alteration in FAK localization at the cell membrane cannot be determined from this data set.

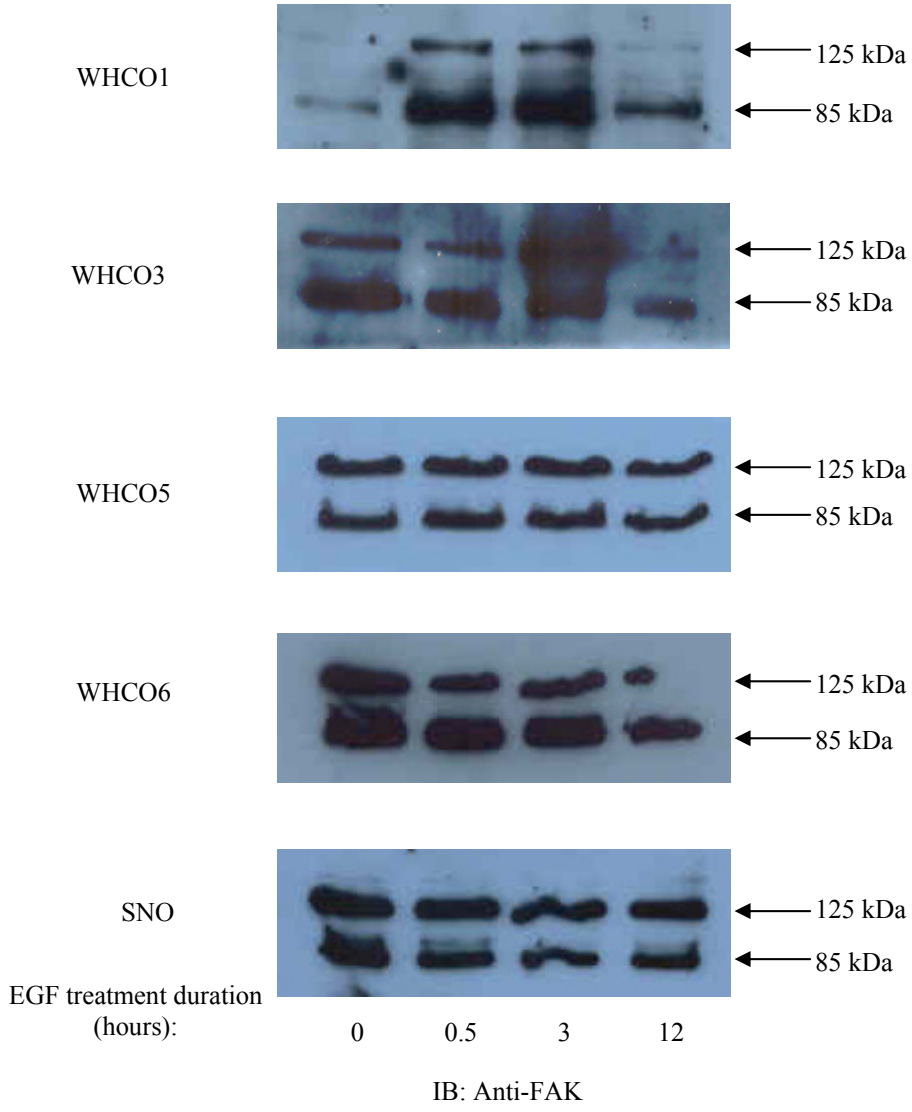
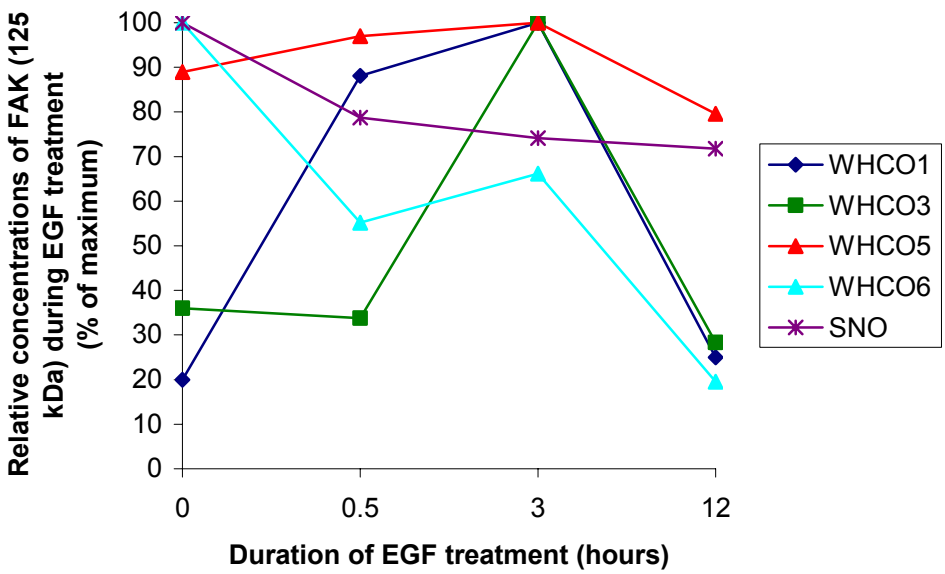


