CHAPTER 3: EGFR ACTIVATION IMPACTS ON FAK PROTEIN EXPRESSION AND PHOSPHORYLATION STATUS IN HOSCC CELL LINES

3.1 Introduction

Developmental processes such as cell migration depend on signals from both the ECM and from soluble growth factors. Evidence shows that the FAK protein is activated in response to both kinds of signals, and is integral in transmitting these signals to the interior of the cell (Ilić *et al.*, 1997; McLean *et al.*, 2005). The implications of integrin-mediated signalling via FAK have already been discussed at length. It now becomes necessary to shed some light on the roles that FAK plays in mediating growth factor signalling.

The role of growth factor-stimulated signalling in cancer progression has long been established. Growth factors are able to maintain the survival of cancer cells and promote tumour-induced angiogenesis. Researchers have recognised that cancer cells maintain high rates of proliferation through producing their own growth factors which act through an autocrine stimulation loop (reviewed in Normanno *et al.*, 2006). The amplification of growth factor signalling may also be due to the increased number of growth factor receptors within the plasma membranes of tumour cells, or to increased sensitivity to low concentrations of growth factors accessible to the cell (Normanno *et al.*, 2006). Since both growth factor receptors and FAK are often upregulated in tumour cells, their synergistic signalling may result in perturbations in the processes often involved in the development of malignancy such as cell proliferation, migration and survival (McLean *et al.*, 2005).

The epidermal growth factor receptor (EGFR) was the first growth factor receptor that drew attention to the link between an activated oncogene and cancer (Downward *et al.*, 1984). EGFR associates and co-localizes with FAK at the cell membrane, with the role that FAK plays in metastasis and cancer progression having already been discussed (Lu *et al.*, 2001). FAK serves as a vital component in linking EGFR

activation to the cellular machinery that promotes cell migration (Sieg *et al.*, 2000). Since upregulated EGFR expression and EGFR-induced signalling have been implicated in the progression to invasion and metastasis in many carcinomas including HOSCC, it becomes imperative to explore the role of the EGFR-FAK complex in signalling in HOSCC (Hanahan and Weinberg, 2000; He *et al.*, 2006; Sutter *et al.*, 2006). Even though EGFR overexpression in the HOSCC cell lines under investigation has been briefly introduced, it is necessary to expand on this by overviewing EGFR signalling.

The 170 kDa EGFR (also known as ErbB-1/HER1) is a member of the ErbB family of receptor tyrosine kinases (RTKs) and is the most common ErbB family member implicated in cancer progression (for reviews see Holbro *et al.*, 2003; Bazley and Gullick, 2005; Normanno *et al.*, 2006). EGFR is constructed of a conserved cytoplasmic PTK domain, a hydrophobic transmembrane domain, and an extracellular ligand-binding domain (Normanno *et al.*, 2006). Receptor activation by the binding of ligands (such as EGF, TGFα, and amphiregulin) leads to the phosphorylation and activation of specific tyrosine residues within the cytoplasmic PTK domain, which serve as binding sites for proteins containing SH2 and phosphotyrosine binding (PTB) domains such as Shc, Grb2, Grb7, Crk, Nck, phospholipase Cγ (PLCγ), Src, and PI3K (Figure 3.1) (Yarden and Ullrich, 1988; Hauck *et al.*, 2001; Bazley and Gullick, 2005; Normanno *et al.*, 2006). EGFR signalling via these proteins leads to increased proliferation through activation of the MAPK pathway and cell survival via the PI3K pathway (Figure 3.1) (Craven *et al.*, 2003; Normanno *et al.*, 2006). In addition to these proteins, various transcription factors such as signal transducer and activator of transcription (STAT), *c-fos*, *c-jun*, *c-myc*, and NF-κB are also activated by EGFR signalling (Normanno *et al.*, 2006). More often than not, many of the above-mentioned EGFR-binding ligands, and affected transcription factors, are implicated in cancer cell signalling (Holbro *et al.*, 2003; Normanno *et al.*, 2006).

Overexpression of EGFR leads to transformation in a variety of cancers cells and increased expression of EGFR occurs at high frequency in most human carcinomas

(Brunton *et al.*, 1997; Gibault *et al.*, 2005; Normanno *et al.*, 2006). EGF receptors are overexpressed at the cell membrane in HOSCC tumours and cell lines, and their overexpression directly correlates with increased metastatic potential and poor patient prognosis (Yamamoto *et al.*, 1986; Veale and Thornley, 1989; Radinsky *et al.*, 1995; Brunton *et al.*, 1997; Hauck *et al.*, 2001; Yamanaka *et al.*, 2003; Gibault *et al.*, 2005; Hanawa *et al.*, 2006; Rothhut *et al.*, 2007).

