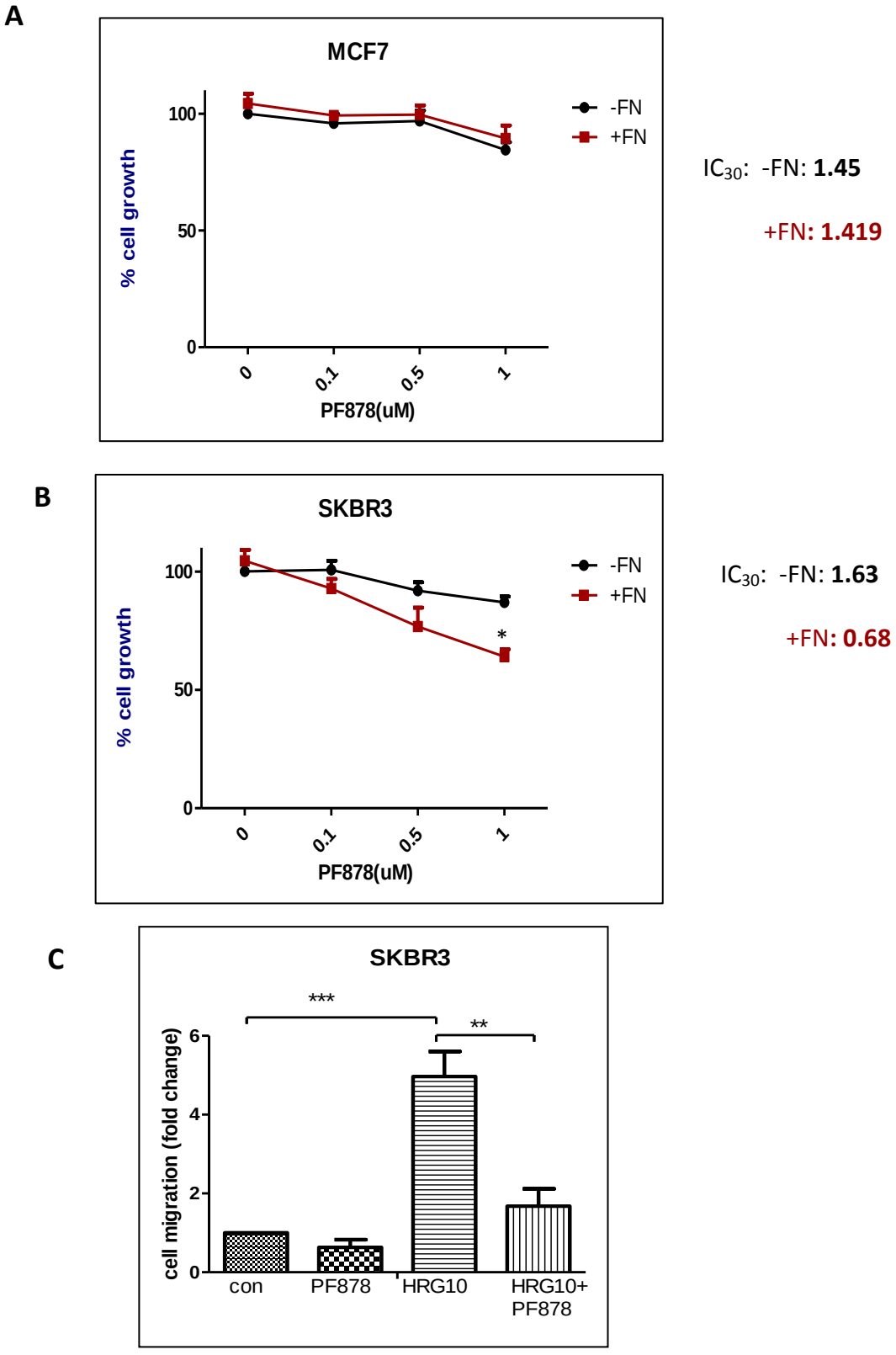


Figure 4.3. Effect of FAK inhibition by PF878 on the growth and migration of MCF7 and SKBr3 cells. (A,B) The growth of MCF7 and SKBr3 cells in response to PF878 (0–1 µM) over a period of 7 days was assessed using Coulter counting (black line). Parallel assays were performed on fibronectin-coated plates (red line). Data are mean cell proliferation values ±SEM (n=3). (C) The effects of PF878 (1 µM) on Heregulin (10ng/ml)-stimulated SKBr3 cell migration over fibronectin coated membranes were assessed using Boyden Chamber assays. Cells were allowed to migrate for 20hrs and subsequently fixed, stained with crystal violet and counted. Data represent the mean numbers of migratory cells/membrane run in duplicates from three

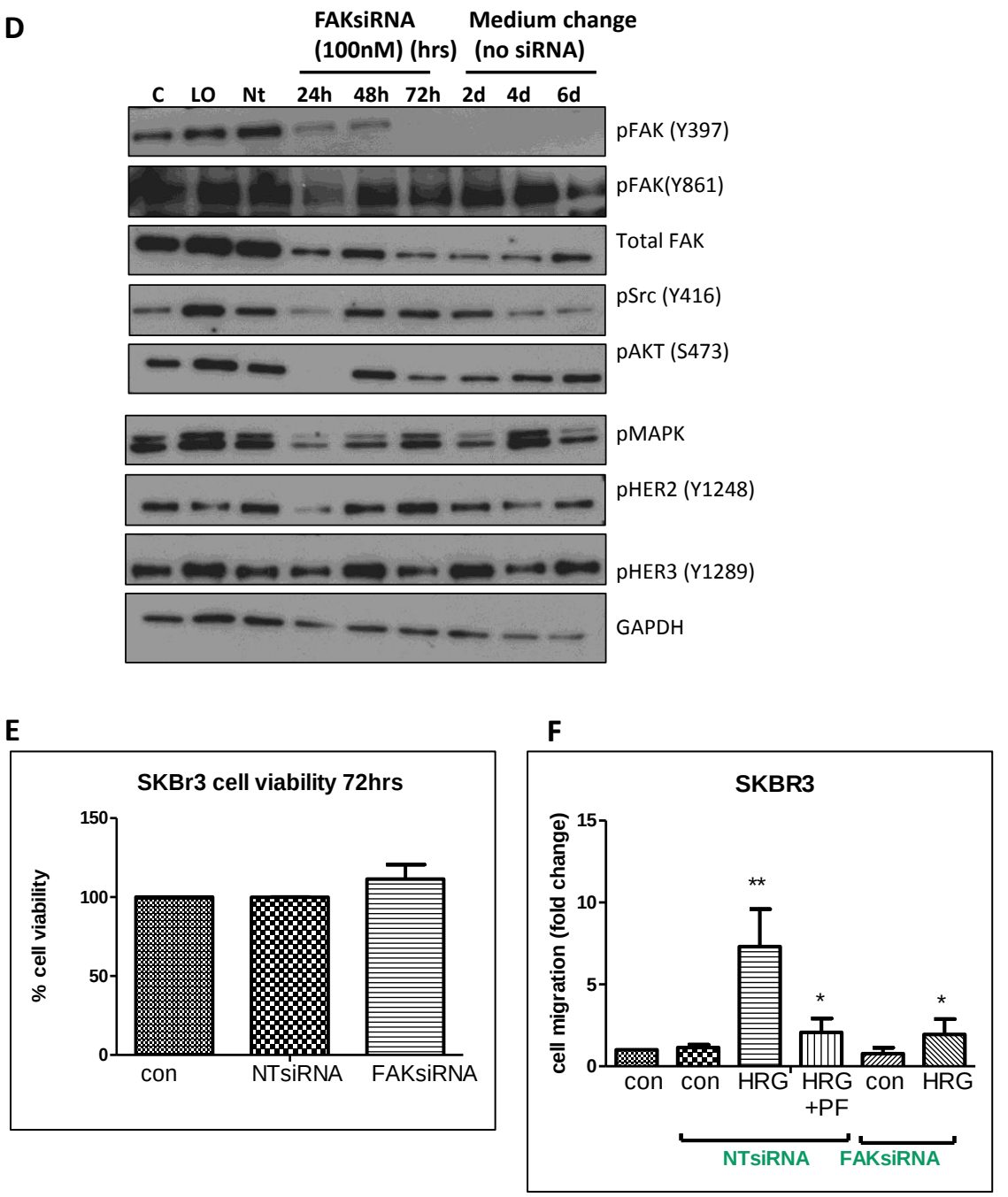


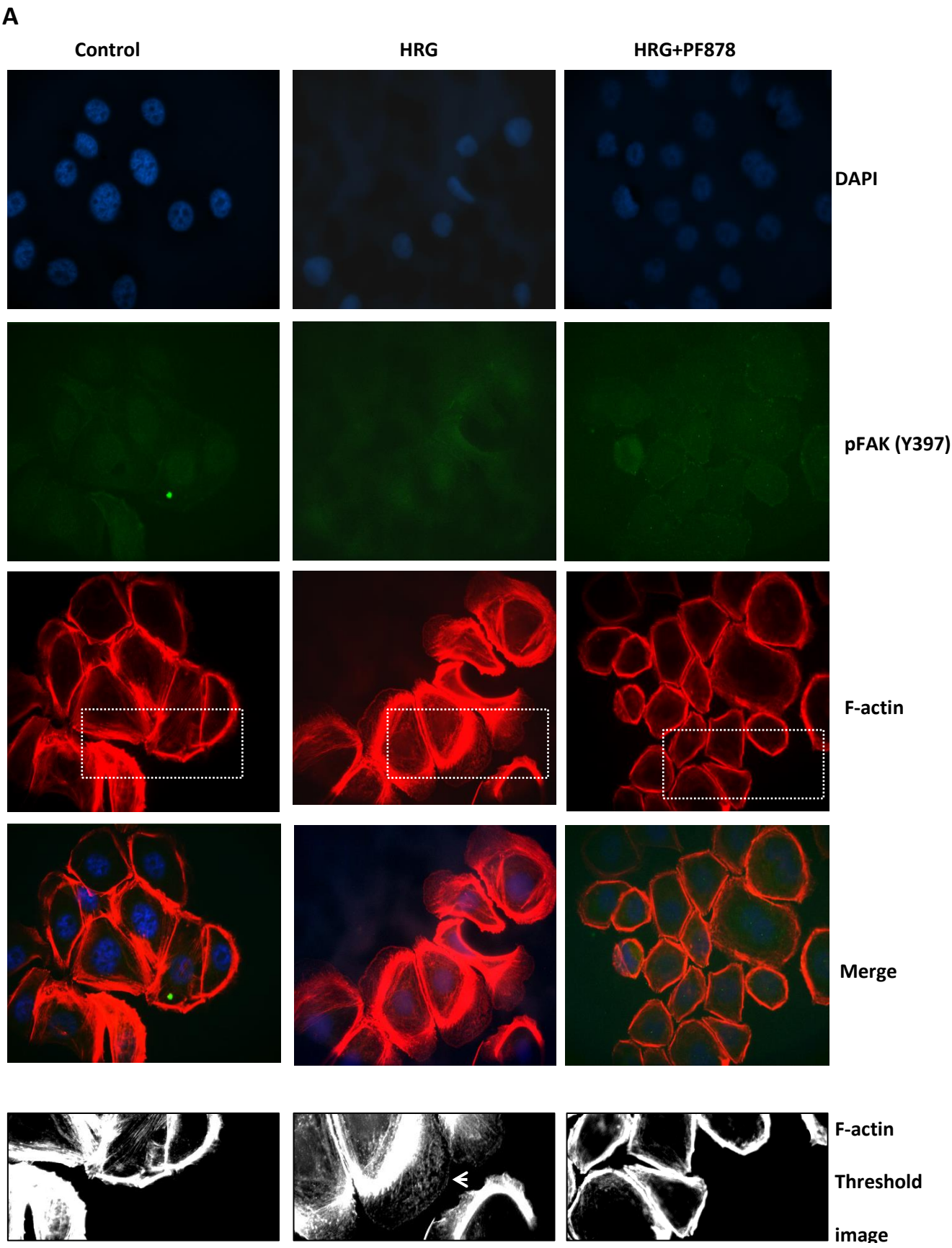
Figure 4.3. (Continued). (D) For validation, SKBr3 cells were transfected with FAKsiRNA. To optimise the siRNA transfection, SKBr3 cells were grown to 50% confluence and treated with 100nM Dharmafect Lipid(LO), NT/FAKsiRNA for 72hrs. Cells were harvested and lysed at indicated time-points up to 6 days post-transfection, processed for Western Blotting and probed for the proteins indicated. (E) Effect of transfection on cell viability was evaluated by direct Coulter counting following transfection of SKBr3 cells with NT/FAKsiRNA for 72hrs. (F) Following optimisation, NT/FAKsiRNA SKBr3 cells were subject to cell migration assays under similar conditions as in (C). Data represent the mean numbers of migratory cells/membrane run in duplicates from three independent experiments. P: *<0.05,**<0.001

4.5 FAK is involved in HRG-induced lamellipodia formation in HER2+/ER- breast cancer cells

FAK is well established as a binding partner for cytoskeletal components where it acts to mediate focal adhesion regulation and actin stress fibre assembly (Mitra et al. 2005; Serrels et al. 2007). In light of this we wished to investigate the effects of pharmacological FAK inhibition on the cytoskeletal system in the context of HRG-induced migration as a possible underlying mechanism through which FAK might regulate HRG-induced migratory responses.

Immunofluorescence staining revealed that in unstimulated SKBr3 cells FAK Y397 levels were generally low (**Figure 4.4A**) in contrast to the levels of Y861 (**Figure 4.4B**). This was also observed in the Western Blotting data (**Figure 4.1**) showing very little pFAK Y397 expression in the SKBr3 cells. In the case of pFAK Y861, punctate staining was observed around the cell periphery and at the end of actin-stress fibres, reminiscent of FA localisation (Parsons et al. 2000). Additionally, actin fibres localised close to and parallel to the cell membrane, indicative of cortical actin which are typically associated with actin cross-linker proteins such as α-actinin and cortactin, that form linkages with the plasma membrane (Stricker et al. 2010). Following HRG stimulation, a small increase in pFAK Y861 was observed together with formation of prominent lammelipodia extensions particularly in cells at the edge of the colony. Moreover, pFAK Y861 was observed to be present at these protrusive structures, suggesting an important role for the activity of FAK in this process. The images were further processed to enhance the visualisation of these membrane protrusions, particularly focusing on areas of interest (**See Figure 4.4B lower panel**). This employs a common processing technique whereby images are converted to a binary image (8-bit) and an automatic threshold range is set to distinguish features of interest apart from the background.

Inhibition of FAK using PF878 suppressed the formation of mature lamellipodia protrusions, with the cells instead producing small projections at the cell periphery in which pFAK Y861 staining remained localised.


A
Control
HRG
HRG+PF878
DAPI
pFAK (Y397)
F-actin
Merge
F-actin
Threshold
image

B

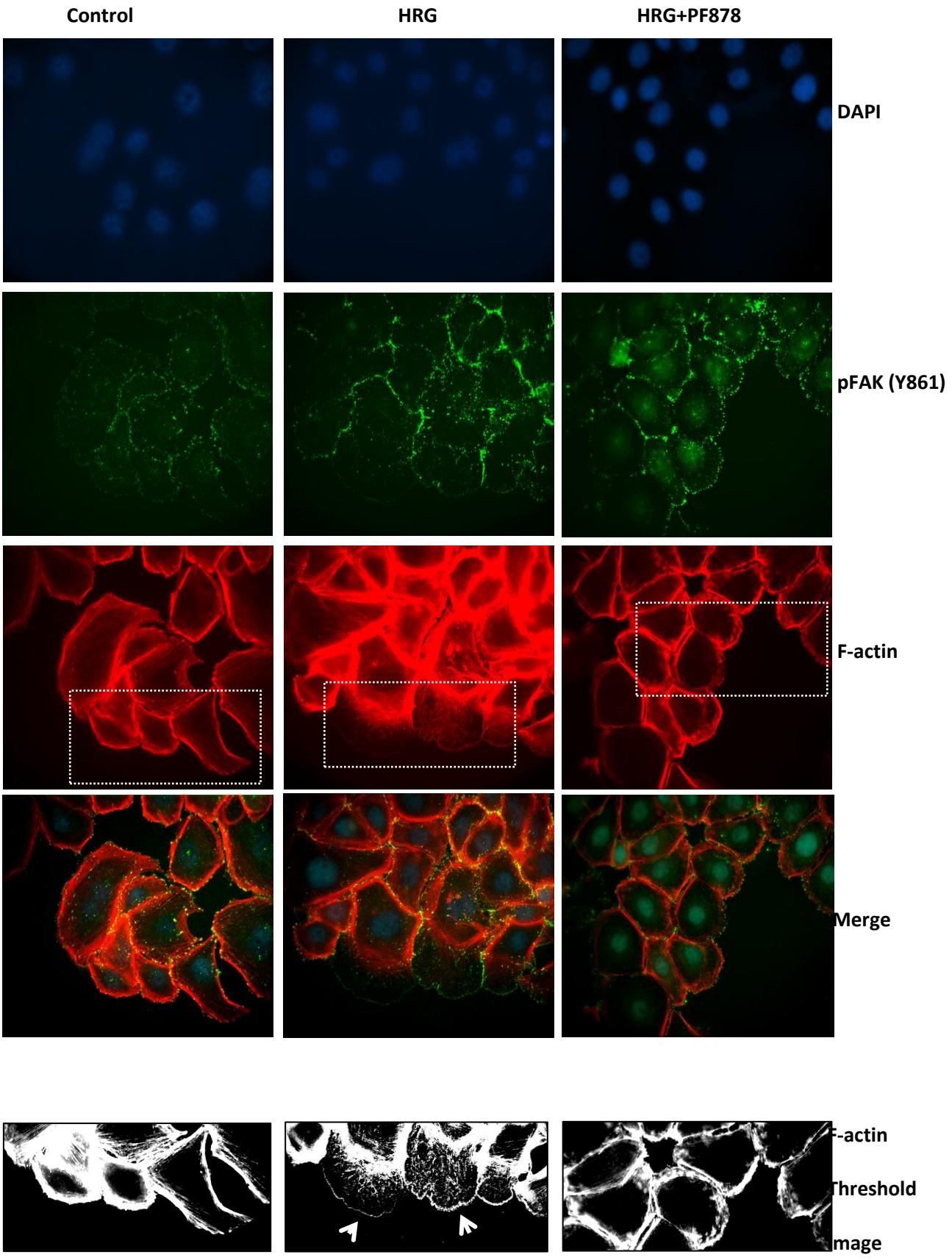

Control
HRG
HRG+PF878
DAPI
pFAK (Y861)
F-actin
Merge
F-actin
Threshold
mage

Figure 4.4. Immunofluorescence images of SKBr3 cells seeded on FN-coated plates following Heregulin-stimulation migration (10ng/ml) ±PF878 (1uM) for 60mins. Cells were stained for primary antibodies: (A) pFAK Y397 (green) + Rhodamine-phalloidin (F-actin) (red), (B) pFAK Y861 (green)+ Rhodamine-phalloidin (F-actin). All cells were stained with appropriate Alexa-Fluor 488-conjugated secondary antibodies and counterstained with DAPI, a nuclear dye (blue). Magnification: x63, oil-immersion objective. To visualise membrane activity, images were processed by converting to 8-bit and subsequently adjusting to a fixed threshold using Image J; resulting images are shown in the white doted boxes.

4.6 Inhibition of FAK attenuates HRG-mediated migratory signalling in HER2+/ER-breast cancer cells

In light of the ability of PF878 to suppress HRG-induced migration in SKBr3 cells, we wished to explore the signalling mechanisms underlying this. SKBr3 cells were stimulated with HRG at various time-points in the presence or absence of PF878 prior to analysis of protein expression and activity with Western blotting. Focusing on the 60minutes time-point, which corresponds with that used the immunofluorescence staining experiments where the onset of pro-migratory focal adhesion and cytoskeletal changes were observed, our Western blot data revealed that HRG stimulation of SKBr3 cells stimulated FAK activity (Y397 and Y861) along with promoting the rapid phosphorylation of Src, AKT and MAPK whilst decreasing the activity of HER3. No change was seen in the phosphorylation state of the other ErbB receptors, EGFR, HER2 or HER4 whilst the activity of HER3 was decreased (**Figure 4.5**). PF878 suppressed both basal and HRG-induced phosphorylation of FAK (Y397>Y861) and Src. Moreover, this was accompanied by a further decrease in the magnitude of HRG-induced suppression of HER3 activity. We did not detect changes in the activity of the other ErbB receptors, AKT or MAPK.

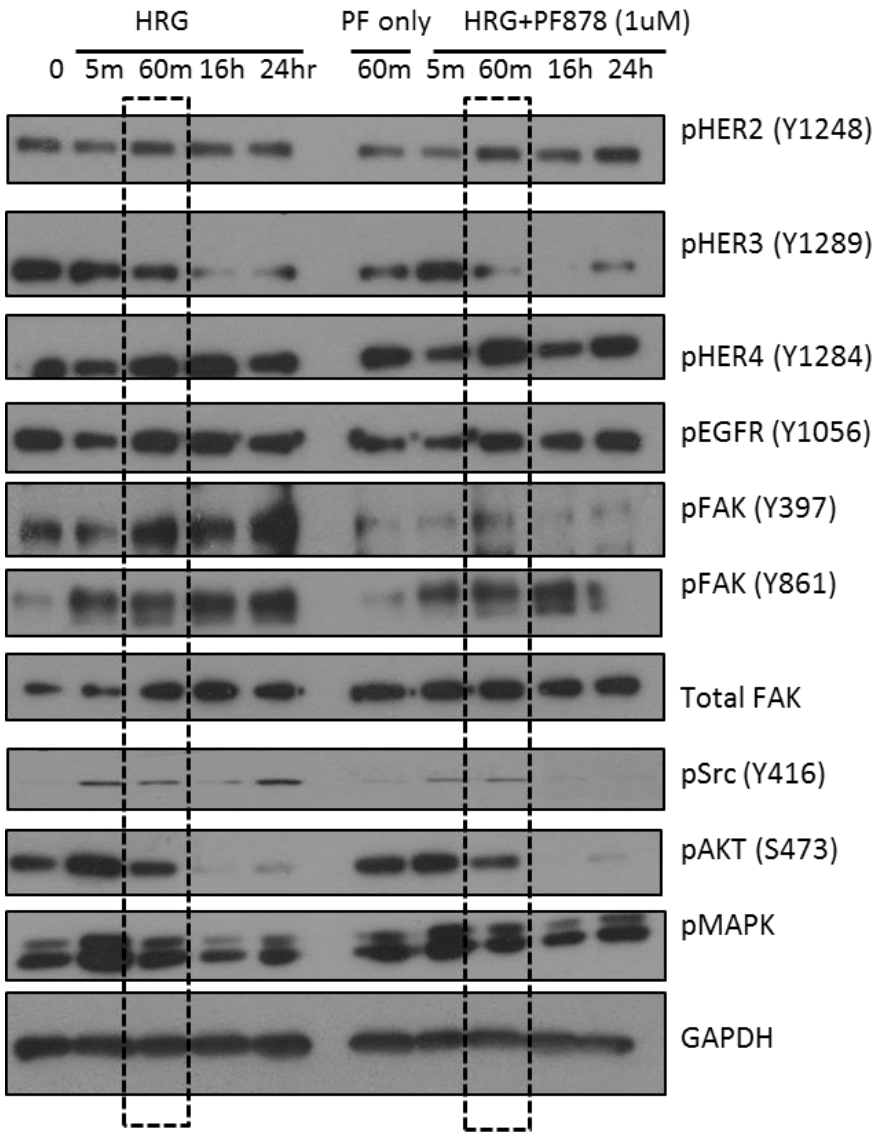


Figure 4.5. HRG stimulates downstream signalling in SKBR3 cells. Cells were seeded on fibronectin-coated plates and cultured to 70% confluence and treated with HRG (10ng/ml) at the indicated time-points ± pre-treatment with PF878 (1μM) for 60mins. Cells were lysed, processed for Western Blotting and immuno-probed for proteins as indicated. GAPDH was used as loading control. Representative blots shown from three independent experiments.

4.7 Discussion

We have previously explored the role of FAK in cell models of HER2+/ER+ disease where it appears to play an important role in regulation of cellular migration with a less prominent role in cell growth. Here we explored the hypothesis that FAK might also play a role, and possibly a more significant one, in HER2+/ER- disease given that this is a more aggressive subtype relative to the luminal tumours (Sørlie et al. 2001; Prat & Perou 2011) and that FAK itself is known to be associated with aggressive tumours. In addition, the HER2+/ER- subtype represents the dominant HER2+ subtype seen clinically.

Initial characterisation of SKBr3 cells confirmed expression of a constitutively active HER2 receptor, as well as the other ErbB receptors, EGFR, HER3 and HER4. Most notable was that these also correlated with increased FAK activity at Y861, as well as the downstream signalling proteins Src, AKT and MAPK. SKBr3 cells also showed an increased sensitivity to PF878 compared to MCF7 cells that were used as a comparator cell line in light of their HER2- status. Interestingly, inhibition of FAK activity in SKBr3 cells promoted an increase in the activities of AKT and MAPK, an effect also observed in FAKsiRNA-expressing cells. This was surprising given that FAK has been established to bind to the p85 domain of PI3K and the Grb2 adaptor protein, major downstream upstream regulators of AKT and MAPK respectively (Zhao & Guan 2009). Our findings here also contradicted our previous observations in HER2+/ER+ cell lines (Chapter 3) and thus suggest potential differences between the regulation of AKT and MAPK signalling between HER2+/ER+ and HER2+/ER- breast cancer cells likely resulting from intrinsic differences in the signalling circuitry in each cell line (Sørlie et al. 2001; Cheang et al. 2009). Importantly, the activation of these pathways may also reflect mechanisms by which cells adapt and defend to external stresses, as supported by studies demonstrating AKT activation in response to chemotherapeutic drugs and cellular stresses (Beitner-Johnson et al. 2001; Alvarez-Tejado et al. 2001). This also might in some part explain the relative insensitivity of SKBr3 cells to PF878 in terms of growth inhibition.

Similarly, we examined the supporting role of the micro-environment particularly in the context of ECM proteins. ECM-cell interactions are mediated by integrin engagement which may in turn activate FAK-mediated signalling (Kornberg et al. 1992) that can potentiate cell proliferation and survival (Stupack & Cheresh 2002). Moreover, there have been reports of

integrin-mediated survival signalling events that occur at levels independent of AKT (Friedland et al. 2007) or MAPK (Matter & Ruoslahti 2001) signalling pathways. Accordingly, we reasoned that FAK plays a more dominant role in terms of regulating growth in the presence of matrix proteins, and therefore hypothesised that inhibition of FAK, may suppress growth in this context irrespective of the increases in AKT and MAPK activity. To do so, cells were seeded on fibronectin-coated plates. This could model, although to a limited extent, the tumour microenvironment where cells are in contact with ECM proteins. The data however showed that the growth inhibitory effects of PF878 remained limited in matrix (fibronectin)-coated surfaces, with the exception at 1 μM, wherein an approximate 30% growth inhibition was observed in SKBr3 cells seeded in fibronectin-coated plates. The implications on FAK activity and related downstream signalling in the presence of fibronectin however are yet to be determined and will require further studies. Moreover, it is also likely that better efficacy is achieved at higher concentrations of the FAK inhibitor. As such, peak serum concentrations of 2-9 μM can be achieved in the clinical setting, which are over and above the concentrations used in our studies (based on Phase 1 studies of a structurally similar FAK inhibitor PF-271 (Infante et al. 2012), and thus the therapeutic targeting of FAK in HER2+/ER-breast cancer needs to be explored in *in vivo* and clinical settings.

As well as investigating a role for FAK in proliferative responses in the HER2+ context, we also wished to determine whether FAK played a role in the migratory responses of this cell type. In agreement with other reports, SKBr3 cells were non migratory basally in agreement with previous observations (Hijazi et al. 2000; Ruan et al. 2012), but responded to HRG which further supports studies that have implicated HRG in stimulating in metastatic tumour progression (Xu et al. 1997; Atlas et al. 2003b; Ruan et al. 2012). In contrast to growth, which was not greatly affected by loss of FAK activity, suppression of FAK activity or expression greatly inhibited HRG-induced migration. Our data suggests that underlying this may be FAK's involvement in the cortical actin cytoskeletal reorganisation. Actin is the main cytoskeletal element in these lamellipodia, fan-like structures that ruffle and move dynamically to sense the surrounding environment and promote persistent directional movement (Stricker et al. 2010). Our data show that HRG triggered a re-localization of pFAK Y861 to the leading edges of the lamellipodia protrusions which appears to be

phosphorylation dependent since PF878 inhibited this, as well as suppressing these cytoskeletal changes. Together these data imply that the recruitment of pFAK Y861 to focal adhesions is important for the establishment and/or persistence of lamellipodia in a migrating cell and are in agreement with the role of FAK as leading-edge organizer based on observations on FAK-siRNA transfected fibroblasts which were shown to be unable to form functional lamellipodia and displayed loss of directional migration (Tilghman et al. 2005). Additionally, an interesting observation was the increased pFAK Y861 staining in a localisation reminiscent of the nucleus in SKBr3 cells when treated with PF878. The evidence of FAK localisation in the nucleus has previously been noted (Lim et al. 2008; Lim 2013). This induction of nuclear FAK localisation in the presence of PF878 may reflect of that suggested by Lim et al. (2008), that loss of FAK from focal adhesions increases the "free" cytoplasmic pool of FAK available for nuclear translocation where it promotes cell survival by facilitating the turnover of the tumour suppressor p53. In consistent, it was also shown that FAK inhibition by PF-271 a triggers nuclear accumulation of FAK which acts to inhibit GATA4-mediated transcription of VCAM-1 (Lim et al. 2012). These and our observation suggest that the loss of FAK activity may correlate with FAK nuclear localisation. However, the significance of such a localisation to the anti-migratory effect of PF878 is yet to be explored.

To delineate the mechanisms underlying the cytoskeletal changes accompanying the migratory behaviour of SKBr3 cells, we performed signalling analysis by Western Blotting. Our data demonstrated that HRG rapidly increased FAK activity at Y861 in SKBr3 cells within 5 minutes and remained elevated up to 24hrs. That FAK is activated in response to HRG is in agreement with other studies in various cellular contexts (Vartanian et al. 2000; Vadlamudi et al. 2002; Vadlamudi et al. 2003). Moreover, the phosphorylation of pFAK Y861 is accompanied by concurrent activation of Src Y416, supporting the close interplay between Src and FAK (Calalb et al. 1996). Previous studies however have also implicated the phosphorylation of another residue, Src Y215, as a unique event following HRG signalling to FAK Y861 in MCF7 and NIH3T3 cells (Vadlamudi et al. 2003). We however failed to detect Src activity at Y215 in SKBr3 cells despite several attempts to pursue this experimentally. Nonetheless, PF878 inhibited FAK activity at Y397 and Y861 as well as of pSrc Y416 suggesting that the formation of lamellipodia protrusions and thus cell migration may be dependent on a pathway involving FAK and Src. We speculate that the resulting

consequences occur at the level of focal adhesions to which the actin filaments are attached. Decreased FAK/Src activity therein may disrupt interactions with and/or activation of multiple signalling molecules that regulate the actin polymerisation machinery that participate in lamellipodia formation, among which, p130cas, Arp2/3, cortactin, N-WASP and vinculin particularly have been implicated to be regulated by FAK and/or Src (Cary et al. 1998; Serrels et al. 2007; W. Wang et al. 2011; Wu et al. 2004).

An increase in the phosphorylation of AKT and MAPK were also observed in response to HRG, in agreement with others (Adam et al. 1998; Yang et al. 2008), albeit transient. PI3K/AKT signalling in particular was implicated in HRG-mediated cytoskeletal re-organisation and migratory response in HER2-overexpressing MCF7 cells (Adam et al. 1998). AKT has also been described to modulate cytoskeletal remodelling by binding to the actin-binding protein Girdin, that regulates the cross-linking and thereby integrity of actin bundles in African monkey kidney (Vero) cells (Enomoto et al. 2005). However, AKT nor MAPK activities were unchanged in the presence of PF878 and therefore do not appear to significantly regulate migratory behaviour of SKBr3 cells in response to HRG.

HRG, to our surprise did not significantly increase HER2 or HER4 activation, and PF878 neither affected their basal activities suggesting that FAK may not direct modulate ErbB receptor activity. In contrast, HRG was observed to promote a decrease in HER3 phosphorylation, an effect that was greater in the absence of FAK.

The lack of a significant increase in ErbB receptor phosphorylation in response to HRG was particularly surprising given that a number of reports have shown strong responses to HRG in terms of phosphorylation of ErbB receptors (Adam et al. 1998; Tan et al. 1999; Vadlamudi et al. 2003). One explanation may be that our lack of HRG response may be due to the over saturation of these receptors from endogenous ligands. Indeed, a lack of response to HRG has been demonstrated in other cell lines proposed to occur through constitutively high levels of receptor phosphorylation resulting in its saturation (Göstring et al. 2012). A further possible explanation for the discrepancy could be due to the cell culture conditions employed. A common approach amongst studies published showing up-regulation of ErbB receptor signalling in response to HRG is that they generally use at least 24hrs period of serum starvation prior to stimulation. In our experiments, a serum starvation period of 60

minutes was used, which may not suppress endogenous signalling to a great extent in order to see a significant difference between control (unstimulated) and HRG-treated samples.

That the HER3 receptor activity was reduced following HRG action was surprising although previous studies on have reported internalisation of ErbB receptors may be triggered in response to EGF and HRG leading to down-regulation of its activity (Hurrell & Outhoff 2013). Whether this is the case with our cell model is unknown; ideally, co-immunofluorescence staining experiments would be performed to determine internalisation of signalling active ErbB receptors and to explore possible co-localisation with FAK in the presence of HRG, though we were not able to do this as the primary antibodies were of the same species.

Taken together from these experiments, our data indicates that FAK plays important roles in actin cytoskeletal remodelling and lamellipodia formation at the leading of SKBr3 cells in response to HRG and to ultimately regulate cell migration. Subsequently, our data demonstrated that inhibition of FAK acts to suppress SKBr3 cell migration, highlighting the potential of targeting FAK to reverse the aggressive nature of this breast cancer subtype.

5. Results (III)

Targeting FAK in HER2+ breast cancer improves response to anti-HER2 targeted therapy

5.1 Introduction

The anti-HER2 monoclonal antibody, Herceptin, represents the standard treatment for patients with HER2+ breast cancer. As a monotherapy however response rates are relatively low varying between 11-26% (Baselga et al. 1996; Cobleigh et al. 1999; Vogel et al. 2001). Better outcomes are achieved in combination with adjuvant chemotherapy. In a study, the inclusion of Herceptin with chemotherapy significantly increased the time to disease progression (TTP) to 7.4 compared to 4.6 months in patients that received chemotherapy alone (Slamon et al. 2001). Despite improvements, this study demonstrated that patients display disease progression within 1 year. Findings from the large Herceptin Adjuvant (HERA) study (1694 patients) demonstrated a significant 8.4% increase in disease-free survival benefit in patients treated with Herceptin for one year following chemotherapy (Piccart-Gebhart et al. 2005) and remained significant at the 3 years follow-up period (Smith et al. 2007). However, of the 1694 patients in the Herceptin-treatment cohort of the HERA study for instance, 7.5% reported recurrent events within the 1 year follow up period, and the majority of these presented as distant metastases. Altogether, these highlight the significant hurdle in treating patients with HER2+ disease.

