THE ROLE OF PTPRK AND PTPRM IN PROSTATE AND BREAST CANCER

by

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Summary

Protein tyrosine phosphatases (PTPs) have been identified that mediate a range of physiological and pathological processes, such as proliferation and tumour metastasis. PTPRK and PTPRM belong to the same subfamily of PTPs. This study aims to investigate the role of PTPRK and PTPRM in cancer development and progression.

Knockdown of PTPRK expression was performed in PC-3 and DU-145 cells. Functional assays were then carried out on these cells in order to determine any changes in their biological properties. Knockdown of PTPRK significantly reduced the growth and adhesion of both PC-3 and DU-145 cells. The experimental results suggested that reduction of cell growth is potential involvement of p53 and/or caspase-3 and -8 and its up-stream molecule JNK.

The decreased expression of PTPRK and PTPRM are associated with poor prognosis and reduced survival. Knockdown of PTPRK resulted in increased adhesive and invasive abilities, and promoted cell proliferation and motility of breast cancer cells. Moreover, PTPRM knockdown resulted in elevated adhesion, invasion, and proliferation of breast cancer cells. Activation of ERK and JNK by tyrosine phosphorylation and consequent elevated MMP9 activity is involved in increased cell migration and invasion by PTPRM knockdown.

These results suggested that PTPRK and PTPRM are involved in the disease progression of prostate and breast cancer by regulating a complex network of pathways and molecules. This provides further proof of the importance of the R2B subfamily, a subgroup of PTP superfamily, in cancer. In addition, it sheds some light on the use of PTPs as prognostic indicators of disease, aiding in diagnosis and treatment. The major effect is the promotion of motility and invasiveness of cancer cells via ERK and JNK pathways. However it can also impair the apoptosis mediated by JNK pathways in certain cancer cells, such as prostate cancer cells. Such contrasting effects on survival and motility require further investigation, and should also be considered when treating cancers by targeting these molecules.

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Abbreviations

APS:ammonium persulphate
ASK1: apoptosis signal-regulating kinase 1
ATF-2: activating transcription factor 2
BCC: basal cell carcinoma
BCCIP: BRCA2 and CDKN1A-interacting protein
BMPs: bone morphogenetic proteins
BSA:bovine serum albumin
CDKs: cyclin-dependent kinases
Co-SMADs: co-operating SMADs
DAB:Diaminobenzidine
DEPC:Diethyl Pryoncarbonate
DMEM:Dulbecco's Modified Eagle's medium
DMSO:Dimethylsulphoxide
EBNA1: Epstein-Barr nuclear antigen-1
EBV: Epstein-Barr virus
ECM: extracellular matrix
EDEM:ER degradation-enhancedα-mannosidase protein
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
EMT: epithelial-mesenchymal transition
ERK: extracellular signal-regulated kinase
FAK: focal adhesion kinase
FBS:foetal bovine serum
GAPDH:glyceraldehyde 3-phosphate dehydrogenase
GDFs: growth and differentiation factors
GnT-V:a substrate of N-Acetylglucosaminyltransferase V
GRB2:Growth Factor Receptor-bound protein 2
GSTM1: glutathione S-transferase Mu 1
GSK-3β: glycogen synthase kinase-3β
HMGA2: High mobility group protein HMGI-C 2
HRP:horseradish peroxidise
IL-6: interleukin-6
INF-γ: interferon-γ
IP:Immunoprecipitation
IQGAP1:RasGTPase-activating-like protein IQGAP1

IRE1:inositol-requiring kinase 1
I-SMADs: inhibitory SMADs
JMML: juvenile myelomonocytic leukaemia
KLK4: kallikrein-related peptidase 4
Lef :lymphoid enhancer factor
MAM:Meprin/A5/ PTPmu
MAPK: mitogen-activated protein kinase
MDM2: E3 ubiquitin-protein ligase Mdm2
MEK3: MAPK/ERK kinase 3
MH1: Mad Homology 1
MIF: Mullerian inhibitory factor
MK2: MAPK-activated kinase 2; also known as MAPK2
MMP: matrix metalloproteinase
MNK1: MAP kinase-interacting serine/threonine kinase 1
MSK1: mitogen- and stress-activated protein kinase 1
OB: osteoblast
PAR-1: protease-activated receptor 1
PCR:Polymerase chain reaction
PDEF: prostate-derived ETS factor
PI:Propidium Iodide
PI3K:phosphatidylinositol 3-kinase
PKC: protein kinase C
PLCγ1: phospholipase C γ 1
pRb: retinoblastoma protein
PS:Phosphatidylserine
PSA: prostate specific antigen
PTEN:phosphatase with tensin homology
PTHrP: parathyroid hormone-related protein
PTKR: receptor protein-tyrosine kinase
PTP: protein tyrosine phosphatase
ROS: reactive oxygen species
RSK: ribosomal S6 kinase
R-SMADs: receptor-associated SMADs
SARA: SMAD anchor for receptor activation
SCC: squamous cell carcinoma
SDS-PAGE:Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SFK:Src family kinase

Snai1/2: Zinc finger protein Snai 1/2
TβRI:TGF-β receptor type I
Tcf :T-cell-specific transcription factor 1
TGF-α:transforming growth factor α
TGFβ: transforming growth factor β
TGFβR: transforming growth factor β receptor
TIEG1: TGF-β-inducible early-response gene
TNF: tumour necrosis factor
TNFα: tumor necrosis factor α
TNFs:Tumour necrosis factors
TRAIL: TNF-related apoptosis-inducing ligan
uPA: urokinas-type plasminogen activator
VEGF: vascular endothelial growth factor
WFDC-1: WAP-type four disulfide core/ps20 proteins
XBP-1:X-box-binding protein 1
ZEB1/2: Zinc finger E-box-binding homeobox 1/2
ZO1: zonaoccludens 1

Chapter 1

Introduction

1.1 Prostate and breast cancer

1.1.1 Prostate cancer

Prostate cancer is the most commonly diagnosed cancer in men in the UK. Approximately a quarter of new cancer cases diagnosed in men are prostate cancer. Several risk factors have been recognised in the development of prostate cancer, the most significant of which include; age, race, diet, family history and place of birth (Wynder *et al.,* 1971).

Around 95% of all prostate cancers are adenocarcinomas and the other 5% of prostate cancers are ductal adenocarcinoma, mucinous carcinoma, and signet ring carcinoma. These rarer prostate cancers are sometimes more difficult to distinguish between different types and they are usually less responsive to hormonal therapy (McWilliam *et al.,* 1997). The most significant histological variant is the neuroendocrine prostate cancer, which is generally classified as either small cell carcinoma or a carcinoid tumour, and represents less than 2% of prostate cancer cases (Grignon 2004).

Prostate cancer is graded by different methods such as the Gleason grading system and TNM (Tumour odal Metastasis) staging system. The Gleason grading system is the most common system for prostate cancer and was first described by Gleason and Mellinger in 1974. The grading is based on the histological pattern of the distribution and growth of the tumour cells in the prostatic stroma, and the degree of glandular differentiation in H&E stained prostatic tissue samples from either a biopsy, or if after radical prostatectomy, a whole prostate (Gleason and Mellinger 1974). The various growth patterns (Figure 1.1) are combined to form 5 basic grade patterns ranging from

1 (least aggressive) to 5 (most aggressive). A histological score ranging from 2-10 is then generated, by the addition of the primary grade pattern; the prevailing one in that area, to the secondary pattern, which is the subsequently most widespread pattern. However, if only one grade is present, or if the second grade covers less than 3% of the total tumour, its value is doubled in order to give a corresponding Gleason score.

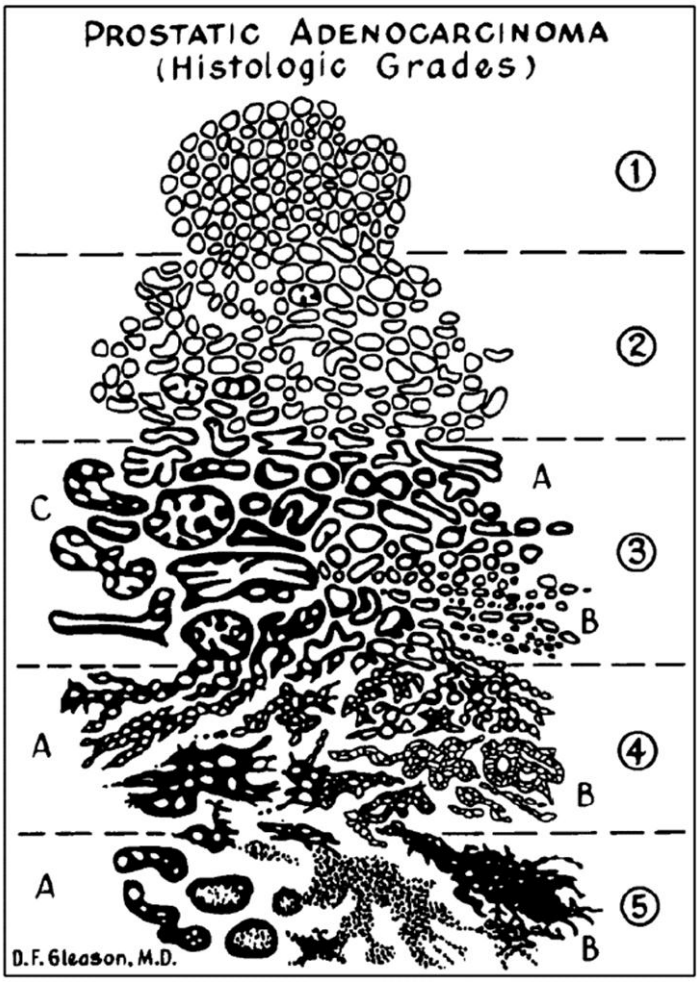


Figure 1.1 Schematic drawing of Gleason histological grading. Taken from (Humphrey *at al*., 2004).

1.1.1.1 Prostate cancer treatment

There are many treatment mehods for prostate cancer including radical prostatectomy, radiation therapy, androgen ablation therapy and chemotherapy. Radical prostatectomy (complete surgical removal of the prostate) is usually carried out on patients with either early stage disease or those who have failed to respond to radiation therapy. It has also been indicated that it is beneficial for patients, who with locally advanced disease, to treat with adjuvant radiation therapy. In contrast, the operation is rarely recommended for elder men with a life expectancy of less than 10 years, because it proves to offer little benefit and can impose significant complications (Bill-Axelson *et al.*, 2005).

Radiation therapy is commonly used to treat all stages of prostate cancer or when surgery has failed. The method uses ionising radiation to damage cellular DNA, and although this occurs in both normal and cancer cells, the cancer cells are less capable of repairing the damage and therefore undergo apoptosis. There are two methods for radiotherapy, external-beam radiotherapy and brachytherapy. External-beam radiotherapy was developed by Bagshaw and colleagues (Bagshaw *et al.*, 1969). They instigated the use of megavoltage linear accelerators as a means of directing a beam of high dose radiation towards the prostate, with lower doses being applied to the surrounding tissue. Possible side effects include inflammation of the bladder, rectum, and urethra, as well diarrhoea and dysuria (Bagshaw *et al.*, 1977). Brachytherapy was introduced by Holm and colleagues (Holm *et al.*, 1983). The procedure involves the insertion of approximately 100 small radioactive rods directly into predetermined positions within the tumour. The patient is placed in the dorsal lithomy position, and remains under general or spinal anaesthesia throughout the whole procedure. The

radioactive source of the rods can either be of the low-dose rate using Iodine-125 or palladium-103 as the source of energy, or as a high-dose rate with Iridium-192; uurrently, LDR is the most commonly used form of brachytherapy.

Androgen ablation therapy of prostate cacner is a way to treat advanced and metastatic prostate cancer due to androgens is important for the cell survival and growth of prostate cancer cells. However, androgen therapy is rarely curative. This is due to the androgen insensitive cells that are present within the tumour, and although initial therapy will reduce tumour size, prolonged therapy can act as a selective pressure for the androgen independent cancer cells to thrive. These cells can then induce re-growth of a tumour which will no longer respond to hormonal therapy (Robson and Dawson 1996).

Chemotherapy is for hormone-refractory prostate cancers. These drugs are cytotoxic, they lead to kill both cancer and normal cells, resulting in considerable toxicity and undesirable side effects. However, scientists have found several newer combination therapies with lower toxicity have been developed. These drugs include Estramustine, Vincristine, Etoposide, Doxorubicin, and the taxanes, Paclitaxel and Docetaxel (Hudes *et al.,* 1997). Other drugs such as Mitoxantrone in combination with corticosteroid are mainly used as a means of palliative treatment in patients with metastatic prostate cancer (Tannock *et al.,* 1996).

1.1.2 Breast cancer

Breast cancer, the same with prostate cacner, is the most common cancer in the UK and the lifetime risk of being diagnosed with breast cancer is 1 in 8 for women in the UK. Several risk factors have been recognised in the development of breast cancer such as age, race, diet, family history and obesity (Wang *et al.,* 2013).

The most common type of breast cancer is ductal carcinoma (70-80%) followed by lobular carcinoma (10-15%) and other type of breast cancers including Inflammatory Breast Cancer and paget's disease of the breast. Paget's disease of the breast is first described by Sir James Paget in 1874 accounting less than 5% of all the breast cancer. It is usually associated with an underlying in situ or invasive ductal carcinoma (Karakas 2011).

Breast cancer is usually graded using TNM staging system. The TNM classification of the prostate was first established in the 1940s by Pierre Denoix, but was updated by the American Joint Committee on Cancer and International Union against Cancer (UICC), in 2002. The system is based on primary tumour (T), lymph node (N), and metastases (M) categories, with each one being built on a series of clinical examinations, radiological imaging, biopsies, and biochemical analyses (Greene and Sobin 2002). Clinical T staging is the most important prognostic factor for clinically localised breast cancer. Regional lymph node metastases meanwhile are strong predictors of progression, and imaging using nomograms is essential for paitents who have a greater risk of developing metastases. The staging is described in full detail in Table 1.1.

Stage characterisation	
Primary tumour (T)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma *in situ*; intraductal carcinoma, lobular carcinoma *in situ*, or Paget;s disease of the breast with no associated tumour
T1	Tumour 2.0 cm or less in greatest dimension
T1mic	Microinvasion 0.1 cm or less in greatest dimension
T1a	Tumour more than 0.1 but not more than 0.5 cm in greastest dimension
T1b	Tumour more than 0.5 but not more than 1.0 cm in greastest dimension
T1c	Tumour more than 1.0 but not more than 2.0 cm in greastest dimension
T2	Tumour more than 2.0 but not more than 5.0 cm in greastest dimension
T3	Tumour more than 5.0 cm in greastest dimension
T4	Tumour of any size with direct extension to chest wall or skin
T4a	Extension to chest wall
T4b	Edema or ulceration of the skin of the breast or satellite skin nodules confined to the same breast
T4c	Both T4a and T4b
T4d	Inflammatory carcinoma
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis to movable ipsilateral axillary lymph node(s)
N2	Metastasis to ipsilateral axillary lymph node(s) fixed to each other or to other structures
N3	Metastasis to ipsilateral internal mammary lymph node(s)
Pathologic classification (pN)	
pNX	Regional lymph nodes cannot be assessed
pN0	No regional lymph node metastasis
pN1	Metastasis to movable ipsilateral axillary lymph node(s)
pN1a	Only micrometastasis (none larger than 0.2 cm)
pN1b	Metastasis to lymph node(s), any larger than 0.2 cm
pN1bi	Metastasis in 1 to 3 lymph nodes, any more than 0.2 cm and less than 2.0 cm in greatest dimension
pN1bii	Metastasis ot 4 or more lymph nodes, any more than 0.2 cm and all less than 2.0 cm in greatest dimention
pN1biii	Extension of tumour beyond the capsule of a lymph node metastasis less than 2.0 cm in greatest dimension
pN1biv	Metastasis to a lymph node 2.0 cm or more in greatest dimension
pN2	Metastasis to ipsilateral axillary lymph node(s) fixed to each or to other structures
pN3	Metastasis to ipsilateral internal mammary lymph node(s)
Distant metastasis (M)	
MX	Presence of distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis present

Table 1.1 The TNM classification for breast cancer.

1.1.2.1 Breast cancer treatment

There are many treatment methods for breast cancer, basically are the same with prostate cancer treatments, including lumpectomy/ mastectomy, radiation therapy, hormone therapy and chemotherapy. Lumpectomy removes the tumor often along with lymph nodes in the armpit and it is suitable for treatment of small local breast cancer. Mastectomy includes total mastectomy and radical mastectomy. Total mastectomy involves removal of the whole breast and sometimes ipsilateral axillary lymph node and radical mastectomy removes the breast, chest muscles, all of the ipsilateral axillary lymph node and some additional fat and skin. Radiation therapy, includes external beam radiation (4-6 weeks) and brachytherapy (less common for breast caner treatment), is carried out following lumpectomy or mastectomy.

In general, paitents with hormone receptor-negative cancers respond better to chemotherapy than paitents with hormone receptor-positive cancers. Adjuvant chemotherapy is administered following surgery and before radiation therapy. It has been also indicated that delaying chemotherapy more than 12 weeks after surgery may increase the risk of breast cancer recurrence and reduce the survival (Lohrisch *et al.*, 2006). For early-statge breast cancer patients, there are many drugs including doxorubicin, cyclophosphamide, fluorouracil, paclitaxel and methotrexate for thay as a combination therapies. Other drugs such as gemcitabine in combination with paclitaxel or capecitabine in combination with docetaxel are mainly used as a means of treatment in patients with advanced metastatic breast cancer (Ferguson *et al.*, 2007; Watanabe 2013). Moreover, combination therapy of trastuzumab, lapatinib and pertuzumab is for HER2-positive breast cancer patients (Dent *et al.*, 2013).

Hormone therapy of breast caner is a way to prevent estrongen form stimulating breast cancer cell proliferation. It is used for patients whose breast cancers are hormone-receptor positive (either estrogen or progesterone receptors). There are three diffenet mechanisms to reach this goal; first of all, blocking estrogen receptors in cancer cells by tamoxifen; second, suppressing estrogen production in the body by aromatase inhibitors (Anastrazole, Exemestane or Letrozole); finally, destroying ovaries to produce estrogen by ovarian ablation (Chemical Ovarian Ablation or Bilateral Oophorectomy) (Coombes *et al.,* 2007; Rao and Cobleigh 2012).

1.2 Cancer metastasis

Cancer metastasis is responsible for most of the mortality of patients with solid tumours. During metastasis, cells from the primary tumour follow a number of seemingly separate but highly related steps in order to acquire the properties necessary for successful metastasis. First of all after breaching the basement membrane, cancer cells need to be able to migrate and invade into the local tissues, which usually involves the process of EMT (epithelial-mesenchymal transition). The cells then invade into blood and/or lymphatic vessels (intravasation), which requires degradation of the ECM (extracellular matrix). The cells need to survive in the circulation until they reach a site conducive to colonisation (homing). In most cases, there is a temporal gap called the metastatic latency between the initial organ infiltration stage and the formation of new secondary tumours at a distant site. During this latency, tumour cells may enter a dormancy stage (Figure 1.1) (Aguirre-Ghiso 2007; Nguyen *et al.,* 2009; Thiery *et al.,* 2009).

1.2.1 Sequential events during cancer metastasis

1.2.1.1 EMT

Cells from the primary tumour that are able to invade the surrounding tissues and disperse to produce metastases usually undergo EMT which provides epithelial cell plasticity. This process plays an important role during foetal and postnatal development and it is regulated by transcription factors such as Twist1, Snai1, and Slug, which inhibit E-cadherin (epithelial-cadherin) expression (Thiery *et al.*, 2009).

EMT contains essential features. First of all, cell polarity is reduced which includes the down-regulation of epithelial cell markers such as E-cadherin, ZO-1 (zonaoccludens-1), and β4-integrin, followed by reorganisation of cytoskeleton structures; following this, the expression of fibroblast markers and genes are up-regulated, such as vimentin, N-cadherin, Twist and α-SMA (α smooth muscle actin). Finally, there is an acquisition of stem cell-like properties and phenotype by cancer cells. The cells become isolated, motile and may also appear to be resistant to *anoikis*. Furthermore, cancer cells undergoing EMT have been found to show an increased resistance to apoptosis and some acquired traits reminiscent of those expressed by stem cells (Miyazono 2009; Xu *et al.*, 2009; Singh and Settleman 2010).

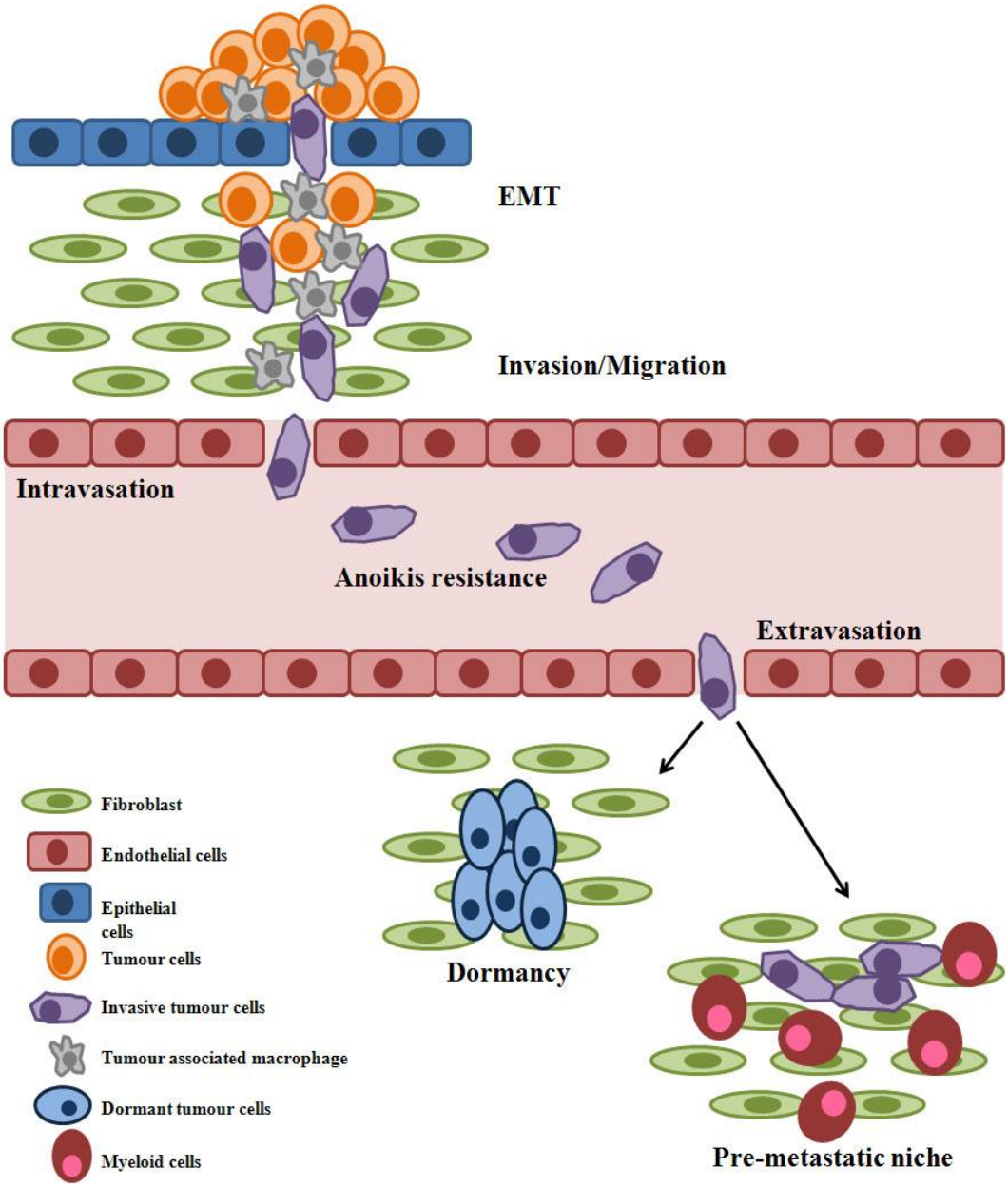


Figure 1.2 The process of metastasis is characterised by several steps; breaching of the basement membrane, EMT, invasion, intravasation, *anoikis* resistance, extravasation, and finally organ colonisation of a secondary site.

1.2.1.1.1 EMT in prostate cancer

EMT plays an important role in prostate cancer progression and expression levels of EMT markers are modulated. Decreased E-cadherin expression is correlated with disease progression and poor prognosis in prostate cancer, and is associated with tumour prognosis factors such as tumour grade, local invasiveness, dissemination into circulatory system and tumour relapse after radiotherapy. In contrast, markers of mesenchymal phenotype including N-cadherin, OB (osteoblast)-cadherin, and WFDC-1 (WAP-type four disulfide core/ps20 proteins) are all up-regulated in certain tumour cells (Tomita *et al.,* 2000; Loric *et al.,* 2001; Mason *et al.,* 2002; McAlhany *et al.,* 2004; Jaggi *et al.,* 2006; Ray *et al.,* 2006). Increased levels of the extracellular domain of N-cadherin have also been shown in the serum of prostate cancer patients (Derycke *et al.,* 2006). The decreased E-cadherin level in prostate cancer cells has been demonstrated to be associated with increased cell motility and protease expression (Chunthapong *et al.,* 2004; Derycke *et al.,* 2006). These changes of epithelial and mesenchymal markers and the loss of prostatic glandular structure are consistent with the general dedifferentiated phenotype of the aggressive prostate cancer cells.

Various factors from the local microenvironment in the prostate are involved in the EMT process, including factors generated from the tumours cells themselves and tumour associated stroma cells. For example, HGF (hepatocyte growth factor) and EGF (epithelial growth factor) can induce EMT in the DU-145 prostate cancer cell line; the action of EGF on E-cadherin is due to its immediate calveolae-dependant endocytosis of E-cadherin and a subsequent up-regulation of Snai1 which in turn represses the transcription of E-cadherin. Such effects can be diminished by blocking EGF signalling

(Lu *et al.,* 2003; Wells *et al.,* 2005; Yates *et al.,* 2007). The transcription factor Twist, represses E-cadherin expression and the up-regulates of N-cadherin levels in prostate cancer cell lines (Kwok *et al.,* 2005; Alexander *et al.,* 2006). Moreover, loss of the epithelium-specific transcription factor, PDEF (prostate-derived ETS factor) which is down-regulated by TGFβ (Transforming growth factor β), induces EMT in PC-3 cells (Gu *et al.,* 2007). It has been further demonstrated that over-expression of PSA (prostate specific antigen) and KLK4 (kallikrein-related peptidase 4), both potential activators of pro-EGF and latent TGFβ2, leads to EMT in PC3 cells (Veveris-Lowe *et al.,* 2005; Whitbread *et al.,* 2006; Gu *et al.,* 2007). Furthermore, the BMP-7 (Bone morphohenetic protein-7) developmental signalling pathway is re-activated in aggressive prostate cancer and can also induce EMT (Yang *et al.,* 2005).

PC-3 and DU-145 prostate cancer cell lines are popular choices for EMT studies and their expression of androgen receptor are absent or at lower levels. Cadherin expression and the invasiveness of prostate cancer cells are reported to be associated with androgen-independence. Inverse correlations between androgen receptor and a number of EMT-related signalling pathways have also been described (Zhau *et al.,* 2008). Therefore, it is likely that the perturbation of the androgen receptor has a permissive effect on EMT, as aggressive prostate cancer cells exhibit increased plasticity and lose their luminal epithelial phenotype during tumour progression (Jennbacken *et al.,* 2006).

1.2.1.2 Migration/Invasion

To enter the blood and lymphatic circulation and thus infiltrate distant organs, tumour cells need to invade through the surrounding tissues. There are a number of reasonably

well defined mechanisms that are associated with invasiveness, such as cell motility and basement membrane degradation, which have been indicated in the intravasation of tumour cells (Nguyen *et al.,* 2009). MMPs (matrix metalloproteinases) are key proteins implicated in ECM remodelling and degradation by metastatic cells (Coussens *et al.,* 2002). For example, the expression of MMP-1, MMP-2, MMP-9, and MMP-13 have been shown to be linked to the aggressiveness of tumours and their expressions are mediated by p38 in prostate, breast, bladder, liver, and skin keratinocyte cell lines (Johansson *et al.,* 2000; Xu *et al.,* 2006; Hsieh *et al.,* 2010; Park *et al.,* 2011).

1.2.1.3 *Anoikis* resistance

Anoikis, derived from the Greek meaning 'homeless and death', defines the phenomenon of cells which undergo cell death or apoptosis after loss of cell-matrix adhesion. Tumour cells in the circulatory system need to survive in an anchorage-independent manner before they disseminate and colonise at a distant organ. Inhibitors of p38 have been shown to induce *anoikis* resistance in human breast cancer cells and murine mammary epithelial cells. The mechanism may be linked to the activation of mitochondrial Bax by the p38 pathway leading to cytochrome *c* release from mitochondria (Lagadec *et al.,* 2009; Owens *et al.,* 2009; Zhang *et al.,* 2009). Furthermore, a recent study shows that p38α induced *anoikis* during mammary mophogenesis by increasing transcription of the death-promoting protein BimEL (Bcl-2-like protein 11) via the transcription factors ATF-2 (activating transcription factor 2) and *c*-Jun (transcription factor AP-1) (Wen *et al.,* 2011).

1.2.1.4 Extravasation

This process describes the process in which circulating tumour cells leave the blood stream by penetrating the vessel wall and is known to involve a variety of adhesion molecules, such as selectins, integrins, and cadherins. It can also be regulated by the MAPK pathways. For example, adhesion of colon cancer cells to E-selectin-expressing endothelial cells leads to the activation of the p38 pathway in both tumour and endothelial cells. This activation induces stress fibre formation resulting in endothelial remodelling by actin-polymerization-mediated phosphorylation of myosin light chain, thus allowing the extravasation of circulating cancer cells (Laferriere *et al.,* 2001; Tremblay *et al.,* 2006). Bone metastasis is the most common metastic site for prostate cancer and it has been reported α_6 integrin plays an important role in bone metastasis. The α_6 integrin cleavage permits extravasation of human prostate cancer cells from circulation to bone (Ports *et al.,* 2009).

1.2.1.5 Organ colonisation

The ability of circulating cancer cells to colonise an organ depends on autonomous cell functions and the barriers imposed by the microenvironment at the site of metastases. Extracellular factors produced by the primary tumour have been shown to prime certain tissues for tumour cell engraftment by way of chemokine production. For example, LLC or B16 tumour cells implanted intradermally into nude mice release VEGF (vascular endothelial growth factor), TGFβ (transforming growth factor β), and TNFα (tumor necrosis factor α), which leads to the expression of the inflammatory chemoattractants S100A8 and S100A9 in lungs. The S100A8 and S100A9 produced by lungs induce p38-mediated migration of tumour cells (Hiratsuka *et al.,* 2006).

Furthermore, activation of angiogenesis is also important for the establishment of metastatic tumours, which can provide tumour cells with nutrition and other necessary materials. For example, blocking of p38 pathway can regulate endothelial cell migration and angiogenesis induced by VEGF (Rousseau *et al.,* 2000).

1.2.1.6 Metastatic dormancy

In some cases, there is a significant delay between tumour cell detatchment from the primary tumour to the colonisation of a distant organ. The time between primary tumour diagnosis and clinically detectable metastatic relapse is defined as metastatic dormancy, which can vary between days and years (Nguyen *et al.,* 2009). Disseminated tumour cells are usually responsible for these distant metastases. When tumour cells are not able to colonise immediately, the cells may enter proliferative dormancy, *i.e.* an arrest at G0/G1 phase of the cell cycle. Alternatively, disseminated tumour cells may develop into a micrometastasis which is unable to spread, but has an acquired equilibrium between cell death and cell proliferation rates. For example, in human head and neck carcinoma cells, the ratio of ERK1/2 activity to p38 activity has been shownto be associated with the ability of tumour cells to proliferate or to enter a state of dormancy (Aguirre-Ghiso 2007).

1.2.2 Key molecules and pathways involved in metastasis

1.2.2.1 MAPK pathway

In mammalian cells, 12 MAPKs (Table 1.1), 7 MAP2Ks, and 20 MAP3Ks have been identified. The MAPK (mitogen-activated protein kinase) pathway plays a key role in cancer development making it an important target for anti-cancer therapy. Dysregulation of the MAPK pathway is common in many human cancers, such as

breast, melanoma, and colorectal cancers (Fang and Richardson 2005; Lin *et al.*, 2010; Leontovich *et al.*, 2012). The three key molecules and signalling pathways of MAPK family, i.e. p38, JNK (stress-activated protein kinase JNK1) and ERK (extracellular signal-regulated kinase) have been studied extensively. These pathways are involved in the regulation of many important cellular processes such as cell proliferation, invasion, metastasis, survival and angiogenesis (Figure 1.2).

MAPKs are evolutionary conserved enzymes, the activation of which requires dual phosphorylation on the Thr-X-Tyr motif which is catalysed by MAP2Ks. MAP2Ks are activated by up-stream MAP3Ks. Following the activation, MAPKs phosphorylate specific serine and threonine residues of the target proteins including other protein kinases and transcription factors. MAPKs are inactivated by both classic phosphatases and dual-specificit phosphatases and are further regulated by scaffold proteins which are usually specific for each of the three major mammalian MAPK pathways (Chang and Karin 2001; Morrison and Davis 2003; Avruch 2007).

Table 1.2 A list of MAPKs and their alternative names.

Name	Alternative names
MAPK1	ERK2 (Extracellular signal-regulated kinase 2), p42-MAPK (MAP kinase isoform p42), MAPK2
MAPK2	The same with MAPK1
MAPK3	ERK1, p44-MAPK
MAPK4	ERK4, p63-MAPK
MAPK6	ERK3, p97-MAPK
MAPK7	ERK5, BMK1 (Big MAP kinase 1)
MAPK8	JNK1 (c-Jun N-terminal kinase 1), SPAK1c (Stress-activated protein kinase 1c)
MAPK9	JNK2, SPAK1a
MAPK10	JNK3, SPAK1b
MAPK11	p38β, SPAK2b
MAPK12	ERK6, p38γ, SPAK3
MAPK13	p38δ, SPAK4
MAPK14	p38α,SPAK2a, CSBP (Cytokine suppressive anti-inflammatory drug-binding protein)
MAPK15	ERK7, ERK8

1.2.2.1.1 p38 signalling

There are four genes encodinge p38 MAPKs which are MAPK14 (p38α), MAPK11 (p38β), MAPK12 (p38γ), and MAPK13 (p38δ). Two alternative splicing isoforms exist for p38α. p38α and p38β are closely related proteins which may have overlapping functions. p38α is highly expressed in most cell types, whilst p38β seems to be expressed at relatively lower levels. The exact function of p38α in the p38 MAPK pathway is still unclear. p38γ and p38δ seem to have more specific functions due to their restricted tissue expression pattern in the body, where p38γ is expressed in skeletal muscle and p38δ in endocrine glands (Ono and Han 2000; Sanz *et al.,* 2000; Cuenda and Rousseau 2007). p38 MAPKs are activated by up-stream MEK3 (MAPK/ERK kinase 3), MEK6, and sometimes MEK4; autophosphorylation can be also indicated in the activation of p38 MAPKs. There are two major groups of proteins that are regulated by p38-mediated phosphorylation. The first group are transcription factors, such as p53, ATF2 (activating transcription factor 2); and the second group are protein kinases including MK2 (MAPK-activated kinase 2; also known as MAPK2), MSK1 (mitogen- and stress-activated protein kinase 1), MNK1 (MAP kinase-interacting serine/threonine kinase 1), and MNK2. p38α acts as a tumour suppressor by both negatively regulating the progression of cell cycle and inducing apoptosis. However, p38α has also been reported to have oncogenic function due to its regulation of cancer progression processes such as invasion and angiogenesis (Ono and Han 2000; Bulavin and Fornace 2004; Mittelstadt *et al.,* 2005; Cuenda and Rousseau 2007; Hui *et al.,* 2007).

p38α can negatively regulate the cell cycle at both the G1/S and G2/M phases by several mechanisms including down-regulation of cyclins, up-regulation of

cyclin-dependent kinase inhibitors, and modulation of tumour suppressor p53 (Thornton and Rincon 2009). This phenomenon is highly conserved amongst different species and is important in many different cells including cardiomyocytes, hepatocytes, fibroblast, haematopoitic cells, and lung cells. This effect of p38α may be mediated by regulation of the JNK/Jun pathway or by down-regulation of the EGFR (epidermal growth factor receptor) pathway (Ambrosino and Nebreda 2001; Engel *et al.,* 2005; Hui *et al.,* 2007; Ventura *et al.,* 2007; Thornton and Rincon 2009). Although p38α activation is usually associated with inhibition of cell proliferation, there are some reports shows that p38α can sometimes positively regulate proliferation in haematopoietic cells and certain other cancer cell types for example, prostate and breast cells (Neve *et al.,* 2002; Platanias 2003; Ricote *et al.,* 2006). p38α also regulates apoptosis via transcriptional and post-transcriptional mechanisms which impact on the death receptors of survival pathways or on the pro- and anti-apoptotic Bcl-2 family proteins. Apoptotic stimuli sometimes trigger p38α activation by a secondary route, such as the production of ROS (reactive oxygen species). This mechanism is important for the suppression of tumour initiation by p38α, which triggers apoptosis in response to the expression of ROS-inducing oncogenes in some immortalised cells (Dolado *et al.,* 2007). However, p38β has been reported as an anti-apoptotic factor in several cell lines for example, endothelial cells. Therefore, p38α is a dual regulator in cell growth and death: it is a general pro-apoptotic factor but acts as a pro-survival factor in certain other cells (Nemoto *et al.,* 1998; Kaiser *et al.,* 2004; Silva *et al.,* 2006; Dolado *et al.,* 2007). The pro-survival function of p38α is mediated by the induction of cell proliferation or anti-apoptosis inflammatory signals such as IL-6 (interleukin-6). In addition, p38α has been implicated in the G2/M phase checkpoint of the cell cycle process which leads to

cell cycle arrest and facilitates DNA repair. This action may diminish chemotherapy-induced DNA damage in cancer cells, which leads to the resistance of apoptosis (Thornton and Rincon 2009). Moreover, down-regulation of p38α in mice has a significant impact on lung homeostasis, probably reflecting the crucial function of p38α in the coordination of proliferation and maintaining the differentiation state of lung epithelial cells (Hui *et al.*, 2007; Ventura *et al.*, 2007).

Several negative regulators of p38 signalling have been found in different human cancer cells, examples include the phosphatases PPM1D and DUSP26, the inhibitors of MAP3K ASK1 (apoptosis signal-regulating kinase 1), GSTM1 (glutathione S-transferase Mu 1) and GSTM2 (Bulavin *et al.*, 2002; Li *et al.*, 2002; Dolado *et al.*, 2007; Yu *et al.*, 2007). In hepatocellular carcinoma, the activity of p38 MAPK and MEK6 are lower than in non-tumorigenic tissues, which supports the observation that increased p38 activity induces apoptosis in hepatoma cell lines. However, increased phosphorylated levels of p38α are also associated with certain malignancies, including head and neck squamous carcinoma, non-small cell lung cancer and breast carcinoma (Greenberg *et al.*, 2002; Elenitoba-Johnson *et al.*, 2003; Esteva *et al.*, 2004; Pomerance *et al.*, 2006; Junttila *et al.*, 2007). These findings collectively suggest that cancer cells with pro-tumorigenic mutations in the p38 pathway have a selective advantage.

1.2.2.1.2 JNK signalling

The JNK proteins are encoded by three genes, MAPK8 (JNK1), MAPK9 (JNK2), and MAPK10 (JNK3), which are alternatively spliced to produce at least ten isoforms. JNK1 and JNK2 are widely expressed in almost all cell types. However, JNK3 is

mainly expressed in the brain (Gupta *et al.*, 1996; Bode and Dong 2007; Cuevas *et al.*, 2007). JNKs can be activated by up-stream MEK4 and MEK7. While there are many down-stream substrates for JNK, the challenge remains to identify the molecular networks regulated by individual JNK family members (Karin and Gallagher 2005; Weston and Davis 2007; Rincon and Davis 2009).

JNKs exert their effects on cell proliferation by interacting with the transcription factor AP-1, a well-known regulator of cell cycle progression (Eferl and Wagner 2003; Sabapathy *et al.*, 2004; Weston and Davis 2007). AP-1 is a dimeric transcription factor complex which contains members of Jun, Fos, ATF and Maf protein families. In non-stimulated cells, JNK2 seems to mainly target *c*-Jun for degradation. However, in stimulated cells, JNK1 phosphorylates and stabilises *c*-Jun resulting in activation of transcription of the target genes. For example, JNK2-knockout fibroblasts grew slightly faster, whilst JNK1-knockout fibroblast cells grew slower compared with the wild-type fibroblasts. This was due to the effect on AP-1 activity and *c*-Jun phosphorylation. The contrasting impacts on proliferation between JNK1 and JNK2 have been observed in fibroblasts and erythroblasts (Sabapathy *et al.*, 2004). Recently, the JNK pathway has been implicated in p53-dependent senescence using a conditional JNK1 allele. Such roles as a negative regulator of the p53 tumour suppressor also supports the idea that activated JNK has an oncogenic role in tumour models (Das *et al.*, 2007). In addition, as cytoplasmic injection of cytochrome c rescues the apoptotic defects of JNK-deficient fibroblasts, JNK pro-apoptotic function is regulated by mitochondrial pathway. JNKs can phosphorylate and regulate the expression of several apoptotic molecules including BAX, BAD and 14-3-3 protein. Phosphorylation of 14-3-3 by JNKs releases the

pro-apoptotic factors BAX and FoxO1, to form inactive complexes leading to an increase of JNK-mediated apoptosis (Tournier *et al.,* 2000; Weston and Davis 2007). Interestingly, inhibition of NF-κB in the mouse model of liver cancer leads to a sustained activity of JNK, increased cell death and cytokine-driven compensatory proliferation (Maeda *et al.,* 2005). Activation of JNK pathway has also been indicated in the biphasic effect of TNFα on the cell survival. Transient activation of JNK was shown to promote cell survival whilst the late and sustained activation of JNK mediated TNFα induced apoptosis via caspase-8 (Ventura *et al.,* 2006).

An important role of the JNK pathway has also been indicated in prostate cancer development. The tumour suppressor PTEN (Phosphatase and tensin homolog) is one of the most commonly mutated tumour suppressors in cancer, and it is frequently lost in prostate cancer (Li *et al.,* 1997). Loss of PTEN leads to Akt activation and increase JNK activity in various human cancer cell lines and human clinical prostate cancer samples. Analysis of gene expression has indicated that several members of the JNK pathway are up-regulated in prostate cancer. In contrast, Transcription factor Jun-B has been identified as an inhibitor of carcinogenesis in the prostate (Vivanco *et al.,* 2007; Konishi *et al.,* 2008; Ouyang *et al.,* 2008).

1.2.2.1.3 ERK signalling

The ERK family is composed of ERK1 (MAPK3), ERK2 (MAPK1), ERK3 (MAPK6), ERK4 (MAPK4), ERK5 (MAPK7), ERK6(MAPK12) and ERK7/8(MAPK15) (Bogoyevitch and Court 2004). ERK1/2 is expressed in virtually all the tissues in the body. In fibroblasts, it is activated by growth factors, serum, ligands which stimulate

heterotrimeric G protein-coupled receptors, cytokines, transforming growth factors, osmotic stress and microtubule disorganisation (Chen *et al.,* 2001). Classic ERK1/2 can be activated by up-stream MEK1 and MEK2. MEK1/2 is activated by up-stream Ras-Raf family members. However, Ras is not the only activator of Raf. PKC (protein kinase C) has also been demonstrated to phosphorylate and activate Raf-1 (Kolch 2005). Nevertheless, the activation of the ERK pathway leads to the regulation of cell functions such as cell survival and apoptosis.

Activation of ERK1/2 has been shown to inhibit apoptosis in response to a wide range of stimuli, some of the notable ones being TNF, Fas ligand, TRAIL (TNF-related apoptosis-inducing ligand), growth factor withdrawal, hydrogen peroxide and matrix detachment (Wang *et al.,* 1998; Erhardt *et al.,* 1999; Le Gall *et al.,* 2000; Tran *et al.,* 2001). First of all, the anti-apoptotic function of ERK1/2 can be regulated indirectly by p38 signalling. Inhibition of p38 stimulates Raf and ERK activities and induces myoblast proliferation. Moreover, activation of p38 induces dephosphorylation of MEK1/2 and apoptosis in human skin fibroblast (Lee *et al.,* 2002; Li *et al.,* 2003). In mammalian cells, for example hepatocytes and leukaemia cells, the ERK1/2 pathway can block apoptosis at levels up-stream and down-stream, as well as events unrelated to the change in mitochondrial transmembrane protein and cytochrome *c* release. The inactivation of the pro-apoptotic Bcl-2 family member BAD is mediated by phosphorylation at Ser112 by ERK-activated p90 RSK (ribosomal S6 kinase) and at Ser136 by PI3K/Akt pathway. BAD phosphorylation at serine 112 by RSK suppresses BAD-mediated neuronal apoptosis and inhibition of ERK1/2 or Akt increases the extent of apoptosis. The ERK-downstream p70 RSK may also be involved in Ser136

phosphorylation and inhibition of BAD functions (Sturgill *et al.,* 1988; Bonni *et al.,* 1999; Erhardt *et al.,* 1999; Scheid *et al.,* 1999; Hayakawa *et al.,* 2000; Harada *et al.,* 2001; Qiao *et al.,* 2001; Mabuchi *et al.,* 2002; Shonai *et al.,* 2002). In addition, it has been shown that inhibition of ERK1/2 up-regulates STAT3/5 phosphorylation and promotes cell apoptosis in melanoma (Krasilnikov *et al.,* 2003).

Although ERK1/2 is a pro-survival factor in MAPK family and regulates cell proliferation and differentiation, under some circumstances ERK1/2 also acts as a pro-apoptotic factor. In the neuronal system, ERK1/2 has been shown to be involved in neuroregeneration. Activation of ERK1/2 is associated with glutamate-induced oxidative toxicity in neuronal cells and the inhibition of ERK1/2 activation protects cells from glutamate toxicity. In addition, ERK1/2 activation promotes low potassium-induced neuronal degeneration predominantly through plasma membrane damage which occurs independent of caspase-3 (Stanciu *et al.,* 2000; Cheung and Slack 2004; Subramaniam *et al.,* 2004). Furthermore, DNA damage also activates ERK1/2 in various cells and inhibition of ERK1/2 activation attenuates apoptosis or G2/M phase arrest. Additionally, suppression of ERK2 activity has been shown to sensitise ovarian carcinoma cells to cisplatin-induced apoptosis (Hayakawa *et al.,* 1999; Persons *et al.,* 2000; Tang *et al.,* 2002; Billecke *et al.,* 2006).

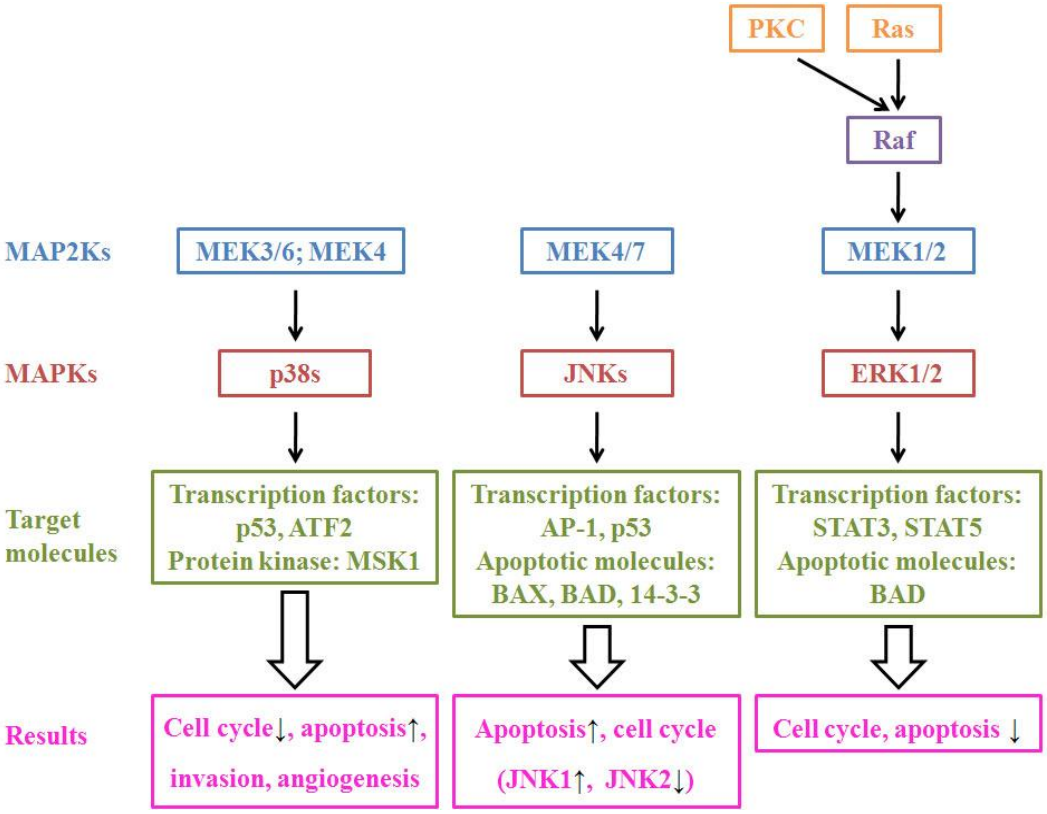


Figure 1.3 The MAPK pathways including p38, JNK, and ERK pathways.

1.2.2.2 TGF-β pathway

The TGF-β superfamily regulates a broad range of cellular functions, including cell growth, differentiation, adhesion and apoptosis. Alteration of TGF-β signalling is involved in a number of pathological conditions, for example it plays a critical role in wound healing and cancer. Prior to tumour initiation and the early stage of cancer progression, TGF-β acts as a tumour suppressor. However, for the most part in the later stage of tumour development, TGF-β usually acts as a tumour promoter.

TGF-β1 was discovered in 1983 as a protein regulating the cell growth of rat fibroblasts. It is now known that TGF-β1 belongs to a large protein family that has thirty-three

members, all of which have a common dimeric structure and a cysteine knot structural domain (Frolik *et al.,* 1983; Galat 2011). The TGF-β superfamily has been divided into several sub-families, including TGF-βs, BMPs (bone morphogenetic proteins), GDFs (growth and differentiation factors), MIF (Mullerian inhibitory factor), activins, and inhibins. There are three isoforms of TGF-βs expressed in mammals, TGF-β1, TGF-β2 and TGF-β3. The BMP family are expressed in both vertebrates and invertebrates and are known to exhibit a wide range of biological effects in different cell types. BMPs regulate the transcription of several genes involved in osteogenesis, neurogenesis and ventral mesoderm specification. GDFs (Growth/differentiation factors) are sometimes classified as part of the BMP subfamily. Activins are structurally related protein involved in the control of cell proliferation, differentiation, apoptosis, homeostasis and endocrine functions. Inhibins are crucial for normal reproductive and endocrine functions as they negatively regulate the synthesis of follicle-stimulating hormone (Lee 1990; Roberts 1998; Stenvers and Findlay 2009; Xia and Schneyer 2009; Miyazono *et al.,* 2010; Rider and Mulloy 2010).

1.2.2.2.1 SMAD-dependent signalling pathway

TGF-β binds to TGFβRI and TGFβRII (type I and II serine/theronine kinase receptors) and the receptors form heteromeric complexes after ligand binding. The co-receptors endoglin and β-glycan (also known as TGFβRIII) interact with TGF-β family ligands but are not directly involved in signalling. These co-receptors may regulate the access of ligands to TGFβRI and TGFβRII. Upon binding to the ligands, TGFβRII recruit and phosphorylates the glycine/serine-rich domain of TGFβRI which forms an activated ligand-receptor complex. The activated TGFβRI then phosphorylates down-stream

molecules such as SMADs (Mothers against decapentaplegic homolog) (Shi and Massague 2003; Kang *et al.*, 2009; Gatza *et al.*, 2010).

