Exploring Focal Adhesion Kinase (FAK) as a therapeutic target in triple negative breast cancer

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Samuel Rhys Jones

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

Abstract

Triple-negative breast cancer (TNBC) is an aggressive cancer subtype that displays poor prognosis due to a lack of targeted therapies and an early pattern of spread. Recent evidence also points to a correlation between cancer "stem-like" cells (CSCs) and the inherently aggressive traits of TNBC. As such, targeting signalling pathways which support metastasis and CSC populations may represent an important therapeutic strategy to treat these tumours and improve current patient outcomes. The non-receptor tyrosine kinase FAK (focal adhesion kinase) is known to influence cancer development and progression, with its upregulation common in several cancer types. Indeed, FAK can regulate various cellular processes associated with disease progression including, cell survival, migration and stem-like behaviours. Therefore, we explored the influence of FAK in TNBC cells and the potential benefit of its targeting in this subtype.

Whilst assessment of FAK expression and activity across a panel of breast cancer cell lines representing the major clinical subtypes revealed that FAK was not significantly augmented in MDA-MB-231 cells (model of TNBC) versus other models, MDA-MB-231 cells displayed a FAK-dependent migratory and invasive behaviour involving FAK-mediated activation of Akt and STAT3. These observations also extended to cell proliferation, with pharmacological or genetic FAK inhibition leading to perturbed cell cycle progression. Whilst FAK did not contribute to the maintenance of a CSC subpopulation, FAK was necessary for their anoikis resistance and mammosphere self-renewal, the latter regulated by FAK-dependent modulation of β-catenin through GSK3β and interaction between the FAK/Wnt signalling pathways.

Using computational modelling, several novel FAK inhibitors that targeted FAK kinase-independent scaffolding function were developed and screened to assess *in vitro* efficacy in TNBC cells. Of all 45 compounds, 'compound 9' showed significantly improved ability to reduce cell proliferation and migration versus the lead compound, chloropyramine. As expected, this agent had little effect of FAK phosphorylation but appeared to reduce focal-adhesion targeting and subcellular distribution of FAK and significantly inhibited cell migration and growth. Our in vitro data support a case for FAK as a promising therapeutic target in TNBC with an ability to suppress both tumorigenic events and those associated with metastasis. Targeting FAK scaffolding function may represent a novel approach to developing FAK inhibitors that can circumvent resistance traditionally associated with kinase inhibitors.

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Contents

List of abbreviations

ABC	ATP-binding cassette
ADCC	Antibody dependent cellular cytotoxicity
ADP	Adenosine diphosphate
AI	Aromatase inhibitor
Akt	Protein kinase B
ALDH	Aldehyde dehydrogenase
APS	Ammonium Persulfate
ARP2/3	Actin related protein 2/3
ASAP1	ADP ribosylation factor (ARF) - GTPase-activating protein (GAP) containing SH3, ANK repeats and PH domain
ATP	Adenosine triphosphate
BAX	Bcl-2-associated X protein
BRCA1/2	Breast cancer type 1/2 susceptibility
BSA	Bovine serum albumin
CAF	Cancer associated fibroblast
cAMP	Cyclic adenosine monophosphate
CD24/44	Cluster of differentiation 24/44
Cdc	Cell division control protein
CDK	Cyclin dependent kinase
c-FLIP	Cellular FLICE-inhibitory protein
CIP/KIP	CDK interacting protein/Kinase inhibitory protein
Crk	Crk adaptor protein
CSC	Cancer stem cell
DAB	Diaminobenzidine
DAPI	4', 6-diamidino-2-phenylindole
DCIS	Ductal carcinoma *in situ*
DEAB	Diethylaminobenzaldehyde
DISC	Death-inducing signalling complex
DMFS	Distant metastasis-free survival
DMSO	Dimethyl sulphoxide
DOCK	Dedicator of cytokinesis
DTT	Dithiothreitol
E2	Oestradiol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
EPAC	Exchange factor directly activated by cAMP
ER	Oestrogen receptor
ERE	Oestrogen responsive element
ERK	Extracellular signal-related kinase

ERM	Ezrin-radixin-moesin
FA	Focal adhesion
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FAT	Focal adhesion targeting
FCS	Foetal calf serum
FERM	4.1-ezrin-radixin-moesin
FITC	Fluorescein isothiocyanate
FRNK	FAK related non-kinase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEF	Guanine nucleotide exchange factor
GFR	Growth factor receptor
Graf	Rho-GTPase activating protein
Grb	Growth factor receptor bound protein
GSK3β	Glycogen synthase kinase 3 beta
HER2/3	Human epidermal growth factor receptor
HRG	Heregulin
HRP	Horseradish peroxidase
HTVS	High-throughput virtual screen
IAP	Inhibitory apoptotic protein
ICC	Immunocytochemistry
IDC	Infiltrating ductal carcinoma
IGF-1R	Insulin-like growth factor 1 receptor
Int	Integrin
IRS	Insulin receptor substrate
KD	Knockdown
LO	Lipid only
LOXL2	Lysyl oxidase homolog 2
LR	Linker region
LRP5/6	Low-density lipoprotein receptor-related protein
MAPK	Mitogen-activated protein kinase
MaSC	Mammary stem cell
MDM2	Mouse double minute 2 homolog
MDR	Multi-drug resistance
MEF	Mouse embryonic fibroblast
MEGM	Mammary epithelial growth medium
MFU	Mammosphere forming unit
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinases
MMTV-PyMT	Mouse mammary tumour virus - Polyoma middle T oncogene
MRP1	Multi-drug resistance-associated protein 1
MS	Mammosphere
MTOR	Mechanistic target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NES	Nuclear export sequence
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear localisation sequence
NOD-SCID	Non-obese diabetic-severe combined immunodeficiency
NS	Non-significant
NT	Non-targeted
p130cas	p130 Crk associated substrate
PARP	Poly-ADP--ribose-polymerase
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PDE	Phosphodiesterase
PDGFR	Platelet derived growth factor receptor
PE	Pleural effusion
PGAP3	Post-GPI Attachment to Proteins 3
PI	Propidium Iodide
Pi3K	Phosphoinositol-3 kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PMSF	Phenylmethylsulphonyl fluoride
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
PTK6	Protein tyrosine kinase 6
Pyk2	Proline-rich tyrosine kinase 2
Rac	Ras-related C3 botulinum toxic substrate
RACK1	Receptor for Activated C Kinase 1
Rap1	Ras-related protein 1
RFS	Relapse-free survival
RhoA	Ras homolog gene family member A
ROCK	Rho-associated protein kinase
RTK	Receptor tyrosine kinase
S	Serine
SDS	Sodium Dodecyl Sulphate
SEM	Standard error of mean
SERD	Selective oestrogen receptor degraders
SERM	Selective oestrogen receptor modulators
Shb	SH2 domain-containing adaptor protein B
siRNA	short-interfering RNA
SPVS	Standard precision virtual screen
SRF	Self-renewal factor
T	Threonine
TAM	Tumour associated macrophage
TBST	TRIS-buffered saline Tween
TEMED	Tetramethylethylenediamine
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta

TNBC	Triple negative breast cancer
VEGF	Vascular-endothelial growth factor
VEGFR	Vascular-endothelial growth factor receptor
WASP	Wiskott-Aldrich syndrome protein
WT	Wild-type
XPVS	Extra precision virtual screen
Y	Tyrosine

1. General Introduction

1.1. Breast Cancer

1.1.1. Incidence and mortality

Breast cancer is one of the most common cancers worldwide, with an estimated 494,000 new cases diagnosed in Europe during 2015: approximately 55,000 of these were diagnosed in the UK (Cancer Research UK 2017). Amongst female patients, cancers of the breast are the most commonly diagnosed malignancies (Miller *et al.* 2016; Siegel *et al.* 2016). Despite this, a study by the National Cancer Institute revealed 89% of patients diagnosed in 2003 were still alive in 2009 (Howlader *et al.* 2013). These results imply that current treatments are relatively successful, yet breast cancer still represents a major health burden in the developed world. This is because a significant portion of all cancer related deaths are linked to breast malignancies. In addition, current treatments frequently fail to cure cancers with a significant percentage of patients suffering from a relapse of the disease (Miller *et al.* 2016). Such patients generally suffer a poorer prognosis upon relapse, owing to an increased chance of metastatic disease compared to newly diagnosed patients (Siddiqi *et al.* 2009). Despite improvements in the 5-year survival rate, there is still a need for the development of novel therapeutics that significantly improve treatment of breast cancer patients with a view to one day completely curing the disease.

1.1.2. Clinical subtypes

Since the early 2000s, treatment of breast cancer patients has become increasingly tailored to the molecular make-up of tumour cells (Perou *et al.* 2000; The Cancer Genome Atlas Network 2012). The use of such screening techniques has meant numerous subtypes of breast cancer have been identified, with different classifications focused on different cellular variations. For example, tumours can be classified by specific gene expression, glycoprotein composition and histological variations (The Cancer Genome Atlas Network 2012). Arguably the most important variation however, is the differences in hormone receptor status.

The presence or absence of these receptors is a central characteristic used in determining prognosis and treatment of breast cancer patients. Using hormone receptor status and the human epidermal growth factor receptor (*HER2*), tumours can be classified into three broad, yet distinct clinical subtypes: oestrogen receptor positive (*ER+*), *HER2+* and triple-negative tumours. The latter most subtype is primarily comprised of cancer cells with the molecular profile *ER-*, *HER2-* and progesterone receptor (PR) negative. Clinically, hormone receptor positive tumours

(i.e. *ER+* or *PR+*) represent the largest proportion found in breast cancer patients (around 70%) (Mehta and Tripathy 2014). The other subtypes have a significantly lower prevalence in patients: *HER2+* cancers represent around 15-20% (Mehta and Tripathy 2014) and triple-negative breast cancers (TNBCs) around 10% (Dent *et al.* 2007).

1.1.2.1. Molecular stratification of breast cancer subtypes

In this molecular age, advancements in genetic screening techniques have further unearthed the complex diversity exhibited by breast cancers, effectively redefining breast cancer taxonomy (Perou *et al.* 2000; Sørlie *et al.* 2001). Analysis of gene expression patterns has resulted in an expansion of the clinical breast cancer subtypes into 4 subcategories: luminal A; luminal B; HER2-enriched (HER2+); and basal tumours.

As they represent a subdivision of ER+ disease, the luminal A and luminal B classes exhibit several similarities, including expression of a number of genes present in cells of the mammary duct lumen such as GATA3 (Creighton 2012). However, multiple differences between these subtypes exist. For example, luminal B cells tend to be more aggressive versus those classified as luminal A, with patients exhibiting significantly worse prognosis (Sotiriou and Pusztai 2009). This is most likely resultant from increased expression of proliferation-associated genes, Ki67 and PCNA, along with increased frequency of abnormalities in several genes directly implicated in cancer progression, such as *TP53*, *PTEN* and *PI3KCA*, compared to luminal A cells (Creighton 2012). The HER2-enriched group represent tumours similar in gene expression patterns to ER-/PR-/HER2+ clinical subtype (Vallejos *et al.* 2010). Typically, this classification of breast cancer is characterised by presence of the HER2-receptor. Although typically this occurs as a result of gene amplification, some such tumours have aberrant HER2-levels resulting from non-amplified, single-copy overexpression (Dai *et al.* 2014). As such, overexpression of genes within the *HER2* amplicon, such as PGAP3 and Grb7, are also used to define this subtype of disease (Dai *et al.* 2014). Basal tumours exhibit expression of specific gene clusters characteristic of basal mammary cells, including laminin, β4-integrin and keratin 5/6/17. Clinically, this subtype is primarily comprised of triple-negative cells, although TNBC has more recently been proposed to be a distinct subclass of its own (discussed further later) (Brenton *et al.* 2005). In terms of gene expression, these tumours display a significantly higher rate of mutations in p53 (82%) versus the other classifications, possibly underpinning the noted prognosis of such cancers. In contrast to the other subtypes, the relevance of normal-like tumour expression profiles to clinical outcome is

poorly understood. This group display comparative gene expression profiles to non-epithelial and adipose tissue cells, whilst also showing lower expression of luminal-like genes (Perou *et al.* 2000).

As one would imagine, further stratification of breast tumours has enabled a more detailed examination of clinical outcome. As seen in **figure 1.1**, luminal A tumours have the greatest overall and relapse- free survival rates versus all other groups, whilst HER2+ and basal tumours have a significantly worse prognosis, likely owing to increased expression of genes associated with proliferation and metastasis (Brenton *et al.* 2005). In addition to its use as a prognostic tool, this expanded taxonomy has also resulted in the development of predictive models of relapse and therapeutic response (Weigelt *et al.* 2008; Parker *et al.* 2009).

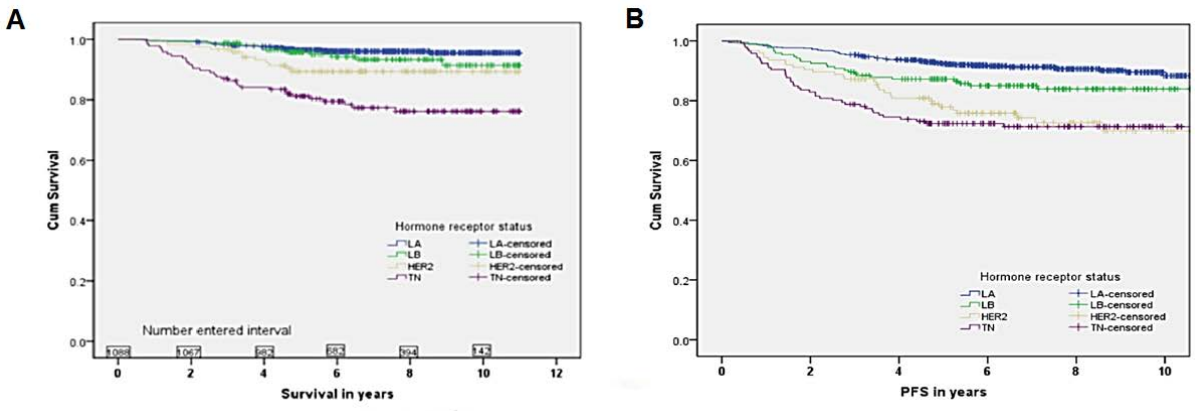


Figure 1.1 – Prognosis of breast cancer patients based on molecular stratification of subtypes – Kaplan-Meir curves showing differences in overall survival **(A)** and progression-free survival (RFS) **(B)** of patients between molecular subtypes. Patients exhibiting triple-negative disease displayed a significantly poorer overall survival, whilst triple-negative and HER2+ had significantly poorer progression-free survival versus other molecular subtypes (X. Wu *et al.* 2016).

Genetic subdivisions of tumours in this manner marks a significant move forward towards a personalised-approach to cancer treatment, with patients being treated based on distinct tumour composition rather than broad classification. Some such technologies are currently in use although to a somewhat limited degree. For example, the Oncotype DX system has been utilised to predict 10-year recurrence risk in patients exhibiting ER+, node-negative tumours

following endocrine and/or chemotherapy (Dai *et al.* 2015). This assay is based on the expression of 21 genes, 16 cancer-related and 5 reference genes, although screens are available which utilise far more. In 2009, Parker et al outlined a classification system based on a 50-gene signature (named PAM50) which also displayed significant prognostic potential (Gnant *et al.* 2014; Dowsett *et al.* 2013; Parker *et al.* 2009), as well as being readily adaptable to a clinical setting (Ades *et al.* 2014).

1.1.3. Therapeutic strategies for breast cancer

As noted, overall prognosis for breast cancer patients is relatively good, with average 5-year survival rates estimated at 89% (Howlader *et al.* 2013). This is largely resultant from the development of successful tailored therapeutic strategies. Indeed, the presence or absence of hormone receptors on tumour cells currently informs treatment regimens, enabling the most appropriate therapies to be utilised based on individual patient requirements. Briefly, ER+ tumours are targeted using endocrine therapy whilst HER2+ patients typically receive antagonists against this receptor (Figueroa-Magalhães *et al.* 2014). In stark contrast to this, triple-negative tumours display lack of specific, targeted treatments, directly resulting from a lack of hormone receptor expression. Consequently, the treatment options in patients diagnosed with such tumours are limited are limited to adjuvant chemotherapy (Dent *et al.* 2007). However, a number of groups are currently exploring alternative methods by which to treat TNBC patients (discussed later).

1.1.3.1 Targeting ER in breast cancer therapy

As noted, patients exhibiting ER+ tumours represent approximately 70% of all diagnosed breast cancer cases. Consequently, the movement to a malignant phenotype in these cells is proposed to be linked to aberrant ER signalling, likely due to increased stimulation with oestrogens. Indeed, ER have been shown to regulate several proteins closely linked to tumourigenesis (Carroll and Brown 2006). For example, stimulation with oestradiol (E2), causes phosphorylation and subsequent dimerization of ERs, which in turn can bind to oestrogen responsive elements (EREs) present in the promoter regions of oestrogen responsive genes and, following the recruitment of coactivators such as A1B1, thus modulate gene expression (Carroll and Brown 2006). Moreover, ER has also been shown to interact with other transcription factors to drive tumourigenic gene expression. Such stimulated genes include *IGF-IR, VEGF, c-myc and cyclin D1*.

In addition to modulating transcription, membrane-associated ER can influence various signalling pathways, such as Pi3K and MAPK (Sun *et al.* 2001; Klinge *et al.* 2005), as well as stimulating growth factors including EGFR and IGF1-R (Levin and Pietras 2008) further promoting tumourigenesis.

Given the role of ER in driving tumourigenic pathways, the primary strategy of therapeutic design for ER+ tumours hinges on preventing oestrogen binding, ER function or reducing oestrogen levels thus impairing disease progression, recurrence risk or death. As such, endocrine therapies have been labelled as the "gold standard" for treatment of ER+ tumours. Below are outlined the three main classifications of endocrine therapies, including their respective benefits and drawbacks.

1.1.3.1.1. Selective oestrogen receptor modulators (SERMs)

These compounds aim to inhibit the interaction between ERs and their stimulating oestrogens. The most well-known of these compounds is Tamoxifen which selectively binds to ERs, blocking stimulation by E2. This drug has been the cornerstone of ER+ tumour treatment, being in use for over 30 years and having a significant impact on patient outcome. For example, a study conducted by the EBCTCG (Early Breast Cancer Trialists' Collaborative Group) found that patients treated with adjuvant Tamoxifen for 5 years exhibited significantly improved recurrence rates and mortality versus control patients over 15 years following initial diagnosis (Davies *et al.* 2011). Subsequent analysis revealed that sustaining Tamoxifen treatment up to 10 years further increases patient outcomes (Davies *et al.* 2013). However, use of this drug has a number of well documented side-effects which include an increased risk of deep vein thrombosis, pulmonary embolisms and endometrial cancer (Swerdlow and Jones 2005; Hernandez *et al.* 2009). The latter is most likely a result of Tamoxifen acting as a partial agonist of ER in endometrial tissues, thus stimulating growth pathways otherwise impaired in breast cells (Davies *et al.* 2011).

1.1.3.1.2. Selective oestrogen receptor degraders (SERDs)

The primary role of these compounds is to trigger the degradation of ER, effectively reducing the amount available for stimulation and resultant regulation. Another well documented compound, Faslodex (Wakeling *et al.* 1991), is an example of this type of agent. Despite appearing to have similar efficacy versus Tamoxifen in the clinic, Faslodex causes a significant higher decrease in ER-driven gene transcription in breast cancer cells. This is likely due to a combination of higher

binding affinity for ER and targeting of cytoplasmic ER, not seen following Tamoxifen treatment (Howell 2006). Additionally, Faslodex appears to significantly reduce tumour PR levels in a dose-dependent manner (Ciruelos *et al.* 2014). Furthermore, Faslodex is referred to as a "pure anti-oestrogen" as it does not demonstrate the partial agonistic effects displayed by Tamoxifen (Howell *et al.* 2000). Unlike Tamoxifen however, this drug is rarely used as a first-line treatment and is more typically prescribed following development of resistance to other hormonal therapies (Ciruelos *et al.* 2014). Indeed, studies have shown Faslodex to be active in breast cancer patients previously treated with tamoxifen or aromatase inhibitors (Chia *et al.* 2008).

1.1.3.1.3. Aromatase inhibitors (AIs)

These compounds are designed to starve tumour cells of oestrogen by reducing levels of circulating oestrogen. This is achieved through binding to the aromatase enzyme responsible for oestrogen production thus impairing synthesis. These compounds fall into 2 classes: non-steroidal AIs, such as anastrozole and letrozole, reversibly bind aromatase, whilst steroidal AIs, such as exemestane, exhibit non-reversible binding (Miller *et al.* 2008). Although this difference in mechanism of action may imply variations in efficacy between the two classes of AIs, very few head-to-head comparisons have been undertaken so data to support any improved efficacy is extremely limited. Regardless, all the mentioned AIs are approved for use in the UK, particularly in the treatment of early or late breast cancer in post-menopausal women (NICE UK). Moreover, these compounds are also approved as an extended treatment for Tamoxifen treated patients. Indeed, a study by Howell et al (2005) revealed use of anastrozole in patients significantly extended disease-free survival versus tamoxifen with patients exhibiting fewer gynaecological or vascular side-effects (although bone fractures and arthralgia was increased) (Howell *et al.* 2005). These observations were subsequently expanded in 2010, where the 10-year analysis of this trial revealed the long-term efficacy of anastrozole over Tamoxifen for hormone-responsive tumours in post-menopausal women (Cuzick *et al.* 2010).

1.1.3.2 Targeting HER2 in breast cancer therapy

HER2 is an ErbB-family, receptor tyrosine kinase (RTK) frequently used in the clinical classification of breast cancer tumours, with around 20% of patients presenting amplified levels (Slamon *et al.* 1987; Mehta and Tripathy 2014). This family of RTKs has been extensively studied and includes members such as HER3, HER4 and EGFR. Moreover, the stimulating ligands for these receptors

have been well documented and include epidermal growth factor (EGF), transforming growth factor alpha (TGFα) and heregulins (HRG) (Olayioye *et al.* 2000). Activation through ligand binding causes a conformational change in these receptors leading to homo/heterodimerisation, subsequently stimulating intrinsic phosphorylation of several residues to provide docking sites for downstream secondary messengers (e.g. PI3K, Src and Crk) and resultant activation of signalling pathways linked to proliferation, cell survival and migration (Schulze *et al.* 2005; Park *et al.* 2008). Consequently, these receptors have been well studied in cancer biology and their aberrant activity is strongly linked to tumour progression and overall patient prognosis.

Although all members of this family have been implicated in cancer, overexpression of HER2 is of particular significance as this protein is used as a prognostic marker and utilised for targeted therapy. Interestingly, unlike its family members, HER2 lacks a functional ligand-binding domain but it represents the preferred heterodimer binding partner of all ErbB receptors (Graus-Porta *et al.* 1997; Park *et al.* 2008). This may be the underlying reason as to why this receptor stands out amongst its family as particularly important and why its overexpression is currently used to guide cancer treatment. Regardless, directly targeting the HER2 receptor is the primary method of treatment used in patients exhibiting the HER2+ class breast cancer.

To that end, the HER2+ antagonist Trastuzumab (Herceptin, Genentech) was developed (Carter *et al.* 1992) and is currently the most widely employed drug used in the treatment of HER2+ breast cancer patients (Figueroa-Magalhães *et al.* 2014). Approved for use in the UK in 2006, this drug is a humanised murine monoclonal antibody that specifically targets HER2 and is believed to impair its function through several mechanisms of action. Primarily, Herceptin prevents the formation of HER2 homo- and heterodimers by disrupting their interaction, leading to dissociation and subsequent reduced stimulation of downstream signalling (Junttila *et al.* 2009). Given its specificity to HER2, homodimerization can be altered to a greater degree than heterodimerization, although the latter is still greatly impaired. It can also impair HER2/Src association preventing Src-mediated inhibition of PTEN, thus restoring suppression of PI3K/Akt survival pathways (Nagata *et al.* 2004). In addition to impairing downstream signalling, Herceptin has also been shown to reduce expression of several genes, particularly associated with angiogenesis *in vivo* (Izumi *et al.* 2002). This led to a significant decrease in tumour blood vessel number and size, as well as reduced tumour growth. Interestingly, Herceptin has been shown to act as an opsonin, facilitating the adaptive immune system in targeting tumours through a

process known as antibody-dependent cellular cytotoxicity (ADCC) (Collins *et al.* 2012). This was demonstrated by Gennari et al (2004) and Arnould et al (2006) who noted significantly increased lymphocyte and natural killer cell infiltration of tumours in patients treated with Herceptin (Gennari *et al.* 2004; Arnould *et al.* 2006).

1.1.3. Triple-negative breast cancer

As noted previously, TNBC are characterised by their absence of ER, PR and HER2 receptors and represent the primary constituents of the basal-like cancers (Brenton *et al.* 2005). Prevalence of this subtype is particularly high in African American women and displays increased diagnosis in younger patients (below 50-years of age) (Bramati *et al.* 2014). Interestingly, patients exhibiting BRCA-1 and -2 mutations, often attributed to hereditary cancer development, regularly develop triple-negative tumours (Badve *et al.* 2011). Although these mutations are not present in the majority of sporadic TNBCs, there is significant correlation in genetic profiles between BRCA1-carrier tumours and those exhibited by TNBCs, likely resultant from irregular BRCA-1 signalling (Turner *et al.* 2004). The significance of this is discussed further later. Of all clinical subtypes, patients exhibiting triple-negative disease have a significantly poorer prognosis versus other subtypes, with many displaying very short disease-free periods prior to relapse (Banerjee *et al.* 2006). Indeed, the risk of recurrence in these patients is highest in the first three years following therapy, with most deaths coming within 5 years (Tischkowitz *et al.* 2007; Dent *et al.* 2007). In addition, progression of triple-negative tumours is fairly rapid with much shorter median times observed between early metastatic disease and death than in other subtypes (Dent *et al.* 2007).

Given their lack of hormone receptor presentation, TNBC lacks tailored treatments with current therapeutic regimens limited to chemotherapy. Typical response rates vary, with neoadjuvant administration of anthracycline alone, or in combination with taxane-based agents showing between 17 and 58% complete pathological response and platinum based chemotherapy 17%. (Sánchez-Muñoz *et al.* 2008; Liedtke *et al.* 2008). Coupled with their increased aggressive tendencies, this lack of therapeutic strategies is proposed as the primary reason for poorer patient outcome in this subtype. Almost seeming to contrast this observation, triple-negative tumours have been shown to respond significantly better to chemotherapy than other breast cancer subtypes, although patients exhibiting residual disease still have a very bleak outcome (Hudis and Gianni 2011). To that end, there has been a large drive to uncover druggable targets within TNBCs to develop new and effective therapeutics. For example, triple-negative tumours

have been shown to exhibit an increased frequency in EGFR overexpression versus all other subtypes (Gumuskaya *et al.* 2010; Liu *et al.* 2012). As a result, studies have been conducted exploring the efficacy of EGFR-antagonists as monotherapies or in combination with chemotherapy (Reeder-Hayes *et al.* 2010). However, this target has proven unfruitful, likely due to abnormalities in PTEN which can not only circumvent anti-EGFR treatment but are also particularly common in TNBC cells (Marty *et al.* 2008).

To date, the most promising novel target in TNBC treatment has been poly ADP (adenosine diphosphate- ribose) polymerase (PARP). This protein plays a central role in mediating cellular responses to DNA damage, particularly single strand breaks. As previously noted, a number of triple-negative tumours exhibit some form of dysfunctional BRCA-1 signalling (Turner *et al.* 2004). Given that BRCA-1 contributes to DNA-repair mechanisms, it stands to reason that targeting TNBCs with DNA damaging agents or further diminishing of DNA repair strategies could represent effective targeted therapies. Indeed, several PARP inhibitors have demonstrated potential as clinical agents in TNBCs. One such compound, Iniparib (also known as BSI-201) was previously identified as a potential compound for treatment of TNBCs in combination with chemotherapy. Randomised phase II clinical trials revealed that treatment with iniparib in combination with carboplatin and gemcitabine resulted in significantly improved overall response rate, survival and progression-free survival versus combination chemotherapy alone (O'Shaughnessy *et al.* 2009). This finding was subsequently validated in a subsequent survival analysis, which revealed median overall survival rate improved from 7.2 to 12.2 months following chemotherapy with an iniparib arm (O'Shaughnessy *et al.* 2011). However, this compound failed to reach pre-specified endpoints for overall or progression-free survival when combined with chemotherapy in a larger, randomised phase III clinical trial (Reeder-Hayes *et al.* 2010). This may have resulted from the inclusion of patients within the study whose tumours were particularly unresponsive to PARP therapy. With that in mind, studies are being undertaken by many groups who are aiming to identify novel biomarkers within TNBCs to further stratify this subtype and identify patients with tumours particularly sensitive to such biological agents (Badve *et al.* 2011). To recapitulate, despite some early successes, EGFR and PARP targeting therapeutics have thus far shown limited efficacy in TNBC treatment. Consequently, new therapeutics are needed, either as monotherapy or to improve existing treatments, in TNBC for which prognosis is poor.

1.2. Focal Adhesion Kinase (FAK)

1.2.1 FAK discovery and structure

In the early 1990s, focal adhesion kinase (FAK) was identified as a 125kDa, non-receptor tyrosine kinase (non-RTK) that is ubiquitously expressed in human tissues (Schaller *et al.* 1992). FAK is conserved across species with mice, *Xenopus* and zebrafish sharing 97%, 90% and 79% sequence homology to human FAK respectively, reflecting the importance of FAK across diverse organisms (Schaller 2010). As noted, FAK is a member of the non-RTK family but, unlike the majority of non-RTKs, FAK lacks both the Src homology (SH) 2 and SH3 domains **(figure 1.2A)** (Luo and Guan 2010). **Figure 1.2B** shows the general structure of FAK, including key features and binding partners. Broadly, the structure of FAK can be broken into five regions, compartmentalised into three broad sections: The N-terminal FERM (band four point one, ezrin, radixin and moesin) domain; the central kinase domain; and the C-terminal domain. The roles of these domains have been characterised on several occasions, although the full function of all these elements remains to be elucidated.

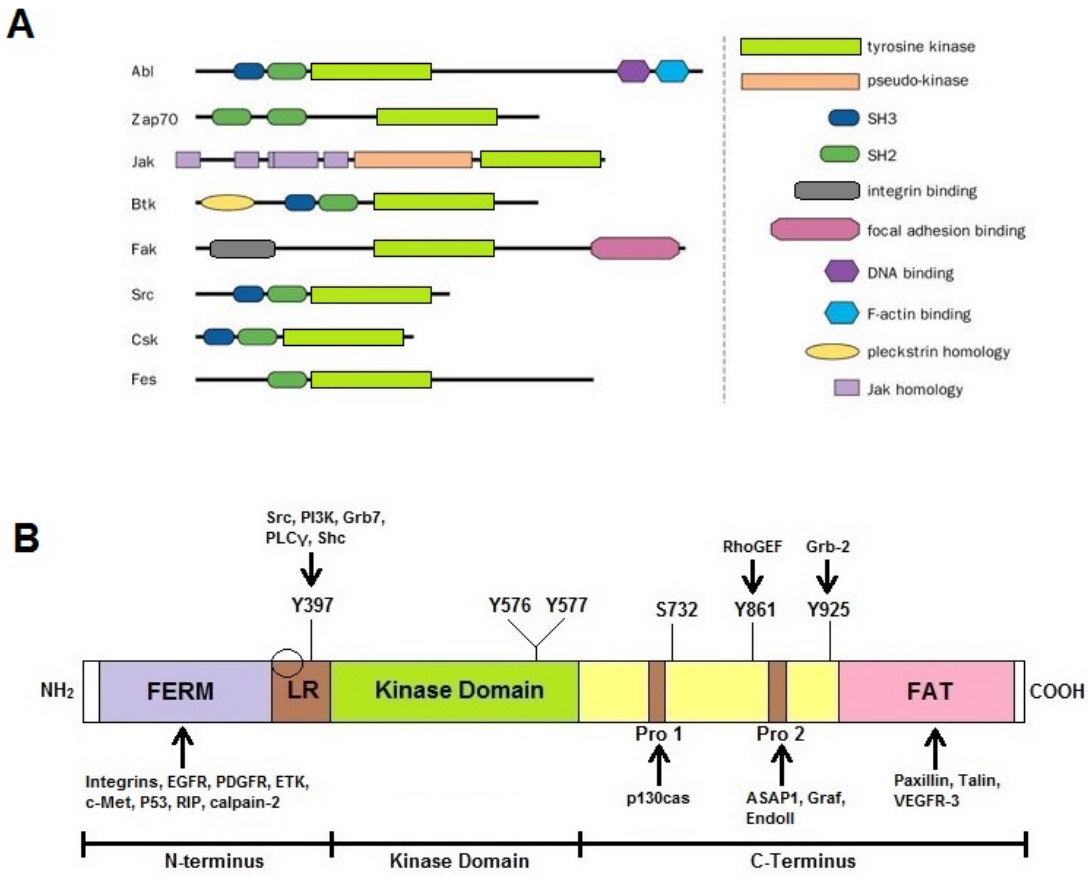


Figure 1.2 – Structural composition of non-receptor tyrosine kinases (non RTKs) and FAK – (A) A basic diagrammatic representation of the major non-RTK families showing key binding and regions of activity. Each family typically contains a tyrosine kinase domain and SH2 and SH3 regions. FAK and JAK family proteins tend to lack the latter of these domains. (image adapted from Voet and Voet 2004) **(B)** A basic outline of the structure of FAK, highlighting the key tyrosine phosphorylation sites (Y), the FERM domain, the FAT sequence, the kinase domain and linker region (LR). Also shown are the proline-rich regions (brown) and the location of the PxxP binding motif (circle in LR). Arrows indicate the locations of molecular binding sites upon activation of FAK. The molecules paxillin and talin are required for localisation to focal adhesions and thus can bind in the absence of activation.

1.2.2. FAK activity and signalling

FAK is primarily located in sites of cell-matrix adhesion termed 'focal adhesions' (FAs) (the structure of which is summarised in **figure 1.3**) reflecting the role of FAK as a central regulator of integrin-mediated signalling (Schaller *et al.* 1992). In recent years, the combination of functional mutation studies and elucidation of the crystal structure of FAK has led to a fairly comprehensive model of integrin-induced FAK activation being developed (Zhao and Guan 2011). This complex regulation is summarised briefly in **figure 1.4.** In brief, upon integrin binding, FAK undergoes a conformational change resulting in the autophosphorylation of FAK^{Y397}. The Y397 site has been postulated as the most important to FAK-kinase activity. This residue represents the major autophosphorylation of FAK, and its stimulation is required for conformational changes which result in Src binding and trans-autophosphorylation to fully active FAK. However, activation of FAK is not only initiated through engagement of integrins. Numerous extracellular stimuli can also cause such activation: for example it can be activated by a number of growth factors (such as platelet-derived and hepatocyte growth factors (Sieg *et al.* 2000; Matsumoto *et al.* 1994)) as well as cytokines and phospholipids (Schlaepfer *et al.* 2007; Seufferlein and Rozengurt 1994). Despite the different nature of the signals, it does appear that the same mechanism is employed to relieve the inhibition of FAK: the signal causes a conformational change alleviating the steric hindrance of Y397, Src binding site and kinase domain.

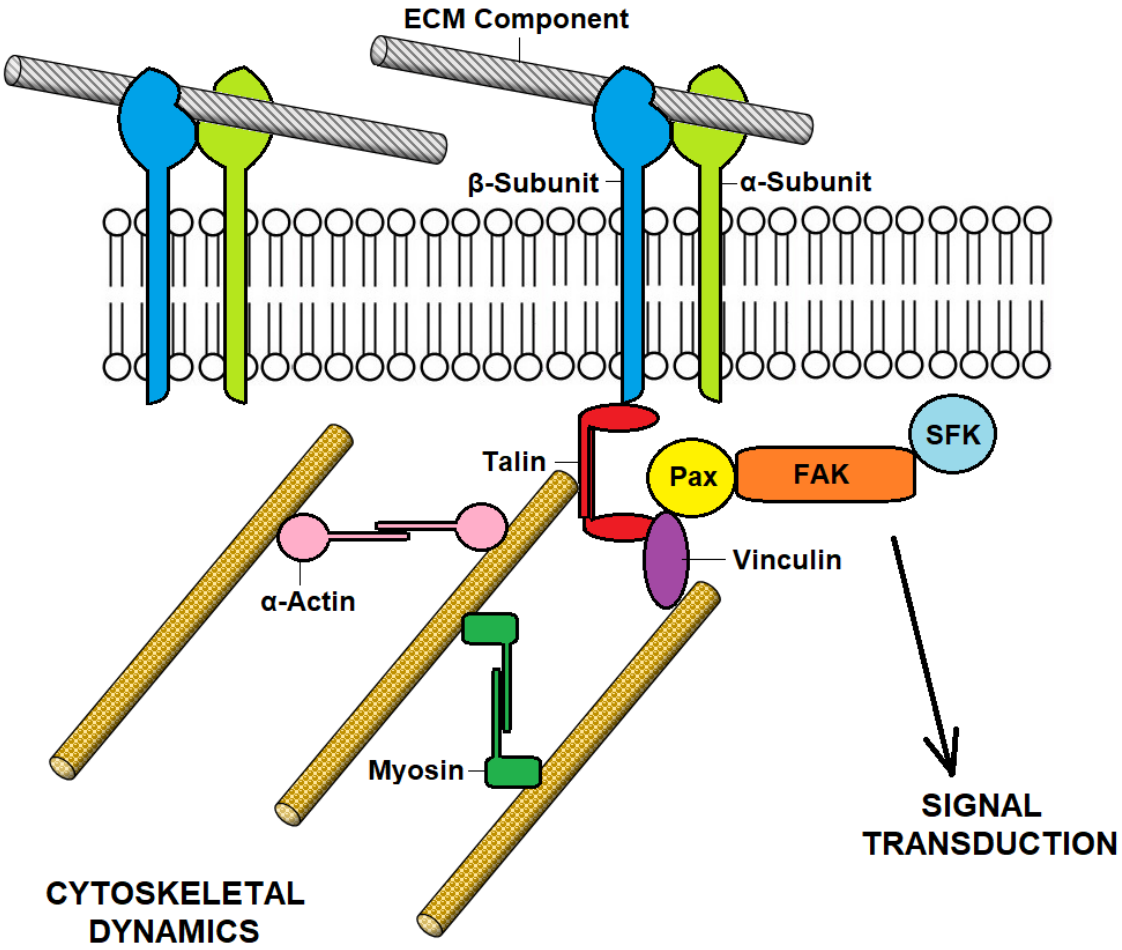


Figure 1.3 – Basic structure of focal adhesions (FAs) including key signalling molecules – FAs represent sites of interaction between the extracellular matrix (ECM) and cells or order to influence cell signalling in response to environmental stimuli. Primarily, these signals are regulated through the binding of specific ECM components (such as fibronectin and collagen) to cell surface receptors known as integrins (heterodimeric protein comprised of an α and β-subunit). Integrins are only able to act in this manner following a conformational change which straightens the protein (Critchley and Gingras 2008). This switch is induced by binding of a protein to the cytoplasmic region of the β-subunit, for example talin. Subsequent conformational changes in integrins due to ECM binding stabilise FAs through talin-mediated binding of actin filaments. The resulting complex then recruits paxillin, FAK and Src-family kinases (SFKs) to allow initial actin polymerisation (Roca-cusachs *et al.* 2012). These molecules can then become activated and phosphorylated leading to a contraction in myosin, leading to the stretching of talin and the exposure of binding sites for vinculin. This vinculin binding is sufficient to cause cellular protrusions often noted in migrating cells (Hirata *et al.* 2014). Resulting nascent adhesions then undergo a phase of growth, which includes cycles of assembly and disassembly, before some evolve and become mature FAs, whilst others are permanently disassembled.

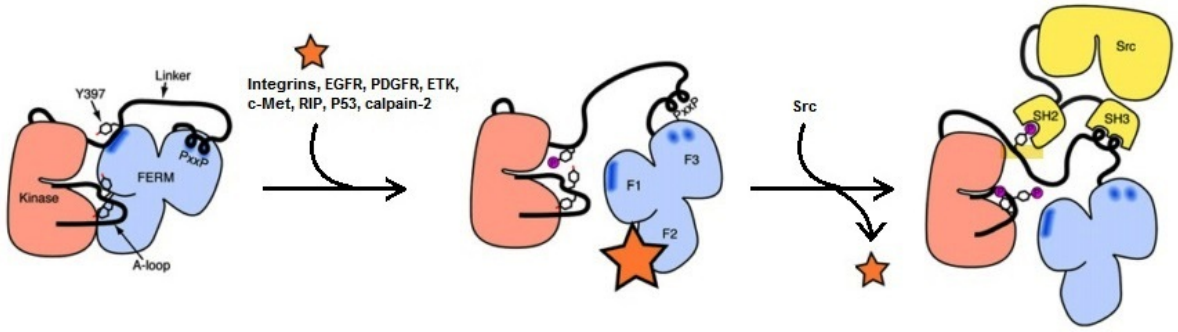


Figure 1.4 – Structural alterations that activate FAK – Lobed regions of the FERM domain bind to the linker region (via F3) and the F596 residue of the kinase domain (via a hydrophobic pocket in the F2 lobe) in the absence of an activating signal. This sterically hinders target protein binding to the kinase domain and sequesters Y397 phosphorylation and SH3-domain protein binding (via PxxP motif). Activating proteins (orange star) bind to the FERM region and cause a conformational change releasing the sequestered regions. This leads to autophosphorylation of Y397 and also enables Src binding via its SH3 domain to the Pxxp-motif. The SH2 domain of Src interacts with phosphorylated Y397 leading to removal of inhibitory phosphorylation at Src^{Y527} and activating phosphorylation of Src^{Y419}. Activated Src subsequently phosphorylates remaining residues leading to maximal activation of FAK. (Image adapted from Lietha et al. 2007).

1.2.2.1. FERM domain regulation of activity

The FERM domain of FAK shows remarkable homology to a number of cytoskeletal proteins, for example the ezrin-radixin-moesin (ERM) and talin (Chishti *et al.* 1998), as well as several other tyrosine phosphatases (Girault *et al.* 1999). The FERM domain acts as the major negative regulator of FAK activity, with its displacement from the kinase region essential for FAK stimulation (X. L. Chen *et al.* 2012). Indeed, early studies revealed that truncation of the FAK FERM domain, which prevented interactions between the FERM and kinase domain, resulted in increased tyrosine phosphorylation and resultant FAK activity (Lim, Mikolon, *et al.* 2008). Moreover, FERM-negative FAK mutants display amplified kinase activity owing to increased Y397 phosphorylation (Cooper *et al.* 2003). Taken together these studies highlight the importance of the FERM-domain in the negative-regulation of FAK.

Additionally, the FERM-domain can directly influence FAK signalling through interactions with a number of secondary messengers, including PIP2 and various growth factor receptors, to enable

their activation by FAK (Cai *et al.* 2008; Chen and Chen 2006). Consequently, the FAK FERM domain can contribute to specific direction of FAK's downstream signalling.

Interestingly, the FERM domain of FAK has been shown to contain potential nuclear-export and nuclear-localisation signals (NES and NLS respectively) (Frame *et al.* 2010). This suggests that the FERM-domain, at least in part, can contribute to transduction of membrane signals to the cell nucleus (Frame *et al.* 2010). Indeed, Lim et al demonstrated that following a reduction in integrin signalling, the FERM domain of FAK can promote cell survival by providing a scaffold for p53/MDM2 interactions within the nucleus resulting in increased ubiquitination of p53 (Lim, Mikolon, *et al.* 2008).

1.2.2.2. Central kinase domain

The central kinase domain of FAK is the core region of catalytic activity and is required for stimulation of several downstream effector molecules. Importantly, this region contains the Y576 and Y577 phosphorylation residues. It has long been established that following FAK-mediated activation, Src phosphorylates these sites leading to the full activation of FAK kinase function (Calalb *et al.* 1995). Indeed, impairing the phosphorylation of these residues is sufficient to cause a significant decrease in cell migration owing to a loss of FA disassembly (Hamadi *et al.* 2005). Moreover, the stimulation of these residues can be achieved through additional molecules, such as the RET receptor, binding to the FERM domain and directly phosphorylating Y576 and Y577 (Plaza-Menacho *et al.* 2011).

1.2.2.3. C-terminus FAT region

The C-terminus of FAK is also central in the regulation of cellular FAK interactions and activity. This domain contains two proline-rich sites **(figure 1.2B)** which serve as binding domains for numerous SH3-containing molecules. Some of these are ASAP1 and p130cas (Liu *et al.* 2005). The binding of molecules to this region has been shown to facilitate subsequent binding of other proteins involved in specific signal cascades (for example see Kondo et al. 2000). Interestingly, very few studies have investigated targeting these proline-rich sites for therapeutic FAK inhibition in cancer or other disease states. The C-terminal domain also contains the focal adhesion targeting sequence (FAT sequence). This four-helix bundle is essential for localisation of FAK to focal adhesions (FAs), being demonstrated as both sufficient and necessary for FAK localisation (Hayashi *et al.* 2002a).

The C-terminal region of FAK is central in enabling multiple protein-protein interactions, for example through its two proline-rich domains. Such interactions are attributed to FAK's kinase-independent functions where its role is as a scaffold protein. Upon Y397 phosphorylation, FAK is able to bind a number of SH3 containing molecules without directly affecting their function. Instead, FAK activated Src acts to phosphorylate these proteins and subsequently lead to the initiation of signalling cascades. Some proteins regulated in this way are the GTPase regulator associated with FAK (Graf) (Hildebrand *et al.* 1996), which regulates actin dynamics through RhoA, and also endophilin A2 (Wu *et al.* 2005), involved in endocytosis. Taken together, this evidence suggests that FAK scaffolding is likely to play an important role in FAK-regulated cell behaviours.

1.2.3. FAK family members

FAK itself is a member of the FAK subfamily of non-receptor tyrosine kinases, which also includes Pyk2. Around the same time that FAK was identified, another structurally similar molecule was also identified. This molecule, Pyk2 (Proline-rich tyrosine kinase), has been found to be around 45% identical to FAK, also sharing a very similar domain structure **(figure 1.5)** (London 1995). However, the relative importance of these two molecules differs significantly. For example, FAK is ubiquitously expressed and transgene studies have shown that it is essential to life. In contrast, Pyk2 is primarily expressed in haematopoietic cells and those of the central nervous system (Lev *et al.* 1995). Moreover, Pyk2 deficient mice survive and are fertile with only minor effects being noted (primarily abnormal morphology of macrophage cells) (Okigaki *et al.* 2003). The regulation of the molecules also differs significantly. FAK is activated through integrins and growth factor receptors present at FAs: Pyk2 is cytoplasmic and its activity initiated via chemokines and G protein-coupled receptors (Dikic *et al.* 1996). Despite these contrasts, the two molecules do not exist independently of each other. A number of studies have shown that Pyk2 has some compensatory function for the loss of FAK (for example see Fan and Guan 2011). Consequently, studies into FAK cannot overlook a potential compensation by Pyk2.

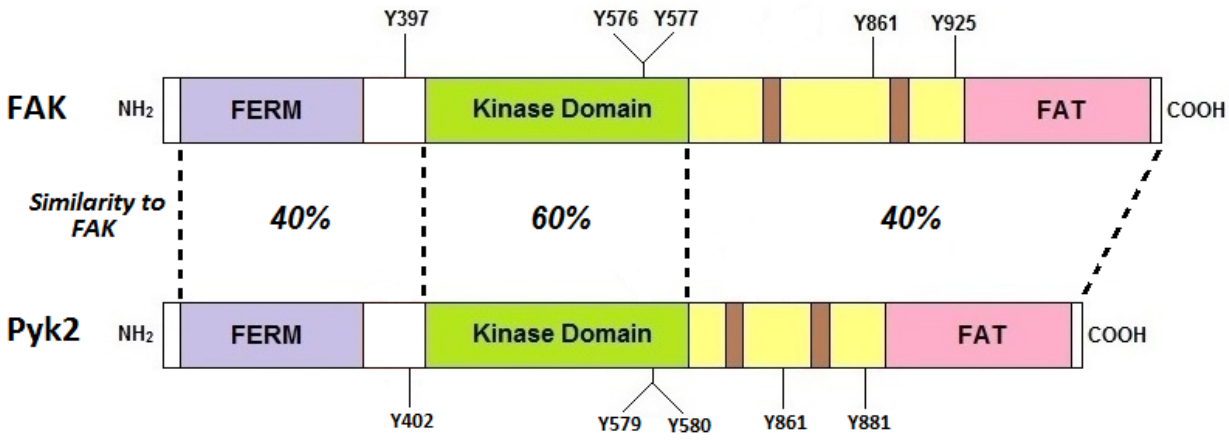


Figure 1.5 – Structural comparison of FAK and Pyk2 – Basic outline of the structures of FAK and Pyk2 highlighting the levels of homology within the previously noted 3 domains.

1.2.4. Functional relevance of FAK signalling

Its close link with integrin-mediated signalling led to the identification of FAK as a central regulator of FA turnover and cytoskeleton remodelling, processes central to cell migration. In support of this, multiple lines of evidence illuminate FAK protein as central in normal animal embryonic development. For example, FAK-null mouse embryos have been shown as embryonically lethal, primarily due to a loss of mesodermal cell movements during embryonic development resulting in perturbed cell localisation (Petridou *et al.* 2013; Furuta *et al.* 1995). However, FAK has also been shown to regulate numerous other cellular processes including: proliferation (Lim, Chen, *et al.* 2008), cell survival (Kurenova *et al.* 2004), migration (Owen *et al.* 1999), invasion (Chan *et al.* 2009), as well as stem-cell like behaviours (Luo *et al.* 2013). An example of the pathways FAK regulates in these processes is shown in **figure 1.6.** Involvement in such processes has directly implicated FAK in pathogenesis of human disease processes: for example cardiovascular associated diseases such as cardiac hypertrophy (Vadali *et al.* 2007). Its role in multiple pathway regulation has also led to the identification of FAK as a central mediator of processes leading to the development and progression of cancer. As a result, FAK represents a major potential target for novel therapeutics that could result in swift and efficient treatment of tumours.

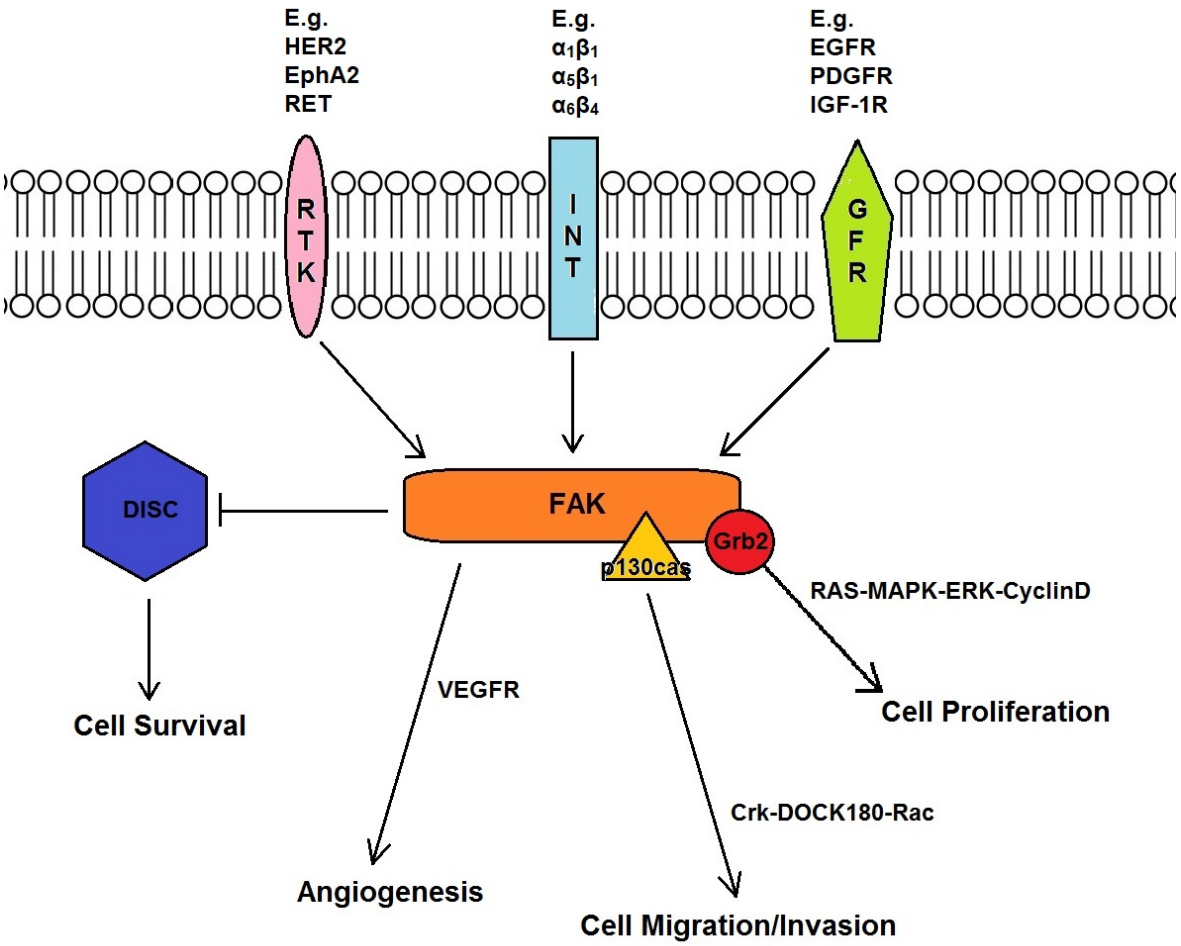


Figure 1.6 – Regulation of signals by FAK – FAK can be activated by a number of signals from cell surface receptors. These do not only include integrins (INT) but also receptor tyrosine kinases (RTKs) and growth factor receptors (GFRs). These lead to induction of FAK activity and, upon association with Src, maximal activation. Fully stimulated FAK can then stimulate a number of different signalling cascades. Active FAK directly interacts with the death-inducing signalling complex (DISC) and inhibits its activity, therefore a loss of caspase-8 mediated apoptosis, so promoting cell survival (L.-H. Xu *et al.* 2000). Moreover, phosphorylation of FAK is essential in order to transmit VEGF-mediated angiogenic signals via integrin αvβ5 and VEGFR3 (Skobe *et al.* 2001; Eliceiri *et al.* 2002). Association of p130cas with FAK following induction leads to recruitment of Crk and its activation via Src. This protein can then activate DOCK180 and subsequently Rac which results in cellular migration (Cho 2000). FAK is also involved in regulating cellular proliferation. Upon maximal activation, Y925 can bind Grb2 which subsequently activates the Ras-MAPK-ERK signalling cascade resulting in upregulation of cyclin D (Zhao *et al.* 2001). This results in progression through the cell cycle and so cell division. It is important to note these are just some of the pathways that FAK regulates in each of the named processes: numerous others exist, for example FAK directly mediates the PI3K/Akt survival pathway, involved in anchorage-independent cell survival.

1.3. FAK and cancer

Given the diversity of signalling programs that FAK is involved in, which control cellular behaviours such as movement, growth and survival, it is not surprising that FAK has been implicated as a key modulator of tumour cell functions promoting tumour development, survival and spread. As such, FAK has been directly implicated in the development and progression of a number of different cancers (Golubovskaya *et al.* 2009). Consequently, research into FAK's role in cancer has seen a major drive in recent years. Given the complex regulation of FAK activity and the role of FAK as a signalling hub for numerous pathways, this implies tightly controlling FAK activity is essential to maintenance normal cellular behaviours. Indeed, aberrant activity can contribute to progression of cells to an aggressive, malignant phenotype. Numerous studies have highlighted oncogenic protein transformations that can induce atypical FAK activity, required for movement to a cancerous phenotype. For example, an early study by Akagi and colleagues (2002) showed that the presence of the oncogenic protein v-Crk induced constitutive phosphorylation of the FAK^{Y397} residue leading to an increase in association between FAK and the p85 subunit of phosphoinositol-3-kinase (PI3K) (Akagi *et al.* 2002). This activation was essential in allowing v-Crk to aberrantly activate the PI3K/Akt pathway as FAK-null cells transformed with this oncoprotein did not exhibit constitutive Akt signalling (Akagi *et al.* 2002). This example also highlights how the activities of many oncogenic proteins hinge on unregulated FAK activity, being unable to induce phenotypic changes without FAK activity.

Interestingly, aberrant stimulation of FAK by oncogenic proteins is not the only means that promote a malignant phenotype, as increased or direct loss of structural regulation within FAK have also been demonstrated to cause cancer. Recently, Fang et al (2014) identified a novel deletion within the FAT sequence in breast and thyroid cancer patients (Fang *et al.* 2014). The effects of this deletion mimicked the phenotype of constitutively phosphorylated FAK. This may result from a loss of steric hindrance of the Y397 site as the region containing the deletion was correlated with the binding sites of the protein talin and paxillin. Given the similarity in structure between the FERM domain and talin, the FAT sequence may no longer be able to interact with this region thus alleviating FERM-kinase domain binding. Coupling studies into FAK's role in cancer, one could suggest numerous mechanisms by which FAK leads to such phenotypic changes (shown in **figure 1.7**).

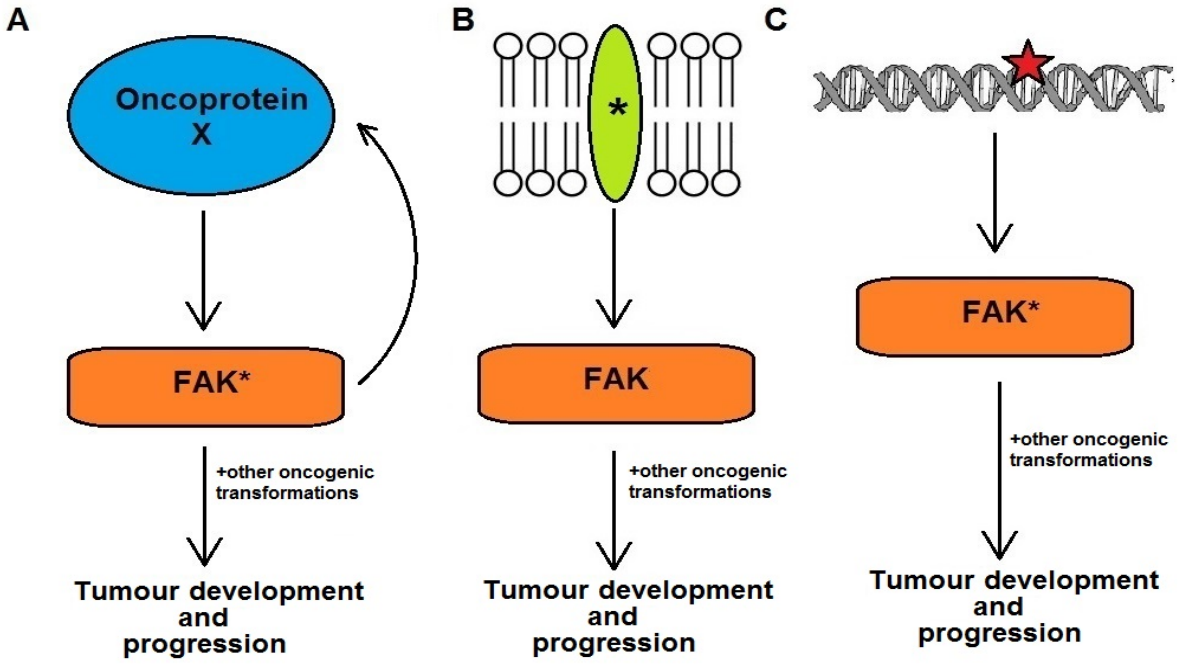


Figure 1.7 – Possible induction of cancer through FAK – Coupled with other malignant transformations, there are a number of possible mechanisms by which FAK contributes to a cancerous phenotype. A) An oncoprotein (e.g. v-Crk or mutant p53) causes a transformation in FAK resulting in it becoming unregulated (FAK*). This then contributes to a malignant phenotype, whilst also maintaining the activity of oncoprotein X so it can continue to affect FAK and aberrant signalling. B) An overexpression or oncogenic transformation of a RTK or GFR leads to constitutive stimulation of FAK activity and thus its associated pathways contributing to cancer development and progression. C) A genetic mutation to either regulatory sequences or the *Fak* gene results in overexpression or hyperactivity of the FAK protein. This then causes aberrant stimulation of FAK-associated signalling pathways influencing malignant transformation. This mechanism may also lead to a sequestering of tumour suppressor proteins (such as p53) further enhancing malignancy.

1.3.1 FAK and tumour initiation – Proliferation and Survival

In normal tissues, FAK has been shown to regulate integrin mediated cell cycle progression. One of the ways it does this is through interacting with the RAS-MAPK-ERK pathway. Upon maximal activation, phosphorylation of FAK^{Y925} occurs which promotes the binding of the Grb2 protein (Schlaepfer *et al.* 1994). This subsequently activates ERK, which in turn causes transcriptional activation of cyclin D1 and, following binding to its corresponding cyclin-dependent kinase (CDK), initiates the events leading to progression from G_1/S phase (Zhao *et al.* 2001). The activation of

FAK not only affects positive cell cycle regulators but also cell cycle inhibitors. For example, expression of the cip/kip family inhibitor p21 has been demonstrated to be downregulated in response to FAK activation (J.-H. Zhao *et al.* 1998). Normally this protein inhibits the interactions of cyclin-CDK complexes in response to DNA damage thus halting the cell cycle in G_1: it also regulates cellular senescence (Gartel and Radhakrishnan 2005). The downregulation of this protein thus means cells are free to progress in the cell cycle through what is seen as the major commitment step (i.e. once G_1 to S-phase is accomplished the cell will undergo a complete cell cycle). Other pathways have also been implicated in FAK regulation of cellular proliferation including the JNK pathway (Oktay 1999). This evidence thus suggests that the loss of FAK regulation would lead to uncontrolled cell division and thus cancer.

Indeed, this is the case as several groups have displayed direct evidence for FAK's regulation of the cell cycle. Recently, a number of these groups have utilised genetically modified mice to observe such effects *in vivo* and compliment *in vitro* studies. One such study demonstrated that by specifically knocking-out FAK in tumour cells, the activity of several proliferation regulators including ERK, cyclin D1 and p130cas were significantly reduced leading to growth arrest of the tumour itself (M. Luo *et al.* 2009). Other groups have also highlighted the importance of specific pathways in FAK-mediated proliferation. In 2002, Aguirre and colleagues showed that FAK inhibition impaired the growth of Hep3 liver cells through downregulation of MAPK/ERK signalling, which could be rescued by an active MEK 1 mutant in FAK-negative mutant cells (Aguirre Ghiso 2002). The evidence presented by these groups not only demonstrates that FAK is central in regulating proliferation, but more importantly inhibition of this over-activity can lead to a disruption of tumourigenesis. As a result, targeting FAK with novel inhibitors could lead to an arrest of this early, tumourigenic event and thus prevent tumours becoming more developed and detrimental to the patient. This idea has not been explored in much detail, particularly in the TNBCs. As such, there is an opportunity with this project to observe whether inhibition of FAK activity in TNBCs leads to an alleviation of the malignant phenotype, possibly through the induction of senescence or arrest of the cell cycle.

FAK may also promote tumour cell survival through inhibition of apoptotic processes. For example, Sonoda et al (2000) reported that FAK overexpression in HL-60 leukaemia cells induced Akt signalling resulting in inhibition of caspase-3-mediated apoptosis. Moreover, FAK was also able to promote activation of the NFkB, another molecular pathway implicated in pro-survival

functions, and inhibitory apoptotic proteins (IAPs) (Sonoda 2000). Numerous studies have also shown that through inhibition of FAK, this pro-survival signal can be overcome and thus enables normal apoptosis. In 2000, it was demonstrated (through inhibition with a dominant negative mutant) that the loss of FAK activity in breast cancer cells induces caspase-8 driven apoptosis (L.-H. Xu *et al.* 2000). This particular paper, along with subsequent related studies (for example Kurenova et al. 2004), demonstrated a direct involvement of FAK in suppressing the activities of the death-inducing signalling complex (DISC). Moreover, this work showed directly that FAK inhibition led to selective destruction of malignant cells without adverse effects to normal cells. This highlights the potential of FAK as a novel therapeutic target as selective elimination of cancer cells without affecting healthy body cells is a key goal for effective cancer treatment. This is particularly relevant for TNBCs where chemotherapy represents the only real treatment option with the ensuing side-effects of targeting normal cells.

1.3.2 FAK and metastatic processes – Migration and Invasion

Undoubtedly, the most important processes in the establishment of advanced malignancies are those governing the migration of tumour cells and subsequent invasion of distal sites. Without the ability to do either process, metastasis cannot occur. Consequently, targeting the regulatory pathways mediating these activities is very desirable, especially in cancers that are prone to aggressive metastatic behaviours (including the TNBCs). More relevantly, FAK has been implicated in the mechanisms that regulate the complex cellular changes required for migration and invasion to occur.

Numerous studies have demonstrated that FAK is a central regulator of cancer cell migration and thus metastasis. However, as for all other functions of FAK, migration does not rely on a single pathway but rather the manipulation of several signalling cascades involved in motility. Due to their importance in cancer and normal development, a number of these pathways have been studied in great detail. Probably the best example of this is the utilisation of p130cas by the FAK/Src complex. Inhibition of p130cas phosphorylation can be seen if FAK is unable to bind Src or p130cas (Cary 1998). Subsequently, p130cas fails to associate with SH2 containing proteins for example Crk, which remains inactive and fails to initiate DOCK180 and Rac activities, thus preventing migration (Cho 2000) Interestingly, this is an example of kinase-independent function, with FAK acting as a scaffold protein for the transmission of cell signals. The kinase function of FAK is also important in mediating the motility of cells. For example, FAK directly

modulates the phosphorylation of Grb7 when it is associated with PI3K (Shen *et al.* 2002). Consequently, both kinase-dependent and independent mechanisms are required for migration, thus the study of both is vital if successful FAK inhibitors are to be developed.

The migration of tumour cells alone is not sufficient to induce a metastatic phenotype. Cells also need to be able to invade into tissues at distal sites. A number of studies has directly implicated FAK in various stages of this process. For example, FAK has a crucial role in coordinating invadopodia dynamics. FAK-null tumour cells develop extra invadopodia but their presence is not complimented by a reduction in FA adhesiveness (Chan *et al.* 2009). However, loss of any of such processes results in a significant reduction of invasive potential and, as such, reduced ability of cancer cells to metastasise. Importantly, this has been demonstrated in model TNBC cells where metastatic potential is significantly reduced when siRNA transfected cells are introduced into mouse mammary fat-pads (Benlimame, He, Jie, Xiao, Xu, Loignon, Schlaepfer and M. A. Alaoui-Jamali 2005). This result was attributed to a loss of invasive properties, implying FAK is crucial to tumour invasion and thus metastasis.

It is important to note that FAK-mediated processes in migration and invasion are not mutually exclusive, often working in parallel. An excellent example of this is seen in studies investigating epithelial-to-mesenchymal transition (EMT). This process is essential to tumour cells if they are to successfully migrate and invade distal tissues. In short, EMT is the alteration of the typical ordered and tightly controlled organisation of cells to a system where cells are free to move with limited (if any) bonds to neighbouring cells. In normal epithelial cells, order is maintained through intercellular junctions, which contain the E-cadherins molecule. In cancer cells however, these adhesion molecules become internalised/lost/dysfunctional as a result of FAK/Src mediated disruption (Avizienyte and Frame 2005). This leads to cellular structures which can be manipulated without impedance from neighbouring cells and thus promote invasion and migration. Loss of maximal FAK activation by Src results in a failure of cancer cells to disrupt such cell contacts through E-cadherins (Avizienyte *et al.* 2002). All evidence in this section shows that FAK is essential for the main processes of metastasis. Consequently, targeting FAK in this context displays exceptional therapeutic potential as reducing metastatic disease will ultimately increase cancer patient prognosis.

1.3.3 FAK and the "cancer stem cell" hypothesis

Since the early 1980s, cancers have been known to be extremely heterogeneous, with a number of different tumour cell types found within a single tumour (Heppner and Miller 1983). Subsequently, a large amount of research has focused on the differences between these cell types in an attempt to discover the roles of the different tumour cells in disease progression. Arguably, the most important and controversial ideas to arise from this research was the identification of a subpopulation of cells that displayed stem cell like characteristics, the aptly named cancer stem cells (CSCs) (Reya *et al.* 2001). Such stem-like properties included, the ability to self-renew, asymmetrically divide and survive anoikis; cellular functions that would be useful attributes for a cancer cell to help it survive and proliferate at distal sites thereby supporting metastatic development **(figure 1.8A)** (Clarke *et al.* 2006). In addition, it has been postulated that CSCs are responsible for the relapse of cancer as they display a remarkable ability to resist a range of therapies and so persist following treatment and lead to new tumour formation (often referred to as "minimal residual disease") **(figure 1.8B)**. For example, Guan et al (2003) showed that in acute myeloid leukaemia a small proportion of CSCs were in a state of quiescence and could consequently persist following chemotherapy (Guan *et al.* 2003). This is understandable given traditional chemotherapeutic agents target rapidly dividing cells. This idea has also been linked to increased resistance to the initial therapeutic strategy following relapse (Vermeulen *et al.* 2008). In this case, CSCs with a specific resistance are possibly being selected for and are subsequently passing resistance on to the remaining bulk cells of the tumour leading to an overall desensitisation of the cancer to treatment **(figure 1.8B)**.

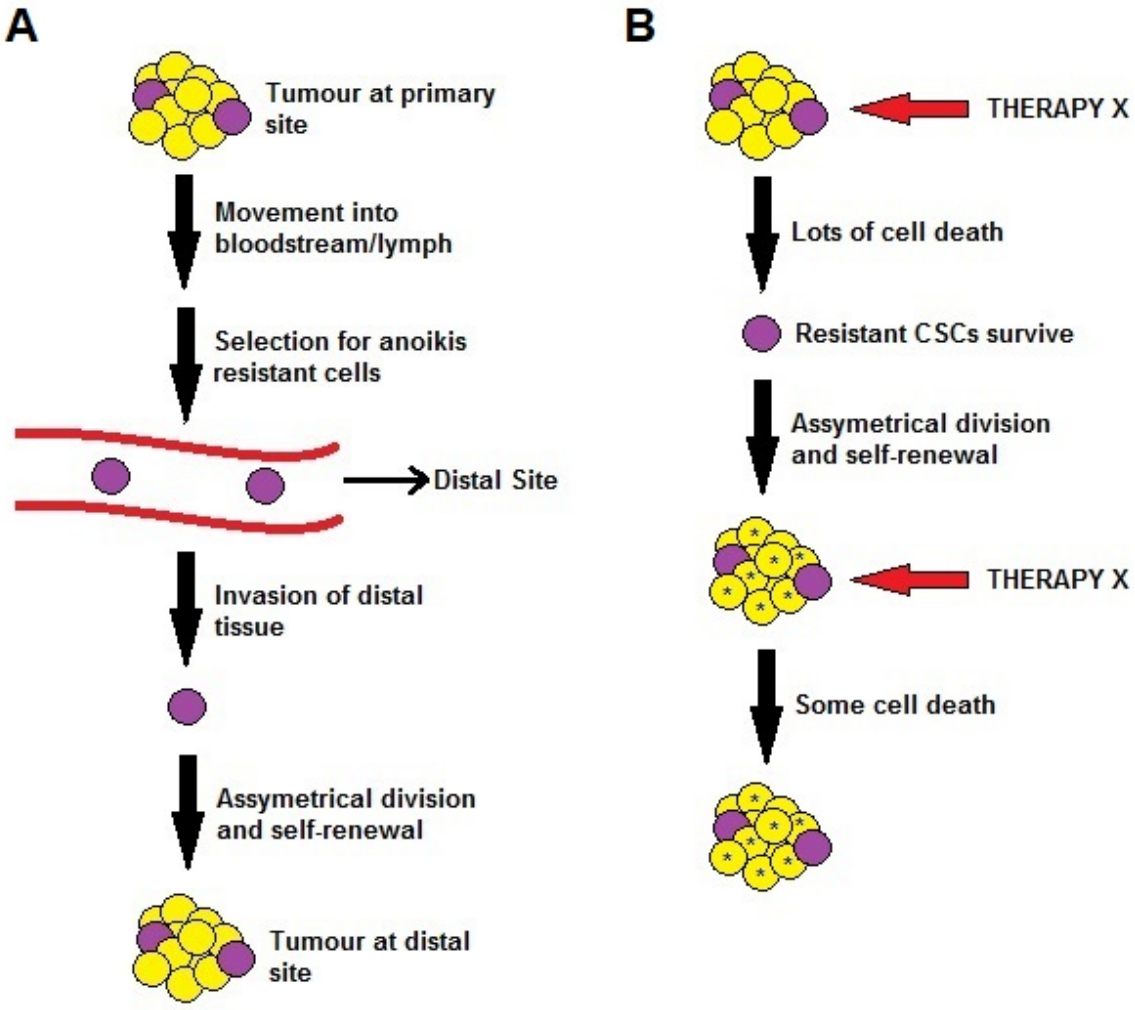


Figure 1.8 – Contribution of CSCs to metastatic disease and cancer relapse – Cancer stem cells are hypothesised to be the basis of metastatic disease and cancer recurrence. **(A)** In order for cells to form complete tumours at distal sites, cells must undertake a variety of different processes. The ability to migrate and invade alone is not sufficient. Cells must also be able to survive anoikis, as well as divide to form complete heterogenous tumours whilst also self-renewing. Such phenotypes are fundamentally stem-like and thus selection of these cells during metastasis is the cause of distal tumours. **(B)** Upon treatment with a therapeutic agent (therapy X) a large number of cells will undergo cell death. Due to the increased resistance of CSCs, such cells persist following treatment. Due to their ability to form all other cells of the tumour and self-renew, these cells soon lead to the reestablishment of complete tumours. The resistant and aggressive characteristics of these cells can be subsequently passed on to daughter cells leading to an increased resistance of new tumours to therapy, along with increased propensity to migrate and cause metastatic disease.

As a result of these observations, it should be a therapeutic priority to remove the CSCs along with the bulk of the tumour to have the best chance of successfully treating patients: particularly in the context of preventing disease relapse and spread. This poses a particular problem in cancer patients exhibiting TNBCs. As previously mentioned, the current treatment for TNBC is very unspecific, with chemotherapy being the only therapeutic option for such patients. Moreover, research has highlighted that TNBC and other aggressive cell populations exhibit extremely high numbers of potential CSCs based on the cell surface marker profile $CD24^{-}/CD44^{+}$ when compared to less invasive subtypes, such as ER+ (Sheridan *et al.* 2006). Consequently, TNBC patients are likely to have increased odds of aggressive relapse compared with those undertaking more targeted treatments, possibly resulting from the sustained presence of the CSCs. Further research is thus needed into the mechanisms that regulate the CSCs present in TNBCs.