Given that FAK plays a key role in the transduction of HER2 signalling in HER2+ cells and that levels of FAK expression strongly correlated with HER2 overexpression in clinical samples (Benlimame et al. 2005; Schmitz et al. 2005), our hypothesis is that FAK may contribute to the limitation of Herceptin response. In support of this are studies which show that resistance to anti-HER2 therapies (Herceptin and Lapatinib) have been associated with increased integrin/FAK signalling (Yang et al. 2010).

In chapters 3 and 4, we demonstrated that targeting FAK with PF878 in both HER2+/ER+ cell lines (BT474, MDA361) and HER2+/ER- cells (SKBr3) had significant anti-migratory effects but only a modest inhibition on growth. In this chapter, we explored whether the combined targeting of FAK alongside the HER2 receptor, using Herceptin would result in a greater suppression of growth, particularly given the supporting evidence for combination versus monotherapy approaches using small molecule inhibitors alongside traditional treatments in the literature (Araujo et al. 2009;Molife et al. 2014; Hudis et al. 2013).

5.2 Combined inhibition of FAK and HER2 in HER2+/ER+ cells results in enhanced suppression of proliferation

We previously demonstrated that for ER+ cells, those which also co-expressed the HER2+ receptor were more sensitive to FAK inhibition as assessed by pFAK Y397 phosphorylation than cell lines which were HER2-. Although the inhibition of FAK activity alone did not significantly affect the proliferation of HER2+/ER+ cells, we hypothesised the FAK inhibitor might be more effective when combined with agents that targeted the HER2 receptor.

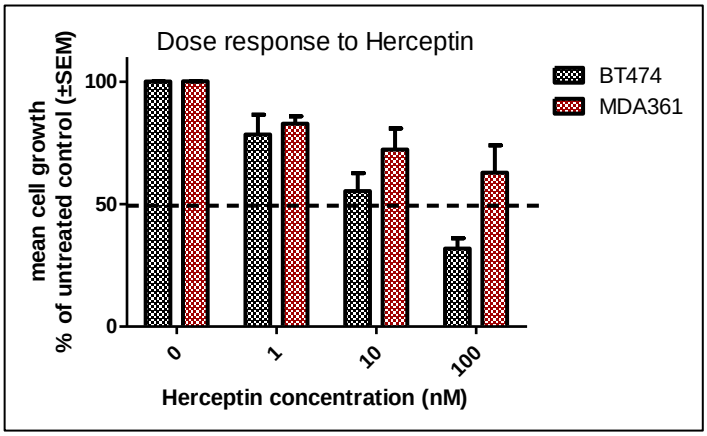
Initial investigations on the two HER2+/ER+ cell lines revealed a differential sensitivity to Herceptin, with BT474 cells being growth inhibited whilst MDA361 cells exhibited limited sensitivity to this agent. IC_{30} values were recorded of 2.59nM (BT474) versus 21.48nM (MDA361) (**Figure. 5.1A**). The combination of Herceptin and PF878 resulted in a significantly enhanced the growth inhibitory effect in BT474 cells (with a low dose of Herceptin) whilst Herceptin-insensitive MDA361 cells appeared to regain Herceptin sensitivity; IC_{30} values for Herceptin now became 1.29nM and 5.9nM for BT474 and MDA361 cells respectively (**Figure 5.1B,C**).

To determine whether the effect of Herceptin and PF878 combined were additive or synergistic, isobologram analysis was carried out, a common approach used to analyse drug interactions (**See Section 2.9**). First, the IC_{30} values for the individual agents were plotted on the X and Y axis of the graph; by connecting the two points, a 'line of additivity' is created. Thereafter, reference combinations of both drugs (dose pairs) that achieve the same level of growth suppression (i.e. 30% growth inhibition) were additionally plotted. As shown in **Figure 5.1D**, this analysis indicated that treatment of BT474 and MDA361 cells with Herceptin and PF878 in combination resulted in a synergistic effect towards the suppression on cell growth.

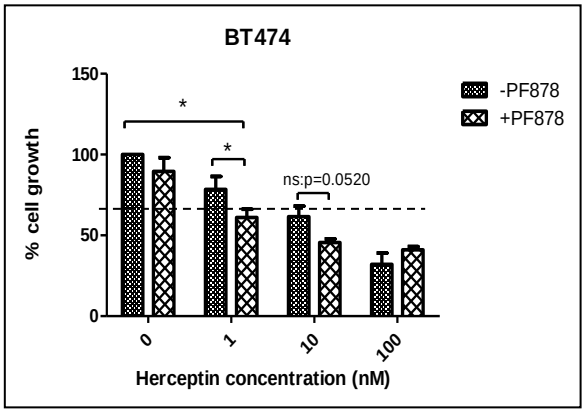
To validate these observations, we compared the effects of Herceptin and PF878 as a monotherapy and in combination on cell proliferation by means of staining for the Ki67 proliferation marker protein. Ki67 is strictly expressed in the active phases of the cell cycle (G1, S, G2, and mitosis), but not in resting cells (G0), and is thus commonly used as a marker for determining proliferative cells in a given cell population (Scholzen & Gerdes 2000). As shown in **Figure 5.1E**, combining Herceptin with PF878 resulted in a greater reduction in

Ki67-positive cells than monotherapies implying a reduction in the number of actively proliferating cells as a result of this treatment approach.

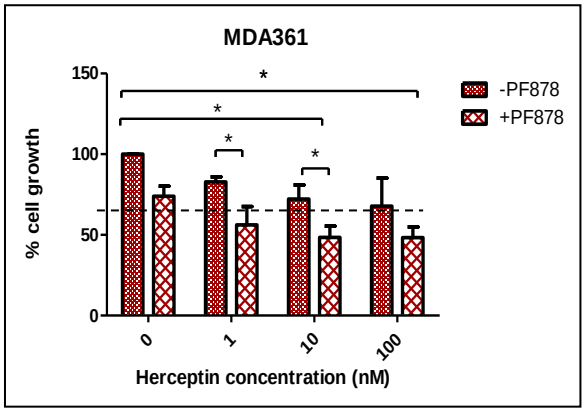
A


Dose response to Herceptin
mean cell growth
% of untreated control (±SEM)
100
50
0
BT474
MDA361
0
1
10
100
Herceptin concentration (nM)

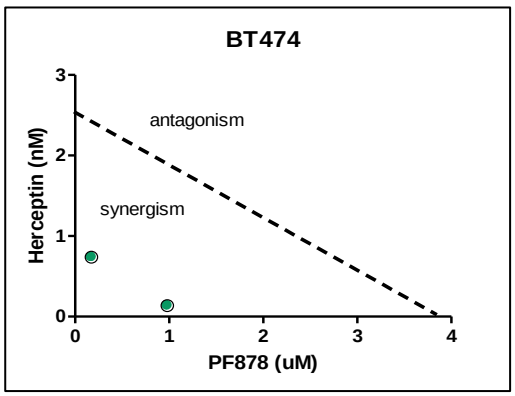
B

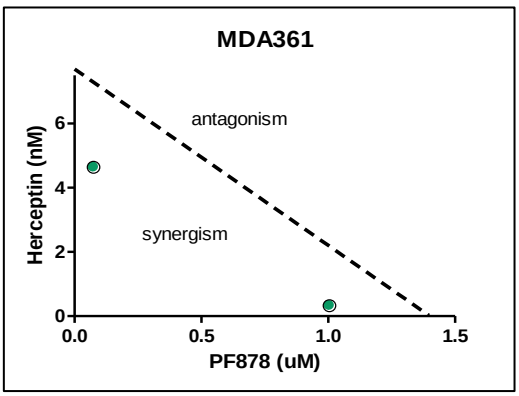

BT474
% cell growth
150
100
50
0
-PF878
+PF878
*
*
ns:p=0.0520
0
1
10
100
Herceptin concentration (nM)

C


MDA361
% cell growth
150
100
50
0
-PF878
+PF878
*
*
*
*
0
1
10
100
Herceptin concentration (nM)

D


BT474
Herceptin (nM)
3
2
1
0
antagonism
synergism
0
1
2
3
4
PF878 (uM)


MDA361
Herceptin (nM)
6
4
2
0
antagonism
synergism
0.0
0.5
1.0
1.5
PF878 (uM)

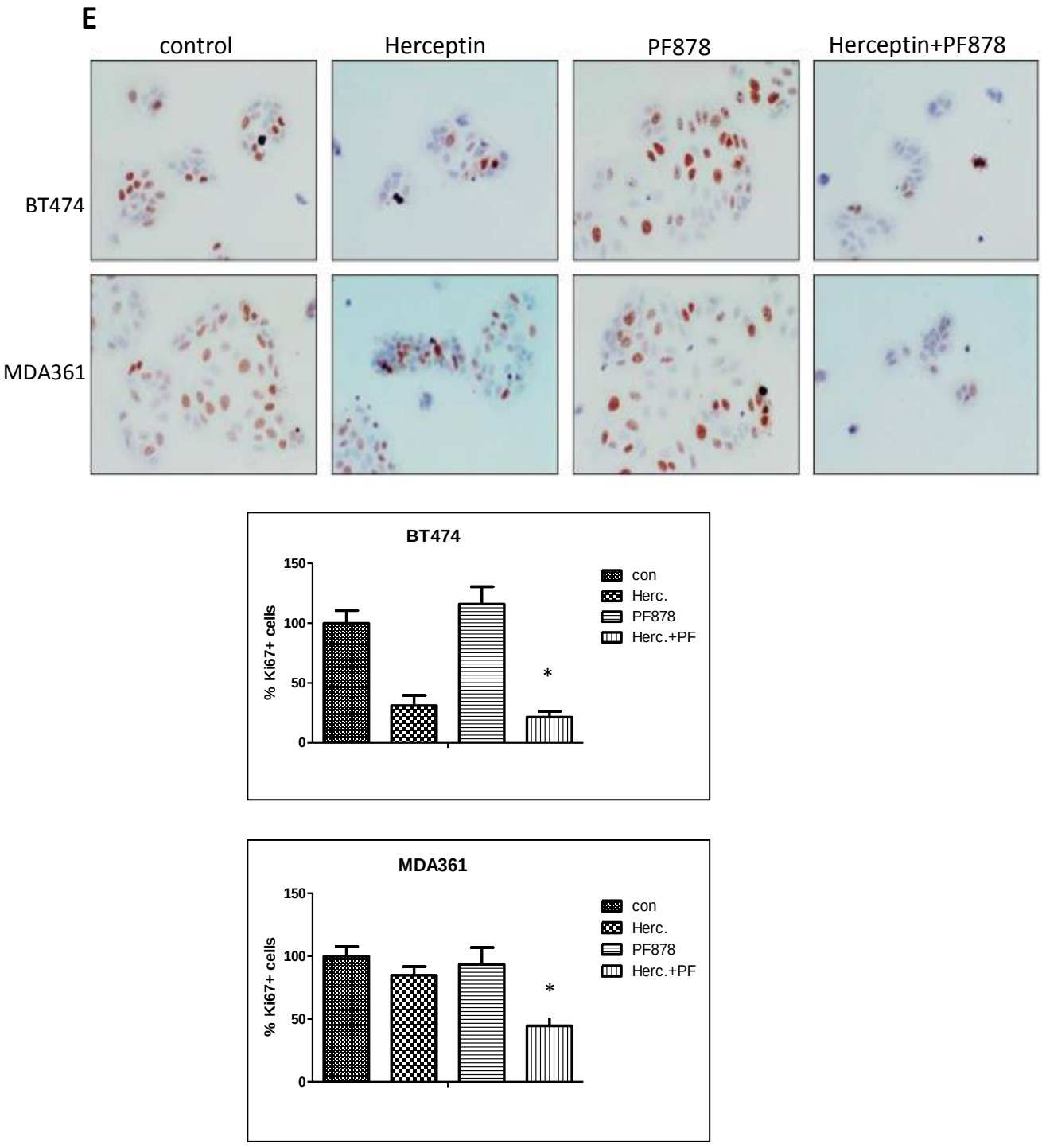


Figure 5.1 Targeting of FAK and HER2 together results in an improved suppression of HER2+/ER+ breast cancer cell proliferation vs monotherapy. (A) The ability of Herceptin to inhibit the proliferation of HER2+/ER+ breast cancer cells was determined by direct Coulter counting (B,C) The anti-proliferative effects of PF878 and Herceptin in combination in both cell lines were subsequently explored. (D) The nature of the interaction between Herceptin and PF878 was evaluated by isobologram analysis .Calculated dose pairs that achieve the IC_{30} falling below the *line of additivity* are regarded to be synergistic whilst those above are antagonistic. In both cell lines, the Herceptin and PF878 appear to act in synergy when given in combination. Data are mean cell proliferation values ±SEM (n=3).(E) Parallel detection of Ki67 by immunocytochemistry on cells treated with Herceptin and PF878 in combination. Accompanying graph shows % mean proliferative counts (+Ki67) per field of view. * p=<0.05

5.3 Combined inhibition of FAK and HER2 suppresses signalling activity and results in PARP cleavage in HER2+/ER+ breast cancer cells

To shed light onto the mechanism for the growth inhibitory effect arising from the combined Herceptin and PF878 treatment we explored the effects of this treatment regimen on FAK and HER2- associated signalling proteins, after a treatment time of 24hrs. Although signalling events could occur rapidly (within minutes), a longer time-point of 24hrs was chosen as it may better reflect signalling changes that mediate the effects on cell growth (**Figure 5.2**).

Herceptin monotherapy resulted in an increase in the activity of HER2, EGFR and HER4 whilst reducing AKT phosphorylation and MAPK (BT474 and MDA361 cells). PF878 alone had no obvious effect on ErbB receptor phosphorylation in either cell line or the activity of AKT and MAPK. However, when PF878 was combined with Herceptin, the effect was to attenuate Herceptin-induced ErbB activation (below control levels in MDA361 cells) and reduce AKT and MAPK to almost undetectable levels.

To determine a potential contribution of apoptosis to the anti-proliferative effects of combination treatment, analysis of PARP cleavage, a surrogate marker for caspase-3 activation and apoptosis, was performed again by Western blotting. These data revealed that the combination of Herceptin and PF878 elevated the amount of the 23kD PARP fragment detected in cell lysates.

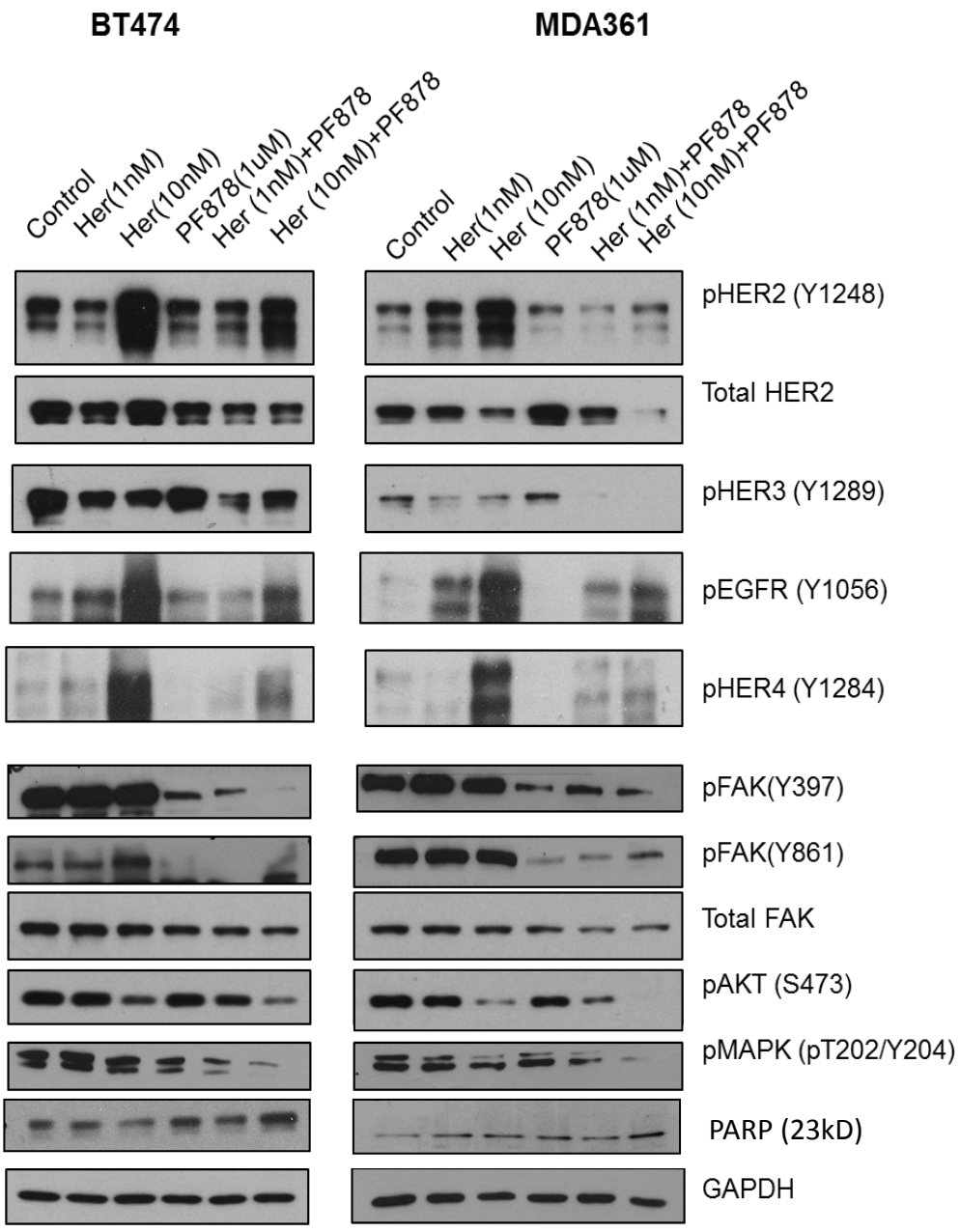


Figure 5.2 Combined targeting of FAK and HER2 in HER2+/ER+ BT474 and MDA361 breast cancer cells and their effects on HER2 and FAK-related signalling components. Cells were treated with PF878 (1 µM), Herceptin (1 and 10 nM) or the combination of both the agents for 24 hrs. Cells were lysed and protein expression and activity were assessed by western blotting. GAPDH was used as a loading control. Representative blots shown from three independent experiments.

5.4 Targeting FAK in HER2+/ER+ breast cancer cells enhances sensitivity to endocrine therapy

According to NICE guidelines (UK), there is variation in clinical practice for HER2+/ER+ tumours, and are evaluated by clinicians on a case-by-case basis. In some patients, treatment regimens can include endocrine therapy; however, given the prevalence of the HER2 receptor in the luminal B group of tumours, there is the potential for *de novo* endocrine resistance, as the HER2 is known to be a limiting factor to endocrine therapies (Houston et al. 1999; Davies et al. 2011). Thus we wished to investigate whether inhibition of FAK might improve endocrine response in HER2+/ER+ models.

As previously demonstrated, BT474 cells were much more sensitive to the growth inhibitory effects of Herceptin versus MDA361 cells. However, the reverse was true when endocrine agents were used, with BT474 cells being much less sensitive to Tamoxifen. Inclusion of PF878 with Tamoxifen further suppressed growth of both cell lines versus monotherapy. This was more profound in BT474 cells suppressing growth from approximately 76% with Tamoxifen treatment alone to approximately 41% when combined with PF878. The combination treatment with the three drugs altogether led to an even further suppression of growth in both cell lines (**Figure 5.3**).

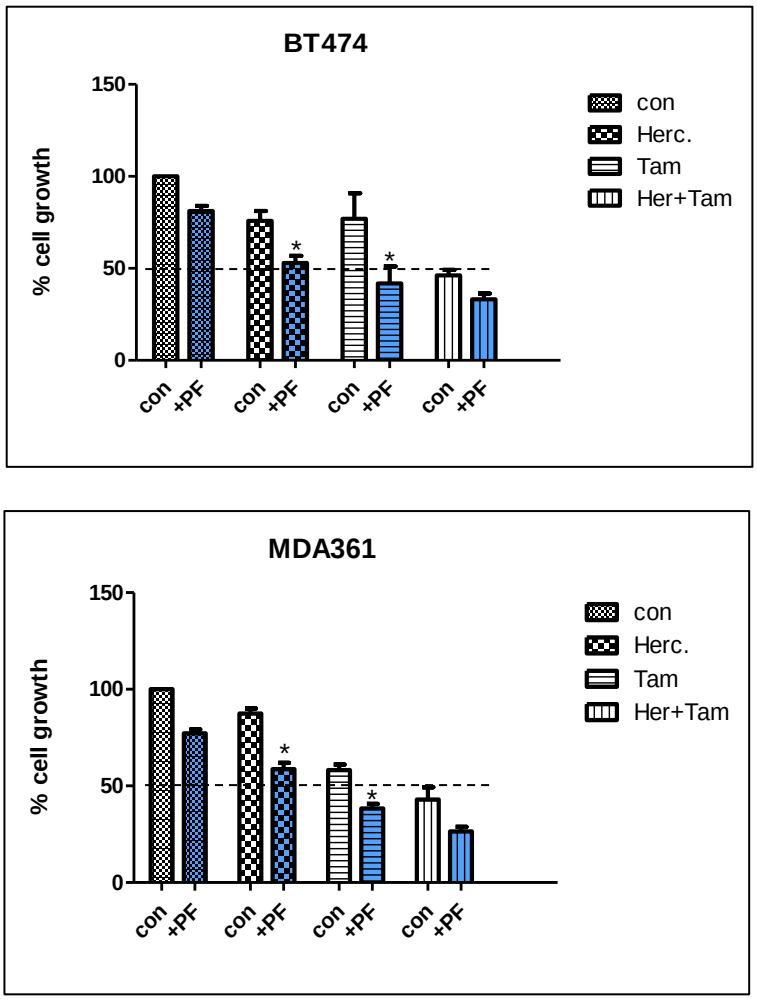


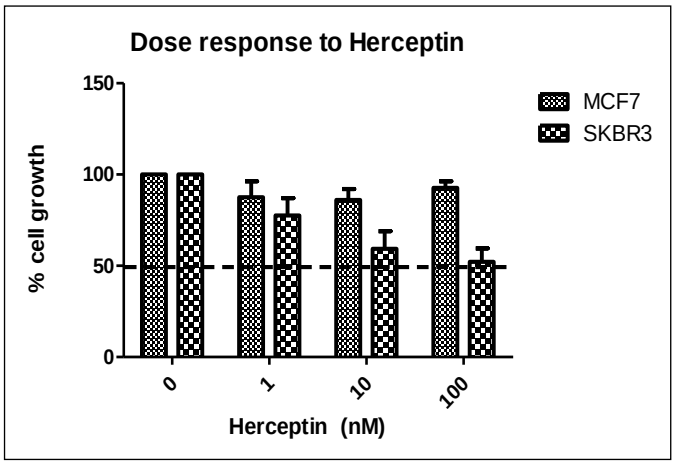
Figure 5.3 Inclusion of endocrine therapy (Tamoxifen (10^{-7}M)) with Herceptin (1nM) and PF878 (1µM) results in a further improved suppression of MDA361 and BT474 cells. Cells were seeded on 24 well plates and treatments as indicated were added after 24hrs. After 7 days, cell proliferation was assessed by direct cell count using a Coulter counter. Data are mean cell count values ±SEM (n=3). * p=<0.05.

5.5 Combined inhibition of FAK and HER2 in HER2+/ER- cells results in enhanced suppression of proliferation

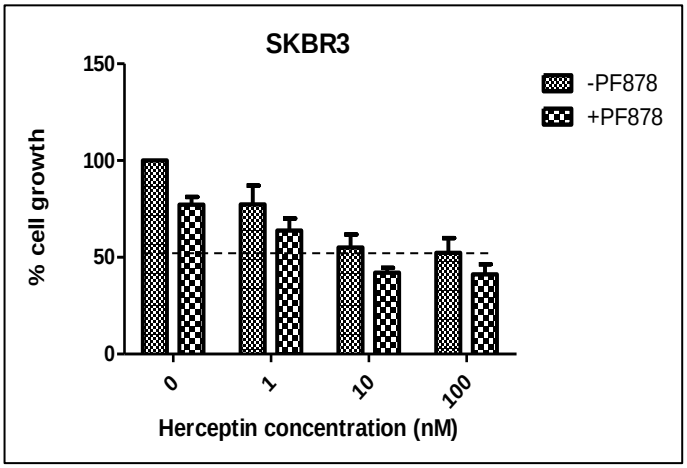
Having seen a benefit from co-targeting FAK and the HER2 receptor in luminal B breast cancer models, we next explored whether the combination of Herceptin with PF878 can provide a significant enhancement of growth suppression in the HER2+/ER- SKBr3 breast cancer model. In the absence of an isogenic control, we employed the HER2-, ER+ cell line, MCF7 as a comparator. Herceptin treatment inhibited SKBr3 cell growth by 50% (IC_{50} 38.5nM) while MCF7 cells were unaffected (**Figure 5.4A**). Combination treatment of Herceptin at 10 and 100nM doses plus 1uM PF878 resulted in further growth suppression although statistically insignificant (**Figure 5.4B**). Parallel immunocytochemical staining also demonstrated that combination treatment (10nM Herceptin + 1μM PF878) led to a greater decrease in Ki67-positive cells (**Figure 5.4D**).

To examine the drug interactions in this context subsequent isobologram analysis was performed and revealed that Herceptin at lower concentrations of 10nM was synergistic with PF878 (**Figure 5.4C**). Increasing the concentration of Herceptin over and above 10nM elicited no further benefit, thus this data was consistent with the isobologram analysis that at higher Herceptin concentrations (100nM), the interaction with PF878 appeared antagonistic.

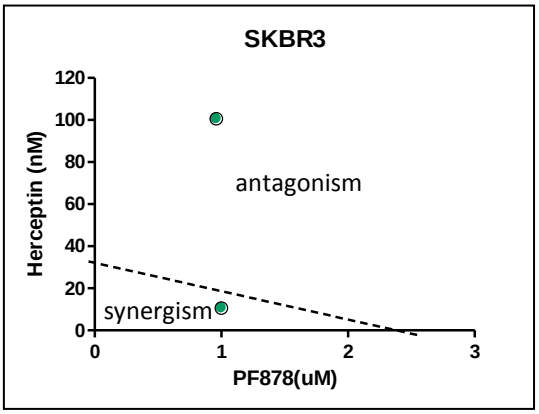
A


Dose response to Herceptin
MCF7
SKBR3
% cell growth
150
100
50
0
0
1
10
100
Herceptin (nM)

B


SKBR3
-PF878
+PF878
% cell growth
150
100
50
0
0
1
10
100
Herceptin concentration (nM)

C


SKBR3
Herceptin (nM)
120
100
80
60
40
20
0
antagonism
synergism
0
1
2
3
PF878(uM)

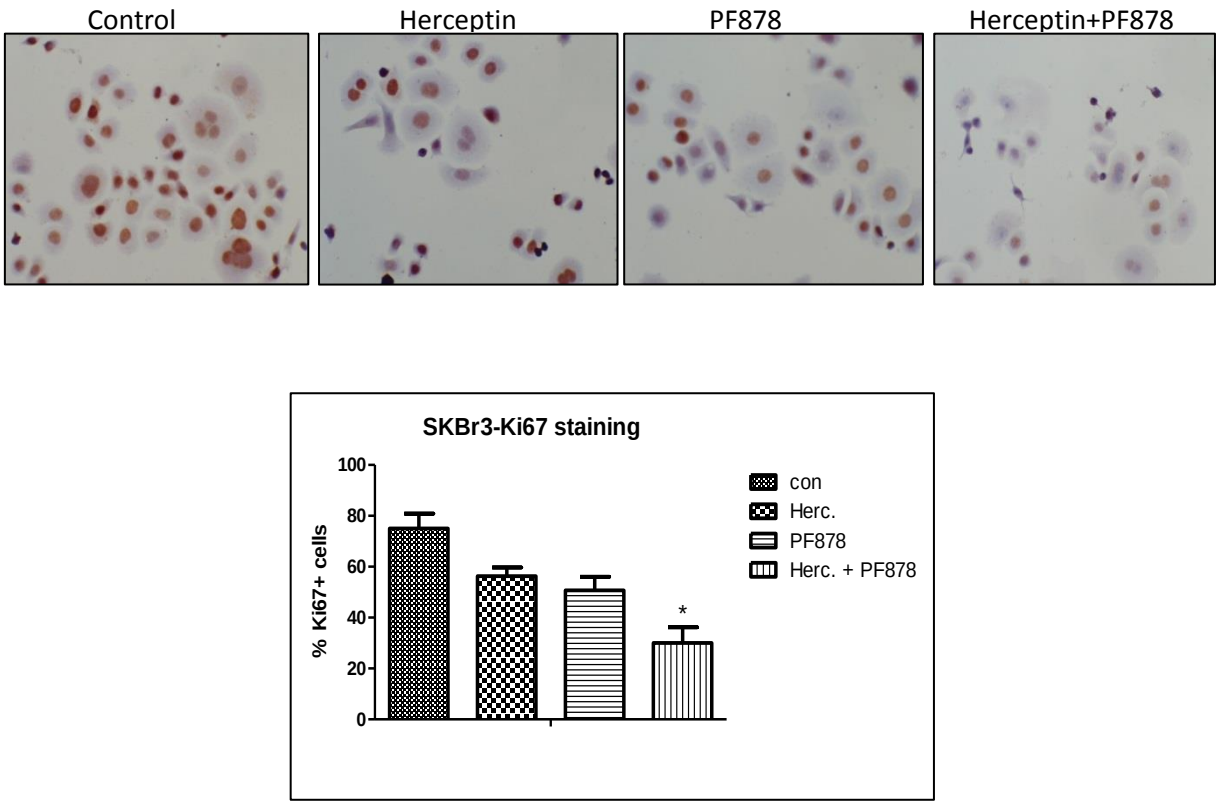


Figure 5.4. Targeting of FAK and HER2 together results in an improved suppression of HER2+/ER- SKBr3 breast cancer cell proliferation. (A) The ability of Herceptin to antagonise the proliferation of MCF7 and SKBr3 breast cancer cells for 7 days was determined by direct cell counting.(B) The anti-proliferative effects of PF878 (1uM) and varying doses Herceptin in combination on SKBr3 cells for 7 days were subsequently analysed by direct Coulter counting (n=3 ±SEM). (C) The nature of the interaction between Herceptin and PF878 was evaluated by Isobologram analysis as in 5.2. (D) Parallel detection of Ki67 by immunocytochemistry on cells treated with Herceptin (10nM) and PF878 in combination. Accompanying graph shows % of mean proliferative counts (+Ki67) per field of view (n=3 ±SEM) * p=<0.05.

5.6 Effect of FAK and HER2 inhibition on SKBr3 signalling pathways

To explore the signalling pathways modulated by Herceptin in combination with PF878, we investigated changes in FAK and HER2-associated signalling proteins known to be involved in regulation of cell survival and growth, by Western Blotting (**Figure 5.5**).

As previously observed in HER2+/ER+ cells, Herceptin again promoted an increase in the activity of HER2, EGFR and HER4. This was also accompanied by an increase in pFAK Y397, and pFAK Y861 as well as AKT and MAPK activity. In contrast, Herceptin resulted in suppression of HER3 phosphorylation. PF878 alone had no obvious effect on EGFR and HER3 receptor phosphorylation but induced activity of HER2 and HER4. Moreover, AKT and MAPK activity were also increased. In particular, PF878 elicited a much greater AKT activation than Herceptin.

When PF878 was combined with Herceptin, the activities of Herceptin-induced increase in HER2, EGFR and HER4 activity were unaffected, whilst AKT and MAPK activities were further increased. Activities of pFAK Y397, Y861 and HER3 were however decreased. Despite these paradoxical increased activation of AKT and MAPK, analysis of PARP cleavage, a surrogate marker for caspase-3 activation and apoptosis, revealed the combination of Herceptin and PF878 elevated the amount of the 23kD PARP fragment detected in cell lysates.

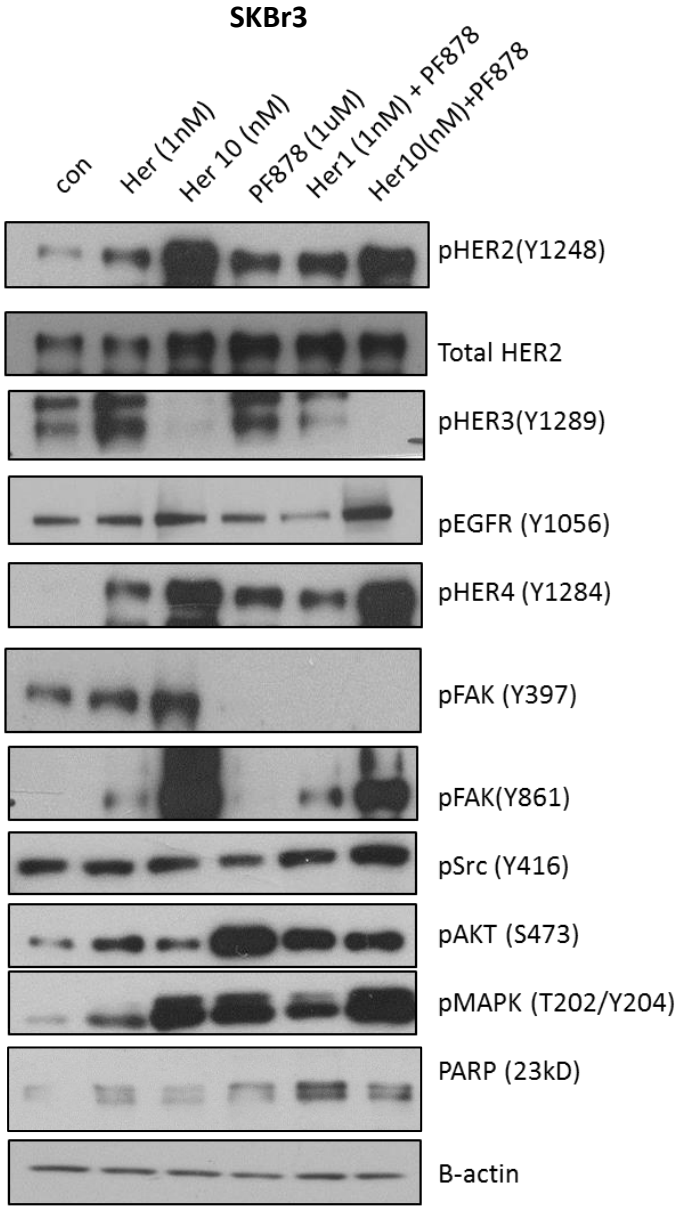


Figure 5.5. Combined targeting of FAK and Her in HER2+/ER- SKBR3 breast cancer cells does not suppress FAK-related signalling components. SKBr3 cells were treated with PF878 (1 μM), Herceptin (1 and 10 nM) or the combination of both the agents for 24hrs. Cells were lysed and protein expression and activity were assessed by western blotting.β-actin was used as a loading control. Representative blots shown from three independent experiments.

5.7 Discussion

Despite the clinical effectiveness of Herceptin in targeting HER2+ tumours, resistance remains a significant hurdle to their effective treatment. A means of improving Herceptin response is therefore warranted given the number of compensatory or resistance mechanisms identified and one opportunity for doing this is through combination treatment approaches (Nahta & Esteva 2007).

Several studies have demonstrated a role for FAK in the progression and spread of HER2+ breast cancers demonstrating that FAK overexpression correlates with HER2 overexpression and also that FAK is key effector of HER2 signalling which mediates oncogenic transformation and invasive behaviour (Schmitz et al. 2005; Benlimame et al. 2005; Xu et al. 2009). Thus targeting FAK alongside the HER2 receptor may have some benefit and represented the focus of this chapter. Although we established that targeting FAK with the small molecule inhibitor PF878 only modestly affected growth of HER2+/ER+ and HER2+/ER- cell lines, we hypothesised that co-targeting both HER2 and FAK using a combination of Herceptin plus PF878 might elicit greater benefit in terms of suppression of cell growth. Indeed, the use of FAK inhibitors in combination treatment regimens is already supported by other studies showing dual FAK and EGFR inhibition in breast cancer and enhanced radio-sensitivity of head and neck cancers (Golubovskaya et al. 2002; Eke & Cordes 2011). Dual targeting of insulin-like growth factor-1 receptor (IGF-1R) and FAK also in pancreatic cancer led to enhanced apoptosis (Zheng et al. 2010).