The SMAD family are divided in to three groups; R-SMADs (receptor-associated SMADs), Co-SMADs (co-operating SMADs), and I-SMADs (inhibitory SMADs). R-SMADs are directly phosphorylated at two serine residues at their C-terminii by TGFβRI. The unphosphorylated R-SMADs are transcriptionally inactive and can be sequestered in the cytoplasm by specific proteins such as SARA (SMAD anchor for receptor activation) and endofin (Tsukazaki *et al.*, 1998; Shi and Massague 2003; Ross and Hill 2008; Huminiecki *et al.*, 2009; Miyazono *et al.*, 2010). Five R-SMADs (SMAD1, SMAD2, SMAD3, SMAD5, and SMAD8) have been identified in humans. SMAD2 and SMAD3 are substrates for receptors activated by TGF-βs and activins, whilst SMAD1, SMAD5, and SMAD8 mediate pathways which are activated by BMPs. After phosphorylation of the R-SMADs, the phosphor-R-SMADs associate with SMAD4 (Co-SMAD). This complex translocates to the nucleus and collaborates with other transcription factors to regulate gene expression. There are two I-SMADs: SMAD6 and SMAD7 which are responsive genes to Smad signalling. The induced expression of these I-SMADs leads to a negative feedback regulation for TGF-β signalling. Furthermore, phosphorylation of R-SMADs can be inhibited by interaction of SMAD7 with type 1 receptors of TGFβ, activin and BMP. For example, SMAD7 competes with R-SMADs for binding to TGFβRI and by recruiting E3 ubiquitin Smurf ligases to the activated receptor and targets this receptor for degradation. In the nucleus, the R-SMAD/Co-SMAD complex can bind to SMAD-binding elements in the DNA via the MH1 (Mad Homology 1) domain. SMAD1, SMAD5, and SMAD8 recognise a

non-consensus GC-rich domain. However, a 30-amino acid insertion in the MH1 domain disables SMAD2 from individual binding to the DNA. The binding of SMAD complexes to DNA is crucial for the transcriptional activation of SMAD target genes, although this binding is low affinity. Additional interactions with other transcription factors are required to form large transcriptional complexes with high affinity for chromatin (Dennler *et al.*, 1999; Shi and Massague 2003; Derynck and Akhurst 2007; Itoh and ten Dijke 2007). Figure 1.3 shows the overall SMADs signalling pathway.

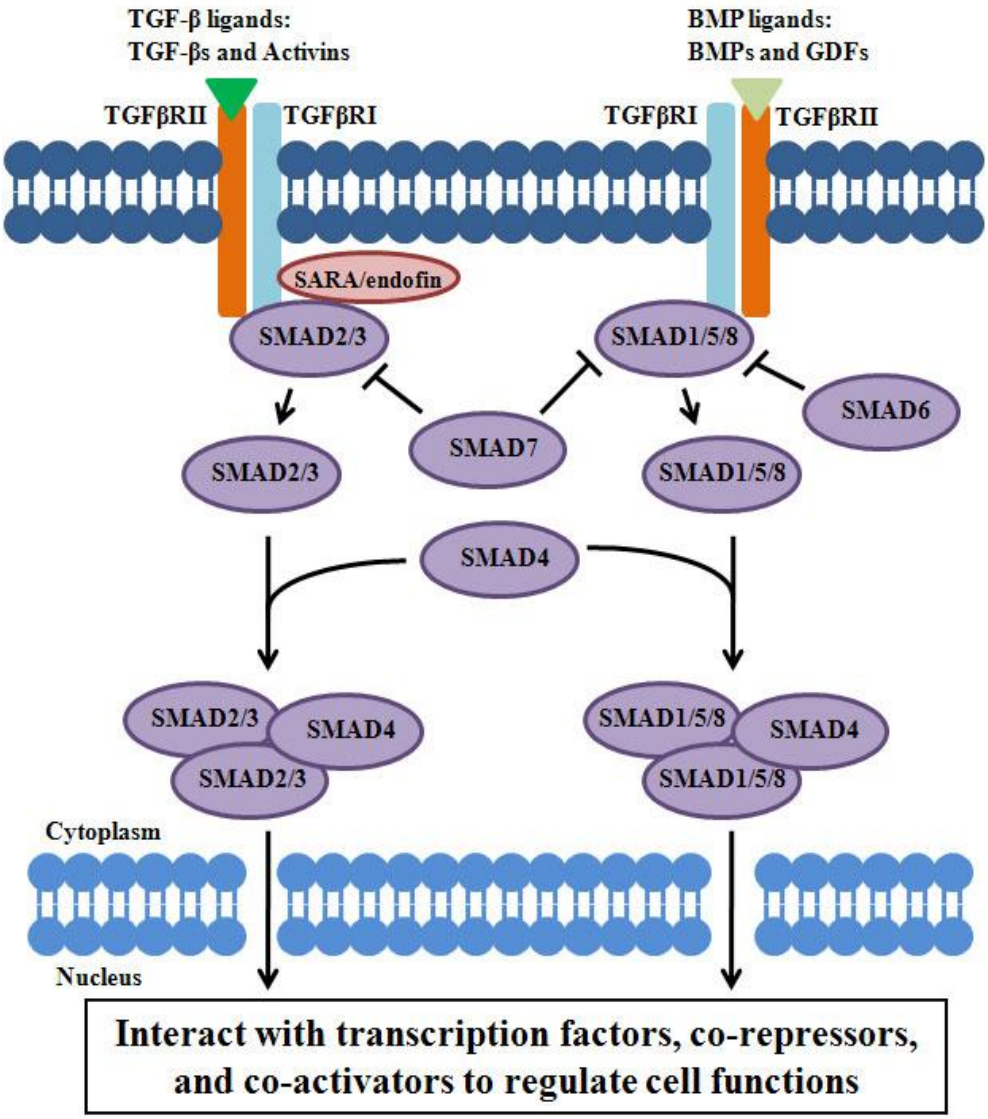


Figure 1.4 SMAD-dependent signalling pathway.

1.2.2.2.1.1 Mutation of SMADs in cancers

Human chromosome 18q21, where *SMAD2* and *SMAD4* are located are often mutated in pancreas and colon cancers. The loss of SMAD4 disrupts the SMAD2/3/4 heteromeric complexes and therefore affects TGF-β signalling. SMAD4 mutations are rare but are present in breast cancer, although. Recent studies have shown that aberrant germline mutation of SMAD4 may be more common in breast cancer than previously described. *SMAD2* mutations have also been found at low levels in colon, head and neck and lung carcinomas. In skin cancer, SMAD2 expression is associated with SCC (squamous cell carcinoma) and BCC (basal cell carcinoma), while SMAD4 expression is only associated with SCC but not BCC (Kretzschmar 2000; Bellam and Pasche 2010; Yang and Yang 2010; Tram *et al.,* 2011; Shao *et al.,* 2012).

SMAD3 mutations have not been found in any specific cancer but expression levels may vary in many cancers during disease progression. The growth of gastric cancer cells which lack SMAD3 expression can be inhibited by TGF-β. Furthermore, the reduction of SMAD2 and SMAD3 in human breast cancer cells resulted in enhanced tumourigenesis with an impaired metastatic capacity. These data show that *SMAD* mutations and aberrant SMAD expression can lead to the development of cancer and metastasis (Tian *et al.,* 2003; Han *et al.,* 2004; Arany *et al.,* 2007).

1.2.2.2.2 TGF-β pathway in cancer

1.2.2.2.2.1 TGF-β-mediated growth inhibition

TGF-β-mediated growth arrest can be mediated by the inhibition of CDKs (cyclin-dependent kinases) and the down-regulation of c-Myc. Cell cycle progression is

regulated by CDKs which is activated by cyclin binding and inhibited by CDK inhibitors. Cells are sensitive to TGF-β during the G1 phase of the cell cycle. If TGF-β is present after the G1 phase, the cell will complete the cell cycle and arrest at the subsequent G1 phase. In most cases, this arrest is reversible and it is associated with terminal differentiation or programmed cell death. TGF-β prevents or inhibits G1 cyclin-CDK activation in a cell type-dependent manner via different mechanisms leading to pRb (retinoblastoma protein) dephosphorylation (Geng and Weinberg 1993; Slingerland *et al.,* 1994; Donovan and Slingerland 2000; Liu and Matsuura 2005). In many cells, TGF-β results in a rapid inhibition of *c*-Myc transcription. Regulation of c-Myc protein is important for G1 to S phase progression. TGF-β induces the down-regulation of c-Myc expression and c-Myc promoter activity through a TGF-β response element. TGF-β mediates the c-Myc transcriptional repression via SMAD signalling. Upon SMAD3 signalling, the complex moves from the cytoplasm to the nucleus and the complex recruits the co-repressor protein, the pRb family member p107 and the transcription factor E2F4. The complex then binds to the c-Myc promoter which is next to the E2F4-binding site and represses transcription (Massague *et al.,* 2000; Chen *et al.,* 2001; Chen *et al.,* 2002; Yagi *et al.,* 2002; Frederick *et al.,* 2004).

Another tumour suppression effect of TGF-β is its ability to induce apoptosis in a cell type-specific manner. SMAD signalling regulates the expression of several genes whose function is directly linked to the apoptosis pathway. Many pro-apoptosis target genes are controlled by the SMAD pathway, such as TIEG1 (TGF-β-inducible early-response gene), the death-associated protein kinases, and the SH2 domain-containing inositol-5-phosphatase.These pro-apoptotic molecules have been indicated as being

important in inhibiting proliferation and inducing apoptosis in different cells. The apoptotic response of normal cells is complex and incorporates both pro-survival and pro-apoptotic pathways. In all cases, the net decision of whether TGF-β will favour apoptosis or survival depends on the expression profile of relevant signalling molecules in certain receipting cells (Tachibana *et al.,* 1997; Jang *et al.,* 2002; Valderrama-Carvajal *et al.,* 2002; Pardali and Moustakas 2007).

1.2.2.2.2.2 TGF-β pathway in cancer progression

Genetic and epigenetic changes in tumour cells are autonomous switch for TGF-β responses, from the inhibition of proliferation and survival to the promotion of growth, motility, and invasion. Interestingly, the p53 tumour suppressor seems to be able to switch the response (Adorno *et al.,* 2009). In a number of human solid tumours, it has been indicated that TGF-β dysregulation has a significant effect on tumour progression and patient prognosis. The association of breast cancer with increased serum TGF-β levels has been reported. A complimentary study has subsequently shown that low levels of TGFβRII gene expression were correlated with an increased risk of breast cancer. Furthermore, endogenous over-expression of TGFβRII in the stromal compartment of human breast cancer is associated with poor prognosis (Gobbi *et al.,* 1999; Grainger *et al.,* 1999; Yokota *et al.,* 2000; Barlow *et al.,* 2003). In addition, a study employing a tissue microarray of 310 colon carcinomas has indicated that stromal TGFβRII expression is an independent prognostic factor for cancer-related survival (Bacman *et al.,* 2007). These observations collectively suggest that the autonomous effects of TGF-β signalling in tumour cell and stromal cell stimulation by TGF-β may contribute to the regulation of the adjacent epithelial cell population in human cancers.

In contrast to the tumour suppressor role of TGF-β1 during tumour initiation and early progression, TGF-β1 may actually promote cancer progression at a later stage (Robson *et al.*, 1996; Tsushima *et al.*, 1996). Moreover, TGF-β1 up-regulation is associated with angiogenesis, metastasis and poor patient prognosis in prostate cancer. Over-expression of TGF-β1 is associated with advanced prostate cancer (Wikstrom *et al.*, 1998; Diener *et al.*, 2010). These results show that TGF-β is able to mediate cell autonomous, local, and systemic responses that regulate the initiation, progression and prognostic outcomes in human cancers.

TGF-β can induce EMT in embryonic epithelial cells. EMT is essential for normal embryonic development and its exploitation during cancer progression is thought to contribute to tumour invasion and metastasis. Tumour cells expressing mesenchymal markers have a greater tendency to be invasive and to metastasise than tumour cells expressing only epithelial markers (Xu *et al.*, 2003; Han *et al.*, 2004). A number of studies have indicated that TGF-β receptor/SMAD signalling and down-stream targets are important modulators of EMT. TGF-β signalling is known to down-regulate claudins, occludins and ZO1 followed by the degradation of tight junctions. It also has been indicated that R-SMADs interacts with EMT-associated transcription factors, such as Snai1 and AP-1 resulting in the formation of TGF-β-induced EMT-promoting SMAD complexes. The expression of E-cadherin is repressed by TGF-β via Snai1/2 (Zinc finger protein Snai 1/2), HMGA2 (High mobility group protein HMGI-C 2), and ZEB1/2 (Zinc finger E-box-binding homeobox 1/2). There is also evidence to show that TGF-β1-induced EMT leads to the loss of E-cadherin and cell-cell adhesion. TGF-β-induced activation of MAPKs is also important to induce EMT. For example,

activation of ERK pathway is important for TGF-β-induced EMT (Xie *et al.,* 2004; Moustakas and Heldin 2007; Xu *et al.,* 2009; Fuxe *et al.,* 2010; Hills *et al.,* 2012). Furthermore, the focal adhesion complexes including β1 and β3 integrins, Src, FAK (focal adhesion kinase), and p130Cas are activated by TGF-β. TGF-β can activate PI3K and its down-stream molecules, Akt and mTOR (Bhowmick *et al.,* 2001; Galliher and Schiemann 2006; Galliher and Schiemann 2007; Lamouille and Derynck 2007; Wendt and Schiemann 2009; Wendt *et al.,* 2009; Lamouille and Derynck 2010). Finally, it has been demonstrated that TGF-β stimulation induces the phosphorylation of PAR6 by TGFβRII, followed by degradation of RhoA (Transforming protein RhoA), a key molecule able to stabilise cell junctions. Meanwhile, microarray studies of human breast cancer show that PAR6 activation correlates with markers of the basal carcinoma sub-type in BRCA1-associated tumours. It has also been reported that destabilisation of p53 by MDM2 (E3 ubiquitin-protein ligase Mdm2) is a key step in the EMT of breast cancer and that the TGF-β1-induced expression of MDM2 is also related to the progression of metastatic breast cancer (Viloria-Petit *et al.,* 2009; Araki *et al.,* 2010).

In summary, occurrence of the cancer metastasis is dependent on the success of inter-related multiple steps which are regulated by many different cytokines and factors. The phosphorylation of diverse receptors and intracellular signalling molecules is crucial for the effect induced by the cytokines and other protein factors. More interestingly, a group of proteins that are embedded in the cellular membrane or exist inside the cells can help to balance and coordinate such phosphorylation. These molecules are phosphatases which can deactivate phosphorylated proteins.

1.3 Protein tyrosine phosphatase (PTP) family

Reversible protein phosphorylation is one of the major systems for regulating cell functions and it was implicated in signalling by paracrine or autocrine-acting growth factors and endocrine hormones such as insulin. PTPs are not only present in eukaryotes but also prokaryotes and viruses, for example, VH1 which is present in the vaccinia virus and the dual specificity enzymes IphP is present in the cyanobacterium *Nostoc commune* (Guan *et al.,* 1991; Potts *et al.,* 1993). This posttranslational modification is involved in many physiological and pathological processes, for example cell differentiation, survival (Fu 1992), cell adhesion and cell-cell contact (Volberg *et al.,* 1992). Current knowledge regarding the interactions between PTPs (protein tyrosine phosphatases) and their substrates is still limited. Contrary to the initial view of signal pathways being a simple linear arrangement of phosphorylation, it is now apparent that is is more complicated due to cross-talk and interactions existing among multiple signalling pathways which help to coordinate biological functions and responses to diverse stimuli. Kinases have been implicated in controlling the amplitude of signalling pathways and phosphatases are thought to play an important role in controlling the rate and duration of the response rather than just acting as passive housekeeping enzymes (Heinrich *et al.,* 2002; Hornberg *et al.,* 2005).

PTPs are divided into the classical phosphotyrosine (pTyr) – specific phosphatases (Figure 1.4) and the dual specificity phosphatases (Figure 1.5) (Alonso *et al.,* 2004; Andersen *et al.,* 2004). Classical pTyr-specific PTPs include transmembrane receptor-like protein tyrosine phosphatases (RPTPs) that have the potential to regulate signalling through ligand-controlled protein tyrosine dephosphorylation. Each PTP is

composed of at least one conserved domain which includes an 11-residue motif (I/V) HCXAGXXR(S/T) G containing the essential catalytic residues, cystenine and agrinine. The sequences of PTPs are different with serine/theronine, acid, or alkaline phosphatases. The diversity in the structure of PTPs is due to the variety of non-catalytic sequences attached to either the NH_2- or COOH- termini of the catalytic domain (Guan and Dixon 1991; Pot *et al.*, 1991; Charbonneau and Tonks 1992). Many of the RPTPs are characterised by variable extracellular multiple domains, followed by a single transmembrane region and one or two intracellular phosphatase catalytic domains. RPTPs exhibit features of cell-adhesion molecules in their extracellular segment and have been implicated in cell-cell and cell-matrix contact via dimerisation, phosphorylation and reversible oxidation.

RPTPs have been classified into eight subfamilies based on their phosphatase domains (Andersen *et al.*, 2001). The eight subfamilies are R1/R6, R2A, R2B, R3, R4, R5, R7 and R8. There are four subfamilies, R2A, R2B, R3 and R4 that play an important role in central nervous system development (Johnson and Van Vactor 2003). The members of the R2 subfamily have very similar protein structures including fibronectin type Ⅲ repeats, Ig-like domains, a siglen transmembrane region, a juxtamembrane domain, followed by one phosphatase domain and one pseudo-phosphatase domain. The R2 subfamily is further divided into two groups. R2B subfamily members have an additional MAM domain (Meprin/ A5/ PTPRM) at the N-terminal of the protein (Beckmann and Bork 1993).

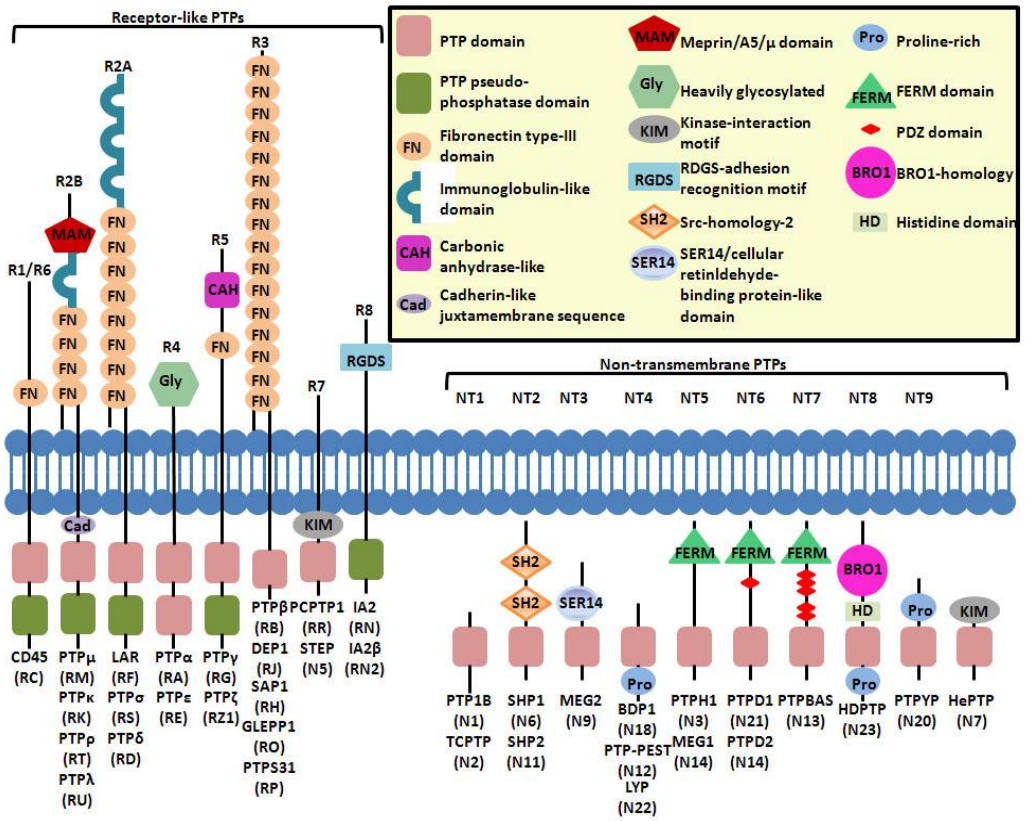


Figure 1.5 The classical PTPs are divided into two groups; receptor-like PTPs and non-transmembrane PTPs. Modified from (Tonks 2006; Soulsby and Bennett 2009)

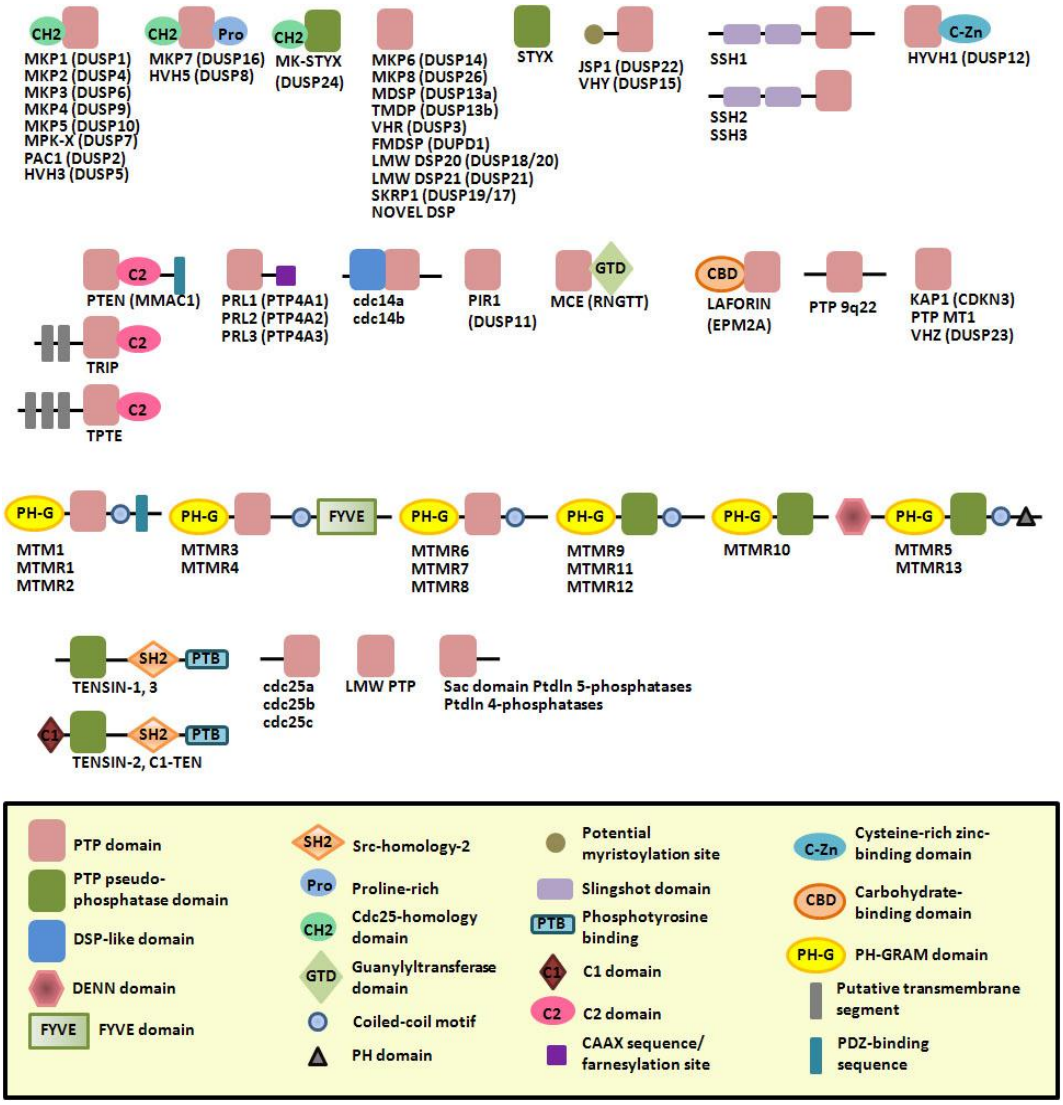


Figure 1.6 The dual specificity phosphatases. Modified from (Tonks 2006; Soulsby and Bennett 2009)

1.3.1 Key members of PTP family

1.3.1.1 PTP1B (PTPN1)

The human placental protein tyrosine phosphatase non-receptor type 1 (PTP1B or PTPN1), a 35KDa soluble enzyme, was purified and its amino acid sequence was partially determined at 1980s (Charbonneau *et al.,* 1988). The placental PTP1B can be activated by EDTA, spermine, spermidine and myelin. Its activity can be inhibited by different reagents including heparin and glutamate/tyrosine, via blocking the cysteinyl group which is an essential part for as PTPase activity (Tonks *et al.,* 1988).

The early identified placental PTP1B was a product with 321 amino acids and subsequently found to be the NH_2-terminal portion derived from a full-length molecule of 435 residues. The conserved PTP domain of PTP1B contains residue 30 to residue 278 and COOH-terminal non-catalytic extension acts as a regulatory function. The COOH-terminal 35 residues target the enzyme on the cytoplasmic surface of endoplasmic reticulum, whereas the preceding 122 residues are predominantly hydrophilic and contain sites for serine phosphorylation. The segment of PTP1B is phosphorylated on seryl residues by PKC *in vivo* and the pattern of phosphorylation is changed in cell cycle-dependent manner (Tonks *et al.,* 1988; Guan *et al.,* 1990; Frangioni *et al.,* 1992; Flint *et al.,* 1993).

PTP1B regulates cell functions via different signalling pathways. For example, PTP1B expression and activity are increased in the mice hindlimb model of angiogenesis. PTP1B negatively regulates the activation of VEGFR2 (vascular endothelial growth factor receptor 2) via directly binding to the VEGFR2 cytoplasmic domain; and

stabilises cell-cell adhesion via decreasing tyrosine phosphorylation of VE-cadherin (Nakamura *et al.,* 2008). PTP1B is positively implicated in the oncogenic properties of breast cancer cells by regulating ERK signalling in an ErbB2-induced breast cancer mouse model. The reduction of PTP1B expression in MCF-7 cells lead to decreased cell growth and ERK phosphorylation. Moreover, IGF-2-induced MCF-7 cell migration was also impaired by the reduced PTP1B expression, an effect independent of the ERK pathway (Julien *et al.,* 2007; Arias-Romero *et al.,* 2009; Blanquart *et al.,* 2010). Down-regulation of PTP1B activity using small molecule inhibitors suppressed cell migration and spreading over fibronectin, increased Tyr^{527} phosphorylation in Src and decreased phosphorylation of FAK, $p130^{Cas}$ and ERK1/2 in the integrin pathway of fibroblasts (Liang *et al.,* 2005). Furthermore, PTP1B and androgen receptor were co-amplified in metastatic tumours and PTP1B amplification was associated with high-risk primary tumours. PTP1B depletion reduced the growth of androgen-dependent human prostate tumours and impaired androgen-induced *in vitro* cell migration and invasion (Lessard *et al.,* 2012).

1.3.1.2 SHP2 (PTPN11)

SHP2 is an intracellular tyrosine phosphatase with two N-terminal Src-homology 2 domains and two tyrosine phosphorylation sites at the C-terminal which are dephosphorylated by receptor and non-receptor PTKs. SHP2 promotes the activation of the Ras/ERK pathway by growth factors and cytokines in different animal models such as *Xenopus* and *Drosophila* and it is an essential component in several oncogene signalling pathways such as leukaemia, breast and gastric cancers (Hatakeyama 2004; Chan *et al.,* 2008). It has been shown that SHP2 dephosphorylates and inhibits RasGAP

and sprout proteins, both being negative regulators of Ras activation. In addition, SHP2 can directly or indirectly dephosphorylate Src kinase, thus negatively regulating the activation of Src and the subsequent Ras pathway (Neel *et al.,* 2003; Hanafusa *et al.,* 2004; Zhang *et al.,* 2004).

Gain-of-function mutations of normal genes are called proto-oncogenes and they generate oncogenes which provide proliferation and survival advantages to the cells. However, loss-of-function mutations of tumour suppressor genes lead to dysregulation of cell proliferation and survival. SHP2 was found to be a proto-oncogene in leukaemia and its gain-of-function mutation has been detected in leukaemia and certain solid tumours (Xu *et al.,* 2005; Zhou *et al.,* 2008). Autosomal-dominant mutations in the human SHP2 have been detected in almost 50% of patients with Noonan syndrome who have higher risk of suffering JMML (juvenile myelomonocytic leukaemia), and somatic mutations constitutively activating SHP2 have also been found in several types of leukaemia (Tartaglia and Gelb 2005). However, SHP2 was also found to act as a tumour suppressor in hepatocellular carcinogenesis. SHP2 ablation enhanced diethylnitrosamine-induced hepatocellular carcinoma development which was diminished by deletion of both SHP2 and Stat3 in hepatocytes (Bard-Chapeau *et al.,* 2011). Furthermore, SHP2 is a predictive marker for good prognosis in colorectal cancer, which is in stark contrast to it role in promoting carcinogenesis in other malignancies (Yu *et al.,* 2011).

Certain pathways have been indicated in the escape of tumour cells from non-immune and immune surveillance system, particularly the PI3K/Akt and Ras/Raf/MEK/ERK

pathways (McCubrey *et al.,* 2006). In gastric cancer, activation of PI3K by inhibition of PTEN leads to an increased cell growth and interferon-γ (INF-γ) resistance. Activation of Akt followed by inactivation of glycogen synthase kinase-3β (GSK-3β) promotes aberrant SHP2, which suppresses the INF-γ signalling and its inhibition on tumour growth. SHP2 also plays an important role in tumorigenesis (Tseng *et al.,* 2012). In addition, reduced SHP2 expression in glioblastoma associates with decreased cell growth which may be due to an increase in cell population arrested at G1 phase (Sturla *et al.,* 2011).

1.3.1.3 LCA (PTPRC)

Leukocyte common antigen (LCA, CD45, PTPRC, Ly-5, T200, or B220) is a type I glycoprotein containing a large extracellular domain, a single transmembrane domain, and a cytoplasmic portion that consists of a wedge-like structure followed by tandem PTP domains and a 79 amino acid C-terminal tail. Only the D1 domain has the enzyme activity, but both D1 and D2 domain are required for the optimal phosphatase function of PTP *in vivo*. The extracellular domain exists as multiple isoforms due to the alternative splicing of exons 4, 5, 6, and potentially 7; it can generate at least 8 different isoforms at mRNA level and five of these protein products have been detected in humans (Streuli *et al.,* 1987; Charbonneau *et al.,* 1988; Streuli *et al.,* 1988; Thomas and Lefrancois 1988; Hermiston *et al.,* 2003). Furthermore, due to the spatial orientations of D1 and D2 domains, D1-D1 interactions cannot be inhibited, suggesting the general inhibitory dimerisation may not be a mechanism for the regulation of receptor-like PTPs (Nam *et al.,* 2005).

The structure of LCA suggests that it is a receptor-like PTP and its activities are controlled by the interaction with specific ligands (Streuli *et al.,* 1989). LCA is composed of several isoforms with their size ranging from 180 to 220 kDa. They are produced by tissue-specific splicing (Streuli *et al.,* 1987; Hall *et al.,* 1988; Streuli and Saito 1989). Inactivation of an LCA protein in which the extracellular domain was replaced by EGFR led to similar results as with inactivation of EGFR which blocks TCR (T cell antigen receptor) signalling (Desai *et al.,* 1993). LCA is thought to dephosphorylate tyrosine residues on the Lck, Fyn and Lyn protein tyrosine kinases. LCA can dephosphorylate both the C-terminal negative regulatory site (PY505 of Lck and PY508 of Lyn) and the positive regulatory site (PY394 of Lck and PY397 of Lyn) in the activation loop (Saunders and Johnson 2010). Lck, Fyn and Lyn are members of the Src family (non-receptor protein tyrosine kinases) and the activation of Src family is essential for initiating downstream signalling responses to the stimulation of T and B cell receptors (Koretzky *et al.,* 1990; Justement *et al.,* 1991). Furthermore, kinases in this family are inhibited by autophosphorylation of a particular tyrosine residue (Tian *et al.,* 1991). However there are no studies indicated that LCA direct interacted with Src kinase family. Other substrates of LCA is Janus kinase (JAK) family. JAKs are hyperphosphorylated in LCA deficient T cells, B cells and most cells in response to IFNα, IL-3 and IL-4. Recombinant LCA D2 domain binds to JAK2 and dephosphorylates the kinase *in vitro*, suggesting that JAKs are direct substrates of LCA. Although Src family kinases do not directly affect JAK phosphorylation, Src family kinase can impact on the JAK/STAT signalling pathway and regulate cytokine signalling. This suggests that LCA plays a key role in regulating the activity of Src family kinase (Irie-Sasaki *et al.,* 2001; Xu *et al.,* 2005; Scapini *et al.,* 2009).

1.3.1.4 PTPRM

The human *protein tyrosine phosphatase μ* (*PTPRM)* gene maps to chromosome 18 and locates p11.2 and predominantly expressed in lung, retina and brain (Suijkerbuijk *et al.*, 1993; Fuchs *et al.,* 1998; Burden-Gulley and Brady-Kalnay 1999). In crystal structure analysis of PTPRM D1, the subunits interact and form a dimer with 2-fold symmetry. The PTPRM D1 domain has 46% similarity with the PTPRA D1 domain and the catalytic site of PTPRM D1 domain is unhindered and adopts an open conformation similar to the PTP1B (Barford *et al.,* 1994; Bilwes *et al.,* 1996; Hoffmann *et al.,* 1997).

The PTPRM protein has a similar structure to some cell-cell adhesion molecules and has been shown to exhibit homophilic binding and to confer cell-cell adhesion in cells including epithelia and cancer cells. However, PTPRM also binds with other subfamily members (PTPRK, PTPRT, and PTPRU) to mediate cell-cell aggregation (Becka *et al.,* ; Brady-Kalnay *et al.,* 1993). Some reports have indicated that the MAM and Ig-like domains are essential for homophilic binding. The MAM domain is the homophilic binding site of PTPRM and contains independent *cis* and *trans* interaction sites; these two different interaction sites play an important role in the promotion of dimerisation of PTPRM at the cell membrane (Cismasiu *et al.,* 2004). Furthermore, the Ig-like domain also contains a homophilic binding site to control the localisation of the enzyme; deletion of Ig-like domain reduced the localisation of PTPRM to the cell-cell contacts in endothelial and epithelial cells (Del Vecchio and Tonks 2005).

Like other PTPs, PTPRM is regulated by the balance between the actions of protein tyrosine kinases (PTK) and PTPs. PTPRM associates with E-cadherin/ α-catenin/

β-catenin complexes in rat heart, lung and brain tissues. Previous studies have shown that the levels of tyrosine phosphorylation of cadherin/catenin complexes are related to the adhesive capacity of the cadherin. PTPRM is associated with the cadherin/ catenin complexes of homotypic cell adhesion molecules. Treatment of cells with PTP inhibitor can result in an increase of tyrosine phosphorylation of cadherins and catenins. It suggests therefore that PTPRM can help to maintain the unphosphorylated state of the cadherin/catenin complexes (Cheng *et al.*, 1997). In fact PTPRM can directly bind to intracellular domain of E-cadherin rather than α-catenin or β-catenin to regulate the phosphorylation of E-cadherin (Brady-Kalnay *et al.,* 1995; Hiscox and Jiang 1998; Freiss and Vignon 2004).

The association of RACK1 (Receptor of activated protein kinase C 1) with protein kinase c (PKC) δ and PKCδ activity, BCCIP (BRCA2 and CDKN1A-interacting protein), and IQGAP1 (Ras GTPase-activating-like protein IQGAP1) are involved in the PTPRM-dependent axonal migration. PKCδ, BCCIP, and PLCγ1 (phospholipase C γ 1) have been reported to be PTPRM substrates. PTPRM regulates cell migration by dephosphorylation PY783 of PLCγ1, resulting in the enzyme being inactive and blocking cytoskeletal changes which are required during cellular migration (Mourton *et al.,* 2001; Rosdahl *et al.,* 2002; Ensslen and Brady-Kalnay 2004; Phillips-Mason *et al.,* 2006; Phillips-Mason *et al.,* 2008; Phillips-Mason *et al.,* 2011). Furthermore, reduced expression of PTPRM resulted in an increased phosphorylation of tyrosine 992 of EGFR (pY992) by EGF, a docking site for PLCγ1 to activate PLCγ1 thus leading to increased cell migration in both scratch wounding assay and chemotaxis assay (Phillips-Mason *et al.,* 2008; Hyun *et al.,* 2011).

1.3.2 PTPRK

Protein tyrosine phosphatase kappa (PTPRK or PTPκ) belongs to the MAM-subfamily (R2B subfamily). The protein contains a MAM (Meprin/A5/ PTPRM) domain in the extracellular region followed by an Ig-like domain and four fibronectin type III repeats, the typical structure in CAM (cell adhesion molecule) proteins. It is also a putative neuronal recognition molecule. The post-translational modification of this molecule is cleavage of its extracellular domain (Streuli *et al.,* 1988). The MAM-subfamily has two cytoplasmic PTP domains and an intracellular juxtamembrane region homologous to the intracellular domain of cadherin (Zondag *et al.,* 1995). There are four members in this subfamily, PTPRM, PTPRK, PTPRT and PTPRL, with just a few variations in the length of their exons. The number and phases of exon/intron junction are highly conserved. It is at the juxtamembrane domain where the biggest variability in genomic organisation and the majority of alternatively spliced exons are found. This domain is a critical region for PTPRK in the regulation of signal transduction. The phylogenetic analysis of complete sequences shows that PTPRM and PTPRT are the most closely related, followed by PTPRK. PTPRL is the most distantly related member in this family (Figure 1.6) (Besco *et al.*, 2004).

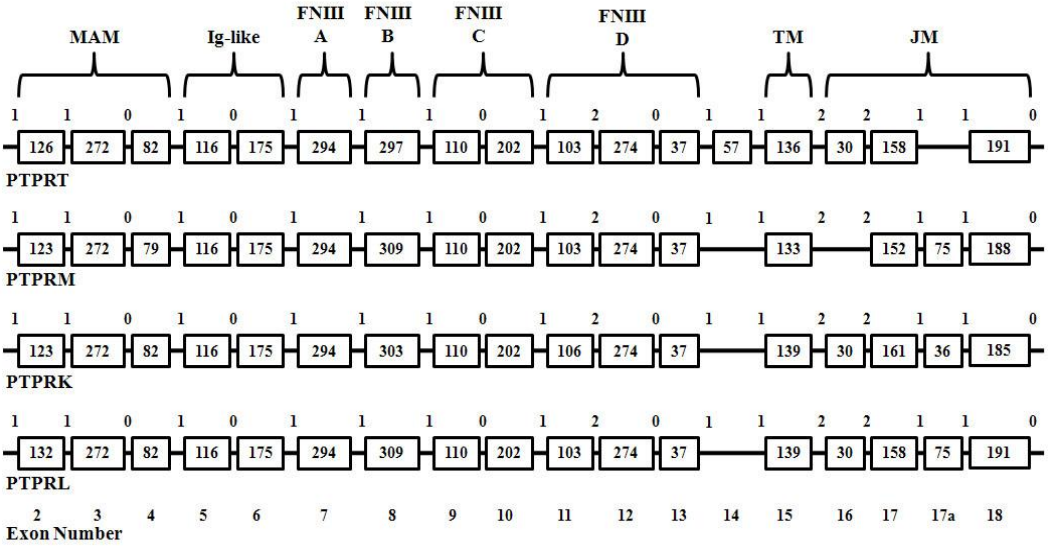


Figure 1.7 Exon sizes of the marine R2b subfamily extracellular and juxtamembrane domains. Boxed numbers indicate the number of nucleotides in each exon; interconnecting horizontal lines represent introns. The numbers between exons indicate intron phase. TM: transmembrane; JM: juxtamembrane. Adapted from (Besco *et al.*, 2004)

PTPRK is widely expressed in normal tissues such as spleen, prostate, ovary, brain, and has also been found in melanocyte and keratinocyte epidermal cell lines with a post-translational cleavage site in extracellular region. Moreover, PTPRK is not expressed in peripheral blood leukocytes and hematopoietic cell lines (Jiang *et al.,* 1993; McArdle *et al.,* 2005). Recent studies have shown that PTPRK is up-regulated by TGF-β and is probably involved in TGF-β dependent anti-proliferation and cell migration effects (Yang *et al.,* 1996). Furthermore, co-localisation of PTPRK with β-catenin at adhesion junctions has been revealed in human embryonic kidney cells (HEK293) which stabilises the E-cadherin/β-catenin complex. This suggests that β-catenin might be a substrate of PTPRK-mediated phosphatase activity. The stabilisation of the β-catenin/E-cadherin complex can suppress wnt/β-catenin-induced transcription of target genes such as cyclin D1 and c-Myc, leading to an inhibition of proliferation and migration in cancer cells. It suggests that PTPRK may be a potential tumour suppressor (Novellino *et al.,* 2008).

In addition, it has also been demonstrated that PTPRK can dephosphorylate EGFR or ErbB1 which belongs to PTKR (receptor protein-tyrosine kinase) superfamily. Abnormal regulation of EGFR has been shown to promote multiple tumourigenic processes by stimulating proliferation, angiogenesis and metastasis. EGFR and its ligands play important roles in some of the most common human cancers (Salomon *et al.,* 1995). It has been shown that PTPRK directly dephosphorylates EGFR *in vitr*o and that increased PTPRK expression decreases both basal and ligand-stimulated EGFR tyrosine phosphorylation. Reduced PTPRK expression leads to an increase in EGFR tyrosine phosphorylation (Xu *et al.,* 2005). Despite the aforementioned biochemical and

cellular functions for PTPRK, the influence that PTPRK may have on cancer and cancer development remains largely unknown. To date, some PTPs have already been shown to act as tumour suppressors, such as PTEN (MMAC1, is a tumour suppressor and is mutated in various human cancers) and PTPρ (PTPRT, potential tumour suppressor in colorectal cancers) (Tonks 2006). Recent studies have shown that PTPRK may possibly act as a putative tumour suppressor in primary central nervous system lymphomas (Nakamura *et al.,* 2003).

1.3.2.1 The *PTPRK* Gene

The mouse *PTPRK* gene maps to chromosome 10 and 19.0 centimorgans (cM) from the centromere and to be highly expressed in liver and kidney. The mouse PTPRK sequence has a 5' untranslated region (1,072 bp), an open reading frame (4,371bp) and a 3' untranslated region (338 bp). The translational initiation codon is identified by a standard environment for initiation of translation (Kozak 1983) and there is an upstream in-frame stop codon (position: -252) which is followed by a hydrophobic region that functions as a signal peptide. The second hydrophobic region is found from amino acid residues 753 to 774. This is followed by a series of predominantly basic residues, which function as a stop transfer sequence. These features show an extracellular region of 752 amino acid residues and an intracellular part of 683 amino acid residues, a PTP domain repeat. The structure is homologous to most PTPRs identified so far. A special feature of PTPRK is the extended distance between the transmembrane segment and the start of the first phosphatase homology domain. This segment is around 70 amino acid residue longer than any other PTPRs with PTPRM being the only exception (Gebbink *et al.,* 1991).The first 170 amino acids of PTPRK show 26% similarity to a region in *Xenopus*

neuronal cell surface protein A5 (position: 30-193). The A5 protein is a class I membrane protein containing two different internal repeats in the extracellular region and is expressed in retinal ganglion cells and visual centre neurons. It is homologous to complement components and coagulation factors in the blood. This protein has been shown to be involved in recognition between input and target neurons in the visual system (Takagi *et al.,* 1991). This domain is followed by an Ig-like domain (C2-type immunoglobulin domain) (position: 195-280) and four putative fibronectin type Ⅲ repeats (position: 290-688). These fibronectin type Ⅲ domains are similar to those in PTPRM (receptor- like protein tyrosine phosphatase μ), LAR (PTPRF, receptor- like protein tyrosine phosphatase F), *Drosophila* PTPRs DLAR and DPTP10D and Drosophila neuroglian (Streuli *et al.,* 1988; Bieber *et al.,* 1989; Streuli *et al.,* 1989; Gebbink *et al.,* 1991; Tian *et al.,* 1991; Yang *et al.,* 1991). The extracellular domain of PTPRK contains the HAV sequence (histidine, alanine and valine; position: 340-342 within the first fibronectin repeat.), a domain seen in most of the Type I cadherins and is central to the cell-cell contact formed by members of cadherin family (Blaschuk *et al.,* 1990). In addition, its extracellular domain contains the RTKR sequence (arginine-threonine-lysine-arginine; position: 640-643), which is the same cleavage site (RXK/RR, arginine-X-lysine/arginine-arginine) for processing by endoprotease furin and PC3. These endoproteases are mammalian homologues of the yeast precursor processing endoprotease Kex2, and are Ca^{2+}-dependent serine proteases. In endocrine cells, the sequence is only cleaved by PC3; however, in non-endocrine cells, furin enhances the precursor cleavage (Hosaka *et al.,* 1991). Other potential post-translational modification sites are the 12 potential N-linked glycosylation sites (position: 100, 139, 210, 415, 423, 435, 461, 551, 585, 589, 606 and 689) and SG (serine-glycine)

sequences which are candidates for chondroitin sulphate attachment (position: 172, 176, 277, 333 and 662) (Kjellen and Lindahl 1991). PTPRK shows a highly similar sequence to mouse PTPRM, with over 77% similarity in their amino acid sequences (Gebbink *et al.,* 1991). There is an 80% sequence similarity in the first PTP homology domain and 74% similarity in the second PTP homology domain (Jiang *et al.*, 1993).

The human *PTPRK* gene maps to chromosome 6 and locates to q22.2-23.3 and is predominantly expressed in the spleen, prostate, ovary and also expressed in the thymus, testis, small intestine and colon. Peripheral blood leukocytes are exceptional and express very little of this PTP. The human *PTPRK* gene has a 98% similarity to the murine *PTPRK* gene. The human *PTPRK* gene contains an open reading frame (4,317bp) coding 1,439 amino acids which includes an extracellular region (position: 27-752), a transmembrane region with a stretch of 22 hydrophobic amino acids (position: 753-774) and a cytoplasmic region (position: 775-1439). The extracellular region of human PTPRK contains a MAM domain (position: 31-194), an Ig-like C2-type domain (position: 196-281), four fibronectin domains (position: 291-680) and twelve potential N-glycoylation site (position: 101, 140, 211, 416, 424, 436, 462, 552, 586, 590, 607 and 690). These features are similar to the cell adhesion molecule (CAM) family. The cytoplasmic region contains two repeats of PTPs (position: 887-1141 and 1173-1435).

1.3.2.2 PTPRK Protein Structure

The molecular weight of the PTPRK protein is 210 kDa. Following posttranslational modifications, it is divided into two subunits, which are a secreted N-terminal 110 kDa

product including most of the extracellular domain and a 100 kDa product including the intracellular sequence and about 100 amino acid residues of extracellular sequence. The potential cleavage site was investigated using furin, a processing endopeptidase. It has been confirmed that the cleavage site, RTKR (position: 640-643) is located 113 amino acids upstream of the transmembrane sequence. This also shows that the RTKR region is the proteolytic cleavage signal for processing of the PTPRK proprotein (Jiang *et al.,* 1993).

The crystal structure of PTPRM D1 domain shows the subunits associate to form a dimer with two-fold structural symmetry. This dimerisation has been proposed to be a result of crystallisation because the protein are monomeric in solution. The dimer exhibits no obstruction to the catalytic site. However, the crystal structure of PTPRA has also been shown as a crystallographic dimer, their active sites occupied by a helix-turn-helix wedge motif of its dyad-related partner (Bilwes *et al.,* 1996) which blocks substrate access to the catalytic site. Although PTPRK belongs to the same subfamily of PTPRM, the PTPRK D1 domain is a monomer in both solution and crystal structure. Furthermore, the crystal structure of catalytically active, monomeric D1 domain of PTPRK is at 1.9Å. The structure of the PTPRK D1 domain and the same domain of other PTP family members has an overall classical PTP structure which comprises twisted mixed β-sheets flanked by α-helices in which the catalytic important WPD (Trp1049-Ala1058) loop is in an open conformation. As in all tyrosine phosphatases, the active site cysteine (Cys1083) is located in the well-conserved phosphate binding loop and an acetate molecule bound to it might reduce the phosphate binding opportunity (Eswaran *et al.,* 2006). In PTPRK, Ile1123 and Asn1124 are

present in the corresponding positions and Asn1124 seems to accommodate the phosphopeptides with a small amino acid in the pY+1 position. The N-terminal β-strand, formed by βx association with βy, is conserved between PTPRK, PTPRM, PTPRA and most of PTPRs. This feature has been suggested to distinguish D1 domain of RPTPs from the cytosolic PTPs and is conserved in the PTPRK structure forming a β-strand at the N-terminal (Bilwes *et al.,* 1996; Hoffmann *et al.,* 1997; Eswaran *et al.,* 2006).

1.3.2.3 Role of PTPRK in physiology

1.3.2.3.1 Role of PTPRK in cell growth/development

PTPRK has been indicated in T cell development. The rat *PTPRK* gene shares 98% similarity with the human and mouse *PTPRK* gene and it is expressed abundantly in the $CD4^-$ $CD8^-$ double-negative stage. Reduced PTPRK expression using RNA interference method has shown a similar phenotype of T helper immunodeficiency which leads to T cell mutation from CD4 and CD8 double-positive to CD4 single positive cells in the thymus (Kose *et al.,* 2007). Furthermore, there is an approximate 380 kb deletion in chromosome 1 of LEC rat (which has a spontaneous mutation in the T helper immunodeficiency) where the *PTPRK* gene is located. It is noted that PTPRK plays a critical role in CD4 single-positive development in thymus (Asano *et al.,* 2007). Most importantly, inhibition of PTPRK suppresses ERK1/2 phosphorylation in T cell hybridoma. PTPRK regulated $CD4^+$ T cell via ERK1/2 signalling pathway and ERK1/2 activity is crucial for CD4 and CD8 T cell. Inhibition of PTPRK suppresses ERK1/2 phosphorylation in T cell hybridoma. ERK1/2 is phosphorylated by MEK1/2. Moreover, PTPRK affects c-Raf (MAP3 kinase) phosphorylation which generally phosphorylates MEK1/2. It has been shown that PTPRK can directly dephosphorylate β-catenin and

EGFR. Although EGFR signalling affects the ERK1/2 pathway, there is no evidence showing EGFR to be involved in the regulation of $CD4^+$ T cell development (Erdenebayar *et al.,* 2009). In addition, PTPRK stimulates growth factor receptor bound-2 (Grb2), a multi-purpose adapter molecule and its down-stream MAPK cascades (Ras/Raf/MEK/ERK) to promote neurite outgrowth from cerebellar neurons whilst EGFR is not required in this event (Drosopoulos *et al.,* 1999).

As already discussed in previous sections, the transforming growth factor-β (TGF-β) family plays an important role in a variety of physiological functions including cell proliferation, adhesion, differentiation, morphogenesis, motility, tissue repair and other functions in many different types of cells. In fibroblasts and epithelial cells, TGF-β1 shows a reversible growth arrest. However, in mesenchymal cells it increases cell proliferation and also up-regulates the expression of several extracellular matrix components and protease inhibitors. Human keratinocytes require EGF and TGF-α for proliferation, whilst TGF-β1 exhibits an inhibitory effect (Shipley *et al.,* 1986; Coffey *et al.,* 1988). Keratinocytes treated with TGF-β1 have a decreased expression of c-myc gene transcript (Pietenpol *et al.,* 1990). Additionally, TGF-β1 inhibits cell growth *via* phosphodiesterase-mediated hydrolysis of phosphatidylcholine (Diaz-Meco *et al.,* 1992). Moreover, TGF-β1 inhibits proliferation of hamster lung fibroblast in which protein kinase C and polyphosphoinositide breakdown are not involved (Chambard and Pouyssegur 1988). TGF-β1 also induces PTPRK mRNA expression in HaCaT cells, leading to an inhibition of proliferation of the human keratinocyte cell line. This can be suppressed by sodium orthovanadate (tyrosine phosphatase inhibitor) and H7 (serine/threonine kinase inhibitor) but not genistein (tyrosine kinase inhibitor) (Yang *et*

al., 1996). RNA interference (RNAi) of PTPRK can accelerate cell cycle progression and enhance the response to EGF-induced EGFR tyrosine phosphorylation which leads to increased downstream ERK activation and abrogates TGF-β-mediated anti-mitogenesis effect. Activation of EGFR is necessary for the induction of signalling pathways including mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K)/ Akt and phospholipase C/ protein kinase C (PKC). Endogenous PTPRK associated with EGFR and HER2 (ErbB2), leading to the suppression of basal and ErbB ligand-induced proliferation and receptor phosphorylation (Wang *et al.,* 2005; Xu *et al.,* 2005). Moreover, activation of EGFR can prevent UV-induced apoptosis *via* activation of PI3K/ Akt pathway in which PTPRK plays an important role in the antioxidant action by directly interacting with EGFR (Xu *et al.,* 2006). PTPRK expression is reduced in EBV (Epstein-Barr virus) infected Hodgkin lymphoma cell lines due to the expression of EBNA1 (Epstein-Barr nuclear antigen-1) and decreased SMAD2 protein expression. EBV infection presses TGF-β induced expression of PTPRK which leads to an increase of in cell proliferation and survival. It has thus been suggested that PTPRK is a putative tumour suppressor in Hodgkin lymphoma (Flavell *et al.,* 2008).

