CHAPTER 1 – GENERAL INTRODUCTION

The loss of cell growth regulation is essential in the initiation and progression of cellular events leading to cancer development, with many of the regulatory circuits that govern normal cell proliferation and homeostasis being defective in cancer cells (Hanahan and Weinberg, 2000; Tilghman and Parsons, 2008). Focal adhesion kinase (FAK) is of particular interest to malignancy as several lines of evidence indicate that increased expression of this protein is integrally involved in mediating many of the cellular alterations required for malignant growth (Tilghman and Parsons, 2008). Integrins and growth factor receptors promote FAK-mediated stimulation of the phosphatidylinositol 3'-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways thereby influencing cell cycle progression, proliferation, differentiation, survival, and apoptosis (Fornaro *et al.*, 2000; Schaller, 2001; Craven *et al.*, 2003). Uncontrolled cell migration, a key process in malignancy, is primarily regulated by FAK which affects focal adhesion turnover at the leading edge of migrating cells (Carragher and Frame, 2004; Tilghman *et al.*, 2005). Studies in several tumour types have emphasized the relationship between FAK expression and cell movement; however, this relationship has yet to be fully elucidated in human oesophageal squamous cell carcinoma (HOSCC), a highly prevalent cancer of poor prognosis in black male South Africans (Owens *et al.*, 1995; Miyazaki *et al.*, 2003; McCabe and Dlamini, 2005). The study presented here highlights some of the cell movement-associated roles that FAK may facilitate in the progression of HOSCC. However, in order to understand and interpret the results of this study a general understanding of tumourigenesis is required.

1.1 Cancer cells are self-sufficient in producing growth signals and in evading apoptosis

Tumourigenesis is a multi-step process in which genetic mutations sequentially steer the transformation of normal human cells into highly malignant ones, with the genomes of tumour cells being altered at multiple sites (Hanahan and Weinberg, 2000). These genetic alterations usually confer some type of growth advantage to

cancer cells which have flaws in regulatory pathways that control the balance between cell proliferation and apoptosis (programmed cell death) (Hanahan and Weinberg, 2000; Sears and Nevins, 2002). In order to move from a quiescent (inactive) to a proliferative state, mammalian cells require extracellular signals in order to activate their transmembrane growth factor receptors (Hanahan and Weinberg, 2000). Tumour cells often generate their own growth signals or factors and/or overexpress growth factor receptors, thus allowing cancer cells to become hyper-responsive to growth factor levels and resulting in uncontrolled proliferation (Hanahan and Weinberg, 2000). Tumour suppressor genes which normally inhibit proliferation and/or stimulate apoptosis often acquire loss-of-function mutations, while gene amplification and overexpression frequently activate oncogenes, leading to continuous proliferation and acquired resistance to apoptosis in tumour cells (Cryns and Yuan, 1998; Hanahan and Weinberg, 2000; Koppert *et al.*, 2005; McCabe and Dlamini, 2005). This acquired resistance often results from mutations in the pro-apoptotic p53 gene which occur in approximately 50% of all human tumours (Hanahan and Weinberg, 2000; Sears and Nevins, 2002; Lehrbach *et al.*, 2003). The p53 pathway is also disrupted by overexpression of murine double minute 2 (MDM2) which targets p53 for ubiquitination (Mayo *et al.*, 2002a; Lehrbach *et al.*, 2003). In addition to the tumour suppressor p53, most anti-proliferative signals within the cell are channelled through the retinoblastoma protein (pRb) which prevents the transcription of genes required for G1/S cell cycle progression. However, disruption of the pRb pathway promotes cell proliferation and renders cells insensitive to anti-growth signals (Hanahan and Weinberg, 2000). It is these aberrations, acquired by most cancers, which co-operatively establish malignant growth (Hanahan and Weinberg, 2000).

At some stage during the development of most human cancers, cells detach from the primary tumour cell mass, invade the surrounding tissue, and travel to distant sites where they form secondary tumours. It is these secondary tumours or metastases that are reported to be the major cause of human cancer deaths (Schwartz, 1997). Invading and metastasizing cells have the same requirements as cells remaining within the primary tumour, except that additional cellular changes are also needed in

order for them to establish themselves in their new microenvironment (Hanahan and Weinberg, 2000). Several classes of proteins involved in linking cells to the surrounding tissue are often altered in metastatic cells, and the impact of changing cell adhesion dynamics is of particular interest in this study (Hanahan and Weinberg, 2000). Therefore it is necessary to understand how cell adhesion molecules function, and of what importance they are to cellular movement and hence metastasis.

1.2 Cell adhesion is essential for the formation and functioning of multicellular organisms

In order for a multicellular organism to form, cells specifically attach to one another as they are organized into tissues which in turn associate with one another to form organs. Animal cells are joined to one another by a relatively loose lattice of organic molecules termed the extracellular matrix (ECM), and by direct cell-to-cell adhesions formed between their adjacent plasma membranes (Alberts *et al.*, 1994). Cell-cell and cell-ECM interactions are facilitated by plasma membrane-bound adhesion receptors which function in cellular communication (Rosales *et al.*, 1995). The four major families of cell adhesion molecules (CAMs) or receptors include the cadherins, the integrins, the immunoglobulin superfamily of cell adhesion molecules (IgCAMs), and the selectins (Rosales *et al.*, 1995). By regulating cell-cell and cell-ECM interactions, CAMs often act in concert with one another in order to control cell growth, motility, migration, signalling, proliferation, differentiation, survival, apoptosis, gene transcription, and immune response (Rosales *et al.*, 1995; Wu and Dedhar, 2001; Defilippi and Vallés, 2002; Nair *et al.*, 2005). As cadherins and integrins are central to epithelial cell functioning, they will be the focus of greater discussion (Defilippi and Vallés, 2002).

1.3 Cadherins mediate interactions between adjacent cells

Cadherins are the transmembrane calcium-dependent adhesion receptors involved in tissue organization as they mediate homotypic cell-to-cell adhesions (Alberts *et al.*, 1994; George and Dwivedi, 2004; Nelson *et al.*, 2004). These CAMs localize to

adherens junctions where they form linkages with the actin cytoskeleton via the alpha- and beta-catenin (α- and β-catenin) proteins (von Schlippe *et al*., 2000; Defilippi and Vallés, 2002). Cadherins also play a significant role in cell recognition, as the set of cadherins expressed within a particular cell is often characteristic of that cell type; for instance, epithelial-cadherin (E-cadherin) is usually concentrated in adhesion belts where it links the actin cytoskeletons of adjacent epithelial cells (Alberts *et al.*, 1994; Rosales *et al.*, 1995; Nair *et al.*, 2005).