Given the phenotype of CSCs, it is likely that FAK activity will be involved to some extent. In addition, CSCs typically display the ability to survive non-adherent conditions. Due to FAK's role in mediating integrin signalling and cell death, it is possible that FAK may contribute to anoikis resistance in these cells. This has been shown previously. For example, loss of FAK function in anoikis resistant breast cancer cells resulted in a significant loss of cells growing in non-adherent culture (L.-H. Xu *et al.* 2000). All these factors imply that FAK may be central to the maintenance and activity of CSCs. Consequently, targeting FAK for therapeutic intervention may provide a means for complete tumour eradication by targeting both CSCs and the remaining cells of the tumours. This will result in significant improvements in patient prognosis resulting from reduced chance of relapse and metastatic disease.

1.3.4 Chemotherapy and the influence of FAK

Currently, treatment of TNBC patients is limited to chemotherapy, along with surgical excision of cancerous tissue. Despite an initial success, the benefits of such therapies are short lived with a high percentage of TNBC sufferers displaying relapse soon after. This is largely due to the nature of the chemotherapeutics as a general treatment whose effects are not limited to the tumour cells. Moreover, chemotherapies tend to cause very dramatic side-effects owing to the killing of healthy body cells. It is essential therefore, that new targeted therapies are developed to improve prognosis and quality of life. However, for a targeted therapy to be widely accepted it will need to be both more financially and therapeutically beneficial than existing treatment options. As such, many groups are focussing on ways to improve the efficacy of chemotherapy

regimens through increasing the sensitivity of tumour cells to chemotherapy agents (for a review in TNBCs see Bramati *et al.* 2014). Agents that can successfully target FAK may provide just such a solution.

1.4. Aims and Objectives

TNBC is highly aggressive breast cancer subtype frequently associated with poor survival, therapeutic response and short disease-free periods before relapse. FAK has a significant role in promoting tumourigenesis, as well as metastatic cell behaviours such as migration, invasion and maintenance and function of CSCs. We hypothesise that FAK could drive TNBC cell proliferation whilst imparting a pro-metastatic phenotype and thus may represent a novel therapeutic target in such disease.

To explore this hypothesis, the following objectives were set:

- Explore the functional relevance of FAK activity, expression and sensitivity to inhibition across breast cancer subtypes to assess potential contributions to aggressive cell behaviour by basal FAK.

- Investigate the role FAK plays in tumourigenic and metastatic-associated processes, including migration and invasion, in models of triple-negative disease through inhibition of kinase activity (using pharmacological inhibitor PF-271) and scaffold functions.

- Assess the contributions of FAK to cancer “stem-like” cell behaviours in triple-negative cells, focussing on how inhibition alters such processes, particularly self-renewal.

- Evaluate a panel of novel FAK inhibitors, primarily targeting the protein-protein interactions and focal adhesion targeting, in a model of TNBC cells.

2. Methodology

2.1. Materials and Reagents

2.1.1. Key drugs and reagents used

- PF-562,271 (**PF271**, purchased from Tocris Bioscience) was used to inhibit FAK activity throughout this project. This compound acts as a specific and potent suppressor of FAK and Pyk2 kinase activity, through ATP-competitive inhibition (Roberts *et al.* 2008). A stock of 5mM was prepared from dried reagent using DMSO and diluted as required. The structure 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) is shown below **(figure 2.1A)**.

- Chloropyramine hydrochloride was also utilised as a FAK inhibitor, as well as being employed as a lead compound for modification to produce novel FAK inhibitors **(chapter 6)**. This drug was first identified as an antihistamine (Vaughan *et al.* 1949) but has more recently gathered momentum as an anticancer agent (Kurenova *et al.* 2009). It has been shown to impair FAK function by interfering with protein-protein interactions, particularly with VEGFR3, hence its classification as a scaffold-inhibitor. **Figure 2.1B** shows the structure of chloropyramine.

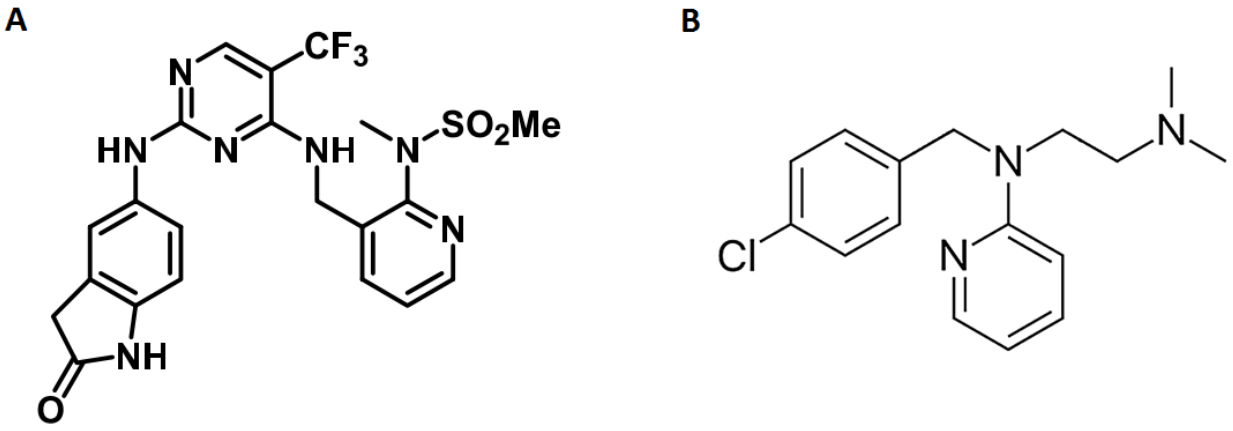


Figure 2.1 – Chemical structure of FAK inhibitors (A) PF271 and (B) Chloropyramine

- Recombinant human Wnt3a protein (Wnt3a-ligand, R&D Systems) was acquired as a lyophilised powder containing bovine serum albumin (BSA) as a carrier protein, which enhances stability and shelf-life of recombinant proteins. Product was reconstituted to in sterile PBS+1% BSA to give a stock of 200µg/ml.

2.1.2. Other materials

All other reagents and their respective suppliers used are compiled in **table 2.1**:

Reagent	Source
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma Aldrich
30% Acrylamide	Sigma Aldrich
50x B-27 Serum-free supplement	Life Technologies Ltd
5x siRNA buffer solution	Thermo Scientific
Aldefluor Detection Kit	Stemcell Technologies
Alexafluor fluorophores-488,594	Invitrogen
Ammonium Persulphate (APS)	Sigma Aldrich
Aprotinin	Sigma Aldrich
Basement Membrane Matrix, Phenol-red free (Matrigel)	VWR International Ltd UK
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)	Invitrogen
Chemiluminescent reagents (ECL, DURA, Femto)	Fisher Scientific UK
Corning Costar Ultra-Low Attachment Plates (96-well)	Fisher Scientific UK
Corning Standard Transwell Inserts (6.5mm diameter, 8µm pore size)	Fisher Scientific UK
Crystal Violet	Sigma Aldrich
Dharmafect Transfection Reagent	Thermo Scientific
Dimethyl sulphoxide (DMSO)	Sigma Aldrich
Di-thiothreitol (DTT)	Sigma Aldrich
Fibronectin (from human plasma); 1mg/ml	Sigma Aldrich
FITC-conjugated Anti-CD24	AbCam
FITC-conjugated Mouse IgG – Isotype Control	AbCam

Foetal Calf Serum (FCS)	Gibco UK
Fungizone	Invitrogen
Glycerol	Fisher Scientific UK
Glycine	Fisher Scientific UK
Human EGF	Sigma Aldrich
Hydrocortisone	Sigma Aldrich
Insulin	Sigma Aldrich
Leupeptin	Sigma Aldrich
MEGM	Lonza group Ltd
Methanol	Fisher Scientific UK
Penicillin/Streptomycin	Invitrogen
Phenylarsine oxide	Sigma Aldrich
Phenylmethylsulphonyl fluoride (PMSF)	Sigma Aldrich
Ponceau S solution (0.1%w/v in 5% acetic acid)	Sigma Aldrich
Precision Blue Protein Marker	BioRad Laboratories Ltd
Propidium Iodide	
siRNA buffer (1x) Diluted in H_20	Sigma Aldrich
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
TRITC-labelled actin phalloidin	Sigma Aldrich
Triton-X-100	Sigma Aldrich
Trizma Base (TRIS)	Fisher Scientific UK
Tween-20	Sigma Aldrich
Vectashield mounting medium (hard-set) containing DAPI	Vectorlabs
Wnt3a Ligand	R&D systems

Table 2.1 – Summary of all reagents used throughout this project

A complete summary of antibodies used for immune-based detection in this project are outlined in **table 2.2**, summarising host species, distributor and product codes:

Target Protein	Use	Species	Distributor	Catalogue Number
Akt^{S473}	Wb	Rabbit	Cell Signalling	4051
AKT (total)	Wb	Rabbit	Cell Signalling	9272
β-actin*	Wb	Mouse	Sigma Aldrich	A5316
Active, non-phospho, β-catenin	Wb	Rabbit	Cell Signalling	8814
β-catenin$^{S33/S37/T41}$	Wb	Rabbit	Cell Signalling	9561
CyclinD1^{T286}	Wb	Rabbit	Cell Signalling	3300
FAK^{Y397}	Wb/IF	Rabbit	Cell Signalling	3283
FAK ^{Y861}	Wb/IF	Rabbit	Invitrogen	44-626-G
FAK^{Y925}	Wb	Rabbit	Cell Signalling	3284
FAK (total)	Wb/IF	Rabbit	Cell Signalling	3285
GAPDH*	Wb	Mouse	SantaCruz	SC-32233
GSK3β^{S9}	Wb	Rabbit	Cell Signalling	9336
Phospho-MAPK42/44	Wb	Rabbit	Cell Signalling	9101
MAPK42/44 (total)	Wb	Rabbit	Cell Signalling	9102
MTOR^{S2448}	Wb	Rabbit	Cell Signalling	2971
PARP	Wb	Goat	R&D Systems	AF-600-NA
PTEN (total)	Wb	Rabbit	Cell Signalling	9552
Pyk2^{Y402}	Wb	Rabbit	Cell Signalling	3291
Pyk2 (total)	Wb	Rabbit	Cell Signalling	3292
STAT3^{S727}	Wb	Rabbit	Cell Signalling	9134
STAT3^{Y705}	Wb	Rabbit	Cell Signalling	9131
Vinculin	IF	Mouse	AbCam	ab73412
Anti-Rabbit IgG**	Wb	Goat	Cell Signalling	7074
Anti-Mouse IgG**	Wb	Sheep	GE Healthcare	NXA931
Anti-Mouse IgG (Alexa-594)**	IF	Goat	Invitrogen	A-11032
Anti-Rabbit IgG (Alexa-488)**	IF	Goat	Invitrogen	A-11008

Table 2.2 – Summary of primary and secondary antibodies used in this project – A complete list of primary and secondary antibodies utilised throughout this body of work. For western blotting analysis, all

primary antibodies were diluted to 1:1000, except FAK^{Y925} and Pyk2^{Y402} which required 1:500 for clarity. Additionally, GAPDH and β-actin were used as loading controls and used at a dilution of 1:15,000. For immunofluorescence, primary antibodies were used at a dilution of 1:100. **Wb:** western blotting; **IF:** immunofluorescence. *HRP-conjugated antibodies; **HRP-conjugated secondary antibodies for western blotting or secondary antibodies bound to fluorophore, located in brackets.

2.2. Cell Culture

The range of *in vitro* breast cancer cell models used throughout this project are summarised in **table 2.3**, outlining routine culture conditions and hormone receptor status. All cell lines were obtained from ATCC.

Cell Line	Hormone Receptors	Clinical Subtype	Source	Tumour Type	Culture Conditions
MDA-MB-231	ER- PR- HER2-	TNBC	PE	Pleural effusion from Breast adenocarcinoma	RPMI + 5%FCS Dilution 1:10
MCF-7	ER+ PR+ HER2-	Luminal A	PE	Invasive ductal carcinoma	RPMI + 5%FCS Dilution 1:8
SkBr3	ER- PR- HER2+	HER2+	PE	Pleural effusion from Breast adenocarcinoma	RPMI + 10%FCS Dilution 1:6
MDA-MB-361	ER+ PR- HER2+	Luminal B	P. Br	Breast adenocarcinoma from brain metastatic site	RPMI + 10%FCS Dilution 1:6
BT474	ER+ PR+ HER2+	Luminal B	P. Br	Invasive ductal carcinoma	RPMI + 10%FCS Dilution 1:6
MDA-MB-468	ER- PR- HER2-	TNBC	PE	Pleural effusion from Breast adenocarcinoma	RPMI + 5%FCS Dilution 1:8

Table 2.3 – Summary of breast cancer cell models used – Characterisation of breast cancer cell lines utilised in this work. Details of hormone receptor status adapted from (Neve *et al.* 2006). **PE:** pleural effusion; **P. Br:** Primary breast cancer. **RPMI:** Roswell park memorial institute medium including phenol-red; **FCS:** Foetal calf serum. Standard culture medium was also supplemented with 10µg/ml streptomycin, 10Units/ml penicillin and 2.5µg/ml fungizone. Dilution representative of seeding density during subculture.

All cell lines were maintained at 37°C in 5% CO_2 and routinely sub-cultured when confluency reached 80-90%. This was achieved by incubating cells with trypsin/EDTA solution (0.05%/0.02% respectively in PBS) in a 37°C, 5% CO_2 humidified incubator, until cells detached from the flasks before serum-containing media was added to neutralise the trypsin solution. This solution was centrifuged at 1,000RPM for 5-minutes to pellet cells, prior to discarding of supernatant and subsequent resuspension in fresh standard culture media. Resultant cells were sub-cultured into flasks at the appropriate density (outlined in **table 2.3**). All work was performed in sterile, laminar flow safety cabinets to prevent contamination of cultures.

To perform stem-cell enrichment (mammosphere) assays, cells were cultured under non-adherent conditions in complete mammosphere media. This was comprised of mammary-epithelial growth media (MEBM) (Lonza), a solution optimised for serum-free, mammary epithelial cell growth supplemented with 5µg/mL Insulin, 1ng/mL Hydrocortisone, 20µg/mL Gentamycin (Sigma), 2% B27 serum-free supplement (Gibco) and 2ng/mL EGF.

2.3. Optimisation of RNAi transfection

We chose to utilise siRNA as a means of transiently suppressing protein expression in our cells. Consequently, we utilised various ON-TARGETplus systems (Thermoscientific) for FAK, Pyk2 and PTEN (details in **table 2.4**), as well as a non-targeted, scrambled control. The siRNA pool for all were obtained as a lyophilised reagent which was reconstituted to a concentration of 20µM in 1x siRNA buffer (diluted from 5x stock in RNAse-free sterile water). All siRNA experiments contained the following controls:

- **Culture medium control** – to provide a baseline **(con)**
- **Lipid-Only Controls** – to affirm that transfection reaction, specifically the lipid, does not perturb cell function, these cells were treated with DHARMAfect transfection reagent only.
- **ON-TARGETplus non-targeting siRNA control** – this is a scrambled siRNA which has no recognised mRNA binding targets and thus allows observation of non-specific effects resulting from siRNA delivery **(NT siRNA)**.

siRNA Target	FAK (PTK2)	Pyk2 (PTK2B)	PTEN	Non-targeting control
Product number	L-003164-00-0005	L-003165-00-0005	L-003023-00-0005	D-001810-10-05
mRNA Target sequences	GGACAUUAUU GGCCACUGU	GGAUCAUCAU GGAAUUGUA	GAUCAGCAUA CACAAAUUA	UGGUUUACAU GUCGACUAA
	UAGUACAGCU CUUGCAUAU	UCAGUGACGU UUAUCAGAU	GACUUAGACU UGACCUAUA	UGGUUUACAU GUUGUGUGA
	GGGCAUCAUU CAGAAGAUA	GAAGAUGUGG UCCUGAAUC	GAUCUUGACC AAUGGCUAA	UGGUUUACAU GUUUUCUGA
	GCGAUUAUAU GUUAGAGAU	GAGGAAUGCU CGCUACCGA	CGAUAGCAUU UGCAGUAUA	UGGUUUACAU GUUUUCCUA

Table 2.4 – Summary of targeted siRNAs used throughout this project – Outlined are details of the four siRNAs (FAK, Pyk2, PTEN and Non-targeting scramble control), including product numbers (GE Healthcare – Dharmacon). All siRNAs were composed of four potent mRNA targeting sequences which are also included.

Prior to transfection, cells were grown to 50% confluency. At which time, 20µM stocks of appropriate siRNA were diluted in 1x siRNA buffer and serum-free RPMI as appropriate to achieve a final working concentration of 100nM. In parallel, DharmFECT1 reagent was diluted in serum-free RPMI in a separate tube. Following 5 minutes, both tubes were combined and incubated at room temperature for 20 minutes, enabling siRNA micelles to form. Resultant mixtures were placed in desired volume of RPMI+5% FCS and added to cell cultures. Cells were re-incubated at 37^{o}C and 5% CO_2 and a lysis performed at 24, 48 and 72 hours, after which siRNA containing media was replaced with complex-free standard culture medium. Cultures were subsequently lysed at 2, 4 and 6-days following incubation in order to evaluate recovery of protein levels post-siRNA. Lysis protocol is outlined in **section 2.5.1.1.**

2.4. Functional Cell Assays

2.4.1. Cell proliferation assays

2.4.1.1. MTT assay

This is a colour-based assay which allows evaluation of proliferation based on conversion of water-soluble, yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan crystals by mitochondrial dehydrogenase enzymes only present in viable, actively-respiring cells. Formazan is subsequently solubilised and evaluated using spectrophotometry, with the optical density directly proportional to the number of viable cells.

Cell cultures were redistributed into 96-well plates at a density of $1x10^6$ cells/plate and allowed to settle for 24-hours prior to treatment with various agents, as required, before culturing for 72-hours at 37°C and 5% CO_2. Resulting cells were gently washed with sterile PBS and 140µl MTT solution (0.5mg/mL in sterile PBS) was added. Plates were re-incubated for 4-hours, after which MTT solution was removed and cells lysed overnight at 4°C in a buffer comprised of 100µl v/v Triton-X100 (Sigma Aldrich) in sterile PBS. Subsequently, plates were analysed in a spectrophotometer (540nm). Mean absorbance values were then calculated and data represented as a percentage of vehicle-only controls.

2.4.1.2. Cell counting assay

In order to detect more discrete changes in proliferation, a counting approach was employed utilising a Coulter counter. This form of electronic counting is based on a single-cell suspension flowing past a sensor located in a small channel between two electrodes, which emits a pulse every time a cell is detected.

Cells were seeded into a 24-well plate ($4.2x10^5$ cells/mL) and, following 24-hours, had fresh media applied (±treatment) (Day 0). Plates were subsequently incubated at 37°C and 5% CO_2 and typically counted every day for up to 7-days. Alternatively, siRNA treated cells were allowed to grow for 72-hours in the presence of desired siRNA (Day 0), before old media was replaced with fresh (±treatment) and cells re-incubated for 72-hours at 37°C and 5% CO_2. At the chosen time-points, medium was removed and cells subsequently treated with trypsin/EDTA (1mL/well) to lift cells. Following dispersion, cells were passed through a 25G needle three-times, to ensure a single-cell suspension, prior to being washed three times with 1mL of Isoton solution. During

each wash, the Isoton was kept in the syringe and the final 4mL mixture (1mL cells+3mL Isoton) was released into a counting cup containing 6mL Isoton. Counting was then performed on a Coulter Multisizer III, with a minimum of two counts taken per well. All experimental conditions were conducted in triplicate, three independent times and mean cell count was represented as a percentage of respective control.

2.4.2. In vitro migration/invasion assays

2.4.2.1. Wound healing Assay

Given its simplicity, yet clear nature, wound healing assays were used in order to initially assess basal migration. Cells were grown in 24-well plates (seeded at $4.2x10^5$cells/mL) until 80% confluent, at which time monolayers were "scratched" with a sterile pipette tip to create a wound. The media was subsequently aspirated and replaced with fresh (±treatment), as well as triplicate images taken at known points in each well (0-hours). Wounded cultures were incubated at 37°C and 5% CO_2 for 18-hours, after which triplicate images were once again taken (24-hours). All experimental conditions were undertaken in duplicate and select wells were fixed in PFA and stained with 0.5% crystal violet to provide clear representative images of cell migration.

2.4.2.2. Boyden Chamber Assay

Migration

To more robustly explore basal cell migration, a Boyden chamber assay was performed. Assays were performed in 24-well, transwell permeable support plates (Corning Lifesciences) containing inserts with a 6.5mm microporous membrane (8µm pore size), which were coated with 10µg/ml fibronectin (1µl:100µl in sterile PBS). Cells were seeded into serum-free RPMI (±treatments) at a density of 50,000cells/ml into the top portion of the inserts, whilst the lower chambers contained FCS containing RPMI as a chemoattractant. Plates were subsequently incubated for 18-hours at 37°C and 5% CO_2 to allow cell migration to the underside of the insert. Migratory cells were fixed with 3.7% PFA and stained with 0.5% crystal violet, whilst non-migratory cells on the topside of the membrane were removed using a cotton bud. Through light microscopy, 5 images were captured from random fields of view, counted and resulting data were expressed

as mean cell counts per field of view. Experimental conditions were performed in duplicate and performed three times.

Invasion

A modified Boyden chamber assay was also adopted for analysis of cell invasion. Inserts were coated with 4mg/ml Matrigel (BD Biosciences), a substance that mimics the ECM forcing cells to actively invade through it, and allowed to set for 2-hours at 37^{o}C, 5%CO_2 in a 24-well transwell permeable support plate. Cells were diluted to a single-cell suspension of 500,000cells/ml in serum-free RPMI (±treatment) and seeded into Matrigel-containing inserts. RPMI+ 5%FCS was placed in the lower chamber as a chemoattractant, to encourage cell movement. Cultures were subsequently re-incubated at 37^{o}C and 5%CO_2 and for 72-hours, after which cell fixing was performed. This involved removal of Matrigel with a cotton bud, prior to incubation in 3.7% PFA for 15 minutes. Fixed membranes were subsequently washed, excised from the inserts using a scalpel blade and mounted onto glass slides using hard-set mounting medium containing DAPI. A coverslip was applied and media was allowed to harden overnight at 4^{o}C in the dark. Cells were viewed using a fluorescence microscope (358/461nm) from which, 5 images representing unique fields of view were acquired at x20 magnification so as to view most of the cells. All experimental conditions were conducted in duplicate and in 3 independent assays.

For both migration and invasion, FAK suppression through siRNA was achieved by incubation of cells with specific siRNA-complexes for 72-hours (as outlined in **section 2.**3), after which cells were collected, diluted and re-seeded into Boyden chamber inserts at the appropriate seeding density.

2.4.3. In vitro mammosphere assays

Cells were seeded in standard culture media into 35mm dishes (density of 100,000cells/dish) and incubated for 24-hours at 37^{o}C and 5% CO_2. Resulting cultures were re-incubated with fresh standard culture media (±treatments) for a further 24-hours. Alternatively, cells were cultured and treated with siRNA for 72 hours (as outlined in **section 2.A**). Cells were subsequently re-seeded in triplicate into 96-well, ultra-low attachment plates (Corning Life Sciences) at a density of 5,000 cells/mL in complete mammosphere media and incubated at 37^{o}C, 5% CO_2 for 7-days, after which cultures were evaluated through light microscopy. These passage 1 spheres were counted and represented as the number of mammosphere-forming units (MFU) as a percentage

of total number of cells seeded. Additionally, 5 representative images were acquired per well and subsequently analyses using imageJ so as to calculate sphere volume; a demonstration of proliferative capacity under anoikis conditions. Sphere volume was calculated as follows:

Mammosphere volume = $(\text{Sphere Width})^2(\text{Sphere length})(0.5)$

The mammospheres were then collected and centrifuged at 3,000RPM for 10-minutes to pellet for subsequent passaging. Pellets were incubated in trypsin/EDTA 37^{o}C for 10-minutes before neutralising with complete standard culture media. Cell were once again pelleted by centrifuging at 3,000RPM for 10-minutes prior to resuspension in complete mammosphere media. The resulting solutions were vigorously pipetted and passed through a 25G needle to ensure a single cell suspension, before reseeding into ultra-low attachment plates (at a density of 5,000 cells/mL). Spheres were then allowed to grow for seven days before being counted and photographed.

2.5. Antibody-based detection

2.5.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting

2.5.1.1. Cell lysis for protein extraction

Cultured cells were placed on ice and washed 3 times in sterile PBS before lysis buffer was added. Fresh protease inhibitors (2mM sodium orthovanadate ($NaVO_4$), 50mM sodium fluoride (NaF), 1mM PMSF, 10mM sodium molybdate, 20µM phenylarsine, 10µg/ml leupeptin and 8µg/ml aproptin) were added to lysis bufferin order to improve yield by preventing protein degradation by various proteases. Moreover, this cocktail prevents dephosphorylation through inhibition of phosphatases, enabling phosphorylated residues to be detected. Dishes were subsequently scraped, harvested into Eppendorf tubes and maintained on ice for a further 5-minutes before centrifuging at 12,000RPM for 15-minutes at 4^{o}C. The protein containing supernatant was then collected and either stored at -20^{o}C until required or immediately analysed for protein content (**section 2.4.1.2.**).

2.5.1.2. Protein Quantification and sample preparation

The protein concentration in the supernatant was assessed using a BioRad DC protein assay. This is a colourimetric assay which produces a distinct blue colour following reduction of a Folin agent in response to the reaction of copper tartrate with protein. Briefly, 5µl of protein lysate was added to 45µl lysis buffer, alongside a series of known concentration, BSA protein standards (0, 0.25, 0.5, 0.75, 1, 1.45mg/mL) to create a standard curve. To both standards and test samples, BioRad reagent A (250µl; plus 20µL/mL BioRad reagent S) were added, followed by 2mL BioRad reagent B. Tubes were mixed gently and allowed to rest for 5-minutes, before being analysed on a spectrophotometer set to 750nm absorbance. BSA protein standard results were used to create a standard curve, from which protein concentration within each test samples could be calculated, subsequently allowing standardisation of protein concentrations from across lysates for their use in SDS-PAGE.

The basis of SDS-PAGE is the separation of proteins based on size. As such, the acquired lysates were used to create samples which were capable of being distributed by an electrical current. To achieve this, the protein concentrations (calculated above) were added to appropriate volumes of 2x or 5x Laemmli sample loading buffer containing 24mg/mL DTT. This buffer contained the following reagents, each with a unique function enabling optimal separation of protein: TRIS which acts as a buffer ensuring maintenance of pH during electrophoresis; glycerol allows sample to remain at the base of wells in acrylamide gels, through increasing sample density; SDS creates a negative charge on proteins by coating them following denaturation, thus enabling separation by an electrical charge. Meanwhile, the freshly added DTT supports SDS-coating by breaking disulphide bridges, thus deteriorating tertiary protein structures. To allow maximum denaturation and binding, prepared samples were boiled at 100^{o}C for 5-minutes.

2.5.1.3. SDS-PAGE

Porous, polyacrylamide gels were prepared on which proteins would be separated. Initially, a resolving gel of between 8-12% (composition of which are summarised in **table 2.5**) was added between two 1.5mm glass plates, held in place by a gel stacking apparatus (BioRad Mini Protean 3), and topped with dH_2O to prevent air perturbing polymerisation and ensure a flat edge. Once gels were set, the water layer was discarded and replaced by a 5% Stacking gel solution (recipe in **table 2.6**), into which a 10/1-well comb was inserted, before being allowed to further

polymerise for approximately 20 minutes or until the gel was set. Prepared gels were subsequently transferred to a electrophoresis, running tank, covered with running buffer (192mM glycine, 25mM TRIS; 0.1%w/v SDS) and wells loaded with protein samples, as well as Precision Plus All Blue marker (BioRad). As shown in **figure 2.1**, this product created a visible standard of protein sizes enabling identification of proteins based of weight. Electrophoresis was then performed at 120V until desired spreading was observed.

Final Gel % (Resolving)	8%	10%	12%
30% Bis-acrylamide	5.4ml	6.6ml	8ml
dH_20	9.2ml	8ml	6.6ml
TRIS pH 8.8	5ml	5ml	5ml
10% SDS	200µl	200µl	200µl
10% APS	200µl	200µl	200µl
TEMED	50µl	50µl	50µl

Table 2.5 – Recipes for resolving gel

Final Gel % (Stacking)	5%
30% Bis-acrylamide (5:1)	1.67ml
dH_20	5.83ml
TRIS pH 8.8	2.5ml
10% SDS	100µl
10% APS	50µl
TEMED	25µl

Table 2.6 – Recipe for stacking gel

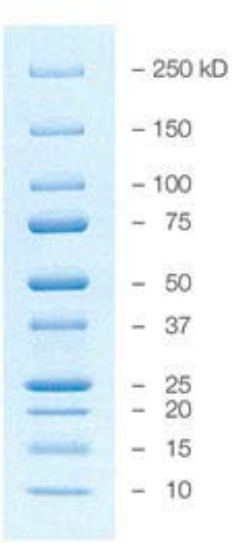


Figure 2.1 – Precision Plus Protein Blue Standard – Representative image of the blue marker as run on an 8% gel with protein weight in kDa denoted on right side.

Resulting gels were transferred to 45µm nitrocellulose membrane (GE Life-Sciences) by incubating with filter paper and sponges (illustrated in **figure 2.2**), before flooding the transfer apparatus with transfer buffer (0.25M TRIS base; 1.92M glycine; 20% methanol) and applying a constant voltage of 100V for 60-minutes. This enabled negatively charged proteins to be drawn onto the nitrocellulose membrane from gels. An ice block was also included to prevent overheating of the apparatus. Immediately after, membranes were rinsed with dH_2O to remove any transfer buffer and stained with Ponceau S to allow the effectiveness of the previous steps to be observed.

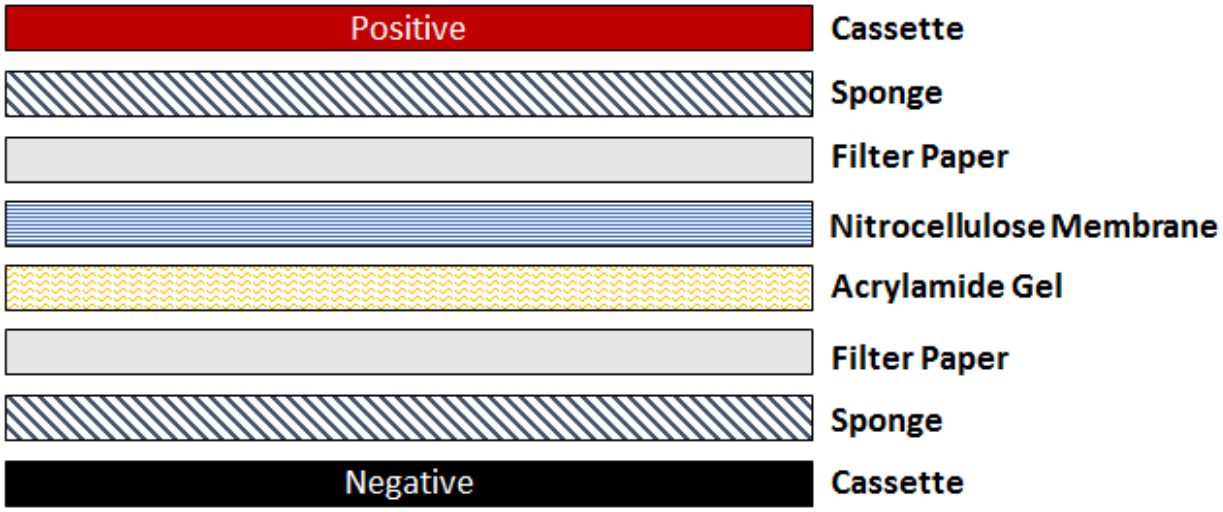


Figure 2.2 – Diagram of set up for wet transfer

This stain was subsequently removed by washing in TBS Tween-20 (TBST) and blocked with 10% non-fat milk powder (marvel) in TBST. Post-blocking, membranes were once again washed in TBST before incubation overnight with desired primary antibodies at 4^{o}C. The resulting membranes were washed in TBST, to remove unbound primary antibodies, and then incubated at room-temperature for 1-hour with horseradish-peroxidase (HRP)-conjugated secondary antibodies diluted 1:1000 in 2% Marvel-solution. A final wash was performed before chemiluminescent detection. This was achieved by treating membranes with a luminol-based chemiluminescence substrate which stimulated the enzymatic activity of HRP-bound secondary antibodies. Protein bands were subsequently visualised by exposing to blue-sensitive X-ray film, under dark conditions, and passing through an automated developer which consisted of fixative and developing solutions.

2.5.1.4. Densitometry

Band intensity was measured using the AlphaDigiDoc software. These integrated intensity values were normalised to the housekeeping proteins GAPDH or β-actin, allowing correction for loading errors. Values were subsequently processed as outlined in the respective results sections.

2.5.1.5. Stripping and re-probing blots

Sometimes, it was necessary to examine membranes for a second protein using a different primary antibody. In cases where the sizes of the initial and second proteins were comparable, membranes were stripped to avoid perturbation of band intensity resulting from the initial analysis. This process entailed immersion of membranes to be stripped in Restore Plus Western Blot Stripping Buffer (Fisher) at room temperature for a maximum of 15-minutes. Resultant blots were washed in TBST, re-blocked in 10% Marvel solution and incubated with primary antibody against the protein of interest (as in **section 2.4.1.3**).

2.5.2. Immunofluorescent staining

Cells were seeded into 35mm dishes containing 0.13-0.17mm thick coverslips (density 70,000cells/ml) and allowed to culture at 37^{O}C, 5% CO_2 until 60-70% confluent. To examine migrating cells, coverslips were coated with fibronectin (1:100 in sterile PBS) prior to seeding. Cells were then allowed to grow until 50% confluent, before being treated with serum-free RPMI for 24-hours and subsequently stimulated to migrate by incubating with RPMI+5% FCS for 1-hour at 37^{O}C and 5% CO_2. Following incubation, cells were washed in sterile PBS (three times) and fixed by submerging in 3.7% PFA (in dH_2O) for 15 minutes. Fixed cells were permeabilised using Triton-X100 (0.2% in PBS) for 8-minutes and blocked for 40-minutes in 10% normal goat serum (in 1%BSA PBS) to minimise non-specific antibody binding. Primary antibody (1:100 dilution in 1%BSA PBS) was subsequently added and left for 30-minutes. For dual-labelling with vinculin (diluted 1:200 in 1%BSA PBS), both primary antibodies were combined prior to addition. Stained cells were washed 3 times in PBS before application of specific fluorophore-bound secondary antibodies (Alexafluor-488, and Alexafluor-594 diluted 1:1000 in 1%BSA PBS). For detection of actin, FITC-conjugated phalloidin (diluted 1:2000 in 1%BSA PBS) was added along with secondary antibodies. Coverslips were then washed thoroughly in PBS and mounted onto glass slides using hard-set Vectashield mounting media containing DAPI (4', 6-diamidino-2-phenylindole) to stain cell nuclei. Subsequent viewing was performed on a Leica DMIRE2 microscope at 63x

magnification and oil immersed. Typically, 5 images were acquired per coverslip for each fluorophore, as well as a merged image. Where changes in the number and size of FAs was evaluated, imageJ was used to count individual FAs and, following a fixed magnification (x3) of the images, measure FA area. The number of FAs was counted in 3 cells per image and represented as mean number of FAs per cell, while 10 random FAs were measured from across all cells in each image.

2.5.3. Flow cytometry analysis

Unless stated otherwise, all preparatory stages for FACS analysis were conducted on ice.

2.5.3.1. Evaluation of cell cycle distribution

Cells were cultured in 35mm dishes for 24 hours, before replacement with fresh standard culture media (±treatment) and subsequent incubation for 3 days. Resulting cultures were harvested washed and resuspended in 1mL of ice-cold PBS at a density of $1x10^6$cells/mL. The resultant cell suspension was added drop-wise to 9mL of 70% ethanol (in PBS) and fixed overnight at -20^oC. Fixed samples were stained with a Propidium iodide (PI) staining solution (4.5mL PBS; 0.1% Triton-X100; 100µL PI solution (1mg/mL); 10%v/v ribonuclease A (0.5mg/mL) at a concentration of $1x10^6$cells per 500µl PI stain (if had fewer cells, adjusted volume accordingly) by incubating at 40^oC for 20 minutes. Stained cells were subsequently filtered through a nylon filter into FACS tubes to ensure a single cell suspension for analysis. The resulting solutions were analysed using FACS verse (BD Biosciences) and subsequent evaluation was performed using FlowJo software.

2.5.3.2. Assessment of stem-like subpopulations

2.5.3.2.1. CD44/CD24 analysis

Cells were seeded into 35mm dishes at 70,000cells/ml and allowed to settle for 24-hours, at which point medium was changed for fresh (±treatment) and re-incubated for a further 24-hours. Resulting cultures were harvested, washed with sterile PBS and resuspended in ice-cold sterile PBS (including 2%FCS and 1% sodium azide) at a density of 200,000cells/ml. The sodium azide and low temperature prevented the loss of fluorescence resulting from modulation and internalisation of cell surface antigens. Resultant solutions were incubated in the dark with primary antibodies on ice for 30-minutes, before being once again washed with ice-cold PBS and fixed by resuspending in 3.7% PFA in PBS. Cells were allowed to fix for 15 minutes at room

temperature and subsequently washed once with PBS containing 3% BSA. Secondary antibodies, where necessary, were then applied (diluted 1:1000 in 3%BSA PBS) and placed on ice for 30 minutes under dark conditions. Final PBS washes were performed and cell suspensions resuspended in ice-cold PBS containing 3%BSA and 1% sodium azide. Subsequent analysis was undertaken using a FACS Verse (BD Biosciences), with resulting data being evaluated through used of Flowing Software. Along with test samples, a number of controls were also performed allowing reliable acquisition of data and accurate gating for distribution analysis **(table 2.7)**.

Condition	Antibodies Present	Reason
Negative Control	No primary or secondary antibodies	Lack of any antibodies allows analysis of background and auto-fluorescence of cells.
Primary Only	CD44 primary only	Enables any fluorescence caused by primary antibody to be seen and accounted for in analysis.
Secondary Only	Alexa647 secondary only	Ensures specific binding of the secondary antibody to the designated primary antibody.
Isotype Control	Mouse IgG + Alexa647 FITC-conjugated Mouse IgG	Observe background binding of the isotype used by the antibody. Signal also used for drawing of quadrants.
CD24 Only	CD24-FITC conjugated only	Allows optimisation of voltage and other factors used to detect CD24
CD44 Only	CD44 + Alexa647	Same as previous except with CD44

Table 2.7 – Outline of control samples used in FACS experiments, the antibodies used in each system and the reason why the controls were used

2.5.3.2.2. ALDH analysis

As a further means of evaluating CSC subpopulations, analysis of ALDH staining was undertaken. Prior to any cell treatments, the Aldefluor substrate needed to be activated. To do this, powdered substrate was incubated with DMSO at room temperature for 1-minute, before adding 2N HCl, mixing well and allowing to rest at room temperature for 25-minutes. It was vital to add DMSO prior to HCl, as the inverse causes the Aldefluor reagent to irreversibly inactivate. Aldefluor Assay buffer was added to the solution and maintained on ice during use: activated solution was aliquoted stored at -20^{O}C for future use.

Cells cultured (as in **section 2.5.3.2.1**) were resuspended in 1ml Aldefluor assay buffer and diluted to a density of 200,000cells/ml. After adjusting concentration, 1ml of cell suspension was placed into a tube labelled "control" and 1ml into tubes labelled "test" in triplicate; this was performed for all experimental conditions. 5µl of 1.5mM DEAB inhibitor (in 95% ethanol) was added to the control tubes, whilst activated Aldefluor substrate was added to each test sample (5µl/ml) and mixed. Immediately, 0.5ml of one "test" tube was added to DEAB control cells, resulting in a final DEAB concentration of 15µM. It is essential that the cell suspension+Aldefluor was added to control tubes immediately to maximise accuracy of this assay, given that the enzymatic reaction of ALDH to Aldefluor begins as soon as the two are introduced. All tubes were subsequently incubated for 45-minutes at 37^{O}C, prior to centrifugation (300xg at 4^{O}C for 5-minutes) and resuspension of each pellet in ice-cold Aldefluor assay buffer. Resultant aliquots were placed on ice and immediately analysed using FACS Verse (BD Bioscience), with at least 100,000 events being examined per sample. Throughout analysis it was essential to keep all samples on ice to minimise efflux of fluorescent products of ALDH activity on Aldefluor reagent.

2.5.4. Immunocytochemistry (ICC) evaluation of Ki67

To examine Ki67 status, cells were cultured for 24-hours in 35mm dishes, prior to addition of fresh FCS containing RPMI (±treatment). Cells were subsequently fixed to glass coverslips following 24, 48 or 72-hours using 3.7% PFA, washed 3 times with PBS and incubated with Ki67 primary antibody at room temperature in a humidified staining chamber for 2-hours. The resultant stained coverslips were again washed in PBS before being treated with appropriate Dako Envision secondary antibody for 2-hours. Finally, cells were stained with 3,3'-diaminobenzidine (DAB) for 5 minutes, counterstained with haematoxylin and mounted onto

glass slides. Subsequent visualisation was undertaken with a light microscope, where 10 images were taken from different fields of view and final counts represented as Ki67 positive cells as a percentage of total cell number.

2.6. Statistical analysis of data

All statistical analysis was performed using Graphpad Prism 5 software. To compare two sets of data, a student's independent T-test was used. One-way analysis of variance (ANOVA) with Tukey's multiple comparison tests (post-hoc) was utilised to compare data with more than two groups, while two-way ANOVA with Bonferroni (post-test) was used for comparing more than two groups with two independent variables. Data was considered significant when $p \leq 0.05$. Where appropriate, error bars are representative of SEM.

3. Results (I)

Characterisation of FAK expression and activity in breast cancer subtypes

3.1. Introduction

The advent of molecular approaches has enabled the in-depth exploration of solid tumours to reveal their molecular heterogeneity. Indeed, genetic profiling of multiple tumour types has revealed them to exist not as one, but as many different tumour subtypes. As such, it is becoming clear that to effectively treat tumours, future treatments will need to address this heterogenicity and targeted therapies that are tailored to an individuals' tumour make up. It has been well established that there are 5 broad classes of breast cancer tumours based on molecular profiling: Luminal A, Luminal B, Normal-like, HER2+ and basal-like (Sørlie *et al.* 2001). These molecular subtypes comprise the three main types of breast cancer diagnosed, namely ER+, Her2+ or triple-negative breast cancer (TNBC). The identification of disease subtype provides vital information pertaining to therapeutic options, disease progression and ultimately prognosis. Despite primarily comprising of basal-like cells, TNBCs have more recently been highlighted as a separate disease based on clinical progression and intrinsic biological characteristics. Many studies have shown that by comparison to other subtypes, triple-negative tumours have significantly poorer prognosis, owing to a more rapid disease progression and much shorter times from relapse to death (Bauer *et al.* 2007).

FAK is essential to many cellular processes, particularly those that govern cell migration in both early development and in mature tissues. In the former case, studies have shown that FAK depletion is embryonically lethal, arising from impaired cellular distribution during embryogenesis (Petridou *et al.* 2013). Additionally, aberrant FAK expression and/or activity has been reported to be associated with the progression of solid tumours, including breast cancer, to a metastatic phenotype (Golubovskaya *et al.* 2014). Given that triple-negative breast cancers exhibit a high tendency for early disease progression and metastatic potential (Bauer *et al.* 2007) and that FAK promotes cellular migration, one hypothesis is that this intrinsic aggressive phenotype of TNBC is driven, at least in part, by FAK.

FAK has one closely related family member, Pyk2, which has also been shown to contribute to cancer cell behaviours (Lipinski and Loftus 2010). Importantly, Pyk2 can provide some functional redundancy for FAK, enabling cells to sustain some FAK-driven processes through upregulation of Pyk2. For example, Pyk2 expression/activity is increased following FAK deletion in mice mammary-tumour cells and is sufficient to restore metastasis *in vivo* (Fan and Guan 2011). As such, Pyk2 may also play a role in TNBC cells.

3.1.1. Aims and Objectives

The primary aims of this chapter were to characterise FAK and Pyk2 expression and activity across breast cancer subtypes to determine if there is a correlation between their expression/activity and the aggressiveness of the subtype, as well as to assess the efficacy of pharmacological inhibition of FAK on cellular behaviour. Consequently, the objectives of this chapter were as follows:

- Explore the association of FAK and Pyk2 expression with prognosis using online gene sets.

- Analyse FAK expression/activity and localisation across a panel of breast cancer cell lines reflective of the dominant clinical subtypes.

- Characterise cell models of major clinical subtypes for their intrinsic proliferative and migratory capacity and correlate the expression/activity of FAK with these behaviours.

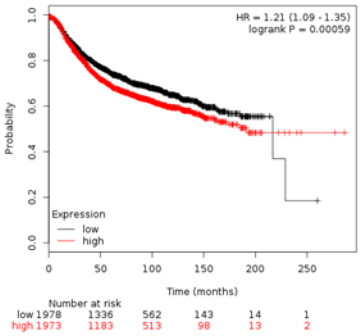
- Investigate the impact of FAK inhibition on the proliferative and migratory capacity of these cell models.

3.2. Analysis of FAK expression with breast cancer patient outcome

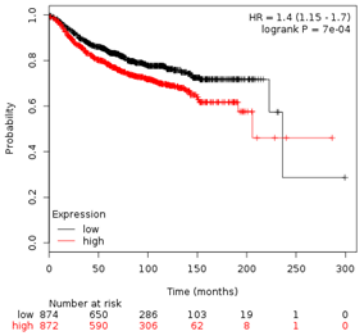
Studies have suggested overexpression of FAK in breast cancer is largely correlated with a highly aggressive phenotype (Lark *et al.* 2005). As such, we initially wished to explore the association FAK and outcome in different breast cancer subtypes using publicly-available, online Kaplan-Meier analysis (www.kmplot.com) (for examples of use see Győrffy et al., 2010 and Szász et al., 2016). An unrestricted comparison (no limitations on tumour grade, lymph-node status or endocrine/chemotherapy treatments) of 3951 breast cancer patient samples, revealed a significant association between FAK expression and patient relapse-free survival (RFS) over 25 years (n=3951; logrank P = 0.00059) **(figure 3.1A)**. High FAK expression was also associated with a reduced distant metastasis-free survival (DMFS) (n=1746; logrank P = 0.0007) **(figure 3.1B)**. When patients were broadly divided by clinical subtypes, no individual subtype showed a significant association between FAK levels and RFS **(figure 3.1C-F)**, although there appeared to be a non-significant trend in TNBC patients for FAK expression and reduced RFS. To further stratify this data, evaluation of RFS in response to FAK expression and individual hormone receptor presence was conducted. Generally, the presence or absence of any one hormone receptor did not alter RFS regardless of FAK levels **(figure 3.1G-L)**. However, high FAK in HER2- patients showed a more positive association versus all others (n=800; logrank P = 0.057). Subtype and hormone analysis was limited to RFS only due to very limited data for DMFS.

To further these investigations, we utilised the database to assess the contributions of the FAK-family member Pyk2 to patient prognosis. In complete contrast to FAK, high expression of Pyk2 was associated with a significant improvement in RFS (n=3951; logrank P = $2.3x10^{-13}$) and, to a lesser degree, an improvement of DMFS (n=1746; logrank P = 0.012) **(figure 3.2A and B)**. Moreover, when divided by clinical subtype, this effect was more prominent, especially in HER2+ (n=251; logrank P = 0.00029) and TNBC (n=255; logrank P = 0.044) patients **(figure 3.2C-F)**. As with the FAK analysis, the relationship between Pyk2 expression and the presence/absence of hormone receptors was evaluated **(figure 3.2G-L)**. High Pyk2 expression in patients absent for ER had very good RFS rates (n=801; logrank P = $2.6x10^{-6}$) whilst no significant trends were observed with any other hormone receptors and Pyk2.

A. All Patients RFS

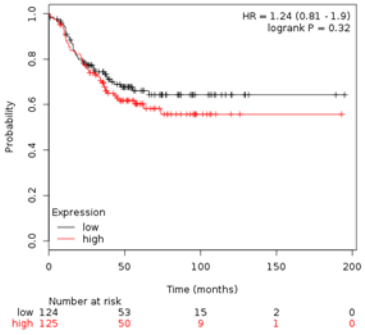


B. All Patients DMFS

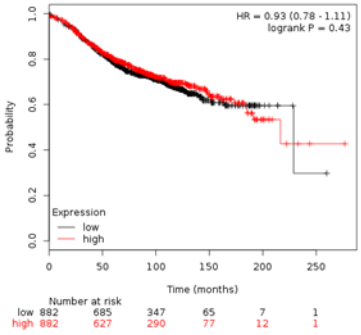


Subtype Analysis

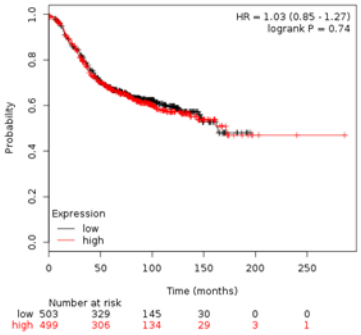
C. Triple-Negative RFS



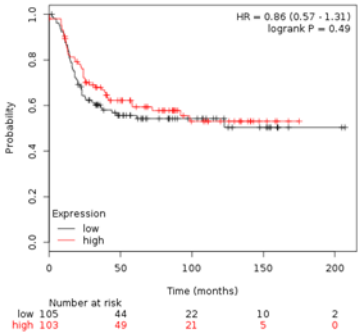
D. Luminal A RFS



E. Luminal B RFS



F. HER2+ RFS



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Hormone Analysis

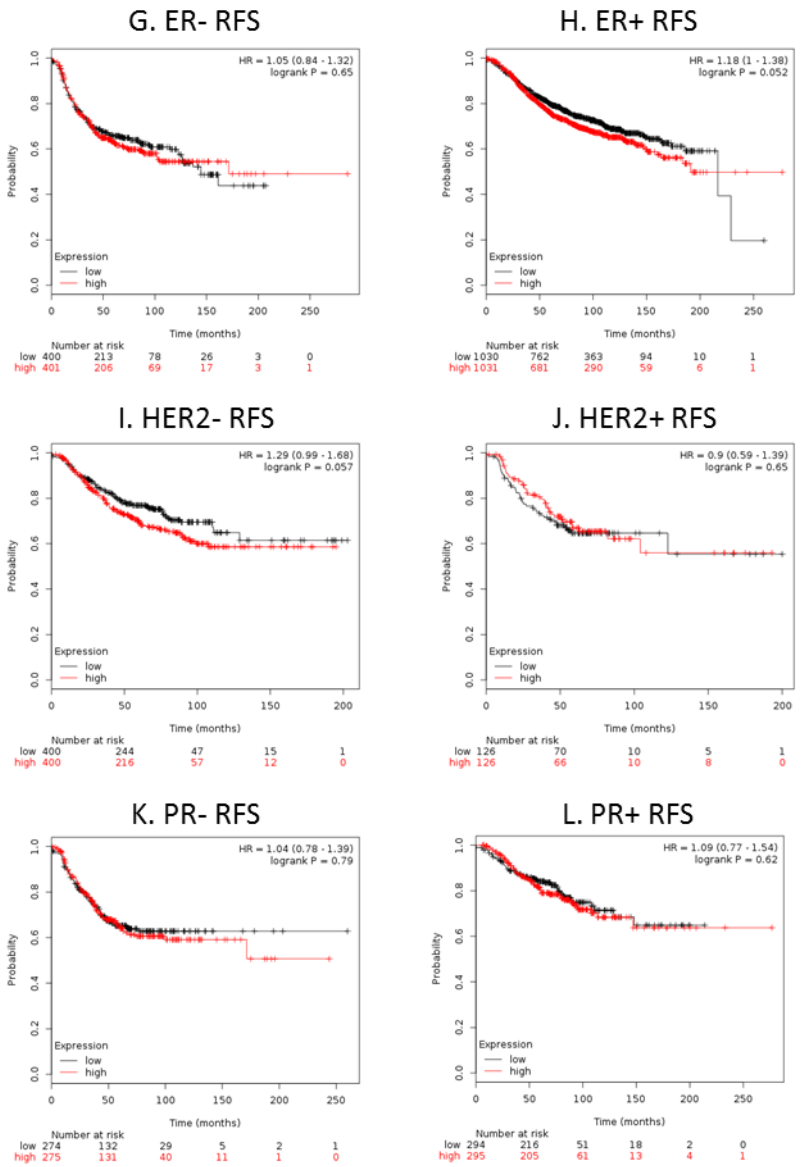
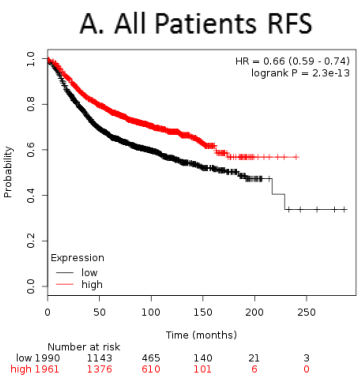
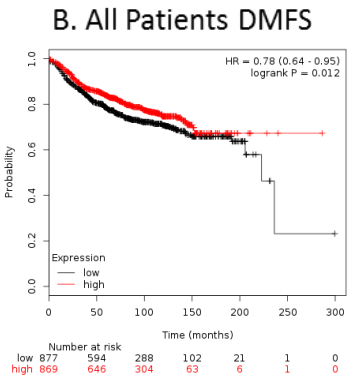
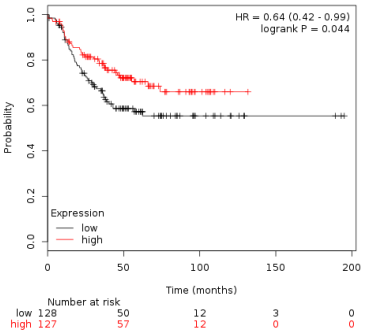


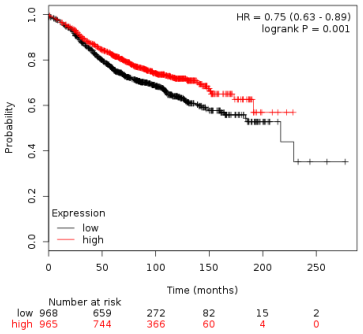
Figure 3.1 – FAK expression has the potential to be used as a prognostic marker of breast cancer – Kaplan-Meier plots (http://www.kmplot.com) were used to assess Affymetrix microarray expression of FAK mRNA (ID: 208820_at) in tumour samples from breast cancer patients. Data was acquired from **(A)** all patients RFS (n=3951; p=0.00059), **(B)** all patients DMFS (n=1746; $p=7x10^{-4}$), **(C)** triple-negative RFS (n=186; p=0.32), **(D)** luminal A RFS (n=1933; p=0.43), **(E)** luminal B RFS (n=1149; p=0.74), **(F)** HER2+ RFS (n=251; p=0.49), **(G)** ER-negative RFS (n=801; p=0.65), **(H)** ER-positive RFS (n=2061; p=0.052), **(I)** HER2-negative RFS (n=800; p=0.057), **(J)** HER2-positive RFS (n=252; p=0.65), **(K)** PR-negative RFS (n=549; p=0.79) and **(L)** PR-positive RFS (n=589; p=0.62). All graphs show hazard ratio (with 95% confidence intervals) with data restricted as follows: grade (all), lymph node status (all), chemotherapy/endocrine therapies (all).

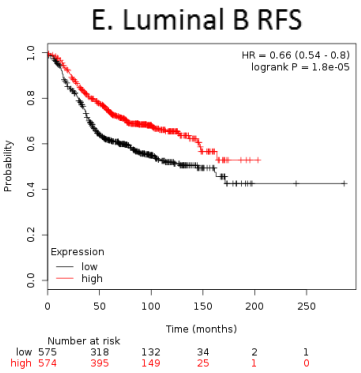


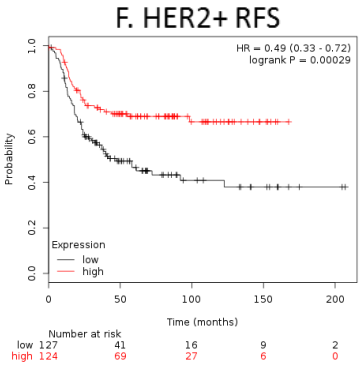


Subtype Analysis









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Hormone Analysis

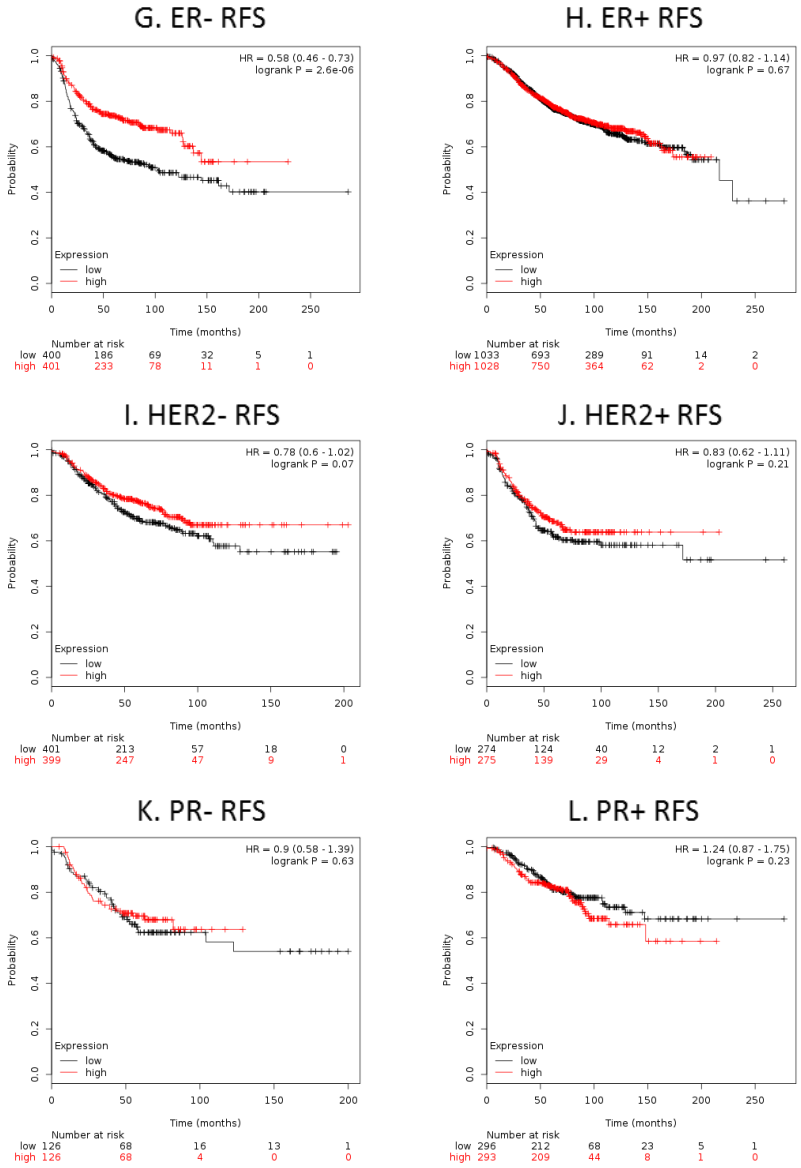


Figure 3.2 – Pyk2 shows great potential as a prognostic marker for breast cancer - Kaplan-Meier plots (http://www.kmplot.com) were used to assess Affymetrix microarray expression of Pyk2 mRNA (ID: 203110_at) in tumour samples from breast cancer patients. Data was acquired from **(A)** all patients RFS (n=3951;p=2.3x10^{-13}), **(B)** all patients DMFS (n=1746; p=0.012), **(C)** triple-negative RFS (n=255; p=0.044), **(D)** luminal A RFS (n=1933; p=0.001), **(E)** luminal B RFS (n=1149; p=1.8x10^{-5}), **(F)** HER2+ RFS (n=251; p=0.00029), **(G)** ER-negative RFS (n=801; p=2.6x10^{-6}), **(H)** ER-positive RFS (n=2061; p=0.67), **(I)** HER2-negative RFS (n=800; p=0.07), **(J)** HER2-positive RFS (n=252; p=0.63), **(K)** PR-negative RFS (n=549; p=0.21) and **(L)** PR-positive RFS (n=589; p=0.23). All graphs show hazard ratio (with 95% confidence intervals) with data restricted as follows: grade (all), lymph node status (all), chemotherapy/endocrine therapies (all).

3.3. Exploration of FAK and Pyk2 in breast cancer models

We next wished to explore the expression and activity of FAK along with its related family member, Pyk2, in a panel of cell lines modelling the major clinical subtypes in order to determine whether there was an association between their expression and cellular characterisation of these cells such as proliferation and migration/invasion behaviour.

Whilst levels of FAK showed some (non-significant) differences between all cell lines, the SkBR3 cells consistently expressed a lower level of protein compared to other models **(figure 3.3A and B, $p<0.05$ for FAK expression in SkBr3 cells versus all others)**. FAK activity at Y397 and Y861 was assessed using phospho-specific antibodies which revealed that all cell lines showed FAK^{Y397} phosphorylation, with highest activity observed in the HER2+ SkBr3 line **(figure 3.3A and C)**. Similarly, phosphorylation at FAK^{Y861} was detected in all models and again highest in SkBr3 cells **(figure 3.3A and D)**. As the primary focus of this work was to explore FAK in TNBCs, all results were normalised to the levels observed in MDA-MB-231s **(figure 3.3E)**.

Investigations into Pyk2 revealed consistently low levels of total protein across all models but with significantly higher expression in SkBr3 cells **(figure 3.4A and B)**. $Pyk2^{Y402}$ activity was considerably more variable than that observed in FAK. All models showed phosphorylation with MDA-MB-361 cells having the highest, relative Y402 activity and SkBr3 cells the lowest **(3.4A and C)**. Taken with our Kmplot data, we decided to focus the remainder of this chapter on FAK given that cellular levels are particularly low and that Pyk2 expression seemed to correlate with better clinical outcome.

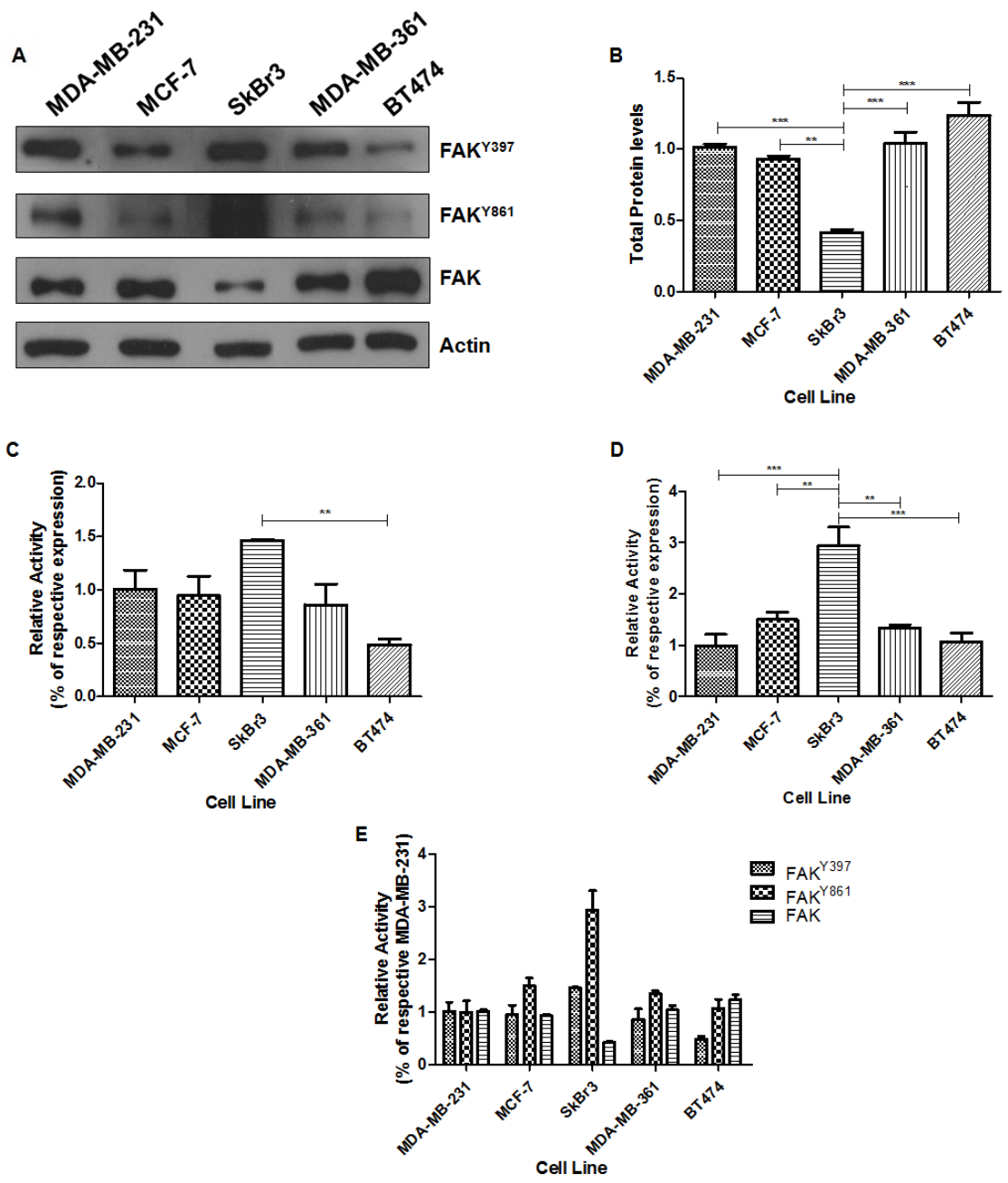


Figure 3.3 – FAK expression and activity in breast cancer models – (A) Western blots showing the basal levels of phosphorylated FAK^{Y397}, FAK^{Y861} and total FAK across the cell lines. Actin was used as a loading control. **(B)** Densitometry analysis of total FAK protein levels across cell lines showing a significantly lower level in SkBr3 cells. **(C)** FAK^{Y397} phosphorylation levels calculated relative to total FAK expression. **(D)** Analysis of densitometry of FAK^{Y861} relative to total FAK levels, showing SkBr3 has significantly higher activity than all other cell lines. **(E)** Summary graph displaying the intensity of each band obtained relative to the respective band in the MDA-MB-231 cell line. All values shown for densitometry are normalised against actin to account for loading error. Error bars represent SEM; n=3. *p=0.05; **p=0.01; ***p<0.001.

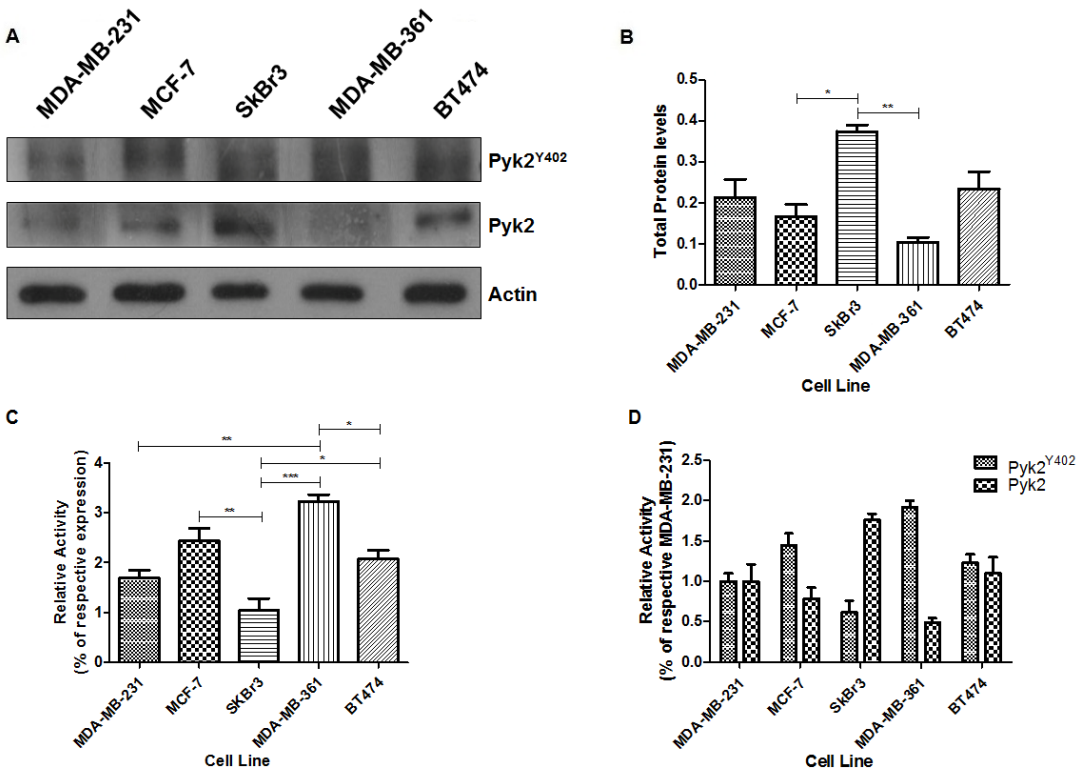
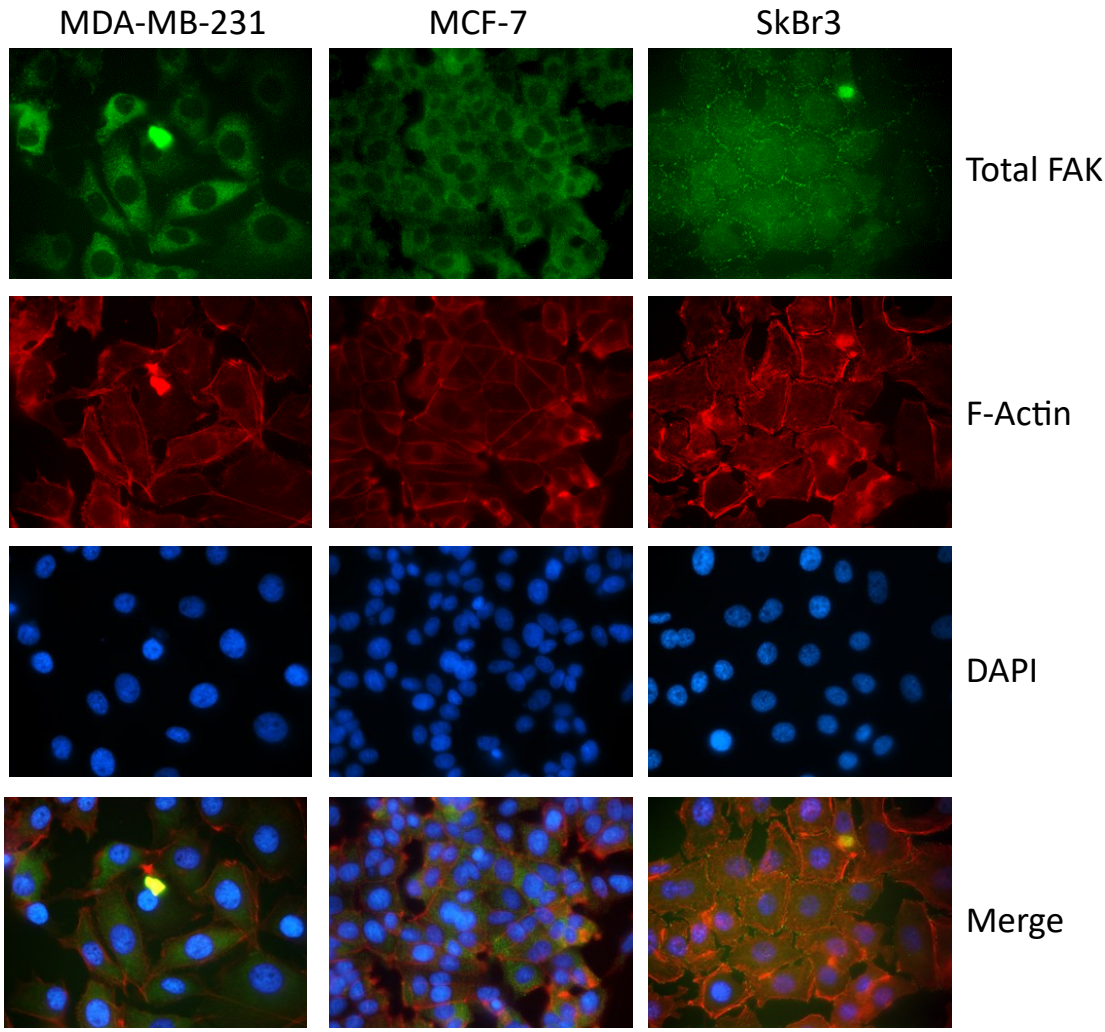


Figure 3.4 – Pyk2^{Y402} expression and activity in breast cancer models - (A) Western blot showing the levels of phosphorylated Pyk2^{Y402} and total Pyk2 across the cell lines. Actin also shown was used as a loading control. **(B)** Evaluation of total Pyk2 protein levels based on densitometry analysis shows significantly higher expression in SkBr3 cells versus all other examined lines. **(C)** Relative activity of Pyk2^{Y402} compared to respective total levels highlights significant variation in activity across cell lines. **(D)** Summary of all densitometry data, with all results normalised to their respective equivalent in MDA-MB-231 cells. All results in this densitometry are normalised to actin. Error bars represent SEM; n=3. *p=0.05; **p=0.01; ***p<0.001.

3.4. Analysis of subcellular distribution of FAK in breast cancer cell models

FAK regulates several cell behaviours through modulating signals present at sites of cell adhesion (Lee *et al.* 2015). Consequently, its localisation to these sites plays a crucial role in directing FAK-dependent signalling cascades. As such, we sought to explore the subcellular distribution of total FAK protein, as well as active, phosphorylated FAK across the cell models to further evaluate how FAK correlates with aggressive cell behaviours. In general, total FAK protein displayed diffuse localisations through the whole cell but SkBr3 and MDA-MB-361 cells both exhibited defined points of expression on the cell periphery **(figure 3.5)**. We also stained the cell lines with active FAK^{Y397} to observe differences in the distribution of active FAK. However, these stains had such high amounts of background that the data was extremely difficult to interpret **(appendix 1)**. As such, we also stained with FAK^{Y861} as a surrogate marker for active FAK **(figure 3.6)**. Active FAK was strongly localised to the cell membranes in all cell lines, whilst MDA-MB-361 cells had particularly large regions of activity at points of actin.



