CHAPTER 4: GENERAL DISCUSSION

4.1 FAK protein expression and cellular localization influences cell behaviour

FAK is a non-receptor tyrosine kinase and its increased expression and aberrant signalling have been reported in many tumours of diverse origin (Beviglia *et al.*, 2003; Gabarra-Niecko *et al.*, 2003; McLean *et al.*, 2005). Upregulated expression may result from deregulation at many levels, as gene amplification resulting in elevated FAK mRNA and protein levels have been observed in invasive and metastatic tumours of the colon, breast, prostate, thyroid, ovary, liver, stomach, oral cavity, and many others (Owens *et al.*, 1995; Aronsohn *et al.*, 2003; Gabarra-Niecko *et al.*, 2003). The relationship between upregulated FAK expression and metastatic ability has been highlighted in many studies, and FAK overexpression is strongly correlated to poor patient survival (Owens *et al.*, 1995; Miyazaki *et al.*, 2003). In addition to being overexpressed in malignant tumours, FAK expression is also upregulated in premalignant lesions which indicates that FAK plays an active role in the progression of cancer (Gabarra-Niecko *et al.*, 2003). The oncogenic effects of elevated FAK expression and activity appear to issue from its adhesion-modulating properties as well as its signalling capabilities and tyrosine kinase activities (Cohen and Guan, 2005b; Mukhopadhyay *et al.*, 2005; Watermann *et al.*, 2005).

Previous studies have detailed FAK expression in many different cancers, although information on its expression in HOSCC tumours has thus far been limited to immunohistochemical staining of formalin-fixed, paraffin-embedded specimens in which comparisons were made between normal and cancerous sections (Miyazaki *et al.*, 2003). Although Miyazaki *et al.* (2003) obtained data relating to FAK expression in normal oesophageal and HOSCC fixed sections, quantitative data of FAK expression in cultured HOSCC cells have thus far not been produced. Therefore, the broad aim of the study presented here is to semi-quantitatively investigate FAK expression and to attempt to elucidate its potential influence in actively growing HOSCC cells in culture.

A comparison between normal oesophageal cell lines and the HOSCC cell lines could not be conducted in the study presented here due to the unavailability world wide of normal oesophageal cell lines in culture. Therefore, whether the FAK protein was over- or under-expressed within the HOSCC cell lines relative to normal oesophageal cells could not be determined. Rather a comparison of FAK expression between the 5 cell lines was conducted, and the semi-quantitative data produced gives a clear indication of the relative levels of FAK expression in each cell line. Protein expression analysis of whole-cell lysates (by western blotting) revealed that the expected 125 kDa FAK polypeptide was expressed in each of the 5 HOSCC cell lines examined (Figure 2.3). The antibody used in the immunoblotting procedure was specific against a peptide mapped at the N-terminus of 125 kDa FAK of human origin. Antibody binding was therefore an indication that full-length (125 kDa) FAK was expressed in each of these HOSCC cell lines, and the size of this polypeptide detected by FAK-specific western blotting of whole-cell lysates was in keeping with previously published literature (Schaller *et al.*, 1992).

The 5 HOSCC cell lines were all derived from moderately differentiated tumours, however, the level of FAK protein expression varied between the cell lines (see graph as part of Figure 2.3). The moderately differentiated category is the broadest pathological category and includes all tumours that are not distinctively 'poor' or 'well' differentiated. Aronsohn *et al.* (2003) determined that FAK expression was related to the degree of tumour differentiation in invasive laryngeal squamous carcinoma paraffin-embedded specimens. Immunohistochemical analysis of these specimens revealed that poorly differentiated tumours yielded more intense FAK staining (indicative of upregulated FAK expression) whereas moderately differentiated tumours demonstrated less intense staining (Aronsohn *et al.*, 2003). Miyazaki *et al.* (2003) confirmed that the level of FAK protein expression correlated with the degree of differentiation in HOSCC tumour sections. They reported that FAK was generally not overexpressed in well differentiated tumour sections, whereas most poorly differentiated tumour sections did overexpress FAK. Approximately 50% of the moderately differentiated HOSCC tumour sections in

their study showed FAK overexpression (Miyazaki *et al.*, 2003). However, the immunohistochemical data obtained from the studies conducted by Aronsohn *et al.* (2003) and Miyazaki *et al.* (2003) was not adequately quantitative. Hence the data produced in the study presented here are unique in that it details FAK protein expression (semi-quantified by densitometric analysis) in actively proliferating HOSCC cell lines in culture.

The level of FAK protein expression varied amongst the 5 HOSCC cell lines examined (Figure 2.3). This data supports the claims made by Aronsohn *et al.* (2003) and Miyazaki *et al.* (2003) that FAK expression either influences or is affected by the degree of tumour differentiation. Although the 5 HOSCC cell lines are classified under the 'moderately differentiated' pathological category, tumours with varying degrees of moderate differentiation are classified in this category. Therefore, it is likely that the WHCO6 and WHCO5 cell lines may be towards the 'more poorly' differentiated end of the moderately differentiated category, as it was quantitatively determined that they expressed FAK at much higher levels than the other 3 HOSCC cell lines. The cell lines that exhibited much lower levels of FAK expression (WHCO1, WHCO3, and SNO) may have been derived from 'more differentiated' tumours within this category (Figure 2.3).

