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The investigation of the integrative function of focal adhesion kinase (FAK) in human oesophagel squamous cell carcinoma cell lines

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CHAPTER 2: PROTEIN EXPRESSION AND LOCALIZATION OF FAK IN HOSCC CELL LINES

2.1 Introduction

FAK is a non-receptor protein tyrosine kinase (PTK) that acts in response to integrin clustering at focal adhesions which are prominent in cells grown in tissue culture (Gabarra-Niecko *et al.*, 2003; McLean *et al.*, 2005; Nagoshi *et al.*, 2006). The human *fak* gene is located on chromosome 8q24 and was initially isolated from a primary human sarcoma, while the 125 kDa FAK protein was originally discovered in v-Src-transformed chicken embryo fibroblasts (Schaller *et al.*, 1992; Weiner *et al.*, 1993; Gabarra-Niecko *et al.*, 2003; Lark *et al.*, 2003). FAK consists of a central catalytic kinase domain situated between the N- and C-terminal non-catalytic domains (refer to Figure 1.1) (Crowe and Ohannessian, 2004; Dunty *et al.*, 2004; Schlaepfer and Mitra, 2004). The N-terminal FERM domain mediates protein-protein interactions with the cytoplasmic domains of integrins and growth factor receptors, while the C-terminal FAT sequence is required for focal adhesion localization as it binds to the cytoplasmic tails of integrins, linking them to other focal adhesion proteins such as talin and paxillin (see Figures 1.1, 1.2 and 2.1) (Toutant *et al.*, 2000; Gabarra-Niecko *et al.*, 2003; Crowe and Ohannessian, 2004; Cox *et al.*, 2006; van Nimwegen and van de Water, 2007).

Invading and metastasizing cancer cells move through changing tissue environments and come across new matrix components (Hanahan and Weinberg, 2000). In response to these new ECM ligands, cancer cells may alter the types of integrins they express, thereby favouring ones that promote proliferation signalling in their new environment (Troussard *et al.*, 1999; Hanahan and Weinberg, 2000; Brakebusch and Fässler, 2003). For instance, in some melanomas and breast carcinomas, expression of the αvβ3 and α6β4 integrins is associated with increased cell invasion and metastasis (Playford and Schaller, 2004). Cell adhesion to the ECM influences the way in which FAK contributes to cellular signalling through regulation of its tyrosine phosphorylation state, activity, and cellular localization (Cohen and Guan, 2005a). Following phosphorylation and activation downstream of integrin signalling, FAK

acts as a platform for the assembly of signal transduction cascades implicated in cell proliferation, survival, and motility (Ilić *et al.*, 1997; Gabarra-Niecko *et al.*, 2003). Aberrant FAK expression and activity leads to upregulation of these signalling pathways and alters the phenotype of cancer cells (McLean *et al.*, 2005).

FAK generally localizes to focal adhesions within the cell membranes of adherent cells, although it is not restricted to these sites in all cell types (Ilić *et al.*, 1997; Toutant *et al.*, 2000; McLean *et al.*, 2005). In neurons, FAK is distributed throughout the cell, while in astroglia FAK is mainly associated with the cytoskeleton (Zhang *et al.*, 1994; Grant *et al.*, 1995; Ilić *et al.*, 1997). Nuclear localization of FAK has been detected in endothelial and glioblastoma cells, as well as in leukaemia and laryngeal squamous cell carcinoma specimens (Lobo and Zachary, 2000; Jones *et al.*, 2001; Aronsohn *et al.*, 2003; Jones and Stewart, 2004). However, because of the absence of a well-defined nuclear localization signal in FAK, the mechanism by which this occurs is unknown (Lobo and Zachary, 2000; Jones *et al.*, 2001). Thus, it appears that the localization of FAK may be cell-type specific.

The cellular localization of FAK influences various signalling cascades, especially those involved in controlling cell migration (Hsia *et al.*, 2003). Cell migration is vital for normal cellular processes such as embryonic development and wound healing, and is also required for the invasion and metastasis of cancer cells (Stewart *et al.*, 2004; Tilghman *et al.*, 2005). Integrin signalling promotes cell migration by inducing changes in cytoskeletal organization leading to increased cellular contractility (Brakebusch *et al.*, 2002). These cytoskeletal alterations involve a dynamic cycle of focal adhesion assembly, actin reorganization, and focal adhesion disassembly (Carragher and Frame, 2004). FAK plays an integral role in cell migration as it is required for the spatial organization of the leading edge (a protrusion in the cell membrane in the direction of cell movement) in migrating cells (Tilghman *et al.*, 2005; McLean *et al.*, 2005; van Nimwegen and van de Water, 2007). At the leading edge of the cell, integrin clustering stimulates Src and FAK to form a complex as they are recruited to the developing focal adhesions (Figure 2.1) (Carragher and Frame, 2004). The FAK-Src complex then stimulates actin polymerization via the Rho-GTPases, resulting in the extension of lamellipodia, which are cytoskeletal actin

projections which drive cell migration at the leading edge of the cell (Figure 2.1) (Small *et al.*, 2002; Carragher and Frame, 2004; Faried *et al.*, 2006). While more stable focal adhesions are located at the cell periphery, smaller short-lived focal complexes occur within cell membranes at the focal contacts in the lamellipodia (Zaidel-Bar *et al.*, 2003). Following lamellar extension at the leading edge, focal adhesion disassembly occurs at the trailing edge of the cell as the integrins in this region detach from the ECM (Carragher and Frame, 2004). This disassembly and detachment (otherwise known as focal adhesion turnover) occurs because the FAK-Src complex influences the MAPK pathway, which enhances calpain and caspase proteolytic activity and hence further focal adhesion deconstruction (Figure 2.1) (Cooray *et al.*, 1996; Carragher *et al.*, 2001; Carragher and Frame, 2004; van Nimwegen and van de Water, 2007).

FAK was first implicated in tumourigenesis when researchers observed that it was one of several highly tyrosine-phosphorylated proteins in Src-transformed fibroblasts (Kanner *et al.*, 1990; Schaller *et al.*, 1992; Miyazaki *et al.*, 2003). Increased FAK gene dosage may also be a common feature in tumours as gene amplification occurs in squamous cell carcinomas of the head and neck, breast, and colon (Gabarra-Niecko *et al.*, 2003). In early studies of FAK expression, elevated levels of FAK mRNA were initially linked to the progression of epithelial and mesenchymal tumours to metastatic phenotypes (Weiner *et al.*, 1993; Lark *et al.*, 2003). The precise role that FAK plays in cancer progression may be as a result of deregulation at many levels, as malignancy is often linked to aberrations in FAK-regulated processes such as cell spreading, proliferation, and survival (Gabarra-Niecko *et al.*, 2003).