Figure 3.8: FAK protein expression in response to EGF stimulation of HOSCC cell lines. Membrane extracts of EGF-treated cells (treated for 0.5, 3, and 12 hrs) and untreated controls (0 hrs) of all 5 HOSCC cell lines were electrophoresed on 10% SDS-PAGE gels, and were transferred to nitrocellulose membranes for western blotting. Both FAK variants (125 kDa and 85 kDa) were present in all the treated and untreated control samples of each cell line and the concentrations of both variants were affected by EGF treatment. IB = Immunoblotting antibody.

a)



b)

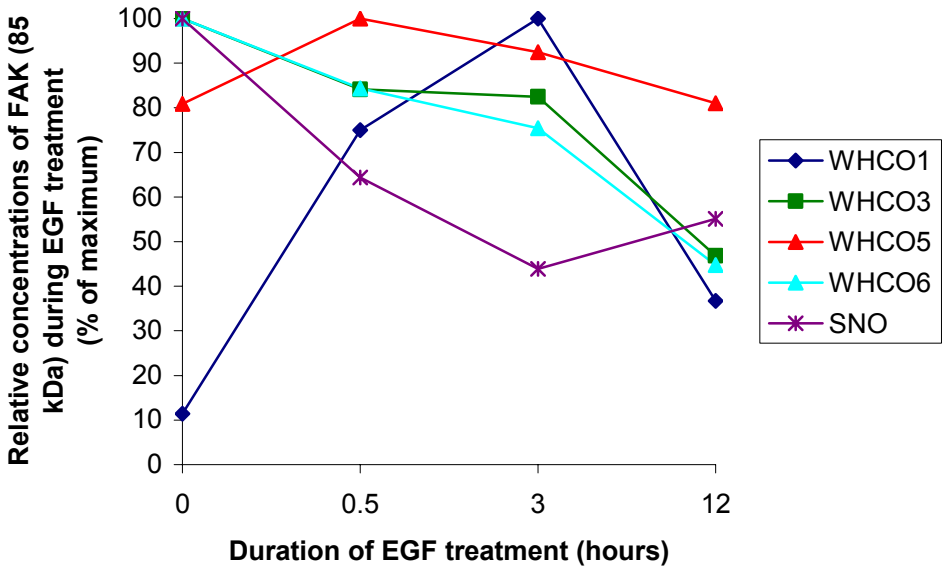


Figure 3.9: EGF treatment affects the protein concentration of both the 125 kDa and 85 kDa FAK variants. Graphical representation of the densitometric analysis of the a) 125 kDa, and b) 85 kDa variants of FAK, showing the changes in FAK concentration at the cell membrane over the duration of EGF treatment.

3.3.6 The 85 kDa variant of FAK co-immunoprecipitates with tyrosine-phosphorylated proteins

The binding of ligands to the extracellular domain of EGFR induces receptor dimerization and subsequent activation of the intrinsic tyrosine kinase domain (Normanno *et al.*, 2006). EGFR activation affects the phosphotyrosine levels of FAK, although opinions differ in the literature as to whether it is an increase or a decrease in the state of tyrosine phosphorylation of FAK that is required for EGF-directed cell migration (Brunton *et al.*, 1997; Hauck *et al.*, 2001; Lu *et al.*, 2001; Yamanaka *et al.*, 2003). Therefore, the phosphotyrosine status associated with FAK following EGF stimulation of EGFR needed to be determined. Triton X-100 membrane extracts of the EGF-treated cells were immunoprecipitated with a specific anti-phosphotyrosine antibody. These immunoprecipitated samples were subsequently separated by 10% SDS-PAGE and subjected to FAK-specific immunoblotting.

The 125 kDa FAK polypeptide band was not detected in the phosphotyrosine-immunoprecipitated samples in the 5 HOSCC cell lines (Figure 3.10). The 125 kDa variant of FAK is possibly either not tyrosine-phosphorylated or does not associate with tyrosine-phosphorylated proteins in these cell lines. However, this is not in keeping with reported data and the possible explanation of this result will be addressed specifically later in this chapter. Surprisingly, the 85 kDa variant of FAK was detected by FAK-specific western blotting of the immunoprecipitated samples, and therefore, appears to be either tyrosine-phosphorylated itself or is associated with other tyrosine-phosphorylated proteins in these cell lines (Figures 3.10).

Because these results were completely unexpected, it was necessary to scrutinize them by comparing them to both positive and negative controls (Figure 3.10). As a positive control, a membrane extract (previously shown to contain both the 125 kDa and 85 kDa FAK variants) was electrophoresed along side the phosphotyrosine-immunoprecipitated samples of each cell line. For the negative control, the above-mentioned immunoprecipitation protocol was followed except that protein was

excluded from this sample, although the anti-phosphotyrosine antibody and the Protein G beads were present. Thus, the absence of bands at 125 and 85 kDa in negative 'no-protein' control and the presence of both the 125 and 85 kDa bands in the positive membrane control confirms that the results obtained for the immunoprecipitated samples are specific for the FAK protein (Figure 3.10). Nonetheless, from the comparison with the positive and negative controls, the data obtained from the immunoprecipitated samples clearly demonstrates that the 85 kDa variant of FAK is tyrosine-phosphorylated or associates with other tyrosine-phosphorylated proteins in these cell lines.