E-cadherin regulates adhesion as well as the cellular location of β-catenin and is thus implicated in cancer progression (George and Dwivedi, 2004). Cellular levels of β-catenin are regulated by its association with a degradation complex consisting of adenomatous polyposis coli (APC), axin, and glycogen synthase kinase 3β (GSK3β) (Nair *et al.*, 2005). This degradation complex competes with E-cadherin for β-catenin binding, and phosphorylates and targets β-catenin for ubiquitination (Shtutman *et al.*, 1999). When GSK3β is phosphorylated upon Wnt signalling, the formation of the APC/axin/GSK3β degradation complex is disrupted (Kishida *et al.*, 2001). Mutations in the molecules that regulate β-catenin turnover or in β-catenin itself (as found in colorectal cancer and adenocarcinomas) compromise its degradation by increasing the cytoplasmic pool of β-catenin which then translocates to the nucleus (Ilyas *et al.*, 1997; Shtutman *et al.*, 1999; Nair *et al.*, 2005). In the nucleus, β-catenin complexes with the lymphoid enhancing factor/T-cell factor (Lef/Tcf) transcription factor complex, subsequently enhancing the expression of specific genes involved in cell cycle progression (such as cyclin D1 and *c-myc*) (Shtutman *et al.*, 1999; Kishida *et al.*, 2001). By playing both a structural and a regulatory role, β-catenin stimulates changes in cell adhesion and junction formation in order to control transmembrane signalling and gene expression (Shtutman *et al.*, 1999).

1.4 Within focal adhesions, integrins mediate interactions between the cell and the extracellular environment

Integrins, the second major family of CAMs, are transmembrane glycoproteins that act as receptors for ECM components such as fibronectin, collagen, and laminin

amongst others (Rosales *et al.*, 1995; Carloni *et al.*, 2001). They are heterodimeric proteins each consisting of an α and a β subunit, with subunit composed of a large extracellular domain, a transmembrane region, and a short cytoplasmic domain (Brakebusch *et al.*, 2002; Qin *et al.*, 2004). Integrin-mediated cell adhesion to the ECM consists of three steps, namely cell attachment, cell spreading, and the formation of focal adhesions (Greenwood and Murphy-Ullrich, 1998).

Focal contacts (immature adhesion complexes) and adhesions (mature adhesion complexes) are the integrin-mediated cell-ECM adhesion sites formed where integrins are able to facilitate cell adhesion links between the extracellular environment and the actin cytoskeleton (Troussard *et al.*, 1999; Nelson *et al.*, 2004; Cox *et al.*, 2006; Gimona and Buccione, 2006). The presence of focal contacts or adhesions is a trademark of migrating cells, and cell adhesion and actin dynamics are simultaneously controlled here as they are both linked to cell movement (Xie *et al.*, 1998; Carragher and Frame, 2004; McLean *et al.*, 2005). The ECM composition governs the recruitment of specific integrins into focal adhesions, suggesting that the conformational changes associated with ligand binding help to regulate integrin-cytoplasmic linkages (Troussard *et al.*, 1999; Brakebusch and Fässler, 2003). Upon ECM ligand binding to the extracellular domain of the integrin, a conformational change is induced within the cytoplasmic domain that extends to its extracellular domain, transforming the integrin from a low to a high-affinity ligand binding state (Qin *et al.*, 2004). High-affinity ligand binding is important for firm attachment of the cell to the ECM. As the cytoplasmic tail does not have detectable enzymatic activity, high-affinity ligand binding and integrin-mediated signalling requires the direct binding of many adaptor molecules and signalling proteins to their cytoplasmic tail (Liu *et al.*, 2000; Brakebusch *et al.*, 2002; Carragher and Frame, 2004; Nelson *et al.*, 2004; Wozniak *et al.*, 2004).

During the adhesion process integrins, as well as cytoplasmic scaffolding proteins, kinases, phosphatases, proteases, and guanine triphosphatases (GTPases), are drafted to the cell-ECM focal contact sites where they link the actin cytoskeleton to the ECM and mediate signal transduction between the cell and its environment (Carragher and

Frame, 2004; Qin *et al.*, 2004; Wozniak *et al.*, 2004). Integrin-mediated interactions are vital to the maintenance of cellular functioning because of their ability to mediate bi-directional signalling, thereby essentially regulating cell attachment, motility, polarity, cell cycle progression, survival, and apoptosis (Ruoslahti and Őbrink, 1996; Liu *et al.*, 2000; Wu and Dedhar, 2001; Brakebusch *et al.*, 2002; Qin *et al.*, 2004; Wozniak *et al.*, 2004; Nair *et al.*, 2005). Extracellular to intracellular (outside-in) signalling initiates the transmission of extracellular stimuli via signal transduction pathways in order to regulate gene expression and stimulate appropriate cellular functions (Liu *et al.*, 2000). Intracellular to extracellular (inside-out) signalling takes place when gene products stimulate signal transduction pathways involved in the regulation of cell adhesion, communication between cells, and cell movement (Liu *et al.*, 2000). Thus, focal adhesion sites act as biochemical signalling centres for many regulatory pathways, and the dynamic regulation of focal adhesion assembly and disassembly is an important determinant of the rate of cell movement (Chen *et al.*, 2003; Carragher and Frame, 2004).

1.5 Cell migration is a prominent feature of malignancy

Cell migration is essential for many cellular processes that depend on the regulated movement of cells during embryonic development, wound-healing, inflammation, tissue maintenance and repair, and generally depends on the dynamic cycle of cell-ECM adhesion and remodelling of the actin cytoskeleton (Carragher and Frame, 2004; Franco and Huttenlocher, 2005; Tilghman *et al.*, 2005). Epithelial cells usually move as a single sheet in which each cell keeps in contact with adjacent cells as well as with the ECM. The structure of the epithelial cell monolayer is maintained by the integrity of the actin cytoskeleton, the reorganization of which is essential for regulating cell-ECM interactions, cell shape and motility (Guvakova and Surmacz, 1999). In polarized epithelial cells, focal adhesion proteins are localized to actin filaments, linking them to cell adhesions. When deprived of adhesion signals, epithelial cells undergo a type of suspension-induced apoptosis termed anoikis (Craven *et al.*, 2003). Unlike normal cells, carcinoma cells are able to overcome this apoptotic signal and move individually as a loss of epithelial polarity during the early

stages of malignancy promotes cell motility (Guvakova and Surmacz, 1999; Craven *et al.*, 2003).

