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Validation of the MEK5 and ERK5 pathway as targets for therapy in prostate cancer and analysis of the ERK5 signalling complex

A thesis by

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Abstract

Extracellular signal-regulated protein kinase 5 (ERK5) is a member of the mitogen activated protein (MAP) kinase family which is specifically activated by mitogen/extracellular signal regulated kinase kinase-5 (MEK5). Over recent years, abnormal MEK5/ERK5 signalling has been shown to be important in prostate carcinogenesis with increased levels of ERK5 immunoreactivity being associated with Gleason sum score (p<0.0001), bone metastases (p=0.0044) and locally advanced disease at diagnosis (p=0.0023). In addition PC3 cells over-expressing ERK5 displayed enhanced proliferation, migration and invasion. Taken together, these data suggest MEK5/ERK5 pathway to be biological important in prostate cancer and a potential target in invasive prostate cancer.

Using siRNA to target ERK5 expression, I found that reduced ERK5 expression significantly inhibited cellular proliferation, motility and invasion in prostate cancer PC3 cells when compared to the controls, (p<0.005). Our group has previously reported upregulated ERK5 expression in primary human prostate cancer specimens. In this study, I was able to validate these results and demonstrate moderate-strong levels of cytoplasmic staining in 63% cases of PIN/PIA. High levels of cytoplasmic (55%) and nucleur (73%) immunoreactivity was also shown in a range of metastatic prostate tumours (n=11).

A number of similarities and interactions between ERK5 and ERK1/2 have recently been identified and there is suggestion that ERK5 may in fact regulate some of the cellular functions originally attributed to ERK1/2. Potential 'cross-talk' between ERK5 and ERK1/2 signalling was investigated using siRNA for each individual isoform of ERK1/2. ERK1 knockdown resulted in increased ERK5 activation in addition to prolonged ERK2 phosphorylation. Proliferation studies were also performed in PC3 cells, the results of which support published data that ERK1 acts as a negative regulator and ERK2 as a positive regulator of cell proliferation.

ERK5 has been shown to regulate the activity of several transcription factors and recent evidence suggests that ERK5 may be heat shock protein (HSP) 90 dependent. To further investigate the ERK5 signalling network and its interacting proteins, I performed mass spectrometry-based quantitative proteomics using SILAC labelled cells. Results from this study support the theory that HSP90 does associate with ERK5 however contrary to published data my results show that it is not involved in ERK5 activation.

Our results validate the importance of the MEK5-ERK5 signalling pathway as a potential target for therapy in prostate cancer and highlight a novel functional and biochemical relationship between ERK1 and HSP 90 with ERK5 signalling.

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List of abbreviations

ADT – Androgen deprivation therapy

AR – Androgen receptor

AS-MDM2 – Antisense murine double minute 2

BAP – Bone alkaline phosphatase

BMK1 – Big MAP kinase 1

BPH – Benign prostatic hypertrophy

CML – Chronic Myeloid Leukaemia

CYP – Cytochrome P

DHT – dihydrotestosterone

DMP - Dimethyl pimelilidate

DRE – Digital rectal examination

EBRT – External beam radiotherapy

EGF / R - epidermal growth factor / receptor

ERK - Extracellular signal-regulated protein kinase

ET – Endothelin

EV – Empty vector

FGF / R- fibroblast growth factor / receptor

GnRH – Gonadotrophin-releasing hormone

HDAC – Histone deacetylase

HDT – Hormone deprivation therapy

HER – human epidermal growth factor receptor

HIFU – High Intensity Focused Ultrasound

CRPC –Castration resistant prostate cancer

HSP – Heat shock protein

IGF-1 / -1R - Type I insulin-like growth factor / receptor

IGFBP-3 – IGF-binding protein-3

IκK – I kappa kinase

IL-1 – Interleukin-1

JNK - c-Jun NH_2-terminal protein kinase

LPS - lipopolysaccharide

mAb – Monoclonal antibodies

MAPK - mitogen activated protein kinase

MDM2 – Murine double minute 2

MEF – Myocyte enhancer factor

MEK - MAP kinase kinase

MEK5 – Mitogen/extracellular signal regulated kinase kinase-5

MS – Mass spectrometry

mTOR – Mammalian target of rapamycin

mTORC1- mTOR-raptor complex

NES – Nuclear export signal

NFκB – Nuclear factor kappa B

NLS – Nuclear localization signal

NSCLC – Non-small cell lung cancer

PC3-ERK5 – PC3 cells stably overexpressing ERK5

PDGF/R – Platelet derived growth factor / receptor

Ph – Philadelphia

PIP2 – phosphatidyl-inositol biphosphate

PIP3 - phosphatidylinositol triphoshate

PI3K - phosphoinositide 3-kinase

PSA –Prostate specific antigen

rbS6 – Ribosomal S6 protein kinase

RCC – Renal cell carcinoma

RRP – Retropubic radical prostatectomy

RTK – Receptor tyrosine kinase

SCLC – Small cell lung cancer

SE – Standard error

SFK – Src family kinases

SILAC - Stable isotope labelling with amino acids in cell culture

SN – Supernatant

STAMPEDE – Systemic Therapy in Advancing or Metastatic Prostate cancer: Evaluation of Drug Efficacy

TEY – Thr-Glu-Tyr

TKI – Tyrosine kinase inhibitors

TMA – Tissuemicroarray

TNF – Tumour necrosis factor

TNM – Tumour-nodes-metastasis

TRAIL – Tumour necrosis factor related apoptosis

TSG – Tumour suppressor genes

TURP –Transurethral resection of the prostate

VEGF/R – Vascular endothelial growth factor / receptor

1   Introduction

1.1   Prostate Cancer

1.1.1   Epidemiology of disease

Prostate cancer is the most commonly diagnosed cancer and the second commonest cause of cancer related death in men in the western world (1) .   In 2005, 34,302 new cases of prostate cancer were diagnosed in the UK (2). A significant increase in incidence has been reported since the 1980's due to an increase in rates of transurethral resection of the prostate (TURP) and prostate specific antigen (PSA) testing (3).

1.1.2   Aetiology of prostate cancer

There is currently no modifiable risk factor identified for prostate cancer however three established risk factors have been found: age, family history and ethnicity. The incidence of prostate cancer increases with age and over 70% of patients with prostate cancer are over the age of 65 years (3). With an aging society, it is therefore inevitable that prostate cancer will become an increasing health burden in years to come.

A family history of prostate cancer is also recognised as a strong risk factor for the disease. Familial forms of prostate cancer, which are those in which at least 2 first degree relatives are affected accounts for approximately 20% of cases and genetic susceptibility has been shown to play a more significant role in younger patients (4).

The worldwide variation in incidence rates suggests that the risk of developing prostate cancer is affected by ethnicity. In the UK, black Caribbean and black African men have 2-3 times the risk of white men in developing or dying from prostate cancer while Asian men generally have a lower risk (5). Migration studies suggest however that lifestyle factors must also play an important role as men who move from a low-risk to a higher-risk country show an increase in incidence (6).

1.1.3   Pathological Staging and Grading

The majority of prostate cancers are adenocarcinomas and mainly occur in the peripheral zone of the prostate. Prostate cancers are classified using the tumour-nodes-metastasis (TNM) staging system (Table I). This system evaluates the size of the tumour, the extent of involved lymph nodes and the presence of any metastasis. The gleason grading system is used to grade prostate tumours and together with TNM staging is used to predict prognosis and guide patient management. A score of 1-5 is given to the cancer based on its microscopic appearance. A higher score indicates a more aggressive tumour with a worse prognosis. A gleason grade is given to the most common tumour and a second score given to the next most common tumour. These grades are added together to give a gleason sum score which is used to guide treatment options for men with prostate cancer.

TABLE I: The TNM grading system

Evaluation of the (primary) tumour 'T'

- **TX**: cannot evaluate the primary tumor
- **T0**: no evidence of tumor
- **T1**: tumor present, but not detectable clinically or with imaging
    - **T1a**: tumor was incidentally found in less than 5% of prostate tissue resected (for other reasons)
    - **T1b**: tumor was incidentally found in greater than 5% of prostate tissue resected
    - **T1c**: tumor was found in a needle biopsy performed due to an elevated serum PSA
- **T2**: the tumor can be felt (palpated) on examination, but has not spread outside the prostate
    - **T2a**: the tumor is in half or less than half of one of the prostate gland's two lobes
    - **T2b**: the tumor is in more than half of one lobe, but not both
    - **T2c**: the tumor is in both lobes
- **T3**: the tumor has spread through the prostatic capsule (if it is only part-way through, it is still **T2**)
    - **T3a**: the tumor has spread through the capsule on one or both sides
    - **T3b**: the tumor has invaded one or both seminal vesicles
- **T4**: the tumor has invaded other nearby structures

Evaulation of the regional lymph nodes 'N'

- **NX**: cannot evaluate the regional lymph nodes
- **N0**: there has been no spread to the regional lymph nodes
- **N1**: there has been spread to the regional lymph nodes

Evaulation of distant metastasis 'M'

- **MX**: cannot evaluate distant metastasis
- **M0**: there is no distant metastasis
- **M1**: there is distant metastasis
    - **M1a**: the cancer has spread to lymph nodes beyond the regional ones
    - **M1b**: the cancer has spread to bone
    - **M1c**: the cancer has spread to other sites (regardless of bone involvement)

1.1.4 Clinical management

Treatment options for men diagnosed with prostate cancer depend on a number of factors including patient performance status, disease status (tumour grade and stage) and social factors (Table II). Prostate cancer diagnosed at an early stage (or when organ confined) are potentially curable and various options are available for these patients. Watchful waiting may be considered if the patient has low grade, small volume disease and a life expectancy of less than 10 years. Follow up for these patients will focus on serial serum PSA measurements and clinical symptoms and if disease progression is evident, the patient can be considered for intervention such as medical treatment.

Active surveillance is offered to patients who are found to have prostate cancer, which is thought to be clinically insignificant, and at low risk of progression. Active surveillance involves PSA measurements and digital rectal examinations (DRE) every 3 months with repeat prostate biopsies to re-grade the cancer 12-24 months following initial diagnosis or if significant changes are found in the PSA level or DRE. If these show that the cancer is progressing then treatment with curative intent will be recommended, in most cases being either surgery or radiotherapy. However, the optimal protocol for active surveillance remains to be validated in a prospective trial.

Radical treatment is offered for patients who have localised disease and good life expectancy. Surgical options include retropubic radical prostatectomy (RRP) with laparoscopic or robotic-assisted approaches gaining popularity in recent years. Whether these novel techniques will translate into better outcome await formal assessment. Conformal external beam radiotherapy (EBRT) or brachytherapy represent radiation based curative options for patients with early disease.

Androgens are the primary regulators of prostate cancer cell growth and differentiation and prostate cancer is often androgen dependent, with the majority regressing following initial androgen ablation treatment. Current medical therapy for patients diagnosed with prostate cancer includes anti-androgens and gonadotrophin-releasing hormone (GnRH) analogues. Anti-androgens block the effect of androgens directly on target cells by inhibiting their binding to the androgen receptor (AR). GnRH analogues work at the level of the pituitary with continued administration producing down regulation of GnRH receptors thereby reducing the release of gonadotophins which leads to inhibition of androgen production. Medical therapy is a treatment option either alone or in addition to radiotherapy in patients with locally advanced disease. Hormone deprivation

is the treatment of choice in patients with metastatic disease most often in the form of medical therapy but some patients may be offered a subcapsular orchidectomy which has the advantage of achieving rapid androgen ablation.

Unfortunately, approximately 20% of patients do not show a favourable response, and even among the responders, there is an 80% risk of relapse at a median period of 24 months following hormone manipulation. Patients are diagnosed with castration resistant prostate cancer (CRPC) when they show evidence of a rising PSA (PSA > or = 2ng/ml above nadir) (7). Treatment for these patients is limited. Secondary (and tertiary) hormonal manipulation such as discontinuation of steroidal or nonsteroidal hormones or the addition of anti-androgens, oestrogens, glucocorticoids or enzymatic inhibitors of the adrenal androgen synthesis pathway may produce a transient biochemical response. In addition, patients with clinically localised disease receiving radical treatment have a significant failure rate over 5-10 years period of follow up (reported rates of 23% post RRP and 63% post EBRT) (8).

Docetaxel has recently been licensed for use in combination with corticosteroid therapy in men with metastatic CRPC following the results of two Phase III trials(9;10). The mean survival benefit in these studies only measured 2 and 2.5 months respectively and timing of this treatment remains controversial. Trials are now focusing on improving the efficacy of docetaxel by combining it with novel biological agents. In addition, there is now an increasing interest in testing the efficacy of novel agents in hormone naive disease, which may result in better overall response and outcome. The STAMPEDE (Systemic Therapy in Advancing or Metastatic Prostate cancer: Evaluation of Drug Efficacy) study, a 5 arm randomised controlled trial, is one example, aimed to recruit patients with high-risk prostate cancer. This large multicentre trial examines the efficacy of combining androgen ablation therapy with a number of agents including docetaxel, zoledronic acid (bisphosphonate) and celecoxib (cox-2 inhibitor) (11).

TABLE II: Treatment options for prostate cancer
(NICE clinical guideline 58)

Early stage (or organ confined disease,$T_{1/2}N_0M_0$)

Conservative managment
- Watchful waiting - Suitable for patients with low grade, small volume disease and life expectancy <10years. Treat if rise in PSA.

- Active surveillance – If disease considered clinically insignificant and at low risk of progression. Involves 3 monthly PSA check and DRE. Repeat biopsy at 12 months.

Radical treatment
- Radiotherapy – External Beam Conformal Radiotherapy or interstitial brachytherapy

- Radical prostatectomy – retropubic, laparoscopic or robotic assisted prostatectomy

- Other experimental methods yet to be validated in formal randomised controlled trials include Cryotherapy, High Intensity Focused Ultrasound therapy (HIFU)

Locally advanced ($T_3N_0M_0$)

- Androgen Deprivation Therapy (ADT) – GnRH analogue and/or anti androgen

- Radiotherapy +/- ADT

Metastatic disease

- ADT

- Subcapsular orchidectomy

Castration Resistant Prostate Cancer (CRPC)

- Second or third line hormonal manipulation – Discontinue steroidal or nonsteroidal hormones or addition of antiandrogen, oestrogen, glucocorticoid or enzymatic inhibitor of adrenal androgen synthesis

- If metastatic disease, consider chemotherapy (docetaxel and prednisolone)

1.2   Androgen receptor signalling in prostate cancer

The androgen receptor (AR) regulates prostate organogenesis as well as the development and progression of prostate cancer. Androgen deprivation leads to apoptosis in a proportion of prostate cancer cells and those which do survive arrest in the G1 phase of the cell cycle (12). However, as prostate cancer progresses cells evolve and develop mechanisms to survive in an androgen depleted environment. This progression is recognised to involve an active AR and various mechanisms in which this altered signalling is implicated in the transition to hormone (or castrate) resistance have been described (13).

Reactivation (or the continued activation) of the AR and AR responsive pathways allow tumours to develop a hormone independent phenotype through altered AR sensitivity, AR amplification and AR mutations (14;15). Mutations in the AR can lead to activation by non-adrogenic steroid molecules and anti-androgens. This may explain why 10-30% of patients who develop resistant cancer following treatment with anti-androgens may show a paradoxical drop in PSA levels when the particular anti-androgen is discontinued (16).

Circulating serum androgen levels are not completely eliminated with hormone deprivation therapy (HDT). While serum testosterone levels are significantly reduced, serum levels of adrenal androgens remain unaffected. Intraprostatic androgens are also reduced sufficiently with HDT to induce a response in untreated prostate cancer cells. It is interesting to note that CRPC tumours have increased endogenous synthesis of androgens (17), along with upregulation of the enzymes required for steroidogenesis (18;19). Hence, tumour cells, particularly in castrate resistant disease, with their increased intracrine androgenic production, may be responsible for tumour progression despite low serum androgen levels.

Growth factors and their signalling cascades such as HER2, IGF-1, and EGF can also activate the AR via the PI3K/Akt and MAPK pathways and reduce or negate the need for ligand binding (Figure 1.1). Overexpression of these coactivators in prostate cancer leads to indirect activation of the AR and many agents currently in trial for use in CRPC target these signalling pathways.

In order for CRPC to establish, prostate cancer cells must also overcome the apoptotic effects of androgen depletion. The induction of AR independent pathways

including receptor tyrosine kinase (RTK) mediated networks allows prostate cancer cells to survive via the upregulation of anti-apoptotic proteins such as survivin and bcl-2.

Genetic alterations are important in the metastatic progression of tumour cells. In addition to these changes alterations in the tumour microenvironment are required to allow local growth and invasion as well as distant metastasis to develop. It has been suggested that cells within the stroma secrete a wide range of growth factors, extracellular matrices, metalloproteinases and/or angiogenic molecules to promote prostate cancer cells into a tumorigenic and invasive phenotype. It is now thought that a more efficacious method of treatment may involve targeting both the tumour and stroma, blocking both the tumour cells' proliferative ability and also the required support from the microenvironment.

FIGURE 1.1   Receptor tyrosine kinase signalling and the androgen receptor
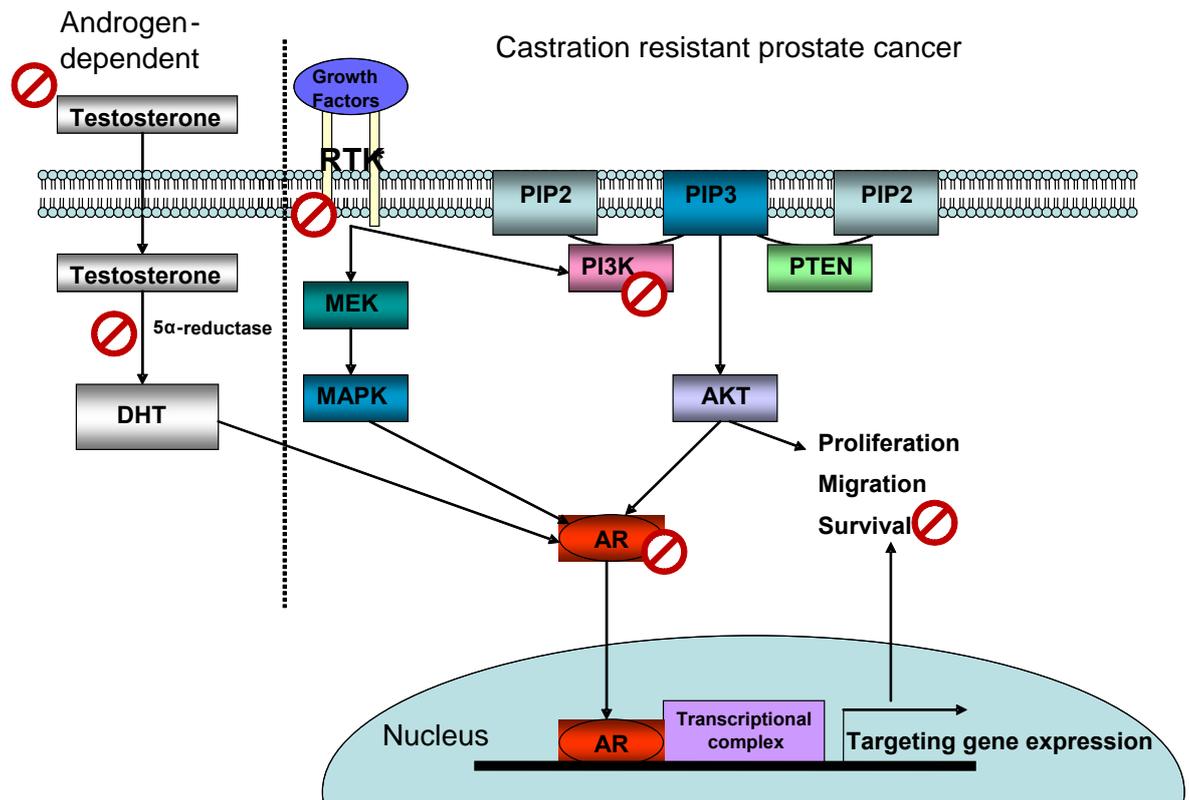
*RTK includes IGF-1R, EGFR and FGFR

In castration resistant prostate cancer (CRPC), androgen receptor remains functional and is thought to significantly contribute to cancer progression. A number of mechanisms of AR activation in an androgen depleted environment have been described (see text for further details) and cross talk from receptor tyrosine kinase signalling pathways plays an important role in CRPC. (Abbreviations: AR - androgen receptor; DHT – dihydrotestosterone; EGFR - epidermal growth factor receptor; FGF / R- fibroblast growth factor receptor; IGF-1 / -1R - Type I insulin-like growth factor / receptor; MAPK - mitogen activated protein kinase; MEK - MAP kinase kinase ; PIP2 – phosphatidyl-inositol biphosphate; PIP3 - phosphatidylinositol triphoshate; PI3K - phosphoinositide 3-kinase; RTK - receptor tyrosine kinase)

The symbol ⊘ signifies the development and evaluation of key inhibitors in clinical trials, including PI3 kinase inhibitors (CCI-779, RAD001); EGFR (Erlotinib, Gefitinib, Cetuximab); EGFR and HER2 dual inhibitor (Lapatinib); IGF-1R inhibitor (A12, CP-751,871); Androgen receptor inhibitor (MDV-3100, BMS-641988); 5α-reductase inhibitor (Dutasteride); apoptosis/survivin (YM155); CYP17 (Abiraterone, to reduce adrenal and intra-tumour androgen biogenesis)(see Table III and text for detail).

1.3   Aberrant signalling pathways involved in prostate carcinogenesis

Abnormal signalling is thought to mediate many of the tumourigenic activities involved in cancer development and progression; discoveries in this field offer potential targets for new drug development. The progression of epithelial prostate cells from a normal differentiated state in which proliferation and apoptosis are tightly balanced to a malignant state involves a combination of events resulting in the activation of oncogenes in addition to the loss of tumour suppressor genes, which critically control aspects of the hallmarks/phenotypes of cancer (Table III) (20). Many signalling pathways have been found to be important in prostate carcinogenesis and in recent years targeted therapy has emerged as a key focus for prostate cancer research.

RTKs for growth factors are essential for the transduction of extracellular signals to their cytoplasmic effectors. RTKs activate several pathways controlling cell proliferation and differentiation as well as migration and apoptosis. In normal cells the activity of RTKs is tightly regulated; however in cancer constitutive activation of RTKs is essential for maintaining the malignant phenotype.

Tumour suppressor genes (TSG) critically regulate the cell cycle, apoptosis, DNA repair, senescence and angiogenesis. Deranged TSG function in carcinogenesis can result from two distinct mechanisms. The function of a TSG can be impaired by (1) mutation or deletion abnormalities, or (2) binding to a regulatory protein which can either inhibit the function or impair the stability of the target TSG. TSGs such as p53 and PTEN are important in prostate carcinogenesis (see section 1.7).

TABLE III: Hallmarks of cancer and the role of novel agents in prostate cancer

Hallmark of cancer	Example of targeted therapy
Self-sufficiency in growth factors	Erlotinib (EGFR inhibitor)
Evading apoptosis	YM155 (Inhibitor of Survivin)
Tissue invasion and metastasis	Dasatinib (Src inhibitor)
Sustained angiogenesis	Bevacizumab (VEGF inhibitor)
Increased cell metabolism	CCI-779 (mTOR inhibitor)

Table III illustrates examples of novel agents that target various phenotypic hallmarks of cancer. Many targeted therapies however act on a number of these phenotypes e.g. Atrasentan (ET-A receptor antagonist) is thought to affect the following functions: evading apoptosis, tissue invasion and metastasis and sustained angiogenesis. Androgen ablation therapy in prostate cancer also affects distinct aspects of carcinogenesis including metabolism, apoptosis and growth.

1.4   Molecular mechanisms of targeted therapy

Different mechanisms have been used to target molecular signalling in cancer with inhibition of RTK signalling offering the most success to date. Currently two classes of compounds are commonly used to inhibit RTK activation: small molecule tyrosine kinase inhibitors (TKI) and monoclonal antibodies (mAb). While both inhibit RTK signalling, they have distinct targeted epitopes and mechanisms of action (21). ATP-dependent TKIs can translocate through plasma membranes and interact with the cytoplasmic domain of cell surface receptors and intracellular signalling molecules. They competitively bind to the ATP-binding site in the catalytic domain of the receptor, inhibiting autophosphorylation and the activation of intracellular signal transducers.

In contrast mAbs can only act on molecules that are expressed on the cell surface or secreted, as they are unable to pass through the cell membrane. Different ways mAbs inhibit RTK signalling are suggested which can be further separated into direct and indirect mechanisms. Direct action includes the following: blocking the function of target signalling molecules or receptors, stimulating function which results in apoptosis and targeting function by conjugating mAbs with toxins, radioisotopes or cytokines. The indirect action described involves the binding of immunoglobulins to the surface of the cells mediating complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity, both of which eventually lead to cancer cell death.

TKIs tend to offer the most efficient method of targeted therapy as they block the kinase activity of the targeted receptor, significantly inhibiting the activation of downstream signalling. Depending on the selectivity of the candidate compounds, they may act as multi target agents as they are prone to bind to different RTKs due to the structure of the ATP-binding pocket being highly conserved within the tyrosine kinase family. In contrast mAbs tend to be specific inhibitors and have been shown to only offer modest antitumour activity when used alone with more significant effects being noted when combined with chemotherapy.

In addition to TKIs and mAbs, other types of targeted therapy have also been used successfully in preclinical studies. Antisense oligonucleotides target specific sequences in the mRNA of interest, implicated to be a causative factor for carcinogenesis, thus inhibiting its expression and protein translation. Antisense oligonucleotides are currently in clinical trial as anti-cancer agents however to date none have been approved for use.

1.5 Molecular targeted therapy – examples of recent success in selected tumour types

1.5.1 BCR-ABL tyrosine kinase in Chronic Myeloid Leukaemia

Chronic Myeloid Leukaemia (CML) is a myeloproliferative disease which is characterised by the expansion of a clone of haemopoietic cells that carry the Philadelphia chromosome (Ph). The Ph chromosome is due to a reciprocal translocation between the long arms of chromosomes 9 and 22. This translocation results in a novel fusion gene BCR-ABL, which encodes a constitutively active protein tyrosine kinase. The treatment of CML has been revolutionised since the discovery of a relatively specific inhibitor of the BCR-ABL tyrosine kinase, imatinib (Glivec). Treatment with this small molecule inhibitor in patients diagnosed with chronic phase CML results in high rates of complete cytogenetic remission (>87%) and molecular remissions with low or undetectable amounts of BCR-ABL transcripts (22). Imatinib is now established as standard therapy for this patient group taking over from its predecessor interferon-α. Results from a 5 year follow up study were recently published confirming durable responses in patients with chronic-phase CML (23). This study followed patients who had been initially treated with imatinib and then were either continued on treatment with this tyrosine kinase inhibitor or given interferon-α and cytarabine. Patients treated with imatinib had high rates of cytogenetic response and the estimated overall survival at 60 months was 89% compared to previous studies of interferon-α plus cytarabine with survival rates around 65% (24).

Approximately 10% of patients treated with imatinib will subsequently develop resistance, and between 50-90% of these cases are associated with mutations in the kinase domain of BCR-ABL (25). Over-expression of Src related kinases has also recently been implicated in treatment resistance, which may explain the efficacy of some second generation BCR-ABL inhibitors such as nilotinib and dasatinib in imatinib relapsed disease (26;27). Novel therapies continue to be developed as advances in the molecular understanding of disease progression in CML emerge.

1.5.2 Trastuzumab (Herceptin) in Breast Cancer

Another example of successful targeted therapy is HER2 mediated therapy in breast cancer. The human epidermal growth factor receptors (HER), namely HER 1-4, are a group of four transmembrane tyrosine kinase receptors that normally regulate cell growth and survival. HER2 gene amplification and protein overexpression is found in 20-30% of invasive breast cancers (28). HER2 positive breast cancer patients in general have decreased overall survival and differential responses to standard chemotherapeutic and hormonal regimes (29).

Trastuzumab (Herceptin) is a monoclonal antibody to the HER2 ectoderm and has been shown to significantly improve the outcome for HER2 positive breast cancer patients. It acts by binding to the extracellular juxtamembrane domain of HER2, resulting in inhibition of proliferation and reduced survival of HER2 dependent tumours. Trastuzumab has been shown to significantly improve patient outcome in both HER-2 positive breast cancer of both early stage and metastatic stage (30;31). Trastuzumab is recommended as treatment for women with early-stage HER2 positive breast cancer following surgery, chemotherapy (neoadjuvant or adjuvant) and radiotherapy (32). It is also recommended that all patients who have HER2 positive advanced breast cancer be considered for treatment with trastuzumab either as monotherapy (if metastatic and had previous chemotherapy) or in combination with chemotherapy and/or hormonal agents (33;34).

1.6   Target signalling in prostate cancer

Despite the success of targeted therapy in other tumour types and the improved understanding of abnormal signalling activities in prostate carcinogenesis, none of the novel agents studied so far have shown adequate efficacy to justify their routine use in prostate cancer. Research and drug development programmes therefore continue to strive for a better understanding of the signalling network involved and in order to develop a more efficacious treatment regime.

1.6.1   PI3K Pathway

The phosphoinositide 3-kinase pathway has been shown to regulate multiple cellular events in prostate cancer. PI3K activation results in the catalytic conversion of phosphatidylinositol biphosphate (PIP2) to phosphatidylinositol triphoshate (PIP3) which in turn activates Akt. Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that regulates cell growth and is involved in tumourigenesis. Akt phosphorylates and activates mTOR to enhance cell growth. PTEN is a tumour suppressor gene which negatively regulates the PI3K pathway. In prostate cancer loss of PTEN and/or Akt activation is coupled with high Gleason score (towards an undifferentiated phenotype), an advanced clinical stage and poorer prognosis (35;36). The PI3K pathway is also associated with hormone resistance and chemotherapeutic insensitivity. High levels of phosphoAkt immunostaining have been shown to be predictive of biochemical recurrence and phosphoAkt-1 expression has been suggested to be an independent prognostic marker of biochemical recurrence-free survival in a subgroup analysis of patients with Gleason score of 6 and 7 (n=488 p=0.0012) (35). *In vitro* studies have shown that PTEN loss is associated with increased resistance to both doxorubicin and paclitaxel and treatment with a PI3K inhibitor reverses this chemoresistance in prostate cancer cells (37).

Inhibition of PI3K signalling has been studied in vitro using two small molecule inhibitors which have been available for some time, namely wortmannin and LY24002. Both of these have demonstrated antitumour effects in prostate cancer cell lines (38), however both have a relatively broad spectrum of activity inhibiting other kinases related to PI3K such as ATM and ATR. There are a large family of PI3Ks including the four class I lipid kinase isoforms p110α, p110β, p110δ and p110γ. Each of these isoforms is thought to have an individual role in cell behaviour. Of note, p110α protein has been found to be overexpressed and mutated in a number of solid tumours including prostate cancer (39). Targeting the p110α isoform in cancer is therefore an attractive strategy in drug

development for prostate cancer. PI-103 inhibits the PI3K pathway at multiple sites including the p110α isoform as well as mTOR-raptor complex (mTORC1) and mTORC2. This dual p110α/mTOR inhibitor has been shown in PC3 cells to reduce proliferation and invasion and has significant anti-tumour activity in xenograft tumour models (40). Formal published results on the use of PI-103 in clinical trial are awaited.

Inhibition of Akt is another important strategy for drug development. A number of small molecule inhibitors including A-443654, Akt-I-1 and Akt-I-2 have been tested *in vitro* and in preclinical *in vivo* models for their anti-tumour effects with promising findings (41). Future investigations of these agents in the clinic will inform us of their potential as novel therapies.

PTEN inactivation results in deregulated signalling through the mTOR pathway. mTOR is the target of the antibiotic rapamycin which is used as an immunosuppressant following renal transplantation. At present there are three rapamycin derivatives in development: temsirolimus (CCI-779), everolimus (RAD001) and deforolimus (AP23573). Both CCI-779 and RAD001 have been shown to have beneficial effects *in vivo*. Transgenic mice with activated Akt or PTEN deficiencies have decreased tumour growth when treated with CC1-779 (42). Similarly, RAD001 has been shown to reverse prostate neoplastic phenotypes in mice expressing human Akt (43). AP23573 has been shown to have promising antitumour activity in sarcoma and selected haematological malignancies. A phase II trial using CCI-779 for patients with CRPC has recently finished recruiting however to date no results are available. Both RAD001 and AP23573 and are currently being assessed in phase II clinical studies.