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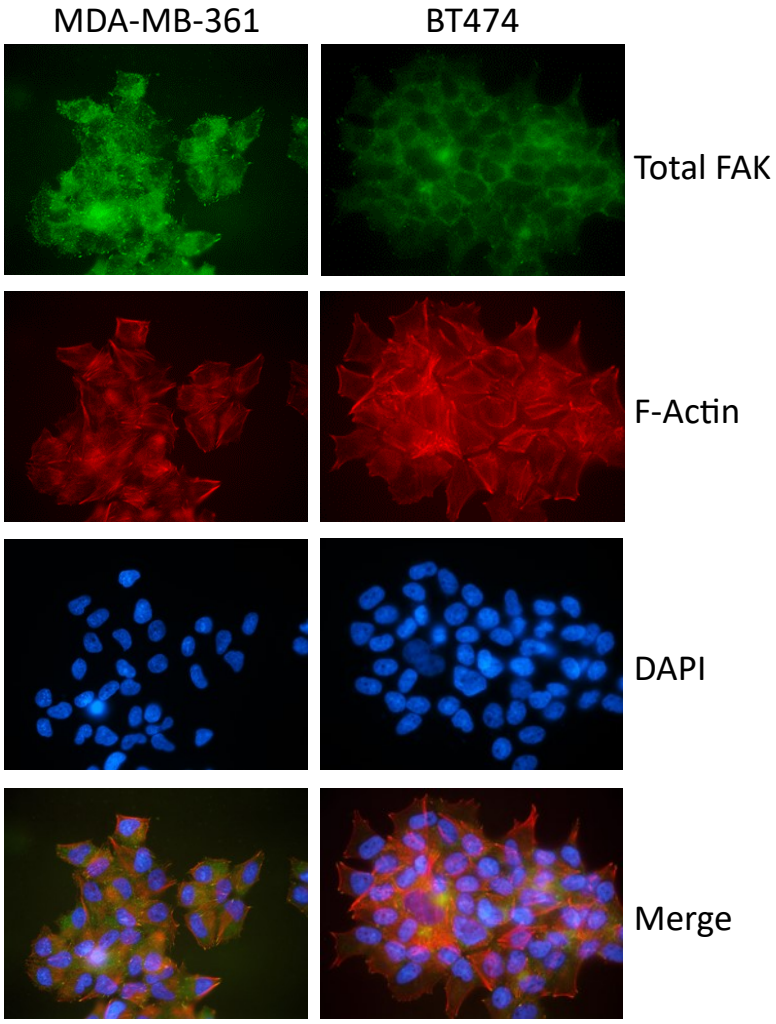
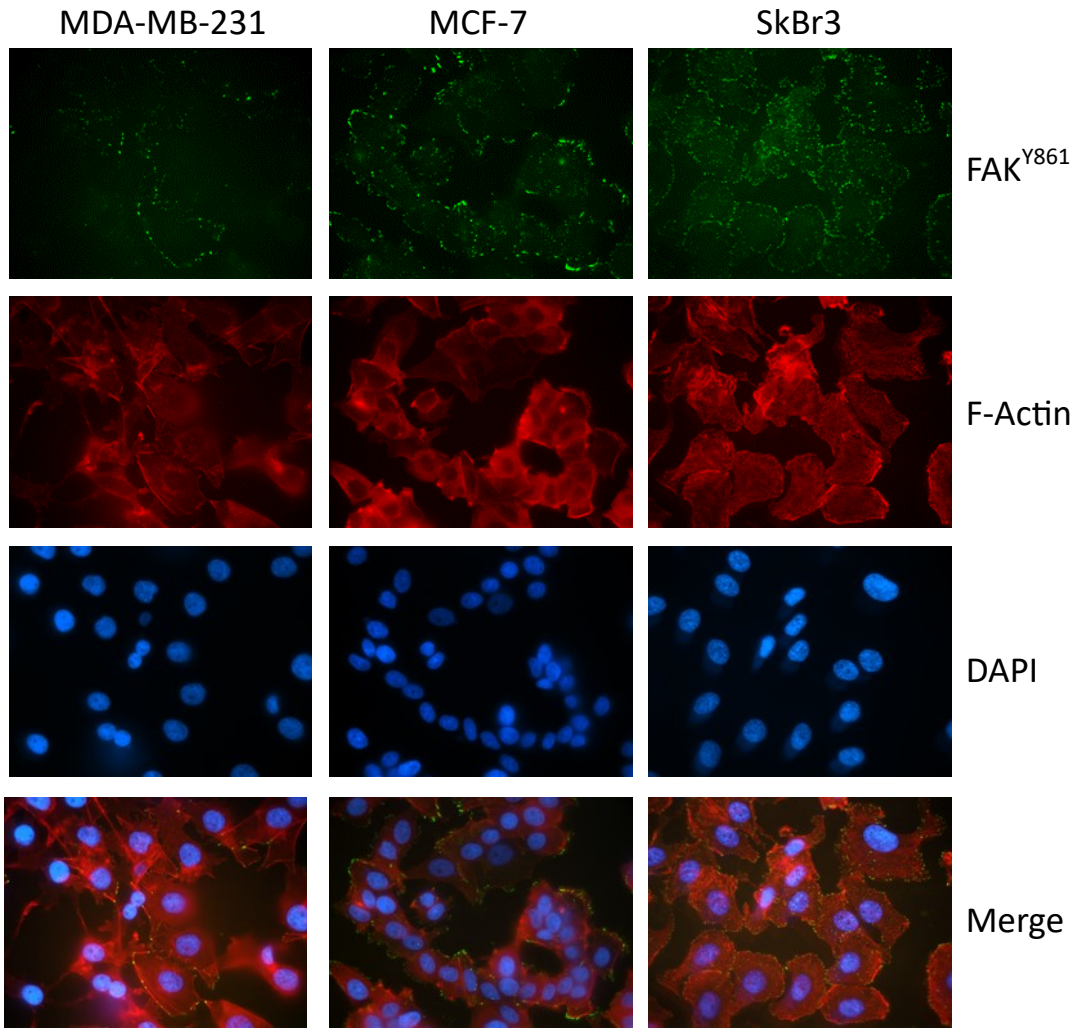


Figure 3.5 – Examination of total FAK distribution throughout breast cancer cell lines – Representative images showing the subcellular distribution of total FAK protein (green), along with actin (red) and nuclear stain DAPI (blue) across breast cancer cell lines. Total FAK is distributed throughout cells, with SkBr3 and MDA-MB-361 cells showing distinct points of high FAK staining localised to the cell periphery. N=3.



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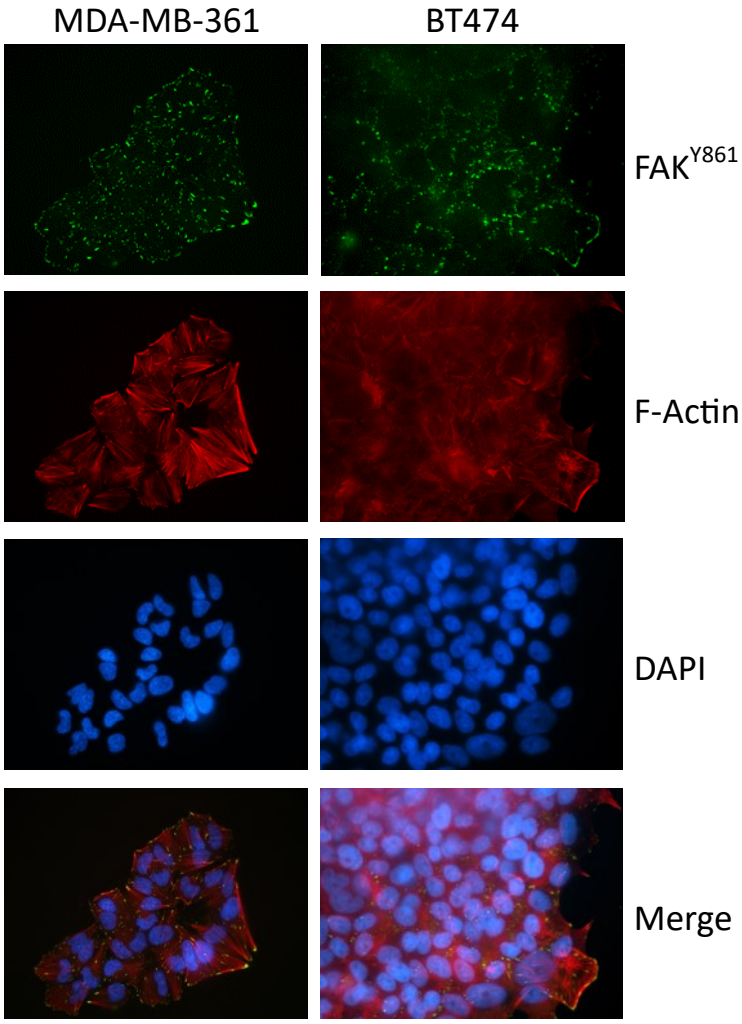


Figure 3.6 – Examination of active FAK^{Y861} localisation in different breast cancer cell models – Representative images showing the localisation of active FAK^{Y861} (green) and actin (red), as well as the nucleus (DAPI – blue) in each of the cell models utilised so far. FAK^{Y861} is primarily localised to the cell periphery with MDA-MB-361 cells exhibiting pronounced regions of activity at defined points of actin convergence. N=3.

3.5. Analysis of proliferative and migratory capacity of breast cancer cell models

Part of our objectives for this chapter were to assess whether FAK expression or activity correlated with the proliferative or migratory characteristics of the cell lies being tested. Having assessed the expression and activity of FAK we therefore next determined the proliferative and migratory capacity of these cell lines. Cell counting experiments performed over a period of 7 days revealed that the TNBC model proliferate significantly more than all other cell models over 7-days **(figure 3.7A)**. Normalisation of cell numbers to those seen in the MDA-MB-231 model also reveals the same trend **(figure 3.7B)**. Unexpectedly, the HER2+ model (SkBr3) displayed the lowest growth rates despite being a typically aggressive disease subtype.

A monolayer wounding assay was used to determine the intrinsic migratory nature of the cells. These data **(figure 3.7C)** revealed that the TNBC cell model (MDA-MB-231) displayed an extremely high propensity to migrate compared to other tested cell lines, with the wound being almost completely closed after a period of 24 hours. SkBr3 and MCF7 cells were both weakly migratory and BT474 cells did not migrate. Consequently, it was decided that all future migration assays would solely focus on the MDA-MB-231 cell migration. It is important to note however, that wound closure may also result from proliferation of cells into the wound and this may account for the observed wound closure in MDA-MB-231 cells.

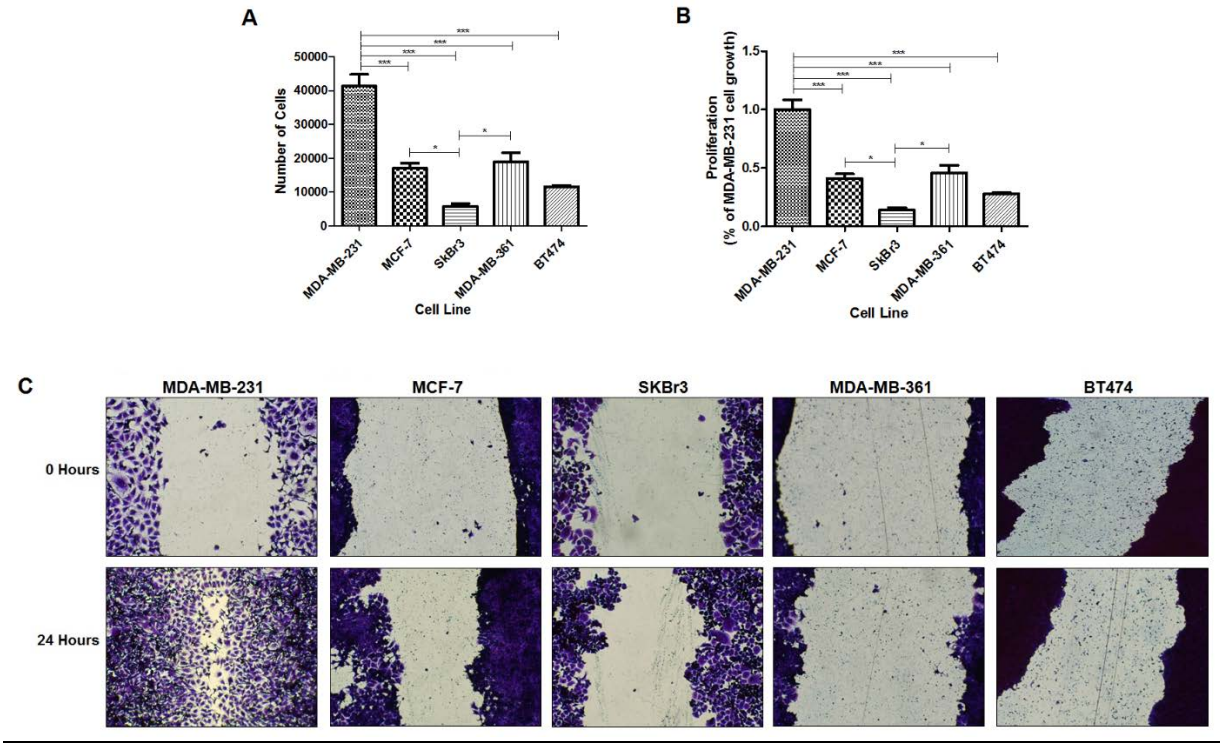


Figure 3.7 - MDA-MB-231 are significantly more migratory than other cell lines – (A) Cell numbers following 7-day growth. MDA-MB-231 cells proliferate significantly more than all other cell lines. **(B)** Evaluation of 7-day cell counts across breast cancer models showing significantly higher proliferation in the TNBC models versus all others. **(C)** Images show the amount of wound healing following 24-hours of a scratch being made through the cell cultures. MDA-MB-231 cells show almost complete wound closure following 24-hours, whilst MCF-7 and SkBr3 cells show limited closure. BT474s display no degree of closure. All error bars are representative of SEM; n=3. *p<0.05; **p<0.01; ***p<0.001.

Having explored levels of FAK and its distribution through the cell models, as well as their respective proliferative and migratory capacities, we next wished to correlate the FAK with intrinsic cell behaviours. **Table 3.1** shows a summary of relative FAK expression/activity (normalised to MDA-MB-231 cells) and a summary of proliferation and migration as determined by the assays outlined previously. Briefly, MDA-MB-231 cells grow significantly faster and exhibit significantly greater migration versus all other models, although FAK is not significantly augmented in this cell line.

Cell Line	Total FAK	FAK^{Y397}	FAK^{Y861}	Proliferation	Basal Migration
MDA-MB-231	**1.00**	**1.00**	**1.00**	**++++**	**++++**
MCF-7	**0.92**	**0.95**	**1.50**	**++**	**++**
SkBr3	**0.41**	**1.46**	**2.94**	**+**	**++**
MDA-MB-361	**1.04**	**0.86**	**1.34**	**++**	**+**
BT474	**1.24**	**0.48**	**1.07**	**+**	**N/A**

Table 3.1 – Comparative summary of FAK expression/activity, proliferation and cell migration across cell lines

3.6. Breast cancer cell models exhibit differential sensitivity to the FAK inhibitor PF271

We next wished to investigate whether these cell models had similar or differential sensitivity to the FAK inhibitor, PF562,271 (PF271) in terms of suppression of FAK activity. This compound is a small molecule, ATP-competitive inhibitor which effectively prevents FAK and Pyk2 phosphorylation and subsequent kinase activity (Roberts *et al.* 2008). Each of the cell lines was treated with PF271, at the doses and times indicated in the figures then lysed and used for Western blotting to assess changes in FAK activity in response to the inhibitor. PF271 treatment resulted in a reduction in FAK^{Y397} activity in all cell lines in a dose dependent manner. However, differential sensitivity was observed across the subtypes **(figure 3.8A and B)**. In contrast, total protein levels of FAK were not significantly affected by PF271 **(figure 3.8A and C)**. IC_{50} values for PF271 against FAK^{Y397} were calculated for each cell line and compared to the data obtained for the TNBC cell model **(figure 3.9)**. These data demonstrated that both luminal B (BT474 and MDA-MB-361) and the TNBC models were the most sensitive to PF271 whilst MCF-7 cells were the least sensitive.

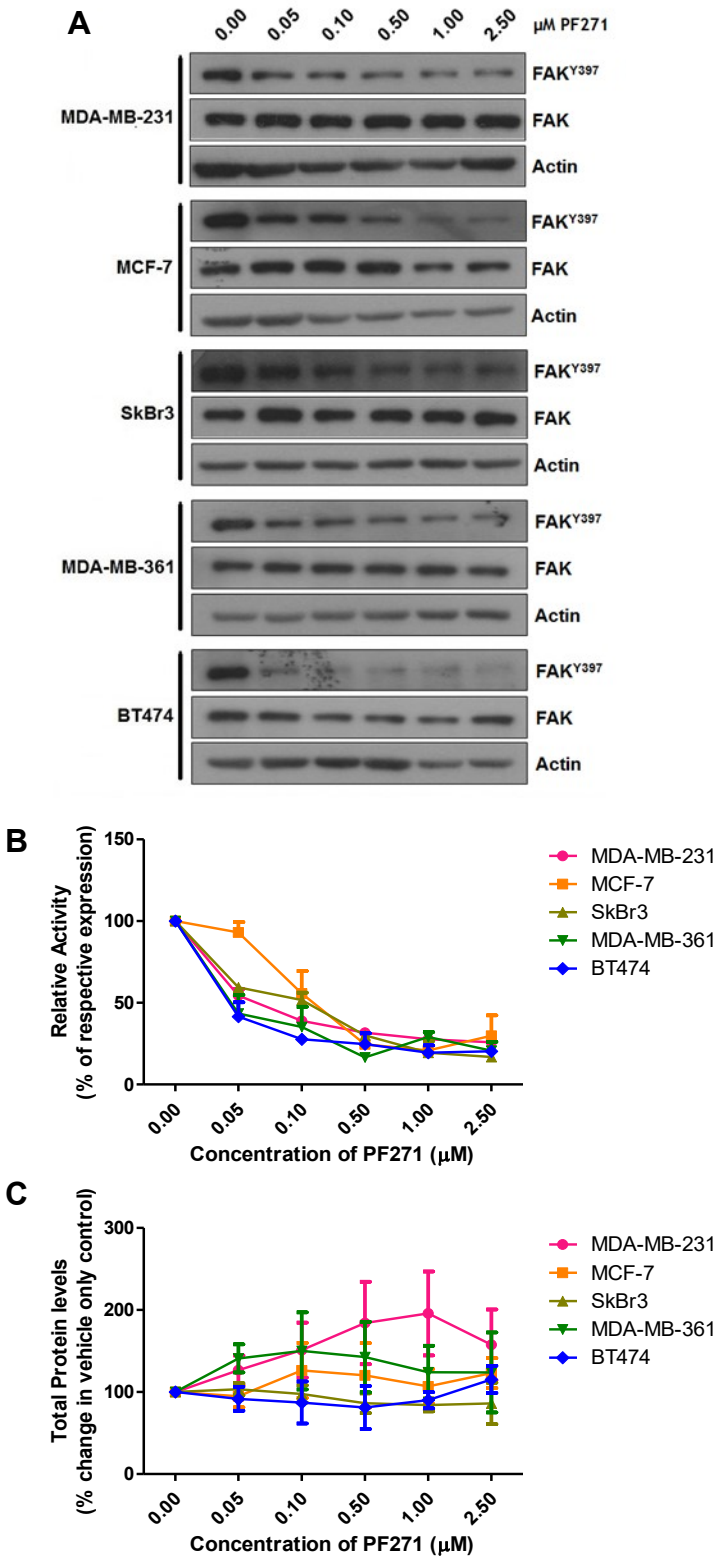


Figure 3.8 – PF271 inhibits FAK activity in breast cancer cell models - (A) Cells were treated with different concentrations of PF271 for 1-hour, lysed and subjected to SDS-PAGE and immunoblotting with FAK (total and Y397) antibodies. Blots show representative trends observed across replicates (n=3) **(B)** Densitometry analysis of all data showing the relative changes in FAK^{Y397} activity over the dose-range explored, normalised to the vehicle-only control (0.0μM) for each respective cell line. **(C)** Total FAK protein levels shown across the dose range normalised to the vehicle-only control. All error bars represent SEM; n=3.

A

Cell Line	IC_{50}/μM	Difference to MDA-MB-231	Relative Sensitivity/%
MDA-MB-231	0.0480	N/A	100.00
MCF-7	0.2230	+0.175	21.52
SkBr3	0.1046	+0.057	34.14
MDA-MB-361	0.0152	-0.033	315.79
BT474	0.0100	-0.038	480.00

B

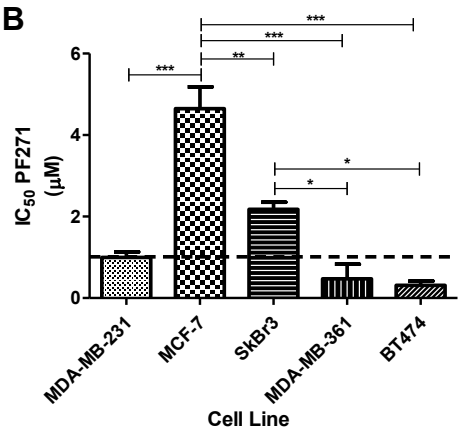
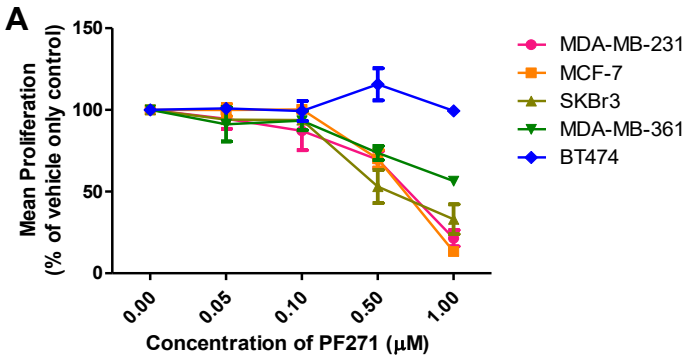


Figure 3.9 – PF271 IC_{50} values show significant variations between breast cancer cell lines – (A) Table summarising the calculated IC_{50} values and relative sensitivity of each cell line to PF271 in comparison to the triple-negative model, MDA-MB-231. **(B)** Graph displaying the relative sensitivities of each cell line to FAK^{Y397} inhibition normalised to MDA-MB-231. Error bars represent SEM; n=3. *p=0.05; **p=0.01; ***p<0.001.

3.7. The effects of FAK inhibition on cellular proliferation

Having demonstrated that FAK could be inhibited in each cell line by PF271, albeit with differential sensitivities, we next wished to determine whether FAK^{Y397} contributed to the proliferation of these cell models given that activation of FAK can promote growth signalling in some tumour types (Lee *et al.* 2015). Cells were seeded into dishes either in the presence or absence of the inhibitor at various concentrations and allowed to grow for 7 days. These data revealed that apart from BT474 cells, PF271 can significantly inhibit proliferation at concentrations above 0.1µM **(figure 3.10A).** Responsive cell lines displayed very similar IC_{50} values, except the MDA-MB-361 cells which were significantly less responsive to PF271-induced inhibition of proliferation **(figure 3.10B and C)**. Interestingly, for all cell lines tested, the IC_{50} values for PF271 against FAK activity were much lower than that for the suppression of cell growth **(figure 3.10D)**.

Although we have explored a range of models representing different clinical subtypes, our primary focus is on TNBC cells. Consequently, we expanded our analysis of MDA-MB-231 cells in order to determine the effects of longer-term (7-day) inhibition, thus allowing us to better observe the contribution of FAK to cell growth. Cells were grown for 24 hours in standard culture media before being subjected to PF271 treatments and daily cell counts performed for 7-days following incubation. Cell numbers were also counted following the 24-hour pre-treatment growth phase, hereafter known as the day 0 counts. Both the vehicle-only control and PF271 cells exhibited log-phase growth, however the treated cells showed a significant decrease in growth rate **(figure 3.11)**. These cells also continued proliferating up to 7-days whereas a drop was observed following day 5 in the vehicle-only control.



B

Cell Line	IC_{50}/μM	Difference to MDA-MB-231	Relative Sensitivity/%
MDA-MB-231	0.605	N/A	100.00
MCF-7	0.535	-0.070	113.08
SKBr3	0.459	-0.146	131.81
MDA-MB-361	1.48	+0.875	40.88
BT474	***	***	***

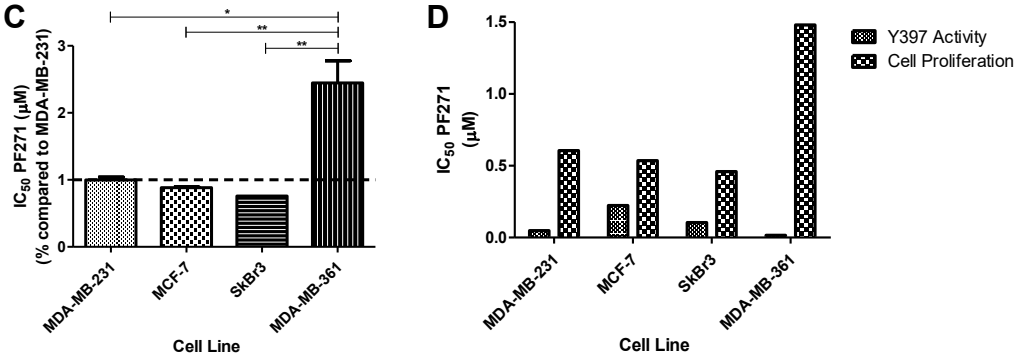


Figure 3.10 – Effects of PF271-mediated FAK inhibition on cell proliferation - **(A)** Relative suppression of proliferation in each cell line following 7 days across the range of PF271 concentrations, normalised to cell numbers in vehicle-only control samples. **(B)** Table summarising the IC_{50} of PF271 on proliferation in each cell line, comparing the relative sensitivities of each subtype to MDA-MB-231. Given that MDA-MB-361 cells failed to reach 50% suppression in proliferation in the allotted 7-days, data was extrapolated to give perceived IC_{50} value. **(C)** Comparative analysis of IC_{50} for proliferation between cell lines normalised to triple-negative sensitivity, displaying the significant lack of response in MDA-MB-361 cells compared to other models. **(D)** Evaluation of FAK^{Y397} activity and proliferation IC_{50} value across each cell line, highlighting the variation in sensitivity between activity and proliferation. All error bars represent SEM; n=4. *p=0.05; **p=0.01; ***p<0.001.

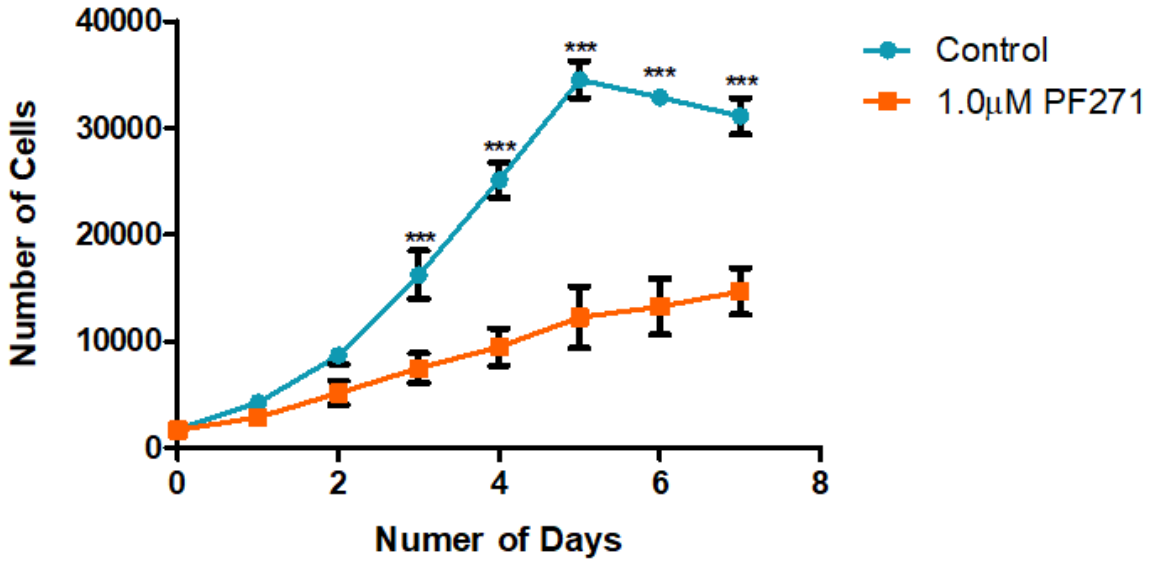


Figure 3.11 – Treatment of MDA-MB-231 cells with PF271 causes a significant reduction in growth rate over 7-days – Growth Curve showing the change in cell numbers in the presence or absence of PF271 for 7-days. Introduction of 1μM PF271 was sufficient to significantly reduce proliferation following 3-days and was sustained up to the final count at day 7. Error bars represent SEM; n=3. *p<0.05; **p<0.01; ***p<0.001.

3.8. The effects of FAK inhibition on TNBC migration and invasion

We next wished to determine whether the migratory/invasive characteristics of the cell models were related to FAK activity by again employing the pharmacological FAK inhibitor, PF271. However, this was only performed on MDA-MB-231 cells since our data revealed that apart from TNBC cells, all the cell lines were weakly or non-migratory and so not an appropriate model to investigate the effects of FAK inhibition on basal migration. As noted previously, wound closure can reflect differences in proliferation rather than cell motility. As such, Boyden chamber migration assays were used to assess migration in a more robust manner. MDA-MB-231 cell migration was significantly reduced following inhibition of FAK^{Y397} activity by 50% and further still with increased PF271 concentration **(figure 3.12)**. Given that metastasis requires cell invasion in addition to migration, we also utilised Boyden chamber assays to explore the contributions of FAK to basal invasion of MDA-MB-231 cells. As with migration, PF271 could significantly attenuate cell invasion, with increased suppression at greater levels of FAK inhibition **(figure 3.13)**.

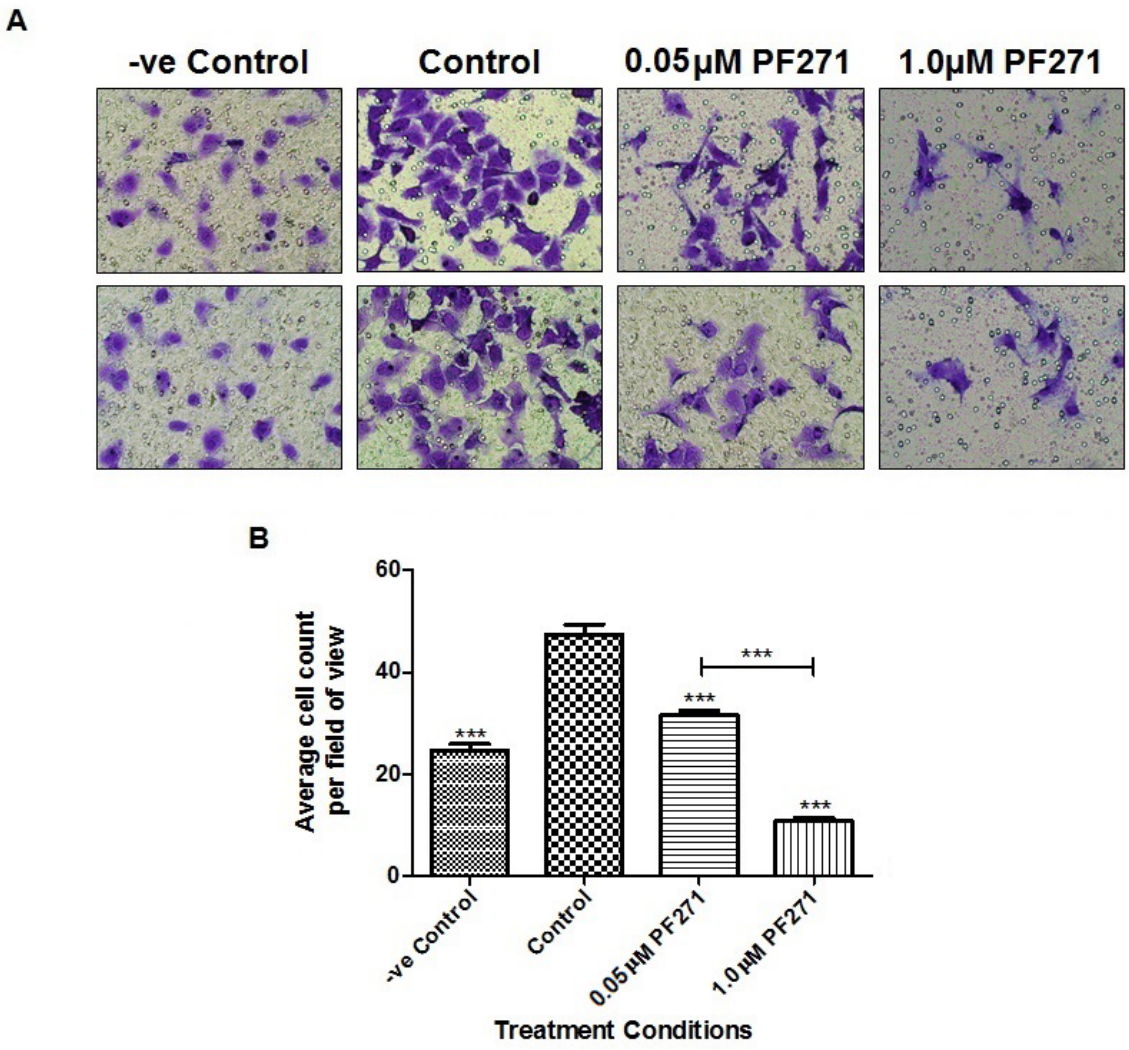


Figure 3.12 – Evaluation of MDA-MB-231 cell migration following treatment with PF271 – (A) Representative images of Boyden chamber assay following 18-hours migration prior to fixing and staining. Negative control (-ve control) represents cells migrating in absence of serum-containing medium in either chamber. **(B)** Analysis of cell migration in response to PF271 treatment revealed a significant decrease in motility following inhibition of FAK function. All error bars represent SEM; n=4. *p<0.05; **p<0.01; ***p<0.001.

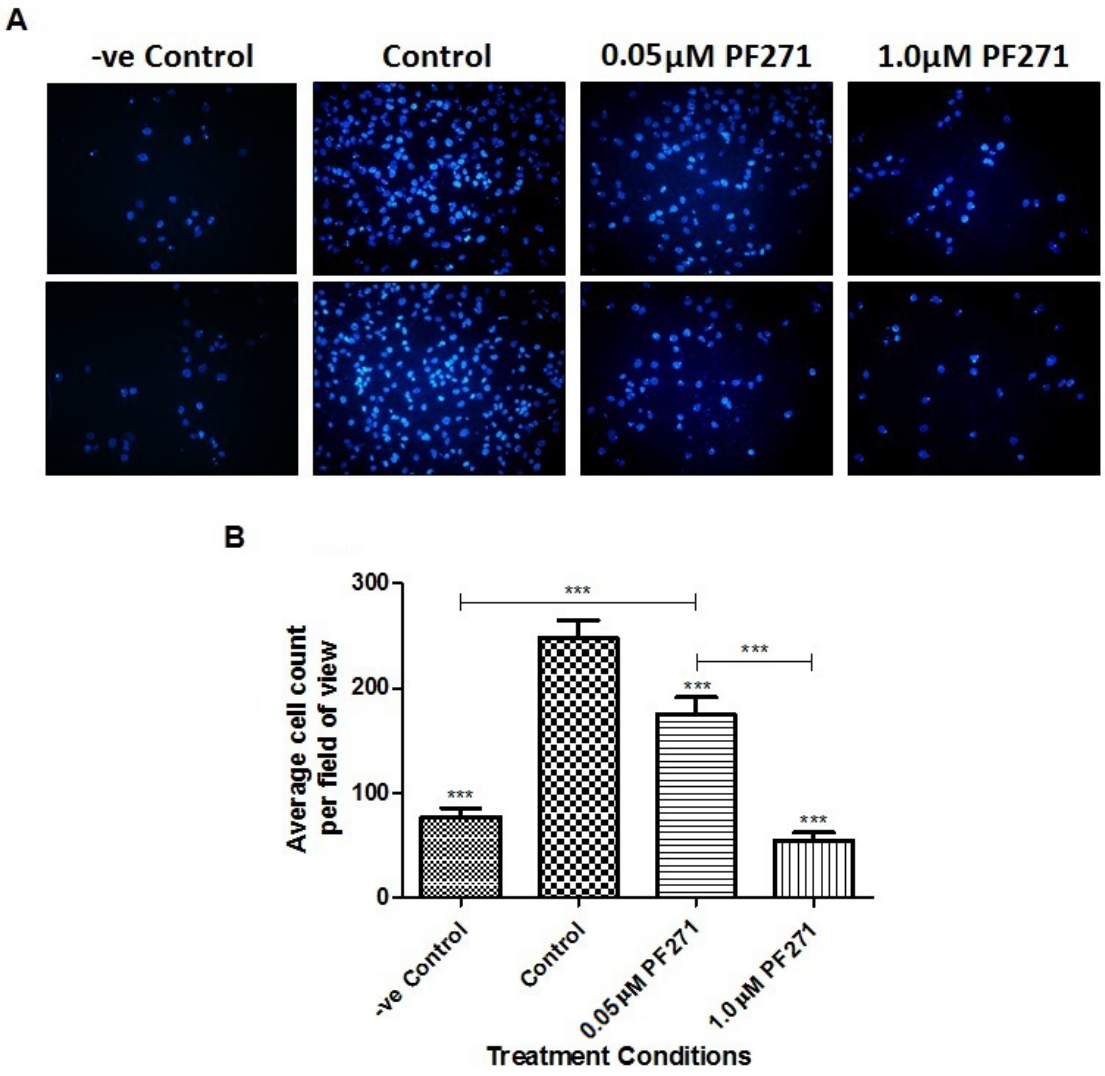


Figure 3.13 – Analysis of changes in MDA-MB-231 cell invasion upon FAK inhibition - (A) Representative images of Boyden chambers following 72-hours invasion through Matrigel. **(B)** Evaluation of FAK's contribution to basal MDA-MB-231 cell invasion showed significantly impaired invasive potential following PF271 inhibition of FAK activity. All error bars represent SEM; n=3. *p<0.05; **p<0.01; ***p<0.001.

3.9 Discussion

TNBCs represent a distinct subtype of basal-like disease characterised by a significantly increased propensity for early metastatic progression and short relapse free periods (Perou *et al.* 2000; Dent *et al.* 2007). Moreover, current therapeutic options are limited to chemotherapy due to the absence of targetable receptors (e.g. ER+ tumours can be therapeutically targeted by tamoxifen), thus target identification remains a priority in this clinical subtype (Bramati *et al.* 2014). This chapter focused on the differences in FAK expression/activity between breast cancer cell models, in order to correlate this data with the intrinsic characteristics of cell lines.

Initial observations of the clinically generated Kaplan-Meier plots (KM plots) revealed that generally FAK overexpression is highly correlated with a decrease in RFS and DMFS. This result reinforces the hypothesis that FAK represents a major therapeutic target in breast cancer, particularly in the context of TNBC where it may both act on cellular growth and disease progression. In terms of TNBC, KM plots revealed a negative relationship between FAK and RFS, although non-significant. However, this may be due to limits of this type of analysis. Due to the prevalence of each subtype, there are very large variations in the number of samples assessed in each experiment; with the triple-negative subtype exhibiting far fewer replicates than the other classifications (n=186 vs. n=1933 for luminal A). It could therefore be argued that if the sample sizes for TNBC were as large as others, the statistical analysis would become stronger and, likely statistically significant. Regardless, our data is in agreement with earlier work by Lark et al (2005) who showed a significant association between FAK expression and poor prognostic markers such as high mitotic index and higher tumour grade (Lark *et al.* 2005). Moreover, Golubovskaya et al (2014) also showed that patients with high FAK-expressing tumours showed significantly worse overall and progression-free survival (Golubovskaya *et al.* 2014). Consequently, FAK represents an opportunity for targeting in TNBC.

By contrast, exploration into Pyk2 revealed a correlation between high expression and improved RFS across breast cancer subtypes, particularly in the more aggressive forms of disease. This data appears to support observations made in prostate cancer by Stanzione et al (2001). They noted that Pyk2 has potential as a tumour-suppressor, as an inverse relationship between the expression of Pyk2 and degree of malignancy exists (Stanzione *et al.* 2001). However, this trend appears to be unique to prostate cancer progression and not reflective of typical behaviour in other solid tumours. For example, high Pyk2 expression in breast cancer has been shown to

potentiate TGF-β mediated EMT and subsequent metastasis, as well as being associated with high tumour grade and increased lymph node metastasis (Verma *et al.* 2015). These findings are in complete disagreement with our data, as they suggest Pyk2 expression is linked to disease progression and thus poorer RFS. However, the relationship between Pyk2 and FAK may account for our results. Previously, decreased FAK expression has been shown to directly correlate with increased Pyk2 levels in both embryonic fibroblasts (Sieg *et al.* 1998) and adult endothelial cells (Weis *et al.* 2008). Consequently, patients who exhibit high levels of Pyk2 could do so as a response to low levels of FAK. Although Pyk2 can compensate for a loss of FAK, it is not a complete substitution and thus reduced activity in several signalling pathways would be observed. For example, Wendt et al. (2013) showed that FAK is essential to TGF-β signalled EMT and, despite some compensation by Pyk2, selective inhibition of FAK causes reduced breast cancer cell invasion (Wendt *et al.* 2013). However, high Pyk2 levels do not always result from reduced FAK expression. Co-overexpression of FAK and Pyk2 has been shown in early stage and invasive HER2+ breast cancers (Behmoaram *et al.* 2008). Therefore, Pyk2 may be influencing prognosis by another means. Despite their similarities and relationship, FAK and Pyk2 do exhibit very different behaviours in cells. For example, Pyk2 induction results in cell cycle suppression through inhibition of the G1 to S-phase transition, whilst increasing FAK expression to similar levels accelerated this progression (Zhao *et al.* 2000). Additionally, FAK and Pyk2 vary in their regulation of apoptosis: FAK expression can protect cells from apoptosis (Lunn *et al.* 2007), whilst increased Pyk2 expression and activity is sufficient to induce cell death (Xiong and Parsons 1997; Wang *et al.* 2011). This inverse relationship in some functions could explain our findings as there could be a balance between FAK/Pyk2 mediating cell behaviours. Therefore, when Pyk2 levels exceed those of FAK, its signals override the typically more dominant FAK-linked signals providing some degree of protection for cancer patients. Whilst interesting, time did not allow the study of FAK/Pyk2 interplay further as the focus for this thesis was FAK, particularly in TNBC.

FAK has previously been highlighted as a central mediator of cell migration and invasion, as well as contributing to cancer cell proliferation (Luo and Guan 2010). Consequently, it could be reasoned that FAK activity and expression would vary across cellular subtypes depending on the intrinsic behaviours of each cell line. Our data contradicts this idea as we show relative consistency in FAK expression between cell models. In addition, the most aggressive cell line, MDA-MB-231, does not show significantly augmented FAK activity as would be expected. However, SkBr3, the model of Her2+ disease, does exhibit significantly higher FAK activity,

particularly FAK^{Y861}. This data directly reflects the work done by Vadlamundi et al (2003) who noted a specific increase in phosphorylation of FAK^{Y861} following the stimulation of the Her2 signalling pathway (Vadlamudi *et al.* 2003). Taken together, our findings and the literature would suggest HER2 as a key mediator of FAK activity. However, the increase in FAK^{Y861} activity seen in SkBr3 cells is not observed in other HER2-positive models explored (MDA-MB-361s). This may suggest that the presence of other hormone receptors (ER and PR) could have a sequestering effect on FAK activity. This is unlikely as several studies have highlighted positive relationships between such receptors and FAK (for examples see Planas-Silva et al. 2006). Alternatively, other proteins present in these cell models may be influencing FAK behaviour. Receptor tyrosine kinases (RTKs) may play a crucial role in this as such molecules have been shown to promote tumour progression through cooperation with integrins; the major upstream activator of FAK (Soung *et al.* 2010). Previous work by Subik et al (2010) has shown that one RTK in particular, epidermal growth-factor receptor (EGFR), is highly upregulated in SkBr3 cells especially if compared to the other models examined here (Subik *et al.* 2010). Furthermore, interactions between EGFR and integrins have been shown to promote metastasis in a FAK-dependent manner (Leng *et al.* 2016). Consequently, it is likely that EGFR may contribute to FAK stimulation in SkBr3 cells and be more important than the presence of the HER2 receptor. However, more work would be needed to confirm this hypothesis.

We also explored differences in Pyk2 activity/expression in order to assess its relationship with FAK in these cells and its possible contribution to cell behaviours. Expression and activity was very low, especially in comparison to FAK suggesting a limited role in modulating cell function. This result seemed to reflect previous reports which suggested Pyk2 has limited usage throughout adult tissues (Behmoaram *et al.* 2008), although some studies have proposed a role for Pyk2 in cancer cells. For example, Pyk2 has been shown to increase migration and EMT associated signals through mediation of EGF-signalling in breast cancer cells (Verma *et al.* 2015). However, the extent of Pyk2's contribution to cancer remain largely unclear, except in terms of FAK compensation. We may be seeing some form of this here as SkBr3 cells which exhibit low levels of hyperactive FAK, also showed high Pyk2 expression with almost complete Pyk2^{Y402} activity. Notably, relative Pyk2 activity was significantly lower than in the other models. This inverse relationship could suggest that in SkBr3 cells, Pyk2 is overexpressed to compensate for downregulated FAK, but high levels of FAK activation mean stimulation of Pyk2 is lower than in cells with less active FAK. This could also mean that although hyperactivation of FAK is sufficient

to modulate some signalling, high Pyk2 levels are required to compensate for loss of kinase-independent scaffold function. Pyk2 does exhibit scaffold function and, given its binding partners overlap with those of FAK, could mediate kinase-independent functions in lieu of FAK (Lipinski and Loftus 2010). However, very few studies have examined the role of kinase-independent Pyk2 in cancer cells and thus more work would be needed to explore its relationships to these functions in FAK.

FAK primarily interacts with signalling cascades at the sites of focal adhesions, requiring a number of such proteins to become activated (Frame *et al.* 2010). Thus, it is not only expression and activation that regulates FAK function but also correct localisation (this is discussed further in **chapter 6**). As such, we went on to explore subcellular distribution of FAK across our cell models, to determine whether this had any correlation with cell behaviours. We reported that activated FAK is primarily localised to the cell periphery (as expected) and that total FAK is generally distributed throughout the cell. However, SkBr3 and MDA-MB-361 cells both showed increased localisation to the cell membrane. Given both these models express the HER2 receptor (Kao *et al.* 2009), it could be reasoned that the presence of this receptor increases peripheral FAK localisation to enhance HER2/FAK signalling. Although studies that directly examine the effect of HER2 activity on FAK localisation are limited, there is some evidence to support this hypothesis. Firstly, a seminal study by Sieg et al (2000) demonstrated that ErbB receptors (including HER2) can physically interact with FAK (Sieg *et al.* 2000), whilst Subsequent studies revealed strong co-localisation between FAK and HER2 in migrating cells (Benlimame, He, Jie, Xiao, Xu, Loignon, Schlaepfer and M. a Alaoui-Jamali 2005). Taken together, these studies could provide evidence for HER2 mediated recruitment of FAK to the cell membrane, although more studies would be needed to confirm if HER2 activation alone is sufficient for increased FAK localisation.

Unfortunately, we were unable to view the distribution of FAK^{Y397} due to high levels of background and non-specific cell staining. This is a limitation as it means we could only view active FAK using FAK^{Y861} as a surrogate marker. Although, a strong substitute due to its correlation with FAK^{Y397} activity **(figure 3.3)**, it is not a perfect replacement as certain factors can increase the activity of this site and potentially create misinterpretations in our results. Indeed, we discussed previously the positive effects of HER2 on FAK^{Y861} phosphorylation (Lazaro *et al.* 2014). This could explain the more diffuse peripheral staining of active FAK in SkBr3 cells, due to

preferential stimulation and localisation with HER2. However, regardless of increased FAK^{Y861} activity in these cells, this marker still largely represents active FAK distribution and thus we concluded that the localisation of this antibody truly reflects active FAK.

We subsequently went on to explore variations between the cell models in terms of basal proliferation and wound healing. Our results highlighted the aggressive nature of the MDA-MB-231 model, with these cells exhibiting significantly greater proliferation and wound closure versus all other models. This closely reflects the clinical presentation of TNBC where patients often display larger tumours at the time of diagnosis, as well as proliferative capacity of these tumours, as characterised by mitotic index (Ho-Yen *et al.* 2012; Rakha *et al.* 2006). This is accompanied by a significantly higher chance of disease recurrence and increased metastasis, reflecting increased cell motility (Dai *et al.* 2015). To further underscore the aggressiveness of such cells, TNBC patients have also been noted to be at a more advanced stage of disease than other subtypes when diagnosed (Aysola *et al.* 2013). Consequently, these studies fully support our cell model as we see a similar reflection of characteristics versus other subtypes.

More interesting was the correlation between these aggressive behaviours and FAK, where despite being highly proliferative and migratory, MDA-MB-231 cells did not show significantly augmented FAK activity or expression. This may infer that FAK has minimal contributions to breast cancer cell phenotypes linked to negative prognosis. Several studies directly contradict these findings as FAK activity and expression has been correlated with movement towards a metastatic phenotype. For example, Pylayeva et al (2009) showed a direct correlation between FAK expression in breast cancer and prognosis (Pylayeva *et al.* 2009). This also seems to contradict our KMplot data where RFS and DMFS was significantly worse in patients with increased FAK levels **(figure 3.1)**. As discussed previously, this data in TNBC is lacking and thus may not reflect the true relationships between FAK and outcome.

Although it can provide some basis for investigation, examining FAK versus traits in of itself is not sufficient to glean the relevance of FAK to breast cancer subtypes. Indeed, despite differences in levels and activity, FAK may be utilised to modulate signalling in a subtype-specific manner. As such, we went on to evaluate the sensitivities of different cell lines to a known FAK antagonist, PF271. It was hypothesised that cell lines with high FAK expression/activity would be particularly susceptible to targeting with such an inhibitor. The observed results contradict this hypothesis as SkBr3 cells which had very high levels of phosphorylated FAK, did not exhibit

increased sensitivity to PF271. This may be because these cells have such high levels of FAK activity that to get a significant suppression of activity would require a high dose of inhibitor. Additionally, MCF-7 cells exhibited a markedly low sensitivity to PF271 compared to all other cell lines. Unlike in SkBr3 cells, this observation could not have resulted from variations in FAK activity/expression. This insensitivity to PF271 may be accounted for by the high prevalence of insulin-like growth factor-1 receptor (IGF-1R) signalling in MCF-7 cells. Typically, luminal A and B tumours have increased levels of IGF-related signalling molecules (Farabaugh *et al.* 2015). Expanding on this, work by Mukohara et al (2009) previously showed that MCF-7 cells display significantly higher levels of IGF-1R and the signalling adaptor protein insulin receptor substrate 1 (IRS-1) when compared to other models of breast cancer representing different clinical subtypes (Mukohara *et al.* 2009). Not only can FAK directly bind to and activate IGF-1R through its FERM domain (Watanabe *et al.* 2008; Zheng *et al.* 2009), but crosstalk exists between the two molecules, mediating function. Indeed, Taliaferro-Smith et al (2015) demonstrated that overexpression of IGF-1R resulted in a significant increase in FAK activity in TNBC cells (Taliaferro-Smith *et al.* 2015). Moreover, pharmacological inhibition of FAK in IGF-1R overexpressing cells still alters cell function but to a lesser degree than in cells with normal expression (Taliaferro-Smith *et al.* 2015). Additionally, IRS-1 can interact with and stimulate FAK via the Shb adaptor protein, whilst also providing a point of convergence for integrin and growth factor-mediated FAK activation (Welsh *et al.* 2002; Holmqvist *et al.* 2003). Taken together, these results suggest that cells with high IGF-1R signalling would be less susceptible to FAK inhibition and thus could account for the observed insensitivity of MCF-7 cells to PF271 treatment.

Interestingly, there was a discrepancy in sensitivities to PF271 in terms of proliferation and FAK stimulation across all cell lines, where IC_{50} values for proliferation were consistently higher than those for FAK activity. This could thus infer that FAK is not the central driver of proliferation in breast cancer cells but rather a contributing factor. One would expect that if FAK was the primary mediator of proliferative signalling that activity and growth would be closely linked, with comparative sensitivities to inhibition. Although several studies implicate FAK as a contributory factor in proliferation (reviewed in Lee *et al.* 2015), to our knowledge no studies implicate FAK as the main driver of cell growth. Moreover, given FAK's role as a signalling node for many pathways (Sulzmaier *et al.* 2014), it stands to reason that its role in cell growth would be contributory, as to be a purely proliferative signal would require FAK to mediate this trait only. Regardless of whether it is the primary factor, FAK inhibition can still significantly alter

proliferation across the cell lines, with sensitivity to PF271 still being very high, therefore we concluded that FAK is a relevant anti-proliferative target in breast cancer.

Given our particular interest in TNBC cells, we went on to further evaluate the relevance of FAK to MDA-MB-231 cell proliferation and migration/invasion. Our results showed that FAK inhibition could significantly reduce growth-rate consistently over 7-days. This was not unexpected as we had already demonstrated that FAK contributes to the proliferation of these cells **(figure 3.10)**, yet we were able to observe trends in growth over the time-course providing us more information on the relevance of FAK. Primarily, it allowed us to determine that the 7-day counts obtained previously were not resultant from alleviated PF271 inhibition, possibly due to loss of efficacy, but rather continuous impedance of log-phase growth. We also showed that FAK significantly contributes to MDA-MB-231 cell migration and invasion and its inhibition causes significant attenuations in both behaviours. Both the proliferation and the migration/invasion studies closely reflect previous studies which have implicated FAK in both these phenotypes (Lee *et al.* 2015). Additionally, these experiments have enabled us to determine that FAK plays a key role in modulation of these behaviours specifically in TNBC cells. Consequently, further studies were undertaken to evaluate the role of FAK function in proliferation and migration in greater detail, exploring both the contributions of kinase dependent/independent functions and the mechanisms by which FAK governs such traits.

3.9.1. Conclusions

Taken together these results initially imply that levels of FAK expression and activity is not the basis for the aggressive tendencies observed in TNBC cells, nor is differential localisation. However, TNBC cells are more migratory and proliferative than other breast cancer subtypes, with FAK contributing to both cell traits. Moreover, the sensitivity of these cells to FAK inhibition provides some evidence for FAK as a therapeutic target in tumourigenic and progressive events. What is not clear is the degree to which triple-negative cells utilise FAK and how inhibited function leads to altered cell behaviours.

4. Results (II)

Mechanistic Exploration of FAK in TNBC proliferation and migration

4.1. Introduction

In the previous chapter, we demonstrated that FAK inhibition by PF271 was able to suppress the proliferative and migratory capacity of TNBC cell models. We next wished to explore the contribution of FAK to each of these cell functions in greater depth.

TNBC tumours display increased aggressive behaviour when compared to other breast cancer subtypes. Consequently such tumour cells tend to exhibit an increased proliferative capacity *in vitro* and patients exhibiting triple-negative disease have significantly larger tumours at time of presentation versus all other subtypes (Dent *et al.* 2007; Bauer *et al.* 2007; Kreike *et al.* 2007). Furthermore, cells within these tumours have demonstrated increased proliferative capabilities versus other subtypes (Elsawaf and Sinn 2011). Anecdotally, during our previous studies (**chapter 3)** it was noted that TNBC cell models grew significantly faster in culture when compared to all other subtypes. This could suggest that therapeutics targeting cell growth pathways could have great potential against TNBCs, given their propensity for rapid cell proliferation.

Moreover, the propensity of TNBC cells to migrate and invade infers an increased metastatic potential versus tumours cells of other subtypes. Interestingly however, the mechanisms that underpin this metastatic potential are poorly understood with many contributing factors that could potentially mediate these processes. FAK is one such candidate as it has previously been demonstrated to underpin several processes essential to cancer cell behaviours that would support metastasis as demonstrated in the previous chapter. As such, here we wished to explore possible mechanisms of action that underpin the role of FAK in modulation of TNBC cell migration, as well as how it influences proliferation.

4.1.1. Aims and objectives

The main focus for this section of this chapter is to explore the contribution of FAK to the proliferative and migratory capacity of TNBC cells and to evaluate the mechanisms by which FAK regulates such functions. To achieve this, the following objectives were set:

- Assess the relative contributions of FAK to the proliferation and migration of MDA-MB-231 cells and the mechanisms that underpin FAK-driven proliferation and migration.

- Evaluate downstream signalling pathways that may contribute to changes in cell behaviours following FAK inhibition.

- Explore the role of FAK in a second model of TNBC (MDA-MB-468 cells).

4.2. Assessment of the contribution of FAK scaffold functions to proliferation

In chapter 3, our data demonstrated that pharmacological inhibition of FAK kinase suppressed MDA-MB-231 cell growth **(figure 3.11)**, however, FAK can also mediate downstream signals through kinase-independent, scaffolding mechanisms. To investigate the broader contribution of FAK, i.e. other than just phosphorylation-mediated events, an siRNA approach was employed to suppress FAK expression thus enabling the exploration of cellular events mediated by both the phosphorylation and scaffolding-functions of FAK. Thus, by comparing pharmacological FAK suppression with siRNA against FAK, we hypothesised that this would reveal the relative contribution that scaffolding plays versus catalytic activity.

4.2.1. Optimisation of FAK siRNA

It was necessary to first optimise the FAK siRNA protocol. As shown in **figure 4.1**, the levels of total and activated FAK significantly decreased with time and were not restored following removal of the siRNA. Neither the non-targeting siRNA (NT-siRNA) nor the lipid-only (LO) control had any significant effect on the total levels of FAK, thus the observed reductions were not a by-product of transfection but specific to targeted siRNA. Although a modest increase in FAK^{Y397} levels was observed in NT and LO controls, a significant reduction was seen in FAK siRNA treated cells. From these experiments, it was decided that 72-hour FAK siRNA treatment was to be used in future experiments as this produced a significant and sustained suppression of FAK.

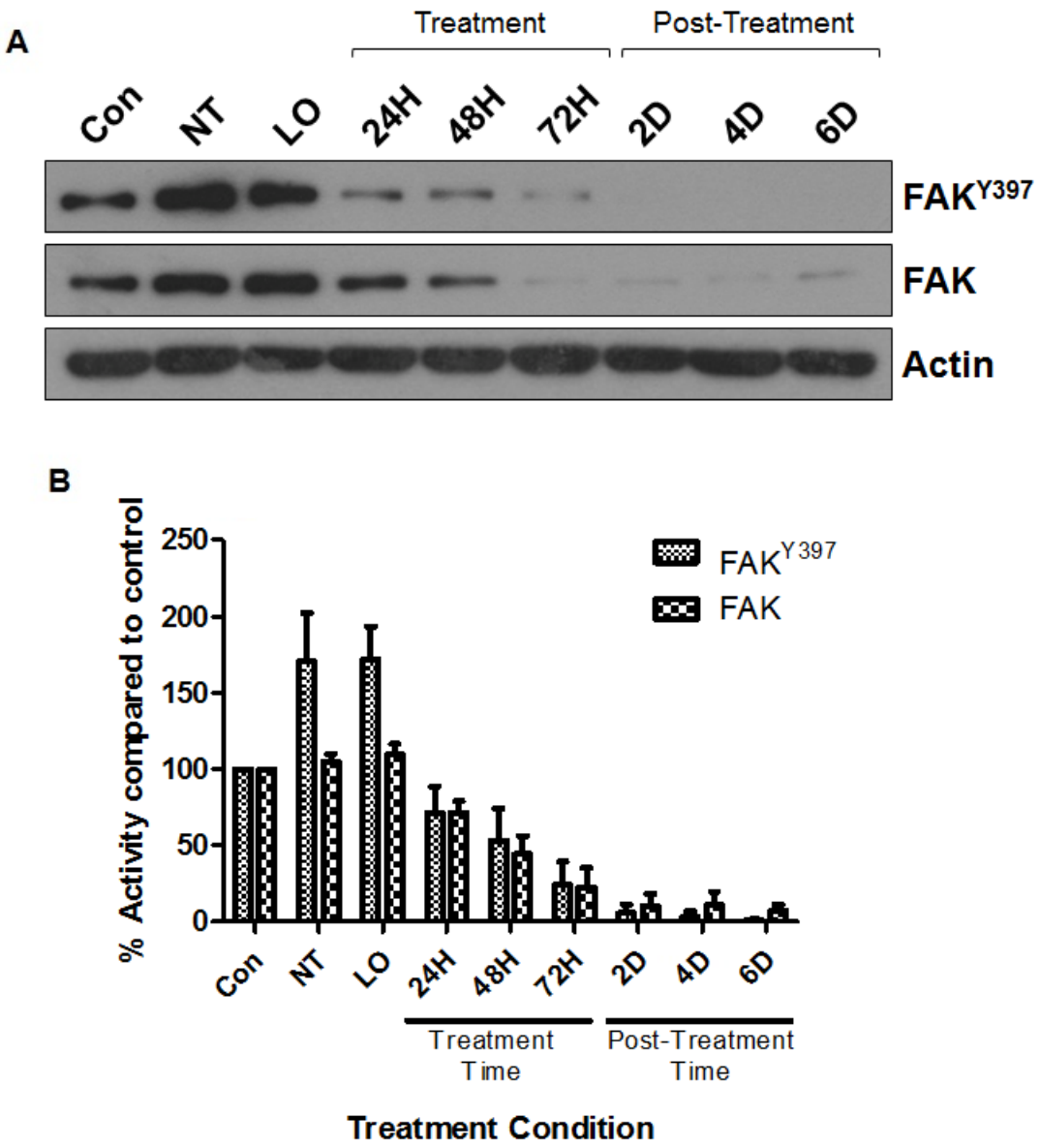


Figure 4.1 – Time-course optimisation of FAK siRNA – (A) Western blot showing the effects of FAK siRNA on Y397 activity and total FAK levels over time and the effects on each following the removal of treatment. Treatment denotes time-period in which cells were in the presence of siRNA complexes, whilst post-treatment represents time-points of cell lysis following replacement of media with complex-free, standard culture medium. The effects of the control, non-transcribing siRNA (NT) and the lipid-only (LO) are also shown, along with actin as a loading control. Times for treatment are notated in hours (H) and post-treatment in days (D). **(B)** Densitometry analysis of FAK^{Y397} and total FAK as a percentage of the control cells. Error bars show SEM; n=3.

4.2.2. Effects of FAK depletion on MDA-MB-231 cell proliferation

Having demonstrated that FAK protein levels can be effectively suppressed using siRNA, cell counting experiments were undertaken. As it was anticipated that introduction of siRNA may impact cell growth, cells were treated with FAK siRNA for 72-hours then growth recorded over the following 3 days. Proliferation was observed in all treatment groups over the 72-hour period although the rate of proliferation was significantly less in cells treated with FAK siRNA **(figure 4.2A and B)**. Given that siRNA will supress both kinase and scaffold functions, changes in proliferation were compared to kinase inhibition alone (PF271), so any additional suppression resulting from siRNA knockdown could be attributed to impaired FAK-scaffolding function. Both approaches resulted in comparable levels of cell growth suppression, whilst combination of the two also had no additional benefit **(figure 4.2C)**. Given the lack of difference with the combination approach, we hypothesised that it was the kinase function of FAK that played the most significant part in growth regulation and thus future experiments focussed on pharmacological inhibition of FAK using PF271 in order to explore FAK-dependent growth regulatory mechanisms.

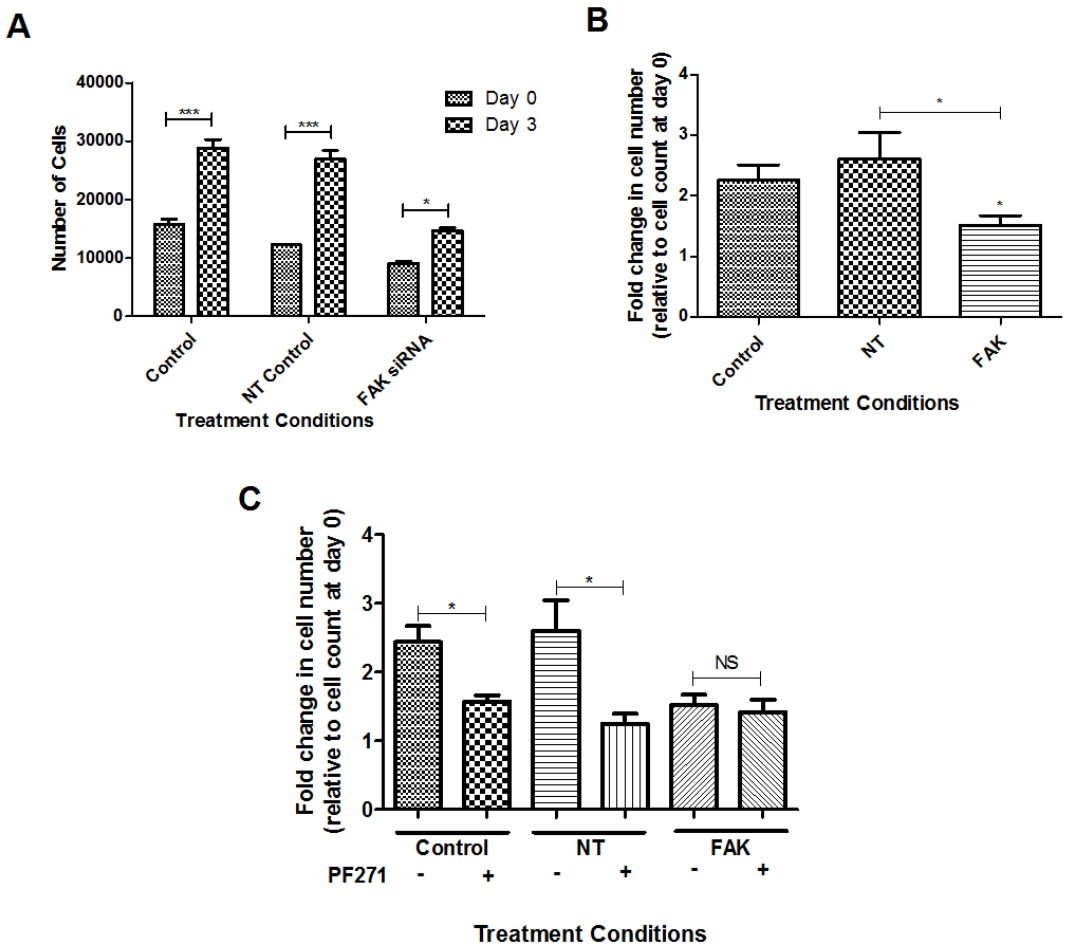


Figure 4.2 – Proliferative response of MDA-MB-231 cells to FAK suppression through siRNA – (A) 72-hour cell counts following incubation with FAK siRNA or relative controls. Day 0 counts are representative of cell number immediately following withdrawal of cells from siRNA containing media. All cells continue to proliferate following siRNA withdrawal, although rate of cell number following 3-days is significantly reduced in cells incubated with FAK siRNA. **(B)** Analysis of the growth rates of siRNA treated cells, representing fold change in cell number between 0 and 3 days. FAK siRNA cells exhibit significant reduction compared to both controls. **(C)** Evaluation of fold-change in cell numbers in MDA-MB-231 cells grown in the presence of siRNA and/or PF271, showing comparable reductions between PF271 and FAK siRNA with no additional benefits from combining inhibitory agents. All error bars are representative of SEM; n=3. *p<0.05; **p<0.01; ***p<0.001.

4.3. Cell growth inhibition is accompanied by a decrease in proliferative marker Ki67

Previous studies have highlighted FAK as a key mediator of both cell proliferation and cell survival. As such, we wished to explore whether PF271-mediated changes to MDA-MB-231 growth was due to impaired proliferation. Consequently, an immunohistochemistry assay for Ki67 (a specific marker of cellular proliferation) was performed following PF271 treatment. FAK inhibition resulted in a significant decrease in Ki67 positive cells compared to controls **(figure 4.3A and B)**; interestingly, this change was noted across all time-points examined. Since FAK has also been implicated as a regulator of apoptosis (Lee *et al.* 2015), we also examined the effects of PF271 on PARP cleavage **(figure 4.3C)**. However, no significant change in the active cleaved form of PARP was observed following FAK inhibition.

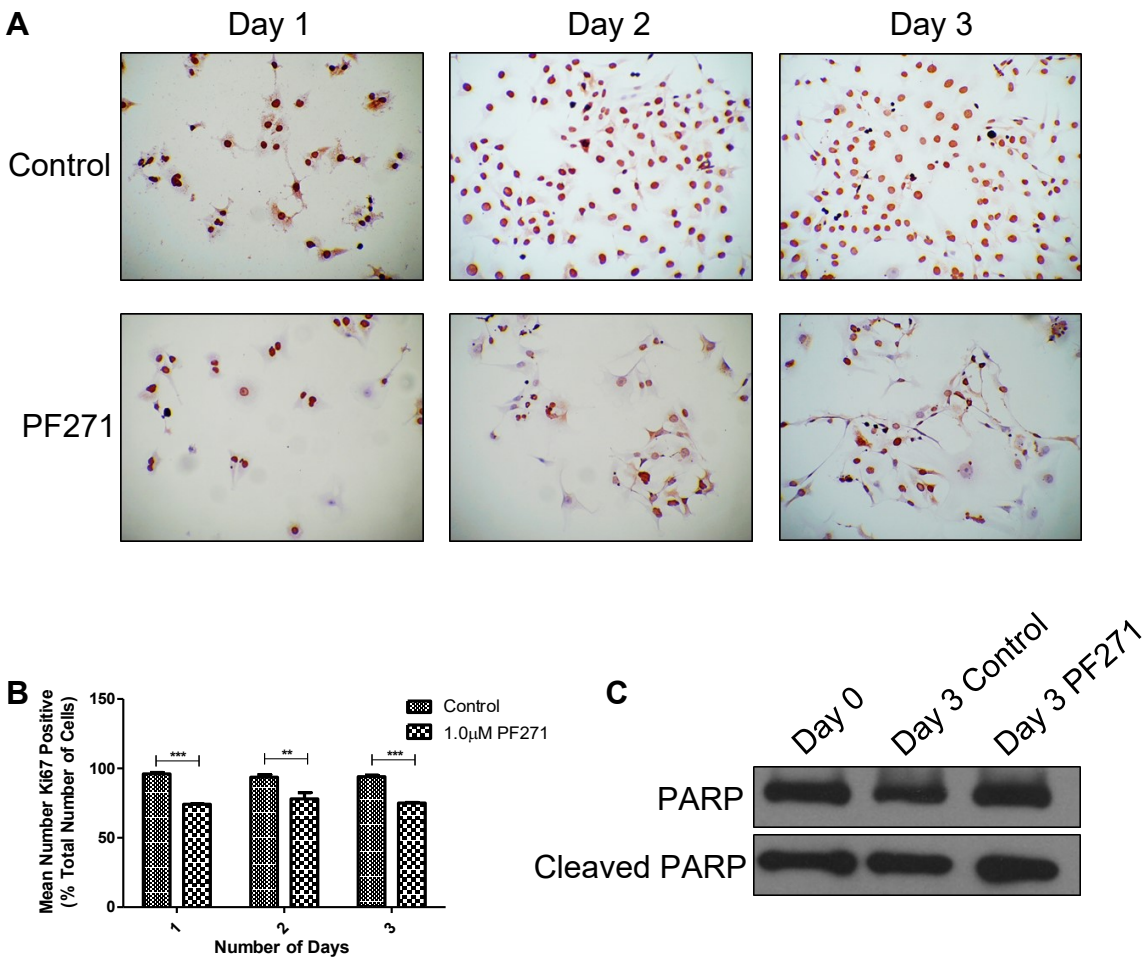


Figure 4.3 – Ki67 staining and western analysis of PARP cleavage in PF271 treated MDA-MB-231 cells – (A) Representative image showing the results of staining MDA-MB-231 cells for Ki67 over 3-days in the presence or absence of PF271. **(B)** Evaluation of Ki67 positive cells represented as a proportion of the total number of cells present on slides. FAK inhibition resulted in a significant reduction in proliferative marker Ki67 across all time-points. **(C)** Western blot showing changes in PARP and active cleaved PARP following 3-days with PF271. Actin was used as a loading control. Error bars represent SEM; n=3. *p<0.05; **p<0.01; ***p<0.001.

4.4. PF271-inhibited proliferation results from perturbed cell cycle progression

To gain further insight into how FAK regulates proliferation, we next explored whether PF271 treatment induced changes in cell cycle **(figure 4.4)**. PF271 treatment resulted in a significant reduction of the percentage of cells in G1 and an increase in S-phase when compared to controls. The proportion of cells in G2/M phase was not significantly different between the two conditions.