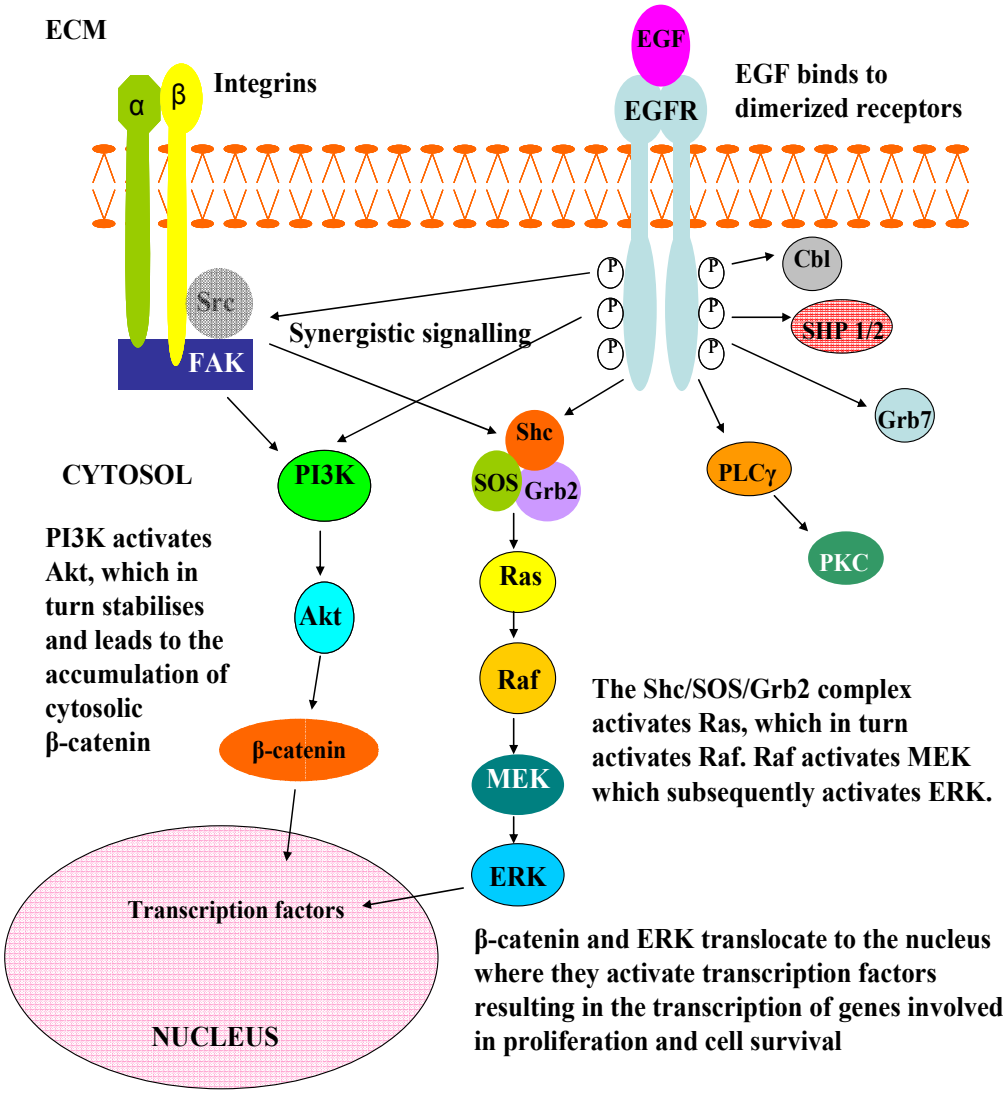


Figure 3.1: EGFR and FAK signalling. EGFR and FAK synergistic signalling leads to enhanced cell proliferation, cell migration, and cell survival via stimulation of the MAPK/ERK and the PI3K pathways.

EGFR activation stimulates cell migration, a key component in tumour cell invasion, by modifying cell-ECM adhesions. FAK associates with EGFR at the cell membrane in order to co-ordinate and promote the signalling events leading to cell migration via stimulation of the MAPK and PI3K signalling pathways (Figure 3.1) (Brunton *et al.*, 1997; Sieg *et al.*, 2000; Hauck *et al.*, 2001; Golubovskaya *et al.*, 2002; Bill *et al.*, 2004). Publications addressing EGFR promotion of cell motility via FAK have thus far reported contrasting results. Some researchers have shown that EGF treatment enhances FAK phosphorylation (and hence promotes its activation and kinase activity), while other reports state that EGF-stimulated dephosphorylation and downregulation of FAK kinase activity is required for cell motility (Brunton *et al.*, 1997; Hauck *et al.*, 2001; Lu *et al.*, 2001; Yamanaka *et al.*, 2003). Still other researchers speculate that it is FAK protein expression, and not its phosphorylation status that is required for EGF-stimulated cell motility (Sieg *et al.*, 2000).

In a study conducted by Brunton *et al.* (1997), an increase in FAK tyrosine phosphorylation following EGF-induced migration of colon carcinoma cell lines was reported. These researchers hypothesized that EGF-induced phosphorylation of FAK plays a role in the recruiting of cellular-Src (c-Src is also rapidly activated by EGF) to focal adhesions (Brunton *et al.*, 1997). This is because FAK activation through phosphorylation at its Tyr-397 residue creates a high affinity binding motif for the SH2 domain of Src-family kinases. Another study conducted on A549 human adenocarcinoma cells determined that FAK activity contributed to EGF-stimulated JNK and MAPK activation, which are both required for promoting cell proliferation and migration (Hauck *et al.*, 2001). The activation of these pathways possibly occurs through FAK activation enabling the recruitment of Src to focal adhesions.

EGFR is highly overexpressed in the human epidermoid carcinoma cell line A431 which reportedly detaches from the ECM following treatment with EGF (Merlino *et al.*, 1985; Lu *et al.*, 2001). Studies conducted by Lu *et al.* (2001) on the A431 cell line revealed that following EGF treatment, FAK was rapidly dephosphorylated and remained hypo-phosphorylated for over 24 hours (Lu *et al.*, 2001). Tyr-397 was dephosphorylated; suggesting that activated EGFR may induce deactivation of FAK

leading to its loss from focal adhesions, and in this manner EGFR may promote focal adhesion turnover and tumour cell motility (Lu *et al.*, 2001). A similar finding was reported by Yamanaka *et al.* (2003) in the cervical adenocarcinoma CAC-1 cell line. These researchers found that EGF rapidly reduced tyrosine phosphorylation of FAK after 30 minutes; however, the tyrosine phosphorylation level was restored after 24 hours. They suggested that an initial decrease in FAK phosphorylation is part of EGF-induced cell migration (Yamanaka *et al.*, 2003).