The mechanism of action of Herceptin is, in part, a consequence of its ability to inhibit downstream signalling of HER2, of which AKT and MAPK play an important role and lead to HER2-mediated cell survival, proliferation and resistance to apoptosis (Marmor et al. 2004; Nahta & Esteva 2007). On investigating Herceptin sensitivity in the HER2+ cell lines, we initially showed that despite Herceptin partially inhibiting AKT and MAPK activity in BT474 and MDA361 cells, only the former were growth inhibited in agreement with a previous study (Yakes et al. 2002). Interestingly, MDA361 cells expressed lower levels of HER2 activity than BT474 cells which may suggest that they are less reliant on HER2 signalling for growth. MDA361 cells may thus represent the subset of HER2+/ER+ tumours that are intrinsically less responsive/resistant to Herceptin. Our data also appears to suggest that AKT or MAPK

are not part of the dominant regulatory pathway in MDA361 cells. There is also a possibility that the intrinsic HER2 hetero/homo dimerization patterns might be different between these two cell lines, thereby providing differential inputs towards regulation of AKT and MAPK signalling, as supported by Ghosh et al. (2011). Alternatively, any residual AKT or MAPK activity (which is still substantial) even after Herceptin treatment may be enough to sustain cell proliferation. Thus, combination strategies such as Herceptin plus PF878 may represent a better approach to target these pathways more effectively, particularly in the context of limited Herceptin sensitivity. Indeed, the combination of Herceptin and PF878 produced greater growth inhibition than the effects of either of the drugs individually in MDA361 and BT474 cells, which concurred with the enhanced suppression of AKT and MAPK activity. Subsequent growth assays employing the use of AKT and MAPK inhibitors confirm that the significant loss of AKT (rather than MAPK) predominantly contributes to this enhanced growth suppression in these cell lines (**See Appendix 8.2**). This is further supported by a study that reports benefits of HER2-targeted therapy in combination with small molecule inhibitors in a mechanism involving suppression of AKT signalling (Yao et al. 2009).

The involvement of AKT is not surprising given its crucial role in regulating proliferation and survival. AKT mediates cell growth by phosphorylating TSC2 and PRAS40 leading to subsequent activation of mTOR complex 1 (mTORC1), a direct regulator of translation initiation. It also regulates the CDK inhibitors p21 and p27, and GSK3 by direct phosphorylation, all of which are critical to cell cycle progression. Other important mechanisms include the negative regulation of pro-apoptotic proteins such as the BH3-only protein BAD and the suppression of FOXO-mediated transcription of pro-apoptotic genes (Reviewed Manning & Cantley 2007). Our data demonstrated that combined treatment with Herceptin plus PF878 resulted in a decrease in the number of proliferative cells and a likely induction of apoptosis, given the increase in PARP cleavage detected. Collectively our data suggest that the anti-proliferative and pro-apoptotic responses seen with Herceptin plus PF878 combined may well occur through suppression of AKT.

Cross talk between the HER2 and ER has been shown to promote resistance to anti-HER2 therapies and studies demonstrate that ER antagonism with endocrine therapy improve outcome on Herceptin (Houston et al. 1999; Y.-C. Wang et al. 2011; Montemurro et al.

2012). Similarly, HER2 overexpression itself is associated with resistance to endocrine therapy (De Laurentiis et al. 2005; Dowsett et al. 2006; Davies et al. 2011). Since we are investigating the role of FAK in BT474 and MDA361 cells that also express ER, from a clinical perspective, it was crucial to explore whether targeting FAK may provide further benefit towards endocrine therapy such as Tamoxifen, widely used in clinical practice (Shiau et al. 1998). Our data revealed that MDA361 cells, were more sensitive to Tamoxifen than BT474 cells. This differential sensitivity between these two HER2+/ER+ cell models suggest that response to endocrine therapy cannot be clearly defined by the ER status alone. This could partially be explained by differences in growth rates, where growth inhibitory effects of drugs are more apparent in faster growing cells, unique differences with regards to post-translational modification or mutations within the ER which may confer differential ER interactions with the DNA, ultimately giving rise to distinct gene expression profiles between the cell lines (Dixon 2014). Encouragingly, inclusion of PF878 with Tamoxifen resulted in further suppression of growth in both cell lines, but was particularly more apparent in BT474 cells which were relatively insensitive to Tamoxifen. This is in further support of the notion that perhaps inhibition of FAK exerts greater benefits in the context of limited sensitivity. Although FAK has previously been implicated in the acquisition of Tamoxifen-resistance in MCF7 cells (Hiscox et al. 2009; Hiscox et al. 2011), this present data also indicates that FAK is also a mediator of intrinsic (*de novo*) resistance to Tamoxifen in these HER2+/ER+ cells. Moreover the inclusion of Tamoxifen with Herceptin resulted in a further suppression of growth in both cell lines, in agreement with previous studies (Argiris 2004; C.-X. Wang et al. 2005), an effect further enhanced in the presence of PF878, thereby strengthening the rationale of combination strategies that include FAK inhibitors.

We also reported that the combination treatment of Herceptin and PF878 similarly resulted in the enhanced growth suppression of the HER2+/ER- SKBr3 cells, although to a much lesser extent. Notably, no further benefits were observed at when increasing Herceptin concentrations to 100nM, indicating a possible saturation of the HER2 receptors at higher concentrations or potential interference with the receptor recycling to the cell surface (Hurrell & Outhoff 2013).

Differences concerning the downstream signalling modulation between HER2+/ER+ and HER2+/ER- cell lines involved also became apparent. Herceptin was also unable to decrease

AKT and MAPK activity, but instead led to their increase in SKBr3 cells. Data from other studies exploring the effect of Herceptin on downstream signalling are conflicting with ours (Longva et al. 2005; Dokmanovic & Wu 2014) whilst others are in agreement (O'Brien et al. 2010; Diermeier-Daucher et al. 2011), and thus firm conclusions cannot be drawn. In addition, we acknowledge a number of additional limitations including the concentration of drugs, variability of treatment time-course points or perhaps the differential growth media used in these studies, ranging from DMEM, McCoys and RPMI as in our case. We further observed that PF878 also induced AKT and MAPK in SKBr3 cells. This was similar to our data in section 4.3 whereby short-term exposure to PF878 (60 minutes) caused a dose-dependent increase in AKT and MAPK activity, despite inhibition of FAK activity. In this regard, at longer exposures to PF878 (24hrs) of SKBr3 cells, the compensatory increase in AKT and MAPK signalling is sustained. Importantly, inclusion of PF878 with Herceptin treatment led to further increases in AKT and MAPK above control levels. These were surprising given our data of enhanced suppression of proliferation and increased apoptosis as detected by Ki67 staining and cleavage of PARP, thereby indicating that the levels of AKT or MAPK activity may not be an important signalling change correlating with cell survival in this cell line and therefore may not always correlate with Herceptin response. However, regarding to the pivotal role of AKT and MAPK in drug resistance (O'Brien et al. 2010; McCubrey et al. 2006), it is reasonable to also acknowledge that their increased activity may contribute to an eventual acquisition of resistance of HER2+/ER- cells to this combination regimen.

The observation of increased AKT activity by Herceptin while growth inhibition ensues was not unprecedented. For instance, a study on MDAMB-468 and ZR75-1 cells demonstrated a transient early induction of AKT phosphorylation following treatment with Herceptin and other therapeutic agents such as Doxorubicin and Tamoxifen (Clark et al. 2002); this induction appeared to correlate with enhanced apoptosis when administered in combinations with LY294002, a PI3K/AKT inhibitor. In this respect, the authors suggested that the increase in AKT activity in response to therapeutic agents might enable them to be more susceptible to inhibition of the AKT pathway by LY294002. Given our evidence that Herceptin and PF878 led to greater suppression of growth, an alternative explanation in the context of SKBr3 cells would be that the inclusion of PF878 increased AKT phosphorylation

the result of which was to confer increased sensitivity to the growth inhibitory effects of Herceptin. However, to fully test this hypothesis further studies are now required that could include a time-course detection over a longer period of AKT activity in response to the combination of Herceptin and PF878 treatment.

A further and surprising finding was that Herceptin itself appeared to induce the phosphorylation of ErbB receptors in BT474, MDA361 and SKBr3 cells. Although this was contrary to our initial assumptions, others have reported similar events in HER2+ cell models (Gijsen et al. 2010; Dokmanovic et al. 2014). Dokmanovic et al., in particular showed that Herceptin-induced HER2 phosphorylation was also coupled with a novel interaction with Csk-homologous kinase (CHK). Here, cooperation with CHK was implicated in the degradation of HER2, the reduction of HER3 and AKT activity and resultant growth inhibition. Interestingly, we observed a small reduction in total HER2 levels following Herceptin treatment in BT474 and MDA361 cells that may represent the early stages of receptor recycling/degradation. Importantly, in BT474 and MDA361 cells, we speculate that FAK is also involved in the direct and/or indirect activation of these ErbB receptors, as indicated by our data that PF878 was able to decrease total HER2 expression and the Herceptin-induced activation of HER2, EGFR and HER4 in these cells.

In SKBr3 cells however, PF878 was unable to suppress the increased HER2, EGFR, HER4 phosphorylation induced by Herceptin. But consistent with another study, Herceptin was able to inhibit HER3 activity (Gijsen et al. 2010). The authors have noted these observations following 1hr Herceptin treatment, which correlated with a decrease in AKT activity, but with prolonged treatment of 2 days, reactivation of AKT occurred. This was attributed to negative-feed loop resulting from Herceptin-mediated AKT suppression which causes an up-regulation of ADAM-17 proteases that function to cleave ErbB ligands from the cell surface, in turn activating other ErbB receptors and subsequently HER2 activation and downstream signalling. Such a mechanism may reflect our findings at 24hrs whereby AKT activity was increased, though more studies will be required to determine whether these are ligand-induced. Importantly, we demonstrated that these observations were strongly paralleled by the increase in FAK activity (Y397, Y861). However, that PF878 potentiated this phenomenon rather than suppress it, suggests the involvement of FAK in regulation of ErbB

signalling and lends a potential yet unidentified mechanism that may substantiate the current mechanisms proposed by others (Gijsen et al. 2010; Dokmanovic et al. 2014).

This finding also raises the question of whether the induction of the ErbB receptors could eventually confer a signalling advantage, and if so, does it present implications in acquiring Herceptin resistance? In this context, previous studies have particularly attributed the increase in EGFR and HER2 activity to Herceptin resistance (Diermeier et al. 2005; Ritter et al. 2007). In contrast, it was also shown that HER2 and EGFR co-expression strongly potentiates apoptotic signalling in response to EGF through both receptors in a variety of cell lines including SKBr3 cells (Tikhomirov & Carpenter 2004). On the other hand, the significance of increased HER4 activity seems to be relatively unclear and paradoxical. Though a study has attributed HER4 expression to Herceptin resistance (Mohd Nafi et al. 2014), many others reports are contradictory. In one study for example, the HER4 receptor intracellular domain (4ICD) was identified to be BH3-only protein, capable of promoting mitochondrial cytochrome C release and subsequent apoptosis (Naresh et al. 2006). Moreover, HER4 activation has been associated with anti-proliferative effects, increased sensitivity to Herceptin and better patient prognosis (Suo et al. 2002; Sassen et al. 2009; Witton et al. 2003). Taken together, these suggest the activation of ErbB receptors we observed in response to Herceptin may determine varying cellular outcomes and are thus worthy of further study as they may have important clinical implications.

Altogether, despite differential signalling observations between the HER2+ breast cancer cells, the data present in this chapter support the approach of targeting elements such as FAK in HER2+ breast cancer alongside the HER2 itself. Importantly, our data suggests that the combination of Herceptin and PF878 are likely to be most effective in the context of *de novo* insensitivity given that Herceptin and PF878 worked best in Herceptin-insensitive MDA361 cells whilst PF878 and Tamoxifen worked best in endocrine-insensitive BT474 cells.

6. Results (IV)

FAK mediates fibroblast-induced HER2+ breast cancer cell migration

6.1 Introduction

Previously, we demonstrated a role for FAK in Heregulin (HRG)-induced breast cancer cell migration (see Chapter 3,4). Given that HRG is known to be present in the tumour microenvironment and that stromal HRG expression correlates with an aggressive tumour behaviour and increased disease recurrence (Visscher et al. 1997; Atlas et al. 2003a) we hypothesised that FAK inhibition might present an effective strategy for suppression of stromal-induced tumour behaviour likely to promote disease progression. Indeed, the role of bi-directional tumour-stroma cross-talk in facilitating tumour spread is increasingly apparent (Hanahan & Weinberg 2011) (**illustrated in Figure 6.1**). These interactions are mediated by a wide spectrum of tumour-secreted factors that include growth factors (e.g. TGFβ, PDGF, VEGF, EGF), cytokines and other soluble factors which may act in an autocrine manner, allowing further activation of the tumour cell itself, or through paracrine modulation of other cell types in the tumour microenvironment including endothelial cells, fibroblasts, resident immune-cells (e.g. lymphocytes, monocytes, mast cells, neutrophils), pericytes and smooth muscle cells. This 'activated' state of stromal cells, similarly observed during the process of wound healing, is characterised by increased secretion of additional growth factors, a range of proteases (e.g. MMP2, MMP9) and ECM components (e.g. tenascin, collagen, elastin, fibronectin) that themselves further induce proliferative and pro-metastatic responses from the tumour, ultimately establishing a permissive environment to promote tumour progression (Tlsty & Hein 2001, Mueller & Fusenig 2004;).

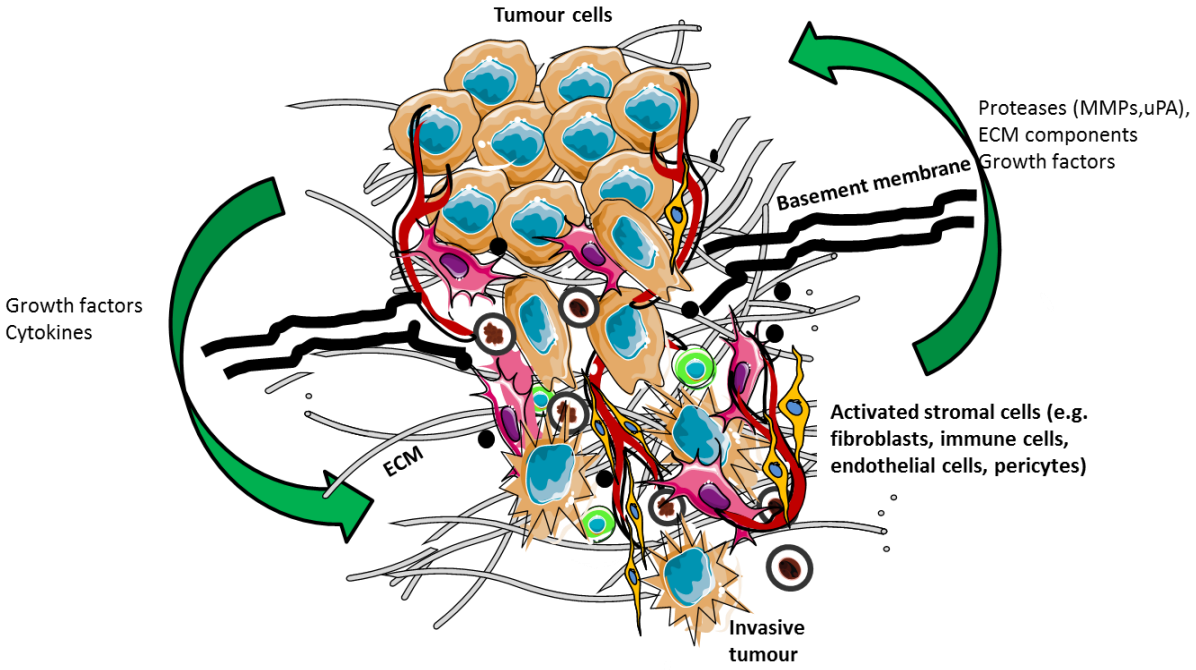


Figure 6.1. Stromal contribution in tumour progression. Tumour cells (in brown) release soluble factors to activate the stroma. Stromal cells subsequently release proteases, additional growth factors and matrix components (e.g. collagen, fibronectin). This leads to breakdown of basement membrane and infiltration of stromal cells into the tumour. Collectively, this results in angiogenesis, tumour growth, invasion through the degraded basement membrane and metastasis.

Of the number of stromal cells involved, the fibroblasts are considered to be predominantly present within the tumour bulk (Kalluri & Zeisberg 2006). The supportive role of fibroblasts in malignancy has been acknowledged for over two decades (van den Hooff 1988) with direct co-culture with fibroblasts, or exposure to fibroblast conditioned-media, being shown to stimulate growth of a number of breast cancer cell lines (Ryan et al. 1993; Lefebvre et al. 1995; Gache et al. 1998; Angelucci et al. 2011). In line with this, a number of *in vivo* studies involving co-inoculation of fibroblasts and pre-neoplastic epithelial cells further demonstrated the ability of fibroblasts to initiate malignancy and promote tumour growth (Camps et al. 1990; Olumi et al. 1999; Yashiro et al. 2005; Orimo et al. 2005). In addition, the interplay between fibroblasts and tumour cells can also invoke reciprocal changes in gene expression profiles such as those involved in cell survival (Rozenchan et al. 2009). More recently, fibroblasts have been demonstrated to play a role in eliciting a favourable tumour microenvironment which supports the development of resistance of melanoma cells to BRAF inhibition (Hirata et al. 2015).

A number of studies also point to a supportive role for fibroblasts in the metastatic behaviour of tumour cells. Breast cancer cell migration is known to be stimulated by co-culture with fibroblasts or exposure to fibroblast-conditioned media (Rossi et al. 1993; Heylen et al. 1998). Conditioned-media from tumour-associated breast fibroblasts was also demonstrated to significantly induce MCF7 cell invasion when compared with those of patient-matched normal breast fibroblasts (Studebaker et al. 2008). Taking a step further, more recent data have shown that fibroblast populations isolated from the invasive tumour front (termed the 'interface zone') were significantly more potent at promoting migration and EMT of breast cancer cells than fibroblast populations isolated from within the tumour bulk (Gao et al. 2010).

Whilst these and other studies strongly suggest that the presence of fibroblasts in the stroma of breast tumours foster tumour progression, the specific contributions of fibroblasts towards HER2+ breast cancer metastasis remains poorly understood. Considering that FAK was shown to be involved in the ligand-induced migratory responses of HER2+ breast cancer cells (Chapters 3 and 4), in this chapter we extended this work to explore our hypothesis that FAK would also represent a key modulator of fibroblast-stimulated HER2+ breast cancer cell migration and invasion.

.

6.2 Optimising the harvest of fibroblast conditioned media

In order to explore fibroblast-induced cell migration *in vitro*, we used conditioned media from the MRC5 lung fibroblast cell line. A standardised collection protocol was used as shown below:

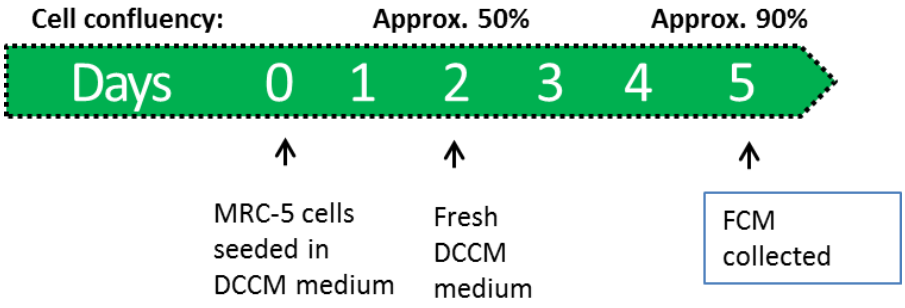


Figure 6.2 Outline of timeline for harvesting conditioned-media from MRC-5 fibroblast cell line.

This helped reduce inconsistencies that may arise from large differences in cell number from which the media was collected from. Since MRC5 fibroblasts displayed a high proliferative rate in culture, cell counts were obtained from culture directly after retrieving conditioned media (i.e. the number of cells contributing to the FCM) to allow normalisation of data where FCM was used collected from different MRC5 passages (to allow for biological replicate experiments, for example). Media was collected and cleared by centrifugation at 1000 rpm for 5 minutes, filtered through a 0.45 µm Millipore filter to remove non-adherent cells and other debris, and kept at -20°C (up to 7days) or -80°C (up to a month) for storage.

Importantly, one further consideration is that of senescence. It is widely reported that MRC5 and other fibroblast cell lines rapidly reach a senescent state due to their high proliferation rate in *in vitro* culture. Once in this state, they are unable to respond to growth stimuli, display morphological changes (cell enlargement) and irreversible growth arrest (Rodier & Campisi 2011). Our own observations also seemed to suggest that such a phenomenon occurs, with MRC5 cell populations at earlier passage number (p4-p7) displaying higher growth rates particularly between days 1-5 when compared to those at a later passage (p8-p11) (**Figure 6.3**). Thus, from these observations and for consistency, it was decided that FCM were collected from MRC5 cells between passage 4-7 and cultured for no more than 5 days and/or are subconfluent.

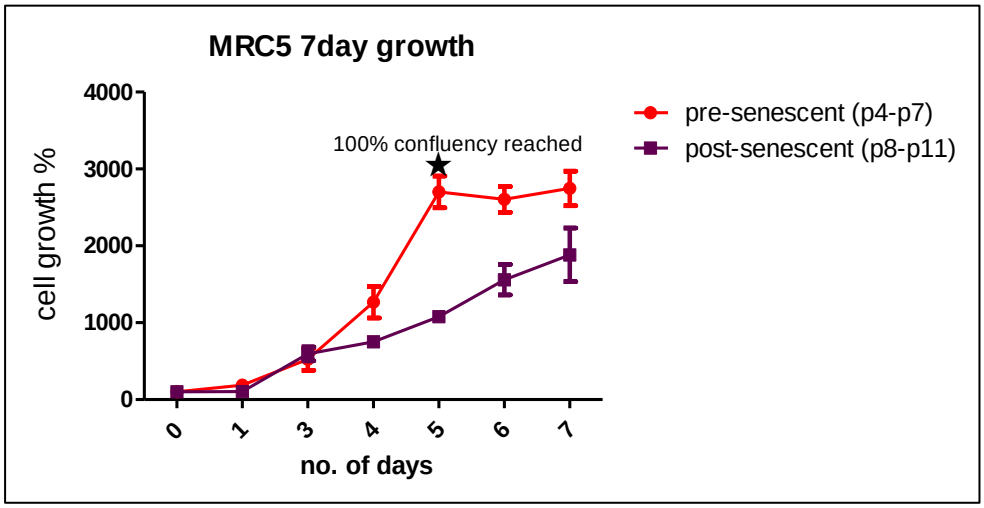


Figure 6.3. MRC-5 fibroblast cells at various passage numbers were grown on 24 well-plates and growth was assessed by direct coulter counting at different time-points over 7 days. Starred: 100% cell confluence was observed.

Further analysis of the protein content of the FCM was undertaken using BioRad DC Coloumetric assay (See section 2.4.1.3) to determine whether the FCM collected from different MRC5 culture passages varied in total protein content. The measured protein content was determined as a function of the total number of MRC5 cells in each flask. This was validated as shown by our obtained values below.

MRC5 FCM harvest (million cells/ml)	**Protein conc. (mg/ml)**
0.3	4.09
0.13	2.485
0.25	2.64
0.20	2.36
Average protein conc:	**2.09**

Figure 6.4. Number of cells contributing to FCM and actual FCM protein concentration as analysed by the BioRad DC Coloumetric protein assay. Protein concentration values obtained taken from four independent FCM harvests.

6.3 Evaluating toxicity of FCM on breast cancer cells

It is a possibility that MRC5-conditioned medium could be toxic to the breast cancer cells since it had been collected over a period of three days and thus may be severely nutrient depleted (albeit that DCCM was used which is a low nutrient media to avoid the use of serum which may confound the data due to presence of multiple growth factors and other elements) and/or the MRC5 cells may secrete factors that could adversely affect breast cancer cell viability. In order to investigate any possible toxicity incurred by the FCM on breast cancer cells, they were cultured with increasing concentrations of FCM ranging from 20% to 100% for 7 days and analysed by direct Coulter counting, as shown (**Figure 6.5**), no significant toxic effects (loss in cell viability) were observed in both MFC7 and SKBr3 cells. There are however indications of possible toxicity to MCF7 cells at 100% FCM. In this regard, to ensure cell viability but at the same time maintain a high concentration of fibroblast-secreted factors, 80% FCM diluted in fresh DCCM growth media was used in subsequent experiments.

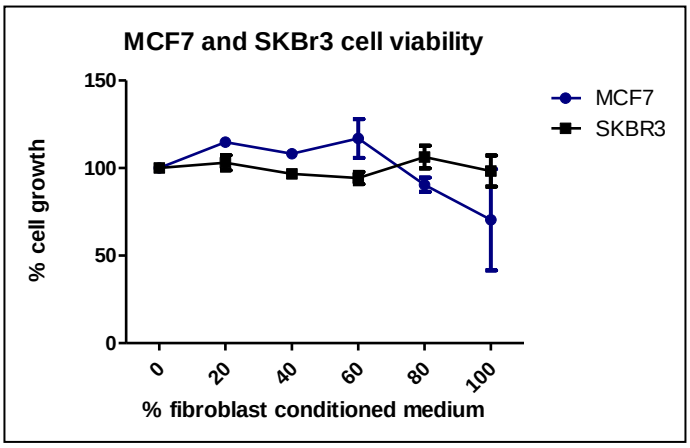


Figure 6.5. Cell viability MCF7 and SKBr3 cells upon exposure to increasing concentration of FCM for 7 days, as assessed by direct Coulter counting. Data are mean cell proliferation values ±SEM. (n=3)

6.4 HER2- and HER2+ breast cancer cells are sensitive to FAK inhibition by PF271

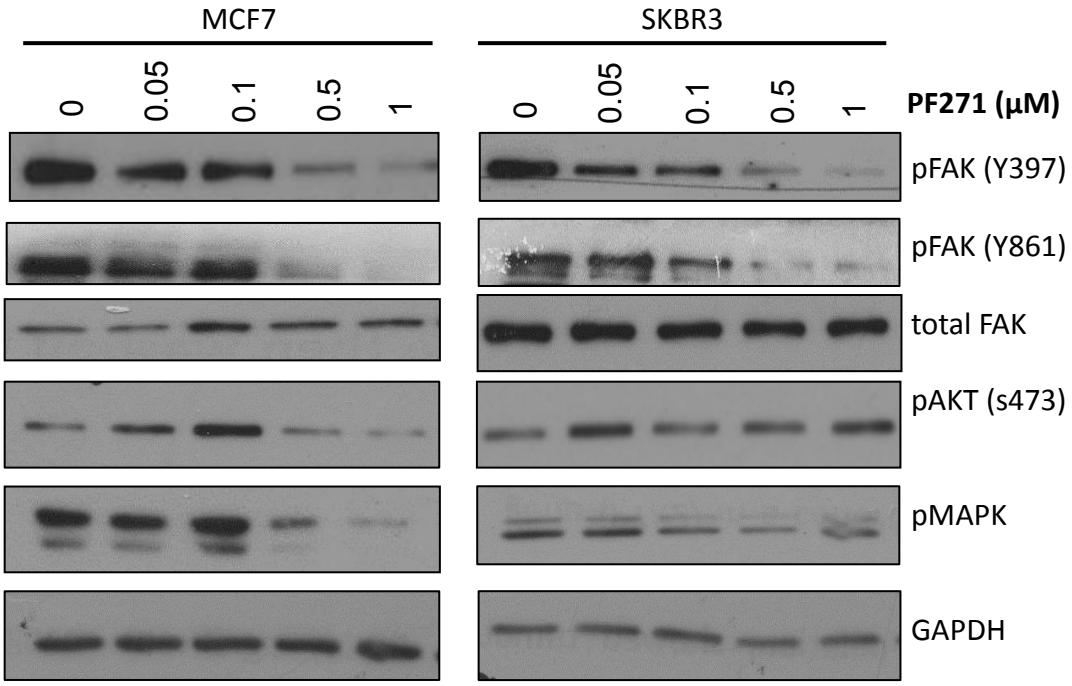
In this chapter where FAK activity is pharmacologically inhibited, we employed the use of the FAK inhibitor 'PF271' (see Chapter 2.1.1). This is a predecessor of PF878 but has a similar chemical structure (one side group has been altered) and mechanism of action. The reason for changing from PF878 to PF271 (Roberts et al. 2008) was that, at the time of the experiments, PF878 was no longer available due to the commercial rights of this compound being acquired by another drug company. Thus for consistency throughout this chapter, PF271 was used.

The sensitivity of MCF7 and SKBr3 cells to PF271 with regards to its target pFAK Y397 was verified using Western blotting. As shown in **Figure 6.6A**, PF271 induced a dose-dependent decrease in pFAK Y397 in both cell lines although neither cell line appeared to be differentially sensitive to PF271 with IC_{50} values of 0.17 µM (MCF7) and 0.10 µM (SKBr3) (**Figure 6.6B**). Accompanying a reduction in FAKY397 was a dose dependent loss of FAK phosphorylation at Y861 (**Figure 6.6A**)

We next investigated whether the suppression of FAK activity resulted in inhibition of intracellular signalling intermediates that are known to be regulated by FAK and/or HER2, and of which, AKT and MAPK have been implicated from our findings in previous chapters. As shown in **Figure 6.6A**, accompanying FAK inhibition was a reduction in the levels of MAPK in both cell lines; in MCF7 cells we also observed a reduction in AKT activity.

Interestingly, in addition to the loss of FAK and signalling pathway activity, we also observed that PF271 treatment resulted in a dose-dependent inhibition of MCF7 and SKBr3 cell proliferation; interestingly, the IC_{50} values for pFAK Y397 inhibition correlated with the IC_{50} required for growth inhibition (**Figure 6.6 C,D**).

A



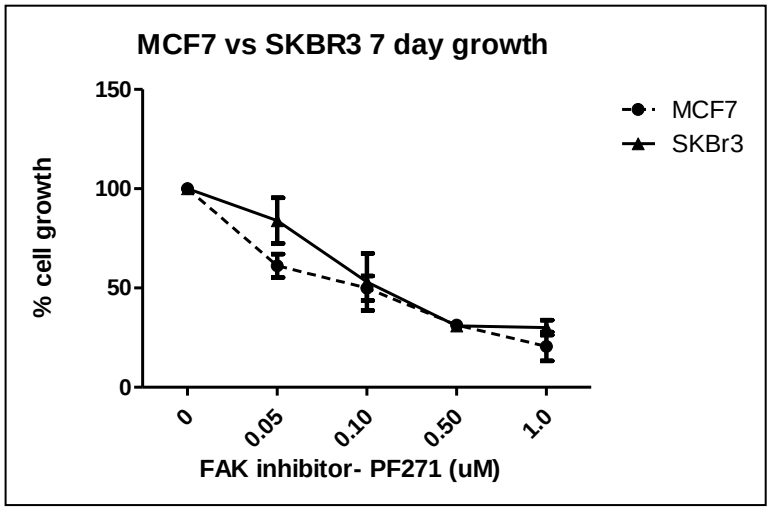
B

pFAK Y397 inhibition

Cell Line	Breast Cancer Molecular Subtype	IC_{50} (µM) (±SEM)
MCF7	HER2-, ER+	0.17± 0.048
SKBr3	HER2+, ER-	0.10± 0.032

Figure 6.6. Sensitivity of ER+/HER2- and ER-/HER2+ breast cancer cells to PF271. (A) MCF7 and SKBr3 lysates treated with the PF271 (0-1µM) for 60mins were probed for proteins as indicated. GAPDH was used as loading control. (B) Densitometric analysis of immunoblots was carried out to calculate the IC_{50} values ± SEM for PF271 with regards to pFAK Y397 inhibition. Representative blots shown are from three independent experiments. Continued.

C



D

Cell growth inhibition

Cell Line	Breast Cancer Molecular Subtype	IC_{50} (μM) (±SEM)
MCF7	HER2-, ER+	0.10± 0.096
SKBr3	HER2+, ER-	0.18 ± 0.17

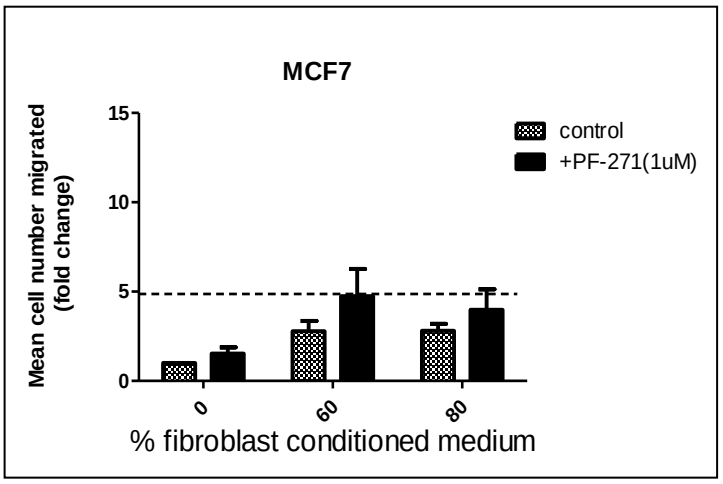
Figure 6.6. (Continued) (C) The growth of MCF7 and SKBr3 cells in response to PF271 (0–1 μM) over a period of 7 days was assessed using Coulter counting. Calculated data are mean cell proliferation values ±SEM (n=3). Plotted dose-response curves were subsequently used to calculate IC_{50} values ± SEM for PF271 with regards to growth inhibition (D).

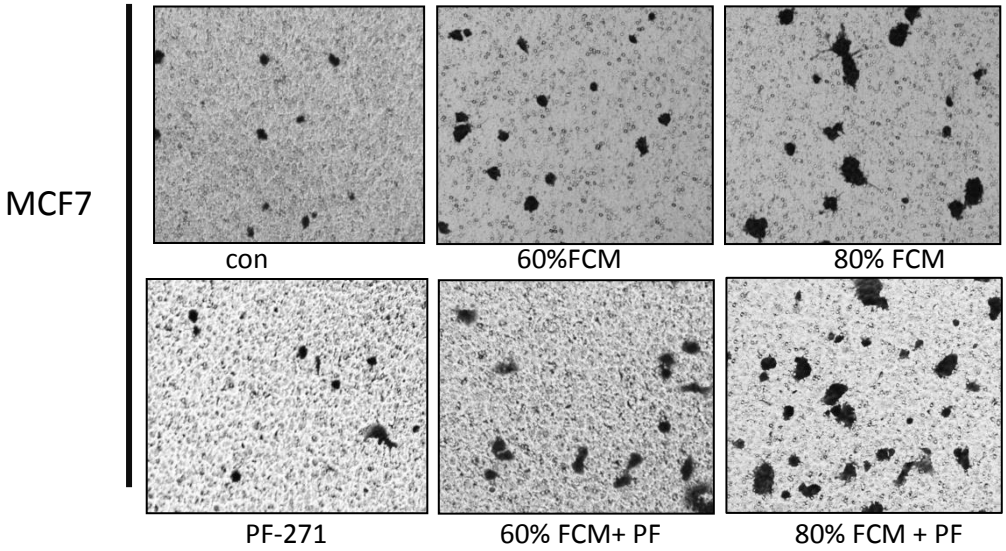
6.5 FAK mediates fibroblast conditioned media-stimulated HER2+ breast cancer cell migration and invasion

In view of our previous observations on the effects of extracellular ligands on cell migration, we next investigated whether the behaviour of MCF7 and SKBr3 cells was influenced by fibroblasts by culturing the cells in the presence of conditioned media from MRC5 cells. The ability of FCM to promote the migration of breast cancer cells was determined using a Boyden Chamber assay in which FCM was used as a chemo-attractant on the lower chamber and MCF7 or SKBr3 cells were seeded into the upper chamber. FCM promoted a concentration-dependent increase in the migratory capacity of SKBr3 cells, whilst having minimal effects on MCF7 cells **(Figure 6.7A**) The effects on SKBr3 cells was significantly inhibited by PF271 (1μM). The function of FAK in this context was further validated by siRNA knockdown of FAK which again inhibited FCM-induced migration (**Figure 6.7D**). Accompanying the fibroblast-induced migration of SKBr3 cells were changes in the cytoskeletal organisation, with the formation of cellular protrusions wherein actin clusters were prominently observed, particularly at the leading edge of the cell. In addition, we also observed that the normally punctate pFAK Y861 localization in focal adhesions were strongly enhanced at the protrusions following stimulation with FCM; inclusion of PF271 with FCM reversed this phenotype (**Figure 6.7C**).