The fact that each cell line generated the same size protein fragment of 125 kDa (see Figure 2.3) indicates that there are no major structural aberrations in the FAK protein. These data also suggests that whichever factor(s) is responsible for controlling FAK protein expression is maintained across these HOSCC cell lines. As mentioned in Chapter 1, gains in *fak* gene copy number have been seen in many tumour types (Agochiya *et al.*, 2005), and fluorescent *in* situ hybridization (FISH) analysis should be done to determine whether there is an increase in the gene copy number of FAK in the HOSCC cell lines. Increased FAK mRNA and corresponding protein levels have been reported in many tumours, thus implying that FAK expression may be controlled at the level of gene transcription (Gabarra-Niecko *et al.*, 2003; Golubovskaya *et al.*, 2004). Both NF-κB and p53 binding sites reportedly occur within the promoter region of FAK, and various studies indicate that NF-κB

positively influences, while p53 negatively affects, FAK transcription (Golubovskaya *et al.*, 2004). In turn, FAK appears to promote its own transcription by inducing NF-κB activation, while inhibiting p53 activity (Sonoda *et al.*, 2000; Golubovskaya *et al.*, 2005; van Nimwegen and van de Water, 2007). Further analysis of the promoter region of the FAK gene is required in order to conclusively establish whether the NF-κB and p53 transcription factors regulate FAK expression in these HOSCC cell lines.

Detection of FAK in these HOSCC cell lines has implications relating to its cellular distribution and subsequently, to its signalling and scaffolding capabilities. Indirect immunofluorescence images clearly show that FAK is present in focal contacts or adhesions situated within the cell membranes of the HOSCC cell lines (Figures 2.4 and 2.5). While more stable focal adhesions are located at the periphery of the cell colonies, smaller short-lived focal contacts occur within the cell membranes in lamellipodia (Figures 2.4 and 2.5) and it is at these focal complexes that FAK is likely to mediate the diverse interactions between the integrins and the actin cytoskeleton (Gimona and Buccione, 2006). As the leading edge comes into contact with the ECM, small focal contacts are formed (Tilghman *et al.*, 2005). It is at these focal contacts that integrin signalling via FAK promotes cell migration by inducing changes in cytoskeletal organization that result in a dynamic cycle of focal adhesion assembly, actin reorganization, and focal adhesion disassembly (Brakebusch *et al.*, 2002; Carragher and Frame, 2004). Actin polymerization at the leading edge provides the force required for the extension of lamellipodia (Rottner *et al.*, 1999; Parsons *et al.*, 2000). As the actin cytoskeleton contracts, the focal adhesions are turned over and the cell moves forwards (Tilghman *et al.*, 2005). The rate of focal adhesion turnover is proportional to the speed of cell migration (Chan *et al.*, 1994; Green *et al.*, 1998). Thus, because FAK controls focal adhesion turnover and disassembly at the rear in migrating cells (through activation of the MAPK pathway), it is a key regulator of cell migration (Webb *et al.*, 2004; McLean *et al.*, 2005; Tilghman *et al.*, 2005; Earley and Plopper, 2006).

The leading edge is observed in the HOSCC cell lines with FAK clearly localized to focal complexes occurring within the lamellar extensions (Figures 2.4 and 2.5). The localization of FAK to these cellular structures therefore suggests that FAK is actively involved in regulating actin polymerization and subsequent cell migration in these HOSCC cell lines (Parsons *et al.*, 2000; Carragher and Frame, 2004; Tilghman *et al.*, 2005). Although the localization of FAK does not contribute to cell motility and invasion independently of other cellular proteins, its localization to focal adhesions is indicative of the role that it plays in cell migration. The displacement of FAK from these areas would reduce cell migration (Agochiya *et al.*, 1999).

Many FAK-containing lamellipodia are especially evident in the WHCO6 and SNO cell lines (Figures 2.4 and 2.5). Therefore, it is possible that these two cell lines may exhibit comparatively higher rates of cell migration (compared to WHCO1, WHCO3, and WHCO5, which have fewer FAK-containing lamellipodia) as FAK is required for the spatial organization of the leading edge of migrating cells (Tilghman *et al.*, 2005). In many tumours, upregulated FAK expression reportedly correlates with increased migratory potential (Akasaka *et al.*, 1995; Owens *et al.*, 1995; Cary *et al.*, 1996; Agochiya *et al.*, 1999). Thus the comparatively high level of FAK expression observed in whole-cell lysates of WHCO6 may also be indicative of a relatively high rate of cell migration in comparison to the other HOSCC cell lines. In the other cell lines (WHCO1, WHCO3, and WHCO5), many FAK-containing focal adhesions were visible within cell membranes of cells at the periphery of the colony (Figure 2.5). These focal adhesions may be more stable and may not be turned over as rapidly as the short-lived focal contacts within the lamellipodia. WHCO1 and WHCO3 had lower levels of FAK expression and fewer lamellipodia in comparison to the other cell lines, thus further supporting the idea that upregulated FAK expression is required for enhanced cell migration (Figures 2.3 and 2.5). Immunofluorescent imaging is not a quantitative procedure whereas western blotting data are quantifiable. Thus, although visually the FAK protein in WHCO3 may appear to stain with similar intensity to the other cell lines, this does not necessarily mean that WHCO3 cells have similar concentrations of FAK protein. Although a certain concentration of total protein from each cell line was loaded onto the SDS-

PAGE gels, western blot data show that a smaller proportion of this total protein is FAK in WHCO3 in comparison with the other cell lines (Figures 2.3 and 2.5).