Still other researchers have determined that FAK protein expression, but not its kinase activity, is required for EGF-stimulated cell motility (Sieg *et al.*, 2000). This indicates that EGF-induced cell migration requires FAK to act as an adaptor or scaffold to recruit other proteins that promote adhesion turnover, such as Src, calpains and caspases, and other proteins involved in the MAPK and PI3K pathways (Sieg *et al.*, 2000, Carragher *et al.*, 2003; Franco *et al.*, 2004; McLean *et al.*, 2005). Thus, FAK must be phosphorylated at Tyr-397 and its other tyrosine residues in order to create binding sites for various signalling and adaptor proteins in order for FAK to link EGFR activation to the cellular machinery that promotes EGF-directed cell migration (Sieg *et al.*, 2000).

Given that the 5 HOSCC cell lines under investigation overexpress EGFR at the cell membrane, EGF stimulation of downstream signalling pathways is likely to be of some importance in the progression of HOSCC (Veale and Thornley, 1989; Driver and Veale, 2006). Recent findings linking FAK with EGFR signalling and the requirement for EGFR activation in HOSCC survival necessitate further examination of the relationship between FAK and EGFR in the 5 HOSCC cell lines in this study (Lu *et al.*, 2001; Yamanaka *et al.*, 2003; Sutter *et al.*, 2006). This chapter details the effects of EGF treatment on the expression and cellular localization of FAK in HOSCC cell lines.

3.2 Methods and Materials

3.2.1 Cell lines and epidermal growth factor treatment

All 5 HOSCC cell lines were cultured as described in Chapter 2. The cell lines were also cultured in tissue culture medium supplemented with EGF (10 ng/ml) for time periods of 30 min, 3 hrs, and 12 hrs at 37ºC in a humid CO_2 incubator. Untreated controls (time = 0 hrs) were also cultured for each cell line.

3.2.2 Antibodies

Western blot detection of the FAK protein in the HOSCC cell lines was completed using polyclonal rabbit anti-FAK (Santa Cruz) primary antibody, and an HRP-conjugated goat anti-rabbit IgG (Sigma) secondary antibody. All antibodies were diluted accordingly in either PBS or Tris-buffered saline (TBS) (see Appendix 5.10). Monoclonal mouse anti-phosphotyrosine antibody (Sigma) was used for immunoprecipitation analysis of Triton X-100 membrane protein-enhanced cell extracts.

3.2.3 Preparation of whole-cell extracts of EGF-treated HOSCC cell lines

Four dishes of EGF-treated cells per HOSCC cell line (1 dish per time treatment plus an untreated control) were cultured to 80% confluency prior to the preparation of whole-cell extracts as described in Chapter 2.

3.2.4 Cell membrane protein-enhancing Triton X-100 extractions

Four dishes of cells per HOSCC cell line (1 dish per time treatment plus an untreated control) were cultured to 80% confluency before being washed 3 times in PBS/phenyl-methyl-sulphonyl fluoride (PMSF)/Trazylol solution (see Appendices 5.4 and 5.5 for solutions). Cells were harvested into sterile eppendorfs which were subsequently centrifuged (1 200 x g) (2 min) in a Tomy Capsule HF-120 bench top

centrifuge. The supernatant of each sample was removed and discarded, and 0.5% Triton X-100 extraction buffer (see Appendix 5.6) was added to the pellet. Following brief vortexing, the samples were incubated on ice for 2 hrs with occasional resuspension of the pellets. Following this, the samples were centrifuged (12 000 x g) at 4ºC for 10 min. The supernatants were aspirated and were stored at –70ºC.

Untreated SNO cells were extracted as above using just the 0.5% Triton X-100 extraction buffer (see Appendix 5.6), while a second sample of SNO cells was extracted using the 0.5% Triton X-100 extraction buffer containing the complete, mini, EDTA-free protease inhibitor cocktail (Roche) (see Appendix 5.7) which inhibits a broad range of serine and cysteine proteases.

3.2.5 Protein estimations

The protein estimation of all whole-cell samples following the method described by Bramhall *et al.* (1969) was completed as described in Chapter 2 (see Appendix 5.16 for the BSA standard curve). However, for the cell membrane protein-enhancing Triton X-100 extracts, the BSA (2µg/µl) was prepared in 0.5% Triton X-100 extraction buffer. The protein concentrations of the samples were determined using the BSA standard curves (see Appendix 5.18 for the standard curve of the Triton X-100 membrane extracts, see Appendix 5.20 for the standard curve of the Triton X-100 membrane extractions of SNO with and without the protease inhibitor cocktail, and see Appendix 5.21 for the standard curve of the Triton X-100 membrane extracts from the EGF-treated cells).

3.2.6 SDS-PAGE separation of cell extracts

Equal amounts of protein from all whole-cell and Triton X-100 extracts of all EGF-treated and untreated samples were separated on 10% SDS-PAGE gels. The gel preparation and electrophoresis procedure was conducted as described in detail in Chapter 2, except that the samples in Triton X-100 extraction buffer were diluted in double lysis buffer (pH 6.8) (Laemmli, 1970) (see Appendix 5.3) and were heated in

a boiling water bath (5 min) in order to linearize the proteins before loading them onto the gel.

3.2.7 Anti-phosphotyrosine Immunoprecipitation

The immunoprecipitation (IP) method involves the purification of a particular protein (or in this case, proteins with a post-translational modification known as tyrosine phosphorylation) along with the proteins that are typically associated with it within the cell. Triton X-100 membrane extracts (250 µg) from the EGF-treated HOSCC cell lines were incubated with 5 µl of anti-phosphotyrosine antibody overnight at 4ºC in IP buffer (pH 8.0) (see Appendix 5.12) in order to ensure the binding of the antibody to the tyrosine-phosphorylated proteins. A negative control was also prepared that did not contain any protein, but only contained anti-phosphotyrosine antibody in IP buffer. Hereafter, the samples containing protein and the 'no-protein' negative control were both subjected to the same procedures. The following day, Protein G agarose beads (Sigma) were washed in IP buffer, and were added to the protein/antibody complexes and to the negative control. These protein/antibody/bead complexes as well as the negative control were incubated at 4ºC overnight to allow the binding of the Protein G beads to the IgG component of the anti-phosphotyrosine antibody. The following day, the complexes were centrifuged (12 000 x g) (30 sec) and the supernatant of each sample was removed and discarded. Thereafter, the pellet was resuspended in IP buffer and was centrifuged (12 000 x g) (30 sec) (this resuspension or washing step was carried out 3 times). After the final washing step, the supernatant was removed and 40 µl of Laemmli lysis buffer (Laemmli, 1970) (see Appendix 5.3) was added to the pellet. The samples were heated in a boiling water bath (5 min) in order to release the tyrosine-phosphorylated proteins from the Protein G beads. Samples were then centrifuged (12 000 x g) for 10 min at 4ºC and were subsequently stored at –20ºC until required for western blot analysis.