To analyse the significance of the HER2 signalling in the migratory response of the HER2+ SKBr3 cells, we employed the use of Herceptin. Indeed, data has shown that HER2 signalling is a key regulator of the migratory response of SKBr3 cells to FCM with Herceptin treatment resulting in inhibition of FCM-induced SKBr3 cell migration. We also found that the combination of Herceptin and PF271 further enhanced the anti-migratory effect (**Figure 6.7E**). Together, these studies suggest that factors present in FCM responsible for induction of cell migration can occur via HER2/FAK signalling, however, there also exists a HER2-mediated migratory signalling that occurs in a FAK-dependent manner. Signalling studies exploring the effect of Herceptin on FAK activity in the presence of FCM would further substantiate this hypothesis.

A


MCF7
Mean cell number migrated (fold change)
15
10
5
0
control
+PF-271(1uM)
0
60
80
% fibroblast conditioned medium


MCF7
con
60%FCM
80% FCM
PF-271
60% FCM+ PF
80% FCM + PF

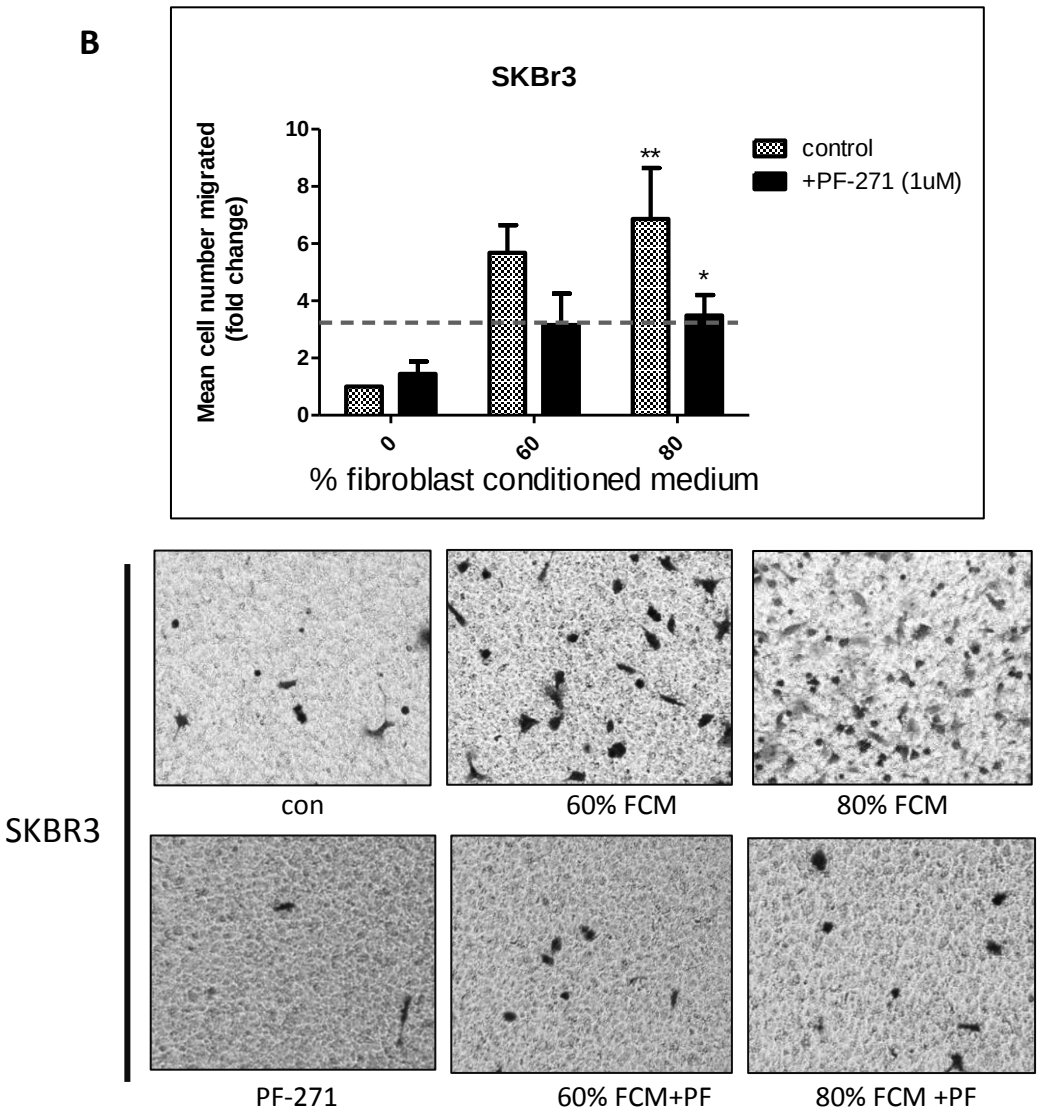


Figure 6.7. (A,B) Inhibition of FAK by PF271 (1µM) impairs SKBr3 cell migration (and not MCF7 cells) through fibronectin-coated Boyden chamber assay in response to two concentrations (60%, 80%) of MRC-5 conditioned media (FCM) over a 20hr period. Cells were subsequently fixed and stained with crystal violet. Experiments were performed in DCCM culture media and data represent the mean numbers of migratory cells/membrane run in duplicates from three independent experiments. Data shown have also been normalized to the MRC-5 cell number of the specific cell passage the FCM was obtained. Representative images of stained migratory cells are shown. P value: *<0.05, ** <0.001. Continued.

C

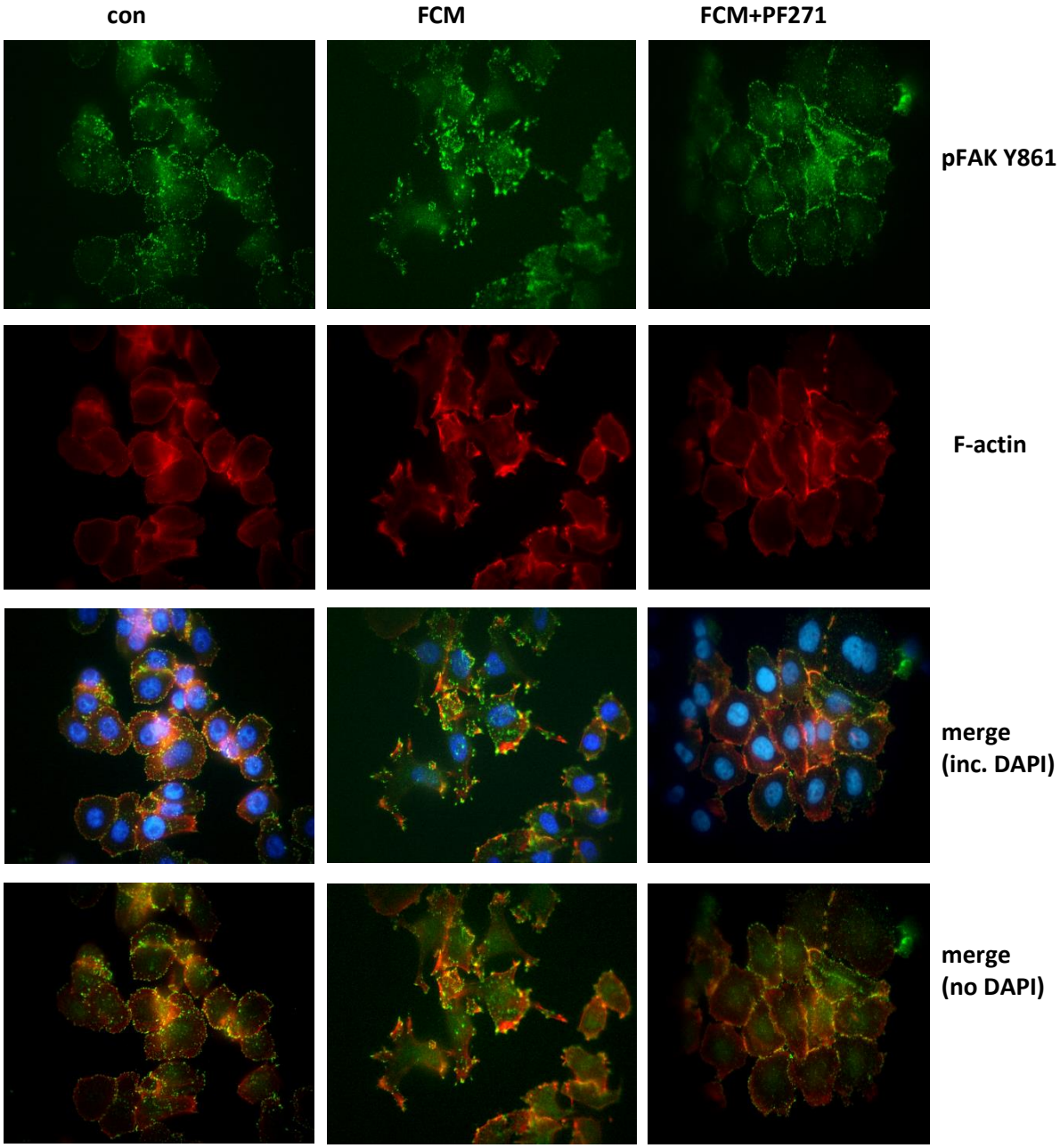


Figure 6.7. (Continued) (C) Immunofluorescence images of SKBr3 cells seeded on FN-coated plates following 80%FCM ±PF271 (1µM) for 60mins. Cells were stained Rhodamine-phalloidin (F-actin) (red), pFAK Y861 (green) and DAPI as a nuclear counterstain (blue). Continued.

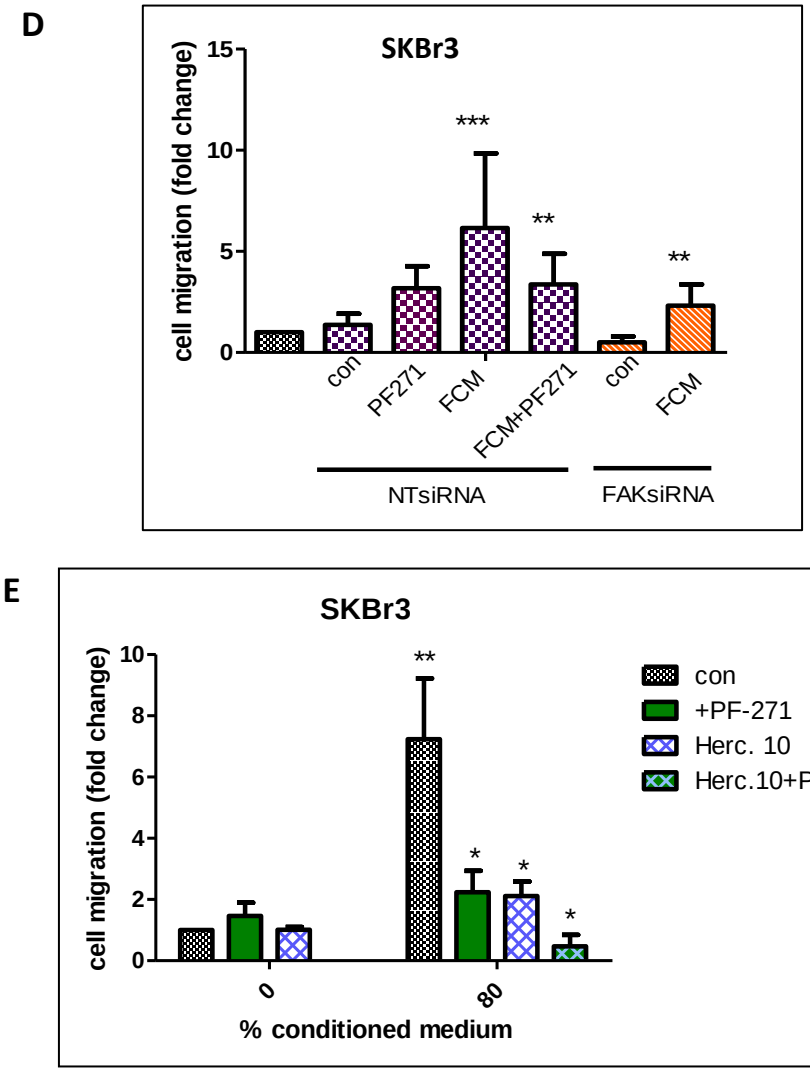


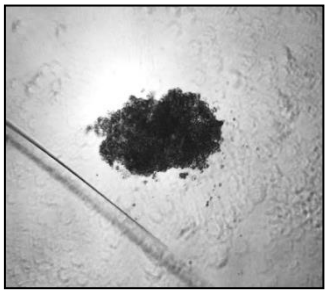
Figure 6.7. (Continued) (D) Parallel migration assays were performed on SKBr3 cells transfected with non-targeting siRNA (NTsiRNA) or FAKsiRNA revealing comparable inhibition of migratory response to FCM. (E) Effect of Herceptin treatment (10nM) alone or in combination with PF271 on SKBr3 cell migration in response to two FCM over a 20hr period. P value:*<0.05, ** <0.001, *** <0.0001.

The effects of FCM on SKBr3 cell invasion were also explored using a 3D basement-membrane extract (BME) invasion assay, wherein cells were pre-formed into spheroids and allowed to invade into the surrounding matrix in the presence of medium containing treatment or chemo-attractants (See section 2.6). This *in-vitro* assay provides the advantage of more closely mimicking *in-vivo* conditions with regards to cell-cell physical interactions and cell-matrix interactions. SKBr3 cells successfully formed spheroid cell-aggregates after Day 1 (**Figure 6.7F**) and subsequent treatment with FCM promoted SKBr3 cell invasion, as demonstrated by the images captured after 3 days incubation which revealed the appearance of budding edges and a number of individual cells detached from the spheroid colony that have invaded into the surrounding matrix (**Figure 6.7F**). Suppression of FAK activity appeared to suppress this invasive behaviour however, due to the irregularity of the colony edges and the single-cell mode of invasion, quantification and accurate comparison between replicate colonies proved challenging. A semi-quantitative analysis attempt based on the direct count of detached cells from the spheroid appeared to confirm that FCM-stimulated SKBr3 cell invasion was also significantly inhibited by PF271.

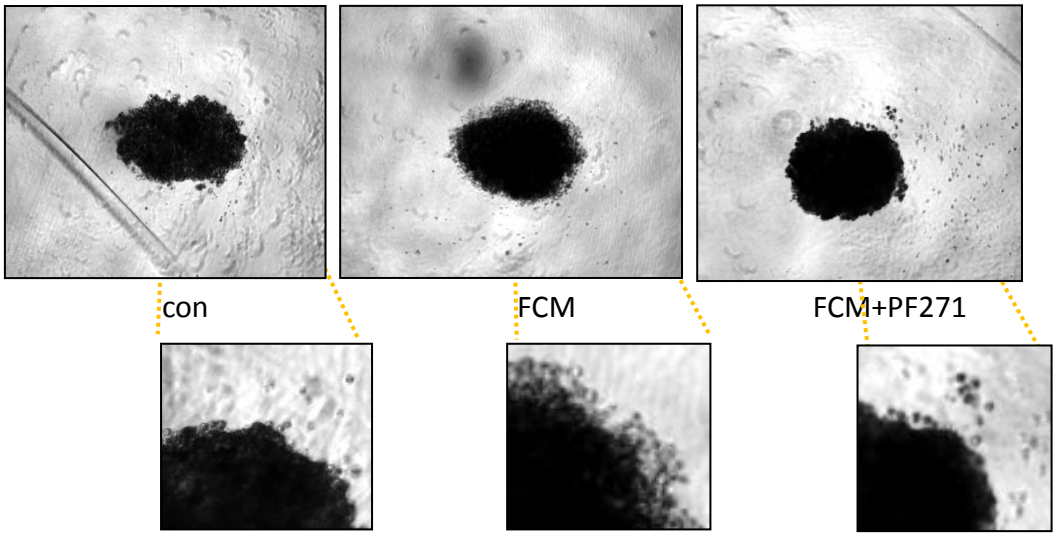
Since invasion required proteolysis of the ECM, we delved and further explored the secretion of MMPs. These proteolytic enzymes, through their ability to degrade ECM are closely associated with cancer cell invasion (Egeblad & Werb 2002). Since secretion of MMPs are known to be regulated by a number of growth factors in the tumour microenvironment (Birkedal-Hansen et al. 1993) and fibroblasts are an abundant source of these soluble factors, we explored the ability of MRC5 cells to stimulate MMP secretion by SKBr3 cells using gel zymography (see section 2.7). FCM contained detectable proteolytic activity to that correlated with MMP-2 which has a molecular weight of 72 kDa. FCM however failed to induce MMP2 activity in SKBr3 cells over and above that likely to be detected from the FCM alone (**Figure 6.7H**). In addition, this method can also identify the proteolytic activity of MMP9 (molecular weight 92kD). As shown, no additional bands appeared in the gel suggesting that no active MMP9 can be detected, neither from fibroblasts or SKBr3 cells.

F

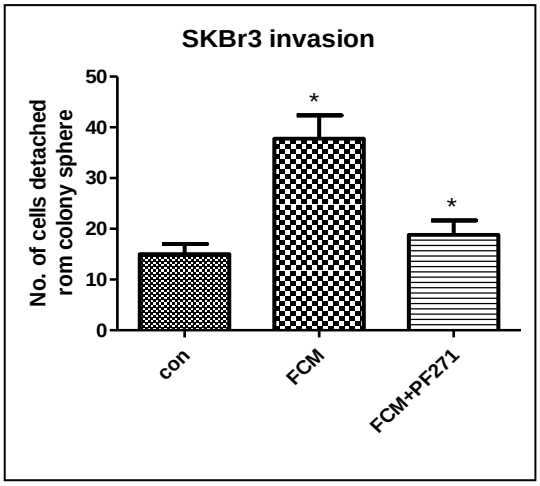
SKBr3 in 3D BME spheroid invasion- **1 day**



SKBr3 in 3D BME spheroid invasion- **3 days**



G



Continued on next page

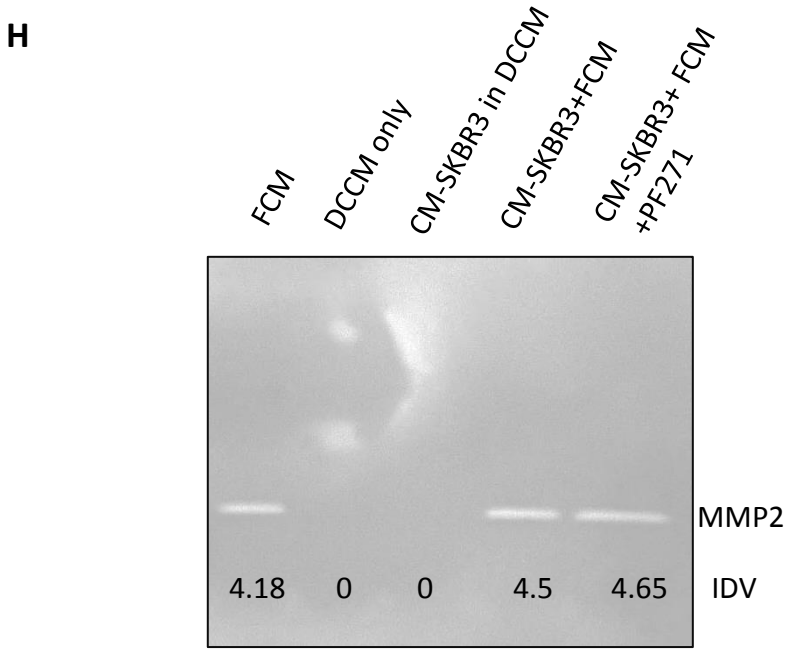


Figure 6.7. (Continued) (F) SKBr3 cell invasion in a 3D-BME assay (Trevigen) in response to 50%FCM over a period of 3 days. Representative photographs are shown. (G) Quantification of E based on images converted to 8-bit grayscale on Image J and processed by auto-threshold to distinctly define cells that appear detached. (H) Gelatin zymography showing MMP-2 activity (72kDA). Integrated density values (IDV) are indicated for quantification of band intensities. P value:*<0.05

6.6 FAK inhibition suppresses fibroblast conditioned media-induced STAT3 activation in SKBr3 cells

We next sought to investigate the signalling pathway(s) involved in fibroblast-induced migratory and invasive responses of SKBr3 cells that might be governed by FAK. SKBr3 cells were stimulated with FCM for different time points and subsequently analysed by Western Blotting.

Whilst little was observed in terms of the activity of the ErbB members HER2, HER3 and HER4, FCM treatment promoted a rapid and transient phosphorylation of AKT and MAPK (**Figure 6.8**). Importantly, FCM also enhanced the phosphorylation of FAK (at Y861 but not Y397); this was accompanied by the strong activation of the transcription factor STAT3 at Y705, and a decreased E-Cadherin expression (boxed).

In the presence of the FAK inhibitor PF271, FCM failed to promote FAK or STAT3 activation indicating that FAK can influence STAT3 phosphorylation. Moreover, the levels of E-cadherin were not affected, but rather appeared much higher at the 24hrs time point.

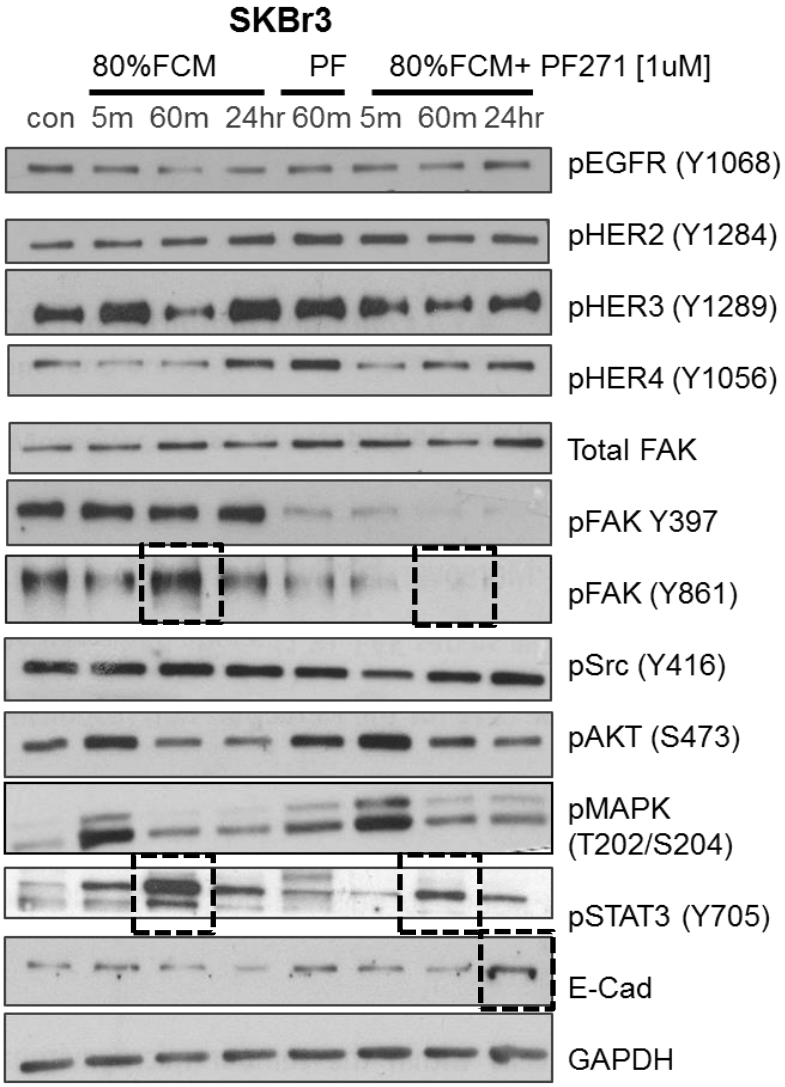
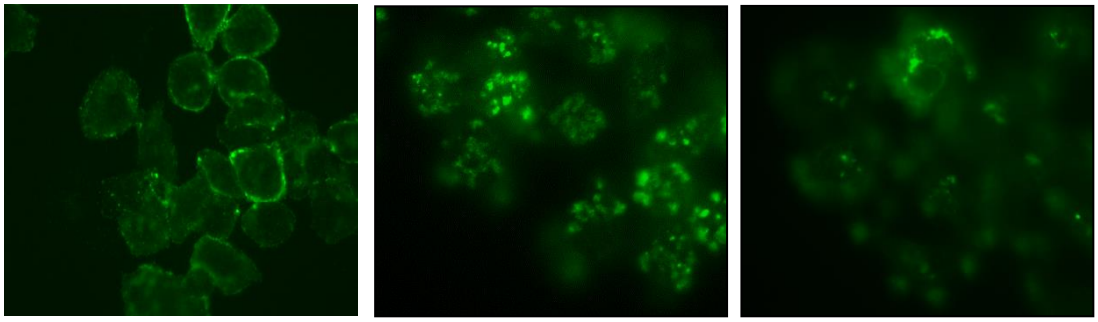


Figure 6.8. FCM stimulates downstream signalling in SKBr3 cells. Cells were grown to 70% confluence and treated with 80% MRC-5 fibroblast conditioned media (FCM) at the indicated time-points ± pre-treatment with PF271 (1µM) for 60mins. Subsequent cell lysates were processed for Western Blotting and immuno-probed for proteins as indicated. GAPDH was used as loading control. Representative blots shown from three independent experiments.

We then investigated whether the FAK was implicated in the nuclear translocation of STAT3 since this is known to be preceded by STAT3 activation and subsequent formation of homo- or heterodimers with other STAT family members; in the nucleus, STAT3 dimers can then bind to DNA elements and regulate transcription of target genes (Ihle 2001). Our immunofluorescence microscopy data demonstrated that FCM stimulation enhanced nuclear translocation of pSTAT3 Y705 (**Figure 6.9A**) Importantly, treatment with PF271 partially prevented this pSTAT3 Y705 nuclear accumulation. This was further confirmed by Western Blot analysis of the SKBr3 cytoplasmic and nuclear fraction, where a higher amount of pSTAT3 Y705 could be detected in nuclear fraction following FCM stimulation, and also reduced in the presence of PF271 (**Figure 6.9B**). Interestingly, a small pool of total FAK protein was also detected in the nuclear fraction suggesting that FAK may have a role in nucleus under certain circumstances.

The localisation of STAT3 to focal adhesions has previously been implicated in ovarian cancer cell migration (Silver et al. 2004) and thus we also sought to investigate this in our cell model. Due to our pSTAT3 and FAK antibodies originating from the same species, we needed to use an alternative marker protein for focal adhesions, namely vinculin. Visualising the cells at a different plane of view as in **Figure 6.9A**, we demonstrated that in the control cells, pSTAT3 co-localised with vinculin at the focal adhesions around the cell periphery (**Figure 6.9C**). When stimulated with FCM, the focal adhesions formed were larger, more prominently localised at the leading edge of the cell, and maintaining strong co-localisation with pSTAT3 Y705. Treatment with PF271 (1µM) decreased pSTAT3 and vinculin staining and more importantly disrupted this co-localisation.

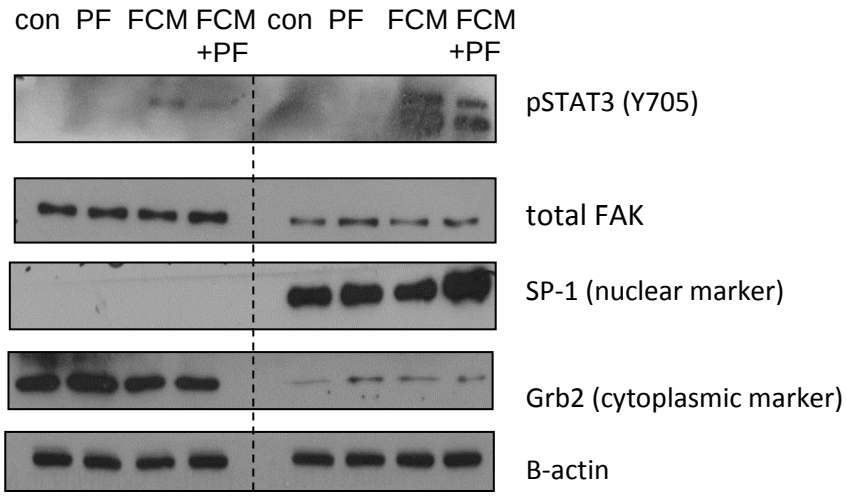
A



pSTAT3 (Y705)

con FCM FCM+ PF271

B



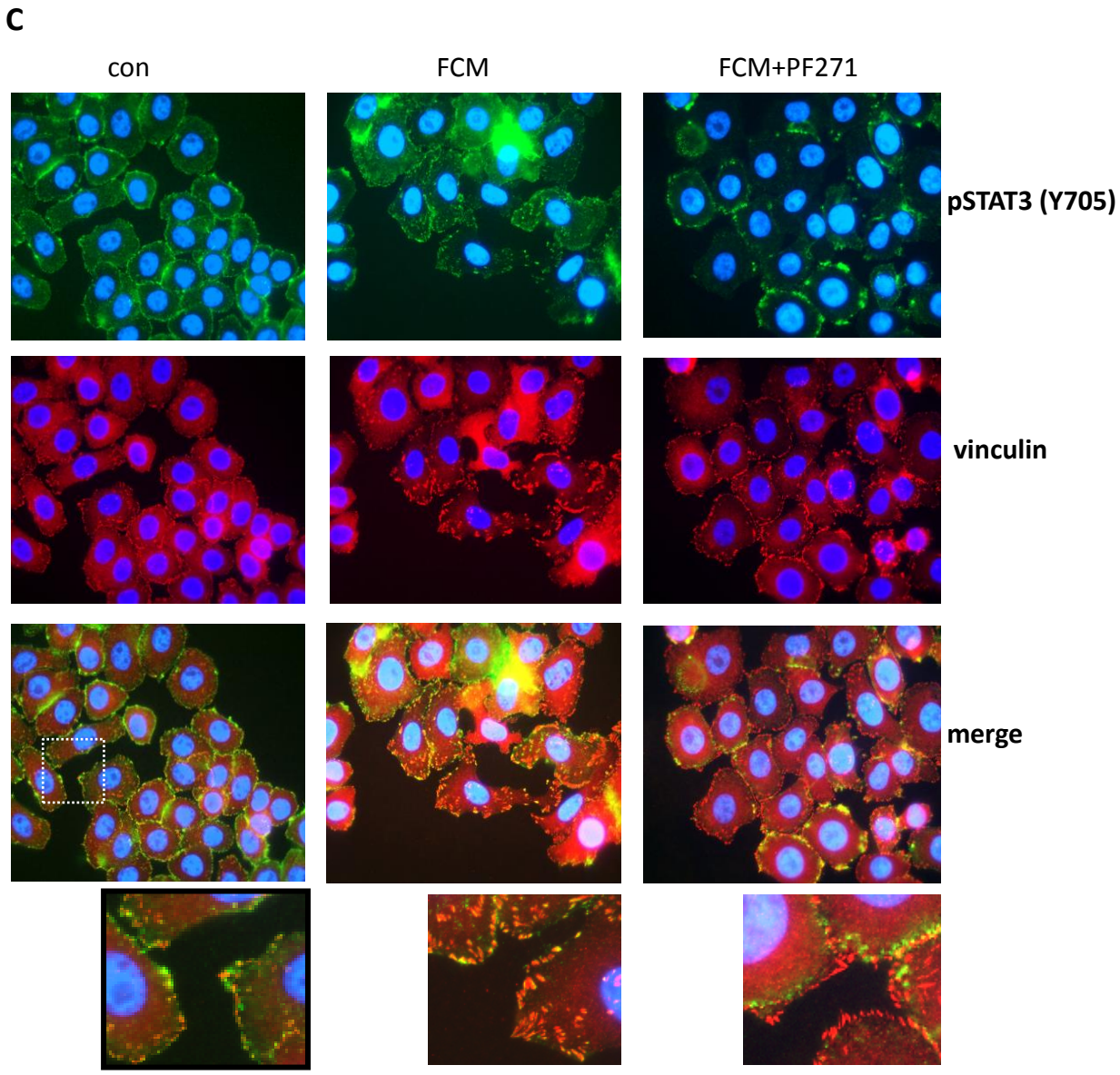


Figure 6.9. (A) Immunofluorescence staining for pSTAT3 Y705 on SKBr3 cells seeded on FN-coated plates demonstrate nuclear accumulation in response to 80% FCM-stimulation migration for 60mins. Pre-treatment with PF271 (1µM) for 60mins suppressed this. (B) Nuclear and cytoplasmic fractionation of SKBr3 cells further support nuclear accumulation in response to 80% FCM as analysed by Western Blotting. SP-1 nuclear transcription factor was used as a nuclear marker, whilst β-actin was used as a loading control. (C) Co-staining of SKBr3 cells for pSTAT3 (Y705) (green) + vinculin (red) and counterstained with DAPI, a nuclear dye (blue). Magnification: x63, oil-immersion objective. White boxes denote areas of interest further magnified directly below. Data represent at least three independent experiments.

To further confirm that STAT3 mediates migratory responses in HER2+ breast cancer cells, we tested the effect of Stattic, an inhibitor of STAT3 activation (Schust et al. 2006), on SKBr3 cell signalling and migration. Initial optimisation experiments demonstrate the 1μM Stattic was sufficient to inhibit FCM-induced STAT3 activation in SKBr3 cells. Subsequent Boyden chamber migration assays show that Stattic was significantly able to inhibit FCM-induced SKBr3 cell migration, though to lesser extent than PF271 (**Figure 6.10A**). When Stattic and PF271 were used in combination this resulted in an additional level of inhibition of SKBr3 cell migration.

Western blotting further revealed that Stattic was not only able to decrease pSTAT3 Y705 activity in response to FCM, but also that of FAK Y861 as well. When Stattic and PF271 were used in combination both pFAK Y861 and pSTAT3 Y705 activity were completely abolished (**Figure 6.10B**).