In addition to being present in focal adhesions, FAK was also noticeably localized to the cytoplasm in the 5 HOSCC cell lines (Figures 2.4 and 2.5). This is in keeping with previously published literature, where FAK was originally described as a non-receptor cytoplasmic tyrosine kinase (Hildebrand *et al.*, 1993; Schaller, 2001). Cell adhesion to the ECM is essential in regulating the tyrosine phosphorylation, activation, and cellular localization of FAK (Cohen and Guan, 2005a). Once integrin-associated activation of FAK has occurred in the HOSCC cell lines, it is likely that FAK associates with various other signalling and scaffolding molecules which subsequently activate a diverse range of cytoplasmic signalling cascades involved in regulating biological processes such as cell spreading, migration, proliferation, and survival (Ilić *et al.*, 1997; Gabarra-Niecko *et al.*, 2003; Schlaepfer *et al.*, 2004; van Nimwegen and van de Water, 2007). The FAK protein may continuously cycle between the focal adhesions and the cytoplasm, depending on its state of phosphorylation. Research groups have confirmed that the autophosphorylated and active form of FAK (FAK pY397) is localized to the cytoplasm (Aronsohn *et al.*, 2003; Carragher *et al.*, 2003; Wozniak *et al.*, 2004; Cohen and Guan, 2005b). Therefore, the autophosphorylated and active form of FAK may constitute a portion of the cytoplasmic staining observed in the HOSCC cell lines in this study.

In the WHCO3 cell line, FAK was predominantly localized to the cytoplasm although some nuclear staining was also observed (Figure 2.5). Originally, FAK was considered to be exclusively a cytoplasmic or membrane-associated protein however; more recent reports have described nuclear FAK (Schaller, 1992; Jones *et al.*, 2001; Aronsohn *et al.*, 2003). Aronsohn *et al.* (2003) discovered a 50 kDa amino-terminal fragment and a 120 kDa FAK variant in the nuclear fraction of endothelial cells, while Jones *et al.* (2001) showed that a 40 to 50 kDa amino-terminal FAK fragment was constitutively present in the nucleus of glioblastoma cells and formed aggregates during apoptosis. Nuclear FAK is generally post-translationally modified by the

covalent addition of a small ubiquitin-related modifier at lysine 152 (Lys152) which is thought to be a prerequisite for the nuclear import of FAK (Angelucci and Bologna, 2007). The precise role of nuclear FAK is unknown, but it is speculated that it could act as a sensor of the physiological state of a cell or may be involved in the regulation of transcription (Aronsohn *et al.*, 2003). Clearly further work is needed to determine the biological importance of nuclear FAK. Bearing in mind that that a relationship exists between FAK expression and localization and the prognosis of many cancer types, it may be of value to further investigate this relationship within the HOSCC cell lines.

4.2 Cell survival and migration in HOSCC cell lines may be influenced by FAK signalling

As autophosphorylated (i.e. active) FAK is reported to occur within the cytoplasm of some tumours specimens, it may comprise a percentage of the total FAK localized in the cytoplasm of the HOSCC cells (Figures 2.4 and 2.5). This active form of FAK probably associates with several kinases, signal transduction molecules, and cytoskeletal proteins within the HOSCC cell lines under investigation. FAK expression and activity are altered in many cancers; therefore FAK may also promote the development of malignancy in HOSCC tumours through its influence on cell migration, proliferation, and survival (Gabarra-Niecko *et al.*, 2003; McLean *et al.*, 2005).

Autophosphorylated Y397 of FAK serves as a binding site for the SH2 domain of p85, the regulatory subunit of PI3K (Zhao *et al.*, 1998; Velling *et al.*, 2004). FAK activates PI3K, leading to the generation of second messenger phosphoinositides which activate the anti-apoptotic PKB/Akt protein (Mayo *et al.*, 2002b). Both integrin and growth factor signalling are able to influences PKB activation via FAK (Wu and Dedhar, 2001; Velling *et al.*, 2004). Active PKB enhances cell survival by preventing p53-dependent apoptosis and by phosphorylating and inhibiting pro-apoptotic proteins such as the caspases, Bcl-2, Bad, and GSK3β (Datta *et al.*, 1997; Troussard *et al.*, 1999; Huang *et al.*, 2001; Mayo *et al.*, 2002a). PKB further

enhances cell survival by activating transcription factors such as NF-κB, which further increases FAK transcription and promotes cell survival via FAK-mediated signalling (Mayo *et al.*, 2002b; Golubovskaya *et al.*, 2004).

FAK also promotes cell survival by directly binding to and inhibiting p53, thereby preventing its pro-apoptotic signalling capabilities (Golubovskaya *et al.*, 2005). Mutation of the p53 gene is the most commonly studied genetic mutation in HOSCC cells (Lam, 2000). Many of the known mutations inhibit the tumour-suppressing activity of p53, thereby enhancing the genomic instability of cancer cells (Montesano *et al.*, 1996). A previous study determined that point mutations occur within a known mutational hotspot within the p53 gene in all 5 of the HOSCC cell lines studied here (Dewar, 2000). Golubovskaya *et al.* (2004) reported that p53 negatively regulates the transcription of the FAK gene; therefore, it is highly likely that mutation of the p53 gene and/or prevention of its tumour-suppressing function may promote FAK transcription in these 5 HOSCC cell lines.