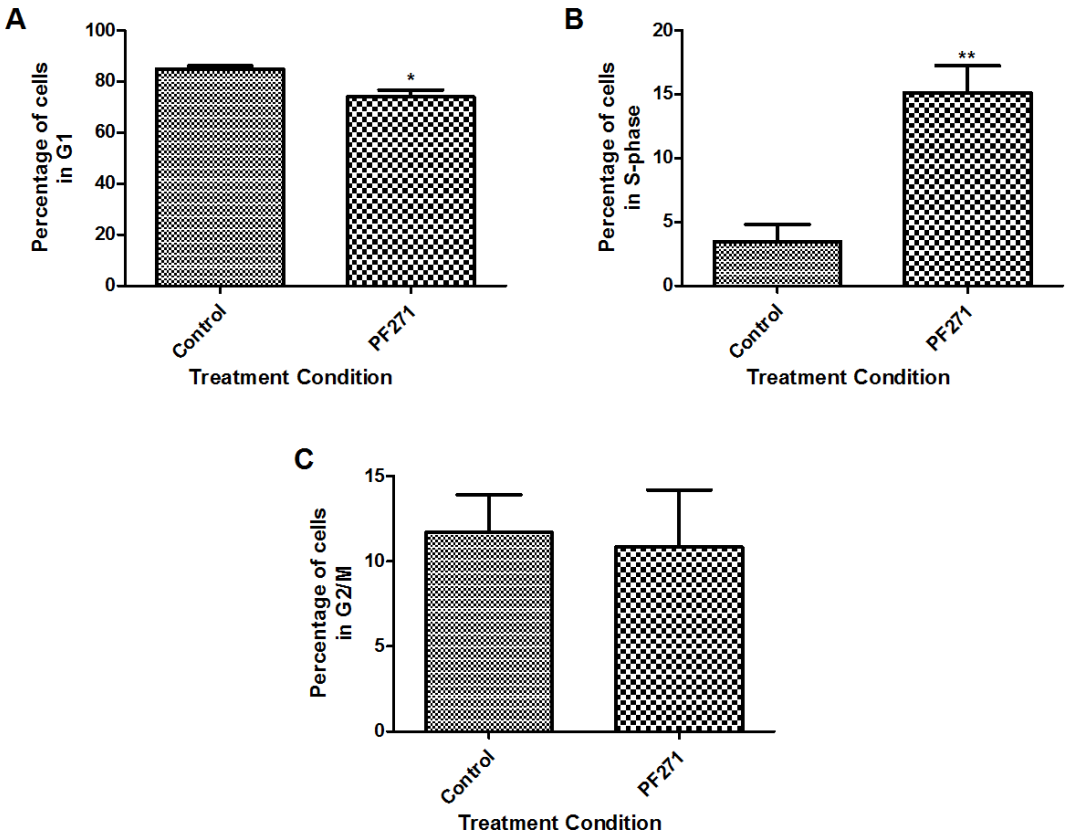
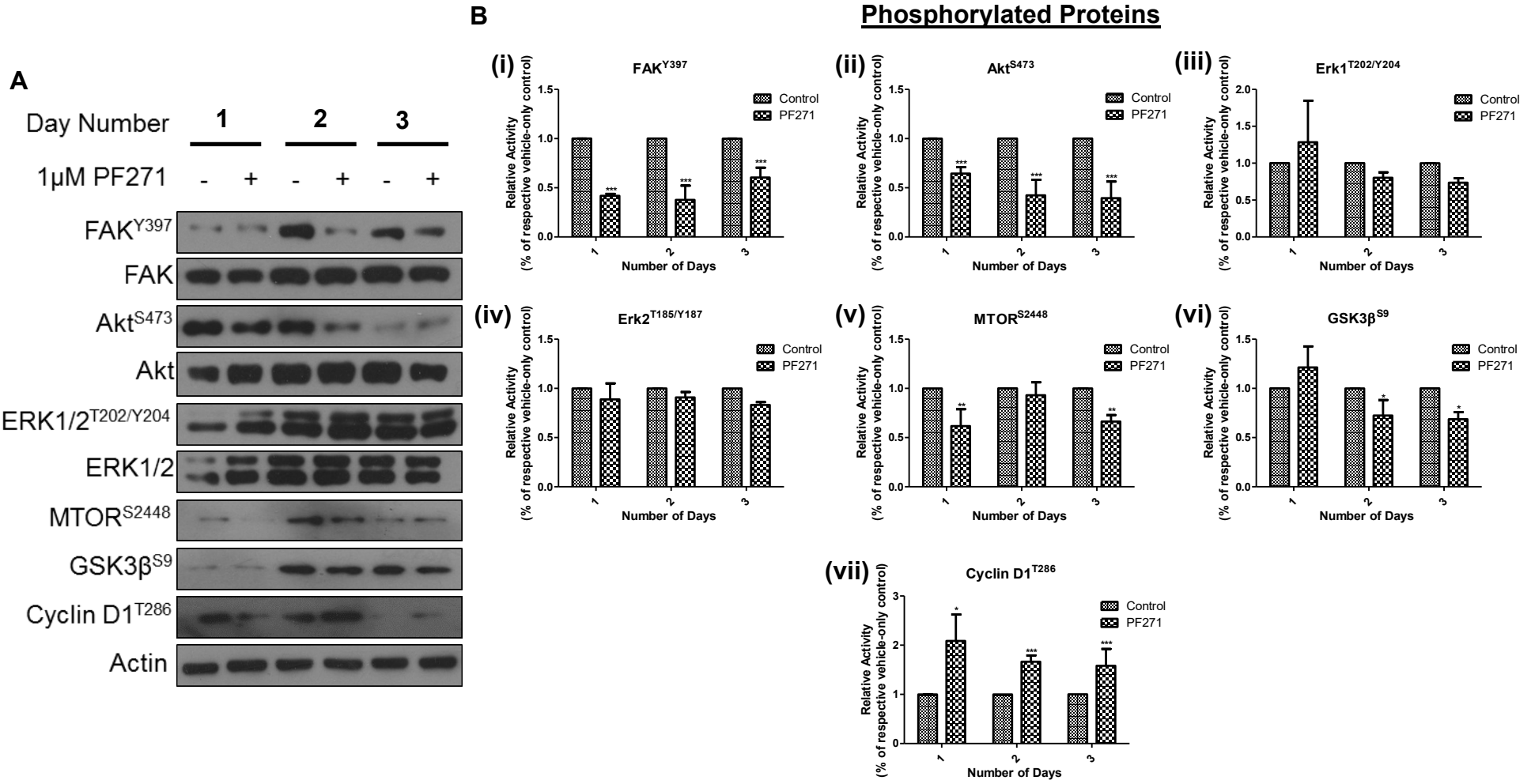


Figure 4.4 – Changes in cell cycle distribution in response to FAK inhibition – (A) Evaluation of how FAK inhibition alters the number of cells in G_1, represented as a proportion of total number of cells. PF271 treatment causes a significant reduction in G_1 cell numbers compared to vehicle-only control. **(B)** Assessment of proportion of cells in S-phase showing a significant increase in the cells this phase of the cell cycle in response to FAK inhibition. **(C)** Relative proportion of cells in G_2/M shows no significant change following treatment with PF271. Error bars represent SEM; n=3. *p<0.05; **p,0.01.

4.5. Inhibiting FAK kinase function significantly impairs downstream pathways associated with proliferation and cell cycle

To further assess examine the mechanisms by which FAK regulates proliferation in this TNBC model, we began to explore the influences of FAK inhibition on downstream signalling pathways previously shown to regulate cell proliferation (reviewed in Lee *et al.* 2015). PF271 treatment resulted in a significant decrease in FAK^{Y397} activity compared to the vehicle-only control **(figure 4.5A and Bi)**. This was accompanied by a significant decrease in phosphorylation of Akt^{S473}, $MTOR^{S2448}$ and $GSK3\beta^{S9}$, as well as a significant increase in inhibitory Cyclin $D1^{T286}$ phosphorylation **(figure 4.5ii, v and vi)**. In contrast, activity of $ERK1/2^{T202/Y204}$, known to be directly influenced by FAK activity in proliferation (Ding *et al.* 2005), was unaltered by PF271 inhibition at any time-point **(figure 4.5iii and iv)**. No significant changes in the total levels of any protein were observed **(figure 4.5C)**.



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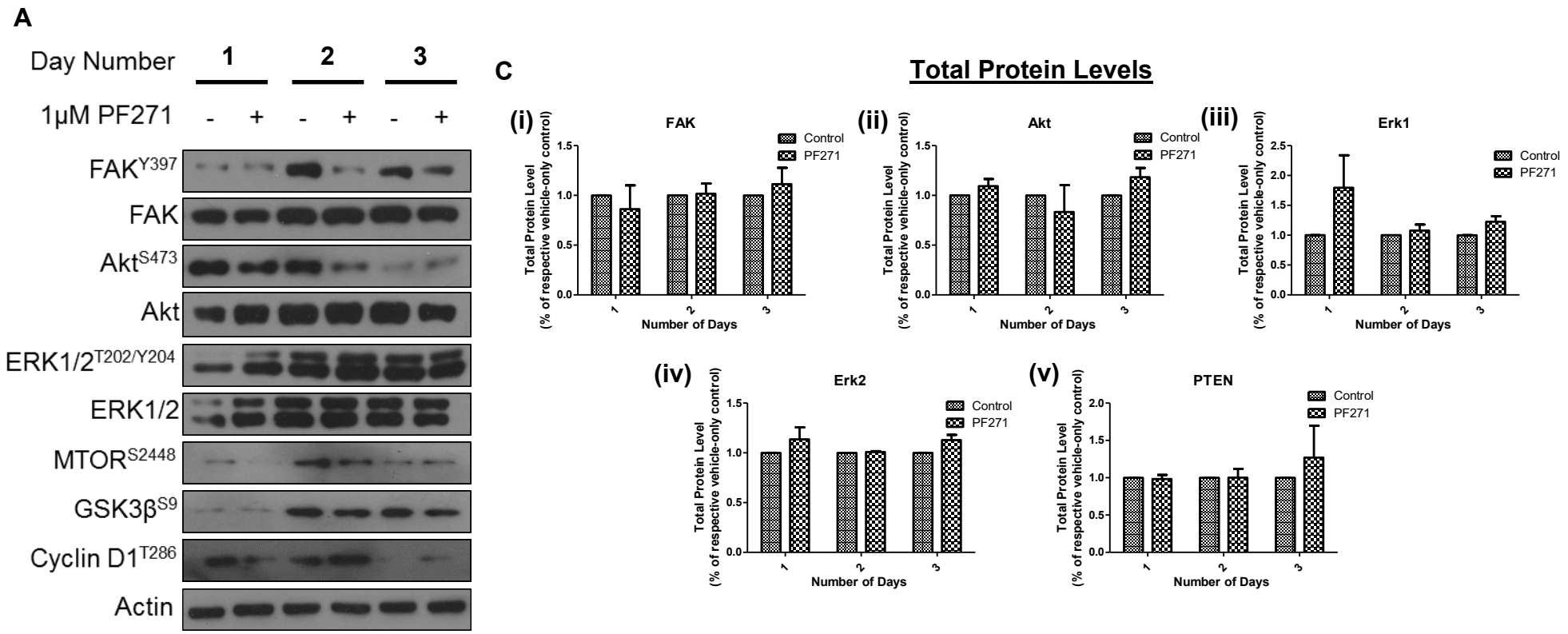


Figure 4.5 – Analysis of changes in phosphorylation and total protein levels over 3-days in the presence or absence of PF271 – (A) Western blot showing response of proteins to FAK-inhibition, measured at 1, 2 and 3-days following introduction of PF271. **(B)** Evaluation of relative activity of phosphorylated proteins (i) FAK^{Y397}; (ii) Akt^{S473}; (iii) Erk1$^{T202/Y204}$; (iv) Erk2$^{T185/Y187}$; (v) MTOR^{S2448}; (vi) GSK3β^{S9}; (vii) Cyclin D1^{T286}, showing significant decreases in active FAK, Akt, and MTOR in response to PF271 versus vehicle-only controls. Results also show a significant decrease in inhibitory GSK3^{S9} and increased inhibition of cyclin D1 through increased phosphorylation of T286 following FAK inhibition. **(C)** Assessment in changes in total protein levels of (i) FAK; (ii) Akt; (iii) Erk1; (iv) Erk2; (v) PTEN. No significant change is noted at any time-point across total proteins. All densitometry results are normalised to the respective actin loading control. Error bars represent SEM; n=3. *p<0.05; **p<0.01; ***p<0.001.

4.6. Evaluation of FAK scaffold function in regulating TNBC migration and invasion

We next wished to apply a similar approach to explore the role of FAK phosphorylation versus scaffolding function in regulating cellular migration and invasion. A wounding assay was used to visualise migratory changes following FAK siRNA treatment **(figure 4.6A)**. Complete wound closure was seen with control an NT-siRNA treated cells after 24-hours treatment, whereas both pharmacological and FAK-siRNA treatments significantly inhibited wound closure. Consequently, cellular migration was further validated using *in vitro* Boyden chamber migration assays **(figure 4.6B)**. FAK siRNA again resulted in a significant reduction of cell migration compared to the NT-siRNA controls **(figure 4.6C)**. In order to assess the contribution of scaffold function to this process changes in migration following FAK-siRNA + PF271 treatment was evaluated. Pharmacological inhibition of FAK caused a significant decrease in migration in all siRNA treated cells **(figure 4.6D)**. FAK siRNA was significantly less effective than PF271 treatment alone in its ability to impede migration, whilst combination of both was no better than sole pharmacological inhibition of kinase function.

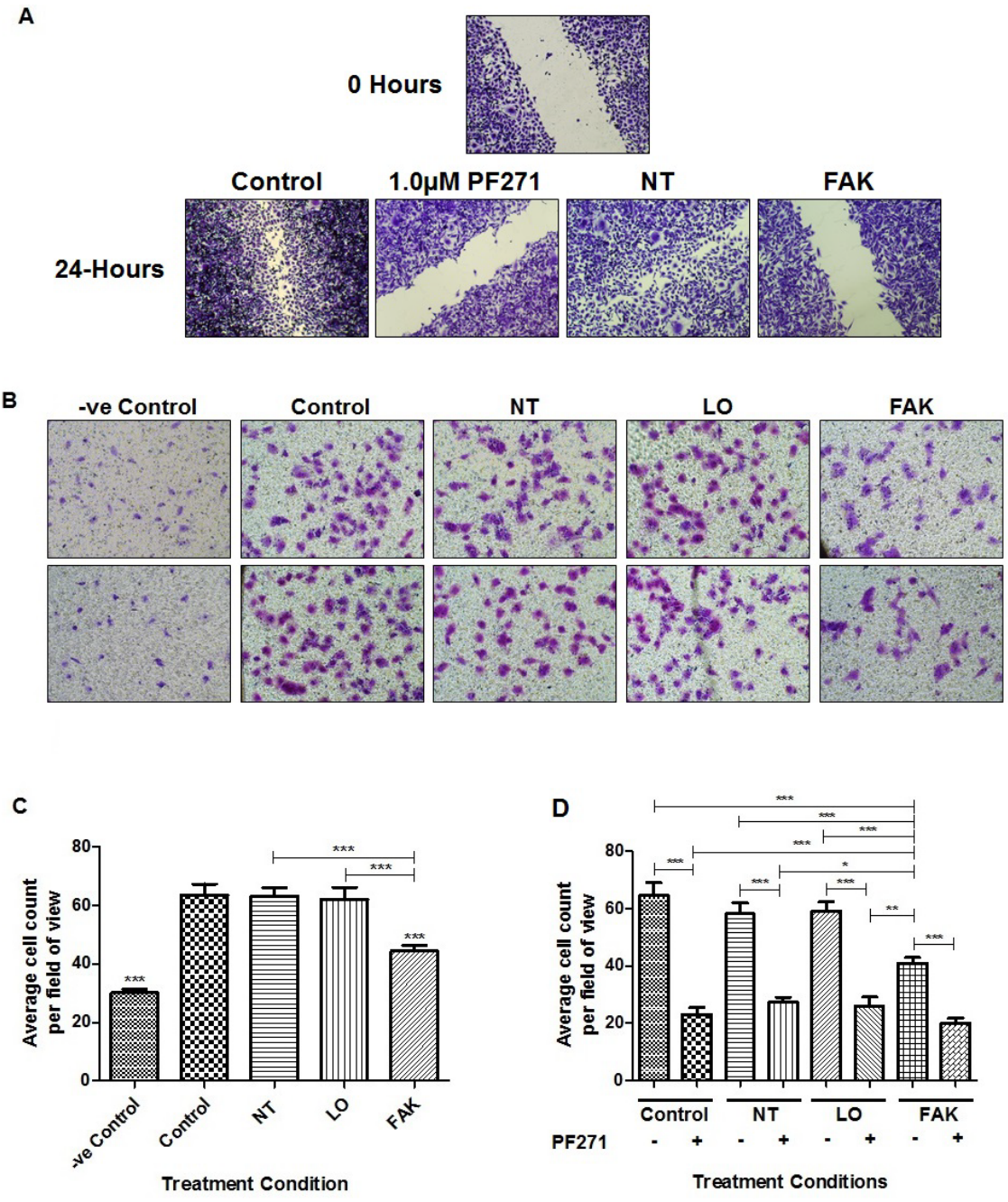


Figure 4.6 – Response of MDA-MB-231 cell migration to siRNA-mediated suppression of FAK – (A) Scratch wound analysis showing impaired wound healing following treatment with PF271 or suppression of FAK through siRNA. **(B)** Representative images of Boyden chamber assay following 18-hours migration prior to fixing and staining. **(C)** Evaluation of migratory capacity in cells transfected with FAK siRNA or respective controls. FAK suppression was caused a significant reduction in cell migration versus non-transfected control, as well as NT and LO controls. Negative control cells exhibited significantly worse migration versus all other cells. **(D)** Assessment of migratory changes in response to siRNA alone or in combination with PF271. FAK inhibition through PF271 significantly reduced migration in all transfected cells. The combination of PF271 and FAK siRNA had no additional inhibitory effects when compared to PF271 alone but showed significantly impaired migration versus FAK siRNA alone. All error bars represent SEM; n=4. *p<0.05; **p<0.01; p<0.001.

We next investigated the effects of FAK siRNA on pharmacological inhibition of cellular invasion. FAK-siRNA inhibited cellular invasion versus control samples **(figure 4.7A and B)**. Subsequent comparative analysis of siRNA and PF271-mediated kinase inhibition revealed that FAK knockdown was as effective at impairing invasion as inhibiting kinase activity **(figure 4.7C)**. Moreover, combination treatment did not demonstrate any additional benefit over FAK-siRNA or PF271 treatment alone. All transfected controls showed decreased invasion in response to PF271. Due to time constraints, future analysis will focus solely on migration.

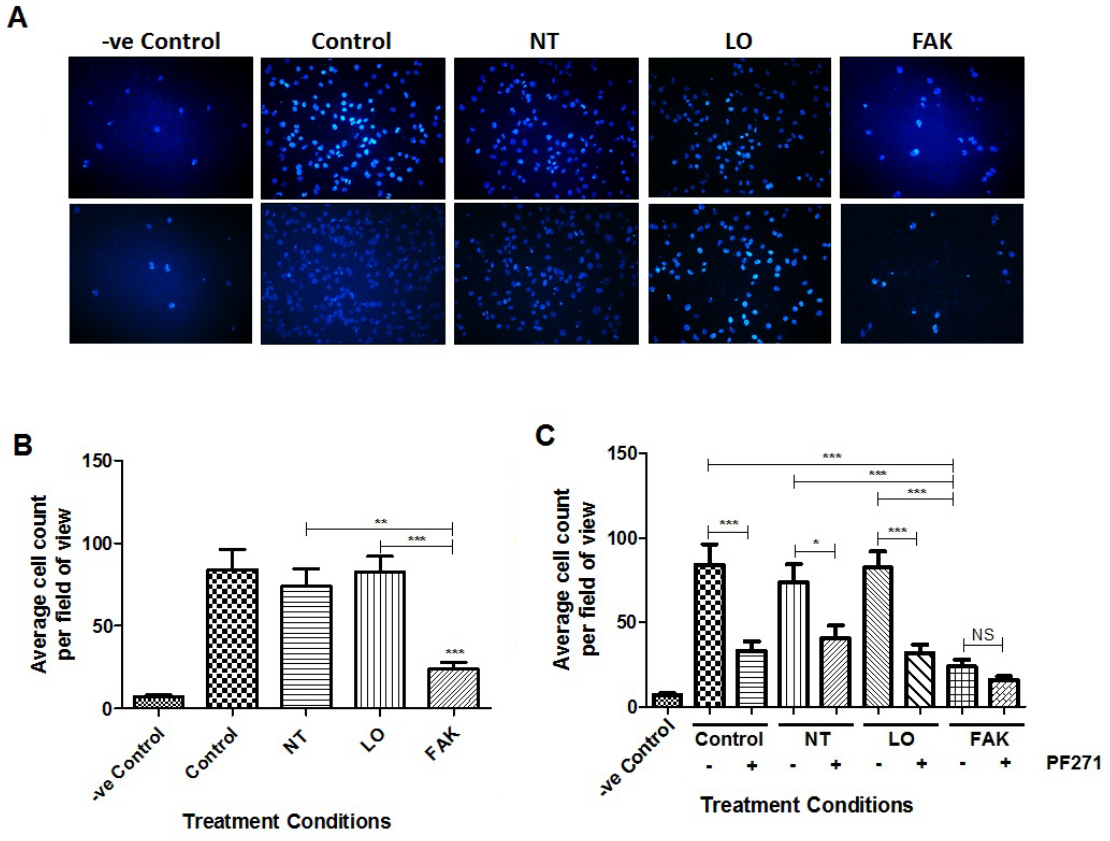


Figure 4.7 – Assessment of TNBC cell invasion following FAK suppression through siRNA – (A) Representative images of Boyden chambers following 72-hours invasion through Matrigel. **(B)** Evaluation of TNBC cell invasion in response to transfection with FAK siRNA and respective controls. Knockdown of FAK caused a significant decrease in invasive capacity versus all control cells, to a level comparable to unstimulated, negative-control cells. Transfection of NT-siRNA or LO had no significant effect on invasion. **(C)** Assessment of the effects on invasion of siRNA suppression or PF271-kinase inhibition of FAK alone or in combination. Knockdown of total FAK was sufficient to cause impaired invasion versus all controls minus PF271. This level was comparable to the negative control. All PF271 treated cells showed significantly attenuated invasion versus the respective vehicle-only control, except those treated with FAK-targeted siRNA. No significant difference in invasion was noted between FAK suppressed cells and those treated with PF271 only. All error bars represent SEM; n=4. *p<0.05; **p<0.01; ***p<0.001.

4.7. Assessing the role of the FAK-family member Pyk2 in regulating migration

The observation that inhibition of FAK kinase function was more effective at suppressing migration than siRNA-mediated knockdown was of particular interest, especially given that this method of inhibition inhibits both scaffolding and kinase function. This suggested that in FAK-silenced cells, a form of compensation may be occurring. PF271 has previously been reported to effectively inhibit both FAK and its family member Pyk2 (Bagi *et al.* 2008). Moreover, previous reports have shown that activity of Pyk2 can provide significant compensation for FAK loss (Fan and Guan 2011). To explore whether Pyk2 compensated for FAK loss, we suppressed Pyk2 using siRNA and determined the effect on cellular behaviour. Pyk2 siRNA was optimised as for FAK. As shown in **figure 4.8A and B** a 24-hour incubation with Pyk2 siRNA was sufficient to almost completely ablate total levels of Pyk2. However, in contrast to FAK, Pyk2 was re-expressed relatively rapidly on the removal of siRNA. Importantly, targeted suppression of Pyk2 did not adversely affect the expression or activity of FAK **(figure 4.8C)**.

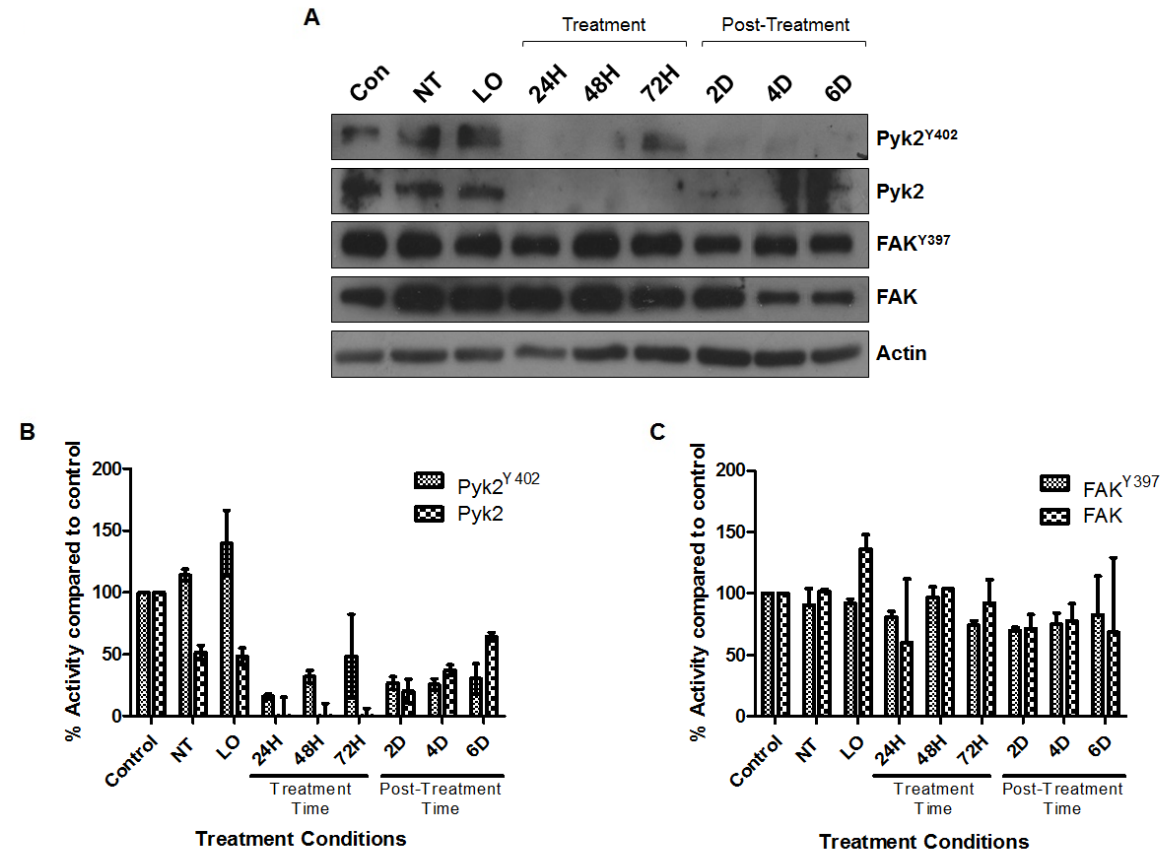


Figure 4.8 – Optimisation of siRNA-mediated Pyk2 inhibition – (A) Western blot showing changes in the protein levels of activated and total Pyk2 and FAK over time when incubated with Pyk2 siRNA and following its removal. A non-targeted siRNA (NT) and lipid only (LO) transfection are also shown for control, along with actin as a loading control. Time course for treatment is in hours (H) and post-treatment in days (D). **(B)** Densitometry data acquired from analysis of Pyk2 blots. Targeted siRNA causes a significant loss of total and activated Pyk2 levels but recovery is achieved following removal from siRNA. **(C)** Evaluation of immunoblots probing for FAK activity and expression. No significant changes are observed. Both densitometry graphs are normalised to the blot acquired for actin. All error bars represent SEM; n=3.

Having optimised Pyk2 suppression, we next performed Boyden chamber assays in order to asses the relative contribution of FAK and Pyk2 in MDA-MB-231 cell migration. We began by using PF271 or PF878 as the FAK inhibitor. PF878 is a second generation FAK inhibitor which was designed to reduce drug-drug interactions sometimes displayed by PF271 (Lee *et al.* 2015). Importantly, it has been shown to have comparable FAK suppressing effects versus PF271 but a greater effect in suppressing Pyk2 activity. Treatment with both agents was sufficient to significantly reduce migration, with both inhibitors displaying comparative levels of suppression **(figure 4.9A)**. However, western blot analysis revealed no significant difference in either FAK or Pyk2 between cells treated with each agent **(figure 4.9B)**. Consequently, we analysed the relative contribution of Pyk2 to migration though introduction of FAK and Pyk2 siRNA alone or in combination. Suppression of FAK was once again sufficient to significantly inhibit migration **(figure 4.9C)**. Unexpectedly, siRNA-mediated knockdown of Pyk2 significantly attenuated migration versus control cells comparative to levels observed when total FAK was suppressed. Interestingly, combination of FAK/Pyk2 siRNA was no better than using either siRNA alone. This suggests that Pyk2 is not compensating for a loss of FAK in migrating TNBC cells, rather it plays an equally important role in cell migration. As such, Pyk2 was not investigated further as the primary focus of this study was the role FAK itself plays in TNBC.

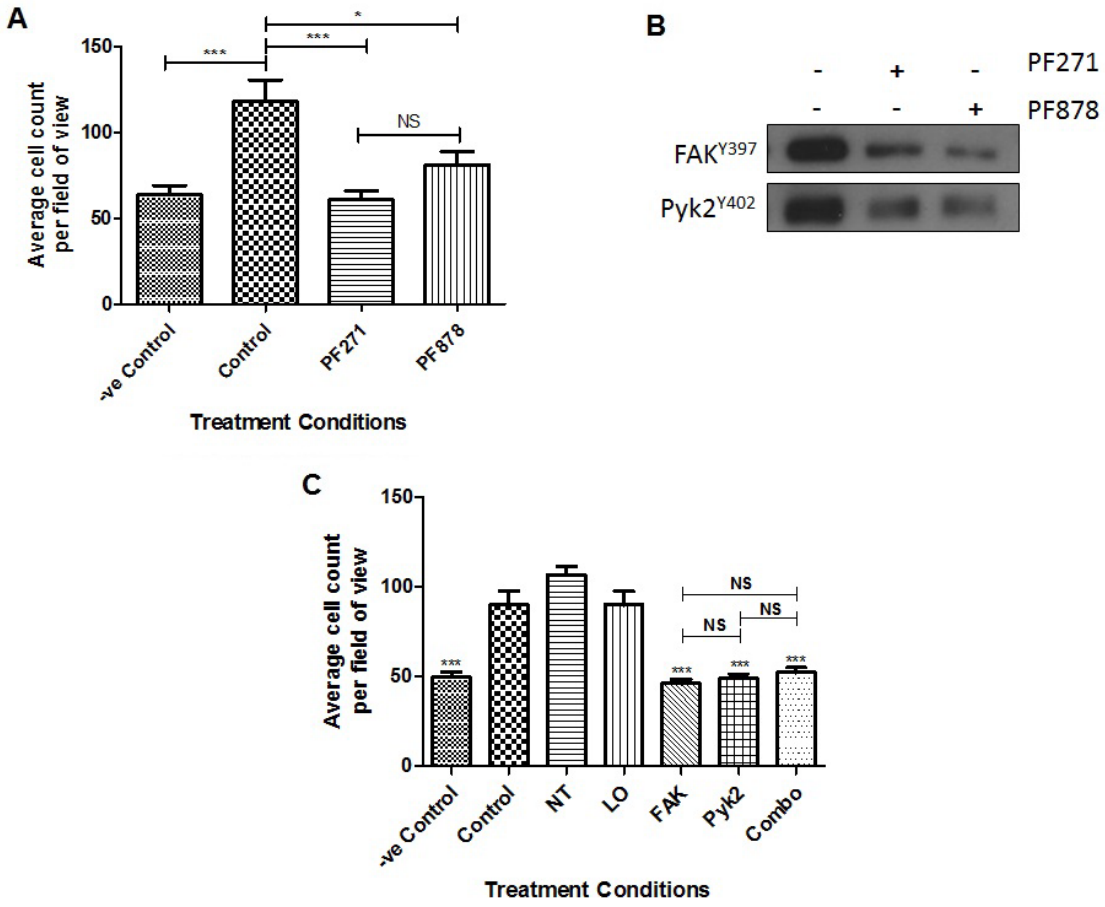
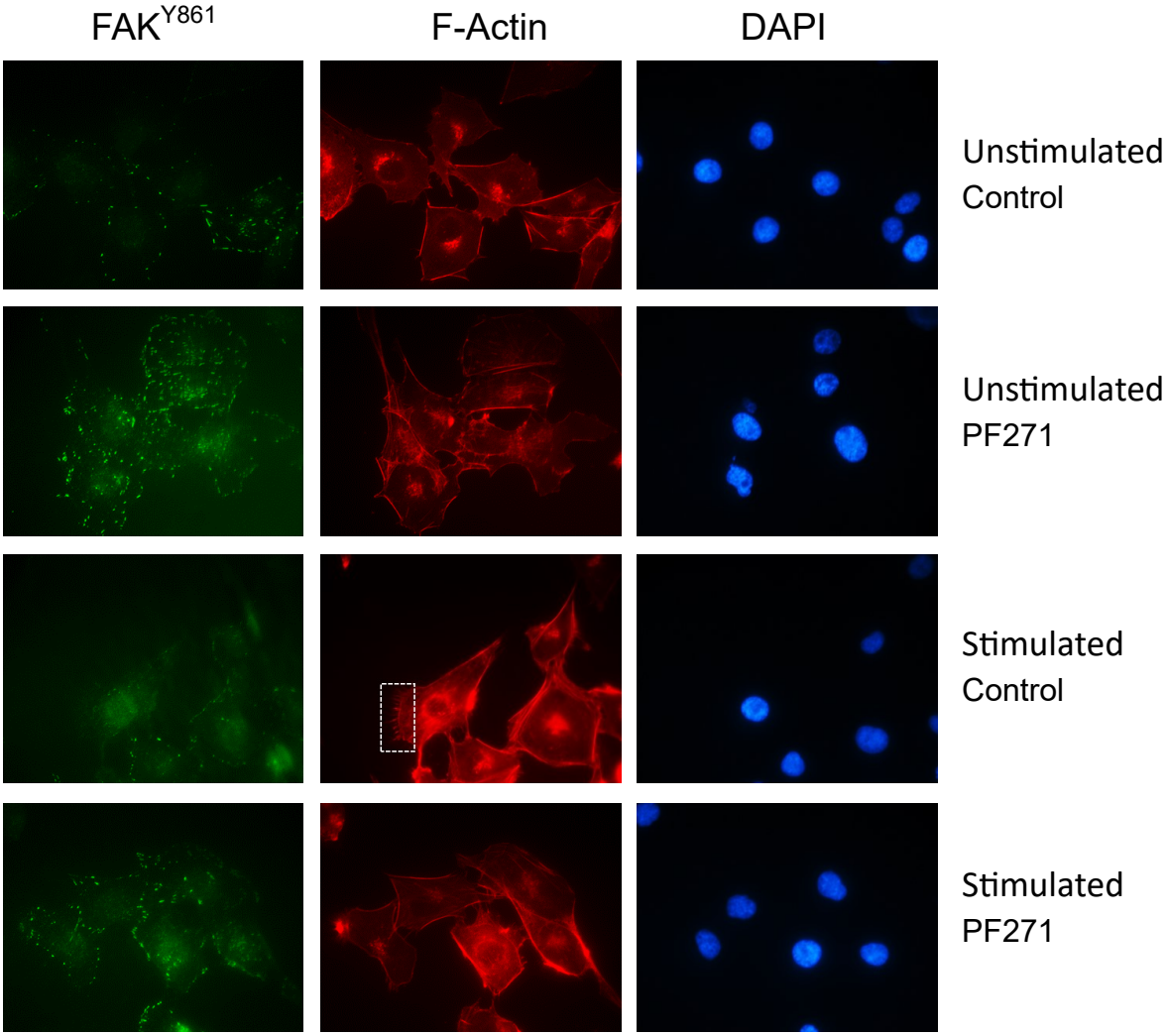


Figure 4.9 – Effects of FAK and Pyk2 siRNA alone or in combination on TNBC cell migration – (A) Representative images of Boyden chambers stained with crystal violet following 18-hour migration. **(B)** Analysis of Boyden chamber migration assay. FAK and Pyk2 siRNA both cause a significant suppression of cell migration, comparable to unstimulated, negative control cells. Combination of FAK and Pyk2 siRNA had no additional effect when compared to sole use of either siRNA. All error bars represent SEM; n=3. *p<0.05; **p<0.01; ***p<0.001.

4.8. Evaluation of changes in cell morphology and FAK distribution in migrating cells following FAK inhibition

We next wished to probe the mechanisms of action underlying FAK-mediated migration of MDA-MB-231 cells. Initially, immunofluorescent staining was performed to observe changes in cell morphology and subcellular localisation of active/total FAK. As shown in **appendix 1**, detection of FAK^{Y397} was particularly difficult due to high levels of background staining potentially masking any true changes in active FAK localisation. Therefore, as previously noted, FAK^{Y861} was used as a surrogate marker for FAK activity given its need for FAK^{Y397} to be activated (Lee *et al.* 2015), its correlation with FAK^{Y397} **(figure 3.3)** and suppression following PF271 treatment. In the absence of serum stimulation cells were significantly less migratory in appearance, lacking polarisation and extension of filopodia-like protrusions **(figure 4.10)**. Additionally, treatment with PF271 caused a significant increase in FAK^{Y861} intensity in both serum-starved and stimulated cells versus their respective vehicle-only control cells. It also seemed to increase the amount of active FAK^{Y861} localised to cortical actin. By contrast, total FAK exhibited little change in localisation following PF271 treatment in both FCS-stimulated and unstimulated cells **(figure 4.11)**. Consequently, further examination of the molecular dynamics of migrating cells would focus only on active FAK changes. When co-stained with vinculin (a marker of focal adhesions (FAs)), we observed that stimulated cells had significantly more FAs across the cell than unstimulated controls **(figure 4.12)**. Indeed, quantification of FA dynamics showed a significant increase in total number and size of FAs following PF271 treatment compared to control cells **(figure 4.13)**, which also correlated with increased localisation of active FAK to these sites.



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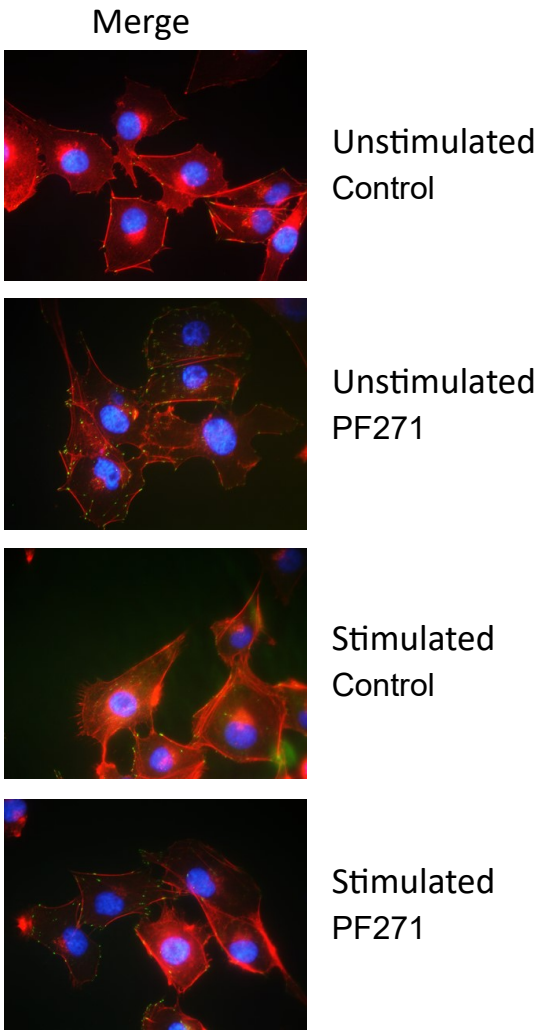
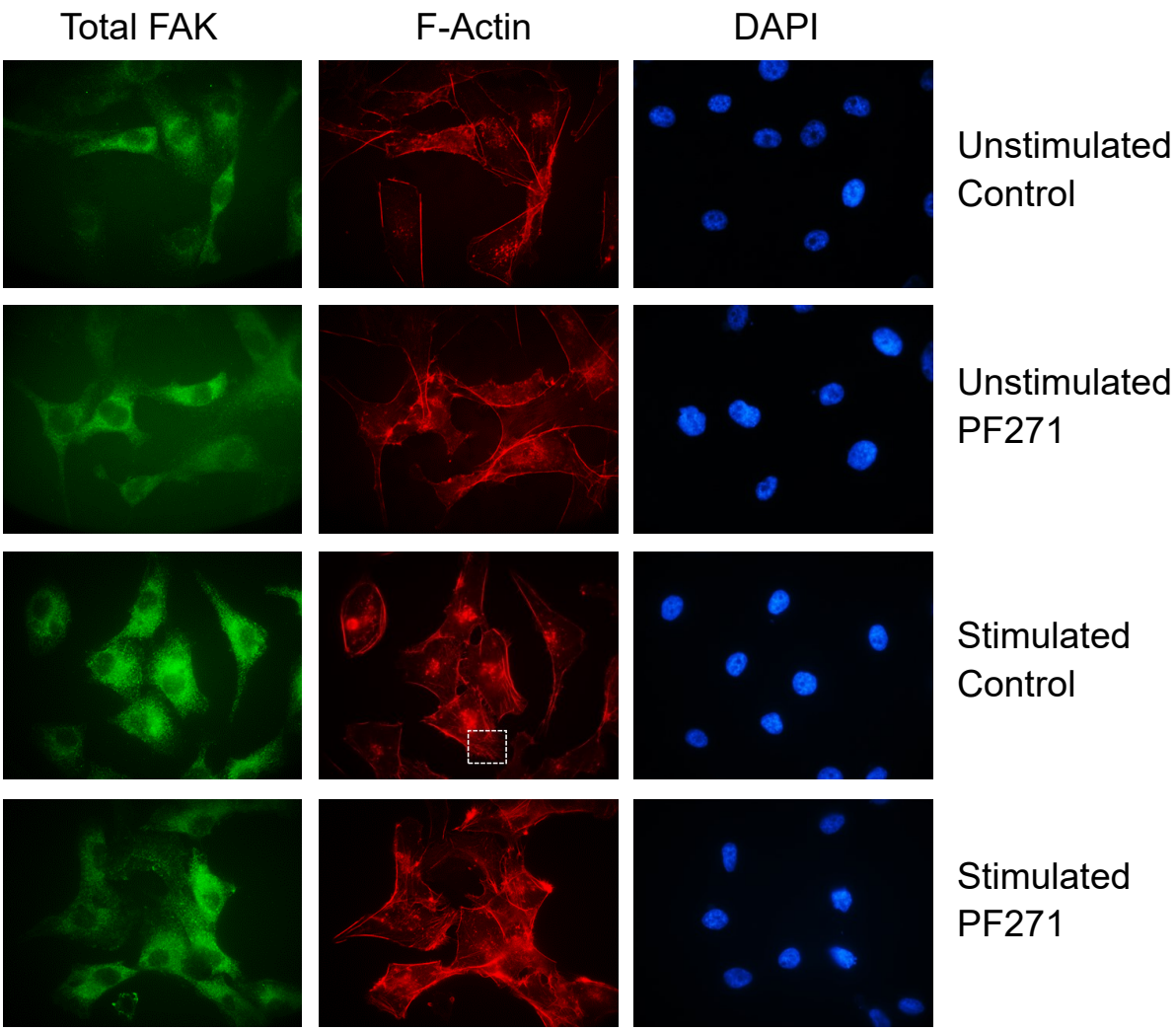


Figure 4.10 – Examination of changes in active FAK and actin in response to PF271 in migrating cells – Serum-starved TNBC cells were either treated with either serum-free RPMI (serum-starved controls) or media including FCS, in addition to PF271. Inhibition of FAK causes increased intensity of active FAK^{Y861} (green) on the cell periphery, suggesting increased localisation to distinct sites, whilst extension of actin (red) protrusions (white box) is also inhibited. Serum-stimulation resulted in increased FAK intensity and lamellipodia extension. Nuclei were stained with DAPI (blue). All images are representative; n=3.



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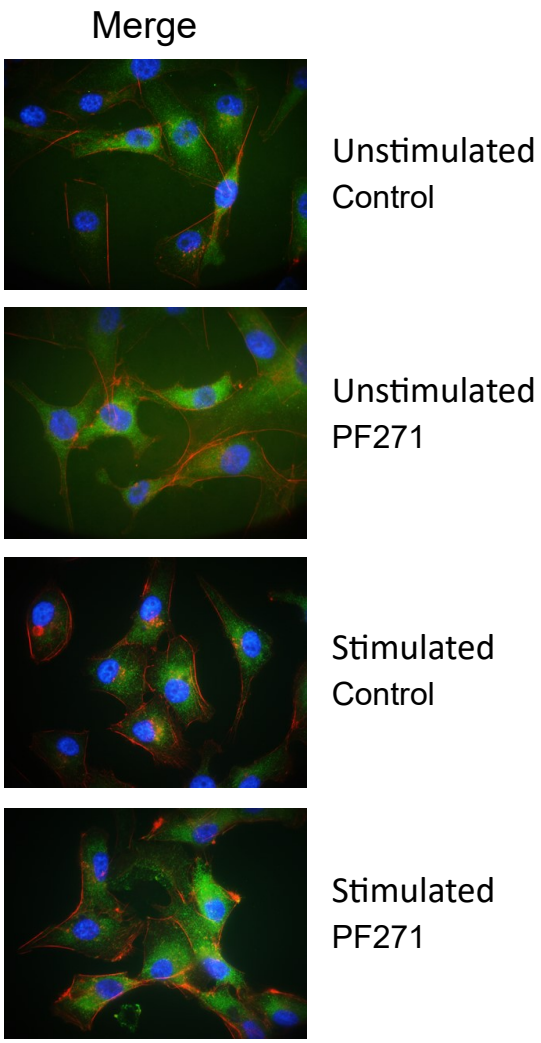
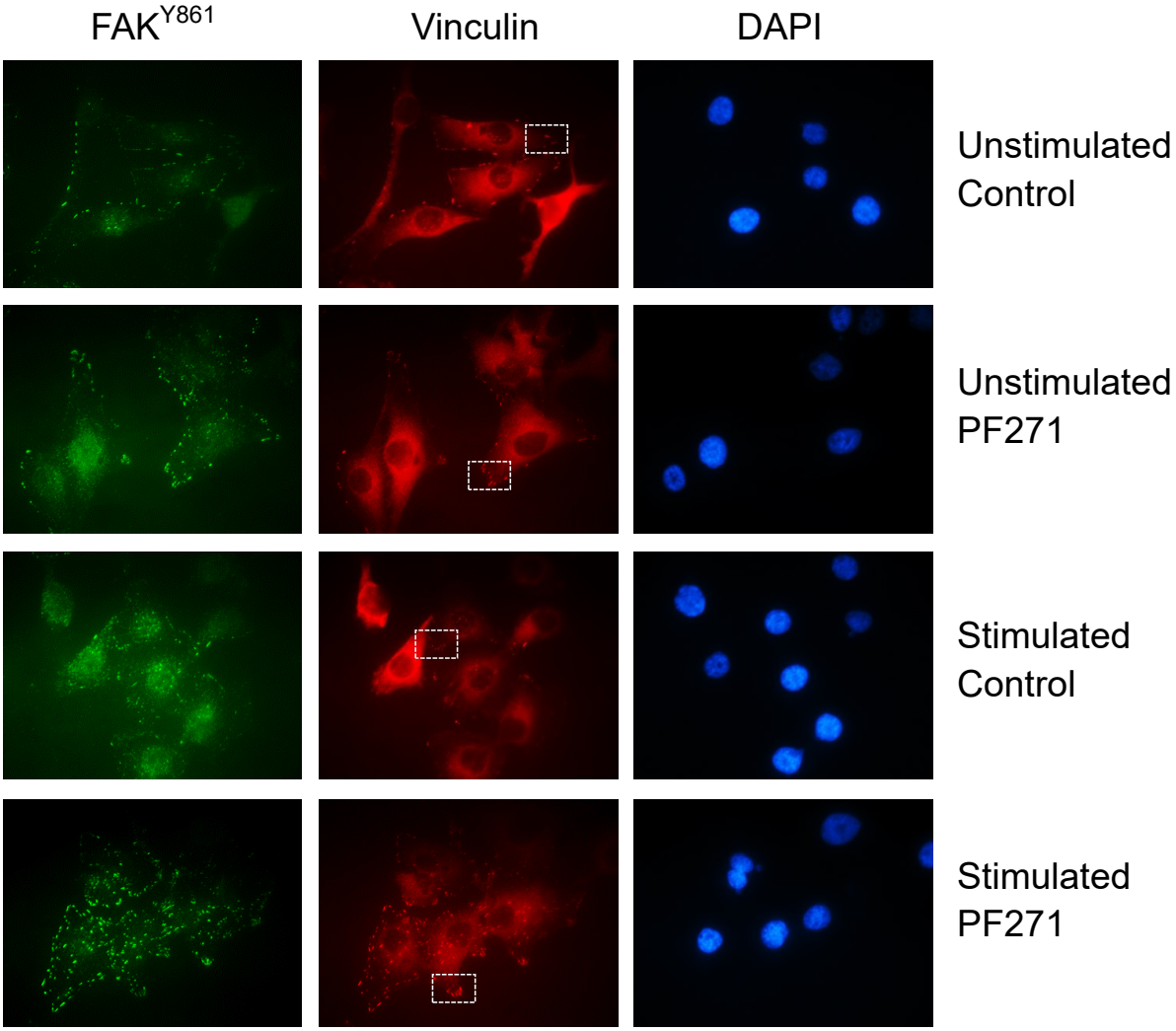


Figure 4.11 – Examination of changes in total FAK and actin in response to PF271 in migrating cells – Serum-starved TNBC cells were either treated with either serum-free RPMI (serum-starved controls) or media including FCS, in addition to PF271. Inhibition of FAK had no significant effect on total FAK (green) distribution. As previously noted extension of actin (red) protrusions (white box) is perturbed by FAK inhibition. Serum-stimulation also had no effect on total FAK, whilst lamellipodia extension was increased. Nuclei were stained with DAPI (blue). All images are representative; n=3.



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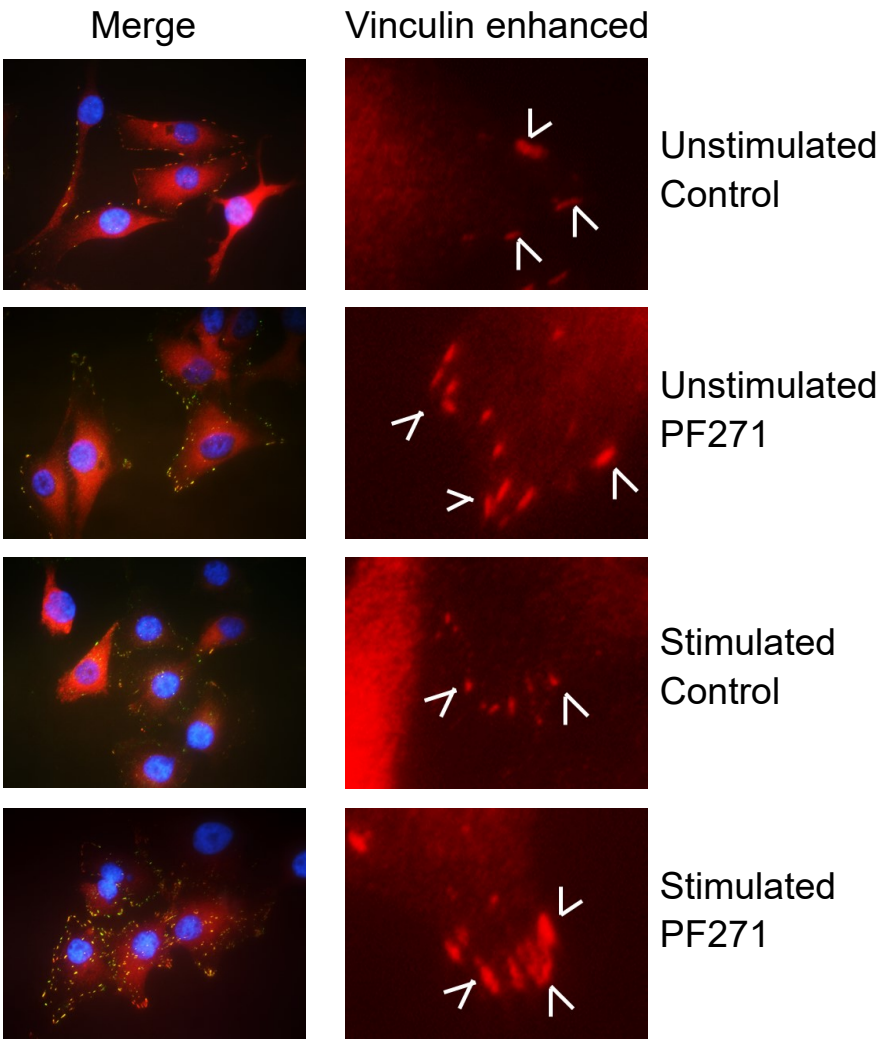


Figure 4.12 – Examination of changes in active FAK and vinculin in response to PF271 in migrating cells – Serum-starved TNBC cells were either treated with either serum-free RPMI (serum-starved controls) or media including FCS, in addition to PF271. As noted previously, inhibition of FAK kinase function causes increased intensity of active FAK^{Y861} (green) on the cell periphery, more so than in serum-stimulated versus starved control cells. FCS treatment caused increased vinculin staining (red) versus starved controls. PF271 inhibition caused further increases in FA number and size and greater colocalization with FAK (yellow). Nuclei were stained with DAPI (blue). All images are representative; n=3.

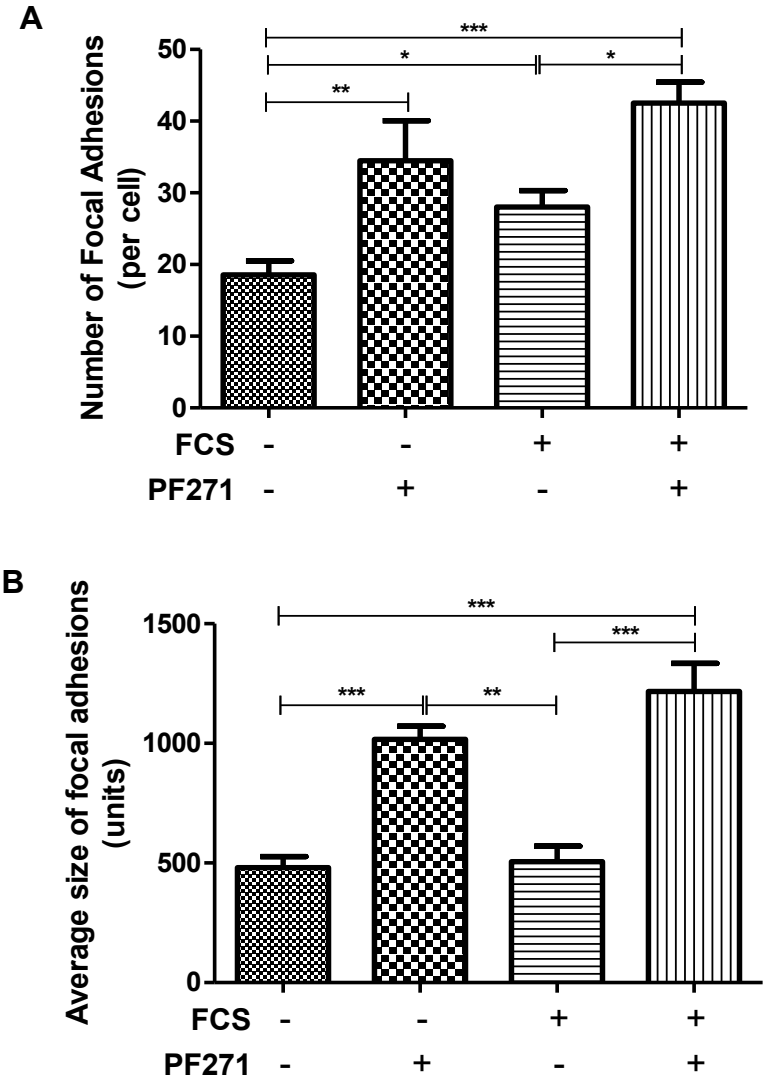
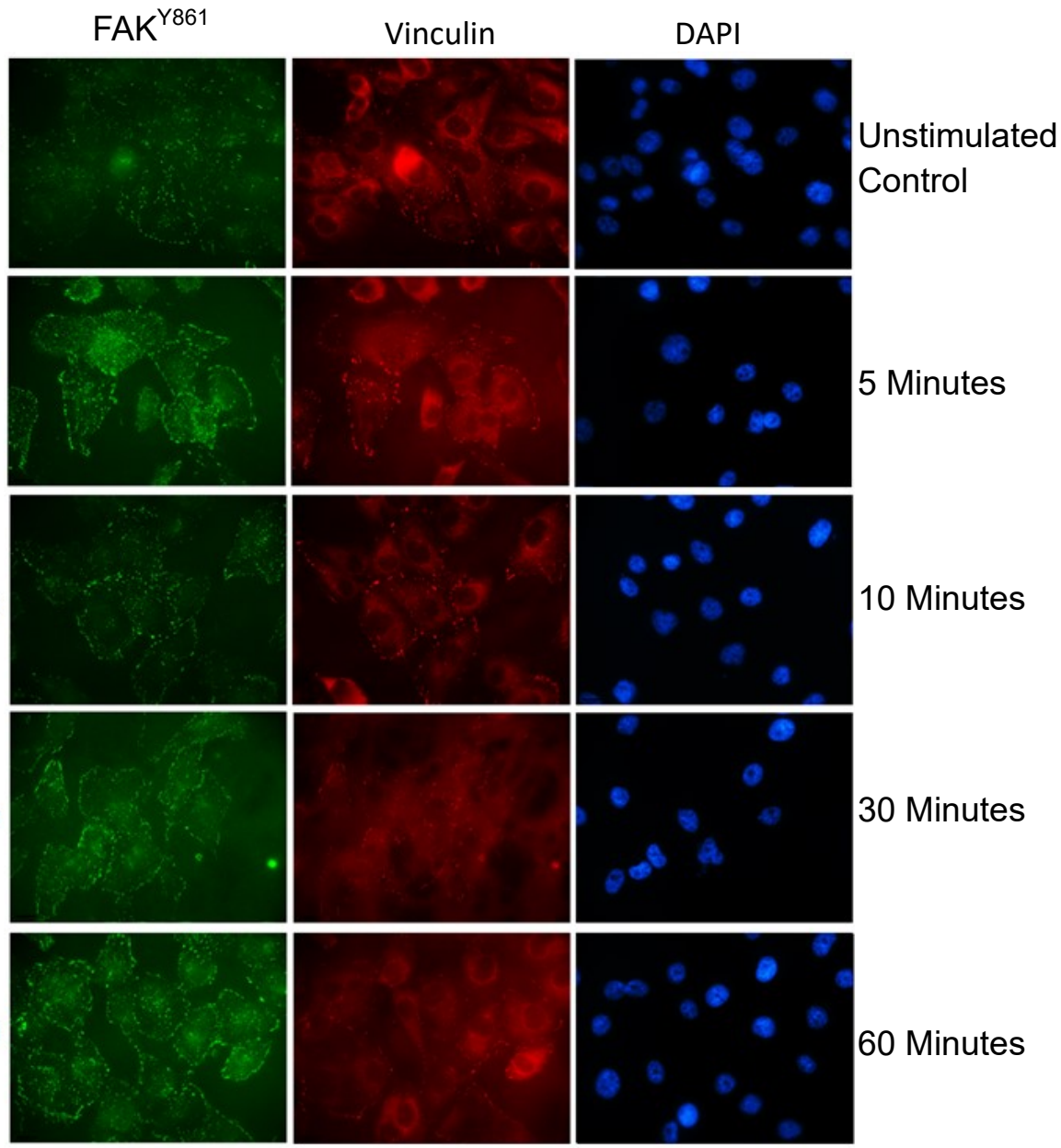


Figure 4.13 – Examination of changes in FA dynamics in migrating cells following PF271 treatment – (A) The average number of FAs per cell was calculated by counting FAs on 3 cells in all 5 acquired images (n=15 technical replicates). Analysis revealed that serum stimulation was sufficient to significantly increase FA numbers. Moreover, inhibition of FAK in stimulated and unstimulated cells caused a significant increase in the number of FAs versus vehicle-only controls. This effect was greatest in cells that were both treated with PF271 and stimulated with FCS. **(B)** Analysis of FA size showed that serum stimulation was not sufficient to alter FA size, whilst introduction of PF271 caused a significant increase in both stimulated and unstimulated cells versus vehicle-only controls. All error bars represent SEM; n=3 independent experiments. *p<0.05; **p<0.01; ***p<0.001.

Previously, it has been shown that changes in FAs happen very quickly. As such, we wished to establish whether by using a 1-hour stimulation we were missing some changes in cell dynamics. As such we performed a time-course immunofluorescent stain over 1-hour with cells co-stained with either FAK^{Y861} or total FAK and vinculin. This would enable us to see if we were missing both changes in FAK distribution and FA dynamics. Following serum stimulation, all cells exhibited significant increases in both vinculin and FAK^{Y861} **(figure 4.14)**. Moreover, 5-minute incubation was all it took to observe this significant change and it was sustained over the 1-hour time-course examined. In contrast, despite significant increases in FAs (as marked by vinculin stain), levels of total FAK were unaltered by serum-stimulation at any time-point versus the unstimulated control cells **(figure 4.15)**. Consequently, it was determined that at 1-hour following serum introduction cells exhibited significant cellular changes and thus our previous findings are not invalidated by using too long a stimulation.



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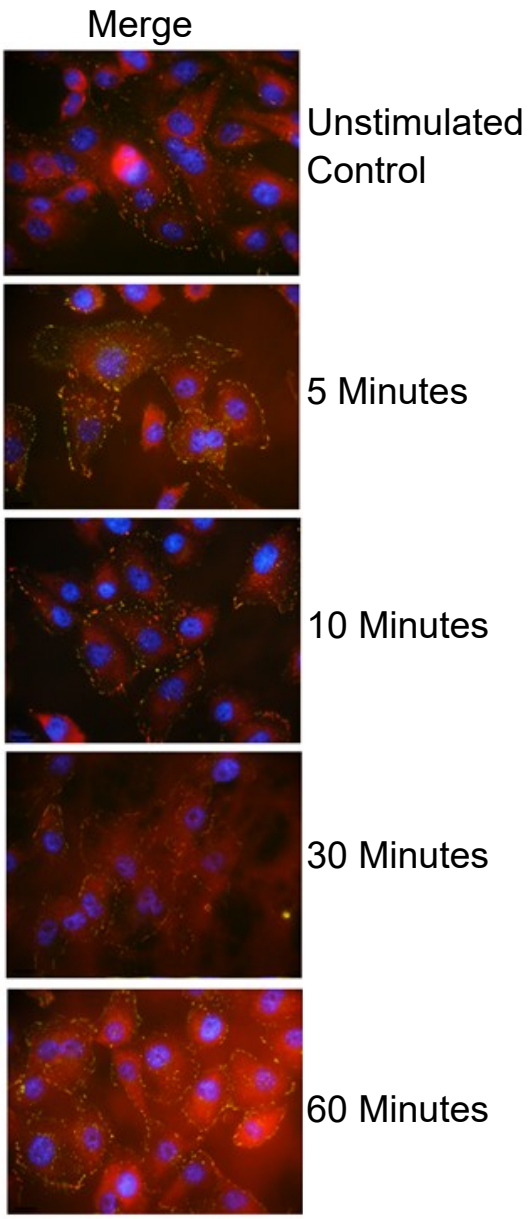
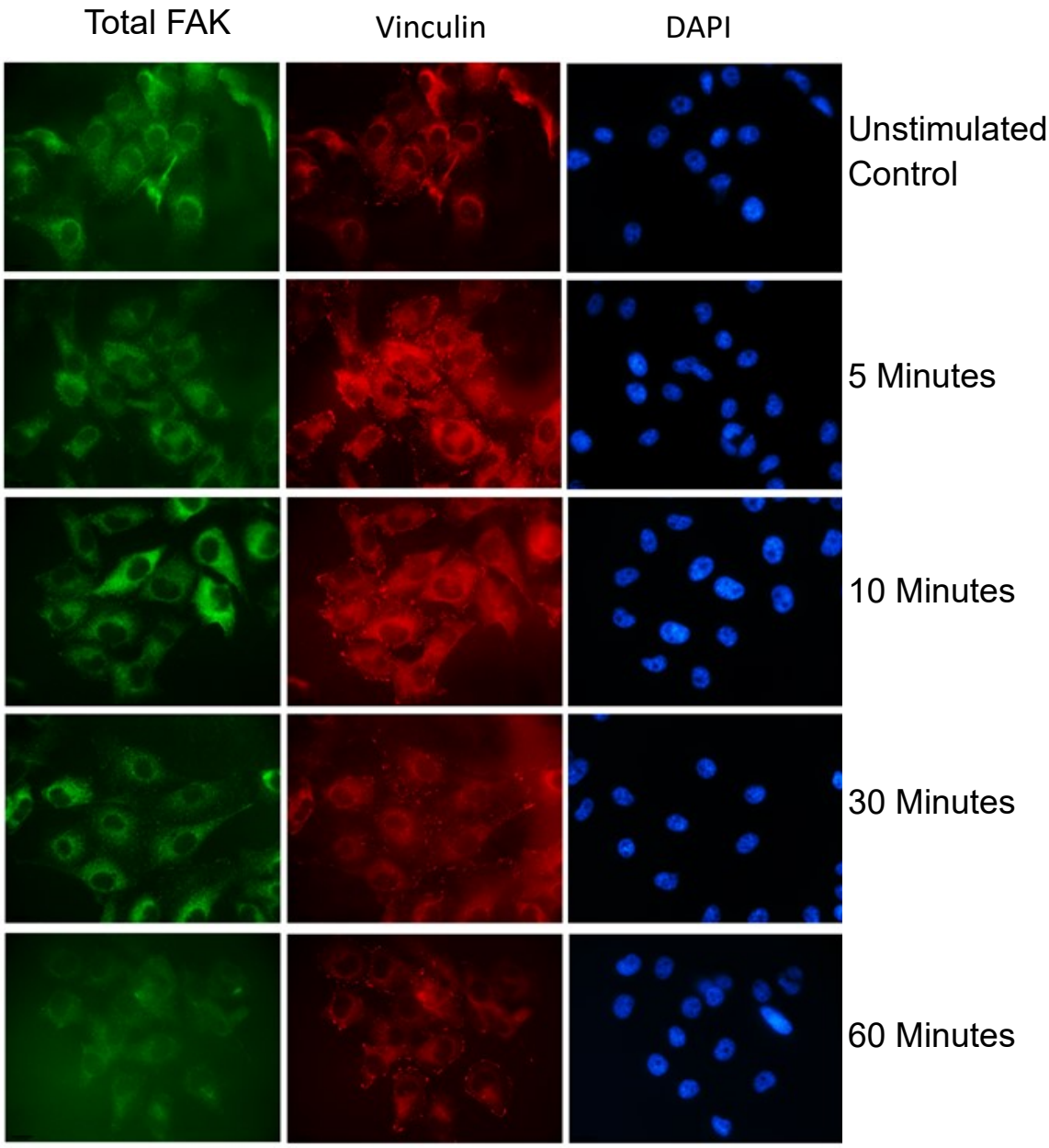


Figure 4.14 – Time-course immunofluorescent assessment of active FAK and focal adhesion dynamics – Serum-starved cells were treated with FCS-containing RPMI prior to fixing and staining at various time-points. Following 5-minutes, serum-stimulated cells exhibited significant increases in vinculin (red) and FAK^{Y861} (green) staining which was sustained through the full 60-minute time-course versus serum-starved controls. All images are representative; n=3.



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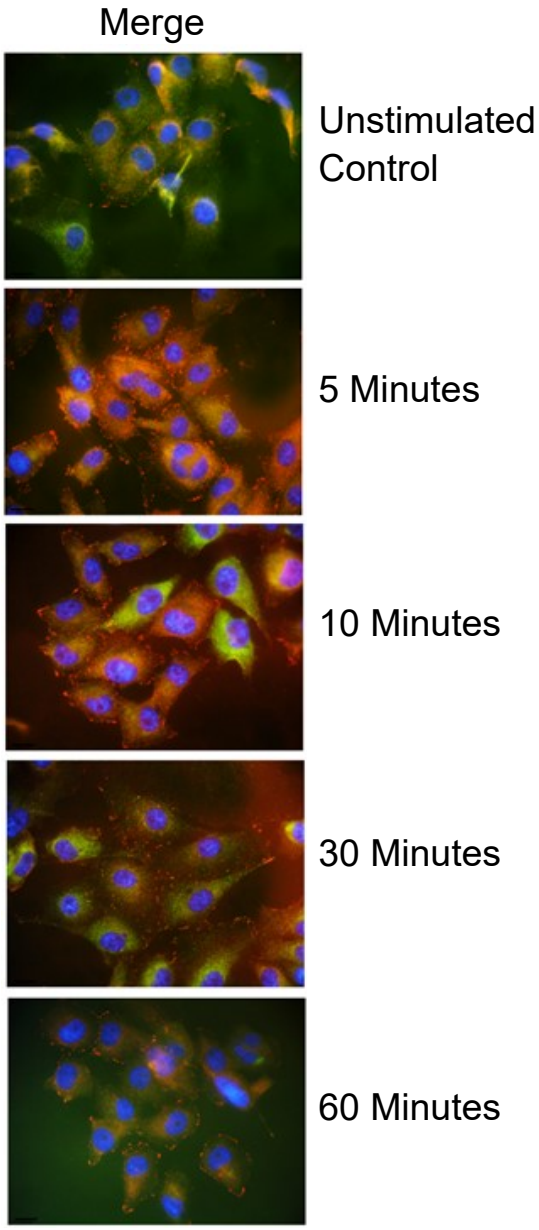
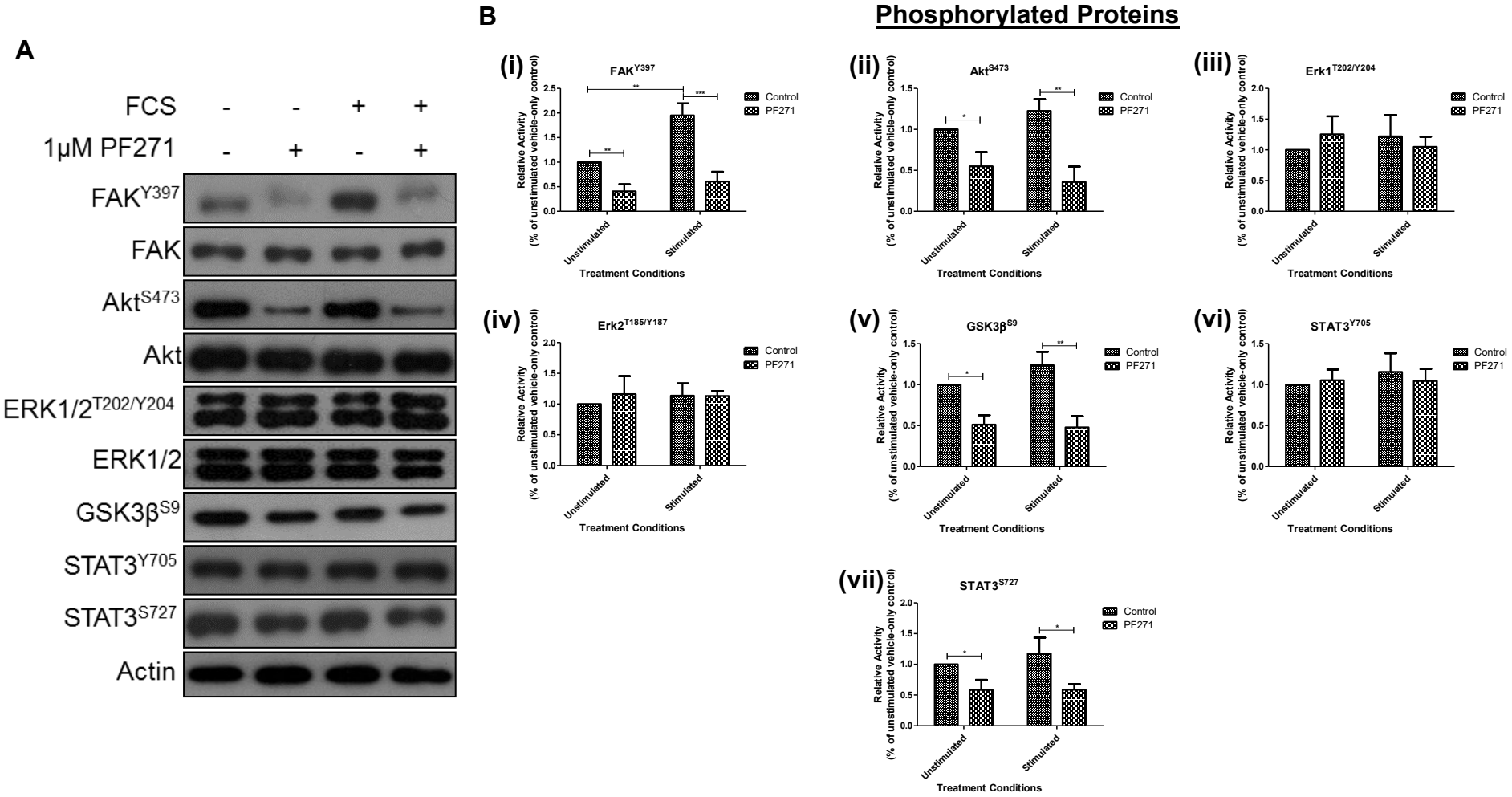


Figure 4.15 - Time-course immunofluorescent assessment of total FAK and focal adhesion dynamics – Serum-starved cells were treated with FCS-containing RPMI prior to fixing and staining at various time-points. Following 5-minutes, serum-stimulated cells exhibited significant increases in vinculin (red) whilst total FAK (green) levels and localisation was unperturbed versus serum-starved controls. All images are representative; n=3.