3.3.7 EGF influences the concentration of the 85 kDa FAK variant associated with tyrosine-phosphorylation at the cell membrane

The concentration of the 85 kDa FAK variant is not constant in the phosphotyrosine-immunoprecipitated samples extracted from each EGF-treated HOSCC each cell line, with both increases and decreases in the concentration of the 85 kDa variant occurring over the assessed 12 hr time interval. These results are complex and thus a common trend across all the cell lines is not clear.

In WHCO1, the concentration of the tyrosine-phosphorylated or –associated 85 kDa FAK variant rose (from 76 to 83%) after 30 min of EGF treatment, reached its maximum association at 3 hrs (100%), and decreased after 12 hrs (87%) (Figures 3.10a and 3.11). In the WHCO5 cell line, the greatest concentration was observed after 30 min of treatment. Thereafter, the concentration dropped substantially to 37% (3 hrs) and remained very low (33% after 12 hrs). Quite contrary to WHCO1 and WHCO5, the initial concentration (73%) of the 85 kDa variant in WHCO3 decreased quite dramatically after 30 min of EGF treatment (37%) before quickly reaching its maximum at 3 hrs, and reduced slightly after 12 hrs (83%) (Figure 3.11). In the WHCO6 cell line, the highest concentration of the 85 kDa FAK variant is observed in the untreated control. Thereafter, the concentration of this FAK variant decreases drastically after 30 min (18%), before increasing after 3 hrs of EGF treatment (54%). The concentration then diminishes again after 12 hrs (21%) (Figure 3.11).

Interestingly, in contrast to the 4 WHCO cell lines, the initial concentration the 85 kDa variant in SNO is extremely low (4%), increasing very rapidly after 30 min of EGF treatment (100%). After 3 hrs, the concentration decreases slightly (76%) before dropping drastically after 12 hrs back to the initial level observed (4%) (Figures 3.10a and 3.11) (see Appendix 5.23 for raw densitometric data of the phosphotyrosine-immunoprecipitated samples). From the data presented here, it is clear that each cell line is very unique in its response to EGF stimulation with regards to the changes in the concentration of the 85 kDa variant of FAK (associated with tyrosine phosphorylation) and thus a trend across the 5 HOSCC cell lines was not evident.

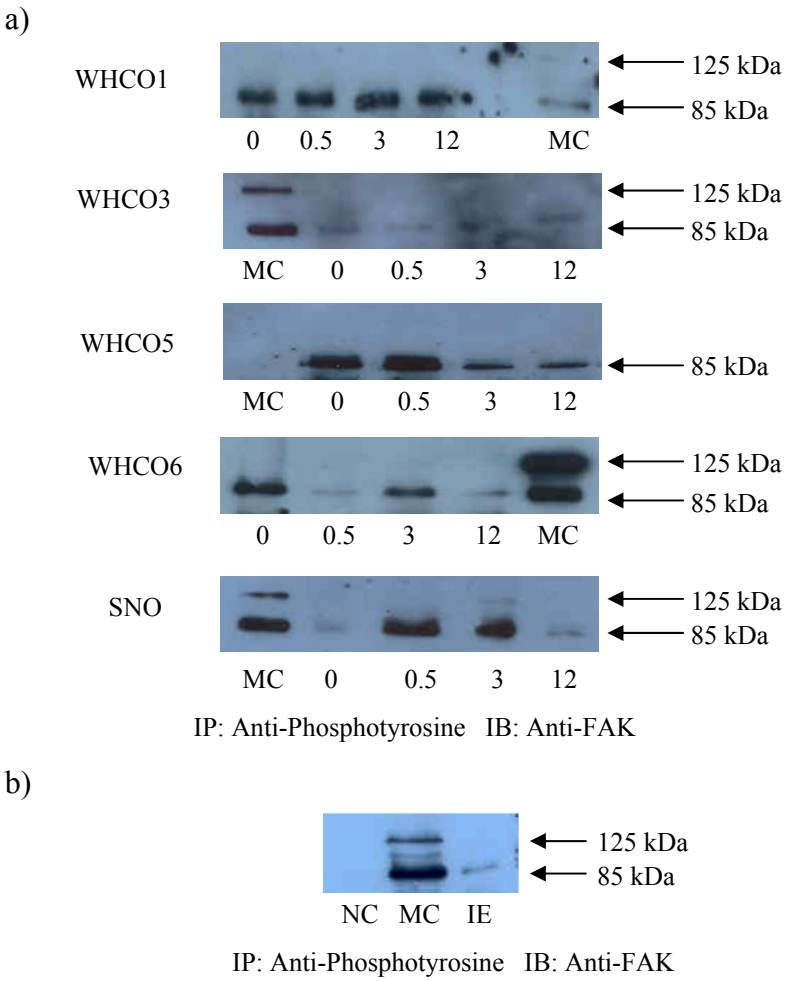


Figure 3.10: EGF alters the concentration of the 85 kDa variant of FAK at the cell membrane in phosphotyrosine-immunoprecipitated samples. a) Untreated controls (0 hrs) and EGF-treated membrane extracts (treated for 0.5, 3, and 12 hrs) were immunoprecipitated with an anti-phosphotyrosine antibody, and an anti-FAK antibody was used for western blot analysis. The 85 kDa variant, but not the 125 kDa variant of FAK immunoprecipitated with anti-phosphotyrosine. The duration (in hrs) of EGF treatment is indicated below each lane in each blot. b) Positive and negative controls for the immunoprecipitation reaction. Both FAK variants were detected in the membrane positive control, while no bands were visible in the 'no-protein' negative control. Only the 85 kDa variant was observed in the immunoprecipitation experimental (IE) sample. MC = Membrane extract positive control, NC = 'No-protein' negative control, IE = Immunoprecipitation Experiment, IP = Immunoprecipitating antibody, IB = Immunoblotting antibody.