The protrusion of the cell membrane in a single direction, usually in response to actin polymerization, is referred to as the leading edge of a cell (Franco and Huttenlocher, 2005; Tilghman *et al.*, 2005). As integrins within the leading edge come into contact with the ECM, small focal contacts are formed. The cell is then able to exert pressure on these focal contacts through contraction of the actin cytoskeleton and hence cell movement is promoted (Brakebusch *et al.*, 2002; Tilghman *et al.*, 2005). The newly formed focal contacts "turn over", while more mature focal adhesions accumulate towards the rear of the cell. As the cell moves forwards, these focal adhesions are disassembled as the trailing edge is retracted (Xie *et al.*, 1998; Tilghman *et al.*, 2005; van Nimwegen and van de Water, 2007). Although focal contacts at the leading edge are important in providing traction for migrating cells, the number of focal contacts is usually inversely proportional to cell migration speed (Chan *et al.*, 1994; Green *et al.*, 1998).

Upon ligand binding, integrins activate signalling molecules, which in turn affect the formation, turnover, and linkage of actin filaments through the stimulation of the Ras homologue-guanine triphosphatase (Rho-GTPase) family (Brakebusch and Fässler, 2003; Faried *et al.*, 2006). RhoA mediates decreased adhesion by the α4β1 and β2 integrins and stress fibre formation, Rac1 facilitates the formation of lamellipodia, and Cdc42 regulates filopodia formation (Brakebusch and Fässler, 2003). In this way, the Rho-GTPases regulate cell movement and focal adhesion turnover and are involved in the pathological processes of transformation and metastasis (Chrzanowska-Wodnicka and Burridge, 1996; Wozniak *et al.*, 2004; Faried *et al.*, 2006).

Many of the cytoplasmic focal adhesion components responsible for cell migration are either tyrosine kinases or their associated substrates (Carragher and Frame, 2004). Studies have demonstrated that tyrosine phosphorylation plays a key role in focal adhesion dynamics, and that tyrosine phosphorylation within focal adhesions

generally occurs in a hierarchical manner following the initial recruitment of FAK, vinculin, and paxillin (Carragher and Frame, 2004). Understanding the role of FAK in particular is crucial to understanding focal adhesion dynamics and cell migration, and it is this focal adhesion-associated molecule that will be the focus of further discussion.

1.6 Focal adhesion kinase mediates integrin-dependent signalling upon focal adhesion formation

Focal adhesion kinase (FAK) is one of the most highly tyrosine-phosphorylated proteins acting in response to integrin clustering in focal adhesions, and was initially identified in cells transformed by the Src oncogene (Kanner *et al.*, 1990; Schaller *et al.*, 1992; Nagoshi *et al.*, 2006). The *fak* gene, located on human chromosome 8q24, encodes a 125 kilodalton (kDa) cytoplasmic protein tyrosine kinase (PTK) that is significantly involved in integrin-stimulated signal transduction pathways (Zhao *et al.*, 1998; Hauck *et al.*, 2001; Gabarra-Niecko *et al.*, 2003). The *fak* gene is highly conserved among vertebrates and gene deletion early on in embryogenesis is lethal, while the FAK protein is ubiquitously expressed throughout development and is present in most adult tissues (Ilić *et al.*, 1997; Maung *et al.*, 1999; Corsi *et al.*, 2006; Hehlgans *et al.*, 2007).

Four main modular domains exist within FAK, as seen in Figure 1.1 (Gabarra-Niecko *et al.*, 2003). The amino-terminal (N-terminal) band four point one, ezrin, radixin, moesin (FERM) domain mediates interactions between FAK and the cytoplasmic domains of integrins and growth factor receptors (Gabarra-Niecko *et al.*, 2003; Cox *et al.*, 2006). The catalytic kinase domain of FAK is located between the N- and carboxy-terminal (C-terminal) domains (Figure 1.1). As the activation loop region of the adenine triphosphate (ATP)-bound FAK kinase domain is disordered, it is likely that conformational changes within this domain are needed for maximal activity (Schlaepfer and Mitra, 2004). The FERM domain plays an auto-inhibitory role as it interacts with and directly inhibits the kinase domain (Cox *et al.*, 2006). This auto-inhibition is alleviated either by post-translational modifications or by

interaction of the FERM domain with target proteins such as the β1 integrin cytoplasmic tail (Figure 1.2) (Wozniak *et al.*, 2004).

The non-catalytic C-terminal FAK-related non-kinase (FRNK) domain consists of two sub-sections, namely the proline-rich regions and the focal adhesion targeting (FAT) domain (Figure 1.1) (Cox *et al.*, 2006). The first proline rich region interacts with Src homology 3 (SH3) domain-containing proteins such as Crk-associated substrate (CAS), and the leucine aspartate repeat 2 (LD2) domain of paxillin, while the second proline rich region binds the SH3 domain of the Rho-GTPases (Parsons, 2003; Wozniak *et al.*, 2004; Cox *et al.*, 2006; van Nimwegen and van de Water, 2007). The FAT sequence, located at the far C-terminus, facilitates the focal adhesion localization of FAK through its association with paxillin, talin, tensin, and vincullin (Figures 1.1 and 1.2) (Zhao *et al.*, 1998; Crowe and Ohannessian, 2004). The FAT domain includes integrin binding sites, as well as sites for integrin-associated proteins such as integrin-linked kinase (ILK) and growth factor receptor binding protein 2 (Grb2) (Nagoshi *et al.*, 2006; Hehlgans *et al.*, 2007; van Nimwegen and van de Water 2007). The localization of FAK within focal adhesions is crucial to its signalling function, as FAK mutants that fail to localize here have impaired autophosphorylation and kinase activity (Gabarra-Niecko *et al.*, 2003; Wozniak *et al.*, 2004). The FRNK domain can be expressed independently of the entire FAK protein through alternative splicing of the FAK gene (Parsons, 2003; Cox *et al.*, 2006). Although FRNK does not exhibit any kinase activity, it acts an inhibitor of FAK as it competes with FAK for focal adhesion localization (Nagoshi *et al.*, 2006).