Stimulation of FAK by both integrins and growth factor receptors (such as EGFR, uPAR, and PDGFR) promotes activation of the MAPK pathway (Schaller, 2001; Normanno *et al.*, 2006). Depending on the desired cellular response, any of three MAPK pathways may be activated namely, ERK, JNK, or p38 (Fornaro *et al.*, 2000). FAK functions as a positive regulator of cell cycle progression by increasing cyclin D1 and D3 expression through MAPK/ERK activation, resulting in pRb hyperphosphorylation and deactivation (Sonoda *et al.*, 2000; Schaller, 2001; van Nimwegen and van de Water, 2007). Aberrant cell cycle regulation is a distinctive characteristic of HOSCC, with mutations occurring in the cyclins, pRb, and p53 (Lehrbach *et al.*, 2003; Huang *et al.*, 2005; McCabe and Dlamini, 2005). As FAK expression contributes to cell cycle progression by influencing the activity of these molecules in other cancers, it may possibly enhance cell cycle progression similarly in the HOSCC cell lines. Abnormal and upregulated cell cycling may contribute to the aggressive nature of HOSCC as tumours with a high proliferative rate generally exhibit more aggressive clinical behaviours (Huang *et al.*, 2005).

Cell migration is vitally controlled by FAK, which is required for focal adhesion turnover at the leading edge and disassembly at the trailing edge of migrating cells (McLean *et al.*, 2005; Tilghman *et al.*, 2005; Earley and Plopper, 2006). Upregulated FAK expression is closely correlated with increased motility in many tumour types including prostate, oral squamous cell, and lung carcinomas, as well as in melanoma cell lines (Akasaka *et al.*, 1995; Schlaepfer *et al.*, 2004; Gabarra-Niecko *et al.*, 2005; Mukhopadhyay *et al.*, 2005). FAK promotes focal adhesion turnover by activating the MAPK pathway and subsequently the calpain-2 protease (Webb *et al.*, 2004). During focal adhesion turnover, calpain-2 cleaves focal adhesion components such as talin, paxillin, Src, β-integrin subunits and FAK, leading to focal adhesion disassembly at the rear of the cell (Oda *et al.*, 1993; Cooray *et al.*, 1996; Carragher *et al.*, 1999; Carragher *et al.*, 2001). Investigations into the significance of calpain activity could yield further information regarding focal adhesion turnover in the HOSCC cell lines.

Once cancer cells have started migrating, proteolytic degradation of the ECM is required for further invasion and metastasis of cancer cells, and MMPs degrade specific ECM components (Stamenkovic, 2000; Schlaepfer and Mitra, 2004; Stewart *et al.*, 2004). In various carcinoma cells, FAK stimulation of ERK or JNK reportedly promotes increases in MMP transcription, leading to ECM degradation (Shibata *et al.*, 1998; Mitra and Schlaepfer, 2006). FAK has been shown to increase the secretion of MMP-2 and -9 in lung adenocarcinoma cells, while overexpression of MMP-1, -3, -7, and -10 have been implicated in the invasive behaviour of HOSCC and other tumour types (Lam, 2000; Hauck *et al.*, 2002; Haskell *et al.*, 2003; McCabe and Dlamini, 2005; Yeudall *et al.*, 2005). Metcalfe (2006) detected the activated form of MMP-7 in these 5 HOSCC cell lines (using western blot analysis). MMP-7 overexpression is significantly correlated with a reduced 5-year patient survival rate (Adachi *et al.*, 2001).

Once the surrounding ECM has been degraded, metastatic cells move free of the primary tumour and travel via the bloodstream to distant sites where they induce angiogenesis in order to ensure their survival (Stewart *et al.*, 2004). The nearby

blood supply induced by angiogenesis ensures cell growth by providing nutrients to and removing toxic waste products from the growing cancer cells (Stewart *et al.*, 2004). FAK activation of ERK enhances VEGF transcription which stimulates angiogenesis and thereby promotes tumour cell growth and survival (Mitra and Schlaepfer, 2006). Invasion, and in particular, the instances of metastasis are of particular importance in HOSCC as they drastically reduce the 5-year survival rate of patients (Kuwano *et al.*, 1997; Stoner and Gupta, 2001).

4.3 FAK is implicated in EGFR signalling

Not only does FAK interact with integrins at the cell membrane, but it is also integral in transmitting signals from growth factor receptors into the cell interior (Ilić *et al.*, 1997; McLean *et al.*, 2005). In particular, the link between EGFR and FAK has been established in many tumours, and EGFR is overexpressed at high frequency in many carcinomas including the HOSCC cell lines used in this study (Veale and Thornley, 1989; Hanahan and Weinberg, 2000; Sieg *et al.*, 2000; Lu *et al.*, 2001; He *et al.*, 2006; Sutter *et al.*, 2006). EGFR overexpression in HOSCC tumours is reported to directly correlate with increased metastatic potential and poor patient prognosis (Gibault *et al.*, 2005).

Veale and Thornley (1989) discovered that EGFR is overexpressed in all 5 of the WHCO and SNO cell lines in this study; hence EGFR activation may enhance downstream signalling effects in these cell lines that are generally associated with HOSCC tumourigenesis. EGFR activation leads to the recruitment of various cytoplasmic proteins, one of them being FAK (Hauck *et al.*, 2001). EGFR signalling via FAK may lead to proliferation and cell migration (via the MAPK pathway) as well as cell survival (via the PI3K pathway) in these HOSCC cell lines (Sieg *et al.*, 2000; Golubovskaya *et al.*, 2002; Bill *et al.*, 2004; Normanno *et al.*, 2006).