FAK itself has not been classified as an oncogene, although elevated protein levels have been reported in a broad spectrum of invasive cancers, including those of the colon, breast, thyroid, liver, oral cavity, ovary, as well as many other invasive squamous cell carcinomas (Owens *et al.*, 1995; Brunton *et al.*, 1997; Agochiya *et al.*, 1999; Aronsohn *et al.*, 2003; Gabarra-Niecko *et al.*, 2003). Increased FAK expression and activity is commonly associated with metastasis and poor patient prognosis (Owens *et al.*, 1995; Schlaepfer *et al.*, 2004; McLean *et al.*, 2005).

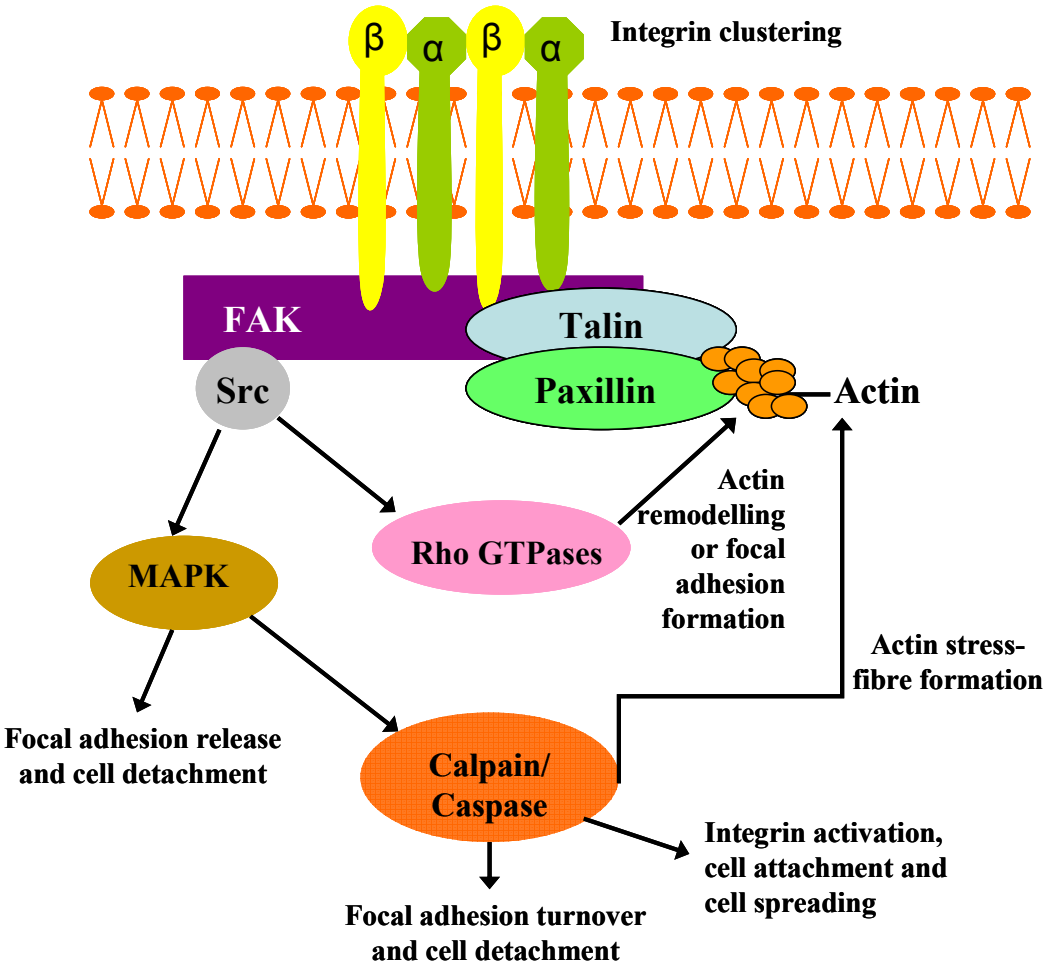


Figure 2.1: Integrin-mediated signalling via FAK results in focal adhesion turnover and cell migration. Cell movement involves a dynamic cycle of focal adhesion assembly, actin reorganization, and focal adhesion disassembly. The FAK protein is a key regulator of focal adhesion turnover. The FAK/Src complex activates the Rho-GTPases which are responsible for remodelling the actin cytoskeleton, leading to focal adhesion turnover. FAK activation of MAPK leads to the activation of proteases which cleave focal adhesion components and further promote focal adhesion turnover.

Although reports show that high levels of FAK expression are associated with human cancers in general, there have been very few investigations on FAK expression in human oesophageal squamous cell carcinoma (HOSCC) (Miyazaki *et al.*, 2003; McLean *et al.*, 2005). Miyazaki *et al.* (2003) investigated FAK expression in HOSCC by immunohistochemical analysis of formalin-fixed, paraffin-embedded specimens. They detected strong FAK immunostaining in the cytoplasm of all HOSCC cells, especially in cells located in the invasive fronts (Miyazaki *et al.*, 2003). They also determined that the mean 5-year survival rate of patients with FAK-overexpressing cancer (38%) was drastically lower than those of patients who did not have FAK-overexpressing cancer (69%). They concluded that FAK overexpression was connected to tumour invasiveness and lymph node metastasis in HOSCC (Miyazaki *et al.*, 2003).

Enquiries into FAK expression and localization in HOSCC have thus far been limited to immunohistochemical analysis of paraffin-embedded specimens, while studies on FAK have not been conducted on actively growing HOSCC cells in culture. This component of the study focuses on the expression and cellular localization of the FAK protein in 5 cell lines derived from moderately differentiated human oesophageal squamous cell carcinomas.

2.2 Methods and Materials

2.2.1 Cell lines

Five South African human oesophageal squamous cell carcinoma (HOSCC) cell lines were obtained from the Cell Biology Research Laboratory, School of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg. These cell lines were derived from moderately differentiated carcinomas obtained from South African patients with well-advanced cancer. These cell lines are termed WHCO1, WHCO3, WHCO5, WHCO6, and SNO (Veale and Thornley, 1989). The cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/Hams F12 (3:1) solution supplemented with 10% foetal calf serum (FCS). The cells were incubated at 37˚C with humidity, with 5% carbon dioxide (CO_2) in air in order to mimic *in vivo* tissue conditions. Trypsin/ethylenediaminetetra-acetic acid (EDTA) was used to harvest the cells (see Appendix 5.1 for all tissue culture solutions).

2.2.2 Antibodies

Polyclonal rabbit anti-FAK primary antibody (Santa Cruz) and a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (Sigma) were used for western blotting. All antibodies were diluted accordingly in phosphate-buffered saline (PBS) (see Appendix 5.2).