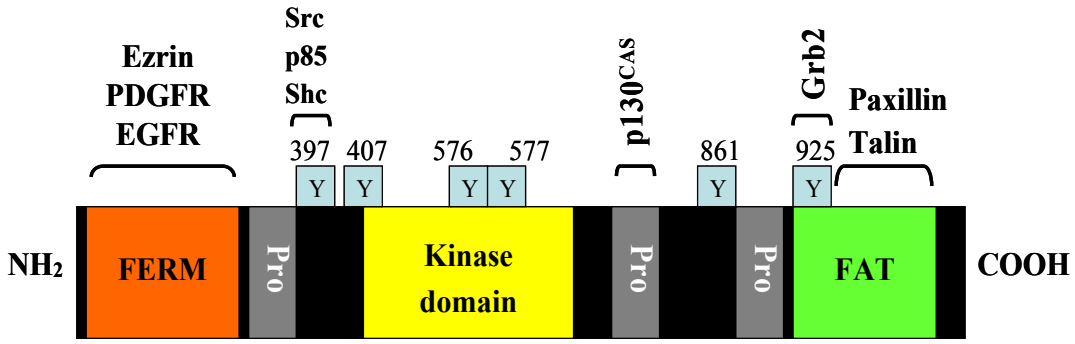

Figure 1.1: FAK structural features and binding partners. The N-terminus of FAK contains the FERM domain which interacts with growth factor receptors. The autophosphorylation site (Y397) of FAK is required for its kinase activity as it interacts with Src, the p85 subunit of PI3K, and Shc. The kinase domain contains Y576/Y577 for maximum kinase activity. The proline rich sequences mediate interactions with SH3 domain containing proteins such as CAS. The C-terminal FAT domain is required for focal adhesion localization, as it links with integrins via talin and paxillin (adapted from van Nimwegen and van de Water, 2007).

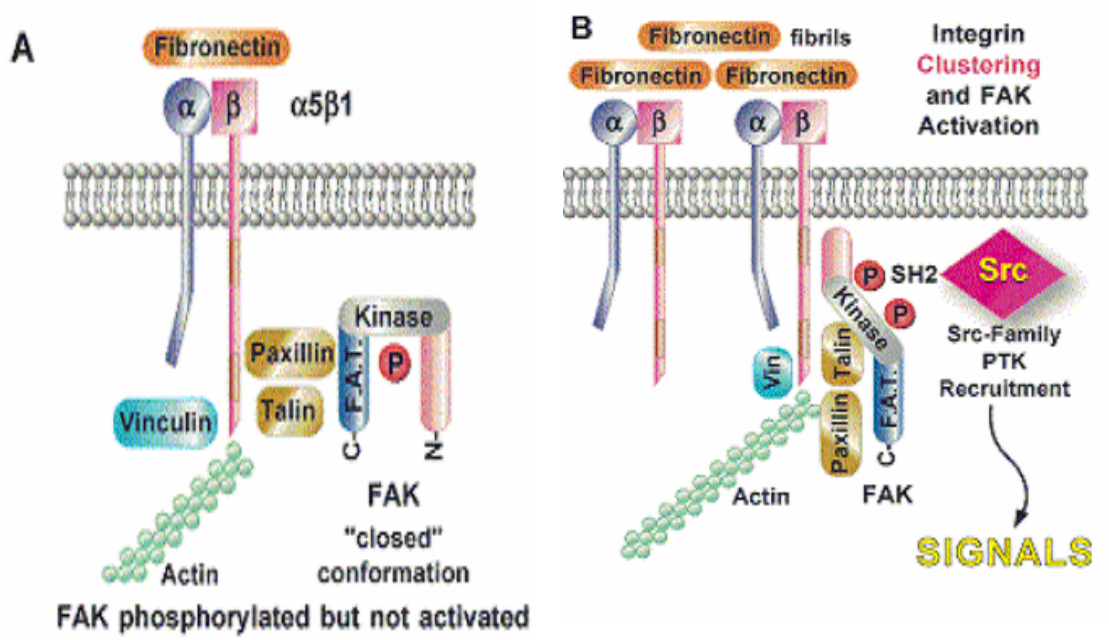

Figure 1.2: A model of β1-integrin-mediated FAK activation (figure taken from Schlaepfer *et al.*, 2004). A) In quiescent and adherent cells, FAK localizes to focal adhesions via interactions with adaptor proteins paxillin and talin. Conformational restraints within the N-terminal FERM domain inhibit FAK activity. B) These conformational restraints are alleviated when the FERM domain of FAK binds to the β1 integrin cytoplasmic tail during integrin clustering. The kinase domain of FAK is then further phosphorylated by Src. Downstream integrin-mediated signalling is then facilitated by FAK.

1.7 Cell adhesion influences the regulation of FAK activity

The way in which FAK contributes to cellular signalling is linked to the regulation of its tyrosine phosphorylation state, activity, and cellular localization, which are all influenced by cell adhesion (Cohen and Guan, 2005a). Cell adhesion-dependent tyrosine phosphorylation of FAK occurs in two stages and six predominant tyrosine phosphorylation sites have been identified, namely tyrosine residues 397, 407, 576, 577, 861, and 925 (Schaller, 2001; Hamadi *et al.*, 2005; McLean *et al.*, 2005). Autophosphorylation at tyrosine 397 (Y397) occurs first as FAK co-localizes with integrins and growth factor receptors at focal adhesions and becomes activated (Gabarra-Niecko *et al.*, 2003; Schlaepfer *et al.*, 2004; McLean *et al.*, 2005). Following autophosphorylation, FAK binds to Src via its SH2 domain (Figure 1.2) (Kanner *et al.*, 1990; Schaller *et al.*, 1992). This binding further promotes Src activation, thereby leading to further phosphorylation of FAK. Other phosphorylation sites in the activation loop (Y576/577) and in the FAT sequence (Y925) are subsequently targeted and phosphorylated by Src family kinases (SFKs) (Gabarra-Niecko *et al.*, 2003; Hamadi *et al.*, 2005). Phosphorylation of these sites is required for the full enzymatic activity of FAK (McLean *et al.*, 2005). Growth factor receptors with intrinsic kinase activity, such as epidermal growth factor (EGF) receptor (EGFR) and platelet-derived growth factor (PDGF) receptor (PDGFR), are also able to directly phosphorylate and activate FAK (van Nimwegen and van de Water, 2007).