1.6.2 Src Family Kinase

The Src family kinases (SFK) are a group of non-receptor protein tyrosine kinases which are involved in tumour adhesion, motility, invasion and angiogenesis. SFK members Src and Lyn are highly expressed in prostate cancer cell lines as well as in the majority of prostate cancer specimens (44;45). Src signalling is involved in androgen induced proliferation of prostate cancer cells and recently it has been suggested that Src is involved in the transition to androgen independent growth (46). Bone metastases occur in the majority of prostate cancer patients with advanced disease. Src inhibition *in vivo* has shown reduced morbidity, lethality and incidence of bone metastases in breast cancer mouse models (47). Therefore a small molecule inhibitor targeting Src may have the

therapeutic advantage in prostate cancer in minimising morbidity associated with bone metastases and possibly improving survival outcome.

Dasatinib is a SFK/Abl inhibitor and has *in vitro* activity in prostate cancer cells. Proliferation, invasion and migration have been shown to be reduced when DU145 cells were pretreated with dasatinib (44). A significant reduction in tumour growth and metastases has also been demonstrated when dasatinib was used in prostate cancer xenograft models. Phase II studies are currently ongoing to assess dasatinib in CRPC as well as combination therapy with docetaxel in metastatic disease. Results from phase II trials in imatinib-resistant CML with dasatinib have shown minimal toxicity thus reducing concerns that Src inhibition may suppress multiple pathways and be associated with high levels of adverse effects (48;49).

AZD-0530, another Src inhibitor, has been shown to inhibit the growth of prostate cell lines and suppress migration in PC3 and DU145 cells (50). It also suppressed the growth and metastasis of androgen-independent LNCaP cells *in vivo* (51). Studies in healthy volunteers have found only mild side effects with AZD-0530 and a phase II trial in CRPC has recently started. Finally Bosutinib, another Src/Abl inhibitor, which has shown *in vitro* and *in vivo* activity in models of CML (52), colon cancer (53) and breast cancer (54); no published results are available for prostate cancer to date.

1.6.3   The ErbB receptor family

The ErbB family of receptor tyrosine kinases include epidermal growth factor receptor (EGFR/ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). Both EGFR and HER2 have critical roles in cell growth, differentiation and the motility of normal and cancer cells through the activation of downstream signalling pathways such as the MAPK and PI3K/Akt pathway. EGFR is highly expressed in primary prostate cancer and associated metastases (55). Overexpression of EGFR is associated with poor prognosis (56) and the transition of androgen sensitive disease to androgen independence (57).

Current anti-EGFR therapies include both TKIs and mAb therapy. Small molecule TKIs have been the most successful method to date in EGFR targeting with Erlotinib (Tarceva) approved for use in the treatment of pancreatic and non-small cell lung cancer (NSCLC) after failure of at least one prior chemotherapy regimen (58;59). Progression free survival in NSCLC however is only improved by 2 months with a reported response rate of 8.9 % (59). Methods have been investigated to improve patient selection with both EGFR mutation and an increase in EGFR copy number recognised as biomarkers for

favourable response to Erlotinib in NSCLC (60;61). Erlotinib has also been studied in CRPC in combination with docetaxel. Results of a phase II study demonstrated no beneficial anti-cancer activity with erlotinib added to docetaxel monotherapy (62). However, the pre-treatment EGFR status was not available. Further studies of erlotinib in non-metastatic prostate cancer and chemotherapy naïve disease are ongoing. EGFR mutation and amplification of the EGFR gene have been shown to occur frequently in advanced prostate cancer however unlike NSCLC there are currently no stratified studies to include either EGFR mutation or gene amplification to improve patient selection for treatment with anti-EGFR therapy (63). Erlotinib combined with bevacizumab (vascular endothelial growth factor inhibitor) has been shown in NSCLC to increase progression free survival and this combination regime is currently being tested in prostate cancer patients following radical prostatectomy (64).

Gefitinib (Iressa) is another orally active EGFR TKI which has been shown to have antiproliferative activity in prostate cancer cell lines(65). EGFR suppression with gefinitib results in significant growth inhibition in PC3 xenografts (66) and has been shown to reduce the incidence of prostate cancer metastasis in nude mice (67). Phase I data of gefitinib monotherapy in a range of solid tumours showed promising antitumour activity (68); however phase II results in non-metastatic CRPC reported no positive response (69;70). EGFR status was assessed in a subset of patients where moderate-strong staining was noted in 12 out of 16 cases (69). There was no correlation between EGFR expression and PSA decline or time to progression suggesting that in prostate cancer EGFR overexpression is not indicative of response to gefitinib.

PD168393 also selectively inhibits EGFR and it has been shown to sensitise prostate cancer cells to the cytotoxic activity of paclitaxel (71). There is currently no *in vivo* or clinical evidence of this small molecule inhibitors effect in prostate cancer.

A number of anti-EGFR monoclonal antibodies have been introduced over recent years with cetuximab being approved for use in patients with colorectal cancer refractory or intolerant to irinotecan and in patients with squamous cell carcinoma of the head and neck (72;73). Phase I results of panitumumab, another EGFR mAb in advanced solid tumours showed treatment was well tolerated, but patients with prostate cancer showed very limited response (74). Patients with CRPC are currently being recruited for phase II trials of cetuximab treatment combined with either docetaxel or mitoxantrone. Taken together, while EGFR inhibitors, namely Erlotinib, Gefitinib and PD168393, all target the kinase domain of EGFR and have encouraging data to support their clinical use in a number of tumour types, it is disappointing that similar efficacy has not been seen in

prostate cancer. Many factors may contribute to this (see Drug development against implicated biological targets, Section 6.1). The lack of stratification among patients according to their EGFR status (namely expression level, mutation and amplification), as an indicator of the significance of abnormal EGFR function as a driving event in prostate carcinogenesis, may partly explain the negative results in a number of trials. Future studies should focus on targeted assessment of these novel agents in prostate cancer sufferers with defined genetic (or epi-genetic) lesions involving EGFR.

Amplification of the HER2 gene and/or overexpression of the HER2 protein occurs in 20-30% breast cancer patients and is associated with an unfavourable outcome (28;29). In contrast, the impact of HER2 in prostate cancer is much less clear, with HER2 overexpression and/or amplification being identified much less frequently in prostate cancer (75). There is also no strong consensus regarding its impact in clinical outcome. This may account for the negative findings in a phase II study using trastuzumab (herceptin) in CRPC (76), which did not support a phase III trial of herceptin.

HER2 is an orphan receptor and functions as a coreceptor. Recent work has shown that HER2/HER3 dimerization and activation may stimulate androgen-receptor mediated signalling in an androgen depleted environment (77). Agents, which may target HER2 dimerisation signalling, include pertuzumab and lapatinib. Pertuzumab is a monoclonal antibody which inhibits HER2 dimerisation with other HER family members including EGFR, HER3 and HER4 (78). Unfortunately, a phase II trial of pertuzumab in CRPC showed no PSA response, while the treatment was well tolerated (79). A phase I study of docetaxal and pertuzumab in solid tumours shows promising results in the CRPC patients with stable disease observed in half of the patients after 4 cycles (12 weeks) of treatment (80). The dual EGFR/HER2 TKI lapatinib is more potent than gefitinib to inhibiting proliferation (81). A phase II trial of lapatinib in recurrent or metastatic prostate cancer is currently being evaluated.

Despite the infrequency of HER2 overexpression, the kinase activity of this receptor is strongly implicated in the progression to CRPC. Tyrosine phosphorylation of HER2 has been shown to play a key role in regulating androgen mediated proliferation in human prostate cancer cells (82). Furthermore the inhibition of HER2 kinase activity by lapatinib impairs AR transcriptional activity (83). The discrepancy for the role of HER2 (and EGFR) between *in vitro* analysis and clinical studies requires attention in future translational trials to incorporate assessment of target (including expression level, mutation and amplification status) and clinical evaluation of efficacy along with validation of drug induced effects on the target.

HER3 plays an essential role in EGFR and HER2 driven tumourigenesis. This ErbB member is also known to have a central role in mediating PI3K/Akt signalling. In contrast to other ErbB receptors, HER3 is kinase inactive and is not a direct target of tyrosine kinase inhibitors. It is suggested that HER3 may be responsible for the drug resistance, which has been observed with EGFR and HER2 inhibitors, and therefore research is currently focussing on HER3 as a new target for anticancer therapies.

1.6.4   The Fibroblast growth factor receptor (FGFR) family

Fibroblast growth factors (FGF) including FGF1, FGF2, FGF6, FGF8 and FGF17 are all expressed at increased levels in prostate cancer (84-89). FGFs signal through fibroblast growth factor receptors (FGFR1-4) activation, which leads to downstream signalling through multiple pathways including the MAPK and PI3K pathways. FGFR4 is overexpressed in prostate cancer with strong expression associated with high grade disease and decreased survival (90). FGFR1 and 2 also show overexpression in prostate cancer when compared to BPH; however FGFR2 shows no correlation with tumour grade or stage (91). Recent transgenic models further validate the role of FGFR signalling as key to prostate carcinogenesis(92;93).

Over recent years work has focussed on selective targeting of the receptors involved in FGF signalling as a novel therapeutic approach in prostate cancer. FGFR inhibition using siRNA to target FGFR4 in prostate cancer cells shows suppression of proliferation and invasion (91). SU5402 potently blocks FGFR1 tyrosine kinase activity while weakly inhibiting PDGF receptor function   *In vivo* SU5402 has been shown to decrease xenograft tumour growth and suppress PSA and promotrilysin expression in a prostate cancer model (94). To date there are no published results of a FGFR inhibitor in a clinical trial.

1.6.5   Type I insulin-like growth factor receptor

Type I insulin-like growth factor receptor (IGF-1R) is activated by insulin-like growth factor I or II (IGF-I/II). The IGF-1R is crucial in maintaining the malignant phenotype with evidence showing its role in proliferation, angiogenesis and apoptosis. IGF-1R activation is ligand dependent and once activated IGF-1R recruits and phosphorylates adaptor proteins, which serve as docking sites for other signalling molecules. This results in activation of intracellular signalling pathways including PI3K and ERK1/2 of the MAPK pathway.

IGF-1R is significantly up-regulated in prostate cancer when compared to benign prostatic epithelium (95) and *in vitro* work has shown that IGF-1R overexpression is associated with androgen independent antiapoptotic and promitotic signalling, processes which drive prostate cancer disease progression (96).

Several approaches have been used to inhibit IGF-1R signalling via reduction or neutralisation of circulating IGF-1 or by inhibition of IGF-1R activation. At present the most promising method is to use antibodies against IGF-1R. The human antibody A12 has been used in prostate xenograft tumours to study the beneficial effects of blocking IGF-1R signalling following castration (97). IGF-1R inhibition enhanced the effect of castration and prolonged tumour-specific survival. Combination treatment was also associated with a decrease in AR signalling and nuclear AR localisation. These results suggest that IGF-1R inhibition in conjunction with androgen ablation enhances the inhibition of signalling through the AR, which remains important even in CRPC. IGF-1R inhibition in xenografts has also been shown to potentiate the activity of cytotoxics (98). A12 and CP-751,871 another monoclonal anti-IGF-1R antibody are currently in phase II trial to test their efficacy in combination with docetaxel and prednisone in the treatment of CRPC.

Both IGF-1 and IGF-binding protein-3 (IGFBP-3) have been reported to be associated with an increased risk of prostate cancer. Conflicting evidence for both biomarkers has been published however with recent evidence showing no observed association for IGFBP-3 (99;100).

1.6.6   Heat Shock Proteins

Heat shock proteins (HSP) are cellular chaperones involved in the regulation and stabilisation of a number of key signal transduction proteins including MAPK, Akt, AR and Src kinases (101).

It has been suggested that HSP27 may be at the centre of many pathways involved in the regulation of the response of a cell to treatment induced stress and targeting it may lead to silencing of multiple survival pathways. Apoptosis resistance is associated with increased expression of multiple HSPs and small HSP such as HSP27 have been found to be important chaperones which protect cancer cells against apoptosis (102). Cytotoxic treatments such as chemotherapy and radiotherapy have a negative effect on cells by inducing apoptosis. HSP27 expression is low or absent in hormone naïve prostate cancer, with increasing levels demonstrated in tumours once treatment is commenced. In CRPC,

HSP27 is uniformly overexpressed and it is thought that this molecular chaperone is important in the progression of prostate cancer from hormone sensitive to resistant (103). The development of hormone resistance is thought to be attributed to decreased apoptotic rates rather than an increase in proliferation (104). The antisense oligonucleotide OGX-427 which targets HSP27 has been shown to inhibit HSP27 expression and synergises with androgen ablation and chemotherapy in prostate cancer xenograft models. *In vitro* work has also been published showing that down regulation of HSP27 radiosensitises prostate cancer cells (105). A phase I clinical trial of the antisense oligonucleotide OGX-427 either alone or with docetaxel is currently recruiting patients.

HSP90 is a key regulator of ligand-independent nuclear localisation and activation of AR in androgen-refractory prostate cancer cells (106). HER2, Raf-1 and Akt are also regulated by HSP90. Geldanamycin and its derivative 17-AAG are anasmycins that interfere with the action of HSP90 leading to the degradation of HSP client proteins. Both have been shown to have anti-tumour effects in prostate cancer cells. Despite its potential use as an effective cancer treatment, geldanamycin presents several major drawbacks as a candidate drug, particularly with hepatotoxicity. 17-AAG however has a more favourable toxicity profile. Using an *in vivo* prostate cancer model, 17-AAG inhibited tumour growth as well sensitised tumour responses to taxol treatment (107).

Phase I data has recently been published of 17-AAG in patients with advanced cancer of which 18 of the 54 patients had CRPC (108). No partial or complete responses were observed with only 1 CRPC patient demonstrating a 25% decline in PSA with treatment. 17-AAG may however be more beneficial in enhancing the effect of cytotoxic therapy rather than as a monotherapy. Phase I and II trials are therefore currently recruiting patients for treatment in combination with docetaxal and other cytotoxics.

1.6.7   Anti-angiogenic agents

Inhibition of angiogenesis has emerged as a promising therapeutic target for a number of solid tumours. Anti-angiogenic agents can reduce intra-tumoural interstitial pressure and increase drug delivery of anti-cancer agents. This mode of anticancer therapy has been successful recently in the treatment of renal cell carcinoma (RCC). Sorafenib is an orally active multikinase inhibitor which seems to have an anti-angiogenic effect in RCC due to its inhibitory effect toward vascular endothelial growth factor receptors (VEGFR) and their targets. Increased production of VEGF is implicated in the progression

of clear-cell RCC (109). Sorafenib has been licensed for maintenance treatment of metastatic RCC following the positive result in trial in advanced clear cell RCC (110).

Over-expression of vascular endothelial growth factor (VEGF) and its receptors are associated with the progression of prostate cancer (111). VEGFR-2 inhibition in orthotopic prostate cancer models reduced tumourigenicity and metastases, supporting the potential use of anti-VEGF agents in prostate cancer (112). Results of phase II trials using Sorafenib in CRPC however have shown minimal effects on PSA response or radiographic appearance of bone metastases (113-115).

Bevacizumab is a humanised murine mAb to VEGF which has been shown to provide clinical benefit in colorectal, NSCLC and breast cancer. Combining bevacizumab with 5-fluorouracil inhibits angiogenesis and tumour growth in mouse prostate cancer models (116). Currently bevacizumab is in Phase II studies for use in high risk cases in combination with medical or radiotherapy. A Phase III trial is also recruiting patients with CRPC to assess the effect of bevacizumab with docetaxel.

Platelet derived growth factor (PDGF) is involved in autocrine stimulation of tumour cells, regulation of stromal fibroblasts as well as tumour angiogenesis (117). Platelet derived growth factor receptor (PDGF-R) is a receptor tyrosine kinase which has 2 subunits, α and β. Upon PDGF binding these subunits either homo- or heterodimerize. Immunohistochemistry has shown PDGF-R α and β to be expressed in 88% of primary prostate tumours and in 80% of bone marrow metastases (118). Inhibition of this signalling pathway appears to be an attractive target in prostate cancer however an initial Phase II trial using the PDGF-R inhibitor SU101 showed minimal effect in CRPC (118).

Imatinib is an inhibitor of PDGF-R signalling as well as the BCR-ABL tyrosine kinase. A number of phase II studies have been done using imatinib in prostate cancer patients who have biochemical relapse following radical (radiotherapy or prostatectomy) treatment. As a single agent imatinib has shown limited biochemical activity with a significant incidence of grade 3 and 4 toxicity leading in some cases to early trial closure (119-121). There has been suggestion that this PDGF-R inhibitor may have a more beneficial role if used in combination with taxane based chemotherapy. Pre-clinical models have demonstrated synergistic effects of imatinib and paclitaxel when used in mouse models of prostate cancer bone metastases (122). Recent published results of a phase II trial combining imatinib and docetaxel in CRPC patients with bony metastases showed no therapeutic benefit despite confirmation of effective p-PDGFR inhibition (123). An osteolytic model of bone metastases was used in the preclinical study as opposed to the

osteosclerotic lesions typically seen in human prostate cancer, which may contribute to the discrepancy in efficacy seen in preclinical model and clinical trial. High levels of grade 3 toxicities were also observed in this phase II trial of imatinib and docetaxel and it has been recommended that further studies of this combination should not be pursued.

Other agents with anti-angiogenic properties which may be of clinical benefit in CRPC include thalidomide and its analogues. Thalidomide has multiple mechanisms of action including immunomodulatory effects on the tumour microenvironment (124). Prostate cancer progression and metastasis has been suggested to be mediated by stromal-epithelial interactions, which could be targeted by thalidomide. Thalidomide is also known to have anti-angiogenic properties from results of pre clinical studies (125). Phase I and II studies showed promising results when thalidomide was used in CRPC patients as well as in studies using combination therapy with docetaxel (126-128). These results support the need for additional studies of thalidomide in CRPC either alone or as combination therapy and current studies are focussing in particular on the potential effect of thalidomide on bone metastases.

1.6.8   The Endothelin Axis

The endothelin axis (ET axis), comprises of the three peptides endothelin (ET)-1, -2, -3 and their receptors ET-A and ET-B. Most of the activities of ET-1 are mediated via the ET-A receptor. ET-1 has important roles in a host of biological functions, including cellular proliferation, apoptosis and angiogenesis.   It also stimulates osteoblast proliferation, leading to osteoblastic bone metastases which is typical of prostate cancer (129).

In metastatic CRPC endothelin receptors are over-expressed and higher levels of endothelin are associated with progressive disease (130). Atrasentan is a selective endothelin-A (ET-A) receptor antagonist that inhibits or reverses the downstream effects of endothelin-1 (ET-1). Phase I and II studies of atrasentan showed encouraging results when used in men with metastatic CRPC (131;132). A phase III trial comparing atrasentan to placebo in patients with metastatic CRPC did not show significant delay in disease progression (133). Bone alkaline phosphatase (BAP) was measured as a biomarker of disease progression and increases from baseline to final BAP were significantly lower in the patients treated with atrasentan. This suggests that atrasentan may have targeted activity in the bone microenvironment and that using this ET-A receptor antagonist may potentially prevent bone metastases formation or slow the onset of skeletal related events

in CRPC patients. A phase III trial studying the possible synergistic effect of atrasentan and docetaxel is currently recruiting patients with CRPC who have bone metastases.

1.6.9 Anti-apoptotic proteins

Accelerated or dysregulated proliferation is well recognised as a major causative factor in tumour development and progression. In addition to this, defective apoptosis (programmed cell death) has been highlighted as a key factor in carcinogenesis. Disruption of these anti-apoptotic signals through selective therapeutic targeting could offer a novel strategy for drug development programmes and a number of potential targets are currently being reviewed. Survivin is a proto-oncogene which is a member of the inhibitor of apoptosis family and has been associated with phenotypically aggressive prostate cancer and androgen resistance (134;135). Inhibition of this pathway would aim to lower the anti-apoptotic threshold in cancer cells. YM155 is a novel small molecule inhibitor of survivin which induces apoptosis in prostate cancer cell lines and regression of tumour growth in CRPC xenografts (136). Phase II trials are currently under way with YM155 and docetaxel in CRPC.

It has recently been shown that IGF-1/ mTOR signalling increases levels of survivin in prostate cancer cells (137). This suggests that suppression of IGF-1/Akt/mTOR signalling may be beneficial to lower an anti-apoptotic threshold maintained by survivin in aggressive prostate cancer.

Bcl-2 is another anti-apoptotic regulatory protein, which is associated with poor therapeutic response and poor clinical outcome in small cell lung cancer (SCLC). There is also strong *in vitro* evidence to show that bcl-2 suppression in SCLC is associated with enhanced chemosensitivity. Disappointingly, a phase II study in patients with advanced SCLC treated with carboplatin and etoposide +/- G3139 (or oblimersan, a bcl-2 antisense oligonucleotide) showed no added effects with the addition of oblimersan; there was in fact a potential negative impact on survival. (138). Of note oblimersan combined with chemotherapy has been shown to improve survival in melanoma patients (139). To explain the negative results in SCLC, two possible explanations are: (1) Despite promising data from *in vitro* and *in vivo* model systems, bcl-2 over-expression does not play a critical role in clinical SCLC, which would argue for more relevant *in vivo* model systems. (2) Oblimersan is not suppressing bcl-2 at a sufficient level for it to enhance chemotherapeutic sensitivity. Tumour biopsy following oblimersan treatment would enable formal assessment of the target status, an important consideration for future trial design. Finally,

it may also be possible that off-target effects such as immunostimulatory effects may be responsible for the effects observed in melanoma but not in SCLC.

Bcl-2 is over-expressed in CRPC (140), and its inhibition results in delayed development of hormone resistance and enhanced effects of chemotherapy in prostate cancer mouse models (141). A Phase II study of docetaxel with oblimersan showed no additional benefit in overall survival and PSA response rates to docetaxel monotherapy in CRPC (142). Protein expression of bcl-2 was analysed in peripheral blood mononuclear cells pre and post treatment in order to assess the pharmacodynamics of oblimersan treatment. No correlations between bcl-2 levels and response rates or survival were noted. However, once again, intra-tumoural biomarker, namely bcl-2 level, was not ascertained. Currently there are no trials recruiting prostate cancer patients for treatment with oblimersan.

1.6.10 Androgen receptor signalling

Targeting the reactivation of AR signalling in CRPC is currently the focus of many drug development programmes and clinical trials. Anti-androgens currently approved for use in prostate cancer (bicalutamide, flutamide, cyproterone acetate) have limited use in hormone resistant disease and all have been observed to convert to agonists in progressive disease (143). A number of novel anti-androgens are currently being introduced which have significantly higher affinity than bicalutamide for the AR. Both novel antiandrogens MDV-3100 and BMS-641988 are currently in Phase I clinical trial in CRPC.

CRPC progression may be due to residual serum androgens as well as upregulated intracrine androgen synthesis from the tumour cells. Therefore methods to further lower androgen levels are under investigation. Ketaconazole is a synthetic anti-fungal agent, which is currently used in some centres for patients with CRPC due to its action as a potent inhibitor of CYP450-dependent adrenal and testicular androgen production. Studies have shown that ketaconazole has a modest activity in CRPC however its use is associated with a rise in adrenal androgen levels at the time of progression (144;145).

Cytochrome P (CYP) 17 is a microsomal enzyme that catalyses two key steroid reactions in both adrenal and tumour intracrine androgen biosynthesis involving 17 alpha-hydoxylase and C (17,20)-lyase. Abiraterone acetate is a selective, irreversible inhibitor of CYP17, suppressing testosterone to castrate levels in the short term; however there is some testosterone recovery with long-term use. Addition of this CYP17 inhibitor to GnRH analogue treatment results in decreases in both testosterone and adrenal androgen levels

(146). Abiraterone has recently been assessed in a phase I study to be safe and may have favourable antitumour activity (147). A phase III study is currently recruiting patients with CRPC who have progressive disease despite docetaxel treatment.

Testosterone is converted to dihydrotestosterone (DHT) in peripheral androgen dependent tissues by two isoforms of 5 α-reductase, SRD5A1 and SRD5A2. The type 2 enzyme has been identified as the dominant type in benign prostate tissue and finasteride, a specific type 2 inhibitor, is approved for use in BPH. It was previously suggested that finasteride may prevent or delay the development of prostate cancer. The Prostate Cancer Prevention Trial studied men who were prescribed either finasteride or placebo for seven years (148). Although a 24.8% reduction in the prevalence of prostate cancer in men on finasteride was observed, treatment with this 5 α-reductase inhibitor was associated with a significant increase in high grade disease. Dutasteride (a dual inhibitor of SRD5A1 and SRD5A2) is currently being evaluated as a chemopreventive agent in prostate cancer as SRD5A1 has been shown to be upregulated in progressive prostate cancer (149). Dutasteride has also been shown to inhibit *in vivo* tumour growth when combined with castration in androgen responsive xenograft models (150). In addition, Phase II and III trials are currently recruiting patients with prostate cancer of various stages for assessment of treatment with dutasteride alone or in combination with androgen deprivation therapy.

1.7 Targeting tumour suppressor genes

In addition to inhibiting oncogenic signalling, drug development programmes are also targeting tumour suppressor genes through either activation or induced expression as an alternative approach for advanced prostate cancer therapy.

1.7.1 p53 and prostate cancer

While the frequency of p53 mutations in early prostate cancer is low, heterozygous loss of function mutations are often observed in advanced disease (151). Furthermore, p53 turnover is maintained by the E3 ubiquitin ligase murine double minute 2 (MDM2) which binds to the c-terminus of p53 and targets it for degradation. Over-expression of this p53 regulator has been observed in several cancer types including prostate cancer (152). Inhibition of the interaction between MDM2 and p53 allows reactivation of p53, and this is currently a promising anti-cancer strategy. Small molecule MDM2 inhibitors such as nutlin-3 have shown promising anti-tumour activity in LNCaP xenograft models (wild type p53) (153). Recent evidence suggests that p53 signalling is also important in androgen signalling with wild type p53 over-expression being associated with decreased androgen function (154). *In vitro* treatment with nutulin-3 has been shown to have a suppressive effect on androgen signalling (155). Antisense MDM2 oligonucleotide (AS-MDM2) is another method of targeting the interaction between MDM2 and p53. Treatment with AS-MDM2 enhanced the in vitro efficacy of radiotherapy and chemotherapy in prostate cancer cells (156). *In vivo* AS-MDM2 sensitises androgen sensitive xenografts to androgen deprivation therapy (157). The MAPK and PI3K pathways are also involved in p53 regulation. p53 can activate the Raf/MEK/ERK pathway and ERK can stabilise p53 by phosphorylation in cervical cancer cells (158). On the other hand, Akt phosphorylates MDM2, enhancing its activity and destabilising p53 (159). The p53, PI3K and MAPK pathways are connected functionally and targeting these signalling systems either in isolation or together may further synergise the effects of specific (conventional) therapies in prostate cancer. Therefore, it is important to incorporate relevant robust patient selection and accurate target validation in the design of future trials in the assessment of novel therapies.

1.7.2 Epigenetics in prostate cancer

Epigenetic changes encompass a number of reversible cellular events including DNA methylation and histone modifications, which can modulate gene expression and alter tumour phenotypes. A number of genes are hypermethylated and silenced in prostate cancer such as GST*pi*, commonly hypermethylated in prostate cancers (>90%) (160). DNA methylation levels can be altered by chemical inhibition of DNA MTase enzymes. However, to date, no demethylating agent has shown significant response in solid tumours (161;162).

Modification of the surrounding histones in which the DNA is packaged is another important epigenetic mechanism involved in carcinogenesis. The expression of histone deacetylases (HDAC) is frequently upregulated in prostate cancer with increased expression being associated with hormone refractory disease (163). HDAC inhibitors induce growth arrest and apoptosis *in vivo* as well as regulate angiogenic and immune functions (164). Limited response however has been observed with HDAC inhibitors in solid tumours (165). Although an increase in histone acetylation was observed in the peripheral-blood mononuclear cells of patients treated, the histone acetylation status in the target organ was not assayed. Overall, HDAC inhibition alone does not appear to be effective as a cancer therapy (165).

1.8   The MAPK signalling pathway

The mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases including the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), ERK5, c-Jun NH_2-terminal protein kinase (JNKs) and p38 MAPKs. The MAPK signaling cascade is involved in various cellular functions including cell proliferation, differentiation and migration. Each subfamily of MAPKs can be stimulated by a separate protein kinase cascade that leads to the activation of a specific MAPK kinase kinase (MAPKKK) and a MAPK kinase (MAPKK), leading to the phosphorylation and activation of the downstream MAPK.   ERK1 and ERK2 are isoforms of the 'classical' MAPK, ERK1/2 which is activated by MEK1/2 following stimulation by a variety of mitogens and is known to be important in cell proliferation.

1.8.1   MEK5/ERK5 signalling pathway

Mitogen/extracellular signal regulated kinase kinase-5 (MEK5) is the most recently identified MAPKK and has been shown to specifically activate ERK5.   ERK5, also known as Big MAP kinase 1 (BMK1) is almost twice the size of other MAPKs (815 amino acids). The N-terminal kinase domain of ERK5 is highly homologous to ERK1/2 however its large C-terminal domain is unique and is thought to be responsible for its unique biological activities.

Cellular stimulation of ERK5 is induced in response to growth factors, oxidative stress, and hyperosmolar conditions. EGF is a potent activator of ERK5 and ERK5 has been shown to be required for EGF-induced proliferation and progression through the cell cycle(166). A number of tyrosine kinases are recognised to activate the ERK5 pathway including the epidermal growth factor receptor (167), HER2/Neu (168)and Src (169). Oncogene products such as Ras (167) and COT (170)can also activate the MEK5/MERK5 pathway.

In resting cells, over-expressed ERK5 localises to the cytoplasm. When co-expressed with constitutively active MEK5 however ERK5 translocates to the nucleus (171). A proposed mechanism for this nuclear shuttling relies on the nuclear localization and export signals (NLS and NES) thought to be located in the C-terminal domain, which determine the subcellular localization of ERK5 depending on its phosphorylation status (see Figure 1.2) (172).

FIGURE 1.2
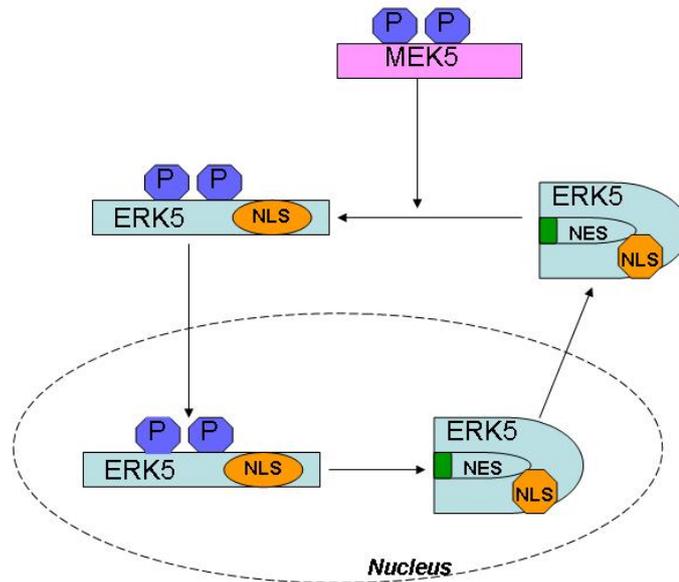
Mechanism controlling ERK5 nucleocytoplasmic transport

ERK5 has a nuclear localisation signal (NLS) in its carboxyl-terminal tail. In unstimulated cells, a nucleur export signal (NES) is produced by the interaction between the amino- and carboxyl-terminal halves, which results in ERK5 being retained in the cytoplasm. Upon stimulation however this interaction is disrupted, abolishing the NES activity and thus ERK5 enters the nucleus.

Modified from Nishimoto et al 2006 (173)

MEK5 phosphorylates and activates ERK5 with activation being shown to be accompanied by the phosphorylation of Thr^{219} and Tyr^{221}, with the former residue being phosphorylated preferentially (174). These residues lie in a Thr-Glu-Tyr (TEY) sequence on the N-terminus of ERK5 which is equivalent to the Thr-Xaa-Tyr motifs of other MAPK members, whose phosphorylation is required for activation. The activation and phosphorylation of these residues results in the phosphorylation of ERK5 downstream effectors as well as the phosphorylation of the C-terminal region of ERK5 itself (see Figure 1.3) (175). The role of this autophosphorylation is unclear but is thought in turn to lead to increased activity of its target molecules (173). Further studies are required to uncover how this C-terminal region may enhance transcriptional activity.