3.2.8 Western Blot Analysis

Untreated controls and EGF-treated whole-cell and membrane-protein extracts for each cell line were analysed on separate western blots (1 blot per cell line). Anti-phosphotyrosine immunoprecipitated samples for each cell line were also analysed on separate western blots (1 blot per cell line). The nitrocellulose membranes were blocked (1 hr) in a milk-powder based blocking buffer (Blotto) (see Appendix 5.10 for all solutions used in the western blotting procedure). Subsequently, the blots were incubated in rabbit anti-FAK primary antibody (1:400) for 1 hr (at room temperature), and were rinsed 6 times (5 min each) in PBS or TBS to remove any unbound primary antibody. TBS was used instead of PBS to rinse the nitrocellulose membranes of the anti-phosphotyrosine immunoprecipitated samples. The blots were 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 or TBS (6 times for 5 min each). The SuperSignal® West Pico Chemiluminescent Substrate kit (Pierce) was used to detect the presence of the secondary antibody bound to the nitrocellulose membrane blots as described in Chapter 2.

3.2.9 Densitometry

Densitometric analysis of the western blots was carried out as described in Chapter 2. Levels of protein association or concentration were represented as a percentage of the maximum association or concentration (100%) for each cell line for each western blot analysed. The results of each densitometric assessment of each western blot were represented graphically.

3.3 Results

3.3.1 EGF stimulation causes alterations in FAK protein expression in HOSCC cell lines

FAK is implicated in EGF-stimulated cell migration (which is important in cancer cell metastasis) as it complexes with activated EGFR at the cell membrane (Sieg *et al.*, 2000). Since the 5 HOSCC cell lines under examination overexpress EGFR, they may potentially have a modified response to EGF stimulation (Veale and Thornley, 1989; Driver and Veale, 2006). Thus the effect of EGF stimulation on FAK expression was examined.

The HOSCC cells were treated with EGF for time periods of 0 hrs (untreated control), 30 min, 3 and 12 hrs prior to extraction. For the sake of simplicity, all protein samples extracted from the EGF-treated HOSCC cell lines will commonly be referred to as "EGF-treated samples". An estimation of the protein concentrations as outlined by Bramhall *et al.* (1969) was conducted on all the EGF-treated samples (whole-cell extracts) extracted from each cell line (see Appendix 5.16 for the BSA standard curve). Hence, equal amounts of protein from the 4 samples of the WHCO1 cell line (3 EGF-treated and 1 untreated control) were separated by 10% SDS-PAGE as this would be representative of the accuracy of the protein estimation of all the samples. As seen in Figure 3.2, each of the sample-containing lanes of the SDS-PAGE gel appeared to have similar amounts of Coomassie Blue staining, indicating that a uniform amount of total protein from each sample was represented on the gel and that the protein estimation was accurate. Numerous polypeptides bands were detected by SDS-PAGE, once again visually supporting the outcome of the quantitative protein estimation.

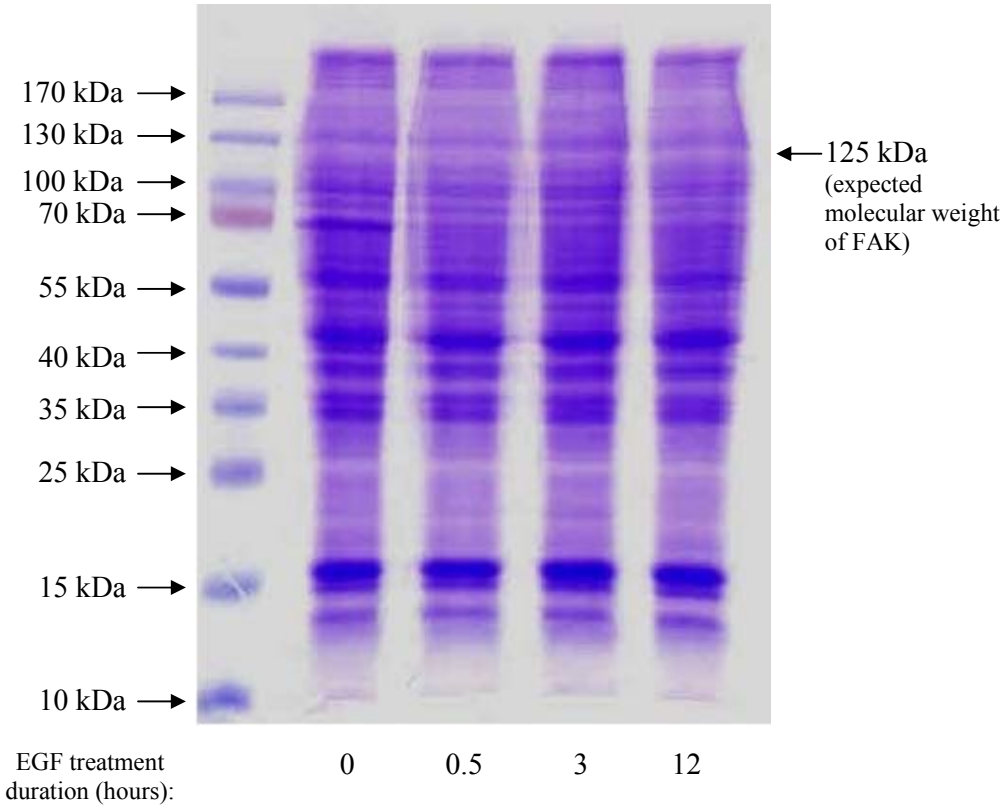


Figure 3.2: Whole-cell lysates of EGF-treated WHCO1 resolved by 10% SDS-PAGE. The WHCO1 cell line was treated with EGF for the time periods indicated. The success of the extraction procedure and the accuracy of the protein estimation were confirmed by the staining of multiple peptide bands of similar intensity in each lane.