Full-length FAK (125 kDa) was expressed in both the untreated control and EGF-treated whole-cell lysates in each HOSCC cell line (Figure 3.3). EGF treatment notably altered the expression levels of 125 kDa FAK within each cell line, although

differences in how EGF treatment affected FAK expression were observed between the cell lines (Figure 3.4). This indicated that EGFR activation altered FAK protein expression uniquely in each cell line. In all the cell lines (with the exception of WHCO1) EGF treatment generally stimulated an overall increase in FAK expression at some point during treatment, followed by an eventual decrease in expression (Figure 3.4). However, once an increase in FAK expression had occurred in the SNO cell line, FAK expression was maintained at a relatively constant level throughout EGF exposure (Figure 3.4).

Although EGF treatment influenced the expression of full-length FAK in these HOSCC cell lines, Yamanaka *et al.* (2003) reported contrasting results in their study as they found that EGF stimulation did not affect the expression of FAK in cervical adenocarcinoma cells. Thus, the mechanisms underlying EGFR regulation of FAK expression may be cell type specific. The question remains: which molecular mechanisms are used by activated EGFR to influence FAK protein expression in the HOSCC cell lines? EGFR signalling reportedly leads to the activation of various transcription factors, such as *c-fos*, *c-Jun*, *c-myc*, STAT, the zinc finger transcription factor family and NF-κB (Normanno *et al.*, 2006). Since Golubovskaya *et al.* (2004) discovered that the promoter region of FAK contains two NF-κB binding sites, it seems likely that EGFR signalling may enhance FAK transcription in the HOSCC cell lines via the activation of the NF-κB transcription factor.

As FAK is activated within focal adhesions it was deemed necessary to establish the status of FAK at the cell membrane in the HOSCC cell lines (Gabarra-Niecko *et al.*, 2003). Full-length FAK (125 kDa) was detected in membrane extracts of the HOSCC cell lines; however, a second FAK variant of 85 kDa was also present at the cell membrane (Figure 3.6). Detection of the second variant in the membrane extracts was unexpected as it was not detected in FAK-immunoblotted whole-cell lysates. In order to achieve clarity as to why this variant was observed in the membrane extracts, additional measures were taken to ensure protein stability during the extraction procedure. It was confirmed that the 85 kDa FAK variant was not an artefact of the experimental procedures (Figure 3.7).

Is the 85 kDa protein expressed independently of the full-length FAK protein? As mentioned earlier, some researchers also discovered the presence of an 85 kDa N-terminal cleavage product of 125 kDa FAK in non-small cell lung cancer, vascular smooth muscle, and HASM cell lines (Wen *et al.*, 1997; Shofuda *et al.*, 2004; Carlin *et al.*, 2005). There are no reports detailing the existence of the 85 kDa protein independently of FAK, but it has been extensively reported that FERM-domain truncations of FAK yield activated FAK constructs (Lim *et al.*, 2008). Therefore, based on these reports, the 85 kDa protein detected in the HOSCC cell lines here must also be an N-terminal cleavage product of full-length FAK. The possibility that the 85 kDa variant contains the N-terminus of FAK was confirmed by the fact that the antibody used for western blotting was specifically targeted to recognize a peptide sequence within the N-terminus of FAK. Yet why was this variant detected in the membrane extracts but not in the whole-cell lysates in the HOSCC cell lines? Carlin *et al.* (2005) produced similar findings to the study presented here in their analysis of FAK expression in HASM cells. They also reported that the 85 kDa variant was unique to the membrane fraction where it constituted a large proportion of the total FAK detected (Carlin *et al.*, 2005). Similarly, in the HOSCC cell lines analyzed here, the 85 kDa variant makes up a great proportion of FAK at the cell membrane; however, in the context of the whole cell it may not constitute a major proportion of total FAK expression, which would explain why the 85 kDa variant was not detected in whole-cell lysates of the HOSCC cell lines. As the Triton X-100 detergent solubilizes membrane-associated proteins but not all of the other cellular proteins, the concentration of the membrane-associated proteins is enhanced by this extraction procedure. Therefore, the reason that the 85 kDa variant is only detected in the membrane samples (and not in the whole-cell extraction) could be a product of the Triton X-100 enhancement of the membrane-associated proteins.

Over time, many researchers have questioned and have set out to establish precisely how the 85 kDa variant of FAK is formed. Some published studies have revealed that an 85-100 kDa FAK cleavage product is produced by either caspases or calpains (or both) in response to apoptotic stimuli in various cell types (Carragher *et al.*, 2001; Carragher *et al.*, 2002; Carlin *et al.*, 2005). FAK is reportedly an early target of caspases-3 and -6, as caspase-mediated cleavage of FAK precedes cell detachment from the ECM (Gervais *et al.*, 1998; Levkau *et al.*, 1998; Carragher *et al.*, 1999; Fischer *et al.*, 2003). Cleavage of FAK differentially affects its association with other signalling molecules of the focal adhesion complex, as p130CAS and vincullin are able to bind to FAK but paxillin is not (Levkau *et al.*, 1998). This initial cleavage coincides with the loss of 125 kDa FAK and paxillin from focal adhesions, leading to focal adhesion disassembly (Levkau *et al.*, 1998; van de Water *et al.*, 1999).