2.2.3 Preparation of whole-cell lysates

HOSCC cell lines were cultured to 80% confluency before the extraction procedure commenced. The cells were rinsed three times in PBS and were harvested into sterile eppendorfs. The samples were centrifuged (1 200 x g) (5 min) in a Tomy Capsule HF-120 bench top centrifuge. After discarding the supernatant, Laemmli lysis buffer (pH 6.8) (Laemmli, 1970) (see Appendix 5.3) was added to the cell lysates before heating them in a boiling water bath (5 min). The samples were centrifuged (12 000 x g) for 20 min at 4ºC and were subsequently stored at –70ºC.

2.2.4 Protein estimation

An estimation of the protein concentration of each sample was conducted as described by Bramhall *et al.* (1969). A Whatman filter paper disc was rinsed in distilled water (dH_2O) (20 min) and was then dehydrated by rinsing it in 95% ethanol (5 min), 99.99% ethanol (5 min), and acetone (5 min). The filter paper disc was air-dried in an extraction hood. Whole-cell lysates of the HOSCC cell lines (2 µl), as well as bovine serum albumin (BSA) samples of known concentration in Laemmli lysis buffer (Laemmli, 1970) (2 µg/µl BSA) equal to 1 µg, 3 µg, 6 µg, 12 µg, 16 µg, and 20 µg, were spotted onto the dried filter paper. These spots were then air-dried and disc was placed in 7.5% tri-chloroacetic acid (TCA) (40 min) in order to fix the proteins onto the filter paper. The filter paper disc was then placed into Coomasssie Blue solution (1 hr) and was then appropriately destained. Each dried protein sample spot was placed into 5 ml of elution solution which was subsequently placed in the dark overnight in order to elute the Coomassie Blue stain bound to the protein (see Appendix 5.8 for all solutions used in the protein estimation procedure). The absorbance of each sample solution was determined by a Beckman DU-64 spectrophotometer at a wavelength of 596 nm. A standard curve of absorbance against protein concentration was constructed using the absorbance readings obtained for the varying BSA standards. The protein concentrations of the samples were then determined from the standard curve.

2.2.5 Separation of proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Each protein sample was separated on a 10% SDS-PAGE gel using the Laemmli (1970) discontinuous buffer system. The Mighty Small™ SE245 Dual Gel Caster (Hoefer Scientific) was used to cast the SDS-PAGE gel. The 10% separating gel was prepared by mixing together acrylamide, separating buffer (pH 8.8), *N',N',*-methylenebis-acrylamide (Bis) solution, sodium dodecyl sulphate (SDS) solution, and dH_2O (see Appendix 5.9 for all solutions used in SDS-PAGE). Immediately before casting the gel, ammonium persulphate (APS) and *N',N',N',N'*-tetramethylene-diamine (TEMED) were added to the separating gel solution in order

to catalyze gel polymerization. The gel was cast between the gel plates and a little 0.1% SDS was laid on top of the separating gel in order to enhance gel polymerization by placing a barrier between the gel and the oxygen in the surrounding air. The separating gel was allowed to polymerize at room temperature for approximately 20 min. Unpolymerized gel and the 0.1% SDS overlay was removed prior to pouring the stacking gel.

The 5% stacking gel was prepared by mixing together acrylamide, stacking buffer (pH 6.8), Bis solution, SDS solution, and dH_2O. APS and TEMED were added just prior to pouring the gel to catalyze polymerization. A 10-well 20 µl comb was placed between the gel-casting plates and the gel was allowed to polymerize (±20 min). The polymerized gel was placed in the Mighty Small™ Electrophoresis Unit which contained the reservoir buffer (pH 8.3). The comb was removed as the wells were filled with reservoir buffer. Various volumes containing 20 µg of each protein sample were loaded into adjacent wells in the gel. The molecular weight (MW) marker (PageRuler™ Prestained Protein Ladder) (Fermentas) (2.5 µl) was loaded into the first well. Electrophoresis was conducted at a constant current (25 mA) for approximately 1 hr. Separated proteins were stained in Coomassie Blue stain for 1 hr, and were then destained appropriately. The gel was scanned using a Hewlett Packard ScanJet 5200c (see Appendix 5.9 for all solutions used in SDS-PAGE).

2.2.6 Western blot analysis

Protein samples were separated on a 10% SDS-PAGE, as described above. Separated proteins were then transferred to Hybond-C nitrocellulose membrane (Amersham Life Science) for 3 hrs using a Bio-Rad Criterion™ Blotter (Bio-Rad) containing western blot transfer buffer (pH 8.3) (see Appendix 5.10 for all solutions used in the western blot procedure). The nitrocellulose membrane was then rinsed and stored in PBS overnight at 4ºC. The following day, the membrane blot was blocked in a milk-powder based blocking buffer (Blotto) for 1 hr at room temperature. Subsequently, the blot was incubated in polyclonal rabbit anti-FAK primary antibody (1:400) (1 hr at room temperature) and was rinsed 6 times (5 mins per rinse) in PBS to remove any unbound primary antibody. The blot was then incubated in HRP-conjugated goat

anti-rabbit secondary antibody (1:5000) (1 hr at room temperature in the dark). Unbound secondary antibody was rinsed off with PBS (6 times for 5 mins each rinse). The SuperSignal® West Pico Chemiluminescent Substrate kit (Pierce), consisting of a luminol:peroxide (1:1) solution, was used to detect the presence of the secondary antibody bound to the nitrocellulose membrane blot. The blot was exposed to Hyperfilm™ MP autoradiography film (Amersham). (Note: all antibodies were diluted accordingly in PBS).

2.2.7 Densitometry

Densitometric analysis of the western blot was conducted using LabWorks™ Image Acquisition and Analysis Software (Version 4.5), which is able to select the bands of interest while eliminating the effects of poor background and smearing problems from affecting the results. Levels of relative protein concentration or expression were presented as a percentage of the maximum protein expression observed (100%). The results of the densitometric assessment of the western blots were represented graphically.