FIGURE 1.3
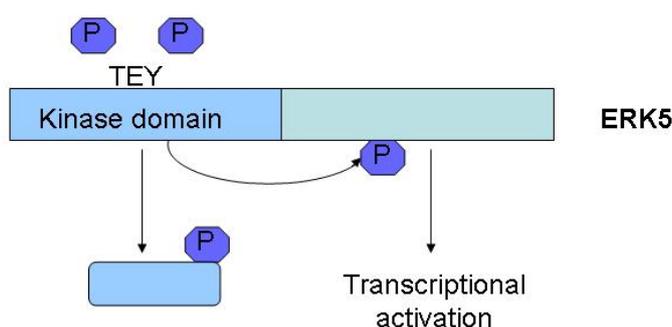
Proposed mechanism by which ERK5 transmits signals to downstream effectors

Upon activation by MEK5, ERK5 phosphorylates both downstream targets and the carboxyl-terminal of ERK5 itself (autophosphorylation), which in turn leads to increased activity of its downstream effectors.

Modified from Nishimoto et al 2006 (173)

1.8.2   Transcriptional activity of ERK5

Upon translocation to the nucleus, ERK5 can control the transcription of downstream effectors to elicit the desired cell response. Three members of the myocyte enhancer factor (MEF) family, are known to be substrates of ERK5; MEF2A, C and D. MEF2D is a specific substrate of ERK5, however both p38 MAPKs and ERK5 mediate the transcription of MEF2A and MEF2C (176). MEF2 activation involves a MEF2-interacting region and a transcriptional activation domain both of which are found in the C-terminal tail of ERK5 (177). Other direct substrates of ERK5 include c-Fos and Fra-1, c-Myc and Sap1a (167;178). RSK p90 ribosomal S6 protein kinase (rbS6) has been implicated in the downstream signalling of ERK5 (179).   RSK is thought to be involved in the regulation of nuclear factor kappa B (NFκB) function which has also been shown to be involved in ERK5 signalling (180;181).

1.8.3   ERK5 and ERK1/2

The ERK5 and ERK1/2 pathways have a number of similarities and interaction between the pathways has previously been suggested (182).   Initial work using the MEK1/2 specific inhibitors PD98059 and U0126 showed that inhibition of the ERK1/2 pathway produced a sustained activation of the MEK5/ERK5 pathway following EGF stimulation in HeLa cells (182). More recently enhanced nucleur accumulation of ERK5 in NIH3T3 cells was observed following suppression of ERK1/2 by low dose (1μM) PD184352 (an MEK1/2 inhibitor) (183).   There has also been suggestion that ERK5 may in fact regulate some of the cellular functions originally attributed to ERK1/2. A number of studies have demonstrated the ability of MEK1/2 inhibitors to block ERK5 activation at high dose (167;182). Treatment with low dose PD184352 (0.3μM) sufficient to block ERK1/2 activation only has been shown to have no significant effect on proliferation whereas treatment with higher dose (3μM) sufficient to block ERK5 significantly inhibited proliferation (184). These results suggest that it is in fact ERK5 rather than ERK1/2, which has the major role in cell proliferation.

1.8.4 ERK5 and cancer

1.8.4.1 Activation of ERK5 by oncogenes

Mutant Ras has been identified in many different cancers and the ERK5 signalling cascade is thought to be among the pathways involved in mediating its oncogenic effects. Raf-1, which is a downstream effector of Ras, has also been shown to enhance ERK5 activity (185). In addition to Ras other oncogenes such as COT and Src have been identified as potential ERK5 activators(169;170;183).

1.8.4.2 ERK5 tumour associated angiogenesis

In vivo studies have shown that ERK5 signalling has a critical role in embryonic angiogenesis with embryonic lethality occurring at 10.5 days post coitum due to cardiovascular defects in ERK5 knockout mice (186) . Xenograft studies have also shown a crucial role of the ERK5 pathway in tumour associated angiogenesis (187). Deletion of the host ERK5 gene significantly inhibited the development of tumour vasculature and growth of tumour xenografts. Studies in endothelial cells suggest that the ERK5 pathway is involved in tumour neovascularisation through its role in regulating the RSK-rbS6 pathway.

1.8.4.3 ERK5 and breast cancer

The MEK5/ERK5 signalling pathway has been shown to be important in human breast carcinoma. As discussed in section 1.5.2, HER2 gene amplification and protein overexpression is found in 20-30% of invasive breast cancers (28). It has been shown that the ERK5 pathway is constitutively activated in cancer cells overexpressing HER2 and that down regulation of this pathway significantly reduces the malignant growth of these cells (188). ERK5 resides in the nucleus of cells that overexpress the HER2 receptor and recent work has found that nuclear ERK5 favours MEF-2 dependant transcriptional activity and inhibits TRAIL(tumour necrosis factor related apoptosis)-induced cell death (189).

Apoptosis is known to be the primary route of cytotoxicity by many forms of anti-cancer therapy however chemoresistance can often become a therapeutic problem. *In vitro* studies have highlighted the importance of the MEK5/ERK5 pathway in mediating breast cancer cells sensitivity to apoptotic inducing events (190). Overexpression of MEK5 in APO- MCF7 cells suggests that this MAPK member may represent a significant potent survival molecule. Further work will confirm if inhibition of this signalling pathway could promote for sensitising breast cancer cells to chemotherapeutic regimens and if successful may be of significance in other chemoresistant cancer.

1.8.4.4   ERK5 and prostate cancer

Over recent years, abnormal MEK5/ERK5 signalling pathway has been shown to be important in prostate carcinogenesis.   Strong MEK5 expression correlates with aggressive disease and activation of MEK5 signalling induced proliferation, motility and invasion in LNCaP prostate cancer cells (191).

More recently, abnormal ERK5 function has also been suggested to contribute to prostate carcinogenesis (184).   ERK5 immunoreactivity is significantly up-regulated in prostate cancer when compared to benign prostatic hyperplasia. Increased levels of ERK5 cytoplasmic signals correlated closely with Gleason sum score (p<0.0001), bone metastases (p=0.0044) and locally advanced disease at diagnosis (p=0.0023), with a weak association with shorter disease-specific survival. A subgroup of 15 (of 81) patients showed strong nuclear ERK5 localisation, which correlated with poor disease-specific survival and, on multi-variant analysis, was an independent prognostic factor (p<0.0001). In addition PC3 cells over-expressing ERK5 displayed enhanced proliferation, migration and invasion.   Taken together, these data suggest MEK5/ERK5 pathway to be biological important in prostate cancer and a potential target in invasive prostate cancer.

1.9   Aims and Objectives of the Study

The specific aims of this study are:

- To study ERK5 expression in resected prostate cancer specimens including primary tumours and metastatic lesions

- To investigate the functional and biochemical effects of ERK5 inhibition in prostate cancer cells

- To explore potential cross talk between ERK1/2 and ERK5 signalling pathways

- To optimise methodology to study the ERK5 interacting proteome

2   Materials & Methods

2.1   Materials

Immunohistochemistry

• Normal rabbit serum	Vector Labs, Peterborough, UK
• Vectastain	Vector Labs, Peterborough, UK
• DAB substrate kit	Vector Labs, Peterborough, UK
• EnVision plus reagents	DAKO, Cambridgeshire, UK

Cell Lines

• PC3 cells	ECACC, Salisibury, UK
• PC3 ERK5	Newcastle University, UK (184)
• PC3 Empty vector	Newcastle University, UK (184)
• HEK 293	BICR, Glasgow, UK

Tissue Culture

• RPMI 1640	Invitrogen, Paisley, UK
• DMEM	Invitrogen, Paisley, UK
• Fetal Bovine Serum	Invitrogen, Paisley, UK
• G418	Invitrogen, Paisley, UK
• L-glutamine	Invitrogen, Paisley, UK
• Recombinant human EGF	R&D systems, Abingdon, UK
• PD184352	Strathclyde University, UK

Western analysis

Protein extraction

• Calbiochem cocktail mixture	Calbiochem, Nottingham, UK
• Bio-rad Protein Assay	Bio-rad Labs, Herts, UK

SDS-PAGE

• Pageruler prestained protein ladder	Fermentas Life Sciences, York, UK
• NuPAGE 10% Bis-Tris Gel	Invitrogen, Paisley, UK
• MOPS Running Buffer	Invitrogen, Paisley, UK

Blotting and detection

- ECL Western Blotting Detection Kit   Amersham, Buckinghamshire, UK
- Immobilon-P   Millipore, Herts, UK
- Super RX medical x-ray film   Fujifilm, Bedfordshire, UK

Antibodies

Antibody against	Company
ERK5, rabbit polyclonal	Cell Signalling, Herts, UK
ERK5, rabbit polyclonal	Upstate, Herts, UK
phospho ERK5, rabbit polyclonal	Cell Signalling, Herts, UK
ERK 1/2, rabbit polyclonal	Cell Signalling, Herts, UK
phospho ERK 1/2, rabbit polyclonal	Cell Signalling, Herts, UK
α tubulin, mouse monoclonal	Santa Cruz, Heidelberg, Germany
actin, mouse monoclonal	Sigma-Aldrich, Dorset, UK
GFP, mouse monoclonal	Living colours, Saint-Germain-en-Laye, France
PCNA, mouse monoclonal	Cell Signalling, Herts, UK
MEK1/2, rabbit polyclonal	Cell Signalling, Herts, UK
NFκB p65, mouse monoclonal	Santa Cruz, Heidelberg, Germany
phospho NFκB p65, rabbit polyclonal	Cell Signalling, Herts, UK
HRP-conjugated anti-mouse IgG	Cell Signalling, Herts, UK
HRP-conjugated anti-rabbit IgG	Cell Signalling, Herts, UK
Biotinylated anti sheep IgG	Vector Labs, Peterborough, UK

Transient transfection

- HiPerfect Transfection Reagent   Qiagen, Crawley, UK
- siRNA suspension buffer   Qiagen, Crawley, UK
- ERK5 siRNA (custom made)   Eurogentec, Seraing, Belgium
- ERK1 siRNA   Dharmacon, Chicago, USA
  (MAPK 3 on-TARGET plus SMARTpool)
- ERK2 siRNA   Dharmacon, Chicago, USA
  (MAPK 1 on-TARGET plus SMARTpool)
- All stars negative control siRNA   Qiagen, Crawley, UK
- Cell line nucleofector Kit V   Amaxa, Cologne, Germany
- Cell line nucleofector Kit L   Amaxa, Cologne, Germany

• Lipofectamine 2000	Invitrogen, Paisley, UK
• pCMV FLAG tag ERK5	Newcastle University, UK (184)
• pCMV FLAG tag EV	Newcastle University, UK(184)
• FLAG peptide	Sigma-Aldrich, Dorset, UK

Invasion assays

• Biocoat Matrigel Invasion Chamber	Becton Dickinson, Oxford, UK

Immunoprecipatation

• Leupeptin	Cambridge Bioscience, UK
• Aprotinin, bovine lung	Sigma-Aldrich, Dorset, UK
• Protein A Sepharose beads	Sigma-Aldrich, Dorset, UK
• Protein G Sepharose beads	Sigma-Aldrich, Dorset, UK
• DMP	Pierce, Rockford, USA
• SimplyBlue SafeStain	Invitrogen, Paisley, UK
• Micro Bio-Spin Chromatography Columns	Bio-rad Labs, Herts, UK
• NuPAGE SDS Running Buffer	Invitrogen, Paisley, UK
• 4x NuPAGE LDS Sample Buffer	Invitrogen, Paisley, UK
• 10x NuPAGE sample reducing agent	Invitrogen, Paisley, UK
• Biomax 5K membrane	Millipore, Herts, UK
• Anti-FLAG M2-Agarose from mouse	Sigma-Aldrich, Dorset, UK

SILAC

• RPMI Media for SILAC	Pierce, Rockford, USA
• DMEM Media for SILAC	Pierce, Rockford, USA
• Dialyzed FBS	Pierce, Rockford, USA
• 13C6 L-Lysine-2HCl	Pierce, Rockford, USA
• L-Lysine-2HCl	Pierce, Rockford, USA
• L-Arginine-HCl	Pierce, Rockford, USA
• 13C6 15N4 L-Arginine	Pierce, Rockford, USA

2.2   Immunohistochemistry

2.2.1   Preparation of agar cell pellets

In order to optimise antibody concentrations for immunohistochemistry experiments agarose cell pellets were made with PC3-ERK5 (PC3 cells stably overexpressing ERK5) and PC3 EV (empty vector) cells (both obtained from Newcastle University (184)). 2×10^7 cells were used per pellet and following trypsinisation, cells were washed twice in PBS before being resuspended in 1 ml neutral buffered formalin and being left at room temperature for 1-2 hours. Following a final spin (5 minutes, 1000rpm) cells were resuspended in 1ml 2.5% agarose which had previously been warmed to 50°C. These samples were then wax embedded and sections cut (4μm) for immunostaining experiments.

2.2.2   ERK5 immunostaining

Archive paraffin wax-embedded sections (4μm) were used from clinical prostate and kidney samples. Xenograft tumours were obtained from Newcastle University (184). Mouse heart sections were used as a positive control and no primary antibody was used as a negative control. All sections had previously been baked overnight at 50°C. Sections were deparaffinised in histoclear and rehydrated through graded alcohols before a final wash in water. Endogenous peroxidase activity was blocked by incubating samples in 0.3% hydrogen peroxide for 15 minutes. Antigen retrieval was achieved by immersion in pre-heated 0.01M pH6 sodium citrate buffer and microwaving for 4.5 minutes at full power in a pressure cooker. Slides were placed into distilled water immediately following this. Sections were then incubated with 150μl of rabbit serum (Vector Labs, Peterborough, UK) in 10ml of 0.1% fraction V BSA in 0.15M NaCl PBS for 1 hour before incubation with the desired primary antibody overnight at 4°C. After washing in 0.1% BSA/PBS slides were incubated with the secondary antibody (Biotinylated anti sheep IgG, Vector, Labs, Peterborough, UK) diluted 1:200 in BSA/PBS for 1 hour at room temperature. Slides were then washed again with BSA/PBS before incubation with Vectastain (Vector Labs, Peterborough, UK) for 1 hour. Following a final wash with BSA/PBS, diaminobenzidine tetrahydrochoride (DAB substrate kit, Vector Labs, Peterborough, UK) was applied to the slides for 5 minutes. The slides were then washed in distilled water before counterstaining with haematoxylin.

2.3   Cells and Cell culture

PC3, PC3M and DU145 cells were maintained in RPMI 1640 (Invitrogen, Paisley, UK) and HEK 293 cells in DMEM growth medium (Invitrogen, Paisley, UK). All medium was supplemented with 10% heat-inactivated FBS (Invitrogen, Paisley, UK) and 1% L-Glutamine (Invitrogen Paisley, UK). The transfected cell lines PC3 ERK5 and PC3 EV were generated as previously described (192), and maintained as the parental cells with the culture medium supplemented with 300ug/ml geneticin (G418)(Invitrogen, Paisley, UK). All cells were incubated at 37°C and 5% CO_2.

2.4   Western blot analysis

2.4.1   Protein extraction

Cultured cells were lysed in buffer (50mM Tris pH 7.6, 150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1mM Na_3VO_4, 1mM NaF, calbiochem protease inhibitor cocktail x1, 0.1µg/ml okadaic acid).

2.4.2   Determination of protein concentration

The protein concentration of the whole cell and nuclear extracts was determined using a colourimetric assay based on the Bradford dye-binding method (193). 5µl of lysate was mixed in a cuvette with 1ml of a 4:1 solution of H_2O: Bio-rad Protein Assay (Bio-rad Laboratories, Herts, UK). The protein concentration was measured using an Eppendorf BioPhotometer (Eppendorf UK Ltd, Cambridge, UK) and the standard curve was drawn from six BSA standards at 80, 100, 200, 400, 1000 and 2000µgml^{-1} in the same buffer as the protein samples being measured.

2.4.3   Polyacrylamide gel electrophoresis of proteins

Proteins were resolved by polyacrylamide gel electrophoresis through a pre cast 10% Bis-Tris polyacrylamide gel (Invitrogen, Paisley, UK), electrophoresed in MOPS running buffer (Invitrogen, Paisley, UK) for 1 hour at 200V. 20µg of protein extract in a volume adjusted to 20µl with dH$_2$O was combined with 10µl of 3x sample loading buffer (0.187M Tris H-Cl pH 6.8, 6.9% SDS, 0.003 % bromophenol blue, 30 %glycerol, 15% mercaptoethanol) and 25µl loaded per lane.

2.4.4   Blotting and hybridisation to antibody

After separation by polyacrylamide gel electrophoresis, proteins were then transferred to Immobilon-P (Millipore, Herts, UK) by semi-dry blotting buffer (3.03g/l Tris base, 14.4g/l glycine, 0.1g/l SDS, 20% methanol). Blots were incubated in blocking

solution (TBST containing 5% Marvel) for 30minutes before being incubated in the primary antibody diluted at the appropriate concentration in 5% BSA in TBST. Blots were washed five times in TBST and then incubated in 5% Marvel containing anti-rabbit, anti-mouse or anti-sheep immunoglobulin horseradish peroxidase-linked whole antibody, diluted as directed by the manufacturer (Cell Signalling, Herts, UK). All incubations with secondary antibody were for 1 hour at room temperature with gentle agitation. Blots were then washed a further five times in TBST. Proteins were visualised using ECL western blotting chemiluminescent detection reagent (Amersham, Buckinghamshire, UK) followed by autoradiography, where the membrane was wrapped in Saranwrap, placed in a medical x-ray cassette, and overlayed with xray film. Exposure was initially for 30seconds to determine optimal exposure times. The autoradiograph was developed using a Kodak X-OMAT 3000RA x-ray processor.

2.5   siRNA transfection

2.5.1   Transfection test

In order to optimise technique an initial transfection test was performed using a fluorescent labelled siRNA, Alexa Fluor 488, and HiPerfect (all Qiagen, Crawley, UK) as the transfection reagant. Two 12 well plates were seeded with 0.6×10^5 PC3 cells the day before and the day of transfection. Different ratios of Alexa Fluor 488, serum free medium and HiPerfect were used to establish the most efficient transfection in PC3 cells. After 24 hours the medium was changed and the cells were observed at 48 hours using a confocal microscope.

2.5.2   HiPerfect transfection

The duplex oligonucleotide 5'-GGTGTTGGCTTTGACCTGGAGGAAT-3' was ordered from Eurogentec, (Seraing, Belgium) (preannealed). All experiments were performed using a final concentration of 10nM ERK5 siRNA. PC3 and PC3 ERK5 cells were used for ERK5 siRNA transfection with HiPerfect as the transfection reagent. All stars negative control siRNA (AS-ve, sequence not provided) was used in parallel to control for non-specific effects. Monitoring of gene silencing was done by Western blotting and siRNA transfected cells were used for proliferation, migration and invasion assays.

2.5.3 Amaxa transfection

siRNA transfection was also carried out using an Amaxa nucleofector kit (Amaxa, Cologne, Germany). Kit V, programme T13 was used for PC3 cells. 1×10^6 cells were used per transfection in 100μl of nucleofector solution (provided by Amaxa). Once combined with the appropriate siRNA, cells were transferred to an amaxa certified cuvette and transfection carried out using a Nucleofector II device (Amaxa, Cologne, Germany). Cells were then resuspended in 500μl of medium and 250μl of this transferred to a 6cm dish already containing 3.7mls full medium. Initial experiments were performed to identify optimal concentrations of ERK1 and ERK2 siRNA (both Dharmacon, Chicago, USA) in PC3 cells. 10nM ERK1 and 100nM ERK2 siRNA was then used for subsequent experiments.

2.6   Proliferation assay

1.5×10^5 cells were seeded in 6 well plates. siRNA transfection was performed and serial cell counts were taken at 24, 48, 72 and 96 hours using a Casey cell counter. Each experiment was repeated three times and 3 wells were used per condition each time.

2.7   Migration assays

Motility assays were performed 48 hours post transfection with ERK5 siRNA. Cells were plated on glass 6 well dishes for optimal visualisation. PC3 ERK5 cells were studied using a Nikon TE2000 time lapse microscope (x20 magnification). Images were taken every 15 minutes over an 18 hour period. Image J software was used to track motility and accumulated and euclidean (direct) distance were measured. Cell velocity and persistance (euclidean distance/accumulated distance) were also calculated. Persistence is an indicator of the randomness in the cells migration. The closer to one, the more directly the cell moved from start to finish. Eight cells were tracked per field and 3 fields were viewed in each experiment (x2).

2.8 Invasion assays

For invasion assays Biocoat Matrigel invasion chambers (Becton Dickinson, Oxford, UK) were used. The matrigel inserts were rehydrated with serum free medium for 2 hours before being transferred to a 24 well companion plate which already has 600μl full medium per well. Overnight serum starved PC3 ERK5 cells were trypsinized and washed twice with serum free medium containing 0.5% BSA. An initial experiment was performed to assess optimal seeding density comparing $5x10^4$, $7.5x10^4$, $1x10^5$ per chamber. The cells were left to attach and invade overnight at 37^oC and under 5% CO_2/ 95% air atmosphere. Cells were seeded in 300ul of serum free medium per insert and after 22 hours the medium was aspirated and the cells on the upper surface of the membrane were removed using a cotton bud. The invaded cells were fixed in methanol and left at -20^oC for 30 minutes prior to staining with haematoxylin. The chambers were left to air dry before the membrane was cut out and mounted on a microscope slide with histomount. The migrated cells were then counted using a light microscope at 10x magnification.
Each experiment was repeated 3 times and each time the mean number of invading cells was taken from 4 chambers.

2.9 EGF stimulation

Cells were serum starved for 24 hours before stimulated with 50ng/ml EGF (R&D systems, Abingdon, UK). Cells were lysed at various time points (15, 30, 60, 90 and 120 minutes).

2.10   Immunoprecipation

Hepes Lysis Buffer

20mM Hepes-NaOH pH 7.5	1mM Na_3VO_4
150Mm NaCl	5ug/ml leupeptin
1% NP-40	2.2ug/ml aprotinin
2mM EDTA	1mM $Na_3P_2O_7$
1mM PMSF	20mM β-glycerophosphate
2mM NaF	

Hepes Wash Buffer

20mM Hepes-NaOH pH 7.5	1mM Na_3VO_4
50mM NaCl	5ug/ml leupeptin
1% NP-40	2.2ug/ml aprotinin
2mM EDTA	20mM β-glycerophosphate
1mM PMSF	
2mM NaF	

Hepes Cross-linking Wash Buffer

100mM Hepes-NaOH pH 8.5

Hepes Cross-linking Buffer

100mM Hepes-NaOH pH 8.5

10mg/ml Dimethyl pimelilidate (DMP)

Glycine Elution Buffer

200 mM glycine-HCl pH 2.5

500mM NaCl

0.01% NP-40

2.10.1 Dimethyl pimelilidate (DMP) cross-linking of antibodies to Protein A / G beads

100ul of beads were pipetted into a 1.5ml microfuge tube and washed 3 times in 1ml Hepes lysis buffer by sequentially mixing the beads with the buffer and then centrifuging the beads to the bottom of the tube. 100µl of ERK5 antibody or 10µl of pre-immunised rabbit serum were then added to the beads. 800µl of Hepes lysis buffer was also added and these were then incubated at $4^{o}C$ overnight. The next day the beads were washed 3 times with 1ml Hepes lysis buffer before washed twice with 1ml Hepes cross-linking wash buffer. The beads were then incubated at room temperature with 1ml cross-linking buffer for 1 hour. Following this the beads were further washed twice in 1ml Hepes cross-linking wash buffer. The reaction was then quenched by adding 1 ml 100mM Tris-HCl pH 7.5 to the beads and mixing at room temperature for 30 minutes. The beads were washed twice with 1ml lysis buffer followed by 2 washes with 1ml elution buffer. Following this the beads were washed twice with 1ml Hepes lysis buffer. 200ul lysis buffer with sodium azide (0.02%) was added to the slurry. The cross linked beads were then stored at $4^{o}C$.

2.10.2 Preparation of lysate for immunoprecipatation from PC3 cells

PC3 cells were grown to 70% confluence on 14cm plates. The plates were then washed once with ice cold PBS and lysed with 500ul Hepes lysis buffer. The lysate was then incubated on ice with occasional vortexing. The lysates were cleared by centrifugation at 132000rpm in a cooled ($4^{o}C$) bench top centrifuge for 10 minutes. The lysate was incubated with the cross-linked antibody beads overnight. The following day the beads were transferred to a spin column and washed 3 times with ice-cold Hepes wash buffer. Each time the beads were mixed with the buffer prior to removing the buffer by centrifugation into a 2ml microfuge tube at 1000rpm. The lysate which was collected following the first spin was collected and labelled as supernatant (SN). After the last wash the dry beads were incubated on ice with two bead volumes of the glycine elution buffer for 5 minutes with occasional vortexing. The eluate was removed by centrifuging (6000rpm) into a clean microfuge tube. This step was repeated once. The combined eluates was neutralised by adding 1/10th of the eluate volume of 2M Tris-HCl pH 9. The total volume of the eluate was then measured and 20µl was aliquoted and kept at $-20^{o}C$ to use for western blotting. To concentrate the eluate a 5kDa cut off membrane was used (Micro Bio-Spin Chromatography Columns, Bio-rad Labs, Herts, UK). The eluate was transferred to this and then centrifuged at $12^{o}C$, due to the temperature sensitive nature of the filter at 16000rpm for 45 minutes.

The concentrated sample was then added to LDS sample buffer and reducing agent. The sample was denatured by heating to 57°C for 15 minutes before electrophoresed on a 10% NuPAGE gel. The samples were run at constant 100V until the dye front reached the end of the gel. The gel was transferred into a 14cm cell culture dish with a lid and washed in 25ml fixing solution (50% ethanol, 10% acetic acid and 40% H$_2$O) for 15 minutes at room temperature. Following this the gel was washed in H$_2$O for 5 minutes, followed by an overnight incubation in Simply Blue coomassie stain (Invitrogen, Paisley, UK). The gel was then destained in 25% methanol for 1 minute before being washed several times in H$_2$O until the background was clear. The subsequent mass spectrometry analysis was carried out by Dr Willy Bienvenut (Proteomics service, Beatson Institute for Cancer Research, Glasgow).

For each experiment a small amount of eluate was kept pre and post concentration and a western blot run to ensure no sample was lost during the concentration step. A sample of the supernatant was also run on the gel to confirm adequate ERK5 pulldown.

2.10.3 Titration of antibody to bead

In order to optimise the bead to lysate ratio an initial titration experiment was performed using the 2 commercially available ERK5 antibodies (Cell Signalling and Upstate) and the phospho ERK5 antibody with both Protein A and G sepharose beads (both Sigma-Aldrich, Dorset, UK). A constant ratio of 100μl of antibody crosslinked to 100μl of beads was used for the titration experiments. For each experiment a constant amount of lysate was used (100μg) and immunoprecipatation performed as described above. All eluated samples with their corresponding supernatant were then analysed by western blotting.

2.10.4 Pre clearing

Pre-clearing was performed to reduce non-specific binding of proteins to the beads. 50μl of normal rabbit serum was added to 1ml of lysate. This was vortexed and incubated on ice for 1 hour. 100μl of protein A sepharose beads were then added to this and incubated at 4°C for 30 minutes with gentle agitation. Prior to adding the beads to the lysate/serum mixture 3 washes with hepes lysis buffer was performed. This was then centrifuged at 132000rpm at 4°C for 10 minutes. The bead pellet was discarded and the supernatant kept for immunoprecipatation.

2.10.5 EGF stimulation for immunoprecipatation

PC3 cells were grown until 70% confluent in 14cm dishes and then serum starved for 24 hours following 2 washes with PBS. Cells were treated with 50ng/ml EGF before the cells were harvested for analysis. Control cells were lysed after 24 hours and immunoprecipatation performed with ERK5 crosslinked beads.

2.10.6 Exogenous ERK5 transfection

PC3 and HEK293 cells were grown in a 14cm plate until 60% confluent and then transfected with 3μg of the plasmid pCMV FLAG ERK5 using Lipofectamine 2000 as a transfection reagent. The plasmid pCMV FLAG EV was used a control. Cells were lysed 48hours post transfection and successful transfection validated by western blotting.

2.10.7 FLAG tag immunoprecipatation

HEK293 cells were used for all FLAG tag immunoprecipatation experiments. An initial experiment was performed with cells grown in full medium on 14cm plates (x10) to confirm pull down of ERK5 by mass spectrometry. Cells were transfected with the pCMV5 FLAG ERK5 plasmid and lysed with 1ml per plate HEPES lysis buffer after 48 hours. After clearing, the lysate was incubated with 200μl of FLAG beads for 1 hour at 4°C.

The beads were then transferred to a spin column and washed 3 times with ice-cold Hepes wash buffer as described previously. After the last wash the dry beads were incubated on ice with 200μl of FLAG peptide (Sigma-Aldrich, Dorset, UK) for 10 minutes with occasional vortexing. The eluate was removed as before and this step was repeated before concentrating the sample to run on the gel.

As before a western was run with a sample of the lysate, supernatant and eluate pre and post concentration to confirm pulldown of ERK5.

2.10.8 Stable isotope labelling with amino acids in cell culture (SILAC)

Heavy ($^{13}C_6$ L-Lysine-2HCl and $^{13}C_6{}^{15}N_4$ L-Arginine-HCl) and light (L-Lysine - 2HCl and L-Arginine-HCl) DMEM media was made up per manufacturers instructions and supplemented with 10% dialysed FBS (all Pierce, Rockford, USA). This was then filtered using a 0.2μm filter before being used for cell culture.

HEK293 cells were grown in each media for a minimum of 5 cell doublings (~7-10 days) before isotope incorporation efficiency was determined.

2.10.9 Determination of Isotope Incorporation Efficiency

$1x10^6$ cells of both heavy and light isotope incorporated cells were plated separately on 9cm dishes and lysed the following day with 500µl reducing sample buffer. The samples were boiled for 5 minutes and clarified by centrifuging at 14000rpm for 1 minute. 25µl of the heavy and light samples were then loaded into two separate wells of a 10% pre-cast polyacrylamide gel and the proteins separated by electrophoresis. The gel was fixed and stained as described previously and incorporation efficiency verified by our proteomics department using mass spectrometry.

2.10.10 EGF stimulation in HEK 293 cells

An initial experiment using the HEK 293 cells confirmed poor adherence following a PBS wash and therefore serum starvation prior to EGF simulation would not be possible. In order to promote cell adhesion 0.1% poly-L-lysine was used to coat the plates the day before cells were plated. 36 hours post transfection cells were serum starved overnight before being stimulated with 50ng/ml EGF. Cells were lysed at various time points (15, 30, 60 and 90 minutes).

2.10.11   Identification of ERK5 associating proteins

HEK 293 cells grown in the corresponding SILAC media were transfected with either pCMV FLAG ERK5 (Experiment 1 – heavy medium, Experiment 2 – light medium) or pCMV FLAG EV (Experiment 2 – light medium, Experiment 2 – heavy medium) plasmid and lysed after 48 hours. Prior to incubating the lysate with the FLAG beads, the protein concentration of each lysate was measured. Equal protein amounts of each cell lysate were mixed in a 15ml falcon before adding the beads as described previously. The sample was run on a 10% gel and given to the proteomic department for analysis once stained.

2.10.12   Identification of ERK5 binding partners

Prior to plating the cells, 14cm plates were coated with 0.1% poly-l lysine. SILAC treated HEK293 cells were plated and grown until 60% confluent before cells were transfected with pCMV FLAG ERK5 and grown for a further 36 hours in full SILAC mediums. Cells were then serum starved overnight before EGF stimulation. Cells were treated with 50ng/ml EGF and lysed at 2 timepoints: time = 0 minutes (Experiment 1 – light medium, Experiment 2 – heavy medium) and time = 15 minutes (Experiment 1 – heavy medium, Experiment 2 – light medium). The lysates were treated as above and the stained gel given for mass spectrometry analysis.

2.10.13   Analysis of data from mass spectrometry study

The Scaffold 2 proteome software (Proteome Software Inc., Oregon, USA) was used to analyse mass spectrometry results. This programme is the industry-standard MS/MS meta-analysis tool and allows comparison of various factors between samples e.g. number of peptide matches, percentage coverage and protein identification probability. The dataset was separated into four separate categories for analysis; 1-non-relevant hits (mostly due to low peptide number or large standard deviation value), 2- potential positive hits (medium number of non-redundant peptides and/or medium ratio and/or low reciprocity between two samples) 3 - clear positive hits (large number of non-redundant peptides with high ratio and high reciprocity between two samples), 4 - remaining proteins.