In addition to being regulated by various kinases, FAK activation is also regulated by phosphatases (Cohen and Guan, 2005a). Site-specific dephosphorylation of FAK may be an effective means to regulate some aspects of FAK signalling independently of others (Schaller, 2001). Distinct tyrosine phosphatases may be responsible for dephosphorylation of different sites thereby leading to either an up- or down-regulation of FAK signalling (Schaller, 2001; Cohen and Guan, 2005a). For instance, the non-receptor protein tyrosine phosphatase (PTP) PTP-PEST dephosphorylates FAK and leads to increased cell motility, whereas the activation of PTPα and PTP1B leads to increased FAK activity (Cohen and Guan, 2005a). Other phosphatases, such

as phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and Src-homology-domain 2-containing tyrosine phosphatase-2 (SHIP-2), are involved in the downregulation of FAK activity (Carragher and Frame, 2004). FAK tyrosine dephosphorylation often results in the loss of FAK from focal adhesions and the inhibition of signal transduction pathways involving integrins (Fornaro *et al.*, 2000; Wozniak *et al.*, 2004).

Proteolytic cleavage is an equally effective method of inhibiting FAK signalling (Schaller, 2001). Different proteases are induced by stimuli that disrupt cell adhesions and/or promote apoptosis. These proteases cleave FAK, thereby generating similar sized proteolytic fragments (Cohen and Guan, 2005a). The major consequence of FAK cleavage is the separation of the catalytic kinase domain of FAK from the FAT sequence, resulting in the disruption of FAK-mediated signalling (Schaller, 2001). Cleavage fragments of FAK may also transmit aberrant signals that promote cell cycle arrest or induce apoptosis, but whether cleavage of FAK simply stops signalling processes or generates fragments that actively promote cell death remains to be established (Schaller, 2001).

Various members of the cysteine-dependent aspartate-directed protease (caspase) and calpain families have been implicated in the proteolytic cleavage of FAK (Schaller, 2001). Caspases cleave FAK into a large N-terminal fragment of 85–100 kDa, which is subsequently cleaved into smaller fragments (Schaller, 2001). Caspase cleavage-induced generation of a C-terminal fragment similar to FRNK may inhibit FAK function, as cleavage of FAK by caspase-3 coincides with the loss of FAK and paxillin from focal adhesion sites and a decrease in cell adhesion (Cohen and Guan, 2005a). In addition to cleavage by caspases, FAK is cleaved by the Ca^{2+}-dependent calpain protease family (Carragher *et al.*, 1999; Cohen and Guan, 2005a). Calpain is targeted to focal adhesions through its interaction with FAK and contributes to focal adhesion turnover through the cleavage of focal adhesion proteins such as FAK, Src, paxillin, and talin (Wozniak *et al.*, 2004).

In its active state, FAK associates with several kinases, signal transduction molecules, and cytoskeletal proteins, many of which have been implicated in cancer development (such as the oncogenes Src, Crk, and PI3K) (Tamura *et al.*, 1999; Gabarra-Niecko *et al.*, 2003). Although FAK is vital for the phosphorylation of some focal adhesion components, kinase-inactive FAK mutants still retain most of their function, suggesting that FAK also plays a role as a scaffolding protein (Wozniak *et al.*, 2004; Cohen and Guan, 2005a). As FAK acts as a platform for the assembly of various signalling cascades, biological processes such as cell spreading, proliferation, survival, and motility are regulated by FAK under normal conditions (Ilić *et al.*, 1997; Gabarra-Niecko *et al.*, 2003). However, as the development of malignancy involves aberrations in the above-mentioned processes, FAK expression and activity are almost certainly altered in cancer cells (McLean *et al.*, 2005).

1.8 Cell proliferation and survival is promoted by FAK signalling

In order for most cells to grow and survive they require adhesion to ECM components as well as growth factors. In an environment where ECM survival signals are absent, normal cells detach from the ECM and undergo anoikis (van Nimwegen and van de Water, 2007). Inhibition of FAK induces anoikis, whereas FAK overexpression ensures cell survival by preventing anoikis (Schaller, 2001; van Nimwegen and van de Water, 2007). As cancer cells are remarkably resistant to anoikis, FAK is possibly (at least partially) responsible for this resistance. The phosphatidylinositol 3'-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways are essentially involved in mediating FAK function and in ensuring cell proliferation and hence survival (Craven *et al.*, 2003). As FAK mediates cross-talk between integrins and growth factor receptors, many stimuli are involved in promoting FAK-mediated stimulation of these two pathways (Figure 1.1) (Zhao *et al.*, 1998; Schaller, 2001; Craven *et al.*, 2003; Velling *et al.*, 2004).

Phosphorylated tyrosine 397 (Y397) of FAK has been mapped as the major binding site for the SH2 domain of the regulatory subunit (p85) of PI3K (Figure 1.1) (Zhao *et al.*, 1998; Craven *et al.*, 2003; Velling *et al.*, 2004). The activation of PI3K results in

the formation of second messenger phosphoinositides, such as phosphatidylinositol-(3,4,5) triphosphate (PIP_3) (Mayo *et al.*, 2002b; van Nimwegen and van de Water, 2007). PIP_3 is involved in the activation of the anti-apoptotic target protein kinase B (PKB)/Akt which protects cells from apoptotic death by phosphorylating and inhibiting certain pro-apoptotic proteins such as caspase-9, Bcl-2, Bcl-2 associated death protein (Bad), and GSK3β (Datta *et al.*, 1997; Troussard *et al.*, 1999; Huang *et al.*, 2001; Mayo *et al.*, 2002b; van Nimwegen and van de Water, 2007). PKB enhances cell survival by preventing p53-dependent apoptosis and by activating certain transcription factors such as nuclear factor kappa B (NF-κB) (Mayo *et al.*, 2002b). In addition to the PI3K pathway, FAK is able to further affect cell survival by directly inhibiting the tumour suppressor p53. The N-terminal domain of FAK interacts with the N-terminal transactivation domain of p53, resulting in reduced transcriptional activity of p53, thereby preventing its pro-apoptotic signalling capabilities (Golubovskaya *et al.*, 2005; van Nimwegen and van de Water, 2007).