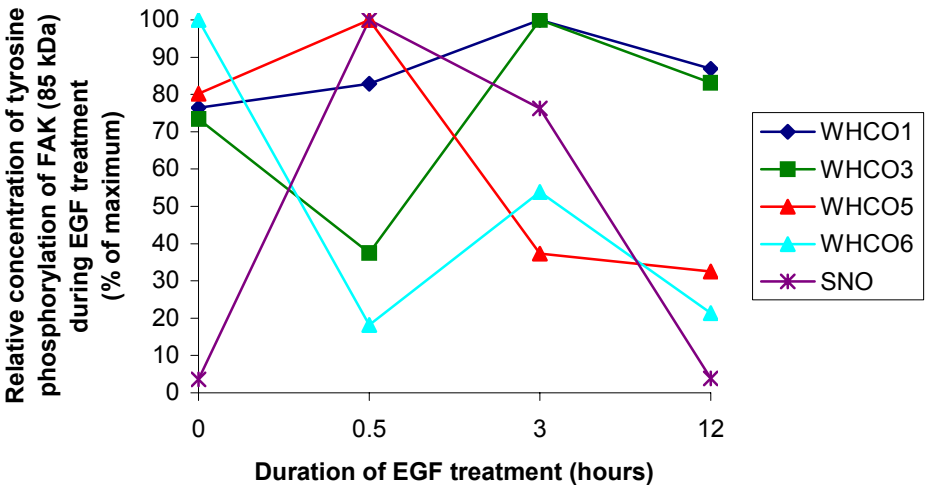


Figure 3.11: EGF stimulation affects the levels of phosphotyrosine associated with the 85 kDa variant of FAK. Graphical representation of the densitometric analysis of western blots of phosphotyrosine-immunoprecipitated, EGF-treated membrane extracts. The concentration of the 85 kDa variant reaches its maximum within 3 hrs of EGF treatment of the HOSCC cell lines.

3.4 Discussion

In this chapter, evidence is provided that EGF stimulation of the HOSCC cell lines affects FAK expression and cell membrane localization. EGFR is reportedly overexpressed in these cell lines; it is therefore highly likely that this overexpression may lead to enhanced downstream signalling effects that are generally associated with tumourigenesis (Ozawa *et al.*, 1987; Veale and Thornley, 1989). EGFR activation and its downstream signalling cascades were discussed earlier in this chapter however, it is necessary to briefly review EGFR signalling here to assist in the understanding of the results.

Upon ligand binding, EGFR activation involves dimerization with other ErbB family members. This results in the phosphorylation of specific tyrosine residues within its cytoplasmic tail and the subsequent recruitment of various cytoplasmic proteins containing SH2 and PTB domains such as Shc, Grb2, Grb7, Src, PI3K, and FAK (Schaller *et al.*, 1992; Hauck *et al.*, 2001; Bazley and Gullick, 2005; Normanno *et al.*, 2006). EGFR signalling via these proteins leads to proliferation signalling via the MAPK pathway and cell survival via the PI3K pathway (Normanno *et al.*, 2006). Active EGFR stimulates cell migration, a key component in tumour cell invasion, via its association with FAK at the cell membrane (Brunton *et al.*, 1997; Sieg *et al.*, 2000; Hauck *et al.*, 2001; Golubovskaya *et al.*, 2002; Bill *et al.*, 2004).

3.4.1 EGFR activation alters FAK protein expression in HOSCC cell lines

The full-length FAK protein (125 kDa) is expressed in both the untreated and EGF-treated whole-cell lysates of each HOSCC cell line analyzed (Figure 3.3). Notably, EGF treatment alters the protein expression levels of FAK, although not in exactly the same way in each of the 5 HOSCC cell lines. Within each cell line, there are peaks and troughs in the level of FAK protein expression at the individually marked time intervals rather than a single simple increase or decrease over the entire 12 hr period (Figure 3.4). EGF treatments were conducted on intact cells rather than extracted or individual proteins. The results of the experiment therefore reflect events

occurring within a complex cell system. Multiple repeats of each EGF treatment were not conducted because the difference in FAK expression between the lowest and highest level over the entire 12 hr treatment period was considerable in each cell line. In WHCO1 and WHCO6, the change in overall expression between the lowest and highest level observed was between 20-30% while in the other 3 cell lines, the difference was much greater (between 45-67%) (Figure 3.4). Thus, it seems that the changes in FAK expression over the time of treatment are not due to natural variation, but are in fact caused by EGF stimulation of the HOSCC cell lines.