2.2.8 Indirect Immunofluorescence

HOSCC cells from exponentially growing cultures were seeded onto sterile glass cover-slips and were permitted to adhere to the cover-slips for approximately 24 hrs. Following this, tissue culture medium was aspirated and the cells were rinsed 3 times with PBS. The cells were fixed in 4% paraformaldehyde (30 min) before being permeabilized by 0.25% Triton X-100 (10 min) (see Appendix 5.11 for all solutions used for indirect immunofluorescence). The cells were rinsed twice in PBS and then once in dH_2O before being allowed to air-dry. Wells were drawn onto the cover-slips with a DAKO pen, and the cells within these wells were rehydrated with PBS (30 min). The experimental wells were incubated in anti-FAK primary antibody (20 µl of 1:50 dilution) while the negative control wells were incubated in 20 µl of PBS (1 hr at room temperature). In order to remove any unbound primary antibody, all the samples were rinsed 5 times with PBS (1 min per rinse) before being incubated in fluorosceine isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody (20 µl

of 1:100 dilution) for 1 hr in the dark (at room temperature). Unbound secondary antibody was removed by rinsing the cells 5 times with PBS (1 min per rinse) prior to the addition of the anti-bleach alvanol/ρ-phenylenediamine solution (10 μl) to each well. The cover-slips were placed onto glass slides, and the cells were viewed immediately with a Zeiss LSM 410 Confocal Microscope (the FITC excitation wavelength is 488 nm and the emission wavelength is 525 nm). (Note: all antibodies were diluted accordingly in PBS).

2.3 Results

2.3.1 FAK protein is expressed in 5 moderately differentiated HOSCC cell lines

The presence of the FAK protein in the 5 HOSCC cell lines was determined by western blot analysis. Prior to western blotting, resolution of whole-cell lysates on a 10% SDS-PAGE served as visual confirmation of the accuracy of the protein estimation (this can be seen in Figure 2.2 as the degree of Coomassie Blue staining in each lane is the same) (see Appendix 5.13 for the standard curve generated for the protein estimation). SDS-PAGE analysis of the whole-cell lysates detected the presence of numerous "bands" which are representative of different polypeptides ranging from higher molecular weights towards the top of the gel to lower molecular weights at the bottom of the gel (Figure 2.2).

Western blotting of the whole-cell lysates resolved one polypeptide band in each cell line, which was representative of the 125 kDa FAK protein (Figure 2.3) (see Appendix 5.14 for the standard curve generated of the log relative MW versus distance migrated by the molecular weight marker within the 10% SDS-PAGE gel). Subsequent densitometric analysis of the western blot showed that the WHCO6 cell line had the highest level of FAK protein expression in comparison to the other cell lines (Figure 2.3). The level of FAK protein expression in the four other cell lines was calculated as a percentage of the maximum protein expression found in WHCO6 (taken as 100%). As seen in Figure 2.3, the WHCO5 cell line expressed the second highest level of FAK protein (66%), while WHCO1 and SNO had relatively similar FAK protein expression levels of 35% and 34% respectively. Although the band in the WHCO3 lane is only faintly visible, the LabWorks™ densitometry software program was able to detect its presence and determine its relative protein expression level. WHCO3 displayed the lowest relative level of only 11% FAK protein expression when compared to the other cell lines (Figure 2.3) (see Appendix 5.15 for raw densitometric data of FAK expression in whole-cell lysates).

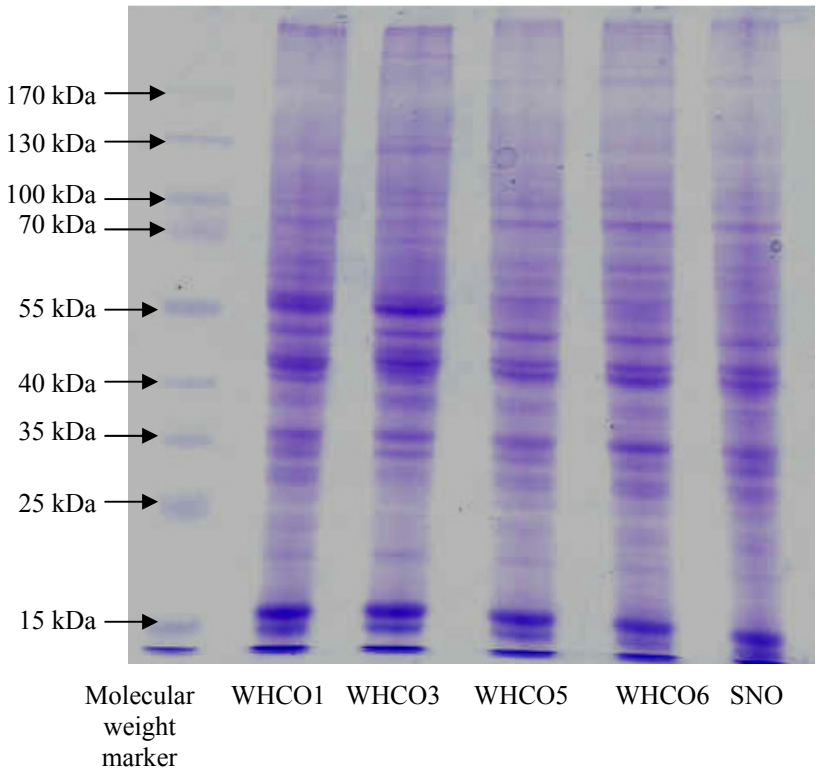


Figure 2.2: Whole-cell lysates of HOSCC cell lines resolved by SDS-PAGE. The occurrence of multiple protein bands of similar intensity throughout the lanes representative of each HOSCC cell line indicates that the samples were extracted correctly and that the protein estimation was accurate.