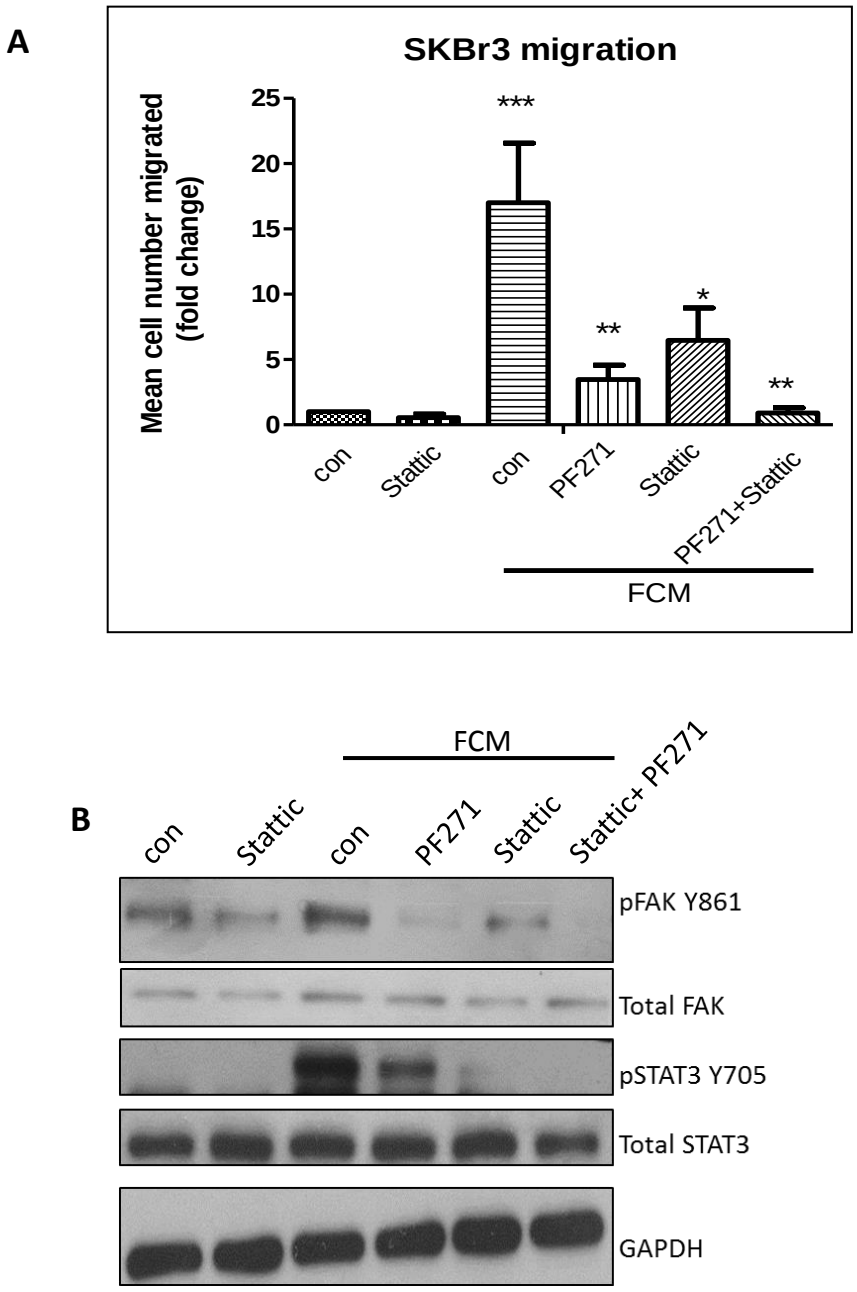


Figure 6.10. Inhibition of STAT3 and/or FAK activation suppresses FCM-induced SKBr3 cell migration. (A) Effects of pSTAT3 and FAK inhibition by Stattic (1µM) and PF271 (1µM) respectively on SKBr3 cell migration in response to two 80% FCM through fibronectin-coated Boyden chamber assay over a 20hrs. Experiments were performed in DCCM culture media and data represent the mean numbers of migratory cells/membrane run in duplicates from three independent experiments. (B) SKBr3 cell lysates treated in parallel were analysed for STAT3 and FAK activity by Western Blotting. GAPDH was used as a loading control. P values: * <0.05, **< 0.01.

6.8 Activated STAT3 physically interacts with FAK upon FCM stimulation

Though our data suggests a link between FAK and STAT3, whether this is a direct physical interaction is not yet known. Thus we performed co-immuno-precipitation (IP) on SKBr3 cells using the STAT Y705 and FAK antibody.

As shown in **Figure 6.11,** pSTAT3 Y705 co-immunoprecipitated with FAK in control (untreated) samples supporting an association between FAK and STAT3. These interactions became more abundant following stimulation by FCM and reduced when treated with PF271. No signal was detected in the negative control which consisted of cell lysate incubated with non-specific IgG antibodies. Altogether, these events further establish a link between FAK and STAT3.

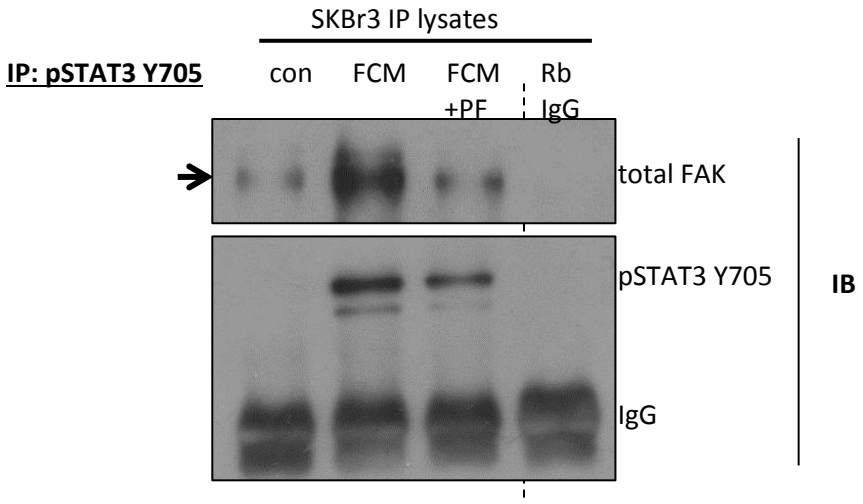
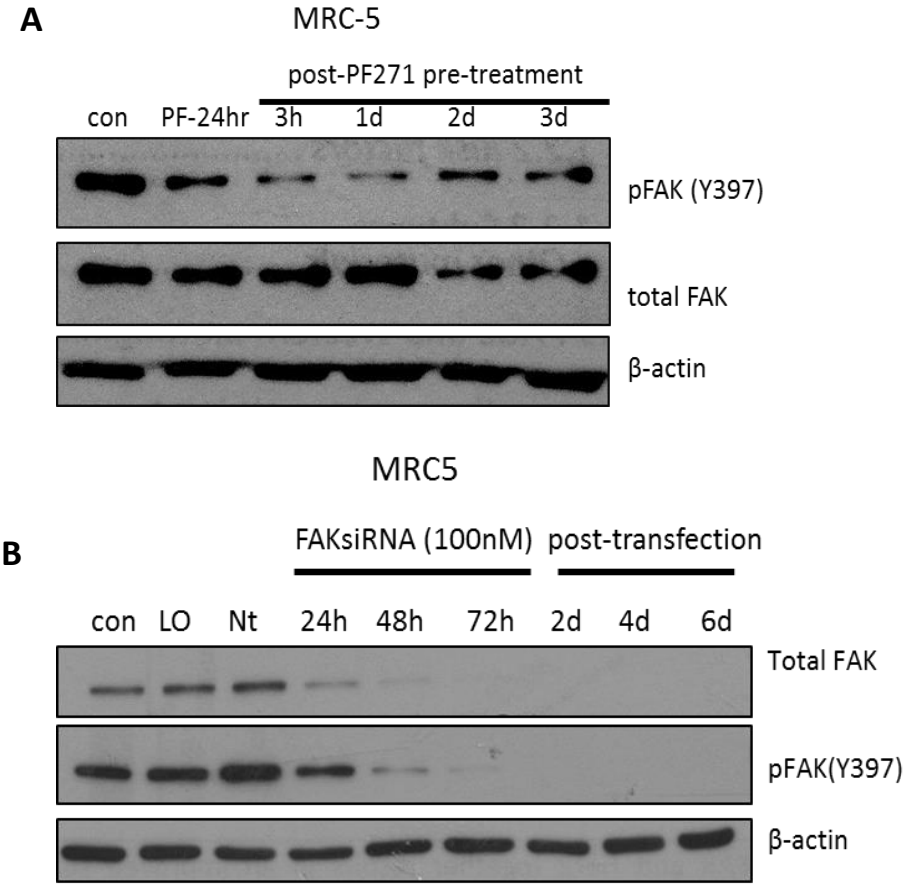


Figure 6.11. Co-immunoprecipitation of pSTAT Y705 with FAK in SKBr3 cells. pSTAT3 Y705 (1.5µg/500µg of lysate) was immunoprecipitated in SKBr3 cell lysates and the complex analysed by Western Blotting for total FAK and pSTAT3 Y705. SKBr3 cells incubated with Rabbit IgG was used as control.

6.9 Inhibition of FAK in MRC5 cells does not affect their pro-migratory effects on HER2+ breast cancer cells

Having established that fibroblasts exert pro-migratory effects towards SKBr3 cells, we examined whether FAK might play a role in the fibroblast cells themselves, for example, by playing a part in signalling pathways that lead to the production of pro-migratory factors secreted by these cells. Importantly, this hypothesis is recently supported by other reports (Barker et al. 2013; Min et al. 2014).

We investigated the effect of FAK inhibition on the ability of MRC5 cells to produce pro-migratory factors. To avoid any confounding effects that may result from the presence of any residual drug in the FCM collected, MRC5 were pre-treated with PF271 (1μM) for 24hrs, and replaced by fresh media thereafter. Parallel Western Blot analysis however demonstrated that inhibition of pFAK Y397 by PF271 was only partial and returned to near basal activity levels 3 days following PF271 pre-treatment, the time period in which FCM was harvested in (**Figure 6.12A**). This suggested that this approach was not appropriate and alternative method for FAK inhibition was sought and siRNA chosen. Optimisation studies confirmed that 100nM FAK siRNA transfection was sufficient to effectively suppress FAK expression and activity for upto 6 days post-transfection in MRC5 cells (**Figure 6.12B**) albeit with some level of toxicity (**Figure 6.12C**). MRC5 transfected with control (non-targeting 'NT') or FAK siRNA were re-seeded onto fresh flasks to ensure equal number of cells, and allowed to adhere for 24hrs. Collection of conditioned media then proceeded as described in 6.1. When tested for its pro-migratory effects, FCM collected from FAK-suppressed MRC5 cells appeared to induce migration to a similar extent as that seen with FCM from control MRC5 cells (**Figure 6.12D**).


A
MRC-5
post-PF271 pre-treatment
con
PF-24hr
3h
1d
2d
3d
pFAK (Y397)
total FAK
β-actin
MRC5
B
FAKsiRNA (100nM)
post-transfection
con
LO
Nt
24h
48h
72h
2d
4d
6d
Total FAK
pFAK(Y397)
β-actin

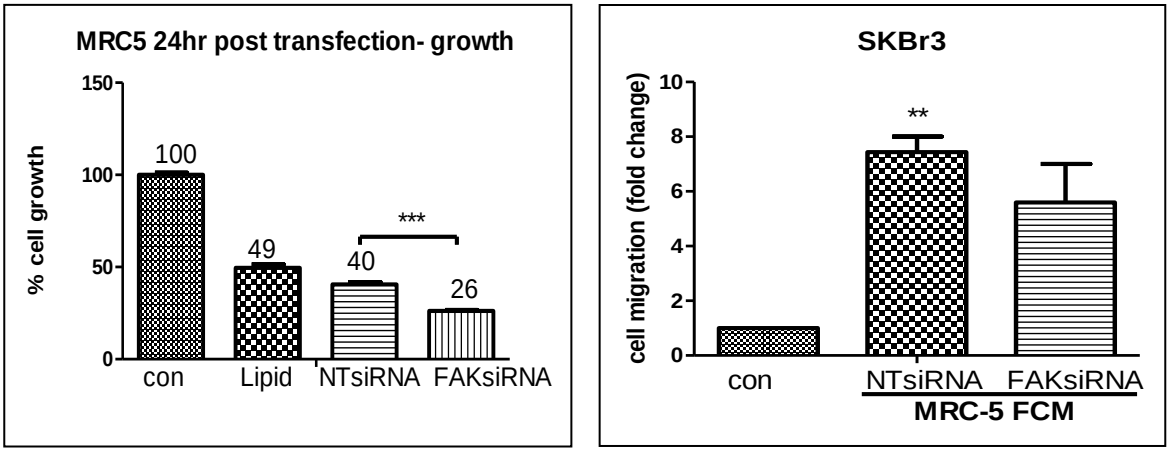

C
MRC5 24hr post transfection- growth
% cell growth
150
100
50
0
100
49
40
26
***
con
Lipid
NTsiRNA
FAKsiRNA
D
SKBr3
cell migration (fold change)
10
8
6
4
2
0
**
con
NTsiRNA
FAKsiRNA
MRC-5 FCM

Figure 6.12. SKBr3 cell migration in response to CM derived from MRC-5 cells in which FAK was inhibited by 24hr PF271 (1μM) pre-treatment or FAKsiRNA 72hr transfection (100nM). Western Blot analysis of MRC-5 cells to determine the extent of sustained FAK inhibition by PF271 (A) or FAKsiRNA post-treatment for up to at least 3 days (B). FAKsiRNA was reasoned to be ideal for sustained FAK inhibition in MRC-5 cells. (C) Toxicity of the lipid and the transfection process was evaluated by treating cells with the DharmaFECT transfection reagent- lipid only (LO) and lipid + the non-targeting siRNA (Nt-siRNA) for 72hrs and effects on cell growth were assessed by Coulter counting. (D) SKBr3 cell migration through fibronectin-coated Boyden chamber over a 20hr period in response to FCM from MRC-5 cells transfected with NT/FAKsiRNA. Experiments were performed in DCCM culture media and data represent the mean numbers of migratory cells/membrane run in duplicates from three independent experiments. P values: * <0.05,**< 0.01, ***< 0.0001. NT: Non-targeting

6.10 FAK plays a role in the cross talk between MRC5 cells and HER2+ breast cancer cells

Our studies to date show that fibroblast conditioned media can promote SKBr3 cell migration in a FAK-dependent manner. However, these studies have used FCM and therefore do not address the bidirectional cross talk suggested to exist between tumour cells and surrounding cells in the tumour microenvironment. Indeed, this is supported by studies that demonstrate the ability of tumour cells to alter or reprogram stromal cells to facilitate tumour progression. For instance, fibroblast MMP9 secretion was significantly enhanced by co-culturing with breast cancer cells, and the level of secretion correlates with the malignancy of the cells (Stuelten et al. 2005). In another study, cancer-associated fibroblasts (CAFs) were shown to secrete higher levels of hepatocyte growth factor (HGF) than normal fibroblasts (NAFs). Pre-co culture of these NAFs with MDA-MB468 breast cancer cells significantly induced their ability to secrete HGF. Subsequent introduction of these pre-co cultured NAFs to mammary fat pad of NOD SCID mice then led to enhanced tumour progression (Tyan et al. 2011).

Therefore, to study the reciprocal influence between fibroblasts and breast cancer cells, 2D co-culture experiments using a transwell (8.0 µm pores insert) Boyden Chamber set up were performed. MRC5 cells were cultured on the lower well whilst SKBr3 cells on the transwell insert. The porous membrane allows for exchange of secreted factors between the compartments and hence the two cell types, schematic illustrated below.

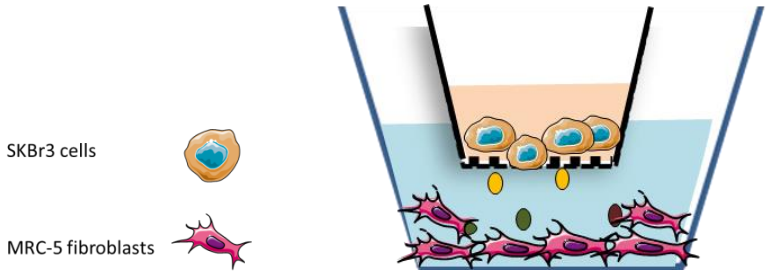
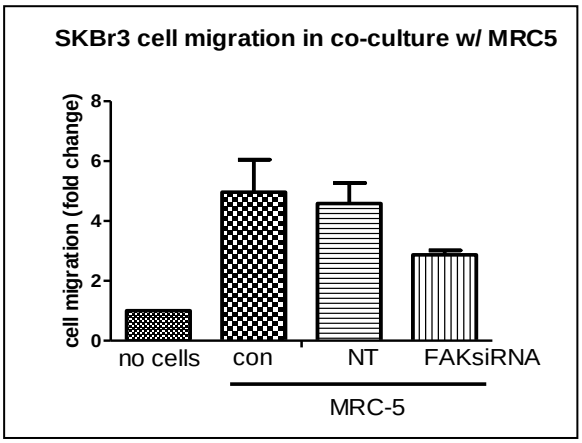


Figure 6.13. Transwell membrane co-culture set up.

We employed the use of siRNA to knock-down FAK in MRC5 fibroblasts or SKBR3 cells. Post-transfected MRC5 cells were seeded onto the lower well of the Boyden Chamber set up (50,000 cells/well) in RPMI +5% FCS media. After 24hrs, media was changed to DCCM media and subsequently after 48hrs, SKBr3 cells were then seeded onto the inserts and allowed to migrate for 20hrs.

Co-culture with MRC5 fibroblasts stimulated SKBr3 cell migration, an effect that was modestly reduced when FAK expression was suppressed (up to 1.7-fold decrease) in MRC5 cells. In contrast, FAK knockdown in SKBr3 cells significantly suppressed their ability to migrate in response to MRC5.

A



B

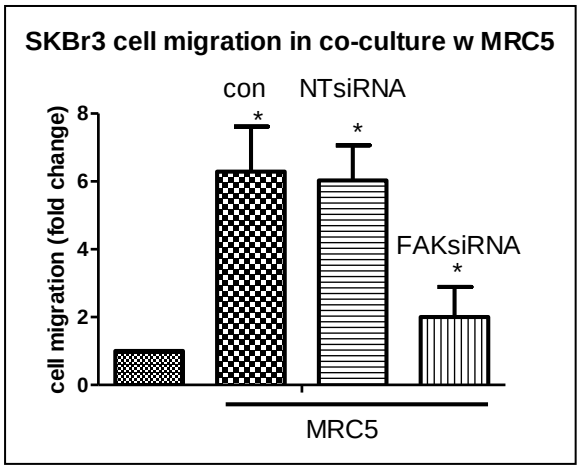


Figure 6.13. Co-culture of SKBr3 cells with MRC-5 in a Boyden Chamber assay set up. (A) FAK was inhibited in (A) MRC5 fibroblasts (lower chamber) or in (B) SKBr3 cells (transwell insert) by FAKsiRNA (100nM) transfection for 72hrs. The effect of FAK knockdown on SKBr3 cell migration was assessed over a 20hr period. Experiments were performed in DCCM culture media and data represent the mean numbers of migratory cells/membrane run in duplicates from three independent experiments. * $p < 0.05$

6.11 Discussion

The extensive cross-talk between the tumour cells and their surrounding environment are now widely reported to support various stages of tumour progression. Tumour cells secrete numerous growth factors, cytokines, interleukins and other soluble factors, acting in an autocrine or paracrine manner to influence the surrounding pre-neoplastic and stromal cells. The subsequent activation and recruitment of these stromal cells consequently leads to secretion of additional factors and in turn, these promote an aggressive tumour behaviour, further activation of nearby stromal cells and concomitant remodelling of the ECM and degradation of the basement membrane. This continuous cycle of reciprocal reprogramming of the stromal cells, tumour cells, surrounding pre-neoplastic cells and ECM altogether assemble into an evolving tumour stroma that will ultimately provide a permissive environment that mediate tumour progression, allowing tumour cell escape and ultimately metastatic dissemination (Mueller & Fusenig 2004).

We reported previously that Heregulin, exogenously added to HER2+ breast cancer cells caused an increase in migratory response in a FAK-dependent manner. In the present chapter, we have extended these observations to show that HER2+ breast cancer cell migration and invasion can also be induced by conditioned media derived from fibroblasts, the most prominent stromal cell type (Kalluri & Zeisberg 2006). Importantly, we explored the hypothesis that, irrespective of the range of growth factors or cytokines likely to be secreted by fibroblasts in the conditioned medium, FAK represented a dominant signalling element in HER2+ cells through which fibroblasts exert their migratory and invasive promoting behaviour.

In these studies, we used 3 day conditioned-media from the MRC5 human lung fibroblast cell line ('FCM') to model fibroblasts effects on HER2+ SKBr3 cells and compared it to the effects seen in HER2- MCF7 cells. The conditioned-media was collected in serum-free DCCM to ensure that only fibroblast-secreted factors would be present in the experimental system as a stimulating/inhibitory factor for the breast cancer cells under test. Further, MRC5 cells represented a good model of fibroblasts for this work given previous reports that conditioned medium from these cells is able to induce migration and invasion of

MDAMB231 cells along with two invasive variants of MCF7 cells (Heylen et al. 1998) and of HER2+ BT474 cells (Studebaker et al. 2008).

In contrast to some other studies that have demonstrated the ability of FCM to promote the growth of a range of breast cancer cells (Studebaker et al. 2008; Yashiro et al. 2005; Gache et al. 1998; Tyan et al. 2011), we did not observe any effects of FCM on MCF7 or SKBr3 cell proliferation. However, it should be noted that growth-promoting effects of FCM are reported either in co-culture or a 3D context. In such settings, interactions between tumour cells and extracellular matrix components may sensitise cells to growth factors and other ligands in the FCM, or the bidirectional crosstalk between tumour cells and fibroblasts may act to promote growth-regulatory ligand secretion from the fibroblasts and/or tumour cells (Cukierman et al. 2001; Baker & Chen 2012). Alternatively, there is a possibility that breast cancer cells are more sensitive accordingly to their subtype-specific fibroblasts. This is supported by significant gene-expression profile differences between fibroblasts isolated from ER+, HER2+ or triple-negative breast tumours (Tchou et al. 2012). In saying that, although MRC5 cells are lung fibroblasts and thus from a different organ, Studebaker et al. have shown that MRC5 cells promote similar behaviour to that of fibroblasts isolated from breast tumour tissues.

In this study, we revealed a differential response to FCM between MCF7 and SKBr3 cells with the migratory and invasive capacity of the latter being significantly augmented in response to FCM. Accompanying this was what appeared to be cytoskeletal remodelling, wherein actin became localised at the number of acquired protrusions all around the edges of the cell. Morphologically, cell-to-cell contacts appear disorganised or disrupted. Altogether, these changes can be attributed to changes in cell polarity and are generally accepted as common feature of cell migration and EMT (Lamouille et al. 2014). Interestingly, these actin cytoskeletal response to FCM appeared different to the larger fan-like lamellipodial protrusions observed following stimulation of SKBr3 cells with HRG (**Chapter 3.7**), suggesting that the actin filament assembly are differentially regulated, such as through a differential actin nucleation mechanism (Le Clainche & Carlier 2008). The Arp2/3 nucleation complex for example can be regulated by either Wiskott-Aldrich syndrome proteins (WASP) and WASP family verprolin homologous (WAVE) family of

proteins which are also differentially regulated by RhoGTPases, phosphorylation and subsequent binding of SH2-domain protein in response to extracellular stimuli (Bompard & Caron 2004). This may indicate that the migratory process in SKBr3 cells can be regulated by various fibroblast-secreted factors and influence distinct changes in actin-cytoskeletal arrangements as we have observed. Importantly, these changes were suppressed when FAK activity was inhibited by PF271, implicating a central role for FAK in FCM-induced SKBr3 cell migration.

Since the migration assays were performed over a short period (20hrs) and therefore the inhibitory effects of PF271 on migration are unlikely to be due to PF271-induced cell death. It should however be noted that it was observed that SKBr3 cells were particularly sensitive to PF271 in terms of growth suppression in a 7 day growth assay (**Fig 6.6c**). Interestingly, this was in contrast to our data obtained with the alternative FAK inhibitor, PF878 (**Chapter 4**). On further investigation it could be seen that the IC_{50} values for PF271-mediated inhibition of FAK phosphorylation at Y397 in of both cell lines correlated with the IC_{50} for growth inhibition whereas this was not the case for PF878 . Since both PF271 and PF878 are ATP-competitive, reversible inhibitors of FAK and almost structurally identical, containing the key amino-methyl pyridinyl sulfonamide group that forms interactions with Asp-Phe-Gly (DFG) motif-containing residues within the FAK activation loop, we hypothesise that the differential effects on cell growth must arise as a consequence of the modification to the side-group in PF271.Whether this side group modification affects binding to FAK, cellular uptake, lipid permeability or a combination of these is not known but these observations highlight the importance that minor differences can make in a drugs' structure, which affect its binding to the target and ultimately the effect on the cell.

Furthermore, SKBr3 cell invasiveness was seen to increase in response to FCM, an effect that was partially inhibited by PF271. This is consistent with previous reports (Benlimame et al. 2005) and further substantiates the widely reported role for FAK in cell invasion (Hauck et al. 2002). One mechanism by which FAK can promote an invasive response is through modulation of MMP production (Sein et al. 2000; Hauck et al. 2001). FCM however failed to induce gelatinase (MMP2) production in SKBr3 cells. Other literature points to the fact that MMP expression is associated with HER2 overexpression (Pellikainen et al. 2004; Tan et al.

1997) and thus it is surprising that no MMPs were detected in these cells. However, only MMP2 and 9 were investigated since these are known to be involved in collagen degradation and are amenable to analysis using zymography with a gelatin (denatured collagen type I) substrate (Toth & Fridman 2001). The major components of BME used in the assay however also included laminin, collagen IV, entactin, and heparin sulfate proteoglycan, thus it is possible that inhibition of FAK modifies other MMPs implicated in the invasive potential of HER2+ breast cancer for instance MMP1 and MMP13 (Bosc et al. 2001; Yong et al. 2010; Westermarck & Kähäri 1999) or perhaps another group of metastasis-associated enzymes reported to regulate ErbB2 driven cell invasion, cathepsins (Rafn et al. 2012). Alternatively, the cellular context might influence MMP production: our invasion assays were performed in a 3D-setting whilst the samples used for gelatin zymography were from conditioned-media harvested from SKBr3 cells grown as monolayers. Indeed, there is evidence to suggest differential MMP expression in 3D culture versus 2D (Mishra et al. 2012).

Nevertheless, our findings that FCM induced migratory responses on SKBr3 cells, led us to investigate the underlying signalling events mediated by FAK. We demonstrated that FCM induced ErbB receptor (HER2, HER4) activation but only at 24hrs post treatment suggesting that effects of fibroblast-secreted factors on these receptors was indirect, possibly in an autocrine or paracrine manner. An example for instance is of the ligand TGFβ, which previously has been reported to up-regulate expression several ErbB ligands in fibroblasts (Andrianifahanana et al. 2010). These observations also suggest that the pro-migratory effects of FCM were not exclusively dependent on ErbB signalling as we originally hypothesised. However, since Herceptin was able to suppress FCM-stimulated SKBr3 cell migration , a role for ErbB signalling cannot be ruled out entirely. The effects of Herceptin were further enhanced when combined with PF271 which might imply differential control of migration through ErbB and FAK; in this context, additional inhibition of FAK activity could potentially further increase these anti-metastatic effects of Herceptin.

Relatively more rapidly, FCM also induced activity at pFAK Y861 (not Y397) in SKBr3 cells. Furthermore, pFAK Y861 activity correlated with the activation of the transcription factor STAT3. Our observations that FCM-induced STAT3 activity was at least in part activated via

FAK as treatment with PF271 reduced the phosphorylation of pSTAT3 Y705. Following activation, STAT monomers are known to form homo/heterodimers and translocate into the nucleus, where it binds to gene promoters to allow transcription of genes involved in cell proliferation, migration, invasion and survival including BCL-2, Mcl-1, Cyclin D1, MMP1 and 2, VEGF and survivin (Hsieh et al. 2005; Snyder et al. 2008). In agreement, our data demonstrated that STAT3 is redistributed to the nucleus following FCM treatment suggested that FCM promotes STAT3-dependent gene transcription that might contribute to the migratory responses seen. Pharmacological inhibition of STAT3 using Stattic also suppressed FCM-induced migration, confirming the role of STAT3 in driving migratory processes. This is consistent with previous studies that established the relationship between STAT and FAK leading to migratory responses (Xie et al. 2001; Silver et al. 2005) although in our cell lines, to be fully confident of this link, FCM could be used to stimulate SKBr3 cells transfected with mutant STAT3 through expression of STAT3 Y705F mutant construct (recently acquired from J. Bromberg).

Co-immunoprecipitation assays provided evidence for a physical interaction between FAK and STAT3 in conditions of STAT3 activation in the presence of FCM. *Some of* these interactions are likely exist in the nucleus as supported by our data that FAK and STAT3 are present in the nuclear fraction of SKBr3 cells. This is in agreement with recent evidences supporting the localisation of FAK in the nucleus where it has been shown to be bind and facilitate turnover of transcription factors including p53,GATA4 and NF-1 (Kweh et al. 2009; Lim 2013). For this reason, we can speculate a possible cooperation between FAK and STAT3 within the nucleus to regulate specific pro-migratory genes yet to be identified. In support of these, a correlation between decreased FAK and STAT3 activity and increased levels of E-cadherin expression was observed, indicating that one such STAT3-regulated gene may be that of E-cadherin, as have been reported to be STAT3-mediated in Ras-transformed MCF10A cells and colorectal cancer cells (Leslie et al. 2010; Xiong et al. 2012).

Apart from our inference that FAK can cooperate STAT3 directly in the nucleus, a previous report also demonstrated that FAK activity is required for *interleukin 6 (IL-6) gene expression (Schlaepfer et al. 2007). As IL-6 is an established activator of STAT3 signalling* to promote metastatic cell phenotypes (Guo et al. 2012; Berishaj et al. 2007; Studebaker et al.

2008), it is also conceivable that FAK may activate STAT3 in an indirect manner by regulation of IL-6 expression in SKBr3 cells, though further studies are required to confirm this. STAT proteins are also known to be activated by various cytokine receptors, growth factor receptors e.g. EGFR, PDGF-R, as well as direct phosphorylation by cytoplasmic kinases such as Src and Abl (Bowman et al. 2000). Whether FAK is involved in these interactions remains to be accomplished, though our data revealing that the combination of PF271 and Stattic led to greater inhibition of migration than either agents alone, indicated some FAK-independent non-redundant STAT3-mediated pathways.

Immunofluorescence studies further revealed the co-localisation of pSTAT3 Y705 with vinculin, a focal adhesion marker, in the presence of FCM (**Fig 6.9d**). This was disturbed in the presence of PF271, providing evidence to suggest that FAK activity is required not only for pSTAT3 activation and the classical paradigm of nuclear accumulation, but also localisation at focal adhesions. Analysis of the cytoplasmic fraction of SKBr3 cells reveals a pool of STAT3 Y705 remaining in the cytoplasm following FCM stimulation. In addition, these immuno-staining experiments were performed following 60mins exposure to FCM, strengthening the interpretation that STAT3 can also have transcription-independent functions to regulate SKBr3 cell migration. This is in agreement with previous studies which demonstrated the involvement of FAK/STAT signalling and their association at focal adhesion to regulate migratory processes in squamous cell carcinoma (Xie et al. 2001) and ovarian cancer cells (Silver et al. 2004; Badgwell et al. 2012). However to our knowledge, our data were the first to demonstrate the interaction and functional cooperation between FAK and STAT3 in HER2+ breast cancer cells.

There are number of studies that support transcription-independent roles of STAT3. In the context of migration, in *Drosophila* for example, STAT3 activation was required for the initiation and maintenance of border cell migration (Silver et al. 2005). STAT3 can modulate microtubule dynamics in migrating cells by interaction with stathmin, a microtubule-destabilizing protein (Ng et al. 2006). The same group subsequently reported that STAT3 activity also regulates directional migratory persistence, membrane ruffling and lamellipodia formation in mouse embryonic fibroblasts by modulation of Rac1-GTPase interaction with βPIX , a RAC-1 GEF (Teng et al. 2009). Some however had opposing observations wherein

STAT3-deficient MEFs displayed reduced actin-stress fibre formation which corresponded with RhoA-GTPase induced migration, thus reflecting that the STAT3 interplay with various RhoGTPases direct differential pro-migratory processes (Debidda et al. 2005). Emerging from these studies is the implication that STAT3 may modulate cell migration through a direct functional interaction with cytoplasmic proteins. Interestingly, a genome-wide study, aimed at identifying STAT3 interacting proteins in human myeloma cell line U266, revealed FAK amongst many others, as a key STAT3 binding protein (Zheng et al. 2012), in further support of our data. By the same approach, another group, though not reporting on FAK/STAT3 binding, identified a number of STAT3-interacting partners are cytoskeleton-related proteins including cortactin, talin-1 and filamin A in HEK293 cells (Blumert et al. 2013). Based on these studies, it is reasonable to assume that following FCM-stimulation, a pool of STAT3 may remain in cytoplasm where it may serve as an adapter to mediate protein-protein interactions critical to the formation of focal adhesion complexes or cytoskeletal arrangements. Moreover, our data demonstrating that Stattic treatment alone suppressed pFAK Y861 activity implicate that activated STAT3 also exert catalytic activities directly towards FAK or via its upstream regulators to mediate signalling events, likely be associated with cytoskeletal regulation and migration.

Having investigated the role of FAK in the HER2+ SKBr3 cells, we asked an alternative approach and examined whether FAK might play a role in the fibroblast cells, for example, by playing a part in signalling pathways that lead to the production of pro-migratory factors secreted by these cells. siRNA-mediated FAK suppression in MRC5 cells resulted in growth inhibition suggesting that FAK played an important growth-regulatory function in these cells as reported by others (Hungerford 1996; Zouq et al. 2009). Interestingly, whilst FCM from FAKsiRNA-treated fibroblasts was still able to promote SKBr3 migration, FAK siRNA in MRC5 cells attenuated their pro-migratory effect towards SKBr3 cells when both cells were cultured together. The differential effects of FAK inhibition in these two culture settings suggest that FAK in MRC5 cells might be involved in the bi-directional crosstalk between them as implicated in other studies (Stuelten et al. 2005; Barker et al. 2013).

Taken together, these findings demonstrate that FAK mediates fibroblast induction of HER2+ breast cancer cell migration potentially via a mechanism involving, STAT3. This data suggests that FAK is an attractive target in HER2+ breast tumours where it may suppress tumour cell migration but additionally can exert added benefits within the tumour microenvironment such as the interference of cross-talk between tumour cells and fibroblasts that can promote migratory tumour behaviour and potentially metastatic progression.

7. General discussion

7.1 HER2+ breast cancer

Up to 25-30% of breast cancer patients are diagnosed with HER2+ tumours which is associated with a poorer prognosis (Slamon et al. 1987). Despite the clinical effectiveness of Herceptin in targeting these HER2+ tumours, a large proportion of patients do not respond (*de novo* resistance) and the majority that do respond display disease progression within a year of treatment initiation, indicative of rapid acquired resistance (see section 1.1.5.2). In light of this there is a clear need for the identification of novel therapeutic targets that may have a potential to improve outcome patients with HER2+ tumours.