EGFR activation by its EGF ligand clearly alters cellular FAK protein expression uniquely in each cell line, because where an increase in expression is noted at a particular treatment time in one cell line, a decrease in expression may occur during the same time interval in another cell line (Figure 3.4). Remarkably, the maximum amount of FAK protein expression is not observed at the same interval of EGF exposure in all of the 5 cell lines. Regardless of the initial (untreated) level of expression, FAK expression tends to increase between 30 min and 3 hrs of treatment with EGF in all 5 HOSCC cell lines. In contrast to some of the WHCO cell lines, in SNO, EGF treatment results in a substantial increase in FAK expression that is maintained at a relatively high level for the duration of EGF exposure (Figure 3.4). With the exception of WHCO3, a general decrease in expression is noticed between 3 and 12 hrs of treatment in the HOSCC cell lines (Figure 3.4). This decrease may be due to either the depletion of EGF in the medium, or to the activation of other cellular processes that may regulate FAK expression, such as proteolytic cleavage by caspases or calpains (Shofuda *et al.*, 2004; Angelucci and Bologna, 2007).

EGFR signalling is also known to activate various transcription factors such as *c-fos*, *c-jun*, *c-myc*, STAT, and NF-κB, and interestingly the FAK promoter region is reported to contain two NF-κB and two p53 binding sites (Golubovskaya *et al.*, 2004; Normanno *et al.*, 2006). Thus, the activation of pathways downstream of EGFR is likely to positively influence the transcription factors responsible for regulating FAK transcription. From the results presented here it is possible to conclude that EGF

stimulation of EGFR plays has an overall positive effect on the regulation of the FAK protein expression.

3.4.2 Full-length FAK, as well as the 85 kDa variant, are detected at the cell membrane of HOSCC cell lines

Two variants of FAK are present in all of the membrane protein extracts of the 5 HOSCC cell lines with molecular weights of 125 kDa and 85 kDa respectively (Figure 3.6). Although reports state that full-length FAK usually has a molecular weight of approximately 125 kDa, some research groups have detailed the existence of an 85 kDa N-terminal cleavage product of the 125 kDa FAK protein (Wen *et al.*, 1997; Shofuda *et al.*, 2004; Carlin *et al.*, 2005). These published findings are consistent with the results presented in this study, as the anti-FAK antibody used for western blotting is designed to target a peptide sequence mapped to the N-terminus of FAK of human origin. Therefore, any protein variant detected by this antibody would obviously contain the N-terminus of FAK.

As the 85 kDa FAK variant is not detected in the whole-cell extracts, it was initially necessary to determine whether this variant was produced as a result of protein degradation during the membrane extraction and storage procedures, or whether this variant was actually produced by cellular processes while the cells were in culture. The membrane extraction buffer used contains PMSF and Aprotinin (Trazylol) which are known to inhibit serine proteases such as trypsin, chymotrypsin, and pepstatin. An additional broad-range serine and cysteine protease inhibitor cocktail (Roche) was added in order ensure that broad-range serine and cysteine proteases were inhibited and were unable to actively cleave full-length FAK during the extraction and storage procedures. The results show that the 85 kDa variant is not an artefact of extraction or storage of the protein samples since the 85 kDa variant was also detected in the sample extracted with the buffer containing the additional serine and cysteine protease inhibitor cocktail (Figure 3.7). Therefore cleavage of full-length FAK must occur via cellular processes occurring within the living cells in culture.

These findings are supported by similar results obtained by Carlin *et al.* (2005) who detected the presence of FAK at two molecular weights in membrane extracts of human airway smooth muscle (HASM) cells: a full-length FAK (125 kDa) and an 85 kDa FAK variant. In parallel with the results obtained for the HOSCC cell lines analysed above, the 85 kDa N-terminal fragment was undetectable in the whole-cell fractions and was only found in the membrane fraction of the HASM cells (Carlin *et al.*, 2005). This fragment was reportedly generated by caspase-6 cleavage, although studies in other cell lines have found this variant to be produced by caspase-3 (Mao *et al.*, 2003; Carlin *et al.*, 2005). Still other authors have reported that Src activity causes calpain cleavage FAK accompanied by focal adhesion disassembly (Carragher *et al.*, 2001; Carragher *et al.*, 2002). In the HASM cells, the 85 kDa variant appeared to be unique to the membrane fraction where it made up a large proportion of the total FAK detected. The relative proportion of the 85 kDa FAK was variable between individual HASM cell cultures, indicating that there could be differences in the rate of proteolytic cleavage within each cell culture (Carlin *et al.*, 2005). In the HOSCC cell lines, Triton X-100 detergent was used to solubilize all of the membrane-associated proteins but not all of the other cellular proteins. Thus, the concentration of the membrane-associated proteins is enhanced by this extraction procedure meaning that because the 85 kDa variant was only detected in the membrane samples (and not in the whole-cell extraction), it could be a product of the Triton X-100 enhancement of the membrane-associated proteins.