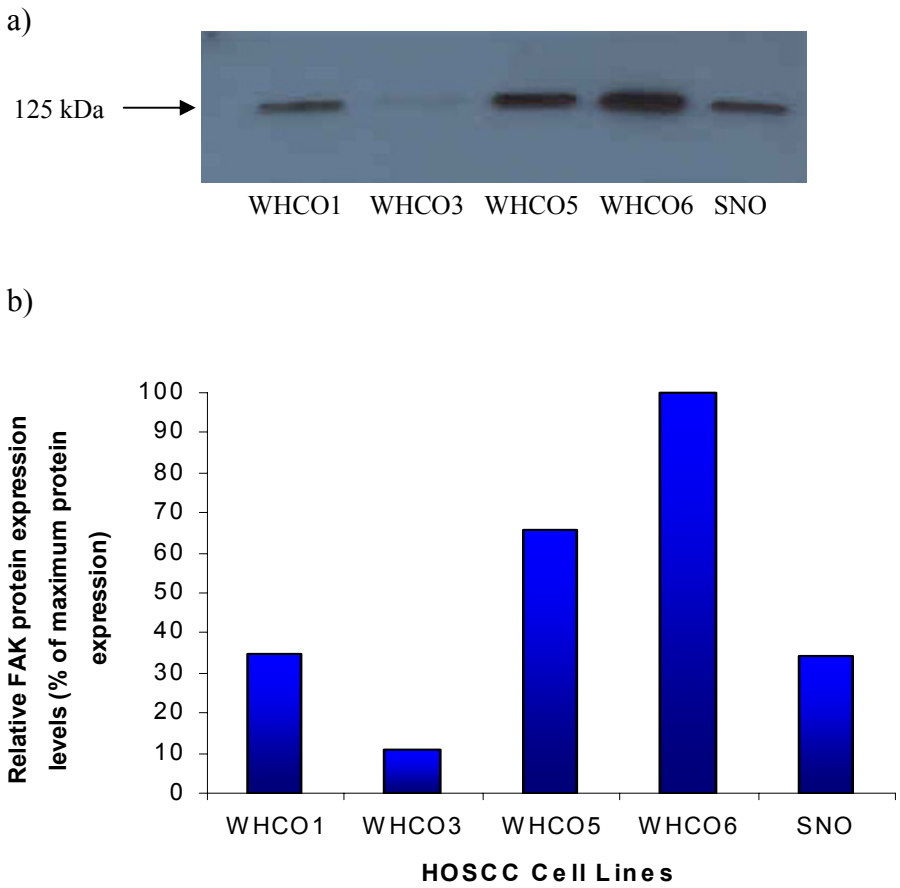


Figure 2.3: The FAK protein is present in whole-cell lysates of HOSCC cell lines. Whole-cell lysates (20 μg) were resolved using 10% SDS-PAGE, and were subsequently transferred to nitrocellulose membrane for western blot analysis. The nitrocellulose membrane was immunoblotted using an antibody raised against a peptide mapped against the N-terminus of the FAK protein. The 125 kDa FAK protein was present at different intensities in whole-cell lysates of all 5 HOSCC cell lines. a) FAK-specific western blot of whole-cell lysates. b) Graphical representation of relative FAK protein expression levels as determined by densitometric analysis of the western blot.

2.3.2 Cellular distribution of FAK in HOSCC cell lines

Immunofluorescent images (see Figures 2.4 and 2.5) provide a clear indication of FAK localization in the 5 HOSCC cell lines examined. Although the fluorescence portrayed in the immunofluorescent images is not considered as "staining" in the strictly histological sense, following common practice for the sake of simplicity this fluorescence will here be referred to as "staining".

In the cell membranes of adherent HOSCC cells, focal adhesions are prominent and it is at these sites that FAK is predominantly localized as indicated by the bright green fluorescent staining (Figures 2.4 and 2.5). In WHCO6, FAK-specific fluorescent staining is bright and quite noticeable at distinct focal adhesions within the cell membranes of the cells occurring around the periphery of the colony (see Figure 2.4 yellow arrow). FAK localization at focal adhesions was noted in WHCO1, WHCO3, WHCO5, and SNO to a lesser extent than in WHCO6. Due to the fact that HOSCC cells in culture generally prefer to grow in colonies, it is sometimes difficult to determine the distribution of FAK within single cells.

In all the cell lines (except WHCO5), FAK is visibly present within the focal contacts of the lamellipodia extending from the periphery of the cell colonies (see yellow arrows in Figures 2.4 and 2.5). In a cell culture context, cells occurring at the periphery of a colony have a free surface that is not attached to other cells. This is why lamellar extension is more noticeable here and FAK staining may be brighter in cells along the colony periphery. These FAK-containing focal contacts are most noticeable in WHCO6 where the cells have numerous lamellipodia (Figure 2.4). FAK-containing lamellipodia were not as clearly observed in WHCO5. Due to the close colony formation observed in WHCO5, the extension of lamellipodia possibly occurred to a lesser extent than in the other cell lines. FAK is also evidently located in the cytoplasm while very little or no FAK fluorescence is detected within the nuclei of the 5 HOSCC cell lines (Figures 2.4 and 2.5).