4.9. Evaluation of signalling changes following PF271-mediated FAK inhibition in migrating cells

To further probe the mechanisms of action governing FAK-mediated migration, western blotting was performed on a range of unstimulated or FCS-stimulated cells. Serum stimulation resulted in increased FAK^{Y397} activity which was blocked in the presence of PF271 **(figure 4.16A and Bi)**. Similar results were also observed for Akt^{S473} and $GSK3\beta^{S9}$ **(figure 4.16Bii and v)**. Although unaltered in proliferating cells **(figure 4.5)**, ERK1/2 activity has been closely linked to FAK in regulating cell migration (Westhoff *et al.* 2004; Carragher *et al.* 2003). However, our results showed that levels of active **(figure 4.16Biii and iv)** and total Erk1/2 **(figure 4.16Ciii and iv)** were unaltered in response to serum or following FAK inhibition. Several studies have highlighted a key role for STAT3 in the promotion of cancer cell migration (Wei *et al.* 2013; Vultur *et al.* 2014). Moreover, previous work from our group has demonstrated a link between FAK and STAT3 in modulating migration of HER2+ breast cancer cells (Lazaro *et al.* 2014). Consequently, we wished to explore the relevance of this FAK/STAT3 signalling axis in the context of MDA-MB-231 cells. $STAT3^{Y705}$ displayed no significant changes in response to serum stimulation and was unaltered by PF271 **(figure 4.16Bvi)**. By contrast, $STAT3^{S727}$ activity was significantly reduced following PF271 treatment, although FCS-stimulation had little effect **(figure 4.16Bvii).**



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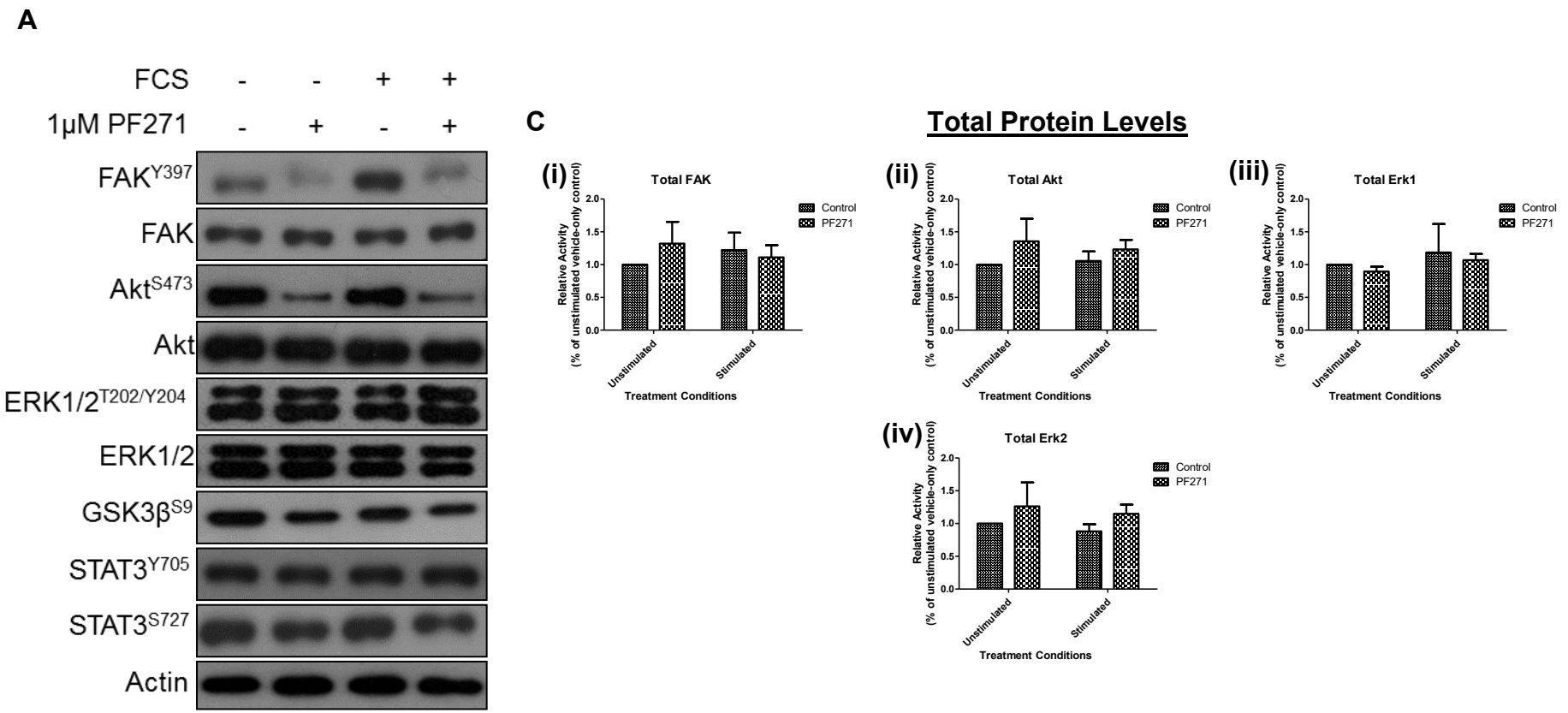


Figure 4.16 – Analysis of changes in phosphorylation and total protein levels over 3-days in the presence or absence of PF271 – (A) Western blot showing response of proteins to FAK-inhibition, in serum starved or stimulated cells grown on fibronectin. **(B)** Evaluation of relative activity of phosphorylated proteins (i) FAK^{Y397}; (ii) Akt^{S473}; (iii) Erk1$^{T202/Y204}$; (iv) Erk2$^{T185/Y187}$; (v) GSK3β^{S9}; (vi) STAT3^{Y705}; (vii) STAT3^{S727}, showing significant decreases in active FAK, Akt, and STAT3^{S727} in response to PF271 versus vehicle-only controls. Results also show a significant decrease in inhibitory GSK3^{S9} and increased activity of FAK^{Y397} in FCS-stimulated cells versus unstimulated controls. **(C)** Assessment in changes in total protein levels of (i) FAK; (ii) Akt; (iii) Erk1; (iv) Erk2. No significant change was noted in response to PF271 or stimulation with FCS. All densitometry results are normalised to the respective actin loading control. Error bars represent SEM; n=3. *p<0.05; **p<0.01; ***p<0.001.

4.10. Validation of FAK-mediated regulation of proliferation and migration in a second model of TNBC

One caveat to using cell models to study cancer biology is that observations may be cell-line specific. In light of this, we wished to validate our findings in the MDA-MB-231 cells by exploring FAK-regulated proliferative and migratory behaviour in a second model of TNBC, namely MDA-MB-468 cells. We first examined how FAK activity/expression compared between these cells and MDA-MB-231s **(figure 4.17A).** Total FAK is significantly higher in MDA-MB-231 cells than MDA-MB-468s, whilst relative activity is comparable between the two models. We went on to explore the sensitivity of these cells to PF271-mediated FAK inhibition. PF271 was able to induce a significant, dose-dependent decrease in FAK^{Y397} activity whilst total protein levels showed no significant alteration **(figure 4.17B-D)**, although when comparing IC_{50} data, MDA-MB-468 cells appeared less sensitive to PF271 versus MDA-MB-231 cells **(figure 4.17E).**

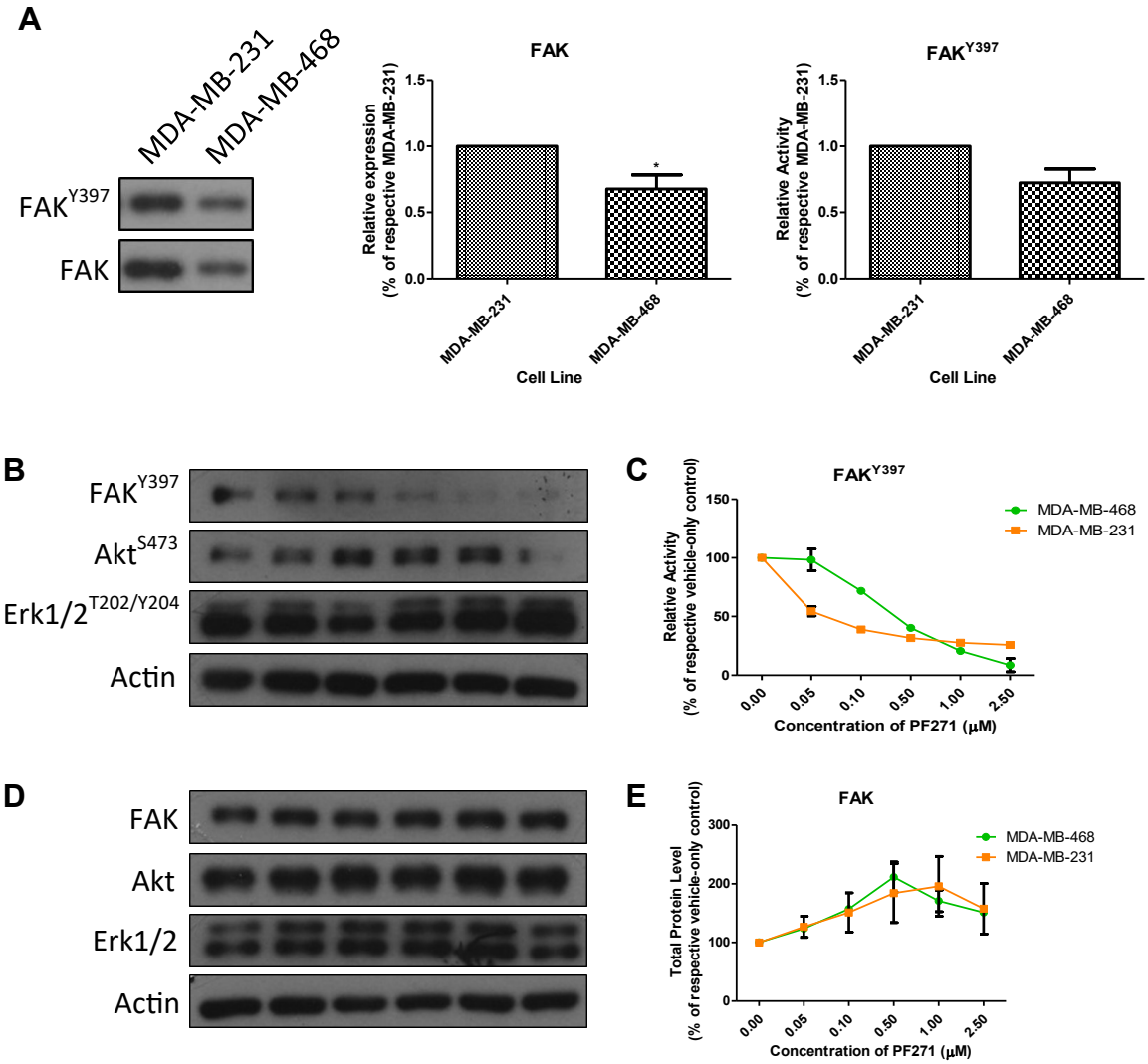


Figure 4.17 – Comparison of FAK expression/activity and PF271 FAK sensitivity in MDA-MB-231 and MDA-MB-468 cells – (A) Western blot and subsequent densitometry of basal FAK expression and activity in MDA-MB-231 and MDA-MB-468 cells showing significantly varying levels of expression, whilst relative activity showed no difference. **(B)** Westerns blots revealing changes in phosphorylated proteins in response to a dose range of PF271. **(C)** Evaluation of the densitometry data from western blots plotted alongside the previously acquired data from MDA-MB-231 cells as a comparison of FAK^{Y397} changes. **(D)** Western blots showing response of total protein levels across the PF271 does range. **(E)** Analysis of densitometry of total FAK level changes in response to PF271 at different doses. No significant changes were observed at any dose. All error bars are representative of SEM; n=3.

In addition to evaluating pharmacological inhibition, we also wished to observe the contribution of scaffolding function. Therefore, we first had to perform a FAK siRNA optimisation in order to establish a robust protocol for total protein knockdown in MDA-MB-468 cells. As seen in **figure 4.18**, FAK-siRNA resulted in significant decrease in FAK expression and subsequent Y397 activity following 72-hours, an effect sustained up to 6 days following removal of siRNA.

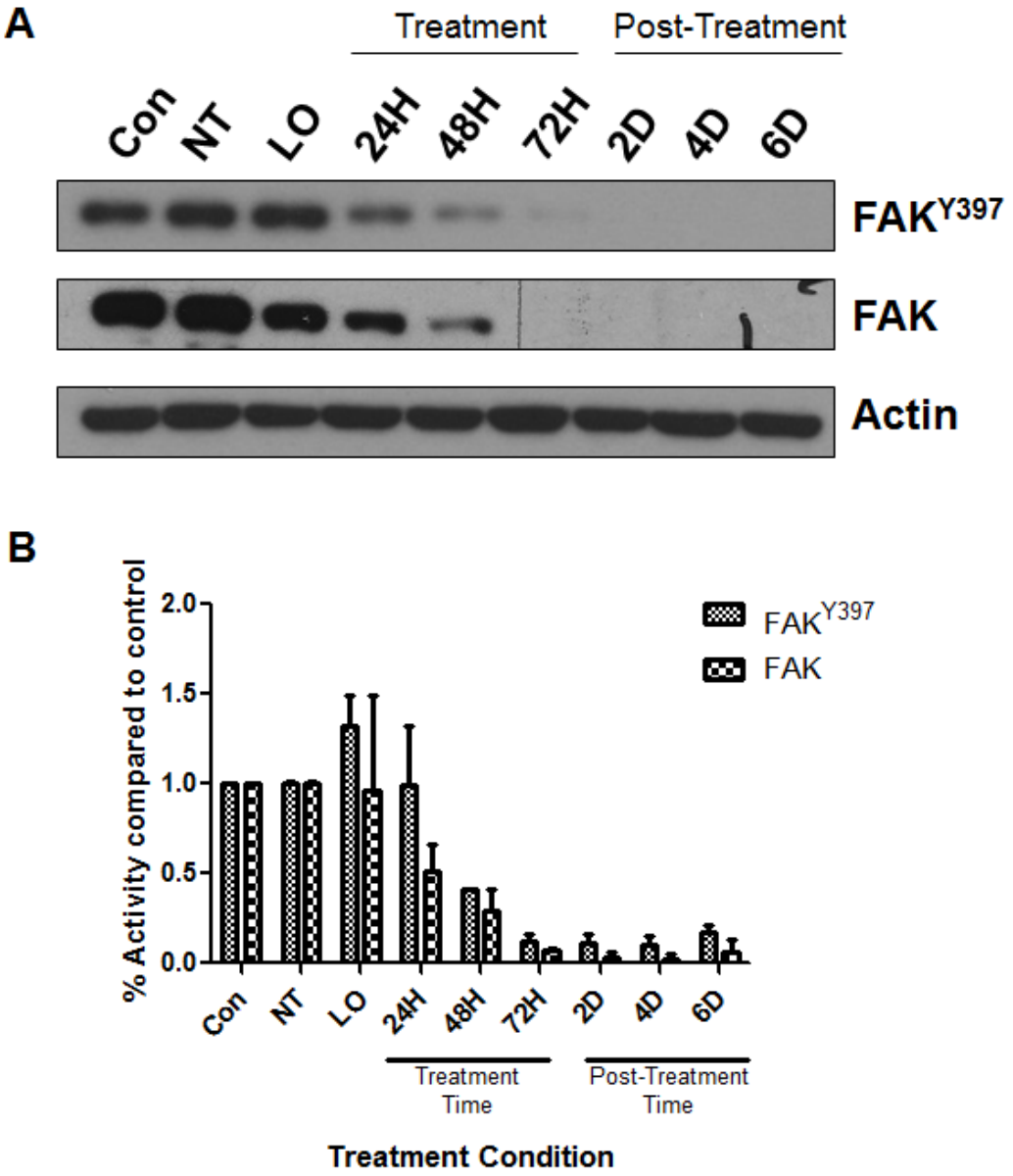


Figure 4.18 – Time-course optimisation of FAK siRNA – (A) Western blot showing the effects of FAK siRNA on Y397 activity and total FAK levels over time and the effects on each following the removal of treatment. The effects of the control, non-transcribing siRNA (NT) and the lipid-only (LO) are also shown, along with actin as a loading control. Times for treatment are notated in hours (H) and post-treatment in days (D). **(B)** Densitometry analysis of FAK^{Y397} and total FAK as a percentage of the control cells. Error bars show SEM; n=3.

4.11. Inhibition or suppression of FAK has limited effects on MDA-MB-468 proliferation

We next sought to explore the effects of FAK inhibition on cellular proliferation in MDA-MB-468 cells and compare this with MDA-MB-231 cells. Using a concentration of 1µM PF271 that effectively reduced FAK activity in both cell lines, we observed that FAK inhibition in MDA-MB-231 cells resulted in a significant reduction in growth over 7-days, whereas proliferation was unaffected in MDA-MB-468 cells **(figure 4.19A)**. To confirm these data FAK was suppressed using siRNA in MDA468 cells prior to undertaking growth analysis as previously described. Again, suppression of FAK did not alter the growth rate of MDA-MB-468 cells **(figure 4.19B and C)**.

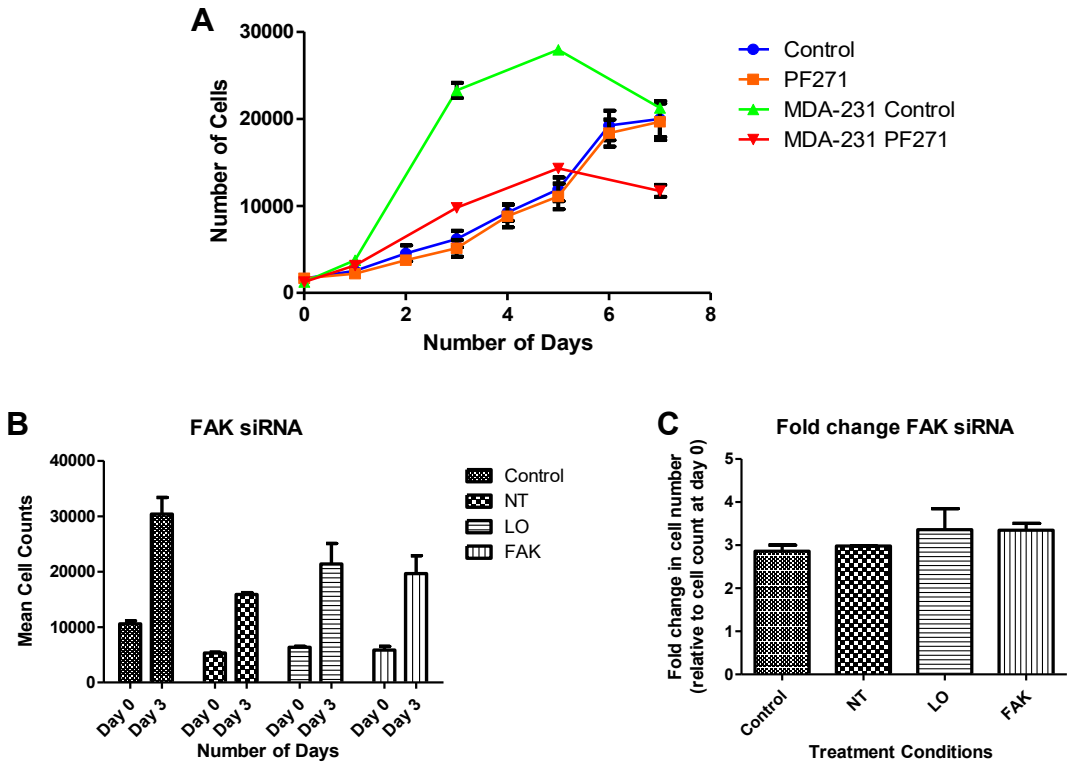


Figure 4.19 – Changes in proliferation of MDA-MB-468 cells in response to PF271 and FAK siRNA – (A) Growth Curve showing the change in cell numbers in the presence or absence of PF271 for 7-days, alongside MDA-MB-231 cells grown in tandem as an experimental control. Introduction of 1µM PF271 was had no significant effect on MDA-MB-468 proliferation, whilst MDA-MB-231 cells exhibited significantly perturbed growth following 3-days and was sustained up to the final count at day 7. **(B)** Total cell numbers of MDA-MB-468 cells in response to suppression of FAK through siRNA. **(D)** Relative changes in cell growth in each treatment condition versus day 0 cell numbers. FAK siRNA has no significant effect of MDA-MB-468 growth rate when compared to all controls. Error bars representative of SEM; n=3 . *p<0.05; **p<0.01; ***p<0.001.

4.12. Levels of Ki67 are unaltered following FAK inhibition in MDA-MB-468 cells

Previously we noted that changes in MDA-MB-231 cell proliferation were accompanied by alterations in levels of Ki67. Since MDA-MB-468 cells were not impaired in response to FAK inhibition, we wanted to further confirm this through immunohistochemical analysis of Ki67 levels in PF271-treated MDA-MB-468 cells **(figure 4.20)**. Whilst an increase in Ki67 positivity was observed over a three-day period, reflective of actively growing cells, this was not affected by PF271.

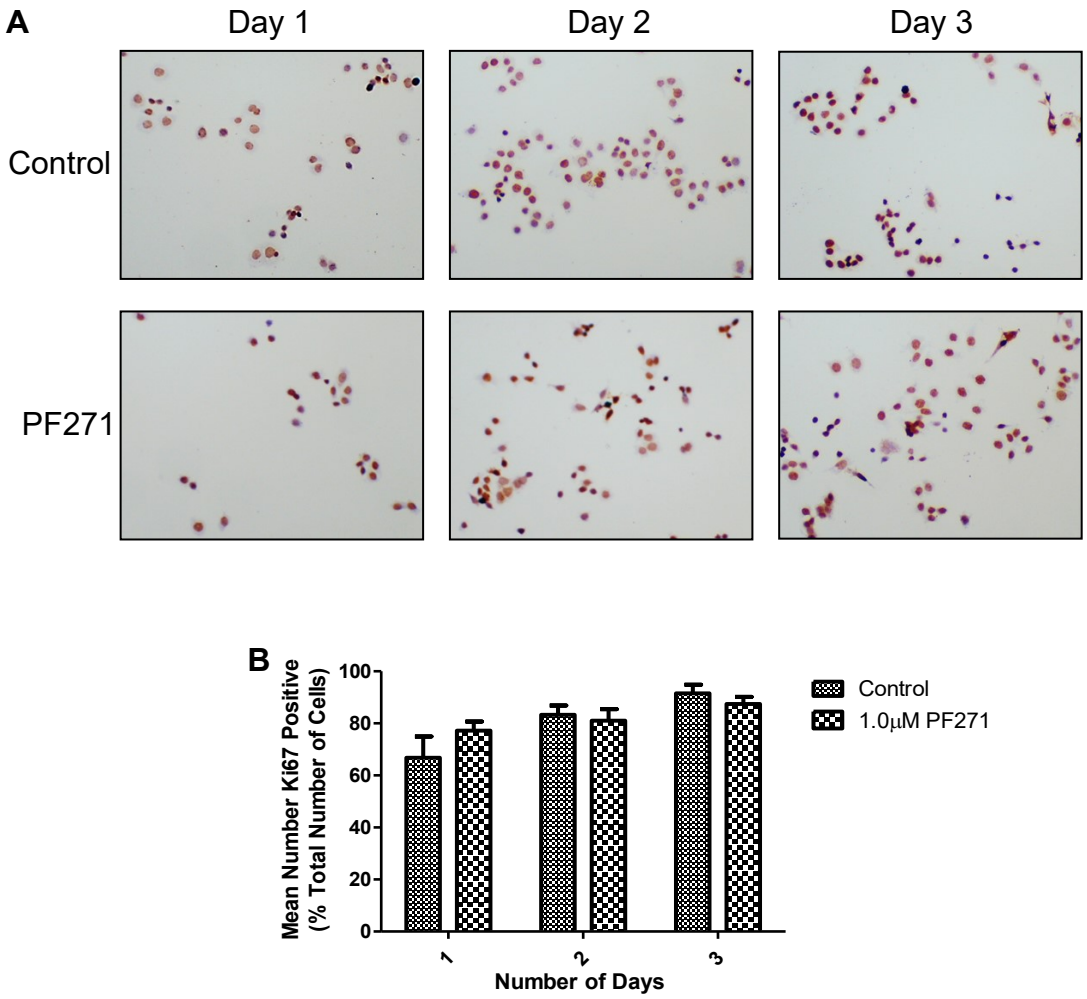


Figure 4.20– Ki67 staining on MDA-MB-468 cells over 3-days in the presence or absence of PF271 – (A) Representative image showing the results of staining MDA-MB-468 cells for Ki67 over 3-days in the presence or absence of PF271. **(B)** Evaluation of Ki67 positive cells represented as a proportion of the total number of cells present on slides. FAK inhibition had no significant effect on proliferative marker Ki67 across all time-points. Error bars are representative of SEM; n=3.

4.13. FAK inhibition does not affect MDA-MB-468 cell migration

We next investigated the effects of FAK inhibition on MDA-MB-468 migration using Boyden chamber assays. As shown in **figure 4.21**, MDA-MB-468 cells are capable of significant migration following 18-hours using FCS containing RPMI as a chemoattractant. However, the use of either 0.5µM (calculated IC_{50} for FAK^{Y397} inhibition) or 1µM PF271 had no significant effects on migration versus control cells. This suggests that inhibition of FAK-kinase function is not sufficient to alter migration in these cells.

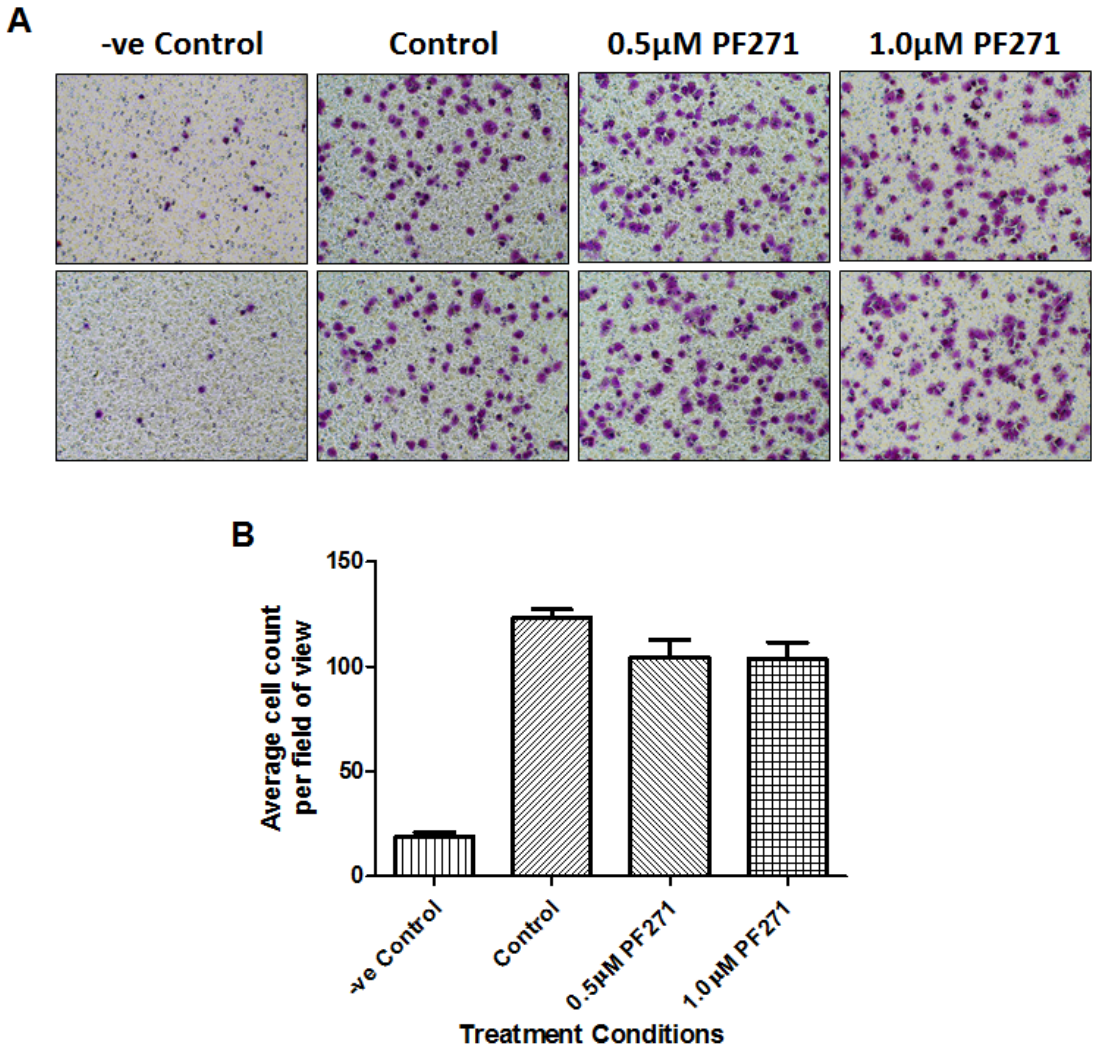


Figure 4.21 – Analysis of migration in MDA-MB-468 cells following PF271 treatment – (A) Representative images of Boyden chamber assays following 18-hours migration, stained with crystal violet. **(B)** Evaluation of changes in migratory capacity following treatment with either 0.5µM or 1 µM PF271. Both concentrations could not significantly attenuate migration when compared to vehicle-only control cells. All cells exhibited significant migration versus negative control. All error bars represent SEM; n=4.

Given that catalytic functions of FAK seem to contribute very little to the migratory phenotype of these cells, we next wished to examine whether scaffolding function of FAK had a role in regulating migration. As such we performed Boyden chamber migration assays on cells in which FAK had been suppressed through targeted siRNA. In FAK-knockdown cells, migration was comparable to the controls (NT siRNA) **(figure 4.22)**. Additionally, all treated cells were capable of migration when compared to unstimulated negative controls.

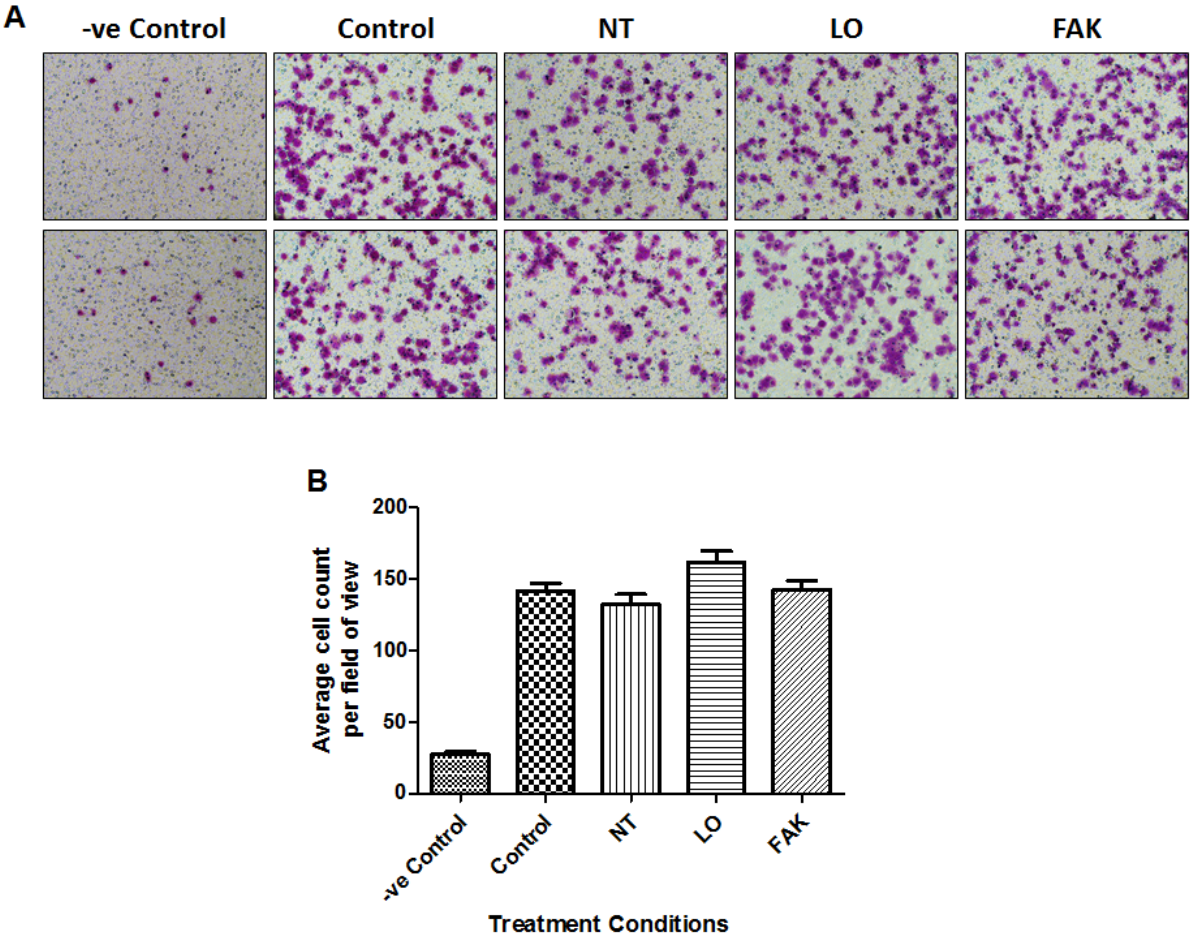
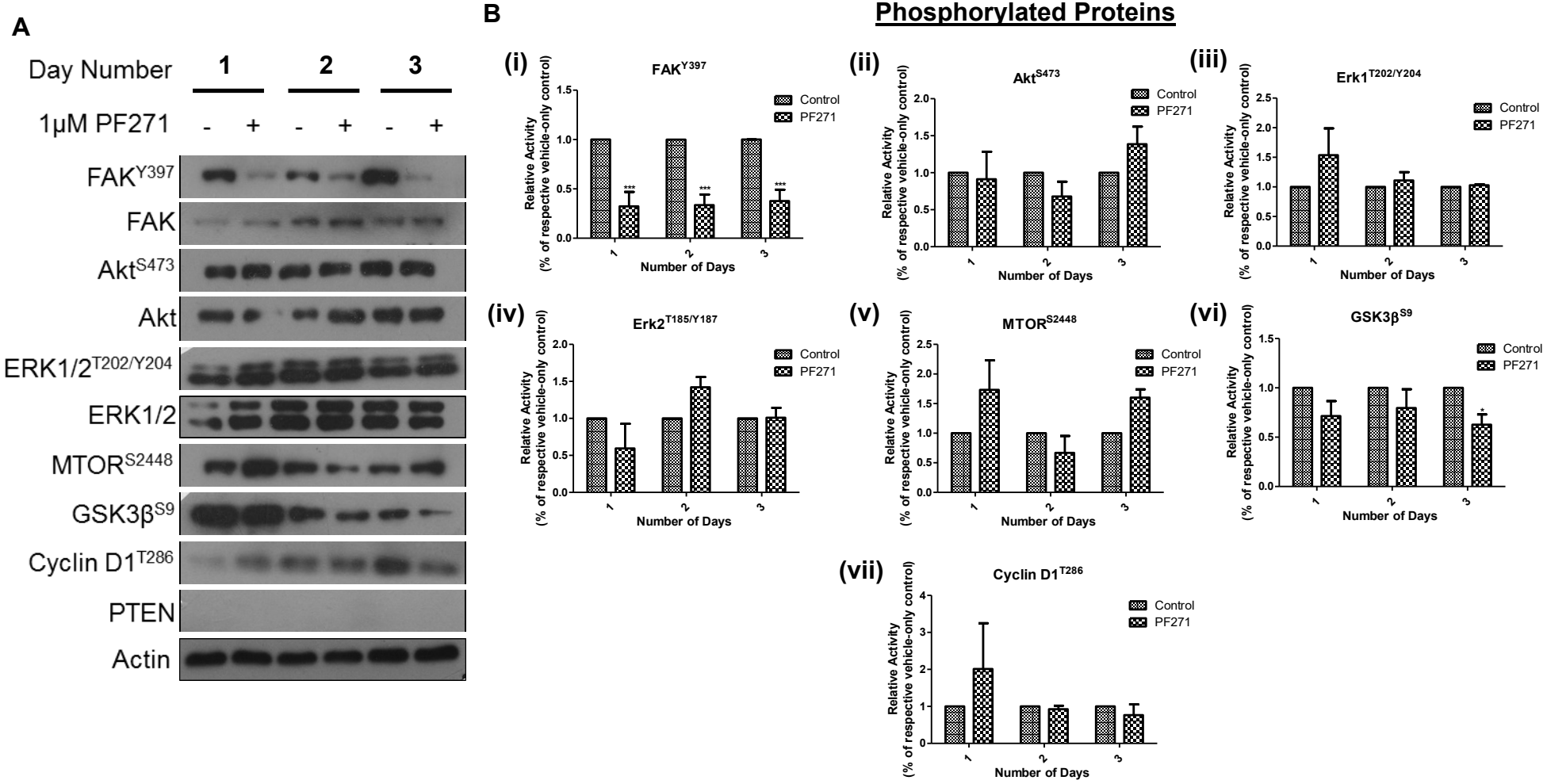


Figure 4.22 – Evaluation of MDA-MB-468 cell migration following siRNA-mediated suppression of FAK – (A) Representative images of Boyden chamber assays following 18-hours migration, fixed and stained with crystal violet. **(B)** Analysis of changes in migration in transfected cells revealed no significant alterations in migration in FAK-suppressed cells versus non-transfected cells and NT/LO controls. All cells showed significant migration comparative to unstimulated, negative controls. Error bars are representative of SEM; n=4.

4.14. Exploration of FAK-regulated signalling pathways in MDA-MB-468 cells

We have demonstrated that FAK inhibition or suppression has limited effects on the proliferation and migration of MDA-MB-468 cells. This is in stark contrast to our findings in MDA-MB-231 cells, in which FAK-kinase function seemed to play a key role in mediating both cell functions. Consequently, we wished to explore possible reasons underlying this observed difference. Previously, MDA-MB-468 cells have been reported as possessing a negative mutation in the *PTEN* gene, resulting in a PTEN-null phenotype (Stemke-Hale *et al.* 2008). PTEN is the central regulator of PI3K/Akt signalling and its activity is essential to negatively regulate this pathway (Wang and Jiang 2008). Given that our data revealed significant changes in Akt activity following FAK inhibition, in both migrating and proliferating cells, we hypothesised that the lack of PTEN in these cells is enabling sustained Akt activity, thus attenuating effects of FAK inhibition. As such, we wished to examine downstream pathway elements in these cells, comparing them to alterations we'd previously observed following FAK inhibition in MDA-MB-231 cells. We began by examining changes in proliferating cells, so western blotting was undertaken on MDA-MB-468 cells, in the presence or absence of PF271 over 3-days. Across all time-points examined, treatment with 1μM PF271 significantly reduced FAK^{Y397} activity **(figure 4.23Bi)**. In contrast, no subsequent changes were seen in any of the downstream phosphorylation targets, previously shown to be altered by FAK-inhibition in MDA-MB-231 cells **(figure 4.23Bii - vii)**. All total protein levels were unaffected by inhibiting FAK-kinase activity **(figure 4.23C)**. Importantly, PTEN was not detectable in MDA-MB-468 cells whereas it was readily observed in the MDA-MB-231 model; anecdotally, Akt^{S473} activity was readily detectable in MDA-MB-468 cells compared to MDA-MB-231 cells, suggesting hyperactivity in the former. These observations led us to hypothesise that PTEN-mutant cells may alter response to FAK-inhibition through sustained activation of Akt and subsequent downstream targets.



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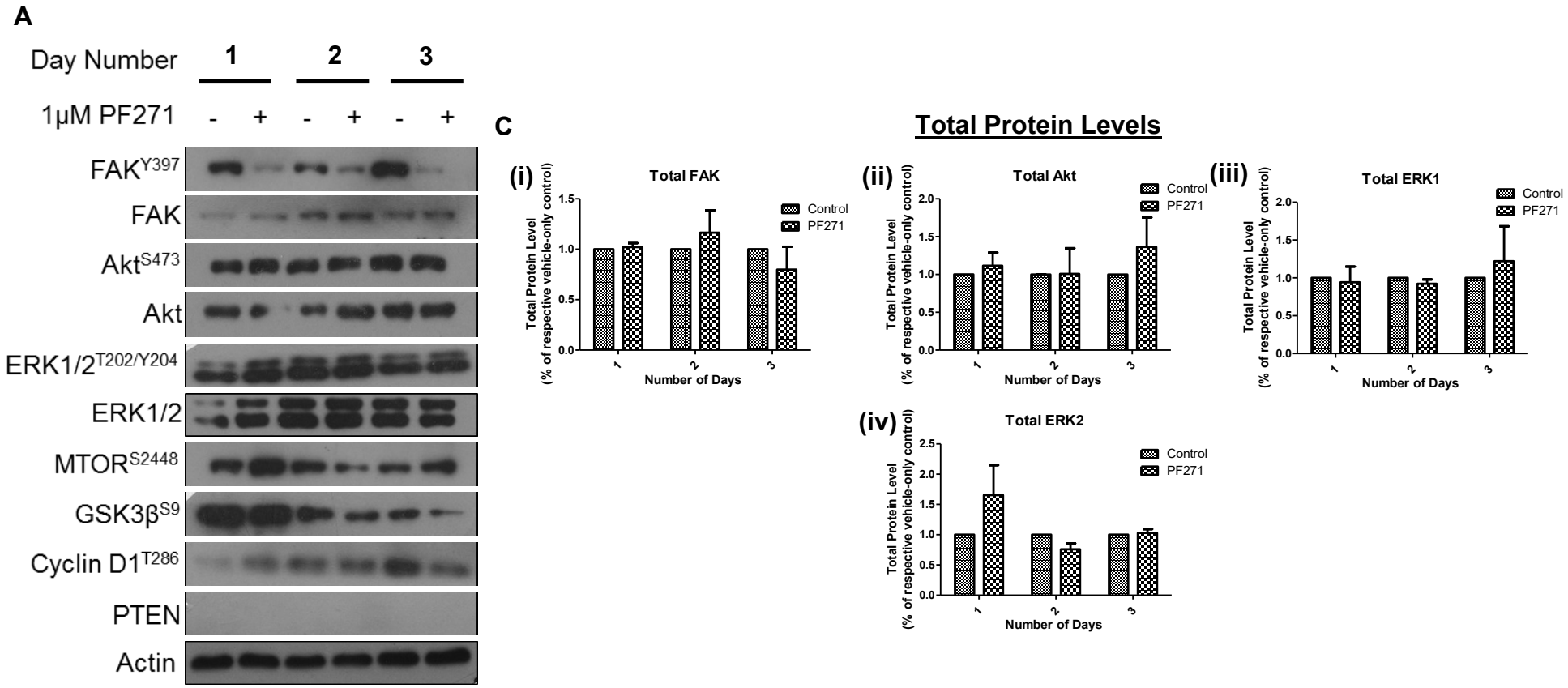


Figure 4.23 – Analysis of protein changes over 3-days in the presence or absence of PF271 in MDA-MB-468 cells - A) Western blot showing response of proteins to FAK-inhibition, measured at 1, 2 and 3-days following introduction of PF271. **(B)** Evaluation of relative activity of phosphorylated proteins (i) FAK^{Y397}; (ii) Akt^{S473}; (iii) Erk1$^{T202/Y204}$; (iv) Erk2$^{T185/Y187}$; (v) MTOR^{S2448}; (vi) GSK3β^{S9}; (vii) Cyclin D1^{T286}, only showing changes in FAK^{Y397} across all time-points and at day 3 in GSK3β^{S9}. **(C)** Assessment in changes in total protein levels of (i) FAK; (ii) Akt; (iii) Erk1; (iv) Erk2. No significant change is noted at any time-point across total proteins. PTEN was undetectable at any timepoint regardless of PF271 treatment. All densitometry results are normalised to the respective actin loading control. Error bars represent SEM; n=3. *p<0.05; **p<0.01; ***p<0.001.

4.15. siRNA-mediated PTEN suppression in MDA-MB-231 cells

4.15.1. PTEN-siRNA optimisation

Our observations imply that in cells with PTEN-negative mutations, FAK inhibition may not be an effective strategy for perturbing proliferation and migration due to hyperactive Akt circumventing FAK-regulation of cellular processes. To explore this hypothesis further, we wished to evaluate whether increased Akt activity through reduced PTEN expression caused a reduced sensitivity to PF271 using a model of PTEN-negative MDA-MB-231 cells **(figure 4.24B)**. We observed that PTEN suppression resulted in a significant increase in Akt^{S473} activity without altering total Akt protein levels **(figure 4.24C)**. Importantly, PTEN has previously been noted to alter the expression and subsequent activity of FAK (L.-L. Zhang *et al.* 2014). Consequently, changes in FAK expression and activity were also explored in this assay. No significant change in either total FAK or phosphorylated FAK^{Y397} was exhibited in PTEN siRNA treated cells **(figure 4.24D)**.

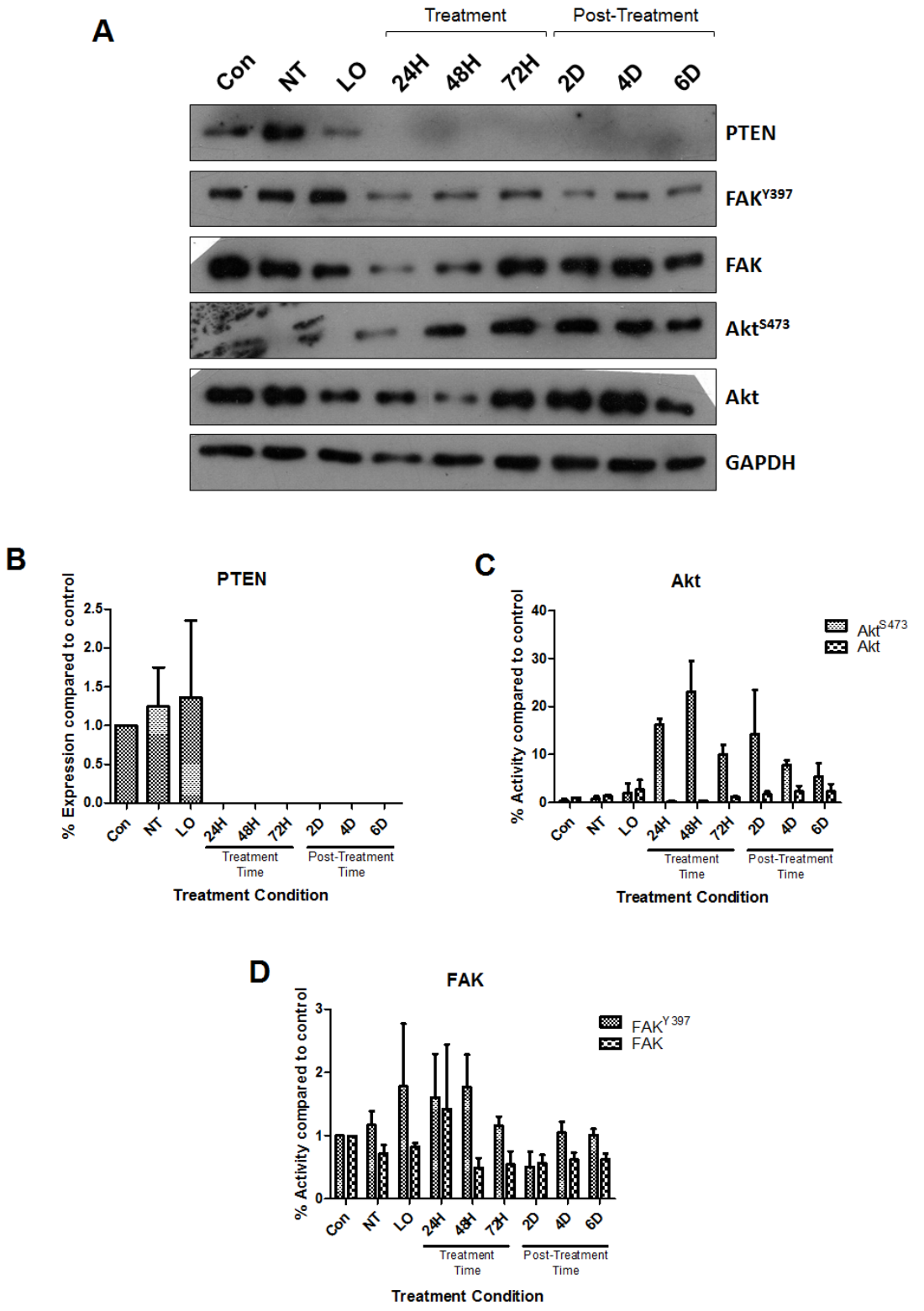


Figure 4.24 – Time-course optimisation of PTEN siRNA in MDA-MB-231 cells – (A) Western blot showing the effects of PTEN siRNA on PTEN, FAK^{Y397} and Akt^{S473}, as well as total FAK and Akt levels over time and the effects on each following the removal of treatment. The effects of the control, non-transcribing siRNA

(NT) and the lipid-only (LO) are also shown, along with actin as a loading control. Times for treatment are notated in hours (H) and post-treatment in days (D). **(B)** Evaluation of densitometry performed on PTEN following siRNA treatment showed complete ablation of PTEN levels from 24-hours. **(C)** Assessment of Akt^{S473} and Akt protein level densitometry showed a significantly increased activity but not total protein levels. **(D)** Densitometry analysis of FAK^{Y397} and total FAK as a percentage of the control cells. No Significant effects were noted. All error bars show SEM; n=3.

4.15.2. Functional assessment of PF271 in PTEN-suppressed MDA-MB-231 cells

We next explored whether siRNA-mediated knockdown of PTEN, which augments AKT activity, was able to attenuate the response of MDA-MB-231 cells to PF271. PTEN siRNA alone had no significant effects on proliferation versus controls **(figure 4.25)** whilst sensitivity to PF271 was apparent for all samples including PTEN-siRNA cells.

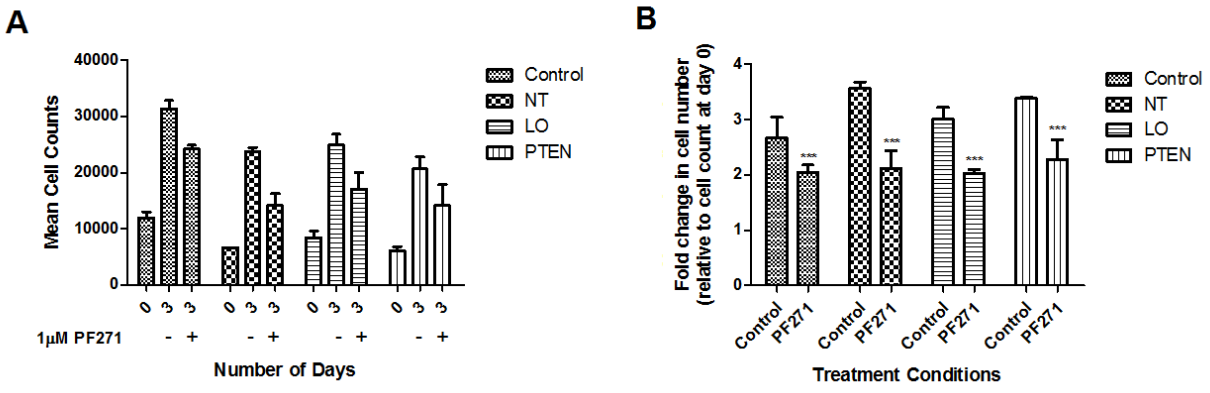


Figure 4.25 – siRNA-mediated suppression of PTEN has no significant effect on the proliferative response of MDA-MB-231 cells treated with PF271 – (A) Mean cell counts after 3-days proliferation following 72-hour treatment with respective siRNA in the presence or absence of PF271. 0-day counts are representative of the cell numbers immediately following withdrawal from siRNA containing media. Significant changes in cell numbers were noted in all treatment conditions when PF271 was introduced. SiRNA alone had no significant effect on proliferation. **(B)** Evaluation of the fold change in cell number, relative to the counts at day 0. Suppression of PTEN had no significant effect on proliferative rate when compared to siRNA controls. Error bars are representative of SEM; n=3. *p<0.05; **p<0.01; p<0.001.

We additionally explored the effects of PTEN suppression on FAK-mediated inhibition of MDA-MB-231 cell migration, again to explore the hypothesis that augmented Akt activity would attenuate PF271 response. However, cells in which PTEN was suppressed exhibited no significant attenuation of migration following PF271 treatment, whilst all control cells are fully responsive showing significantly impaired migration versus the respective vehicle-only control cells **(figure 4.26)**. Cellular migration in PTEN siRNA vehicle-only controls and those treated with PF271 exhibit migration comparative to non-transfected and NT siRNA cells.

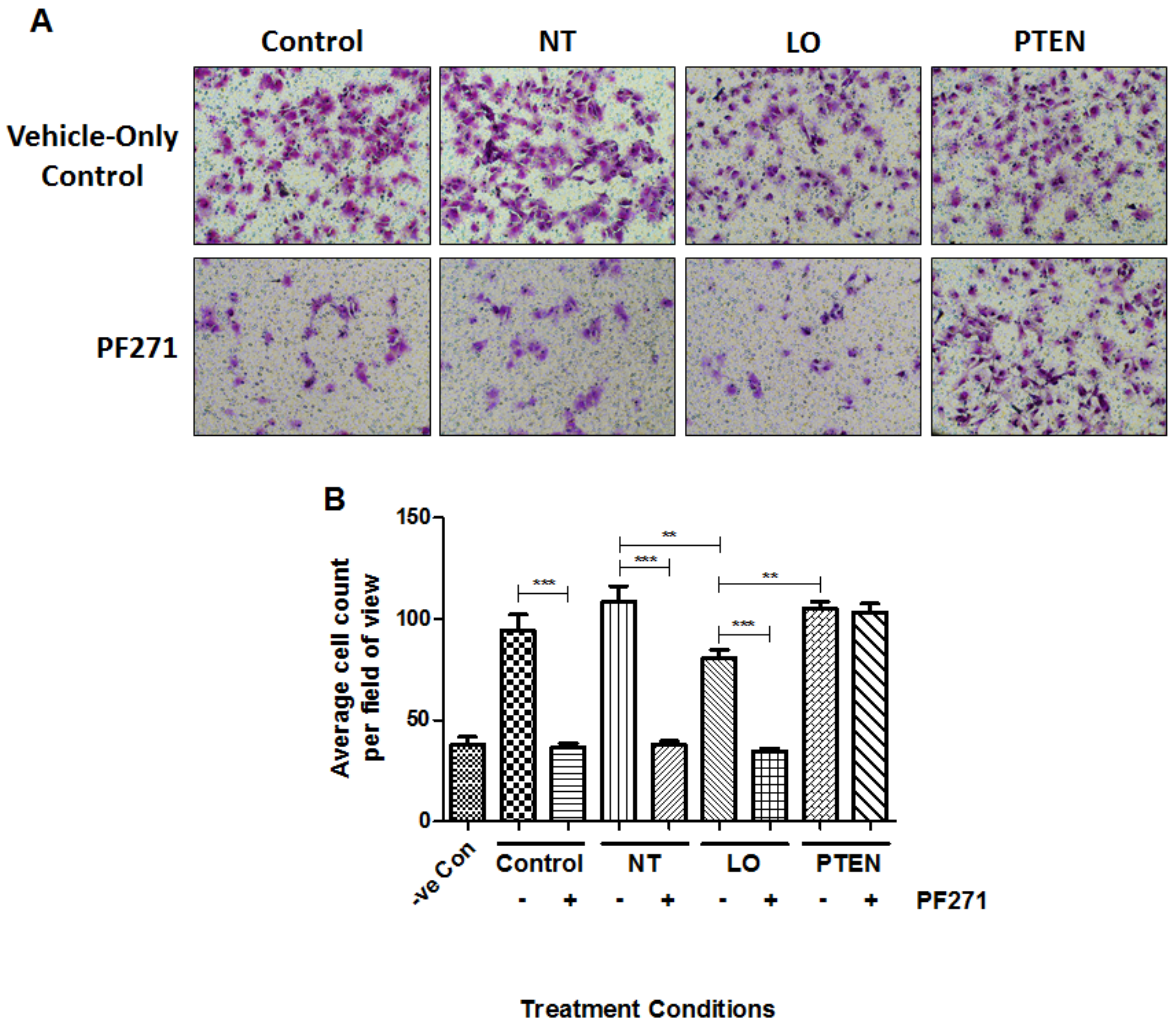
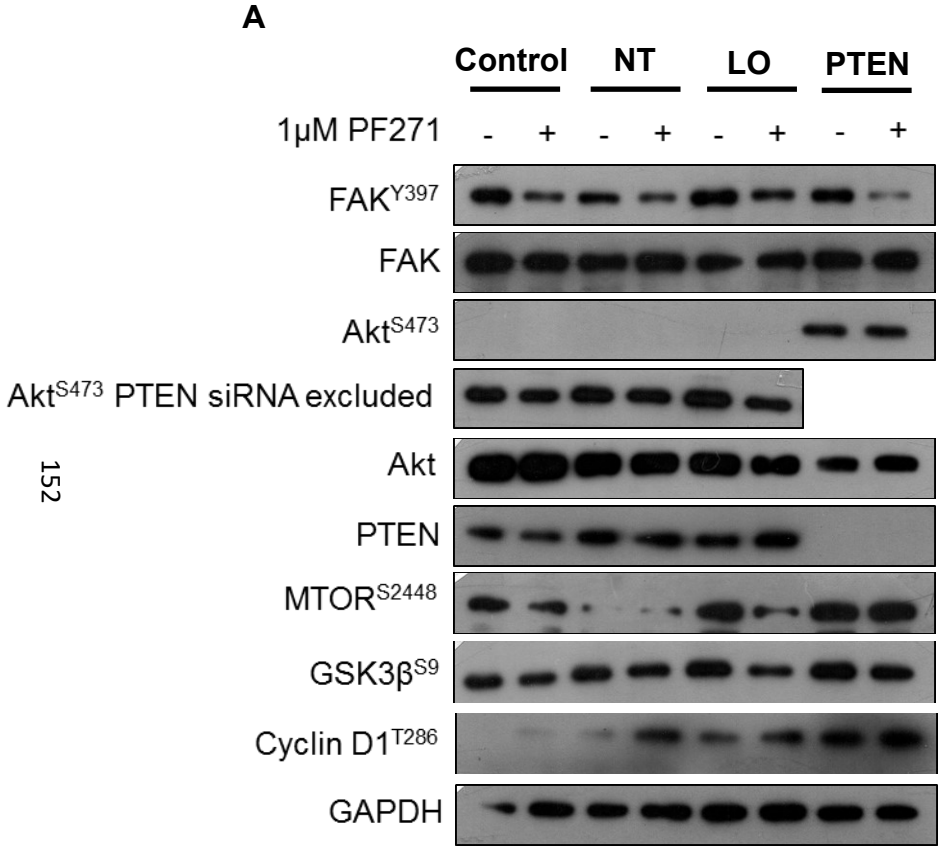
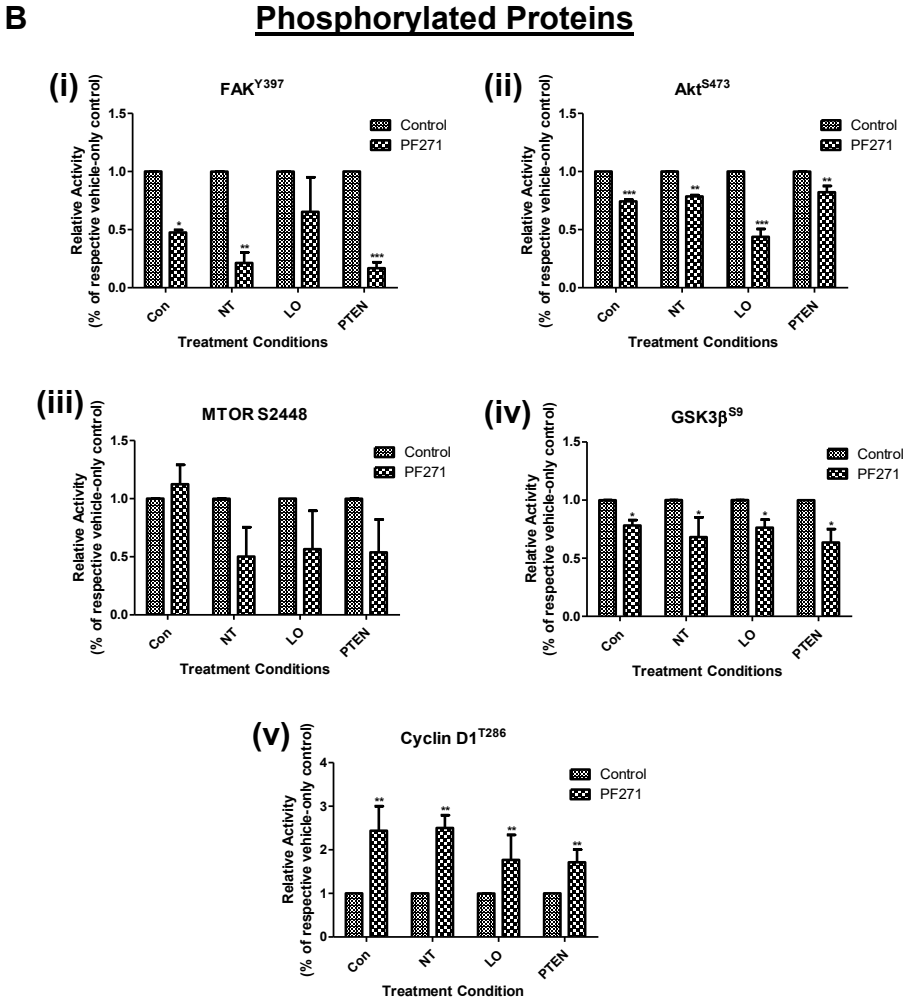


Figure 4.26 – Analysis of the effects of combining siRNA-mediated PTEN suppression with PF271 FAK inhibition on the migration of MDA-MB-231 cells – (A) Representative images of siRNA transfected cells in Boyden chamber assays following 18-hours migration in the presence or absence of PF271. **(B)** Evaluation of the combinatorial effects of PTEN suppression and inhibition of FAK-kinase inhibition. All transfected cells migrate compared to negative control. PF271 treatment significantly augments migration in transfection controls, whilst PTEN knockdown cells exhibit no change in migration following FAK inhibition versus their respective vehicle-only control. All error bars represent SEM; n=3. *p<0.05; **p<0.01; ***p<0.001.

4.16. Exploration of signalling changes in PTEN-suppressed cells following PF271 treatment

Having failed to demonstrate that PTEN suppression is sufficient to rescue proliferation in FAK inhibited cells, we wanted to explore changes in the downstream pathway elements previously highlighted as potential mediators of FAK-dependent proliferation. As expected, PTEN siRNA caused an almost complete loss of PTEN accompanied by a significant increase in active Akt^{S473}, so much so that separate blots needed to be acquired to assess the effects of PF271 on Akt activity in control cells **(figure 4.27)**. Despite hyperactivation, Akt^{S473} was still significantly inhibited in PTEN siRNA treated cells versus vehicle-only control, although activity was still significantly higher than in all siRNA controls. Inhibition of FAK^{Y397} was observed in all transfected cells treated with PF271 **(figure 4.27Bi)**. FAK inhibition also caused a significant reduction in $GSK3\beta^{S9}$ and increased cyclin $D1^{T286}$ activity **(figure 4.27Biv and v)**. It is important to note that these molecules were inhibited to comparable levels in control, NT, LO and PTEN siRNA treated cells. Although some difference was noted, $MTOR^{S2448}$ activity was not significantly reduced in all cell types **(figure 4.27Biii)**. Treatment with PF271 resulted in no significant alterations in total protein levels of FAK or Akt, regardless of transfection **(figure 4.27Ci and ii)**. These results suggest FAK can still influence downstream targets regulating proliferation independent of Akt activity.





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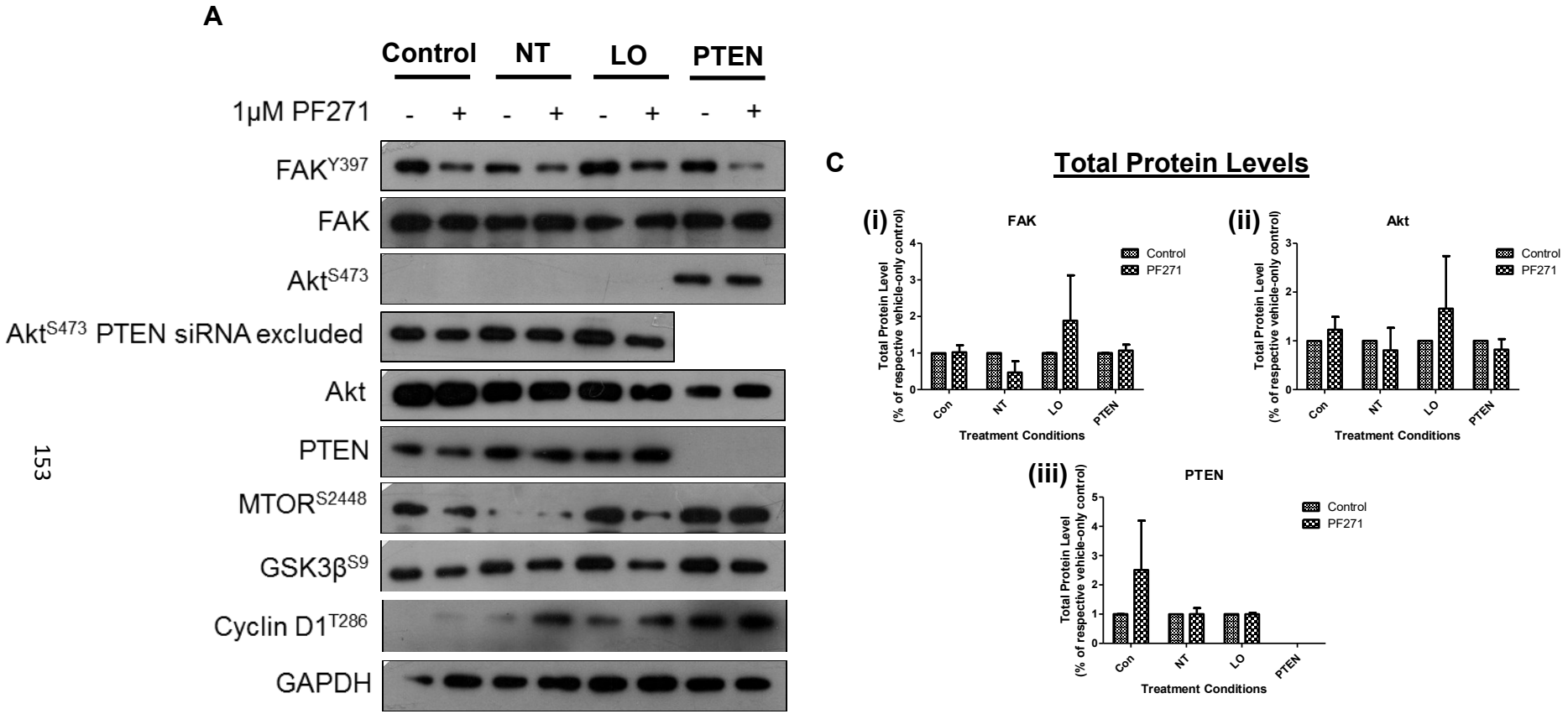
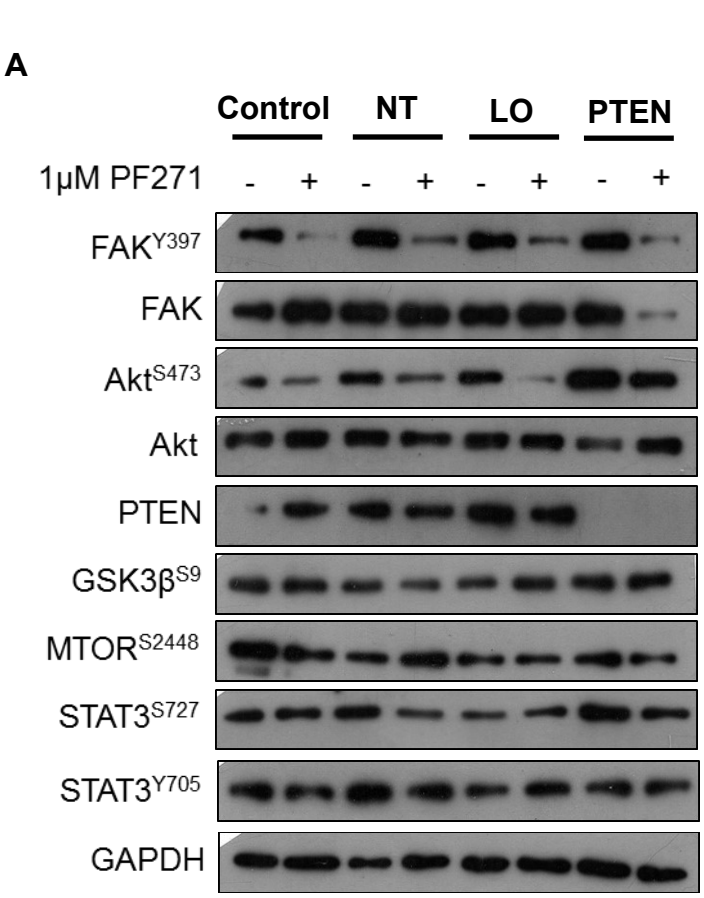
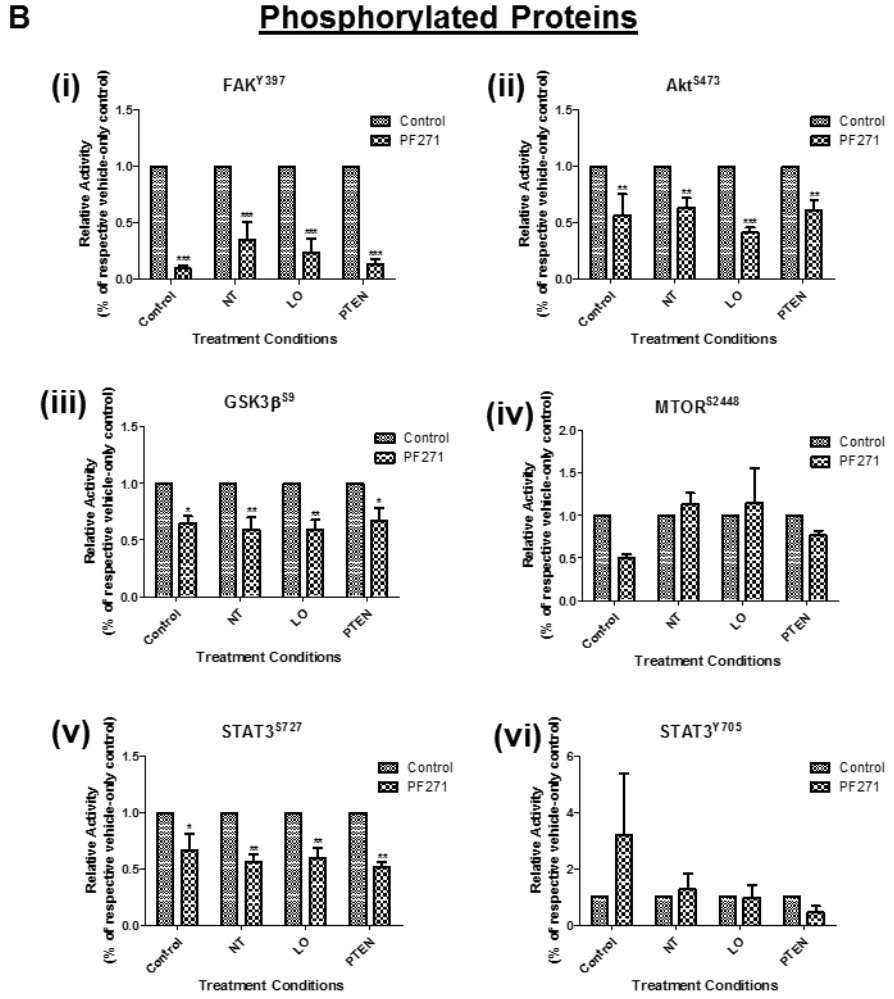


Figure 4.27 – Examination of downstream signalling changes in response to PF271 in PTEN siRNA cells – (A) Western blot showing the effects of PF271 on cells treated with PTEN siRNA and associated controls. **(B)** Evaluation of changes in phosphorylated protein levels of (i) FAK^{Y397}; (ii) Akt^{S473}; (iii) MTOR^{S2448}; (iv) GSK3β^{S9}; (v) Cyclin D1^{T286}, showing significant decreases in the activity of FAK, Akt and GSK3β in response to PF271. All treatment conditions show significantly increased Cyclin D1^{T286}. PTEN siRNA rescues inhibitory effect of PF271 treatment on MTOR^{S2448}. **(C)** Densitometry analysis of total protein levels of (i) FAK; (ii) Akt; (iii) PTEN, following FAK inhibition. No total protein levels were attenuated by PF271 whilst PTEN siRNA completely ablated PTEN protein levels. Error bars are representative of SEM; n=3. *p<0.05; **p<0.01; ***p<0.001.

Having examined how PTEN siRNA alters PF271-driven signalling changes in proliferating MDA-MB-231 cells, we also wished to evaluate these changes in migrating cells. Again, PTEN siRNA almost completely ablated PTEN expression and was concomitant with increased Akt^{S473} **(figure 4.28)**. However, Akt^{S473} levels were significantly decreased by PF271 treatment, as were FAK^{Y397}, GSK3β^{S9} and STAT3^{S727} **(figure 4.28Bi-v)**. Akt^{S473} showed comparative activity between PF271+PTEN siRNA cells and vehicle-only, siRNA controls. Levels of MTOR^{S2448} and STAT3^{Y705} were unaffected **(figure 4.28Biv and vi)**, with total FAK and Akt also showing no significant response regardless of siRNA **(figure 4.28C)**.





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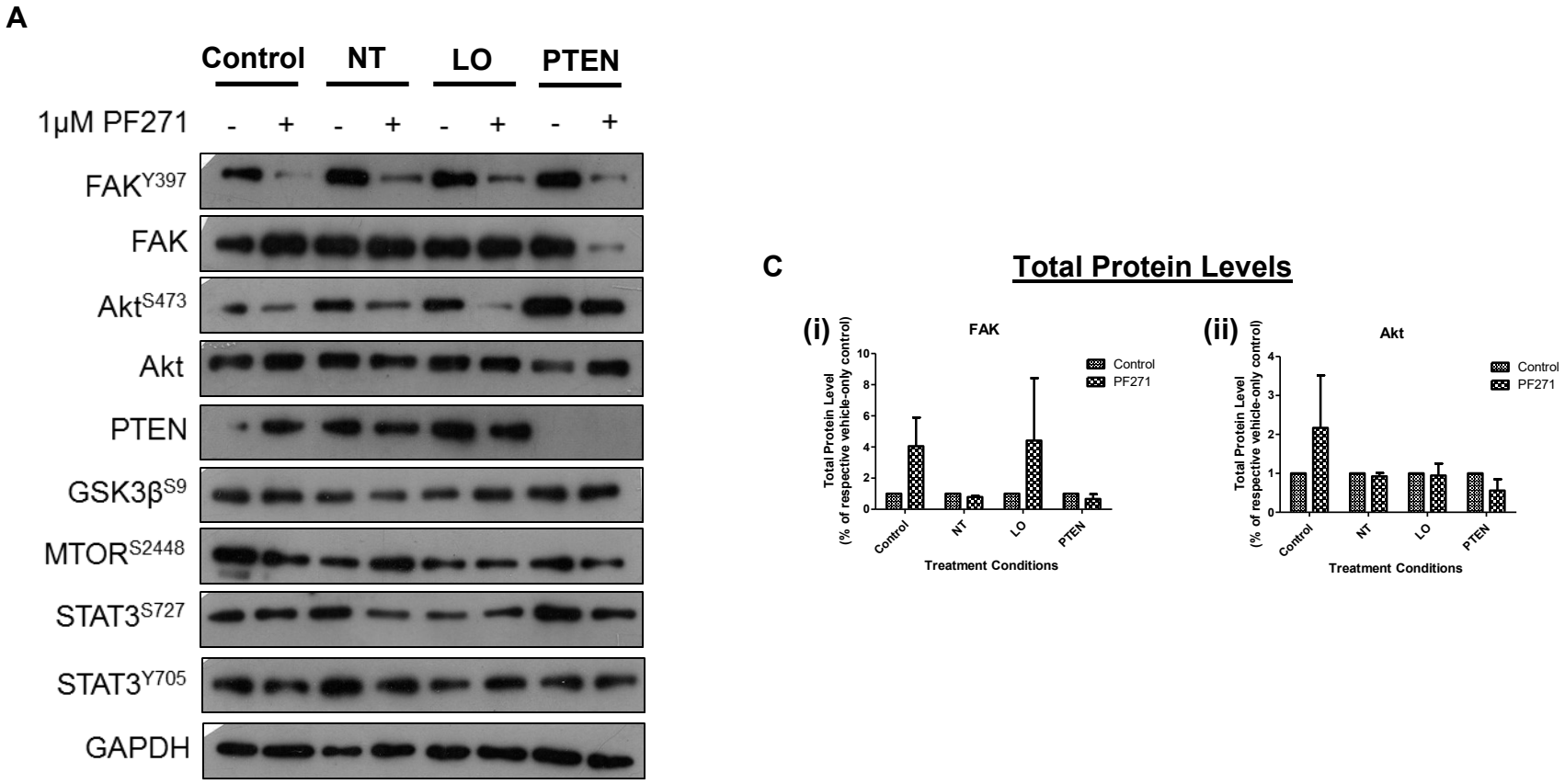


Figure 4.28 – Exploration of signalling changes in migratory, PTEN siRNA cells in response to FAK inhibition – (A) Western blot showing the effects of PF271 on cells treated with PTEN siRNA and associated controls. **(B)** Evaluation of changes in phosphorylated protein levels of (i) FAK^{Y397}; (ii) Akt^{S473}; (iii) GSK3β^{S9}; (iv) MTOR^{S2448}; (v) STAT3^{S727}; (vi) STAT3^{Y705} showing significant decreases in the activity of FAK, Akt, GSK3β and STAT3^{S727} in response to PF271, whilst MTOR and STAT3^{Y705} were unaltered. **(C)** Densitometry analysis of total protein levels of (i) FAK; (ii) Akt, following FAK inhibition. No total protein levels were attenuated by PF271 whilst PTEN siRNA completely ablated PTEN protein levels. Error bars are representative of SEM; n=3. *p<0.05; **p<0.01; ***p<0.001.