2.11   Statistical analysis

Statistical analysis was performed using SPSS version 15. Student's t-test was used to compare to compare data that followed the normal (Gaussian) distribution. A statistical significant difference was defined to be a p-value < 0.05. Where error bars are represented on a graph they signify the standard error (SE) of the mean value plotted.

3 Target validation – in prostate cancer

3.1 Introduction

Molecular targets which are identified as being potentially involved in the development of cancer must be validated prior to the initiation of drug development programmes. The target of interest must be shown to be critical in the disease process as well as being a valuable point of intervention. Various methods of target validation are used including downregulation of gene expression, protein inhibition and cellular assays. Other molecular tools which are commonly used to prioritise targets of interest include analysis of proteomic and microarray data.

3.1.1 MEK5/ERK5 signalling in prostate cancer

Aberrant MEK5/ERK5 signalling is recognised to play an important role in prostate carcinogenesis. Strong MEK5 expression correlates with the presence of bone metastases, the most common site of distant metastasis in prostate cancer (191). *In vitro* work has also shown that MEK5 expression increases matrix metalloproteinase (MMP) 9 expression, an enzyme that is implicated in enhancing the metastatic potential of prostate cancer. In addition, immunohistochemistry experiments on human prostate tumours identified increased ERK5 cytoplasmic expression to correlate with the presence of bone metastases (P=0.0044) (184). Analysis of ERK5 staining in matched tumour samples (pre and post hormone relapse) showed ERK5 nuclear expression to be associated with hormone resistant disease (P=0.0078). ERK5 expression is also upregulated in an androgen independent LNCaP subline when compared with the androgen dependent parental cell line.

In order to investigate the role of ERK5 in advanced prostate cancer a tissue microarray (TMA) containing both primary and metastatic prostate cancer specimens was examined. The biological effect of suppressing ERK5 expression in prostate cancer was also investigated using small interfering RNA (siRNA) in PC3 cells in a number of functional assays.

3.1.2 NFκB and ERK5

There is accumulating evidence implicating the transcription factor Nuclear Factor *kappa* B (NFκB) in carcinogenesis (194). NFκB is a transcription factor of the Rel protein family, which include the p50, p52, p65 (RelA), c-Rel and RelB proteins, and its most widely studied form is the p50/p65 heterodimer (195;196). NFκB is sequestered in the cytoplasm bound in an inactive complex with inhibitory IκB proteins, including IκBα and

IκBβ (196)[6]. A wide variety of agents activate NFκB, including tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1) and lipopolysaccharide (LPS) (195). Most employ the canonical pathway of I *kappa* Kinase (IκK) activation, which induces phosphorylation and degradation of IκB (see Figure 3.1). This exposes a nuclear translocation signal on the p65 subunit and permits NFκB transport into the nucleus. There, NFκB controls the transcription of numerous genes vital for cell immunity and inflammation, e.g. TNF-α, IFNγ, IL-1 and IL-8 (194). Fundamental for tumorigenesis, NFκB also regulates genes encoding growth factors (IL-2, IL-6), cell adhesion molecules (intercellular adhesion molecule-1, vascular cell adhesion molecule-1), angiogenic factors (VEGF) and antiapoptotic factors, (bcl-2 and survivin) (197;198). It also relates with numerous components of the cell cycle, including c-myc, cyclins and p53, and acts on the G1/S transition (195;199) .

NFκB is has been shown to be constitutively activated in human prostate cancer, and this overexpression, especially of p65, correlates with increased cell invasion and tumour stage (200-202). NFκB blockade in PC3M cells decreased invasion, by downregulating MMP 9 and collagenase activity, and angiogenesis, through inhibiting VEGF and IL-8 expression (203). Inhibition of NFκB signalling *in vivo* has also shown reduced tumour growth and metastatic potential (203).

The NFκB pathway has recently been implicated as a downstream target for ERK5 signalling. ERK5 is believed not to directly bind to NFκB, but activate it through a non-canonical pathway (see Figure 3.1). An intermediate kinase suggested may mitogen-activated p90 ribosomal S6 protein kinase (RSK) 2, which phosphorylates IκB, targeting it for degradation (204;205). This role of RSK2 has been confirmed recently, however it is thought that another unidentified kinase is also required for the activation of NFκB by ERK5 (206). ERK5 has been shown to be essential for G2/M progression with NFκB being involved at this checkpoint (206). In fibroblast cells, overactivity of MEK5 and MEK1, upstream activators of ERK5 and ERK2 respectively, demonstrated a synergistic approach in activating an NFκB-sensitive reporter (207). ERK5 predominantly targets the NFκB p65 component, and has been shown to be essential for NFκB-induced survival in leukaemic T cells (208).

Given the fact that NFκB has itself been reported to play an important role in prostate cancer, particularly in hormone resistant disease, we aimed to investigate if ERK5 mediated signalling in prostate cancer cells involves the NFκB pathway.

FIGURE 3.1
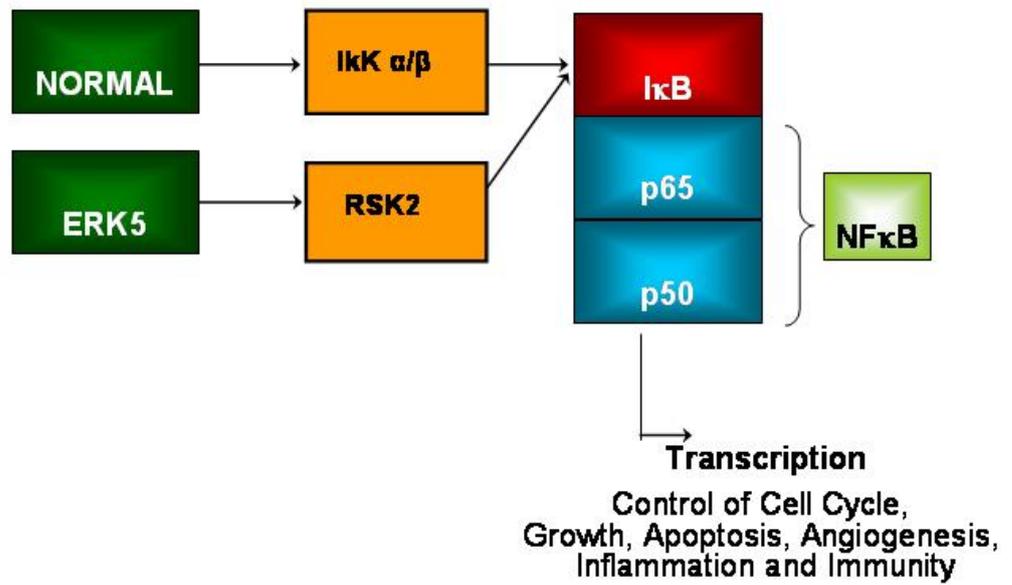
Normal and alternative pathway for NFκB activation

Most agents which activate NFκB do so via the canonical pathway of IκK activation which induces phosphorylation and degradation of IκB. Activation by ERK5 however is thought to be via an intermediate kinase (RSK2) which phosphorylates IκB, targeting it for degradation. Activated NFκB can then translocate to the cell nucleus and act as a transcription factor.

Modified from Cude et al 2007 (206)

3.2 Results

3.2.1 Antibody optimisation

Initial experiments were performed using PC3 ERK5 (PC3 cells overexpressing ERK5) and PC3 EV (PC3 cells transfected with the corresponding empty vector) cells to confirm the specificity of the ERK5 antibody used. This confirmed strong ERK5 immunoreactivity in the PC3 ERK5 cells with weaker immunostaining observed in the PC3 EV cells (Figure 3.2). All subsequent immunohistochemistry experiments were performed using a sheep polyclonal antibody raised against human ERK5 provided by Professor Philip Cohen, University of Dundee (182). Immunostaining with the commercial ERK5 and phospho ERK5 antibodies available was also assessed however poor results were observed.

### 3.2.2 ERK5 expression in human prostate cancer and metastases

To further define the patient cohorts, including those with metastatic lesions that may benefit from manipulation of ERK5 status, ERK5 immunoreactivity was studied in a prostate cancer TMA consisting of 48 primary prostate tumours. Consistent with data from previous TMA analysis, strong cytoplasmic ERK5 expression was found in prostate cancer with high levels of positive nuclear staining (moderate-strong cytoplasmic staining = 29%, positive nuclear staining = 62.5%). Though not statistically significant, there was a trend between high levels of cytoplasmic ERK5 expression and high Gleason sum score (>7) and high serum PSA levels (>10) (data not shown). Moderate-strong levels of cytoplasmic staining were observed in 63% cases of PIN/PIA. Within the TMA, eleven cores of metastatic prostate tumour were examined, including liver (n=5), lung (n=2), lymph node (n=1) and soft tissue (n=3). Among these metastatic lesions, ERK5 expression was consistently upregulated with both cytoplasm and nucleus immunoreactivity observed at high levels (73%, 8 out 11, Figure 3.3). Two liver metastases and 1 soft tissue metastases showed no nuclear ERK5 immunoreactivity. Hence, our expression analysis supports ERK5 as a potential target for therapy in prostate cancer sufferers particularly in those with castrate resistant and/or metastatic disease.

FIGURE 3.2　Optimisation of ERK5 antibody for immunohistochemistry
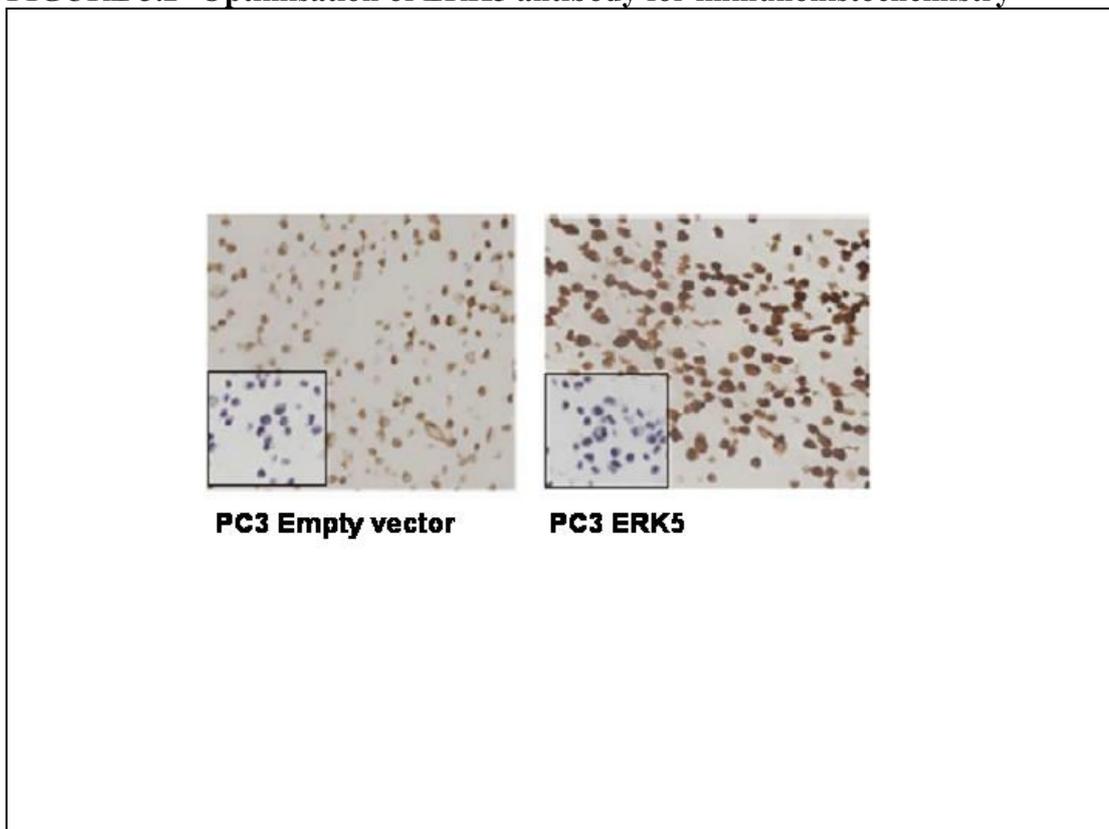
Immunohistochemical analysis of cell pellets (x40 magnification) showing upregulated ERK5 expression in PC3-ERK5 cells under optimal growth condition compared to PC3 cells transfected with empty vector control. Insets represent control staining omitting primary antibody.

FIGURE 3.3 Immunohistochemistry of ERK5 expression in human prostate cancer and metastases
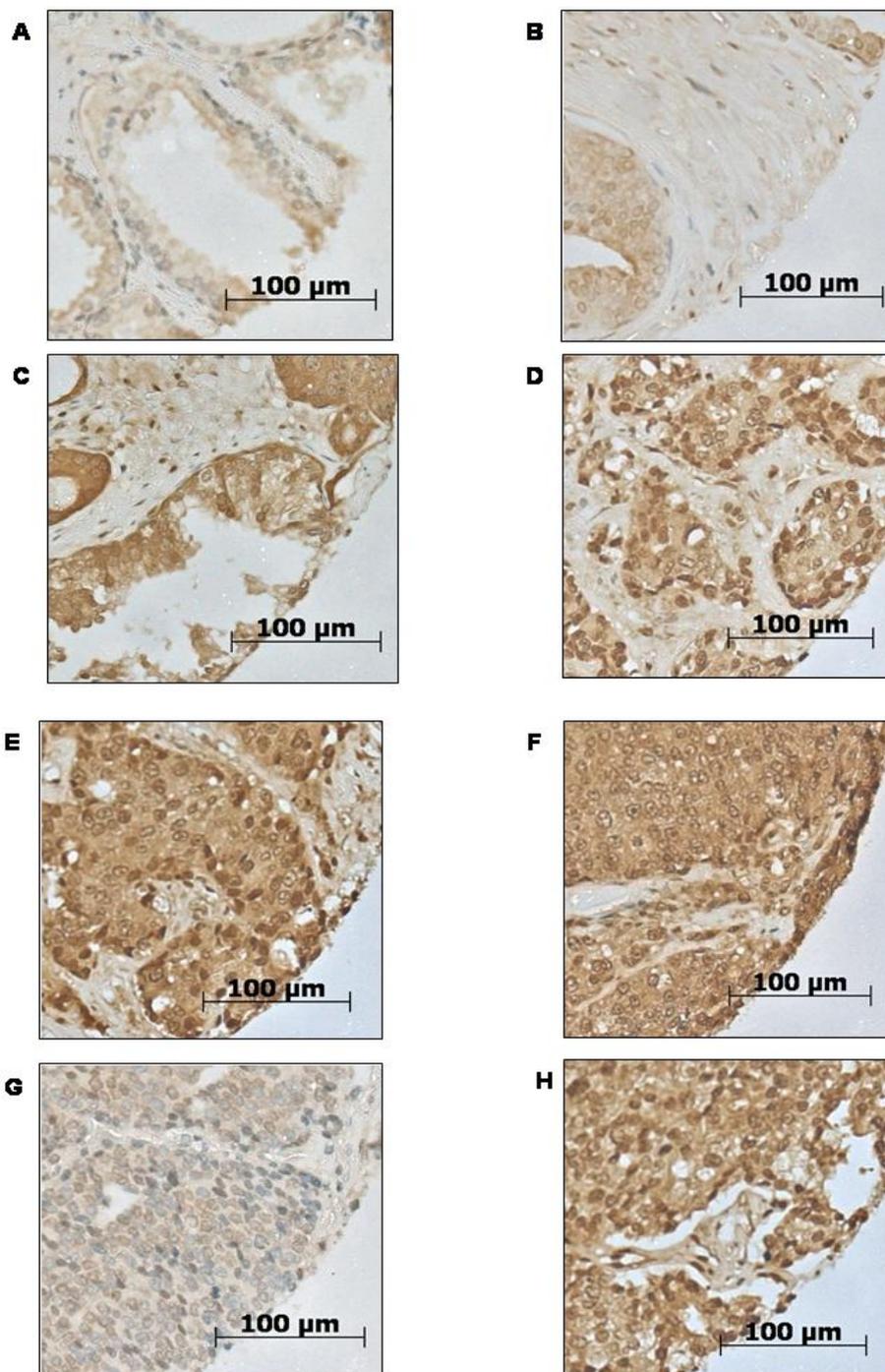
A   BPH (1+ immunoreactivity)

B   PIN (1+ immunoreactivity)

C   Gleason 3+4 prostate cancer (3+ immunoreactivity with positive nuclear staining)

D   Gleason 5+4 prostate cancer (3+ immunoreactivity with positive nuclear staining)

E   Liver metastasis (3+ immunoreactivity with positive nuclear staining)

F   Soft tissue metastasis (2+ immunoreactivity with positive nuclear staining)

G   Lung metastasis (1+ immunoreactivity with positive nuclear staining)

H   Lymph node metastasis (2+ immunoreactivity with positive nuclear staining)

3.2.3 ERK5 in prostate cancer cell lines

ERK5 expression was examined in a panel of human prostate cancer cell lines maintained in full culture medium (Figure 3.4). DU145 demonstrated the strongest level of ERK5 expression. As shown previously ERK5 is present in both androgen sensitive and androgen insensitive LNCaP cells however there is marginal stronger expression in the androgen insensitive cell line (184). Exogenous ERK5 expression in the PC3 ERK5 cell line is shown at a higher molecular weight than the endogenous ERK5 due to the presence of a GFP tag in the fusion protein, increasing its size by approximately 27kDa.

High levels of both endogenous and exogenous ERK5 are demonstrated in PC3 ERK5 cells. However, with increased passage, I observed that the level of exogenous expression decreases (Figure 3.5). All experiments with this cell line therefore were performed with low passage number to ensure optimal levels of ERK5 overexpression. There was no GFP expression evident on western blotting in the PC3 ERK5 cell line even at low passage. Low expression levels of GFP were observed using the confocal microscope. The reason for the discrepancy in levels of GFP: ERK5 is unclear. PC3 EV cells showed a decrease in GFP expression with increased passage.

3.2.4 ERK5 siRNA - Transfection test

Using the confocal microscope green fluorescent siRNA (Alexa Fluor 488) transfection was observed. Optimal conditions were regarded as those in which the highest rate of transfection was achieved with the lowest rate of cell death, assessed by cell adherence. Various conditions were tested, altering the concentrations of siRNA and the transfection reagent (HiPerfect) in each. Using a 10nM siRNA concentration with the lowest volume of transfection reagent appeared to have the highest rate of transfection with no significant loss of cells. Figure 3.6 shows the images taken from this optimal transfection well.

Successful knockdown of exogenous and endogenous ERK5 was achieved from 48 hours onwards in PC3 ERK5 cells with no associated change in ERK1/2 levels (Figure 3.7). Knockdown of endogenous ERK5 was also observed in the parental PC3 cells when transfected with ERK5 siRNA. ERK5 phosphorylation status was examined in PC3 cells 72 hours post siRNA transfection. Phosphorylation levels were suppressed in the ERK5 siRNA cells compared with the controls (Figure 3.8).

FIGURE 3.4 ERK5 expression in prostate cancer cell lines
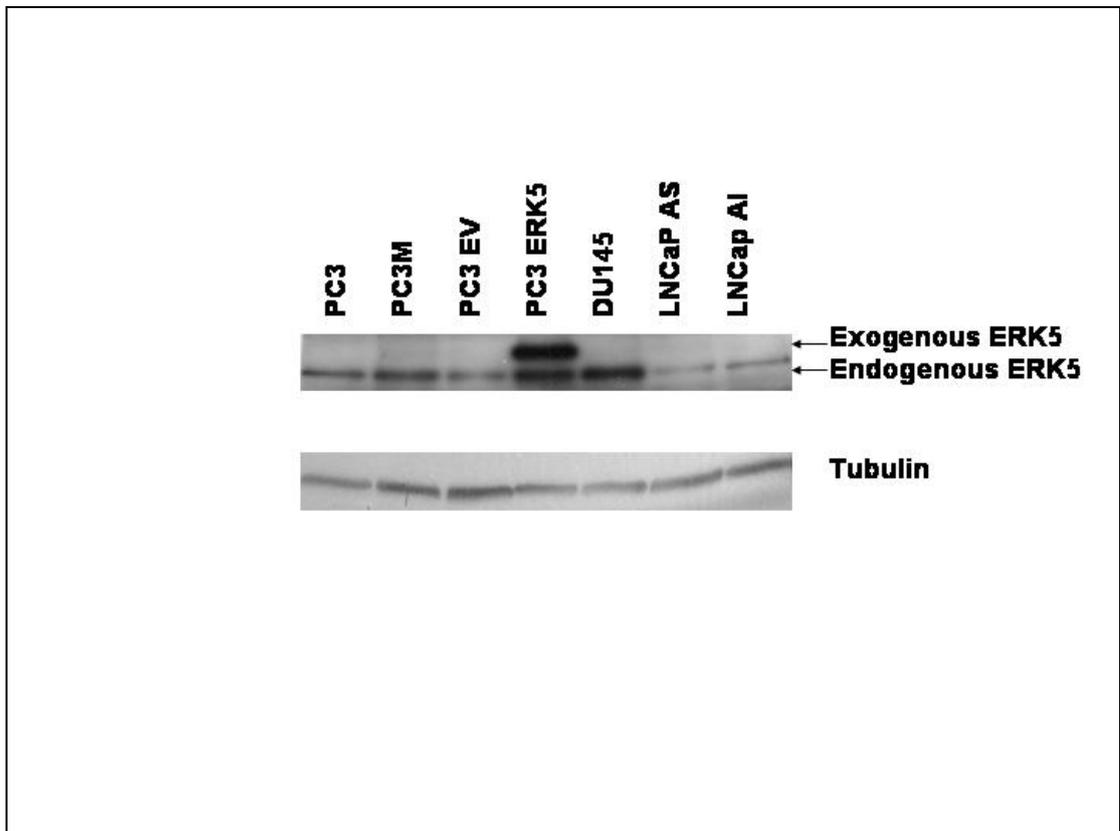
**FIGURE 3.5 Exogenous ERK5 expression in PC3 ERK5 cells**
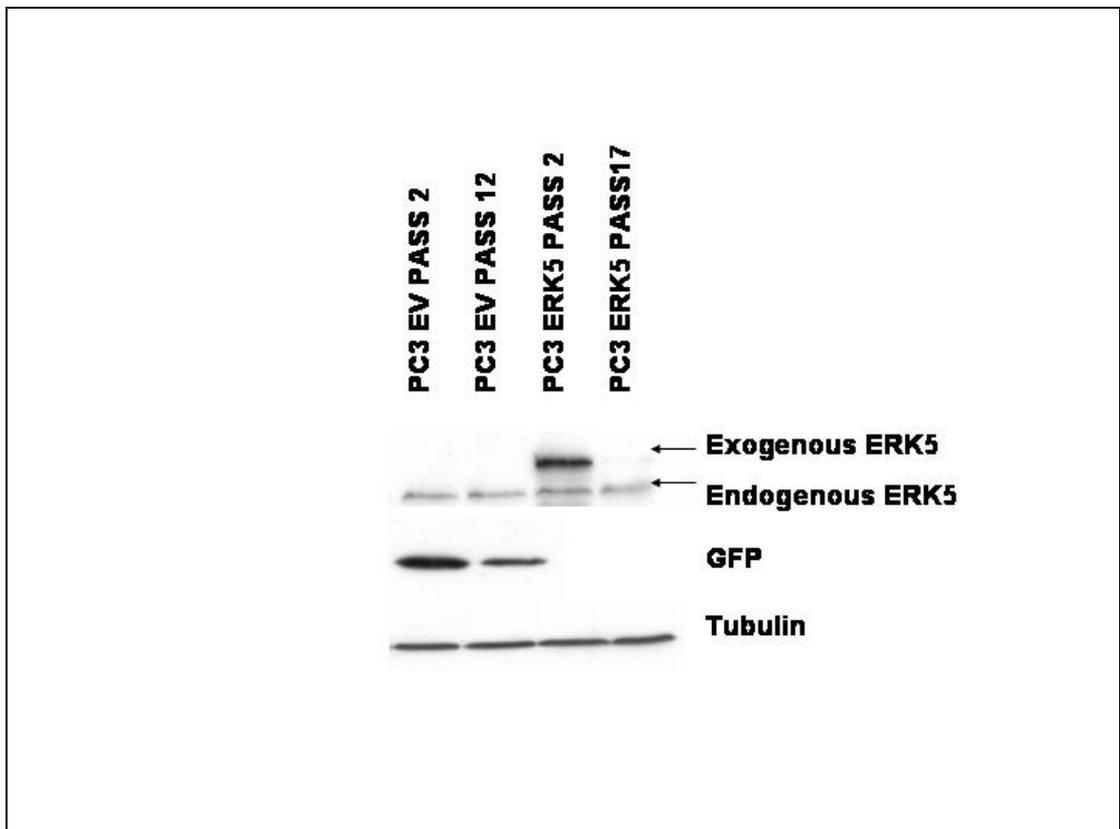
**FIGURE 3.6 Optimal transfection with green fluorescent siRNA (Alexa Fluor 488)**

Fluorescence and phase   Fluorescence   Phase

Scalebar = 20nm
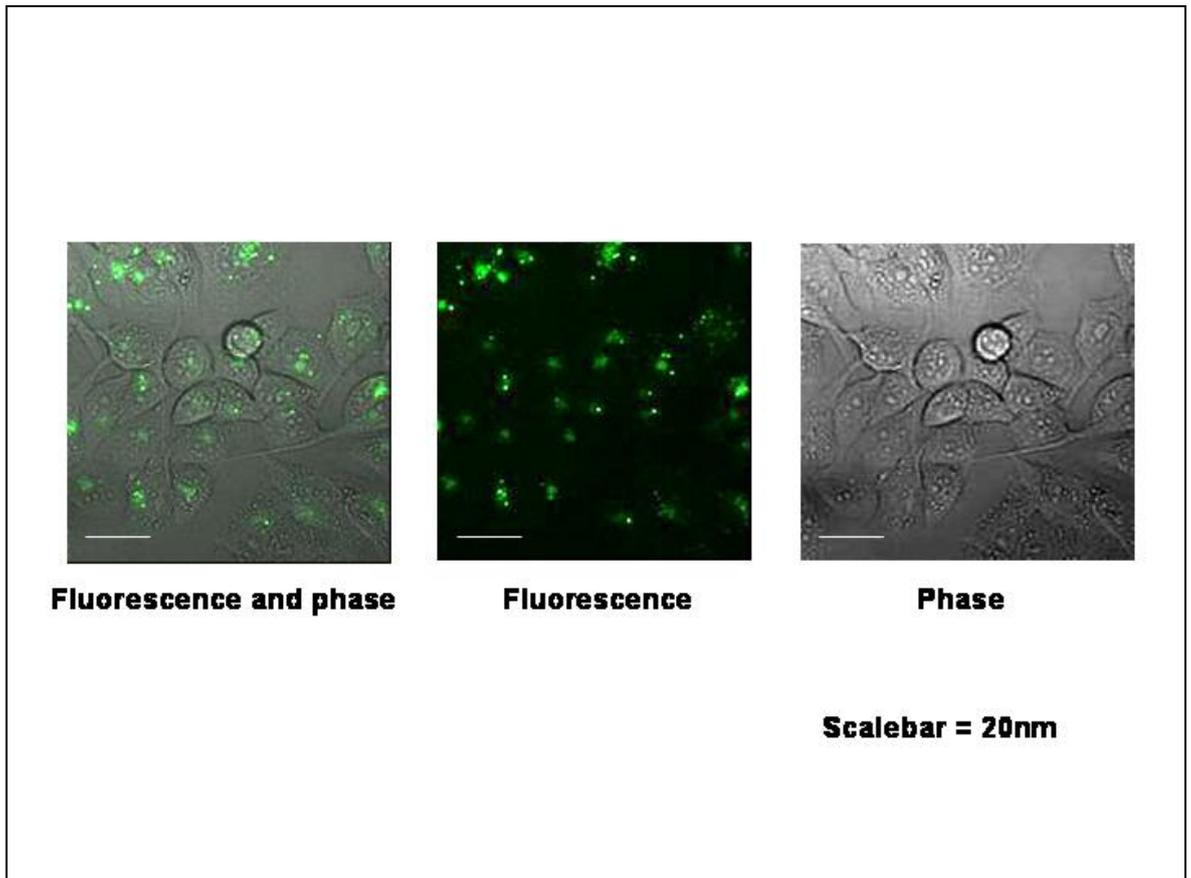
**FIGURE 3.7 ERK5 knockdown in PC3 and PC3 ERK5 cells**
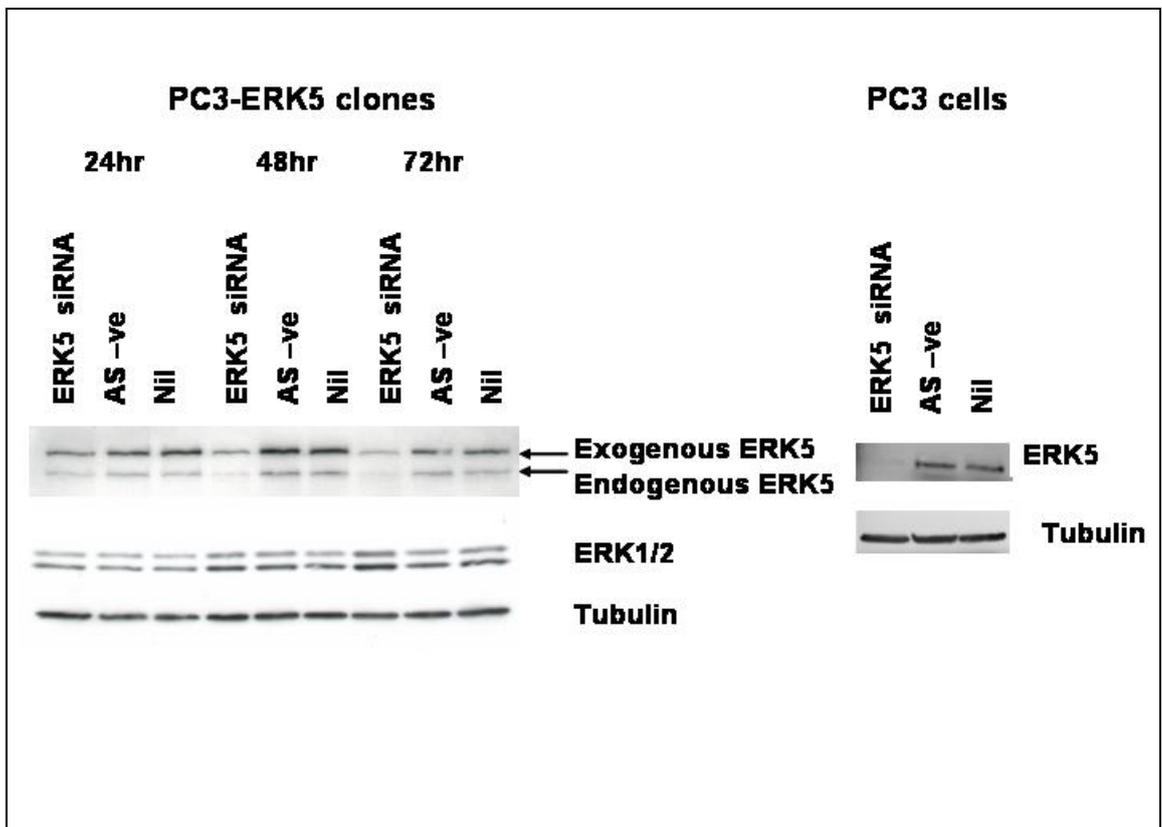
FIGURE 3.8 ERK5 siRNA inhibits ERK5 phosphorylation
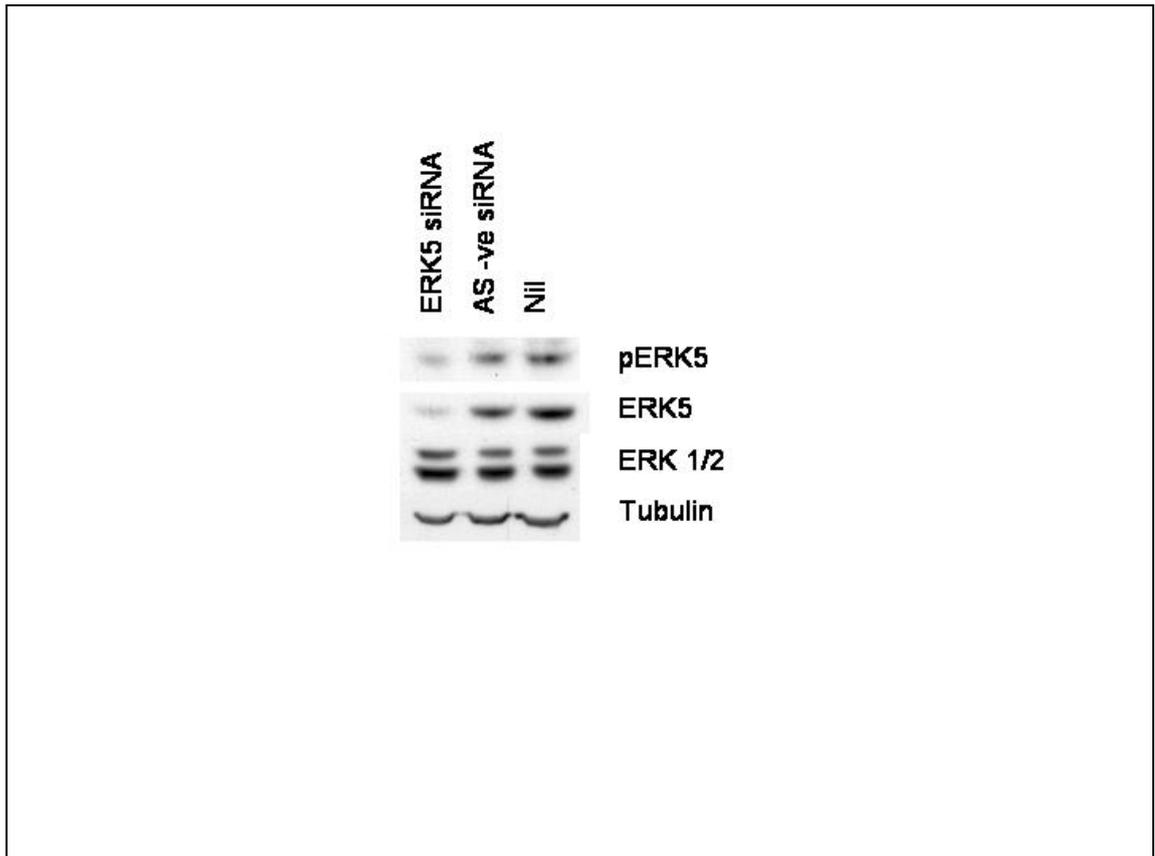
3.2.5   Proliferation assay

As ERK5 overexpression has been shown to be associated with increased proliferation *in vitro*, I investigated the effects of siRNA mediated ERK5 knockdown in a proliferation assay. Transfected PC3 ERK5 cells were counted at 24 hour intervals post siRNA transfection and a significant reduction in the rate of proliferation was observed upon ERK5 knockdown, when compared to the controls, $p < 0.005$ (34.9% (non silencing siRNA) and 31.2% (no transfection) at 96 hours post transfection, Figure 3.9).