Although all of the HOSCC cell lines are derived from tumours of similar pathological grading (i.e. moderately differentiated), distinctly different levels of the FAK protein variants are observed in membrane extracts of these cell lines (Figure 3.6). Membrane extracts of the WHCO6 cell line exhibit the greatest concentrations of both FAK variants (85 and 125 kDa) when compared to the other cell lines. However, in the other cell lines the expression level of the 85 kDa variant is not necessarily aligned with that of the 125 kDa FAK protein. For instance in the WHCO5 cell line, which has a relatively high concentration of 125 kDa FAK, a small percentage of the full-length product may be cleaved resulting in a low level of

85 kDa FAK being produced. Those cell lines (SNO, WHCO1, and WHCO3) that have comparatively lower concentrations of the 125 kDa variant could possibly have higher levels of the 85 kDa variant because a larger concentration of the 125 kDa isoform is being cleaved (Figure 3.6). In the case of WHCO6, where the concentrations of both variants are very high, a large concentration of FAK (125 kDa) may still be present after extensive cleavage processes have taken place. This could be due to the increased amount of FAK expressed in this cell line; this is supported by the immunoblotting results presented in Chapter 2 where WHCO6 expressed the highest concentration of FAK in whole-cell lysates (Figure 2.3). Therefore, the observed differences in expression of the two FAK variants may result from the activity of various cellular factors involved regulating FAK expression in the 5 HOSCC cell lines.

3.4.3 EGFR activation may influence the expression and localization of the FAK variants in the HOSCC cell lines

Full-length FAK complexes with activated EGFR at the cell membrane and together their synergistic signalling is implicated in EGF-stimulated cell migration (Sieg *et al.*, 2000). The results presented here further indicate that EGFR activation by its common ligand (EGF) affects the concentrations of both of the FAK variants at the cell membrane. In the WHCO1, WHCO3, and WHCO5 cell lines, an initial increase in the expression of full-length FAK during EGF treatment; thereafter the concentration decreases considerably (Figure 3.9). In the WHCO6 and SNO cell lines, EGF treatment induces a general decrease in the concentration of full-length FAK at the cell membrane. Hence, EGFR activation may influence the concentration of membrane-associated full-length FAK uniquely (at different time intervals) within each cell line.

The fact that EGFR interacts with the N-terminal FERM domain of full-length FAK is well documented (van Nimwegen and van de Water, 2007). Thus, it stands to reason that EGFR could also possibly interact with, and subsequently affect, the concentration of the N-terminal 85 kDa variant (which would also contain the FERM

domain) of FAK at the cell membrane. EGF treatment of the WHCO3, WHCO6, and SNO cell lines induces a general decrease in the concentration of the 85 kDa variant at the cell membrane, whereas in the WHCO5 and WHCO1 cell lines an increase in the concentration of this variant is generally observed. As observed for the 125 kDa isoform, after the initial peak in concentration is reached in each cell line, the levels of 85 kDa decrease substantially with time (Figure 3.9). Once again, the changes in concentration of this variant do not always correspond to those noted above for full-length FAK.

Yet how and why does EGF stimulation cause the observed differences in the levels of the full-length and 85 kDa FAK variants among the HOSCC cell lines? One plausible explanation stems from observations made by Driver and Veale (2006), who suggested that EGF receptor recycling in these HOSCC cell lines was responsible for abrupt changes in ILK expression. Therefore it is reasonable to suggest that EGF receptor recycling in these HOSCC cell lines may also cause variations in the concentration of the FAK variants depending on the duration of EGF exposure. Although EGFR is predominantly localized at the cell membrane, the receptors are constantly internalized and cycle between the plasma membrane and the endosomal compartment (for a review on EGFR trafficking see Wiley, 2003). The constitutive degradation rate of EGFR is slower than other ErbB family members, and only EGFR alters its trafficking pattern in response to ligand binding resulting in accelerated internalization and increased lysosomal targeting. This disrupted trafficking could alter signalling patterns which are commonly observed as a consequence of receptor overexpression (Wiley, 2003). The rate of recycling of EGFR can be increased by EGF stimulation, however, once internalized the receptors may be rapidly recycled back to the cell membrane (Wiley, 2003; Driver and Veale, 2006). A ligand-induced change in EGFR trafficking also results in a decrease in the fraction of receptors that recycle. The saturable process of lysosomal sorting of internalized EGFR involves multiple rounds of internalization, sorting, recycling and reinternalization (French *et al.*, 1994; Wiley, 2003). Studies suggest that c-Cbl-mediated ubiquitination appears to be necessary for the internalization and degradation of EGFR, although other reports indicate that this is not always the case

(reviewed in Wiley, 2003). Although all internalized receptors are ultimately degraded, the rate of degradation is much slower than the rate of internalization, resulting in the accumulation of intracellular pools of receptors (Wiley, 2003).

Each of the 5 HOSCC cell lines may exhibit different rates of EGFR recycling which may be responsible for the observed changes in FAK expression during the course of EGF treatment. EGFR signalling at the cell membrane is likely to activate transcription factors such as NF-κB, which in turn is likely to regulate FAK transcription in these HOSCC cell lines (Normanno *et al.*, 2006). Increased FAK concentrations may result from an abundance of EGF receptors at the cell membrane (leading to increased NF-κB activation), whereas decreases in FAK concentration at the cell membrane could be attributed to EGFR internalization and reduction at the cell membrane (leading to decreased NF-κB activity). The degree to which internalized receptors remain active is unclear, and internalized EGFR has been shown to associate with Shc, Grb2, and SOS in rat liver cells (Wiley, 2003). EGFR signalling through the PLC-γ, PI3K, and Ras pathways seems to occur from both the cell surface and intracellular compartments (Wiley, 2003). However, it is unlikely that internalized EGFR is associated with FAK signalling. Moreover, because FAK is activated at the cell membrane, EGFR may very well need to be membrane-anchored in order to channel its signalling processes through active FAK. Clearly, a more detailed understanding is needed of the regulated trafficking of active and inactive EGF receptors in order to predict how alterations in trafficking can alter signalling in HOSCC and other cell lines overexpressing these receptors.