PTPRK, an N-linked glycoprotein, has been identified as a substrate of N-Acetylglucosaminyltransferase V (GnT-V) in colon cancer (Kim *et al.,* 2006). GnT-V overexpression does not only change the glycosylation of PTPRK but also decreases its protein expression. PTPRK is involved in the regulation of β-catenin signalling and downstream target genes such as c-myc and cyclin D1 which may promote the proliferation of human hepatoma SMMC-7721 cells (Wang *et al.,* 2010). However, in

melanoma cells, PTPRK exhibits an inhibition of β-catenin protein expression and a disruption of the β-catenin/Tcf-mediated cyclin D1 expression, resulting in a reduction of cell proliferation (Novellino *et al.,* 2008).

1.3.2.3.2 Role of PTPRK in cell-cell/cell-matrix adhesion

The extracellular region of PTPRK contains one Ig-like domain, four fibronectin type Ⅲ repeats and a MAM domain. A combination of fibronectin type Ⅲ repeats and Ig-like domains are often highly expressed in epithelial and neuronal tissues and PTPRK behaves similarly to members of the N-CAM family. Some proteins of the Ig superfamily can mediate both homophilic and heterophilic interactions by the presence of MAM domain. However, it is very interesting to note that PTPRK fails to interact with PTPRM, which belongs to the same subfamily of PTPs. PTPRK only promotes homophilic interactions such as cell aggregation and cell-cell adhesion. Furthermore, it has been reported that PTPRK-mediated intercellular adhesion does not need PTPs activity or post-translational modification of PTPRK protein and it is also calcium independent (Sap *et al.,* 1994; Brady-Kalnay and Tonks 1995; Zondag *et al.,* 1995).

GnT-V overexpression down-regulates cell calcium-independent adhesion and increases the tyrosine phosphorylation of β-catenin (Wang *et al.,* 2010). The increased tyrosine phosphorylation level of β-catenin can lead to a decrease of cadherin-bound β-catenin, and an increase of β-catenin in the cytoplasmic and nuclear pool (Gujral *et al.,* 2008). β-catenin in the nuclear pool has been found to serve as a key component in the Wnt pathway in which it interacts with Tcf (T-cell-specific transcription factor 1)/ Lef (lymphoid enhancer factor) family to activate target genes such as, c-myc and cyclin D1

(He *et al.,* 1998; Tetsu and McCormick 1999). PTPRK can negatively regulate β-catenin transcriptional signalling in melanoma cells. PTPRK is also able to re-locate β-catenin/E-cadherin complexes to cytoskeleton-linked phase alongside cell-cell contact surface and to inhibit cytosolic and nuclear accumulation of both wild type β-catenin and the oncogenic form of β-catenin (Novellino *et al.,* 2008).

1.3.2.3.3 Role of PTPRK in cell motility

Previous studies have shown that excess production and activation of TGF-β in tumour cells can accelerate cancer progression by a combination of autocrine and paracrine mechanisms which include enhancement of tumour cell motility and survival, increase in tumour angiogenesis, production of extracellular matrix and peritumoral proteases and inhibition of immune responses in tumour host (Derynck *et al.,* 2001; Wakefield and Roberts 2002). It has been reported that SHP-1 and SHP-2 are activated by TGF-β to regulate T cell functions and PTPRK is also activated by TGF-β to reduce keratinocytes cell proliferation (Yang *et al.,* 1996; Choudhry *et al.,* 2001). Moreover, TGF-β pathway not only regulates cell proliferation but also other cell functions. The negative effect of TGF-β-induced PTPRK expression on Src and HER2 signalling plays a profound role in coordinating the cell motility and adhesion (Wang *et al.,* 2005; Kim *et al.,* 2006).

Overexpression of GnT-V induces an addition of β1, 6GlcNAc branch to N-glycan of PTPRK and also decreases the protein level of PTPRK. This change of PTPRK leads to an increased phosphorylayion of EGFR at tyrosine 1068 and subsequently increased phosphorylation of ERK and FAK which results in an increase in cell migration.

Glycosylation of PTPRK tends to be aberrant in the GnT-V overexpressed cells and glycosylated PTPRK is easy to be broken by proteolytic cleavage. All these events would lead to an enhanced cleavage of PTPRK and reduced capacity of the homophilic binding which eventually results in an increase in cancer cell migration. (Wang *et al.*, 2009).

1.3.2 PTP family in cancer/disease

There have been overwhelming reports on the abnormalities of a number of the PTP family members in human cancer, ranging from gene mutations, abnormalities in gene expression at message and proteins levels. Tables-1.2 to 1.4 summarise some of the key observations in various human cancers. The following sections discuss some of the most interesting members of this family in cancer.

1.3.2.1 PTPs and apoptosis

Apoptosis, also known as programmed cell death, can be induced by both internal and external pathways. TNFs (tumour necrosis factors) and their receptors trigger apoptosis via the external pathway and its down-stream transcriptional factors are also involved in the internal pathway of apoptosis. The Bcl-2 superfamily plays a crucial role in the control of apoptotic signalling in mitochondria. Moreover, Bcl-2 and anti-apoptotic family proteins such as the Bax/ Bak subfamily promote cell survival. The Bax/ Bak subfamily is able to mediate apoptotic signalling and also help to release the pro-apoptotic factors such as cytochrome *c* and Smac/ Diablo, from mitochondria (Adams 2003; Roset *et al.*, 2007).

The intrinsic mitochondria pathway of apoptosis is regulated by several molecules, for example XIAP (inhibitors of apoptosis) and the Bcl-2 family. Activation of Bcl-2 family members leads to mitochondrial outer membrane permeabilisation; cytochrome *c* is released and bound with APAF-1 and caspase-9 to form an apoptosome (Siu *et al.,* 2008). After apoptosome formation, caspase-9 is activated and there is also regulation of down-stream molecules such as caspase-3 (Ohtsuka *et al.,* 2003). Tumour cells can inhibit apoptosis by regulation of anti-apoptotic mechanisms such as upregulation of the apoptotic suppressors Bcl-2 and Bcl-XL and downregulation of the caspase cascade (Elmore 2007). The extrinsic pathway involves the promotion of apoptosis *via* the ligand-activation of death receptors (Wallach *et al.,* 2008). For example, TNF-α binding to its receptors leads to the formation of two complexes; one can lead to activation of the pro-survival NF-κB pathway and another one activates the apoptosis pathway through FADD (Fas associated death domain) and activation of caspase-8 (Wajant and Scheurich 2011).

PTPs deficiency leads to several physiologic abnormalities such as embryonic developmental defect (PTP-PEST), impairment of the immune system (TC-PTP) and inhibition of tumourigenesis and metastasis (PTP1B) (Sirois *et al.,* 2006; Bourdeau *et al.,* 2007; Julien *et al.,* 2007). Dysregulation of apoptosis by PTP deficiency may play a profound role in these disorders. PTP-PEST makes cells more sensitive to anti-Fas and TNFα induced apoptosis. PTP-PEST is cleaved by caspase-3 which increases its catalytic activity and changes its protein structure. Furthermore, PTP-PEST proteolysis facilitates cellular detachment during apoptosis (Halle *et al.,* 2007). Unlike PTP-PEST, PTP1B has no caspase cleavage site and is not cleaved by caspases during apoptosis.

However, there is a report showing activated PTP1B contributes to STAT3 dephosphorylation and induces apoptosis in human glioma cells (Akasaki *et al.,* 2006; Halle *et al.,* 2007). PTP1B and its catalytic activity are required for IRE1 (inositol-requiring kinase 1) signalling which activates JNK and p38 MAPK, XBP-1 (X-box-binding protein 1) splicing, and EDEM (ER degradation-enhanced α-mannosidase protein) transcription. Moreover, ER-induced apoptosis is decreased in cells lacking PTP1B and PTP1B null mice are resistant to Fas-induced liver damage because of absence of PTP1B-mediated suppression of pro-survival NFκB and ERK signalling (Gu *et al.,* 2004; Sangwan *et al.,* 2006). In addition, p53 is required for TC-PTP overexpression induced apoptosis. In MCF-7 breast cancer cells, the accumulation of p53 in response to TC-PTP overexpression directly increases the expression of Apaf-1 and the pro-apoptotic α-isoform of caspase-1 (Radha *et al.,* 1999; Gupta *et al.,* 2002). SHP-1 disrupts anti-apoptotic pathway through the regulation of Lyn, Tyk2 and PI3K upon death receptor stimulation. In addition, SHP-1 favours Bim/ Bod expression and *c*-jun phosphorylation, resulting in increased apoptosis. However, SHP-1 dephosphorylates TrkA which in turn inhibits NGF-mediated PLC1 and Akt phosphorylation, thus producing a reduced TrkA survival signal (Marsh *et al.,* 2003; Yousefi and Simon 2003).

1.3.2.2 PTPs and cancers

Tyrosine and serine/threonine phosphorylation are important in the regulation of biological functions and is mainly regulated by kinases and phosphatases. Many phosphatases have been reported to participate in the regulation of cancer development. Furthermore, it has been shown that some phosphatases are tumour suppressors, such as

PTEN (deletion in many cancers) and PTPRH (down-regulation in hepatocellular carcinoma). However, some of them are thought to act as proto-oncogenes/oncogenes, for example SHP2 (leukaemia) and PTPRF (over-expression in thyroid and breast cancers). Other examples will be discussed in the following sections.

1.3.2.2.1 PTPRJ

PTPRJ (DEP1) was initially identified as a potential tumour suppressor in breast cancer. This was demonstrated when re-expression of PTPRJ in breast cancer cells resulted in a five- to ten-fold reduction of cell proliferation. Similar findings in pancreatic, thyroid and colon cancers were reported later (Keane *et al.*, 1996; Trapasso *et al.*, 2000; Iuliano *et al.*, 2003; Trapasso *et al.*, 2004).

Loss of heterozygosity, occurring in the absence of acquired mutation in the remaining allele has been found in breast, colon, lung and thyroid cancers, implying that PTPRJ alone may not be sufficient as a transforming mechanism in cancerous cells. According to those reports, different allelic variants of PTPRJ have been identified, which result in different structures in the extracellular domain of PTPRJ variants. In colon cancer, analyses of tumours of heterozygous patients indicated that the preferential loss of one of those alleles (Q276 of the Q276P polymorphism) occurred during tumour growth, suggesting functional differences may exist between these alleles (Ruivenkamp *et al.*, 2002; Ruivenkamp *et al.*, 2003; Iuliano *et al.*, 2004; van Puijenbroek *et al.*, 2005). However, similar experiment in patients with thyroid cancer failed to detect any loss of the Q276 allele. The D872allele (D872E polymorphism) has been detected more frequently in patients with thyroid carcinoma. The D872 variant may increase the

ability to form dimmers resulting in an inhibition of PTP function (Iuliano *et al.*, 2004). Furthermore, a significant association has been demonstrated between breast cancer risk and the PTPRJ haplotype which does not require Q276P allele (Lesueur *et al.*, 2005).

Various signalling pathways have been implicated in the function of PTPRJ. PTPRJ plays an antagonistic role in growth factor signalling pathways; it can directly dephosphorylate PDGFR, VEGFR and cMET (Kovalenko *et al.*, 2000; Grazia Lampugnani *et al.*, 2003; Palka *et al.*, 2003). In addition, PTPRJ substrates including Src, p120 catenin and PLCγ, have been indicated in its tumour suppressing action. Another tumour suppressing mechanism of PTPRJ is that it promotes G1 phase arrest via acyclin-dependent-kinase-inhibitor p27. Additionally, a pro-apoptotic effect has also been indicated in the anti-cancer functions of PTPRJ (Holsinger *et al.*, 2002; Trapasso *et al.*, 2004; MacKeigan *et al.*, 2005).

1.3.2.2.2 PTPRA

PTPRA (PTPα) is a member of the transmembrane subfamily of PTPs and is widely expressed in various human tissues. PTPRA participates in the regulation of several pathways controlling cell proliferation and differentiation. For example, PTPRA can down-regulate insulin-increased prolactin gene activity via a dephosphorylation of insulin receptor (Jacob *et al.*, 1998). Over-expression of PTPRA in rat embryo fibroblasts leads to activation of ERK and -Jun (Zheng and Pallen 1994). Therefore, PTPRA plays a critical role in the proliferation and differentiation of different cell types.

PTPRA may have an oncogenic role in gastric cancer progression by promoting

migration and anchorage-independent cell growth, an effect achieved by dephosphorylation of proteins that are involved in these processes (Wu *et al.,* 2006). PTPRA is required for the activation of SFKs (Src family kinases) following integrin ligation, an event necessary for adhesion to occur. PTPRA binds to integrin α5β3 and regulates integrin signalling and cell migration via the dephosohorylation of Src on pY527, which increases the association of Src with FAK (Harder *et al.,* 1998). PTPRA can also potently transform mouse fibroblasts (Zheng *et al.,* 1992). Furthermore, over-expression of PTPRA has been found in late-stage human colon carcinoma and breast cancer (Tabiti *et al.,* 1995; Ardini *et al.,* 2000). However, it has been demonstrated that PTPRA over-expression is correlated with low tumour grade and positive oestrogen-receptor expression. PTPRA has also been shown to inhibit transformation of breast cancer cells associated with increasing accumulation in G0 and G1 phases leading to a delayed tumour growth and metastasis (Ardini *et al.,* 2000).

1.3.2.2.3 PTEN

PTEN (phosphatase with tensin homology) is a recognised tumour suppressor in many cancers and it has been identified as a gene mutated in many tumour types. PTEN is a dual specificity phosphatase; it not only has dephosphorylated tyrosine but also dephosphorylated serine/threonine residues (Myers *et al.,* 1997). PTEN has been shown to act as a tumour suppressor by negatively regulating the PI3K pathway, an important route for the regulation of cell proliferation and cell survival (Stambolic *et al.,* 1998).

There are two important subunits of PI3K required for activation of the downstream Akt signalling pathway (to protect cell from apoptosis and increase cell proliferation), which are a 85 kDa regulatory subunit (p85 subunit) and a 110 kDa catalytic subunit (p110

subunit). There are a number of ways for PI3K to activate downstream pathways. The first is direct binding of the p85 subunit to phospho-YXXM motifs of the RTK which then activates the p110 subunit. Another route requires an adaptor protein, GRB2 (Growth Factor Receptor-bound protein 2) for binding to the RTK phosphor-YXN motifs and then GRB2 binds to the scaffolding protein GAB (GRB2-associated binding protein) which subsequently binds to the p85 subunit (Domchek *et al.,* 1992; Ong *et al.,* 2001; Pawson 2004). The p110 subunit of PI3K can be activated by interaction with Ras (Rodriguez-Viciana *et al.,* 1994). In endometrial tumours and melanoma cell lines, mutations of Ras and PTEN genes are mutually exclusive. In comparison wit PTEN mutations, Ras mutations are more commonly seen in pancreatic, lung and colon cancers. Such mutations have seldom been found in glioblastomas. In the mouse model of chemically-induced skin carcinogenesis, Ras mutations are increased in $PTEN^{+/+}$ mice and decreased in $PTEN^{+/-}$ mice. Tumours that lack Ras mutations tend to lose the wild-type PTEN allele. It has been suggested that Ras activation and loss of PTEN may have a similar impact during tumorigenesis (Liu *et al.,* 1997; Ikeda *et al.,* 2000; Tsao *et al.,* 2000; Simpson and Parsons 2001; Downward 2003; Mao *et al.,* 2004).

The tumour suppressor p53 activates PTEN and functions as a negative regulator of the PI3K signalling pathway (Stambolic *et al.,* 2001). Down-regulation of the PI3K pathway by p53 activation is due to p53-mediated transcriptional inhibition of the gene encoding the p110 subunit of PI3K. Transcriptional regulation of the PI3K pathway by p53 eliminates PI3K-mediated cell survival and proliferation and provides additional protection for DNA replication in the presence of genotoxic stress (Singh *et al.,* 2002). p53 mutation is one of the most common mutations in human cancer and the p53 apoptotic response requires the down-regulation of the PI3K pathway via the

transcriptional activation of PTEN (Stambolic *et al.*, 2001). Meanwhile, inhibition of the PI3K pathway and activation of apoptosis by p53 downstream signalling might have synergistic effects. Moreover, drug induced activation of p53 or Ras may also synergistically increase the anti-tumour effects of drugs that target PI3K or TOR (Cully *et al.*, 2006).

Table 1.3 Receptor-like PTPs and Cancers.

Receptor-like PTPs			
PTPs	**Negative regulator**	**Positive regulator**	**Reference**
PTPRA	Breast$^{\downarrow}$	Colorectal$^{\uparrow}$, head and neck$^{\uparrow}$, gastric$^{\uparrow}$	(Tabiti *et al.*, 1995; Berndt *et al.*, 1999; Ardini *et al.*, 2000; Wu *et al.*, 2006)
PTPRB		Glioma$^{\uparrow}$, gastric$^{\uparrow}$	(Foehr *et al.*, 2006; Wu *et al.*, 2006; Hagerstrand *et al.*, 2008)
PTPRD	Glioma*, melanoma*, colorectal$^{\downarrow}$, liver*, head and neck*, lung*		(Nair *et al.*, 2008; Solomon *et al.*, 2008; Veeriah *et al.*, 2009; Laczmanska and Sasiadek 2011; Nalesnik *et al.*, 2012)
PTPRF	Colorectal*	Thyroid$^{\uparrow}$, breast$^{\uparrow}$	(Yang *et al.*, 1999; Levea *et al.*, 2000; Konishi *et al.*, 2003; Laczmanska and Sasiadek 2011)
PTPRG	Head and neck*, breast$^{\downarrow}$, ovarian$^{\downarrow}$, lung$^{\downarrow}$, gastric, lymphoma	Glioma$^{\uparrow}$, gastric$^{\uparrow}$, lymphoma$^{\uparrow}$	(van Niekerk and Poels 1999; Liu *et al.*, 2002; Wang *et al.*, 2004; van Doorn *et al.*, 2005; Vezzalini *et al.*, 2007; Wang and Dai 2007; Cheung *et al.*, 2008)
PTPRH	Liver$^{\downarrow}$	Gastrointestinal$^{\uparrow}$, colorectal$^{\uparrow}$	(Matozaki *et al.*, 1994; Seo *et al.*, 1997; Nagano *et al.*, 2003)
PTPRJ	Breast*, lung*, colorectal, thyroid		(Ruivenkamp *et al.*, 2002; Iuliano *et al.*, 2004; Ding *et al.*, 2010; Mita *et al.*, 2010)
PTPRK	Melanoma$^{\downarrow}$, lymphoma		(Zhang *et al.*, 1998; McArdle *et al.*, 2001; Nakamura *et al.*, 2003)
PTPRM	Glioma$^{\downarrow}$, melanoma$^{\downarrow}$	Ovarian	(McArdle *et al.*, 2001; Gyorffy *et al.*, 2008; Burgoyne *et al.*, 2009; Burgoyne *et al.*, 2009)
PTPRO	Breast, lung, leukaemia, colorectal		(Mori *et al.*, 2004; Motiwala *et al.*, 2004; Motiwala *et al.*, 2007; Motiwala *et al.*, 2009; Ramaswamy *et al.*, 2009)
PTPRS	Colorectal*		(Korff *et al.*, 2008)
PTPRT	Colorectal*, gastric*, lung*		(Wang *et al.*, 2004)
PTPRZ		Glioma$^{\uparrow}$, melanoma$^{\uparrow}$, gastric$^{\uparrow}$	(Goldmann *et al.*, 2000; Ulbricht *et al.*, 2003; Foehr *et al.*, 2006; Wu *et al.*, 2006; Hagerstrand *et al.*, 2008)

*: deletion or mutation of gene. $^{\downarrow}$: expression decreased. $^{\uparrow}$: expression increased.

Table 1.4 Non-transmembrane PTPs and Cancers.

Non-transmembrane PTPs			
PTPs	**Negative regulator**	**Positive regulator**	**Reference**
PTPN1	Oesophageal$^{\downarrow}$	Breast$^{\uparrow}$, ovarian$^{\uparrow}$, gastric, pancreatic, prostate$^{\uparrow}$	(Wiener *et al.*, 1994; Wiener *et al.*, 1994; Warabi *et al.*, 2000; Mahlamaki *et al.*, 2002; Yang *et al.*, 2005; Wu *et al.*, 2006)
PTPN2	T cell acute lymphoblastic leulaemia*		(Kleppe *et al.*, 2010)
PTPN3	Breast*, lung*, colorectal*	Gastric$^{\uparrow}$, oesophageal$^{\uparrow}$	(Warabi *et al.*, 2000; Wang *et al.*, 2004; Wu *et al.*, 2006)
PTPN5	Colorectal*		(Korff *et al.*, 2008)
PTPN6	Gastric, prostate, lymphoma, myeloma, leukaemia	Ovarian$^{\uparrow}$	(Oka *et al.*, 2002; Zapata *et al.*, 2002; Chim *et al.*, 2004; Reddy *et al.*, 2005; Cariaga-Martinez *et al.*, 2009; Ksiaa *et al.*, 2009)
PTPN7	Lymphoma$^{\downarrow}$	Leukaemia$^{\uparrow}$	(Zanke *et al.*, 1994; Fridberg *et al.*, 2008)
PTPN11	Liver$^{\downarrow}$	Leukaemia*	(Chan *et al.*, 2008; Bard-Chapeau *et al.*, 2011)
PTPN12	Breast*, kidney*		(Streit *et al.*, 2006)
PTPN13	Breast$^{\downarrow}$, colorectal*, lung, liver, lymphoma		(Lee *et al.*, 1999; Wang *et al.*, 2004; Yao *et al.*, 2004; Yeh *et al.*, 2006; Ying *et al.*, 2006; Korff *et al.*, 2008; Revillion *et al.*, 2009; Mita *et al.*, 2010)
PTPN14	Breast*, lung*, gastric*, colorectal*		(Wang *et al.*, 2004)
PTPN18	thyroid		(Guimaraes *et al.*, 2006)
PTPN21	Colorectal*	Colorectal*	(Korff *et al.*, 2008)

*: deletion or mutation of gene. $^{\downarrow}$: expression decreased. $^{\uparrow}$: expression increased.

Table 1.5 Dual specificity phosphatases and Cancers.

Dual-specificity phosphatases			
PTPs	**Negative regulator**	**Positive regulator**	**Reference**
PTP4A1		Pancreatic$^{\uparrow}$, oesophageal$^{\uparrow}$, colorectal$^{\uparrow}$	(Wang *et al.*, 2007; Liu *et al.*, 2008; Stephens *et al.*, 2008)
PTP4A2		Pancreatic$^{\uparrow}$, colorectal$^{\uparrow}$	(Wang *et al.*, 2007; Liu *et al.*, 2008; Stephens *et al.*, 2008)
PTP4A3		Head and neck$^{\uparrow}$, oesophageal$^{\uparrow}$, breast$^{\uparrow}$, gastric$^{\uparrow}$, ovarian$^{\uparrow}$, lung$^{\uparrow}$, liver$^{\uparrow}$, colorectal$^{\uparrow}$, glioma$^{\uparrow}$, myeloma$^{\uparrow}$	(Saha *et al.*, 2001; Bardelli *et al.*, 2003; Kato *et al.*, 2004; Peng *et al.*, 2004; Wang *et al.*, 2006; Kong *et al.*, 2007; Miskad *et al.*, 2007; Fagerli *et al.*, 2008; Mollevi *et al.*, 2008; Zhao *et al.*, 2008; Ren *et al.*, 2009; Xing *et al.*, 2009; Zhou *et al.*, 2009)
DUSP1	Liver$^{\downarrow}$, ovarian$^{\downarrow}$, prostate	Nead and neck$^{\uparrow}$, breast$^{\uparrow}$, grastric$^{\uparrow}$, pancreatic$^{\uparrow}$, bladder$^{\uparrow}$, ovarian$^{\uparrow}$, prostate$^{\uparrow}$, lung$^{\uparrow}$, colorectal$^{\uparrow}$	(Loda *et al.*, 1996; Magi-Galluzzi *et al.*, 1997; Bang *et al.*, 1998; Denkert *et al.*, 2002; Manzano *et al.*, 2002; Liao *et al.*, 2003; Wang *et al.*, 2003; Vicent *et al.*, 2004; Rauhala *et al.*, 2005; Tsujita *et al.*, 2005; Colombo *et al.*, 2009)
DUSP2	Leukaemia$^{\downarrow}$	ovarian$^{\uparrow}$	(Kim *et al.*, 1999; Givant-Horwitz *et al.*, 2004)
DUSP3		cervical$^{\uparrow}$	(Henkens *et al.*, 2008)
DUSP4	Breast*	Breast$^{\uparrow}$	(Wang *et al.*, 2003; Armes *et al.*, 2004)
DUSP6	Pancreatic, ovarian$^{\downarrow}$		(Furukawa *et al.*, 2003; Xu *et al.*, 2005)
DUSP7		leukaemia$^{\uparrow}$	(Levy-Nissenbaum *et al.*, 2003; Levy-Nissenbaum *et al.*, 2003)
DUSP16	Prostate*		(Kibel *et al.*, 2004)
DUSP26	Glioma$^{\downarrow}$	thyroid	(Yu *et al.*, 2007; Tanuma *et al.*, 2009)
CDKN3	Glioma	Breast$^{\uparrow}$, prostate$^{\uparrow}$, liver*	(Lee *et al.*, 2000; Yeh *et al.*, 2000; Yu *et al.*, 2007)
MTMR6		leukaemia$^{\uparrow}$	(Vallat *et al.*, 2003)

*: deletion or mutation of gene. $^{\downarrow}$: expression decreased. $^{\uparrow}$: expression increased.

1.4 Aims and objectives

Cancer metastasis is a complicated and fatal problem. Although there have been an increasing number of studies with a focus on cancer metastasis, understanding of the essential molecular and cellular mechanisms involved remains poor. Metastasis is a multi-stage process involving EMT, angiogenesis, cell invasion/migration, intravasation, extravasation and organ colonisation. To accomplish metastasis, many signalling pathways are involved as they participate in the regulation of different cell functions. Such pathways are the PI3K, ERK, EGF and HGF pathways, etc. kinases and phosphatases also play crucial roles in the regulation of these pathways.

The PTPs family is a group of these key regulators which can dephosphorylate the target molecules and inactivate certain critical pathways involved in cancer progression. PTPRK is one member of this family and has been indicated as a possible tumour suppressor in primary central nervous system lymphomas and colorectal cancer. Recent studies have shown that PTPRK participates in both TGF-β and EGFR pathways in the regulation of cell growth. Furthermore, PTPRK also performs homophilic interaction to promote cell aggregation and cell-cell adhesion. PTPRM belongs to the same subfamily with PTPRK and has been indicated regulation of cell migration via PLCγ in glioma. The implication of PTPRK and PTPRM in prostate and breast cancer remains unknown.

Therefore, this study determined to characterise the role played by PTPRK and PTPRM in the development and disease progression of prostate and breast cancer.

The aims of this study were:

1. To screen and identify the expression profile of PTPRK and its associated down-stream molecules in different prostate cancer cell lines.

2. To construct hammer-head ribozymze targeting human PTPRK and to establish PTPRK knockdown cell lines for examining the influence of PTPRK on the biological functions of cancer cells.

3. To understand how PTPRK regulates apoptosis in prostate cancer, such as the expression of the apoptosis relevant genes in the PTPRK knockdown cells and to elucidate the potential pathways involved in the effect by PTPRK using small inhibitors.

4. To screen and identify the expression profile of PTPRK and PTPRM in breast cancer cell lines and breast cancer tissues and carry out a clinical cohort study.

5. To establish PTPRK or PTPRM knockdown in breast cancer cells and to investigate the *in vitro* cell functions and possible downstream signalling pathway of cancer metastasis involved in the effect by PTPRK or PTPRM knockdown in breast cancer.

Chapter 2

Methodology

2.1 Materials

2.1.1 Cell lines

The current study used seven prostatic cell lines, two breast cancer cell lines and one fibroblast cell line. PC-3, MRC5, MDA-MB-231 and MCF-7 were acquired from the European Collection of Animal Cell Culture (ECACC, Salisbury, UK). DU-145, CA-HPV-10, LNCaP, PZ-HPV-7, BT474 and BT482 were all obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA) and PNT-1A and PNT-2C2 were generously given by Professor Norman Maitland (University of York, England, UK). Full details of these cell lines are supplied in Table 2.1. Cells were maintained in DMEM-F12 Media supplemented with 10% foetal bovine serum (FBS) and 1X antibiotics.

2.1.2 Collection of human prostate and breast cancer tissues

Fresh tissue samples were collected immediately after surgery and stored at -80℃ until use, with approval of the Bro Taf Health Authority local research ethics committee. All patients were informed and participated with written consent. All the specimens were verified by a consultant pathologist. Breast cohort study please refers to section 2.9.

2.1.3 Primers

All the primers used in the current study were designed with the use of the Beacon Design Programme (Biosoft International, Palo Alto, California, USA) and were synthesised by either by Invitrogen (Paisley, UK) or Sigma (Poole, Dorset, UK). Details of the primers used for PCR and qPCR are provided in Table 2.2. Details of the primers used for generating hammerhead ribozymes are found in Table 2.3.

Cell line	Origin	Cell morphology	Tissue type	Metastatic site	Hormone seceptors
PC-3	62 year old Caucasian male	Epithelial	Adenocarcinoma (highly tumourigenic)	Bone	Androgen receptor negative
DU-145	69 year old Caucasian male	Epithelial	Carcinoma	Brain	Androgen receptor negative
LNCaP	50 year old Caucasian male	Epithelial	Carcinoma	Left supraclavicular lymph node	Androgen and Estrogen receptor positive
CA-HPV-10	63 year old Caucasian male	Epithelial	Non-tumourigenic cell line derived from prostatic adenocarcinoma, transformed HPV18 transfection		Androgen receptor negative
PZ-HPV-7	70 year old Caucasian male	Epithelial	Non-tumourigenic epithelial cell line transformed through HPV18 transfection		
PNT-1A	35 year old Caucasian male	Epithelial	Normal prostatic epithelial cell line, transfected and immortalised with SV40 DNA with a defective replication origin.		Androgen receptor positive
PNT-2C2	33 year old Caucasian male	Epithelial	Normal prostatic epithelial cell line, immortalised with SV40 DNA		Androgen receptor negative
MRC-5	14 weeks gestation Caucasian male	Fibroblast	Normal lung fibroblast cell line		
MDA-MB-231	51 year old Caucasian female	Epithelial	Adenocarcinoma (tumourigenic)	pleural effusion	Extrogen receptor negative
MDA-MB-436	43 year old Caucasian female	Epithelial	Adenocarcinoma (non-tumourigenic)	pleural effusion	Extrogen receptor negative
MCF-7	69 year old Caucasian female	Epithelial	Adenocarcinoma	pleural effusion	Extrogen receptor positive
BT474	60 year old Caucasian female	Epithelial	ductal carcinoma (tumourigenic)		Extrogen receptor positive
BT482	23 year old Caucasian female	Epithelial	ductal carcinoma (tumourigenic)		Extrogen receptor positive

Table 2.1 Details of cell lines used in this study.

Table 2.2 Primers for conventional RT-PCR and Q-PCR (Primers were designed using Beacon Design programme (Palo Alto, California, USA) and were synthesised by Invitrogen (Paisley, UK) and Sigma (Poole, Dorset, UK)).

Gene	**Primer name**	**Primer sequence (5'-3')**	**Product size (bp)**
GAPDH	GAPDHF8	GGCTGCTTTTAACTCTGGTA	475
	GAPDHR8	GACTGTGGTCATGAGTCCTT	
GAPDH	GAPDHF2	CTGAGTACGTCGTGGAGTC	93
	GAPDHZR2	**ACTGAACCTGACCGTACA**CAGAGATGATGACCCTTTTG	
PTPRK	PTPRKF11	AATTACAATTGATGGGGAGA	427
	PTPRKR11	CCACTTTTCCACCTGAAGTA	
PTPRK	PTPRKF11	AATTACAATTGATGGGGAGA	90
	PTPRKZR11	**ACTGAACCTGACCGTACA**TATTGTGTGACGATGAAAGC	
PTPRM	PTPRMF8	TGCAGGAAACCATCTATGA	500
	PTPRMR8	CTCTGTTTGCACCATGTTC	
PTPRM	PTPRMF1	CCTTCTCCCCTATAAAAGCTA	87
	PTPRMZR1	**ACTGAACCTGACCGTACA**GCCACTTGGACACAGTCTAT	
c-Myc	Myc1F	TGCTCCATGAGGAGACAC	450
	Myc1R	TTTCATTGTTTTCCAACTCC	
c-Myc	Myc1F	TGCTCCATGAGGAGACAC	128
	MycZR	**ACTGAACCTGACCGTACA**TGATCCAGACTCTGACCTTT	
ID1	ID1F1	TCAACGGCGAGATCAG	57
	ID1ZR	**ACTGAACCTGACCGTACA**GATCGTCCGCAGGAA	
p53	p53F2	ATCCTCACCATCATCACACT	85
	p53ZR	**ACTGAACCTGACCGTACA**CAGGACAGGCACAAACAC	
Caspase-3	Cas3F1	GGCGTGTCATAAAATACCAG	115
	Cas3ZR1	**ACTGAACCTGACCGTACA**ACAAAGCGACTGGATGAA	
Caspase-8	Cas8F1	AGAAAGGAGGAGATGGAAAG	113
	Cas8ZR1	**ACTGAACCTGACCGTACA**GACCTCAATTCTGATCTGCT	
Caspase-9	Cas9F1	AAGCCCAAGCTCTTTTTC	102
	Cas9ZR1	**ACTGAACCTGACCGTACA**GTTACTGCCAGGGGACTC	
MMP9	MMP9F1	AACTACGACCGGGACAAG	357
	MMP9R1	ATTCACGTCGTCCTTATGC	

MMP9	MMP9F1	AACTACGACCGGGACAAG	106
	MMP9ZR1	**ACTGAACCTGACCGTACA**GGAAAGTGAAGGGGAAGA	

Table 2.3 Primers used for ribozyme synthesis.

Gene	**Primer name**	**Primer sequence (5'-3')**
PTPRK ribozyme-2	PTPRKrib2F	CTGCAGGATGATAGGACCATCGCCAATCTGATGAGTCCGTGAGGA
	PTPRKrib2R	ACTAGTGATCCAACTAAATGCCAACTCGATTTCGTCCTCACGGACT
PTPRK ribozyme-3	PTPRKrib3F	CTGCAGTTTGCTCTTTTTTACAATTAATATCTGATGAGTCCGTGAGGA
	PTPRKrib3R	ACTAGTTCATCCTCCTTCTCCTAGTTGTTTCGTCCTCACGGACT
PTPRM ribozyme	PTPRMribF	CTGCAGCCTGTCTCCTGCCACGGTCCTGCCCTGATGAGTCCGTGAGGA
	PTPRMribR	ACTAGTGCTACCTTCCAGTGCAGTGCCATTTCGTCCTCACGGACT
pEF/His TOPO TA plasmid vector	T7F	TAATACGACTCACTATAGGG
	BGHR	TAGAAGGCAGTCGAGG
Ribozyme clone verification	RbBMR	TTCGTCCTCACGGACTCATCAG
	RbTPF	CTGATGAGTCCGTGAGGACGAA

2.1.4 Antibodies

2.1.4.1 Primary antibodies

Full details of primary antibodies used in this study are provided in Table 2.4.

2.1.4.2 Secondary antibodies

The secondary antibodies used for Western blotting were horseradish peroxidise (HRP) conjugated anti-goat IgG, goat anti-rabbit IgG, and rabbit anti-mouse IgG antibodies, all supplied by Sigma (Poole, Dorset, UK). Those used for immunofluorescent studies are goat anti-rabbit IgG TRITC conjugated secondary antibodies, and FITC conjugated goat anti-rabbit IgG FITC conjugated secondary antibodies, which are all supplied by Sigma (Poole, Dorset, UK).

Table 2.4 Primary and secondary antibodies used in this study.

Antibody name	Molecular weight (kDa)	Supplier	Product code
Mouse anti-GAPDH	37	SANTA CRUZ BIOTECHNOLOGY	SC-47724
Rabbit anti-PTPRK	162	SANTA CRUZ BIOTECHNOLOGY	SC-28906
Mouse anti-p38	38	SANTA CRUZ BIOTECHNOLOGY	SC-7972
Rabbit anti-JNK	46	SANTA CRUZ BIOTECHNOLOGY	SC-571
Rabbit anti-ERK1/2	42/44	SANTA CRUZ BIOTECHNOLOGY	SC-93
Mouse anti-c-Myc	48	SANTA CRUZ BIOTECHNOLOGY	SC-70465
Rabbit anti-ID1	15	SANTA CRUZ BIOTECHNOLOGY	SC-734
Rabbit anti-Caspase-3	32	SANTA CRUZ BIOTECHNOLOGY	SC-7148
Mouse anti-Caspase-8	57	SANTA CRUZ BIOTECHNOLOGY	SC-70501
Mouse anti-Caspase-9	46	SANTA CRUZ BIOTECHNOLOGY	SC-17784
Mouse anti-Phosphotyrosine	Dependent on protein	SANTA CRUZ BIOTECHNOLOGY	SC-508
Mouse anti-Phosphoserine	Dependent on protein	Sigma-Aldrich	P-5747
Mouse anti-Phosphothreonine	Dependent on protein	Sigma-Aldrich	P-6623
Rabbit anti-mouse IgG peroxidise conjugate		Sigma-Aldrich	A-9044
Goat anti-rabbit IgG peroxidise conjugate		Sigma-Aldrich	A-9169
Rabbit anti-goat IgG peroxidise conjugate		Sigma-Aldrich	A-5420

2.2 Standard reagents and solutions

2.2.1 Solutions for use in cell culture

0.05M EDTA

1g KCl (Fisons Scientific Equipment, Loughborough, UK), 5.72g Na_2HPO_4 (BDH Chemical Ltd., Poole, England, UK), 1g KH2PO4 (BDH Chemical Ltd., Poole, England, UK), 40g NaCl (Sigma-Aldrich, Inc., Poole, Dorset, England, UK) and 1.4g EDTA (Duchefa Biochemie, Haarlem, The Netherlands) were dissolved in 5L distilled water, adjusted to pH of 7.4, autoclaved, and stored until further use.

Trypsin (25mg/ml)

500mg trypsin was dissolved in 0.05M EDTA and then filtered through a 0.2μm minisart filter (Sartorius, Epsom, UK). This solution was aliquoted and stored at -20^{0}C until further use. When trypsin was required for cell culture work, 250μl of the trypsin stock was diluted in 10ml 0.05M EDTA and used to detach the cells.

Balanced Saline Solution (BSS)

79.5g NaCl, 2.1g KH_2PO_4, 2.2g KCl and 1.1g of Na_2HPO_4 were dissolved in 10L of distilled water, and the pH adjusted to 7.2, using NaOH (Sigma-Aldrich, Inc., Poole,

Dorset, England, UK).

100X Antibiotics

5g streptomycin, 3.3g penicillin and 12.5mg amphotericin B in DMSO, were dissolved in 0.5L of BSS and filtered. 5ml of the antibiotics was then added to a 500ml bottle of DMEM media.

2.2.2 Solutions for use in molecular biology

Tris-Boric-Acid-EDTA (TBE)

TBE solution (5X) (1.1M Tris; 900mM Borate; 25mM EDTA; pH 8.3) was made by dissolving 540g Tris-HCl (Melford Laboratories Ltd, Suffolk, UK), 275g Boric acid (Melford Laboratories Ltd, Suffolk, UK) and 46.5g of EDTA in 10L of distilled water. The pH was adjusted to 8.3 using NaOH and then stored at room temperature. It was diluted in distilled water to 1X TBE for preparing agarose gel and DNA electrophoresis.

DEPC water

250ml Diethyl Pyroncarbonate (DEPC) (Sigma-Aldrich, Inc., Poole, Dorset, England, UK) was added to 4.75ml of distilled water and then autoclaved .

Loading buffer (used for DNA electrophoresis)

25mg of bromophenol blue (Sigma-Aldrich, Inc., Poole, Dorset, England, UK) and 4g sucrose (Fisons Scientific Equipment, Loughborough, UK) were dissolved in 10ml of distilled water and stored at 4^{o}C.

Ethidium Bromide

The stock solution of 10mg/ml ethidium bromide was prepared by dissolving 200mg of ethidium bromide powder (Sigma-Aldrich, Inc., Poole, Dorset, England, UK) in 20ml distilled water. In order to protect the solution from sunlight, it was stored in a tube covered with aluminium foil.

2.2.3 Solutions for gene cloning

LB agar

LB agar was prepared by dissolving 10g tryptone (Duchefa Biochemie, Haarlem, Netherlands), 5g yeast extract (Duchefa Biochemie, Haarlem, Netherlands), 15g agar, and 10g NaCl, in 1L of distilled water then adjusting the pH to 7.0 and autoclaving the solution. The solution was melted antibiotics added and solution poured into $10cm^2$

Petri dishes (Bibby Sterilin LTd., Staffs, UK). These dishes were left to cool and harden before use.

LB broth

10g tryptone, 10g NaCl and 5g yeast extract were dissolved in 1L of distilled water. The pH of the solution was adjusted to 7.0 and then autoclaved. After cooling down, antibiotics were added and the solution stored at 4^{o}C until use.

2.2.4 Solutions for Western blot

Lysis Buffer

This was made up by dissolving NaCl 150mM (8.76g), Tris 50mM (6.05g), Sodium azide 0.02% (200mg), Sodium deoxycholate 0.5% (5g), Triton X-100 1.5% (15ml), Aprotinin 1µg/ml (1mg), Na_3VO_4 5mM (919.5mg), Leupeptin 1µg/ml (1mg) in 1 litre of distilled water. The solution was then stored at 4^{o}C for further use.

10% Ammonium Persulphate (APS)

1g APS was dissolved in 10ml distilled water and then stored at 4 ^{o}C for further use.

Tris Buffered Saline (TBS)

10X TBS (0.5M Tris, 1.38 M NaCl, pH 7.4) stock solution was prepared by dissolving 606g of Tris and 765g of NaCl (Melford Laboratories Ltd., Suffolk, UK) in 10L distilled water. The pH was adjusted to 7.4 using HCl and stored at room temperature.

10X Running buffer (for SDS-PAGE)

10X running buffer (0.25M Tris, 1.92M glycine, 1% SDS, pH8.3) was prepared by dissolving 303g of Tris, 1.44kg of Glycine (Melford Laboratories Ltd., Suffolk, UK) and 100g SDS (Melford LaboratoriesLtd., Suffolk, UK) in 10L distilled water. It was diluted to 1X running buffer with distilled water before use.

Transfer buffer

15.5g Tris and 72g glycine were dissolved in 4L distilled water. 1L of methanol (Fisher Scientific, Leicestershire, UK) was added to make a final volume of 5L.

Ponceau S stain

Supplied by Sigma.

2.2.5 Solutions for use in immunocytochemical staining

Diaminobenzidine (DAB) chromagen

2 drops (approximately 50µl) of wash buffer, 4 drops of DAB (Vector Laboratories, Inc., Burlingame, USA) and 2 drops of H_2O_2 were added to 5ml of distilled water and mixed well before use.

ABC Complex

The ABC complex is prepared by using a kit provided by Vector Laboratories Inc. 4 drops of each reagent A and B were added to 20ml of wash buffer before being mixed thoroughly and left at room temperature for 30 minutes before use.

2.2.6 Solutions for use in galetine zymography

Wash buffer

This was made up by 2.5% Triton X-100 and 0.02% NaN_3

Incubation buffer

This was made up by 50mM Tris-HCl, 5mM $CaCl_2$ and 0.02% NaN_3. The pH was adjusted to 8.0 and stored at room temperature.

Destain buffer

This was made up by 100ml Acetic acid and 250ml Ethanol in 1 litre of distilled water.

2.3 Cell culture, maintenance, and storage

2.3.1 Preparation of growth media and cell maintenance

- Dulbecco's Modified Eagle's medium (DMEM/ Ham's F-12 with L-Glutamine; Sigma-Aldrich, Inc., Poole, Dorset, England, UK), pH 7.3 containing 2mM L-glutamine and 4.5mM $NaHCO_3$, supplemented with 10% heat inactivated Foetal Bovine Serum (Sigma-Aldrich, Inc., Poole, Dorset, England, UK) and antibiotics, was used to routinely culture the cells.
- Cell lines transfected with the pEF6/His plasmid or constructed plasmid vectors were subjected to a selection of 2 weeks using 5µg/ml blasticidin (Melford laboratories ltd, Suffolk, UK), the resultant transfectants were then cultured in medium supplemented with 0.5µg/ml blasticidin for maintaining purpose. The transfectants were then verified and used for subsequent studies.

- All the cell lines were cultured in 25cm^2 and 75cm^2 culture flasks (Greiner Bio-One Ltd, Gloucestershire, UK) with a loosed cap in incubator at 37^{o}C, 95% humidification, and 5% CO_2.
- Cell confluency was visually measured with a light microscope and estimating the percentage of cells covering the surface of the tissue culture flasks. If needed for experimental work, the cells were left to grow until they reached sub-confluency (2-3 days). All handling of cells was carried out using a Class II Laminar Flow Cabinet with autoclaved and sterile equipment (manufactor). Cells were routinely sub-cultured when they had reached a confluency of 80-90% as explained in section 2.3.2.

2.3.2 Trypsinisation (detachment) of adherent cells and cell counting

Once the cells had reached a confluency of approximately 80-90%, the medium was aspirated then briefly rinsed with sterile EDTA BSS buffer in order to remove any remaining serum which has an inhibitory effect on the action of trypsin.

- Adherent cells were then detached from the tissue culture flask using 1-2ml of Trypsin/ EDTA (0.01% trypsin and 0.05% EDTA in BSS buffer) and incubating the cells at 37^{o}C for approximately 5 minutes.

- Once detached, the cell suspension was then collected into 30ml universal containers (Greiner Bio-One Ltd, Gloucestershire, UK) and centrifuged at 1,780rpm for 8 minutes in order to collect the cell pellet.
- The supernatant was removed and the cell pellet resuspended in an appropriate amount of medium. Cells were either counted for using in immediate experiments, or transferred into fresh tissue culture flasks for re-culturing.
- Cells were counted with a Neubauer haemocytometer counting chamber (Mod-Fuchs Rosenthal, Hawksley, UK) using a light microscope at a magnification of 10x10 (Reichert, Austria). A haemocytometer allows for the calculation of the number of cells in a predetermined volume of fluid in order to obtain the quantity of cells per millilitre, and an accurate estimation of cell density for use in *in vitro* and *in vivo* cell function assays.
- The haemocytometer chamber is divided into 9 squares with dimensions of 1mm x 1mm x 0.2mm. For consistency of cell density, three of these 9 squared areas were counted. This allowed for determination of cell number by using the following equation:

  Cell number/ml= (number of cells in three 9 squares÷ 6) x (1×10^4).

2.3.3 Storing cells in liquid nitrogen and cell resuscitation

Cell stocks of low passage number that needed to be stored were done so in liquid nitrogen.

- Cells were trypsinised as described in section 2.3.2 and resuspended in medium with 10% Dimethylsulphoxide (DMSO; Fisons, UK) at a cell density of $1x10^6$cells/ml.
- This cell suspension was then divided into 1ml aliquots and transferred into pre-labelled 1ml CRYO.S^{TM} tubes (Greiner Bio-One, Germany) and the tubes were wrapped in protective tissue paper, stored overnight at -80^oC, and then transferred to liquid nitrogen tanks for long term storage .
- In order to resuscitate the cells, they were removed from liquid nitrogen and left to rapidly thaw before being transferred into a universal container containing 5ml pre-warmed medium and incubated for 10 minutes at 37^oC.
- This was followed by centrifugation at 1,600 rpm for 5 minutes to remove any excess DMSO, before re-suspending the cells in 5ml media. The cell suspension was then transferred into a fresh 25cm^2 tissue culture flask and kept in an incubator.

2.4 Methods for RNA detection

2.4.1 Total RNA isolation

There are three types of Ribonucleic acid (RNA) in the cytoplasm known as; ribosomal RNA (rRNA), transfer RNA (tRNA), and messenger RNA (mRNA). Despite its critical role of carrying the genetic information required for specific protein formation, cellular mRNA only constitutes 1-2% of the total cellular RNA. It is this type of RNA that is normally used in research, and is used in this study.

- The protocol followed for RNA isolation was using the Tri Reagent kit (Sigma-Aldrich, Inc., Poole, Dorset, England, UK).
- Cells were grown in a monolayer of 85-90% confluency before the medium was aspirated and replaced with RNA reagent (1ml per 5-10 x 10^5 cells), in order to induce cell lysis.
- This homogenate was transferred into a 1.5ml eppendorf, and incubated at 4^oC for 5 minutes.
- This was followed by the addition of 0.2ml (per 1ml of RNA reagent) chloroform (Sigma-Aldrich, Inc., Poole, Dorset, England, UK), 15 seconds of shaking, and incubated at 4^oC for 5 minutes.
- The resulting homogenate was centrifuged at 12,000rpm for 15 minutes at 4^oC

(Boeco, Wolf laboratories, York, UK). Under these acidic conditions, the homogenate separated into three phases; a lower pink organic phase contains protein, an inter phase contains DNA, and an upper aqueous phase contains RNA.

- The aqueous phase should constitute around 40-50% of the total volume, was carefully transferred into a fresh eppendorf, then an equal volume of isopropanol was added (Sigma-Aldrich, Inc., Poole, Dorset, England, UK) and incubated for 10 minutes at 4^{o}C. After centrifuging the samples at 12,000rpm for 10 minutes at 4^{o}C, the RNA precipitates as a white pellet on the bottom of the eppendorf.
- The supernatant was discarded, and the RNA pellet washed twice with 75% ethanol in DEPC water. Each wash was carried out by adding 1ml 75% ethanol, vortexing and centrifuging the samples at 7,500rpm for 5 minutes at 4^{o}C.
- After the final wash, the RNA pellet was briefly dried at 55^{o}C for 5-10 minutes, in a Techne, Hybridiser HB-1D drying oven (Wolf laboratories, York, UK), to remove any remaining traces of ethanol.
- The final step was to dissolve the RNA pellet in 50-100µl (dependent on pellet size) of DEPC water by vortexing. DEPC is used as it is a histidine specific alkylating agent and inhibits the effect of RNAases, which require histidine active sites to carry out their function.

2.4.2 RNA Quantification

- Once RNA isolation was completed, the concentration and purity of the resulting RNA was measured with the use of a UV1101 Biotech Photometer (WPA, Cambridge, UK). This spectrophotometer had been previously set to detect single strand RNA (μg/μl) at a dilution of 1:10, measuring the difference of absorbance between the RNA sample and DEPC water (blank) at a wavelength of 260nm.
- By using a ratio of A260nm/A280nm an estimation of the purity of the RNA was determined, pure RNA reaching an optical density of 2.0, and RNA contaminated with ethanol or protein, reaching optical density values less than 1.5.
- The samples were measured using Starna glass cuvettes (Optiglass limited, Essex, UK). The RNA samples were used immediately for reverse transcription (RT) or placed at -80^{o}C for long term storage.