3.2.6   Migration assay

We next tested the effects of ERK5 knockdown on cell motility. Untransfected and control transfected PC3-ERK5 cells display high levels of random motility with a combination of amoeboid and mesenchymal movement.   Amoeboid movement is a crawling-like type of cell movement accomplished by protrusion of the cytoplasm of the cell involving the formation of pseudopodia. Mesenchymal cells on the other hand are characterized by a small cell body with a few cell processes that are long and thin. Reduction of ERK5 significantly decreased cell motility, when compared to the non-silencing siRNA and untransfected cells (Figure 3.10A).   Both the accumulated and euclidean (directional) distances were significantly reduced when compared to the controls ($p < 0.005$, Figures 3.10 B & C). Hence, ERK5 function in PC3 appears to regulate cellular motility.

FIGURE 3.9   ERK5 knockdown results in reduced proliferation in PC3 ERK5 cells

*1.5x10^5 PC3 cells were transfected with ERK5 siRNA and counted at 24 hour intervals up to 96 hours. Non silencing siRNA (AS-ve) was used a control in addition to HiPerfect only. A significant reduction in proliferation was observed in the ERK5 siRNA transfected cells compared to both controls. Each experiment was performed in triplicate and repeated 3 times. *p<0.005*
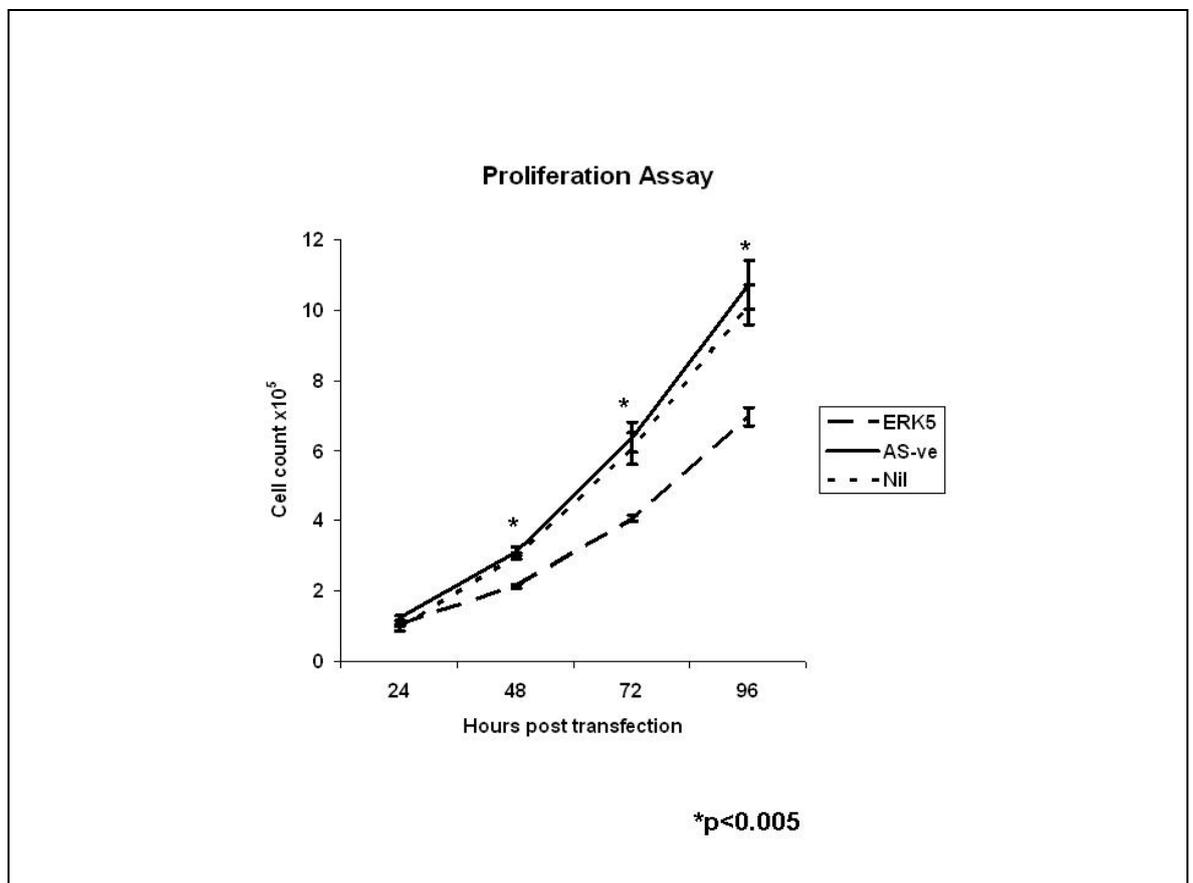
FIGURE 3.10A   **Migration plots for transfected cell lines**
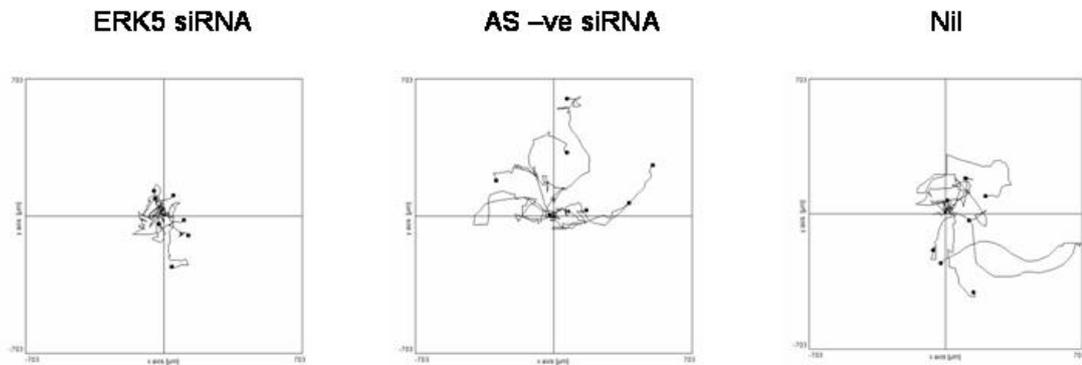
FIGURE 3.10 B & C
ERK5 knockdown reduces accumulated and euclidean distance in PC3 ERK5 cells
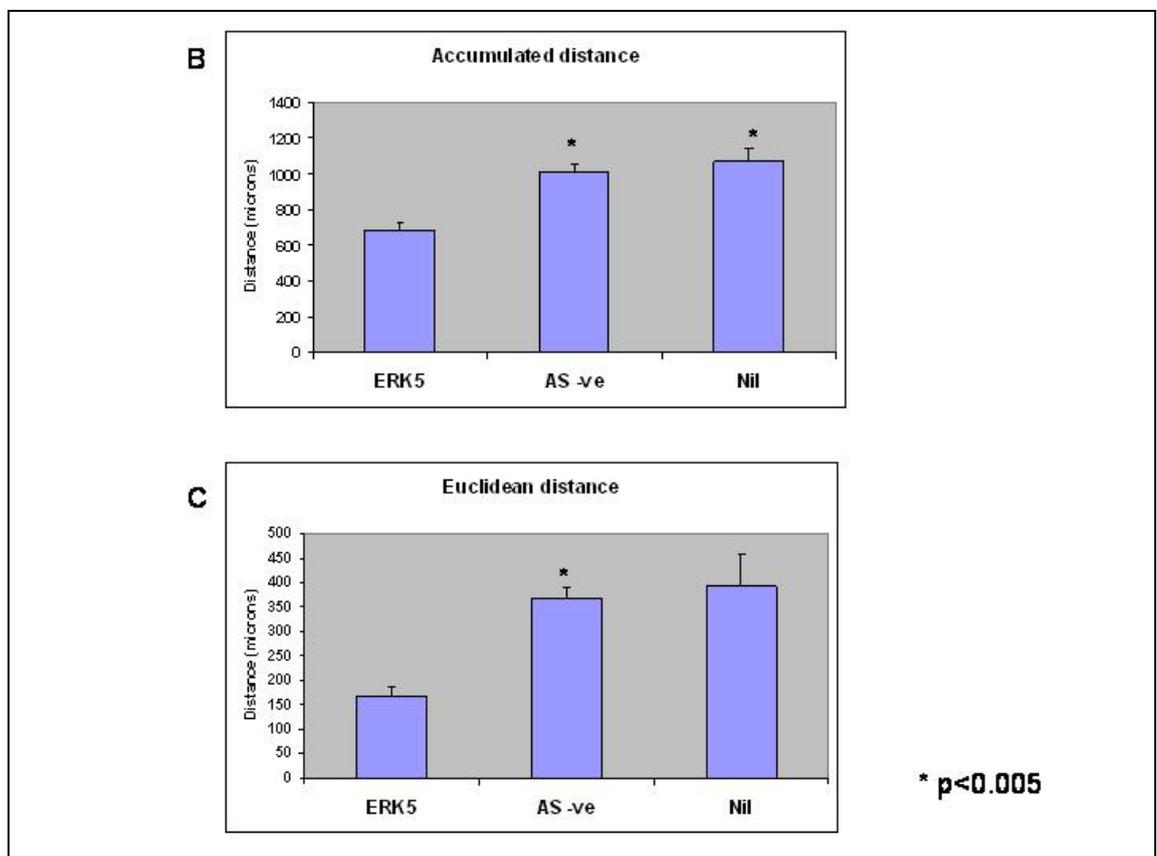
3.2.7 Invasion assay

Data from our laboratory have recently revealed that ERK5 over-expression is closely associated with the presence of metastatic prostate cancer and unfavourable patient survival outcome (184). I therefore wanted to test if ERK5 drives cellular invasion in a dose dependent manner and performed *in vitro* invasion assays using both parental PC3 and PC3-ERK5 cells. In keeping with our previous observation, PC3-ERK5 cells have an enhanced invasive phenotype when compared to parental PC3 cells, with around 25 (27+/-15/9) and 10 (10+/-4) cells per field respectively (Figure 3.11A and B). Upon transfection with ERK5 targeted siRNA, the invasive capability of PC3 was significantly inhibited: 2.1 and 1.97 fold reduction when compared with control siRNA transfected and untransfected PC3 cells ($p < 0.005$) (Figure 3.11 A). It was interesting to note that PC3-ERK5 cells are proportionally more sensitive to ERK5 manipulation in the invasion assay, with a 3.5 and 3.4 fold reduction when ERK5 expression was knockdown, when compared with control siRNA and untransfected PC3-ERK5 cells ($p < 0.005$)(Figure 3.11 B).

FIGURE 3.11 ERK5 inhibition reduces invasion in PC3 and PC3 ERK5 cells
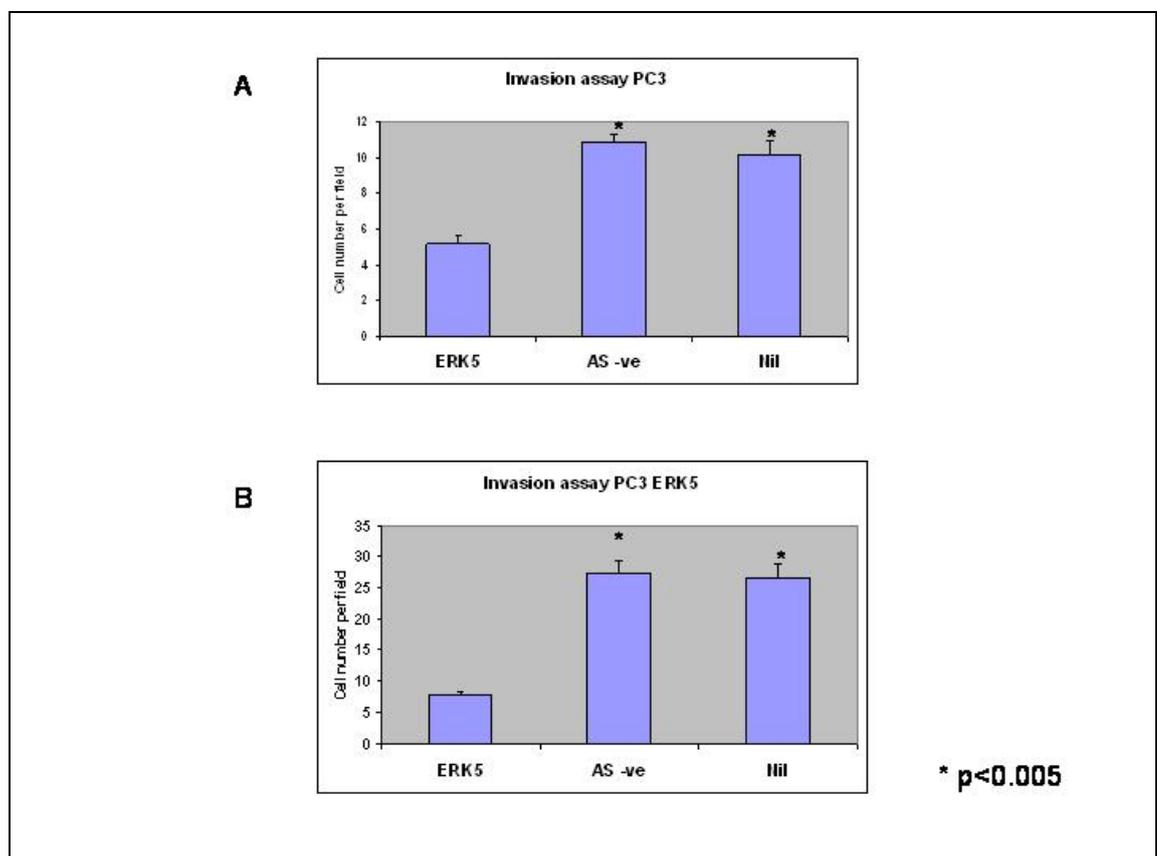
3.2.8   ERK5 over- or under-expression does not alter NFκB signalling

Using PC3 cells as a model system, we tested if siRNA meditated knockdown of ERK5 expression may result in reduced NFκB activities, as assessed by the level of phospho-p65. The phospho-p65 antibody (Cell Signalling #3031) used detects p65 only when phosphorylated at serine 536 and does not cross-react with the p50 subunit or other related proteins. While ERK5 targeted siRNA drastically suppressed ERK5 expression, the level of total and phospho-p65 remained unchanged when compared to negative control siRNA and untransfected PC3 cells (Figure 3.12A). In addition, in PC3-ERK5 cells, the level of total and phospho-p65 did not appear to be affected by the presence of increased ERK5 expression (Figure 3.12B).

FIGURE 3.12   **ERK5 expression does not change p65 levels**
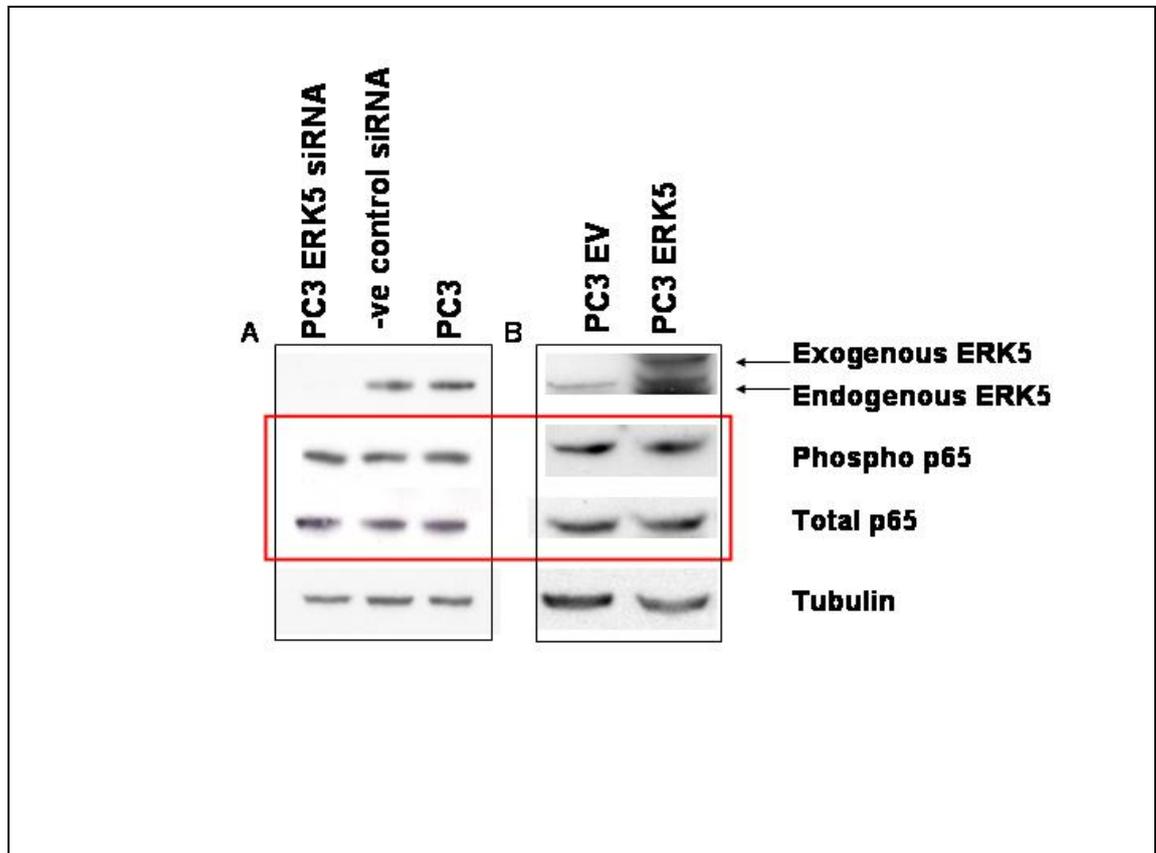
3.2.9   ERK5 overexpression and p65 levels - Xenograft studies

In order to examine if there was nuclear co-localisation of ERK5 and NFκB we used PC3-ERK5 generated subcutaneous xenograft tissue for immunostaining. This tissue was selected as our group have previously demonstrated that ERK5 promotes both *in vitro* and *in vivo* growth of these cells (184). Overall, 8 xenografts were studied, at both high (x20) and low magnification (x5) and serial sections were stained for ERK5 and total p65 expression. p65 immunoreactivity in the PC3-ERK5 xenografts was noted to vary widely with different staining intensities within individual tumours and also among different tumours. In addition, there did not appear to be any correlation between p65 and ERK5 staining patterns. In Figure 3.13, there was strong and fairly homogeneous nuclear ERK5 immunoreactivity in the PC3-ERK5 cells, which contrast to the infrequent expression of nuclear (activated) p65 with the majority of ERK5 positive expressing detectable p65. Xenografts derived from PC3 EV cells were also stained for p65 and did not reveal any trend for association between nuclear ERK5 and p65 expression (data not shown). These results suggest that there is no significant relationship between ERK5 and NFκB p65 protein expression in these prostate cancer xenografts and corroborates the results from Western blotting. This work was carried out by a BSc student whom I supervised during her time in the laboratory.

FIGURE 3.13   ERK5 and p65 immunostaining in PC3 ERK5 xenografts
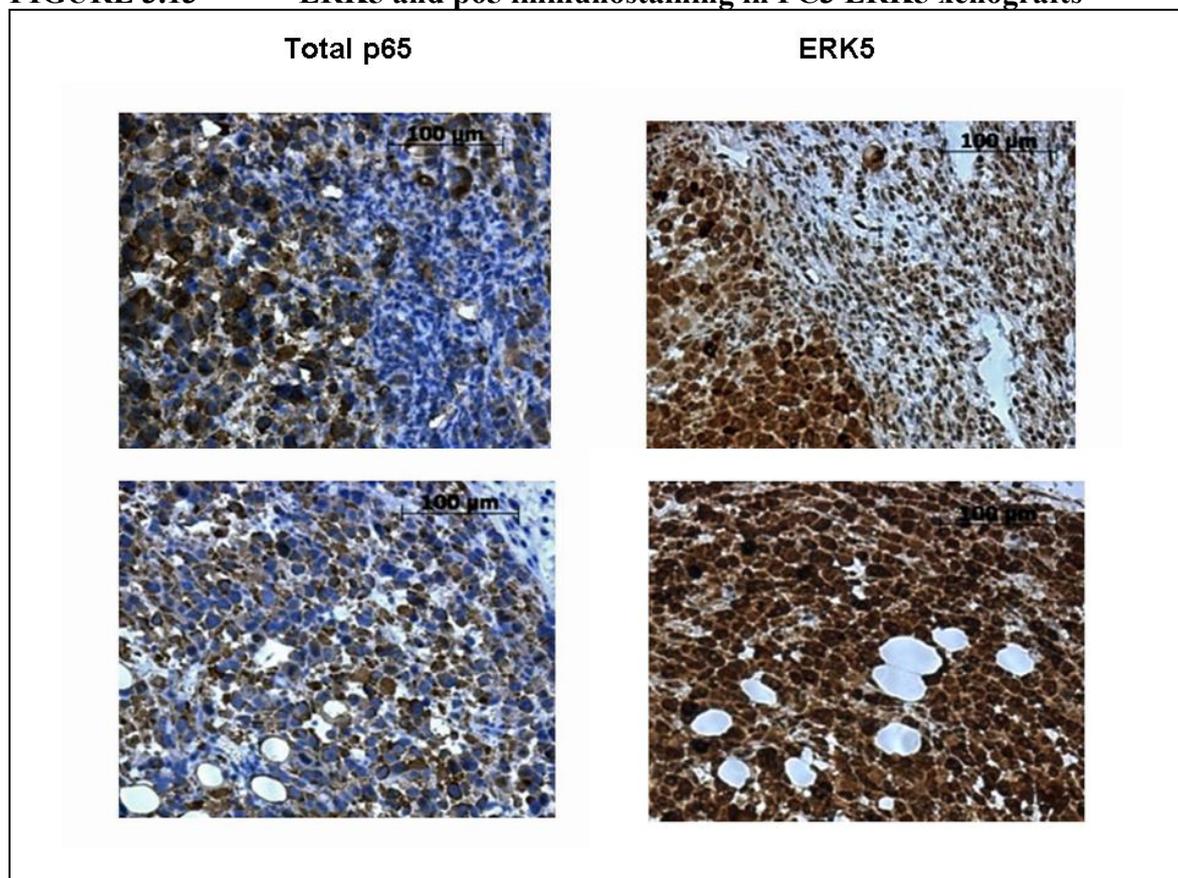
3.2.10 Potential downstream markers of ERK5 activity

In order to monitor ERK5 activity in future drug trials involving an ERK5 inhibitor it is necessary that a reliable downstream marker is identified. Several downstream substrates for ERK5 have been described in the literature in addition to NFκB including p70S6 kinase, GSK-3, ribosomal-S6 and BAD(209;210).

Therefore, in order to assess the potential role of these as a marker of activity in prostate cancer, their expression levels were examined in PC3 cells where ERK5 expression was inhibited using siRNA transfection. Surprisingly none the known ERK5 targets were altered when ERK5 levels were suppressed. (Figure 3.14) It may be that these targets previously described are not downstream of ERK5 in prostate cancer cells or that other upstream pathways which would not be affected by ERK5 knockdown continue to have contributory effects. Complete knockdown of ERK5 was not achieved with ERK5 siRNA and this may also lead to lack of effect noted on the downstream markers.
Due to time constraints this was not explored further. Given more time it would of been interesting to reassess the effect of ERK5 knockdown on these known targets by using serum starved cells and by performing a timecourse experiment with EGF stimulation.

FIGURE 3.14   **Potential downstream markers of ERK5 signalling**
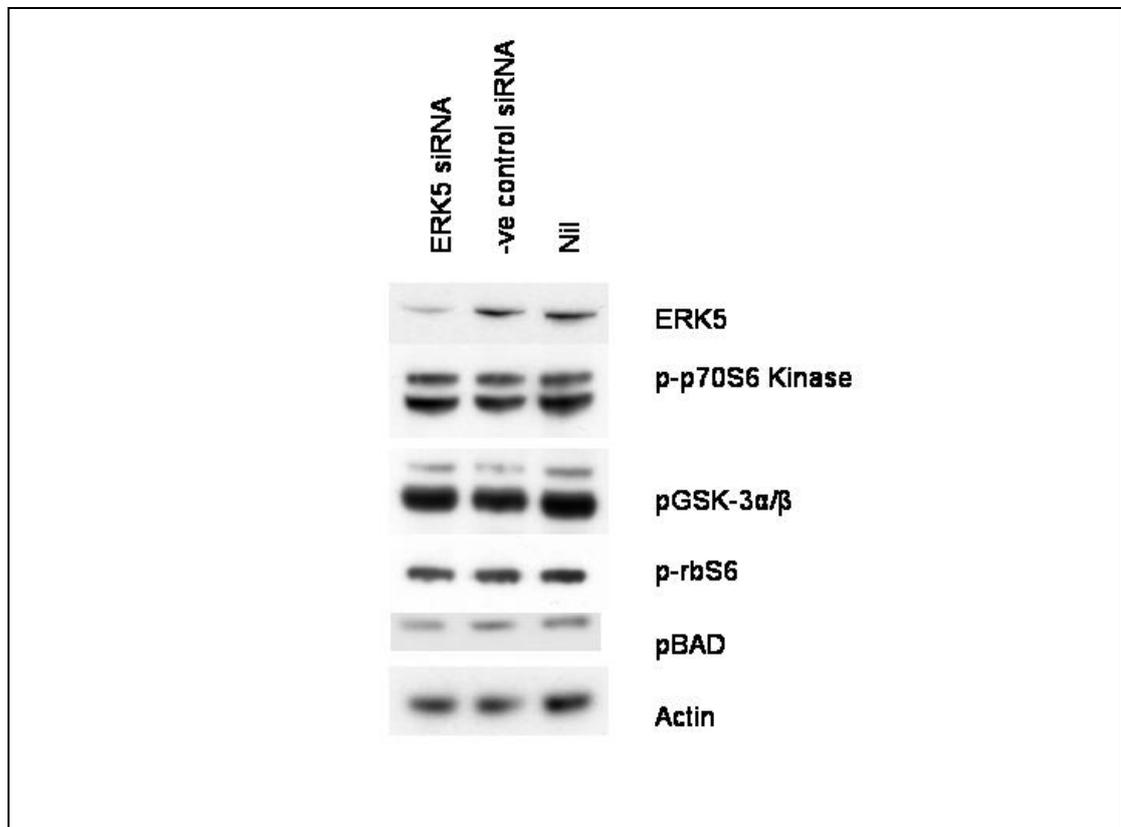
3.3 Discussion

Consistent with previous data, ERK5 nuclear expression is shown to be associated with aggressive prostate cancer in this study. In addition strong levels of immunostaining have been shown in a range of metastatic tissue suggesting that ERK5 inhibition may be of therapeutic interest in both advanced prostate cancer and metastatic disease. ERK5 overexpression has previously been shown to enhance proliferation, migration and invasion in PC3 cells (184). By using siRNA as a method of suppressing ERK5, we have shown that ERK5 inhibition results in a significant reduction in proliferation, migration and invasion *in vitro*. This data confirms that targeting ERK5 in prostate cancer is an attractive therapeutic candidate. A Cancer Research Technology Drug Development programme is currently focusing on ERK5 as an anti-cancer target. Of particular interest, two MEK5 inhibitors (BIX02188 and BIX02189) have recently been identified. Successful inhibition of ERK5 phosphorylation was achieved using these novel compounds in HeLa cells (211). There is currently no data of either of these MEK5 inhibitors *in vivo*. Future work will therefore focus on the biological effect of these MEK5/ERK5 inhibitors on prostate cancer both *in vitro* and in murine models to assess if suitable for a future clinical trial.

NFκB has been shown to be constitutively active in prostate cancer and recent evidence suggests that ERK5 and NFκB may interact in tumourigenesis. The ERK5 pathway has been shown to mainly target the localisation and activation of the p65 component of NFκB (208).

This study is the first to examine the relationship of ERK5 and NFκB in prostate cancer cells. Immunohistochemistry and Western blotting were used to assess the effect on NFκB by altering ERK5 expression in PC3 cells. Expression levels of p65 protein were measured because the p65/p50 heterodimer is the most abundant and transcriptionally active form of NFκB (196) . Contrary to previous studies, there was no evidence of changes in the total NFκB p65 expression caused by over- or under-expression of ERK5. In addition, phospho-p65 level was also unchanged by ERK5 siRNA. Consistent with this *in vitro* data, NFκB p65 and ERK5 staining did not correlate in human prostate cancer xenografts.

4 The relationship between ERK1/2 and ERK5 mediated signalling in prostate cells

4.1 Introduction

The MAPK family consists of at least 4 members: ERK 1/2, JNK, p38 and ERK5. All are activated by extracellular stimuli such as growth factors and environmental stresses and are recognised to have important roles in cell proliferation, migration and differentiation. It is now increasingly appreciated that complex cross talk further refine and control the activities of these and other signalling pathways.

Similarities between the ERK 1/2 and ERK5 pathways are recognised both in their activation modes and functions. It has recently been suggested that there may be cross-talk between these pathways as treatment with high dose (10μM) PD184352, a specific MEK1 inhibitor prevents ERK5 activation (182). Treatment with low dose (1μM) PD184352 has also been shown to increase nuclear accumulation of ERK5 and MEF2 promotor driven luciferase activity (183) suggesting that when ERK1/2 activation is suppressed, ERK5 activation is enhanced.

The current considered model of ERK1/2 regards each isoform as interchangeable due to their similar regulation and downstream effects. It has recently been proposed however that there may be significant functional differences between each isoform with ERK1 acting as a negative regulator and ERK2 as a positive regulator of cell proliferation (212;213). It is thought that ERK1 may affect the overall signalling by antagonizing ERK2 activity.

My aim in this chapter was to explore if individually ERK1 and ERK2 may 'cross-talk' with ERK5 in prostate cancer cell signalling. siRNA mediated knock down of each isoform was performed and their effects examined, including the effect on phosphorylation status as well as ERK5 activation by western blotting. The functional effects of ERK1 and ERK2 knockdown on PC3 proliferation were also assessed.