A single band, representative of the full-length 125 kDa FAK protein, was detected by means of western blotting of the EGF-treated whole-cell samples of each HOSCC cell line (Figure 3.3). Once again, the words "band" or "bands" will here be used for simplicity when describing the linearized polypeptides visualised by SDS-PAGE or detected by western blotting. The graph in Figure 3.4 was generated using the densitometric data generated from the western blots and depicts the changes in FAK expression in response to EGF treatment over time (see Appendix 5.17 for raw densitometric data). Notably, EGF treatment was found to alter the protein expression levels of FAK, although it did not have the same effect in each of the 5 HOSCC cell lines (Figures 3.3 and 3.4). The level of FAK protein expression within each sample was calculated as a percentage of the maximum expression (100%)

detected in each cell line. Remarkably, maximum FAK protein expression was not observed at the same time of EGF exposure in each cell line. There were both increases and decreases in FAK protein expression over the 12 hr period, rather than a single simple increase or decrease over the measured time period (Figures 3.3 and 3.4).

Maximum FAK protein expression was observed in the untreated control in the WHCO1 cell line, thus indicating that EGF stimulation leads to a general decrease in FAK expression in this cell line. Intriguingly in the WHCO3 cell line, maximal FAK protein expression was observed after 12 hours of EGF treatment, thus indicating that EGF induces an overall increase in FAK expression in this cell line (Figure 3.4).

In the WHCO5, WHCO6, and SNO cell lines, maximum expression of the 125 kDa FAK protein was observed after 3 hrs of EGF treatment (Figure 3.4). In the WHCO5 cell line, FAK protein expression in the untreated control (70%) increased marginally after 30 min of treatment (75%) before reaching its maximum at 3 hrs. After 12 hrs of EGF treatment, FAK expression dropped fairly sharply to 40%. The initial expression (89%) of FAK observed in the WHCO6 cell line dwindled slightly to 85% after 30 min of treatment. After reaching the maximum expression level at 3 hrs of EGF treatment, FAK expression was reduced to 70 % after 12 hrs of treatment (Figure 3.4).

In contrast to the WHCO cell lines, the untreated control of SNO had a relatively low level of FAK expression (35%). This increased sharply to 84% after 30 min, reached maximum expression at 3 hrs, and decreased to 75% after 12 hrs of EGF treatment (Figure 3.4). Hence, it appears that in the SNO cell line, EGF treatment generally induces a marked increase in FAK protein expression when compared to the untreated control (see Appendix 5.17 for all raw densitometric data for the EGF-treated whole-cell lysates). In all of the HOSCC cell lines (except WHCO3) there is a decrease in FAK expression between 3 and 12 hrs of EGF treatment. For WHCO5, WHCO6, and SNO, this decrease was somewhat substantial, whereas for WHCO1 this decrease was not as drastic as in the other 3 cell lines (Figure 3.4).

From the data presented here, it is evident that the expression of the 125 kDa FAK protein is variably affected by EGF treatment in these HOSCC cell lines, and an overall trend in expression between the cell lines is not obvious.