2.4.3 Reverse Transcription of RNA for production of cDNA

Reverse transcription (RT) is a simple and sensitive technique for mRNA analysis and acts as an enhanced and more rapid alternative to the more conventional methods of RNA examination.

- In this study, RT is carried out by converting 0.5μg/μl of RNA into complementary

DNA (cDNA) using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, California, USA). The protocol that was carried out is outlined below:

- Each reaction was set up in thin-walled 200µl PCR tubes (ABgene, Surrey, UK) as follows:

Component	*Volume per reaction*
5X iScript Reaction Mix	4µl
iScript Reverse Transcriptase	1µl
Nuclease-free water	xµl
RNA template (0.5µg/µl)	xµl
Total Volume:	*20µl*

- The complete reaction was mixed and then incubated in a T-Cy Thermocycler (Creacon Technologies Ltd, The Netherlands) at the following temperatures:

  5 minutes at 25 ^{o}C

  30 minutes at 42 ^{o}C

  5 minutes at 85 ^{o}C

- Once the process was completed, the cDNA was diluted 1:4 with PCR water, and either used immediately as a template for conventional PCR, or stored at -20^{o}C for

further use.

2.4.4 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) was initially devised in 1983 by Karey Mullis as a method for detecting and amplifying a specific target sequence of nucleic acids.

- In this study, a particular gene of interest was amplified by PCR using the REDTaq™ ReadyMix PCR Reaction mix (Sigma-Aldrich, Inc., Poole, Dorset, England, UK). The reactions were set up in PCR tubes in aliquots of 16µl as follows:

Component	*Volume*
cDNA template	2µl
Forward primer (working concentration of 1 µM)	1µl
Reverse primer (working concentration of 1 µM)	1µl
2X REDTaq^{TM} ReadyMix	8µl
PCR H_2O	4µl

A negative control containing PCR water instead of the template (cDNA) was run alongside the test samples to ensure there was no contamination.

- The PCR reactions were then briefly mixed before being placed in a 2720 Thermo Cycler (Applied Biosystems) set to the following conditions;

  Initial denaturation: 94^{o}C for 5-10 minutes

  Followed by 30-42 cycles of:

  Denaturation: 94^{o}C for 40 seconds

  Annealing: gene/primer specific temperature (55 ^{o}C) for 30-40 seconds

  Extension: 72^{o}C for 40 seconds

  And finally:

  Final extension: 72^{o}C for 7-10 minutes

  The cycling conditions are dependent on different sets of primers and the size of the PCR product.

- The products were stained with ethidium bromide and visualised using agarose gel electrophoresis system (section 2.4.5). In order to demonstrate a representative and reliable expression pattern, each RT-PCR was repeated three times.

2.4.5 Agarose gel electrophoresis and DNA visualisation

Agarose gel electrophoresis is the simplest and the most common method of DNA separation and analysis. It works by using an electrical current to separate the amplified

DNA according to charge and size. Depending on the product size, the samples were either run on a 0.8% agarose gel (used for large DNA fragments 1-10kb) or a 2% gel (used for smaller fragments less than 1kb).

- The agarose gels were prepared by adding the required amount of agarose (Melford Chemicals, Suffolk, UK) to 400ml of (1x) TBE solution before being heated to completely dissolve the agarose. An appropriate amount of this solution was then poured into electrophoresis cassettes (Scie-Plas Ltd., Cambridge, UK) containing well forming combs, before the gel was left to set at room temperature for 30-40 minutes.
- Once gel set, (1X) TBE buffer was poured over the gel until it reached to a level 5mm above the gel surface. The comb was removed, and approximately 8µl of 1Kb DNA ladder (Cat No. M106R; GenScript USA Inc.), or 8-10µl of each PCR reaction, was loaded into the wells by placing the gel loading tip just over the well and dispensing the product into it.
- A power pack (Gibco BRL, Life Technologies Inc.) was then used to run the samples electrophoretically through the gel at a constant voltage of around 100V for approximately 30-50 minutes until the samples had migrated to as far as required for that particular product size.

- The DNA was then visualised by the addition of ethidium bromide which acts as a DNA intercalator and fluoresces once intercalated, thereby absorbing invisible UV light and transmitting the energy into visible orange light. The gel was placed in ethidium bromide stain diluted in the (1X) TBE buffer and left for 15 minutes with constant agitation to ensure even staining, before being visualised under UV light using a VisiDoc-It imaging system (UVP).
- Images were captured with the use of an UV camera imaging system (UVP), and printed with a SONY thermo printer (SONY UK, London, UK) or saved as a TIFF file. If insufficient staining was found, the gel was returned to the ethidum bromide stain for additional staining or destaining in water to remove any background staining if required.

2.4.6 Quantitative RT-PCR (Q-PCR)

Unlike conventional RT-PCR, which only allows for semi-quantification, Q-PCR is a method of detecting extremely small amounts of cDNA template within a sample while also determining an accurate and reliable value of the template copy number in each sample. This method works due to the use of a sequence specific DNA based fluorescent reporter probe which only quantifies the levels of DNA containing the probe

sequence, thereby greatly increasing specificity.

This current study uses the Amplifluor™ Uniprimer™ Universal system (Intergen company® , New York, USA) to quantify transcript copy number. The amplifluor probe consists of a 3’ region specific to the Z-sequence (ACTGAACCTGACCGTACA) present on the target specific primers and a 5’ hairpin structure labelled with a fluorophore (FAM). When in this hairpin structure, the fluorophore is linked to an acceptor moiety (DABSYL) which acts to quench the fluorescence emitted by the fluorophore, preventing any signal from being detected.

During PCR, the probe becomes incorporated and acts as a template for DNA polymerisation in which DNA polymerase uses its 5’-3’ exonuclease activity to degrade and unfold the hairpin structure, thereby disrupting the energy transfer between fluorophore and quencher, allowing sufficient floorescence to be emitted and hence detected. The fluorescent signal emitted during each PCR cycle can then be directly correlated to the amount of DNA that has been amplified. This process is illustrated in Figure 2.1.

- Each reaction to be amplified was set up as follows:

Component	*Volume*
Forward primer	0.3µl (10pmol/µl)
Reverse Z primer	0.3µl (1pmol/µl)
Amplifluor probe	0.3µl (10pmol/µl)
2X iQ™ Supermix	5µl
cDNA and PCR H_2O	4µl

- Each sample was then loaded into a 96 well plate (BioRad laboratories, Hemel Hampstead, UK), alongside standards (ranging from copy numbers of 10^1-10^8), covered with optically clear Microseal® (Biorad laboratories, California, USA) and this was placed in an iCyclerIQ thermal cycler and detection software (BioRad laboratories, Hemel Hampstead, UK) at the following conditions:

  Initial denaturation: 94°C for 5 minutes

  Followed by 80-90 cycles of:

  Denaturation: 94°C for 10 seconds

  Annealing: 55°C for 35 seconds

  Extension: 72°C for 20 seconds

- The fluorescent signal is detected at the annealing stage by a camera where its geometric increase directly correlates with the exponential increase of product. This is then used to determine a threshold cycle (TC) for each reaction and the transcript copy number depends on when fluorescence detection reaches a specific threshold.
- The degree of fluorescence emitted by a range of standards with a known transcript copy number is used to compare the amount emitted by each sample, allowing for the transcript copy number in each sample to be accurately calculated.
- Furthermore, the transcript copy number of each sample was normalised against the detection of β-actin or GAPDH copy numbers. The procedure was repeated at least three times, and representative data are demonstrated as expressional trends. Figure 2.2 shows how transcript levels are quantified.

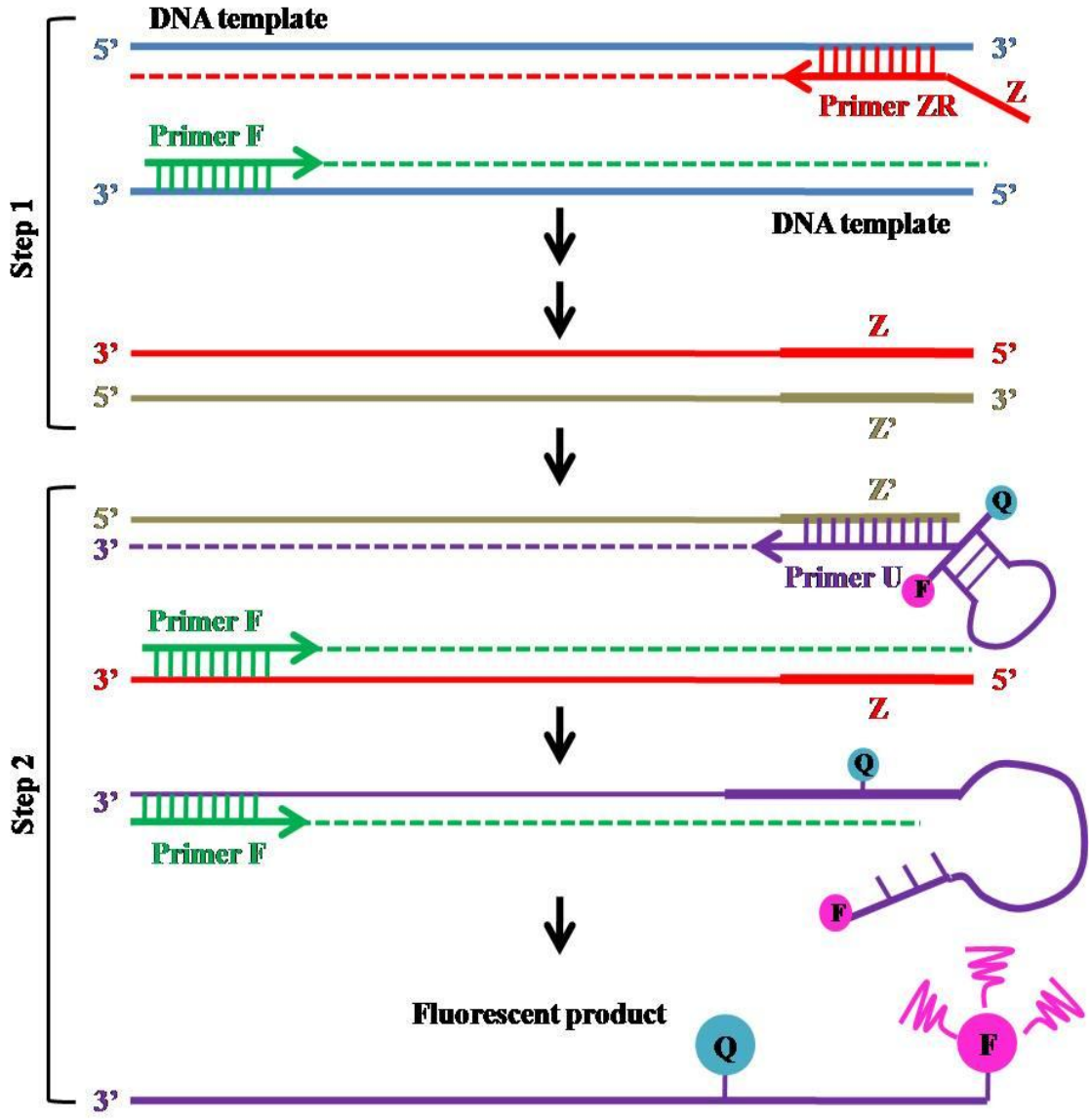


Figure 2.1 Diagram showing function of the u-probe during DNA amplification using Q-PCR.

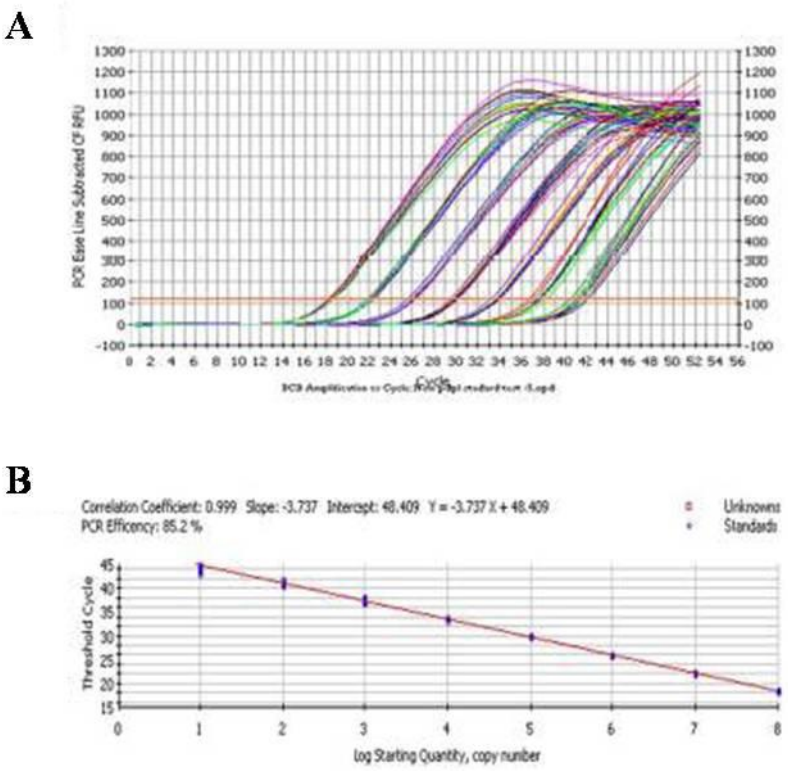


Figure 2.2 (A) Detection of transcript levels from a range of standard samples (10^8 to 10^1 copy number) using the iCyclerIQ thermal cycler. (B) Subsequent generation of a standard curve from these samples.

2.5 Methods for protein detection

2.5.1 Protein extraction and preparation of cell lysates

- Once cells had reached an adequate confluency, the cell monolayer was scraped off in BSS using a cell scraper. The cell suspension was then transferred into a universal tube.
- This was followed by centrifugation at 1,800rpm for 8 minutes before the supernatant was poured off, and the cell pellet resuspended in 200-300µl (depending on total cell number, approximately 100µl/10^6 cells) of lysis buffer.
- This was transferred into a 1.5ml eppendorf tube and placed for an hour at room temperature on a Labinco rotating wheel (Wolf laboratories, York, UK) to allow for cell lysis.
- The resulting suspension was then centrifuged at 13,000rpm for 15 minutes so any unwanted cell debris formed a pellet. The supernatant was transferred to a fresh eppendorf tube and the pellet discarded.
- The protein samples were then either quantified for SDS-PAGE as explained below, or stored at -20^{o}C until further use.

2.5.2 Protein quantification and preparation of samples for SDS-PAGE

In order to standardise the protein sample concentration for western blotting, the amount of protein in each sample was quantified following the protocol outlined in the Bio-Rad DC Protein Assay kit (Bio-Rad laboratories, Hemel Hampstead, UK).

- In a 96 well plate, 50mg/ml of bovine serum albumin (BSA) standard (Sigma, Dorset, UK) was serially diluted in lysis buffer to a concentration of 0.78mg/ml and used to set up a standard curve of protein concentration.
- Five microliters of either protein sample or standard was added into well, before 25µl of 'working reagent A' (prepared by adding 20µl of reagent S per millilitre of reagent A), followed by 200µl of reagent B to each well.
- After samples were mixed, the plate was left at room temperature for 30-45 minutes to allow the colorimetric reaction to take place. Once this was complete, the absorbance of each sample was measured at 620nm using the ELx800 plate reading spectrophotometer (Bio-Tek, Wolf laboratories, York, UK).
- A standard curve and an equation to calculate protein concentration based on absorbance were established using the scatter line chart of Excel. Protein concentration of each sample was determined using the corresponding absorbance and the equation of the standard curve. The samples were then diluted in an

appropriate amount of lysis buffer in order to normalise them to the required final concentration of 1.0-2.0mg/ml.

- This was then further diluted with 2X Lamelli sample buffer concentrate (Sigma-aldrich, St Louis, USA) in a ratio of 1:1 before the samples were denatured by boiling at 100^{o}C for 5-10 minutes and either loaded onto a SDS-PAGE gel or stored at -20^{o}C until further use.

2.5.3 Preparing immunoprecipitates

Immunoprecipitation (IP) can be used for analysing intracellular phosphorylation that occurs in downstream signalling cascades. The process involves adding a specific antibody targeting a protein of interest within a cell lysate. This is then mixed with sepharose or agarose bonded staphylococcal protein A, protein G, or both, in order to collect the resulting protein-antibody complexes. These complexes are then centrifuged to precipitate the protein-IgG-proteinA/G beads. After being denatured at 100 ^{o}C for 10 minutes, the samples were separated on an SDS-PAGE gel, and evaluated using immunoprobing. The process was carried out as follows:

- Antibody targeted against a protein of interest was added to the cell lysate samples before being incubated at 4^{o}C for 1 hour on a Labinco rotating wheel.

- Following incubation, 20µl of conjugated A/G protein agarose beads (Santa Cruz Biotechnology, supplied by Insight Biotechnologies Inc, Surrey, England, UK) were added to each sample and placed back on the wheel for another hour to allow for the antibody-protein complexes to bind to the beads.
- Centrifugation at 8,000rpm for 5 minutes then acts as a way of removing any unbound protein or excess antibodies present in the supernatant. The protein pellet was subsequently washed twice with 300µl of lysis buffer before being resuspended in 40-60µl of 1x sample buffer and boiled for 10 minutes. The resulting samples were then run on SDS-PAGE gels as explained below.

2.5.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

- The system used to carry out SDS-PAGE in this study was the OmniPAGE VS10 vertical electrophoresis system. Resolving gels of a required percentage (depending on protein size) were prepared in 15ml aliquots (enough for 2 gels) by adding all the constituents at the amounts indicated below:

Table 2.5 Ingredients for resolving gel.

Component	**8% Resolving gel (proteins size over 100kDa)**	**10% Resolving gel (proteins size below 100kDa)**
Distilled water	6.9ml	5.9ml
30% acrylamide mix (Sigma-Aldrich, St Louis, USA)	4.0ml	5.0ml
1.5M Tris (pH 8.8)	3.8ml	3.8ml
10% SDS	0.15ml	0.15ml
10% Ammonium persulphate	0.15ml	0.15ml
TEMED (Sigma-Aldrich, St Louis, USA)	0.009ml	0.006ml

- The resulting mixture was then poured in between two glass plates held in place by a loading cassette, until at a level 1.5cm below the top edge of the plate, to prevent gel oxidation the top of the resolving gel was covered with a 0.1% SDS solution.
- The gels were then left to polymerise at room temperature for approximately 30 minutes, or until fully set. The excess SDS solution was then poured off before adding a sufficient amount of stacking gel in its place, prepared as shown below:

Table 2.6 Ingredients for stacking gel.

Component	**Stacking gel**
Distilled water	3.4ml
30% acrylamide mix (Sigma-Aldrich, St Louis, USA)	0.83ml
1.0M Tris (pH 6.8)	0.63 ml
10% SDS	0.05ml
10% Ammonium persulphate	0.05ml
TEMED (Sigma-Aldrich, St Louis, USA)	0.005ml

- After the addition of the stacking gel, a well forming Teflon comb was inserted before allowing the gel to polymerise at room temperature for around 20 minutes.
- Once set, the loading cassette was transferred into an electrophoresis tank and covered with 1X running buffer before the well comb was removed, and by use of a 50µl syringe (Hamilton), 10µl of Broad range markers were loaded (Santa Cruz Biotechnology, supplied by Insight Biotechnologies Inc, Surrey, England, UK), followed by 10-15µl of the required protein samples.
- The gels were then run at 100-120V, 50mA, and 50W for a length of time appropriate for the size of the protein of interest, in order to separate the proteins according to charge and molecular weight.

2.5.5 Western blotting: transferring proteins from gel to nitrocellulose membrane

- Once SDS-PAGE was completed, the protein samples were transferred onto a nitrocellulose membrane by western blotting. The electrophoresis equipment was disassembled and the gel pried out from the two glass plates before discarding of the stacking gel. The resolving gel was then placed on the bottom graphite base electrode in a SD20 SemiDry Maxi System blotting unit (SemiDRY, Wolf Laboratories, York, UK) on top of 3 pieces of 1X transfer buffer pre-soaked filter paper (Whatman International Ltd., Maidstone, UK), and 1 sheet of Hybond nitrocellulose membrane (Amersham Biosciences UK Ltd., Bucks, UK).
- An additional 3 sheets of pre-soaked filter paper were placed on top of the gel to form a sandwich arrangement in an order as from anode to cathode; filter paper- membrane- gel- filter paper, as shown in Figure 2.3. Electroblotting was then carried out at 15V, 500mA, and 8W for 20-60 minutes (depending on protein size). Once the proteins had been sufficiently transferred, the membranes were blocked over night at 4^{o}C in 10% skimmed dry milk solution (10% milk powder and 0.1% polyoxyethylene (20) sorbitan monolaurate (Tween 20) (Sigma-Aldrich, St Louis, USA) in TBS. This acts to block the proteins onto the membrane to avoid forming non-specific binding of primary antibody.

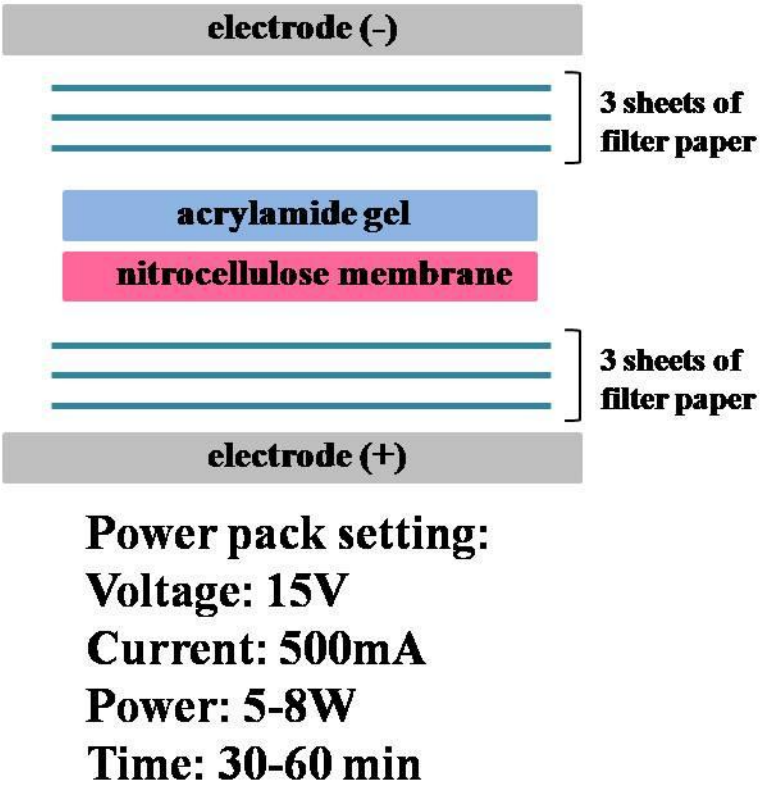


Figure 2.3 Diagram depicting process of western blotting; the transferring of proteins onto a nitrocellulose membrane.

2.5.6 Protein staining

2.5.6.1 Staining membranes in Ponceau S

Ponceau S is a reversible and re-usable protein stain that does not interfere with any subsequent immuno-probing. Its main use is to confirm a successful transfer of proteins from the polyacrylamide gel onto the nitrocellulose membrane, but can also be used to aid in membrane sectioning for multiple immune-probing. The protocol was carried out as follows:

- After the transfer was completed and before probing began, the membrane was immersed in Ponceau S solution for a few minutes at room temperature.
- The solution was then washed off with distilled water until the bands become visible. If required, the membrane was then cut into several sections using a sharp and sterile scalpel.
- Once the staining was completely washed off it was placed in 10% milk solution.

2.5.6.2 Coomasie blue staining of polyacrylamide gels

Coomasie blue is used to stain polyacrylamide gels following SDS-PAGE. This allows for visualisation of protein bands if no immuno-probing is required and can be used as a way of semi-quantifying the volume of the protein bands. The protocol was carried out

as follows:

- The gel was immersed in Coomassie blue stain solution for approximately 30 minutes before being repeatedly washed in destaining solution until the background staining disappeared, and the protein appeared as blue bands. The gel was then photographed or analysed.

2.5.7 Protein detection using specific immuno-probing

- Once staining was completed, the membrane was transferred into 50ml falcon tubes (Nunc, Fisher-Scientific, Leicestershire, UK) ensuring that the membrane surface that had been in contact with the gel was facing upwards. 10% milk blocking solution was added to the membranes and incubated for an hour at room temperature on a roller mixer (Stuart, Wolf-Laboratories, York, UK).
- Once this was done, the 10% milk solution was poured off and replaced with 10ml of 3% milk solution (3% milk powder, 0.1% Tween 20 in TBS) for 15 minutes. This was followed by incubation of the membranes for an hour at room temperature, with primary antibody diluted 1:500 in 5mls of 3% milk solution.
- After the probing with primary antibody, any remaining unbound antibody was then washed off twice in 3% milk solution at 15 minute intervals.

- The membranes were further incubated with 5ml of 1:1000 HRP conjugated secondary antibody diluted in 3% milk for one hour with continuous rotation.
- This was followed by two 15 minute washes in 10ml of 3% milk solution, and two 15 minute washes with Tween TBS (0.2% Tween 20 in TBS), in order to wash off any unbound secondary antibody.
- The membrane was then washed twice in TBS before the chemiluminescent detection.

2.5.8 Chemiluminescent protein detection

Chemiluminescent protein detection was carried out using the chemiluminescence detection kit (Luminata, Millipore), which consists of a highly sensitive chemiluminescent substrate that detects the horseradish peroxidise (HRP) used during the western blot procedure. The protocol was carried out as follows:

- 1ml regent was added on to the membrane and incubated for 5 minutes at room temperature with constant agitation.
- Excessive solution on the membrane was then drained over a piece of tissue paper and the membrane was transferred into a plastic tray. The chemiluminescent signal was detected using an UVITech Imager (UVITech Inc., Cambridge, UK) which

contains both an illuminator and a camera linked to a computer.

- Each membrane was subjected to varying exposure times until the protein bands were sufficiently visible. These images were then captured and further analysed with the UVI band software package (UVITEC, Cambridge, UK), which allows for protein band quantification.
- In this study, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control and ran alongside any other proteins to be detected, so as to allow for additional normalisation of the samples and to compensate for any other biological variaions which may have occurred during the process. The GAPDH is used due to its highly abundant and conserved nature within eukaryotic cells, and is one of the most widely employed and accepted internal controls in determining mRNA and protein expression.
- In order to verify the results, each western blot was carried out three times and the protein bands quantified and standardised separately.

2.5.9 Immunohistochemical staining

This study followed a previously reported immunohistochemical staining procedure.

- The cryosection was taken from -80^{o}C freezer then the cryosection was laid on

bench wrapped in foil and allowed it adapt to room temperature.

- The foil was removed and slides were labled in pencil with the appropriate information.
- Slides were fixed in acetone for 15 minutes followed by three 5 minutes washes with 1X TBS.
- After the fixing and washing, the slide was blocked with a blocking solution containing horse serum (Vector Laboratories Inc., Burlingame, USA) for 30 minutes at room temperature. To make a blocking solution, 1-2 drops of horse serum were added into 5ml of 1x OptiMax Wash Buffer (BioGenex, San Ramon, USA).
- After blocking, the cells were washed three times with wash buffer before being incubated for an hour at room temperature with primary antibody (1:100, the dilution may vary depending on a specific antibody used) diluted in blocking solution.
- Any unbound antibody was subsequently washed off with wash buffer. This was repeated three times before incubating the cells for a further 30 minutes at room temperature, with the corresponding secondary antibody diluted 1:1000 in blocking solution.

  After three washes with wash buffer, the cells were incubated with 200µl of

working VECTASTAIN® Universal ABC complex (Vector Laboratories Inc., Burlingame, USA). The ABC complex was made up 30 minutes before use by mixing 4 drops of the supplied reagent A, with 4 drops of reagent B.

- The ABC solution was subsequently removed by washing three times with wash buffer before a few drops of DAB chromogen (Vector Laboratories Inc., Burlingame, USA) were added to the cells and left to incubate for 5 minutes in darkness. DAB was made up as follows; 2 drops of the provided buffer (pH 7.5), 4 drops of DAB, and 2 drops of hydrogen peroxide diluted in 5ml of distilled water.
- Following DAB addition, the solution should turn brown and once this had occurred, the DAB was washed off using distilled water before the cells were counterstained with Mayer's haemotoxylin for approximately 5 minute.
- After three washes with distilled water, slides need to be dehydrated for 5 minutes in each condition including 50% ethanol, 70% ethanol, 90% ethanol, 100% ethanol, 50% ethanol/ 50% xylene, 100% xylene.
- After dehydration, slides were mounted with DPX and leaved to dry.

2.5.10 Immunochemical staining

This study followed a previously reported immunohistochemical staining procedure

(Jiang *et al*.2005).

- 20,000 cells in 200µl DMEM medium were seeded into each well of a chamber slide (Nalge, NUNC International, LAB-TEK® , USA) and incubated overnight at 37 ^{o}C with 5% CO_2.
- The cells were then fixed in ice-cold pure ethanol for 30 minutes at 4 ^{o}C, before being rehydrated with BSS for 30 minutes at room temperature.
- This was followed by cell permeabilisation in 0.1% TritonX100 in TBS for 5-10 minutes at room temperature.
- Following the permeabilisation, the slide was blocked with a blocking solution containing horse serum (Vector Laboratories Inc., Burlingame, USA) for 30 minutes at room temperature. To make a blocking solution, 1-2 drops of horse serum were added into 5ml of 1× OptiMax Wash Buffer (BioGenex, San Ramon, USA).
- After blocking, the cells were washed three times with wash buffer before being incubated for an hour at room temperature with primary antibody (1:100, the dilution may vary depending on a specific antibody used) diluted in blocking solution.
- Any unbound antibody was subsequently washed off with wash buffer. This was repeated three times before incubating the cells for a further 30 minutes at room

temperature, with the corresponding secondary antibody diluted 1:1000 in blocking solution.

- After three washes with wash buffer, the cells were incubated with 200µl of working VECTASTAIN® Universal ABC complex (Vector Laboratories Inc., Burlingame,

  USA). The ABC complex was made up 30 minutes before use by mixing 4 drops of the supplied reagent A, with 4 drops of reagent B.
- The ABC solution was subsequently removed by washing three times with wash buffer before a few drops of DAB chromogen (Vector Laboratories Inc., Burlingame, USA) were added to the cells and left to incubate for 5 minutes in darkness. DAB was made up as follows; 2 drops of the provided buffer (pH 7.5), 4 drops of DAB, and 2 drops of hydrogen peroxide diluted in 5ml of distilled water.
- Following DAB addition, the solution should turn brown and once this had occurred, the DAB was washed off using distilled water before the cells were counterstained with Mayer's haemotoxylin for approximately 1 minute.

2.5.11 Gelatine zymography

- Cells were counted and $1x10^6$ seeded in a tissue culture flask and incubated

overnight.

- Following incubation, samples were washed once with 1x BSS followed by a wash with serum-free DMEM and then either incubated in serum-free DMEM control or treated medium for 4 hours.
- After 4 hours the conditioned medium was collected and samples were prepared in non-reducing sample buffer.
- Protein samples were separated using SDS-PAGE on gels containing 1% gelatine (Sigma-Aldrich Inc, USA).
- After SDS-PAGE, gels were renatured for 1 hour at room temperature in washing buffer then incubated at 37^{o}C in incubation buffer for 36 hours.
- Following incubation, the gels were stained with coomassie blue for 1 hour and washed in destain buffer for another 1 hour. The brightness of clear bands where MMP2 and MMP9 were located and gelatine was degraded.

2.6 Alteration of gene expression

2.6.1 Knocking down gene expression using Ribozyme Transgenes

In order to knockdown the expression of PTPRK, hammerhead ribozyme transgenes which specifically target and cleave the PTPRK mRNA transcript are employed. The

hammerhead ribozyme was first described by Forster and Symons in 1987 as a self-cleaving region in the RNA genome of various plant viroids and virusoids. The hammerhead motif was subsequently integrated into short synthetic oligonucleotides, transforming it into a turnover catalyst capable of cleaving various RNA targets (Uhlenbeck, 1987; Haseloff and Gerlach, 1988).

Hammerhead motifs contain a conserved secondary structure that consists of three helical stems (I, II, and III) enclosing a junction known as the catalytic core, typified by various invariant nucleotides. The best codons demonstrated to be suitable for cleavage are AUC, GUC and UUC (Figure 2.4). In order to generate a ribozyme transgene specific to PTPRK, primers were designed using Zuker's RNA mFold programme (Zuker, 2003) according to the secondary structure of the gene (Figure 2.5). Subsequently, an appropriate GUC ribozyme target site was chosen according to the secondary structure of PTPRK mRNA and the ribozyme created to specifically bind the sequence adjacent to this GUC codon (Figure 2.4). This made it possible for the hammerhead catalytic region of the ribozyme to bind to and specifically cleave the GUC sequence within the target mRNA transcript.

Following ribozyme design, the sequences were ordered from invitrogen as

sense/antisense strands (as shown in Table 2.4) and the transgenes were then synthesised using touchdown PCR of the following conditions:

Initial denaturation: 94 ^{o}C for 5 minutes

Followed by 8 cycles of each annealing temperature (total of 48 cycles):

Denaturation: 94 ^{o}C for 10 seconds

Various annealing steps: 70^{o}C for 15 seconds, 65^{o}C for 15 seconds,

60^{o}C for 15 seconds, 57^{o}C for 15 seconds, 54^{o}C for 15 seconds and 50^{o}C for 15 seconds.

Extension: 72^{o}C for 20 seconds

Final extension: 72^{o}C for 7 minutes

- Subsequently, the transgenes were run on a 2% agarose gel in order to verify their presence as well as size before being cloned into the pEF6/His plasmid as described in section 2.7.

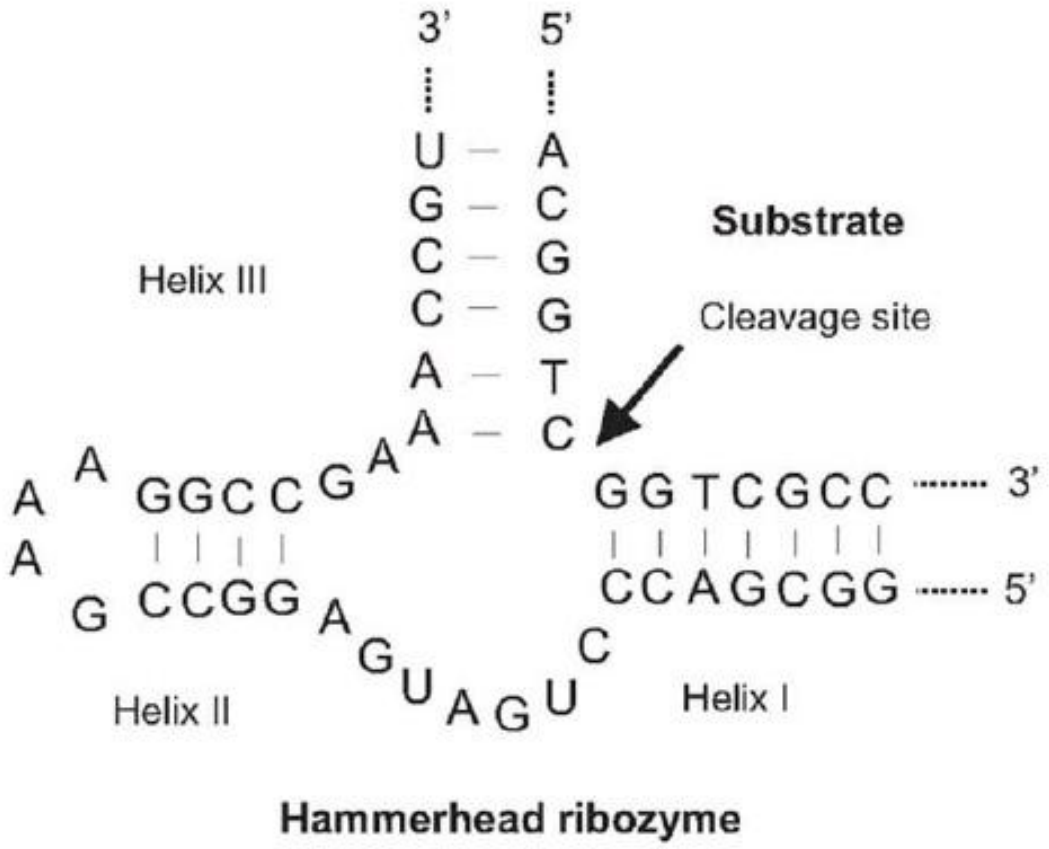


Figure 2.4 Secondary structure of hammerhead ribozyme with bound substrate.

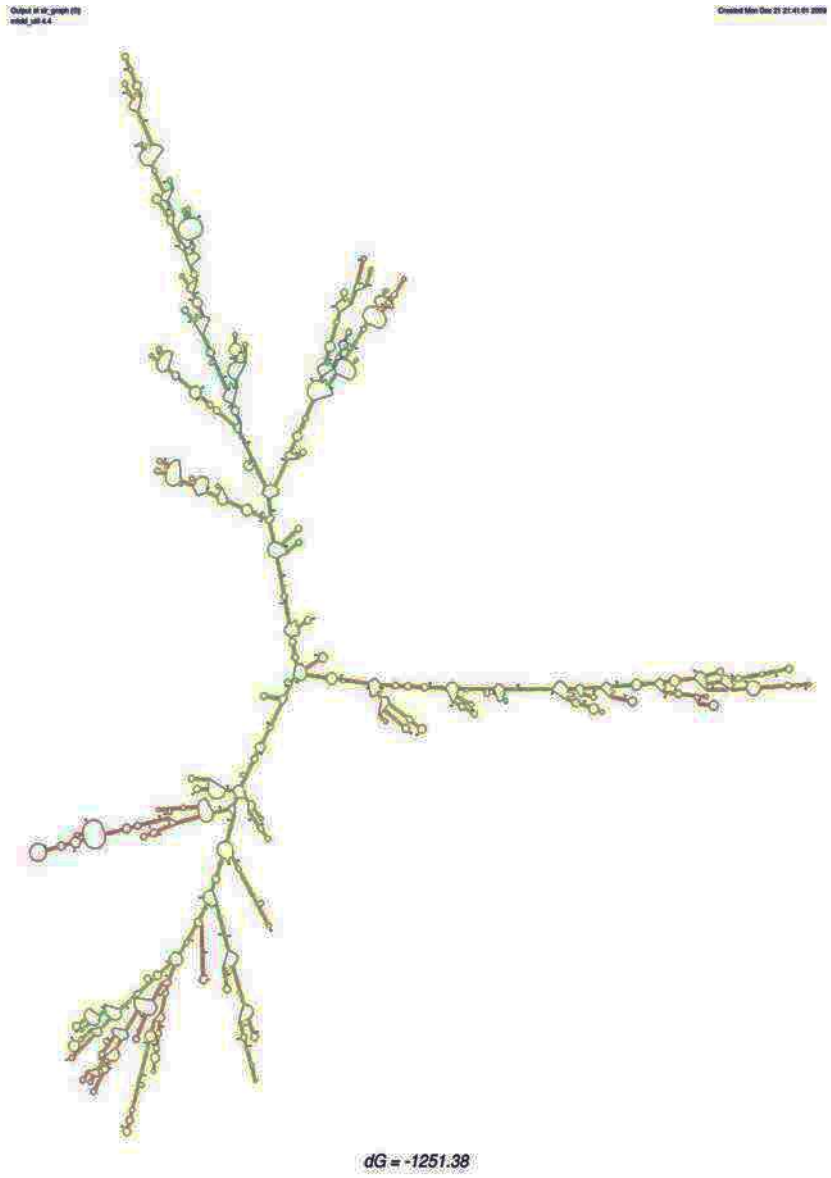


Figure 2.5a The predicted secondary structure of human PTPRK mRNA. dG: free energy; which is the default parameters that are used by the mfold programme.

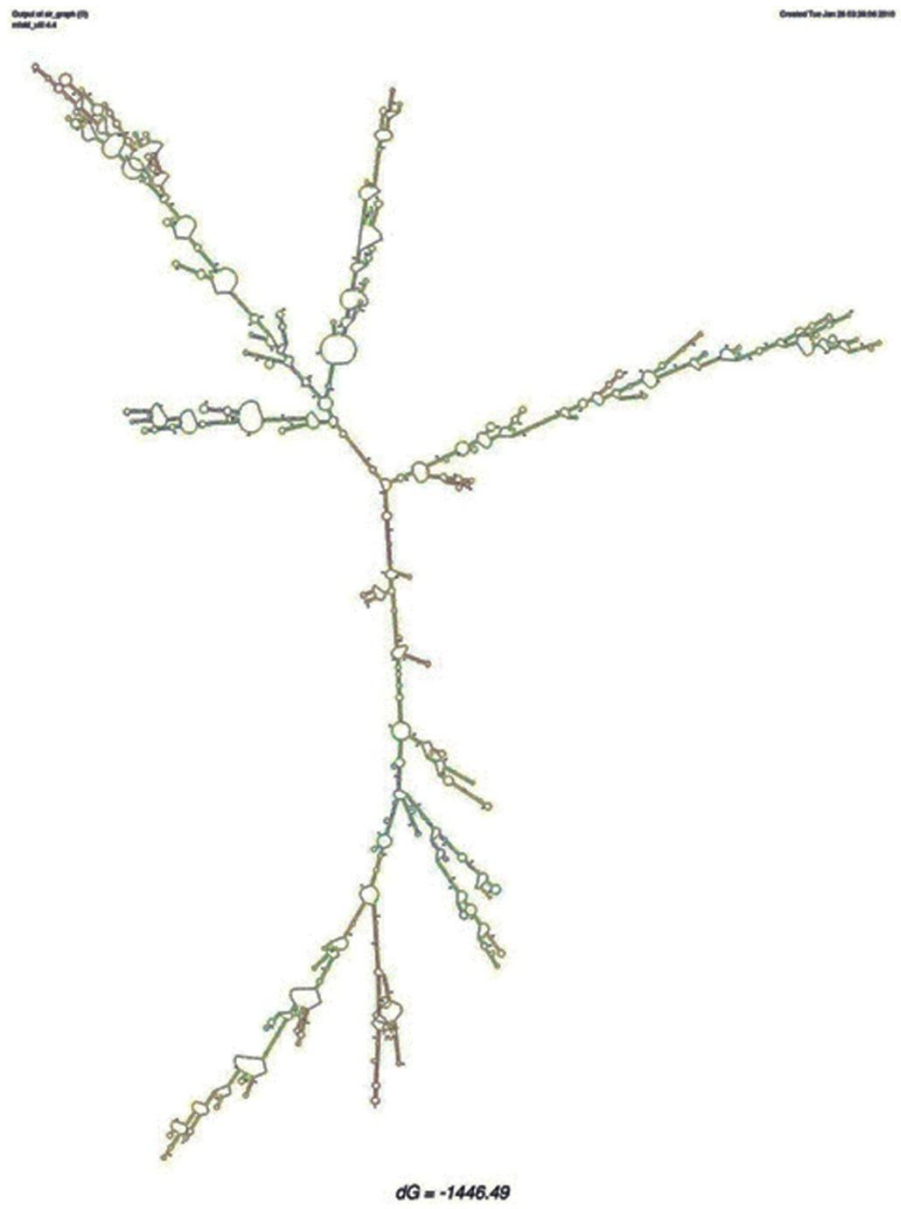


Figure 2.5b The predicted secondary structure of human PTPRM mRNA. dG: free energy; which is the default parameters that are used by the mfold programme.

2.7 TOPO TA gene cloning and generation of stable transfectants

The TOPO TA expression system provides a highly efficient and simple one step cloning approach without the requirement of ligases, specific PCR primers, or any post PCR procedures. The process involves the direct insertion of *Taq* polymerase amplified PCR products into a plasmid vector suited for high level and constitutive expression in mammalian host cell lines, following transfection.

The current study uses the pEF6/V5-His-TOPO plasmid vector (Invitrogen Inc., Paisley, UK) which is provided linearised with a single 3' Thymidine (T) overhang for TA cloning, and a covalently bound Topoisomerase (Figure 2.6). Due to its template independent terminal transferase activity, *Taq* polymerase catalyses the addition of a single deoxyadenosine (A) to the ends of PCR products this allows for efficient ligation of the PCR product into the plasmid vector due its 3' T overhang mentioned above (the process of cloning is summarised in Figure 2.7).

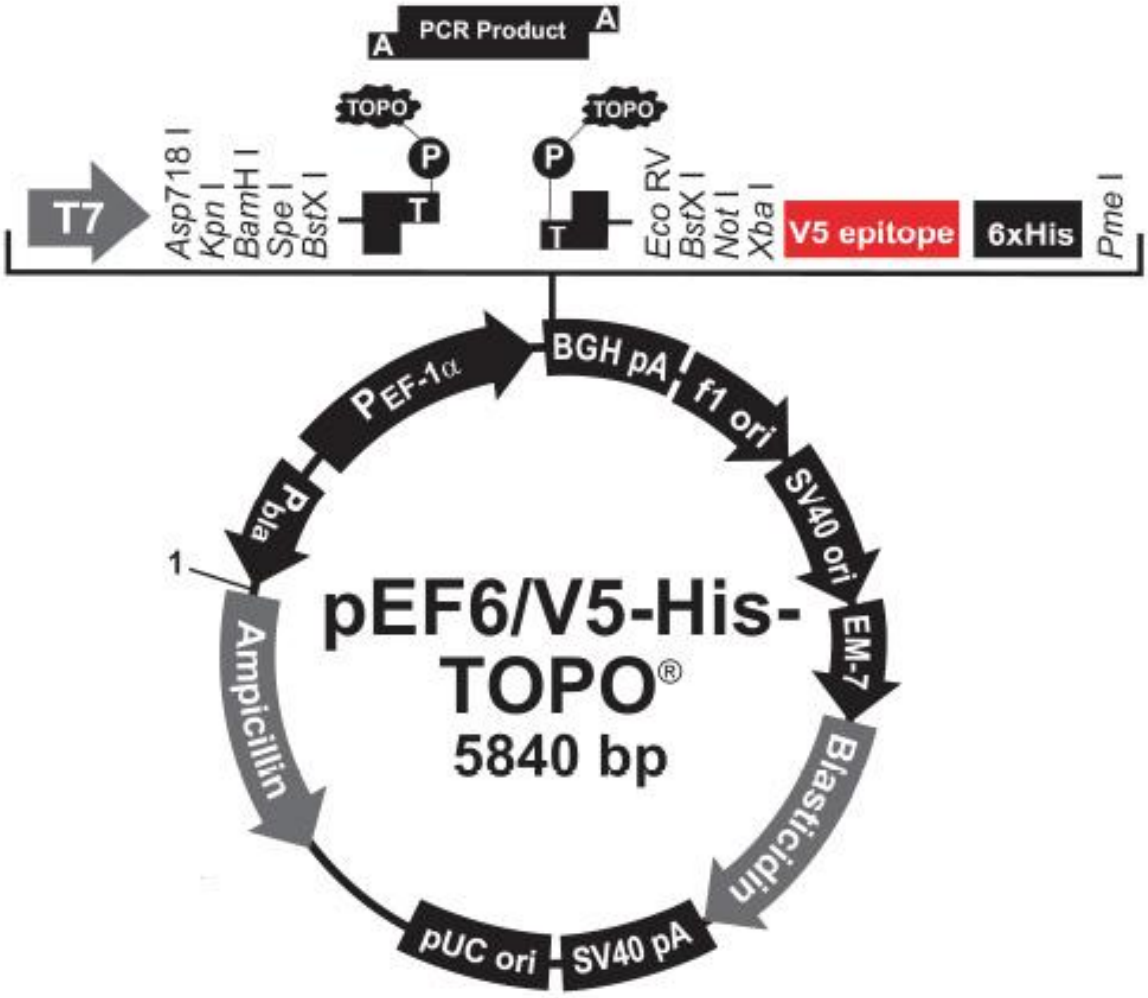


Figure 2.6 Schematic of the pEF6 plasmid (taken from pEF6/V5-His TOPO TA Expression Kit protocol).

2.7.1 TOPO cloning reaction

- The following reagents were placed in a pre-labelled eppendorf tube and mixed gently before being incubated for 5 minutes at room temperature.

  4µl of PCR product

1µl salt solution

1µl TOPO vector

2.7.2 Transformation of chemically competent E. coli

- A 5µl cloning reaction was set up (section 2.7.1) and was mixed in a vial with chemically competent One Shot™ TOP10 E.Coli (Invitrogen Inc., Paisley, UK) before being incubated on ice for 30 minutes.
- This was followed by a heat-shock at 42^{o}C for 30 seconds in a water bath, and immediate incubation back onto ice for 2 minutes.
- SOC medium (250µl warmed to room temperature) was then added to the cell suspension and left to shake horizontally at 200rpm on an orbital shaker (Bibby Stuart Scientific, UK) at 37^{o}C for an hour.
- Following this incubation period, the *E.coli* mixture was spread onto pre-warmed selective LB-agar plates (with 100µg/ml ampicillin) at high and low seeding densities, before being incubated at 37 ^{o}C over night.
- As the pEF plasmid has antibiotic resistance genes to both ampicillin and blasticidin, only the cells that contain the plasmid are capable of growing on the agar. This is a way of selecting only the colonies positive for the plasmid containing a gene of

interest. However, to confirm that the gene sequence has been inserted in the correct orientation, the colonies need to be further tested (explaining in section 2.7.3).

2.7.3 Colony selection and analysis

- In order to confirm that a right product will be produced, the colonies were analysed to verify that the gene sequence had been ligated into the vector in the correct orientation.
- Two PCR reactions were performed to analyse each of 10 isolated colonies. The first reaction used a reverse primer specific for the plasmid vector (BGHR) and a forward primer specific for the insert (RbTPF). BGHR primer ends around 173bp downstream of the inserting site and so if the insert has been ligated in the correct orientation, the resulting product should be around 50bp bigger than the predicted product size. The second reaction contains a forward primer specific for the plasmid vector (T7F) and a forward primer specific for the insert (RbTPF). If a product was seen in the second reaction, it indicated the insert was ligated in the wrong orientation. If a band is seen for both reactions then the colonies contain a mixture of plasmids with both correctly and incorrectly inserted sequences. Finally, in order to verify that the full sequence has been inserted without degradation, a further

reaction with T7F and BGHR is carried out on the colonies.

- Individual colonies were examined by lightly touching a labelled colony with a pipette tip, and mixing it into each PCR reaction before specific amplification of the desired sequence. The thermal cycler was set to the following conditions:

  Initial denaturation: 94 ^{o}C for 10 minutes

  Followed by 35 cycles of:

  Denaturation: 94 ^{o}C for 30 seconds

  Annealing: 56 ^{o}C for 40 seconds

  Extension: 72 ^{o}C for 1.5 minutes

  And finally:

  Final extension: 72^{o}C for 7 minutes

2.7.4 Plasmid purification and amplification

- Following colony analysis, the ones deemed to have the insert in the correct orientation were picked from the plate and inoculated in 10ml of LB broth with 100μg/ml ampicillin and then being incubated at 37^{o}C over night with constant agitation.
- The amplified *E.coli* were then pelleted by centrifugation at 4^{o}C for 15 minutes at

6,000rpm, and then used for plasmid extraction. This was carried out using the Sigma GenElute Plasmid MiniPrep Kit (Sigma-Aldrich, USA) according to the provided protocol, outlined below.

- The bacterial pellet was resuspended in 200µl of resuspension fluid (containing RNase A) before being mixed thoroughly and transferred into the provided 2ml collection tubes.
- This was followed by the addition of 200µl lysis solution and gentle mixing by inverting the tubes 5-6 times. The resulting mixture was left at room temperature for 5 minutes before adding 350µl of neutralisation solution.
- The tubes were inverted several times, and centrifuged at 12,000rpm for 10 minutes. The resulting supernatant was then transferred into a fresh collection tube containing a Mini Spin Column, which binds the plasmid DNA after centrifugation at 12,000rpm for 1 minute.
- The flow through was discarded before the column was washed with 700µl of wash solution (containing ethanol) and centrifuging at 12,000rpm for 1 minute. The flow through was discarded once more, before the column was dried by another centrifugation of one minute.
- The column was then transferred into a fresh collection tube for elution. This was

carried out by adding 100µl of elution solution and centrifuging at 12,000rpm for 1 minute. The resulting flow through containing the purified plasmid was collected, and around 4µl was run on a 0.8% agarose gel in order to confirm the presence and purification of the plasmid.