Calpains, which are a highly conserved family of non-lysosomal calcium-dependent cysteine proteases, have also been shown to cleave FAK and induce focal adhesion disassembly during v-Src-induced transformation (Carragher *et al.*, 2001). Westhoff *et al.* (2004) determined that FAK can act as a scaffold thereby permitting the assembly of a complex consisting of calpain 2 and ERK. EGF is able induce ERK activity, which is required for maximal activation of calpain 2 at the cell membrane. In this way, EGF stimulates focal adhesion turnover and cell motility (Westhoff *et al.* 2004). Calpain-mediated disassembly of focal adhesions reduces adhesion strength, thereby promoting tumour cell motility (Carragher *et al.*, 2001). Calpains also regulate the levels of p53 and thereby influence cell cycle progression (Carragher *et al.*, 2002). As the promoter region of FAK contains two p53 binding sites, calpain activity may play an integral role in influencing FAK transcription (Golubovskaya *et al.*, 2004). Whether calpains or caspases are responsible for generating the 85 kDa FAK variant in the HOSCC cell lines needs to be established.

The localization of FAK to focal adhesions is crucial to its signalling function. The focal adhesion targeting (FAT) sequence of FAK is located at the far C-terminus of the protein, and it is this region that facilitates the localization of FAK to focal adhesions through its association with paxillin and talin (Gabarra-Niecko *et al.*,

2003; Wozniak *et al.*, 2004). Since the 85 kDa FAK variant does not contain the C-terminal FAT sequence and therefore cannot bind to paxillin and talin, how is it able to localize to focal adhesions? As the N-terminal FERM domain reportedly contains both growth factor and integrin binding sites, it is highly likely that the binding of the N-terminal 85 kDa variant to these membrane-bound proteins anchors it to the cell membrane (Gabarra-Niecko *et al.*, 2003; Cox *et al.*, 2006).

Although the 85 kDa variant was initially identified in cells undergoing apoptosis (Carlin *et al.*, 2005), it can be concluded that the expression of the 85 kDa variant is exclusively observed in apoptosing cells, as it has been detected in many actively proliferating cell lines including those HOSCC cell lines studied here. However, likewise, there is no indication in the literature that the expression of the 85 kDa variant is necessarily significant in cancer cell characterisation (i.e. in actively proliferating cells) with or without increased EGF exposure. Thus, it is difficult to deduce whether the expression of the 85 kDa variant of FAK is characteristic of cancer cell development in the HOSCC cell lines.

EGFR activation affects the concentration of both FAK variants at the cell membrane differently in each of the HOSCC cell lines, and thus a single uniform trend was not distinguishable (Figures 3.8 and 3.9). Also, the changes in concentration of one variant do not necessarily correspond to those noted for the other FAK variant at the same interval of EGF treatment in each cell line (Figure 3.9). As previously suggested in Chapter 3, EGF receptor recycling may be partly responsible for the detected changes in full-length and 85 kDa FAK expression. Briefly, EGF stimulation may cause increased rates of EGFR internalization and rapid recycling back to the cell membrane (Wiley, 2003; Driver and Veale, 2006). EGFR signalling at the cell membrane increases NF-κB activity, promoting the transcription of FAK. EGFR internalization would attenuate NF-κB activity, leading to downregulation of FAK transcription.

Although EGFR activation affected total FAK protein expression in the HOSCC cell lines, this did not completely account for the changes in the concentration of either

FAK variant at the cell membrane. Changes in total FAK expression were not necessarily reflected as changes in membrane localization at a particular EGF treatment interval. This may have resulted from EGF-induced calpain activity, which would lead to cleavage of FAK, resulting in its loss from the cell membrane and focal adhesion turnover (Westhoff *et al.*, 2004). As there was a concomitant decrease in full-length FAK expression over the time of EGF-treatment (Figures 3.8 and 3.9), one would expect to notice an increase in the cleaved 85 kDa FAK variant. However, this was not the case as a general decrease in 85 kDa protein expression was noted by 12 hours of EGF treatment (Figure 3.9b). This is possibly due to further cleavage of the 85 kDa variant into smaller fragments of 77 and 44 kDa by caspases-3 and 6 as noted in previous studies conducted by Wen *et al.* (1997) and Gervais *et al.* (1998); however, this was not explored further in the HOSCC cell lines examined here. As discussed in Chapter 3, EGFR activation influences both p53 and NF-κB activity, which can either upregulate (via NF-κB) or downregulate (via p53) FAK expression. Currently, however, the exact mechanisms by which this up-or down-regulation occurs in these EGF-treated HOSCC cell lines has not been fully elucidated and further studies in this area may uncover the exact molecular mechanisms which influence FAK expression.

Full-length FAK was expected to be tyrosine phosphorylated in the untreated controls (and possibly EGF-treated samples) in the HOSCC cell lines, as it is this form of FAK that mediates its signalling and scaffolding functions (van Nimwegen and van de Water, 2007). Contrary to what was expected, the 85 kDa FAK cleavage product was detected by FAK-specific immunoblotting of the phosphotyrosine-immunoprecipitated membrane extracts in all of the untreated and EGF-treated samples, while full-length FAK was not detected (Figure 3.10). Thus, at first glance it appears that full-length FAK was not tyrosine-phosphorylated in these HOSCC cell lines. However, this is highly unlikely. Aronsohn *et al.* (2003) made a similar observation when immunohistochemically staining laryngeal carcinoma specimens as they expected to see large amounts of cytoplasmic FAK pY397 staining – however, this was not the case. They concluded that the presence of proteins that physically associate with activated FAK may mask the antigen that reacts with anti-

FAK pY397 (Aronsohn *et al.*, 2003). Proteins such as Src, PI3K, Grb2, and Grb7 are known to bind to tyrosine-phosphorylated residues on FAK (van Nimwegen and van de Water, 2007). Therefore, it is similarly concluded in this study that within the HOSCC cell lines, FAK-associated proteins may block, or mask, the tyrosine residues (on full-length FAK) that would bind to the anti-phosphotyrosine antibody, therefore leading to an apparent lack of immunoreactivity.