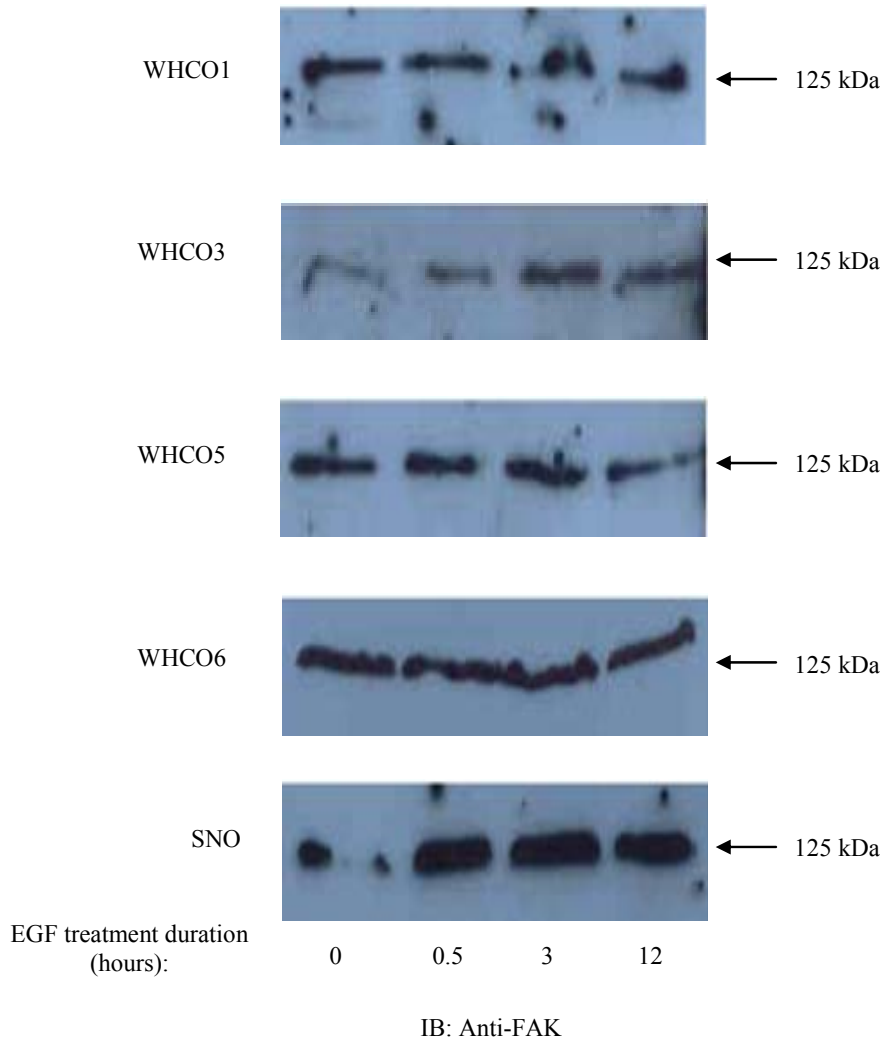


Figure 3.3: Cellular expression of the 125 kDa FAK protein following EGF treatment of HOSCC cell lines. HOSCC cell lines were treated with EGF for varying time durations (0, 0.5, 3, and 12 hours). FAK-specific immunoblotting detected the presence of the FAK protein at 125 kDa in all the untreated controls and EGF-treated whole-cell lysates of each cell line. IB = Immunoblotting antibody.

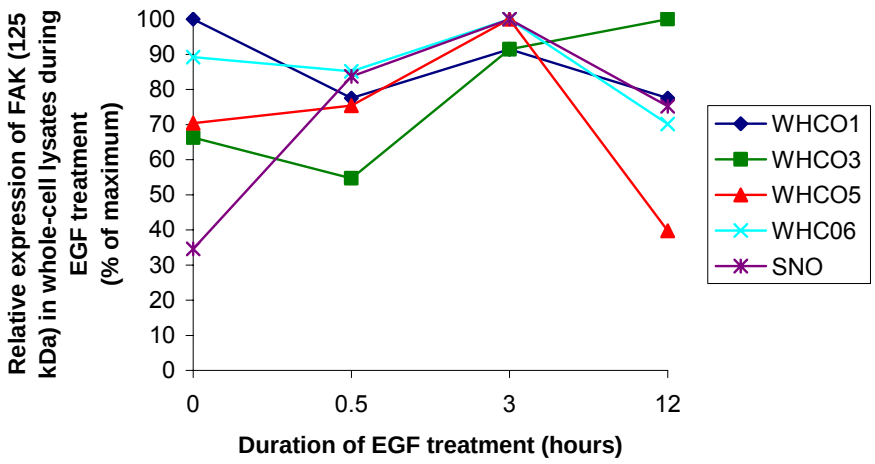


Figure 3.4: EGF treatment affects the expression of the 125 kDa FAK protein detected in whole-cell extracts of HOSCC cell lines. Graphical representation of the densitometric analysis of FAK-specific western blotting of EGF-treated whole-cell lysates. The highest protein expression level in each EGF-treated cell line was taken as 100%, and all other expression levels were represented relative to the maximum expression observed.

3.3.2 Membrane protein-enhanced extracts of HOSCC cell lines constitute an extensive diversity of polypeptides

As FAK functions primarily within focal adhesions in the plasma membrane, its status at the cell membrane needs to be established. Triton X-100 was included in the extraction buffer used here, as this detergent disrupts the cell membrane thus releasing the cell membrane-embedded or -associated proteins but does not solubilize all of the other cellular proteins. Thus the ratio of membrane-associated proteins to other cellular proteins is far greater in this extraction than in the Laemmli (1970) extraction which solubilizes all of the cellular proteins. Thus, the concentration of plasma membrane-associated proteins solubilized is enhanced by this extraction procedure. Although the procedure used does not solubilize cell membrane-associated proteins exclusively of all other cellular proteins, for the purpose of clarity these samples will be referred to as simply as 'membrane extracts'. The cell membrane extracts from each HOSCC cell line were resolved on a 10% SDS-PAGE

gel to visually confirm the accuracy of the protein estimation (Figure 3.5) (see Appendix 5.18 for the BSA standard curve generated for the protein estimation). SDS-PAGE analysis of the membrane extracts detected the presence of numerous polypeptides of varying molecular weights, possibly including FAK and its associated binding and signalling partners.