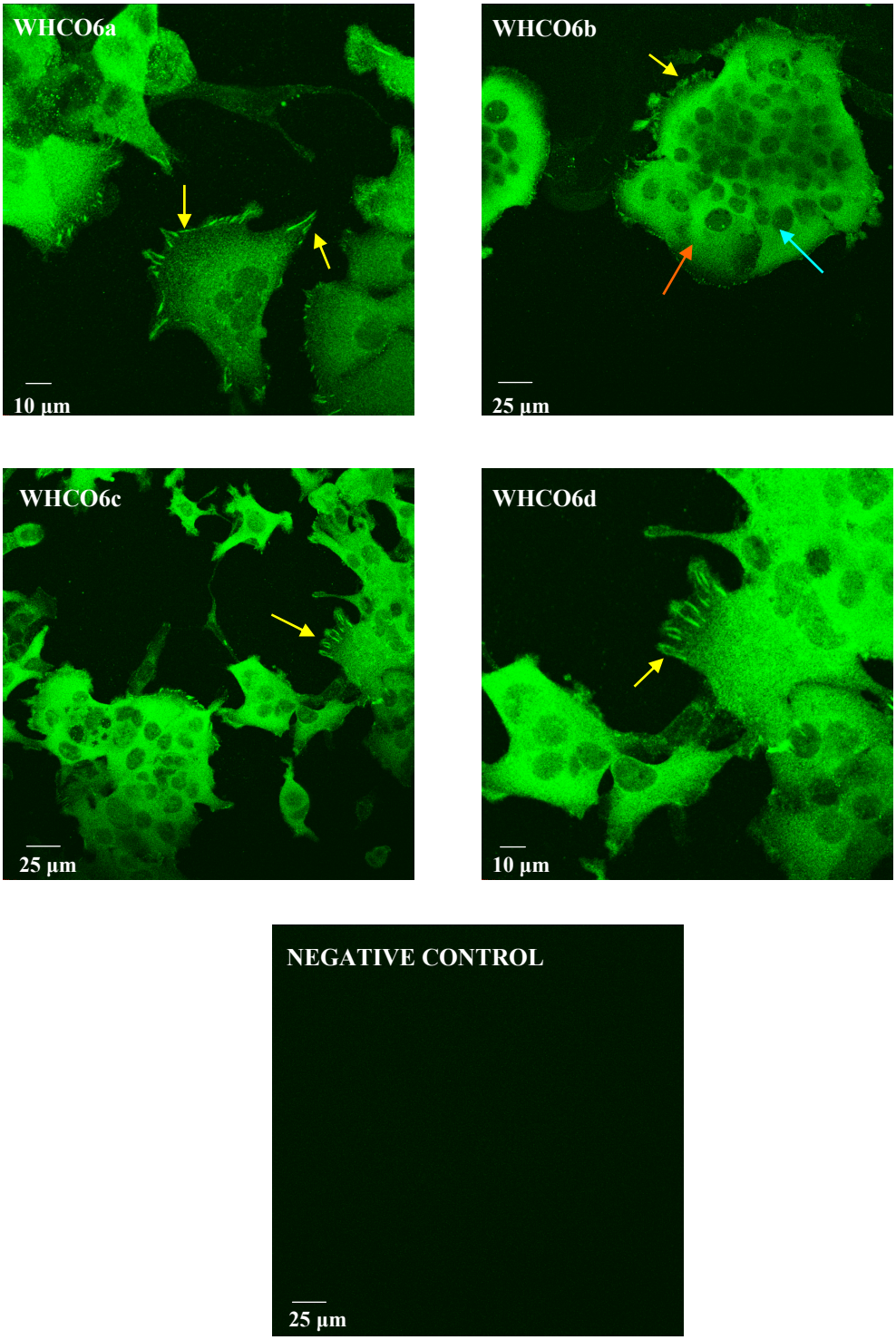
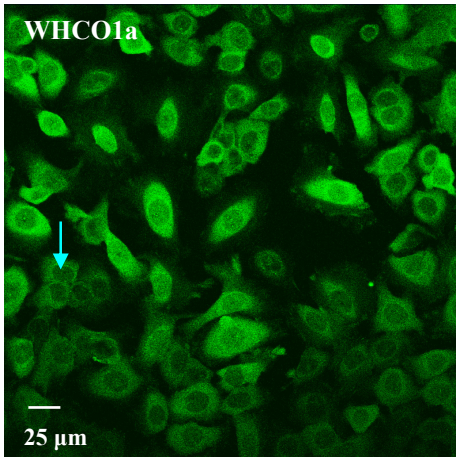
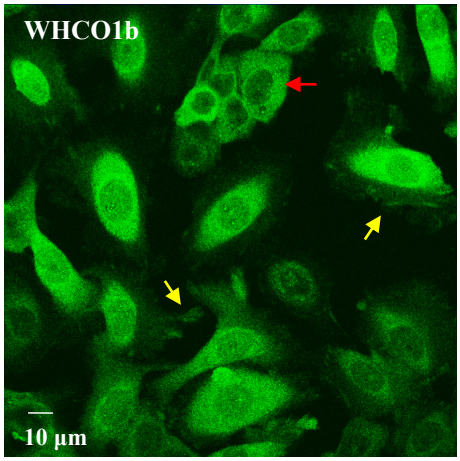
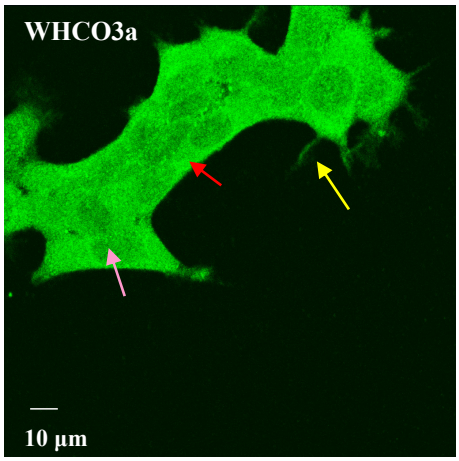
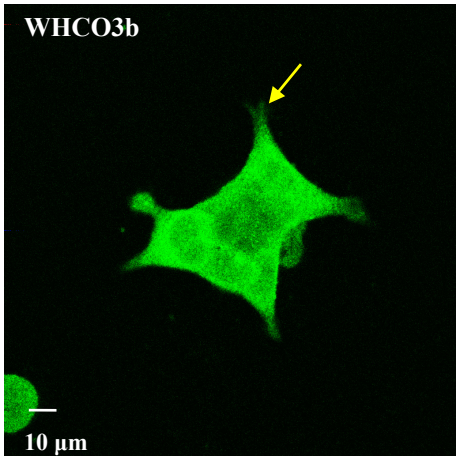


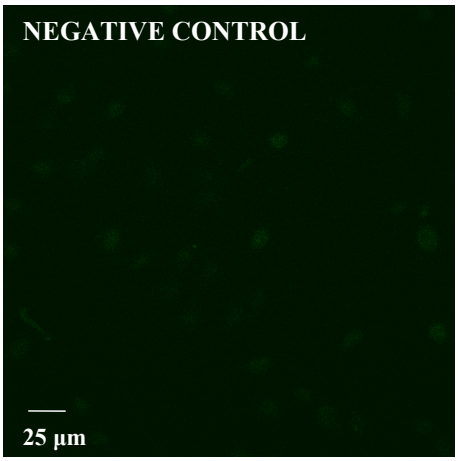
Figure 2.4: The cellular distribution of FAK in WHCO6 was examined by indirect immunofluorescence using an antibody targeted to the N-terminus of FAK. (a-d) Bright fluorescent staining indicates that FAK localizes predominantly to focal adhesions within the lamellipodia (yellow arrow) of migrating cells at the colony periphery. FAK is also present in the cytoplasm (orange arrow), while it appears to be almost entirely excluded from nuclei (blue arrow). WHCO6d is a magnified version of WHCO6c. Cells were viewed at 40x magnification.