In addition to downregulating FAK activity, PTEN also dephosphorylates and deactivates PIP_3 (Tamura *et al.*, 1999; Schlaepfer *et al.*, 2004; van Nimwegen and van de Water, 2007). PTEN activity thus leads to a decrease in PKB phosphorylation, thereby promoting p53 activity and the commitment of cells to apoptosis (Mayo *et al.*, 2002a; Mayo *et al.*, 2002b; Leslie and Downes, 2004). PTEN activity effectively inhibits cell growth, invasion, migration, and the formation of focal adhesions; however, mutations occurring in PTEN are associated with a variety of cancer types (Tamura *et al.*, 1999; Schaller, 2001; Leslie and Downes, 2004).

While PI3K and PKB mainly regulate survival signalling, cell proliferation is primarily influenced by activation of the MAPK pathway (Craven *et al.*, 2003). FAK has been implicated in activating the MAPK pathway following stimulation by the urokinase-type plasminogen activator receptor (uPAR), PDGFR, as well as EGFR (Schaller, 2001; Normanno *et al.*, 2006). MAP kinases are often elevated in cancer cells and their activation is important in tumour progression (Craven *et al.*, 2003). FAK activates Ras and the MAPK pathway by binding to the adaptor proteins CAS or Src homology containing protein (Shc), which in turn recruit the Grb2/son of

sevenless (SOS) complex (Schaller, 2001; Angelucci and Bologna, 2007). Depending on the desired cellular response, upstream signals may induce the activation of any of three MAPK pathways: Jun N-terminal kinase (JNK), p38, or extracellular signal-regulated kinase (ERK) (Fornaro *et al.*, 2000). The JNK pathway inhibits apoptosis and promotes proliferation, while the p38 pathway promotes cell survival in response to cellular stress. The ERK pathway is mainly involved in the regulation of genes involved in cellular proliferation, development, and differentiation (Fornaro *et al.*, 2000).

Cell cycle progression from G1 to S phase is regulated by many cyclin-dependent kinases (CDKs), which in turn are regulated themselves by cyclins and CDK-inhibitors (van Nimwegen and van de Water, 2007). Integrin-mediated cell adhesion controls cell cycle progression by regulating the expression and activities of cyclins, CDKs, and CDK-inhibitors, and is required for sustained ERK activation and the induction of cyclin D1 expression by growth factors (Zhao *et al.*, 1998). Upregulated cyclin D1 expression is an important aspect of anchorage-independent growth of transformed cells. FAK functions as a positive regulator of cell cycle progression by increasing cyclin D1 expression through its activation of the MAPK/ERK pathway (Zhao *et al.*, 1998; Sonoda *et al.*, 2000; Schaller, 2001). FAK is also involved in the upregulation of cyclin D3, resulting in pRb phosphorylation and deactivation, thereby further enhancing cell proliferation (van Nimwegen and van de Water, 2007).

1.9 Cell spreading and migration are regulated by FAK

FAK signalling is linked to cell spreading and migration as it controls the dynamic regulation of focal adhesions, cadherin-dependent cell-cell adhesions, and actin structures (McLean *et al.*, 2005). FAK is required for focal adhesion turnover at the leading edge and disassembly at the rear in migrating cells (Tilghman *et al.*, 2005; Earley and Plopper, 2006). Since this cycle of assembly and disassembly of focal adhesions controls the rate of cell movement, FAK is a key regulator of cell migration (McLean *et al.*, 2005). FAK promotes focal adhesion turnover as it

activates both the MAPK pathway and the calpain-2 protease (Webb *et al.*, 2004). Calpain-2 is an intracellular cysteine protease that cleaves focal adhesion components, such as talin, paxillin, and FAK, during focal adhesion turnover (Carragher *et al.*, 1999; Carragher *et al.*, 2001). Since FAK is both an activator and a substrate for calpains, the interplay between these proteins is important in the disassembly of focal adhesions at the rear of the cell during migration (van Nimwegen and van de Water, 2007). FAK expression is also required for PDGF- and EGF-stimulated cell motility as its adaptor function promotes the assembly of a complex containing calpain-2, FAK, Src, and ERK (Carragher and Frame, 2004; McLean *et al.*, 2005).

Cell migration and proteolytic degradation of the ECM is required for the invasion and metastasis of cancer cells (Schlaepfer and Mitra, 2004). In order for cells to metastasize, ECM barriers are often degraded through the secretion of matrix metalloproteases (MMPs), which are a family of zinc-dependent endopeptidases with wide substrate specificities for ECM components such as collagen, laminin, and fibronectin (Stamenkovic, 2000; Stewart *et al.*, 2004). FAK-enhanced activation of ERK or JNK is required in order to promote transcription-associated increases in MMP expression in carcinoma cells (Shibata *et al.*, 1998; Mitra and Schlaepfer, 2006). Remodelling of the actin cytoskeleton is an essential element of cell migration (McLean *et al.*, 2005). FAK contributes to the regulation of actin dynamics and adhesion by influencing the Rho-GTPase pathway. Phosphorylated FAK is also able to promote Rac1 activation via the CAS and Crk adaptor proteins (Brakebusch *et al.*, 2002). PI3K also phosphorylates other cytoskeletal proteins, causing a decrease in the concentration of the actin cytoskeleton, leading to increased cell spreading (Tamura *et al.*, 1999). PI3K contributes to the activation of Rac1, which interacts with and activates p21-activated kinase (PAK), thereby stimulating migration by increasing focal adhesion turnover (Brakebusch *et al.*, 2002).

Once free from the primary tumour, these metastatic cells then enter the bloodstream. They then attach to organs at a secondary site, where they induce angiogenesis in order to ensure their survival (Stewart *et al.*, 2004). Angiogenesis, the establishment

of a nearby blood supply, must occur in order to provide essential nutrients for the growing cancer cells and to ensure the removal of toxic waste products (Stewart *et al.*, 2004). FAK signalling promotes angiogenesis via activation of the MAPK pathway. Nuclear translocation of ERK2 enhances the transcription of vascular endothelial growth factor (VEGF) and thereby enhances tumour cell growth by stimulating angiogenesis (Mitra and Schlaepfer, 2006).