2.7.5 Transfection of mammalian cells using electroporation

- Following plasmid extraction, the empty plasmid, and the plasmid containing the ribozyme transgene was used to transfect both PC-3 and DU-145 prostate cancer cell lines, MDA-MB-231 and MCF-7 breast cancer cell lines, and HECV endothelial cell line respectively.
- The method of transfection used in this study was electroporation using an electroporator (Easyjet, Flowgene, Surrey, UK).
- Once the cells had reached confluency they were detached from their tissue culture flasks, counted, and approximately $1x10^6$ cells were resuspended in 1ml of media.
- 300µl of the cell suspension was then added into an electroporation cuvette (Eurgenetech, Southampton, UK), and following the addition of 3-5µl of plasmid, left at room temperature for 5 minutes.
- The cuvette was placed into the electroporator and subjected to an electrical pulse of

290-310V before immediately being transferred into a fresh tissue culture flask containing media.

2.7.6 Establishment of a stable expression mammalian cell line (Figure 2.7)

- Following transfection, in order to obtain a stable cell line carrying the constructed vector, the cultured cells needed to be selected.
- As mentioned before, the pEF plasmid contains a resistance gene against blasticidin and this is used as a selection of mammalian cell. Therefore, the cells were cultured in selection media containing 5μg/ml blasticidin for around 1-2 weeks which only allowed the cells containing the plasmid to survive.
- After selection, the cells were transferred into maintenance media containing 0.5μg/ml blasticidin. In order to verify the knockdown of the gene of interest, the cells were analysed using RT-PCR and western blotting.
- Once the cells had been verified to stably express the desired molecule; they were subjected to various *in vitro* assays in order to test the effect of altering the expression of that molecule on the biological properties of the cells. These assays are outlined in section 2.8.

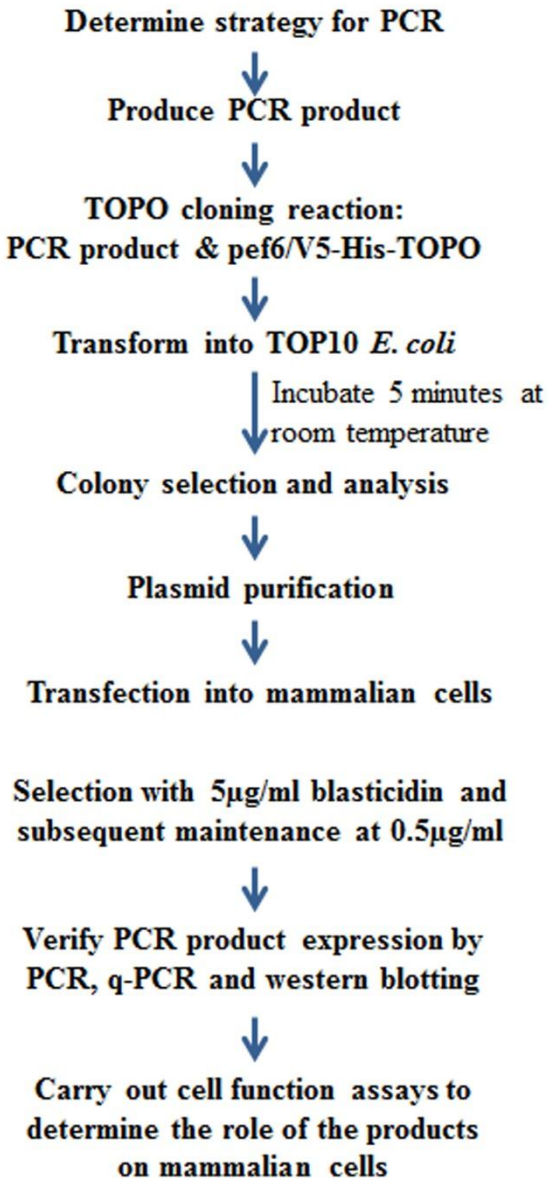


Figure 2.7 Flow chart summarising cloning and production of a stable expression mammalian cell line.

2.8 *In vitro* cell function assays

These assays can be used to quantify cell density as they adhere to multi-well plates, with or without Matrigel (a basement membrane substitute). After a set amount of time, the cells are stained with crystal violet (0.2% mixed with 2% ethanol, and dissolved in distilled H2O), which stains DNA. The cells can then either be counted, or the amount of crystal violet that the cells have absorbed can be quantified, by solubilising the dye and measuring the absorbance at 540nm using a spectrophotometer.

2.8.1 *In vitro* cell growth assay

- The protocol by Jiang *et al*. was followed (Jiang *et al.*, 2005). 200µl of media containing 3,000 cells was seeded into four 96 well plates and if required, cells were treated with a protein of interest in serum free media.
- These plates were then left to incubate at 37^{o}C, with 5% CO_2, for a period of 24 hours and 120 hours respectively.
- After the incubation, the cells were fixed with 4% formalin for 10-20 minutes, before being subsequently stained with 0.5% crystal violet for 10 minutes. The dye was washed off with water, and the plates left to dry.
- The dye was then solubilised using 200µl acetic acid, and cell growth was assessed

by measuring the absorbance at 540nm using a spectrophotometer (BIO-TEK, Elx800, UK). The growth rate was calculated as a percentage, using the absorbance taken at 24 hours as a baseline.

2.8.2 *In vitro* adhesion assay

- Wells in a 96 well plate were coated with 100µl of serum free media with 5µg (stock concentration 0.05µg /µl) Matrigel (BD Matrigel™ Matrix, Magrigel™ Basement Membrane Matrix, Biosciences). The Matrigel was then left to dry for 2 hours at 55^{o}C.
- To rehydrate the Matrigel, 200ul of sterile water was added to each well and left for 45 minutes at room temperature.
- The media was aspirated and 20,000 cells diluted in 200µl media, were seeded into each well and left to adhere on the Matrigel over a 40-minute culture at 37^{o}C, with 5% CO_2.
- After the incubation, the wells were washed with BSS to remove any non-adherent cells. The adherent cells were fixed with 4% formalin for 10-20 minutes, and then stained with 0.5% crystal violet for 10 minutes. Following washes, the plates were left to dry before counting the adherent cells and taking photos under a microscope.

Due to the fluid dynamics within the small sized wells of a 96 well plate, matrigel sets unevenly, causing cell aggregation around the edges of the well. Therefore, in order to avoid these areas, only the cells which had adhered to the central area of the well were counted.

2.8.3 *In vitro* invasion assay

- 8µm pore Transwell inserts (FALCON®, pore size 8.0µm, 24 well format, Greiner Bio one, Germany) were placed into wells of a 24 well plate (NUNC™, Greiner Bio one, Germany), using sterile forceps in order to prevent contamination.
- Each insert was subsequently coated with 100µl serum free media containing 50µg Matrigel, and left to dry for 2 hours at 55 ^{o}C.
- The Matrigel was then rehydrated with 100µl sterile water for an hour at room temperature.
- The water was discarded, and 20,000 cells in 200µl media were seeded into each insert. 600µl media was then added to the lower chamber of each well (outside the inserts) and the cells were incubated for a maximum of 96 hours, with 5% CO_2 at 37 ^{o}C. This setup is shown in Figure 2.8.
- After 72 hours incubation, the invasive cells migrate through the Matrigel, and the

porous membrane to the other side of the insert. The Matrigel layer and the non-invasive cells were then removed from inside the insert using tissue paper. If this step is not carried out, the Matrigel would also be stained with crystal violet, making it difficult to distinguish between the background and invading cells.

- The invasive cells were then fixed with 4% formalin for 10-20 minutes, and then stained with 0.5% crystal violet for 10 minutes. The crystal violet was washed off, the plates left to dry, and the stained cells photographed and counted under a microscope.

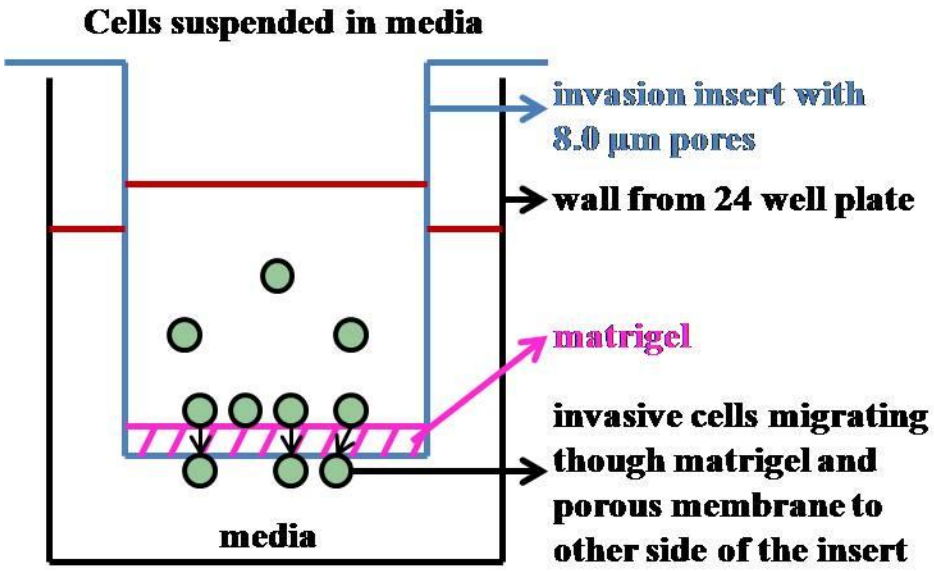


Figure 2.8 Schematic diagram showing *in vitro* invasion assay.

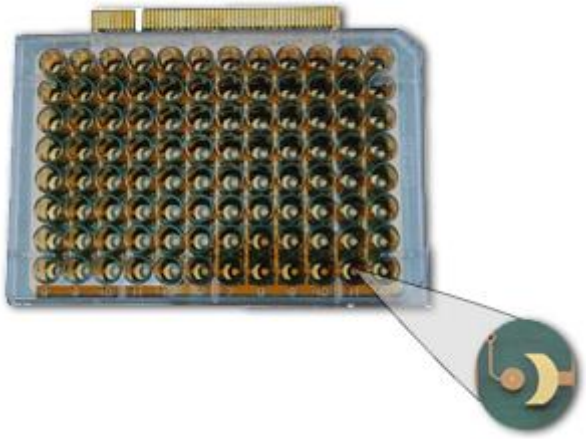
2.8.4 *In vitro* motility assay

- The protocol by Jiang *et al*. was followed (Jiang *et al.*, 1995). 700µl of media containing 200,000-300,000 cells are seeded into a 24 well plate and cells were treated with a protein of interest in serum free media if required.
- These plates were then left to incubate at 37^{o}C, with 5% CO_2, for a period of 24 hours to allow cells to form a confluent monolayer then the cells were wounded and took photos using a microscope with camera at 0.25, 1, 2, and 3 hours after wounding. Migration distances were measured using ImageJ software (National Institutes of Health, USA).

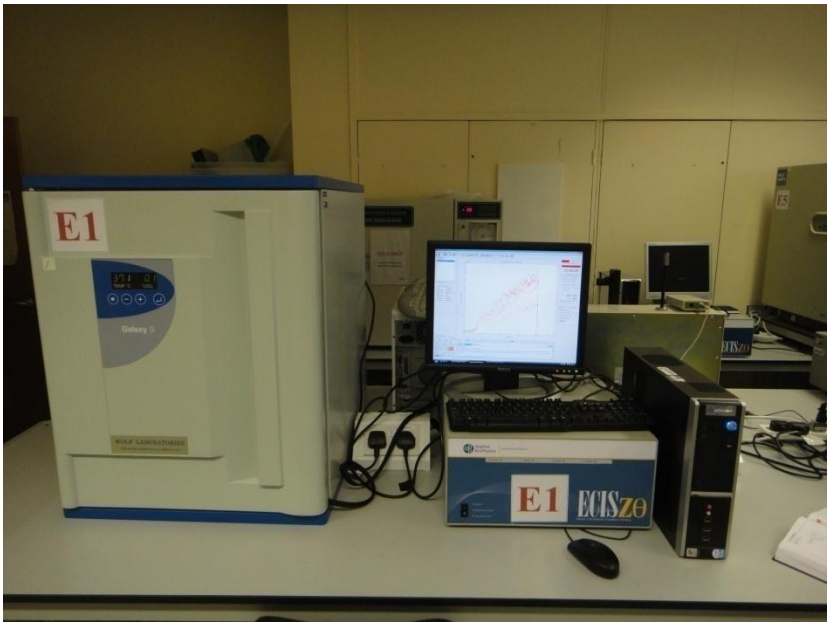
2.8.5 Electrical cellular impedance sensing (ECIS)

- Electrical Cellular Impedance Sensing (ECIS) is a novel method used as an alternative to the conventional function assays mentioned above. It works using an array of 96 wells, each containing a gold electrode. These measure the current and voltage across this electrode, calculating the impedance and resistance. From the impedance changes, effects on cell attachment and motility can be examined (Keese *et al.*, 2004). Using 96W1E arrays (ECIS™ cultureware, Applied Biophysics, Inc., NY, USA), each well was stabilised at room temperature for around 20 minutes

using 200µl electrode stabilising solution (ECIS™, Applied Biophysics, Inc., NY, USA).



- The solution was then aspirated and replaced with 200µl DMEM F12 media containing Hepes buffer (Lonza, Verviers, Belgium) and left until needed.
- The media was aspirated, and 40,000 cells diluted in 200µl DMEM were seeded into each well, and treated with a protein of interest if required.
- The array was then placed into an ECIS™ CO_2 incubator (RS biotech 9600, R galaxy R+) which is connected to the ECIS™ Model 9600 Controller (Applied Biophysics, Inc., NY, USA). The software was set up so that resistance to the current flow was measured at 400Hz. Data was normalised using resistance from the first time point.



2.8.6 Analysing apoptosis using flow cytometer

Phosphatidylserine (PS), a phospho-lipid component of the cell membrane is normally located on the cytoplasmic side of cell membranes in normal live cells. However, when the cells become apoptotic, PS translocates from the inner to the outer leaflet of the cell membrane, resulting in it being exposed to the external cellular membrane. In leukocytes, this functions as a trigger for macrophages to phagocytose the apoptotic cells.

In order to detect apoptotic cells, this current study uses the Annexin V Kit (Santa Cruz Biotechnology) to carry out an apoptosis assay. This kit contains recombinant fluorescent conjugated annexin V (FITC annexin V) and Propidium Iodide (PI) solution.

The calcium dependent phospholipid-binding protein annexin V, binds to PS with a high affinity, and hence when labelled with a fluorophore or biotin, can be used to detect apoptotic cells by binding to the PS on the outer leaflet of the cell membrane. PI on the other hand, is a red-fluorescent intercalating dye, capable of permeating only through cell membranes of non-viable cells and binding to their nucleic acids. Therefore, any cells that have lost their membrane integrity will be stained red by the PI, whereas the apoptotic cells, which are impermeable to PI, will be stained green by the FITC annexin V only. Any live cells present in the solution will show little or no fluorescence. This partial staining allows for easy identification with the Partec CyFlow® SL flow cytometer and the accompanying FloMax software package (Partec GmbH, Munster, Germany). The protocol was carried out as follows:

- Both the adherent cells and those floating in the culture medium, were harvested and washed in PBS. The cell solution was then centrifuged, before $5x10^5$ cells were resuspended in 200µl 1X annexin-binding buffer, sufficient for 100µl per assay.
- 1µl of FITC annexin V (0.01µg/ml) and 1µl of PI working solution (100µg/ml) were subsequently added to each 100µl cell fraction and the cells were left at room temperature for 30 minutes.
- Following incubation, 400µl of 1X annexin binding buffer was added and mixed

gently then placed on ice.

- The stained cells were analysed immediately using the flow cytometer and FlowMax software package, measuring any fluorescence emission at 530nm (FL1) and 575nm (FL3).

2.9 Clinical cohort study

2.9.1 Breast cancer

A total of 160 breast samples were collected immediately after surgery and stored at -80^{o}C until use, with approval of the Bro Taf Health Authority local research ethics committee. All patients were informed and participated with written consent. The cohort included 127 breast cancer tissues and 33 background normal breast tissues (Table 2.7). All the specimens were verified by a consultant pathologist. A routine follow-up was carried out after surgery. The median follow-up period was 120 months.

Table 2.7 Sample numbers of breast cohort study.

Clinical/pathological features	Sample numbers
Tissue sample	
Normal	33
Tumour	127
Grade	
1	23
2	41
3	56
NPI	
1 (<3.5)	68
2 (3.5-5.4)	38
3 (>5.4)	16
TNM	
1	61
2	40
3	7
4	4
Clinical outcome	
Disease-free	87
Poor outcome	28
With metastasis	7
With local recurrence	5
Died of breast cancer	16
Histology	
Ductal	88
Lobular	14
Others	8
Lymph node status	
Negative	65
Positive	55
Estrogen receptor	
ERα (-)	75
ERα (+)	38
ERβ (-)	91
ERβ (+)	24

2.10 Statistical analysis

Statistical analysis was performed using SPSS18 (SPSS Inc., Chicago, USA). Data were calculated as the mean ± SD and Mann-Whitney U-test was used for analysis of non-normally distributed data, while the Student's t-test was used for normally distributed data. Each assay was performed at least three times. Chi-squared test was used for comparison of two independent groups and Fisher's exact test was used for certain samples analysis. Survival data was analysed using the Kaplan-Meier survival analysis. P-value $<$ 0.05 were considered statistical significant.

Chapter 3

PTPRK expression in prostate tissues and cell lines

3.1 Introduction

Protein tyrosine phosphatase kappa (PTPRK or PTPκ) belongs to the MAM-subfamily of the classic protein tyrosine phosphatases (PTPs). PTPRK was identified in 1993 by Jiang *et al*. (Jiang, Wang *et al*. 1993) and it contains a MAM (Meprin/A5/ PTPmu) domain in the extracellular region followed by one Ig-like domain and four fibronectin type III repeats. The MAM-subfamily has two cytoplasmic PTP domains (the second PTP domain is a pseudophosphatase) and an intracellular juxtamembrane region homologous to the intracellular domain of cadherin (Zondag *et al.,* 1995). PTPRK is expressed in normal tissues such as spleen, prostate, ovary, brain, and has also been found in melanocyte and keratinocyte epidermal cell lines (Jiang *et al.,* 1993; McArdle *et al.,* 2005).

Recently it has been suggested that PTPs have already been shown to act as tumour suppressors, such as PTEN (MMAC1, a tumour suppressor which is mutated in various human cancers) and PTPρ (PTPRT, a potential tumour suppressor in colorectal cancers) (Tonks 2006). PTPRK has been demonstrated to act as a potential tumour suppressor in primary central nervous system lymphomas (Nakamura *et al.,* 2003). However, the role played by PTPRK in cancer remains largely unknown.

The study presented in this chapter was aimed to investigate the implication of PTPRK in prostate cancer, by examining its expression profile in prostate cancer tissues and cell lines, and subsequently to establish *in vitro* cell models to further explore its effect on functions of the cancerous cells.

3.2 Materials and methods

3.2.1 Prostate cancer tissues

Prostate samples were collected immediately after surgery and stored at -80℃ until further use, with approval of the Bro Taf Health Authority local research ethics committee. All patients were informed and participated with written consent. All the specimens were verified by a consultant pathologist.

3.2.2 Antibody and primers

Polyclonal rabbit anti-PTPRK antibody (SC-28906) was obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). All the primers used were synthesised and provided by Invitrogen (Paisley, UK). Primer sequences are shown in Tables 2.2 and 2.3.

3.2.3 Cell lines

PC-3, DU-145, LNCaP, CAHPV-10, PZHPV7, PNT1A, PNT2C2 and MRC-5 cells were routinely cultured in DMEM-F12 medium as described in section 2.3.

3.2.4 RNA isolation, cDNA synthesis, and RT-PCR

RNA was isolated from cells using Tri Reagent (Sigma-Aldrich, Inc., Poole, Dorset, England, UK), and converted into cDNA by reverse transcription using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, California, USA), as described in section 2.4. RT-PCR was carried out at the following conditions; 94˚C for 5 minutes, followed by 30 to 42 cycles of 94˚C for 30 seconds, 56˚C for 30 seconds, and 72˚C for 1 minute and a final extension of 7 minutes at 72˚C. The products were run on an agarose gel and visualised using ethidium bromide.

3.2.5 Protein extraction, SDS-PAGE, and Western blot analysis

Protein was extracted and was then quantified using the DC Protein Assay kit (BIO-RAD, USA). After SDS-PAGE, the proteins were transferred onto nitrocellulose membranes which were then blocked and probed with the specific primary (anti-PTPRK 1:500) and the corresponding peroxidise-conjugated secondary antibodies

(1:1000). All of the antibodies used in this study are listed in Table 2.4. The protein bands were eventually visualised using the chemiluminescence detection kit (Luminata, Millipore).

3.2.6 Immunochemical staining of PTPRK

Immunochemical staining of PTPRK in prostate cancer cells and tissues were carried out using specific primary antibody for the protein, followed by secondary antibody. For the detailed procedure refer to Section 2.5.9.

3.3 Results

3.3.1 Prostate tissues and cell lines screened for PTPRK expression

The expression of PTPRK was examined in seven prostate cell lines, including PC-3, DU-145, LNCaP, CA-HPV-10, PZ-HPV-7, PNT-1A and PNT-2C2 and fibroblast cell line, MRC-5. The first four are prostate cancer cells. PC-3 is derived from bone metastatic lesions originating from prostate cancer. DU-145 is from a brain metastasis of prostate cancer. LNCaP is from lymphatic metastasis and CA-HPV-10 from a localised prostate carcinoma. The remaining three are immortalised prostatic epithelial cells.

Figure 3.1 shows that PTPRK is consistently expressed in all the prostate cell lines, except PNT-1A, where it appears to have a lower level of PTPRK mRNA. Figure 3.2 shows that PTPRK is more frequently expressed in prostate cancer tissues (12/18, 66.7% positive, p=0.143 vs. normal using Fisher exact test) than normal prostate tissues at mRNA level (4/11, 36.7% positive). Furthermore, Figure 3.3 also shows a higher PTPRK expression level in prostate cancer tissues compared with normal prostate tissues using IHC staining. Figure 3.4 shows the overall PTPRK protein expression in two prostate cancer cell lines (PC-3 and DU-145) and one fibroblast cell line (MRC-5) using ICC staining. Figure 3.5 shows PTPRK protein expression in different cell lines using western blot. PTPRK was consistently expressed in all the cell lines except in PNT-1A and PNT-2C2 where a lower protein expression level of PTPRK was seen.

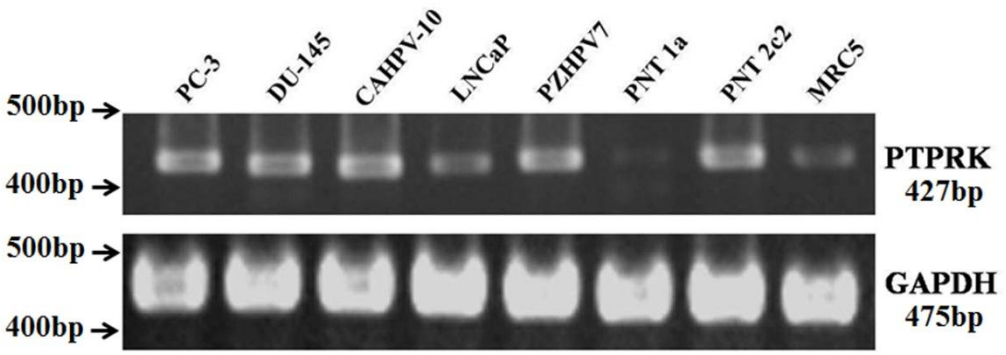


Figure 3.1 Cell line screen for PTPRK mRNA expression. Seven prostate cell lines and one fibroblast cell line were screened for mRNA expression of PTPRK using conventional PCR techniques.

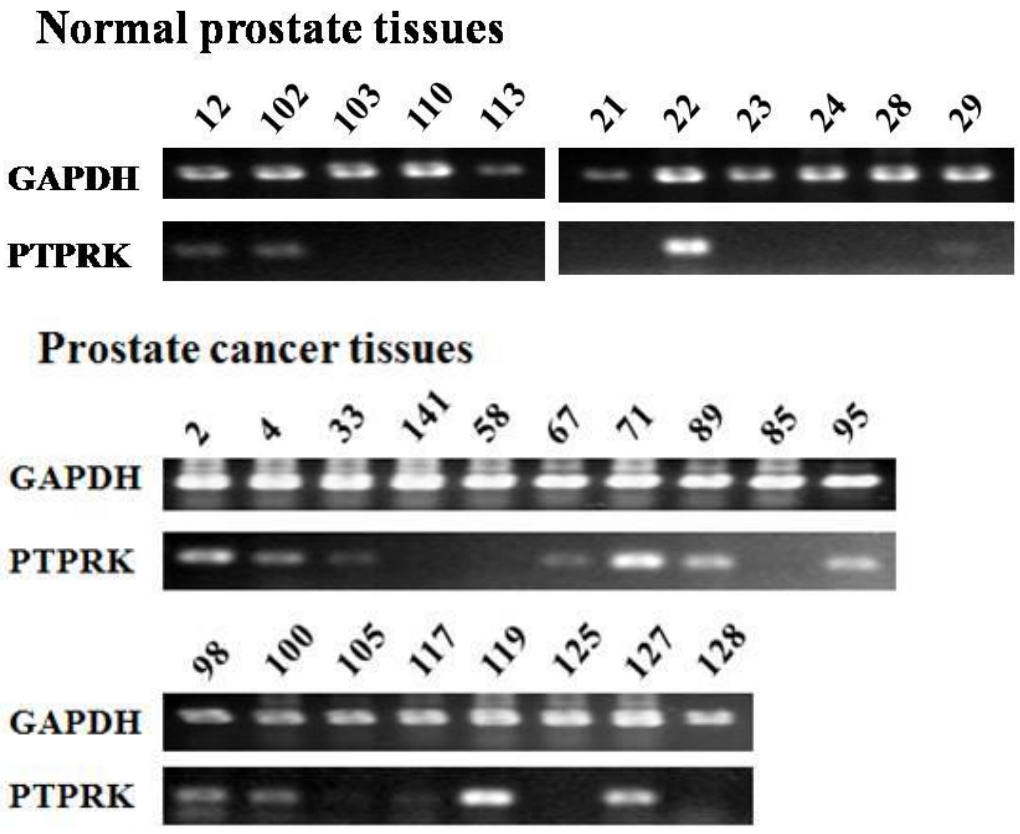


Figure 3.2 Prostate tissues screen for PTPRK. 29 prostate tissues were screened for mRNA expression of PTPRK using conventional PCR techniques. Different nombers

were stood for different patients.

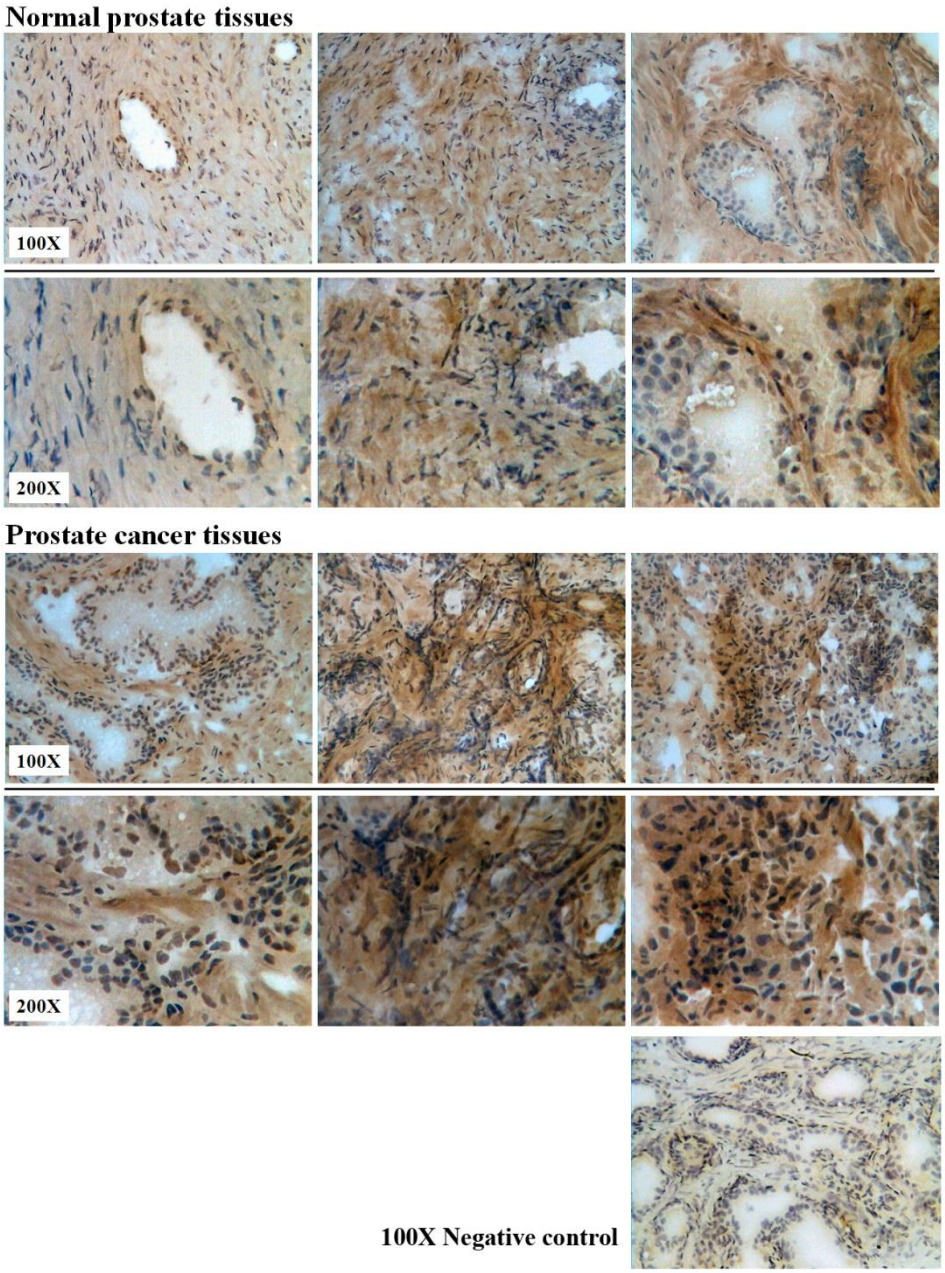


Figure 3.3 IHC staining for PTPRK in normal prostate and prostate cancer tissues.

Three normal prostate tissues and three prostate cancer tissues were screened for the PTPRK protein expression in tissues using IHC staining.

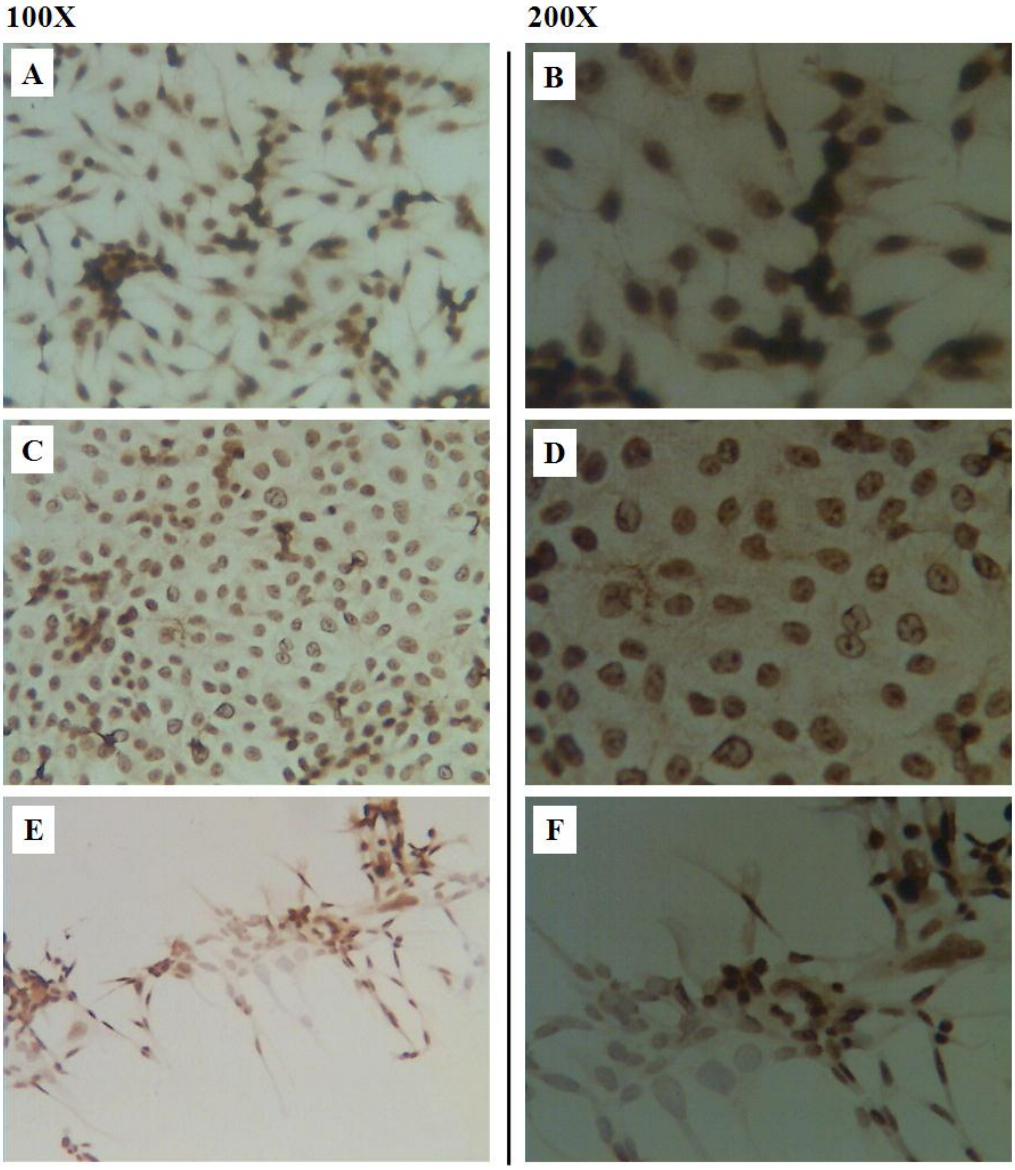


Figure 3.4 ICC staining for PTPRK in cell lines. Two prostate cancer cell lines (PC-3 and DU-145) and one fibroblast cell line (MRC-5) were screened for the PTPRK expression in tissues using ICC staining. A-B show PC-3 cells, C-D show DU-145 cells and E-F show MRC-5 cells.

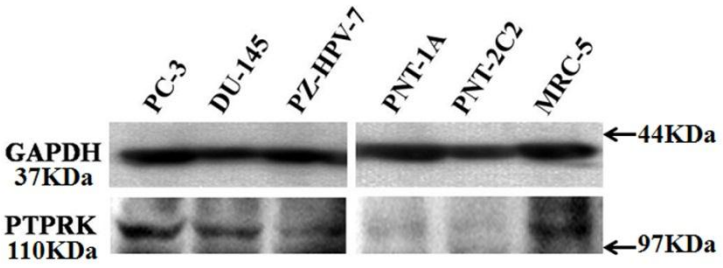


Figure 3.5 Cell line screen for PTPRK protein expression. Five prostate cell lines and one fibroblast cell line were screened for protein expression of PTPRK using western blot.

3.4 Discussion

PTPRK is a poorly studied protein phosphatase in the field of cancer progression. The limited information available in the literature indicates that PTPRK probably behaves in a similar manner with its closest homologue: PTPRM. In fact, PTPRM and PTPRK both prefer homophilic binding; PTPRM not only binds to PTPRM but also recognises other subfamily members, such as PTPRK and PTPRT to mediate cell-cell aggregation. However, PTPRK does not interact with PTPRM. Expression of PTPRK only promoted homophilic interactions which include cell aggregation and cell-cell adhesion. Furthermore, it has been reported that PTPRK-mediated intercellular adhesion does not need PTPase activity or post-translational modification of PTPRK protein and is calcium independent (Brady-Kalnay *et al.*, 1993; Sap *et al.*, 1994; Zondag *et al.*, 1995; Becka *et al.*, 2010). Additionally, PTPRM has been shown to drive cancer progression (Gyorffy *et al.*, 2008), however PTPRK has been reported as a tumour suppressor in the primary lymphoma of central nervous system and associated with colorectal cancer and pancreatic islet tumour (Lu *et al.*, 1998; Nakamura *et al.*, 2003; Cady *et al.*, 2008; Starr *et al.*, 2009). No other studies have attempted to define the function of PTPRK in prostate cells and its potential involvement in cancer metastasis.

In the current study, we firstly demonstrated the presence of PTPRK expression in seven prostatic cell lines and one human endothelial cell line. The cell lines used here have extensively been used as models for *in vitro* studies on prostate cancer. PC-3, DU-145 and LNCaP are aggressive prostatic carcinomas derived from metastatic sites of the bone, brain and lymph nodes respectively. CA-HPV-10 represents a less aggressive prostatic adenocarcinoma cell line immortalised with HPV-18. PZ-HPV-7, PNT-1A, and PNT-2C2 are immortalised prostatic epithelial cell lines.

Its expression in tissues shows that PTPRK expression slightly higher in prostate cancer tissues than in normal prostate tissue samples in both mRNA and protein levels but the stastical analysis dose not reach significant difference. According to the mRNA levels of PTPRK, its expression appears at a similar level of that seen in prostate cell lines. The only exception was PNT-1A which appeared to have lower PTPRK expression. Furthermore, the protein levels of PTPRK in cell lines are similar with mRNA expression as well. PTPRK expression in PNT-1A and PNT-2C2 are both very weak compared with other cell lines. However, the current assessment of PTPRK expression in prostate cancer is limited and not sufficient to reach any solid conclusion. Hopefully, the implication of PTPRK in disease development and progression of prostate cancer

can be elucidated by further investigations using a large clinical cohort of prostate cancer.

The staining location of PTPRK was seen in all pictures to be located mainly at the cell nuclei using ICC staining. As PTPRK is believed to be a transmembrane protein phosphatase, the location of the staining appears to against this belief. It may be due to the distribution change PTPRK even if no certain contact with ECM suggesting PTPRK itself may be a molecule which is participated in regulation of down-stream signalling pathway. However, the activation and changes should be further investigated.

This screening process also aided the determination of which cell lines to use for the remainder of the study. As previously mentioned, PTPRK is a cell surface tyrosine phosphatase with a possible role in intracellular signal transduction, as well as being in place to inactivate other cell surface proteins. Since the mRNA expression levels are similar in all prostate cells, we chose aggressive prostate cancer cell lines, PC-3 (highly tumourigenic) and DU-145; these cell lines were selected to transfect with the ribozyme transgene to determine the consequences of PTPRK knockdown on *in vitro* cellular functions. The findings of the function based studies are presented in the next chapter.

Chapter 4

Knockdown of PTPRK and the effect on the functions of prostate cancer cells

4.1 Introduction

A cancer cell relies on varying vital biological processes to establish itself in its environment and to subsequently metastasise. The most important of these include aberrations in cell proliferation, adhesion, invasion and migration. Cancer cells can influence these processes in several ways, most significantly by altering the expression of molecules that play key roles in controlling these cellular functions. PTPRK has been previously shown to inhibit cell proliferation by regulating EGFR and the TGF-β signalling pathway in different cells. The homophilic binding ability of PTPRK is similar to PTPRM. Furthermore, PTPRK regulates cell migration by TGF-β signalling pathway via positively regulated Src. This chapter attempts to determine whether the knockdown of PTPRK can affect the behaviour of prostate cancer cells.

PTPRK was knocked down in PC-3 and DU-145 cells using a ribozyme transgene and the effect on cellular functions was subsequently investigated.

4.2 Materials and methods

4.2.1 Cell lines

PC-3 and DU-145 prostate cancer cell lines were used in this chapter, including empty plasmid control and transfected cell lines. Cells were continuously maintained in

DMEM media with 10% FBS and antibiotics. The stable transfected cells were maintained in the same media and supplemented with 0.5μg/ml blasticidin.

4.2.2 Generation of PTPRK ribozyme transgenes

Hammerhead ribozymes targeting PTPRK were designed using Zuker's mRNA Fold programme (Zuker, 2003), based on the secondary structure of PTPRK mRNA. Primers containing restriction sites were then generated (Table 2.3). The ribozymes were then synthesised using a touch-down PCR procedure at the following conditions; 94˚C for 5 minutes, followed by 8 cycles at each annealing temperature (total of 48 cycles): 94˚C for 10 seconds, 70˚C, 65˚C, 60˚C, 57˚C, 54˚C, and 50˚C for 15 seconds, 72˚C for 20 seconds and a final extension of 7 minutes at 72˚C. Subsequently, the transgenes were run on a 2% agarose gel in order to verify their presence as well as size, before being cloned into a plasmid vector.

4.2.3 TOPO TA cloning of PTPRK fragments or transgenes into a pEF6/His TOPO plasmid vector

Following verification, the PTPRK transgene was cloned into a pEF6/V5-His-TOPO plasmid (Invitrogen Inc., Paisley, UK), followed by transformation of constructed

plasmid into *E.Coli*. The correct constructs were then amplified and purified using the Sigma GenElute Plasmid MiniPrep Kit (Sigma-Aldrich, USA).

4.2.4 Prostate cancer cell transfection and generation of stable transfectants

Following plasmid verification using DNA electrophoresis, the plasmids were transfected into target cells using the electroporation at 310V. The transfectants were then selected with 5μg/ml blasticidin for a period of two weeks. Empty plasmid vectors were also used to transfect the same cells and used as a control for the following experiments. After the selection, the cells were verified for PTPRK knockdown using RT-PCR, Q-PCR, and Western blotting. Full details of the cloning process have been given in section 2.7.

4.2.5 RNA isolation, cDNA synthesis, RT-PCR, and Q-PCR

RNA was isolated from the cells using the Tri Reagent kit (Sigma-Aldrich, Inc., Poole, Dorset, England, UK) and converted into cDNA by reverse transcription using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, California, USA), as described in section 2.4. RT-PCR was carried out at the following conditions; 94 ^{o}C for 5 minutes, followed by 30 to 35 cycles of 94 ^{o}C for 30 seconds, 56 ^{o}C for 30 seconds, and 72 ^{o}C for

1 minute, and a final extension of 7 minutes at 72 ^{o}C. The products were run on an agarose gel and visualised using ethidium bromide. Q-PCR was performed following the conditions; 94^{o}C for 5 minutes, followed by 80 to 90 cycles of 94^{o}C for 10 seconds, 55^{o}C for 15 seconds, and 72^{o}C for 20 seconds.

4.2.6 Protein extraction, SDS-PAGE, and Western blot analysis

Protein was extracted and was then quantified using the DC Protein Assay kit (BIO-RAD, USA). After the SDS-PAGE, the proteins were transferred onto nitrocellulose membranes which were then blocked and probed with the specific primary (anti-PTPRK 1:500) and the corresponding peroxidise-conjugated secondary antibodies (1:1000). All of the antibodies used in this study are listed in Table 2.4. The protein bands were eventually visualised using the chemiluminescence detection kit (Luminata, Millipore).

4.2.7 *In vitro* cell growth assay

The cells were seeded into three 96 well plates, and incubated for 1 and 5 days respectively, as described in section 2.8.1. Following incubation, the cells were fixed and stained with crystal violet before the absorbance was measured in order to

determine cell number.

4.2.8 *In vitro* cell Matrigel adhesion assay

The cells were seeded into a 96 well plate coated with matrigel as described in section 2.8.2. The cells were left to adhere for a period of 40 minutes, before being fixed and stained with crystal violet and the cells counted.

4.2.9 *In vitro* cell motility assay

The protocol followed is described by Jiang (Jiang *et al*., 1995b). Cells are seeded into a 24 well plate and these plates were then left to incubate at 37^{o}C, with 5% CO_2, for a period of 24 hours to allow cells to form a confluent monolayer then the cells were wounded and photos taken using a microscope with camera at 0.25, 1, 2 and 3 hours after wounding. Migration distances were measured using ImageJ software (National Institutes of Health, USA)

4.2.10 *In vitro* cell Matrigel invasion assay

The cells were seeded into transwell inserts with 8μm pores coated with 50 μg matrigel, in a 24 well plate and were incubated for a period of 3 days. Following incubation, the

cells which had migrated through the matrigel to the other side of the insert were fixed in formalin, stained with crystal violet and counted.

4.3 Results

4.3.1 Generation of a PTPRK ribozyme transgene

In order to silence the expression of PTPRK in prostate cancer cells, ribozyme transgenes targeting PTPRK were generated based on the secondary structure of human PTPRK mRNA (figure 2.5a) and cloned into a pEF6/His plasmid. Figure 3.6 depicts the series of PCR reactions carried out to construct a plasmid containing a PTPRK ribozyme transgene. Based on the secondary structure of PTPRK, an appropriate targeting site for the ribozyme was first ascertained.

This was followed by ribozyme synthesis through touchdown PCR, and cloning into a pEF6/His plasmid. In order to verify correct orientation of the ribozyme transgene, primers RbTPF and RbBMR were paired with T7F, respectively. These primers were specific to the ribozyme transgenes.

If the transgene is correctly orientated, a PCR product of around 140bp (T7F promoter

starts 90bp before insert and ribozyme sequence is around 50bp) should arise for the T7F+RbBMR reaction. However, if it is incorrectly orientated, a product of a similar size should appear for the T7F+RbTPF reaction. Following colony analysis, all those found to be positive for the transgenes underwent further amplification and finally, plasmid extraction. Colonies 2 and 10 were shown to have the highest levels of correctly orientated ribozyme transgene. The plasmids were then verified using DNA electrophoresis, in order to demonstrate successful isolation of correctly sized plasmids (Figure 4.1).

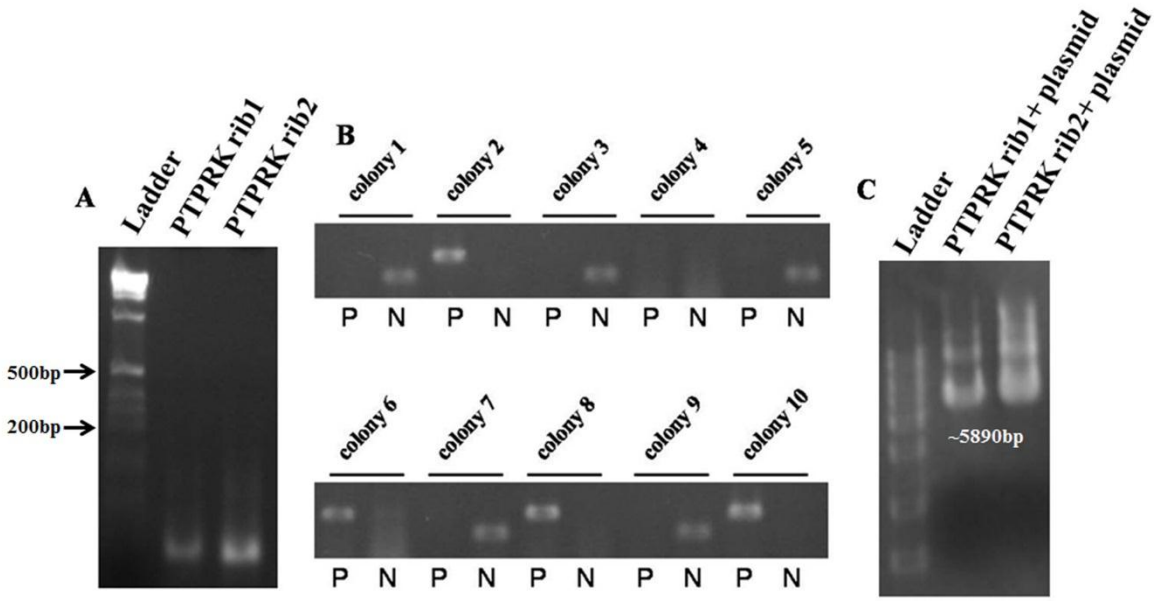


Figure 4.1 Ribozyme transgene synthesis. A. The ribozymes were generated using touchdown PCR and run on an agarose gel. **B.** After transformation into *E.coli* cells, the colonies were analysed using PCR in order to verify correct orientation of the transgene. P for correct orientation (50bp ribozyme sequence plus 173bp sequence downstream of the insert site), which refers to a PCR reaction that uses a plasmid specific reverse primer (BGHR) and a ribozyme specific reverse primer (RbTPF) and N stands for incorrect orientation (50bp ribozyme sequence plus 90bp sequence upstream of the insert site), where a plasmid specific forward primer (T7F) is coupled with a ribozyme forward primer (RbTPF), in a PCR reaction. **C.** The plasmids were extracted from the correct colonies and verified with DNA electrophoresis.

4.3.2 Verification of PTPRK knockdown in PC-3 and DU-145 cells

RT-PCR, Q-PCR and western blots were carried out to ensure that the knockdown of PTPRK was successful at both mRNA and protein levels using conventional RT-PCR, quantitative real time PCR and Western blot in both PC-3 (Figure 4.2) and DU-145 (Figure 4.3) cells. Figure 4.3A shows knockdown of PTPRK in mRNA level using RT-PCR. Figure 4.3B shows PTPRK mRNA volume of four repeats which was normalised against corresponding internal control (GAPDH) using Quantitative real time PCR. PTPRK expression was decreased in PTPRK knockdown cells compared with empty plasmid pEF control. Figure C shows knockdown of PTPRK in protein level using western blots. Figure 4.3D shows PTPRK protein bands volume of three repeats which is normalised against corresponding internal control. PTPRK expression was decreased in PTPRK knockdown cells compared with pEF control.

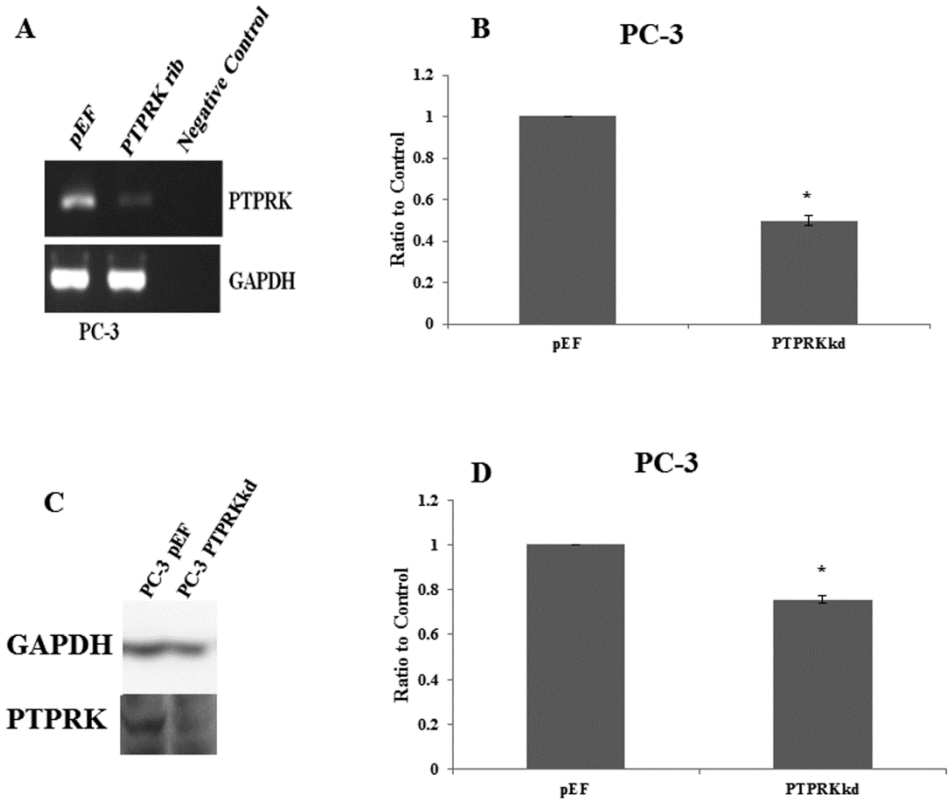


Figure 4.2 Confirmation of knockdown of PTPRK in PC-3 cells. A. RT-PCR showing reduced levels of PTPRK in mRNA in PC-3 cell compared with PC-3^{pEF} control cell. **B.** Quantitative real time PCR shows PTPRK mRNA volume of four repeats which is normalised against corresponding internal control (GAPDH). PTPRK expression was decreasing in PC-$3^{PTPRKkd}$ cells compared with PC-3^{pEF} ($p<0.05$). **C.** Western blot showing reduced levels of PTPRK protein in the PC-$3^{PTPRKkd}$ cell compared to the PC-3^{pEF} control cell. **D.** PTPRK protein bands volume of three repeats which is normalised against corresponding internal control (GAPDH). PTPRK expression was decreasing in PC-$3^{PTPRKkd}$ cells compared with PC-3^{pEF} ($p<0.05$).