4.2   Results

4.2.1   ERK5 and PD184352

PD184352 is a MEK1 inhibitor, which suppresses ERK1/2 activity at low doses (1-3μM). It has previously been reported that PD184352 also suppresses ERK5 activity at higher dosage (10μM)(182;184).

ERK5 is activated 15 minutes post EGF stimulation with neglible levels observed in serum-starved cells (Figure 4.1). Serum starved PC3 cells were therefore treated with 5-50μM PD184352 prior to EGF stimulation in order to assess its role as an ERK5 inhibitor. ERK5 phosphorylation is maximal 15 minutes post EGF stimulation and therefore cells were harvested at this timepoint in order to highlight the effective reduction in phosphorylation when using PD184352.   Complete pERK1/2 inhibition was achieved with 5μM treatment with a significant reduction in ERK5 observed at 10μM (Figure 4.2).

FIGURE 4.1 EGF activates ERK5 in serum starved PC3 cells
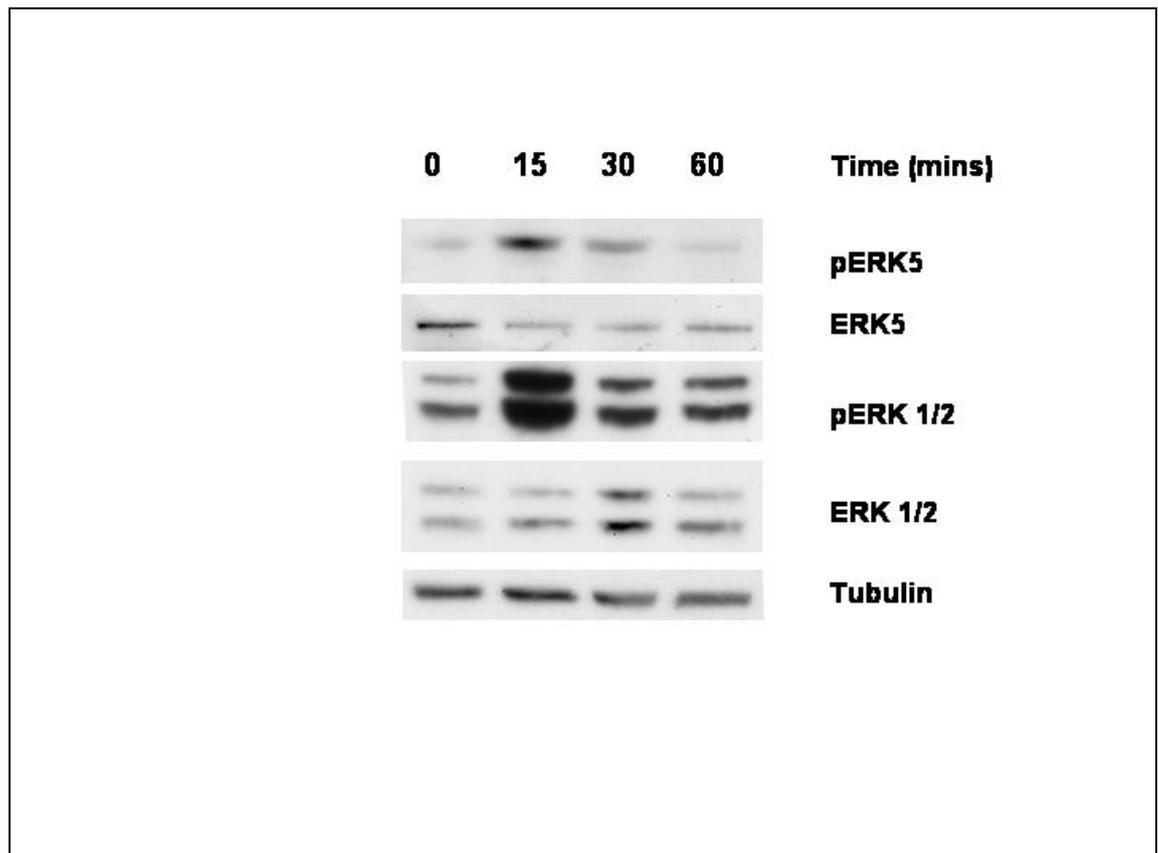
FIGURE 4.2 Inhibition of ERK1/2 and ERK5 in PC3 cells by PD184352 (0-50µM) at 15 minutes post EGF stimulation
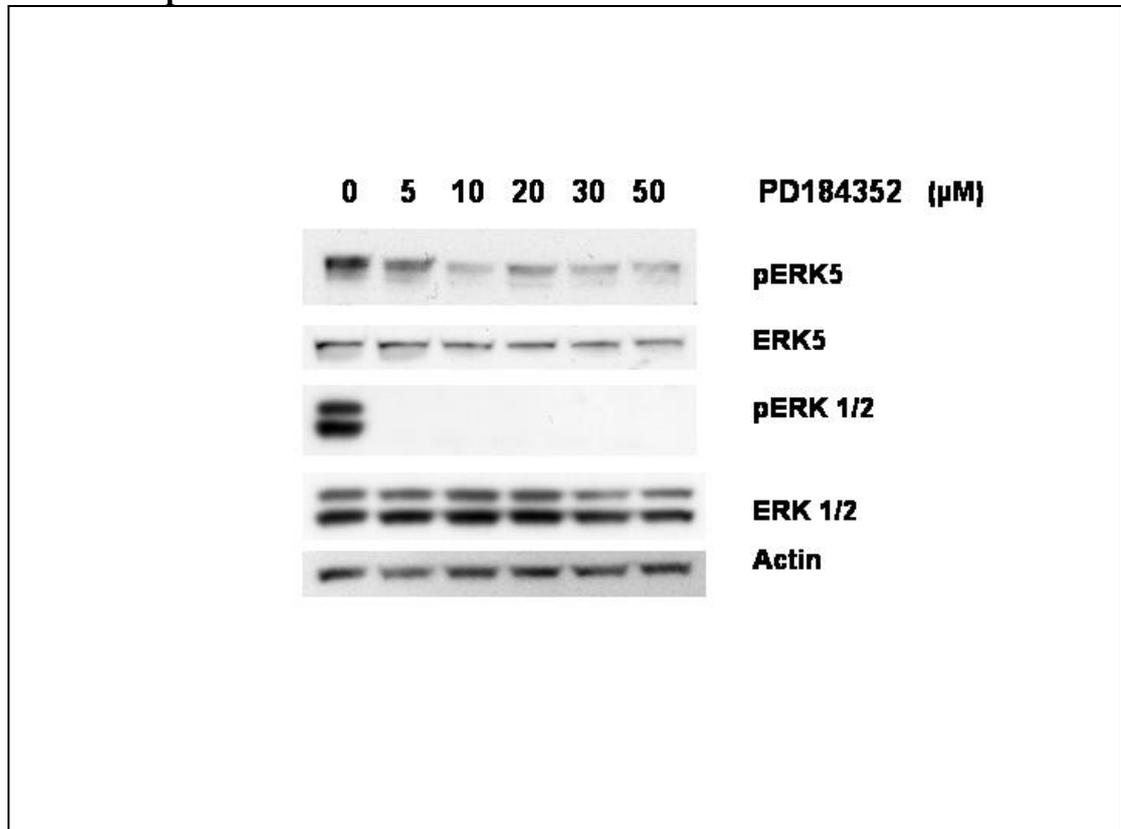
4.2.2   ERK1 and ERK2 knockdown in prostate cancer cell lines

Successful knockdown of ERK1, ERK2 and ERK1/2 was achieved in PC3 cells and using both the HiPerfect and Amaxa method of siRNA transfection (Figure 4.3). Analysis of ERK5 status showed that in full medium ERK5 activation was upregulated when ERK1 expression was suppressed by siRNA. Initial results suggested that ERK5 phosphorylation may be suppressed when ERK2 was knocked down however when repeated (2 further times), no change in ERK5 activation levels were observed. As this result was not reproducible it is thought to not be real.

4.2.3 ERK1 and ERK5 phosphorylation

To further examine the potential relationship between ERK1 and ERK5 signalling, a timecourse experiment with EGF stimulation was performed on the ERK1 and ERK2 knockdown cells (Figure 4.4). As reported previously (212), ERK1 knockdown resulted in enhanced ERK2 phosphorylation. The duration of ERK5 activation also appeared prolonged in the ERK1 knockdown cells when compared to the non-silencing siRNA control. In contrast when ERK2 was knocked down ERK1 activation was enhanced however no significant alteration in ERK5 activation status was observed. ERK5 phosphorylation status was also assessed in PC3 cells in which ERK1 and ERK2 were simultaneously knocked down. Surprisingly, there was no alteration in ERK5 activation observed.

In order to further investigate the association between ERK5 and ERK1/2, a timecourse experiment with ERK5 siRNA transfected PC3 cells was performed (Figure 4.5). ERK1/2 phosphorylation appears to suppressed in the ERK5 knockdown cells. Due to time constraints this experiment was only performed once and further work would need to be done in order to validate this preliminary finding.

FIGURE 4.3 Effects of siRNA mediated ERK1 and/or ERK2 knockdown on ERK5 status
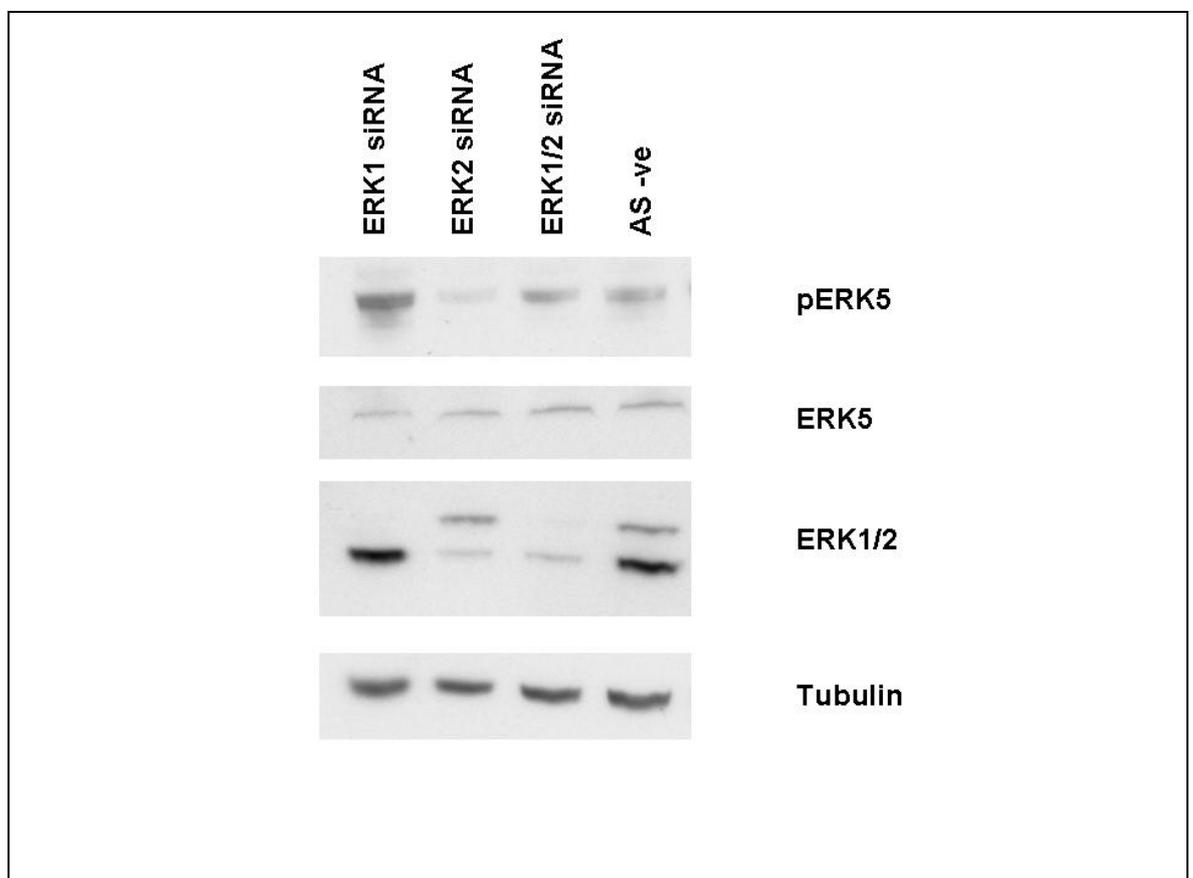
FIGURE 4.4 ERK1 knockdown results in prolonged ERK5 phosphorylation
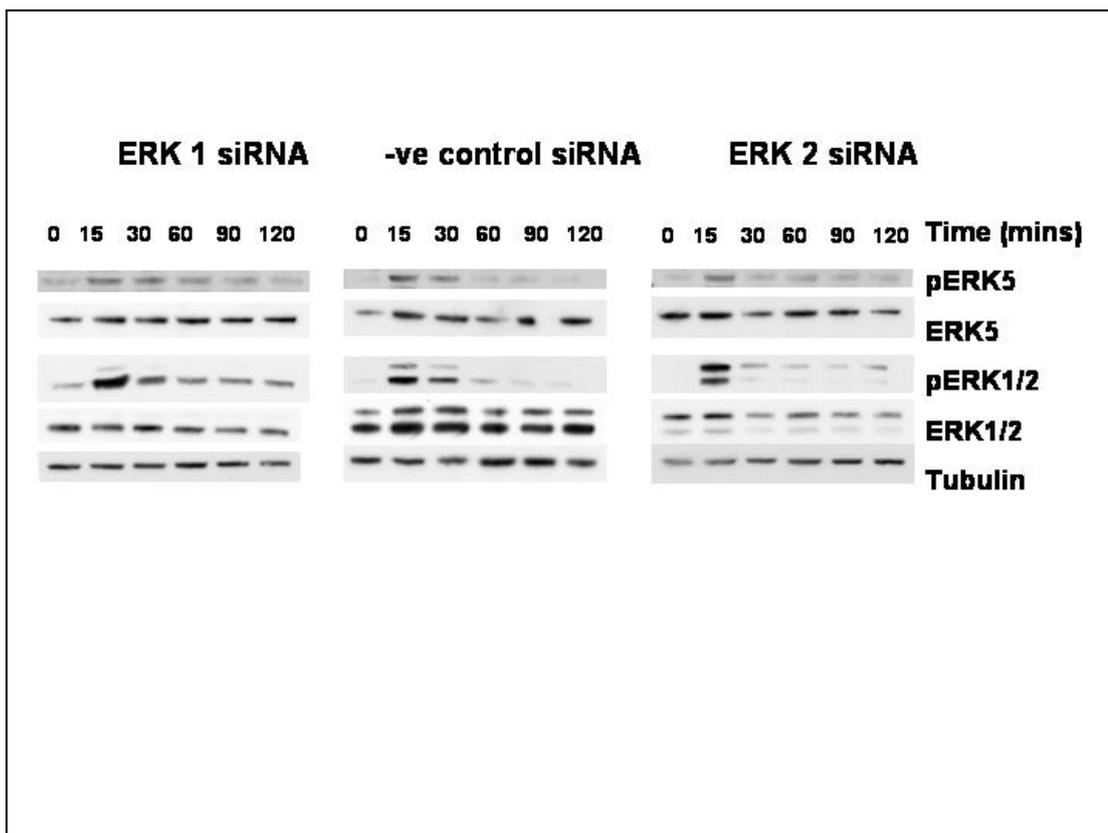
FIGURE 4.5 ERK5 knockdown has no effect on ERK1 phosphorylation
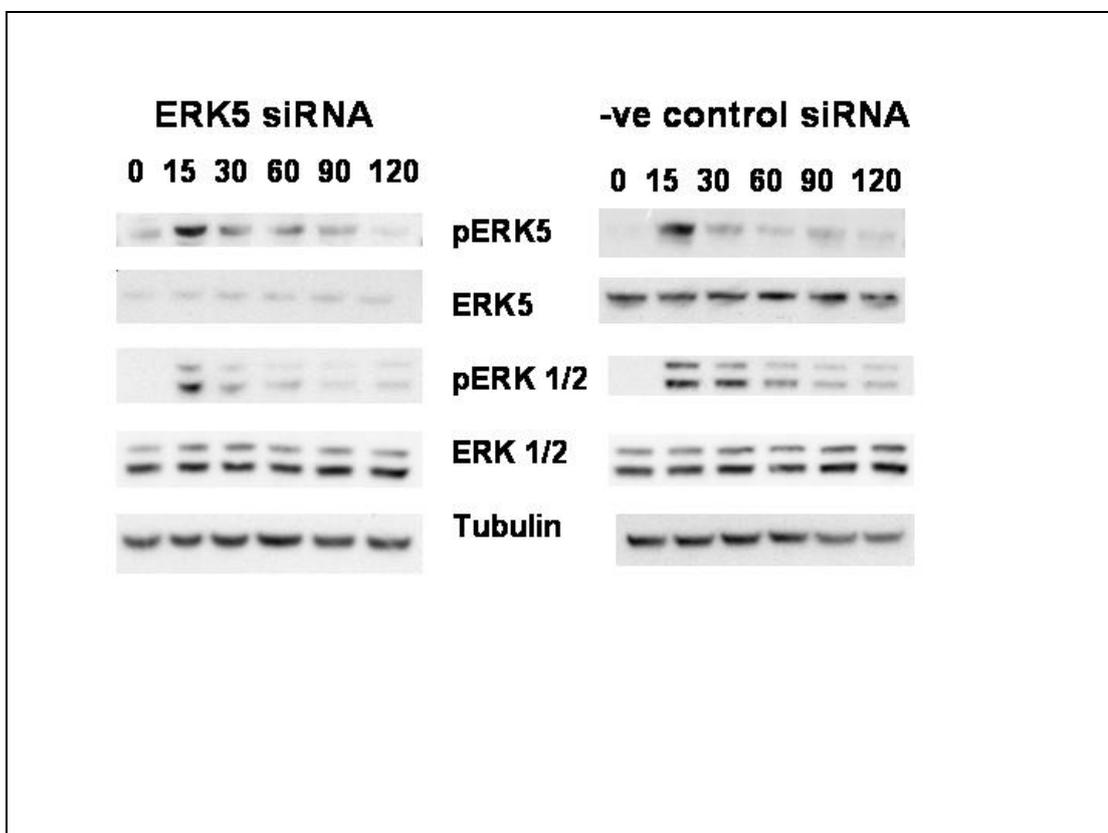
4.2.4   ERK1 and ERK2 knockdown have separate effects on PC3 proliferation

Recent published data suggests that ERK1 may be a negative regulator and ERK2 a positive regulator of proliferation. In order to study the effects of each individual isoform on prostate cancer cell proliferation, serial cell counts were performed on transfected cells at 24-hour intervals.   An increase in proliferation was observed in ERK1 knockdown cells ($p < 0.005$).   A decrease in proliferation was noted in ERK2 knockdown cells when compared to the negative control siRNA ($p < 0.005$) however this was only observed at 96 hours post transfection (Figure 4.6).

FIGURE 4.6 ERK1 and ERK2 have individual effects on PC3 proliferation rates
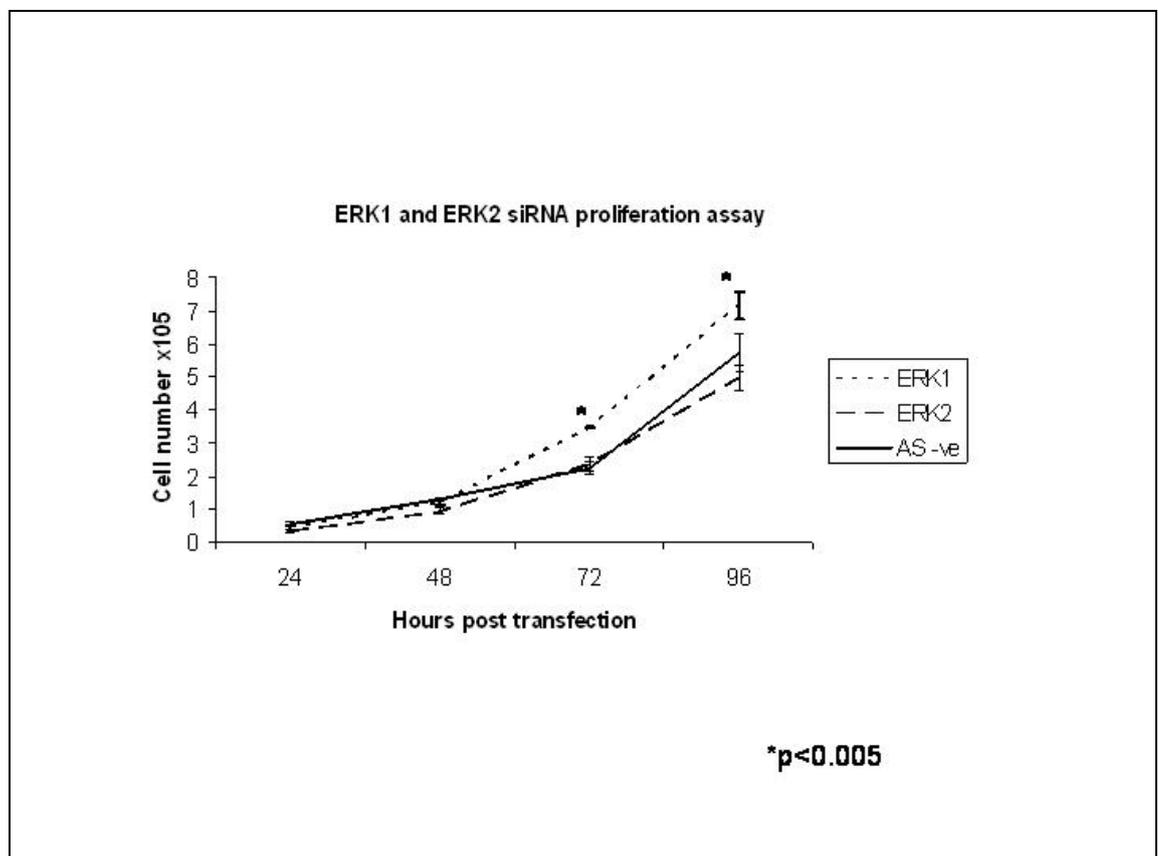
4.3   Discussion

Both the ERK1/2 and ERK5 MAPK pathways share a number of activators such as the EGF receptor (167), HER2 (215) and Src (169) as well as oncogene products such as Ras (167) and COT (170). Studies using MEK inhibitors previously implied roles for ERK1/2 activation downstream of Ras and Raf in neoplastic transformation. Recent studies have shown however that these inhibitors block ERK5 activation as well at various concentrations making it difficult to conclude that only ERK1/2 activation is involved (182).

To date little is known of the relationship between ERK1/2 and ERK5 signalling. Previous work suggests that inhibiting ERK1/2 enhanced MEK5 signalling through ERK5. Treatment with low dose PD184352 enhanced nuclear accumulation of ERK5 and led to a twofold increase in MEF2 promoter driven luciferase activity in Src transformed cells (183).

The results of this study highlight a novel biochemical and functional relationship between ERK1 and ERK5. Future experiments of this project would involve quantitation of downstream signalling e.g. by transfection of an ERK5 specific MEF2 luciferase construct in addition to ERK1 siRNA in PC3 cells. In addition immunofluorescence of ERK5 in ERK1 knockdown cells may allow further understanding of the effect of ERK1 on ERK5 phosphorylation and its nuclear trafficking.

5   ERK5 proteomics

5.1   Introduction

ERK5 has been shown to regulate the activity of several transcription factors including MEF2, c-Fos and Fra-1, Sap-1, c-Myc and NFκB (167;170;171;216;217). In order to further investigate the ERK5 signalling network I attempted to immunoprecipate endogenous ERK5 in PC3 cells and use mass spectrometry analysis to characterise its interacting proteins.

Exogenous ERK5 was also studied using FLAG tag ERK5 and stable isotope labelling with amino acids in cell culture (SILAC) which is a method used to study *in vitro* mass spectrometry (MS)-based quantitative proteomics. SILAC involves incorporating a given 'light' or 'heavy' form of the amino acid into the proteins (218). The method relies on the incorporation of amino acids with substituted stable isotopic nuclei (e.g. deuterium, 13C, 15N) (See Figure 5.1). In an experiment two cell populations are grown in culture media that is identical with the exception that one will contain a 'light' and the other a 'heavy' form of a particular amino acid (e.g. ^{12}C and ^{13}C labelled L-lysine, respectively). The labelled form of the amino acid is then incorporated into all new synthesised proteins and after a number of cell divisions this amino acid will be replaced by its isotope labelled analogue. The cells behave exactly like the control cells since there is negligible difference between the labelled and natural amino acid isotopes.

FIGURE 5.1 Overview of SILAC protocol
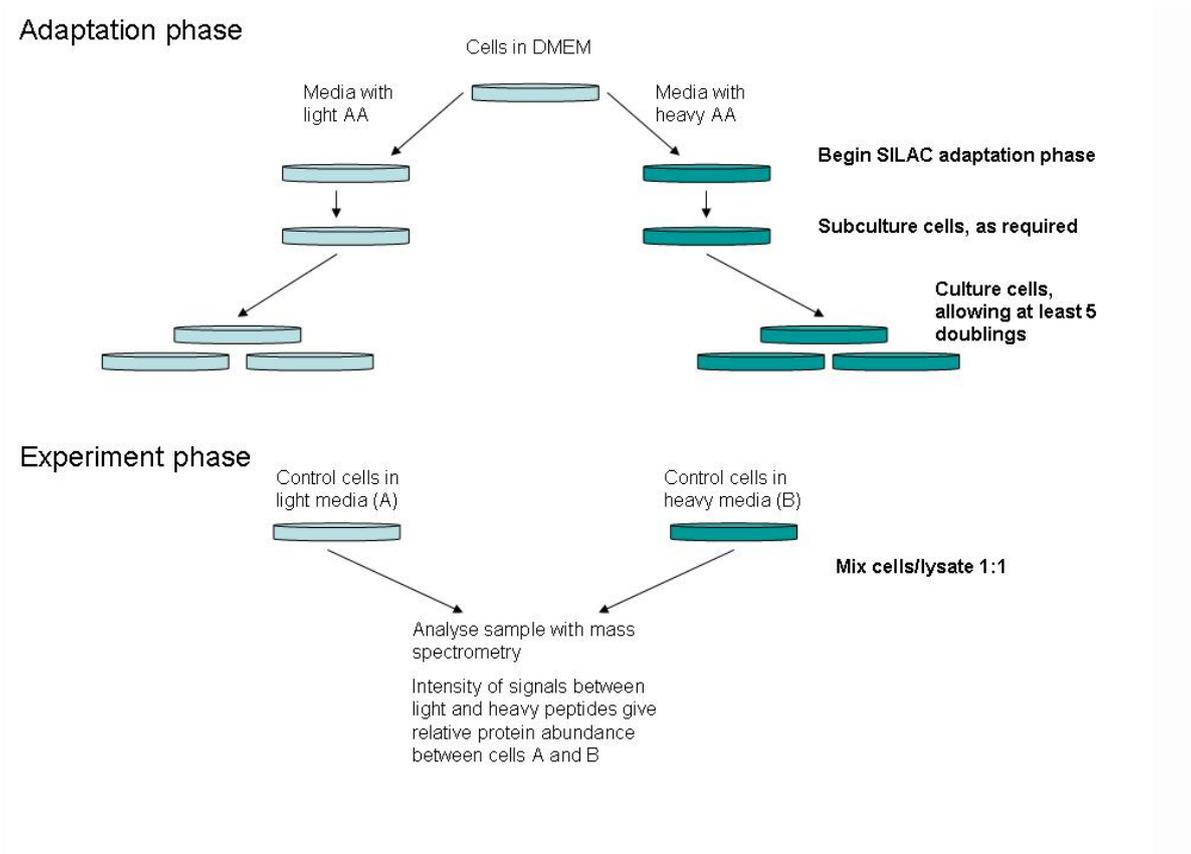
The SILAC experiment consists of two distinct phases; an adaptation and an experimental phase. During the adaptation phase, cells are grown in light and heavy SILAC media until the cells grown in the heavy media have fully incorporated the heavy amino acids (confirmed by MS). Cell number can also be expanded during this phase to accomodate the required number for the desired experiment. In the experimental phase, the two cell populations are treated differently in order to induce changes in the proteome. The samples are then mixed equally and analysed by MS for protein identification and quantification.

Modified from Ong et al 2007(219)

5.2   Results

5.2.1   Titration experiments

Initial immunoprecipatation experiments involved optimising experimental technique and reagents. Both total and phospho-specific ERK5 antibodies (Cell signalling and Upstate) were tested with protein A and G sepharose beads. For each titration experiment a constant antibody:bead ratio was maintained while increasing volumes of bead:PC3 lysate were used. Neither ERK5 antibody worked successfully with protein G beads as smearing was observed at higher bead volumes on western blot analysis (Figure 5.2). Protein A beads and the phospho ERK5 antibody also demonstrated smearing in the bead lane with no significant reduction in the supernatant (SN) lane as the bead concentration increased (Figure 5.3). Successful pulldown of ERK5 was achieved when the Cell Signalling antibody and Protein A beads were cross linked resulting in significant reduction in total ERK5 levels in the supernatant lane being observed with increasing bead concentration (Figure 5.4). Subsequent experiments were therefore performed using the antibody:bead ratio 5:1.