1.10 Deregulation of FAK protein expression and activity leads to disease

The deregulation of several of the above-mentioned processes is associated with malignancy (Gabarra-Niecko *et al.*, 2003). Although FAK itself does not appear to be an oncogene, enhanced FAK protein expression levels and aberrant regulation of FAK signalling have been reported in epithelial tumours of the breast, colon, prostate, thyroid, cervix, rectum, ovary, and oral cavity (Gabarra-Niecko *et al.*, 2003; McLean *et al.*, 2005). FAK overexpression in cancerous cells may be caused by deregulation at many levels. FAK mRNA (messenger ribonucleic acid) and protein levels were reported to be elevated in various carcinomas, and FAK gene dosage was increased in squamous cell carcinomas of the head, neck, and lung. FAK gene amplification was also suggested to be a common feature in these tumours (Gabarra-Niecko *et al.*, 2003). Gene amplification often gives tumour cells a selective advantage by enhancing the protein's response to levels of growth factor or ligand that would not normally trigger proliferation (Weber, 2002). Changes in integrin expression occur in many cancers, and FAK, as a major integrin-associated signalling molecule, is involved in the transformation of cancerous cells (Gabarra-Niecko *et al.*, 2003). Growth factor receptor pathways also influence cell behaviour via FAK. Many of these pathways are upregulated in cancer, thus FAK may influence the altered growth of tumour cells as well as their responses to autocrine and paracrine stimuli through its interaction with oncogenic growth factor receptors (Gabarra-Niecko *et al.*, 2003; McLean *et al.*, 2005). The role that FAK plays in anchorage-independent growth and survival of cancer cells seems to confer these advantages in some tumours. The deregulation of the adhesion-dependent growth,

survival, and motility processes usually regulated by FAK is critical for transformation (Gabarra-Niecko *et al.*, 2003).

FAK upregulation or overexpression may be an early event in invasive tumour development as FAK protein expression has also been found in premalignant lesions (Gabarra-Niecko *et al.*, 2003). FAK overexpression accelerates the G1/S phase transition, increases cyclin D1 levels, and decreases p21 expression (Fornaro *et al.*, 2000). FAK protein expression may be important at different points during tumour progression, for example, in normal epithelium and benign hyperplasias FAK protein expression is usually low (Owens *et al.*, 1995; Gabarra-Niecko *et al.*, 2003). With time, cells within the hyperplastic lesion acquire mutations that lead to the establishment of the pre-invasive phenotype. FAK overexpression occurs in pre-invasive tumours, where cells no longer require adhesion to the basement membrane in order to survive and grow (Gabarra-Niecko *et al.*, 2003). In squamous cell carcinoma and lung adenocarcinomas, FAK overexpression has been shown to promote cell invasion, while increased expression of FAK correlates with increased cell motility in melanoma cell lines (Akasaka *et al.*, 1995; Schlaepfer *et al.*, 2004; Mukhopadhyay *et al.*, 2005).

Phosphorylation of FAK at certain sites has been reported to occur within different tumour types. Phosphorylation of Y397 was reported in invasive tumours but not in normal epithelium of the ovary (McLean *et al.*, 2005). Also, hyperphosphorylation of FAK in response to hepatocyte growth factor/scatter factor (HGF/SF) signalling corresponds with the increased motility of oral squamous cell carcinoma cells (Gabarra-Niecko *et al.*, 2003). Hess and Hendrix (2006) discovered that more aggressive melanoma cell lines contain phosphorylated FAK whereas poorly aggressive melanoma cell lines do not. In many cancer types, malignancy and metastasis as well as poor patient prognosis have been linked to increased FAK expression, and it is likely that FAK protein levels as well as its phosphorylation state may be useful prognostic markers (Owens *et al.*, 1995; Schlaepfer *et al.*, 2004; McLean *et al.*, 2005).

1.11 Human oesophageal squamous cell carcinoma (HOSCC) is a multi-factorial disease with an aggressive phenotype

Oesophageal carcinoma is one of the most highly malignant gastrointestinal cancers found in humans and is reported to be the ninth most common malignancy worldwide (Lam, 2000; Lehrbach *et al.*, 2003; Koppert *et al.*, 2005; McCabe and Dlamini, 2005). Reportedly, oesophageal carcinoma is associated with a 5-year survival rate of less than 25%, and surgical cure rates are compromised as most patients are diagnosed at such a late stage of disease that metastases and organ infiltration have often already occurred (Koppert *et al.*, 2005; Nair *et al.*, 2005). Human oesophageal squamous cell carcinoma (HOSCC) and adenocarcinoma are the two prevalent histological types of oesophageal carcinoma as they have highest mortality rates (Lehrbach *et al.*, 2003; Koshy *et al.*, 2004; Holmes and Vaughan, 2006). HOSCC develops in the squamous cells occurring in the upper oesophagus, while adenocarcinoma develops in the glandular tissue in the lower oesophagus.

The occurrence of oesophageal cancer varies greatly with geographical location. Oesophageal adenocarcinoma, which usually develops from Barrett's oesophagus, is more prevalent than HOSCC is in the western hemisphere, while HOSCC occurs more frequently in third world countries (Lehrbach *et al.*, 2003; Koshy *et al.*, 2004; Koppert *et al.*, 2005). Overall though, HOSCC is the most frequent subtype of oesophageal cancer worldwide and occurs at high incidences in China, Hong Kong, Japan, southern and eastern Africa, regions of South America, Iraq, Iran, and France (Stoner and Gupta, 2001; Lehrbach *et al.*, 2003; Koshy *et al.*, 2004; Zhang *et al.*, 2004; Driver and Veale, 2006; Sutter *et al.*, 2006).

In South Africa, HOSCC is the second most common cancer among all men combined and is the most common cancer in black men (McCabe and Dlamini, 2005). The instances of metastasis are of particular importance in HOSCC, and the recurrence of the disease due to the presence of micro-metastases dramatically reduces the 5-year survival rate, which is less than 10% (Stoner and Gupta, 2001). Two major risk factors of HOSCC are the use of tobacco and alcohol, which have a

synergistic effect (Lee *et al.*, 2007). Tobacco exposure has been linked to a tenfold higher risk for HOSCC in heavy smokers relative to non-smokers, and the risk is directly related to the duration of tobacco exposure (Koshy *et al.*, 2004). In addition to these risks, dietary factors (a lack of fruit, vegetables, vitamins, antioxidants, magnesium, and zinc), the consumption of carcinogens (such as nitrosamines released by *Fusarium moniliforme* fungus on maize), and viruses (such as human papilloma virus – HPV) also appear to be contributing factors in the development of HOSCC (Lam, 2000; Lehrbach *et al.*, 2003; Koshy *et al.*, 2004; Isaacson, 2005; McCabe and Dlamini, 2005).