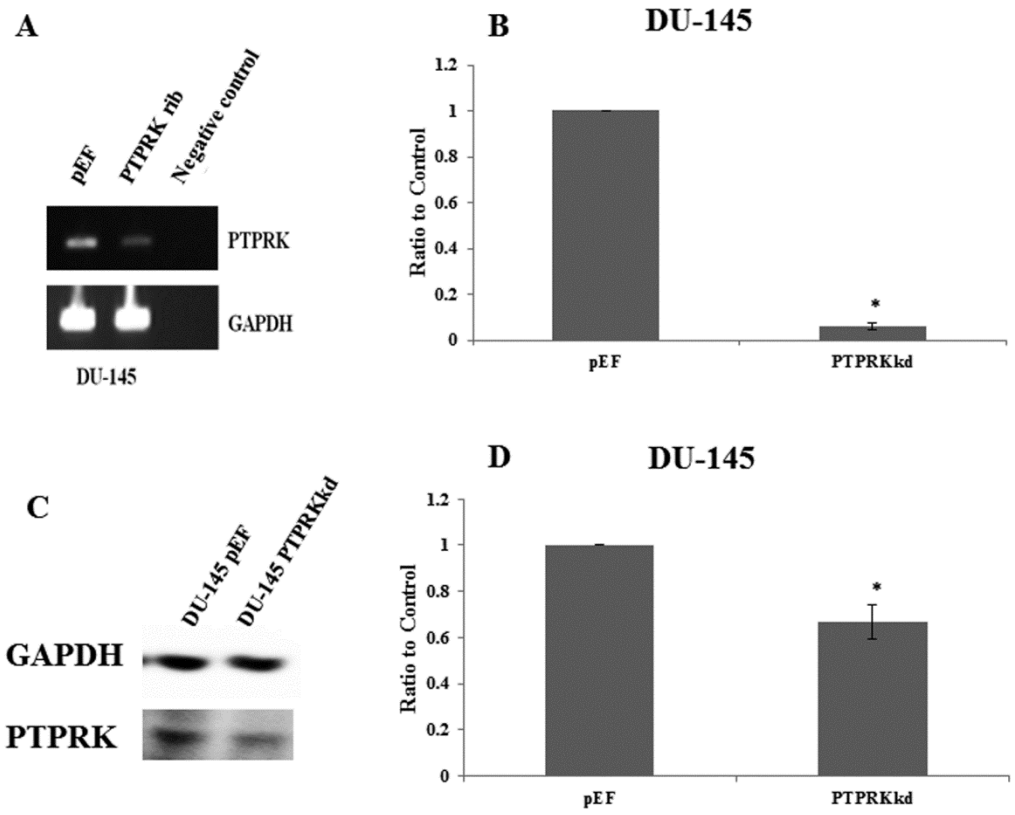


Figure 4.3 Confirmation of knockdown of PTPRK in DU-145 cells. A. RT-PCR showing reduced levels of PTPRK in mRNA in DU-145 cell compared with DU-145^{pEF} control cell. **B.** Quantitative real time PCR shows PTPRK mRNA volume of four repeats which is normalised against corresponding internal control (GAPDH). PTPRK expression was decreasing in DU-145^{PTPRKkd} cells compared with DU-145^{pEF} ($p<0.05$). **C.** Western blot showing reduced levels of PTPRK protein in the DU-145^{PTPRKkd} cell compared to the DU-145^{pEF} control cell. **D.** PTPRK protein bands volume of three repeats which is normalised against corresponding internal control (GAPDH). PTPRK expression was decreasing in DU-145^{PTPRKkd} cells compared with DU-145^{pEF} ($p<0.05$).

4.3.3 Effect of PTPRK knockdown on the growth of prostate cancer cells

The cell lines displaying knockdown of PTPRK were used in an *in vitro* cell growth assay along with the empty plasmid controls. There was a significant decrease in the growth of the PC-3^{PTPRKkd} cells (Figure 4.4). Cell growth at 5 days was significantly reduced in the PC-3^{PTPRKkd} cells (634.33±58.76) $p<0.001$, compared with the control PC-3^{pEF} (739.35±24.14).

There was also a significant decrease in the growth seen in the DU-145^{PTPRKkd} cells (Figure 4.5). The growth rate at 5 days was significantly reduced in the DU-145^{PTPRKkd} cells (667.23±6.91) compared with the control DU-145^{pEF} (817.22±46.69) $p<0.001$.

4.3.4 Effect of PTPRK knockdown on *in vitro* cell-matrix adhesion

The PC-3 cells were further analysed for their adhesive capacity using an *in vitro* matrigel adhesion assay. The cells with a knockdown of PTPRK displayed a significant decrease in adhesive capability compared to its controls. Representative images of adhered PC-3 cells (Figure 4.6A). There was a significant decrease in cell adhesion of PC-3^{PTPRKkd} cells (23.95±7.47) $p<0.001$ compared to the control PC-3^{pEF} (53.95±11.94) (Figure 4.6B).

The DU-145 cells were also analysed for their adhesive capacity. The cells with a knockdown of PTPRK displayed a significant decrease of adhesive capability compared to its controls. Figure 4.7A **was** representative images of adhered DU-145 cells. Figure 4.7B demonstrated that **t**here was a significant decrease in cell adhesion with DU-145^{PTPRKkd} cells (18.87±3.95) compared to the control DU-145^{pEF} (29.67±8.16) p=0.008.

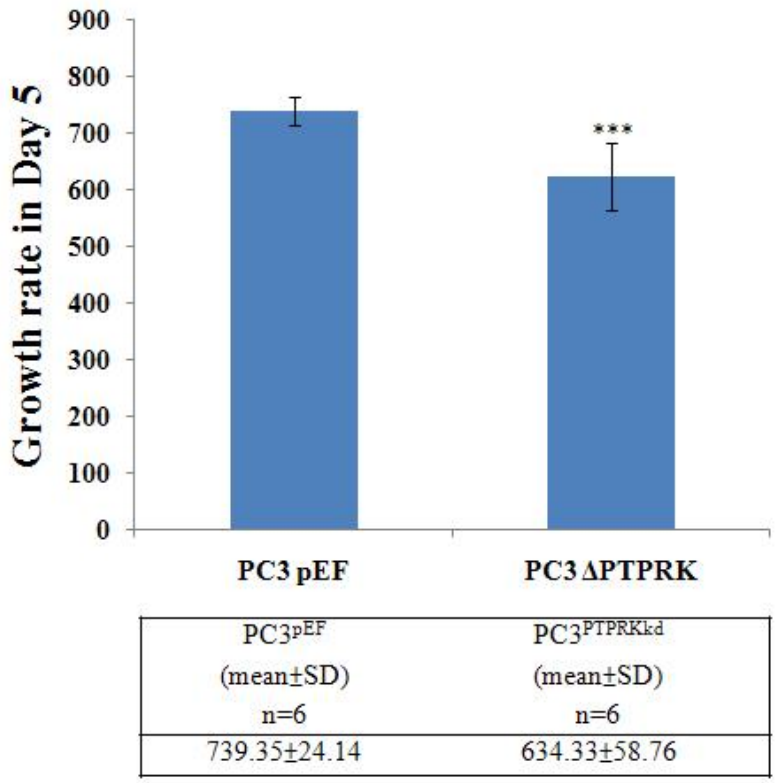
4.3.5 Effect of knockdown of PTPRK in the cell motility

The cells were further analysed for their motility using scratch or wounding assay. The cells with knockdown of PTPRK displayed a significant increase in motility compared to control cells. Figure 4.8 shows that **t**here was a significant increase in cell motility with PC-3^{PTPRKkd} cells (96.9±27.9) compared to the PC-3^{pEF} cells (62.1±16.0) $p<0.05$. There was no significant difference in cell motility of DU-145^{PTPRKkd} cells compared with the control DU-145^{pEF} (Figure 4.9).

4.3.6 Effect on invasion of prostate cancer cells by PTPRK knockdown

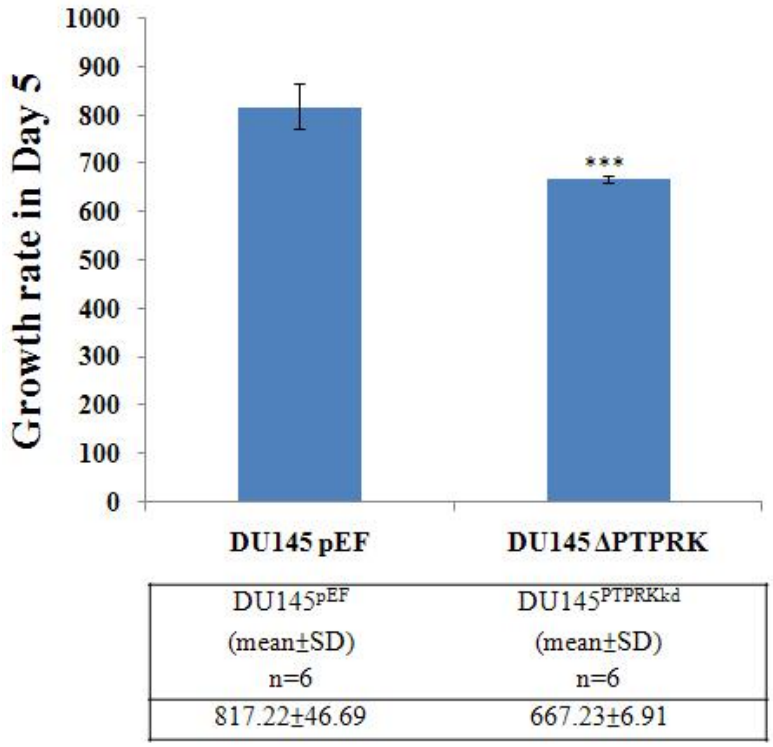
PTPRK knockdown cells displayed a significant reduction of invasion compared to the controls. The number of invaded cells were 168.72±15.25 after PTPRK knockdown

$p<0.05$ compared to that of the control (190.83±12.25). However, an increase of invasion was seen in DU-145^{PTPRKkd} cells (207.67±40.26), $p<0.001$ compared to the control DU-145^{pEF} (92.42±18.37).



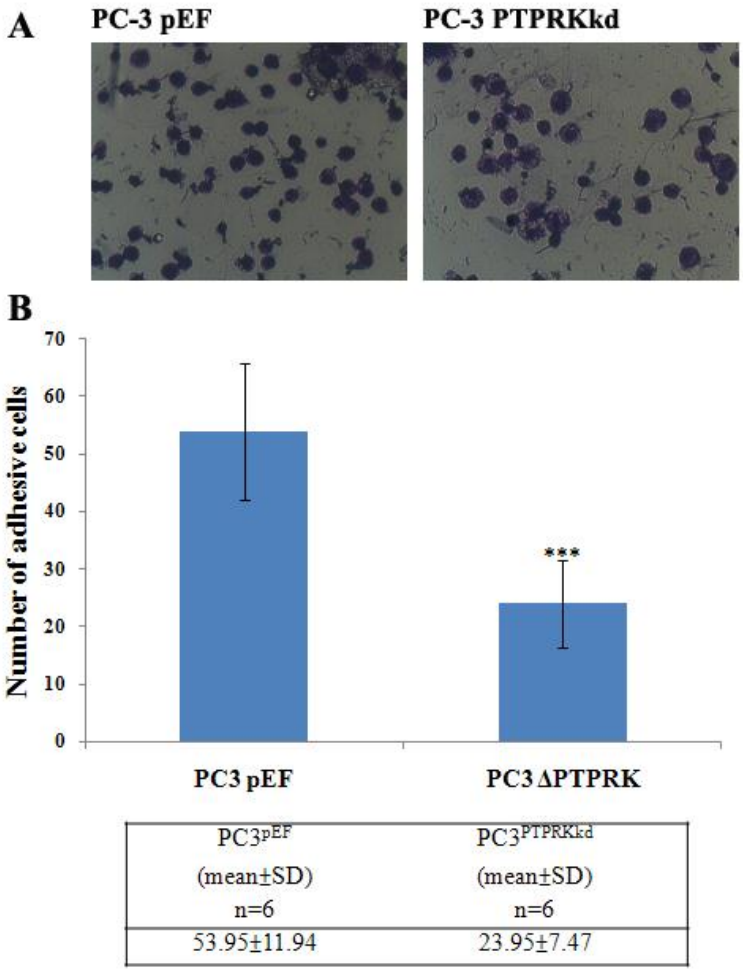
$PC3^{pEF}$ (mean±SD) n=6	$PC3^{PTPRKkd}$ (mean±SD) n=6
739.35±24.14	634.33±58.76

Figure 4.4 Knockdown of PTPRK has a significant decrease in the growth of the PC-3^{PTPRKkd} cells. After 5 days incubation there was a significant decrease in the PC-3^{PTPRKkd} cells (634.33±58.76) compared with the control PC-3^{pEF} (739.35±24.14) ($p<0001$). Data shown is representative of at least 6 independent repeats. Error bars represent standard deviation. The 1 day time point was used as a baseline to normalise the data. Growth rate (%) = Day1 absorbance/ Day 5 absorbance x100.



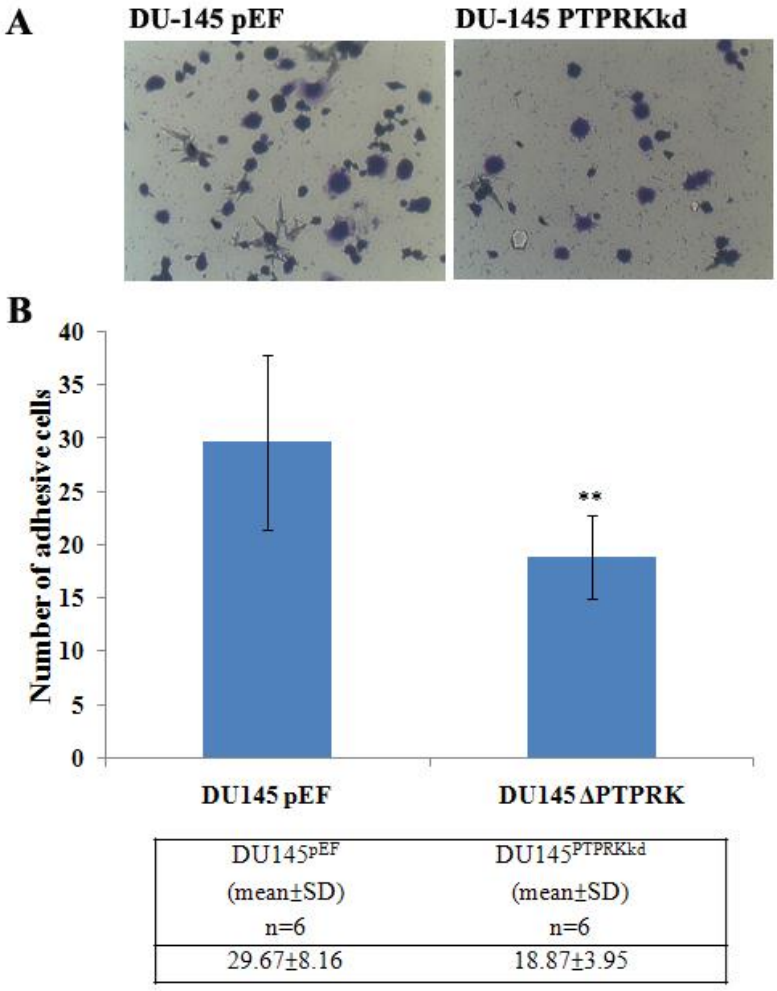
$DU145^{pEF}$ (mean±SD) n=6	$DU145^{PTPRKkd}$ (mean±SD) n=6
817.22±46.69	667.23±6.91

Figure 4.5 Knockdown of PTPRK has a significant decrease in the growth of the DU-145^{PTPRKkd} cells. After 5 days incubation there was a significant decrease in the DU-145^{PTPRKkd} cells (667.23±6.91) compared with the control DU-145^{pEF} (817.22±46.69) ($p<0001$). Data shown is representative of at least 6 independent repeats. Error bars represent standard deviation. The 1 day time point was used as a baseline to normalise the data. Growth rate (%) = Day1 absorbance/ Day 5 absorbance x100.



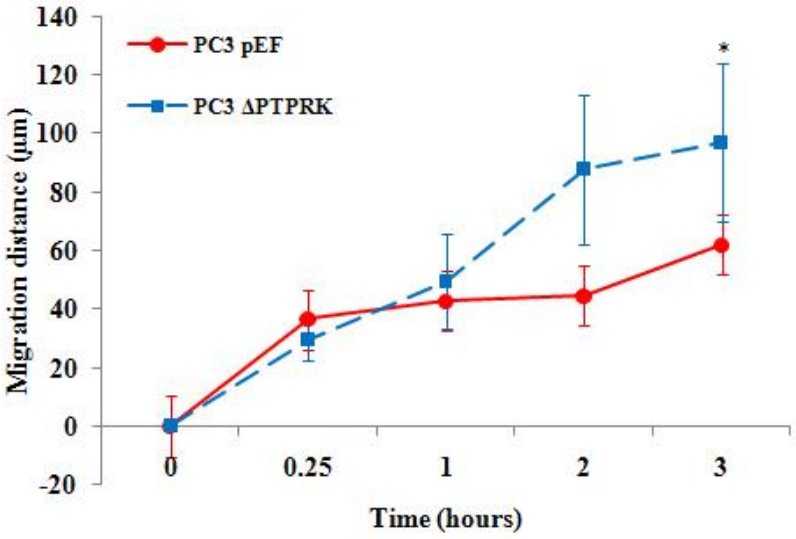
$PC3^{pEF}$ (mean±SD) n=6	$PC3^{PTPRKkd}$ (mean±SD) n=6
53.95±11.94	23.95±7.47

Figure 4.6 Knockdown of PTPRK in PC-3 cells displayed a significant decreasing in adhesive capability compared to its controls. A. Representative images of cells following staining. **B.** After 40 minutes incubation of the cells on an artificial matrigel basement membrane, there was a significant decrease in adhesion was seen between the PC-$3^{PTPRKkd}$ cells (23.95±7.47) compared to the control PC-3^{pEF} (53.95±11.94) ($p<0.001$). Data shown is representative of at least 6 independent repeats. Error bars represent standard deviation.



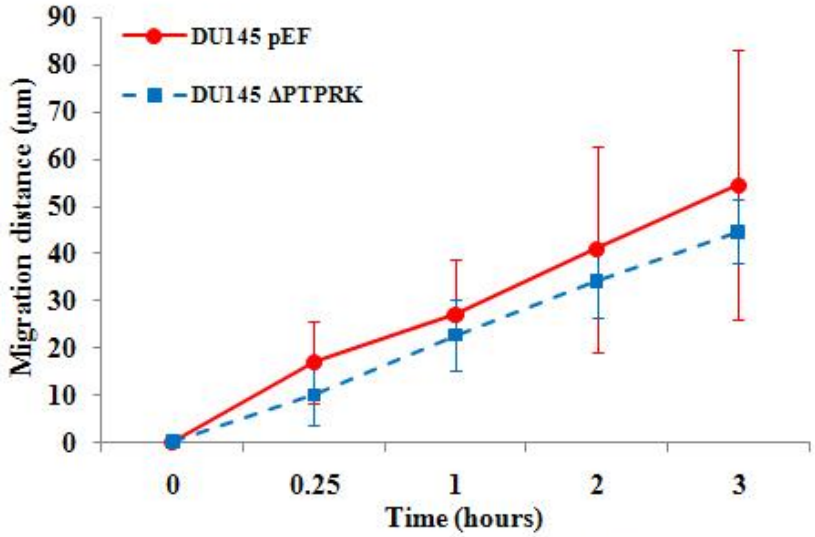
$DU145^{pEF}$ (mean±SD) n=6	$DU145^{PTPRKkd}$ (mean±SD) n=6
29.67±8.16	18.87±3.95

Figure 4.7 Knockdown of PTPRK in DU-145 cells also displayed a significant decreasing in adhesive capability compared to its controls. A. Representative images of cells following staining. **B.** After 40 minutes incubation of the cells on an artificial matrigel basement membrane, there was a significant decrease in adhesion was seen between the DU-$145^{PTPRKkd}$ cells (18.87±3.95) compared to the control DU-145^{pEF} (29.67±8.16) (p=0.008). Data shown is representative of at least 6 independent repeats. Error bars represent standard deviation.



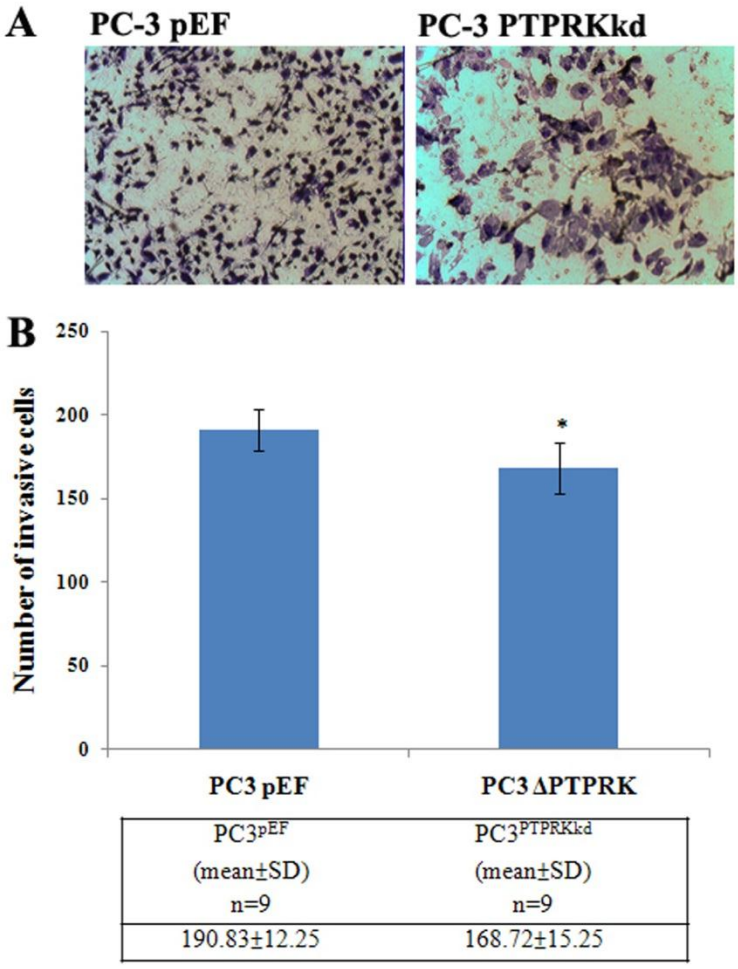
$PC3^{pEF}$ (mean±SD) n=6	$PC3^{PTPRKkd}$ (mean±SD) n=6
62.1±16.0	96.9±27.9

Figure 4.8 Knockdown of PTPRK increases the motility of PC-3 prostate cells. The knockdown of PTPRK caused a significant increase in the motility of the PC-$3^{PTPRKkd}$ cells (96.9±27.9) compared to the PC-3^{pEF} cells (62.1±16.0) ($p<0.05$). Data shown is representative of at least 6 independent repeats. Error bars represent standard deviation.



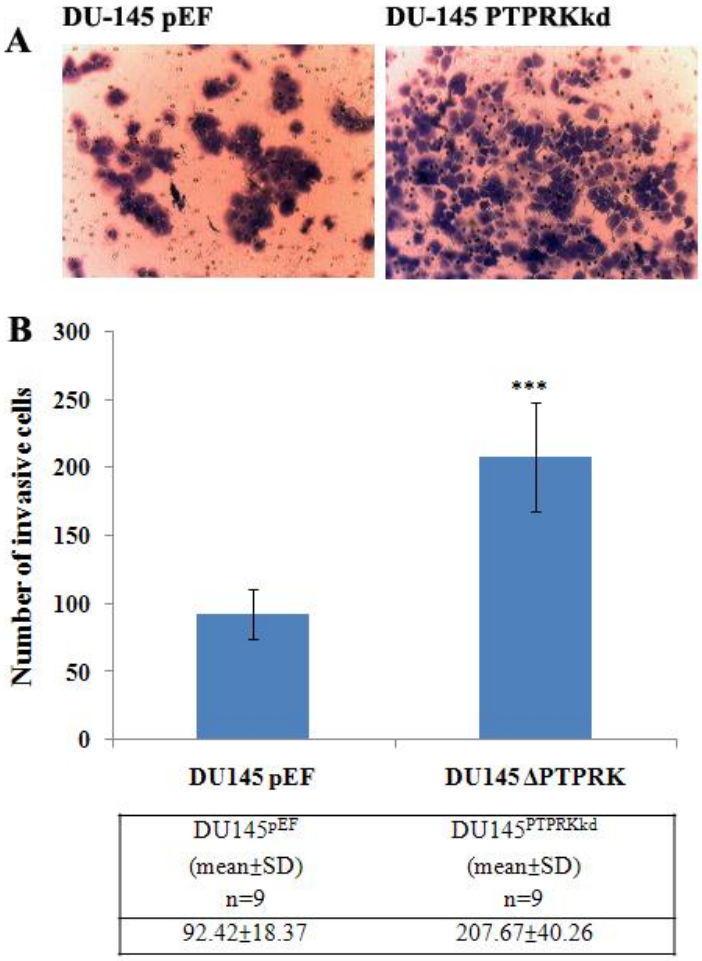
$DU145^{pEF}$ (mean±SD) n=6	$DU145^{PTPRKkd}$ (mean±SD) n=6
54.5±28.7	44.6±6.6

Figure 4.9 Knockdown of PTPRK has no effect in the motility of DU-145 prostate cells. There was no significant difference in cell motility of DU-$145^{PTPRKkd}$ cells (44.6±6.6) compared with the control DU-145^{pEF} (54.5±28.7). Data shown is representative of at least 6 independent repeats. Error bars represent standard deviation.



$PC3^{pEF}$ (mean±SD) n=9	$PC3^{PTPRKkd}$ (mean±SD) n=9
190.83±12.25	168.72±15.25

Figure 4.10 Knockdown of PTPRK decreases the invasive ability of PC-3 prostate cancer cells. A. Representative images of cells following staining. **B.** Following a 3 day incubation of the cells on an artificial matrigel basement membrane there was a significant decrease in the invasion of the PC-$3^{PTPRKkd}$ cells (168.72±15.25) compared to the control PC-3^{pEF} cells (190.83±12.25) ($p<0.05$). Data shown is representative of at least 3 independent repeats. Error bars represent standard deviation.



$DU145^{pEF}$ (mean±SD) n=9	$DU145^{PTPRKkd}$ (mean±SD) n=9
92.42±18.37	207.67±40.26

Figure 4.11 Knockdown of PTPRK increases the invasive ability of DU-145 prostate cancer cells. A. Representative images of cells following staining. **B.** Following a 3 day incubation of the cells on an artificial matrigel basement membrane there was a significant decrease in the invasion of the DU-$145^{PTPRKkd}$ cells (207.67±40.26) compared to the control DU-145^{pEF} (92.42±18.37) ($p<0.001$). Data shown is representative of at least 3 independent repeats. Error bars represent standard deviation.

4.4 Discussion

Several members of the PTP proteins have been linked to cancer progression. PTPRM has been reported to play a role in cancer cell migration and adhesion and associated with ovarian carcinoma carcinogenesis (Zondag *et al.,* 1995; Gyorffy *et al.,* 2008). PTPRK is in the same sub-family with PTPRM and has similar structure of cell adhesion molecule (CAM). PTPRK has been identified as a possible tumour suppressor in primary lymphoma of central nervous system lymphomas, in which the locus that the *PTPRK* gene resides is frequently deleted (Nakamura, Kishi *et al*. 2003). Over-expression of PTPRK in Hodgkin Lymphoma cells resulted in a decrease in cell proliferation and survival and knockingdown PTPRK caused the opposite effect (Flavell et al 2008). A similar suppressing role was observed in human keratinocyte, adding extra TGF-β1 to treat HaCaT cell induced PTPRK expression and final lead to anti-proliferation phenomenon (Yang *et al.,* 1996). In contrast, another study demonstrated that blocking of PTPRK expression accelerated cell cycle progression, enhanced response to EGF-induced EGFR tyrosine phosphorylation, then increased downstream ERK activation, and abrogated TGF-β-mediated antimitogenesis (Wang *et al.,* 2005; Xu *et al.,* 2005). Together, the limited available literature has presented a rather mixed, if not confusing, picture with regard the role of PTPRK in cancer and

cancer development.

The present study attempted to investigate the role of PTPRK in prostate cancer. The ribozyme transgene proved effective at knockdown the expression of PTPRK at both the mRNA and protein level. These tools of genetic manipulation aid in the generation of useful *in vitro* models allowing for the subsequent investigations of the impact on cellular functions.

When PTPRK was knocked down in PC-3 and DU-145 cells lines, they demonstrated decreased cell growth and adhesion compared to their controls. However, knockdown of PTPRK in PC-3 cells showed significant increases of cell motility. There was no effect on the motility in DU-145 PTPRK knockdown cells compared with their control cells. Furthermore, in the cell invasion assay, knockdown of PTPRK in PC-3 and DU-145 showed completely different trends. In PC-3 cells, knockdown of PTPRK reduces the invasiveness compared with control cells, while PTPRK knockdown in DU-145 cells promotes the invasive ability. The exact reason for the differential effect of PTPRK on the cell migration and invasion is currently unknown but is likely to be due to differences in the expression patterns of other proteins between PC-3 and

DU-145, particularly the interacting molecules and pathways of PTPRK.

The inhibition of cell growth in PC-3 and DU-145 cells by PTPRK knockdown is probably linked to the balance between EGFR proliferation and TGF-β anti-proliferation pathways. It has been shown that PTPRK directly dephosphorylates EGFR *in vitro* and increased PTPRK expression decreases both basal and ligand-stimulated EGFR tyrosine phosphorylation, and reduced PTPRK expression leads to an increase in EGFR tyrosine phosphorylation (Xu *et al.*, 2005). Moreover, PTPRK is up-regulated by TGF-β1 which is probably involved in mediation TGF-β dependent anti-proliferation and cell migration effects (Yang *et al.*, 1996). Furthermore, co-localisation of PTPRK with β-catenin at adhesion junctions has been revealed in human embryonic kidney cells (HEK293) which stabilise E-cadherin/ β-catenin complexes. It suggests that β-catenin might be a substrate of PTPRK-mediated phosphatase activity. The stabilisation of β-catenin /E-cadherin complexes can suppress wnt/ β-catenin-induced transcription of target genes, such as cyclin D1 and c-Myc, leading to an inhibition of proliferation and migration in cancer cells. It provides evidence that PTPRK may be a potential tumour suppressor (Novellino *et al.*, 2008).

In addition, knockdown of PTPRK decreases cell adhesion in both PC-3 and DU-145 cells due to the extracellular region of PTPRK, which contains one Ig-like domain. Some molecules of the Ig superfamily can mediate both homophilic and heterophilic interactions. However, PTPRK fails to interact with PTPRM which belong to the same subfamily of PTP. Expression of PTPRK only promoted homophilic interactions which include cell aggregation and cell-cell adhesion. Furthermore, it has been reported that PTPRK-mediated intercellular adhesion does not need PTP activity or post-translational modification of PTPRK protein and is calcium independent (Sap *et al.*, 1994; Zondag *et al.*, 1995).

This study suggests that PTPRK is an important regulator of the biological properties of prostate cancer cells, which is possibly involved in the disease progression. In order to further determine the role of PTPRK in cancer, the following chapter will focus on the mechanisms of the inhibitory effect on growth of prostate cancer cells.

Chapter 5

Knockdown of PTPRK induces apoptosis in prostate cancer cells and the involvement of the MAPK pathway

5.1 Introduction

The previous chapter has shown that knockdown of PTPRK reduces cell proliferation in prostate cancer cell lines. Unlimited cell proliferation is essential for cancer progression. Most oncogenic factors can regulate cell proliferation *via* two different ways; disrupting the cell cycle and/or inhibiting apoptosis.

PTP deficiency leads to several physiologic abnormalities such as embryonic developmental defect (PTP-PEST), impairment of the immune system (TC-PTP), inhibition of tumourigenesis and metastasis (PTP1B) and others (Sirois *et al.,* 2006; Bourdeau *et al.,* 2007; Julien *et al.,* 2007). Dysregulation of apoptosis is one of the mechanisms underlying these conditions. PTP-PEST renders cells more sensitive to anti-Fas and TNFα apoptotic stimulation. PTP-PEST is cleaved by caspase-3, leading to an increased catalytic activity and simultaneous changes in its protein structure. Furthermore, PTP-PEST proteolysis facilitates cellular detachment during apoptosis (Halle *et al.,* 2007). In contrast to PTP-PEST, PTP1B does not possess a caspase-cleavage site. Activated PTP1B contributes to STAT3 dephosphorylation and results in apoptosis in human glioma cells (Akasaki *et al.,* 2006; Halle *et al.,* 2007). In the breast cancer cell line MCF-7, overexpression of PTPRB induces apoptosis *via* a

direct increase in the expression of Apaf-1 and the pro-apoptotic α-isoform of caspase-1 (Radha *et al.*, 1999; Gupta *et al.*, 2002).

The previous chapter has shown that PTPRK knockdown resulted in inhibition of *in vitro* growth of prostate cancer cells. Together with the role of PTP family proteins in the regulation of apoptosis, it was hypothesised that one of the mechanisms by which PTPRK influence the growth rate of prostate cancer cells is vis the action on apoptosis.

5.2 Materials and methods

5.2.1 Materials

The primers used in this chapter are shown in Table 2.2, including caspase-3, caspase-8, caspase-9, p53, c-Myc, ID1, and cyclin-D1. The antibodies used in this chapter including anti-caspase-3, caspase-8, caspase-9, p38, JNK, ERK, and phospho-tyrosine are shown in Table 2.4.

5.2.2 Investigating the expression of apoptotic molecules using PCR and Western blotting

Both RNA and protein were extracted from stable transfected cells and were used in

either Western blot analysis or RT-PCR in order to investigate expression levels of molecules involved in apoptosis. These methods were carried out as previously described in sections 2.4 and 2.5.

5.2.3 Immunoprecipitation and detection of tyrosine phosphorylated ERK, JNK and p38

Protein extracted from PC-3 cells was immunoprecipitated with anti-p38, JNK, and ERK antibody in order to detect phosphorylation of these proteins. These immunoprecipitated protein samples were then run on an SDS-PAGE and blotted with anti-phosphotyrosine antibody. Please refer to section 2.5.3 for further details on immunoprecipitation method.

5.2.4 Apoptosis analysis using flow cytometry

Both the transfected PC-3^{pEF} cells and PC-$3^{PTPRKkd}$ cells were treated with or without 100nM p38 and JNK inhibitor over 72-hour culture before analysis of apoptosis. In order to detect apoptotic cells, the current study used an Annexin V Kit (Santa Cruz Biotechnology) which contained recombinant flourescein conjugated annexin V (FITC annexin V) and propidium iodide (PI) solution. The cells were stained with both FITC

annexinV and PI solution, followed by the detection of apoptotic cells using a flow cytometer (PartecCyFlow® SL, Partec GmbH, Munster, Germany). This was achieved by measuring any fluorescence emission at 530nm (FL1) and>575nm (FL3).

5.3 Results

5.3.1 PTPRK knockdown affected apoptosis in prostate cancer cells

There were an increased proportion of apoptotic cells (both early and late) in PC-$3^{PTPRKkd}$ cells (34.90%) compared to the PC-3^{pEF}control (16.14%) and PC-3 wild-type (8.05%) cells (Figure 5.1).

5.3.2 Expression of caspases in the PTPRK knockdown cells

Caspase-3 is an indicator of apoptosis at the end-stage. Caspase-8 and caspase-9 are up-stream key factors of apoptosis; caspase-8 is normally activated by external signalling, and caspase-9 is activated by internal signalling. Therefore, in order to further determine whether PTPRK knockdown has an effect on prostate cancer cell apoptosis, levels of caspase-3, caspase-8, and caspase-9 were examined in the transfected PC-3 cells using PCR, Q-PCR, and western blotting analysis.PC-$3^{PTPRKkd}$ cells demonstrated significantly higher expression levels of caspase-3 and caspase-8,

but not caspase-9, compared with PC-3^{pEF} controls cells in both mRNA and protein levels (Figure 5.2 and 5.3).

5.3.3 Expression of other genes/molecules relevant to apoptosis and/or the cell cycle

As PTPRK knockdown was shown to promote the progression of apoptosis, the expression of a number of relevant genes was examined using RT-PCR. An up-regulation of p53 was also seen in the PTPRK knockdown cells, whilst a down-regulation of ID1 appeared in the same cells (Figure 5.4). No effect on the expression of c-Myc was observed in the PC-3^{PTPRKkd} cells compared with the control.

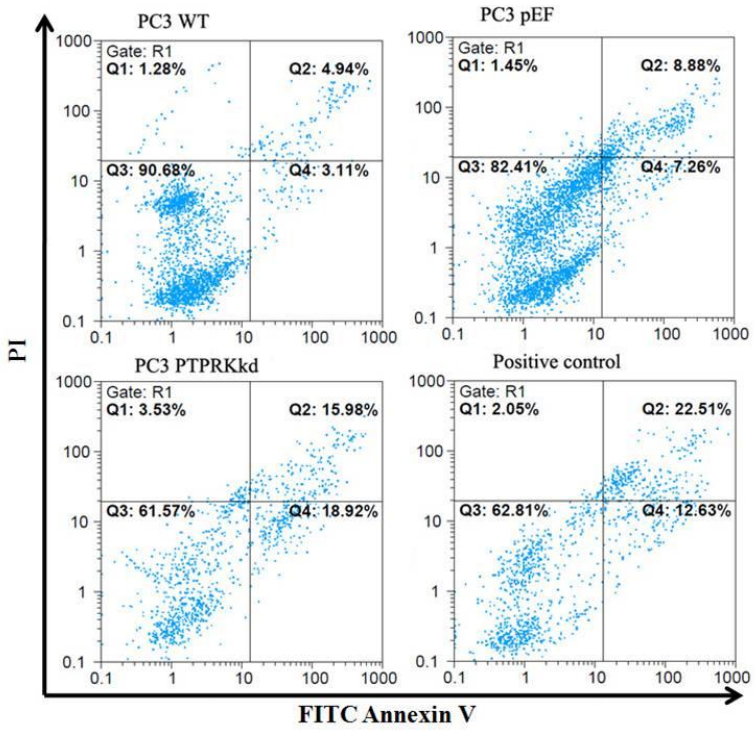


Figure 5.1 The apoptotic population in PC-3 cells was analysed using flow cytometry after 72 hours incubation, in order to induce apoptosis. Segment Q1 of the quadrants refers to non-specific PI staining which are alive cells ; Q2 indicates late apoptotic and necrotic cells which are stained with both annexinV and PI; Q3 includes healthy and alive cells with low staining of both PI and annexinV; and Q4 to early apoptotic cells with high annexinV but low PI staining. Apoptotic index refers to total apoptotic population including both late apoptotic cells (Q2) and early apoptotic cells (Q4). The positive control was PC-3^{WT} cells treated with H_2O_2. H_2O_2 will induce peroxidation of cell membrane and break lipid bilayer structure resulting in aopotosis. Data shown are representative data of three independent experiments.

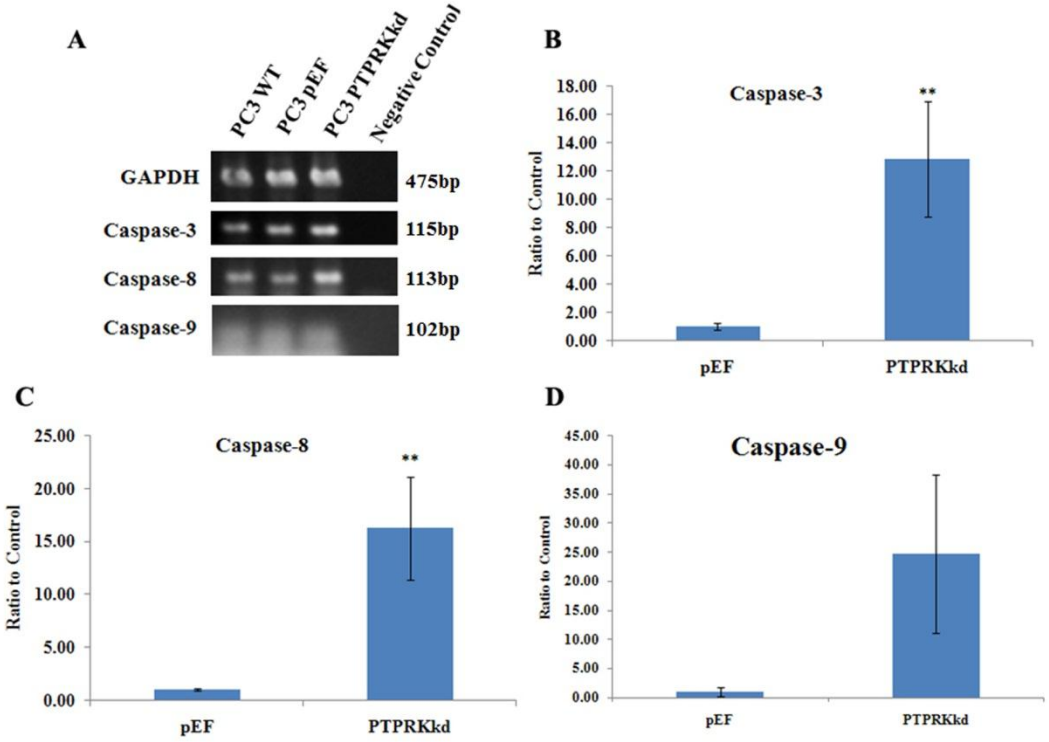


Figure 5.2 The effect of PTPRK knockdown on Caspase-3, -8, and -9 expressions were analysed in mRNA level using PCR and Q-PCR. A. PCR results of Caspase-3, -8, and -9 mRNA expressions. Expression of caspase-3 **(B)** and caspase-8 **(C)** was increased in PC-$3^{PTPRKkd}$ cell vs. PC-3^{pEF} control using Q-PCR. However, there is no significant difference ofcaspase-9 **(D)** seen inPC-$3^{PTPRKkd}$ cell using Q-PCR. Q-PCR result was normalised with GADPH. **p<0.01.

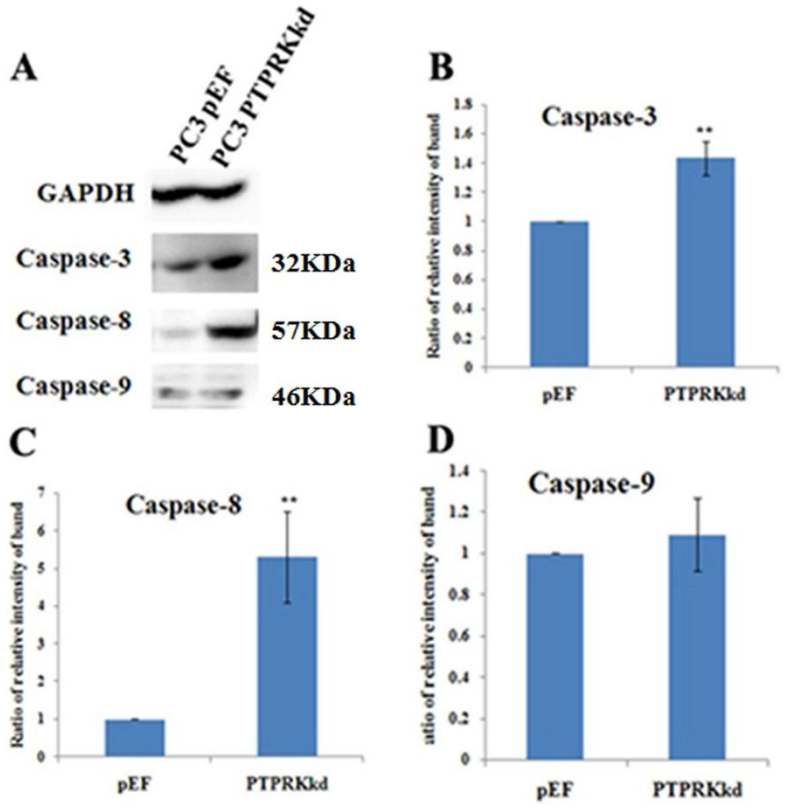


Figure 5.3 The effect of PTPRK knockdown on Caspase-3, -8, and -9 expressions were analysed in protein level using western blot. A. The protein expression of Caspase-3 and -8 were also increased in PC-3 cells by PTPRK knockdown, and also no similar change was seen in the Caspase-9 expression. Intensity of bands from three western blots was analysed using Image J software for caspase-3 **(B)**, caspase-8 **(C)**, and caspase-9 **(D)**.Bar graphs shown quantifications of three Western blots of each molecule. The intensity shown is integrated band intensity (intensity×area) and was normalised against the corresponding GAPDH signal. **p<0.01.

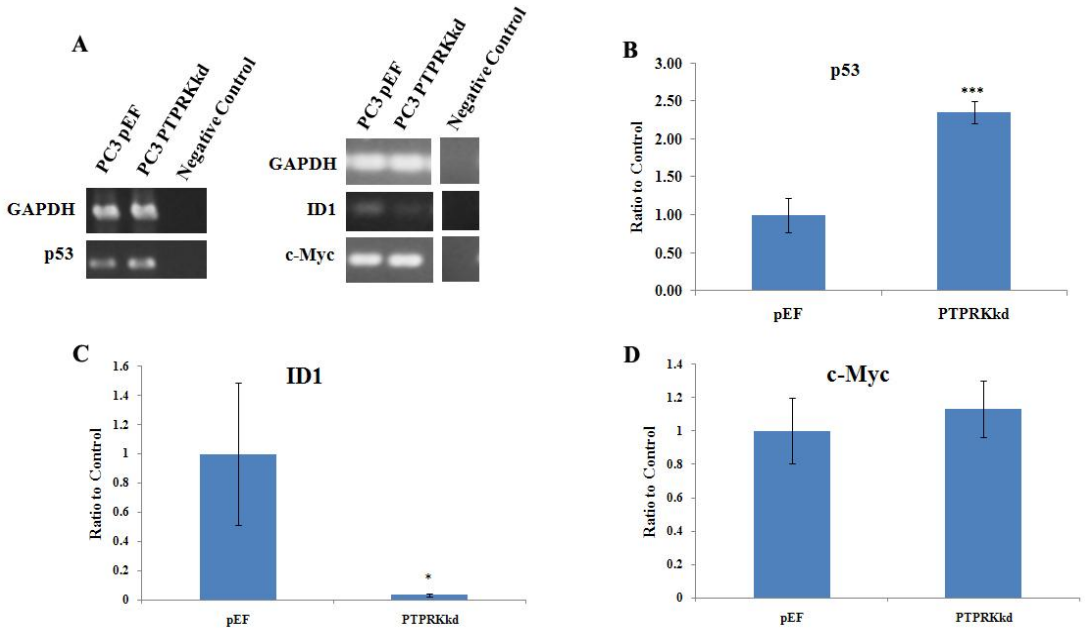


Figure 5.4 The effect of PTPRK knockdown on p53, ID1, and c-Myc expressions were analysed in mRNA level using PCR and Q-PCR. A. PCR results of p53, ID1, and c-Myc, it demonstrated that expression of p53 was increased and expression of ID1 was decreased in PC-3^{PTPRKkd} cell compared with their controls. **B.** Expression of p53 was increased in PC-3^{PTPRKkd} cell vs. PC-3^{pEF} control using Q-PCR. **C.** Expression of ID1 was decreased in PC-3^{PTPRKkd} cell vs. PC-3^{pEF} control using Q-PCR. **D.** There is no significant difference in c-Myc expression of PC-3^{PTPRKkd} cell using Q-PCR. Q-PCR result was normalised with GADPH expression. *$p<0.05$ and ***$p<0.001$.

5.3.4 The role of JNK in PTPRK knockdown-associated apoptosis

The mitogen-activated protein kinase (MAPK) pathway is the major signalling pathway involved in cellular proliferation and it affects both apoptosis and the cell cycle. As knockdown of PTPRK has been shown to impact on the apoptosis, its effect on the expression of three key molecules of the MAPK pathway, p38, JNK, and ERK in PC-3 cells was analysed. The PC-3^{PTPRKkd} cell showed a similar level of protein expression in overall p38, JNK and ERK compared with their pEF control. Furthermore, levels of phosphorylated p38, JNK, and ERK were analysed using immunoprecipitation and Western blotting. A marked increase inactive p-JNK (Tyr) was seen in the PTPRK knockdown cells and also to a less degree, an increased level of active p38, suggesting that JNK and p38 may play a role in the regulation of apoptosis in the PC-3^{PTPRKkd} cell (Figure 5.5).

Additionally, PC-3 cells were treated with p38 and JNK inhibitors for 48 hours and analysed for apoptosis using flow cytometry. The PC-3^{pEF} control cells showed no effect on treatment of cells with p38 and JNK inhibitors, but apoptosis of untreated PC-3^{PTPRKkd} cells (26.70%±2.87) was dramatically increased compared with PC-3^{pEF} cells (14.61%±1.74), $p<0.001$. The PC-3^{PTPRKkd} cells treated with p38 inhibitor (26.75%±4.80) exhibited similar apoptotic levels to the untreated cells (13.36%±2.69).

However the PC-3^{PTPRKkd} cells treated with JNK inhibitor (18.76%±4.28) exhibited significant reduction of apoptotic cells compared with untreated PC-3^{PTPRKkd} cells, $p<0.001$ (Figure 5.6). The addition of the p38 inhibitor did not inhibit the effect on apoptosis, suggesting that p38 was unlikely to be involved. However, the addition of the JNK inhibitor diminished the apoptotic effect of PTPRK knockdown. This suggests that PTPRK knockdown may utilise a signalling pathway via JNK to impact on apoptosis and to thereby inhibit cell proliferation.