7.2 FAK as an anti-cancer target in HER2+ breast cancer

FAK is now emerging as a promising therapeutic target in many tumour types (Golubovskaya 2010), due to its overexpression in tumour cells and association with various cellular processes involved in cancer progression (Owens et al. 1995; Lark et al. 2005; Schaller 2010). More recently, it has also been implicated in tumour metabolism and maintenance of cancer stem cells (Luo et al. 2009; Zhang & Hochwald 2013; Guan 2010). In addition to integrin mediated signalling, FAK is also involved downstream of growth factors receptors such as the HER2 (Vadlamudi et al. 2003; Benlimame et al. 2005; Xu et al. 2009) with a number of studies reporting that that HER2 and FAK overexpression are co-expressed in breast cancer (Schmitz et al. 2005; Lark et al. 2005). High levels of FAK activity are both associated with progression in ductal carcinoma in situ (DCIS) and infiltrating ductal carcinoma (IDC) lesions, as well as in invasive and metastatic lesions (Owens et al. 1995; Lark et al. 2005; Lightfoot et al. 2004). In our HER2+ breast cancer cell models, although we were unable to show increased levels of total FAK protein levels relative to HER2- cells, analysis of FAK activity (particularly at Y861) appeared to be elevated in HER2+ cells versus HER2- cells suggesting a potential link between FAK activity and HER2+ breast cancer.

The suggested role of FAK in cancer progression has led to the development of a number of inhibitors for FAK such as PF271 (Pfizer, see Table 1.3) is a small ATP competitive inhibitor of FAK (and Pyk2) and been shown to inhibit tumour growth in multiple tumour xenograft models including pancreatic, prostate and lung (Roberts et al. 2008). In mouse models of pancreatic ductal adenocarcinoma (PDA), PF271 was shown to significantly inhibit tumour growth and reduce the incidence of metastasis (Stokes et al. 2011) in addition to reducing

the fibroblast and macrophage infiltration into the stroma. Importantly systemic administration of PF271 into mice has not been reported to result in severe side effects which is encouraging given that FAK is ubiquitously expressed in most tissues and thus its inhibition has the potential to disrupt key FAK-mediated signalling important for physiological processes, although given that the expression of FAK are reported to be greatest during embryonic development (Turner et al. 1993) suggests that FAK inhibition in adults may have less consequence. Clinically, this inhibitor has been tested in a Phase 1 clinical trial (Infante et al. 2012) which, despite yielding no firm conclusions on efficacy largely due to the heterogeneity of tumour types involved, did result in stable disease in 34% of patients (n=99) tested after 6 weeks of treatment initiation. Given the favourable toxicology and pharmacokinetic profiles of these agents in human studies, clinical trials on small molecule FAK inhibitors are ongoing (See Table 1.3); of these, PF878 (now VS-6063), a chemical relative of PF271 but having more favourable oral availability, appears to be progressing well (See Table 1.3) with encouraging pre-clinical data in xenograft studies (Xu et al. 2014).

Given the emerging prominence of FAK in cancer and its relationship with growth factor signalling, in this thesis we have explored the role of FAK in HER2+ breast cancer and investigated the potential for improvement of current therapies for this breast cancer subtype using inhibitors of FAK.

Our initial findings revealed that targeting the Y397 auto-phosphorylation site of FAK using PF878 in two HER2+/ER+ cell models (BT474 and MDA361 cells) and HER2+/ER- cells (SKBr3) did not directly correlate with growth inhibition despite inhibition of FAK activity. This was particularly interesting in MDA361 cells where FAK inhibition led to concomitant decrease in AKT and MAPK activity, key regulators of cell survival, apoptosis and proliferation (Marmor et al. 2004; Nahta & Esteva 2007). In contrast, FAK inhibition in SKBr3 cells resulted in paradoxical increases in AKT and MAPK. These combined data lead us to speculate that the changes in downstream signalling towards AKT and MAPK were not useful markers of sensitivity to PF878.

Interestingly, our data shows that the effects of different inhibitors on the same cells can vary. The unavoidable decision to switch from PF878 to PF271 for the last set of

experiments (Chapter 6) revealed that, despite both compounds inhibiting pFAK Y397 and being produced by the same company, the latter was able to exert modest suppression on SKBr3 cell growth compared to the former. Both PF271 and PF878 are ATP-competitive, reversible inhibitors of FAK and almost structurally identical, containing the key amino-methyl pyridinyl sulfonamide group that forms interactions with Asp-Phe-Gly (DFG) motif-containing residues within the FAK activation loop. PF878 was developed from its predecessor PF271 with minor side-group modifications for increased oral availability. Our data indicates however that the differential effects on cell growth must arise as a consequence of this modification. Whether this side group modification affects binding to FAK, cellular uptake, lipid permeability or a combination of these is not known but these observations highlight the importance the minor differences can make in a drugs' structure, which affect binding to the target and ultimately modify its effects on the cell. This is a recognised phenomenon referred to as 'Structure Activity Relationship' (SAR) paradox; *'that it is not the case that all similar molecules have similar activities'* (Brown N. 2012). Importantly, this underscores the importance of fully testing inhibitors to explore their effects on signalling and cell function and also may suggest that when used in the clinic, may have differential effectiveness.

Overall, our *in vitro* data suggests that inhibition of FAK may not represent an effective strategy to inhibit HER2+ tumour growth although, as discussed later in this chapter, the strength of FAK inhibitor monotherapy may lie in its ability to suppress the aggressive tumour cell behaviour associated with metastasis.

7.3 FAK inhibitor in combination with Herceptin as a therapeutic strategy

In light of the lack of effect of FAK inhibition of cell growth, we explored in the potential of combining PF878 with Herceptin, an established treatment for HER2+ breast cancer. Initial studies revealed that BT474 cells were relatively more sensitive to Herceptin than MDA361 cells in agreement with a previous study (Yakes et al. 2002). This could reflect the fact that MDA361 cells expressed lower levels of HER2 activity than BT474 cells which may suggest that they are less reliant on HER2 signalling for growth. One study on a number of HER2+ cell lines suggested that Herceptin sensitivity correlates with HER2 phosphorylation, however, the authors acknowledged the challenge of predicting the relation between the HER2 phosphorylation and the clinical benefit from Herceptin due to the significant

influence of other factors including the expression of other ErbB receptors and RTKs, the potential input from cross-talk and alternate signalling mechanisms, as well as the regulation of downstream signalling proteins for example of AKT by PTEN inactivation (Ginestier et al. 2007). In this regard, MDA361 cells may reflect a subset of HER2+/ER+ tumours that display *de novo* resistance to Herceptin and that together with BT474 cells, these cell lines may therefore provide a unique opportunity to begin to identify treatment-regulated genes that may potentially explain the differential sensitivity of these cells to Herceptin, for example, by initially examining their gene expression profiles by microarray analysis; with the ultimate goal of enabling to select those individuals most likely to benefit from FAK inhibitors.

Encouragingly, our data suggested that combination treatment of HER2+ breast cancer cells with Herceptin and PF878 resulted in a synergistic inhibition of cell growth that appeared to be linked to the suppression of AKT, at least for BT474 and MDA361 cells. AKT has been reported to play a key growth regulatory role in these cell lines where its inhibition results in suppression of cell proliferation (Yakes et al. 2002; Will et al. 2014). Although Herceptin alone separately reduced AKT activity in both HER2+/ER+ cells (with the effects in MDA361 greater than BT474 cells), this was not to the extent seen when both Herceptin was used in combination with PF878. Thus this data argues for a threshold of AKT activity below which cell growth may be affected; this is further supported by our data showing that treatment of these cells with the AKT inhibitor, MK2206, led to a significant decrease in cell number (Appendix 8.2). In addition, loss of AKT may result in an inhibition of cell cycle progression and induction of apoptosis, as supported by the loss of Ki67 staining and gain in PARP cleavage. Interestingly, this synergistic growth suppression was more significant in MDA361 cells, which were relatively insensitive to Herceptin monotherapy, than BT474 cells, suggesting that this combinatorial strategy may prove more beneficial in the context of refractory diseases.

In light of this data, it could be further argued that AKT itself might represent a potential target in HER2+ disease. As such various PI3K/AKT inhibitors under development have shown encouraging data in a pre-clinical study to enhance efficacy of existing targeted therapies (Clark et al. 2002 ; Karaca B et al. 2014). Targeting mTOR, a downstream effector of AKT and a critical regulator of protein synthesis is also an emerging target currently being

tested in a number of clinical trials (Reviewed Vicier et al. 2014). For example, an ongoing phase III trial will evaluate the combination of an mTOR inhibitor everolimus with endocrine treatment for patients with ER+/HER2- breast cancer (NCT01805271). The BOLERO-1 trial is also an ongoing phase III randomised trial in patients with HER2+ advanced breast cancer to evaluating the role of everolimus to overcome *de novo* Herceptin resistance (NCT00876395). The outcome of such trials are critical, however, it is not clear whether this strategy would provide the additional benefits of the suppression of cell migration seen with FAK inhibitors.

In addition to Herceptin, HER2+/ER+ breast cancers may also receive endocrine therapy. The effectiveness of these is limited by HER2/ER cross talk, as this is a well-known mechanism of endocrine resistance (Houston et al. 1999; Dowsett 2001; Shou et al. 2004; Montemurro et al. 2012). Herceptin and Tamoxifen together has been previously shown to be beneficial in pre-clinical (Witters et al. 1997; Kunisue et al. 2000) and clinical studies (Reviewed Prat & Baselga 2008). Our data supports this in the context of HER2+/ER+ breast cancer but, importantly, demonstrates that inclusion of FAK in the treatment regimen further enhances growth suppression, particularly in BT474 cells that were relatively Tamoxifen-insensitive. This is in further support of the recurring theme that perhaps inhibition of FAK exerts greater benefits in the context of limited drug sensitivity.

Clinical studies evaluating the combination of Herceptin and endocrine therapies have clearly demonstrated improvement in relapse and overall survival and similar benefits have been achieved in patients that had been treated with the combination of chemotherapy and Herceptin (Piccart-Gebhart et al. 2005; Marty et al. 2005). And as such, current treatment practice for patients with HER2+/ER+ tumours based on NICE guidelines state that these patients are more likely to receive anti-HER2 therapy plus chemotherapy whilst endocrine therapy only becomes an option if the patient presents an adverse reaction to these (NICE UK).

The combination of Herceptin with PF878 similarly proved beneficial in the HER2+/ER- SKBr3 cells, though statistically insignificant and only occurring at the Herceptin concentration of 10nM. This enhanced growth suppression was however not correlated with AKT suppression, and in fact, the combination of Herceptin and PF878 induced AKT

activation. Thus, the effects of dual targeting of HER2 and FAK on downstream signalling likely vary from cell line to cell line. However, we have yet to prove whether this increased AKT activity is only transient and whether activity levels declined at time-points longer than 24hrs, in which our growth assays were performed in. Interestingly no further benefits were observed when the concentration of Herceptin used was increased from 10 to 100 nM and it may be that at high concentrations, saturation of the HER2 receptors or potential interference with the receptor recycling to the cell surface (Hurrell & Outhoff 2013) could explain this phenomenon. Nonetheless, in the short term, we have data to support that the combined treatment suppressed proliferation and promoted apoptosis in the context of enhanced AKT and MAPK activity. Whether such effects are unique to this cell line or mediated by the HER2 needs to be verified. As such, studies can be extended to another HER2+/ER- cell lines such as ZR751. However, there is the likelihood of these different HER2+/ER- cell lines to have acquired mutations during the course of tumour progression (intrinsic differences) or during *in vitro* culturing, making direct comparison between cell lines difficult. An alternative approach is to generate an isogenic cell line model such as that of MCF7 modified to express HER2.

7.4 FAK as an anti-cancer target to reduce metastasis

Anti-metastatic therapies have focused on MMP inhibitors that have largely failed in clinical development. This has been attributed to various reasons including the number of MMPs involved in metastasis and that they are produced by various stromal cells, making effective drug delivery a challenge (Reveiwed Zucker & Cao 2009). Although there are an increasing number of targets identified to be associated with metastatic progression, no anti-metastatic drugs are clinically licensed to date.

Herein, we demonstrated that pharmacological inhibition of FAK activity might represent a potential method to reduce metastatic progression given that FAK appears to be involved in the migratory responses of HER2+ breast cancer cells. Indeed, stimulation of these cells with Heregulins resulted in a change in the localisation of FAK at focal adhesions and FAK-dependent cytoskeletal rearrangements, in agreement with the established role of FAK in these processes (Mitra et al. 2005). In MDA361 cells in particular, HRG also promoted the loss of peripheral E-Cadherin staining which favours dissociation of single cell from the

colony as we have observed. Deregulated E-cadherin localisation is a described marker of epithelial-mesenchymal transition (EMT), a process ascribed to the initial stages of metastatic dissemination (Kalluri & Weinberg 2009). That PF878 was able to reverse this, suggest the role of FAK in this critical process as reported by others (Serrels et al. 2011).These anti-migratory effects were further confirmed with the use of FAKsiRNA. Such an approach has also allowed us, to a limited extent, to explore the accumulating evidence that the kinase-independent, scaffolding functions of FAK mediate several important intercellular events (Sieg et al. 1999; Fan et al. 2013). The data that FAKsiRNA transfection exerted no differential magnitude of migratory inhibition when compared with PF878 suggests the dispensable scaffolding functions of FAK in regulating the migratory behaviour of these cells.

Our data also showed that in MDA361 cells, HRG was associated with the increase the activity of HER2 receptor, which is likely to occur via hetero-dimerisation with the other ErbB receptors and literature suggests that signals emanating from HER2/HER3 heterodimers are likely to be formed in response to HRG (Wallasch et al. 1995). Studies have also shown that in HER3 is an obligate partner for HER2 in HER2+ breast tumour cells and provides an potent oncogenic input, (Alimandi et al. 1995) whilst expression of HER2 and HER3 in non-tumourigenic MCF10A cells induces their migratory behaviour in response to HRG (Aceto et al. 2012). Importantly, the activation of ErbB receptors in MDA361 cells was accompanied by an increase in FAK activity (Y397,Y861) supporting a the role for ErbB signalling in cell migration through regulation of focal adhesions (Xu et al. 2009).

In SKBR3 cells, HRG induced migration was associated with the FAK-dependent formation of lamellipodia structures together with activation of FAK (Y397,Y861) and Src. HRG however had only minor effects on the activation of ErbB receptors contrary to other reports (Adam et al. 1998; Tan et al. 1999; Vadlamudi et al. 2003). The elevated basal phosphorylation of the ErbB receptors may result from these cells having established an autocrine loop to secrete ErbB ligands such as HRG, and will thereby have high ErbB receptor activity as has been reported (Venkateswarlu et al. 2002; Li et al. 2004). These saturating conditions are more likely to reflect the tumour micro-environment where they are exposed to such ligands however to understand the underlying mechanisms, a better experimental approach should have been adopted wherein at least a 24hrs period of serum starvation, rather than

60 minutes, prior to additional HRG stimulation. Alternatively, our data may indicate that the spatial localisation of FAK is more important for HRG-induced migratory response rather than global changes in downstream HER2 signalling.

An important point to take in account is that our data supported the role of FAK activity in HRG-induced HER2+ migration, while our immunofluorescence data can only confirm this for activated FAK at Y861 and not Y397. We however cannot discount or validate the involvement of pFAK Y397, which were particularly tricky to detect by immunofluorescence with our particular antibody. However, that pFAK Y861 is expressed at higher levels than Y397 in HER2+ breast cancer cells supports the drive for development of inhibitors that target other tyrosine residues of FAK. This is starting to be explored by others in the group currently.

HRG is known to be present in the tumour microenvironment and its expression correlates with an aggressive tumour behaviour and increased disease recurrence (Visscher et al. 1997; Atlas et al. 2003). Stemming from our data that FAK is involved in HRG-mediated HER2+ cell migration, in Chapter 6, we extended our work to explore whether FAK represented a dominant signalling element in HER2+ cells through which stromal cell components, specifically fibroblasts exert their pro-metastatic behaviour. Fibroblasts represent a prominent stromal cell component (Kalluri & Zeisberg 2006), and supports tumour progression by paracrine signalling towards pre-neoplastic and other stromal cell types (Mueller & Fusenig 2004).

Our data demonstrated that the migratory capacity of SKBr3 breast cancer cells was stimulated in a FAK-dependent manner in agreement with previous reports on HER2+ breast cancer cells (Heylen et al. 1998; Studebaker et al. 2008). In this context, our data suggested that FAK acts through a mechanism involving activation of STAT3 (at Y705), a key effector of cytokine and growth factor signalling (Yu et al. 2009). This may involve a physical association between FAK and STAT3, as supported by our co- immune-precipitation data which in turn may facilitate nuclear accumulation of STAT3, supporting its established role as a transcription factor to regulate pro-metastatic genes (Carpenter & Lo 2014). It was therefore encouraging to detect FAK in the nuclear fraction of FCM-stimulated SKBr3 cells in agreement with the emerging role of FAK in the nucleus where it has been shown to

regulate transcription factors such as p53 (Lim 2013). In this regard, it is likely that FAK could cooperate with STAT3 in the nucleus though this subject needs further exploration. The first of which is to optimise the cellular fractionation assay to be fully confident of the presence of FAK in the nuclear fraction. As though minimal, a small amount of Grb2 protein (cytoplasmic marker) can be detected in the nuclear fraction, and thus the FAK detected in the nuclear fraction my reflect contamination from the cytoplasmic fraction proteins.

The role of FAK in STAT3 activation is consistent with previous studies demonstrating the cooperation and interactions between STAT proteins and FAK to regulate migratory responses (Xie et al. 2001; Silver et al. 2005). Whether these physical interactions are phosphorylation dependent cannot be confidently confirmed. Further studies to explore whether FCM could stimulate interactions between STAT3 and FAK in SKBr3 cells transfected with a phospho-mutant of FAK termed FRNK (FAK-related non-kinase domain). Generation of mutants by site-directed mutagenesis of the key FAK phosphorylation sites may further reveal which residue(s) are critical for its cooperation with STAT3. Conversely, SKBr3 cells could also be transfected with STAT3 Y705F mutant construct (recently acquired from J. Bromberg), to further complete the picture; focusing on the implications on migratory behaviour. Our immunofluorescence data also showed that localisation of activated STAT3 at focal adhesions (co localised with vinculin) upon exposure to FCM was disrupted by PF271, thereby suggesting an additional transcription-independent role of STAT3. Such a localisation of STAT proteins at focal adhesions has previously been reported (Xie et al. 2001; Silver et al. 2004) but to our knowledge we are the first to document this in HER2+ breast cancer cells. Likely, FAK may phosphorylate STAT3 by direct interactions. Dual immunofluorescence staining with FAK and STAT3 using appropriate antibodies however are required to confirm this at the least, or a proximity-ligation assay (DuoLink), which has been recently been optimised by colleagues in the lab. On the other hand, FAK may also be indirectly involved by interaction with JAK2 as has been reported (Zhu et al. 1998), and that FAK also exist in a complex with JAK and STAT3 is also worth exploring.

In spite of this we demonstrated the linkage between FAK and STAT3 and that FAK activity was a requirement for STAT3 at focal adhesions. The role of STAT3 therein could be further examined. Some potential insights have been reported in the literature, wherein STAT3 has been shown to physically interact with proteins associated with cytoskeletal regulation (Ng

et al. 2006; Teng et al. 2009; Debidda et al. 2005) and other focal adhesion proteins such as paxillin (Silver et al. 2004). Moreover, at the time of these experiments, it was reported that STAT3 localised at focal adhesions in a complex with FAK and NEDD9 in ovarian cancer cells (Xiao & Connolly 2014). NEDD9 is a non-catalytic scaffolding protein also associated with an aggressive tumour behaviour (Sima et al. 2013; Izumchenko et al. 2009). This supports the idea that STAT3 and FAK exist in a complex with other proteins and is thus capable of interplay. Our data demonstrating that Stattic treatment alone suppressed pFAK Y861 activity implicate that activated STAT3 also exert direct activities towards FAK or via its upstream regulators to mediate signalling events associated with migration. This part of the study can extended by investigating the possibility of FAK and STAT3 existing in a complex of other focal adhesion proteins, by co-immunoprecipitation, and the effects of PF271 and/or Stattic on these interactions and/or activity to further sought the role of STAT3 in HER2+ cell migration.

The association between FAK and STAT3 is also primarily observed to be dependent on FCM-derived factors and therefore requires a receptor-input in order to activate these cytoplasmic proteins. However, we observed that ErbB receptors are minimally affected by FCM stimulation, suggesting that these receptors may be irrelevant in the context of SKBr3 cell migration. In agreement, it has been demonstrated that HER2-induced STAT3 activation promoted EMT and cancer stem cell traits only in breast cancer cells that also express ER (HER2-overexpressing MCF7 cells) implicating the critical role of HER2-ER-STAT3 pathway (Chung et al.2012).

In relation to the above, from a mechanistic view, exploring the receptor(s) that mediate SKBr3 cell migratory responses would be interesting to pursue. Potential candidates include PDGFR, IL-6R/GP130 and C-Met, known to require STAT3 to mediate a wide variety of cellular responses (Zhang et al. 2002; Yu et al. 2009;). It is also likely that FAK may represent a critical mediator of cross-talk between various cytokine, interleukin or growth factor receptors; crosstalk between the IL-6/GP130 receptor and the RET receptor was described to be mediated by FAK (Morandi & Isacke 2014).

Finally, we further explored the role of FAK in fibroblasts. Our data demonstrated that though FAK was critical to their survival. Importantly, FAKsiRNA-transfected MRC-5

suppressed its pro-migratory role towards SKBr3 in a co-culture setting, whilst no changes were observed when only the conditioned media was used. The differential effects of FAK inhibition in these two culture settings suggest that FAK in MRC5 cells might be involved in the bi-directional crosstalk between them as implicated in other studies (Stuelten et al. 2005; Barker et al. 2013). Having explored this crosstalk between fibroblasts and breast cancer cells in paracrine manner, this work could be extend to determine whether additional or differential effects following cell-to-cell contact. A recent study for instance demonstrated that a transmembrane protein TSPAN12 in fibroblasts promoted cancer cell proliferation and invasion of non-small cell lung carcinoma cell line, which only occurred in a direct-contact co-culture system (Otomo et al. 2014).

It is not enough to understand the internal mechanisms that FAK is involved in to regulate cell migration. These cells exist within the tumour surrounded by various external cues which others and we have shown to affect their signalling and migratory behaviour and thus propensity to metastasize. Our studies have contributed to understanding these, but importantly yielding the conclusion that targeting FAK in HER2+ breast cancer is a potentially a viable strategy suppress tumour cell migration but additionally interfering with fibroblasts and their cross-talk with tumour cells.

Recent studies exploring the role of FAK in other cell types however raised very important issues. In an *in vivo* study, loss of FAK in the haematopoetic environment led to increased circulating granulocytes which contributed to enhanced experimental metastasis (Batista et al. 2014). Low dosage pharmacological inhibition of FAK in mice models was able to promote tumour growth and angiogenesis (Kostourou et al. 2013). These indicate that the beneficial effects of FAK inhibition are therefore context and dose dependent and together highlights the careful analysis of the role of FAK not only in tumour cells but in the surrounding tissues as well. Extensive studies will enable the understanding of the mechanisms underlying these paradoxical effects. Considerations in exploring the possibility to counteract or minimize these effects or optimising FAK inhibitor dosing regimens will ensure the successful incorporation of FAK inhibitors in combination treatment approaches.

7.5 Experimental limitations and considerations

Although we have provided evidence for an important role of FAK in HER2+ breast cancer particularly in the response of these cells to treatment with Herceptin, additional studies are required to resolve the limitations of this current study, of which some are outlined below.

(1) Our current data are based on 2D *in vitro* growth studies which do not wholly represent the micro-environment of the tumours *in vivo* and the potential for interactions with ECM and stromal cells which themselves may alter drug response. Indeed, studies have demonstrated that tumour cells grown in suspension or in 3D cell culture systems are more sensitive to the growth inhibitory effects of a FAK inhibitor (Tanjoni et al. 2010) and Herceptin (Pickl & Ries 2009). Pickl and colleagues specifically described the differential signalling in 3D cell cultures of SKBr3 cells, wherein there is an increase in HER2 phosphorylation, accompanied by a decrease in AKT activity and an increase in MAPK activity, and has strong implications for our data. These data underscore the importance of not extrapolating the effects of inhibitors in 2D culture into 3D/in vivo systems. In this regard, it would be very interesting to it is likely that the benefits of Herceptin and PF878 in combination are much greater than we actually have seen.

(2) Longer term clonogenic assays would also address the question of whether these effects of enhanced growth suppressive effects yielded by the Herceptin and PF878 combination are transient or are maintained over a much longer term. This is particularly important in HER2+/ER- SKBr3 cell lines, where the increased AKT and MAPK activity may contribute towards development of resistance to the combination treatment in the long term.

(3) There is the suggestion that the timing and order of administration of combinatorial drug regimens can be critical to efficacy. In our studies, cells were subjected to the simultaneous treatment of Herceptin and PF878. However, others have indicated that treatment with the first drug may alter signalling networks that make the cells more susceptible to the effects of the second drug (Lee et al. 2012). Modifications of the treatment schedules may further improve the synergistic growth inhibitory effects of Herceptin and PF878, although at this point, we can only speculate.

(4) Acquired resistance to Herceptin has been attributed to a number of cellular processes including a compensatory increase in growth factor receptor signalling pathways that can also involve integrins (Huang et al. 2011). As FAK plays a role these signalling pathways, we hypothesised that FAK may also be an important mediator of such phenomenon. An interesting question therefore is whether the combination of FAK inhibitor with Herceptin might delay or prevent the acquisition of Herceptin resistance? One such strategy would be to generate Herceptin-resistant derivatives of our parental-HER2+ breast cancer cell lines by long-term culture with chronic exposure to Herceptin. This approach has previously been performed in our lab to generate Tamoxifen and Faslodex-resistant (TamR, FasR) derivatives of the MCF7 cell line (McClelland et al. 2001; Knowlden et al. 2003). Interestingly, these resistant derivatives were shown to have elevated levels of FAK activity (Y397, Y861) compared to their parental MCF7 cell counterparts. Importantly, inhibition of FAK activity partially restored the sensitivity of TamR cells to the anti-proliferative effects of Tamoxifen (Hiscox et al. 2011). Given our data showing that Herceptin induced the activation of FAK activity (Y397, Y861) in SKBr3 cells, it is reasonable to hypothesise that FAK acts a regulatory signalling protein that may in the long term represent as an escape mechanism to the growth suppressive effects of Herceptin.

(5) Further examination of our hypotheses in *in vivo* models would prove beneficial and will also address other characteristics that are unique to an *in vivo* setting, such as tumour hypoxia and angiogenesis that may also contribute to drug responses.

7.6 Final conclusions

A wealth of studies have emerged over the last decade supporting a role for FAK in tumour progression and the prominence of FAK in this context is reflected by the number of ongoing clinical trials with small molecule FAK inhibitors. In this thesis we have added further data that supports a rationale for targeting FAK in the context of HER2+ breast cancer. Importantly, our studies have shown that FAK inhibition may have benefit in Herceptin-insensitive breast cancer when used alongside Herceptin and that anti-FAK monotherapies represent a potential strategy to suppress stromal-induced cellular migration of breast cancer cells. In this latter context, our data suggests that FAK may act through a STAT3-dependent mechanism to regulate migration exogenously-stimulated migratory and invasive responses (**Figure 7.1**).

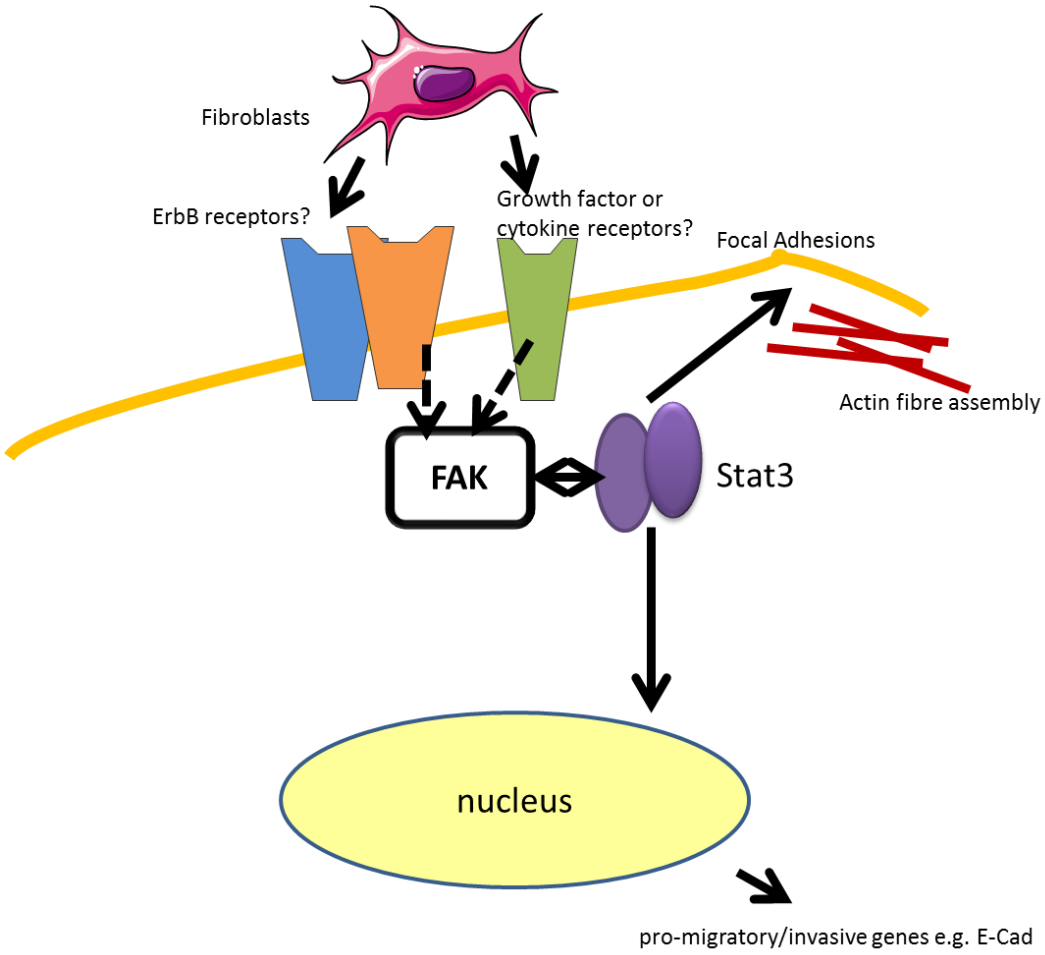
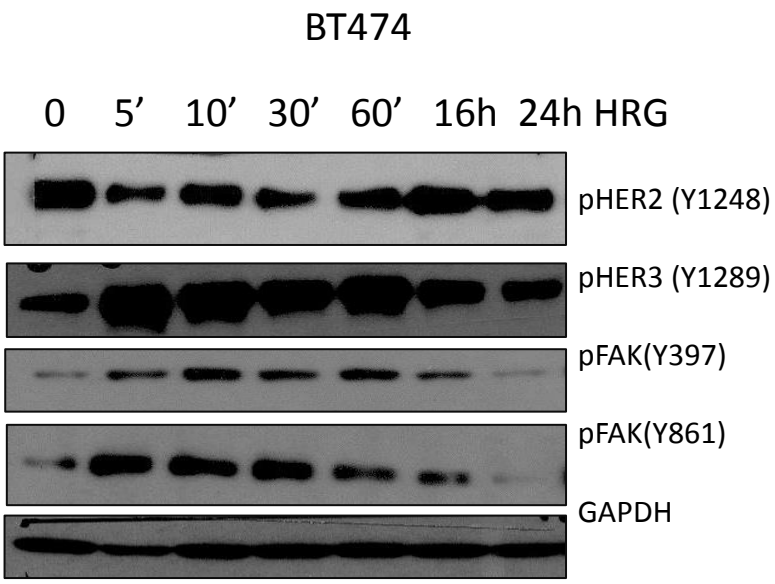
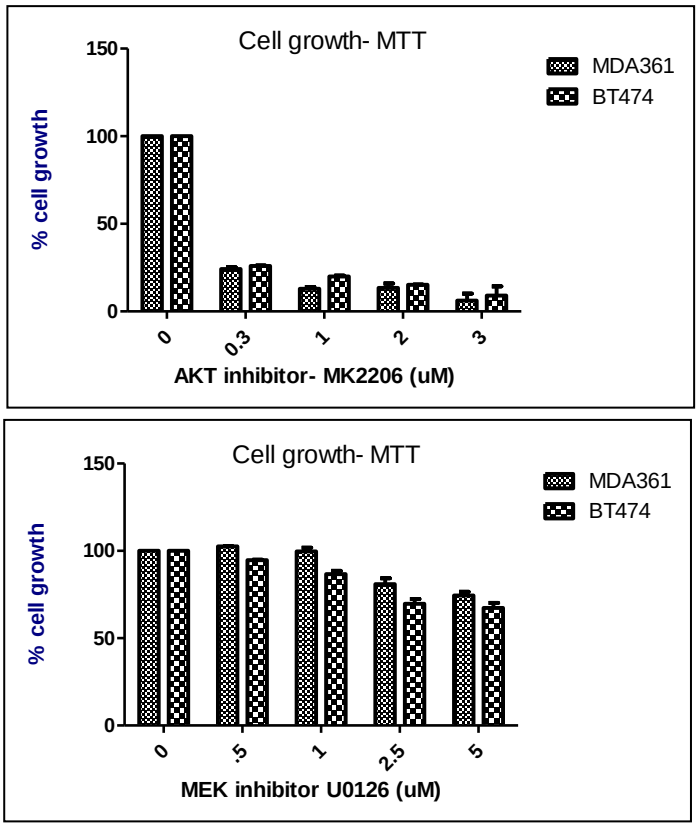


Figure 7.1 Schematic hypothesis of the role of FAK in fibroblast driven HER2+ breast cancer cell migration/invasion.

8. Appendix



Appendix 8.1 BT474 cells stimulated with 100ng/ml HRGβ1 at various time points for up to 24hrs, subsequently lysed and analysed by Western Blotting



Appendix 8.2 BT474 and MDA361 treated with increasing dosage of AKT (MK2206) and MEK (U0126) inhibitor for 5 days and cell growth assessed by the MTT viability assay. (n=3, ±SEM)

9. References

Abbi, S. et al., 2002. Regulation of focal adhesion kinase by a novel protein inhibitor FIP200. *Molecular biology of the cell*, 13(9), pp.3178–91.

Abu-Ghazaleh, R. et al., 2001. Src mediates stimulation by vascular endothelial growth factor of the phosphorylation of focal adhesion kinase at tyrosine 861, and migration and anti-apoptosis in endothelial cells. *The Biochemical journal*, 360(Pt 1), pp.255–64.

Aceto, N. et al., 2012. Co-expression of HER2 and HER3 receptor tyrosine kinases enhances invasion of breast cells via stimulation of interleukin-8 autocrine secretion. *Breast cancer research : BCR*, 14(5), p.R131.

Adam, L. et al., 1998. Heregulin regulates cytoskeletal reorganization and cell migration through the p21-activated kinase-1 via phosphatidylinositol-3 kinase. *The Journal of biological chemistry*, 273(43), pp.28238–46.

Agochiya, M. et al., 1999. Increased dosage and amplification of the focal adhesion kinase gene in human cancer cells. *Oncogene*, 18(41), pp.5646–53.

Alimandi, M. et al., 1995. Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. *Oncogene*, 10(9), pp.1813–21.

Almeida, E.A.C., 2000. Matrix Survival Signaling: From Fibronectin via Focal Adhesion Kinase to c-Jun NH2-terminal Kinase. *The Journal of Cell Biology*, 149(3), pp.741–754.

Alvarez-Tejado, M. et al., 2001. Hypoxia induces the activation of the phosphatidylinositol 3-kinase/Akt cell survival pathway in PC12 cells: protective role in apoptosis. *The Journal of biological chemistry*, 276(25), pp.22368–74.

Angelucci, C. et al., 2011. Breast cancer cells and fibroblasts in co-culture: reciprocal influences on cell adhesion, membrane fluidity and migration. *Italian Journal of Anatomy and Embryology*, 116(2), p.12.