1.12 Several genes are implicated in the pathology of HOSCC

The development of oesophageal cancer is a multi-step, progressive process involving alterations in intercellular adhesion, cell cycle regulation, and growth factor activity (Lehrbach *et al.*, 2003; Koppert *et al.*, 2005). Aberrant cell cycle regulation is a typical characteristic of HOSCC with mutations occurring in oncogenes such as EGFR, cyclin A, cyclin D1, c-myc, MMP-7, and MDM2, and in tumour suppressor genes such as pRb, p53, p16, $p14^{ARF}$, p21, and telomerase, which affect the G1 restriction point (Lehrbach *et al.*, 2003; Wang *et al.*, 2003; Koshy *et al.*, 2004; Gibault *et al.*, 2005; Huang *et al.*, 2005; McCabe and Dlamini, 2005). The genetic mutations affecting oncogenes often result in an upregulation of function, whereas mutations affecting tumour suppressor genes often cause a loss of function (Lehrbach *et al.*, 2003). Previous analysis of tumour cell cycling indicates that tumours with a high proliferative rate exhibit more aggressive clinical behaviours compared to tumours with a low proliferative rate (Huang *et al.*, 2005). Thus, aberrant and upregulated cell cycling may account for the aggressive nature of HOSCC.

As mentioned earlier, p53 and pRb are significantly involved in cell cycle regulation. In response to DNA damage, p53 transactivates genes encoding effectors of cell cycle arrest, DNA repair, and apoptosis (Montesano *et al.*, 1996). Mutation of the p53 gene is the most common genetic alteration in human cancers and is the most

frequently studied genetic mutation in HOSCC (Lam, 2000). Many of the known p53 mutations are point mutations and are associated with the intake of carcinogens such as cigarette smoke and aflatoxins (Montesano *et al.*, 1996; Lam, 2000). These point mutations prevent the tumour-suppressing activity of p53 and enhance the genomic instability of cancer cells (Montesano *et al.*, 1996).

Some growth factors, such as EGF and transforming growth factor α (TGFα), have been shown to function in an autocrine loop in HOSCC (Lam, 2000). HOSCC patients with EGFR overexpressing tumours have a worse prognosis as EGFR overexpression is associated with minimal response to chemoradiotherapy (Lam, 2000). Both gene amplification and overexpression of EGFR have been detected in HOSCC, and EGFR overexpression is significantly correlated with p53 mutations (Veale and Thornley, 1989; Montesano *et al.*, 1996). Cyclin D1 is also overexpressed in oesophageal cancers and genetic alterations leading to the constitutive action of CDK/cyclin D1 pathway are frequent in HOSCC (Montesano *et al.*, 1996). These alterations include amplification and overexpression of the cyclin D1 gene and deletion and inactivation of pRb, resulting in uncontrolled cell proliferation (Montesano *et al.*, 1996). Deletions or mutations in pRb are common in many tumours and may play a role in the development of HOSCC (Busatto *et al.*, 1998; Lam, 2000).

In oesophageal carcinoma, invasion and metastasis play a key role in influencing patient survival (Nair *et al.*, 2005). Generally, invasive events require alterations in integrin- and cadherin-controlled cell adhesion. Miller and Veale (2001) showed that HOSCC cell lines express the αv integrin subunit strongly whereas normal oesophageal tissue does not, thus suggesting that αv is a major indicator of the transformed phenotype. This suggestion is supported by evidence that αv has been implicated in promoting proliferation and preventing apoptosis in some carcinoma cell types (Miller and Veale, 2001). These researchers also found that the α2 and β1 integrin subunits are downregulated in HOSCC, and they suggest that due to the prevalence of the α2β1 heterodimer, there may be a decrease in cell adhesion and an increase in the migratory potential of these carcinoma cells (Miller and Veale, 2001).

The α2β1 integrin binds to collagen and laminin, and its expression is reportedly correlated to invasive potential in many carcinomas (Hynes, 1992). The cadherins have also been frequently studied in HOSCC, and reduction of E-cadherin and α-catenin expression was shown to be notably associated with increased metastatic potential and poor prognosis in patients with HOSCC (Lam, 2000; Nair *et al.*, 2005).

1.13 The exact molecular pathogenesis of HOSCC is not clearly defined

Despite improvements in surgery, chemotherapy, and radiotherapy, the prognosis of HOSCC remains poor (Kawaguchi *et al.*, 2006). Although a number of genes associated with HOSCC pathology have been identified, the precise pathogenesis and the exact molecular mechanisms that govern the aggressive behaviour of the disease are still largely uncertain (Hu *et al.*, 1999; Nair *et al.*, 2005). In order to create a more dynamic approach to clinical diagnosis and therapeutic intervention, a greater understanding of the processes underlying HOSCC malignancy is needed.

FAK is reportedly amplified and/or overexpressed in human tumours of diverse origin including breast, colon, head and neck, lung, and thyroid tumours (Owens *et al.*, 1995; Agochiya *et al.*, 1999; Beviglia *et al.*, 2003). Studies in some of these tumours highlight the relationship between FAK expression and metastatic ability in tumour cells (Owens *et al.*, 1995). Although numerous studies have been conducted on FAK expression in many different cancer cell lines and tissues, very few reports detail FAK expression in HOSCC.

Invasion, and in particular, lymph node metastasis are associated with poor prognosis in HOSCC (Kuwano *et al.*, 1997). As FAK expression directly influences these processes, FAK may be a useful therapeutic target in preventing HOSCC cells from invading other organs and spreading to the lymph nodes (Miyazaki *et al.*, 2003). Therefore, the goal of this study is to investigate the expression and potential role(s) of FAK in HOSCC cells with the hope of shedding light on some of the key molecular processes that characterize the aggressive and invasive phenotype observed in HOSCC.

1.14 Research aims and objectives

The specific aims and objectives of this study conducted in 5 HOSCC cell lines are detailed below. The aims were to:

1) Detect and quantify FAK protein expression in the HOSCC cell lines.
2) Determine the cellular distribution of FAK in the HOSCC cell lines.
3) Investigate the predominance of FAK variants.
4) Establish the effects of EGF stimulation on FAK protein expression and cellular distribution.
5) Establish the status of FAK at the cell membrane in the HOSCC cell lines.
6) To determine the phosphorylation status of the FAK variants at the cell membrane in the HOSCC cell lines.