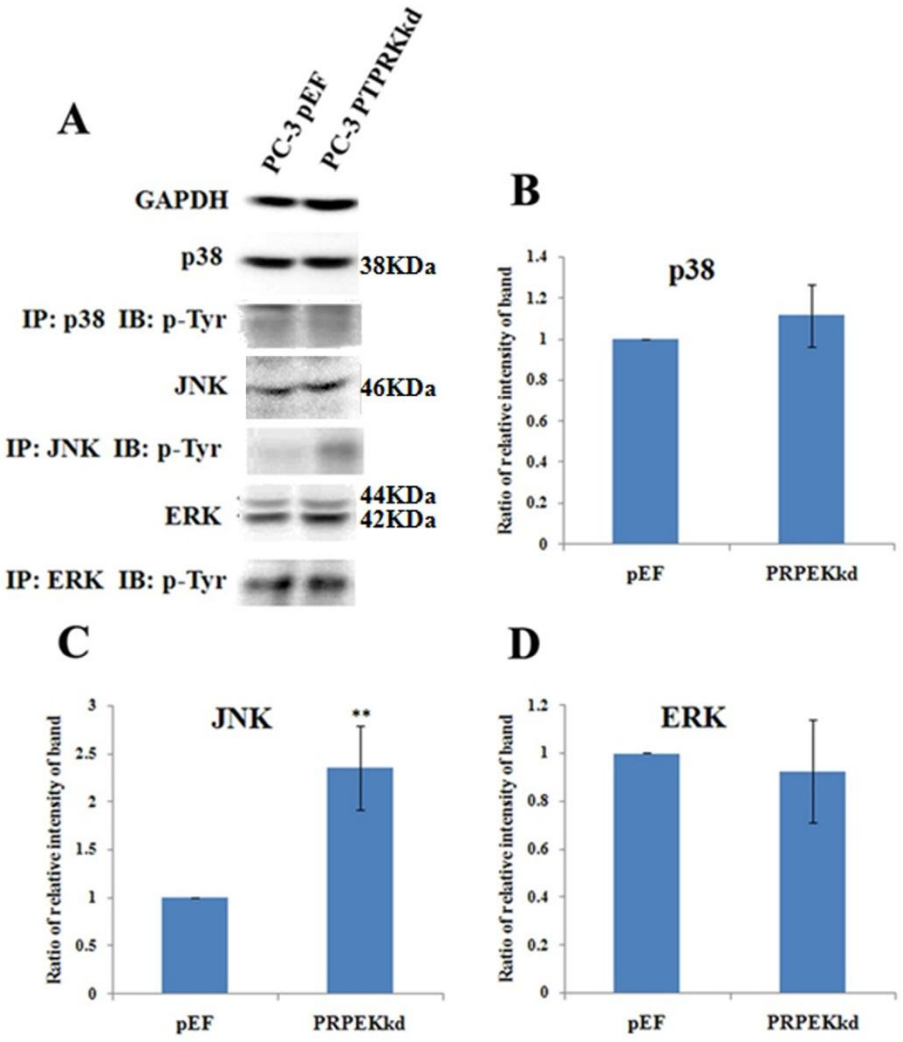
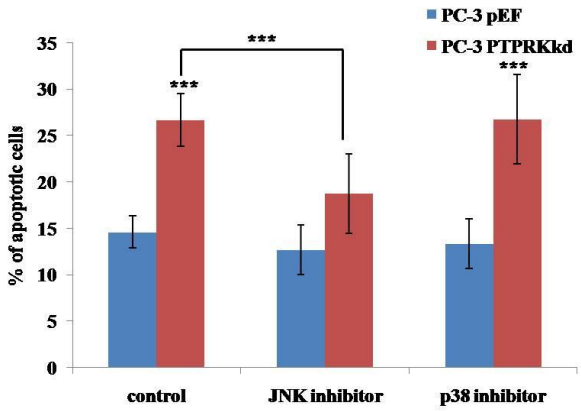


Figure 5.5 PTPRK signalling via MAPK pathway in PC-3 cells. A. The overall expression of p38, JNK, and ERK in PC-3^{PTPRKkd} cells were showed similar amount in protein levels compared with their pEF controls. In addition, there is no change in both active p-38 and p-ERK; however, the active p-JNK was increased significantly compared with the PC-3^{pEF} cells. Relative intensity of bands from three Western blots was analysed using Image J software for p38 **(B)**, JNK **(C)**, and ERK **(D)**. **p<0.01.



PC-3 (n=4)	pEF	pEF +JNK inhibitor	pEF +p38 inhibitor	PTPRKkd	PTPRKkd +JNK inhibitor	PTPRKks +p38 inhibitor
Mean	14.60525	12.6925	13.36425	26.70083	18.76083	26.7525
SD	1.738993	2.706835	2.69211	2.872415	4.27612	4.798424

Figure 5.6 The role of p38 and JNK in PTPRK knockdown associated apoptosis. PC-3^{PTPRKkd} cells treated with JNK inhibitors exhibited a significantly decreased apoptotic population compared with untreated control, $p<0.001$. There was no change in apoptotic effect induced by PTPRK knockdown seen in the cells treated with the p38 inhibitor. The bar graph demonstrates the mean apoptotic percentage of four independent experiments. Error bars represent standard deviation. ***$p<0.001$.

5.4 Discussion

PTPs have been shown to exhibit different effects on tumour apoptosis (Julien *et al.*, 2011). In the case of PTPRK, the previous chapter has shown that it is capable of inhibiting the growth of prostate cancer cells through endogenous alteration of expression. This current chapter further investigates the mechanisms behind this growth reduction.

In general, cell population is control by the regulation and balance between cell proliferation (cell cycle) and cell death (necrosis and apoptosis). PTPRK knockdown promoted the apoptosis in prostate cancer cells and suppressed *in vitro* growth.

Moreover, apoptosis was associated with the activation of Caspase-3, -8, and -9 and an increasing Bax:Bcl-2 ratio, followed by a releasing of cytochrome-c (Halle *et al.*, 2007). Caspase-3 is a key molecule in the late stage of apoptosis; caspase-8 normally is activated by extrinsic receptor-mediated pathway; caspase-9 is activated by intrinsic mitochondrial-mediated pathway (Lee *et al.*, 2011). In order to analyse how these molecules were affected in PC-3^{PTPRKkd} cells, the expression of caspase-3, -8, and -9 was analysed using PCR, Q-PCR, and western blotting. There was a significant increase in both caspase-3 and -8 expressions, but not in caspase-9 following the knockdown of

PTPRK. This result showed that the increased apoptosis in PC-3^{PTPRKkd} cells was related with extrinsic signalling rather than mitochondrial signalling.

Most PTPs play a role in promoting apoptosis. For example, PTP1B has been reported to play a role in the activation of MAPKs. PTP1B activates JNK and P38 pathway via inositol-requiring kinase 1 (IRE1) signalling and lack of PTP1B resulted in decreased levels of ER-induced apoptosis (Gu *et al.*, 2004; Sangwan *et al.*, 2006). However, SHP-1 dephosphorylates TrkA which in turn inhibits NGF-mediated PLCγ1 and Akt phosphorylation, reducing the TrkA survival signal (Marsh *et al.*, 2003). In contrast, osteoclastic PTP (PTP-oc) has been reported to promote *c*-Src-mediated activation of NFκB and JNK leading cell protection from apoptosis (Amoui *et al.*, 2007). In the present study, the reduction of PTPRK expression in PC-3 cells shows a promotion of apoptosis and an involvement of MAPK signalling pathway. The tyrosine phosphorylation of JNK was increased in PC-3^{PTPRKkd} cells and promotion of apoptosis in PC-3^{PTPRKkd} cells was diminished after treating cells with the JNK inhibitor (SP600125). These data suggest that PTPRK down-regulates apoptosis in prostate cancer cells by suppressing the JNK pathway.

We also investigated other apoptosis-related molecules such as p53, ID1 and *c*-Myc. These molecules play crucial roles in the regulation of cell proliferation. Expression of c-Myc in tumours helps cancerous cells pass through check-points and progress to the G2/M phase of cell cycle. In addition, expression of ID1 activates NFκB, which then induces Bcl-2 to inhibit apoptosis. In contrast to these anti-apoptotic factors, p53 acts as a promoter of apoptosis. Furthermore, mRNA expression of p53 was increased and expression of ID1 was decreased. p53 regulates both cell cycle and apoptosis. There are some reports that show that expression of p53 is associated with the induction of apoptosis in different cancer cells (Pietsch *et al.,* 2008). p53 silencing was able to suppress cadmium-induced apoptosis in prostate cells (Aimola *et al.,* 2012). Additionally, activation of JNK can also up-regulatep 53 expression, leading to the accumulation of Bax which induces cell apoptosis in HeLa cells (Cao *et al.,* 2010). In contrast, ID1 expression increases NF-κB expression which is associated with the anti-apoptotic pathway. NF-κB activates Bcl-2 to initiate the mitochondrial mediated anti-apoptotic effect and also activates XIAP to inhibit the activities of caspase-3 and -9 (Ling *et al.,* 2003; Peng *et al.,* 2012). These results indicate a complex network affected by PTPRK which participates in the coordination of cellular functions, making further investigations into the protein interactions between PTPRK and the network protein an

interesting area to explore in future.

In conclusion, PTPRK knockdown resulted in increased apoptosis leading to the inhibition of *in vitro* growth of prostate cancer cells. PTPRK is a key factor in coordinating apoptosis via the regulation of MAPK pathways, in particular the JNK pathway in prostate cancer cells.

Chapter 6

PTPRK is negative regulator of adhesion and invasion of breast cancer cells and is associated with poor prognosis of breast cancer

6.1 Introduction

It has been demonstrated that most PTPs act as tumour suppressors in cancer, such as PTPRT and LAR in colorectal cancer (Wang *et al.,* 2004). However, certain PTPs have been shown as oncogenic factors, for example PTPRA in breast and colon cancers (Tabiti *et al.,* 1995; Ardini *et al.,* 2000).

PTPRK has been reported to regulate cell proliferation by coordinating EGFR pathway and TGF-β pathway (Xu *et al.,* 2005). Activation of EGFR may trigger downstream cascades including MAPKs, PI3K/ Akt, and PLC/PKC. PTPRK affects the activation of both EGFR (ErbB1) and HER2 (ErbB2) by suppressing phosphorylation of the receptors and consequently reduces the basal and ErbB ligand-induced proliferation (Wang *et al.,* 2005; Xu *et al.,* 2005).

The previous chapters have shown that PTPRK knockdown resulted in an inhibition of the *in vitro* growth of prostate cancer cells by promotion of apoptosis via JNK. To investigate if this pathway is a general phenominan and applicable to other tumour type(s) and if the PTPRK regulation of cancer cell functions may have a clinical relevance, we chose another tumour type, namely breast cancer, due to the hypothesised

impact of PTPRK on the tumour type and the available tumour cohort. The current chapter aims to investigate the role played by PTPRK in breast cancer, particularly the link to disease progression and impact on functions of breast cancer cells.

6.2 Materials and methods

6.2.1 Breast cancer tissues

Breast samples were collected immediately after surgery and stored at -80℃ until use, with approval of the Bro Taf Health Authority local research ethics committee. All patients were informed and participated with written consent. All the specimens used in the current study were verified by a consultant pathologist. A routine follow-up was carried out after surgery and details were stored in a database. The median follow-up period was 120 months.

6.2.2 Antibody and primers

Polyclonal rabbit anti-PTPRK antibody (SC-28906) was obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). All the primers used were synthesised and provided by Invitrogen (Paisley, UK). Primer sequences are located in Tables 2.2 and 2.3.

6.2.3 Cell lines

MDA-MB-231, MCF-7, MDA-MB-436, BT474 and BT483 were routinely cultured in DMEM-F12 medium as described in section 2.3.

6.2.4 Generation of PTPRK ribozyme transgenes

Hammerhead ribozymes targeting PTPRK were designed based on the secondary structure of PTPRK mRNA, and synthesised following a previously described method in section 2.7. Primer details are listed in the Table 2.3.

6.2.6 Breast cancer cell transfection and generation of stable transfectants

Anti-PTPRK hammerhead ribozyme transgenes and empty plasmids were transfected into the breast cancer cells, followed by a selection using blasticidin (5µg/ml). The transfectants were verified for PTPRK knockdown using RT-PCR, Q-PCR and Western blotting. Full details of the cloning process have been given in section 2.7.

6.2.7 RNA isolation, cDNA synthesis, and RT-PCR

RNA was isolated from the cells using the Tri Reagent kit (Sigma-Aldrich, Inc., Poole, Dorset, England, UK). For tissue RNA extraction, all frozen tissues were sectioned

using a cryostat (Leica DM40). Up to 50 frozen sections from each sample were collected, combined and homogenised in 1ml Tri Reagent solution, using a handhold homogenizer (Fisher Scientific). Total RNA was extracted according to the manufacturer's instruction. cDNA was synthesised by reverse transcription using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, California, USA), as described in section 2.4. RT-PCR was carried out at the following conditions; 94˚C for 5 minutes, followed by 30 to 42 cycles of 94˚C for 30 seconds, 56˚C for 30 seconds, and 72˚C for 1 minute and a final extension of 7 minutes at 72˚C. The products were run on an agarose gel and visualised using ethidium bromide.

6.2.8 Protein extraction, SDS-PAGE, and Western blot analysis

Protein was extracted and was then quantified using the DC Protein Assay kit (BIO-RAD, USA). After SDS-PAGE, the proteins were transferred onto nitrocellulose membranes which were then blocked and probed with the specific primary (anti-PTPRK 1:500) and the corresponding peroxidise-conjugated secondary antibodies (1:1000). All of the antibodies used in this study are listed in Table 2.4. The protein bands were eventually visualised using the chemiluminescence detection kit (Luminata, Millipore).

6.2.9 Immunochemical staining of PTPRK

Immunochemical staining of PTPRK in breast cancer tissues were carried out using the anti-PTPRK antibody for the protein (1:100), followed by secondary antibody. For the detailed procedure refer to Section 2.5.9.

6.2.10 *In vitro* cell growth assay

The cells were seeded into two 96 well plates, and incubated for 1 and 5 days respectively, as described in section 2.8.1. Following incubation, the cells were fixed and stained with crystal violet before the absorbance was measured in order to determine cell number.

6.2.11 *In vitro* cell Matrigel adhesion assay

The cells were seeded into a 96 well plate coated with matrigel as described in section 2.8.2. The cells were left to adhere for a period of 40 minutes, before being fixed and stained with crystal violet. The adherent cells were then photographed and counted under a microscope.

6.2.12 *In vitro* cell motility assay

The protocol followed is described by Jiang (Jiang *et al*., 1995b). Cells are seeded into a 24 well plate and cells were treated with a protein of interest in serum free media if required. These plates were then left to incubate at 37^{o}C, with 5% CO_2, for a period of 24 hours to allow cells to form a confluent monolayer. Subsequently, the cells were wounded and photos taken using a microscope with camera at 0.25, 1, 2 and 3 hours after wounding. Migration distances were measured using ImageJ softwear (National Institutes of Health, USA)

6.2.13 *In vitro* cell Matrigel invasion assay

The cells were seeded into transwell inserts with 8μm pores coated with 50 μg matrigel, in a 24 well plate and were incubated for a period of 3 days. Following incubation the cells which had migrated through the matrigel to the other side of the insert were fixed in formalin, stained with crystal violet and counted.

6.3 Results

6.3.1 Expression of PTPRK in breast cancer.

The expression of PTPRK was examined in breast cancer cell lines and a cohort of breast cancer tissues. The expression of PTPRK mRNA in breast cancer cell lines was determined using RT-PCR. PTPRK was expressed similarly in five different breast cancer cell lines (Figure 6.1A). We quantified PTPRK transcript levels in the breast specimens using Q-PCR, in which expression of PTPRK appeared to be higher in breast tumour tissues compared with the normal mammary background tissues (Figure 6.1B). IHC revealed a stronger staining of PTPRK in tumour tissues compared with normal background tissues (Figure 6.1C) which is consistent with PTPRK mRNA expression. Staining intensity analysis confirmed higher PTPRK expression in breast cancer (Figure 6.1D).

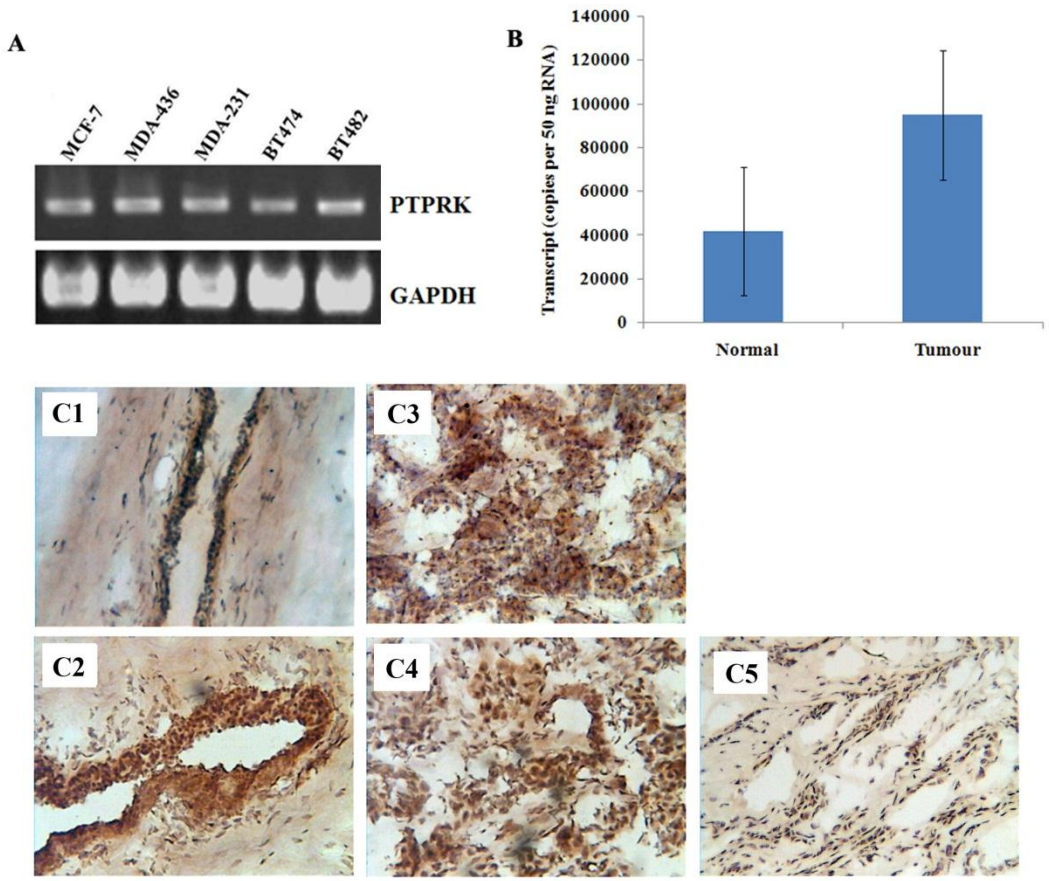


Figure 6.1 Expression of PTPRK in breast cancer. A, Expression of PTPRK mRNA in breast cancer cell lines using RT-PCR. **B**, PTPRK transcript levels were increased in human breast cancer compared with normal breast tissues. **C**, The immunohistochemical staining revealed an increase staining of PTPRK in breast cancer tissues compared with normal tissues. **C1**, Normal breast. **C2**, Ductal carcinoma *in situ*. **C3** and **C4**, Ductal carcinoma. C5, negative control. *, $P < 0.05$.

6.3.2 Correlation of PTPRK expression with histological type, tumour grade, lymph node involvement, and tumour-node-metastasis stage.

Levels of PTPRK transcripts were analysed against the corresponding clinical and pathological data (Table 6.1). PTPRK levels appeared to be lower in well differentiated tumours, but there was no significant differences compared with its expression in moderately differentiated tumours and poorly differentiated tumours. There was no correlation observed between PTPRK expression and lymph node involvement. Furthermore, PTPRK levels were lower in tumours of early TNM stage and were up-regulated in the advanced tumours, particularly the most advanced tumours of TNM4, $p= 0.0096$ compared with TNM1.

6.3.3 Prognostic relevance and clinical outcomes of PTPRK in breast cancer.

According to the Nottingham Prognostic Index (NPI) which takes into consideration of tumour grade, tumour size and nodal status, we further analysed the relationship between prognosis and PTPRK expression. PTPRK was expressed at similar levels in the groups of NPI 1 (NPI score<3.5, good prognosis) and NPI 2 (NPI score=3.5-5.4, moderate prognosis). A decreased expression of PTPRK was seen in the tumour of NPI 3 (NPI score>5.4, poor prognosis) compared with NPI 1, $p= 0.039$. PTPRK transcript

levels were decreased in patients who had metastases or died from breast cancer p=0.0039 and p= 0.0075 compared respectively with that of patients being disease free. Furthermore, the relationships between PTPRK expression and oestrogen receptors (ERs) had also been analysed. There was no difference observed in PTPRK transcript levels among tumours with different ERα expression. PTPRK expression appeared to be higher in ERβ positive group which was not statistically significant (Table 1). The Kaplan-Meier survival model was used to analyse the overall survival and disease-free survival status of patients. The average level of PTPRK transcripts in NPI 2 group was used as threshold. It was found that patients with higher PTPRK transcript levels had a longer overall survival (n=35, 138.8 months, 95% CI= 130.1-147.5 months), p= 0.016 vs. that of patients with lower transcript levels (n= 49, 122.0 months, 95% CI= 105.3-138.6 months) (Figure 6.2A). The patients displaying higher expression of PTPRK also had longer disease-free survival (n=35, 135.5 months, 95% CI= 124.9-146.1 months), p= 0.014 vs. that of patients with lower transcript levels (n= 49, 114.6 months, 95% CI= 97.5-131.8 months) (Figure 6.2B).

Table 6.1 Transcript levels of PTPRK in breast cancer

Clinical/pathological features	Mean± SD (copy no.)	*P*-value
Tissue sample		
Normal	41727±29372	
Tumour	94853±29454	0.20
Grade		
1	49406±26362	
2	58798±30155	0.82
3	91732±79298	0.15
NPI		
1 (<3.5)	101232±35303	
2 (3.5-5.4)	133681±76599	0.70
3 (>5.4)	21029±14034	**0.039**
TNM		
1	66529±24657	
2	138704±78190	0.38
3	91732±79298	0.77
4	402±131	**0.0096**
Clinical outcome		
Disease-free	93337±29257	
Poor outcome	104467±87823	0.91
With metastasis	5327±4270	**0.0039**
With local recurrence	604691±533976	0.41
Died of breast cancer	10392±7684	**0.0075**
Histology		
Ductal	114036±36747	
Lobular	4542±2265	
Others	86319±74256	0.75
Lymph node status		
Negative	101232±35303	
Positive	97728±52643	0.96
Estrogen receptor		
ERα (-)	96298±32787	
ERα (+)	96323±71111	1.00
ERβ (-)	78492±26375	
ERβ (+)	164555±118437	0.49

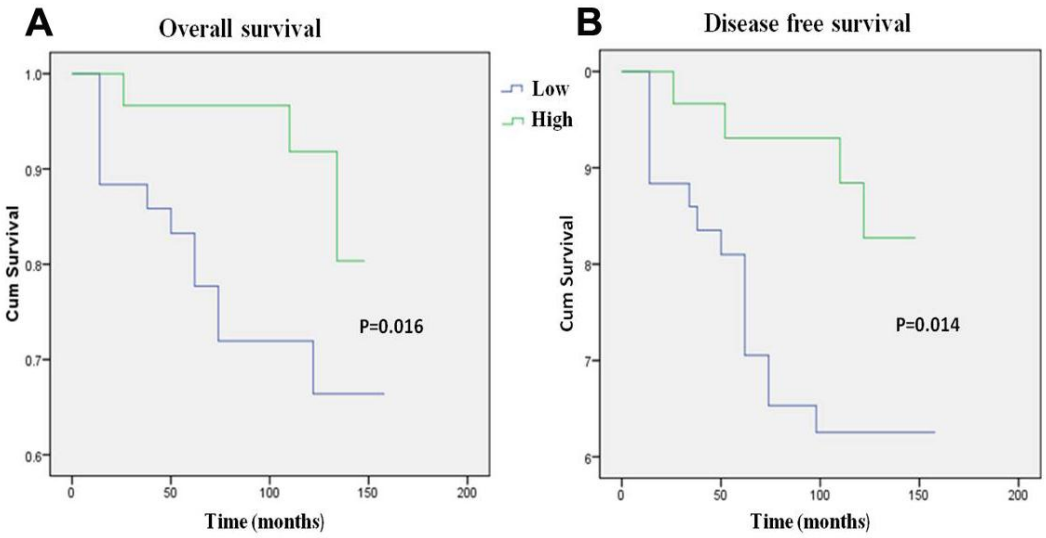


Figure 6.2 The Kaplan-Meier survival model demonstrated the impact of PTPRK expression in breast cancer. Higher levels of PTPRK expression (n=35; vs lower expression, n=49) in primary breast tumours correlate with longer overall survival (A) and disease free survival (B). Groups of PTPRK expression level were divided by NPI2.

6.3.4 Knockdown of PTPRK in breast cancer cell lines.

The expression of PTPRK was knocked down using ribozyme transgenes targeting human PTPRK mRNA. This was performed in two breast cancer cell lines, MDA-MB-231 (ER negative) and MCF-7 (ER positive), which expressed PTPRK (Figure 6.1A). The knockdown of PTPRK was verified in the transfectants using RT-PCR (Figure 6.3A), real-time quantitative PCR (Figure 6.3B) and Western blot (Figure 6.4). Decreased expression of PTPRK was seen in both MDA-MB-231^{PTPRKkd} and MCF-7^{PTPRKkd} cells which were transfected with ribozyme transgenes, compared to their corresponding controls.

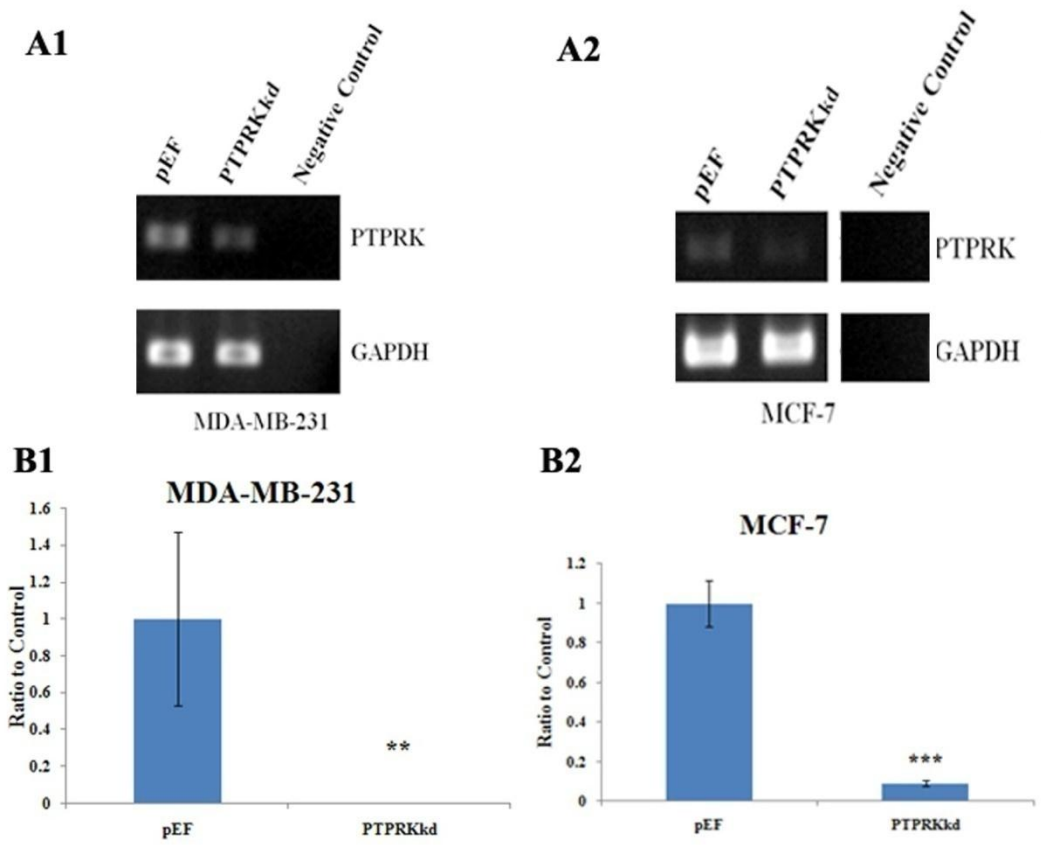


Figure 6.3 Knockdown of PTPRK transcript levels in breast cancer cells. Knockdown of PTPRK was seen in both MDA-MB-231^{PTPRKkd} (**A1**) and MCF-7^{PTPRKkd} (**A2**) cells using RT-PCR compared with their empty plasmid control (MDA-MB-231^{pEF} and MCF-7^{pEF}) cells. Knockdown of PTPRK in MDA-MB-231 (**B1**) and MCF-7 (**B2**) cells was also verified using real-time quantitative PCR compared with pEF control cells. **, $P < 0.01$ and ***, $P < 0.001$.

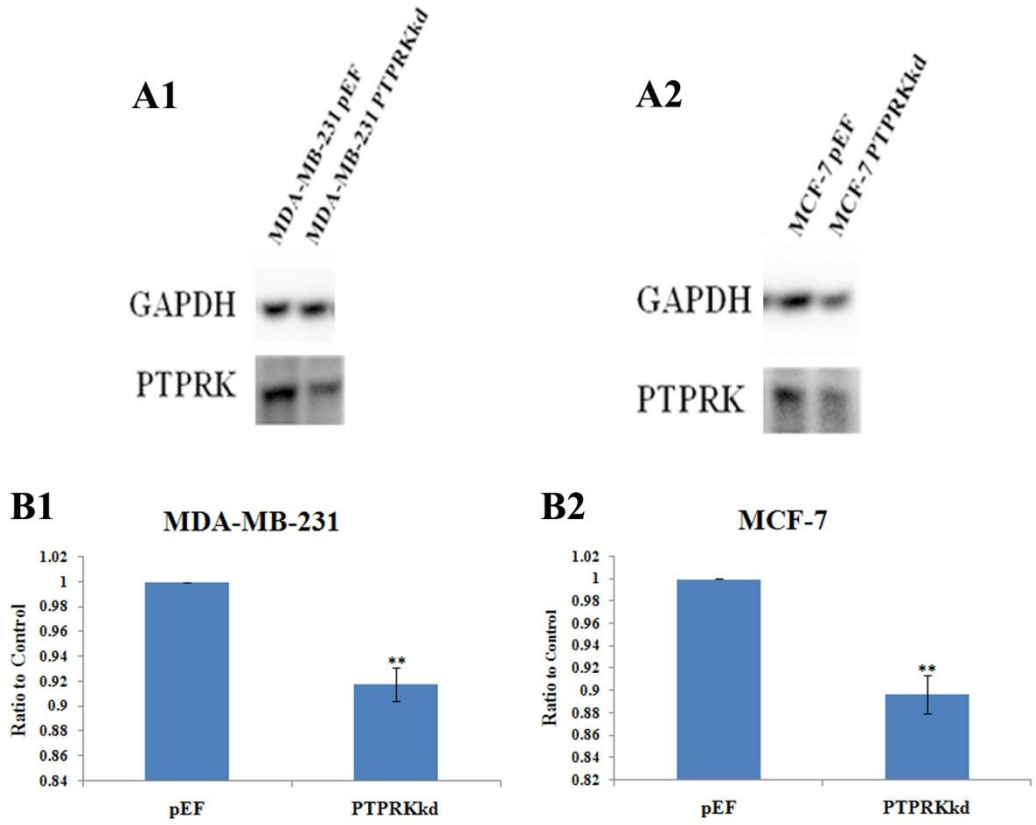


Figure 6.4 Knockdown of PTPRK protein levels in breast cancer cells. A, Knockdown of PTPRK in MDA-MB-231 and MCF-7 cells was confirmed using western blot in comparison with pEF control. **B**, The Western blot band intensity of PTPRK. **, $P < 0.01$.

6.3.5 Knockdown of PTPRK promotes *in vitro* cell functions.

The effect on *in vitro* cell functions by PTPRK knockdown was examined including cell growth, adhesion, invasion and migration. Knockdown of PTPRK in both MDA-MB-231 and MCF-7 cells exhibited an impact on cell growth. The MDA-MB-231^{PTPRKkd} cells showed an increased growth rate at day 3 (621.94±12.87, P<0.001) compared with MDA-MB-231^{pEF} (502.87±23.51) (Figure 6.5A1). Similarly, MCF-7^{PTPRKkd} cells showed the same trend, with a marked increase of growth rate at day 3 (326.36±23.29, P<0.001) compared with MCF-7^{pEF} (223.16±16.49) (Figure 6.5A2). Knockdown of PTPRK in both MDA-MB-231 and MCF-7 cells had a significant influence on cell matrix adhesion. The MDA-MB-231^{PTPRKkd} cells (237.64±52.55 cells) exhibited an increased adhesion, P<0.05 compared with MDA-MB-231^{pEF} cells (154.17±23.67) (Figure 6.5B1). An enhanced adhesion was also seen in MCF-7^{PTPRKkd} cells (315.17±28.34 cells, p<0.001) compared with MCF-7^{pEF} (233.67±30.10 cells) (Figure 6.5B2). Invasiveness of both MDA-MB-231 and MCF-7 cells were also promoted after knockdown of PTPRK. Both MDA-MB-231^{PTPRKkd} and MCF-7^{PTPRKkd} cells showed a remarkably increased invasive capacity compared with respective controls (Figure 6.6A). Finally, in MDA-MB-231 cells, knockdown of

PTPRK increased motility compared with MDA-MB-231^{pEF} (Figure 6.6B). No obvious effect of PTPRK knockdown was seen on the migration of MCF-7.

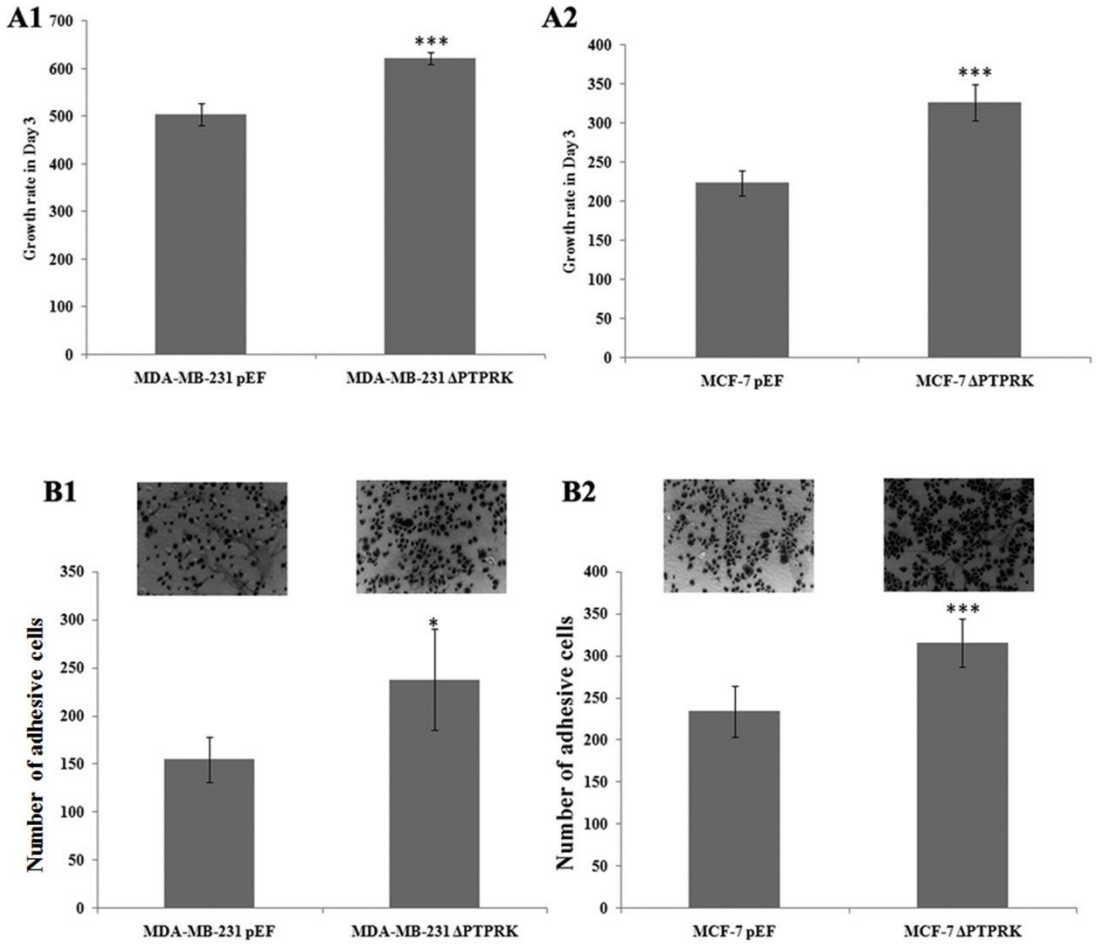


Figure 6.5 The effects of PTPRK knockdown on cell growth and adhesion of breast cancer cells. Knockdown of PTPRK increased the *in vitro* growth of breast cancer cells, MDA-MB-231 (**A1**) and MCF-7 (**A2**). Knockdown of PTPRK promoted cell-matrix adhesion in both MDA-MB-231 (**B1**) and MCF-7 (**B2**) cells. *, $P < 0.05$ and ***, $P < 0.001$. Growth rate (%) = Day1 absorbance/ Day 3 absorbance x100.

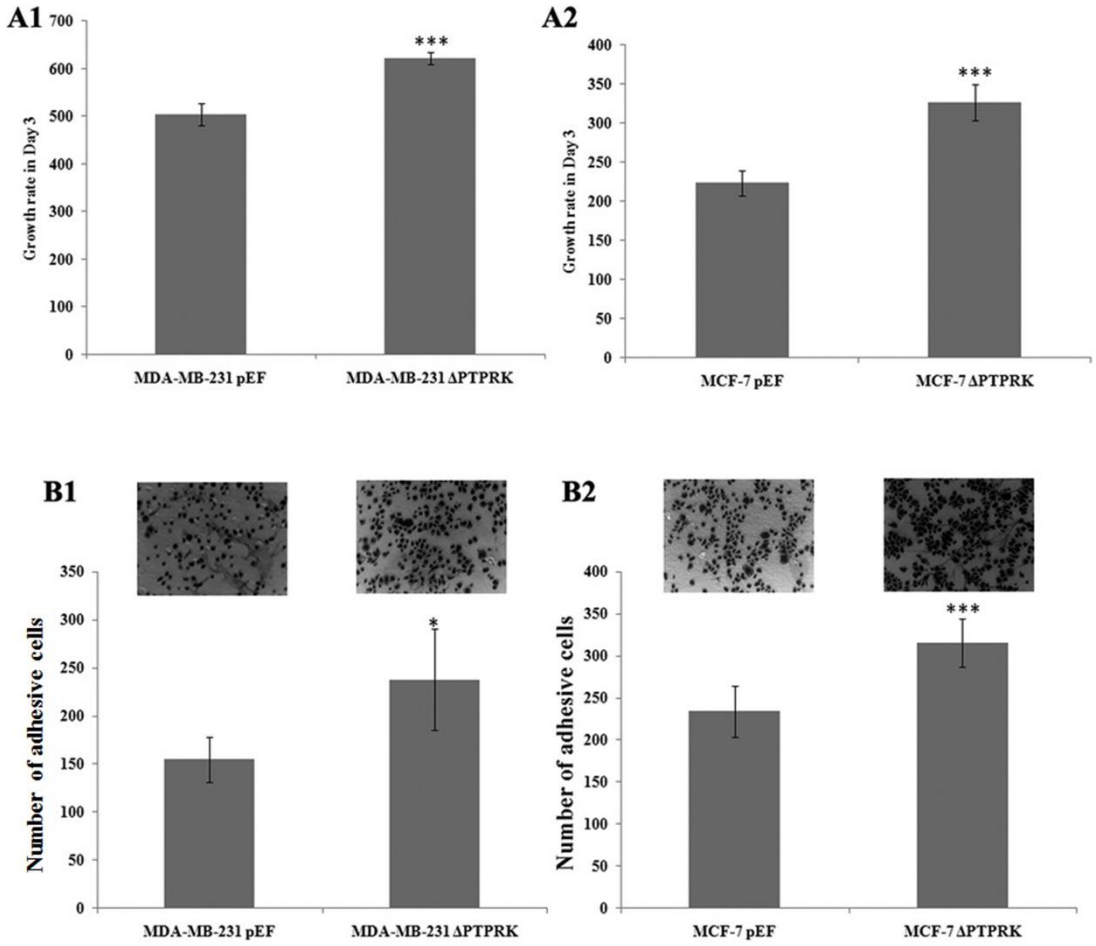


Figure 6.6 The effects of PTPRK knockdown on cell invasion and motility of breast cancer cells. Knockdown of PTPRK in MDA-MB-231 (**A1**) and MCF-7 (**A2**) cells increased cell invasive ability. **B1**, Knockdown of PTPRK in MDA-MB-231 cells increased cell motility. **B2**, Knockdown of PTPRK had no effects on cell motility in MCF-7 cells ***, $P < 0.001$.

6.4 Discussion

Previous studies have already shown that breast cancer is associated with different PTPs, such as PTPγ, LAR, PTPα, PTP1B, and DEP-1. PTPγ has been report as a tumour suppressor in kidney and lung adenocarcinoma and its expression has also been seen to be reduced in breast cancer tissues compared with normal tissues. Furthermore, expression of PTPγ is reduced in tumours compared with normal tissues, which is also related to higher ERα and lower ERβ expression. It is suggested that PTPγ may be an estrogen-regulated tumour suppressor in human breast cancer (LaForgia *et al.,* 1991; Zheng *et al.,* 2000; Liu *et al.,* 2002). However other PTPs may play very different roles in malignancies. For example, LAR expression is increased and detected as a neuronal-type splicing format in breast tumours (Yang *et al.,* 1999). PTPα is able to induce fibroblast transformation via regulation of *Src*, in which overexpression of PTPα in cells is accompanied with increased *Src* activity and blockage of PTPα leads to a reduced activity of *Src* (Su *et al.,* 1999). Overexpression of PTPα has also been indicated in poorly differentiated breast cancer and also the ER positive tumours. Overexpression PTPα in MCF-7 resulted in increased *Src* activity and an inhibition of cell growth which may due to an arrest of cell at G0/G1 phase (Zheng *et al.,* 1992; Ponniah *et al.,* 1999; Su *et al.,* 1999; Ardini *et al.,* 2000). Moreover, PTP1B is

up-regulated in human breast cancer cells by *neu* oncogene. PTP1B also activates *c-Src* in breast cancer cells (MDA-MB-435) (Zhai *et al.,* 1993; Wiener *et al.,* 1994; Bjorge *et al.,* 2000).

Although the levels of PTPRK transcript were higher in tumour versus normal (Table 6.1), there was a distinct association between loss of PTPRK and increasing NPI within the tumour tissues themselves. This supports a role for PTPRK in tumours as a potential tumour suppressor. In the current study, levels of PTPRK transcript in a breast cancer cohort were analysed against the corresponding clinical and pathological data. The results showed that patients with advanced breast cancer and poor prognosis, especially patients with metastases and who died from breast cancer had relatively lower levels of PTPRK expression. Moreover, the patients with lower expression of PTPRK had shorter overall survival and disease-free survival compared with those with higher expression. Certain PTPs have been shown to be differentially expressed in breast tumours with links to oestrogen receptors (LaForgia *et al.,* 1991; Zheng *et al.,* 2000; Liu *et al.,* 2002). Although PTPRK expression is slightly higher in ERβ positive than ERβ negative, there was no statistical difference. Similarly, there was no correlation between PTPRK and ERα status seen in the current study.

Our present study has indicated that there is a profound role played by PTPRK in breast cancer cells. In line with the findings from the breast cancer cohort, knockdown of PTPRK, in both MDA-MB-231 and MCF-7 cell lines leads to increased *in vitro* cell proliferation, adhesion and invasion. Currently there are a few studies indicating that overexpression PTPs, such as PTPα, LAR, and DEP-1, inhibit cell proliferation (Zhai *et al.,* 1993; Keane *et al.,* 1996; Ardini *et al.,* 2000). Furthermore, recent studies have shown that PTPRK is up-regulated by TGF-β and probably involved in TGF-β dependent anti-proliferation and cell migration effects. Co-localisation of PTPRK with β-catenin at adhesion junctions has been revealed in human embryonic kidney cells which stabilise E-cadherin/β-catenin complexes. The stabilisation of β-catenin/E-cadherin complexes can suppress wnt/β-catenin-induced transcription of target genes, such as cyclin D1 and c-Myc, leading to an inhibition of proliferation and migration in cancer cells (Yang *et al.,* 1996; Novellino *et al.,* 2008). In addition, it has been shown that PTPRK directly dephosphorylates EGFR and increased PTPRK expression decreases both basal and ligand-stimulated EGFR tyrosine phosphorylation, and reduced PTPRK expression leads to an increase in EGFR tyrosine phosphorylation. Blocking PTPRK expression accelerated cell cycle progression which may due to the

enhanced EGF-induced tyrosine phosphorylation of EGFR and HER2 (ErbB2), and the downstream ERK activation (Wang *et al.*, 2005; Xu *et al.*, 2005). However, the involvement of these pathways in the inhibitory effect of PTPRK on the growth, adhesion and invasion of breast cancer cells has yet to be investigated.

In conclusion, lower expression levels of PTPRK are correlated with poor prognosis and reduced overall survival and disease free survival. Moreover, knockdown of PTPRK resulted in increased adhesive and invasive abilities and promoted the cell proliferation and motility of breast cancer cells. This suggests that PTPRK may be a potential tumour suppressor in breast cancer.

Chapter 7

PTPRM is a negative regulator of proliferation and invasion of breast cancer cells and is associated with disease prognosis in breast cancer

7.1 Introduction

PTPs are characterised by variable extracellular multiple domains and exhibit features of cell adhesion molecules in their extracellular segment. It has been implicated in cell-cell and cell-matrix contact via dimerisation, phosphorylation and reversible oxidation (Angers-Loustau *et al.*, 1999; Stoker 2005). PTPRM belongs to the same PTP R2B subfamily with PTPRK and its basically shares the some extracellular domains including Ig-like domain, MAM domain, and fibronectin type-III repeats.

PTPRM has a similar structure to cell-cell adhesion molecules and has been shown to exhibit homophilic binding and confer cell-cell adhesion in cells including epithelial and cancer cells. Moreover, PTPRM also recognises other subfamily members to mediate cell-cell aggregation (Brady-Kalnay *et al.,* 1993; Becka *et al.,* 2010). Like other PTPs, PTPRM is regulated by the balance between the actions of protein tyrosine kinases (PTKs) and PTPs. Reduced expression of PTPRM resulted in an increased phosphorylation of tyrosine 992 of EGFR (pY992) by EGF, a docking site for PLCγ1 to activate PLCγ1, thus leading to increased cell migration in both wounding and chemotaxis assays (Phillips-Mason *et al.,* 2008; Hyun *et al.,* 2011).

The previous chapters have shown the effects of PTPRK knockdown in breast cancer cells and PTPRK expression associates with prognosis in breast cancer. It has been reported that PTPRM is associated with carcinogenesis of ovarian carcinoma (Gyorffy *et al.*, 2008). However, the role played by PTPRM in breast cancer remains unknown. In order to investigate the PTPRM in breast cancer, the disease progression and impact of PTPRM on functions of breast cancer cells were analysed.

7.2 Materials and methods

7.2.1 Breast cancer tissues

The collection and preparation of breast tissue samples have been introduced in the previous chapter section 6.2.1.

7.2.2 Antibody and primers

The primers used in this chapter are shown in Table 2.2 including PTPRM and MMP9. The antibodies used in this chapter including anti-PTPRM, PLCγ, JNK, ERK and phospho-tyrosine are shown in Table 2.4.

7.2.3 Cell lines

MDA-MB-231 and MCF-7 were routinely cultured in DMEM-F12 medium as described in section 2.3.

7.2.4 Generation of PTPRM ribozyme transgenes

Hammerhead ribozymes targeting PTPRM were designed based on the secondary structure of PTPRM mRNA and synthesised following a previously described method in section 2.7. Primers' details are listed in the Table 2.3.

7.2.6 Breast cancer cell transfection and generation of stable transfectants

Following plasmid verification using DNA electrophoresis, the plasmids were transfected into target cells using the electroporation at 310V. The transfectants were then selected with 5μg/ml blasticidin for a period of two weeks. Empty plasmid vectors were also used to transfect the same cells in order to use as controls. After the selection, the cells were verified for PTPRM knockdown using RT-PCR, Q-PCR, and western blotting. Full details of the cloning process have been given in section 2.7.

7.2.7 RNA isolation, cDNA synthesis, and RT-PCR

RNA was isolated from the cells using the Tri Reagent kit (Sigma-Aldrich, Inc., Poole, Dorset, England, UK), and converted into cDNA by reverse transcription using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, California, USA), as described in section 2.4. RT-PCR was carried out at the following conditions; 94˚C for 5 minutes, followed by 30 to 42 cycles of 94˚C for 30 seconds, 56˚C for 30 seconds and 72˚C for 1 minute, and a final extension of 7 minutes at 72˚C. The products were run on an agarose gel and visualised using ethidium bromide.

7.2.8 Protein extraction, SDS-PAGE, and Western blot analysis

Protein was extracted and was then quantified using the DC Protein Assay kit (BIO-RAD, USA). After the SDS-PAGE, the proteins were transferred onto nitrocellulose membranes which were then blocked, and probed with the specific primary (1:500) and the corresponding peroxidase-conjugated secondary antibodies (1:1000). All of the antibodies used in this study are listed in Table 2.4. The protein bands were eventually visualised using the chemiluminescence detection kit (Luminata, Millipore).

7.2.9 Immunoprecipitation and detection of tyrosine phosphorylated ERK, JNK, and PLCγ

Protein extracted from MDA-MB-231 cells was immunoprecipitated with anti-PLCγ, JNK, and ERK antibody in order to detect phosphorylation of these proteins. These immunoprecipitated protein samples were then run on an SDS-PAGE and blotted with anti-phosph-tyrosine antibody. Refer to section 2.5.3 for further details on immunoprecipitation method.

7.2.10 *In vitro* cell growth assay

The cells were seeded into three 96 well plates, and incubated for 1 and 5 days respectively, as described in section 2.8.1. Following incubation, the cells were fixed and stained with crystal violet before the absorbance was measured in order to determine cell number.

7.2.11 *In vitro* cell Matrigel adhesion assay

The cells were seeded into a 96 well plate coated with matrigel as described in section 2.8.2. The cells were left to adhere for a period of 40 minutes, before being fixed and stained with crystal violet and the cells counted.

7.2.12 *In vitro* cell motility assay

The protocol followed is described by Jiang (Jiang *et al*., 1995b). Cells are seeded into a 24 well plate and cells were treated with a protein of interest in serum free media if required. These plates were then left to incubate at 37^{o}C, with 5% CO_2, for a period of 24 hours to allow cells to form a confluent monolayer then the cells were wounded and took photos using a microscope with camera at 0.25, 1, 2 and 3 hours after wounding. Migration distances were measured using ImageJ software (National Institutes of Health, USA)

7.2.13 *In vitro* cell Matrigel invasion assay

The cells were seeded into transwell inserts with 8μm pores coated with 50 μg matrigel, in a 24 well plate and were incubated for a period of 3 days. Following incubation, the cells which had migrated through the matrigel to the other side of the insert were fixed in formalin, stained with crystal violet and counted.

7.2.14 Electric cell-substrate impedance sensing (ECIS)

The ECIS system (9600 model, Applied Biophysics Inc., USA) was used to quantify cell migration as previously reported (Jiang *et al.,* 2008). 96W1E arrays were used in

this study. Cells were seeded at 40,000 cells per well in 200µl of DMEM medium alone or medium supplemented with 200nM PLCγ (U-73122), JNK (SP600125), ERK (FR180204) small inhibitors (MERCK, Germany). The resistance at 30 kHZ was recorded for 6 hours after wounded and the data was analysed using an ECIS-9600 software package.

7.2.15 Gelatin zymography assay

1×10^6 cells were counted and seeded to a tissue culture flask and incubated overnight. Following incubation, samples were washed once with 1x balanced salt solution followed by a wash with serum-free DMEM and then either incubated in serum-free DMEM control or treated medium for 4 hours. The treatments consisted of a 200 nM JNK or ERK inhibitors. After 4 hours the conditioned medium was collected. Protein samples were prepared in non-reducing sample buffer. Samples were separated using SDS-PAGE on gels containing 1% gelatine (Sigma-Aldrich Inc, USA). Gels were renatured for 1 hour at room temperature in washing buffer and incubated at 37^oC in incubation buffer for 36 hours. The gel was stained with coomassie blue. The brightness of clear bands, where MMP9 was located and gelatine was degraded, was analysed using densitometry.

7.3 Results

7.3.1 Expression of PTPRM in breast cancer

A lower level of transcript expression was seen in breast cancer cells and the breast cancer tissue compared with normal mammary background tissue. Although transcript levels of PTPRM appeared to be reduced in breast cancer tissue in comparison with normal mammary tissues, the difference was not statistically significant (Figure 7.1).

7.3.2 Association of PTPRM with tumour grade and TNM staging

Levels of PTPRM transcripts were analysed against the corresponding clinical and pathological data (Table 7.1). PTPRM levels were higher in well differentiated tumours and were decreased in moderately differentiated tumours (p=0.011) and poorly differentiated tumours (p=0.031). Furthermore, PTPRM levels were higher in tumours of early TNM stage and decreased in the TNM2 (p=0.032). TNM3 and TNM4 tumours also tended to have decreased levels of PTPRM, compared to TNM1 tumours, but this was not statistically significant. This lack of significance was likely due to a smaller number of samples at advanced stages (TNM3, n=7 and TNM4, n=4). Tumours with lymphatic involvement appeared to have lower levels of PTPRM transcripts than the node negative tumours, but were not statistically significant.