Although it was demonstrated that EGFR affects total FAK protein expression, this does not account completely for the changes in concentration of the FAK variants observed in the membrane extracts. An increase or decrease in the FAK expression levels in the whole-cell lysates does not necessarily correlate with a corresponding increase or decrease in the expression of full-length FAK observed in the membrane extracts. Firstly, as seen in the WHCO1, at times when a high level of full-length FAK was detected in the whole-cell lysates, much lower concentrations were observed in the membrane extracts (Figures 3.4 and 3.9). Although FAK expression

is upregulated by EGFR signalling, receptor internalisation may account for low concentrations of FAK at the cell membrane. Secondly, in other instances, such as in the WHCO3 and WHCO5 cell lines, when a low expression level is detected in whole-cell lysates, the corresponding membrane extracts may exhibit a high concentration of FAK, and vice versa (Figures 3.4 and 3.9). This could indicate that that if many EGF receptor molecules are present in the cell membrane at a particular time, the concentration of FAK at the cell membrane need not be directly proportional to the overall percentage of FAK expressed in the whole cell. Although a relatively small amount of FAK is detected in the whole-cell lysates, most of the FAK present in the cell could be localized to the cell membrane at that time. Thirdly, in complete contrast to this possibility, in the WHCO6 cell line the expression of FAK in whole-cell lysates does seem to correspond to the concentration of FAK present in the membrane extracts. This is possibly due to high concentrations of EGFR in the plasma membrane. In this cell line, high FAK expression levels compounded with a high level of FAK associated with EGFR at the cell membrane could further enhance the stimulation of cell proliferation, cell survival, and cell migration.

In the SNO cell line, there seems to be a relatively delayed response in that following good membrane localization of FAK (and possibly a high percentage of membrane-associated EGFR), the overall expression of FAK in the whole-cell lysates seems to increase during the subsequent time treatment interval (Figures 3.4 and 3.9). Thus, EGFR signalling via FAK at the cell membrane may induce an increase in FAK expression a while later. Similarly, when the presence of FAK is lower at the cell membrane, it is usually followed by a decrease in FAK expression at a later time treatment interval due to reduced EGFR-FAK membrane association. Thus it seems that within each cell line, different responses are induced that affect the overall expression of FAK in response to EGFR-FAK membrane-associated signalling. In turn, FAK expression also affects the amount of FAK that is able to localize at the cell membrane in order to associate with membrane-bound EGFR. Receptor recycling also plays a role in FAK localization and expression, although different

effects result within each of the HOSCC cell lines due to the differential rates of receptor recycling between the cell lines.

As was mentioned earlier in this chapter, full-length FAK may be cleaved by various proteases, which would also account for the changes in the levels of detectable expression of the full-length FAK protein. Thus, the results obtained in this study are complex in nature and it seems that the differences noted in the expression and localization of the FAK protein within the HOSCC cell lines have a multifaceted origin.

3.4.4 EGFR signalling appears to influence the phosphorylation state of FAK in the HOSCC cell lines

Overexpression of EGFR directly correlates with increased metastatic potential as EGFR activation stimulates cell migration by modifying cell-ECM interactions (Sieg *et al.*, 2000; Hauck *et al.*, 2001). EGFR and full-length FAK are co-ordinately involved in promoting cell migration signalling, although opinions differ as to whether FAK tyrosine phosphorylation and activation are required for EGF-stimulated cell migration. FAK contains many tyrosine phosphorylation sites, and it also associates with other tyrosine-phosphorylated proteins such as EGFR, PI3K, and MAPK (Schaller, 2001; Velling *et al.*, 2004; van Nimwegen and van de Water, 2007). It has become of interest to assess how the phosphotyrosine status of FAK in the HOSCC cell lines is affected by activation of the receptor tyrosine kinase EGFR.

In the immunoprecipitation study, it was assumed that increased binding of the phosphotyrosine antibody to FAK would be indicative of a similar increase in the phosphotyrosine status of FAK. Contrary to the results expected, FAK-specific western blotting of the phosphotyrosine-immunoprecipitated membrane extracts detected the presence of the 85 kDa but not the full-length variant of FAK (Figure 3.10). Both variants were detected in the positive membrane extract control, while no FAK bands were present in the ‘no-protein’ negative control. The full-length FAK variant was expected to be present in the phosphotyrosine-immunoprecipitated

samples because it is this form of FAK that is known to mediate growth factor and integrin signalling, as well as acting as a scaffold for the binding of other proteins in focal adhesions (van Nimwegen and van de Water, 2007). It is not known what role, if any, the 85 kDa variant of FAK plays in cellular signalling.