FIGURE 5.2 ERK5 (Cell Signalling and Upstate) and Protein G beads
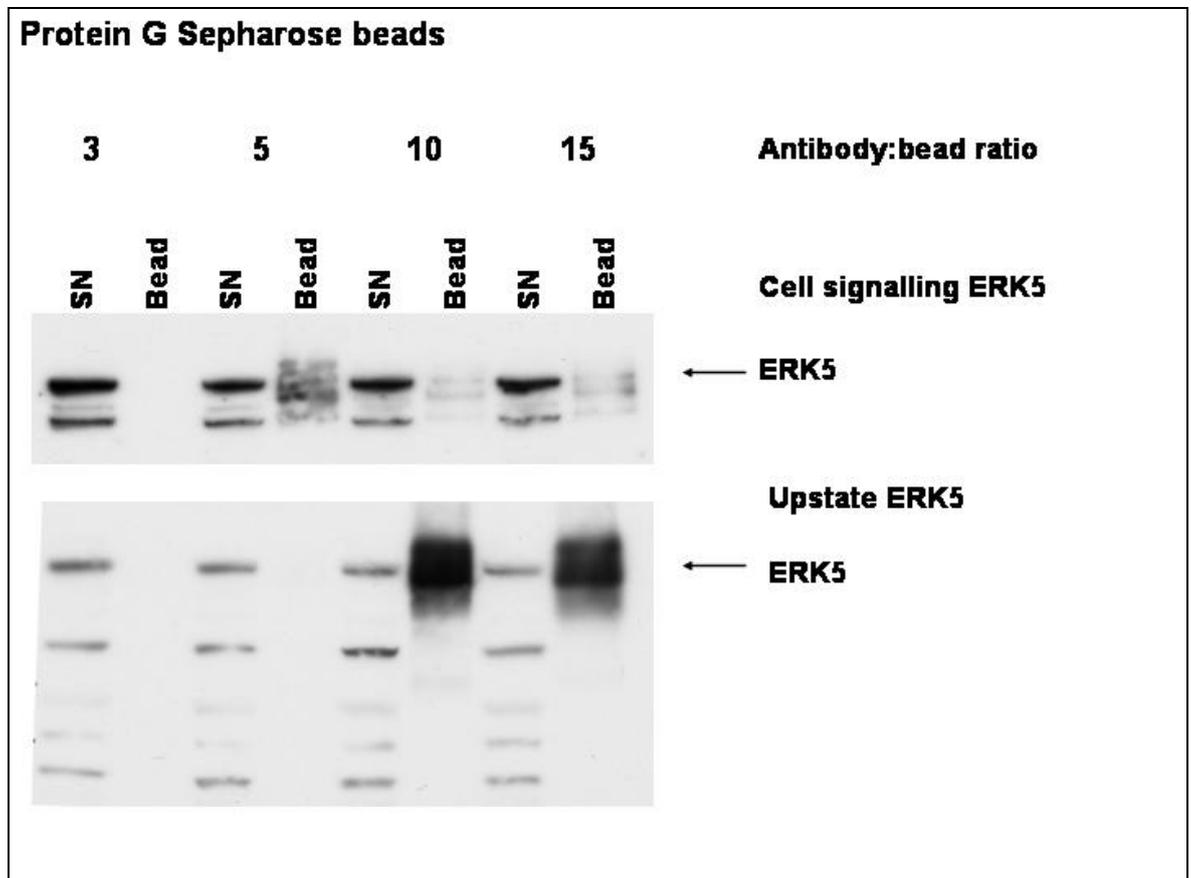
FIGURE 5.3 Phospho ERK5 and Protein A beads

Protein A Sepharose beads
Cell Signalling phospho ERK5

← pERK5
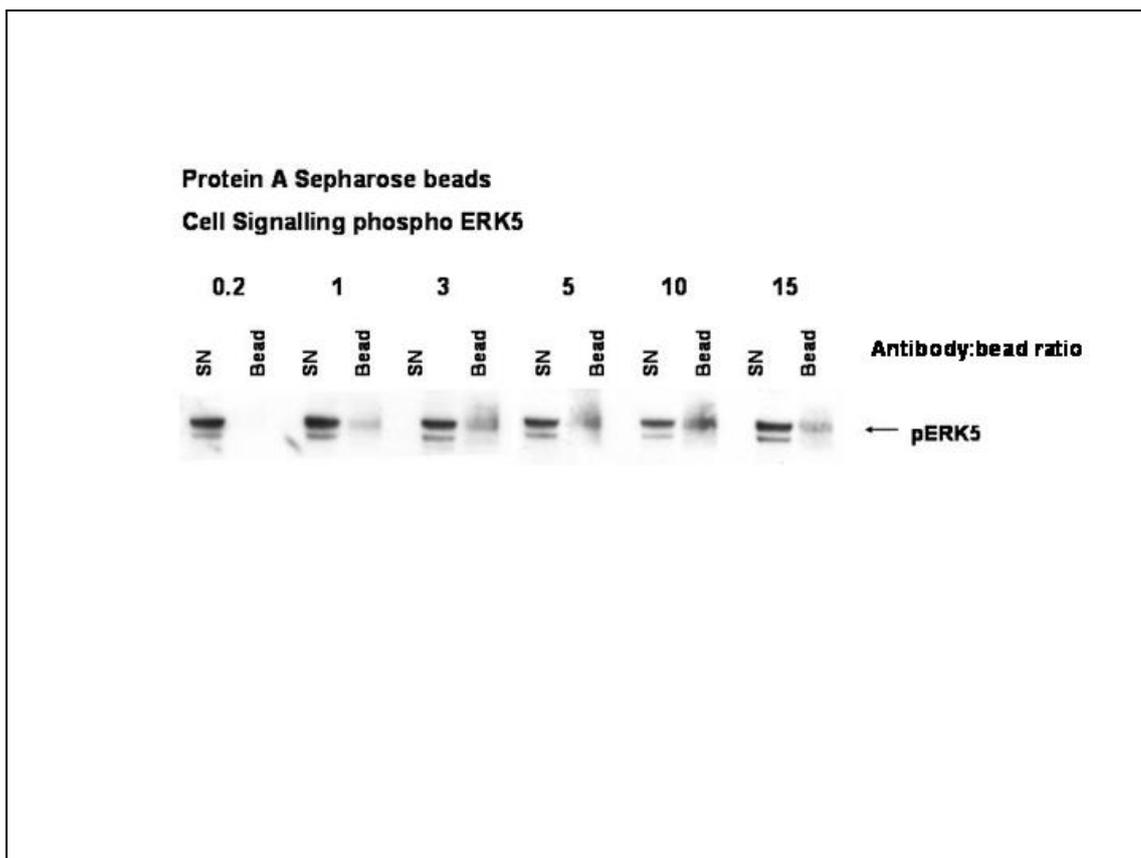
FIGURE 5.4 ERK5 (Cell Signalling) and Protein A beads

Protein A Sepharose beads
Cell Signalling ERK5
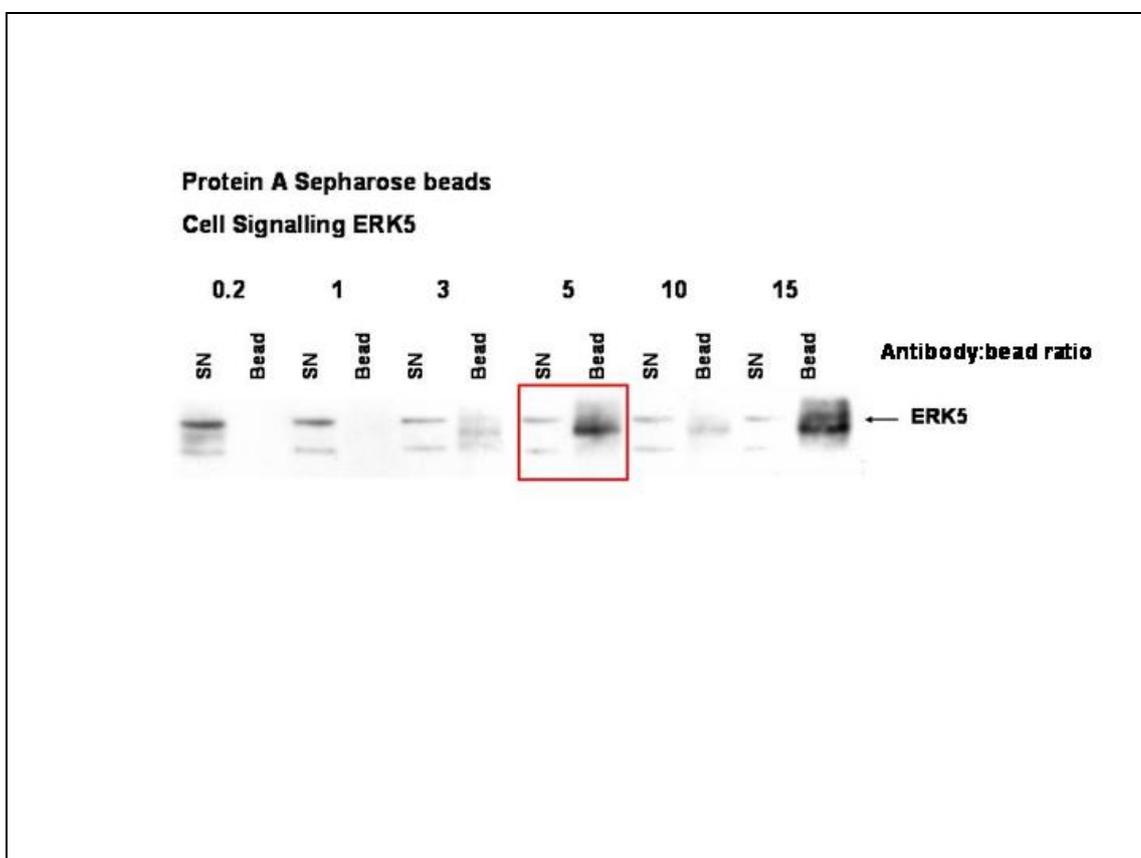
← ERK5

5.2.2 ERK5 identification

In order to confirm adequate ERK5 pull down for mass spectrometry, an initial immunopreciptation experiment was performed using the results of the titration experiment. PC3 cells maintained in full medium were lysed, incubated with the ERK5 or Rabbit IgG cross linked beads overnight and the eluated sample run on a gel. Samples of the lysate post incubation and the eluate pre and post concentration were analysed by western blotting in parallel to confirm successful pulldown and optimal technique when concentrating the final sample (see section 2.10.2) (Figure 5.5). Mass spectrometry analysis of the area of interest (approximately 95-130kda) confirmed identification of ERK5 (Mitogen-activated protein kinase 7 - Homo sapiens) (Figure 5.6). ERK5 was not however the most abundant protein with only 8 peptide matches and 12% sequence coverage observed.

FIGURE 5.5 Confirmation of successful ERK5 pulldown and satisfactory concentration step
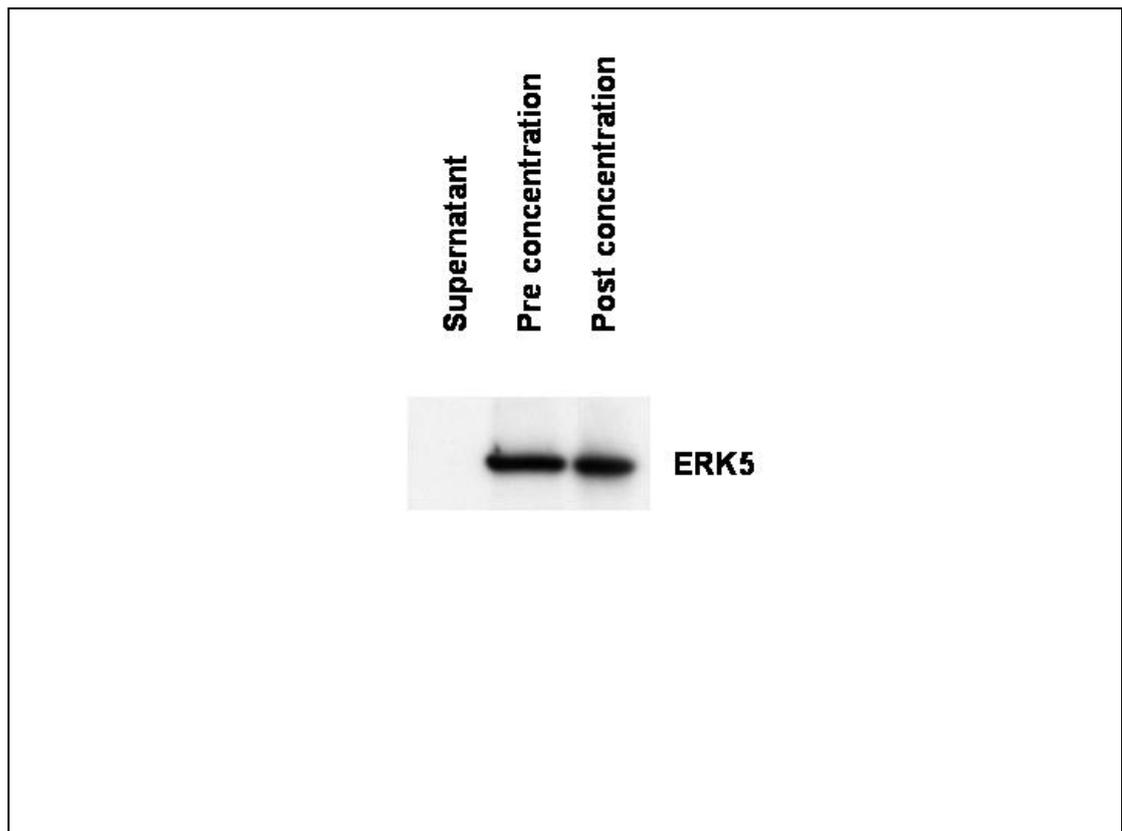
FIGURE 5.6 Analysis of initial endogenous ERK5 pulldown

Results

<table>
<tr><th colspan="6">Band 1 (results from SwissProt Human search)</th></tr>
<tr><th>Hit</th><th>Protein Description</th><th>Protein Score</th><th>Protein Mass</th><th>Number of peptide matches</th><th>Sequence coverage %</th></tr>
<tr><td>1</td><td>AP-2 complex subunit alpha-1 - Homo sapiens</td><td>984</td><td>108570</td><td>22</td><td>21.8</td></tr>
<tr><td>2</td><td>Alpha-actinin-1 - Homo sapiens</td><td>928</td><td>103563</td><td>18</td><td>24.4</td></tr>
<tr><td>3</td><td>AP-2 complex subunit beta-1 - Homo sapiens</td><td>684</td><td>105398</td><td>16</td><td>14.9</td></tr>
<tr><td>4</td><td>Heat shock protein HSP 90-beta - Homo sapiens</td><td>601</td><td>83554</td><td>13</td><td>17.7</td></tr>
<tr><td>5</td><td>Alpha-actinin-4 - Homo sapiens</td><td>552</td><td>105245</td><td>10</td><td>13.4</td></tr>
<tr><td>6</td><td>Nucleolin - Homo sapiens</td><td>539</td><td>76625</td><td>11</td><td>14.6</td></tr>
<tr><td>7</td><td>Splicing factor, proline- and glutamine-rich - Homo sapiens</td><td>526</td><td>76216</td><td>12</td><td>15.8</td></tr>
<tr><td>8</td><td>Endoplasmin precursor - Homo sapiens</td><td>390</td><td>92696</td><td>8</td><td>10.5</td></tr>
<tr><td>9</td><td>Importin subunit beta-1 - Homo sapiens</td><td>373</td><td>98420</td><td>6</td><td>7.4</td></tr>
<tr><td>10</td><td>Heat shock protein HSP 90-alpha - Homo sapiens</td><td>316</td><td>85006</td><td>6</td><td>8.1</td></tr>
<tr><td>**11**</td><td>**Mitogen-activated protein kinase 7 - Homo sapiens[1]**</td><td>**296**</td><td>**89151**</td><td>**8**</td><td>**12**</td></tr>
<tr><td>12</td><td>Transitional endoplasmic reticulum ATPase -Homo sapiens</td><td>271</td><td>89950</td><td>7</td><td>8.9</td></tr>
<tr><td>13</td><td>Keratin, type II cytoskeletal 1 - Homo sapiens</td><td>259</td><td>66149</td><td>5</td><td>6.7</td></tr>
<tr><td>14</td><td>Elongation factor 2 - Homo sapiens</td><td>201</td><td>96246</td><td>4</td><td>4.8</td></tr>
<tr><td>15</td><td>Interleukin enhancer-binding factor 3 - Homo sapiens</td><td>155</td><td>95678</td><td>5</td><td>6.6</td></tr>
</table>

1. ERK-5 is equivalent to Mitogen-activated protein kinase 7

• BSA was also identified with a score of 636 and a 20% sequence coverage in an expanded search

5.2.3   Pre clearing

A pre clearing step with rabbit serum was performed prior to immunoprecipatation in order to reduce contamination. An initial experiment was performed using PC3 cell lysate incubated with rabbit serum however significant smearing was noted and analysis not feasible. Therefore a second pre clearing experiment was performed with four samples of lysis buffer (1ml) instead of cell lysate. Increasing volumes of rabbit serum were incubated with the buffer (5, 10, 25 and 50ul) prior to incubation with a constant volume of Protein A beads (100ul). These samples were then run on a gel and stained with coomassie blue (Figure 5.7). All four lanes demonstrated significant levels of the heavy chain and therefore this optimisation step was abandoned.

FIGURE 5.7 Pre clearing optimisation experiment
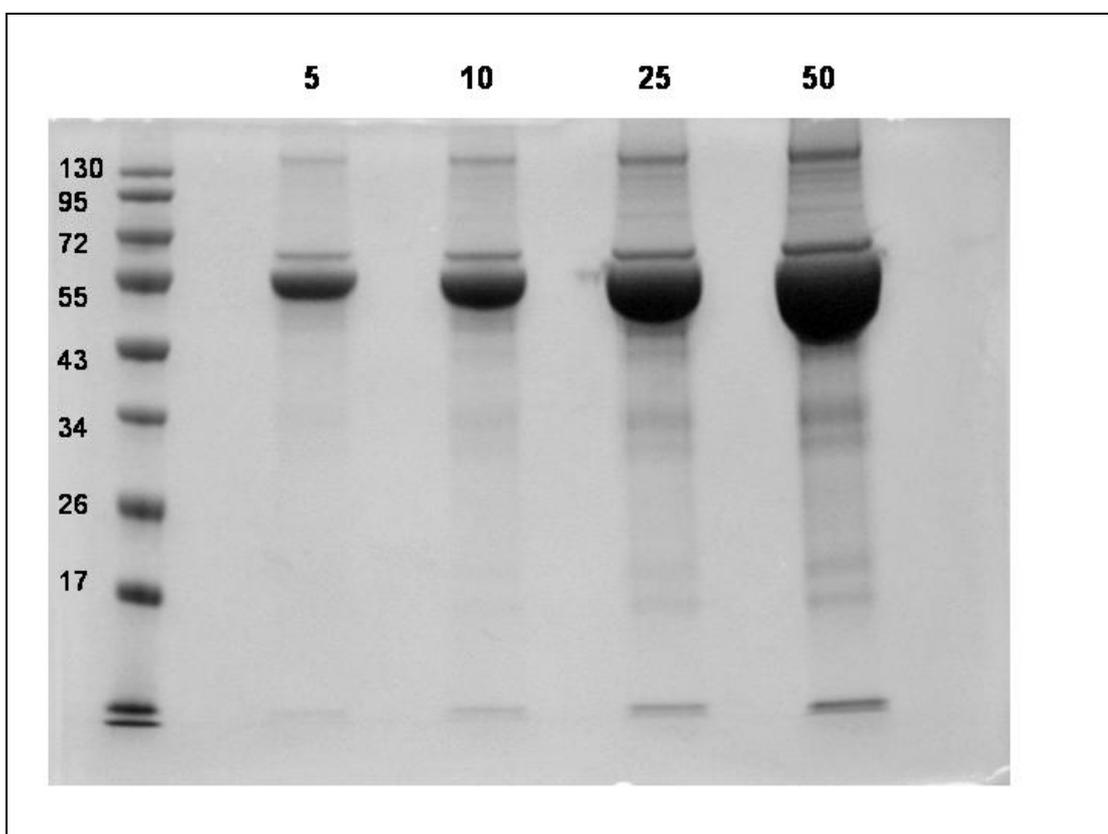
5.2.4   EGF stimulation

As shown previously ERK5 is activated 15 minutes post EGF stimulation with neglible levels observed in serum-starved cells (Figure 4.1). Therefore I chose to perform immunoprecipatation with EGF treated PC3 cells at 2 time points (0 and 15 minutes) as a method of analysing potential ERK5 interacting proteins. Western blotting was performed in parallel to confirm successful ERK5 pull down (Figure 5.8). Streaking and spreading of the samples was observed following coomassie staining, which meant that the sample was not suitable for analysis below 34kDa (Figure 5.9). Our proteomics facility (supervised by Dr Willy Bienvenut) analysed the section of the gel above 34kDa and ERK5 was once again identified. However, only 2 unique peptide matches were found for ERK5 in the 0 minute sample with 6 being identified in the 15 minute sample (Figure 5.10) making it unclear if any pulled down proteins were authentic.

FIGURE 5.8 Successful activation of ERK5 post EGF stimulation and corresponding ERK5 immunoprecipatation
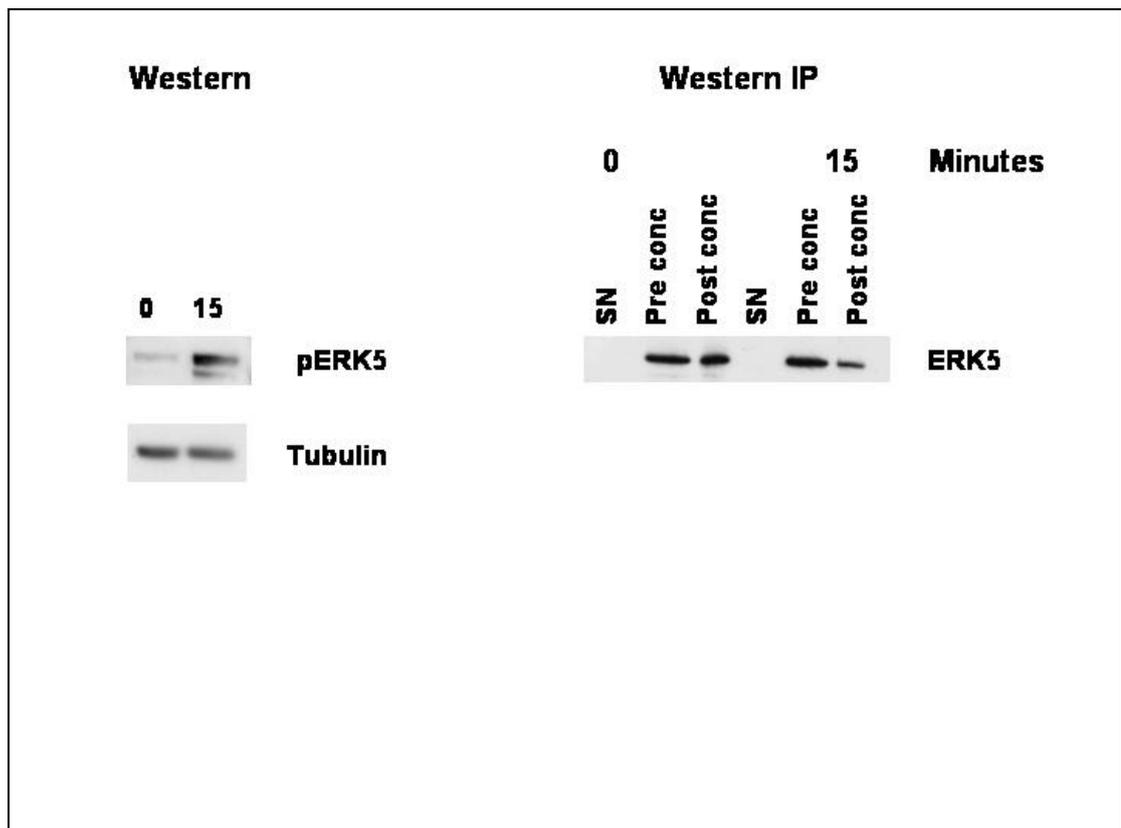
**FIGURE 5.9 EGF stimulation gel for mass spectrometry with marked area of analysis**
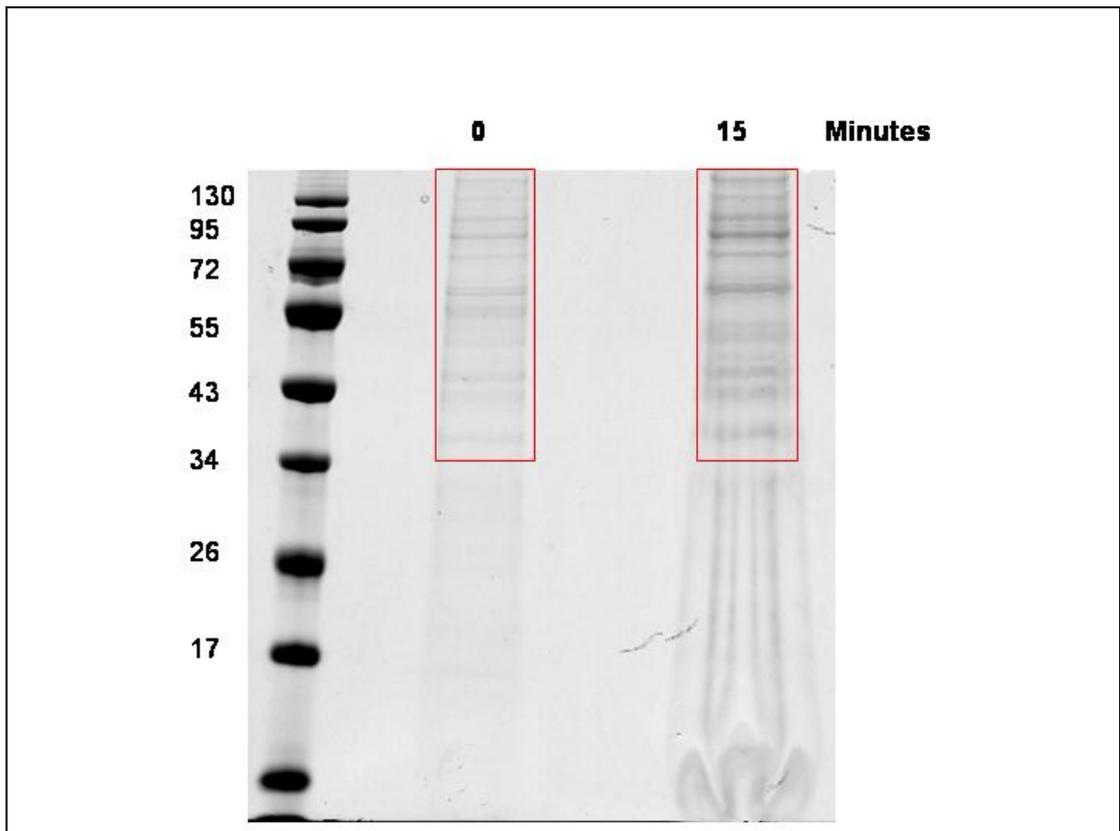
FIGURE 5.10 Results from exogenous EGF immunoprecipatation experiment

5.2.5 Exogenous ERK5

As endogenous ERK5 pulldown was suboptimal it was decided to try transient transfection using a FLAG tag ERK5 expression construct. Transient transfection was assessed in both PC3 and HEK 293 cells. Western blotting confirmed inefficient expression of ERK5 in PC3 cells transfected with the pCMV FLAG tag ERK5 plasmid; however high levels of transfected ERK5 expression were noted in the HEK 293 cells (Figure 5.11). HEK293 cells are well recognised to transfect readily and their transfection efficacy was therefore evaluated alongside PC3 cells. Due to time constraints the transfection efficacy of other prostate cancer cell lines were not assessed. The drawback of using HEK293 for transfection is that it does not allow assessment of ERK5 in prostate cancer and only allows assessment of ERK5 overexpression. SILAC can also be performed using other tagged proteins and therefore I initially considered using the PC3 ERK5 cells discussed in Chapter 3. These cells would have been preferable as the primary cells would have been a prostate cancer cell line. GFP expression was not abundant in these cells however and pull down would not have been as efficient as the FLAG tag protein.

In order to confirm adequate ERK5 pull down for mass spectrometry analysis an initial sample was run on a gel for analysis (Figure 5.12). After elution with the FLAG peptide the beads were boiled in sample buffer in order to assess efficiency of the peptide elution step. The corresponding western is shown in figure 5.13.

Mass spectrometry analysis of band 1 confirmed identification of ERK5 (Mitogen-activated protein kinase 7 - Homo sapiens) (Figure 5.12). ERK5 was the most abundant protein in this band with 39 peptide matches and 37% sequence coverage observed. Band 2 identified heat shock protein (HSP) 90 α and β with 43 and 24 peptide matches respectively. Keratin was also observed in this band, which is presumed to be a contaminant. As a pilot experiment, no control was included in this initial experiment, and therefore it would not be appropriate to compare the relative association between different HSP90 subunits and ERK5.

Following successful pull down of exogenous ERK5, HEK293 cells were grown in SILAC media. After 7 days, cells in both the light and heavy medium were lysed and run on a gel (figure not shown). Mass spectrometry analysis of the heavy labelled lysate showed no significant presence of unlabelled material, confirming successful incorporation for subsequent experiments.

FIGURE 5.11 Transient transfection of FLAG tag ERK5 in HEK293 and PC3 cells
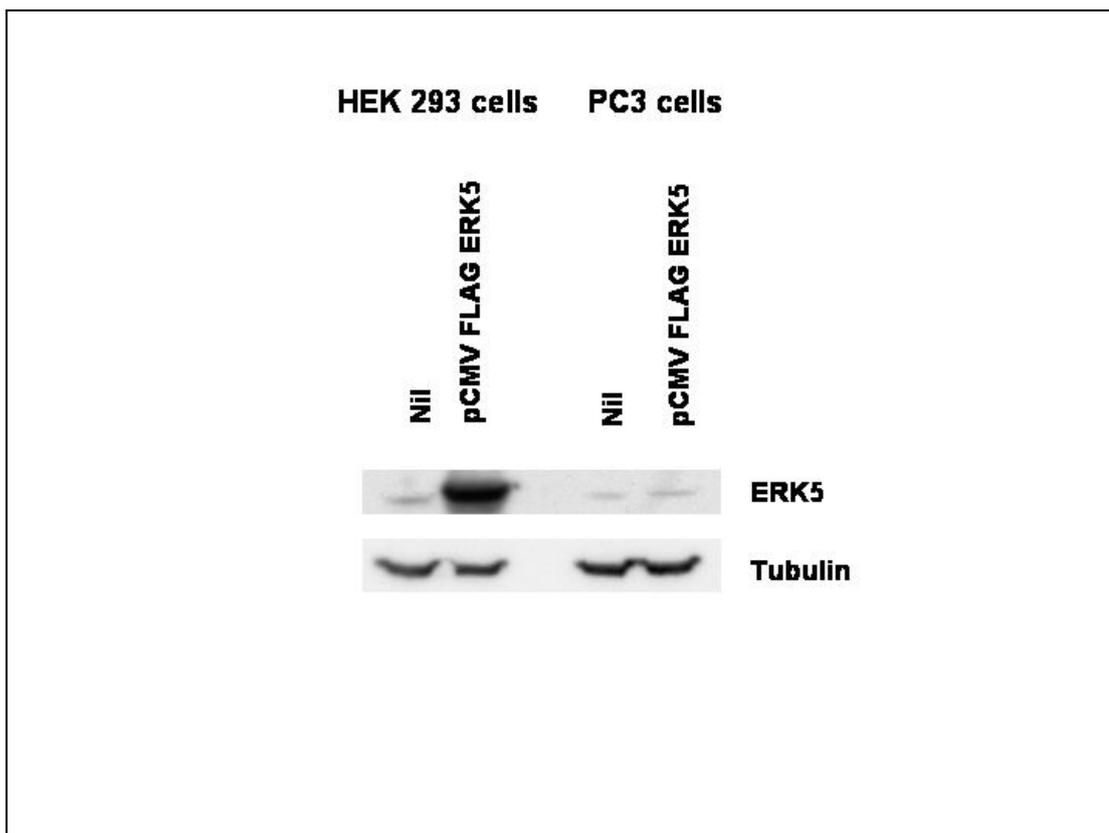
FIGURE 5.12 FLAGtag ERK5 transfection in HEK293 cells allows successful pulldown of ERK5 for mass spectrometry
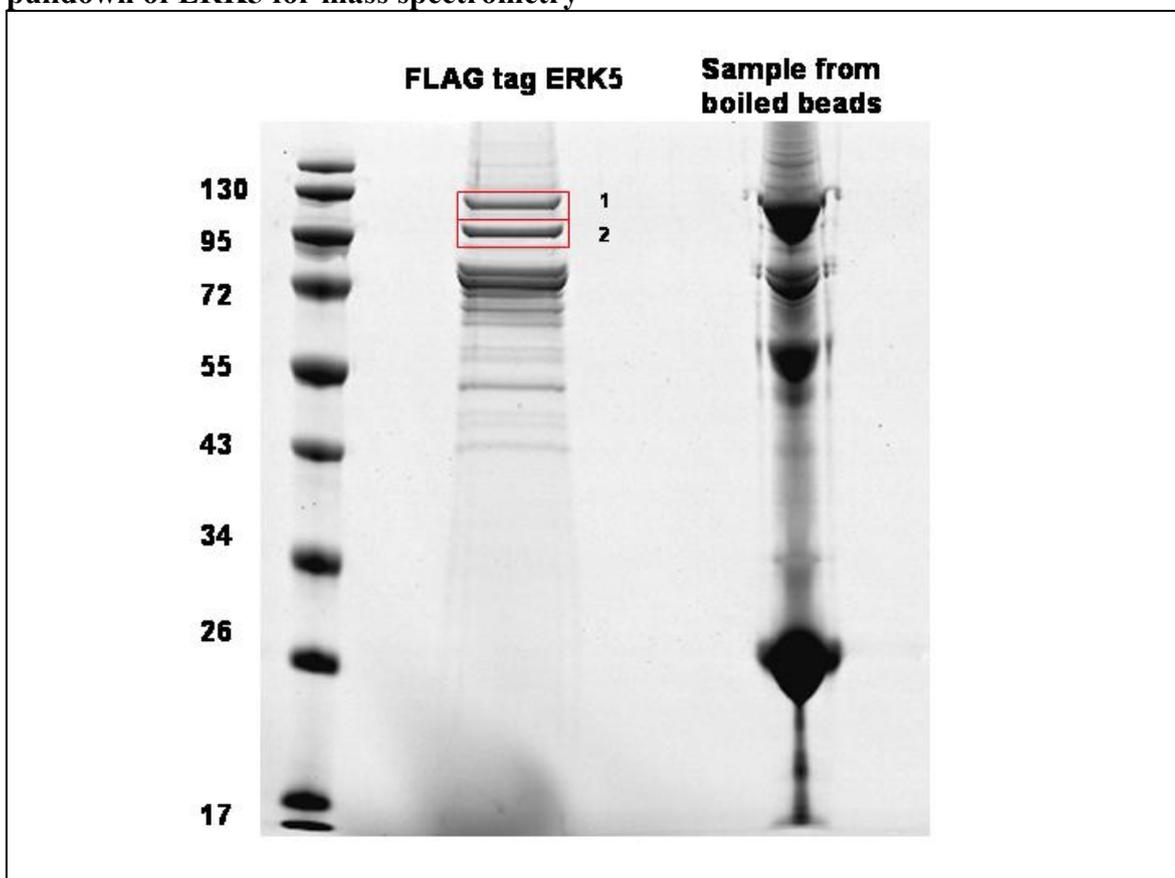
FIGURE 5.13 Transient transfection and pulldown of ERK5 in HEK293 cells
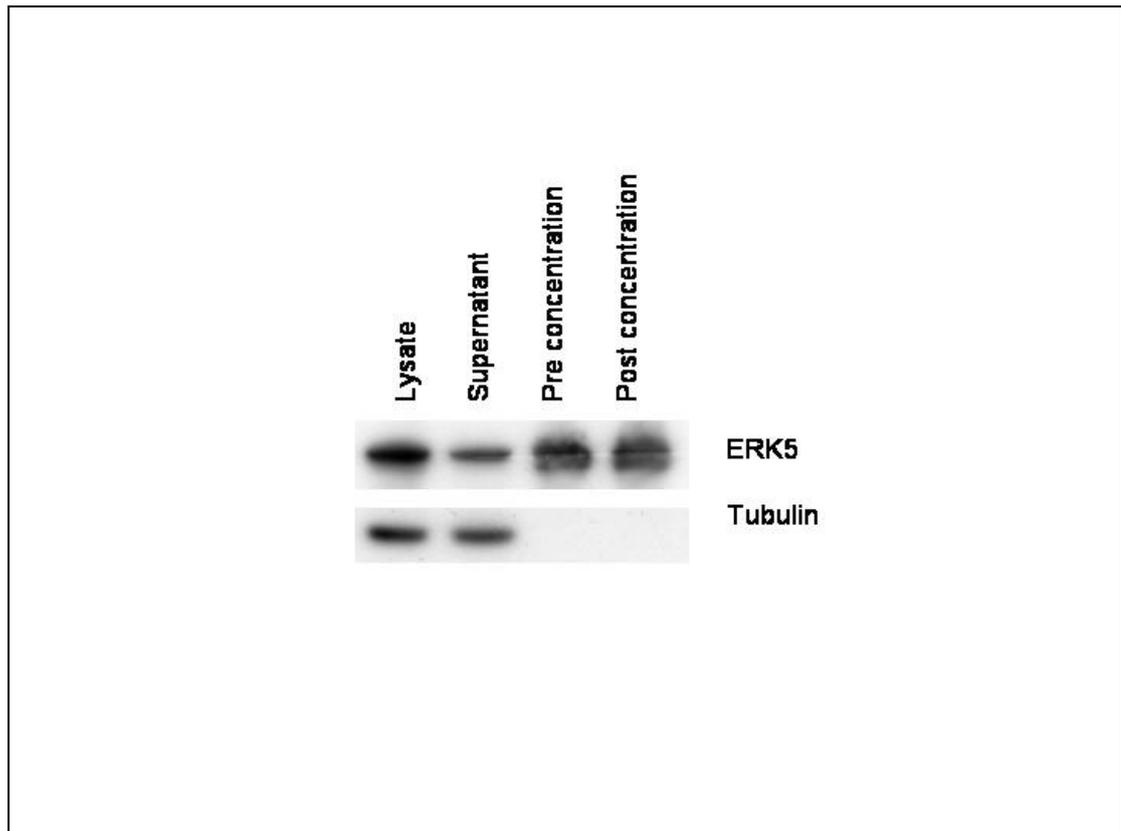
5.2.6   ERK5 vs EV

In order to identify ERK5 associating proteins an initial SILAC experiment was performed comparing 293 cells transfected with either the pCMV FLAG ERK5 (Experiment 1 – heavy medium, Experiment 2 – light medium) or pCMV FLAG EV (Experiment 1 – light medium, Experiment 2 – heavy medium) plasmid).   Mass spectrometry identified unequal loading of samples in experiment 1 which then meant that only experiment 2 could be used for analysis and unfortunately due to time constraints I was unable to repeat this experiment. This experiment identified a number of interesting proteins including Heat Shock Protein 90 (HSP 90), which has previously been identified as being associated with ERK5 activation (220).