4.17. Discussion

In chapter three we demonstrated that FAK appeared to be a regulator of MDA-MB-231 cell proliferation and migration/invasion: in this chapter I sought to evaluate the degree to which FAK could mediate these behaviours and their subsequent mechanisms of action. We observed that inhibition or suppression of FAK induced a significant reduction in cell proliferation. Interestingly, FAK siRNA alone or in combination with PF271 did not cause any additional decrease versus the inhibitor alone, despite both kinase dependent and independent functions being attenuated. Consequently, we propose that FAK-mediated cell proliferation of MDA-MB-231 cells is primarily driven by kinase activity with limited contribution from kinase-independent functions. This directly contradicts several studies which suggest that scaffolding function of FAK is essential for proliferation in some cell types. For example, Lim et al (2010) showed that unlike FAK-null mouse embryonic fibroblasts (MEFs), kinase-dead knock-in cells could still proliferate *ex vivo*, thus suggesting FAK catalytic activity is dispensable provided scaffolding function is intact (Lim *et al.* 2010). Moreover, kinase-dead endothelial cells display no impaired proliferation despite a significant decrease in VEGF-mediated vascular permeability (X. L. Chen *et al.* 2012). In contrast, FAK kinase function has been demonstrated to regulate breast cancer cell proliferation as introduction of kinase-dead FAK to FAK-deficient mammary tumour cells was not sufficient to rescue tumour growth *in vivo*, whist FAK-WT significantly restored tumourigenic potential (Pylayeva *et al.* 2009). This study aligns with our data suggesting kinase function is essential for proliferation. However, the noted mechanism is this study was attributed to a decrease in Src-mediated phosphorylation of p130cas. Indeed, reintroduction of a FAK-mutant unable to bind p130cas also exhibited impaired tumourigenesis (Pylayeva *et al.* 2009). As FAK-p130cas interaction is kinase independent (Luo and Guan 2010), it suggests that these breast cancer cells utilise both kinase dependent and independent activity to mediate proliferation. Our data could reflect this as significant PF271-inhibition of kinase activity may be sufficient to reduce FAK-dependent proliferation to its maximum level. The siRNA-mediated knockdown of FAK could mirror this effect through both inhibition of catalytic and scaffold function. Therefore, we suggest that FAK activity and scaffolding may function equally contribute to TNBC proliferation and the significant reduction in one of these behaviours is sufficient to reduce proliferation. Further experiments using scaffolding specific inhibition would be required to confirm or refute this hypothesis.

We hypothesised that changes in TNBC cell growth primarily resulted from impaired proliferative signalling and cell cycle progression. Indeed, our data showed a significant decrease in Ki67 staining in FAK inhibited cells. As Ki67 is absent in cells no longer undergoing proliferation, this data suggests that inhibiting FAK-kinase function is sufficient to induce a movement of cells into a state of either quiescent or senescent cell cycle arrest (G_0). A seminal study by Zhao and colleagues in 1998 highlighted FAK as a central mediator of cell cycle progression in response to integrin signalling (J.-H. Zhao *et al.* 1998). They noted that functional FAK-kinase activity is essential for controlling G_1 to S-phase transition, with inhibition leading to decreased cell cycle progression through downregulation of cyclin D1 and an increase in the inhibitory protein p21 (J.-H. Zhao *et al.* 1998). Subsequent studies have reinforced this role of FAK in mediating cell cycle in a variety of cells. For example, FAK-kinase function was shown to be key in the movement of glioblastoma cells from G_1 to S-phase through upregulation of cyclin D1 and E, whilst simultaneously reducing expression of cell cycle inhibitory proteins (Ding *et al.* 2005). Moreover Serrels et al (2012) reported that squamous cell carcinoma cells grown in 3D culture exhibited a significant loss of proliferative capacity following FAK ablation, attributed to reduced cyclin D1 expression and resultant blocking of G_1/S transition (Serrels *et al.* 2012). Taken together, these studies seemed to support our data and substantiated our hypothesis that FAK contributes to MDA-MB-231 cell cycle progression, subsequently modulating proliferation.

We thus sought to explore changes in the cell cycle and, although changes in distribution were noted, our data showed a significant decrease in the percentage of cells in G_1 and an increase in S-phase proportions. This differs from previous findings and seems to undermine the idea of FAK as a G_1/S-phase regulator in MDA-MB-231 cells. Conversely, we also noted a significant increase in inhibitory phosphorylation of cyclin D1 following PF271 intervention, suggesting some form of influence at the G_1/S-phase transition by FAK. When combined, these findings may suggest FAK can influence both the G_1/S-phase transition and the movement of cells from S-phase to G_2.

It has long been established that cyclin D1 is essential for the movement out of G_1 and into S-phase, with its phosphorylation at T286 resulting nuclear export and subsequent ubiquitin-mediated proteolysis (Diehl *et al.* 1997). Consequently, the noted increase in cyclin $D1^{T286}$ should reflect impaired G_1/S transition and subsequent move into G_0. This does support our Ki67 data but appears to contrast our flow cytometry results. However, propidium iodide detection of cell cycle distribution has limitations due to the nature of the stain. The assay works on the premise

that propidium iodide binds DNA in a stoichiometric manner: the dye binds proportionally depending on the amount DNA present. As such, cells in G_1 and G_0 are indistinguishable in this assay as both have equivalent DNA content. Consequently, it is unclear what proportion of cells in the G_0/G_1 section contribute to each of these distinct stages of the cell cycle. Apart from the Ki67 staining, confirming whether cells are in arrest would require further profiling of G_0 associated proteins.

Nevertheless, if progression to S-phase were inhibited one would still expect an increase in the percentage of cells in G_1. Our results showed an opposing significant decrease in G_1 cell numbers and an increase in S-phase. As early as 1974, it was demonstrated that a restriction point (R-point) exists within G_1, after which cells are committed to entering a full mitotic cycle (Pardee 1974). Consequently, if the number of cells leaving G_1 is reduced, one would expect a decrease in both S-phase and G_2/M. Yet, our results show an increase in S-phase with no associated increase in G_2/M. Despite cells being committed to a mitotic cycle, the rate at which they move through the remainder of the cell cycle can be influenced by multiple factors, several of which are key to progression through S-phase. For example, during DNA replication (the primary event of S-phase) the recruitment of various replication factors to DNA can be inhibited thus causing S-phase delay. Consequently, if FAK activity influenced some of these recruiting factors it could slow progression and thus account for the increase in the proportion of cells in S-phase we observed. Indeed, FAK has been previously linked to at least one such factor, Cdk2. This cyclin-dependent kinase has long been known to bind cyclin E, enabling G_1/S transition, as well as cyclin A, required for movement through S-phase (Morgan 1997). Additionally, blocking the removal of inhibitory phosphorylation of Cdk2 prevents recruitment of DNA polymerase to replication origins through perturbed Cdc45 association (Falck *et al.* 2002). In support of this molecule as a medium of slowing S-phase in our cells, FAK has also been shown to directly regulate Cdk2 in hepatocytes, with inhibition of FAK-kinase function sufficient to reduce cell cycle progression through impaired Cdk2 expression (Flinder *et al.* 2013). Consequently, we propose that the inhibition of FAK's catalytic function causes a decrease in normal cellular proliferation through cyclin D1 mediated induction of cell cycle arrest, in tandem with a significant S-phase delay. However, more work is needed to fully validate this model which explores molecular changes in S-phase in response to FAK inhibition.

Having demonstrated that FAK-inhibition can significantly alter cell cycle progression, we wished to analyse downstream signalling that may cause such alterations. Previously, the Ras-Erk pathway has been identified as a central downstream regulator of cell cycle machinery in response to FAK-dependent integrin signalling. This was first postulated in 1998 when introduction of the dominant-negative FRNK mutant was noted to cause a significant decrease in Erk activation, coupled with reduced cyclin D1 induction and an increase in p21 expression (a Cdk-specific inhibitory protein) (J. H. Zhao *et al.* 1998). Indeed, subsequent studies have also shown the importance of FAK-kinase function in this interaction with overexpression of kinase-dead mutant FAK being sufficient to reduce proliferation, coupled with decreased Erk activation (Ding *et al.* 2005). This has since been shown to be a result of subsequent FAK^{Y925} activation, as introduction of mutant FAK^{Y925F} caused a significant decrease in melanoma cell proliferation, resulting from decreased Grb2 recruitment and subsequent Ras-mediated activation of Erk (Kaneda *et al.* 2008). Our findings appear to rule this pathway out as the mechanism for FAK-mediated MDA-MB-231 proliferation, as PF271 had no significant impact on either Erk1 or Erk2 activity or expression at any time-point. Therefore, increased inhibitory phosphorylation of cyclin D1^{T286} must result from another FAK-linked signalling pathway.

It has long been established that FAK interacts with PI3K, subsequently acting as an upstream regulator of the PI3K/Akt pathway (Lee *et al.* 2015). Additionally, it has been shown that FAK-mediated regulation of this pathway has a vital role in certain cancer types. For example, FAK stabilised β-catenin through PI3K/Akt/GSK3β resulting in hyperproliferation of skin cancer cells *in vivo* (Samuel *et al.* 2011). In breast cancer, PI3K-dependent tumourigenesis has been shown to require FAK, inhibition of which results in reduced proliferation and tumour burden (Pylayeva *et al.* 2009). Consequently, we sought to investigate this pathway in our TNBC model. Our results are in concurrence with these studies as PF271 inhibition resulted in a significant reduction in active Akt^{S473}. Moreover, we also noted a significant reduction in GSK3β^{S9} activity. This residue is known to be inhibitory and its phosphorylation by Akt blocks downstream substrate activation (Dajani *et al.* 2001). Taken together this suggests that reduced FAK activity leads to decreased stimulation of Akt^{S473} and alleviation of the resultant inhibitory phosphorylation of GSK3β^{S9}. In further support of this mechanism of action, GSK3β has been shown to negatively regulate cyclin D1 by increasing phosphorylation of T286, leading to nuclear export and proteolytic degradation (Diehl *et al.* 1997), thus perturbing cell cycle progression and cellular proliferation. We showed such an increase cyclin D1^{T286} following PF271 treatment, thus implying FAK can contribute to

cyclin D1 stabilisation. Taken together, we propose that FAK-kinase activity stabilises cyclin D1 through modulating Akt stimulation and resultant inhibition of GSK3β, leading to perturbed cell cycle progression and decreased proliferation of MDA-MB-231 cells.

In addition to regulating GSk3β, Akt has been marked as a master regulator of cellular metabolic pathways associated with cell growth, with its interaction with MTOR closely linked to anabolic cell growth pathways, such as lipid synthesis (Deberardinis *et al.* 2008). Importantly, FAK has been shown to influence this pathway, enhancing proliferation and tumourigenesis of intestinal cells in Apc heterozygous mice (Ashton *et al.* 2010). As such we also examined changes in MTOR signalling in response to FAK-inhibition. As with Akt^{S473}, $MTOR^{S2448}$ activity was significantly reduced in response to PF271 treatment, inferring that FAK not only modulates cell cycle progression, but also metabolic growth through Akt in MDA-MB231 cells. Consequently, Akt may form an integral part of FAK-dependent cell proliferation by coordinating regulation of both proliferation and cell growth. The link between FAK and Akt is in concordance with numerous studies who all suggest FAK as a major upstream regulator of Akt signalling (discussed previously). However, the model proposed here suggests this FAK/Akt relationship could greatly impact proliferation unlike most studies which suggest the primary role of this pathways is regulation of cell survival (Sulzmaier *et al.* 2014).

We subsequently went on to explore how FAK functions mediate cell migration. Notably, we observed that FAK siRNA was significantly less effective than PF271 in impairing MDA-MB-231 cell migration. As siRNA suppression decreased kinase-dependent and independent functions, we hypothesised that FAK knockdown would be comparable, if not better than PF271 treatment owing to impaired kinase function, similar to that caused by pharmacological inhibition. Therefore, we hypothesised that in FAK siRNA cells, potential compensation was enabling partial sustaining of the migratory phenotype. Given PF271 has been shown to significantly impair FAK and its family member Pyk2 (Roberts *et al.* 2008), we further theorised that this dual inhibition was responsible for the improved attenuation of migration versus FAK knockdown. Pyk2, the only other member of the FAK-family of non-RTKs, has been shown to regulate its own distinct signalling pathways, whilst also sharing some functional overlap with FAK (Lipinski and Loftus 2010). As such, it was previously proposed that Pyk2 activity may be able to compensate for a loss of functional FAK. Indeed, deletion of FAK stimulated expression of endogenous Pyk2, sufficient to compensate for lost FAK activity in embryonic fibroblasts and adult endothelial cells

(Sieg *et al.* 1998; Weis *et al.* 2008). More recently, Pyk2 activity has been demonstrated to compensate for a loss of FAK in breast cancer, with this activity essential to maintaining metastatic potential *in vivo* (Fan and Guan 2011). These reports seem to support our hypothesis; however, we did not observe a complete restoration of migration following FAK suppression as might be expected if Pyk2 could completely compensate for a loss of FAK. This could be due to limited compensation as, although Pyk2 has some redundancy with FAK, it is not a completely complimentary and thus reduced activity in several signalling pathways would be observed. For example, Wendt et al. (2013) showed that FAK is essential to TGF-β signalled EMT and, despite some compensation by Pyk2, selective inhibition of FAK causes reduced breast cancer cell invasion (Wendt *et al.* 2013).

Taken together, this led us to hypothesise that Pyk2 activity was sustaining migration in FAK siRNA cells. Consequently, we explored migration in Pyk2 siRNA cells and observed that such suppression was comparable to FAK siRNA in inhibiting migration, whilst the combination had no additional benefit. This suggests that Pyk2 may be as important to MDA-MB-231 cell migration as FAK. Previous studies have shown that Pyk2 activity plays a major role in metastatic behaviours of breast carcinoma cells, with inhibition sufficient to significantly attenuate migration and invasion (Verma *et al.* 2015). Moreover, upregulation of Pyk2 caused a significant increase in both processes as well as significantly potentiating EMT through prolonged endosomal-derived receptor signalling (Verma *et al.* 2015). Our data seems to align with these findings and reinforces the relevance of Pyk2 as a molecule of interest within this model. However, our findings in this experiment contradict our initial hypothesis as combination of FAK/Pyk2 siRNA resulted in a comparable inhibition of migration versus either agent alone. Moreover, it appears to contradict the dogma that Pyk2 can compensate for loss of FAK as presented by the literature. This may not necessarily be the case here due to the strong anti-migratory effects of both siRNAs because, if FAK and Pyk2 contribute to MDA-MB-231 cell migration through similar pathways, suppression of one may cause the maximum anti-migratory effects. As such, combination of both will have no additional effects as FAK or Pyk2-driven migration is impaired to the lowest levels these pathways will allow. To explore this further, experiments should be performed in which FAK and Pyk2 levels are suppressed significantly less (e.g. to 50%), this would allow additive effects on pathway inhibition to be observed when the dual knockdown is performed. Nevertheless, these experiments have shown that Pyk2 is not providing any compensation for the ablation of FAK in our experiments and thus something else

might be accounting for the difference in migratory reduction between knockdown and kinase-inhibited cells.

Despite FAK siRNA providing us with limited information on scaffold functions, combination of siRNA and kinase-inhibition does enable us to draw some conclusions regarding kinase-independent functions of FAK in regulating migration. As with proliferation, PF271+FAK siRNA has no additional benefit when compared to kinase inhibition alone, suggesting that scaffold functions have a limited role in regulating MDA-MB-231 cell migration. However, several studies have directly implicated FAK scaffolding function in migration. For example, kinase-independent p130cas recruitment to FAK promotes lamellipodia formation through directing of guanine nucleotide exchange factor (GEF) signalling via Rac (McLean *et al.* 2005). Yet this pathway does require some contribution from kinase-function as p130cas binding to FAK is enhanced by Src-mediated FAK^{Y861} phosphorylation. In addition, embryonic cells and mouse embryonic fibroblasts with kinase-dead FAK can still migrate comparable to cells expressing WT FAK (Shen *et al.* 2005). Such roles for FAK scaffolding have also been shown in TNBC cells. In 2009, Sawhney and colleagues demonstrated that FAK regulates integrin-mediated ERK5 activation, independent of kinase-function, to modulate haptotactic motility and cell adhesion (Sawhney *et al.* 2009). Consequently, suggesting kinase-independent functions of FAK play little part in cell migration is presumptuous, yet our results do enable us to conclude that targeting kinase-dependent and independent functions in tandem has no additional benefit versus catalytic function alone. As with proliferation, we suggest that scaffolding does play a role in MDA-MB-231 cell migration, but kinase activity is essential for maximal motility. Further studies using scaffold-specific inhibition would need to be conducted to assess relative contributions by FAK protein-protein interactions on migration.

In addition to migration, we utilised Boyden chamber assays to explore FAK in invasion. As noted in proliferation, FAK siRNA was as effective as PF271 kinase inhibition at preventing invasion, with no additional reduction observed in dually-treated cells. This again suggests that FAK scaffolding function has limited contribution to this phenotype in MDA-MB-231 cells, thus kinase function is the primary mediator of FAK-driven invasion. Whilst many studies acknowledge the importance of FAK catalytic activity to invasive signalling pathways, much fewer exist examining scaffold function. Although much fewer in number, these studies do highlight some key roles for FAK scaffolding in invasion and as such may contradict our findings. For example, it has been

demonstrated that cells with catalytically inactive FAK can sustain F-actin polymerisation, a key factor in invasion, through continued association with Arp2/3 and subsequent cooperation with N-WASP via the FAK-FERM domain (Serrels *et al.* 2007; Tang *et al.* 2013). In this context, FAK serves to direct the activity of ARP2/3 to cellular protrusions prior to FA maturation.

FAK has previously been shown to contribute to a change in molecular profiles attributed to epithelial to mesenchymal transition (EMT) (Kim *et al.* 2010; Cicchini *et al.* 2008). Such programs underpin cell motility and invasion and thus contribute to aggressive tumour cell behaviours. Indeed, scaffolding functions of FAK have been shown to contribute to changes in EMT markers. One particular example of these markers are the matrix metalloproteinases (MMPs). These proteins are essential to tumour cell metastasis and invasion as they degrade ECM components thus enabling space for cell migration, whilst facilitating invasion of mobile cells (Page-McCaw *et al.* 2007). Consequently, changes in MMP status are clearly of vital importance to invasion and proteins which can cause such alterations must play a crucial role. Whilst FAK activity has been shown to increase expression of MMP9 and subsequent metastasis of breast cancer cells *in vivo* (S. Mitra *et al.* 2006), scaffolding function also plays a significant role in MMP regulation. For example, interaction of the FAK scaffold with endophilin A2 increases its phosphorylation, leading to alterations in MMPs (amongst other EMT markers) and direct modulation of breast tumour progression *in vivo* (Fan *et al.* 2013). These studies serve to highlight that FAK scaffolding function does contribute to cell invasion. As such, when taken together with our findings we cannot conclusively say that FAK scaffolding function is second to kinase activity in regulating invasion of TNBC. We can however state that inhibiting kinase function alone can reduce FAK-mediated invasion to its maximal level. Therefore, we can conclude that FAK plays a significant role in invasion and whilst kinase function appears crucial to this process, scaffolding contributions are either of comparative or less importance. Specific targeting of FAK scaffolding function will be required to determine conclusively its relative importance versus catalytic function in TNBC cell invasion.

Considering our findings, we went on to investigate potential mechanisms by which FAK regulates MDA-MB-231 cell migration. We began by exploring changes in cell morphology and subcellular distribution of FAK following inhibition through immunofluorescent microscopy. These results showed several changes that seemed to affect all steps of the migratory process. To successfully migrate in 2D culture, a few events need to occur. Initially cells must become

polarised to establish the direction of movement, subsequently enabling cells to undergo actin polymerisation at the leading edge, resulting in lamellipodia extension and attachment to the substratum. Finally, cells must be able to disassemble focal adhesions (FAs) at the trailing edge to allow cell contraction and movement towards the leading edge. The combination of these processes enables effective, directional cell migration all of which seem to be impaired in some way following FAK inhibition.

Our results showed that cells appeared to lose their leading edge in response to FAK inhibition, as well as reduced filopodia-like protrusions which could represent impaired lamellipodia production. Together, these observations suggest that FAK-kinase inhibition disrupts polarization of cells in tandem with reducing leading cell dynamics, marked by reduced lamellipodia extension. Such changes are in coherence with several other studies which highlight FAK as a regulator of cell polarity. For example, Tomar and colleagues (2009) demonstrated that disruption of integrin-mediated FAK phosphorylation could perturb directional cell movement through failure to recruit p120RasGAP resulting in disrupted of transient decreases in RhoA activity (Tomar and Schlaepfer 2009). Interestingly, the coordinated actions of the Rho family of GTPases are essential to mediating cell polarity and several of these proteins, namely Rac, Rho and Cdc42, are well documented to be under the tight control of FAK (Nobes and Hall 1995; Nobes and Hall 1999). Further evidence of FAK's role in polarity was demonstrated by Serrels et al (2011) who demonstrated that FAK is part of a "direction sensing" complex, comprised of FAK, RACK1 and PDE4D5, which signals to EPAC (a member of the GEF family) and G protein Rap1 to regulate cell polarisation of malignant keratinocytes (Serrels *et al.* 2011). Additionally, multiple studies have also highlighted a central role for FAK in lamellipodia formation. For instance, FAK-bound p130cas enables Crk recruitment following activation, which redirects GEF activity towards Rac thus promoting lamellipodia extension (McLean *et al.* 2005). Interestingly, this mechanism relies on both scaffolding and kinase function as FAK/Src-mediated phosphorylation of FAK^{Y861} significantly enhances p130cas activation. Our results align with this study as PF271-kinase inhibition resulted in perturbed lamellipodia formation. Taken together we concluded that inhibition of FAK catalytic function could disturb leading edge dynamics impairing directional migration.

We also showed that PF271 treatment caused a significant increase in the number of FAs and their size versus control cells. This implies that FAK is contributing to FA disassembly and its

inhibition is sufficient to reduce their turnover. Indeed, a significant amount of studies align with this finding which show normal FAK function is essential for FA turnover. For example, FAK-kinase function is essential for recruitment of calpain-2, a protease which cleaves molecular components of FAs and FAK, in order to enable disassembly and turnover of FAs (Chan *et al.* 2010). Again, FAK's relationship with Rho family GTPases, specifically Rho, has been proposed to play a key role in FA disassembly at the trailing edge (Gupton and Waterman-storer 2006). Indeed, stimulation of FAK results in activation of RhoA and ROCK, causing increased cell contraction and turnover of FAs to enable cell movement towards the leading edge (Iwanicki *et al.* 2008). Taken with our data, we believe that FAK regulates FA turnover through its kinase activity, with its inhibition effectively preventing detachment of cells from the substratum (in this case fibronectin) thus resulting in impaired migration and the more pointed appearance of PF271 treated cells. However, it seems likely that the combination of perturbed leading and trailing edge dynamics is what causes the change in morphology and is not due to FAK changes at either pole.

To further explore changes in cell motility programs, we used western blotting to explore signalling in migrating cells and found several alterations in response to PF271 treatment that reflected the observed changes in cellular morphology and subsequent migration. First, stimulating cells with FCS containing RPMI caused a significant increase in active FAK^{Y397} versus serum-starved control cells, whilst PF271 treatment reduced activity, comparative to unstimulated controls. The latter of these groups represents the negative controls in our Boyden chamber assays which exhibit significantly lower propensity for migration than stimulated cells. Taken together this suggests that FCS stimulates migration through increased FAK^{Y397} phosphorylation and resultant kinase activity. In addition, immunofluorescent cells lacking stimulation exhibit poorer polarisation, lamellipodia extension and FA formation. This reinforces the importance of FAK in mediation of cell migration and strongly supports the several studies presented to this point.

Along with FAK changes, we also observed significant impairment of Akt^{S473} phosphorylation following PF271 treatment. This infers that decreased FAK catalytic function impairs Akt stimulation and subsequent signal transduction. As noted previously, FAK and Akt have long been known to interact through PI3K bound to active FAK. In terms of cell migration, PI3K/Akt has been shown to contribute to cell movement through enhancing remodelling of the actin

cytoskeleton (Qian *et al.* 2003). This study showed that expression of active PI3K increased activation of Akt and caused significant actin filament remodelling, stimulating cell migration. The converse is also true, whilst this phenotype can be rescued by expression of active Akt. Moreover, a loss-of-function study by Seo et al (2014) further highlighted the importance of PI3K/PTEN/Akt signalling in cell movement (Seo *et al.* 2014). After identifying multiple genes that contributed to cell motility, they determined that a significant portion of these impacted migration through modulation of PI3K/PTEN/Akt signalling thus emphasising the importance of this pathway as a convergence point for signals mediating cell motility. Such observations also extend to the migration and metastatic potential of cancer cells. For example, Elloul and collagues (2014) demonstrated that Akt can regulate breast cancer cell migration through phosphorylation of the adherens junction protein Afadin, causing its subcellular redistribution to the nucleus causing increased cell mobility (Elloul *et al.* 2014). As such, it is reasonable to conclude from our data that FAK-mediated Akt stimulation could be mediating cell migration.

As with proliferating cells, PF271 treatment also alleviated inhibitory GSK3β^{S9} phosphorylation. Interestingly, GSK3β has been shown to regulate cell-matrix adhesion dynamics through modulation of paxillin and FAK (Sun *et al.* 2009) which seem to reflect both our western blotting and immunofluorescence analysis. As outlined, FAK inhibition seemed to impair FA turnover with increased numbers and sizes noted in PF271 treated cells. In 2005, Bianchi et al showed that GSK3β could phosphorylate FAK at S722, significantly impairing both its kinase activity and cell mobility (Bianchi *et al.* 2005), thus implying that active GSK3β could impair FA disassembly. Additionally, GSK3β has been implicated in FA formation. Indeed, Cai et al (2006) showed that GSK-dependent phosphorylation of paxillin was required for macrophage cell spreading (Cai *et al.* 2006). Taken together, these studies suggest a dual role for GSK3β in cell migration. As such, we concluded that PF271-mediated reduction in inhibitory GSK3β^{S9} phosphorylation may be contributing to decreased migration of MDA-MB-231 cells through simultaneously facilitating formation and impairing destruction of FAs. It is important to note that in HeLa cells, GSK3 association with the cytoplasmic cAMP phosphodiesterase (PDE) prune has been shown as necessary for activation of FAK, postulated to be resultant from interaction of GSK3/prune with an unknown tyrosine kinase (Sun *et al.* 2009; Kobayashi *et al.* 2006). However, such an observation may not extend to TNBC cells as the role of this complex in FAK has been largely unexplored in other cancers.

FAK inhibition also lead to a significant reduction in STAT3^{S727} signalling within migratory cells. Typically, STAT3 exists as a monomer within cells but activation causes homo/heterodimerisation, which enables nuclear translocation where it can function as a transcription factor for specific genes, some of which are linked directly to cell migration (Snyder *et al.* 2008). The mechanism of STAT3 activation hinges on phosphorylation of Y705 to engage dimerization and nuclear localisation, where phosphorylation of S727 enables maximal function as a transcription factor (Banerjee and Resat 2016) , whilst possibly contributing to nuclear localisation (Sakaguchi *et al.* 2012). We did not note a significant change in STAT3^{Y705} implying that FAK does not alter translocation but rather transcriptional activity of STAT3 through reducing S727. In support of this, FAK is known to directly interact with STAT3 at FAs to modulate motility signalling (Silver *et al.* 2004). However, this group did not explore changes in STAT3 phosphorylation, so we cannot be sure how FAK influences these activities, but we can speculate that given the relevance for STAT3^{Y705} for localisation it is likely that FAK would act with this site. This would contradict our findings as STAT3^{Y705} was not affected by PF271 treatment.

Although we cannot rule out FAK modulating STAT3^{S727} in the cytoplasm, given the disparities surrounding S727 mechanisms of action in the literature, we hypothesise that these effects are mediated within the nucleus of MDA-MB-231 cells. To support this, FAK has been shown to have nuclear activity and directly regulate several nuclear proteins (Lim 2013). Given that FAK can directly interact with STAT3 (Lazaro *et al.* 2014; Silver *et al.* 2004) and the canonical view of STAT3^{S727} activity as primarily nuclear, it stands to reason that nuclear FAK could modulate STAT3 activation and thus migration through decrease expression of a yet unknown, pro-migration gene. One such gene may be E-cadherin as this protein was observed to have STAT3-dependent expression in MCF10A cells (Leslie *et al.* 2010), as well as increased levels following FAK and STAT3 inhibition in HER2+ breast cancer cells (Lazaro *et al.* 2014). More studies would be needed to explore this idea, which may include immunoprecipitation of FAK and STAT3 in nuclear samples and examination of STAT3-mediated gene expression in response to FAK inhibition. Nevertheless, our data demonstrates that FAK inhibition in migrating cells can negatively alter STAT3^{S727} activity, suggesting a possible mechanism by which FAK mediates MDA-MB-231 cell motility.

To validate our findings and expand on our proposed mechanisms, we next sought to examine FAK's role in MDA-MB-468 TNBC cells. We initially saw that MDA-MB-231 and MDA-MB-468 cells

showed comparative relative FAK activity, but MDA-MB-231 cells had more total FAK protein and were significantly more sensitive to PF271 in terms of FAK^{Y397} activity. Despite this difference in sensitivities, the chosen concentration of 1μM was sufficient to reduce FAK activity in both cell lines to a comparable and very low level. By contrast, this level of FAK-kinase inhibition had very different effects on proliferation and migration, with MDA-MB-468 cells largely unaffected by PF271 treatment. Such differences between these cell lines in response to FAK silencing has previously been noted (Pylayeva *et al.* 2009). This study noted that MDA-MB-231 cells exhibit reduced proliferation and growth arrest in response to repressed FAK expression, whilst MDA-MB-468 cells only displayed inhibited proliferation. Importantly, whilst our observations in MDA-MB-231 cells hold, this study contradicts the results obtained from MDA-MB-468 cells. Moreover, this study used shRNA to silence FAK thus removing both kinase-dependent and independent functions. If taken with our PF271 data, this could imply a more important role for scaffolding function in this cell model. However, our siRNA results contradict this idea as suppression of FAK in MDA-MB-468 cells is not sufficient to cause significant reductions in proliferation.

We hypothesised that these differences were resultant from the inherent PTEN mutation in MDA-MB-468 cells. We initially confirmed that these cells indeed had no detectable levels of PTEN and detection of active Akt^{S473} was elevated versus MDA-MB-231 cells. Our data further reinforced this idea as the effects of FAK inhibition on Akt^{S473} activity in the absence of PTEN is negligible in both migrating and proliferating cells. Additionally, previously identified downstream elements of Akt signalling are largely unaltered by PF271 treatment. Therefore, we initially proposed that hyperactive Akt can circumvent FAK inhibition in TNBC cells and so sustain high levels of proliferation and migration. In terms of cell growth, there is some evidence in the literature which could support this. Previous studies have demonstrated a significant relationship between FAK and Akt in mediating anti-apoptotic survival signals. For example, Zheng et al (2013) showed that phosphorylation of FAK by protein tyrosine kinase 6 results in stimulation of anti-anoikis pathways, dependent on Akt activation (Zheng *et al.* 2013). Additionally, FAK inhibition can sensitise ovarian cancer cells to paclitaxel-induced cell death, through Akt-mediated dephosphorylation of Y-box-binding protein 1 (Kang *et al.* 2013). Taken together, this could suggest that in MDA-MB-468 cells, the PTEN-null phenotype and subsequent Akt hyperactivation may be enabling cell survival usually disrupted by FAK inhibition. However, our results in MDA-MB-231 cells contradict this idea as we demonstrated that cell number

changes were as a result of proliferative changes not increased apoptosis. Indeed, the lack of change in cell cycle, Ki67 and proliferative signals in MDA-MB-468 cells implies that FAK acts through Akt to regulate proliferation, as well as cell metabolism through Akt/MTOR signalling.

Our hypothesis regarding PTEN/Akt-mediated rescue following FAK inhibition also coincides with the literature in terms of cell migration. For example, early studies showed that PTEN overexpression can significantly reduce cell migration through suppression of FAK activity (Tamura *et al.* 1998). Additionally, PTEN can attenuate the invasion and metastatic potential of gastric cancers through downregulation of FAK expression (L.-L. Zhang *et al.* 2014). These studies highlight a strong link between FAK/PTEN in regulating migration and indeed may suggest why MDA-MB-468 cells are less migratory than MDA-MB-231 cells. However, if PTEN suppression of FAK was underpinning reduced responsiveness to PF271, one would still expect to see some decrease in MDA-MB-468 cell migration following treatment, even if not comparable to the effects noted in MDA-MB-231 cells. However, PTEN reduction also causes a significant increase in Akt activity and thus may explain sustained migratory potential. Indeed, the previously highlighted studies both demonstrated that attenuation of FAK phosphorylation was correlated with inhibited Akt activity thus suggesting a link between the two molecules regulated through PTEN. In cancer cells, it has been demonstrated on several occasions that FAK can influence Akt signalling to modulate cell motility. For example, treatment with the chemopreventative agent 4HPR causes significant mitigation of prostate cancer cell migration through via decreased FAK/Akt activity and subsequent increase in β-catenin degradation (Benelli *et al.* 2010). Similar results have also been noted in breast cancer cells with overexpression of microRNA-200 (miRNA-200) in MDA-MB-231 cells causing a significant increase in cell migration resulting from a stimulation of VEGF-A and subsequent activation of FAK/PI3K/Akt signalling (Choi *et al.* 2016). Taken together these studies seem to validate our hypothesis that FAK acts through Akt to stimulate migration, a process which can be perturbed by removal of endogenous PTEN activity.

Although this hypothesis seems rational given the MDA-MB-468 data, the noted changes may result from some other fundamental difference between the two TNBC models, especially given that several such differences can exist between models of identical disease states. Therefore, we needed to explore the effects of PTEN suppression and resultant Akt activity in MDA-MB-231 cells to validate our model of FAK-mediated proliferation and migration. Initial suppression studies revealed that PTEN siRNA had no significant effect on FAK. This provided an essential

validation that enabled subsequent FAK inhibitory studies to be undertaken but also seemed in contrast with some studies which suggest a direct role for PTEN in the regulation of FAK in cancer cells (e.g. the previously outlined studies by Zhang *et al.* 2014; Tamura *et al.* 1998). Our PTEN siRNA cells displayed no significant decrease in either expression or activity of FAK, inferring no regulatory role for PTEN in MDA-MB-231 cells.

In contrast to our hypothesis, the PTEN suppression and associated increase in Akt activity was not sufficient to rescue proliferation in FAK inhibited cells. This also contradicts the observations in MDA-MB-468 cells, where hyperactive Akt appeared to alleviate the proliferative changes in response to PF271. This would suggest that some other protein differentially expressed or activated between MDA-MB-468 and MDA-MB-231 cells may contribute to FAK-dependent proliferation. For example, both MDA-MB-468 and 231 cells have been reported to have high levels of EGFR expression, but are significantly higher in MDA-MB-468 cells (Subik *et al.* 2010; Kao *et al.* 2009; Konecny *et al.* 2006). In 2001, Mariotti et al showed that in keratinocytes, EGFR is able to combine with β4 integrins and cause a significant amplification of their activity via Fyn kinase (Mariotti *et al.* 2001). Given this pathways role in regulating proliferation, it stands to reason that cells with higher EGFR can more readily utilise this relationship to sustain propagation. More importantly, as this relationship does not require the canonical regulation of integrin signals via FAK, this integrin β4-EGFR pathway could sustain MDA-MB-468 proliferation in FAK inhibited cells. Subsequently, this may highlight a potential issue with targeting FAK-kinase activity in high EGFR expressing cells. Although more studies would be needed to confirm at what point this became an issue (given that MDA-MB-231 cells also have high EGFR but are FAK sensitive), it serves to emphasise the relevance of patient stratification with regards to an effective therapeutic regime for cancer treatment.

To further explore the role of Akt in FAK-mediated proliferation, we evaluated downstream signalling changes in PTEN supressed cells in response to PF271 following 3-days. As seen previously, Akt^{S473} activation is significantly higher in PTEN treated cells than all controls. Interestingly, inhibition of FAK is still sufficient to induce a decrease in Akt^{S473}, although activity remains significantly higher than in cells with normal PTEN expression. However, downstream inhibition of $GSK3\beta^{S9}$ and subsequent increase in inhibitory cyclin $D1^{T286}$ are unchanged in PTEN suppressed cells treated with PF271, suggesting that FAK can mediate these signals independent of Akt. A potential mechanism could be through p130cas. This protein is one of the major targets

for FAK activity, with its activity linked to several signalling pathways (Lee *et al.* 2015). Indeed, a seminal study by Oktay et al (1999) showed that FAK-dependent stimulation of p130cas promoted activation of JNK and c-Jun, leading to increased cell proliferation (Oktay *et al.* 1999). Further studies in genetically engineered mice revealed that mammary-specific deletion of FAK caused a significant decrease in proliferation, dependent on reduced p130cas (Pylayeva *et al.* 2009). As such, p130cas could be enabling sustained cyclinD1 inhibition in hyperactive Akt cells treated with PF271, thus maintaining suppression of proliferation. Further experiments would be needed to directly assess how FAK inhibition alters p130cas and JNK activity in "normal" and PTEN-suppressed MDA-MB-231 cells.

In contrast to proliferation, PTEN suppression and resultant Akt hyperactivity was capable of rescuing migration following FAK inhibition. Moreover, when signalling in these cells is examined PF271 still causes some effect on Akt^{S473}, $GSK3\beta^{S9}$ and $STAT3^{S727}$ activity but levels in inhibited cells are comparable to vehicle-only control cells without PTEN knockdown. This suggests that FAK requires Akt and downstream signals to mediate its effects on migration. Interestingly, given the theorised role of Akt activity in mediating FAK-driven cell migration, one would expect that hyperactivation of Akt would lead to an increase in cell motility versus cells without increased activity. However, we noted that PTEN knockdown cells have comparable migration to all siRNA control cells. This seems to contradict several studies which links aberrant PTEN expression and activity to increased migratory potential. For example, Dong et al (2013) showed that angiotensin II could induce vascular smooth muscle migration through reducing expression and activity of PTEN (Dong *et al.* 2013). Additionally, negative regulation of PTEN through TGF-β1 activation of SMAD and ERK1/2 significantly increased motility of type II endometrial cancer cells (Xiong *et al.* 2016). These studies would suggest that hyperactivation of Akt through PTEN loss would be sufficient to increase migration. Interestingly, both these studies noted an increase in FAK activity following loss of PTEN, suggesting FAK as the underlying driver of migration via Akt. In our studies, this was not seen and suggests that for hyperactive Akt to increase motility, cells also require tandem increases in FAK function and subsequent stimulation of downstream Akt. This could account for the observed differences between previous studies and the results presented here. Additionally, it could be argued that increased Akt alone is not sufficient to drive migration as several other signalling pathways utilise Akt as a central modulator of cell function (Mayer and Arteaga 2016) and thus its activity is being directed towards regulation of other behaviours. Rather you need recruitment of Akt to FAK-bound PI3K in order to direct signalling

towards migration. It would be interesting to explore both Akt localisation through immunofluorescence and changes in molecular interactions through immunoprecipitation in PTEN siRNA cells to better clarify how FAK/Akt signalling can drive cell motility.

4.17.1. Conclusions

The data in this chapter shows that inhibition of FAK-kinase activity can significantly alter TNBC cell proliferation through mediation of signalling proteins linked to cell cycle. Moreover, these changes are independent of either the Akt or Erk signalling pathways. Additionally, we have demonstrated that FAK is a key driver of MDA-MB-231 cell migration, through perturbation of focal adhesion turnover and resultant disruption of normal cytoskeletal dynamics. This seems to be dependent on activation of Akt-signalling and possibly STAT3. Taken together, our data suggests that FAK could represent a major therapeutic target for the inhibition of cellular proliferation and migration in TNBC cells and thus is a valid target for prospective drug design.

5. Results (III)

Mechanistic evaluation of FAK in TNBC Cancer Stem Cells (CSCs)

5.1 Introduction

Tumour heterogeneity is a well-documented phenomenon and one that has been linked with therapeutic failure. At the broadest level, tumours are comprised of cells from the epithelia, stroma and immune system. However, each of these "types" can exhibit a further level of heterogeneity giving rise to cellular subtypes. It has been postulated that within cancer cell subtypes exists a hierarchal system whereby a single cell has the potential to divide and differentiate to give rise to the variety of cell types observed within the tumour (Huntly and Gilliand 2005). This is the so-called "cancer-stem cell (CSC) hypothesis". In contrast to the traditional view of tumour development, the CSC hypothesis suggests that this rare cell population has the capacity to self-renew endlessly, whilst being able to create all other cell types present within a tumour. These cells have thus been labelled as key drivers of metastatic potential as they have the potential to form complete tumours following extravasation into distal tissues. Several lines of evidence exist that support this hypothesis in solid cancers, including breast cancer. For example, Al-Hajj et al (2003) identified a subpopulation of human breast carcinoma cells which could initiate tumour growth and development following implantation into mice (Al-Hajj *et al.* 2003). These cells were identified as being immunoreactive for CD44 or had little/no CD24 presentation, a profile now utilised as marker of CSCs. Moreover, enrichment of $CD44^{+}/CD24^{low/-}$ cells demonstrated an 10 to 50-fold increase in tumour forming capacity versus the total cell population further providing validation of a stem-like population of cells within breast cancers (Al-Hajj *et al.* 2003). This profile of cell surface markers was also used to identify mammary somatic stem-cells capable of re-forming mammary glands following introduction of a single $Lin^{-}/CD29^{High}/CD24^{+}$ cell *in vivo* (Shackleton *et al.* 2006). Moreover, CSCs have been suggested to be responsible for disease recurrence due to CSCs being intrinsically more resistant to a range of cancer therapeutics versus other tumour cells. As such, therapies that fail to target these cells could allow retention of CSCs within patients and facilitate disease recurrence. Indeed, treatment with conventional chemotherapies has been shown to enrich the CSC subpopulation, whilst causing increased metastasis and bestowing chemo-resistance to the initial therapeutic agent (Carrasco *et al.* 2014). Taken together, these traits have led to CSCs being earmarked as the key drivers of disease progression and resultant patient outcome. In this section, we have defined CSCs as cells which have the potential to survive and proliferate under anoikis conditions and self-renew.

Given the propensity of TNBCs towards metastatic disease and short, disease-free periods prior to relapse (Dai *et al.* 2015), it may be that this breast cancer subtype might exhibit increased numbers of stem-like cancer cells. Indeed, reports have identified a subpopulation of cells with CSC-like characteristics; self-renewal, non-adherent survival and increased chemo-resistance, in *in vitro* cell cultures (French *et al.* 2015). Moreover, such cells have been isolated from TNBC patient tumours and subsequently reintroduced *in vivo* where they establish new, fully-diverse tumours, reflective of triple-negative disease (Kolev *et al.* 2017).

Intriguingly, FAK has been shown to contribute to stem-like characteristics of cells, for example, through facilitating anchorage independent growth (Sulzmaier *et al.* 2014). The influence of FAK on such traits has led to its direct implication in the maintenance and regulation of CSCs. For example, through supressing FAK in mammary epithelial cells, Luo and colleagues (2009) significantly reduced tumourigenesis *in vivo*, consistent with inhibition of a CSC subpopulation demonstrating reduced numbers, self-renewal and sphere formation *in vitro* (M. Luo *et al.* 2009).

5.1.1. Aims and Objectives

Given the role of FAK in stem-like cell behaviours and the fact that TNBCs may harbour such a subpopulation, here we wished to ask the question whether the MDA-MB-231 cell line has a CSC-like subpopulation and whether these cells are sensitive to FAK inhibition. As such, we set out the following objectives for this chapter:

- Identify whether a CSC subpopulation exists within MDA-MB-231 cells and explore the responses of these cells to FAK inhibition.

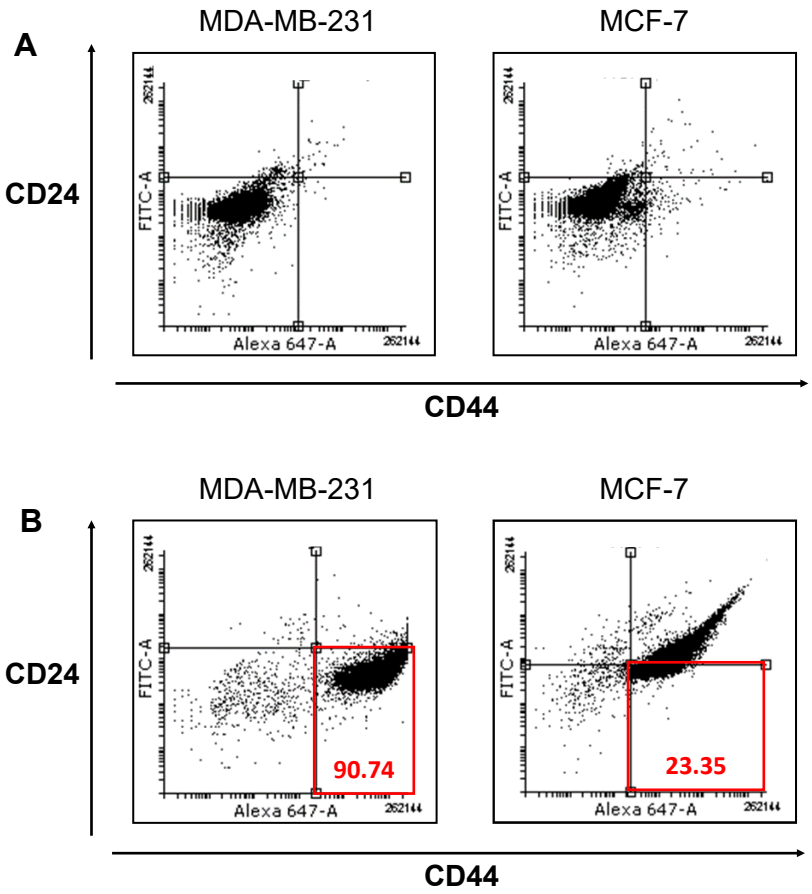
- Analyse the contribution of FAK to stem-like characteristics including, anoikis-resistance and self-renewal.

- Evaluate signalling changes that could result in perturbed stem-like phenotypes.

5.2. Evaluation of FAK contribution to maintenance of the CSC subpopulation

The first element of this work was to explore whether a subpopulation of cells with CSC-like characteristics existed within the MDA-MB-231 parental line. Consequently, we used flow cytometry to probe for the expression of the cell surface markers CD44 and CD24. As noted previously, the relative presence or absence of these markers (as $CD44^{+}/CD24^{low/-}$) is indicative of a cell population with enhanced tumour initiating and stem-like capabilities (Al-Hajj *et al.* 2003). Initially, this analysis required the establishment of true negative cells in order to set up quadrants for population analysis. Briefly, cells were incubated with isotype controls and appropriate secondary antibody. These negative controls will not stain positive for markers, whilst containing the isotypes found in antibodies under experimental conditions thus providing the best representation of background fluorescence to allow quadrants to be placed on subsequent data **(figure 5.1A)**. Flow cytometry analysis revealed that MDA-MB-231 cells exhibited a high number of cells with a $CD44^{+}/CD24^{low/-}$ phenotype; MCF-7 cells (used as a comparative control) had significantly fewer cells with stem-like molecular profile **(figure 5.1B)**.

To investigate whether the existence of this subpopulation of cells was FAK-dependent, we treated the parental cells with the FAK inhibitor for 24hrs before performing FACS analysis. Inhibition of FAK kinase function caused no significant change in stem-like cell proportions in either MDA-MB-231 or MCF-7 cells **(figure 5.1C and D)**.



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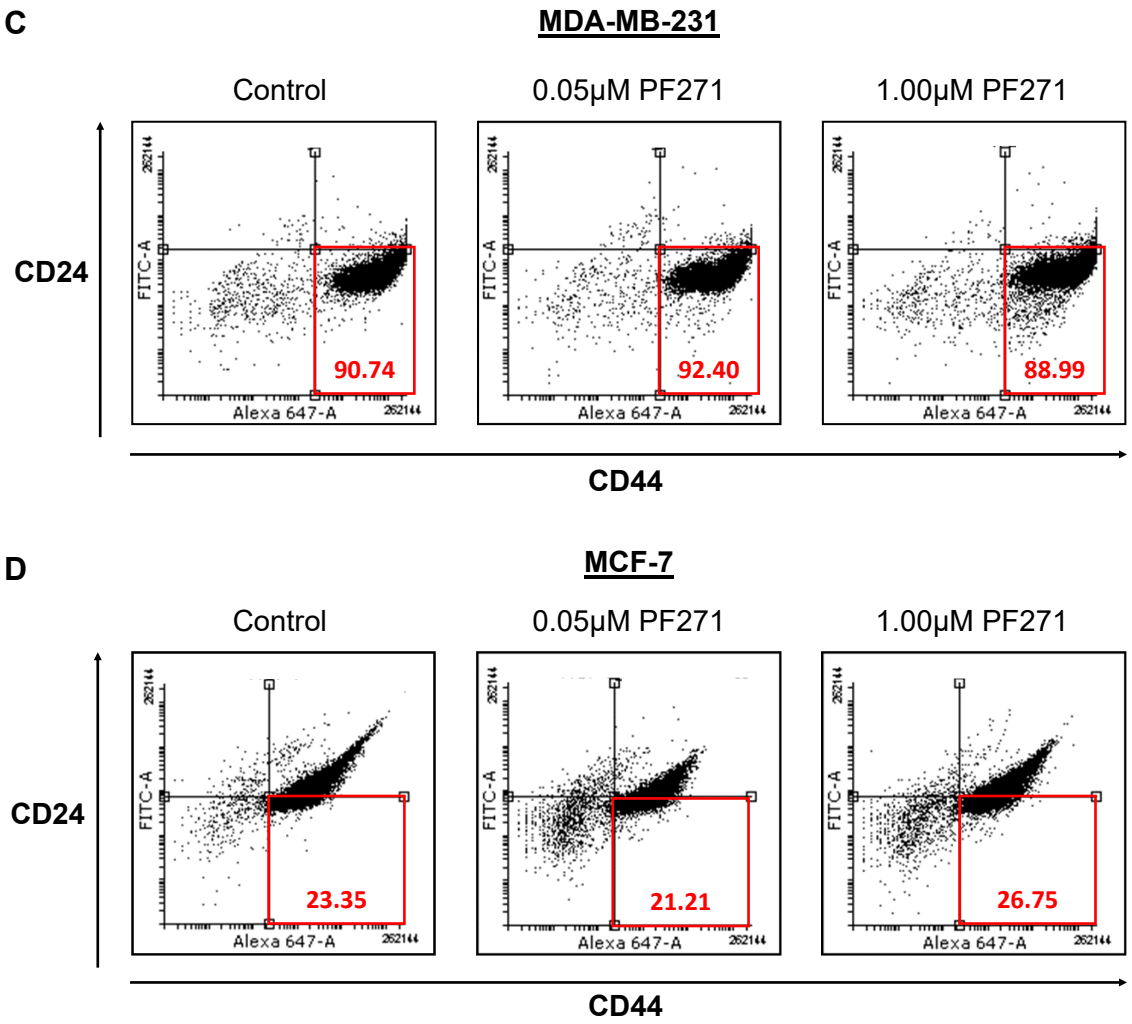


Figure 5.1 – Flow cytometry analysis of CSC composition of breast cancer cell models in response to FAK inhibition – (A) Representative scatter plots of isotype controls used to determine quadrants. Such controls will not recognise CD44/24 and thus points represent background fluorescence which were subsequently utilised for drawing of quadrants. **(B)** Evaluation of CD44 (alexa 647) and CD24 (FITC) presentation on MDA-MB-231 and MCF-7 cells shows both cell models contain a stem-like subpopulation (red box), whilst MDA-MB-231 cells have significantly higher potential CSCs than MCF-7s. **(C)** Exploration of the effects of FAK inhibition on CD44 and CD24 presentation in MDA-MB-231 cells shows no significant changes following PF271 treatment. **(D)** Assessment of changes in CD44 and CD24 in response to PF271 treatment in MCF-7 cells had no signifcant effect on stem-like subpopulation of cells. Numbers included in red boxes represent mean percentage of cells in this population quadrant; n=3.

Given the extremely high number of stem-like cells defined by a $CD44^{+}/CD24^{low/-}$ presentation, we decided to attempt to validate our findings using an alternative marker of CSCs, ALDH (Ginestier *et al.* 2007). Breast cancer cells positive for expression of ALDH represent CSCs, enriched populations of which are capable of forming tumours following transplantation into NOD-SCID mice (Ginestier *et al.* 2007). We first needed to determine the gating for ALDH positive cells **(figure 5.2A)**. In brief, a proportion of cells were treated with a specific inhibitor of ALDH, diethylaminobenzaldehyde (DEAB), which prevents the conversion of Aldefluor™ reagent into the detectable substrate. From these cells, background fluorescence can be determined and appropriate gating applied. Following gating, our results showed that MDA-MB-231 cells exhibit a small number of $ALDH^{+}$ cells **(figure 5.2B)**. Again, inhibition of FAK activity through PF271 had no significant effect on the proportion of cells represented in this subpopulation **(figure 5.2C and D)**.

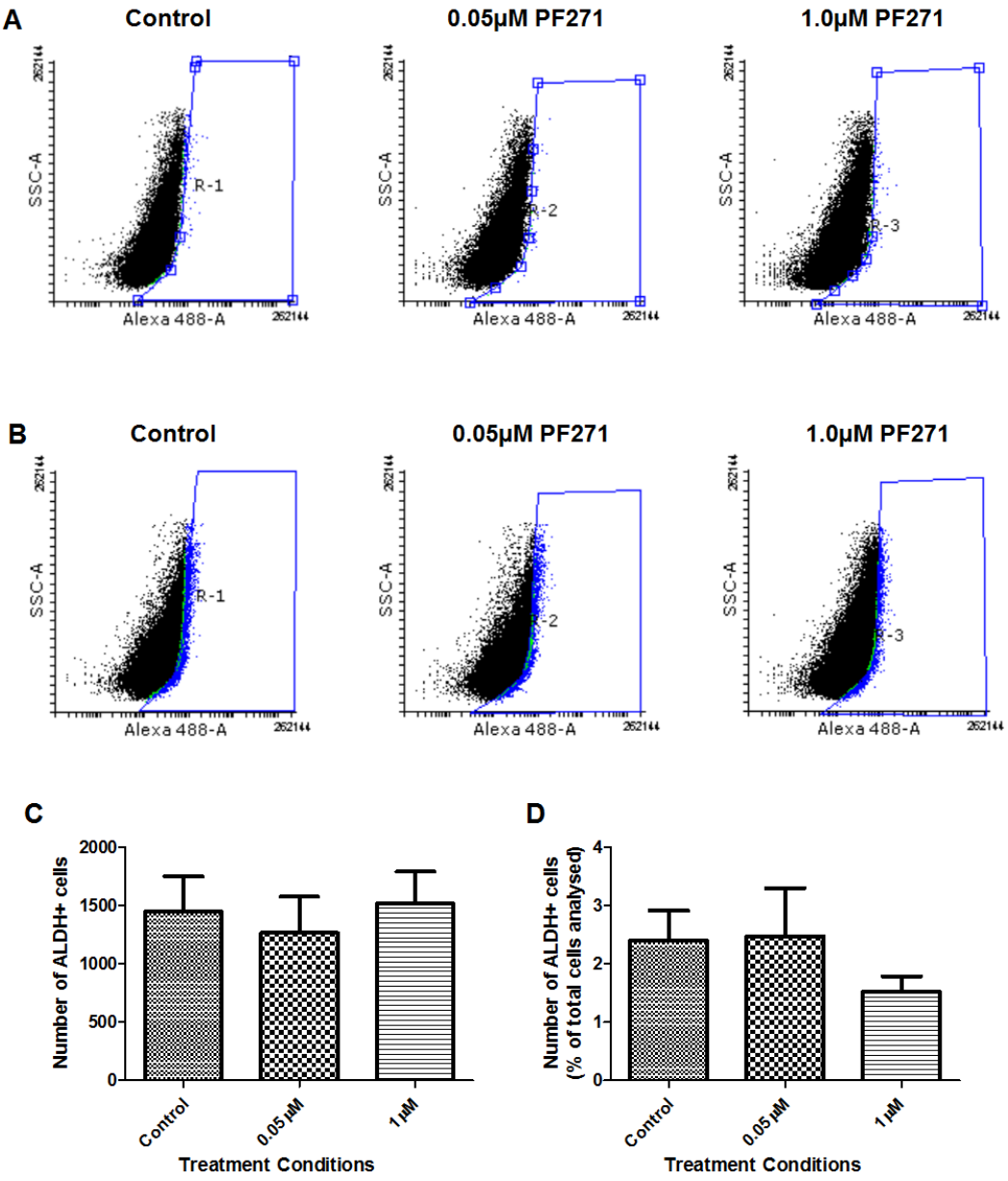


Figure 5.2 – Analysis of ALDH CSC marker by flowcytometry in cells treated with PF271 – (A) Representative scatter plots of cells treated with DEAB, a specific inhibitor of ALDH function. These plots of ALDH1 (Alexa 488) versus side-scatter (SSC) show levels of background fluorescence within cell populations, subsequently gated (blue polygon) to enable observation of true ALDH+ cells. **(B)** Representative scatter plots for ALDH1 versus SSC with gates encompassing ALDH+ cells as determined through used of a negative-control. **(C)** Evaluation of the number of cells in each condition that exhibited ALDH-positive staining. Inhibition of FAK had no significant effects on the number of positively expressing cells. **(D)** Analysis of ALDH-positive cells represented as a percentage of the total cell population analysed, showing no significant attenuation of stem-like cells following PF271 treatment. All error bars represent SEM; n=3.

5.3. Functional assessment of the role of FAK in stem-like characteristics

To examine the functional contribution of FAK to the CSC phenotype, a mammosphere assay was performed. The number of spheres present (represented as mammosphere forming units (MFU)) allows changes in anoikis resistance to be observed, along with the number of cells capable of forming new tumours. Inhibition of FAK resulted in a significant decrease in passage 1 MFUs, coupled with a significant decrease in mammosphere volume **(figure 5.3A-C)**. Given that we defined a mammosphere as being larger than 50µm, the change in size following PF271 treatment may be masking changes in the number of MFUs. As such, we also assessed mammosphere-forming potential with spheroids defined primarily by morphology, with a low minimum size requirement (>20µm). These data revealed no significant attenuation in the number of MFUs in FAK inhibited cells **(figure 5.3D)**.

A limitation to measuring the initial mammosphere forming capability was that this only determines anoikis survival and non-adherent proliferation. However, subsequent passages of mammospheres better represent stem-like characteristics as they permit assessment of anoikis resistance, proliferation and self-renewal capability. Consequently, this assay was extended and mammospheres passaged further and the number of spheres counted **(figure 5.3E)**. When self-renewal was assessed (passage 2 MFU numbers relative to passage 1 counts), PF271 caused a significant decrease in self-renewal factor (SRF) versus vehicle-only controls, regardless of the size used to determine mammospheres **(figure 5.3F)**.

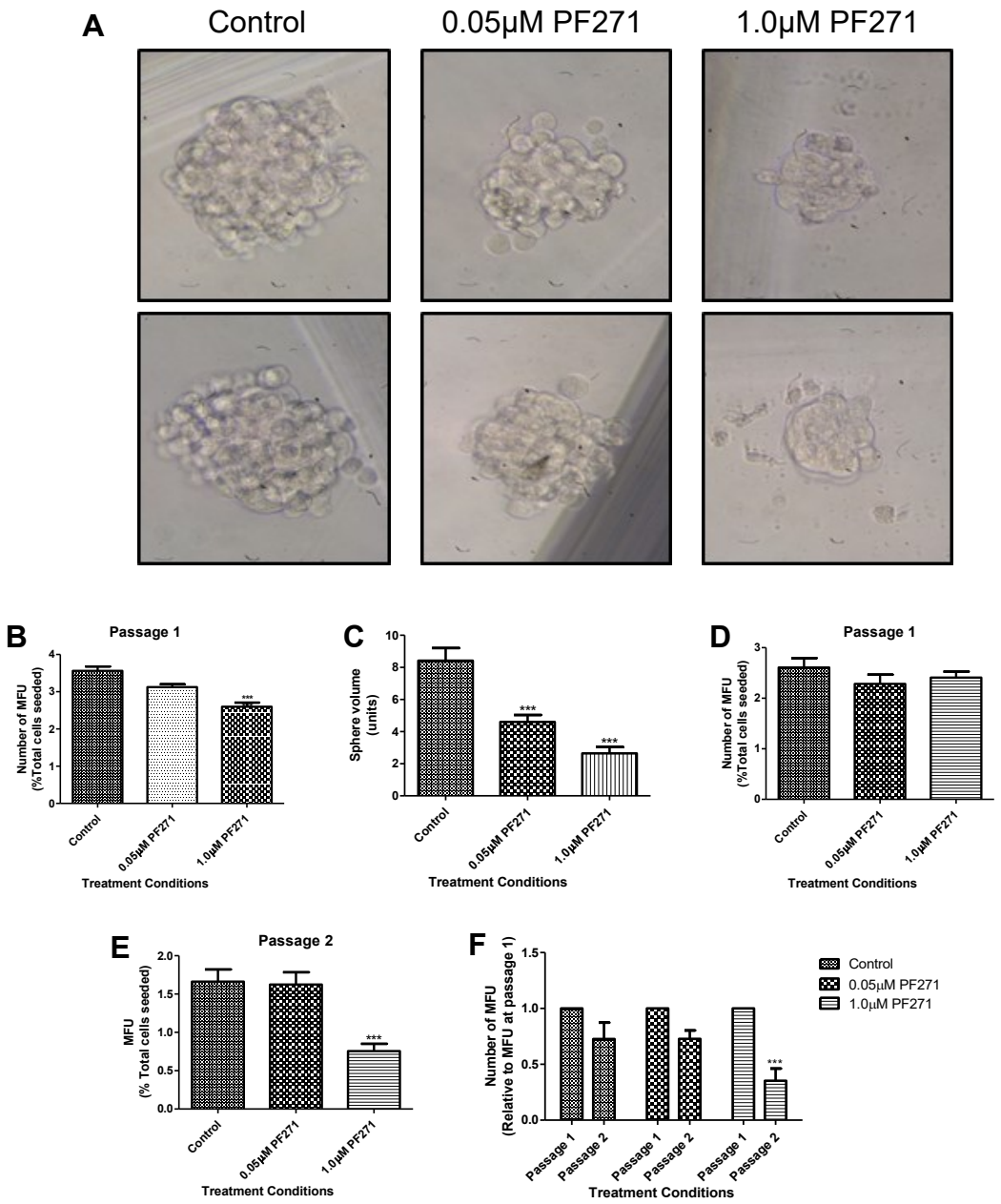


Figure 5.3 – Functional assessment of FAK contribution to stem-like traits of CSCs through PF271 inhibition – (A) Representative images of mammospheres showing clear decrease in tumour volume in response to FAK inhibition. **(B)** Evaluation of the number of mammospheres (represented as mammosphere-forming units (MFUs)) >50μm at passage 1 in response to PF271 inhibition of FAK kinase activity. Reduction of FAK activity caused a significant decrease in mammosphere forming capability. **(C)** Assessment of mammosphere volume in response to PF271 treatment showed that FAK inhibition can cause a significant reduction in size versus vehicle-only control. **(D**) Investigation of changes in MFUs in passage 1 defined by spheroid structures >20μm showed no significant changes in mammosphere-forming capacity in response to FAK inhibition. **(E)** Probing changes in mammosphere forming capabilities following seeding into a second passage in response to FAK inhibition. PF271 treatment resulted in a significant decrease in the number of passage 2 MFUs. **(F)** Self-renewal capacity assessment in response to FAK inhibition by representing passage 2 MFU numbers as a proportion of those observed in passage 1. Self-renewal is significantly impaired by PF271 treatment. All error bars represent SEM; n=5. *p<0.05; **p<0.01; ***p<0.001.

To further explore whether mammosphere formation was FAK-dependent, FAK was suppressed through siRNA and the assay repeated. FAK suppression caused a significant decrease in the number of mammospheres detected that were >50μm in diameter and also the overall mammosphere volume **(figure 5.4A-C)**. Again, as size was affected we also assessed MFUs at a much lower minimum size **(figure 5.4D)**. FAK siRNA had no significant effect on mammosphere-forming potential in this assessment. As with pharmacological FAK inhibition, the number of MFU at passage 2 was significantly reduced in FAK siRNA cells, accompanied by a significant decrease in SRF, irrespective of the definition of mammospheres **(figure 5.4E and F)**.

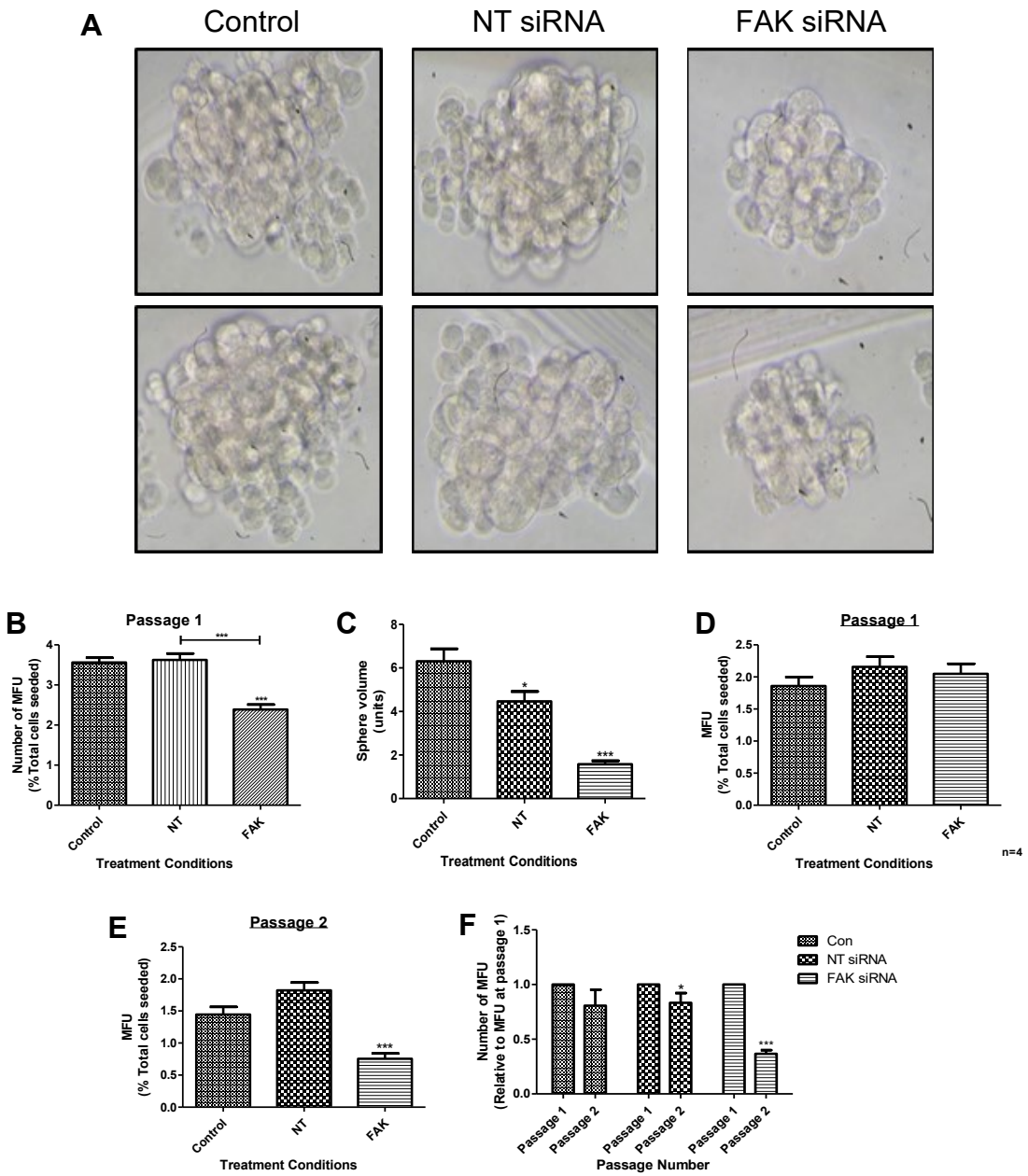


Figure 5.4 – Functional assessment of FAK contribution to stem-like traits of CSCs through siRNA suppression – (A) Representative images of mammospheres showing clear decrease in tumour volume in response to FAK repression. **(B)** Evaluation of the number of mammospheres (represented as mammosphere-forming units (MFUs)) >50µm at passage 1 in response to FAK siRNA. Suppression caused a significant decrease in mammosphere forming capability. **(C)** Assessment of mammosphere volume in response to siRNA treatment showed that decreasing total FAK levels can cause a significant reduction in size versus non-transfected and NT controls. **(D**) Investigation of changes in MFUs in passage 1 defined by spheroid structures >20µm showed no significant changes in mammosphere-forming capacity in response to FAK siRNA. **(E)** Probing changes in mammosphere forming capabilities following seeding into a second passage in response to FAK suppression. Targeted FAK siRNA resulted in a significant decrease in the number of passage 2 MFUs versus controls. **(F)** Self-renewal capacity assessment in response to FAK siRNA by representing passage 2 MFU numbers as a proportion of those observed in passage 1. Self-renewal is significantly impaired in FAK siRNA cells. All error bars represent SEM; n=5. *p<0.05; **p<0.01; ***p<0.001

Given that CSCs comprise a very small proportion of the total cell population, the effects of treatment in heterogeneous, adherent culture may impair targeting of the stem-like subpopulation. To address this potential issue, cells were treated in adherent and/or anoikis culture, allowing assessment of the most effective way to targeting FAK function in MDA-MB-231 CSCs. As seen in **figure 5.5A**, PF271 was sufficient to cause a significant loss of MFUs regardless of when treatment was administered. This effect was not significantly amplified in cells treated under anoikis conditions or following dual administration of PF271. Self-renewal was also significantly impaired in all conditions, where alterations in time of treatment had no significant impact on decreasing SRF **(figure 5.5B)**.

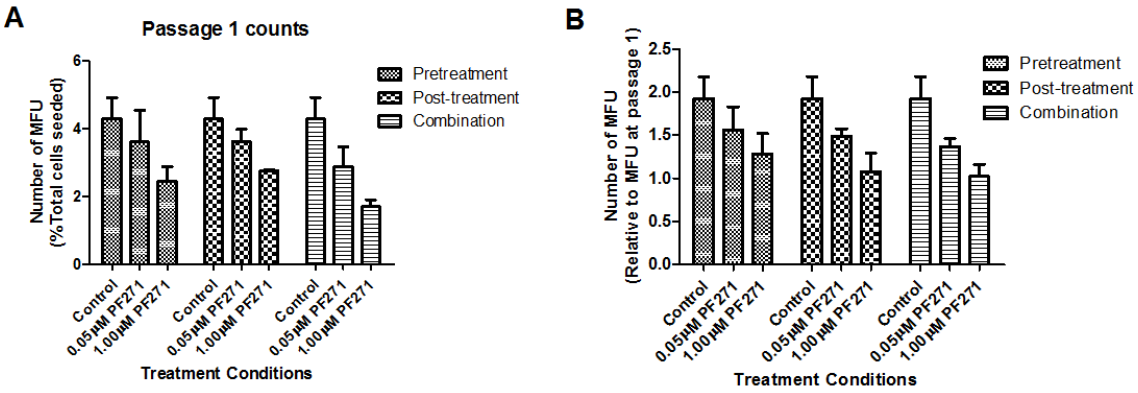
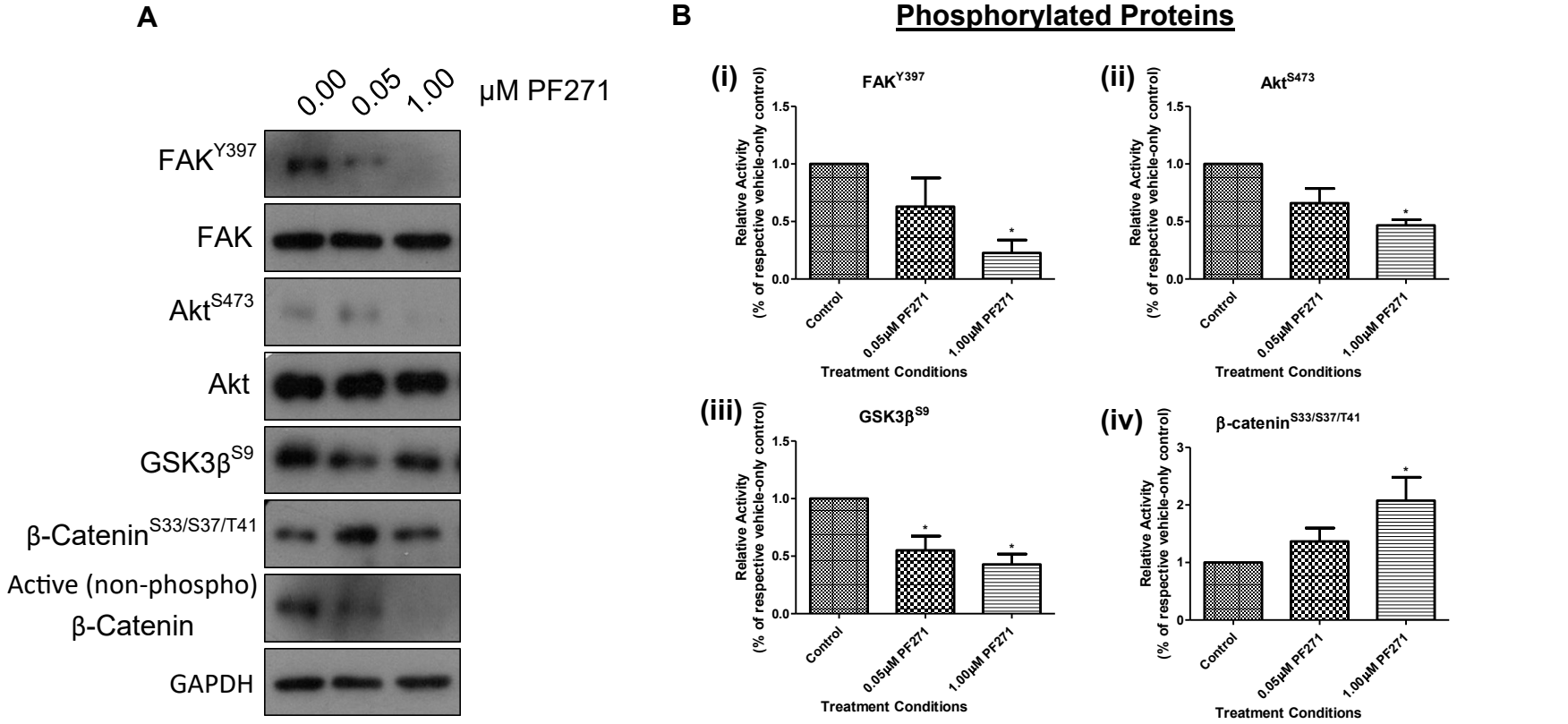


Figure 5.5 – Evaluation of changes in MFU and self-renewal in response to PF271 administered before and/or after seeding into anoikis conditions – (A) Evaluation of the changes in passage 1 MFUs, as a percentage of total cells seeded, in response to PF271 treatment administered to adherent cultures prior to seeding into anoikis conditions (pre-treatment), whilst suspended in non-adherent culture (post-treatment) or at both time-points (combination). FAK could significantly impair MFUs, whilst time of inhibitor administration caused no significant changes in response. **(B)** Exploration of self-renewal of MDA-MB-231 mammospheres treated with PF271 at varying time-points. Inhibition of FAK catalytic function significantly attenuated SRF, not affected by conditions of treatment. All error bars represent SEM; n=3.