Araujo, J. et al., 2009. Dasatinib and docetaxel combination treatment for patients with castration-resistant progressive prostate cancer: A phase I/II study (CA180086). *ASCO Meeting Abstracts*, 27(15S), p.5061.

Argiris, A., 2004. Synergistic Interactions between Tamoxifen and Trastuzumab (Herceptin). *Clinical Cancer Research*, 10(4), pp.1409–1420.

Arnould, L. et al., 2006. Trastuzumab-based treatment of HER2-positive breast cancer: an antibody-dependent cellular cytotoxicity mechanism? *British journal of cancer*, 94(2), pp.259–67.

Arribas, J. et al., 2011. p95HER2 and breast cancer. *Cancer research*, 71(5), pp.1515–9.

Atlas, E. et al., 2003. Heregulin Is Sufficient for the Promotion of Tumorigenicity and Metastasis of Breast Cancer Cells in Vivo. *Mol. Cancer Res.*, 1(3), pp.165–175.

Baker, B.M. & Chen, C.S., 2012. Deconstructing the third dimension: how 3D culture microenvironments alter cellular cues. *Journal of cell science*, 125(Pt 13), pp.3015–24.

Barker, H.E. et al., 2013. Tumor-secreted LOXL2 Activates Fibroblasts Through FAK Signaling. *Molecular cancer research : MCR*, pp.1541–7786.MCR–13–0033–T–.

Baselga, J., Bradbury, I., et al., 2012. Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): a randomised, open-label, multicentre, phase 3 trial. *Lancet*, 379(9816), pp.633–40.

Baselga, J., Cortés, J., et al., 2012. Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. *The New England journal of medicine*, 366(2), pp.109–19.

Baselga, J. et al., 1996. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, 14(3), pp.737–44.

Batista, S. et al., 2014. Haematopoietic focal adhesion kinase deficiency alters haematopoietic homeostasis to drive tumour metastasis. *Nature communications*, 5, p.5054.

Behmoaram, E. et al., 2008. Focal adhesion kinase-related proline-rich tyrosine kinase 2 and focal adhesion kinase are co-overexpressed in early-stage and invasive ErbB-2-positive breast cancer and cooperate for breast cancer cell tumorigenesis and invasiveness. *The American journal of pathology*, 173(5), pp.1540–50.

Beitner-Johnson, D. et al., 2001. Hypoxia activates Akt and induces phosphorylation of GSK-3 in PC12 cells. *Cellular signalling*, 13(1), pp.23–7.

Benlimame, N. et al., 2005. FAK signaling is critical for ErbB-2/ErbB-3 receptor cooperation for oncogenic transformation and invasion. *The Journal of cell biology*, 171(3), pp.505–16.

Beral, V., 2003. Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet*, 362(9382), pp.419–27.

Berns, K. et al., 2007. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. *Cancer cell*, 12(4), pp.395–402.

Bhargava, R. et al., 2011. Semiquantitative hormone receptor level influences response to trastuzumab-containing neoadjuvant chemotherapy in HER2-positive breast cancer. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc*, 24(3), pp.367–74.

Birkedal-Hansen, H. et al., 1993. Matrix metalloproteinases: a review. *Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists*, 4(2), pp.197–250.

Bose, R. et al., 2006. Phosphoproteomic analysis of Her2/neu signaling and inhibition. *Proceedings of the National Academy of Sciences of the United States of America*, 103(26), pp.9773–8.

Boyd, N.F. et al., 2003. Dietary fat and breast cancer risk revisited: a meta-analysis of the published literature. *British journal of cancer*, 89(9), pp.1672–85.

Braren, R. et al., 2006. Endothelial FAK is essential for vascular network stability, cell survival, and lamellipodial formation. *The Journal of cell biology*, 172(1), pp.151–62.

Burcak Karaca; Emir Bozkurt; Harika Atmaca; Zeki S, Effect of trastuzumab/AT-101 combination on apoptosis and cytotoxicity in HER2-positive breast cancer cells: A novel combination to effect resistance to anti-HER2 therapy through inhibition of PI3K signaling. | 2014 ASCO Annual Meeting | Abstracts | Meeti. Available at: http://meetinglibrary.asco.org/content/128696-144 [Accessed May 8, 2015].

Calalb, M.B. et al., 1996. Focal adhesion kinase tyrosine-861 is a major site of phosphorylation by Src. *Biochemical and biophysical research communications*, 228(3), pp.662–8.

Calalb, M.B., Polte, T.R. & Hanks, S.K., 1995. Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. *Molecular and cellular biology*, 15(2), pp.954–63.

Camps, J.L. et al., 1990. Fibroblast-mediated acceleration of human epithelial tumor growth in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, 87(1), pp.75–9.

Canel, M. et al., 2013. E-cadherin-integrin crosstalk in cancer invasion and metastasis. *Journal of cell science*, 126(Pt 2), pp.393–401.

Canel, M. et al., 2010. Quantitative in vivo imaging of the effects of inhibiting integrin signaling via Src and FAK on cancer cell movement: effects on E-cadherin dynamics. *Cancer research*, 70(22), pp.9413–22.

Carlson, J.J. & Roth, J.A., 2013. The impact of the Oncotype Dx breast cancer assay in clinical practice: a systematic review and meta-analysis. *Breast cancer research and treatment*, 141(1), pp.13–22.

Carpenter, R.L. & Lo, H.-W., 2014. STAT3 Target Genes Relevant to Human Cancers. *Cancers*, 6(2), pp.897–925.

Carragher, N.O. & Frame, M.C., 2004. Focal adhesion and actin dynamics: a place where kinases and proteases meet to promote invasion. *Trends in cell biology*, 14(5), pp.241–9.

Carraway, K. 3rd et al., 1994. The erbB3 gene product is a receptor for heregulin. *J. Biol. Chem.*, 269(19), pp.14303–14306.

Carroll, J.S. & Brown, M., 2006. Estrogen receptor target gene: an evolving concept. *Molecular endocrinology (Baltimore, Md.)*, 20(8), pp.1707–14.

Carter, P. et al., 1992. Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proceedings of the National Academy of Sciences of the United States of America*, 89(10), pp.4285–9.

Cary, L.A. et al., 1998. Identification of p130Cas as a mediator of focal adhesion kinase-promoted cell migration. *The Journal of cell biology*, 140(1), pp.211–21.

Cary, L.A., Chang, J.F. & Guan, J.L., 1996. Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. *Journal of cell science*, 109 (Pt 7, pp.1787–94.

Cary, L.A. & Guan, J.L., 1999. Focal adhesion kinase in integrin-mediated signaling. *Frontiers in bioscience : a journal and virtual library*, 4, pp.D102–13.

Chandarlapaty, S. et al., 2011. AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity. *Cancer cell*, 19(1), pp.58–71.

Cheang, M.C.U. et al., 2009. Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. *Journal of the National Cancer Institute*, 101(10), pp.736–50.

Chen, H., 1994. Stimulation of Phosphatidylinositol 3 ” Kinase Association with Focal Adhesion Kinase by Platelet-derived Growth Factor *. , 269(49), pp.31229–31233.

Chen, H.C. et al., 1995. Interaction of focal adhesion kinase with cytoskeletal protein talin. *The Journal of biological chemistry*, 270(28), pp.16995–9.

Chen, S. et al., 2012. Abstract 3714: Characterization of a selective focal adhesion kinase (FAK) inhibitor in a panel of glioblastoma cell lines identify rational drug-drug combination strategies. *Cancer Research*, 72(8 Supplement), pp.3714–3714.

Chen, Y. et al., 2010. The effect of focal adhesion kinase gene silencing on 5-fluorouracil chemosensitivity involves an Akt/NF-kappaB signaling pathway in colorectal carcinomas. *International journal of cancer. Journal international du cancer*, 127(1), pp.195–206.

Cho, S.Y. & Klemke, R.L., 2002. Purification of pseudopodia from polarized cells reveals redistribution and activation of Rac through assembly of a CAS/Crk scaffold. *The Journal of cell biology*, 156(4), pp.725–36.

Chu, P.-Y. et al., 2009. Tyrosine phosphorylation of growth factor receptor-bound protein-7 by focal adhesion kinase in the regulation of cell migration, proliferation, and tumorigenesis. *The Journal of biological chemistry*, 284(30), pp.20215–26.

Chung, S. S., Giehl, N., Wu, Y., & Vadgama, J. V. (2014). STAT3 activation in HER2-overexpressing breast cancer promotes epithelial-mesenchymal transition and cancer stem cell traits. *International Journal of Oncology*, *44*(2), 403–11.

Clark, A.S. et al., 2002. Constitutive and Inducible Akt Activity Promotes Resistance to Chemotherapy, Trastuzumab, or Tamoxifen in Breast Cancer Cells. *Mol. Cancer Ther.*, 1(9), pp.707–717.

Clark, A.S. et al., 2002. Constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab, or tamoxifen in breast cancer cells. *Molecular cancer therapeutics*, 1(9), pp.707–17.

Cobleigh, M.A. et al., 1999. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, 17(9), pp.2639–48.

Codony-Servat, J. et al., 1999. Cleavage of the HER2 ectodomain is a pervanadate-activable process that is inhibited by the tissue inhibitor of metalloproteases-1 in breast cancer cells. *Cancer research*, 59(6), pp.1196–201.

Collins, D.M. et al., 2012. Trastuzumab induces antibody-dependent cell-mediated cytotoxicity (ADCC) in HER-2-non-amplified breast cancer cell lines. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*, 23(7), pp.1788–95.

Cooper, L.A., Shen, T.-L. & Guan, J.-L., 2003. Regulation of focal adhesion kinase by its amino-terminal domain through an autoinhibitory interaction. *Molecular and cellular biology*, 23(22), pp.8030–41.

Corsi, J.-M. et al., 2006. Organization and post-transcriptional processing of focal adhesion kinase gene. *BMC genomics*, 7(1), p.198.

Creighton, C.J., 2012. The molecular profile of luminal B breast cancer. *Biologics : targets & therapy*, 6, pp.289–97.

Cuevas, B.D. et al., 2003. MEKK1 regulates calpain-dependent proteolysis of focal adhesion proteins for rear-end detachment of migrating fibroblasts. *The EMBO journal*, 22(13), pp.3346–55.

Davies, C. et al., 2013. Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. *Lancet*, 381(9869), pp.805–16.

Davies, C. et al., 2011. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet*, 378(9793), pp.771–84.

Debidda, M. et al., 2005. A role of STAT3 in Rho GTPase-regulated cell migration and proliferation. *The Journal of biological chemistry*, 280, pp.17275–17285.

Dickson, R.B. & Stancel, G.M., 2000. Estrogen receptor-mediated processes in normal and cancer cells. *Journal of the National Cancer Institute. Monographs*, (27), pp.135–45.

Diermeier, S. et al., 2005. Epidermal growth factor receptor coexpression modulates susceptibility to Herceptin in HER2/neu overexpressing breast cancer cells via specific erbB-receptor interaction and activation. *Experimental cell research*, 304(2), pp.604–19.

Diermeier-Daucher, S. et al., 2011. Modular anti-EGFR and anti-Her2 targeting of SK-BR-3 and BT474 breast cancer cell lines in the presence of ErbB receptor-specific growth factors. *Cytometry. Part A : the journal of the International Society for Analytical Cytology*, 79(9), pp.684–93.

Dittmar, T. et al., 2002. Induction of cancer cell migration by epidermal growth factor is initiated by specific phosphorylation of tyrosine 1248 of c-erbB-2 receptor via EGFR. *FASEB journal : official*

publication of the Federation of American Societies for Experimental Biology, 16(13), pp.1823–5.

Dixon, J.M., 2014. Endocrine Resistance in Breast Cancer. *New Journal of Science*, 2014, pp.1–27.

Dokmanovic, M. et al., 2011. Trastuzumab regulates IGFBP-2 and IGFBP-3 to mediate growth inhibition: implications for the development of predictive biomarkers for trastuzumab resistance. *Molecular cancer therapeutics*, 10(6), pp.917–28.

Dokmanovic, M. et al., 2014. Trastuzumab-induced recruitment of Csk-homologous kinase (CHK) to ErbB2 receptor is associated with ErbB2-Y1248 phosphorylation and ErbB2 degradation to mediate cell growth inhibition. *Cancer biology & therapy*, 15(8), pp.1029–41.

Dokmanovic, M. & Wu, W.J., 2014. Trastuzumab-induced HER2 phosphorylation: exploring the mechanisms and implications. *Receptors & Clinical Investigation*, pp.1–5.

Dowsett, M. et al., 2006. Benefit from adjuvant tamoxifen therapy in primary breast cancer patients according oestrogen receptor, progesterone receptor, EGF receptor and HER2 status. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*, 17(5), pp.818–26.

Dowsett, M., 2001. Overexpression of HER-2 as a resistance mechanism to hormonal therapy for breast cancer. *Endocrine-related cancer*, 8(3), pp.191–5.

Egeblad, M. & Werb, Z., 2002. New functions for the matrix metalloproteinases in cancer progression. *Nature reviews. Cancer*, 2(3), pp.161–74.

Eke, I. & Cordes, N., 2011. Dual targeting of EGFR and focal adhesion kinase in 3D grown HNSCC cell cultures. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology*, 99(3), pp.279–86.

Eliceiri, B.P. et al., 2002. Src-mediated coupling of focal adhesion kinase to integrin alpha(v)beta5 in vascular endothelial growth factor signaling. *The Journal of cell biology*, 157(1), pp.149–60.

Enomoto, A. et al., 2005. Akt/PKB regulates actin organization and cell motility via Girdin/APE. *Developmental cell*, 9(3), pp.389–402.

Fan, H. & Guan, J.-L., 2011. Compensatory function of Pyk2 protein in the promotion of focal adhesion kinase (FAK)-null mammary cancer stem cell tumorigenicity and metastatic activity. *The Journal of biological chemistry*, 286(21), pp.18573–82.

Fang, X.-Q. et al., 2013. *Somatic mutational analysis of FAK in breast cancer: A novel gain-of-function mutation due to deletion of exon 33*,

Fedi, P. et al., 1994. Efficient coupling with phosphatidylinositol 3-kinase, but not phospholipase C gamma or GTPase-activating protein, distinguishes ErbB-3 signaling from that of other ErbB/EGFR family members. *Molecular and cellular biology*, 14(1), pp.492–500.

Frame, M.C. et al., 2010. The FERM domain: organizing the structure and function of FAK. *Nature reviews. Molecular cell biology*, 11(11), pp.802–14.

Friedland, J.C. et al., 2007. alpha6beta4 integrin activates Rac-dependent p21-activated kinase 1 to drive NF-kappaB-dependent resistance to apoptosis in 3D mammary acini. *Journal of cell science*, 120(Pt 20), pp.3700–12.

Frisch, S.M. et al., 1996. Control of adhesion-dependent cell survival by focal adhesion kinase. *The Journal of cell biology*, 134(3), pp.793–9.

Furuyama, K. et al., 2006. Clinical Significance of Focal Adhesion Kinase in Resectable Pancreatic Cancer. *World Journal of Surgery*, 30(2), pp.219–226.

Gabarra-Niecko, V. et al., 2003. FAK regulates biological processes important for the pathogenesis of cancer. *Cancer and Metastasis Reviews*, 22(4), pp.359–74.

Gabarra-Niecko, V., Keely, P.J. & Schaller, M.D., 2002. Characterization of an activated mutant of focal adhesion kinase: "SuperFAK". *The Biochemical journal*, 365(Pt 3), pp.591–603.

Gabriel, B. et al., 2006. Weak expression of focal adhesion kinase (pp125FAK) in patients with cervical cancer is associated with poor disease outcome. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 12(8), pp.2476–83.

Gache, C. et al., 1998. Positive regulation of normal and tumoral mammary epithelial cell proliferation by fibroblasts in coculture. *In vitro cellular & developmental biology. Animal*, 34(4), pp.347–51.

Gao, M. et al., 2010. Stromal fibroblasts from the interface zone of human breast carcinomas induce an epithelial – mesenchymal transition-like state in breast cancer cells in vitro.

Gennari, R. et al., 2004. Pilot study of the mechanism of action of preoperative trastuzumab in patients with primary operable breast tumors overexpressing HER2. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 10(17), pp.5650–5.

Geyer, C.E. et al., 2006. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *The New England journal of medicine*, 355(26), pp.2733–43.

Ghosh, R. et al., 2011. Trastuzumab has preferential activity against breast cancers driven by HER2 homodimers. *Cancer research*, 71(5), pp.1871–82.

Gijsen, M. et al., 2010. HER2 phosphorylation is maintained by a PKB negative feedback loop in response to anti-HER2 herceptin in breast cancer. *PLoS biology*, 8(12), p.e1000563.

Gill, J., 2000. THE EFFECTS OF MODERATE ALCOHOL CONSUMPTION ON FEMALE HORMONE LEVELS AND REPRODUCTIVE FUNCTION. *Alcohol and Alcoholism*, 35(5), pp.417–423.

Ginestier, C. et al., 2007. ERBB2 phosphorylation and trastuzumab sensitivity of breast cancer cell lines. *Oncogene*, 26(50), pp.7163–9.

Girault, J.-A. et al., 1999. The N-termini of FAK and JAKs contain divergent band 4.1 domains. *Trends in Biochemical Sciences*, 24(2), pp.54–57.

Gluz, O. et al., 2009. Triple-negative breast cancer--current status and future directions. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*, 20(12), pp.1913–27.

Goldhirsch, A. et al., 2011. Strategies for subtypes--dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*, 22(8), pp.1736–47.

Golubovskaya, V. et al., 2002. Dual inhibition of focal adhesion kinase and epidermal growth factor receptor pathways cooperatively induces death receptor-mediated apoptosis in human breast cancer cells. *The Journal of biological chemistry*, 277(41), pp.38978–87.

Golubovskaya, V.M., Nyberg, C., et al., 2008. A small molecule inhibitor, 1,2,4,5-benzenetetraamine tetrahydrochloride, targeting the y397 site of focal adhesion kinase decreases tumor growth. *Journal of medicinal chemistry*, 51(23), pp.7405–16.

Golubovskaya, V.M., 2010. Focal adhesion kinase as a cancer therapy target. *Anti-cancer agents in medicinal chemistry*, 10(10), pp.735–41.

Golubovskaya, V.M. et al., 2014. High focal adhesion kinase expression in breast carcinoma is associated with lymphovascular invasion and triple-negative phenotype. *BMC cancer*, 14(1), p.769.

Golubovskaya, V.M. et al., 2013. Pharmacologic blockade of FAK autophosphorylation decreases human glioblastoma tumor growth and synergizes with temozolomide. *Molecular cancer therapeutics*, 12(2), pp.162–72.

Golubovskaya, V.M., Virnig, C. & Cance, W.G., 2008. TAE226-induced apoptosis in breast cancer cells with overexpressed Src or EGFR. *Molecular carcinogenesis*, 47(3), pp.222–34.

Gong, C. et al., 2011. Up-regulation of miR-21 mediates resistance to trastuzumab therapy for breast cancer. *The Journal of biological chemistry*, 286(21), pp.19127–37.

Göstring, L. et al., 2012. Cellular effects of HER3-specific affibody molecules. *PloS one*, 7(6), p.e40023.

Graf, K. et al., 1997. Mitogen-activated protein kinase activation is involved in platelet-derived growth factor-directed migration by vascular smooth muscle cells. *Hypertension*, 29(1 Pt 2), pp.334–9.

Graham, J.D. et al., Nuclear receptor conformation, coregulators, and tamoxifen-resistant breast cancer. *Steroids*, 65(10-11), pp.579–84.

Graus-Porta, D. et al., 1997. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *The EMBO journal*, 16(7), pp.1647–55.

Guan, J.-L., 2010. Integrin signaling through FAK in the regulation of mammary stem cells and breast cancer. *IUBMB life*, 62(4), pp.268–76.

Guan, Z. et al., 2013. Randomized trial of lapatinib versus placebo added to paclitaxel in the treatment of human epidermal growth factor receptor 2-overexpressing metastatic breast cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, 31(16), pp.1947–53.

Győrffy, B. et al., 2010. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. *Breast cancer research and treatment*, 123(3), pp.725–31.

Halder, J. et al., 2005. Focal adhesion kinase silencing augments docetaxel-mediated apoptosis in ovarian cancer cells. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 11(24 Pt 1), pp.8829–36.

Hamadi, A. et al., 2005. Regulation of focal adhesion dynamics and disassembly by phosphorylation of FAK at tyrosine 397. *Journal of cell science*, 118(Pt 19), pp.4415–25.

Han, D.C. & Guan, J.-L., 1999. Association of Focal Adhesion Kinase with Grb7 and Its Role in Cell Migration. *Journal of Biological Chemistry*, 274(34), pp.24425–24430.

Han, D.C., Shen, T.L. & Guan, J.L., 2000. Role of Grb7 targeting to focal contacts and its phosphorylation by focal adhesion kinase in regulation of cell migration. *The Journal of biological chemistry*, 275(37), pp.28911–7.

Hanahan, D. & Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. *Cell*, 144(5), pp.646–674.

Haque, R. et al., 2012. Impact of breast cancer subtypes and treatment on survival: an analysis spanning two decades. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*, 21(10), pp.1848–55.

Hauck, C.R., 2002. FRNK blocks v-Src-stimulated invasion and experimental metastases without effects on cell motility or growth. *The EMBO Journal*, 21(23), pp.6289–6302.

Hauck, C.R. et al., 2001. Inhibition of focal adhesion kinase expression or activity disrupts epidermal growth factor-stimulated signaling promoting the migration of invasive human carcinoma cells. *Cancer research*, 61(19), pp.7079–90.

Hauck, C.R., Hsia, D.A. & Schlaepfer, D.D., 2002. The focal adhesion kinase--a regulator of cell migration and invasion. *IUBMB life*, 53(2), pp.115–9.

Heffler, M. et al., 2013. Focal adhesion kinase autophosphorylation inhibition decreases colon cancer cell growth and enhances the efficacy of chemotherapy. *Cancer biology & therapy*, 14(8), pp.761–72.

Hernandez, R.K. et al., 2009. Tamoxifen treatment and risk of deep venous thrombosis and pulmonary embolism: a Danish population-based cohort study. *Cancer*, 115(19), pp.4442–9.

Heylen, N. et al., 1998. Effect of MRC-5 fibroblast conditioned medium on breast cancer cell motility and invasion in vitro. *Clinical & experimental metastasis*, 16(2), pp.193–203.

Hijazi, M.M. et al., 2000. Heregulin regulates the actin cytoskeleton and promotes invasive properties in breast cancer cell lines. *International journal of oncology*, 17(4), pp.629–41.

Hildebrand, J.D., Schaller, M.D. & Parsons, J.T., 1993. Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125FAK, to cellular focal adhesions. *The Journal of cell biology*, 123(4), pp.993–1005.

Hirata, E., Girotti, M. R., Viros, A., Hooper, S., Spencer-Dene, B., Matsuda, M., Sahai, E. (2015). Intravital Imaging Reveals How BRAF Inhibition Generates Drug-Tolerant Microenvironments with High Integrin β1/FAK Signaling. *Cancer Cell*, *27*(4), 574–588.

Hiscox, S. et al., 2009. Dual targeting of Src and ER prevents acquired antihormone resistance in breast cancer cells. *Breast cancer research and treatment*, 115(1), pp.57–67.

Hiscox, S. et al., 2011. Inhibition of focal adhesion kinase suppresses the adverse phenotype of endocrine-resistant breast cancer cells and improves endocrine response in endocrine-sensitive cells. *Breast cancer research and treatment*, 125(3), pp.659–69.

Van den Hooff, A., 1988. Stromal Involvement In Malignant Growth. *Advances in Cancer Research*, 50, pp.159–196.

Houston, S.J. et al., 1999. Overexpression of c-erbB2 is an independent marker of resistance to endocrine therapy in advanced breast cancer. *British journal of cancer*, 79(7-8), pp.1220–6.

Howell, A., 2006. Pure oestrogen antagonists for the treatment of advanced breast cancer. *Endocrine-related cancer*, 13(3), pp.689–706.

Howell, A. et al., Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet*, 365(9453), pp.60–2.

Hsia, D.A. et al., 2003. Differential regulation of cell motility and invasion by FAK. *The Journal of cell biology*, 160(5), pp.753–67.

Huang, C. et al., 2011. β1 integrin mediates an alternative survival pathway in breast cancer cells resistant to lapatinib. *Breast cancer research : BCR*, 13(4), p.R84.

Hudis, C. et al., 2013. A phase 1 study evaluating the combination of an allosteric AKT inhibitor (MK-2206) and trastuzumab in patients with HER2-positive solid tumors. *Breast cancer research : BCR*, 15(6), p.R110.

Hurrell, T. & Outhoff, K., 2013. The in vitro influences of epidermal growth factor and heregulin-β1 on the efficacy of trastuzumab used in Her-2 positive breast adenocarcinoma. *Cancer cell international*, 13(1), p.97.

Ihle, J.N., 2001. The Stat family in cytokine signaling. *Current opinion in cell biology*, 13(2), pp.211–7.

Ilić, D. et al., 1995. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature*, 377(6549), pp.539–44.

Infante, J.R. et al., 2012. Safety, Pharmacokinetic, and Pharmacodynamic Phase I Dose-Escalation Trial of PF-00562271, an Inhibitor of Focal Adhesion Kinase, in Advanced Solid Tumors. *Journal*

of clinical oncology : official journal of the American Society of Clinical Oncology, 30(13), pp.1527–33.

Izaguirre, G. et al., 2001. The cytoskeletal/non-muscle isoform of alpha-actinin is phosphorylated on its actin-binding domain by the focal adhesion kinase. *The Journal of biological chemistry*, 276(31), pp.28676–85.

Izumchenko, E. et al., 2009. NEDD9 promotes oncogenic signaling in mammary tumor development. *Cancer research*, 69(18), pp.7198–206.

Izumi, Y. et al., 2002. Tumour biology: herceptin acts as an anti-angiogenic cocktail. *Nature*, 416(6878), pp.279–80.

Jean, C. et al., 2014. Inhibition of endothelial FAK activity prevents tumor metastasis by enhancing barrier function. *The Journal of Cell Biology*, 204(2), pp.247–263.

Jones, K.L., 2004. A review of adjuvant hormonal therapy in breast cancer. *Endocrine Related Cancer*, 11(3), pp.391–406.

Junttila, T.T. et al., 2009. Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. *Cancer cell*, 15(5), pp.429–40.

Kahana, O. et al., 2002. The focal adhesion kinase (P125FAK) is constitutively active in human malignant melanoma. *Oncogene*, 21(25), pp.3969–77.

Kahlert, S. et al., 2000. Estrogen receptor alpha rapidly activates the IGF-1 receptor pathway. *The Journal of biological chemistry*, 275(24), pp.18447–53.

Kalluri, R. & Zeisberg, M., 2006. Fibroblasts in cancer. *Nature reviews. Cancer*, 6(5), pp.392–401.

Kasahara, T. et al., 2002. Antiapoptotic action of focal adhesion kinase (FAK) against ionizing radiation. *Antioxidants & redox signaling*, 4(3), pp.491–9.

Kauraniemi, P. et al., 2003. Amplification of a 280-kilobase core region at the ERBB2 locus leads to activation of two hypothetical proteins in breast cancer. *The American journal of pathology*, 163(5), pp.1979–84.

Key, T.J., Verkasalo, P.K. & Banks, E., 2001. Epidemiology of breast cancer. *The lancet oncology*, 2(3), pp.133–40.

Kim, J. et al., 2013. HRG-β1-driven ErbB3 signaling induces epithelial-mesenchymal transition in breast cancer cells. *BMC cancer*, 13(1), p.383.

Kim, S.-H., Turnbull, J. & Guimond, S., 2011. Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor. *The Journal of endocrinology*, 209(2), pp.139–51.

Klemke, R.L., 1997. Regulation of Cell Motility by Mitogen-activated Protein Kinase. *The Journal of Cell Biology*, 137(2), pp.481–492.

Klingbeil, C.K. et al., 2001. Targeting Pyk2 to beta 1-integrin-containing focal contacts rescues fibronectin-stimulated signaling and haptotactic motility defects of focal adhesion kinase-null cells. *The Journal of cell biology*, 152(1), pp.97–110.

Klinge, C.M. et al., 2005. Resveratrol and estradiol rapidly activate MAPK signaling through estrogen receptors alpha and beta in endothelial cells. *The Journal of biological chemistry*, 280(9), pp.7460–8.

Knowlden, J.M. et al., 2003. Elevated Levels of Epidermal Growth Factor Receptor/c-erbB2 Heterodimers Mediate an Autocrine Growth Regulatory Pathway in Tamoxifen-Resistant MCF-7 Cells. *Endocrinology*, 144(3), pp.1032–1044.

Kolev, V.N. et al., 2014. Abstract A39: FAK inhibitors VS-6063 and VS-4718 preferentially target ovarian cancer stem cells. *Clinical Cancer Research*, 19(19_Supplement), pp.A39–A39.

Kornberg, L. et al., 1992. Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase. *The Journal of biological chemistry*, 267(33), pp.23439–42.

Kostourou, V. et al., 2013. FAK-heterozygous mice display enhanced tumour angiogenesis. *Nature communications*, 4, p.2020.

Kunisue, H. et al., 2000. Anti-HER2 antibody enhances the growth inhibitory effect of anti-oestrogen on breast cancer cells expressing both oestrogen receptors and HER2. *British journal of cancer*, 82(1), pp.46–51.

Kuramochi, Y., Guo, X. & Sawyer, D.B., 2006. Neuregulin activates erbB2-dependent src/FAK signaling and cytoskeletal remodeling in isolated adult rat cardiac myocytes. *Journal of molecular and cellular cardiology*, 41(2), pp.228–35.

Ladjemi, M.Z. et al., 2010. Anti-HER2 vaccines: new prospects for breast cancer therapy. *Cancer immunology, immunotherapy : CII*, 59(9), pp.1295–312.

Lark, A.L. et al., 2005. High focal adhesion kinase expression in invasive breast carcinomas is associated with an aggressive phenotype. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc*, 18(10), pp.1289–94.

De Laurentiis, M. et al., 2005. A meta-analysis on the interaction between HER-2 expression and response to endocrine treatment in advanced breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 11(13), pp.4741–8.

Lee, J. et al., 2010. Conditional deletion of the focal adhesion kinase FAK alters remodeling of the blood-brain barrier in glioma. *Cancer research*, 70(24), pp.10131–40.

Lee, M.J. et al., 2012. Sequential application of anticancer drugs enhances cell death by rewiring apoptotic signaling networks. *Cell*, 149(4), pp.780–94.

Lefebvre, M.F. et al., 1995. Influence of tumor derived fibroblasts and 1,25-dihydroxyvitamin D3 on growth of breast cancer cell lines. *Breast cancer research and treatment*, 33(3), pp.189–97.

Lewis, G.D. et al., 1996. Growth regulation of human breast and ovarian tumor cells by heregulin: Evidence for the requirement of ErbB2 as a critical component in mediating heregulin responsiveness. *Cancer research*, 56(6), pp.1457–65.

Lewis Phillips, G.D. et al., 2008. Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. *Cancer research*, 68(22), pp.9280–90.

Li, Q., Ahmed, S. & Loeb, J.A., 2004. Development of an autocrine neuregulin signaling loop with malignant transformation of human breast epithelial cells. *Cancer research*, 64(19), pp.7078–85.

Liang, J. & Slingerland, J.M., Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. *Cell cycle (Georgetown, Tex.)*, 2(4), pp.339–45.

Liekens, S., De Clercq, E. & Neyts, J., 2001. Angiogenesis: regulators and clinical applications. *Biochemical pharmacology*, 61(3), pp.253–70.

Lietha, D. et al., 2007. Structural basis for the autoinhibition of focal adhesion kinase. *Cell*, 129(6), pp.1177–87.

Lightfoot, H.M. et al., 2004. Upregulation of focal adhesion kinase (FAK) expression in ductal carcinoma in situ (DCIS) is an early event in breast tumorigenesis. *Breast cancer research and treatment*, 88(2), pp.109–16.

Lim, S.-T. et al., 2008. Nuclear FAK promotes cell proliferation and survival through FERM-enhanced p53 degradation. *Molecular cell*, 29(1), pp.9–22.

Lim, S.-T. et al., 2012. Nuclear-localized focal adhesion kinase regulates inflammatory VCAM-1 expression. *The Journal of Cell Biology*, 197(7), pp.907–919.

Lim, S.-T.S., 2013. Nuclear FAK: a new mode of gene regulation from cellular adhesions. *Molecules and cells*.

Lim, Y. et al., 2004. Phosphorylation of focal adhesion kinase at tyrosine 861 is crucial for Ras transformation of fibroblasts. *The Journal of biological chemistry*, 279(28), pp.29060–5.

Lim, Y. et al., 2008. PyK2 and FAK connections to p190Rho guanine nucleotide exchange factor regulate RhoA activity, focal adhesion formation, and cell motility. *The Journal of Cell Biology*, 180(1), pp.187–203.

Liu, E. et al., 1992. The HER2 (c-erbB-2) oncogene is frequently amplified in in situ carcinomas of the breast. *Oncogene*, 7(5), pp.1027–32.

Longva, K.E. et al., 2005. Herceptin-induced inhibition of ErbB2 signaling involves reduced phosphorylation of Akt but not endocytic down-regulation of ErbB2. *International journal of cancer. Journal international du cancer*, 116(3), pp.359–67.

Lu, Y. et al., 2001. Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). *Journal of the National Cancer Institute*, 93(24), pp.1852–7.

Luo, M. et al., 2009. Mammary epithelial-specific ablation of the focal adhesion kinase suppresses mammary tumorigenesis by affecting mammary cancer stem/progenitor cells. *Cancer research*, 69(2), pp.466–74.

Mañes, S. et al., 1999. Concerted activity of tyrosine phosphatase SHP-2 and focal adhesion kinase in regulation of cell motility. *Molecular and cellular biology*, 19(4), pp.3125–35.

Manning, B.D. & Cantley, L.C., 2007. AKT/PKB signaling: navigating downstream. *Cell*, 129(7), pp.1261–74.

Marmor, M.D., Skaria, K.B. & Yarden, Y., 2004. Signal transduction and oncogenesis by ErbB/HER receptors. *International journal of radiation oncology, biology, physics*, 58(3), pp.903–13.

Marty, M. et al., 2005. Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer administered as first-line treatment: the M77001 study group. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, 23(19), pp.4265–74.

Matter, M.L. & Ruoslahti, E., 2001. A signaling pathway from the alpha5beta1 and alpha(v)beta3 integrins that elevates bcl-2 transcription. *The Journal of biological chemistry*, 276(30), pp.27757–63.

McCawley, L.J. & Matrisian, L.M., 2000. Matrix metalloproteinases: multifunctional contributors to tumor progression. *Molecular Medicine Today*, 6(4), pp.149–156.

McClelland, R.A. et al., 2001. Enhanced epidermal growth factor receptor signaling in MCF7 breast cancer cells after long-term culture in the presence of the pure antiestrogen ICI 182,780 (Faslodex). *Endocrinology*, 142(7), pp.2776–88.

McCubrey, J.A. et al., 2006. Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. *Advances in enzyme regulation*, 46(1), pp.249–79.

McPherson, K., Steel, C.M. & Dixon, J.M., 2000. ABC of breast diseases. Breast cancer-epidemiology, risk factors, and genetics. *BMJ (Clinical research ed.)*, 321(7261), pp.624–8.

Meric-Bernstam, F. & Hung, M.-C., 2006. Advances in targeting human epidermal growth factor receptor-2 signaling for cancer therapy. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 12(21), pp.6326–30.

Millar, E.K.A. et al., 2009. Prediction of local recurrence, distant metastases, and death after breast-conserving therapy in early-stage invasive breast cancer using a five-biomarker panel. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, 27(28), pp.4701–8.