Currently, it is not known what role (if any) the 85 kDa variant of FAK plays in cellular signalling in HOSCC. Early studies report that FERM-domain truncations of FAK yield proteins with increased tyrosine phosphorylation (Chan *et al.*, 1994; Schlaepfer and Hunter, 1996; Lim *et al.*, 2008). Mutations within the binding surface of the FAK FERM or kinase domains can generate activated FAK constructs. The binding of proteins or phospholipids to the FAK FERM domain can increase FAK Y397 phosphorylation in these mutated constructs (Poullet *et al.*, 2001; Dunty *et al.*, 2004; Lietha *et al.*, 2007; Cai *et al.*, 2008; Lim *et al.*, 2008). This may explain why the 85 kDa variant appears to be tyrosine-phosphorylated in the HOSCC cell lines. As the 85 kDa variant does not contain the FAT sequence, it also does not contain all of the tyrosine residues that are required for binding to other proteins such as Grb2, paxillin, talin, tensin, and vincullin (Zhao *et al.*, 1998; Brakebusch and Fässler, 2003; Crowe and Ohannessian, 2004; van Nimwegen and van de Water, 2007). Therefore, this variant would not be able to mediate all of the signalling and scaffolding functions required of full-length FAK. As full-length FAK was present in the HOSCC cell lines, protein-binding interactions and cellular functions would preferentially be mediated through the full-length protein rather than the 85 kDa cleavage product. Thus, the few tyrosine residues that are present in the 85 kDa isoform in the HOSCC cell lines may not be bound by other proteins, leaving them free to interact with the anti-phosphotyrosine antibody.

The tyrosine phosphorylation levels of the 85 kDa variant are variably affected by EGFR activation across the HOSCC cell lines (Figure 3.11). The full-length and 85 kDa FAK variants may associate with EGFR at the cell membrane via the FERM domain; therefore, EGFR may directly affect the tyrosine phosphorylation levels of

both variants in the HOSCC cell lines. EGFR and FAK are jointly involved in modifying cell-ECM interactions in order to promote cell migration, although reports differ on whether FAK phosphorylation and activation are required for this process (Sieg *et al.*, 2000; Hauck *et al.*, 2001).

Studies conducted in human carcinoma cell lines have reported that EGF-stimulated cell proliferation and migration requires an increase in FAK tyrosine phosphorylation (Brunton *et al.*, 1997; Hauck *et al.*, 2001). EGF-induced phosphorylation of FAK leads to the recruitment of Src to focal adhesions, leading to EGF-stimulated JNK and MAPK activation which are required for cell proliferation and migration (Brunton *et al.*, 1997; Hauck *et al.*, 2001). In contrast, in the human epidermoid carcinoma cell line A431, FAK was rapidly dephosphorylated following treatment with EGF (Lu *et al.*, 2001). Deactivation of FAK led to its loss from focal adhesions, resulting in focal adhesion disassembly, detachment of cells from the ECM, as well as enhanced cell motility, invasion, and metastasis (Lu *et al.*, 2001). However, once cells started reattaching to the ECM, integrin signalling restored FAK activity, which could not be attenuated by further EGF stimulation (Lu *et al.*, 2001). An initial decrease in FAK phosphorylation leading to cell detachment and increased motility, followed by integrin-reactivation during re-adhesion, is required for EGF-induced cell migration, invasion, and metastasis (Lu *et al.*, 2001; Yamanaka *et al.*, 2003).

Other researchers suggest that FAK may promote cell motility via its adaptor protein function by linking upstream signals to downstream effector proteins, and that it is FAK expression, but not its kinase activity, that is required for EGF-stimulated cell motility (Sieg *et al.*, 2000; Carragher and Frame, 2004). However, tyrosine phosphorylation of FAK is still required in order to create binding sites for these downstream signalling molecules (Sieg *et al.*, 2000). FAK phosphorylation in response to EGF may be cell type specific, suggesting that phosphorylation of FAK is not directly related to its role in cell movement (Park *et al.*, 2007).

The influence of EGFR signalling on FAK expression and activation is complex. To our knowledge, this is the first study detailing the effects of EGF stimulation on the

expression, cellular localization, and phosphorylation of FAK in HOSCC cell lines. Further data needs to be generated in order to construct a complete picture of the role of EGFR/FAK complex signalling in HOSCC tumour progression. The expression of both FAK and EGFR are upregulated in cancers of multiple origins (Agochiya *et al.*, 1999; He *et al.*, 2006). Therefore, cancer treatments targeting FAK and/or EGFR may exert positive results across the spectrum of cancer types.

4.4 Clinical trials are exploring the advantages of FAK-specific cancer therapies

Despite improvements in surgical techniques and chemotherapy, the prognosis of patients with HOSCC remains poor (Kawaguchi *et al.*, 2007). Molecular-targeted therapy using small inhibitors of tyrosine kinases or humanised monoclonal antibodies are needed for HOSCC patients (Kawaguchi *et al.*, 2007). FAK has been causally implicated in tumour development, and contributing effects may stem from cell cycle and survival signalling, effects on cell adhesion molecules, migration and invasion, MMP production, and on other cancer-related processes (McLean *et al.*, 2005). As FAK is a strong mediator of survival signalling, tumour cells that overexpress FAK could be more resistant to classic anti-cancer therapies (van Nimwegen and van de Water, 2007). The causal nature of FAK in tumour development, as well as numerous reports correlating FAK expression with poor clinical outcome, indicates that targeting and inhibiting FAK may prove to be a useful anti-cancer strategy (McLean *et al.*, 2005).