5.4. Analysis of CSC signalling changes in response to FAK inhibition

Given that our data suggested a role for FAK in CSC self-renewal, we investigated potential mechanisms of action underpinning this process at a cell signalling level. Western blot analysis was performed on mammospheres ±PF271 in an enriched stem cell population. It has long been established that β-catenin plays an integral role is sustaining stem-cell self-renewal, whilst GSK3β can modulate this through phosphorylation of β-catenin which leads to its degradation (Yost *et al.* 1996). Previous studies have highlighted a role for FAK activity in stabilising β-catenin to regulate stem-like characteristics, although the mechanism behind this is unclear (Kleinschmidt and Schlaepfer 2017). Given our previous observation that FAK influences Akt signalling in MDA-MB-231 cell migration, we hypothesised that Akt-mediated inhibition of GSK3β could be underlying FAK-dependent stabilisation of β-catenin to regulate MDA-MB-231 mammosphere self-renewal. In the mammosphere cultures we observed that FAK^{Y397} and Akt^{S473} activity was significantly reduced by PF271 treatment **(figure 5.6Bi and ii)**, whilst total protein levels were unaltered **(figure 5.6Ci and ii)**. In addition, decreased FAK kinase activity was accompanied by a loss of $GSK3\beta^{S9}$ and an associated increase in ubiquitinoylation-targeted β-$catenin^{S33/37/T41}$ **(figure 5.6Biii and iv)**. In tandem, levels of active β-catenin significantly decreased in mammospheres treated with PF271 **(figure 5.6Bv)**.



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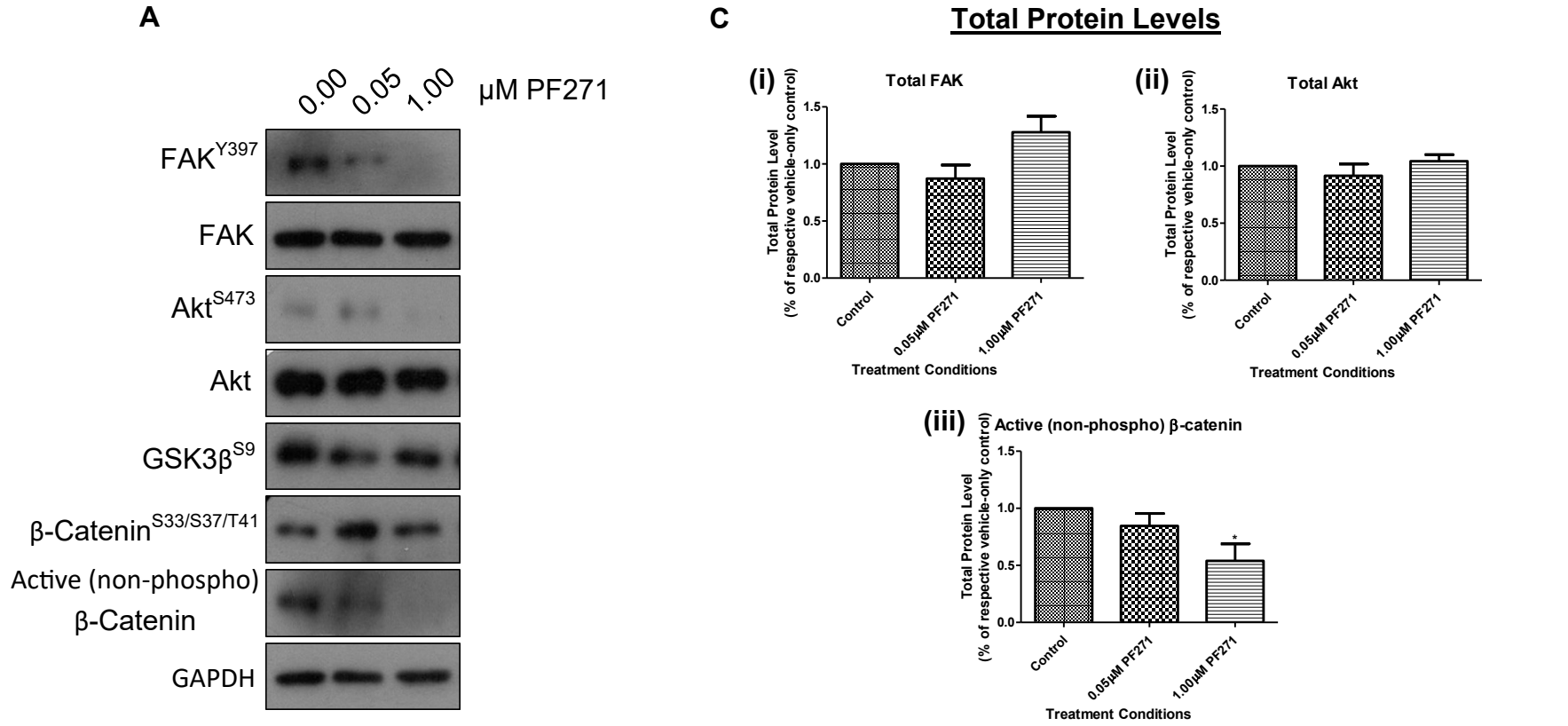


Figure 5.6 – Evaluation of signalling pathways in mammospheres treated following FAK kinase inhibition (A) Western blot showing response of proteins to FAK-inhibition in mammospheres grown under anoikis conditions. **(B)** Evaluation of relative activity of phosphorylated proteins (i) FAK^{Y397}; (ii) Akt^{S473}; (iii) GSK3β^{S9}; (iv) β-catenin$^{S33/37/T41}$. Phosphorylated FAK, Akt and GSK3β displayed a significant decrease following PF271 treatment versus vehicle-only control, whilst β-catenin$^{S33/37/T41}$ levels significantly increased. **(C)** Assessment in changes in total protein levels of (i) FAK; (ii) Akt and (iii) Active (non-phospho) β-catenin. No significant change was noted for total FAK or Akt following FAK inhibition, whilst active β-catenin decreased significantly. All densitometry results are normalised to the respective GAPDH loading control. Error bars represent SEM; n=3. *p<0.05; **p<0.01; ***p<0.001.

5.5. Exploration of the FAK/Wnt relationship in CSC self-renewal

Self-renewal in normal breast cancer stem cells has been shown to be directly influenced by the activity of Wnt signalling (Lee *et al.* 2014). Moreover, evidence exists for a FAK-Wnt signalling crosstalk in embryonic development, as well as in a cancer context (Roarty and Rosen 2010; Fonar *et al.* 2011). Indeed, FAK inhibits Wnt3a signalling and stimulates Wnt5-dependent β-catenin stabilisation leading to deregulated growth in haematopoietic cells which is then thought to contribute to AML development (Despeaux *et al.* 2012). In line with this, our data also suggests a role for FAK in modulation of β-catenin activity which may affect its stability and potential contribution to MDA-MB-231 mammosphere self-renewal. Consequently, we wished to explore whether Wnt3a contributes to FAK-dependent stabilisation of β-catenin to sustain self-renewal in MDA-MB-231 stem-like cells. As such, we investigated whether stimulation of mammospheres with Wnt3a could rescue self-renewal in PF271 treated cells. In passage 1 and 2, treatment with the FAK inhibitor caused a significant decrease in the numbers of MFUs >50μM as previously observed **(figure 5.7A and B)**. Moreover, the addition of Wnt3A caused no significant recovery of passage 1 mammosphere-forming capacity in PF271 treated cells regardless of dose administered. In contrast, the numbers of passage 2 MFU were significantly increased when stimulated with Wnt3A in a dose dependent manner **(figure 5.7B)**, a result reflected by a significant recovery in SRF following Wnt3a treatment in FAK inhibited mammospheres **(figure 5.7C)**.

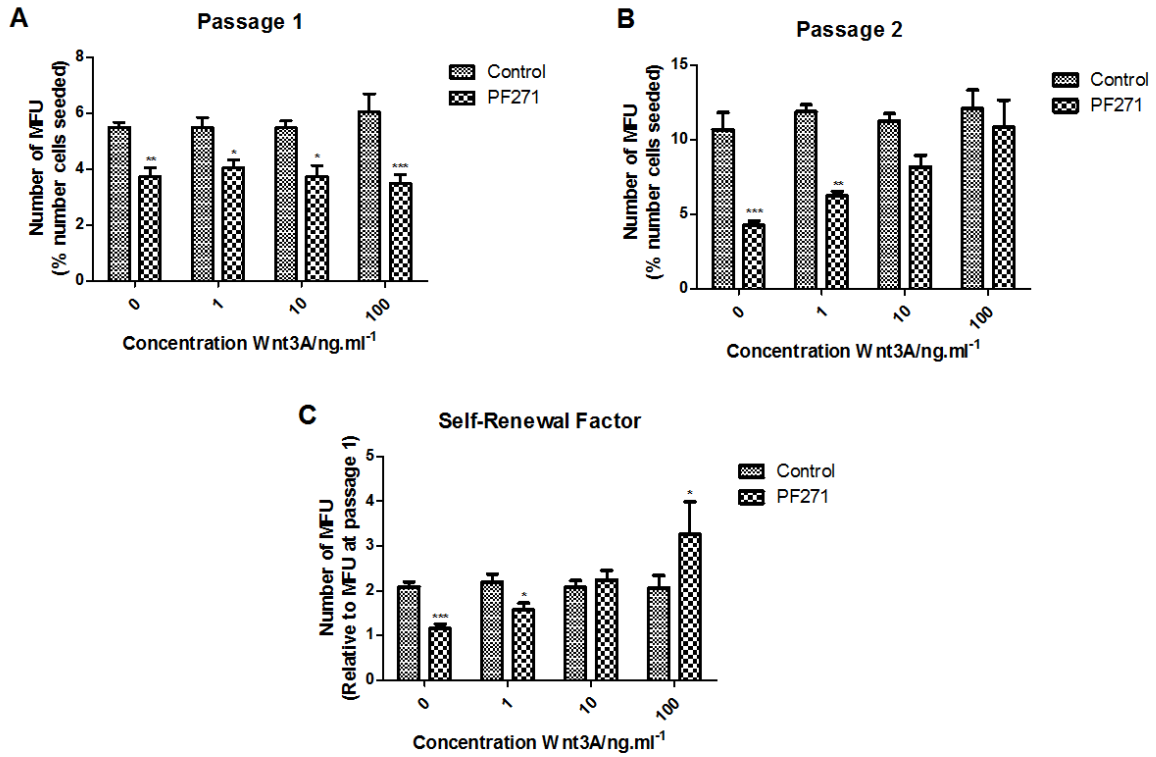


Figure 5.7 – Analysis of the effects of Wnt3a on mammosphere forming potential and self-renewal in FAK inhibited cells – (A) Passage one counts of MFUs as a percentage of total cells seeded showed a significant decrease in mammosphere-forming capacity in PF271 cells versus vehicle only controls. Addition of Wnt3a at any of the described doses had no additional or opposing effects of MFUs. **(B)** Analysis of passage 2 MFU numbers showed a significant reduction in spheres formed following PF271 treatment. Addition of Wnt3a could restore mammosphere-forming potential in FAK inhibited spheres in a dose-dependent manner. **(C)** Evaluation of SRF showed that PF271 attenuation of self-renewal could be rescued by Wnt3a stimulation in a dose dependent manner. All error bars represent SEM; n=3. *p<0.05; **p<0.01; ***p<0.001.

5.6. Discussion

CSCs represent a significant obstacle to therapeutic intervention and disease progression. As such, the identification of novel therapeutic targets in this subpopulation of cells is essential to maximise treatment efficiency and minimise the risk of disease recurrence. To this end, we began by demonstrating that MDA-MB-231 cells exhibited a subpopulation of CSC-like cells defined by the $CD44^{+}/CD24^{low/-}$ cell surface markers. Interestingly the number of cells positive for this event was extremely high suggesting that most cells in this TNBC model are CSC-like. Given the proposed role for CSCs in driving metastatic events, this could explain the aggressive nature of MDA-MB-231 cells. However, if TNBC tumours, such as those from which the MDA-MB-231 cell model is derived, had this many CSCs, one would image such patients would be extremely unresponsive to traditional chemotherapies due to the inherent chemo-resistant nature of such cells. In practice, a large number of patients diagnosed with TNBC actually display very good responses to chemotherapeutic intervention thus undermining this idea (Bramati *et al.* 2014). Moreover, our subsequent exploration into CSCs revealed that when ALDH1 is used as a marker, the number of stem-like cells is significantly lower versus $CD44^{+}/CD24^{low/-}$ cells, with a correlation between the numbers $ALDH1^{+}$ cells and those exhibiting mammosphere-forming potential. These data bring into question the validity of using $CD44^{+}/CD24^{low/-}$ based identification criteria as a reflection of CSC subpopulations.

Others have shown similar results when exploring CSC markers in breast cancer cell lines. For example, Sheridan et al (2006) showed a very similar proportion of $CD44^{+}/CD24^{low/-}$ cells in the MDA-MB-231 model (reported at 85%) to those presented here (Sheridan *et al.* 2006), whilst Charafe-Jauffret *et al.* (2009) similarly demonstrated that MDA-MB-231 cells have very low numbers of ALDH+ cells. These studies agree with our data thus validating the results presented here as genuine reflections of marker presentation and not due to experimental error. Moreover, it highlights a limitation of this study, namely how to define CSCs by surface marker presentation. In general, this is an issue seen throughout CSC research, largely due to variations in individual protein expression which may result in the identification of false CSCs. This is seen here as MDA-MB-231 cells have been shown to express very high levels of CD44 (Ricardo *et al.* 2011), which has resulted in an extremely high number of "CSCs" being identified. Such cell-specific variations in "CSC marker" expression has led to much debate around the universality of such profiles, leading to increased identification of new markers and thus repeated refinement

of the CSC population profiles. As such, the question remains open as to how many markers would be required to truly define CSC populations universally. On an individual basis however, the use of limited marker profiles can be utilised. In MDA-MB-231 cells for example, ALDH+ cells do reflect a stem-like population given the proportions of these cells closely mirror those observed in mammosphere assays (as presented here) and the increased metastatic potential of this subpopulation (Croker *et al.* 2009).

We went on to investigate whether FAK was an important player for the presence for CSCs in the parental cell population. Our data suggested that FAK activity was not key to the number of CSCs detected. This appears to be contradictory to other studies that suggest that FAK is required for sustained proliferation of stem cells, both normally and in the cancer-context. In patient-derived xenograft models of mesothelioma, treatment with the FAK inhibitor VS-4718 eliminated CSCs that were enriched by cisplatin and pemetrexed chemotherapy treatment (Shapiro *et al.* 2014). The same holds true in breast cancer, where mammary-epithelial specific ablation of FAK in MMTV-PyMT mice considerably reduced progenitor and CSC subpopulations in primary tumours (M. Luo *et al.* 2009). Although differing to our observations, neither of these studies explored TNBC and, given the intrinsic differences between subtypes, may not reflect the behaviours of cells reflected by our model. Moreover, as both studies were conducted *in vivo*, decreases in the CSC-subpopulation may be an indirect result of FAK inhibition/removal. Given these models have the full influence of biology, i.e. they have a tumour microenvironment and immune system, these auxiliary cells may be influencing CSC numbers through increased targeting of this subpopulation following FAK suppression.

We went on to observe that MDA-MB-231 cells have the capacity to form anoikis-resistant spheroids in the CSC-enriching mammospheres assays. This came as no surprise as it has been well established that these cells have stem-like subpopulations which lead to formation of mammospheres. When mammospheres were defined by volume (as proposed by Shaw *et al.* 2012) we saw that FAK suppression or inhibition could significantly attenuate mammosphere-formation. This is in concordance with others who have highlighted a role for FAK in mammosphere formation. For example, Williams and colleagues (2015) demonstrated that repression or inhibition of FAK kinase function perturbs capability of breast cancer cells *in vitro* to form mammospheres over 60µm in volume (Williams *et al.* 2015). However, we also noted that inhibiting FAK activity also impaired mammosphere volume, an observation that may be

reflective of a decreased ability for non-adherent proliferation. As such, defining mammospheres by a minimum acceptable size may not truly reflect the number of MFUs and their changes in response to target inhibition. Consequently, we repeated these experiments defining mammospheres primarily by morphology, although a low minimum size limit was used. Our results demonstrated that inhibition of FAK does not perturb the number of MFUs at passage one suggesting that FAK activity does not contribute to the establishment of anoikis-resistant spheroids that arise from CSCs but, in contrast to Williams *et al*. 2015, rather impairs their subsequent proliferation.

In the study by Williams and colleagues (2015) they used the SUM225 cell line as their established model of breast cancer. This model is defined as epithelial-like whereas our MDA-MB-231 cells are more mesenchymal in nature (Grigoriadis *et al.* 2012). This difference is important as it has been proposed that FAK contributes differently to CSCs based on their genetic signatures. Luo et al (2013) explored the relative contribution of FAK kinase function to stem/progenitor cell functions through use of a kinase-dead (KD) mutant knock-in (Luo *et al.* 2013). These experiments revealed that luminal-like progenitor cells rely on FAK kinase function for proliferation, whereas mammary stem-cell like cells (MaSC) were supported by kinase-independent activities. Consistent with this, using a breast cancer cell model which reflected luminal-progenitor or MaSCs, they showed that FAK kinase function is essential to proliferation and mammosphere forming potential of luminal-like cancer cells but not those reflective of MaSCs. Given that MDA-MB-231 fall into the latter category, this work infers that FAK kinase function would be dispensable for spheroid formation and proliferation in this model. Although reflective of our work in terms of mammosphere formation, this study seems to contradict our observation that FAK-kinase inhibition perturbs non-adherent proliferation of MDA-MB-231 mammospheres.

As FAK has a well-established role in mediating integrin-driven attachment signals (Desgrosellier and David 2015), it stands to reason that it would be involved in anoikis response. Indeed, inhibition of FAK results in apoptosis when cells are grown in anchorage independent conditions. For example, in acute lymphoblastic leukaemia, precursor B-cells lacking functional Ikaros, a transcription factor that negatively-regulates non-adherent proliferation, exhibit a restoration of anoikis following FAK inhibition (Joshi *et al.* 2015). The inverse is also true where stimulation of FAK activity can enable cells to survive conditions of anoikis. Indeed, stimulation of FAK by

upstream protein tyrosine kinase 6 (PTK6) enables anoikis survival in breast cancer cells (Zheng *et al.* 2013). Consequently, although more experiments are needed for confirmation, FAK may provide a means by which MDA-MB-231 cells can survive and propagate under non-adherent conditions.

Having established that FAK contributes to initial mammosphere forming potential and anoikis proliferation of MDA-MB-231 cells, we next wished to explore the contribution of FAK to self-renewal of CSCs. As such initial spheroids were passaged into subsequent assays to observe self-renewal capacity alongside non-adherent cell survival and proliferation. Following this procedure, we observed a significant reduction in passage 2 MFUs derived from kinase inhibited or FAK suppressed cells versus passage 1 numbers, as well as significantly smaller mammospheres. This was regardless of whether mammospheres were defined by size or morphology. These data infer that self-renewal capabilities are significantly attenuated by a loss of normal FAK function. Our data aligns with several others who have also demonstrated a role for FAK in mediation of self-renewal. For example, ablation of FAK in mammary epithelial cells of MMTV-PyMT mice caused a significant reduction in self-renewal of the CSC subpopulation when grown *in vitro* (M. Luo *et al.* 2009). Moreover, progenitor/CSCs from these mice displayed significantly impaired tumourigenicity, coupled with impaired ability to sustain these subpopulations within tumours, further highlighting both impaired self-renewal and the potential for targeting FAK as a means of reducing breast cancer CSCs (M. Luo *et al.* 2009). Interestingly, the previously highlighted study by Luo et al (2013) suggested that inhibition of catalytic function had no effect on the mammosphere-forming capabilities of MDA-MB-231 cells (Luo *et al.* 2013). This observation also held true in MaSCs and was expanded to show that self-renewal was also unimpaired by loss of FAK kinase activity. From this, one might assume that FAK kinase inhibition may not be sufficient to impair self-renewal in MDA-MB-231 cells given their similarity to MaSCs, a result in contrast to our data. However, the cancer model studies did not explore self-renewal, unlike the examination of progenitor and MaSC subpopulations. Thus, it may be that MaSC-like cancer cells actually do exhibit impaired self-renewal following kinase inhibition, unlike their non-tumourigenic counterparts, especially as cancer cells exhibit multiple differences versus comparative healthy cells.

We wished next to examine the mechanisms of action that underpinned the stem-like characteristics of MDA-MB-231 cells. Specifically, we chose to focus on evaluating potential

mechanisms by which FAK controls self-renewal, given that FAK appeared to modulate CSC self-renewal more-so than mammosphere forming ability. Consequently, we explored changes in downstream signalling events that have been previously linked to the self-renewal of normal and cancer stem-like cells. In **chapter 4,** we demonstrated that FAK contributed to cell migration through modulating Akt^{S473} activity. Again here, we demonstrated that FAK inhibition decreases AKT^{S473} activity. Due to its position in the hierarchy of several signalling pathways, Akt activity in response to reduced FAK^{Y397} activity could justifiably mediate downstream signals which lead to impaired MDA-MB-231 CSC self-renewal. Indeed, Akt has been shown to contribute to self-renewal in several cell types. For example, neural stem-cells are unable to self-renew in Pi3K/Akt inhibited cells (Le Belle *et al.* 2011). Furthermore, in breast cancer cells, IGF-1R driven self-renewal relies on activation of Pi3K/Akt/mTOR pathway, the inhibition of which can significantly attenuate stem-like subpopulations, consistent with decreased self-renewal (Chang *et al.* 2013). These studies support our results, reinforcing the hypothesis that FAK acts through Akt to modulate self-renewal of MDA-MB-231 CSCs.

Although it may play some role, the activity of Akt in of itself cannot directly regulate self-renewal, thus we sought to explore downstream signals which can. As seen in migrating cells, PF271 decrease of Akt was accompanied by a significant alleviation of inhibitory phosphorylated $GSK3\beta^{S9}$. Moreover, this was associated with a decrease in active, non-phosphorylated β-catenin and a concomitant increase in inhibitory phosphorylation of $\beta\text{-catenin}^{S33/37/T41}$. The effects of GSK3β on β-catenin activity are well documented, with active GSK3β causing destabilisation and degradation of β-catenin through phosphorylation of S33/37 and T41 (Yost *et al.* 1996). It has long been established that β-catenin is a central protein in the regulation of stem-like cell traits. For example, He et al (2004) demonstrated that intestinal stem-cell self-renewal is negatively influenced by downregulating Wnt/ β-catenin signalling (He *et al.* 2004). Additionally, this paper highlighted a link between Akt and activity of β-catenin further supporting the data presented here. Importantly, previous studies have highlighted an axis by which FAK can regulate β-catenin through Akt. For example, in periodontal ligament cells, mechanical load-induced β-catenin activation was significantly impaired following pharmacological inhibition of FAK, dependent on suppression of Akt (Premaraj *et al.* 2013). Furthermore, administration of anticancer agent excisanin A could significantly reduce invasion and MMP production in MDA-MB-231 or SkBr3 cells, resulting from a downregulation of β-catenin due to decreased activity of FAK and Akt, as well as an increase in active GSK3β (Qin *et al.* 2013). Taken together with our data, we

hypothesise that FAK regulates MDA-MB-231 CSC self-renewal by stabilising β-catenin through Akt-dependent suppression of GSK3β. Moreover, these data highlight the therapeutic relevance of targeting FAK for attenuation of CSC self-renewal.

Our data demonstrated that FAK can contribute to the stability of β-catenin in MDA-MB-231 mammospheres, possibly contributing to self-renewal. To assess this potential mechanism further, we stimulated mammospheres with Wnt3a to explore its contribution to FAK-dependent stabilisation of β-catenin. Indeed, Wnt3a treatment could rescue self-renewal in FAK-inhibited mammospheres, indicating a possible contribution of Wnt-signalling downstream of FAK in MDA-MB-231 CSCs. This in agreement with several studies that demonstrate a significant contribution of Wnt signalling to stem-like traits of breast cells, including CSCs. For example, dynamic changes in Wnt/β-catenin signalling governs cell fate in mammary epithelial cells, particularly through mediation of stem and progenitor cells (van Amerongen *et al.* 2012). Moreover, Lamb and colleagues (2013) demonstrated increased activation of Wnt signalling in breast CSCs versus normal stem-like cells, whist impairing such signals led to a preferential decrease of patient-derived metastatic breast CSCs compared to normal cells (Lamb *et al.* 2013). Together these clearly showed the relevance of Wnt signalling in CSCs. Moreover, this paper demonstrated that tumours that are basal-like in nature (of which TNBCs are primarily) have increased levels of the Wnt receptors LRP5 and LRP6 inferring a greater potential for utilisation of these signalling pathways (Lamb *et al.* 2013). Taken together these studies support a role for Wnt signalling in MDA-MB-231 breast CSCs. Specifically in terms of self-renewal, PF271 inhibition of DCIS-derived or SUM225 (Her2+ model) mammosphere cultures caused a significant reduction in SRF which could be rescued through stimulation of Wnt3a (Williams *et al.* 2015). Further luciferase reporter studies demonstrated Wnt activity is specifically increased in PF271 treated cells following Wnt3a stimulation. Although not in a model of TNBC, these results directly mirror our findings and lend credence to our hypothesis that Wnt3a contributes to FAK-driven self-renewal, through β-catenin stabilisation in MDA-MB-231 CSCs. In further support of our proposed mechanism of action, this paper also demonstrated that *in vivo* xenograft models of DCIS exhibited reduced active β-catenin in response to impaired FAK stimulation (Williams *et al.* 2015). This was comparable to what we observed in PF271 treated mammospheres.

However, one limitation to the studies undertaken here is that due to time-constraints we did not explore the influence Wnt3a stimulation of mammospheres had on FAK-linked signalling.

Our data suggested that Wnt3 acts downstream of FAK, as it can rescue self-renewal in inhibited cells, but Wnt may be restoring the phenotype through some other means. For example, stimulation with Wnt3a could be acting on FAK to restore activity and thus circumvent inhibition. Indeed, studies have shown that active Wnt signalling can influence FAK activity (Pandur *et al.* 2002). This scenario is unlikely however, as if FAK activity was increased in the presence of Wnt3a, initial mammosphere-forming capacity would also be restored, given that this trait is also reduced by PF271 treatment. Interestingly, FAK has been shown to regulate Wnt3a production and subsequent activity of the Wnt pathway (Fonar *et al.* 2011). Consequently, FAK may play a dual role in stabilising β-catenin thorough influencing GSK3β-mediated degradation and directly influencing Wnt-dependent activation.

5.6.1. Conclusions

Within this chapter we have demonstrated that FAK plays an essential role in the mediation of stem cell-like traits in MDA-MB-231 cells, particularly self-renewal. This hinged on FAK's ability to regulate Akt activity and subsequent modulation of GSK3β and β-catenin to sustain symmetrical cell division. Moreover, we showed that Wnt3a likely acts downstream of FAK, likely through influencing the activity of β-catenin, to contribute to MDA-MB-231 CSC self-renewal. This chapter also highlighted the relevance of FAK as a therapeutic target in TNBC cells, particularly in the function of a stem-like subpopulation, hypothesised to mediate aggressive behaviours and disease relapse.

6. Results (IV)

Development and screening of novel FAK inhibitors for TNBC

6.1. Introduction

Our data so far suggests that FAK plays a central role in the maintenance of the TNBC phenotype in terms of its ability to contribute to proliferation and metastatic-supporting characteristics (migration and invasion). Moreover, FAK appears to support a "stem-like" phenotype in these cells. Consequently, targeted inhibition of FAK may represent an effective therapeutic strategy for this breast cancer subtype. Indeed, several FAK inhibitors have been developed and have moved into various stages of clinical assessment. It is important to recognise however, the majority of FAK inhibitors available are ATP-competitive kinase inhibitors. A caveat to using small molecule FAK inhibitors of FAK kinase activity is that there is potential for non-specific inhibition of alternative kinases with associated toxicities (Hartmann *et al.* 2009) limiting their clinical application. Moreover, FAK is known to regulate numerous cellular functions, independent of kinase activity (Lim, Chen, *et al.* 2008; Luo *et al.* 2013); the use of kinase-inhibitors will show limited actions against these behaviours. A potential strategy in developing novel FAK inhibitors that target alternative parts of the molecule may prove useful in inhibiting the scaffolding function of FAK; such inhibitors may prove to be useful agents in the future in place of, or alongside, inhibitors of FAK kinase activity. This may be of particular significance in diseases such as TNBCs where targeted therapies are currently limited and target identification for new agents is yet to create effective clinical agents. Taking these points into consideration, a drug discovery project was initiated in order to design and screen novel inhibitors that target alternative regions of FAK as potential agents for TNBC treatment.

6.1.1. Chloropyramine as a potential starting point for novel FAK-compound development

FAK has several regions outside the traditional kinase sites that may be suitable for therapeutic targeting, including sites situated near functional residues of activity (e.g. Y577 and Y861). Evaluation of the structures present within FAK (through Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank), reveals a very promising structural pocket for drug targeting within the FAT-region, around tyrosine residue 925 **(figure 6.1)**. Interestingly, a previous study has identified a compound (chloropyramine) that targets FAK protein-protein interactions in very close proximity to this region of interest (Gogate *et al.* 2014). Chloropyramine (structure shown in **figure 6.1B**) was synthesised in 1949 as a first-generation antihistamine (Vaughan *et al.* 1949) and is commonly prescribed as such in Eastern European countries under the brand name Suprastin. Additionally, close structural derivatives of

chloropyramine are approved for use in the USA for a range of health complaints including pre-menstrual cramps (available over the counter as Midol®), rhinitis and asthma (Wilton *et al.* 2016). In cancer biology, chloropyramine has been shown to disrupt the physical interaction between FAK and VEGFR-3 whilst reducing the phosphorylation of both molecules (Kurenova *et al.* 2009). This resulted in a significant suppression of tumour cell growth *in vitro* and *in vivo* across a range of cancer cell types, also being able to act synergistically with a traditional chemotherapeutic agent (doxorubicin) (Kurenova *et al.* 2009). In addition, in 2011 the US FDA approved a grant for further study into its use as a therapeutic in pancreatic cancer (Wilton *et al.* 2016). This compound therefore provides a good lead compound for further investigation into novel FAK inhibitors for triple-negative breast cancer, as it exhibits good anti-cancer properties whilst exhibiting a structure shown to be metabolically stable and has already received FDA approval as a therapeutic treatment.

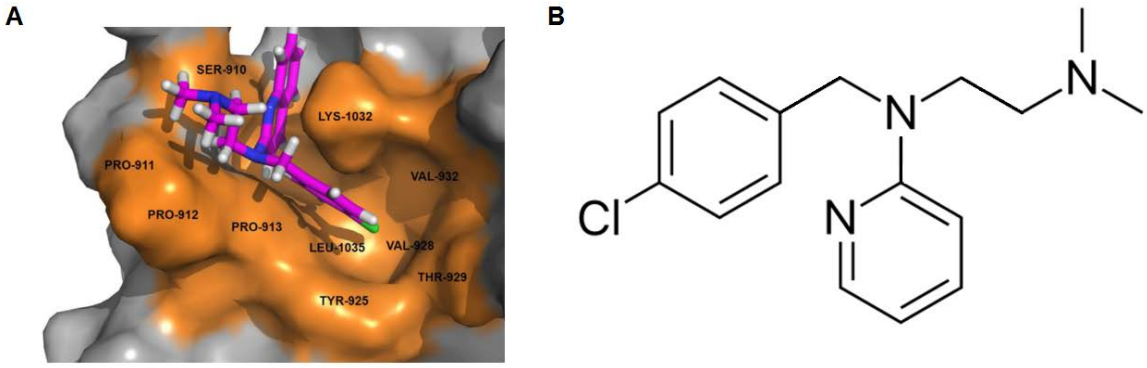


Figure 6.1 – The FAT-region of FAK contains a promising region for drug targeting – (A) Surface representation of the FAT-region of FAK (grey) with druggable pocket highlighted orange containing a chloropyramine analogue (adapted from (Gogate *et al.* 2014)). **(B)** Linear structure of potential starting compound chloropyramine.

6.1.2. Outline of design processes used for development of novel FAK inhibitors in TNBC

In order to design new therapeutic agents as efficiently as possible, it was decided that two separate approaches of rational drug design would be used. The first collection of inhibitors (herein know as batch 1) were derived using virtual-screening of numerous structures from a database of known compounds (SPECS library) that had a strong degree of fit within the aforementioned pocket in the FAK-FAT sequence. Briefly, analysis was conducted using a "computational funnel" which selects molecules from a virtual library based on increasingly stringent criteria to yield a manageable number of potential, top performing agents. This approach (summarised is **figure 6.2A**) began with a total of 210,103 potential compounds and was reduced through the highlighted process to a final group of 25 compounds. These compounds were subsequently purchased and tested *in vitro*. The second approach involved lead compound optimisation based on the structure of chloropyramine. Essentially, the central structure of chloropyramine was maintained and its functional groups (highlighted in **figure 6.2B**) altered to improve its binding affinity for FAK and hopefully its ability to alter normal triple-negative cell behaviours. These compounds were then synthesised in-house by Dr Sahar Kandil of the Medicinal Chemistry department.

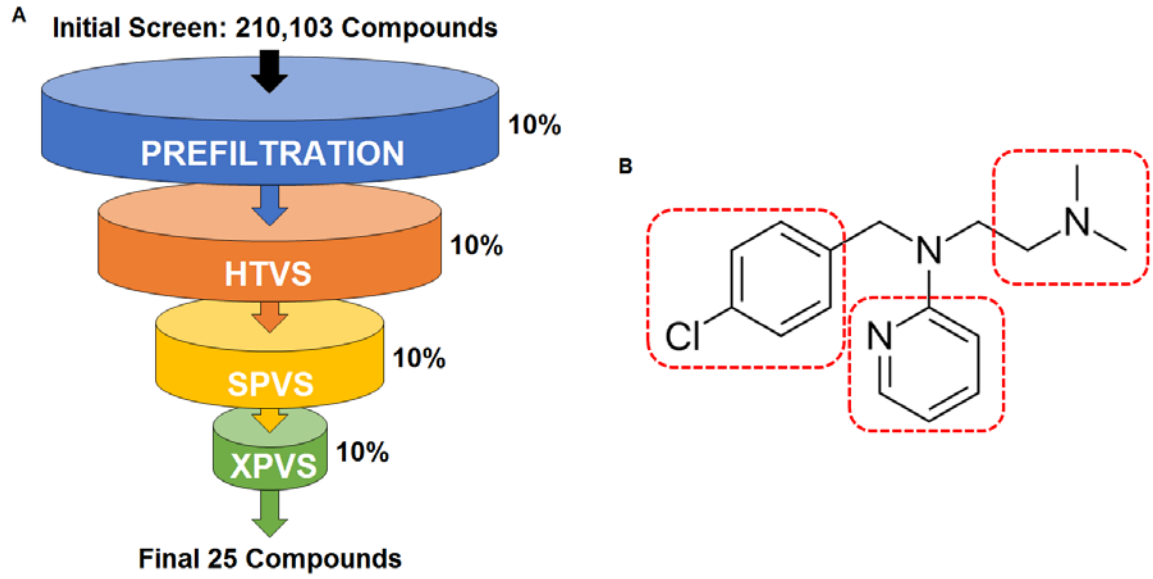


Figure 6.2 – Summary of the two approaches used to generate novel FAK inhibitors targeting protein-protein interactions – (A) Initial screening of compounds targeting the FAT-domain pocket revealed 210,103 potential compounds that could target this site. These compounds were then sequentially filtered through increasing stringent virtual screens to yield a manageable number of compounds to be synthesised and tested. HTVS, high-throughput virtual screening; SPVS, standard-precision virtual screening; XPVS, extra-precision virtual screening. **(B) –** The linear structure of chloropyramine showing the 3 functional regions (red boxes) that were altered in an attempt to optimise this compound as a FAK inhibitor in TNBC cells.

6.1.3. Aims and Objectives

This chapter aimed to explore the potential of these novel compounds in inhibition of FAK-mediated cell behaviours in TNBC cells. It also began to examine mechanisms by which potential agents alter FAK-function thus supporting lead compound identification to support subsequent development. To do this, the following objectives were evaluated:

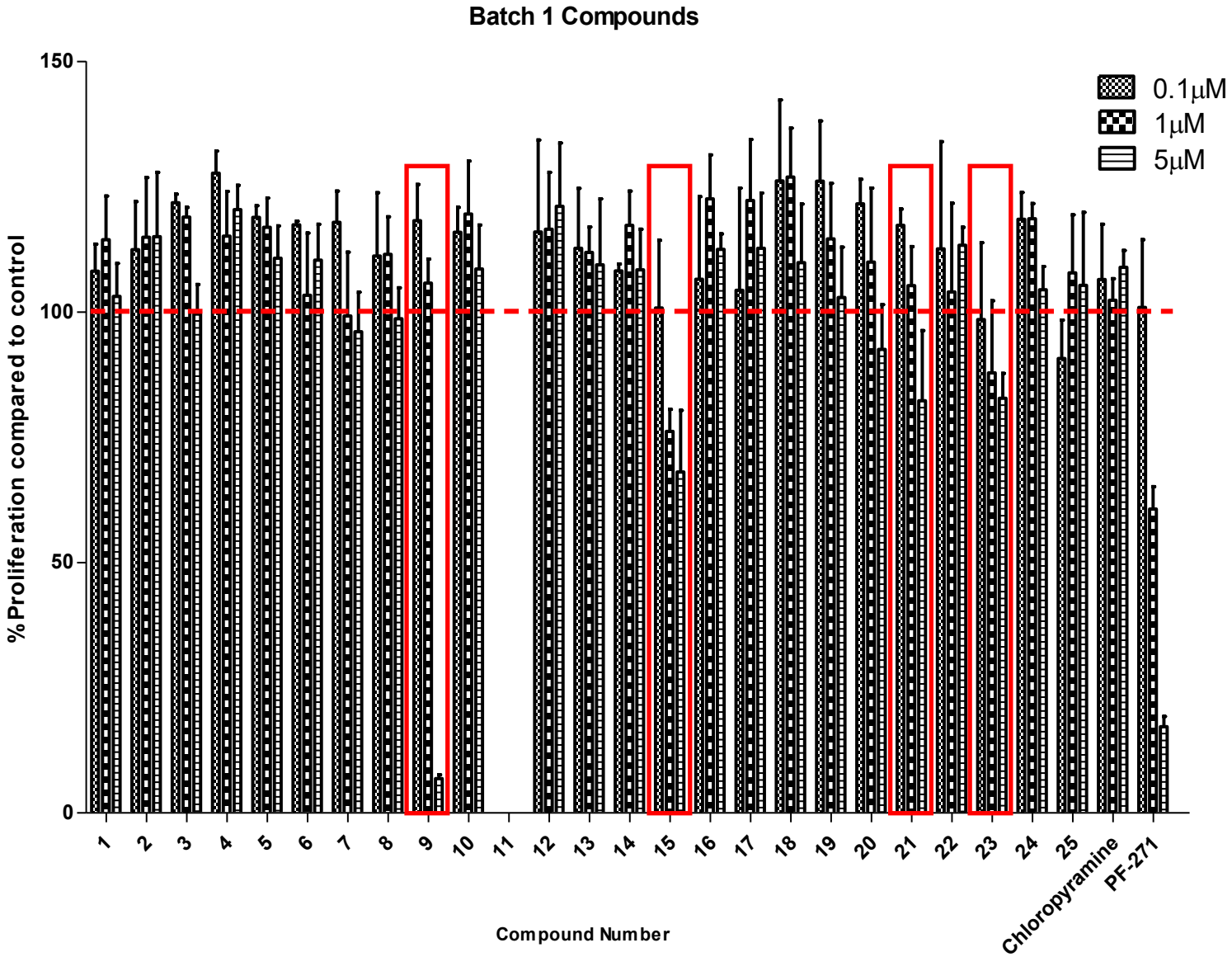
- Assess novel compounds for their capacity to inhibit normal TNBC proliferation through a series of MTT assays.

- Evaluate the efficacy of the new agents in suppressing the highly-migratory phenotype of MDA-MB-231 cells.

- Explore how select compounds alter FAK expression/activity, as well as cellular localisation.

6.2. Screening of novel anti-FAK agents against MDA-MB-231 cell proliferation

Having demonstrated that FAK can contribute to TNBC proliferation **(chapter 3.7)**, we first wished to determine whether the newly synthesised compounds could adversely affect cell proliferation in MDA-MB-231 cells. To achieve this, MTT assays were performed following 72-hour incubation with 3 doses of each compound, along with chloropyramine and PF271 as a comparative control. The first batch of compounds (designed through virtual screening) contained several compounds that could significantly reduce cell number at one or more concentrations when compared to the vehicle only controls **(figure 6.3A)**. As expected, PF271 significantly suppressed cellular proliferation in the triple negative cells, whilst the effects of chloropyramine were negligible. In contrast, no compounds in batch 2 were sufficient to reduce cell numbers even at the highest concentration **(figure 6.3B)**. PF271 once again displayed significant changes in proliferation.



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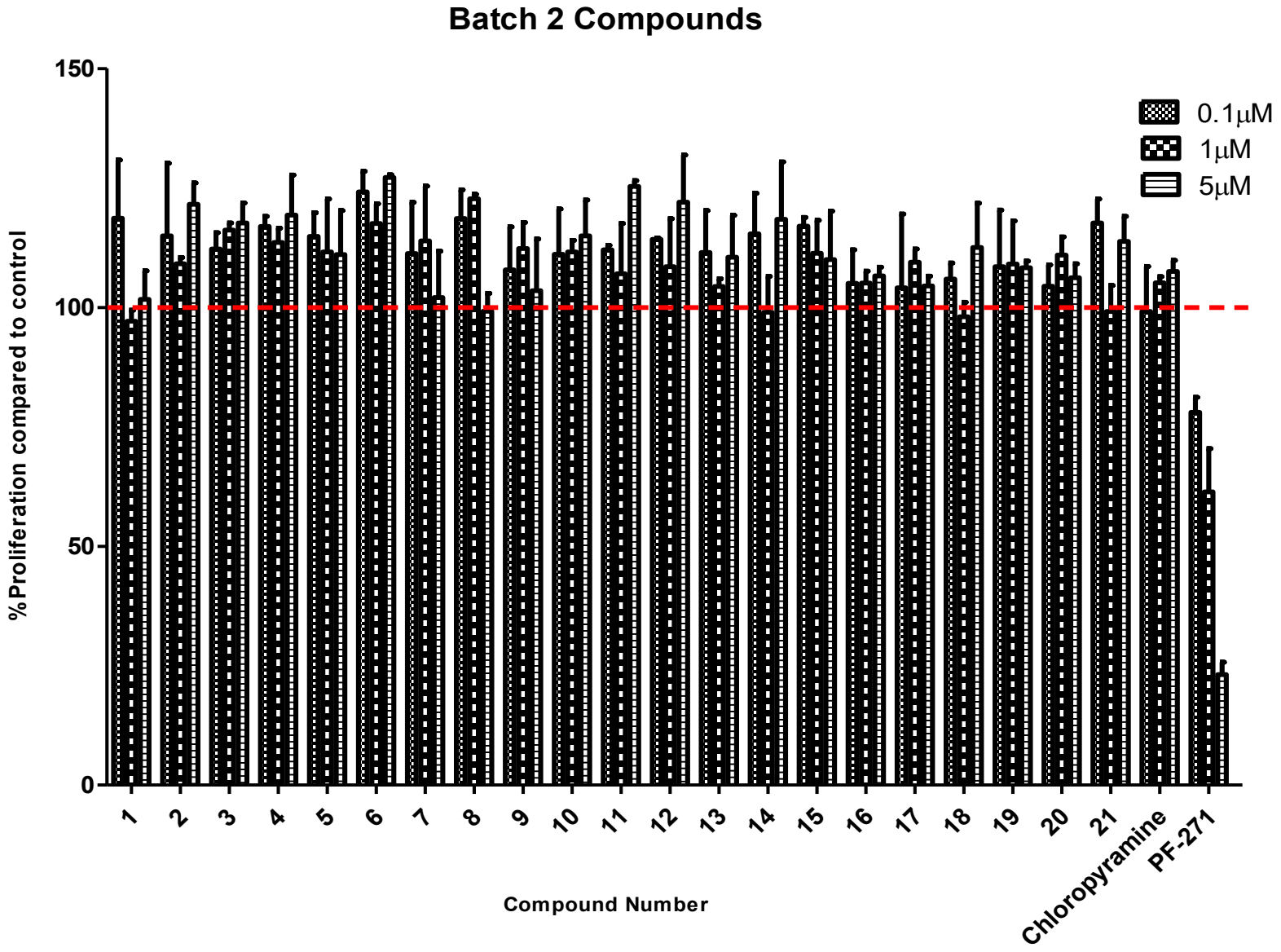


Figure 6.3 – Analysis of proliferation changes in response to novel inhibitor treatment – (A) Relative changes in cell proliferation following 72-hours incubation with batch 1 novel inhibitors at different concentrations, normalised to vehicle-only control. Red boxes highlight top 4 anti-proliferative compounds for subsequent analysis. **(B)** Proliferation changes caused by batch 2 compounds compared to vehicle-only control. All error bars represent SEM; n=3. Red dashed line marks level of proliferation by vehicle-only control.

Having identified the top 4 compounds capable of suppressing proliferation, further MTT assays were performed using a wider dose range over the same treatment time **(figure 6.4)**. Very limited response was noted across the novel compounds, especially at lower dose-ranges. However, compound 9 displayed a significant ability to inhibit TNBC proliferation, which was comparative to the effects seen in PF271 treated cells. Chloropyramine again showed negligible effects.

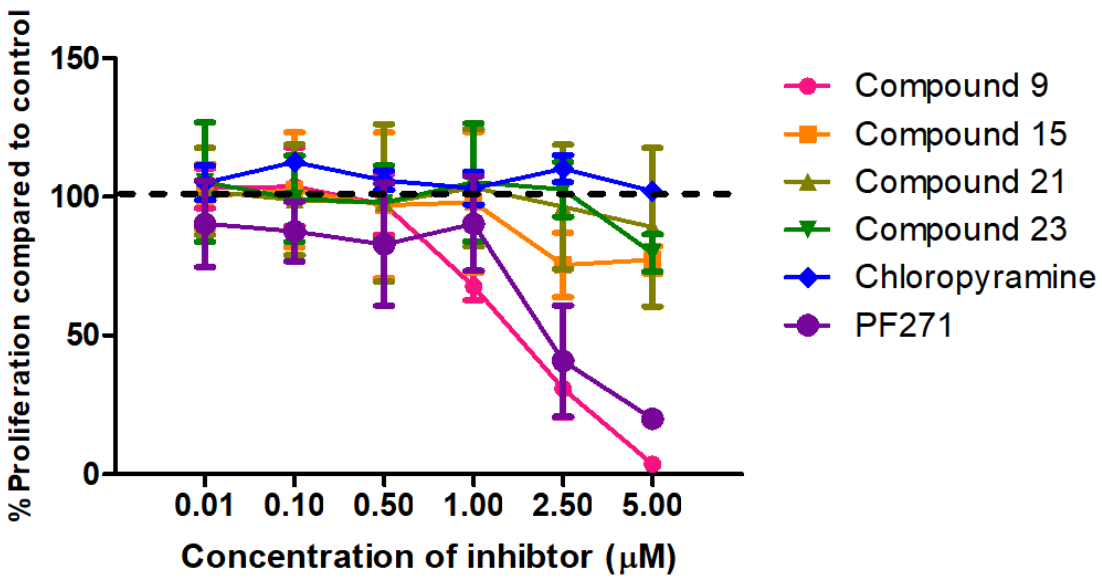


Figure 6.4 – Dose response of the top 4 proliferation inhibiting compounds – MTT analysis on the top 4 proliferation inhibiting compounds following 72-hour incubation with each compound normalised to vehicle-only control cells. All bars represent SEM; n=3.

6.3. Several batch 1 compounds can significantly inhibit wound healing capability in triple-negative cells

In addition to examining the novel compounds for anti-proliferative capabilities, their potential to inhibit migration was also assessed. This was to allow for the identification of compounds that could be utilised as inhibitors in one or both capacities, broadening the selection criteria for "successful" compounds. The batch 1 agents showed several compounds capable of significantly reducing migration linked to wound healing **(figure 6.5A)**, including the base compound chloropyramine, previously shown to have limited effects on TNBC proliferation. PF271 once again significantly reduced migration as in earlier experiments. Interestingly, compound 9 (previously identified as a very promising anti-proliferative agent) showed not only a significant ability to inhibit migration but suppression was comparable to that caused by PF271-mediated inhibition (no significant difference was observed between these compounds). In contrast to batch 1, the second batch of compounds exhibited no significant ability to reduce wound healing, while PF271 and chloropyramine caused significant reductions in migratory ability **(figure 6.5B)**.

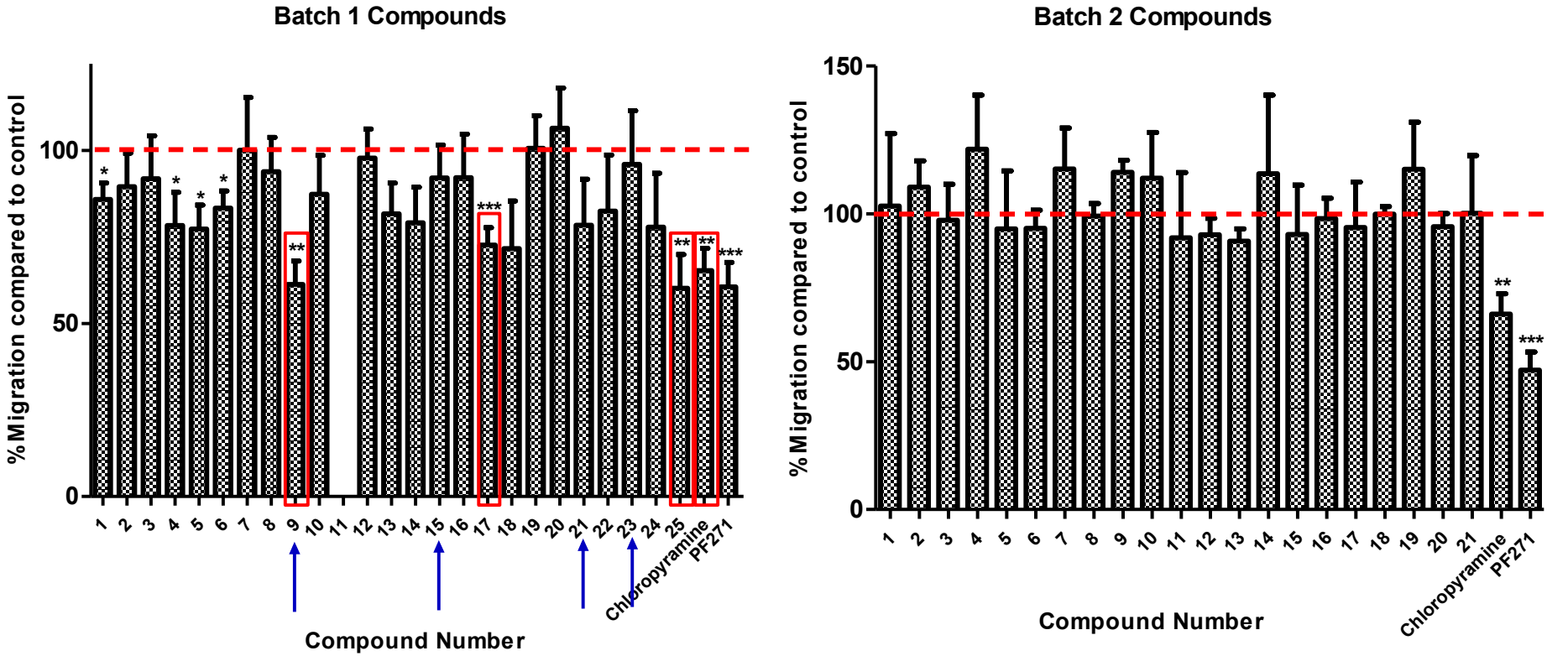


Figure 6.5 – Analysis of differences in wound healing in response to novel inhibitor treatment – (A) Relative changes in cell migration, represented as percentage of wound closure, following 24-hour incubation with batch 1 novel inhibitors, normalised to vehicle-only control. Red boxes highlight top 4 anti-migratory compounds., while blue arrows represent the previously identified top anti-proliferative agents. **(B)** Wound healing changes caused by batch 2 compounds compared to vehicle-only control. All error bars represent SEM; n=3. Red dashed line marks level of proliferation by vehicle-only control. *p<0.05; **p<0.01; ***p<0.001.

6.4. Compound 9 inhibits TNBC cell proliferation and migration

So far, compound 9 has shown potential as an inhibitor for the suppression of multiple phenotypes within a TNBC cell model. To further explore the relevance of this compound as a new lead for a novel FAK inhibitor, more robust analyses were performed to support the previous findings and give a better comparison to PF271. As previously shown, proliferation was significantly suppressed versus vehicle-only control by compound 9 following 72-hours incubation **(figure 6.6A)**. Prior to this time point, no significant change in proliferation was observed. Additionally, this perturbation in proliferation was sustained until 5 days post-treatment, after which point cell death from over-confluency became a factor. When the efficacy of compound 9 as an anti-proliferative agent is compared to that of PF271, it was seen to be less effective as an inhibitory agent, especially in later stage sustained inhibition. Inhibition of FAK through PF271 was sufficient for long-term reductions in proliferative capacity.

Compound 9 has also shown potential as an antimigratory agent for triple-negative breast cancer cells. Consequently, Boyden chamber assays were performed to allow for an in-depth assessment of the efficacy of compound 9 to alter cell migration in comparison to the existing inhibitor PF271. When compared to the vehicle only control, compound 9 and PF271 exhibited significant suppression of MDA-MB-231 cell migration **(figure 6.6B and C)**. In contrast to the observations in **section 6.3 (figure 6.5A)**, the more robust analysis undertaken here reveals that PF271 is significantly better at inhibiting migration than compound 9.

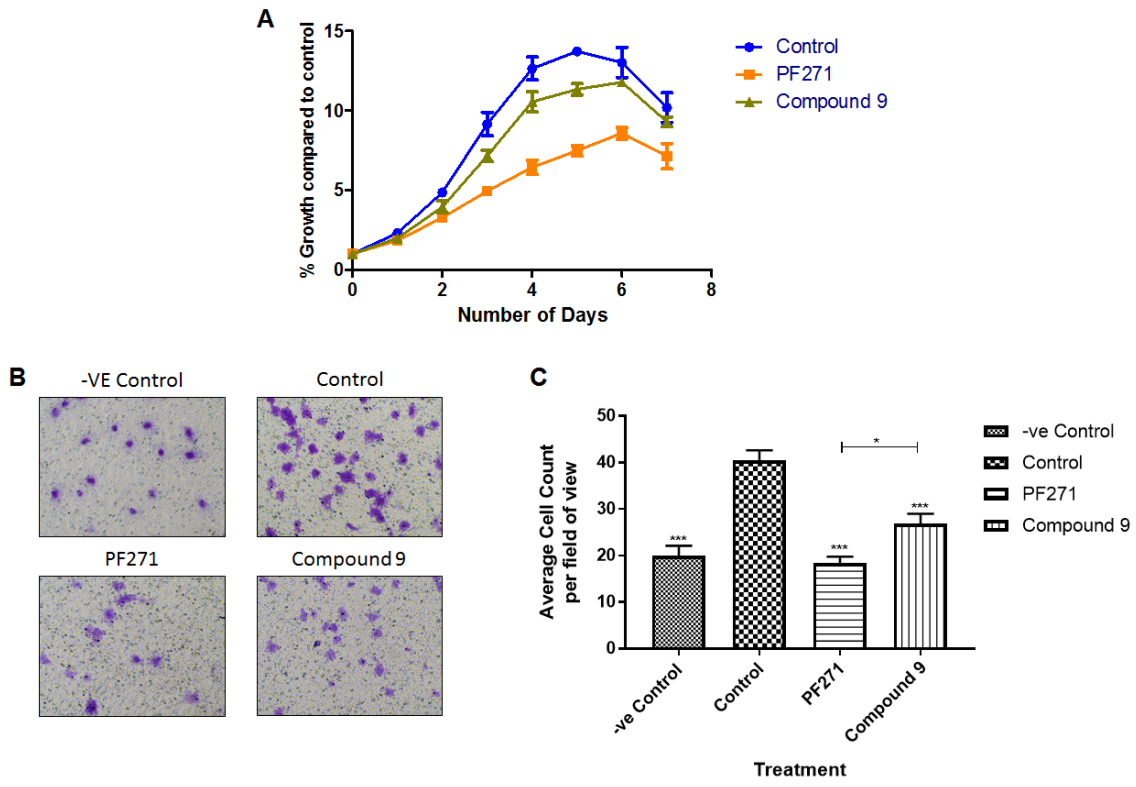


Figure 6.6 – Compound 9 significantly augments TNBC cellular proliferation and migration – (A) Relative rate of MDA-MB-231 cell proliferation over 7-days in the presence of compound 9 or PF271 (1µM), normalised to cell numbers in the vehicle-only control. **(B)** Resulting images of Boyden chambers stained with crystal violet following 18-hours serum-stimulated migration in the presence of compound 9 and PF271 (1µM). Negative (-ve) control shows cell migration without serum stimulation. **(C)** Quantification of Boyden chamber assays, showing average number of cells present following 18-hour migration. Data shows average number of cells/membrane from duplicate wells across 3 separate replicates All error bars represent SEM; n=3. *p=0.05; **p=0.01; ***p<0.001.

6.5. Compound 9 does not alter the activity and expression of FAK and associated kinases

Given that compound 9 is supposed to inhibit protein-protein interactions, we wanted to explore if this compound had any additional effects on FAK activity/expression. Moreover, as we had previously identified Akt as a key downstream target of FAK in our models of TNBC, thus it was decided that the expression/activity of Akt would also be assessed in response to compound 9 treatment. Western blots were performed on cells treated with chloropyramine, PF271 and compound 9 for 1-hour. Compared to vehicle-only treated cells, all compounds caused no significant changes in total protein levels of FAK or Akt inferring that expression is unaltered **(figure 6.7).** Additionally, no change in activity of FAK^{Y397} or Akt^{S473} when exposed to chloropyramine or compound 9, whilst PF271 treatment was sufficient to cause a significant decrease in phosphorylation. As compound 9 targets the FAT-sequence of FAK, the activity of FAK^{Y925} (located in this region) was also evaluated. Whilst PF271 significantly augmented activity at this site, compound 9 and chloropyramine had no effect.

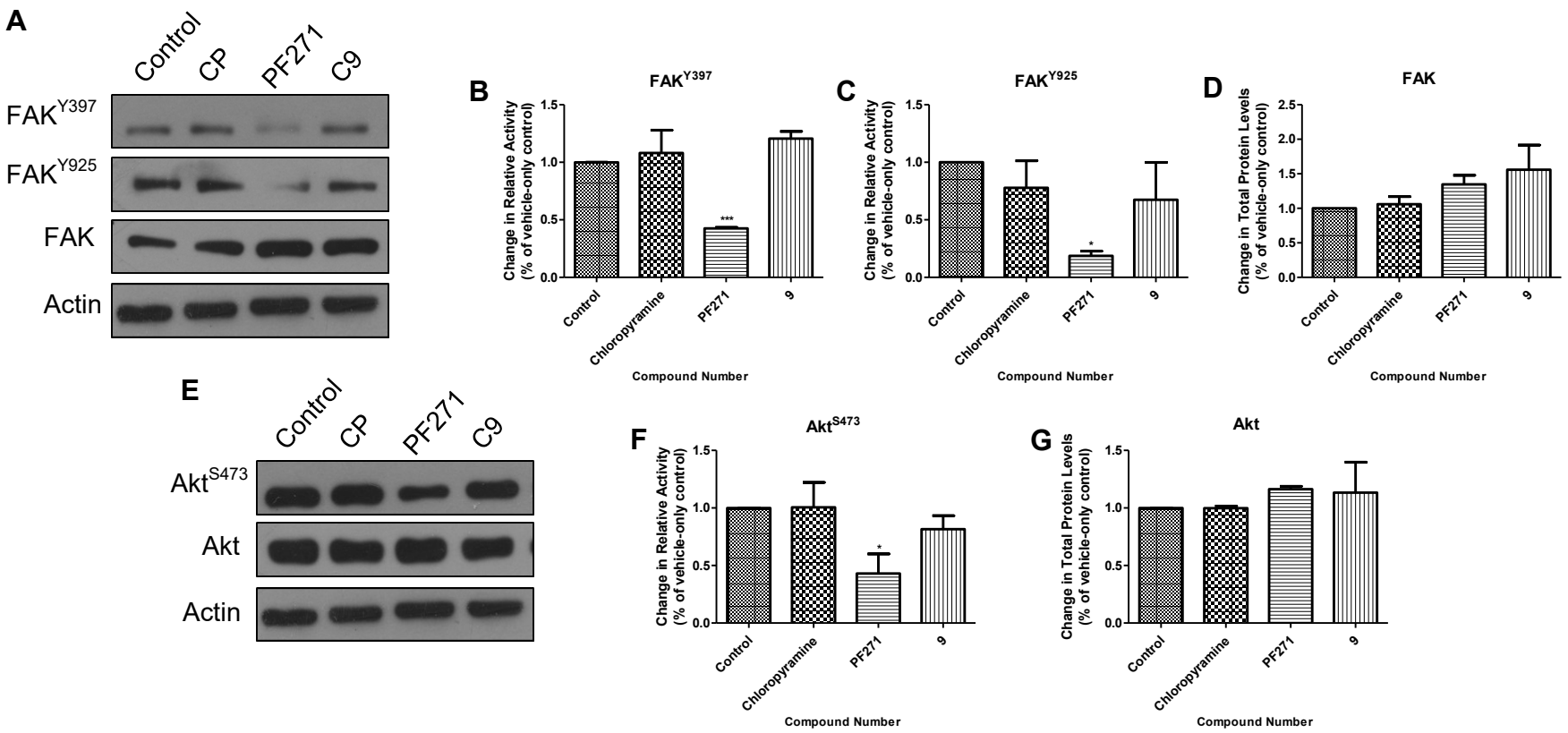


Figure 6.7 –The effects of compound 9 on FAK and Akt expression and activity – (A) Western blots showing changes in FAK^{Y397}, FAK^{Y925} and total FAK levels in response to 1µM chloropyramine (CP), PF271 and compound 9 (C9). Actin was used as a loading control. **(B)** Analysis of FAK^{Y397} activity calculated relative to vehicle-only control, showing significant reductions in PF271 treated cells. **(C)** FAK^{Y861} activity normalised to vehicle-only control cells with only PF271 showing significant changes in phosphorylation. **(D)** Densitometry analysis of total FAK protein levels with no changes observed following treatment with any compound. **(E)** Western blots assessing changes in activity of Akt^{S473} and total Akt levels. **(F)** Evaluation of changes in Akt^{S473} relative to activity in vehicle-only control cells. PF271 was the only compound to cause a significant decrease in phosphorylation. **(G)** Densitometry analysis of total Akt levels with no treatment causing any significant effect. All error bars are representative of SEM; n=3. *p<0.05; **p<0.01; ***p<0.001.

6.6. Treatment with compound 9 causes changes in cellular morphology and cellular localisation of active FAK

Previously, it has been demonstrated that normal function of the FAK FAT-domain is essential for correct targeting to FAs, inhibition of which results in perturbed activation by integrins and associated signalling proteins (Lee *et al.* 2015). Given that compound 9 targets this region it stands to reason that it may alter localisation of active FAK despite not influencing phosphorylation. As such, immunofluorescence was performed to examine subcellular localisation of FAK in response to treatment with 1µM of each compound. As noted previously, PF271 causes a perturbation in the "normal" polarisation of the cells: the cells treated with the inhibitor become more pointed and lose the typical leading edge characterised by lamellipodia extension during migration. This observation also holds true in compound 9 treated cells, although to a lesser degree **(figure 6.8)**. These changes in actin were also accompanied by alterations in the distribution of active FAK^{Y861}. PF271 treatment resulted in an increase in discrete FAK^{Y861} localisation to the cell periphery, particularly along cortical actin. Regions of FAK activity are also larger, as seen by increased intensity of the observed fluorescent FAK^{Y861} signal. Compound 9 also caused an increase in FAK^{Y861} at the cell periphery, although reduced when compared to the PF271 treated cells. Moreover, compound 9 caused an increase in diffuse cytoplasmic FAK^{Y861} than either vehicle-only or PF271 cells.

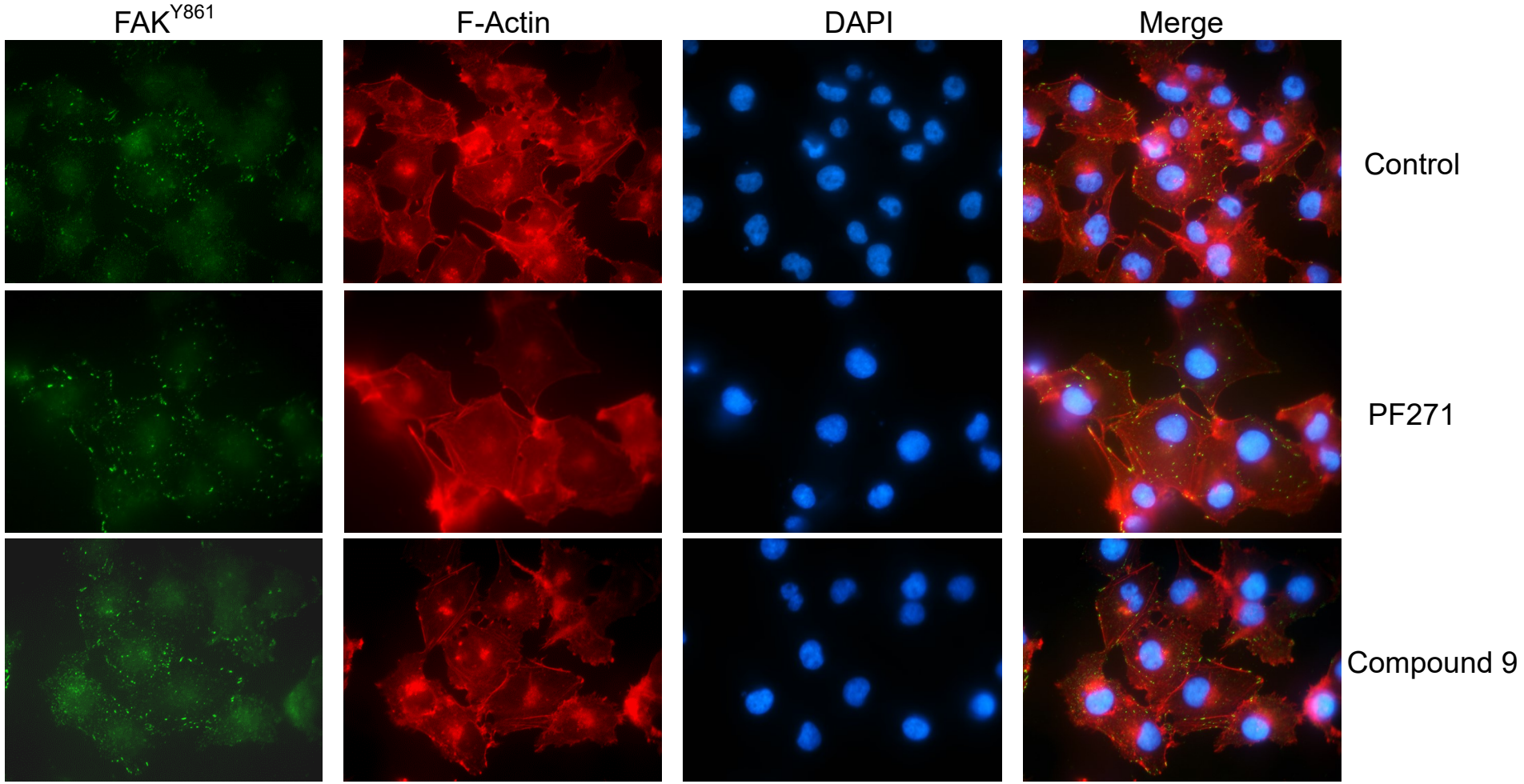


Figure 6.8 – Examination of changes in active FAK and actin in response to PF271 and compound 9 – MDA-MB-231 cells were treated with either PF271 or compound 9. Inhibition of FAK by either agent caused an increase in intensity of active FAK^{Y861} (green) on the cell periphery versus vehicle-only controls, whilst compound 9 treated cells had less active FAK than PF271 but a more diffuse cytoplasmic localisation. Extensions of actin (red) protrusions was reduced by both agents but to a greater extent in PF271-treated cells. Nuclei were stained with DAPI (blue). All images are representative; n=3.

To further explore the ability of compound 9 to perturb normal subcellular localisation of active FAK, a co-stain with vinculin was performed, enabling the relationship between FAK and focal adhesions to be explored **(figure 6.9)**. Control cells exhibited relatively few focal adhesions, whilst considerably more were seen in PF271 and compound 9 inhibited cells **(figure 6.10A)**. However, despite an increase in numbers of FAs, compound 9 did not cause any changes in the size of these structures **(figure 6.10B)**. Both compound 9 and PF271 had an impact on FAK^{Y861} localisation. PF271 caused a substantial increase in FAK^{Y861} activity on cell periphery, coupled with a significant increase in colocalization with focal adhesions suggesting impaired turnover at these sites. Compound 9 also caused an increase in peripheral FAK^{Y861} localisation when compared to control cells. However, this was accompanied by an increase in the number of FAs in these cells and subsequent colocalization of FAK^{Y861} and vinculin. Again, the amount of active FAK localised to the cytoplasm was higher than in vehicle-only and PF271 treated cells.

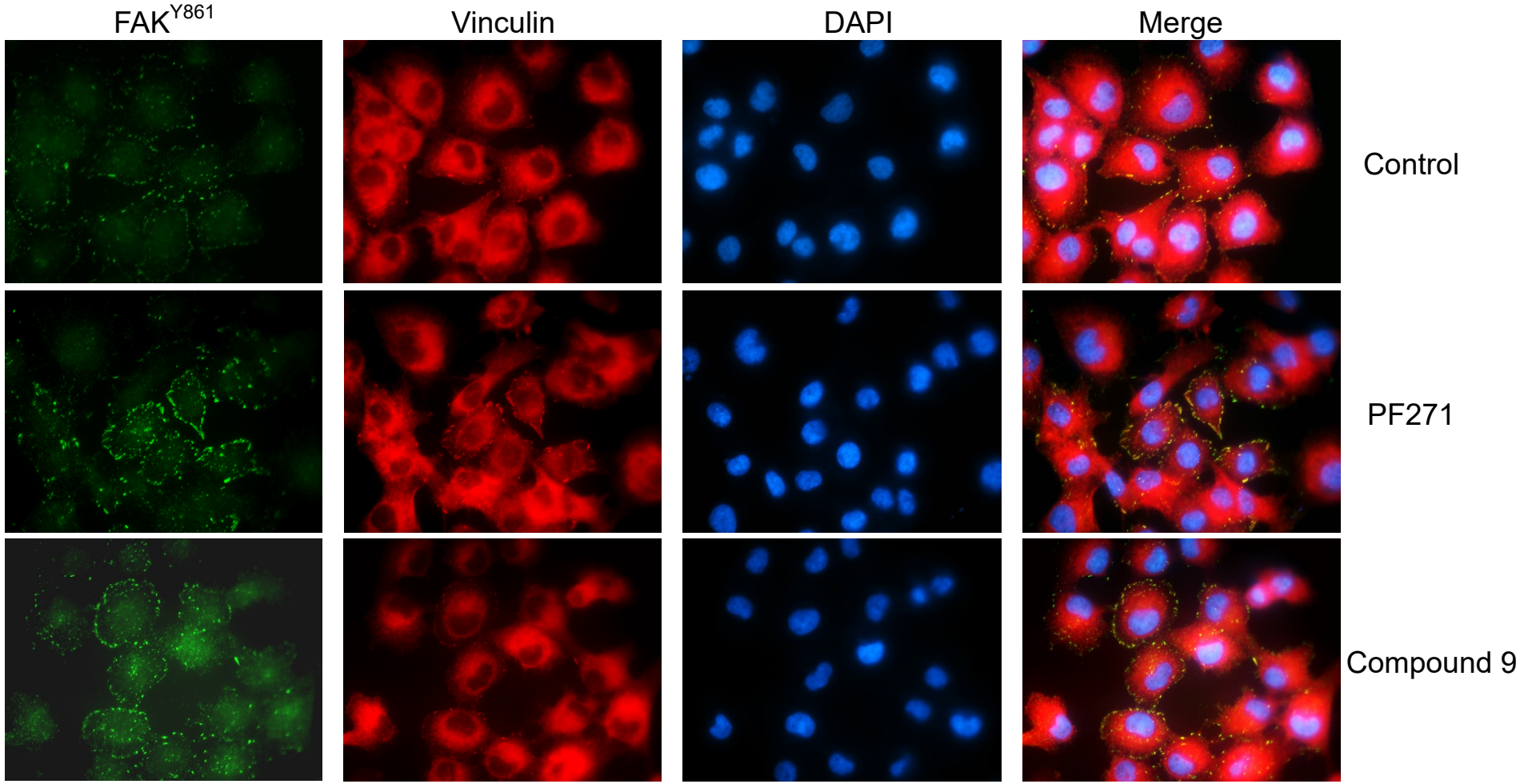


Figure 6.9 – Examination of changes in active FAK and FA-marker vinculin in response to PF271 or compound 9 treatment – Cells were treated with either PF271 or compound 9. As previously observed, both agents increased the levels of active FAK^{Y861} (green) on the cell periphery versus vehicle-only controls. Compound 9 exhibited an increase in cytoplasmic localisation of active FAK compared to PF271. Number of FAs, as represented by vinculin (red) were increased following PF271 or compound 9 treatment, though only PF271 caused an increase in FA size. Nuclei were stained with DAPI (blue). All images are representative; n=3.