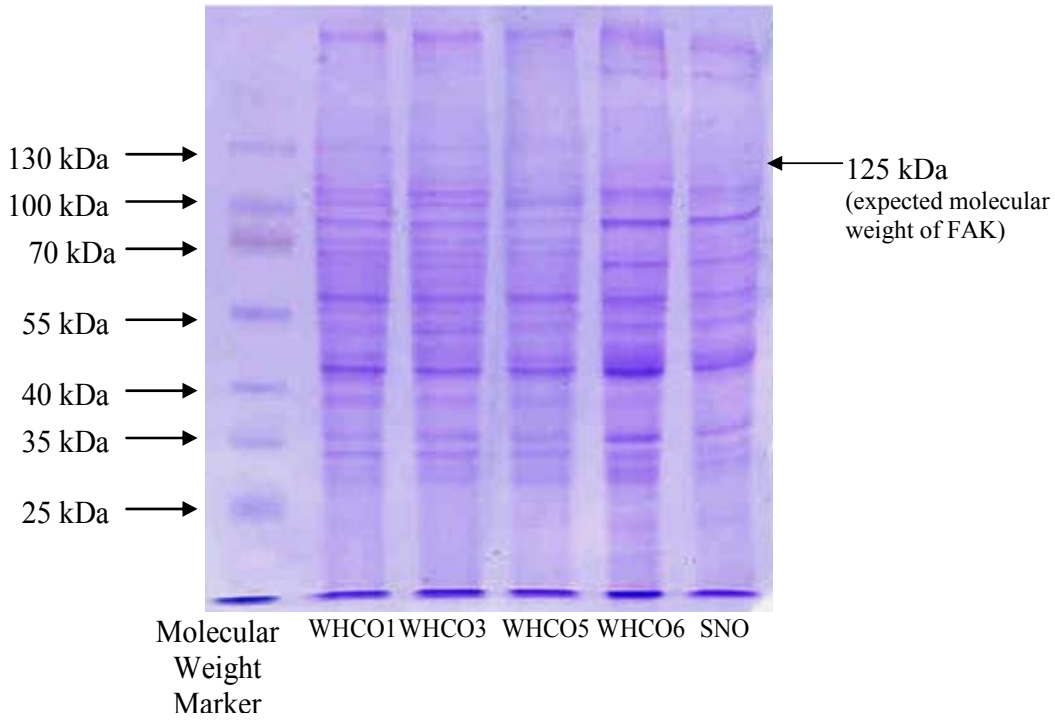


Figure 3.5: Triton X-100 membrane extracts of the HOSCC cell lines resolved by 10% SDS-PAGE. Multiple polypeptide bands of similar intensity occur throughout the lanes of each cell line, thus indicating that the samples were extracted correctly and that the quantitative protein estimation was accurate. Numerous polypeptides were detected in the plasma membrane extracts in the HOSCC cell lines analysed.

3.3.3 Two variants of the FAK protein are observed at the cell membrane in the HOSCC cell lines

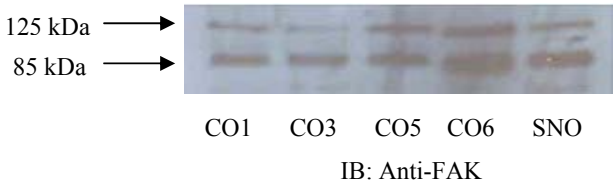
Two predominant polypeptide bands were resolved in each HOSCC cell line by FAK-specific western blotting of membrane extracts (Figure 3.6a). The higher molecular weight band occurred at 125 kDa (corresponding to full-length FAK) while the lower band had a relative molecular weight of ~85 kDa. Published studies report that the full-length FAK protein usually has a relative molecular weight of 125 kDa, although some reports have detailed the existence of an 85 kDa N-terminal cleavage product of 125 kDa FAK often formed during apoptosis (Wen *et al.*, 1997; Shofuda *et al.*, 2004; Carlin *et al.*, 2005). The 85 kDa band detected in this study must also be an N-terminal truncation of the full-length FAK protein as the FAK-specific antibody used for western blotting is targeted to a peptide sequence occurring within the N-terminus of FAK.

The graph included as part of Figure 3.6 was generated using densitometric data obtained from the western blot and shows the relative concentrations of the two FAK variants (see Appendix 5.19 for raw densitometric data). The WHCO6 cell line had the highest concentrations of both the 125 kDa and 85 kDa FAK protein variants. As was done in Chapter 2, the relative concentrations of the FAK protein variants in the four other cell lines were calculated as a percentage of the maximum FAK protein concentration detected in the WHCO6 cell line (taken as 100%) (Figure 3.6b). The WHCO5 cell line expressed the second highest relative concentration of the 125 kDa variant (80%), followed by SNO (49%), WHCO1 (41%), and WHCO3 (30%) (Figure 3.6b).

As mentioned, the WHCO6 cell line also exhibited the highest concentration of the 85 kDa variant of FAK. However, in contrast to the protein concentrations observed for the 125 kDa variant, SNO exhibited the next highest relative concentration of 58%, closely followed by WHCO5 (57%). WHCO3 (37%) had a slightly higher relative concentration of the 85 kDa variant than WHCO1 (36%) (Figure 3.6b). As

seen in the data presented, the concentration of the 85 kDa variant need not necessarily be aligned with that of the 125 kDa variant within each cell line.

a)



b)

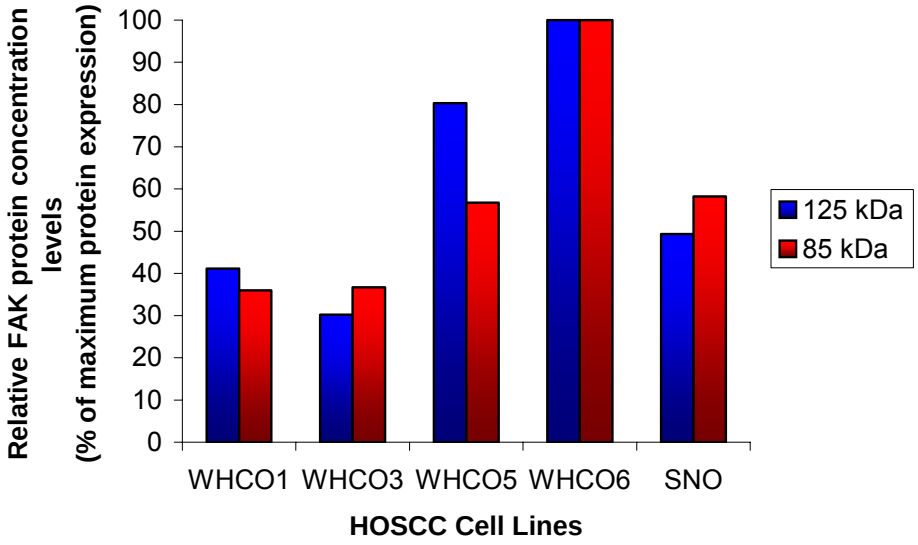


Figure 3.6: Two FAK variants are present in Triton X-100 membrane extracts of all 5 HOSCC cell lines. a) The western blot of the membrane extracts was probed using an antibody specifically targeted to the N-terminus of FAK. The presence of two polypeptide bands indicates that there are two N-terminal variants of FAK present in each HOSCC cell line (at 125 kDa and 85 kDa). b) Graphical representation of densitometric analysis of the 125 kDa and 85 kDa variants of FAK. Protein concentrations of both variants were highest in the WHCO6 cell line (taken as 100%), and the concentrations of the variants in all other cell lines were represented as a percentage relative to WHCO6. (Note: CO1, CO3, etc. represents the cell lines WHCO1, WHCO3, etc.). IB = Immunoblotting antibody.