5.2.7 Identification of ERK5 binding partners upon activation of the MEK5-ERK5 Pathway

Initial optimisation experiments using HEK293 cells for EGF stimulation revealed that when serum starved the 293 cells had problems with adherence. Plates were therefore coated with poly-l lysine prior to adding the cells. EGF stimulation of HEK293 transfected with pCMV ERK5 at 0 and 15 minutes (Figure 5.14) demonstrated a similar pattern of phospho ERK5 activation to that seen in prostate cancer cells (Figure 4.1).

Cells were treated with 50ng/ml EGF and then lysed at 2 time points: 0 minutes (Experiment 1 – light medium, Experiment 2 – heavy medium) and 15 minutes (Experiment 1 – heavy medium, Experiment 2 – light medium). Mass spectrometry identified unequal loading of samples in experiment 1 which then meant that only experiment 2 could be used for analysis and unfortunately due to time constraints I was unable to repeat this experiment. Once again the sample was analysed by our in house proteomic facility and a list of potential ERK5 binding partners generated for review (Figure 5.15 and 5.16). Equal levels of ERK5 were noted in both timepoints (middle ratio=0.99, variation=0.01). Interestingly HSP 90 was not significantly upregulated between the serum starved and EGF stimulated cells indicating that although HSP 90 does associate with ERK5 it is not involved in its activation.

Overall, 302 proteins were positively identified, and among 125 of these were shown to be recruited by at least 2 fold. Based on a combination of number of peptide matches and confidence of match a further (n=64) subgroup of proteins were thought to represent the interacting proteome of activated ERK5. Further bioinformatics and validation analysis will be required to formally characterise the individual interacting protein of ERK5 and their potential role in carcinogenesis.

FIGURE 5.14

EGF stimulation of HEK293 pCMV ERK5 cells leads to ERK5 phosphorylation
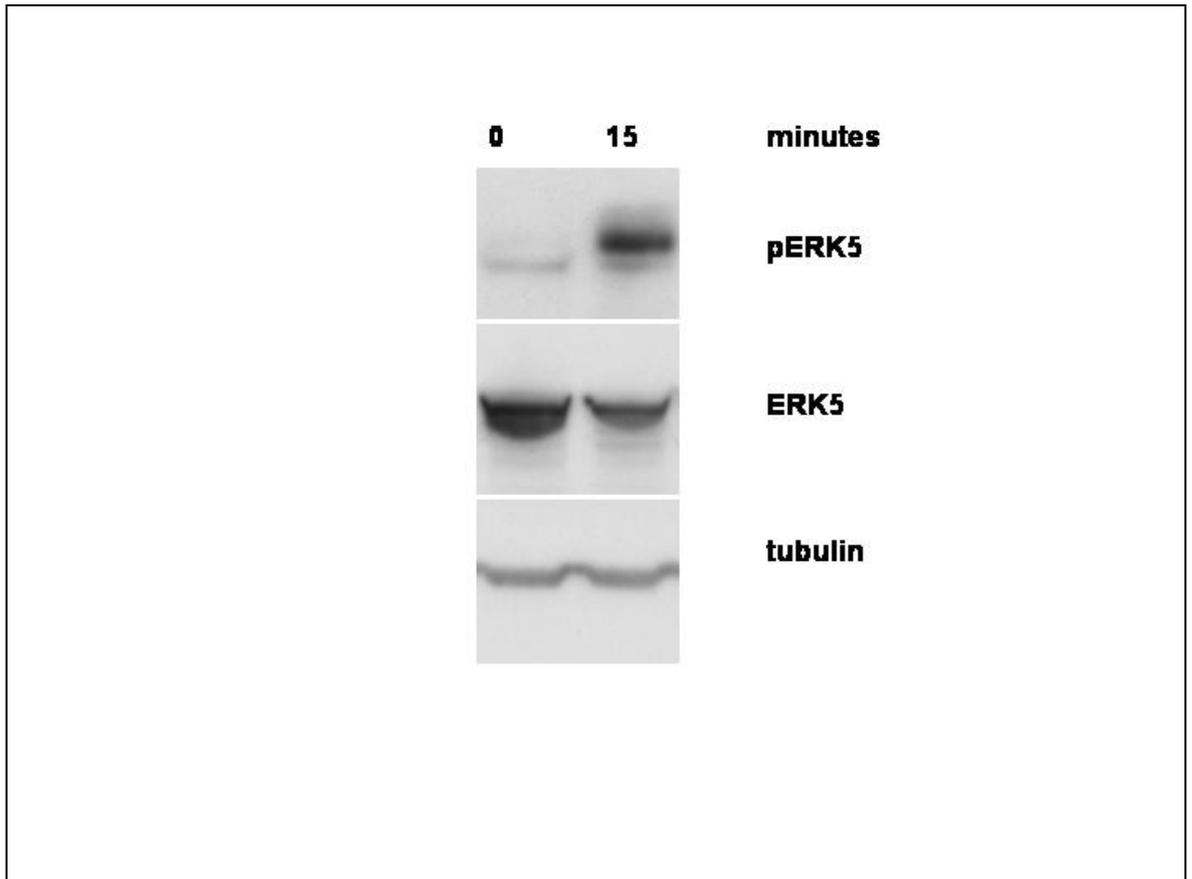
FIGURE 5.15 Clear positive matches from EGF stimulation experiment

Figure 5.15 and 5.16. Summary of proteins identified from MS study on EGF stimulation experiment   Studied proteins were those with recruitment at 15 minutes following EGF stimulation (~2 fold or more) as indicated by signal (peak height) from individual peptide hits in MS analysis. Data from experiments 1 and 2 are presented to show the respective fold of recruitment to ERK5. Identified proteins with a large number of non-redundant peptides and a high recruitment ratio are considered to be 'clear positive' and are listed in figure 5.15. Other identified proteins with only a medium number of non-redundant matches are considered as 'potential hits' and are listed in figure 5.16.

	Experiment 1	Experiment 2
Heterogeneous nuclear ribonucleoprotein D0	8.30	5.10
Stress-induced-phosphoprotein 1	5.62	3.62
Proliferation-associated protein 2G4	6.18	2.92
Heterogeneous nuclear ribonucleoprotein K	4.81	4.08
Caprin-1	4.42	3.42
ATP-dependent DNA helicase 2 subunit 1	4.67	3.15
Poly [ADP-ribose] polymerase 1	4.58	3.25
Actin, cytoplasmic 1	3.58	4.12
Heat shock 70 kDa protein 4	4.29	3.31
Nucleolin	4.27	3.04
Probable ATP-dependent RNA helicase DDX17	4.08	2.70
ATP-dependent RNA helicase DDX3X	3.54	3.14
ATP-dependent DNA helicase 2 subunit 2	4.10	2.40
Eukaryotic translation initiation factor 4B	2.97	3.42
Putative elongation factor 1-alpha-like 3	3.44	2.94
ATP-dependent RNA helicase A	3.37	2.88
Elongation factor 1-gamma	3.41	2.39

FIGURE 5.16 Potential positive matches from EGF stimulation experiment

	Experiment 1	Experiment 2
RNA-binding protein EWS	7.19	2.83
ELKS/RAB6-interacting/CAST family member 1	0	4.50
Heterogeneous nuclear ribonucleoproteins A2/B1	1.99	6.13
Nuclease-sensitive element-binding protein 1	3.76	0
KH domain-containing, RNA-binding, signal transduction-associated protein 1	0	3.57
Structural maintenance of chromosomes protein 1A	3.57	0
Spliceosome RNA helicase BAT1	3.42	0
Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1	0	3.01
Heterogeneous nuclear ribonucleoprotein M	2.80	3.12
Eukaryotic translation initiation factor 2 subunit 1	0	2.83
Stress-70 protein, mitochondrial	3.18	2.48
Insulin-like growth factor 2 mRNA-binding protein 3	2.93	2.68
Polypyrimidine tract-binding protein 1	2.32	3.15
Insulin-like growth factor 2 mRNA-binding protein 1	3.10	2.34
Probable ATP-dependent RNA helicase DDX5	3.12	2.31
Eukaryotic translation initiation factor 3 subunit I	2.77	2.58
Eukaryotic initiation factor 4A-I	2.67	0
60S ribosomal protein L3	2.62	0
Kappa-actin	2.62	0
40S ribosomal protein S3	2.19	2.97
Elongation factor 2	2.75	2.41
Heterogeneous nuclear ribonucleoprotein R	2.74	2.36
Heat shock protein 105 kDa	2.71	2.38
60 kDa heat shock protein, mitochondrial	2.97	1.98
Polyadenylate-binding protein 4	3.23	1.71
Heterogeneous nuclear ribonucleoprotein U	2.65	2.16
Eukaryotic translation initiation factor 3 subunit B	2.83	1.92
78 kDa glucose-regulated protein	2.41	2.26
Transitional endoplasmic reticulum ATPase	2.42	2.22
Trifunctional enzyme subunit alpha, mitochondrial	2.30	0
Nucleolar RNA helicase 2	2.67	1.81
Heat shock 70 kDa protein 4L	1.82	2.66
Interleukin enhancer-binding factor 3	2.21	2.23
FACT complex subunit SSRP1	2.22	0
Polyadenylate-binding protein 1	2.66	1.76
Histone H1.4	1.95	2.46
Eukaryotic translation initiation factor 3 subunit A	2.24	2.14
Inosine-5'-monophosphate dehydrogenase 2	2.58	1.78
26S proteasome non-ATPase regulatory subunit 11	2.23	1.99
T-complex protein 1 subunit alpha	2.50	1.64
Heterogeneous nuclear ribonucleoprotein H	2.06	2.06
Clathrin heavy chain 1	2.26	1.75
Glyceraldehyde-3-phosphate dehydrogenase	1.29	2.71
26S proteasome non-ATPase regulatory subunit 2	2.24	1.74
DNA replication licensing factor MCM3	2.01	1.95
Eukaryotic translation initiation factor 3 subunit E	1.97	0
T-complex protein 1 subunit theta	2.35	1.58

5.3   Discussion

Endogenous protein immunoprecipatation is the method of choice when analysing protein interactions as it allows the most accurate analysis of events in the cell line chosen. Unfortunately in our experiments as is often the case inadequate amounts of endogenous protein were available despite numerous efforts to increase sample size.

As an alternative to endogenous pulldown experiments, exogenous tagged cell lines can be used to facilitate a larger volume of protein complexes for analysis. We used FLAG-tag epitope labelling, which is one of the most commonly used tags for immunoprecipation experiments.

My experiments highlight some potential interacting proteins however these are very early results and future work is required to validate potential interacting proteins. Once these were confirmed their functional significance could be explored. The main aim of this section of my work was to develop the methodology required for future ERK5 proteomics work.

HSP 90 is part of a chaperone complex for multiple proteins involved in cell signalling, proliferation and survival such as HER2, RAF and Src (221). Previously it was thought that HSP90 was not involved in MAP kinase activities however recent evidence published suggests that ERK5 may be HSP90 dependent (220). ERK5 kinase activity was abolished by HSP90 inhibition *in vitro* and ERK5 activity was lost when examined in a mutant HSP90 yeast strain.

Inhibition of HSP90 is currently the focus of a number of phase I and II clinical trials. As mentioned previously 17-AAG, a derivative of geldanamycin, is being investigated as an anti-cancer drug however initial results have shown no positive response following treatment (222).

Our results support the theory that HSP90 does associate with ERK5 however contrary to Truman et al our results show that it is not involved in ERK5 activation. The initial SILAC experiment performed with FLAG tag empty vector and ERK5 highlighted strong association between ERK5 and HSP90. In the subsequent experiment with EGF stimulated FLAG tag ERK5 cells, HSP90 was confirmed to be present in both the unstimulated and stimulated cells in equal proportions. This indicates that it is not involved in the activation of ERK5 in these cells.

6   Discussion

6.1   Overview of project and summary of results

Abnormal MEK5/ERK5 signalling has been shown to be important in prostate cancer and is thought to represent a potential target in for novel therapies in invasive disease (184). The primary aim of this study was to validate previous expression data in human prostate cancer specimens and to investigate the effect of ERK5 inhibition both functionally and biochemically in prostate cancer cancer cell lines.

As previous work has shown ERK5 to be overexpressed in CRPC (184) experiments in my project were performed using the PC3 cell line which is androgen independent aswell as the PC3-ERK5 overexpressing clone. In order to validate these results repeat experiments using an androgen resistant cell line should have been performed.

Cellular proliferation, motility and invasion was significantly reduced ($p<0.005$) when ERK5 was knocked down using siRNA. Previous expression analysis performed by our group showed upregulated ERK5 expression in primary human prostate cancer specimens (184). In this study, these results were validated and in addition high levels of cytoplasmic (55%) and nucleur (73%) immunoreactivity was also shown in a range of metastatic prostate tumours (n=11).

Potential 'cross-talk' between ERK5 and ERK1/2 signalling was investigated in this study using siRNA for each individual isoform of ERK1/2. My results showed that ERK1 knockdown resulted in increased ERK5 activation in addition to prolonged ERK2 phosphorylation. Proliferation studies were also performed in PC3 cells, the results of which support published data that ERK1 acts as a negative regulator and ERK2 as a positive regulator of cell proliferation.

The final aim of my project was to develop a methodology to investigate the ERK5 interacting proteome. In order to do this a stepwise approach was adopted to first identify the correct antibody: bead ratio and optimal cell line to use. As discussed in Chapter 5, I was unable to pulldown sufficient levels of endogenous ERK5 for immunoprecipation and therefore exogenous levels were examined. A number of potential interacting proteins were identified however these preliminary results require validation by repeat experiments and further investigation.

A more detailed discussion of results is included at the end of each of the results chapters (3-5). The remainder of this chapter discusses ERK5 related biology and drug development as well as the potential role of an ERK5 inhibitor in clinical practice.

6.2 Drug development against implicated biological targets

Previous clinical trials designed to evaluate novel agents (biological or small molecules) were frequently allocating patients to treatment regimes under investigation without prior analysis of gene expression and/or genotype. High throughput gene expression profiling can be used to assess gene expression signatures from individual tumours. Such methods of molecular profiling may offer an opportunity to link the oncogenic process with potential therapeutic strategies in the context of improved patient outcome (223). Such a global approach will hopefully shed light on how individual biological cascades, including various signalling networks, interact to drive prostate carcinogenesis – this will have important implications in drug development.

Another crucial aspect of drug development is the availability of biomarker(s) for a number of purposes, including their use in risk assessment, diagnosis - prognostic, stratification and therapy monitoring. It is relevant to highlight the following requirements for drug development: (1) Robust method for *in vivo* assessment of quantifiable effects on the target of interest by the candidate agents is important, (2) Patient selection and stratification as guided by biologically relevant biomarker(s) will provide strong rationale for design of future clinical trials, (3) Clinical effects of the novel treatment on prostate cancer growth and progression will be assessed by the appropriate (surrogate) end points. Studies using many of the novel agents discussed in Chapter 1 (Sections 1.6 and 1.7) have attempted to identify markers as readouts for target inhibition or downstream signalling to guide patient selection and follow up e.g. pEGFR and pMAPK with EGFR inhibitors. Repeat tissue sampling however is not always feasible; more acceptable methods of sampling such as serum or urine would be preferable and may provide useful alternatives. Hence, there is an urgent need for validated urine or serum markers to evaluate the status of target of interest as well as the tumour as a whole. Serum PSA is currently used as a biomarker in prostate cancer. Its increasing use in recent years has facilitated early diagnosis of prostate cancer, reflected by a global rise in the incidence of prostate cancer since the 1990s. PSA measurement has a number of drawbacks as it can also be elevated due to BPH or prostatitis leading to a significant number of false positive results. There is a need for more specific biomarkers for prostate cancer, which can be used alone or in conjunction with PSA. A number of biomarkers (serum and urine) are currently in trial. (224). Prostate cancer antigen 3 (PCA3) is a gene which is overexpressed in prostate cancer and has been shown to have a higher specificity than PSA for malignancy (225). It should be measured in the first-catch urine after prostatic massage and has been shown to be useful in patients to predict the presence of malignancy in men undergoing repeat

prostate biopsies (226). Currently it is not funded for use in the National Health Service in the UK.

Epigenetic changes have also been studied as both diagnostic and prognostic tools in prostate cancer. It is apparent that genetically identical tumours have differing phenotypes due to altered epigenetic arrangements, which encompass a wide range of abnormalities at cell and molecular level, including post-translational modification (e.g. methylation, acetylation, ubiquitination, sumoylation), gene silencing (e.g. promoter hyper-methylation) and growth arrest (e.g. senescence). Hence, analysis of such assays for methylation changes or histone modifications in individual tumours has the potential to allow clinicians to predict patient prognosis and subsequent treatment stratification or direct targeted therapy can be considered.

Targeted therapy in haematological cancers has proved more amenable than in solid tumours. Haematological malignancies often arise from specific genetic mutations, e.g. Philadelphia chromosome with specific oncogenic translocation in *bcl-abl*, offering single molecular candidates for drug targeting. In contrast solid tumours tend to be heterozygous and/or multi-focal involving multiple pathways. Hence, the identification of the 'single' molecular target is much more difficult in solid tumours. Indeed, for the vast majority of clinical solid tumour types, the knowledge of a single genetic or epigenetic lesion that critically drives carcinogenesis is missing. In prostate cancer however recent studies have shown the majority of patients have a chromosomal rearrangement that fuses the gene for an androgen-regulated prostate-specific serine protease, *TMPRSS2*, with a member of the ETS family of transcription factors, most commonly *ERG* (227). The clinical implications of the fusion products remains unclear however it is anticipated that once this is understood *TMPRSS2:ERG* gene fusions may be used as a screening test or molecular target for novel therapies.

Targeting a single molecule or pathway therefore may not be sufficient to significantly influence the malignant phenotype as signalling through other pathways may compensate for the effects of a single target affected by treatment. Different pharmacological strategies have therefore been pursued to inhibit multiple pathways or multiple steps within the same pathway for use in advanced solid tumours. Multi targeted agents or a combination of single targeted drugs may maximise effective target inhibition and have a complementary impact on downstream signalling.

Sorafenib is a multi targeted TKI which is approved for clinical use in RCC (110). Sorafenib blocks receptor tyrosine kinase signalling (VEGFR, PDGFR, c-Kit and b-RAF)

and inhibits downstream signalling through the MAPK pathway preventing tumour growth by anti-angiogenic, anti-proliferative and /or pro-apoptotic effects. Other novel muti-target inhibitors, which have been developed, include sunitinib, imatinib and lapatinib. Multi targeted TKIs avoids drug-drug interactions and better compliance may be achieved with administration a single compound. On the other hand, combining specific TKIs may increase treatment efficacy with exact titration for each agent allowing optimal target inhibition. However, drug-drug interactions may potentially lead to altered responses. Another method, which may increase antitumour activity, is to combine different classes of inhibitors e.g. a monoclonal antibody and a TKI against the same single target. *In vitro* and *in vivo* evidence has shown that combining monoclonal antibody therapy and TKI to target different molecular domains of the same receptor can potentiate cellular toxicity due to non-overlapping mechanisms of action and partially overcome acquired resistance to any single inhibitor. A number of trials are currently studying the potential effects of combination regimes of targeted therapies with docetaxel. Docetaxel improves survival in patients with hormone refractory disease by a mean of only 2.5 months (9;10). Minimum survival improvements in this patient group would therefore convey significant results compared to docetaxel monotherapy, which is currently the only effective treatment option available.

Understanding of the signalling pathways involved in prostate carcinogenesis has lead to the development of a number of potential new drugs with many reaching clinical trials. To date none of the targeted therapy have shown adequate efficacy for approval for routine usage. The discrepancy between preclinical and clinical findings may be a reflection of the fundamental differences between the currently available preclinical (usually murine) models and clinical cancer. Current research on the development of a mouse model of prostate cancer focuses on using the Cre-loxP technology which would provide a conditional gene-knockout model. This method is a more favourable model to study than traditional single or multi- gene knockout or knock-in models as it overcomes the problems of embryonic lethality, premature death or concerns that the cancer develops due to developmental defects (228). Development of a relevant prostate cancer transgenic mouse model driven by validated molecular lesions may provide a meaningful model for clinical prostate cancer and may allow more clinically relevant assessment of novel anti-cancer treatments prior to clinical trial.

Several promising targets and agents are continuing to emerge and it is imperative that multi-disciplinary teams incorporating urological surgeons, oncologists and laboratory scientists are involved in bringing these novel treatments to clinical trial.

6.3 ERK5 as a drug target

6.3.1 Which tumour types and which patient groups would be suitable for ERK5 inhibition?

Aberrant ERK5 expression has been demonstrated in a range of tumour tissue types, however the most significant data focuses on prostate and breast cancer.

Based on data obtained by my study and previous data from our group, both metastatic and hormone (castrate) resistant prostate cancer have upregulated ERK5 expression. These data would argue for benefit for ERK5 targeted therapy in prostate cancer. When a clinical candidate for ERK5 inihibitor is made available, the patient cohort with either metastatic or castration resistant disease would be suitable for recruitment to a clinical trial. The presence of ERK5 overexpression in tumour/metastatic tissue may indicate which patients should be targeted with an ERK5 inhibitor. Patients would require repeat biopsies to assess ERK5 expression levels if therapy was to be commenced once castration resistant or metastatic disease developed.

MEK5/ERK5 signalling has been shown to also be important in breast carcinoma. High levels of ErbB2, a member of the ErbB family of tyrosine kinases, correlates with aggressive growth properties of breast cancer and is found in 20-30% of patients (29). It has been shown that the MEK5/ERK5 pathway is constitutively activated in breast cancer cells overexpressing ErbB2 and that down regulation of this pathway significantly reduces the malignant growth of these cells (215). ERK5 resides in the nucleus of cells that overexpress the ErbB2 receptor and recent work has shown that nuclear ERK5 favours MEF-2 dependant transcriptional activity and inhibits TRAIL (tumour necrosis factor related apoptosis)-induced cell death (229).

Apoptosis is the primary route of cytotoxicity by many forms of anti-cancer therapy. Chemoresistance is a major therapeutic problem, allowing tumour cells the capability to progress and grow further resulting in a more aggressive metastatic phenotype. Recent *in vitro* studies have highlighted the importance of the MEK5/ERK5 pathway in mediating breast cancer cells sensitivity to apoptotic inducing events (230). Overexpression of MEK5 in APO- MCF7 cells suggests that this MAPK member may represent a significant potent survival molecule. Further work will confirm if inhibition of this signalling pathway could promote for sensitising breast cancer cells to chemotherapeutic regimens and if successful this may be of significance in chemoresistant prostate cancer.

6.3.2   How would an ERK5 inhibitor be used: single agent or in combination?

Many issues arise when developing targeted therapies for use in cancer.   A selective small molecule inhibitor of ERK5 would most likely be trialled initially in advanced prostate cancer patients in combination with docetaxel.   Docetaxel is currently the recommended treatment for men with metastatic CRPC.   Clinical trials for this patient group would therefore focus on comparing current recommended therapy against and in combination with the novel therapy under review.   As ERK5 has been shown to be overexpressed in castration resistant disease it would most likely also be used in clinical trials involving castration refractory cases either alone or in combination with other targeted therapies in order to tackle the heterogeneity of the disease.

Crosstalk between ERK5 and ERK1/2 is a focus of current research and is discussed in Chapter 4.   Combining ERK5 inhibition with a MEK1/2 inhibitor may provide a more significant effect than single agent use.   By using combination therapy it may be that a lower dose of the MEK inhibitor could be used, increasing patient tolerability.

6.3.3   Downstream biomarkers for ERK5 signalling

A number of substrates of ERK5 are recognised however to date there is no specific and reliable downstream biomarker.   Further work is required to identify a downstream biomarker of which could be measured following treatment with an ERK5 inhibitor.   Assessment of downstream targets of ERK5 was not studied in detail in this project however is recognised as an essential area which requires further work.

6.3.4   Why would an ERK5 inhibitor work when MEK1/2 inhibitor in the past have not worked?

The MEK/ERK signalling pathway plays a central role in the regulation of many of the cellular processes involved in carcinogenesis.   A number of MEK 1/2 inhibitors have entered phase I and II clinical trials.   However, these selective inhibitors have shown limited clinical effects in cancer patients with significant associated toxicities.   The first MEK 1/2 inhibitor to enter clinical trial was PD-184352 (also known as CI-1040).   One partial response in a pancreatic cancer patient was reported with 25% of patients achieving disease stabilization for 4 months or longer (231).   These results supported progression into a broad phase II study however no objective responses were documented.   Pharmacokinetic studies showed up to 100-fold variation in drug exposure in different patients and the lack of efficacy in this phase II trial was therefore attributed to poor drug

bioavailability (214). Second generation MEK inhibitors (PD0325901 and ARRY-142886) were developed with reported superior potency and pharmacologic properties. Phase II trials of PD0325901 were abandoned due to severe toxicity. This MEK inhibitor has very high potency and it is thought that these severe side effects suggested that complete inhibition of this pathway in normal tissues were not desirable. ARRY-142886 (AZD6244) however has shown more promising results with a lower incidence of reported toxicities and phase II trials are currently recruiting patients (232).

The main issue, which raises concerns about the use of MEK1/2 inhibitors in cancer, is that MEK cannot be inhibited consistently in tumours at tolerable inhibitor doses. It also remains unclear whether successful inhibition correlates with clinical outcome. MEK inhibitors have been shown to have heightened sensitivity in tumours with B-Raf mutation and *in vitro* data shows that an activating RAS mutation showed much lower and more variable sensitivity. Analysis of the tumour gene expression signature may therefore be useful to predict pathway activation and suitable patient selection.

It may be that targeting the MEK/ERK5 pathway would not have the same toxicity profile due to their selective target inhibition. Initial in vitro studies of the MEK5 inhibitor BIX02188 and BIX02189 showed inhibition of ERK5 phosphorylation in a dose dependant manner without affecting the phosphorylation of ERK1/2 (211).

6.4 ERK5 related biology

Details of ERK5 mediated carcinogenesis remains to be fully elucidated. It is likely that it plays key roles in cellular migration/invasion, survival (including chemoresistance), and angiogensis (186;187;230). My work on the ERK5 interacting proteome has provided a wealth of data on how ERK5 may function and the signalling partners involved. Future validation will need to be performed and their functional significance in ERK5 driven phenotype tested. It is particularly interesting to observe the binding of a number of RNA binding proteins to ERK5. This raised the possibility that ERK5 complexed with RNA binding proteins may be important in the processing of RNA species. Hence, future work in this topic and additional validation experiments of the ERK5 interacting proteome may yield key insight into the biology of ERK5 mediated signalling which may in turn provide hints for important downstream biomarkers that may be exploited as 'read-outs' for the functional state of ERK5 activities. This will be very useful in the development and evaluation of novel ERK5 inhibitors.

6.5   ERK5 signalling interactions

Increased understanding of ERK5 signalling is required. A number of activators of the ERK5 pathway have been identified including growth factors, tyrosine kinases and oncogene products such as Ras and COT (See section 1.8.1) however downstream targets of ERK5 are less well understood.

ERK5 has been implicated in NFκB signalling which has itself been reported to play a role in prostate carcinogenesis. To date there is no published data on their interaction in prostate carcinogenesis and therefore I investigated if ERK5 mediated signalling in prostate cancer cells involved the NFκB pathway. Contrary to previous studies, NFκB levels in both *in vitro* and *in vivo* experiments did not show any correlation with ERK5 expression in this study.

ERK5 and ERK 1/2 have recognised similarities in their activation modes and functions and crosstalk between these MAPK family members has been suggested (182;183). This study is the first to demonstrate a novel relationship between ERK1 and ERK5 signalling in prostate cancer. Such interaction may have clinical implications, and future trials of novel compounds will need to incorporate such cross talk into the design to ensure the *in vitro* and *in vivo* mode of action of any candidate compounds are adequately examined.

6.6   Conclusion

This study validates the importance of the ERK5 signalling pathway as a potential target for therapy and highlights a novel functional and biochemical relationship between ERK1 and ERK5 signalling. A suitable, reliable downstream biomarker is essential for the development of an ERK5 inhibitor and although this study does not highlight such a specific target it does provide useful preliminary results for further work in this area.

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Appendix: Publications

Publications

McCracken SR, Ramsay A, Heer R, Mathers ME, Jenkins BL, Edwards J et al. Aberrant expression of extracellular signal-regulated kinase 5 in human prostate cancer. Oncogene 2008; 27(21):2978-2988. doi:10.1038/sj.onc.1210963
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Ramsay AK, Leung HY (2009) Signalling pathways in prostate carcinogenesis: potentials for molecular-targeted therapy *Clinical Science*, **117** 209-228.
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