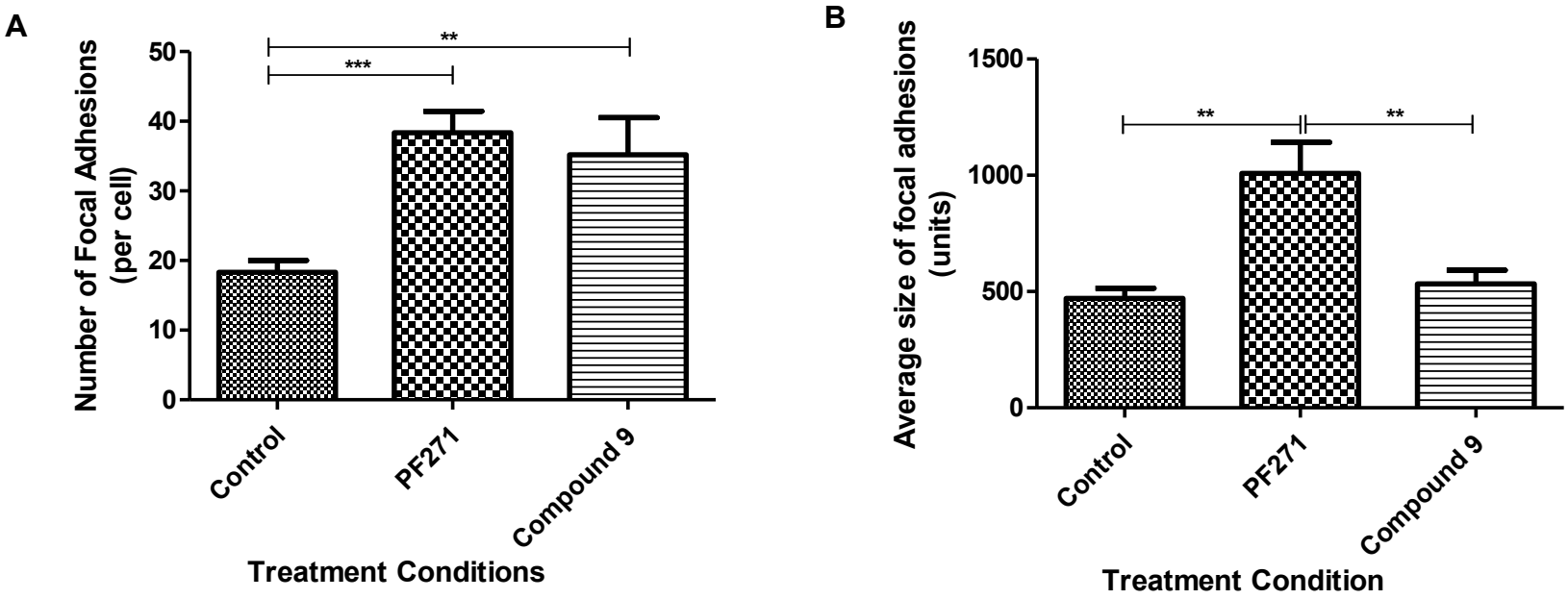


Figure 6.10 – Analysis of focal adhesion changes in response to PF271 and compound 9 – (A) Analysis of changes in the numbers of FA in response to PF271 or compound 9 showed a significant increase following treatment with either agent, whilst no significant difference was observed between PF271 and compound 9. **(B)** Evaluation of the size of FAs revealed that PF271 treatment significantly increases FA size versus both vehicle-only control and compound 9. No significant size change was noted between compound 9 and control cells. All error bars represent SEM; n=3. *p<0.05; **p<0.01; ***p<0.001.

The localisation of total FAK was also examined to see if changes in active FAK localisation coincided with overall changes in FAK distribution **(figure 6.11)**. As previously described, total FAK displayed a very diffuse localisation throughout the whole cell. Very little changes were observed following either treatment, although some increase in perinuclear staining was observable in the compound 9 or PF271 cells.

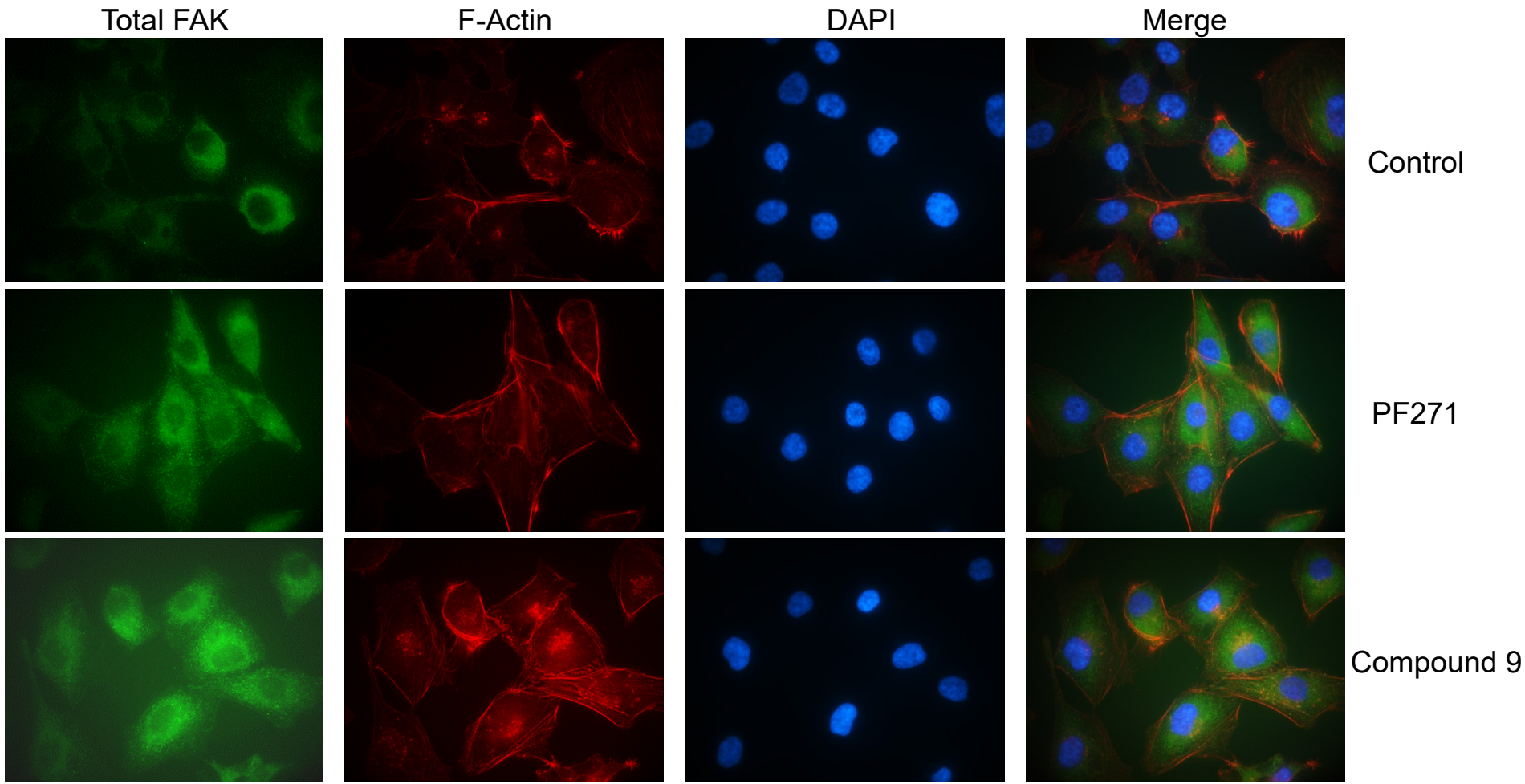


Figure 6.11 – Examination of changes in total FAK and actin in response to PF271 and compound 9 – MDA-MB-231 cells were treated with either PF271 or compound 9. Total FAK (green) is unaffected when treated with either agent, with cells exhibiting a diffuse signal throughout the cytoplasm. Both inhibitors caused a loss of protruding actin (red), which was more pronounced in PF271 treated cells. Nuclei were stained with DAPI (blue). All images are representative; n=3.

6.7. Discussion

In this chapter, we have explored a panel of novel FAK inhibitors, believed to target protein-protein interactions rather than kinase activity, to assess their validity as lead compounds in TNBC cells for subsequent development. From this we have determined that one compound, **compound 9**, can effectively inhibit proliferation and migration of a TNBC cell model, although with less potency than the well-established PF271 inhibitor. Further studies on this compound have been undertaken by Cyprotex to explore metabolic stability *in vitro* across a range of breast cancer subtypes. The results showed that this compound is metabolically stable and further enhances the potential of compound 9 as a FAK-scaffold inhibitor.

Scaffolding functions of FAK have been shown in numerous studies to be vital to normal cell function. Consequently, interference with such relationships could have a dramatic effect on "normal" cancer cell physiology. This has been shown on several occasions with a study by Fan et al (2013) providing a particularly fascinating example where the role of the FAK-scaffold in Src phosphorylation of endophilin A2 was explored (Fan *et al.* 2013). FAK directly binds endophilin A2 through its Pro-rich domain, enabling Src-mediated phosphorylation to occur promoting the epithelial-mesenchymal transition (EMT) and mammary CSC self-renewal. In a mouse, mammary tumour model of human breast cancer containing a P878/881A knock-in mutation, the FAK-endophilin A2 complex was significantly disrupted and resulted in a subsequent decrease in phosphorylation of endophilin A2. This was accompanied by a significant lessening of mammary tumour growth and EMT. Additionally, perturbation of this interaction appears specific to tumour cells as normal mammary gland development was unaffected. This may suggest that targeting scaffold function may be specific to tumour cells. An earlier study by Xu et al (2000) also noted a similar result (L. H. Xu *et al.* 2000). They showed that FRNK (FAK-related non-kinase) transduction in breast cancer cells was sufficient to degrade and dephosphorylate FAK and induce cell apoptosis. However, introduction of FRNK into normal mammary epithelia without altering adhesion or viability of the cells (although FAK dephosphorylation was noted). As an aside, FRNK represents a recombinant form of the FAK C-terminal, which acts as a dominant negative form of FAK through displacement of FAK at FAs (Richardson *et al.* 1997). When overexpressed in cells, FRNK results in a significant decrease in FAK phosphorylation and resulting cell processes including cell cycle progression (J. H. Zhao *et al.* 1998) and migration (Gilmore and Romer 1996). Together, these studies further highlight the

benefits of targeting FAK-scaffold function as cellular requirements for such processes appear distinct between tumour and normal cells resulting in increased target specificity.

Despite the potential targeting FAK-scaffold function possesses, current inhibitors against such interactions are limited. This may be due to concerns regarding the efficacy of such inhibitors to cause significant, beneficial changes in patients either alone, or in tandem with other agents. One such issue concerns the specific nature of targeting protein-protein interactions, with regards to whether the sites of interaction are a feasible size for antagonist targeting. In addition, concerns have also been raised about whether targeting these sites is sufficient to induce a cell-wide effect owing to potential compensation by another cell complex. Despite these concerns however, several inhibitors are available that target protein-protein interactions. As outlined in section 6.1, chloropyramine is a FAK inhibitor that has been shown to have potent effects on the FAK-VEGFR3, resulting in strong perturbation of "normal" tumour cell traits (Kurenova *et al.* 2009). Moreover, a group of inhibitors known as Roslins exist which also target protein-protein interactions of FAK. Previously, FAK has been shown to directly interact with p53 through binding in its N-terminal FERM domain (Golubovskaya *et al.* 2005). Indeed, inhibition of this complex removes FAK-mediated suppression of p53 activity, restoring downstream signals, such as p21, BAX and MDM2, as well as reinstating p53-mediated apoptosis (Golubovskaya *et al.* 2005; Lim, Chen, *et al.* 2008). Roslins (specifically R2) were designed to target this interaction in tumour cells (Golubovskaya, Ho, *et al.* 2013). Treatment with this compound not only decreased cancer cell viability and clonogenicity *in vitro*, but was also sufficient to cause significant reduction in tumour growth *in vivo*. It also caused an increase in p53 transcription and increased expression of genes containing p53-responsive promoter elements (Golubovskaya, Ho, *et al.* 2013). Taken together, these compounds show that pharmacological inhibition of FAK scaffolding is possible and further highlights the potential of therapeutically targeting such interactions. Our data reinforces this idea and further validates FAK-complex formation as a viable target for therapeutic intervention in the context of TNBC.

Indeed, we demonstrated that several novel scaffold-targeting agents were capable of suppressing migration and proliferation in MDA-MB-231 cells, whilst the initial compound, chloropyramine, was particularly poor as an anti-proliferative agent in this cell line. In contrast, previous studies have noted that chloropyramine is capable of suppressing proliferation in several cancer cell models (Sulzmaier *et al.* 2014). Although this seems contradictory to our

findings, work by Kurenova and colleagues (2009) does provide justification for this discrepancy. This study explored the efficacy of chloropyramine in reducing cell viability across a range of breast cancer cell models and noted that MDA-MB-231 cells had an IC_{50} value of around 20μM, far lower than that used here (Kurenova *et al.* 2009). A further study by Gogate et al (2014) also showed that this model requires a high concentration of chloropyramine for statistically significant results (Gogate *et al.* 2014). Taken together, these studies may suggest that our experimental methods were flawed, given we used a maximum concentration of 5μM for all compounds tested. This is not the case however, as our objective was to identify novel agents which showed comparable or improved effects versus PF271, which we showed to be effective at 1μM. Indeed, this approach allowed us to identify several compounds which had significant anti-proliferative effects *in vitro*.

The most promising novel agent was compound 9, which showed significantly improved anti-proliferative activity versus chloropyramine in MDA-MB-231 cells. In order to understand this increased efficacy, a number of docking simulations using Glide SP in Maestro (Glide, Version 9.5, Schrödinger, LLC, New York, NY. http://www.schrodinger.com) were performed by Dr. Sahar Kandil (Department of Medicinal Chemistry, Cardiff School of Pharmacy and Pharmaceutical Sciences). These analyses revealed that compound 9 contained a number of structural features which allowed enhanced interactions with residues within the target pocket compared with those displayed by chloropyramine. However, although initial MTT assays revealed comparative decreases in cell numbers between compound 9 and the kinase-inhibitor PF271, latter cell counting experiments revealed that PF271 was better at impairing MDA-MB-231 cell proliferation, possibly implying that inhibiting kinase activity is a more effective way of targeting proliferation than impairing scaffold functions. This also seems to support our previous findings that FAK siRNA had limited benefits against proliferation when combined with PF271 **(chapter 4)**, further suggesting a limited contribution by FAK scaffolding versus kinase activity. However, the purpose of these experiments was to identify compounds that could be used as a basis for further development of novel inhibitors of FAK scaffolding functions. As such, this compound has not been optimised in the same manner as PF271 and may exhibit increased effectiveness on subsequent modification. Combined with its anti-migratory properties, we concluded that compound 9 is a promising lead candidate for future development as an anti-cancer agent in TNBC cells.

Despite having limited influence on proliferation, we did show that chloropyramine could effectively reduce cell migration comparable to that observed in PF271 treated cells. We believe this to be a novel finding, largely due to most previous studies focusing on cell viability rather than anti-migratory effects. However, there is evidence to suggest that chloropyramine, and similarly functioning scaffold inhibitors, could function effectively as anti-migratory agents. As noted previously, the proposed mechanism of action of chloropyramine in cancer cells is through disruption of the FAK-VEGFR3 interaction, leading to reduced activity of both proteins (Kurenova *et al.* 2009). Given the role of FAK in cell migration it is clear that reduction in its activity would reduce cell motility (Lee *et al.* 2015), whilst the VEGF-family receptors have also been shown to promote metastasis *in vivo* (Smith *et al.* 2010; He *et al.* 2005). Moreover, interaction between active VEGF-receptors and FAK contributes to disease spread, with selective FAK-KD being sufficient to reduce metastatic burden (Jean *et al.* 2014). Although the exact mechanism underlying FAK-VEGFR driven metastasis in unclear, these experiments imply it is likely due to increased vascular permeability and tumour cell extravasation rather than through promotion of cell motility, especially given that FAK-KD results in significantly reduced transmigration across endothelial cell barriers *in vivo* (Jean *et al.* 2014). However, further studies have hinted that the relationship between FAK and VEGFRs can directly impair migration. For example, Bhattacharya and colleagues (2016) showed that depletion of stimulating VEGF significantly attenuated both migration and invasion of colorectal cancer cells *in vitro*, whilst also reducing phosphorylation of FAK (Bhattacharya *et al.* 2016). Taken together these studies provide justification for chloropyramine disrupting FAK-VEGFR interactions leading to reduced migration. Given that the region on FAK with which chloropyramine interacts has binding partners beyond VEGFR3, it is also possible that the noted anti-migratory effects could be due to perturbed interaction with a yet unidentified downstream molecule. Nevertheless, we surmised that chloropyramine alone may prove to be a useful anti-migratory compound in TNBC cells, although further experiments are needed to confirm the mechanism of action and to explore whether this observation extends to other models of triple-negative disease.

As previously highlighted, FAK activity is essential for the disassembly of focal adhesions and inhibition of this process results in perturbed migration owing to failure of cells to detach from ECM. Our data once again supports this as PF271, a potent FAK-kinase inhibitor, caused cells to have a greater number of focal adhesions and an increase in their respective size. Moreover, it was markedly more difficult to find cells positive for FAs in the vehicle-only control than those

treated with either inhibitor. However, compound 9 treatment caused an increase in FA numbers without effecting size. It also increased diffuse cytoplasmic activity of FAK. This suggests that this inhibitor is influencing cell behaviours through altering correct FAK localisation, effectively sequestering active FAK in the cytoplasm. This result is in concordance with several other who show that failure of FAK to localise to FA significantly alters FAK's ability to modulate migration and growth. Indeed, early studies by Shen et al (1999) showed that interference of FAK targeting to FAs through mutation of the C-terminal FAT-domain was sufficient to significantly impair cell adhesion-dependent FAK activity and stimulation of downstream targets (Shen and Schaller 1999). Subsequent studies utilising FRNK (FAK-related non-kinase) have further highlighted the essential nature of correct FAK localisation. Additionally, this localisation has been shown to play a key role in cancer cells with aberrant targeting linked to reduced invasion in lung adenocarcinoma cells (Hauck *et al.* 2001) and impaired angiogenesis in breast cancers (S. K. Mitra *et al.* 2006). As such, if compound 9 is inhibiting FAK localisation it is warranted this could be leading to the noted results on proliferation and migration.

Compound 9 could possibly be exerting this effect through disruption of the interaction between the FAT-domain of FAK and the adaptor protein paxillin. Although not explored here, it has long been established that paxillin plays a central role in FA dynamics (Turner 2000), largely due to interactions with FAK (Shen and Schaller 1999; Bertolucci *et al.* 2005). Indeed, Scheswohl et al (2008) showed that paxillin contains two FAK-binding sites, both of which are essential for activation of FAK and subsequent downstream signalling (Scheswohl *et al.* 2008). This interaction is not only essential for FAK function but also localisation to FA. Mutations in FAK that prevent FAK-paxillin binding led to complete loss of FAK targeting to FA, along with subsequent reductions in adhesion, migration and invasion (Deramaudt *et al.* 2014). Importantly, the paxillin binding domain of FAK is within the FAT-domain, near the proposed binding region of compound 9 (Deramaudt *et al.* 2014). Consequently, it could be reasoned that the use of this compound leads to impaired FA targeting through disruption of normal FAK-paxillin interactions, thus causing the observed effects on migration and proliferation.

Interestingly, our results also showed an increase in the number of focal adhesions without altering size. This could suggest that compound 9 causes a disruption in the processes governing FA turnover and growth. During initiation, integrin receptors on the cell surface cluster in response to ECM ligand binding. These initial clusters are subsequently stabilized by talin-

mediated linking of the actin cytoskeleton and integrin-ECM bonds (reviewed in Critchley and Gingras 2008). Resulting integrin-talin-actin complexes recruit paxillin, FAK and Src-family kinases, providing a basis for initial actin polymerisation (Roca-cusachs *et al.* 2012). Following a cascade of kinase activation and phosphorylation events of these early FA molecules, contraction of myosin II occurs leading to talin-stretching revealing binding sites for vinculin; the recruitment of which is sufficient to promote cellular protrusions associated with cell motility (Hirata *et al.* 2014). FA then go through a growth phase, which includes ephemeral transitions between assembly and disassembly before a fraction evolve and become mature. The sequential recruitment and activation of proteins means that as FA assembly progresses, distinct molecular profiles are present. Vinculin levels reach their highest at the end of this nascent-phase, prior to FA growth and evolution (Zimerman *et al.* 2004). Subsequently, high levels of vinculin are noted in later nascent adhesions, following activation of FAK. As a result, our data appears to suggest that nascent FA formation is uninhibited by compound 9 treatment. However, treatment may be causing impaired turnover and growth through weakened interactions between FAK and another FA-associated protein.

Integrin clustering is essential to the growth of FAs. As such interactions between FAK and a known influencer of clustering could be being altered by compound 9. Talin may represent one such protein as it has been linked to grouping of active integrins (Shattil *et al.* 2010). Additionally, studies have shown direct interactions between talin and FAK (Zhang *et al.* 2008; Frame *et al.* 2010), with the site of interaction seen to reside within the c-terminal FAT sequence of FAK, particularly close to the predicted binding site of compound 9 (Chen *et al.* 1995; Lawson *et al.* 2012). Indeed, mutations within residues associated with talin-FAK binding prevents subsequent talin recruitment resulting in a reduction in talin-mediated reinforcement of FAs (Lawson *et al.* 2012). It is important to note that alternative mechanisms for localisation of talin to FAs exist (Franco *et al.* 2006; Wang *et al.* 2010) and its subsequent interaction with FAK is not essential for FAK targeting to FAs (Lawson *et al.* 2012). Consistent with this, our results still show active FAK localisation to nascent FAs, although in greater numbers. However, Lawson et al (2012) noted that cells in which talin-FAK binding was prevented, cells showed both increased numbers and size of FAs (Lawson *et al.* 2012), a result not dissimilar from PF271-mediated inhibition of FAK. This suggests that FAK-talin interactions are essential for FA turnover, an observation in contrast to our findings as we saw increased numbers without an increase in size. This does not mean however that compound 9 does not inhibit FAK-talin interaction. The outlined study by

Lawson et al (2012) completely removed the ability of FAK and talin to interact whereas our compound may impair the binding enough to slow rather than completely stop FAK-talin-mediated FA disassembly. Furthermore, this level of inhibition could prevent focal adhesion growth in treated cells through inhibiting FAK mediated recruitment of talin and subsequent clustering of active FAs. The association between FAK and paxillin may also be contributing to the observed results. Previously, Wu et al (2016) proposed a mechanism of sequential kinase activation at FAs and suggested the time between initial assembly and subsequent activation of FAK could be a major determinant in the fate of nascent FAs (Y. Wu *et al.* 2016). They noted that mature FAs demonstrated nearly concurrent FA assembly and FAK kinase activity, whilst FAs with a much greater time between these two events tended to be disassembled (Y. Wu *et al.* 2016). Consequently, it could be suggested that slowing FAK recruitment to early assembling FAs could result in an increased likelihood of turnover. As such, if the interaction between FAK and paxillin (essential for FAK localisation) was inhibited, the recruitment of FAK to FAs could be slowed sufficiently to induce disassembly. Taken together, compound 9 may be impairing turnover through reduced interaction and recruitment of talin, creating more focal adhesions, whilst sufficiently slowing FAK targeting to FAs so that once active, they're targeted for disassembly. This mechanism is currently very hypothetical as our work on compound 9 is in the very early stages of study and several association experiments are needed to explore changes, if any, in interactions between FAK and other FA-linked proteins.

6.7.1. Conclusions

Taken together our results show the potential of compound 9 as a novel inhibitor of TNBC cell proliferation and migration through altering FA-targeting and protein-protein interactions. Given the evidence that FAK-scaffold inhibitors have many therapeutic benefits over their traditional kinase-targeting equivalents, the subsequent development of compound 9 to a refined pharmacological inhibitor could provide a means by which sustainable FAK inhibition may be achieved through alternating between readily available kinase inhibitors and the much rarer scaffold inhibitors. Our results also reinforce the concept of scaffold inhibitors as viable options for subsequent research and development, overcoming some of the stigma associated with such agents.

7. General Discussion

7.1. TNBC

Of all patients diagnosed with breast cancer, around 10-15% display triple-negative disease (Dai *et al.* 2015). Despite good initial responses to chemotherapy, a significant number of these patients rapidly relapse following therapy and develop metastatic disease (Bramati *et al.* 2014). The need for better treatments that specifically target TNBC tumours is clear and offers the opportunity to prevent or at least delay TNBC progression.

7.2. Relevance of FAK as a therapeutic target in cancer

In recent years, FAK has been identified as a potential target for therapeutic intervention in a host of cancers (Lee *et al.* 2015). This is largely due to a combination of its overexpression being correlated with disease progression and its ability to modulate several aggressive characteristics of malignant cancer cells (Schaller 2010). FAK has also been shown to contribute to initial events of tumourigenesis, as well as cancer cell metabolism (Zhang and Hochwald 2014). In breast cancer, increased FAK activity is observed in metastatic lesions and correlates with the progression of both ductal carcinoma *in situ* (DCIS) and infiltrating ductal carcinoma (IDC) (Lark *et al.* 2005; Lightfoot *et al.* 2004). FAK may therefore play a significant role in TNBC, given their aggressive behaviour. Although we did not observe that TNBC cancer cells have the greatest levels of FAK protein or activity versus other breast cancer subtypes, FAK was clearly a key factor regulating the proliferative and migratory capacity of TNBC cells along with contributing to maintenance of a cancer stem cell-like phenotype. The inference is that FAK may therefore represent a promising candidate for therapeutic intervention in TNBC, where targeted therapies are currently lacking (Bramati *et al.* 2014).

There are a number of potential benefits to therapeutically targeting FAK, not only in TNBCs but in a range of cancers. Arguably the most significant of these is that FAK acts a signalling hub for numerous upstream pathway elements, as well as influencing several phenotypic traits (Sulzmaier *et al.* 2014). Consequently, therapeutic targeting of FAK will alter a range of cell behaviours rather than altering growth or migration alone. Indeed, we demonstrated in this work that PF271 inhibition of FAK could significantly attenuate proliferation, as well as cell motility. Additionally, other groups have reported similar multi-potencies of FAK inhibition. For example, in ovarian cancer cells pharmacological suppression of FAK activity resulted in impaired growth and tumour volume, along with reduced migration and endothelial tube formation,

indicative of angiogenesis (Stone *et al.* 2014). More importantly, targeting signal-junction proteins can overcome the functional redundancy of input signals by simultaneously inhibiting several pathways regulating a single cell behaviour. This reduces the risk of developing resistance by limiting the effects of compensatory mechanisms employed by cancer cells to circumvent inhibition.

Despite being a major signalling hub, FAK is not completely immune to functional redundancy and a major challenge for FAK therapeutics is to establish the extent of Pyk2 compensation in specific tumour types. We showed in **chapter 4** that Pyk2 does not seem to compensate for FAK inhibition in MDA-MB-231 migration, rather its suppression is as effective as FAK in modulating cell motility. Meanwhile, other studies have demonstrated a very pronounced role of Pyk2 in compensating loss-of-FAK activity (Fan and Guan 2011), with others showing a more prominent role for Pyk2 in certain cancer subtypes (Lipinski and Loftus 2010). This discrepancy suggests a tumour/subtype specific role of Pyk2 alone or in relation to FAK. As such, further research is needed to identify specific tumour types in which FAK activity is strongly compensated by Pyk2 to identify patients that will be FAK-sensitive with limited functional redundancy from Pyk2. In patients where compensation is an issue, the use of dual-specificity inhibitors, such as PF271, could be utilised effectively and thus the importance of such compounds cannot be overlooked when designing new FAK inhibitors. Investigation into FAK-Pyk2 relationships will inevitably lead to the identification of tumours which may benefit from Pyk2 therapeutics without the need for targeting FAK. Despite these limitations, FAK remains a promising target for therapeutic targeting, especially given its diverse range of signalling effects and its potential to mediate various cancer cell behaviours.

Additionally, the broad signal regulation of FAK could have the added downfall of increased toxicity by impairing a range of signalling pathways in other, non-cancerous cells. However, targeting of FAK has several factors which suggest this is not the case. Indeed, systemic administration of PF271 or conditional knockout of FAK *in vivo* has limited effects on healthy tissues and overall physiology of the mice (Stokes *et al.* 2011a; Fan and Guan 2011). Given that FAK activity is most prominent during embryogenesis, evidenced by embryonic lethality of FAK-null mice (Cai *et al.* 2008), it could be suggested that FAK actually plays a very limited role in adult tissues and thus off-target effects arising through FAK inhibition might be minimal. Indeed, FAK conditional knock-out in adult mice tissues had very limited effects on normal physiology

(Sulzmaier *et al.* 2014). Moreover, FAK inhibitors in phase-I clinical trials are typically associated with good side-effect profiles. For example, Phase-I trials of PF271 (used in this thesis) showed very limited adverse effects, with the most common complaint being nausea (42% grade 1; 18% grade 2), although low-grade GI issues (vomiting and diarrhoea), headaches and fatigue were also reported (Infante *et al.* 2012). No grade 4 or 5 treatment-related toxicities were reported and the only grade 3 events reported were fatigue (3 of 33 patients) and headaches (3 of 33 patients) occurring at the recommended Phase-II dose. Cancer cells may also have increased susceptibility to FAK inhibition versus normal cells as FAK is commonly upregulated in malignant cells contrasted with their healthy counterparts (Lee *et al.* 2015). Although our data showed that FAK expression/activity is not significantly augmented in MDA-MB-231 cells versus other breast cancer subtypes, studies have shown its upregulation in TNBC cells versus normal mammary tissue. Consequently, this suggests that anti-FAK drugs could have a strong therapeutic index and be utilised for various cancer types, not limited to TNBCs.

7.2.1. Targeting FAK as a strategy to reduce tumour metastasis

In recent years, advances in surgery, radio- and chemotherapy have led to significant improvements in disease management, even leading to complete curing (Miller *et al.* 2016). However, the mortality rate for patients with metastatic disease remains high, with metastasis representing the primary cause of cancer patient death (Wells *et al.* 2013). As such, limiting cancer spread represents a major goal for therapeutic intervention. Some effort has been made to target the mechanisms underlying metastatic disease, particularly through targeting of MMPs. However, these inhibitors have so far been limited in their clinical efficacy, likely due to poor drug delivery strategies and the vast array of MMPs that participate in metastasis limiting single-target efficacy (Zucker and Cao 2009).

7.2.1.1. FAK as an anti-migratory target

One of the central cellular processes underlying metastasis is that of tumour cell migration (Wells *et al.* 2013). As such, agents which can suppress this behaviour may have benefit in preventing or suppressing cancer spread. To target this type of cellular behaviour, several approaches have been taken with respect to selecting an appropriate target, each with its own benefits and disadvantages. These include: design of agents versus migration-linked proteins that are overexpressed or hyperactivated in specific cancers (e.g. MMPs); targeting motility signals that

are common across several cancer types (e.g. tenascin-C); or impedance of signalling node proteins common to several processes regulating migration (such as those linking contractility to adhesion to polarity).

However, there are several limitations that apply to targeting metastasis. First, by their very nature anti-migratory therapies may not be effective monotherapies for reducing tumour burden, unless they contain a cytotoxic component. As such they must be utilised in combination with surgery and/or regiments that kill tumour cells. In addition, long-term inhibition of cell behaviours can be difficult to achieve, as one may need to sustain almost complete suppression to achieve the desired therapeutic effect. Indeed, partial inhibition may prove counterproductive, with some cancer cells displaying adverse consequences for incomplete reduction in activity. For example, breast and prostate cancer cells have been shown to have increased invasiveness upon incomplete myosin light chain kinase (MLCK) inhibition, whereas full impairment causes significantly reduced motility and dissemination (Kharait *et al.* 2007). MLCK is a cellular kinase which phosphorylates the regulatory light chain of myosin-II, to induce actomyosin contraction and the mechanical forces required for directional cell migration (Chen *et al.* 2014). Another significant limitation to anti-metastatic compounds is that at time of diagnosis, tumour cells may have already spread (Cristofanilli *et al.* 2004), even if disseminated cells exist is a state of quiescence and are yet to form secondary lesions (Taylor *et al.* 2013). Although, in such cases it seems that targeting metastasis may be futile, anti-metastatic compounds still have some merit in preventing further disease spread, given that secondary tumours (or dormant metastatic cells) can also disseminate to distal tissues (Wells *et al.* 2013).

In this thesis, we have demonstrated a potential role for FAK in driving MDA-MB-231 migration and thus highlighted its therapeutic potential as an anti-metastatic target in TNBC. One of the biggest advantages for targeting FAK is that it meets all three of the outlined means of targeting cell migration described above, most importantly functioning as a migratory signalling node. As shown in **chapter 4**, FAK contributes to several migration-associated traits of MDA-MB-231 cells. This is in agreement with many others that imply FAK regulates cell motility through mediating several distinct processes that enable migration, as well as effecting expression of genes which contribute to metastatic cell behaviours. For example, Taliaferro-Smith *et al.* (2015) demonstrated that specific inhibition of FAK caused a significant downregulation of EMT-markers, whilst directly impairing migration and invasion. Consequently, anti-FAK agents may

have the potential to overcome functional redundancy through increasing the amount of signalling changes for compensation. In addition, FAK plays a key role in linking mechanical and molecular signalling (e.g. from actin contractility to adherence signalling) to coordinate cell motility. Given that proteins which can coordinate such signals are less common than those that regulate molecular signals alone, mean the loss of FAK is even more difficult to overcome. However, one may postulate that targeting signalling hubs that control migratory responses may be fraught with the same problems as targeting signalling nodes common to many cell behaviours, primarily off-target toxicity. Contradictorily, affecting migration in this way does not have the same problems, largely because induced tumour cell migration is not comparative to routine haptotaxis of healthy cells, although it is somewhat reflective of motility observed in migration-driven wound healing (Wells 1999). FAK as a target can also circumvent this issue due to its patterns of expression/activity in normal versus malignant tissue which ultimately improves its therapeutic index. Taken with our data, these factors all support the use of FAK as an anti-motility treatment in TNBC and other cancers.

7.2.1.2. FAK as an anti-CSC target

Despite improvements in cancer therapies over the past few decades, many malignant cells remain resistant to chemo- and radiotherapies resulting in reduced efficacy to anti-cancer agents. This lack of sensitivity also means that such regimens can be toxic to healthy cells whilst leaving CSCs intact (Chen *et al.* 2013). Given their inherent resistance to traditional therapeutics, CSCs have been implicated as a major cause of disease recurrence (Carrasco *et al.* 2014). Additionally, these cells have been demonstrated as key drivers of metastasis (Chen *et al.* 2013) it is essential that anti-cancer treatments can effectively eliminate CSCs to maximise patient outcomes. As such, much effort has been put into unearthing the traits of CSCs and several therapeutic strategies have been employed, with varying degrees of success. Broadly, the approaches utilised so far fall into four categories: targeting cell surface markers; targeting signal cascades; targeting efflux transporters; targeting the CSC microenvironment niche.

As noted previously, CSCs differentially express cell surface markers compared with “normal” stem cells, as well as non-stem-like cells (de Beca *et al.* 2012). Therefore, marker-targeting provides a means by which to reduce side effects, whilst simultaneously enhancing effectiveness of a compound versus CSCs. As is common practice, the attachment of such targeting ligands or antibodies to therapeutic agents has been utilised to enhance efficacy of various treatments. For

example, conjugation of an anti-CD44 antibody (CSC marker) with a gold nano-rod resulted in a successful targeting of MCF-7 CSCs (Alkilany *et al.* 2012). Subsequent irradiation with near infrared light resulted in a localised increase in temperature and specific photo-ablation of $CD44^{+}$ cancer cells. Despite the reported efficacy of this approach, a major issue still surrounds utilising cell surface markers for anti-CSC strategies. Primarily, the lack of a universal definition of CSC markers, as well as the possibility that such markers may evolve and change during disease progression.

In addition to unique cell surface markers, CSCs also display several alterations in signalling cascades of which, due to modern molecular biology techniques, several have been identified while more are still being uncovered. Strategies that target such pathways are particularly interesting as they can suppress specific stem-like traits of these cells with a number of groups reporting promising results when such an approach is taken. Particularly, focus has centred around altering the balance between pro- and anti-apoptotic signals as a means of killing CSCs. In 2011, Piggott and colleagues demonstrated that suppression of c-FLIP, long known to be an inhibitor of the death inducing signalling complex (DISC) and thus extrinsic apoptosis, could selectively sensitise breast CSCs to the cytotoxic agent TRAIL *in vitro* and *in vivo* (Piggott *et al.* 2011). Subsequent studies further illustrated that cytoplasmic localisation of c-FLIP underpinned TRAIL-resistance, while nuclear activity was associated with increased Wnt-signalling and increase stemness (French *et al.* 2015). These studies not only highlight that CSCs exhibit altered signal pathways versus the bulk tumour cells, but also that these pathways can be effectively targeted to specifically eliminate the stem population. They may also suggest that such proteins can act as biomarkers to inform of disease progression and likely response to therapy.

The inherent chemoresistance of CSCs has previously been suggested to be as a direct result of drug efflux, mediated by ATP-driven cellular pumps (Broxterman *et al.* 2009). Indeed, studies have shown that ATP-binding cassette (ABC) transporters, which can actively remove small molecules from cells, appear to be highly expressed in CSCs thus implying their participation in multi-drug resistance (MDR) (Chen *et al.* 2013). Consequently, the activity of these pumps has gained significant attention recently and numerous methods have been developed by which the effects of such pumps can be minimalized, with several pharmacological agents against ABC transporters currently available (Ritchie *et al.* 2011). For example, in clinical trials, MS-209 can overcome drug resistance in breast cancer, amongst other solid tumours, through inhibiting

activity of both the P-gp and MRP1 ABC transporters (Saeki *et al.* 2007). Additionally, downregulation of ABC transporters has also been explored as a therapeutic strategy. Indeed, a study by Sims-Mourtada and colleagues demonstrated that inhibition of Hedgehog signalling through cyclopamine significantly reduced expression of MDR1 and ABCG2 resulting in improved response to treatment (Sims-Mourtada *et al.* 2007). This latter study is of particular significance to CSCs, given the importance of Hedgehog signalling to these cells (Takebe *et al.* 2015). However, research thus far has been focussed on improving chemotherapeutic response in general, with very few studying such approaches specifically on CSCs. Nevertheless, we believe that ABC transporters as therapeutic targets have great potential in CSCs, with a view to improving therapeutic sensitivity and overall patient outcome.

It is known that although CSCs are inherently chemoresistant, the microenvironment surrounding tumours can create an ideal niche in which CSC can be nursed and protected from drug-induced killing (Korkaya *et al.* 2011). For example, evidence has been presented showing that accessory stromal cells in bone marrow and secondary organs promote malignant B-cell growth and drug resistance through stromal-induced activation of anti-apoptotic signals (Burger and Peled 2009). By antagonising the target receptors of these signals, leukaemic cells can be sensitised to cytotoxic agents. In addition to microenvironment cells, vascularisation of tumours has also been linked to CSC survival. Indeed, inhibition of the well-known pro-angiogenic factor VEGF perturbs vascularisation leading to a significant decrease in glioblastoma CSCs *in vivo* (Burkhardt *et al.* 2012; Calabrese *et al.* 2007). However, the exact mechanism by which reduced angiogenesis results in a disruption of the CSC niche is not well understood and could possibly be due to impaired function of non-malignant cells within the tumour microenvironment.

In this project, we explored the role of FAK in maintenance of a CSC population in TNBC cells, along with examining whether FAK could contribute to stem-like cell behaviours, particularly self-renewal. In **chapter 5** we demonstrated that although FAK does not appear essential to maintain a stem-like subpopulation, it plays a central role in anoikis survival and propagation, as well as self-renewal of TNBC CSCs. This means that FAK inhibition could be utilised to inhibit specific CSC traits and may even represent a potential marker of this subpopulation. Although more experiments would be required to explore this hypothesis, the fact that high FAK expression is correlated with disease progression (Lee *et al.* 2015) could infer increased stemness of such tumours. Moreover, given that FAK activity is associated with metastasis and reduced

relapse-free survival (de Heer *et al.* 2008) also seems to reinforce this hypothesis as these characteristics of disease are closely tied to CSCs. Despite the importance of FAK to stem-like behaviours and correlations with CSC-associated disease states, some evidence appears to suggest that active FAK may not be indicative of stemness. In 2005, Baumann and colleagues showed that FAK and Src activity could be stabilised by the cell surface marker CD24 in breast cancer, even suggesting that $CD24^{+}$ cells drove disease progression (Baumann *et al.* 2005). Given that lack of CD24 presentation is a well cited marker of CSCs, this may suggest that FAK activity would be lessened in the stem-like subpopulation. However, FAK is clearly important to CSC functions, as evidenced by our work amongst others, so suggesting its role in stem-like cells is independent of CD24 stabilisation and therefore could still represent a marker of CSCs.

Even if FAK does not represent a hallmark protein in CSCs, therapeutically targeting it still has merit in targeting these cells. Although we are not unique in noting this, our demonstration of FAK as an essential mediator of MDA-MB-231 self-renewal is noteworthy as very few studies highlight FAK as key to this behaviour. Targeting FAK to impede CSC functions also has the added benefit of not only directly targeting the stem-like subpopulation but also microenvironment cells comprising the CSC niche. Although not studies here, numerous studies have linked FAK to the biology of tumour-associated stromal cells and angiogenesis. For example, FAK activity is known to directly contribute to the immune response effector cells, tumour-associated macrophages (TAMs) (Sulzmaier *et al.* 2014). Infiltration of such cells into tumours has been shown to drive of cancer-related inflammation and disease progression, with inhibition of FAK directly disrupting infiltration leading to decreased pancreatic and breast tumour volumes *in vivo* (Stokes *et al.* 2011b; Wendt and Schiemann 2009). Beyond immune cells, FAK has also been shown to regulate cancer-associated fibroblasts (CAFs). Indeed, fibroblast activation by lysyl oxidase-like 2 (LOXL2) has been shown to be FAK-dependent in a model of breast carcinoma (Barker *et al.* 2013). Comparative to TAMs, inhibition of FAK decreases CAF activity and tumour-association resulting in reduced tumour size (Stokes *et al.* 2011b). FAK has also been shown to contribute to angiogenesis through interaction with VEGF in breast cancer (S. K. Mitra *et al.* 2006). Suppression of this interaction decreases vascular permeability and leads to a perturbation of the tumour microenvironment. Taken together these studies not only show that FAK can directly influence stromal cell behaviours which may nurse and protect CSCs, but that its targeting can provide an effective means by which to inhibit stroma-driven cancer progression. However, very few studies have directly explored how FAK-driven behaviours of the

microenvironment contribute to CSCs. Nevertheless, the combined effects of tumour cell and stromal FAK inhibition could provide an effective means by which to sensitise CSCs to cytotoxic agents, while impeding stem-like cell traits that could lead to metastasis.

Given the evidence presented in this thesis, FAK could be used as a target for the development of novel anti-metastatic compounds. If such agents are developed, arguably the biggest obstacle they will face is in the designing of clinical trials because they are not inherently designed for the testing of anti-metastatic drugs but tend to focus on changes in tumour size as the measurable outcome. Consequently, this raises the question of how one would measure the success of therapeutic agents targeting migration. This could be overcome through use of overall-survival or progression-free survival as endpoints in early stage trials, although this may be inefficient due to difficulties in sufficient patient recruitment. Alternatively, one could limit research on these compounds to particularly invasive or rapidly progressing cancer types, such as glioblastoma. Given their aggressive nature this could allow more short-term observations of clinical efficacy though the associated increase in proliferation could pose issues, as well as issues with metastasis at time of diagnosis. FAK inhibitors may be able to avoid this issue, given that we, amongst others, have demonstrated that FAK also influences tumour growth and survival (Golubovskaya 2014). Could scientists and regulatory agencies work to overcome these obstacles, we postulate that subsequent trial recruitment could be improved owing to the low toxicity of such compounds and that they could be given in tandem with therapeutic regimens already approved for cancer treatments. The necessity for this advancement is underscored by the fact that several trials are currently underway, focussed on motility-targeting agents (clincaltrials.gov). For example, phase-I trials are currently ongoing investigating the safety profiles and subsequent determination of the recommended phase-II dose of Foxy-5, a formylated 6 amino acid peptide fragment which impairs epithelial cancer cell migration through mimicking Wnt5a (clinical trial: NCT01589900) (Sebio *et al.* 2014). Although such studies are interventional, the specific effects on migration are limited, with progression-free survival the only indicator of reduced migratory potential. In order for such trials to progress, issues such as those previously described must be addressed so less potential compounds are disregarded due to poor or limited trial design.

7.2.2. Clinical challenges of FAK inhibition

FAK appears to be a promising target not only for TNBC but for a wide-range of solid malignancies. However, like all novel anti-cancer targets, FAK has a number of clinical challenges moving forward to maximise its therapeutic efficacy. One of the biggest issues surrounds identification of tumour types, and even individual patients, that will exhibit sensitivity to FAK inhibitors. Interestingly, our data (**chapter 4**) revealed that hyperactivation of Akt can rescue migration in FAK inhibited MDA-MB-231 cells. Such modulation of target efficacy by levels of a compensatory protein is not unique to FAK but is often seen in novel therapeutics and potential targets. For example, Buzzai and colleagues showed that the presence of function p53 in colon cancer cells could mitigate the inhibitory effects of metformin (Buzzai *et al.* 2007). However, such challenges have resulted in progression towards "personalised medicine" where patients are treated based on molecular profiles of tumours rather than the broader classifications currently used (Jackson and Chester 2015). Although being used to limited degree, this process is in its infancy and, due to its precise nature, will require significantly more investigation to extrapolate its use to a large-scale. In order to progress FAK as a therapeutic target under the umbrella of personalised medicine will most likely require the identification of biomarkers predictive of response which may include FAK expression/activity itself or surrogate. Indeed, a recent study by Davis et al (2017) strongly indicated FAK expression as a marker of disease progression in colorectal cancer patients and, given variations in FAK levels between early and advanced disease, suggested a therapeutic window for effective FAK (Davis *et al.* 2017). A further example of such work is that conducted by Shah and colleagues in 2014, who explored the relevance of the protein merlin as a biomarker for therapeutic response to FAK inhibition in ovarian cancer cells (Shah *et al.* 2014). This group noted that low expression of merlin resulted in increased sensitivity to pharmacological FAK inhibition, a finding also noted in mesothelioma (Shapiro *et al.* 2014). Taken together, this could suggest merlin as a promising marker for stratification of patients likely to be highly responsive to FAK inhibition. Further identification and correlation of such markers with treatment response and prognosis should enable more accurate design of clinical trials, as well as recognition of cancer patients who would benefit from anti-FAK agents.

7.3. Therapeutic strategies for anti-cancer targeting of FAK

7.3.1. Current FAK-targeting therapeutics

As shown so far, FAK activity forms an integral part of a multitude of cell signalling pathways, many of which are aberrant in cancer cells. Coupled with the highlighted correlations between FAK expression and cancer cell progression, it stands to reason that FAK has recently been emerging as a highly lucrative therapeutic agent in several cancer types. **Table 7.1** below contains a brief summary of current FAK targeting compounds and their relative progression in clinical and preclinical development.

Name	Alternative Names	Target	Type	Trial Details	References
PF-573,228	PF-228	FAK	Kinase-I	• Preclinical efficacy in a range of cancer cells, particularly in lung, breast and prostate	(Howe *et al.* 2016) (Slack-Davis *et al.* 2007)
TAE226	NVP-226	FAK Pyk2 IGF1-R	Kinase-I	• Preclinical efficacy shown in lung, oesophageal, breast and glioma cells	(Otani *et al.* 2015) (Wang *et al.* 2008) (Vita M. Golubovskaya *et al.* 2008) (Shi *et al.* 2007)
NVP-TAC544		FAK	Kinase-I	• Preclinical studies only	(Weis *et al.* 2008)
GSK2256098		FAK	Kinase-I	• Phase I clinical trials in advanced solid tumours, melanoma and mesothelioma • Preclinical efficacy also shown in glioblastoma and pancreatic ductal adenocarcinoma	(Soria *et al.* 2016) (J. Zhang *et al.* 2014) (S. Chen *et al.* 2012)
VS-4718	PND-1186	FAK Pyk2	Kinase-I	• Ongoing Phase I trials in patients exhibiting non-	(Churchman *et al.* 2016)

				haematological malignancies • Preclinical effects noted in several cancer types including pancreatic, breast, lung, leukaemia and ovarian	(Jiang *et al.* 2016) (Tancioni *et al.* 2014) (Tanjoni *et al.* 2010) (Walsh *et al.* 2010)
VS-6062	PF-562,271 PF-271	FAK Pyk2	Kinase-I	• Completed Phase I clinical trials in prostatic, pancreatic and head and neck neoplasms • Preclinical responses noted in lung, breast, ovarian, skin, pancreatic and colon cancers	(Howe *et al.* 2016) (Tancioni *et al.* 2015) (Stone *et al.* 2014) (Infante *et al.* 2012) (Serrels *et al.* 2012) (Stokes *et al.* 2011a) (Roberts *et al.* 2008)
VS-6063	PF-04554878 PF-878 Defactinib	FAK Pyk2	Kinase-I	• Ongoing Phase I/Ib for patients with advanced ovarian cancer • Completed Phase II trials in lung cancer (non-small cell carcinoma and mesothiloma) • Completed Phase I clinical trials in non-haematological malignancies • Preclinical efficacy shown in breast, ovarian and thyroid cancers.	(Xu *et al.* 2017) (Shimizu *et al.* 2016) (Gerber *et al.* 2015) (Kindler *et al.* 2015) (Kang *et al.* 2013)
Y15	Compound 14	N/A	Other	• Preclinical studies undertaken in breast, thyroid, colon, pancreatic, melanoma, glioblastoma and lung cancer	(O'Brien *et al.* 2014) (Golubovskaya, Huang, *et al.* 2013) (Heffler *et al.* 2013) (Vita M Golubovskaya *et al.* 2008)

C4	Chloropyramine hydrochloride	N/A	Scaffolding	• Preclinical studies in breast	(Burgess *et al.* 2016) (Kurenova *et al.* 2009)
R2	Roslins	N/A	Scaffolding	• Preclinical studies limited to colon cancer	(Golubovskaya, Ho, *et al.* 2013)
Y11		FAK	Scaffolding	• Limited preclinical studies in breast and colon cancer	(Golubovskaya *et al.* 2012)

Table 7.1 – Examples and details of current FAK targeted inhibitors in cancer – Several FAK targeting compounds are currently in various stages of development as novel cancer therapeutics. Most are ATP-competitive kinase inhibitors (Kinase-I), although some are being used targeting scaffolding function (scaffolding) or through alternative mechanisms (Y15 is an allosteric FAK inhibitor shown to have some effects on altering normal scaffolding behaviours).

Primarily, these agents target the kinase activity of FAK, particularly through preventing FAK^{Y397} autophosphorylation. Given its position in the hierarchy of FAK activation and its importance for enabling full activation (Lee *et al.* 2015) its justifiable that this site has been the primary focus for therapeutic targeting. Indeed, as demonstrated throughout this thesis, the use of PF271 can significantly reduce FAK^{Y397} activity and subsequently impair MDA-MB-231 cell proliferation, migration/invasion and stem-like phenotypes. Moreover, the amount of clinically successful FAK-inhibitors that utilise this approach **(table 7.1)** reinforces the relevance of targeting FAK catalytic function. However, limiting exploration to perturbing the enzymatic activity of FAK may prove problematic, as this narrow-view of novel compound design appears to have two major flaws: development of resistance and incomplete FAK inhibition. As such, although already being undertaken to a limited degree, alternative means of targeting FAK-function for therapeutic intervention need to be explored.

7.3.2. Strategies for targeting FAK

7.3.2.1 Targeting scaffolding function and kinase activity

As noted previously, FAK exhibits several kinase-independent functions in which various regions of the FAK protein act as a molecular scaffold for secondary messenger activation (Sulzmaier *et al.* 2014). Consequently, limiting the design of anti-FAK agents to kinase functions alone may be missing some of these regions and thus restricting the efficacy of potential inhibitors. However,

some effort has been made to target FAK-scaffolding function (as shown in **table 7.1**), although this is to a limited degree. Moreover, as outlined in **chapter 6**, we sought to design and synthesise a series of novel FAK inhibitors that would target FAK scaffold functions in a model of TNBC. Although studies into the mechanism of action of these inhibitors were limited due to time available, our screening approaches did identify one compound (compound 9) which showed potential to inhibit proliferation and migration of MDA-MB-231 cells, independent of inhibiting kinase function.

Given that we designed our compounds to target the pocket surrounding FAK^{Y925}, we hypothesised that our compounds may also influence activity of this site. Although this was not noted for compound 9, it is interesting to speculate as to what effects inhibiting activity at alternate residues could have on FAK function. FAK contains a number of amino acids which can be phosphorylated and subsequently interact with specific secondary messengers to influence behaviour (Lee *et al.* 2015). For example, phosphorylation of FAK^{Y295} is essential to enable FAK interaction with Grb2 to mediate proliferation and angiogenesis through MAPK stimulation (Schlaepfer *et al.* 1994). One could therefore hypothesise that specific impairment of FAK^{Y925} activation may have a profound effect on certain cell behaviours and thus may provide an effective strategy by which to target specific signalling pathways and cell traits through FAK inhibition. However, the number of proteins which interact with FAK, it seems likely that inhibiting one phosphorylation site's activity could be ineffective due to compensation by other residues. One caveat to this would be if the pathway was the dominant, FAK-dependent driver of a specific behaviour such as migration. As FAK is utilised to various degrees in different tumours and subtypes of cancer, such an approach would require extensive research across various cancers to determine specific residue functions and their effectiveness as therapeutic targets. This may be why the primary focus of FAK-therapeutics has been to target FAK^{Y397}, given its importance to FAK activation and function (Lee *et al.* 2015).

Although it could be assumed that FAK^{Y397} is a more promising site than other phosphorylation sites, FAK also contains 2 phosphorylation sites that may be as essential to FAK function as FAK^{Y397}, thus making them of particular interest for therapeutic targeting. The FAK$^{Y576/577}$ residues are located within the activation loop of FAK's kinase domain and importantly, phosphorylation of both is required for full catalytic activity of FAK (Calalb *et al.* 1995). This is postulated to result from stabilisation of the subdomain loop in an active conformation allowing protein substrate

binding and subsequent stimulation. Consequently, one would hypothesise that preventing the activation of these residues could have serious implications on the formation of FAK-dependent signalling pathways, especially those dependent on catalytic function. Indeed, mutation of these amino acids to phenylalanine, thus removing the capacity for phosphorylation, causes a significant preclusion of kinase activity (70% of wild-type if either is impaired, increased to 50% if double mutants) (Calalb *et al.* 1995). Although studies exploring the effects of impairing $FAK^{Y576/577}$ phosphorylation are extremely limited, these residues have been shown as important to cancer progression. For example, tumours have been shown to exhibit increased levels of $FAK^{Y576/577}$ activity versus normal tissues, whilst paired analysis revealed a further significant increase in bone metastatic legions (Conti *et al.* 2014). This study highlights the potential contribution of $FAK^{Y576/577}$ to tumour cell metastasis. However, these data are largely correlative and may not truly represent functionality, although they do provide a basis for further investigation into the functional role $FAK^{Y576/577}$. Interestingly, $FAK^{Y576/577}$ also contributes to the structural regulation of FAK. Indeed, phosphorylation of these residues is sufficient to impair FERM-domain docking and resultant FAK inhibition (Lee *et al.* 2015). As such, therapeutic targeting of $FAK^{Y576/577}$ may exhibit the added benefit of sustaining inactive FAK, providing such a compound did not physically prevent FERM-kinase interaction. Taken together, this demonstrates the significant potential of $FAK^{Y576/577}$ as a target for inhibition and justifies further exploration into its functions and therapeutic potential.

7.3.3 Alternative targeting strategies for targeting FAK

7.3.3.1. Impairing the FAK FERM-domain

Although many of its binding partners are known, lack of information demonstrating the relative significance of these partners as mediators of FAK-dependent cell behaviours means targeting kinase functions of any active residue may not be the best approach to target FAK (Siesser and Hanks 2006). However, the intrinsic regulation of FAK could well prove promising as an alternative therapeutic strategy. As previously noted, the FERM-domain of FAK is one of the most important regulators of FAK activity FERM normally holds FAK in an inactive conformation where Y397 phosphorylation is inhibited. Upon integrin/growth factor receptor binding, a change in the structural conformation of FERM occurs, alleviating intrinsic inhibition of Y397 leading to full FAK activity (Lietha *et al.* 2007). Given the wealth of structural information on the autoinhibitory FERM-kinase interaction (Frame *et al.* 2010), it may be possible to design allosteric inhibitors

which stabilise this interface, thus precluding FAK^{Y397} autophosphorylation. In addition to increased suppression of FAK activity, such agents could also prove more effective than the widely-used ATP-competitive inhibitors given the specific nature of the area being targeted. To a limited degree, one such compound was developed by Golubovskaya and colleagues. Their agent, compound 14, was targeted to the FAK^{Y397} site, but also hit surrounding regions within the FERM-domain that interact with this residue thus sustaining autoinhibition (Vita M Golubovskaya *et al.* 2008).

Prevention of protein-protein interactions between the FERM-domain of FAK and the various activating signals may also provide a novel means of targeting FAK activity. If the link between the two proteins could be impaired, that would prevent FERM displacement and thus maintain FAK in its inactive conformation. Given the role of the FERM domain in directing FAK signalling from specific activator proteins, one could argue that this approach may enable more precise targeting of FAK-dependent signalling pathways and thus the development of more specific inhibitors. One of the biggest obstacles that faces this approach is the identification of the FERM binding partners as, despite some being well characterised, the identification of such kinase-displacing signals remains incomplete (Sulzmaier *et al.* 2014). Coupled with the notorious difficulties associated with targeting protein-protein interactions, targeting FAK in this way may seem ambitious at best. However, aptamer-based screening technologies may be capable of revealing specific and effective FERM-activator complexes (Frame *et al.* 2010). For example, the use of biosensors, real-time imaging and aptamer-based molecular screening could illuminate the FAK FERM-domain interactome, as well as the location and timing of such FAK stimulation. Despite being in its infancy, studies (such as those presented here) indicate that targeting FAK through FERM-domain perturbation is feasible and has potential as a novel therapeutic strategy.

7.3.3.2. Targeting the FAT-region and subcellular localisation

In addition to the FERM-domain, the C-terminus FAT-region has also been demonstrated to contribute to the structural regulation of FAK activity and thus may also represent a potential target for drug design. This region contains a four-helix bundle, H1/H4 and H2/H3 on opposite sides of FAK, which form the primary binding site for the LD-motif of paxillin (Bertolucci *et al.* 2005). Of particular interest, within H1 is located the Y925 residue which, when phosphorylated, binds to Grb2 (Schlaepfer *et al.* 1994). Interestingly, the structural conformation of this region seems to hide this amino acid making it unavailable for phosphorylation and subsequent Grb2

interaction (Hayashi *et al.* 2002b). As a result, it was postulated that the FAT-domain must undergo some form of conformational change to allow activation of this site. Indeed, several studies have demonstrated that activation of FAK increases tension in the hinge region between H1 and H2, enabling the establishment of an open conformation that exposes FAK^{Y925} (Arold *et al.* 2002). However, it was only recently that the biological relevance of this transition was uncovered. Kadaré *et al.* 2015 compared the biological characteristics of cells containing wild-type FAK with FAK mutants which had improved or hindered H1 opening (Kadaré *et al.* 2015). They showed that "closed FAT" mutant cells (i.e. cells which contained FAK protein with impaired FAT-opening) exhibited significantly impeded FAK function associated with decreased FAK^{Y925} phosphorylation, paxillin binding and focal adhesion turnover. In converse was noted with "open FAT" mutants, with the additional observation of enhanced phosphorylation of Y861. Despite not exploring changes in cell behaviours of FAK mutants, this study hints at the potential benefits for impairing FAT-domain opening. As a side-note, we had postulated maybe compound 9 (**chapter 6**) could be altering conformational dynamics but the lack changes in Y925 phosphorylation seemed to disprove this hypothesis. Nevertheless, if a closed conformation could be sustained pharmacologically it could prove effective as a means of targeting FAK. Most likely, such an inhibitor would either need to prevent tension in the hinge region (possibly through allosteric inhibition) or enhance interaction between H1 and H2 helices to prevent opening. Additionally, it is unknown whether specific FAT-region binding partners modulate H1 opening in cells, although if some were characterised this would open a new avenue for pharmacological inhibition.

In addition to altering FAK functionality, the above study also showed that changes in FAT-region conformation also impacted localisation of FAK to FAs (Kadaré *et al.* 2015). This observation was also reflected in our work, where compound 9 appeared to impair subcellular distribution of active FAK (**chapter 6**). As a result, these studies imply a potential therapeutic benefit of perturbed localisation of FAK. Previously, we discussed the relevance of the FAT-domain to this process but other forms of FAK targeting exist that may be feasible foci for development of novel anti-FAK agents. For example, FAK contains at least one nuclear localisation sequence (NLS) and two nuclear export sequences (NES), suggesting that FAK can be readily undergo nucleo-cytoplasmic shuttling (Golubovskaya 2014). Several studies have shown that FAK plays an active role in the nucleus of a number of cancers. Indeed, nuclear FAK can directly enhance cell survival through enhancing p53 degradation (Lim, Chen, *et al.* 2008). In addition, the traditional view of

FAK in gene expression involves the stimulation of downstream signalling cascades, however nuclear FAK, in coordination with an ever-expanding library of binding partners, can directly modulate gene expression (Lim *et al.* 2012; S.-W. Luo *et al.* 2009; Arold *et al.* 2002). Interestingly, such interactions are mostly driven by the FERM-domain (Lim 2013), although the FAT-region has also been implicated in some cell types. For example, in response to mechanical stress in cardiomyocytes, FAK's FAT-region directly complexes with the transcription factor MEF2 to increase gene transcription (Cardoso *et al.* 2016). In terms of cancer biology, increased nuclear localisation of FAK has been implicated in disease progression of several cancers, such as squamous cell carcinoma where nuclear FAK activity is significantly higher than non-cancerous keratinocytes (Serrels *et al.* 2015). Interestingly, localisation of FAK to the nucleolus also contributes to breast cancer biology through protection of the CSC marker nucleostemin from degradation (Tancioni *et al.* 2015).

Given the importance of both membrane and nuclear localisation of FAK, if we could disrupt the processes governing both we may be able to inhibit FAK function. This could be achieved in a number of ways. First, given the involvement of the FERM and FAT domains in FAK targeting, disrupting the activity at these sites (as described previously) may impact both nuclear and membrane FAK function, effectively preventing interaction with activating stimuli at either site. This approach has the added benefit that if localisation is unaffected, protein-protein interactions linked to FAK function at either the cell membrane or nucleus could be impaired, mimicking the effects of perturbed localisation. Alternatively, directly targeting the NES or NLS sequences may provide a means by which to perturb active FAK distribution. Pharmacologically, this could entail masking these regions to prevent their interactions with shuttle proteins. Despite the potential of such an approach, very limited studies have actually explored localisation as a means of preventing FAK activity. We explored it to a certain degree in **chapter 6**, although compound 9 was not specifically designed to alter FAK localisation. Significantly more work is needed, particularly on the role of FAK in the nucleus, if targeting subcellular distribution is to be considered as a therapeutic approach for anti-cancer FAK inhibitors.

7.3.4. FAK inhibitors in combination treatment strategies

So far, we have discussed the use of FAK as a target for monotherapy which is important given the role of FAK as a key component of a wide-ranging signalling network: this approach could hit several pathways and cell behaviours simultaneously. However, a further use of FAK inhibitors

might be in the context of combination treatment to circumvent the acquisition of resistance frequently observed when certain targeted agents, such as RTK-inhibitors or endocrine therapies, are used (De Marchi *et al.* 2016; Corcoran and O'Driscoll 2015). This hypothesis is supported in breast and colon cancer cells, where combination of a dominant-negative FAK mutant with AG-1478 or PP2 (EGFR and Src inhibitor respectively) was sufficient to increase apoptosis and cell detachment in breast and colon cancer cells (Golubovskaya *et al.* 2003; Golubovskaya *et al.* 2002). This observation also holds true upon dual-pharmacological inhibition. In fact, dual targeting of FAK and Src shows increased effectiveness versus single agent treatment alone (Bolós *et al.* 2010). This is not only limited to ATP-competitive inhibitors of FAK, as the allosteric inhibitor Y15 in combination with PP2 significantly decreased the viability of colon cancer cells (Heffler *et al.* 2013). Such benefits are also noted when FAK is inhibited along with upstream receptors. For example, the antiangiogenic sunitinib (SU11248) blocks, amongst other angiogenesis-linked receptors, VEGF and in combination with PF271 effectively reduced hepatocellular carcinoma growth *in vivo*, likely through increased suppression of blood vessel formation (Bagi *et al.* 2009). Indeed, work from within our lab has highlighted the benefits of combining FAK inhibition with endocrine therapies in HER2+ breast cancer. Although endocrine-resistant cells exhibited little change in proliferation when treated with FAK inhibitor PF228 or tamoxifen alone, combination treatment restored tamoxifen sensitivity in previously resistant cells (Hiscox *et al.* 2011). Further work also demonstrated that a FAK inhibitor alondside trastuzumab was significantly better at impairing proliferation and migration versus either agent alone (Lazaro *et al.* 2014) These studies highlight the potential of combining FAK inhibitors with those that target other molecular signals.

Alternative signalling inhibition is not the only means by which to target tumours. Agents that can improve responsiveness of cancer cells to standard chemotherapeutics are also desirable, especially in tumours lacking targeted therapies such as TNBC. Increased sensitivity of these cells can also serve to alleviate some of the unpleasant side-effects associated with traditional chemotherapy due to a reduction in doses required for effective tumour cell killing. FAK inhibitors have also been utilised in this context. For example, combination of docetaxel and the FAK inhibitor TAE-226 significantly prolonged the survival of ovarian tumour-bearing mice, postulated to result from decreased tumour growth and increased apoptosis of associated endothelial cells (Halder *et al.* 2007). *In vitro* this inhibitor has also been shown to significantly improve radiosensitivity of head and neck cancer cells (Hehlgans *et al.* 2009). Again, this

observation extends to FAK-scaffold inhibitors. Combination of chloropyramine with doxorubicin effectively inhibited *in vivo* breast tumour growth and angiogenesis, whilst the FAK inhibitor also sensitized tumours to doxorubicin in subcutaneous xenograft models of breast cancer (Kurenova *et al.* 2009). The lucrative potential of this approach is underscored by the fact that Phase I clinical trials are currently being undertaken by Verastem exploring the potential of VS6063 (PF878) in combination with paclitaxel in advanced ovarian cancer (Xu *et al.* 2017). Consequently, it may be worth considering the use of FAK as a combination treatment, with either chemotherapy or alternative signalling inhibitors, as opposed to a monotherapy.

7.4. General study limitations and considerations

In this study we present data that suggests that FAK is a key player in the aggressive phenotype of TNBC cells and that targeting FAK may represent a beneficial therapeutic strategy. It is worth noting that further elements could be considerations for future research in this area:

- To date, all work undertaken has been conducted in 2D *in vitro* studies. Although highly beneficial to understanding cancer biology, these models are not the best representation of overall tumour biology as they lack protein which comprise the tumour micro-environment, such as stromal cells or the ECM. The presence of such elements may alter drug interactions overall behaviours of cancer cells. Indeed, it has been demonstrated that tumour cells grown in 3D cultures do show increased reductions in migration and growth following FAK inhibition versus cells treated in 2D-adherent culture (Tanjoni *et al.* 2010). Although this study was not conducted on a model of TNBC cells, it serves to highlight that observations in 2D culture must be substantiated in 3D/*in vivo* models to best reflect true biology.

- The primary focus of this study was the role of FAK in the TNBC model MDA-MB-231. Although a second cell line was used, MDA-MB-468, this model did not validate observations of FAK function in MDA-MB-231 cells. Our data suggested that this resulted from hyperactive Akt in MDA-MB-468 cells. The use of another or multiple models of TNBC need to be used to better reflect how FAK influences the behaviours of this breast cancer subtype as a whole and not only in a specific cell model.

- In addition to exploring FAK kinase function we also began to explore the role of scaffolding in MDA-MB-231 cells. To achieve this, we used FAK siRNA to suppress total protein levels

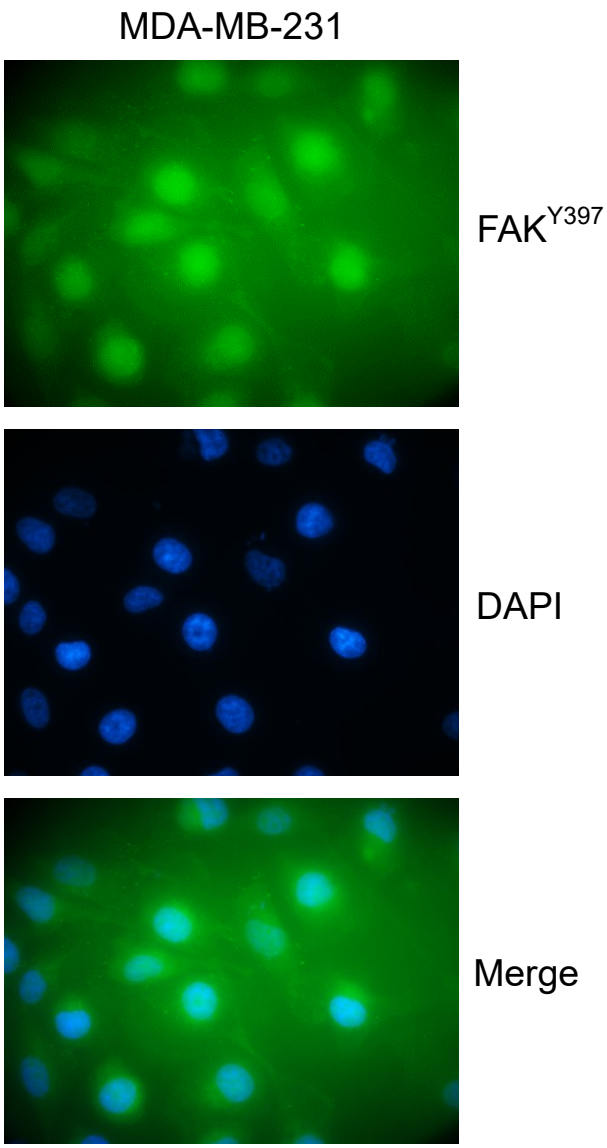
and compared changes in cell behaviour to targeted kinase inhibition to gain some insight into kinase-independent functions. However, this approach meant we lost both kinase dependent and independent functions. Although this may have hinted at a role of scaffolding to TNBC cell biology, the relative contributions could not be specifically addressed. In the absence of specific and truly effective scaffold inhibitors, future experiments should adopt a mutational approach where key regions of secondary messenger binding are no longer able to interact with other proteins.

- Expanding the work undertaken here into *in vivo* models would be highly beneficial to confirm the relevance of our findings to complete tumour biology. This is particularly important in terms of CSC behaviours because, although we have used a robust *in vitro* model of stem-like traits, the gold standard is to test tumour initiating capacities through tail-vein injection and monitor subsequent tumour establishment. Such mouse studies would also enable the expansion of this work to understand FAK's role in cell behaviours unique to *in vivo* systems, including hypoxia and angiogenesis. Although in its infancy, future experiments on compound 9 will require the use of mouse models in order to establish a range of factors, for example *in vivo* efficacy, toxicity and pharmacokinetics profiles.

7.5 Final Conclusions

The data in this thesis provides additional evidence for the role of FAK in aggressive triple-negative breast cancers where it acts to control migration and proliferation. Moreover, our data also highlights a little-reported role of FAK as a mediator of cancer stem-like cell behaviour, particularly their ability to self-renew through FAK-mediated modulation of β-catenin. These data suggest that FAK may represent an important therapeutic target in breast cancer, particularly in the context of TNBC. To this end we have also undertaken preliminary drug discovery work to develop a FAK inhibitor that impairs FAK scaffolding function and have identified a novel compound which is has been taken forward for further development.

8. Appendix



Appendix 1– Examination of FAK^{Y397} in MDA-MB-231 cells – MDA-MB-231 cells were stained for active FAK^{Y397} (green), along with DAPI (blue) to show cell nuclei. FAK^{Y397} stain exhibited significant non-specific, background staining making results difficult to interpret. As such, FAK^{Y861} was used as a surrogate marker for active FAK in future immunofluorescent assays.

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