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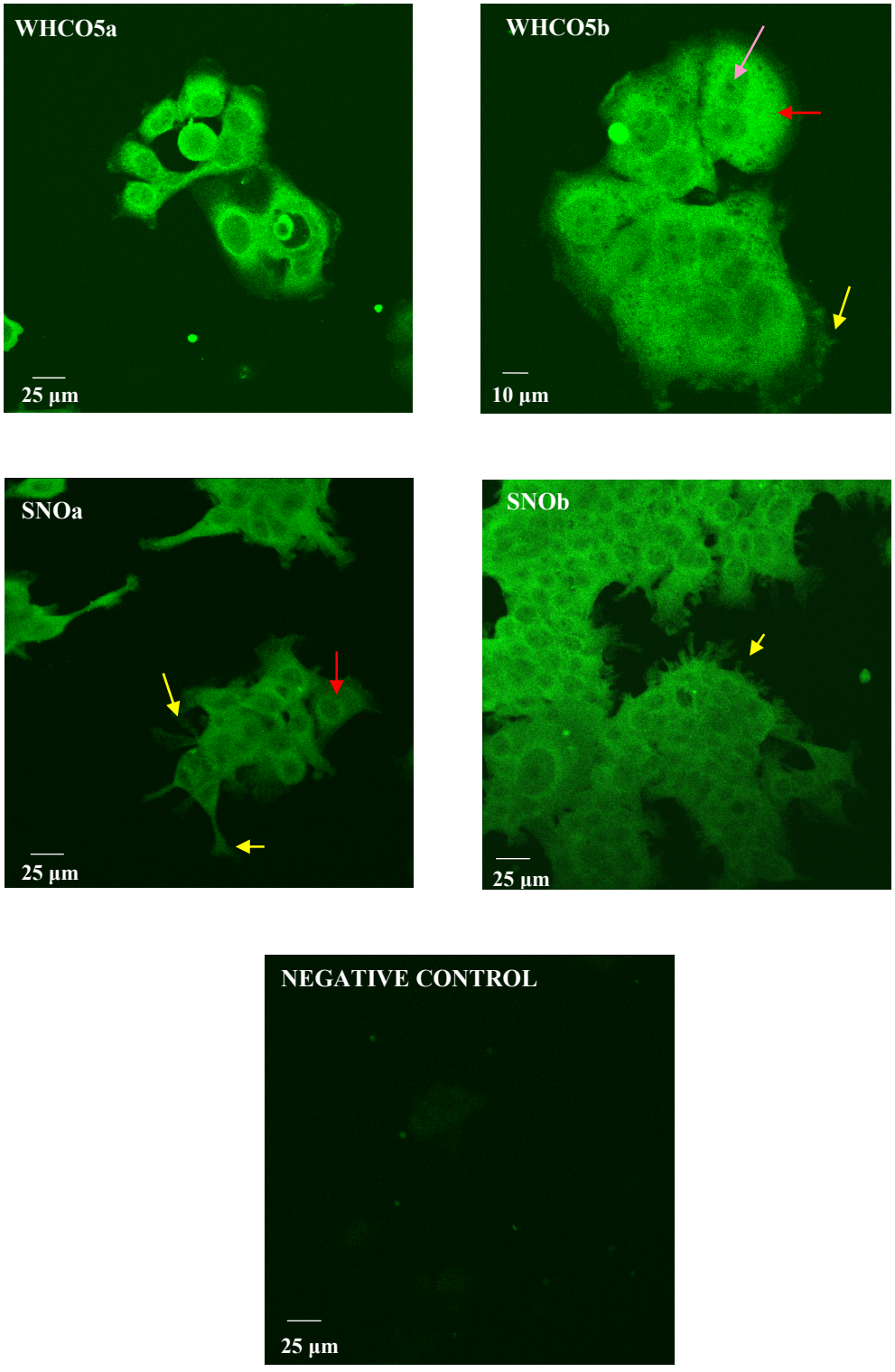


Figure 2.5: FAK-specific indirect immunofluorescence conducted on the WHCO1, WHCO3, WHCO5 and SNO HOSCC cell lines. A similar localization pattern was observed for WHCO1, WHCO3, WHCO5, and SNO as was seen in WHCO6. FAK was localized at focal adhesions (yellow arrow) of migrating cells, as well as in the cytoplasm (orange arrow). FAK appeared to be present in the nuclei in WHCO3 and WHCO5 (pink arrow), while being absent in the nuclei of WHCO1 (blue arrow). WHCO1b is a magnified version of WHCO1a. Cells were viewed at 40x magnification.

2.4 Discussion

FAK protein expression is often linked to clinicopathological factors including invasion, metastasis, and poor patient prognosis, which are associated with many types of tumours including HOSCC (Owens *et al.*, 1995; Kuwano *et al.*, 1997; Miyazaki *et al.*, 2003). In this study, it was demonstrated that the FAK protein is expressed in 5 human oesophageal squamous cell carcinoma cell lines derived from moderately differentiated tumours.

Western blotting indicates that the FAK protein is expressed in all five HOSCC cell lines at a relative molecular weight of 125 kDa (Figure 2.3). This corresponds to the size of full-length FAK reported in previously published literature (Schaller *et al.*, 1992). In order to compare the protein expression levels of FAK in the 5 HOSCC cell lines, densitometric analysis of the western blot was completed. This analysis revealed that WHCO6 has the highest level of FAK protein expression compared to the other four cell lines. In relation to WHCO6, WHCO5 demonstrated the second highest FAK protein expression level, while WHCO1 and SNO had much lower FAK expression levels. WHCO1 and SNO had comparatively similar levels of FAK expression. WHCO3 displayed the lowest relative level of FAK protein expression (Figure 2.3).

Distinctively varying levels of FAK protein expression were observed amongst the 5 cell lines even though they are all derived from oesophageal tumours of the same pathological grading (i.e. moderately differentiated). This may be as a result of the broad range of tumours included in the 'moderately differentiated' category, with some tumours being either more or less moderately differentiated than others within this category. Miyazaki *et al.* (2003) also observed differences in the FAK protein expression level by immunohistochemically staining well, moderately, and poorly differentiated HOSCC tumours. In their study, well differentiated tumours generally did not overexpress FAK, while most poorly differentiated tumours did when compared to normal epithelium of the same section. Approximately half of the moderately differentiated tumours included in the study overexpressed FAK (Miyazaki *et al.*, 2003). From their study it was concluded that the degree of tumour

differentiation of HOSCC cell lines either influences or is influenced by the level of FAK protein expression. Normal cultured oesophageal cells were not available for comparison in the data presented here on the WHCO and SNO cell lines, and thus a comparison of over- or under-expression compared to normal oesophageal cells could not be conducted. However, based on the observations noted by Miyazaki *et al.* (2003), it is plausible that because the level of FAK expression is greater in WHCO6 and WHCO5 than in the other cell lines, these cell lines may be derived from "more poorly" differentiated tumours (within the moderately differentiated category). Thus WHCO1, SNO, and WHCO3 which exhibited comparatively much lower levels of FAK expression may have been derived from better differentiated tumours.

What controls FAK expression and precisely how is it controlled in the HOSCC cell lines? Although FAK expression has been established in a number of malignancies, the particular mechanism(s) by which FAK expression is regulated requires further investigation. In a study conducted by Agochiya *et al.* (1999), *in situ* hybridization of cell lines derived from invasive squamous cell carcinomas revealed an increased dosage of the *fak* gene. In addition, the *fak* gene was elevated in frozen sections of the squamous cell carcinomas, proving that increased *fak* was not a consequence of cell growth in culture and had occurred *in vivo* during tumour progression (Agochiya *et al.*, 1999). However, even though these cell lines had increased *fak* gene copy number, it did not necessarily lead to a related increase in protein in all cases. These researchers suggest that in some malignant cell lines additional controls may regulate the level of FAK protein (Agochiya *et al.*, 1999).