Min, A. et al., 2014. Focal Adhesion Kinase Knockdown in Carcinoma-Associated Fibroblasts Inhibits Oral Squamous Cell Carcinoma Metastasis via Downregulating MCP-1/CCL2 Expression. *Journal of biochemical and molecular toxicology*.

Mitra, S.K. et al., 2006. Intrinsic FAK activity and Y925 phosphorylation facilitate an angiogenic switch in tumors. *Oncogene*, 25(44), pp.5969–84.

Mitra, S.K., Hanson, D.A. & Schlaepfer, D.D., 2005. Focal adhesion kinase: in command and control of cell motility. *Nature reviews. Molecular cell biology*, 6(1), pp.56–68.

Mohd Nafi, S.N. et al., 2014. Nuclear HER4 mediates acquired resistance to trastuzumab and is associated with poor outcome in HER2 positive breast cancer. *Oncotarget*, 5(15), pp.5934–49.

Molife, L.R. et al., 2014. Phase 1 trial of the oral AKT inhibitor MK-2206 plus carboplatin/paclitaxel, docetaxel, or erlotinib in patients with advanced solid tumors. *Journal of hematology & oncology*, 7(1), p.1.

Molina, M.A. et al., 2002. NH(2)-terminal truncated HER-2 protein but not full-length receptor is associated with nodal metastasis in human breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 8(2), pp.347–53.

Molina, M.A. et al., 2001. Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. *Cancer research*, 61(12), pp.4744–9.

Moll, R. et al., 1993. Differential loss of E-cadherin expression in infiltrating ductal and lobular breast carcinomas. *The American journal of pathology*, 143(6), pp.1731–42.

Montemurro, F. et al., 2012. Hormone-receptor expression and activity of trastuzumab with chemotherapy in HER2-positive advanced breast cancer patients. *Cancer*, 118(1), pp.17–26.

Morandi, A. & Isacke, C.M., 2014. Targeting RET-interleukin-6 crosstalk to impair metastatic dissemination in breast cancer. *Breast cancer research : BCR*, 16(1), p.301.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods*, 65(1-2), pp.55–63.

Mueller, M.M. & Fusenig, N.E., 2004. Friends or foes - bipolar effects of the tumour stroma in cancer. *Nature reviews. Cancer*, 4(11), pp.839–49.

N, B., Bioisosteres in Medicinal Chemistry - Wiley Online Library. Available at: http://onlinelibrary.wiley.com/book/10.1002/9783527654307 [Accessed March 13, 2015].

Nagata, Y. et al., 2004. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer cell*, 6(2), pp.117–27.

Nagy, P. et al., 2005. Decreased accessibility and lack of activation of ErbB2 in JIMT-1, a herceptin-resistant, MUC4-expressing breast cancer cell line. *Cancer research*, 65(2), pp.473–82.

Nahta, R. et al., 2007. Lapatinib induces apoptosis in trastuzumab-resistant breast cancer cells: effects on insulin-like growth factor I signaling. *Molecular cancer therapeutics*, 6(2), pp.667–74.

Nahta, R. & Esteva, F.J., 2007. Trastuzumab: triumphs and tribulations. *Oncogene*, 26(25), pp.3637–43.

Nahta, S.S. and R., 2011. *Breast Cancer - Current and Alternative Therapeutic Modalities* E. Gunduz, ed., InTech.

Neve, R.M. et al., 2006. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer cell*, 10(6), pp.515–27.

Ng, D.C.H. et al., 2006. Stat3 regulates microtubules by antagonizing the depolymerization activity of stathmin. *The Journal of cell biology*, 172(2), pp.245–57.

Van Nimwegen, M.J. et al., 2006. Focal adhesion kinase and protein kinase B cooperate to suppress doxorubicin-induced apoptosis of breast tumor cells. *Molecular pharmacology*, 70(4), pp.1330–9.

Van Nimwegen, M.J. & van de Water, B., 2007. Focal adhesion kinase: a potential target in cancer therapy. *Biochemical pharmacology*, 73(5), pp.597–609.

Nista, A. et al., 1997. Functional role of alpha4beta1 and alpha5beta1 integrin fibronectin receptors expressed on adriamycin-resistant MCF-7 human mammary carcinoma cells. *International journal of cancer. Journal international du cancer*, 72(1), pp.133–41.

O'Brien, N.A. et al., 2010. Activated phosphoinositide 3-kinase/AKT signaling confers resistance to trastuzumab but not lapatinib. *Molecular cancer therapeutics*, 9(6), pp.1489–502.

O'Brien, S. et al., 2014. FAK inhibition with small molecule inhibitor Y15 decreases viability, clonogenicity, and cell attachment in thyroid cancer cell lines and synergizes with targeted therapeutics. *Oncotarget*, 5(17), pp.7945–59.

Okigaki, M. et al., 2003. Pyk2 regulates multiple signaling events crucial for macrophage morphology and migration. *Proceedings of the National Academy of Sciences of the United States of America*, 100(19), pp.10740–5.

Okoshi, R. et al., 2011. Heregulin β-1 induces loss of cell-cell contact and enhances expression of MUC1 at the cell surface in HCC2998 and MKN45-1 cells. *PloS one*, 6(12), p.e29599.

Olayioye, M.A. et al., 2000. The ErbB signaling network: receptor heterodimerization in development and cancer. *The EMBO journal*, 19(13), pp.3159–67.

Olayioye, M.A., 2001. Update on HER-2 as a target for cancer therapy: intracellular signaling pathways of ErbB2/HER-2 and family members. *Breast cancer research : BCR*, 3(6), pp.385–9.

Olumi, A.F. et al., 1999. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer research*, 59(19), pp.5002–11.

Onitilo, A.A. et al., 2009. Breast cancer subtypes based on ER/PR and Her2 expression: comparison of clinicopathologic features and survival. *Clinical medicine & research*, 7(1-2), pp.4–13.

Orimo, A. et al., 2005. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell*, 121(3), pp.335–48.

Osborne, C.K. & Schiff, R., 2011. Mechanisms of endocrine resistance in breast cancer. *Annual review of medicine*, 62, pp.233–47.

Otomo, R. et al., 2014. TSPAN12 is a critical factor for cancer-fibroblast cell contact-mediated cancer invasion. *Proceedings of the National Academy of Sciences of the United States of America*, 111(52), pp.18691–6.

Owen, J.D. et al., 1999. Induced focal adhesion kinase (FAK) expression in FAK-null cells enhances cell spreading and migration requiring both auto- and activation loop phosphorylation sites and inhibits adhesion-dependent tyrosine phosphorylation of Pyk2. *Molecular and cellular biology*, 19(7), pp.4806–18.

Owens, L. V et al., 1995. Overexpression of the focal adhesion kinase (p125FAK) in invasive human tumors. *Cancer research*, 55(13), pp.2752–5.

Pachter, J.A. et al., 2014. Abstract 4797: VS-6063 (defactinib) targets cancer stem cells directly and through inhibition of tumor-associated macrophages and cytokine production. *Cancer Res.*, 74(19_Supplement), p.4797–.

Palazzo, A.F. et al., 2004. Localized stabilization of microtubules by integrin- and FAK-facilitated Rho signaling. *Science (New York, N.Y.)*, 303(5659), pp.836–9.

Park, J.W. et al., 2008. Unraveling the biologic and clinical complexities of HER2. *Clinical breast cancer*, 8(5), pp.392–401.

Parker, J.S. et al., 2009. Supervised risk predictor of breast cancer based on intrinsic subtypes. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, 27(8), pp.1160–7.

Parsons, J.T. et al., 2000. Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. *Oncogene*, 19(49), pp.5606–13.

Pedersen, K. et al., 2009. A naturally occurring HER2 carboxy-terminal fragment promotes mammary tumor growth and metastasis. *Molecular and cellular biology*, 29(12), pp.3319–31.

Peiró, G. et al., 2014. Src, a potential target for overcoming trastuzumab resistance in HER2-positive breast carcinoma. *British journal of cancer*, 111(4), pp.689–95.

Peng, X. et al., 2004. Overexpression of focal adhesion kinase in vascular endothelial cells promotes angiogenesis in transgenic mice. *Cardiovascular research*, 64(3), pp.421–30.

Perou, C.M. et al., 2000. Molecular portraits of human breast tumours. *Nature*, 406(6797), pp.747–52.

Pharoah, P.D. et al., 1997. Family history and the risk of breast cancer: a systematic review and meta-analysis. *International journal of cancer. Journal international du cancer*, 71(5), pp.800–9.

Piccart-Gebhart, M.J. et al., 2005. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *The New England journal of medicine*, 353(16), pp.1659–72.

Pickl, M. & Ries, C.H., 2009. Comparison of 3D and 2D tumor models reveals enhanced HER2 activation in 3D associated with an increased response to trastuzumab. *Oncogene*, 28(3), pp.461–8.

Plaza-Menacho, I. et al., 2011. Focal adhesion kinase (FAK) binds RET kinase via its FERM domain, priming a direct and reciprocal RET-FAK transactivation mechanism. *The Journal of biological chemistry*, 286(19), pp.17292–302.

Plowman, G.D. et al., 1993. Heregulin induces tyrosine phosphorylation of HER4/p180erbB4. *Nature*, 366(6454), pp.473–5.

Ponnusamy, M.P. et al., 2008. MUC4 activates HER2 signalling and enhances the motility of human ovarian cancer cells. *British journal of cancer*, 99(3), pp.520–6.

Pontiggia, O. et al., 2012. The tumor microenvironment modulates tamoxifen resistance in breast cancer: a role for soluble stromal factors and fibronectin through β1 integrin. *Breast cancer research and treatment*, 133(2), pp.459–71.

Pradip, D. et al., 2013. Dissecting GRB7-mediated signals for proliferation and migration in HER2 overexpressing breast tumor cells: GTP-ase rules. *American journal of cancer research*, 3(2), pp.173–95.

Prat, A. & Baselga, J., 2008. The role of hormonal therapy in the management of hormonal-receptor-positive breast cancer with co-expression of HER2. *Nature clinical practice. Oncology*, 5(9), pp.531–42.

Prat, A. & Perou, C.M., 2011. Deconstructing the molecular portraits of breast cancer. *Molecular oncology*, 5(1), pp.5–23.

Provenzano, P.P. et al., 2008. Mammary epithelial-specific disruption of focal adhesion kinase retards tumor formation and metastasis in a transgenic mouse model of human breast cancer. *The American journal of pathology*, 173(5), pp.1551–65.

Razandi, M. et al., 2003. Proximal events in signaling by plasma membrane estrogen receptors. *The Journal of biological chemistry*, 278(4), pp.2701–12.

Razis, E. et al., 2011. Evaluation of the association of PIK3CA mutations and PTEN loss with efficacy of trastuzumab therapy in metastatic breast cancer. *Breast cancer research and treatment*, 128(2), pp.447–56.

Recher, C. et al., 2004. Expression of focal adhesion kinase in acute myeloid leukemia is associated with enhanced blast migration, increased cellularity, and poor prognosis. *Cancer research*, 64(9), pp.3191–7.

Reiske, H.R. et al., 1999. Requirement of phosphatidylinositol 3-kinase in focal adhesion kinase-promoted cell migration. *The Journal of biological chemistry*, 274(18), pp.12361–6.

Ren, X.D. et al., 2000. Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. *Journal of cell science*, 113 (Pt 2, pp.3673–8.

Ritter, C.A. et al., 2007. Human breast cancer cells selected for resistance to trastuzumab in vivo overexpress epidermal growth factor receptor and ErbB ligands and remain dependent on the ErbB receptor network. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 13(16), pp.4909–19.

Roberts, W.G. et al., 2008. Antitumor activity and pharmacology of a selective focal adhesion kinase inhibitor, PF-562,271. *Cancer research*, 68(6), pp.1935–44.

Rodier, F. & Campisi, J., 2011. Four faces of cellular senescence. *The Journal of cell biology*, 192(4), pp.547–56.

Rodrik-Outmezguine, V.S. et al., 2011. mTOR kinase inhibition causes feedback-dependent biphasic regulation of AKT signaling. *Cancer discovery*, 1(3), pp.248–59.

Rossi, L. et al., Fibroblasts regulate the migration of MCF7 mammary carcinoma cells in hydrated collagen gel. *Anticancer research*, 14(4A), pp.1493–501.

Rozenchan, P.B. et al., 2009. Reciprocal changes in gene expression profiles of cocultured breast epithelial cells and primary fibroblasts. *International journal of cancer. Journal international du cancer*, 125(12), pp.2767–77.

Ruan, S.-Q. et al., 2012. Heregulin-β1-induced GPR30 upregulation promotes the migration and invasion potential of SkBr3 breast cancer cells via ErbB2/ErbB3-MAPK/ERK pathway. *Biochemical and biophysical research communications*, 420(2), pp.385–90.

Ryan, M.C., Orr, D.J. & Horgan, K., 1993. Fibroblast stimulation of breast cancer cell growth in a serum-free system. *British journal of cancer*, 67(6), pp.1268–73.

Sáez, R. et al., 2006. p95HER-2 predicts worse outcome in patients with HER-2-positive breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 12(2), pp.424–31.

Sanchez, A.M. et al., 2013. Estrogen Receptor-α Promotes Breast Cancer Cell Motility and Invasion via Focal Adhesion Kinase and N-WASP. *Molecular Endocrinology*.

Schaller, M.D. et al., 1994. Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Molecular and cellular biology*, 14(3), pp.1680–8.

Schaller, M.D., 2010. Cellular functions of FAK kinases: insight into molecular mechanisms and novel functions. *Journal of cell science*, 123(Pt 7), pp.1007–13.

Schaller, M.D. et al., 1995. Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains. *The Journal of cell biology*, 130(5), pp.1181–7.

Schaller, M.D., 2001. Paxillin: a focal adhesion-associated adaptor protein. *Oncogene*, 20(44), pp.6459–72.

Schaller, M.D. et al., 1992. pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proceedings of the National Academy of Sciences of the United States of America*, 89(11), pp.5192–6.

Schaller, M.D., Borgman, C.A. & Parsons, J.T., 1993. Autonomous expression of a noncatalytic domain of the focal adhesion-associated protein tyrosine kinase pp125FAK. *Molecular and cellular biology*, 13(2), pp.785–91.

Schlaepfer, D.D., Broome, M.A. & Hunter, T., 1997. Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130cas, and Nck adaptor proteins. *Molecular and cellular biology*, 17(3), pp.1702–13.

Schlaepfer, D.D., Mitra, S.K. & Ilic, D., 2004. Control of motile and invasive cell phenotypes by focal adhesion kinase. *Biochimica et biophysica acta*, 1692(2-3), pp.77–102.

Schmitz, K.J. et al., 2005. High expression of focal adhesion kinase (p125FAK) in node-negative breast cancer is related to overexpression of HER-2/neu and activated Akt kinase but does not predict outcome. *Breast cancer research : BCR*, 7(2), pp.R194–203.

Scholzen, T. & Gerdes, J., 2000. The Ki-67 protein: from the known and the unknown. *Journal of cellular physiology*, 182(3), pp.311–22.

Schulze, W.X., Deng, L. & Mann, M., 2005. Phosphotyrosine interactome of the ErbB-receptor kinase family. *Molecular systems biology*, 1, p.2005.0008.

Schust, J. et al., 2006. Stattic: a small-molecule inhibitor of STAT3 activation and dimerization. *Chemistry & biology*, 13(11), pp.1235–42.

Serrels, A. et al., Src/FAK-mediated regulation of E-cadherin as a mechanism for controlling collective cell movement: insights from in vivo imaging. *Cell adhesion & migration*, 5(4), pp.360–5.

Serrels, B. et al., 2007. Focal adhesion kinase controls actin assembly via a FERM-mediated interaction with the Arp2/3 complex. *Nature cell biology*, 9(9), pp.1046–56.

Sharma, A. & Mayer, B.J., 2008. Phosphorylation of p130Cas initiates Rac activation and membrane ruffling. *BMC cell biology*, 9(1), p.50.

Shattuck, D.L. et al., 2008. Met receptor contributes to trastuzumab resistance of Her2-overexpressing breast cancer cells. *Cancer research*, 68(5), pp.1471–7.

Shen, T.-L. et al., 2005. Conditional knockout of focal adhesion kinase in endothelial cells reveals its role in angiogenesis and vascular development in late embryogenesis. *The Journal of cell biology*, 169(6), pp.941–52.

Shi, Q. et al., 2007. A novel low-molecular weight inhibitor of focal adhesion kinase, TAE226, inhibits glioma growth. *Molecular Carcinogenesis*, 46(6), pp.488–496.

Shiau, A.K. et al., 1998. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell*, 95(7), pp.927–37.

Shono, T., Kanetake, H. & Kanda, S., 2001. The role of mitogen-activated protein kinase activation within focal adhesions in chemotaxis toward FGF-2 by murine brain capillary endothelial cells. *Experimental cell research*, 264(2), pp.275–83.

Shou, J. et al., 2004. Mechanisms of Tamoxifen Resistance: Increased Estrogen Receptor-HER2/neu Cross-Talk in ER/HER2-Positive Breast Cancer. *JNCI Journal of the National Cancer Institute*, 96(12), pp.926–935.

Sieg, D., Hauck, C. & Schlaepfer, D., 1999. Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. *J. Cell Sci.*, 112(16), pp.2677–2691.

Sieg, D.J. et al., 2000. FAK integrates growth-factor and integrin signals to promote cell migration. *Nature cell biology*, 2(5), pp.249–56.

Sieg, D.J. et al., 1998. Pyk2 and Src-family protein-tyrosine kinases compensate for the loss of FAK in fibronectin-stimulated signaling events but Pyk2 does not fully function to enhance FAK- cell migration. *The EMBO journal*, 17(20), pp.5933–47.

Sieg, D.J., Hauck, C.R. & Schlaepfer, D.D., 1999. Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. *Journal of cell science*, 112 (Pt 1, pp.2677–91.

Silver, D.L. et al., 2004. Activated signal transducer and activator of transcription (STAT) 3: localization in focal adhesions and function in ovarian cancer cell motility. *Cancer research*, 64(10), pp.3550–8.

Sima, N. et al., 2013. The overexpression of scaffolding protein NEDD9 promotes migration and invasion in cervical cancer via tyrosine phosphorylated FAK and SRC. *PloS one*, 8(9), p.e74594.

Slack-Davis, J.K. et al., 2007. Cellular characterization of a novel focal adhesion kinase inhibitor. *The Journal of biological chemistry*, 282(20), pp.14845–52.

Slamon, D.J. et al., 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science (New York, N.Y.)*, 235(4785), pp.177–82.

Slamon, D.J. et al., 2001. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *The New England journal of medicine*, 344(11), pp.783–92.

Smith, I. et al., 2007. 2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial. *The Lancet*, 369(9555), pp.29–36.

Sørlie, T. et al., 2001. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proceedings of the National Academy of Sciences of the United States of America*, 98(19), pp.10869–74.

Sotiriou, C. et al., 2003. Breast cancer classification and prognosis based on gene expression profiles from a population-based study. *Proceedings of the National Academy of Sciences of the United States of America*, 100(18), pp.10393–8.

Sperinde, J. et al., 2010. Quantitation of p95HER2 in paraffin sections by using a p95-specific antibody and correlation with outcome in a cohort of trastuzumab-treated breast cancer patients. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 16(16), pp.4226–35.

Stokes, J.B. et al., 2011. Inhibition of focal adhesion kinase by PF-562,271 inhibits the growth and metastasis of pancreatic cancer concomitant with altering the tumor microenvironment. *Molecular cancer therapeutics*, 10(11), pp.2135–45.

Stricker, J., Falzone, T. & Gardel, M.L., 2010. Mechanics of the F-actin cytoskeleton. *Journal of biomechanics*, 43(1), pp.9–14.

Studebaker, A.W. et al., 2008. Fibroblasts isolated from common sites of breast cancer metastasis enhance cancer cell growth rates and invasiveness in an interleukin-6-dependent manner. *Cancer research*, 68(21), pp.9087–95.

Stuelten, C.H. et al., 2005. Breast cancer cells induce stromal fibroblasts to express MMP-9 via secretion of TNF-alpha and TGF-beta. *Journal of cell science*, 118(Pt 10), pp.2143–53.

Stupack, D.G. & Cheresh, D.A., 2002. Get a ligand, get a life: integrins, signaling and cell survival. *Journal of cell science*, 115(Pt 19), pp.3729–38.

Sun, M. et al., 2001. Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor alpha (ERalpha) via interaction between ERalpha and PI3K. *Cancer research*, 61(16), pp.5985–91.

Swerdlow, A.J. & Jones, M.E., 2005. Tamoxifen treatment for breast cancer and risk of endometrial cancer: a case-control study. *Journal of the National Cancer Institute*, 97(5), pp.375–84.

Tallarida, R.J., 2001. Drug Synergism: Its Detection and Applications. *J. Pharmacol. Exp. Ther.*, 298(3), pp.865–872.

Tamura, M. et al., 1998. Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. *Science (New York, N.Y.)*, 280(5369), pp.1614–7.

Tamura, M. et al., 1999. Tumor suppressor PTEN inhibition of cell invasion, migration, and growth: differential involvement of focal adhesion kinase and p130Cas. *Cancer research*, 59(2), pp.442–9.

Tan, M., Grijalva, R. & Yu, D., 1999. Heregulin {beta}1-activated Phosphatidylinositol 3-Kinase Enhances Aggregation of MCF-7 Breast Cancer Cells Independent of Extracellular Signal-regulated Kinase. *Cancer Res.*, 59(7), pp.1620–1625.

Tanjoni, I. et al., 2010. PND-1186 FAK inhibitor selectively promotes tumor cell apoptosis in three-dimensional environments. *Cancer biology & therapy*, 9(10), pp.764–77.

Tavora, B. et al., 2010. Endothelial FAK is required for tumour angiogenesis. *EMBO molecular medicine*, 2(12), pp.516–28.

Taylor, J.M. et al., 2001. Selective expression of an endogenous inhibitor of FAK regulates proliferation and migration of vascular smooth muscle cells. *Molecular and cellular biology*, 21(5), pp.1565–72.

Teng, T.S. et al., 2009. Stat3 promotes directional cell migration by regulating Rac1 activity via its activator betaPIX. *Journal of cell science*, 122(Pt 22), pp.4150–9.

Tikhomirov, O. & Carpenter, G., 2004. Ligand-induced, p38-dependent apoptosis in cells expressing high levels of epidermal growth factor receptor and ErbB-2. *The Journal of biological chemistry*, 279(13), pp.12988–96.

Tilghman, R.W. et al., 2005. Focal adhesion kinase is required for the spatial organization of the leading edge in migrating cells. *Journal of cell science*, 118(Pt 12), pp.2613–23.

Tlsty, T.D. & Hein, P.W., 2001. Know thy neighbor: stromal cells can contribute oncogenic signals. *Current opinion in genetics & development*, 11(1), pp.54–9.

Tomar, A. et al., 2009. A FAK-p120RasGAP-p190RhoGAP complex regulates polarity in migrating cells. *Journal of cell science*, 122(Pt 11), pp.1852–62.

Turner, C.E., Schaller, M.D. & Parsons, J.T., 1993. Tyrosine phosphorylation of the focal adhesion kinase pp125FAK during development: relation to paxillin. *Journal of cell science*, 105 (Pt 3, pp.637–45.

Tyan, S.-W. et al., 2011. Breast cancer cells induce cancer-associated fibroblasts to secrete hepatocyte growth factor to enhance breast tumorigenesis. X. Guan, ed. *PloS one*, 6(1), p.e15313.

Vadlamudi, R.K. et al., 2002. Differential regulation of components of the focal adhesion complex by heregulin: role of phosphatase SHP-2. *Journal of cellular physiology*, 190(2), pp.189–99.

Vadlamudi, R.K. et al., 2003. Heregulin and HER2 signaling selectively activates c-Src phosphorylation at tyrosine 215. *FEBS letters*, 543(1-3), pp.76–80.

Vadlamudi, R.K. et al., 2003. Heregulin and HER2 signaling selectively activates c-Src phosphorylation at tyrosine 215. *FEBS letters*, 543(1-3), pp.76–80.

Vartanian, T. et al., 2000. Neuregulin induces the rapid association of focal adhesion kinase with the erbB2-erbB3 receptor complex in schwann cells. *Biochemical and biophysical research communications*, 271(2), pp.414–7.

Venkateswarlu, S. et al., 2002. Autocrine heregulin generates growth factor independence and blocks apoptosis in colon cancer cells. *Oncogene*, 21(1), pp.78–86.

Vicier, C. et al., 2014. Clinical development of mTOR inhibitors in breast cancer. *Breast cancer research : BCR*, 16(1), p.203.

Visscher, D.W. et al., 1997. Clinicopathologic analysis of amphiregulin and heregulin immunostaining in breast neoplasia. *Breast cancer research and treatment*, 45(1), pp.75–80.

Vogel, C.L. et al., 2001. First-line Herceptin monotherapy in metastatic breast cancer. *Oncology*, 61 Suppl 2, pp.37–42.

Wakeling, A.E., Dukes, M. & Bowler, J., 1991. A potent specific pure antiestrogen with clinical potential. *Cancer research*, 51(15), pp.3867–73.

Wallasch, C. et al., 1995. Heregulin-dependent regulation of HER2/neu oncogenic signaling by heterodimerization with HER3. *The EMBO journal*, 14(17), pp.4267–75.

Walsh, C. et al., 2010. Oral delivery of PND-1186 FAK inhibitor decreases tumor growth and spontaneous breast to lung metastasis in pre-clinical models. *Cancer biology & therapy*, 9(10), pp.778–90.

Wang, C.-X. et al., 2005. In vitro and in vivo effects of combination of Trastuzumab (Herceptin) and Tamoxifen in breast cancer. *Breast cancer research and treatment*, 92(3), pp.251–63.

Wang, S.E. et al., 2005. Transforming growth factor {beta} (TGF-{beta})-Smad target gene protein tyrosine phosphatase receptor type kappa is required for TGF-{beta} function. *Molecular and cellular biology*, 25(11), pp.4703–15.

Wang, W., Liu, Y. & Liao, K., 2011. Tyrosine phosphorylation of cortactin by the FAK-Src complex at focal adhesions regulates cell motility. *BMC Cell Biology*, 12(1), p.49.

Wang, Y.-C. et al., 2011. Different mechanisms for resistance to trastuzumab versus lapatinib in HER2-positive breast cancers--role of estrogen receptor and HER2 reactivation. *Breast cancer research : BCR*, 13(6), p.R121.

Wang, Z.G. et al., 2008. TAE226, a dual inhibitor for FAK and IGF-IR, has inhibitory effects on mTOR signaling in esophageal cancer cells. *Oncology reports*, 20(6), pp.1473–7.

Ward, K.K. et al., 2013. Inhibition of focal adhesion kinase (FAK) activity prevents anchorage-independent ovarian carcinoma cell growth and tumor progression. *Clinical & experimental metastasis*, 30(5), pp.579–94.

Watermann, D.O. et al., 2005. Specific induction of pp125 focal adhesion kinase in human breast cancer. *British journal of cancer*, 93(6), pp.694–8.

Webb, D.J. et al., 2004. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nature cell biology*, 6(2), pp.154–61.

Weigelt, B. et al., 2008. Refinement of breast cancer classification by molecular characterization of histological special types. *The Journal of pathology*, 216(2), pp.141–50.

Weingaertner, I.R., Koutnik, S. & Ammer, H., 2013. Chronic morphine treatment attenuates cell growth of human BT474 breast cancer cells by rearrangement of the ErbB signalling network. *PloS one*, 8(1), p.e53510.

Weis, S.M. et al., 2008. Compensatory role for Pyk2 during angiogenesis in adult mice lacking endothelial cell FAK. *The Journal of cell biology*, 181(1), pp.43–50.

Wilken, J.A. & Maihle, N.J., 2010. Primary trastuzumab resistance: new tricks for an old drug. *Annals of the New York Academy of Sciences*, 1210, pp.53–65.

Will, M. et al., 2014. Rapid induction of apoptosis by PI3K inhibitors is dependent upon their transient inhibition of RAS-ERK signaling. *Cancer discovery*, 4(3), pp.334–47.

Witters, L.M. et al., 1997. Enhanced anti-proliferative activity of the combination of tamoxifen plus HER-2-neu antibody. *Breast cancer research and treatment*, 42(1), pp.1–5.

Wong, C.-W. et al., 2002. Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with Src/Erk phosphorylation cascade. *Proceedings of the National Academy of Sciences of the United States of America*, 99(23), pp.14783–8.

Worzfeld, T. et al., 2012. ErbB-2 signals through Plexin-B1 to promote breast cancer metastasis. *The Journal of clinical investigation*, 122(4), pp.1296–305.

Wu, X. et al., 2005. FAK-mediated src phosphorylation of endophilin A2 inhibits endocytosis of MT1-MMP and promotes ECM degradation. *Developmental cell*, 9(2), pp.185–96.

Wu, X. et al., 2004. Focal adhesion kinase regulation of N-WASP subcellular localization and function. *The Journal of biological chemistry*, 279(10), pp.9565–76.

Xiao, F. & Connolly, D.C., 2014. Abstract 2095: FAK mediates STAT3 activation, migration and invasion in ovarian carcinoma cells. *Cancer Res.*, 74(19_Supplement), p.2095–.

Xie, B. et al., 2001. Focal adhesion kinase activates Stat1 in integrin-mediated cell migration and adhesion. *The Journal of biological chemistry*, 276(22), pp.19512–23.

Xu, F.J. et al., 1997. Heregulin and agonistic anti-p185(c-erbB2) antibodies inhibit proliferation but increase invasiveness of breast cancer cells that overexpress p185(c-erbB2): increased invasiveness may contribute to poor prognosis. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 3(9), pp.1629–34.

Xu, Q. et al., 2014. Abstract 3908: Focal adhesion kinase (FAK) inhibitor VS-6063 (defactinib) preferentially targets cancer stem cells in triple negative breast cancer. *Cancer Res.*, 74(19_Supplement), p.3908–.

Xu, W. et al., 2007. Loss of Hsp90 association up-regulates Src-dependent ErbB2 activity. *Molecular and cellular biology*, 27(1), pp.220–8.

Xu, Y. et al., 2009. Regulation of focal adhesion turnover by ErbB signalling in invasive breast cancer cells. *British journal of cancer*, 100(4), pp.633–43.

Yakes, F.M. et al., 2002. Herceptin-induced Inhibition of Phosphatidylinositol-3 Kinase and Akt Is Required for Antibody-mediated Effects on p27, Cyclin D1, and Antitumor Action. *Cancer Res.*, 62(14), pp.4132–4141.

Yamamoto, D. et al., 2003. FAK overexpression upregulates cyclin D3 and enhances cell proliferation via the PKC and PI3-kinase-Akt pathways. *Cellular signalling*, 15(6), pp.575–83.

Yang, C. et al., 2008. Heregulin beta1 promotes breast cancer cell proliferation through Rac/ERK-dependent induction of cyclin D1 and p21Cip1. *The Biochemical journal*, 410(1), pp.167–75.

Yang, X.H. et al., 2010. Disruption of laminin-integrin-CD151-focal adhesion kinase axis sensitizes breast cancer cells to ErbB2 antagonists. *Cancer research*, 70(6), pp.2256–63.

Yao, E. et al., 2009. Suppression of HER2/HER3-mediated growth of breast cancer cells with combinations of GDC-0941 PI3K inhibitor, trastuzumab, and pertuzumab. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 15(12), pp.4147–56.

Yarden, Y., 2001. Biology of HER2 and its importance in breast cancer. *Oncology*, 61 Suppl 2, pp.1–13.

Yashiro, M. et al., 2005. Effect of organ-specific fibroblasts on proliferation and differentiation of breast cancer cells. *Breast cancer research and treatment*, 90(3), pp.307–13.

Yoeli-Lerner, M. et al., 2005. Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. *Molecular cell*, 20(4), pp.539–50.

Yom, C.K. et al., 2011. Clinical significance of high focal adhesion kinase gene copy number and overexpression in invasive breast cancer. *Breast cancer research and treatment*, 128(3), pp.647–55.

Yu, H. et al., 1996. Activation of a novel calcium-dependent protein-tyrosine kinase. Correlation with c-Jun N-terminal kinase but not mitogen-activated protein kinase activation. *The Journal of biological chemistry*, 271(47), pp.29993–8.

Yu, H., Pardoll, D. & Jove, R., 2009. STATs in cancer inflammation and immunity: a leading role for STAT3. *Nature reviews. Cancer*, 9(11), pp.798–809.

Zeng, L. et al., 2003. PTP alpha regulates integrin-stimulated FAK autophosphorylation and cytoskeletal rearrangement in cell spreading and migration. *The Journal of cell biology*, 160(1), pp.137–46.

Zhai, J. et al., 2003. Direct interaction of focal adhesion kinase with p190RhoGEF. *The Journal of biological chemistry*, 278(27), pp.24865–73.

Zhang, J. et al., 2014. A small molecule FAK kinase inhibitor, GSK2256098, inhibits growth and survival of pancreatic ductal adenocarcinoma cells. *Cell cycle (Georgetown, Tex.)*, 13(19), pp.3143–9.

Zhang, J. & Hochwald, S.N., 2013. The role of FAK in tumor metabolism and therapy. *Pharmacology & Therapeutics*.

Zhang, S. et al., 2011. Combating trastuzumab resistance by targeting SRC, a common node downstream of multiple resistance pathways. *Nature medicine*, 17(4), pp.461–9.

Zhang, X. et al., 1999. Focal adhesion kinase promotes phospholipase C- 1 activity. *Proceedings of the National Academy of Sciences*, 96(16), pp.9021–9026.

Zhang, Y.-W. et al., 2002. Requirement of Stat3 signaling for HGF/SF-Met mediated tumorigenesis. *Oncogene*, 21(2), pp.217–26.

Zhao, J. et al., 2003. Identification of transcription factor KLF8 as a downstream target of focal adhesion kinase in its regulation of cyclin D1 and cell cycle progression. *Molecular cell*, 11(6), pp.1503–15.

Zhao, J. & Guan, J.-L., 2009. Signal transduction by focal adhesion kinase in cancer. *Cancer metastasis reviews*, 28(1-2), pp.35–49.

Zhao, J., Pestell, R. & Guan, J.L., 2001. Transcriptional activation of cyclin D1 promoter by FAK contributes to cell cycle progression. *Molecular biology of the cell*, 12(12), pp.4066–77.

Zhao, Y. et al., 2011. Overcoming trastuzumab resistance in breast cancer by targeting dysregulated glucose metabolism. *Cancer research*, 71(13), pp.4585–97.

Zheng, D. et al., 2010. A novel strategy to inhibit FAK and IGF-1R decreases growth of pancreatic cancer xenografts. *Molecular carcinogenesis*, 49(2), pp.200–9.

Zheng, Y. & Lu, Z., 2014. Paradoxical roles of FAK in tumor cell migration and metastasis. *Cell Cycle*, 8(21), pp.3474–3479.

Zhu, T., Goh, E.L.K. & Lobie, P.E., 1998. Growth Hormone Stimulates the Tyrosine Phosphorylation and Association of p125 Focal Adhesion Kinase (FAK) with JAK2: FAK IS NOT REQUIRED FOR STAT-MEDIATED TRANSCRIPTION. *Journal of Biological Chemistry*, 273(17), pp.10682–10689.

Zhuang, G. et al., 2010. Elevation of receptor tyrosine kinase EphA2 mediates resistance to trastuzumab therapy. *Cancer research*, 70(1), pp.299–308.

Zucker, S. & Cao, J., 2009. Selective matrix metalloproteinase (MMP) inhibitors in cancer therapy: ready for prime time? *Cancer biology & therapy*, 8(24), pp.2371–3.