3.3.4 The 85 kDa variant of FAK is formed as a result of cellular processing prior to the protein extraction procedure

Two possibilities exist to explain how the 85 kDa variant of FAK was formed. Firstly, the 85 kDa variant may be present within the cells in culture prior to the membrane extraction procedure or secondly, may result from degradation of full-length FAK during the extraction procedure and subsequent storage of the samples. Using the SNO cell line as an example, the Triton X-100 membrane extraction was performed both in the absence and presence of an additional serine and cysteine protease inhibitor cocktail (Roche) in the extraction buffer in order to discover which possibility was correct.

Equal amounts of total protein from both SNO extracts (in the absence and presence of the protease inhibitor cocktail) were separated by 10% SDS-PAGE and analysed by FAK-specific western blotting (see Appendix 5.20 for standard curve of protein estimation). As seen in Figure 3.7, both the 125 kDa and 85 kDa variants are present in both extracts, thus indicating that the 85 kDa variant is present as a result of cleavage within the living cells prior to the extraction procedure, and was not an artefact produced during extraction or storage of the samples.

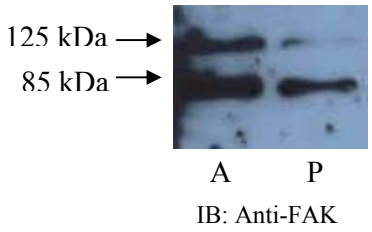


Figure 3.7: FAK protein stability in Triton X-100 membrane extracts. Membrane protein-enhancing extractions of SNO were completed both in the absence (indicated as lane A) and presence (indicated as lane P) of a protease inhibitor cocktail in 0.5% Triton X-100 extraction buffer. Both variants of FAK (125 kDa and 85 kDa) are present in both the extractions, indicating that FAK is cleaved to the 85 kDa variant within the living cells in culture prior to the protein extraction procedure. IB = Immunoblotting antibody.

3.3.5 EGF treatment affects the concentration of the FAK variants at the plasma membrane

In the data presented in this chapter, it was shown that EGF stimulation of the HOSCC cell lines alters the expression of the full-length FAK protein in whole-cell lysates. Thus, two questions remain to be answered. Firstly, are the changes in expression of the full-length FAK variant reflected at the cell membrane, and secondly, does EGF treatment affect the membrane concentration of the 85 kDa variant?

Both the full-length (125 kDa) and the 85 kDa cleaved variant of FAK were detected in all of the untreated and EGF-treated HOSCC cell lines by FAK-specific western blotting (Figure 3.8) (see Appendix 5.21 for standard curve of protein estimation). The graphs in Figure 3.9 were generated from the densitometric analysis of the western blots and depict changes in the concentration of the two FAK variants for the duration of EGF exposure (see Appendix 5.22 for raw densitometric data). Within each cell line, the sample with the highest concentration of each variant was designated as having a relative concentration of 100%, and the FAK concentrations in the 3 remaining samples in each cell line were represented as a percentage relative to that sample. Once again, the maximum amount of FAK protein expression was not observed at the same time of EGF exposure in each cell line and the concentrations of each variant were not necessarily allied with one another. There were both increases and decreases in FAK protein expression of both variants over the 12 hr period for each cell line, rather than a single simple increase or decrease over the measured time period (Figure 3.9).

The general trend noticed (in 3 of the 5 cell lines: WHCO1, WHCO3, and WHCO5) was an increase in the concentration of the 125 kDa full-length variant of FAK in the membrane extracts after 3 hours of EGF treatment (Figures 3.8 and 3.9a). In the WHCO1 cell line, the FAK concentration increased from an initial concentration of 20% to 88% after 30 min of EGF treatment, 100% after 3 hrs, and then decreased to 25% after 12 hrs. A similar tendency is observed for WHCO5, where the initial

concentration of FAK (89%) increased after 30 min (97%), reached 100% after 3 hrs, and decreased to 80% after 12 hrs. The initial concentration of 125 kDa FAK was much lower in WHCO3 (36%) than in all the other cell lines. In contrast to WHCO1 and WHCO5, after 30 min the 125 kDa concentration in WHCO3 decreased slightly (34%) before dramatically increasing after 3 hrs (100%) of treatment. After 12 hrs the concentration decreased drastically to 28% (Figure 3.9a).

In contrast to the other three cell lines, the highest concentration of the 125 kDa FAK variant was detected in the untreated control samples of WHCO6 and SNO (Figures 3.8 and 3.9a). The concentration of this variant then decreased to 55% (WHCO6) and 79% (SNO) after 30 min of EGF treatment. In WHCO6, the concentration of this variant then improved to 66% (3 hrs) before decreasing to 20% (12 hrs), whereas in SNO the concentration decreased to 74% (3 hrs) and then dropped very slightly to 72% after 12 hrs of EGF treatment (Figure 3.9a).

The concentrations of the 85 kDa variant also varied with EGF treatment, although the changes in concentration are not necessarily related to those noted for the 125 kDa variant in each cell line (Figures 3.8 and 3.9b). In the WHCO1 cell line, the protein concentration of the 85 kDa variant increased from an initial 11% to 75% (30 min), and then to 100% (3 hrs) before dropping to 37% after 12 hrs of treatment with EGF. The initial concentrations (untreated controls) of the cell WHCO3, WHCO6, and SNO cell lines were the greatest (100%) when compared to the subsequent EGF-treated concentrations for the 85 kDa variant over time (Figures 3.8 and 3.9b). In WHCO5 however, the untreated control had a relatively high concentration of 85 kDa FAK (81%), while the maximum concentration for this cell line was observed after 30 min of treatment. The concentration started to decline thereafter (Figures 3.8 and 3.9b).

From the data presented here, it is evident that EGF treatment causes changes in the concentration of both FAK variants at the cell membrane. Whether these changes in concentration are only as a result of modified FAK protein expression or also of an