Doxorubicin treatment of the rat mammary adenocarcinoma cell line MTLn3 induces the formation of well-defined focal adhesions and stress fibres in association with early activation of PKB, later followed by focal adhesion loss during the onset of apoptosis (van Nimwegen *et al.*, 2006). As mentioned earlier, the non-catalytic C-terminal domain of FAK termed FAK-related non-kinase (FRNK) can be expressed independently of the entire FAK protein through alternative splicing of the FAK gene (Parsons, 2003; Cox *et al.*, 2006). FRNK acts an inhibitor of FAK as it competes with FAK for focal adhesion localization (Nagoshi *et al.*, 2006).

Expression of FRNK sensitizes MTLn3 cells to doxorubicin-induced apoptosis, as FRNK interferes with the focal adhesion localization of FAK, thereby inhibiting focal adhesion signalling and turnover (van Nimwegen *et al.*, 2006). Inhibition of PI3K with wortmannin as well as transient overexpression of PTEN (both of which inhibit doxorubicin-induced activation of PKB) enhances doxorubicin-induced cell death (van Nimwegen *et al.*, 2006).

Reportedly, extensive effort is being put into the development of tyrosine-kinase inhibitors as cancer treatments, although, in the case of FAK, the requirement for kinase activity in the development of cancer has not been unequivocally proven (McLean *et al.*, 2005). This is in part due to studies that have shown that FAK regulation of cell migration and invasion cannot be entirely attributed to its kinase function (Cary *et al.*, 1996; Sieg *et al.*, 2000; Lu *et al.*, 2001; McLean *et al.*, 2005). Therefore, it is not clear whether targeting the kinase domain of FAK will have any therapeutic advantage (McLean *et al.*, 2005).

Besides possessing kinase activity, FAK is also an important molecular scaffold in many protein complexes. Blocking the interaction between FAK and its chief binding partners may serve as a key therapeutic strategy (McLean *et al.*, 2005). For instance, the interaction between signalling molecules and the Y397 and Y925 tyrosine residues on FAK may be targeted in order to blockade downstream signalling pathways (van Nimwegen and van de Water, 2007). However, this approach has not had much success due to relatively low-affinity interactions and other problems (McLean *et al.*, 2005). *In vivo*, other kinases (such as Src) could take over the function of FAK, still resulting in the activation of the downstream signalling pathways (van Nimwegen and van de Water, 2007).

RNA interference (RNAi) may be a valuable therapeutic tool, as there is some evidence that FAK small interfering RNA (siRNA) inhibits the spread of pancreatic cancer in mice (McLean *et al.*, 2005). FAK siRNA causes gemcitabine-induced cytotoxicity *in vivo* and increases tumour susceptibility to chemotherapy (Duxbury *et*

al., 2003). Therefore, therapeutic approaches could be useful in combination with cytotoxic agents (Duxbury *et al.*, 2003; McLean *et al.*, 2005).

In HOSCC, specific therapies have been designed against EGFR and VEGF, while new genetic therapies are being developed in order to restore the physiological function of p53 (Gibault *et al.*, 2005). Much attention has recently been given to the development of clinical therapies against EGFR (Kawaguchi *et al.*, 2006). EGFR can be targeted by specific monoclonal antibodies such as *IMC-C225*, which is targeted against the extracellular domain of EGFR (Gibault *et al.*, 2005). Phase-II and –III trials are being conducted with this molecule (in conjunction with chemotherapy) in patients with colorectal, head, or neck cancers (Gibault *et al.*, 2005). SH2 inhibitors are also currently being developed. They may prevent Grb2 binding to EGFR, thereby prevent downstream activation of Ras and limiting the growth of tumour cells (McLean *et al.*, 2005).

Cetuximab (Erbitux™), a high-affinity anti-EGFR antibody, has been approved for use in patients with colorectal cancers (Kawaguchi *et al.*, 2006). This antibody is able to block the binding of EGFR ligands, thereby inducing the internalization and downregulation of EGFR, inhibition of EGFR kinase activity, inhibition of cell cycle progression, and increased levels of pro-apoptotic molecules (Kawaguchi *et al.*, 2006). Cetuximab is able to induce antibody-dependent cellular cytotoxicity in EGFR-overexpressing HOSCC cells (Kawaguchi *et al.*, 2006). Thus the application of Cetuximab in the treatment of HOSCC is being encouraged and the enhancement of its antitumour effect may be necessary to properly treat HOSCC patients (Kawaguchi *et al.*, 2006).

4.5 FAK may be an important molecule contributing to the clinicopathological factors associated with HOSCC

Human oesophageal squamous cell carcinoma (HOSCC) exhibits particularly aggressive behaviour and is the most common cancer occurring in black South African men (Hu *et al.*, 1999; McCabe and Dlamini, 2005). Despite improvements in

surgery, chemotherapy, and radiotherapy, the prognosis of HOSCC remains poor as the exact molecular pathogenesis of the disease is still unknown (Hu *et al.*, 1999; Kawaguchi *et al.*, 2006). FAK contributes to many aspects of cancer cell behaviour, many of which have been discussed here. Based on the data presented in this study, it is suggested that modifiers of FAK expression (such as EGFR) may well contribute to the aggressive malignant behaviour that is characteristic of HOSCC. Further study may confirm whether FAK expression and signalling are central to the progression of HOSCC, thereby leading to the development of FAK-specific therapeutic targets in this and other squamous cell cancers.