Increased FAK mRNA levels reportedly correlate with elevated FAK protein levels in colon, breast, prostate and liver tumours (Gabarra-Niecko *et al.*, 2003; Lark *et al.*, 2003). This suggests that the control of elevated FAK expression may occur at the transcriptional level (Golubovskaya *et al.*, 2004). In attempting to test the validity of this suggestion, Golubovskaya *et al.* (2004) identified two NF-κB and two p53 transcription factor binding sites in the promoter region of the *fak* gene. They showed that NF-κB positively regulates while p53 negatively regulates FAK transcription (Golubovskaya *et al.*, 2004). In the human leukaemia cell line HL-60, FAK

reportedly induces NF-κB activation by stimulating the PI3K cell survival pathway (Sonoda *et al.*, 2000). Activation of NF-κB led to the marked induction of inhibitor-of-apoptosis proteins (IAPs), thereby rendering cells resistant to apoptotic stimuli (Sonoda *et al.*, 2000).

The role of p53 in the promotion of apoptosis is well documented; however, p53 is often mutated or lost in many cancers including HOSCC (Lam, 2000). In the SNO cell line, Dewar (2000) used western blot analysis to detect the p53 protein with an extended half-life, a characteristic of the mutant p53 protein (Blagosklonny, 1997). Using polymerase chain reaction (PCR) analysis they detected a missense guanine (G) to adenine (A) mutation at codon 175 in exon 5 of p53 in the SNO cell line, while in the WHCO cell lines a G to A transition was found 4 bases upstream of exon 5 (Dewar, 2000). Codon 175 in exon 5 is a known mutational "hotspot" occurring in the sequence-specific DNA-binding domain of p53 (Harris, 1991; Harris, 1993; Dewar, 2000). This mutation may prevent apoptosis, as SNO cells transfected with wildtype p53 complementary DNA (cDNA) experienced rapid cell death (Dewar, 2000).

As p53 is mutated and possibly inactivated in the WHCO and SNO cell lines, transcription of the *fak* gene may be increased in these cell lines. FAK has been shown to prevent p53 activity in breast carcinomas by binding to its N-terminal transactivation domain, thereby inhibiting its tumour-suppressing function (Golubovskaya *et al.*, 2005; van Nimwegen and van de Water, 2007). Therefore, it is possible that FAK may also enhance its own expression in the HOSCC cell lines in a positive feedback loop involving either NF-κB activation or p53 inactivation, or both. The fact that FAK is able to synergistically inhibit p53 while enhancing NF-κB transcriptional activity explains why FAK-overexpressing tumours are resistant to apoptosis (Golubovskaya *et al.*, 2005; van Nimwegen and van de Water, 2007).

Focal adhesions are reportedly prominent in the cell membranes of cells that are grown in tissue culture, and it is at these sites that FAK is predominantly localized (McLean *et al.*, 2005). FAK is noticeably detected by indirect immunofluorescence at focal contacts within the cell membranes in the lamellipodia of the migrating

HOSCC cells in this study (see yellow arrows in Figures 2.4 and 2.5). Here, FAK mediates the diverse interactions between the integrins and the actin cytoskeleton. Subsequently, cell migration is controlled by focal complex formation at the leading edge of lamellipodia and destruction at the trailing edge of migrating cells (McLean *et al.*, 2005).

FAK lamellar localization is especially evident in the WHCO6 cell line, where bright fluorescent staining indicative of FAK localization is observed in cell membranes of the lamellar extensions as well as at the cell membrane of most cells at the periphery of the colony (Figure 2.4). Long lamellipodia are also observed in many cells in the SNO cell line (Figure 2.5). The abundance of these long FAK-containing lamellipodia in WHCO6 and SNO suggests that these cell lines may be somewhat migratory. This suggestion is based on published evidence that FAK is required for the spatial organization of the leading edge of migrating cells, whereas the displacement of FAK from focal adhesions reduces cell migration (Agochiya *et al.*, 1999; Tilghman *et al.*, 2005). Focal complexes are formed at the leading edge in the lamellipodia of the migrating cells, while they are turned over at the trailing edge in these HOSCC cell lines. The expression level of FAK may also determine the rate of cell movement (Agochiya *et al.*, 1999). Thus the level of FAK expression in WHCO6 may also be indicative of a higher rate of migration (in comparison to the other HOSCC cell lines). The presence of numerous FAK-containing lamellipodia in this cell line further suggests that FAK protein expression is correlated to the rate of cell migration.

FAK is also localized to the focal contacts in the cell membranes in the lamellipodia of WHCO1, WHCO3, and WHCO5 (see yellow arrows in Figure 2.5). In comparison to WHCO6 and SNO, notably fewer FAK-containing lamellipodia are observed in the other three cells lines, thus indicating that they are potentially exhibit less migratory behaviour. However, many FAK-containing focal adhesions are clearly visible within the cell membranes in WHCO1 and WHCO5. These focal adhesions are possibly more stable and may not be turned over as rapidly as those observed in the cell lines that exhibit greater migratory behaviour.

FAK is also visibly distributed in the cytoplasm in all 5 HOSCC cell lines (see orange arrows in Figures 2.4 and 2.5). This is not surprising, as FAK was originally described as a non-receptor cytoplasmic tyrosine kinase (Hildebrand *et al.*, 1993; Schaller, 2001). In the WHCO3 cell line, FAK localization appeared to be primarily cytoplasmic with some possible nuclear staining. FAK was also detected in the cytoplasm of the WHCO5 cell line, and to a lesser extent in the nuclei. WHCO1 and WHCO6 also exhibited some cytoplasmic staining, but FAK seemed to be excluded from the nuclei in these cell lines. The SNO cell line showed cytoplasmic and peri-nuclear FAK staining (Figures 2.4 and 2.5).

Aronsohn *et al.* (2003) conducting immunohistological staining on squamous cell carcinoma of the larynx specimens and showed that FAK was localized to the cytoplasm and cell membrane, whereas the autophosphorylated and active form of FAK (FAK pY397) was either nuclear, cytoplasmic, or both (Aronsohn *et al.*, 2003). Other research groups have confirmed that depending on its phosphorylation state, the FAK protein may continuously cycle between focal adhesions and the cytoplasm (Carragher *et al.*, 2003; Wozniak *et al.*, 2004). Based on these findings, it seems logical to suggest that a portion of the cytoplasmic staining observed in the HOSCC cell lines may be the phosphorylated and activated form of FAK (FAK pY397). The status of Src in these HOSCC cell lines is unknown, yet it may in fact play a role in the cytoplasmic distribution of FAK in these cell lines by influencing FAK phosphorylation.

The data presented here unequivocally demonstrate that the full-length FAK protein is expressed and is localized within migratory structures (such as lamellipodia) as well as within the cytoplasm in each HOSCC cell line under investigation. The considerable potential implications of FAK expression in these cell lines are diverse, considering that FAK expression in other human tumours is strongly correlated with cell motility, invasion, survival and other metastatic capabilities related to poor patient prognosis. In the following chapter, modulation of FAK protein expression by active EGFR in these HOSCC cell lines will be explored further.