Due to the fact that the 85 kDa FAK variant is an N-terminal cleavage product of full-length FAK, it possibly contains the N-terminal FERM domain. It is speculated that EGFR is able to bind to the FERM domain present within the 85 kDa FAK variant, as EGFR is known to bind to the FERM domain of full-length FAK (van Nimwegen and van de Water, 2007). The results presented here show that EGFR signalling affects the concentration of the 85 kDa variant that is tyrosine-phosphorylated or is associated with other tyrosine-phosphorylated proteins at the cell membrane. In all the cell lines except for WHCO6, a peak in concentration of the 85 kDa variant is reached between 30 min and 3 hrs of EGF treatment. The untreated control of WHCO6 exhibits the highest concentration in this cell line. Generally speaking, once the peak in concentration has been reached the concentration of the 85 kDa variant thereafter drops quite drastically in all 5 cell lines. Once again, receptor recycling may be partly responsible for the noted changes in concentration of this variant at the cell membrane.

Full-length FAK is not detected in the phosphotyrosine immunoprecipitated samples, however, it is highly unlikely that full-length FAK is not tyrosine-phosphorylated or is not associated with any other tyrosine-phosphorylated proteins in these cell lines. Full-length FAK may be tyrosine-phosphorylated in these cell lines, but the concentration of tyrosine-phosphorylated FAK may be too little to detect by western blot analysis. Another more plausible explanation is that the tyrosine-phosphorylated sites on FAK and its associated binding partners may be blocked by the binding of other proteins, thus rendering the sites inaccessible to the phosphotyrosine antibody. This explanation is highly likely, as it is well known that the phosphotyrosine sites on FAK are residues which facilitate the binding of proteins such as Src, the p85 subunit of PI3K, Grb2, and Grb7 (van Nimwegen and van de Water, 2007). Because the N-terminal 85 kDa variant of FAK does not contain some of these residues or the

focal adhesion targeting (FAT) sequence, signalling proteins may not bind to this variant as it is unable to facilitate the same degree of signalling that full-length FAK normally would. Thus, the tyrosine residues that are present in the 85 kDa variant, such as Y397 and Y407, are "free" and are not bound by any other proteins and are thereby readily accessible to the phosphotyrosine antibody. As phosphotyrosine antibodies directed against specific tyrosine residues on FAK are now commercially available, using them would provide more detailed data on the phosphorylation status of each tyrosine residue within the FAK protein.

If the immunoprecipitation experiment had worked according to plan, what results would be expected? This is a difficult question to answer, as published studies have produced contrasting results on how EGF treatment of cell lines affects the tyrosine phosphorylation status of FAK (Brunton *et al.*, 1997; Lu *et al.*, 2001; Yamanaka *et al.*, 2003). As mentioned in the introduction to this chapter, EGF treatment of colon carcinoma cell lines causes an increase in FAK tyrosine phosphorylation and leads to cell migration (Brunton *et al.*, 1997). In contrast Lu *et al.* (2001) showed that EGF treatment of the human epidermoid carcinoma cell line A431 caused FAK to be rapidly dephosphorylated and to remain in a hypo-phosphorylated state. Yamanaka *et al.* (2003) showed that EGF treatment induced rapid tyrosine dephosphorylation of FAK in cervical carcinoma cell lines, but that the tyrosine phosphorylation level was restored later on. Thus, from these somewhat contradictory reports, it is difficult to predict how EGF treatment of the HOSCC cell lines would affect the tyrosine phosphorylation status of FAK.

Miyazaki *et al.* (2003) showed that the level of FAK phosphorylation at Y397 was similar to the amount of FAK protein expressed in 7 HOSCC cell lines in their study. These cell lines were not treated with EGF, so it would be difficult to predict if EGF would alter the tyrosine phosphorylation status of FAK. However, it would be logical to predict that if the tyrosine phosphorylation status of FAK is proportional to the amount of FAK expressed, that EGF treatment of the 5 HOSCC cell lines in the study presented here would lead to similar changes in both the expression level and tyrosine phosphorylation status of FAK. Nonetheless, this does not appear to be the

case with the 85 kDa variant of FAK, as the changes in expression levels and tyrosine phosphorylation levels do not correlate with each other within any of the 5 HOSCC cell lines (see graphs in Figures 3.9b and 3.11). It is also important to remember that the expression levels of the 125 and 85 kDa variants were not necessarily similar to each other in the 5 cell lines (see Figure 3.9), therefore; the tyrosine phosphorylation levels of the two variants would not necessarily be aligned either. Thus, there is the possibility that the tyrosine phosphorylation status of full-length FAK may parallel the changes in expression in the 5 EGF-treated HOSCC cell lines.

Taken together, the results show that the influence of EGFR signalling on FAK is complex. EGF-stimulation of the HOSCC cell lines has a marked effect on both the overall expression levels and the membrane localization of the full-length and 85 kDa FAK variants. Many converging influences may be responsible for the noted changes in FAK protein expression, membrane localization and possible tyrosine phosphorylation, and data from dissection of the compilation of individual pathways will be necessary to finally elucidate the potential implications of EGFR/FAK signalling in terms of the transformed status in these human squamous cell carcinoma cell lines.