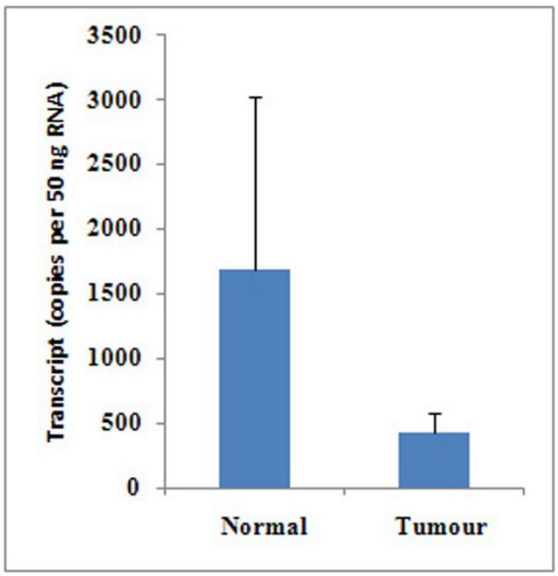


Figure 1. Expression of PTPRM in breast cancer tissues. The PTPRM transcript level was decreased in human breast cancer compared with normal breast tissues using quantitative PCR, *p*=0.36.

Table 7.1. Transcript levels of PTPRM in breast cancer.

Clinical/pathological features	**Mean± SD (copy no.)**	***P*-value**
Tissue sample		
Normal	1687±1337	
Tumour	428.8±148.8	0.36
Grade		
1	472±114	
2	288.8±110	**0.011**

3	92.5±279	**0.031**
NPI		
1 (<3.5)	605.4±277	
2 (3.5-5.4)	339±132	0.39
3 (>5.4)	83.8±25.7	0.06
TNM		
1	423±105	
2	172.8±45	**0.032**
3	212.7±208	0.27
4	165.5±109	0.13
Clinical outcome		
Disease-free	510±209	
Poor outcome	219±66	0.19
With metastasis	209±163	0.27
With local recurrence	432±298	0.84
Died of breast cancer	170±55	**0.012**
Lymph node status		
Negative	605.4±277	
Positive	257.7±91.9	0.24

7.3.3 Reduced PTPRM is associated with poor prognosis

According to the Nottingham Prognostic Index (NPI), we further analysed the relationship between prognosis and PTPRM expression. Patients with moderate prognosis (NPI 3.5-5.4, p=0.39) and poor prognosis (NPI >5.4, p=0.06) exhibited lower levels of PTPRM compared to the good prognosis group (NPI<3.5). According to clinical outcome from our follow-up data, PTPRM transcript levels were decreased in patients who died from breast cancer when compared with that of disease free patients (p=0.012). To investigate whether PTPRM expression levels were correlated with long-term survival, we divided the patients into two groups according to the average PTPRM transcript levels of patients with moderate prognosis of NPI 3.5-5.4. Kaplan-Meier survival analysis demonstrated that the expression of PTPRM transcripts was significantly associated with disease free survival (Figure 7.2). The patients with lower expression of PTPRM had shorter survival (median=109.5 months, 95% CI=94.1-124.9), p=0.029 vs. that of patients with higher expression levels (median=142.5 months, 95% CI=129.6-155.4 months).

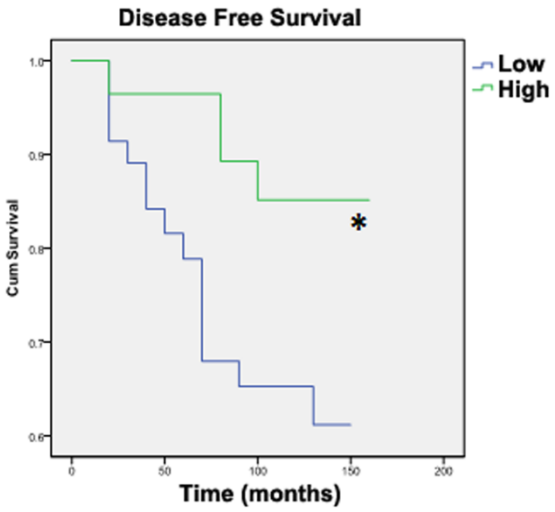


Figure 7.2 The Kaplan-Meier survival model of PTPRM in breast cancer. Higher levels of PTPRM expression (n=35; vs lower expression, n=49) in primary breast tumours correlate with longer disease free survival. *, $p < 0.05$. Groups of PTPRM expression level were divided by NPI2.

7.3.4 Knockdown of PTPRM in breast cancer cells

The expression of PTPRM was knocked down using ribozyme transgenes targeting human PTPRM mRNA. Two different breast cancer cell lines were employed in the current study. MDA-MB-231 cells are oestrogen receptor (ER) negative and appear to be more aggressive compared to MCF-7 cells which are ER positive. The knockdown of PTPRM in these two cell lines was verified in the transfectants using RT-PCR, real-time quantitative PCR and Western blot (Figure 7.3). Marked reduction of PTPRM expression was seen in both MDA-MB231$^{\Delta PTPRM}$ and MCF-7$^{\Delta PTPRM}$ cells which were transfected with ribozyme transgenes, compared to their corresponding wild type and empty plasmid control.

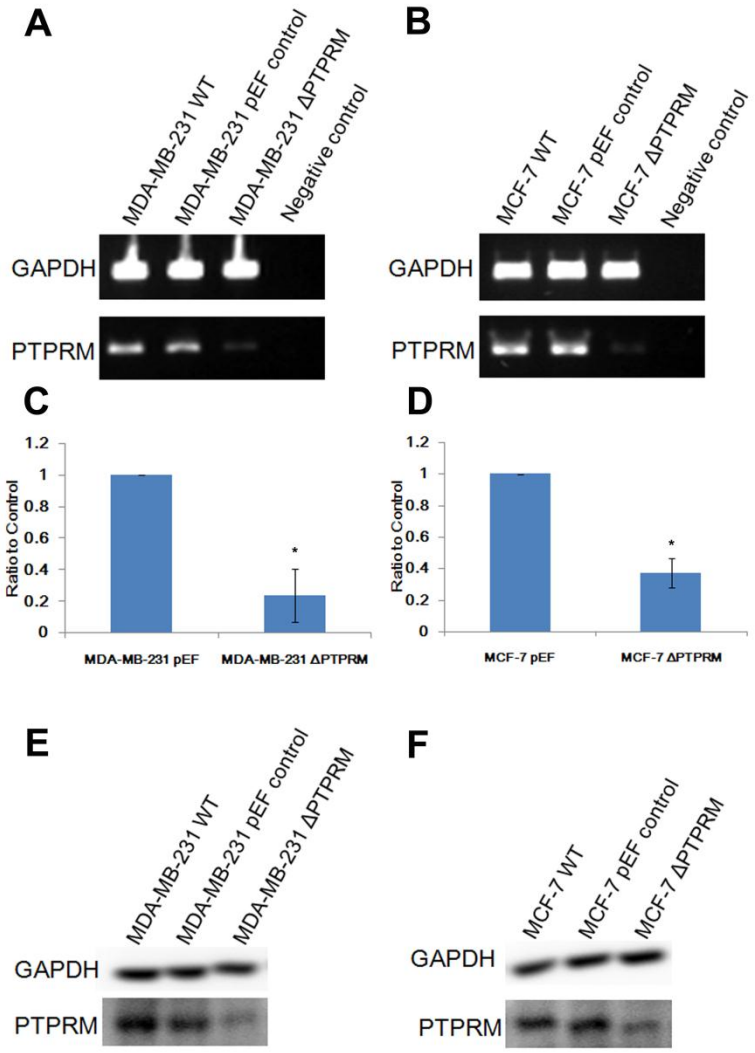


Figure 7.3 Knockdown of PTPRM in breast cancer cells. Knockdown of PTPRM was seen in both MDA-MB-231$^{\Delta PTPRM}$ (**A**) and MCF-7$^{\Delta PTPRM}$ (**B**) cells using RT-PCR compared with their wild type and (MDA-MB-231^{WT} and MCF-7^{WT}) empty plasmid control (MDA-MB-231^{pEF} and MCF-7^{pEF}) cells. Knockdown of PTPRM in MDA-MB-231$^{\Delta PTPRM}$ (**C**) and MCF-7$^{\Delta PTPRM}$ cells (**D**) was also verified using real-time quantitative PCR compared with pEF control cells. Knockdown of PTPRM in MDA-MB-231$^{\Delta PTPRM}$ (**E**) and MCF-7$^{\Delta PTPRM}$ (**F**) cells was confirmed using Western blot in comparison with wild type and pEF control. *, $p < 0.05$.

7.3.5 Effect on breast cancer cell functions by knockdown of PTPRM

The effect on *in vitro* cell functions (cell growth, adhesion, invasion and migration) by PTPRM knockdown was examined. Knockdown of PTPRM in both MDA-MB-231 and MCF-7 cells exhibited a significant impact on *in vitro* cell growth. Both MDA-MB-231$^{\Delta PTPRM}$ and MCF-7$^{\Delta PTPRM}$ cells showed an increased growth rate at day 3 compared with empty plasmid control (Figure 7.4A and 4B). Knockdown of PTPRM in both cell lines had a significant influence on cell matrix adhesion. Both cell lines exhibited a significantly stronger ability to adhere to matrix compared to empty plasmid control (Figure 7.4C and 4D). The invasiveness of both MDA-MB-231$^{\Delta PTPRM}$ and MCF-7$^{\Delta PTPRM}$ cells were also enhanced after knockdown of PTPRM. *In vitro*, both MDA-MB-231$^{\Delta PTPRM}$ and MCF-7$^{\Delta PTPRM}$ cells shown an increased invasive capacity compared with respective controls (Figure 7.5A and 5B). Finally, MDA-MB-231 cells, knockdown of PTPRM also enhanced cell motility (p<0.001) compared with MDA-MB-231^{pEF} (Figure 7.5C).

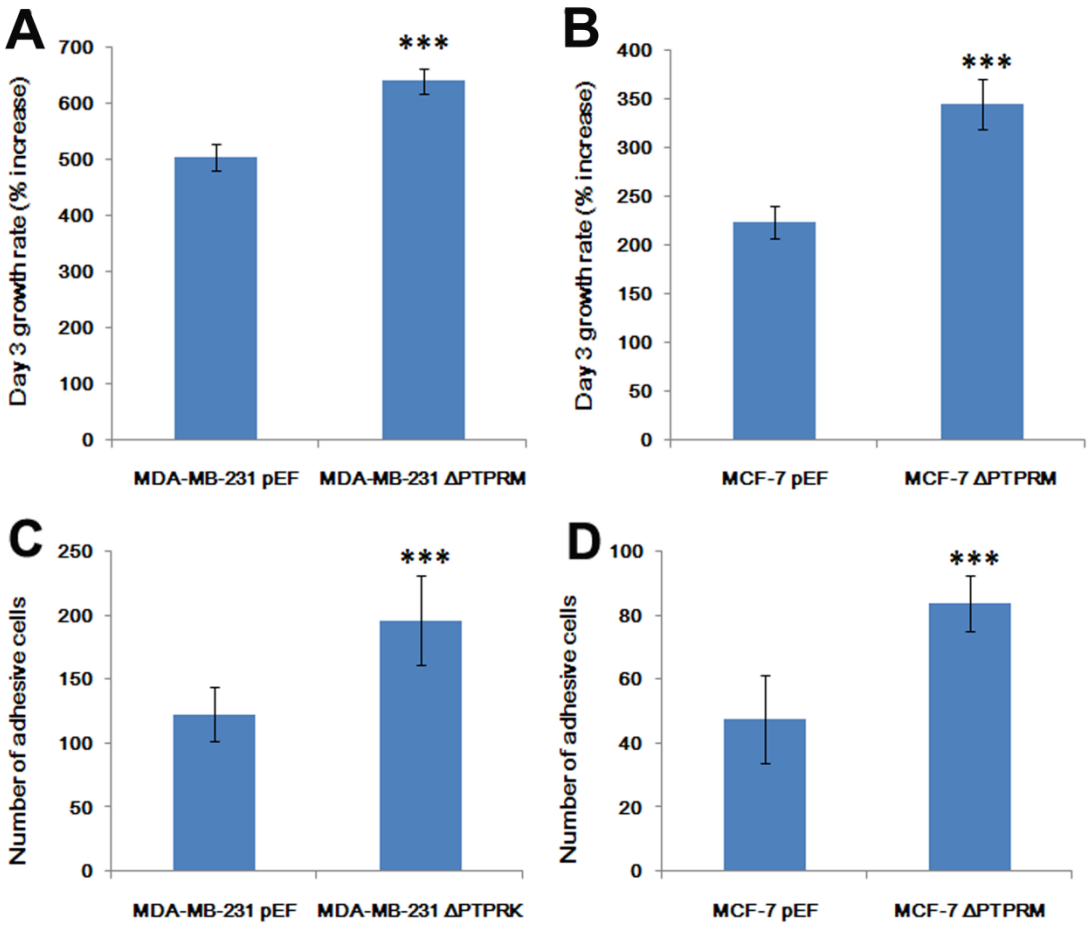


Figure 7.4. The effects of PTPRM knockdown on biological functions of breast cancer cells. A and **B**, Knockdown of PTPRM increased the *in vitro* growth of breast cancer cells. **C** and **D**, Knockdown of PTPRM promoted cell-matrix adhesion in both MDA-MB-231 and MCF-7 cells. ** $p < 0.01$ and *** $p < 0.001$. Growth rate (%) = Day1 absorbance/ Day 3 absorbance x100.

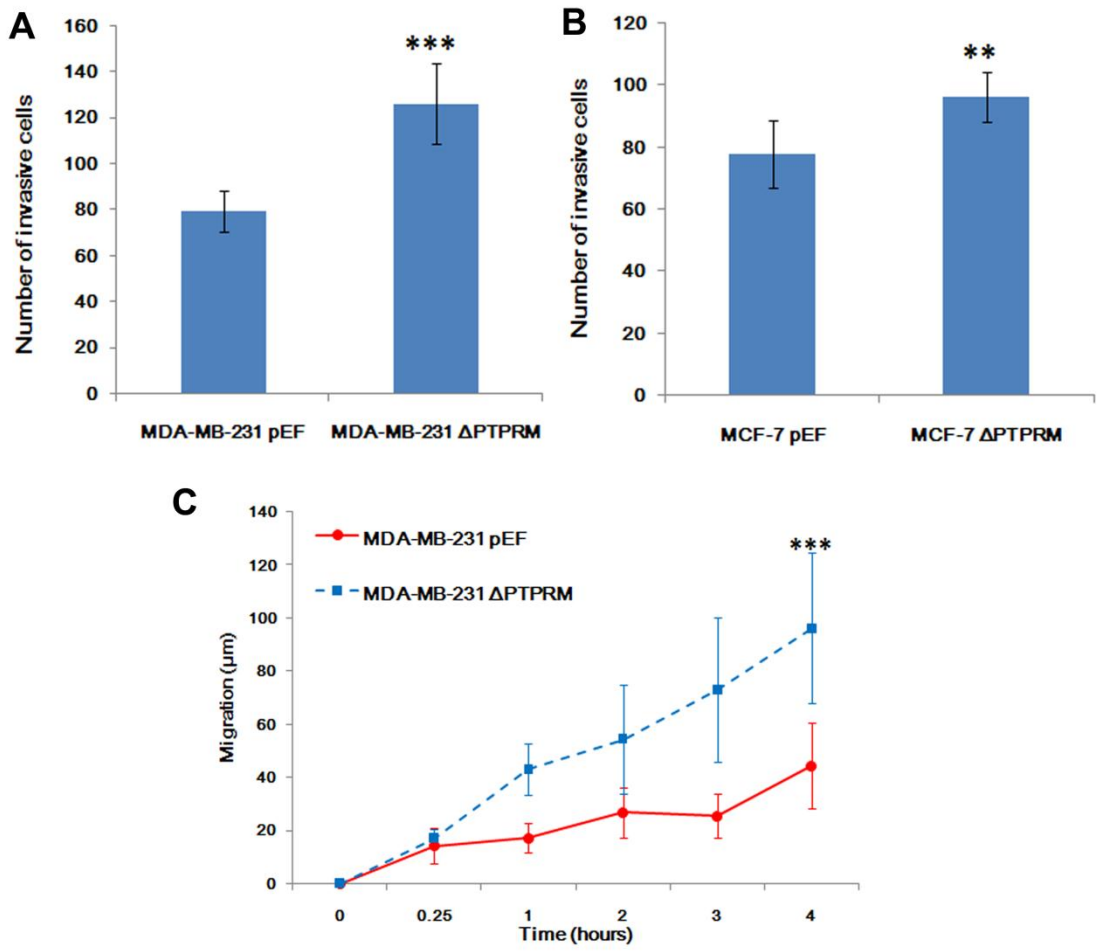


Figure 7.5. The effects of PTPRM knockdown on biological functions of breast cancer cells. A and **B**, Invasiveness of both MDA-MB-231 and MCF-7 cells were also promoted after knockdown of PTPRM. **C**, *in vitro* wounding assay showed that MDA-MB-231$^{\Delta PTPRM}$ cells promoted cell migration. ** $p < 0.01$ and *** $p < 0.001$.

7.3.6 Tyrosine phosphorylation of JNK and ERK were increased by PTPRM knockdown

Recent reports have shown that a reduction in expression of Protein Tyrosine Phosphatase-1B (PTP1B) and T-Cell Protein Tyrosine phosphatase (TC-PTP) reduced ERK phosphorylation in MCF-7 cells (Blanquart *et al.,* 2010) and PTPRM suppressed glioma cell migration by dephosphorylation of PLCγ1 (Phillips-Mason *et al.,* 2011). To determine the relationship between these proteins, immunoprecipitation (IP) and western blotting was used to investigate the impact of PTPRM on the tyrosine phosphorylation of PLCγ1, JNK and ERK in MDA-MB-231 cells (Figure 7.6A). Results showed that tyrosine phosphorylation of both ERK (Figure 7.6B) and JNK (Figure 7.6C) were increased in MDA-MB-231$^{\Delta PTPRM}$ cells compared with MDA-MB-231^{pEF} cells (p<0.05). Our data also suggests that there is no change in tyrosine phosphorylation of PLCγ1 in PTPRM knockdown cells compared to control cells (Figure 7.6D).

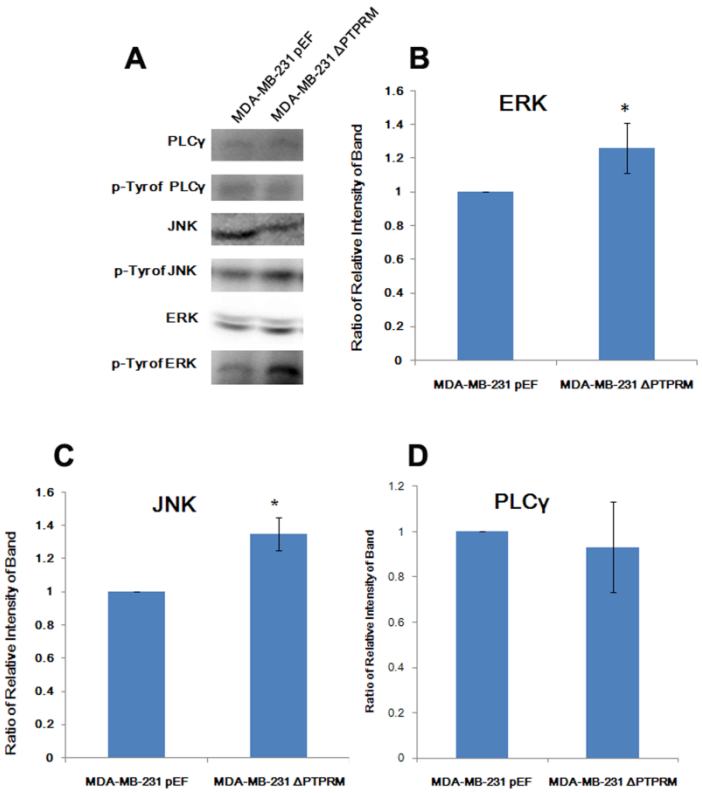


Figure 7.6 Impact on tyrosine phosphorylation of JNK and ERK. A, immunoprecipitation and western blot showed tyrosine phosphorylation of JNK and ERK were increased in PTPRM knockdown cells, which exhibited no effect on PLCγ phosphorylation. Relative intensity of bands from three western blots was analysed using Image J software for PLCγ (**B**), JNK (**C**), and ERK (**D**). *, $p < 0.05$.

7.3.7 Involvement of JNK and ERK pathway in PTPRM impact on cell migration

In order to investigate breast cancer cell migration we used the ECIS system to analyse cell motility. MDA-MB-231 cells were treated with 200nM PLCγ1, ERK and JNK small inhibitors. As shown in Fig. 7.7, a decrease in cell motility was observed in MDA-MB-231$^{\Delta PTPRM}$ following incubation with JNK (Figure 7.7A) and ERK (Figure 7.7B) small inhibitors compared to control cells, as shown by a slower rise in resistance compared to untreated knockdown cells, indicative of reduced migration onto the electrode. No effect was seen following the addition of PLCγ1 inhibitor (Figure 7.7C). Figure 7.7D shows the statistical analysis of resistance on the fifth hour following wounding and indicates that MDA-MB-231$^{\Delta PTPRM}$ cells migrated faster than MDA-MB-231^{pEF} cells in both control and PLCγ1 inhibitor treated groups, $p<0.05$. However, this effect on the migration was diminished in MDA-MB-231$^{\Delta PTPRM}$ cells when exposed to JNK or ERK inhibitors.

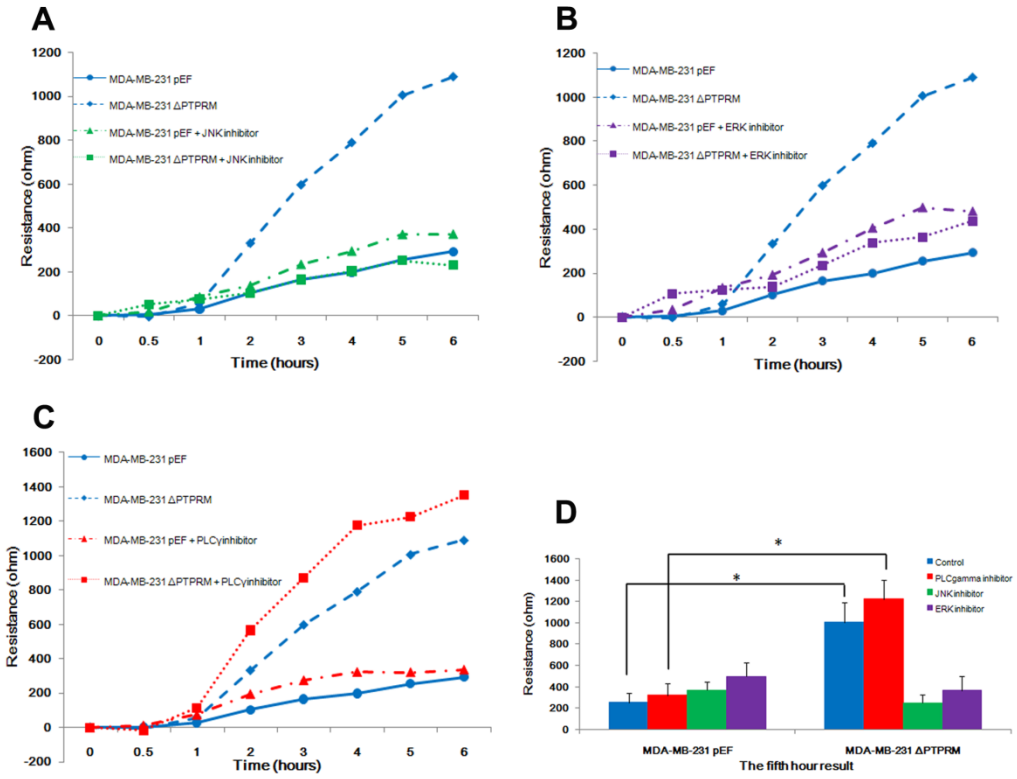


Figure 7.7 The knockdown of PTPRM in MDA-MB-231 cell resulted in increased cell motility via JNK and ERK pathways. Incubation of MDA-MB-231$^{\Delta PTPRM}$ cells with JNK small inhibitor (**A**) and ERK small inhibitor (**B**) diminished such effect. **C**, incubation of MDA-MB-231$^{\Delta PTPRM}$ cells with PLCγ small inhibitor had no effect on cell migration using ECIS. **D**, Overall changes of resistance on the fifth hour with statistical analysis. *, $p < 0.05$ and ***, $p < 0.001$.

7.3.8 Enzyme activity of MMP9 was increased via ERK in PTPRM knockdown cells.

Recently, several reports have shown that MMP9 expression and activity were up-regulated by the ERK signalling pathway in different human cells (Liu and Wilson 2012). In order to investigate MMP9 activity in breast cancer cells, we used gelatine zymography to analyse enzyme activity. Knockdown of PTPRM resulted in an increase of both active MMP9 and MMP2 in MDA-MB-231 cells, which was consistent with increased invasiveness. We further treated MDA-MB-231 cells with 200 nM JNK and ERK small inhibitor, respectively. The elevated MMP9 and MMP2 activity in PTPRM knockdown cells was reduced to a similar level seen in the control cells, especially after treatment with ERK inhibitor (Figure 7.8A). Furthermore, MMP9 gene expression is also increased in PTPRM knockdown cells (Figure 7.8B and 8C).

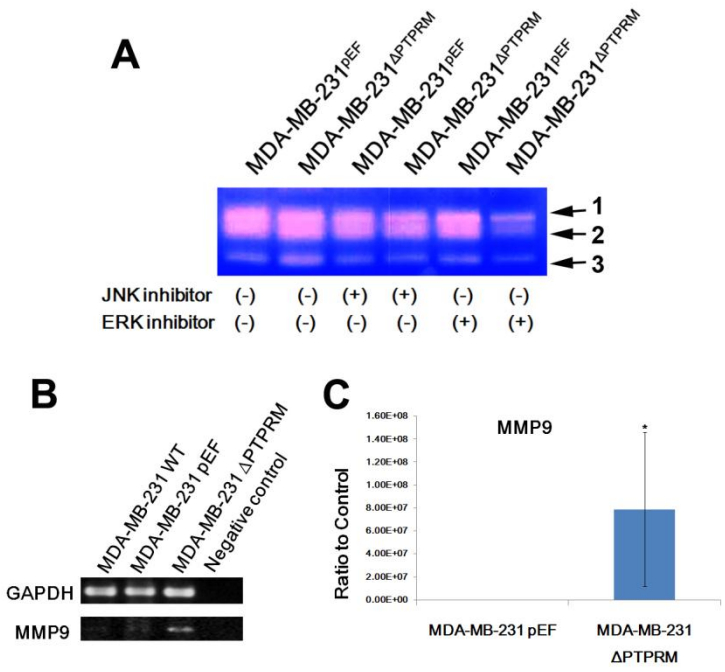


Figure 7.8 MMP9 expression and activity in MDA-MB-231 cell. A, gelatine zymography indicated the reduced enzyme activity of MMP9 in cells treated with ERK inhibitors.The overall MMP9 gene expression was increased in MDA-MB-231$^{\Delta PTPRM}$ cells using (**B**) RT-PCR and (**C**) real-time quantitative PCR. 1: pro-MMP9, 2: MMP9, and 3: MMP2. * $p < 0.05$.

7.4 Discussion

Previous studies looking at the different PTPs have in general indicated that increased expression of PTPs is associated with breast cancer (Hiscox and Jiang 1998). This finding is not universal as Zheng *et al* (Zheng *et al.,* 2000) showed that PTPRG was more highly expressed in normal tissue than in breast tumour tissue. PTPRM appears to have a similar tumour suppressing role and, for the first time, we demonstrate that in breast cancer there is a significant reduction in transcript levels. In the current study, levels of PTPRM transcript in a breast cancer cohort were analysed against the corresponding clinical and pathological data. The results showed that patients with higher tumour grade had relatively lower levels of PTPRM expression. Furthermore, the patients with lower expression of PTPRM had shorter disease-free survival compared with those with higher expression.

Our present study has indicated profound roles played by PTPRM in breast cancer cells. Knock-down of PTPRM in both MDA-MB-231 and MCF-7 cell lines led to increased *in vitro* cell proliferation, adhesion and invasion. There was a study indicating that PKC is involved in PTPRM-dependent signalling. PTPRM, RACK1, and PKCδ exist in a complex in cultured retinal cells and retinal tissue. PKCδ is required for neurite

outgrowth of retinal ganglion cells on a PTPRM substrate (Rosdahl *et al.,* 2002). Furthermore, PTPRM regulates the PKC pathway to restore E-cadherin-dependent adhesion via its interaction with RACK1 (Hellberg *et al.,* 2002). Activation of PLC γ 1 is associated with increased invasion of cancer cells. A recent study has shown that PLCγ1 is a target of PTPRM and dephosphorylation of PLCγ1 is a major pathway by which PTPRM suppresses glioma cell migration (Phillips-Mason *et al.,* 2011). However, in our current study no change in the activated PLCγ1 was seen in the PTPRM knockdown cells and the promoted migration was not affected by a PLCγ1 small inhibitor. This suggests that the PLCγ1 pathway is not involved in the effect on these breast cancer cells by knockdown of PTPRM.

In addition to the PLCγ1 pathway, ERK and JNK pathways have been indicated in the functions of certain PTPs. It has been shown that reduction in expression of Protein Tyrosine Phosphatase-1B (PTP1B) and T-Cell Protein Tyrosine phosphatase (TC-PTP) reduced ERK phosphorylation in MCF-7 cells to regulate cell migration (Blanquart *et al.,*). Inhibition of JNK and ERK1/2 reduced PTP1B protein expression (Sarmiento *et al.,*) and the absence of PTP1B in endoplasmic reticulum (ER) resulted in an activation of JNK leading to a suppression of apoptosis (Gu *et al.,* 2004). Moreover, a 45kDa

variant of TC-PTP (TC45) exits in the nucleus upon EGFR activation and inhibits the EGFR-dependent activation of JNK and consequent activation of PI3K/Akt pathway, but elicits no impact on activation of ERK2 (Tiganis *et al.*, 1999). To examine the involvement of ERK and JNK pathways in the effect of PTPRM knockdown on breast cancer cell motility, we treated the cells with small inhibitors for these pathways. The effect of PTPRM knockdown on cell migration was diminished by blocking these pathways individually. This indicates that the dephosphorylation of ERK and JNK by PTPRM plays a critical role in coordinating functions of breast cancer cells.

It has been suggested that MMP9 activity is related to the ERK pathway, as tumour necrosis factor (TNF) stimulates proMMP9 production in human chorionic trophoblast cells through ERK1/2 pathway but not JNK and p38 pathway (Li *et al.*, 2010) and miR-520c and miR-373 up-regulated MMP9 expression by activation of Ras/Raf/MEK/ERK in human fibrosarcoma cells (Liu and Wilson 2012). Our data has thus indicated that MMP9 activity in MDA-MB-231$^{\Delta PTPRM}$ was increased compared with pEF control and this increase was diminished by the treatments with ERK and JNK inhibitors. MMP9 activity in the JNK inhibitor-treated PTPTM knockdown cells was reduced to a level similar to the corresponding control cells. This implies that

activation of JNK pathway resulting from PTPRM knockdown has contributed to the increased MMP9 activity. Surprisingly, in the ERK inhibitor-treated PTPRM knockdown cells, MMP9 activity was reduced to a level lower than the control cells and that this appeared to be much less affected by the small inhibitors. This may be due to the dual actions by the ERK pathway on the MMP9, *i.e.* activation and transcriptional regulation. It also suggests that the activity of MMP9 tends to be more dependent on the MAPK pathway in the PTPRM knockdown cells. Additionally, the potency and unknown effect of the small inhibitors used in the current study may also be a reason for such a difference. However, the exact mechanism and other unknown interacting molecules involved in this effect require further investigation.

In summary, results from this study suggest lower expression levels of PTPRM to be a characteristic of breast cancer. Lower expression levels of PTPRM are correlated with poor prognosis and reduced disease free survival. Moreover, knockdown of PTPRM resulted in elevated adhesion, invasion and proliferation of breast cancer cells. Activation of ERK and JNK by tyrosine phosphorylation and consequent elevated MMP9 activity is involved in increased cell migration and invasion by PTPRM knockdown.

Chapter 8

General discussion

Tyrosine phosphorylation is essential and dispensable for the signal transductions of many pathways involved in the tumourigenesis and metastasis. For example, tyrosine phosphorylation is important for EGFR, cMet and MAPK pathways to fulfil their regulation on cell proliferation, apoptosis, migration and morphology. EGFR is one of the most common targets of cancer therapy due to the involvement of EGFR in tumour growth and metastasis. The PI3K/ Akt pathway, downstream of EGFR, plays an important role in cell growth, apoptosis, and cell motility (Vivanco and Sawyers 2002). It has also been reported that PTEN (phosphatase with tensin homology), a well-known tumour suppressor and a phosphatase, negatively regulates the PI3K/ Akt pathway (Stambolic *et al.,* 1998). It indicates that protein tyrosine phosphatases also play crucial roles cancer.

8.1 PTPRK, PTPRM, and cancer

PTPRK has been reported that PTPRK participates in the regulation of cell functions, mainly via EGFR and TGF-β pathways. These two pathways are involved in the regulation of most biological functions, for example, the EGFR pathway regulates cell proliferation via downstream PI3K/ Akt pathway and cell motility by FAK and also affects the MAPK pathway. PTPRM and PTPRK belong to the same subfamily and it

has been reported that PTPRM regulates cell migration by dephosphorylation of PLCγ1, consequently suppressing the cytoskeletal changes of migration (Rosdahl *et al.,* 2002; Ensslen and Brady-Kalnay 2004; Phillips-Mason *et al.,* 2008; Phillips-Mason *et al.,* 2011).

Recent studies have shown that PTPRK and PTPRM both play important roles in cancer development (Nakamura *et al.,* 2003; Gyorffy *et al.,* 2008). The molecular mechanisms involved in the activation of PTPRK and PTPRM in prostate and breast cancer remain largely unknown. There is very little information in the literature concerning the specific involvement of PTPRK and PTPRM in cancer. This project aimed to provide more information about the function of PTPRK, PTPRM and the mechanism by which it exerts its effects on cancer cells.

8.2 The role of PTPRK in prostate cancer

Human PTPRK is widely expressed in different organs such as spleen, prostate and ovary and the expression of some PTPs is increased in prostate cancer patients such as PAcP (human prostatic acid phosphatase, a neutral PTP) (Yang *et al.*, 1997; Veeramani *et al.,* 2005). The IHC staining of PTPRK in tumour tissues appeared to be more

intensive compared to the normal prostate tissues. However, such difference was not statistically significant, when determined semi-quantitively. This may be due to the limited number of specimens available at the time of the study. The staining of PTPRK was seen mainly at the cell nuclei. Although PTPRK is a transmembrane protein phosphatase, there is a report that the post-translational modification of PTPRK is through cleavage of its extracellular domain (N-terminal) and RPTPs, PTPRM and LAR have also been shown to undergo post-translational modification (Gebbink *et al.,* 1991; Streuli *et al.,* 1992; Jiang *et al.,* 1993). The anti-PTPRK antibody we used was raised against amino acids 27-101, mapping within an N-terminal extracellular domain of PTPRK of human origin. Due to most of the PTPRK ICC staining being located at the cell nuclei, it indicated that after post-translational modification of PTPRK, the extracellular domain may be a molecule which participated in regulation of down-stream signalling pathways. However, the location of PTPRK should be further examined using IF staining with confocal microscopy analysis. The mechanism of this phenomenon warrants further investigation.

PTPRK knockdown inhibited the growth and adhesion of both PC-3 and DU-145 cells. Moreover, knockdown of PTPRK resulted in a significant promotion of cell motility in

PC-3 cells, but not in DU-145 cells. In cell invasion assays, knockdown of PTPRK in PC-3 and DU-145 cells showed a completely different cell response. In PC-3 cells, knockdown of PTPRK reduced the invasiveness compared with control cells, while PTPRK knockdown in DU-145 cells promoted the invasive ability. The exact reason for the differential effect of PTPRK on the cell migration and invasion is currently unknown, but is likely to be due to differences in the expression patterns of other proteins involved in PTPRK function. Recent studies shows that JNK and ERK play important roles in regulation of metastasis, JNK and ERK activation is associated with promotion of MMPs activities in prostate cancer cells (Sekine *et al.*, 2010; Chen *et al.*, 2011). In the current study, the phosphorylation of JNK was increased in PC-3 PTPRK knockdown cells however there was no effects on ERK phosphorylation. The mirgation mechanisms between PC-3 and DU-145 cells needs to be further investigated, with particular focus on the interacting molecules and pathways of PTPRK.

Cell growth is important for cancer development. In order to elucidate the mechanism(s) by which PTPRK can inhibit the growth of prostate cancer cells, the apoptotic population was analysed using a flow cytometric assay. PC-3^{PTPRKkd} cells showed a significant promotion in the percentage of apoptotic cells under serum starvation

compared to the control PC-3^{pEF} cells, suggesting that PTPRK may act as an anti-apoptotic factor protecting PC-3 cells from undergoing apoptosis. Most PTPs play promoting roles in apoptosis. PTP1B has been reported that to be associated with MAPKs activation. PTP1B activates JNK and p38 pathways via IRE1 (inositol-requiring kinase 1) signalling (Gu *et al.,* 2004). However, the osteoclastic PTP (PTP-oc) has been reported to promote c-Src-mediated activation of NFκB and JNK leading to a protection of the cells from apoptosis (Amoui *et al.,* 2007). In this study, PTPRK knockdown in PC-3 cells resulted in promotion of apoptosis. Moreover, the tyrosine phosphorylation of JNK was increased in PC-3 knockdown cells and the increased apoptosis in PC-3^{PTPRKkd} cells was diminished through treatment with a JNK inhibitor (SP600125). This suggests that PTPRK suppresses the apoptosis in prostate cancer cells by deactivating the JNK pathway.

In general, apoptosis is associated with the activation of the caspase cascade (Halle *et al.,* 2007). PC-3^{PTPRKkd} cells showed significantly higher expression levels of caspase-3 compared to the empty plasmid control. Additionally, the upstream caspase-8, of the extrinsic cascade, was also increased in the cells. No change was seen in the expression of caspase-9. It has been demonstrated that caspase-8 is normally activated by extrinsic

receptor-mediated pathway and caspase-9 is activated by intrinsic mitochondrial-mediated pathway (Lee *et al.,* 2011). The present study showed that the increased apoptosis in PC-3^{PTPRKkd} cells was more related with extrinsic signalling rather than mitochondrial apoptosis signalling. At the same time, we also investigated other apoptosis-related molecules including p53, ID1 and c-Myc. There are reports in the literature showing that expression of p53 is associated with the induction of apoptosis in different cancer cells (Pietsch *et al.,* 2008). Silencing of p53 was able to suppress cadmium-induced apoptosis in prostate cells. Additionally, the activation of JNK can also up-regulate p53 expression leading to the accumulation of Bax which induces apoptosis of HeLa cell (Cao *et al.,* 2010; Aimola *et al.,* 2012). In contrast, ID1 expression increases NF-κB expression, which is associated with the anti-apoptotic pathway (Ling *et al.,* 2003; Peng *et al.,* 2012). In PTPRK knockdown cells, the expression of p53 was increased whilst the expression of ID1 was reduced. These results indicate a complex network affected by PTPRK which participate in the coordination of apoptosis.

In summary, activation of JNK by tyrosine phosphorylation and consequently increased p53, caspase-8/caspase-3 expression, and reduced ID1 expression are involved in the

differential rates of apoptosis of prostate cancer cells following PTPRK knockdown (Figure 8.1).

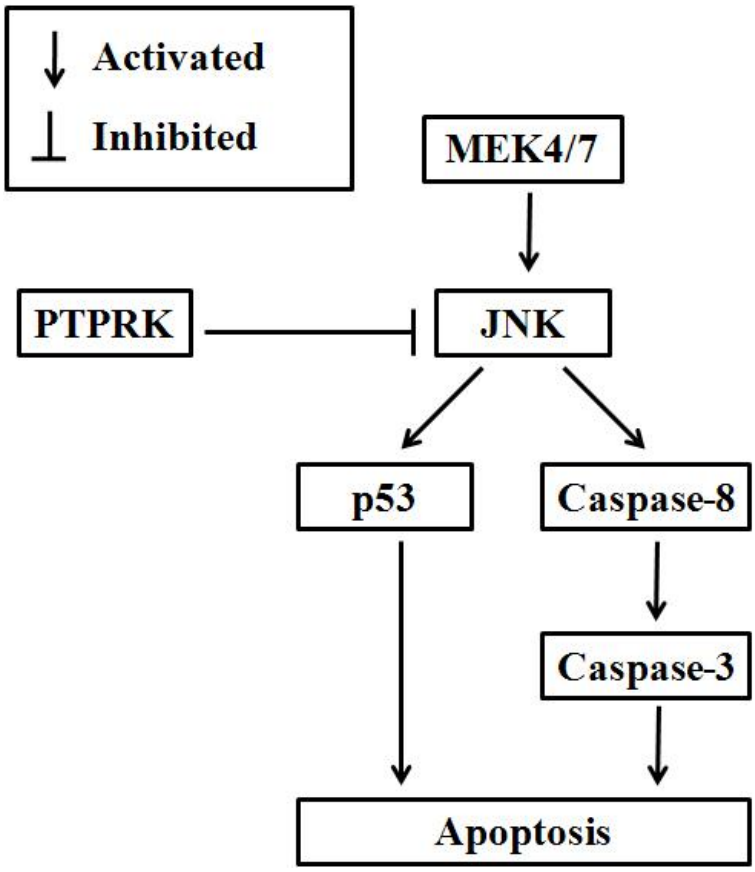


Figure 8.1 Potential interacting pathways and molecules involved in the functions of PTPRK in prostate cancer cells.

8.3 The role of PTPRK in breast cancer

Previous studies have already demonstrated that breast cancer is associated with different PTPs, for example PTPγ, LAR, PTPα, PTP1B and DEP-1. However, the expression of PTPs exhibits certain variations in different malignancies. For example,

PTPγ expression is reduced in breast cancer tissues and is associated with higher ERα and lower ERβ expression (Zheng *et al.,* 2000; Liu *et al.,* 2002), whilst the expression of LAR is increased in the breast cancer (Yang *et al.,* 1999). According to the current study, PTPRK expression appears to be increased in breast cancer which is yet to be validated larger cohort of breast cancer tissues. The clinical findings have been supported by the *in vitro* results in the present study. PTPRK knockdown promoted most cell functions of breast cancer cells including cell growth, adhesion, invasion and migration.

However and interestingly, *in vitro* data showed almost completely opposite effects on the cellular functions of breast cancer cells compared with the prostate cancer cells tested. The sharp contrasts for the functions of PTPRK in the two tumour types are intriguing. There are previous studies which have indicated that ERK1/2 is the most relevant MAPK to breast cancer (Santen *et al.,* 2002) and JNK is more relevant to prostate cancer (Kumar *et al.,* 2010; Hubner *et al.,* 2012; Sielicka-Dudzin *et al.,* 2012). Thus, different downstream and upstream pathways to PTPRK in different tumour types and indeed in different cell lines of the same tumour type, may respond to PTPRK in a different fashsion, as seen in the present study. This can at least partially explain the

different effects of PTPRK knockdown seen in the breast cancer cells and prostate cancer cells. However, whether and how the ERK pathway is involved in the effect of PTPRK knockdown in breast cancer cells would be an exciting study to pursue in future.

PTPRA expression is also increased in the poorly differentiated breast cancer and ER positive tumours. Overexpression of PTPRA in MCF-7 cells resulted in increased *Src* activity and an inhibition of cell growth (Ponniah *et al.,* 1999; Ardini *et al.,* 2000). The current study showed that patients with advanced breast cancer and poor prognosis, especially patients with metastases and who died from breast cancer, had low levels of PTPRK expression. Patients who had lower expression of PTPRK had shorter overall survival and disease-free survival compared with those with higher expression. Certain PTPs have been shown to be differentially expressed in breast tumours with links to oestrogen receptor status (LaForgia *et al.,* 1991; Zheng *et al.,* 2000; Liu *et al.,* 2002). PTPRK expression was slightly higher in ERβ positive tumours than the ERβ negative tumours though this was not found to be statistically significant. Similarly, there was no correlation between PTPRK and ERα status seen in the current cohort of breast cancer.

Above all, lower expression levels of PTPRK were correlated with poor prognosis, reduced overall survival and disease free survival. Moreover, knockdown of PTPRK resulted in increasing adhesive and invasive abilities and promoted cell proliferation and motility of breast cancer cells. Together, this data suggests that PTPRK may be a negative regulator in breast cancer progression.

8.4 The role of PTPRM in breast cancer

PTPs expression is seen to vary in cancers, for example, the expressions of certain PTPs are increased in breast cancer but PTPRG expression is decreased (Hiscox and Jiang 1998; Zheng *et al.*, 2000). In the current study, we demonstrated a significant reduction of PTPRM transcripts in breast cancer. The results showed that patients with higher tumour grade had relatively lower levels of PTPRM expression. Furthermore, patients with lower expression of PTPRM had shorter disease-free survival compared with those with higher expression.

PTPRM Knockdown in both MDA-MB-231 and MCF-7 cell lines led to increased *in vitro* cell proliferation, adhesion and invasion. Currently, PTPRM has been identified to interact with molecules such as PKC, PLCγ and EGFR. Firstly, PTPRM, RACK1 and

PKCδ exist in a complex form in cultured retinal cells and retinal tissue (Rosdahl *et al.,* 2002) and secondly PLCγ1 has been reported as a target of PTPRM and activation of PLCγ1 is associated with increased invasion of cancer cells. It has been reported that PTPRM reduces glioma cell migration via inactivation of PLCγ1 (Phillips-Mason *et al.,*). Thirdly, EGFR is dephosphorylated by PTPRM at pY992, the docking site for PLCγ1 activation. If this tyrosine is dephosphorylated by PTPRM, the activation of PLCγ1 is reduced and this may finally lead to a decrease in cell migration (Hyun *et al.,* 2011; Phillips-Mason *et al.,* 2011). However, in the current study, PTPRM knockdown exhibited little effect on the activation of PLCγ1 and the promoted cell migration was also not affected by the PLCγ1 inhibitor. Apart from the PLCγ1 pathway, ERK and JNK pathways have also been demonstrated to play a role in the regulation of the cell functions which can be coordinated by PTPs. For example, PTP1B and TC-PTP have been reported to regulate the migration of breast cancer cells (MCF-7) via ERK pathway (Blanquart *et al.,* 2010). In the current study, the tyrosine phosphorylation of ERK and JNK were increased in the PTPRM knockdown cells but not the PLCγ1. We further treated PTPRM knockdown cells with ERK and JNK inhibitors and found the promotion of cell migration was diminished by inhibition of these pathways. Taken

together, the data suggests that PTPRM can regulate the migration of breast cancer cells via ERK and JNK rather than PLCγ1.

MMPs are zinc dependent proteolytic enzymes that can cleave their target ECM and non-matrix substrates such as growth factors. MMPs are crucial for tumour metastasis due to their ECM degrading activity. It has been indicated that MMP9 activity is related to the ERK pathway. This has been implicated in a study where TNF (tumour necrosis factor) was found to stimulate proMMP9 production in human chorionic trophoblast cells through ERK1/2 pathway but not JNK and p38 pathway (Li *et al.,* 2010). miR-520c and miR-373 also up-regulate MMP9 expression by activation of Ras/Raf/MEK/ERK in human fibrosarcoma cells (Liu and Wilson 2012). The ERK pathway regulates proliferation, invasion, and MMP9 expression in colon cancer cells (CT-26) and a similar effect has also been seen with the JNK pathway which regulates the expression of both MMP2 and MMP9 (Son *et al.,* 2010). In this study, we found the MMP9 activity wass increased in PTPRM knockdown cells and this increase was diminished by treating cells with ERK and JNK inhibitors. Treating cell with JNK and ERK individually both resulted in decreased MMP9 activity. MMP9 activity in PTPRM knockdown cells was reduced to a similar level of the control cells when treated by the

JNK inhibitor. However, the MMP9 activity in the PTPRM knockdown cells was reduced to a lower level compared with the control. Together, it indicates that MMP9 activity is regulated by both JNK and ERK pathways in PTPRM knockdown cells but may be more dependent on ERK pathway. This may be due to the dual actions of the ERK pathway on MMP9, i.e. activation and transcriptional regulation. However, the exact mechanism and other unknown interacting molecules involved in this effect require further investigation.

In summary, results from this study suggest lower expression levels of PTPRM to be a characteristic of breast cancer. Lower expression levels of PTPRM are correlated with poor prognosis and reduced disease free survival. Moreover, knockdown of PTPRM resulted in elevated adhesion, invasion, and proliferation of breast cancer cells. Activation of ERK and JNK by tyrosine phosphorylation and consequent elevated MMP9 activity is involved in increased cell migration and invasion by PTPRM knockdown (Figure 8.2).

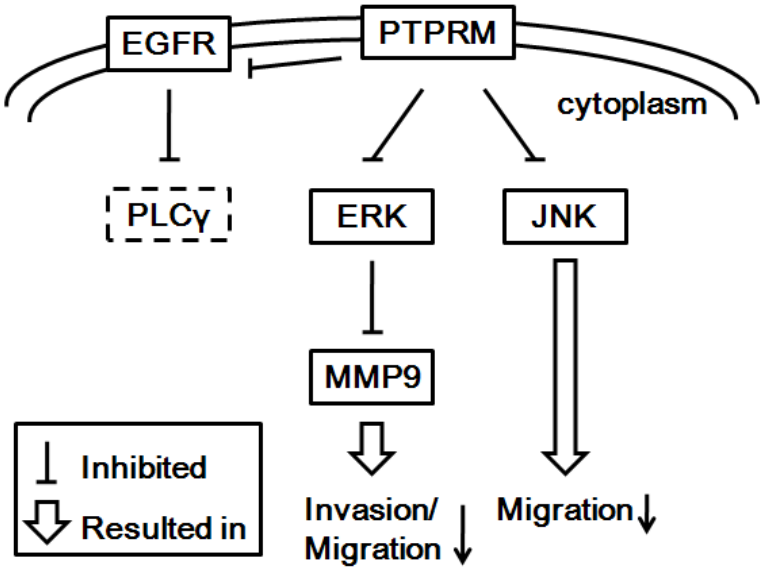


Figure 8.2 Potential interacting pathways and molecules involved in the functions of PTPRM in breast cancer cells.

8.5 Future work

Further investigation into the relationship between PTPRK and prostate cancer is essential. The findings from this study have added further weight to the theory that PTPRK is a potential oncogene/proto-oncogene in prostate cancer but the precise mechanism of action is yet to be fully understood. First of all, from the results of this study and previous studies, the interplay between PTPRK and EGFR and TGF-β should be investigated. The present study on PTPRK in prostate cancer only used a small number of clinical samples, making it difficult to draw a solid clinical conclusion. This was limited by the number of quality samples available to this study. A large cohort

study supported by full clinical, pathological and followup information will be highly desirable.

The different impacts on *in vitro* cell functions between prostate and breast cancer should be further investigated. ERs, ARs, and MAPKs play an important role in breast and prostate cancer development. In the current study, we already demonstrated that tyrosine phosphorylation of JNK was increased in prostate cancer cells and it has been reported JNK activation is more relevant to prostate cancer. Further investigation of MAPK and the crosstalk with hormones such as oestrogen and androgen and their receptors may shed further light on the differential responses to PTPRK and PTPRM seen in the breast and prostate cancer cells.

The relationship between PTPRM and breast cancer is important as well. The findings from this study indicate that PTPRM is a potential tumour suppressor in breast cancer and its mechanism of action must be fully investigated. Previous studies indicated that PLCγ1, a proposed substrate of PTPRM, may be the key molecule to regulate cell migration in cancers. In this study, however, ERK and JNK play crucial roles in the regulation of cell migration in breast cancer cells. Further investigations focusing on the

ERK and JNK pathways in other cell functions and in relationship to the PTP family will be very valuable.

Chapter 9

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